Phospholipid Synthesis in a Membrane Fraction Associated with Mitochondria*

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A crude rat liver mitochondrial fraction that was capable of the rapid, linked synthesis of phosphatidylserine (PtdSer), phosphatidylethanolamine (PtdEtn), and phosphatidylcholine (PtdCho) labeled from [3-3H]serine has been fractionated. PtdSer synthase, PtdEtn methyltransferase, and CDP-choline:diacylglycerol cholinephosphotransferase activities were present in the crude mitochondrial preparation but were absent from highly purified mitochondria and could be attributed to the presence of a membrane fraction, X. Thus, previous claims of the mitochondrial location of some of these enzymes might be explained by the presence of fraction X in the mitochondrial preparation. Fraction X had many similarities to microsomes except that it sedimented with mitochondria (at 10,000 × g). However, the specific activities of PtdSer synthase and glucose-6-phosphate phosphatase in fraction X were almost twice that of microsomes, and the specific activities of CTP:phosphocholine cytidylyltransferase and NADPH:cytochrome c reductase in fraction X were much lower than in microsomes. The marker enzymes for mitochondria, Golgi apparatus, plasma membrane, lysosomes, and peroxisomes all had low activities in fraction X. Polyacrylamide gel electrophoresis revealed distinct differences, as well as similarities, among the proteins of fraction X, microsomes, and rough and smooth endoplasmic reticulum.

The combined mitochondria-fraction X membranes can synthesize PtdSer, PtdEtn, and PtdCho from serine. Thus, fraction X in combination with mitochondria might be responsible for the observed compartmentalization of a serine-labeled pool of phospholipids previously identified (Vance, J. E., and Vance, D. E. (1986) J. Biol. Chem. 261, 4486-4491) and might be involved in the transfer of lipids between the endoplasmic reticulum and mitochondria.

In mammalian cells the majority of phospholipid biosynthesis occurs on membranes of the endoplasmic reticulum (1-4) although certain phospholipid-biosynthetic enzymes are restricted to the mitochondria (5-7), for example PtdSer' decarboxylase, cardiolipin synthase, and phosphatidylglycerol synthase. In addition, the Golgi apparatus contains many enzymes of phospholipid biosynthesis that have specific activities as high, or higher than, those in endoplasmic reticulum (1). In terms of total cellular phospholipid biosynthesis, however, the Golgi probably contributes less than 5% (1, 4). Thus, mechanisms must exist for the transfer of phospholipids from their sites of synthesis to other subcellular organelles for membrane biogenesis. Although the detailed mechanisms of interorganelle protein trafficking are now emerging (8), the mechanisms of the intracellular movement of lipids are still not well understood. Models of assembly of lipids into membranes must explain how net transfer of lipid occurs and how both the interorganelle and transbilayer asymmetric lipid composition of membranes is established and maintained. Several models have been proposed that are supported by some experimental evidence (9). For example, in vitro protein-mediated phospholipid transfer from one organelle to another by the action of specific and nonspecific transfer proteins has been demonstrated. A second mechanism proposed for lipid movement within cells is a vesicle-mediated transfer whereby vesicles bud from a donor membrane and fuse with an acceptor membrane. A third plausible mechanism for the movement of phospholipids between membranes is one in which there are regions of continuity between the donor and acceptor membranes, thereby allowing the lateral flow of lipids from one membrane to the other. In spite of the very rapid movement of phospholipids within animal cells (10, 11) there is also evidence that defined intracellular pools of phospholipids exist. Such compartmentalization was demonstrated when pools of PtdEtn and PtdCho biosynthesized from PtdSer were preferentially assembled into lipoproteins (12). In contrast, there was discrimination against the use of PtdEtn and PtdCho derived from CDP-ethanolamine for lipoprotein secretion (12). In addition, there was a selection of newly synthesized, rather than pre-existing, phospholipids for assembly into hepatic lipoproteins (13, 14). Other studies have also suggested the existence of specific membrane domains of phospholipids. For example, only a fraction (1/6-1/5) of the hepatocyte pool of PtdSer is apparently used for decarboxylation to PtdEtn (15), and only a small pool of phospholipid is active in the Ca2+-stimulated base exchange reaction with choline, ethanalamine, and serine (16). Likewise, compartmentalization of separate pools of PtdEtn in rat liver microsomal membranes has been proposed (17). Moreover, in the plasma membrane of rat liver 10-90 min after labeling with [3H]serine the specific activity of PtdSer was approximately double that in the endoplasmic reticulum (18), even though the latter is the major site of PtdSer synthesis (1, 4). In the same study (18), phospholipids of the various subcellular membranes (endoplasmic reticulum, Golgi, plasma membrane, and mitochondria) were labeled to different extents depending on their routes of biosynthesis. Examples of cellular lipid compartmentalization are not restricted to phospholipids. There are also reports that di
acetylglycerol (19, 20), cholesterol (21), triacylglycerols (22), and even water-soluble phospholipid precursors (23–25) are compartmentalized. Thus, there appears to be incomplete mixing of cellular (phospho)lipids, either of newly synthesized with pre-existing, or of lipids synthesized from different biosynthetic routes. The mechanism by which individual pools of lipids are generated and maintained is not understood.

An interesting example of lipid movement in eukaryotic cells is the formation of PtdEtn from PtdSer (Fig. 1). PtdSer, made in the endoplasmic reticulum, is translocated to the outer aspect of the inner mitochondrial membrane, where PtdSer decarboxylation to PtdEtn occurs (26, 27). Subsequently, for the methylation of serine-derived PtdEtn to PtdCho, the PtdEtn must be returned to the endoplasmic reticulum (or Golgi). PtdSer transfer from the endoplasmic reticulum, and decarboxylation in the mitochondria, can apparently occur without the addition of any cytosolic proteins or other cofactors such as ATP (28). The movement of PtdEtn between mitochondria and endoplasmic reticulum is reportedly much slower than that of PtdCho (29–32). The mechanism of the intermembrane movement of PtdEtn is at present not clear (5) although some evidence suggests that a monomeric, rather than a vesicle-mediated, movement through the aqueous phase may be responsible for PtdEtn equilibration (5, 11, 33).

How a pool of PtdEtn and PtdCho, more highly labeled from serine than the bulk of PtdEtn and PtdCho in the endoplasmic reticulum, can be compartmentalized throughout these transverse and used for lipoprotein (12) or membrane (18) assembly, is an enigma.

In the present study a unique membrane fraction, X, from rat liver has been isolated that is associated with mitochondria and has high specific activity for several, but not all, phospholipid biosynthetic enzymes that are usually ascribed to the endoplasmic reticulum. The protein profile of the membrane fraction on SDS-polyacrylamide gel electrophoresis was significantly different from that of mitochondria and either rough or smooth endoplasmic reticulum. When associated with mitochondria, the membrane fraction was capable of the concerted synthesis of PtdSer, PtdEtn, and PtdCho from labeled serine.

MATERIALS AND METHODS

Chemicals—UDP-[6-3H]galactose (specific activity 14.5 Ci/mmol), [methyl-3H]choline (specific activity 15 Ci/mmol), cytidine-5'-diphospho-[methyl-3H]choline (specific activity 51 Ci/mmol), [3-3H]serine (specific activity 23 Ci/mmol), and [4,5-3H]leucine (45 Ci/mmol) were purchased from Amersham Corp. Cytidine-5'-diphospho-[1,2-14C]ethanolamine (specific activity 68 Ci/mmol) obtained from ICN Radiochemicals as was SDS. Phosphatidyl-(U-14C)-serine (0.5 Ci/mmol) was kindly provided by Dr. Dennis Voelcker, National Jewish Hospital, Denver, CO. The anion exchange resin, AG-2X8 (200–400 mesh, Cl- form) was from Bio-Rad, as were reagents for polyacrylamide gel electrophoresis. Boehringer Mannheim (Canada) supplied NADPH and S-adenosylmethionine, and Aldrich was the source of titanium sulfate (TlSO4·H2SO4·H2O). Disaccharide used for the cholinephosphotransferase assays was prepared by phospholipase C digestion of egg PtdCho (1). Asolectin (95% purified soy phospholipid) was purchased from Associated Concentrates, Woodside, NY. Triton X-100 (Sigma) was purified according to the method of Chang and Boek (36). Du Pont-New England Nuclear provided ENHANCE, Smac 350 and Hionic-Fluor were from Packard. Thin layer chromatography plates (Silica Gel-G, 0.25-mm thickness) were purchased from BDH Chemicals, and Protein A-Sepharose CL-4B and Percoll were from Pharmacia Fine Chemicals, Sweden. The standard phospholipids PtdSer, PtdEtn, PtdCho, and PtdIns were from Avanti Polar Lipids, Birmingham, AL. All other chemicals were from Sigma or Fisher.

Preparation of Subcellular Fractions—Female Sprague-Dawley rats (approximately 200 g) were provided with standard diet and drinking water ad libitum but were fasted overnight before being decapitated. Livers were quickly removed and immersed in ice-cold isolation medium (250 mM mannitol, 5 mM HEPES, pH 7.4, 0.5 mM EDTA, and 0.1% bovine serum albumin). The liver was minced with scissors and homogenized gently by four strokes in a Potter-Elvehjem motor-driven homogenizer. The homogenate was centrifuged twice at 100,000 × g for 5 min to remove large debris and nuclei. The supernatant was centrifuged for 10 min at 10,300 × g to pellet the crude mitochondria. The resultant supernatant was centrifuged at 100,000 × g for 1 h in a Beckman Ti-70 rotor to pellet the microsomes. For further purification of mitochondria, the crude mitochondrial pellet was suspended by hand homogenization in approximately 4 ml isolation medium, and the suspension was layered on top of 20 ml of medium containing 225 mM mannitol, 25 mM HEPES, pH 7.4, 1 mM EGTA, 0.1% bovine serum albumin, and 30% (v/v) Percoll, in each of four 30-ml polycarbonate ultracentrifuge tubes. The tubes were centrifuged for 30 min at 50,000 × g, after which a dense band, containing purified mitochondria, was recovered from approximately 0.5 ml below the tube. The mitochondria were removed with a Pasteur pipette, diluted with isolation medium, and washed twice by centrifugation at 6,300 × g for 10 min to remove the Percoll. The final pellet was resuspended in isolation medium and stored at −70°C.

Fraction X was isolated from the Percoll gradient immediately above the mitochondria, by centrifugation first at 5,000 × g for 10 min, then further centrifugation of the supernatant at 100,000 × g for 1 h in a Beckman Ti-70 rotor. The pellet, designated fraction X, was resuspended in approximately 0.5 ml of buffer containing 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, and 0.1 mM phenylmethylsulfonyl fluoride, and stored at −70°C.

For subfractionation of mitochondria into inner and outer membranes, the pure mitochondrial pellet was suspended in buffer (20 mg/ml) containing 70 mM sucrose, 200 mM mannitol, and 2 mM HEPES, pH 7.4. The mitochondria (2.5 mg) were mixed gently with 125 µl of 0.6% digitonin solution made in the above buffer and incubated on ice for 15 min. The mixture was diluted with the above buffer containing 90 mg of bovine serum albumin (100 µl), then centrifuged for 10 min at 12,000 × g. The supernatant was enriched in mitochondrial outer membranes, and the pellet was enriched in inner membranes.

For the experiments in which Golgi, plasma membrane, and rough and smooth endoplasmic reticulum fractions were studied, the subcellular fractions were isolated by the method of Croze and Morris (37) as modified (1), with the endoplasmic reticulum I and II fractions being designated "rough" and "smooth" endoplasmic reticulum, respectively.

A membrane fraction enriched in lysosomes was prepared from a rat treated 5 days prior to killing with a single, intraperitoneal dose of TR-55 (300 mg/kg) and TR-1539 (0.5 ml of a 20% solution, i.p.). The rat was fasted overnight before being killed. The liver was minced with scissors and homogenized by four strokes in a Potter-Elvehjem motor-driven homogenizer in five volumes of buffer containing 0.5 M sucrose, 1% dextran, and 37.5 mM Tris-HCl, pH 6.5. The homogenate was centrifuged at 65,000 × g for 10 min, and the supernatant was the following morning at a volume of 10 ml/g liver, with homogenization buffer. The supernatant was centrifuged at 34,000 × g for 10 min in a Beckman Ti-70 rotor which produced a tight, brown pellet that was resuspended by hand homogenization in 5 ml of homogenization buffer/g liver. The suspension was then centrifuged for 10 min at 34,000 × g to yield a pellet (containing primarily lysosomes and peroxisomes) that was washed by homogenization in approximately 7 ml/liver of 45% sucrose in 3 mM HEPES, pH 7.4, 1 mM EDTA, and 0.1% ethanol. The suspension was placed in the bottom of each of two ultracentrifuge tubes, and 16 ml of 34.5% sucrose, then 14.5% sucrose (both made up in 3 mM HEPES, pH 7.4, 0.1% ethanol, 1 mM EDTA, and 0.1% bovine serum albumin), was layered on top. The tubes were centrifuged in a Beckman SW 28 rotor for 2 h at 110,000 × g.

Lysosomes were enriched in the white band at the interface between the 14.3 and 34.5% sucrose layers. The lysosomes were washed with homogenization buffer and pelleted by centrifugation at 110,000 × g for 1.5 h in the SW 28 rotor. The pellet was resuspended in 0.5 ml of buffer containing 0.5 M sucrose, 10 mM Tris-HCl, pH 7.4, and 0.1% bovine serum albumin, and stored at −70°C.

Peroxisomes were isolated by the method of Völkl and Fahimi (38) from the livers of normal, untreated rats.

Characterization of Subcellular Fractions Membrane fractions were enriched for the following enzymes by standard procedures (1): glucose-6-phosphate phosphatase, NADPHcytochrome c
Phospholipid Synthesis in Mitochondria-associated Membrane

Results

Phospholipids Made from Serine by Crude Mitochondrial Membranes—There is some evidence that phospholipids derived from PtdSer are compartmentalized into distinct pools (12, 14–16). One possible explanation for this finding is that isolated mitochondria may have the ability to synthesize pools of PtdSer, PtdEtn, and PtdCho from serine (see Fig. 1). Hence, rat liver mitochondria were examined for their content of PtdSer synthase (exchange enzyme), PtdSer decarboxylase, and PtdEtn N-methyltransferase. Mitochondria were prepared by preliminary centrifugation of a rat liver homogenate for 5 min at 600 x g to remove nuclei and cell debris and subsequent centrifugation for 10 min at 10,300 x g to pellet the mitochondria. As indicated in Table I, crude mitochondria prepared by this method contained all three of these phospholipid biosynthetic activities. The presence of PtdSer de-carboxylase in mitochondria agrees with previous reports (1, 26), but the apparent presence of PtdSer synthase and PtdEtn N-methyltransferase was surprising because these enzymes are thought to be primarily microsomal (1, 4). The specific activity of PtdSer synthase in the mitochondrial preparation was as high as that in microsomes prepared from the same livers. The identity of the products of the enzymatic reactions, PtdSer, PtdEtn, and PtdCho, was confirmed by thin layer chromatography.

According to the marker enzymes for endoplasmic reticulum (NADPH-cytochrome c reductase and glucose-6-phosphate dehydrogenase), contamination of the crude mitochondrial fraction by endoplasmic reticulum membranes was only 17 and 15%, respectively (Table II), as calculated by comparison of the specific activities of these two enzymes in the crude mitochondria and microsomal membranes. Thus, the activities of PtdSer synthase and PtdEtn N-methyltransferase could not be attributed to contamination by endoplasmic reticulum as measured by these two marker enzymes. Enrichment of the mitochondrial marker enzyme, cytochrome c oxidase, was 4-fold compared with the homogenate.

Mitochondria prepared as described above were incubated at 37 °C with [3H]serine. After various incubation times up to 1 h, the incorporation of radioactivity into PtdSer, PtdEtn, and PtdCho was measured (Fig. 2A). Initially, the majority of radioactivity was in PtdSer, but at later times PtdSer was rapidly converted into PtdEtn. Subsequently, the PtdEtn was methylated to PtdCho; the identity of PtdCho as the labeled product was confirmed by thin layer chromatography. Since label from [3H]serine is incorporated into both the polar head groups and the diacylglycerol moieties of PtdEtn and PtdCho in cultured hepatocytes, PtdEtn and PtdCho were hydrolyzed by phospholipase C so that incorporation of 3H into the ethanolamine, choline, and diacylglycerol moieties could be assessed. From these hydrolysates, approximately 98% of the radioactivity in PtdEtn and PtdCho was in the polar head groups. In addition, the rate of formation of PtdSer, PtdEtn, and PtdCho was approximately linear with time (Fig. 2A) and with the amount of protein, at least up to 1.5 mg protein/200 μl assay (data not shown). For comparison, microsomes were incubated with [3H]serine under the same conditions (Fig. 2B), and as expected, at all time points, the majority of radioactivity was in PtdSer. The small amount of [3H] in PtdEtn was probably due to the microsomes being approximately 9% contaminated with mitochondria, according to the specific activity of cytochrome c oxidase (Table II). The addition of cytosolic proteins (a potential source of phospholipid exchange proteins) did not increase the rate of the
Phospholipid Synthesis in Mitochondria-associated Membrane

**TABLE I**

Subcellular membrane fractions were prepared from rat liver and assayed for phospholipid biosynthetic enzymes as described under "Materials and Methods." The units of enzyme activities are: PtdSer synthase (exchange enzyme), nmol of PtdSer synthesized/h/mg protein. The activities of the other enzymes are expressed as nmol/min/mg protein. All values are means ± S.D. The numbers in parentheses (n) give the numbers of individual subcellular fractionations. ND, not determined.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity in</th>
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<tr>
<td></td>
<td>Crude mitochondria</td>
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<tr>
<td>PtdSer synthase</td>
<td>1.92 ± 0.55</td>
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<td></td>
<td>(n = 6)</td>
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<tr>
<td>PtdSer decarboxylase</td>
<td>0.22 ± 0.04</td>
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<td></td>
<td>(n = 4)</td>
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<tr>
<td>PtdEtn N'-methyltransferase</td>
<td>1.57 ± 0.45</td>
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<td>(n = 9)</td>
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<td>CDP-choline:1,2-diacylglycerol cholinephosphotransferase</td>
<td>1.60 ± 0.13</td>
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<tr>
<td>CTP-phosphocholine cytidylyltransferase</td>
<td>0.01 ± 0.01</td>
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<td>(n = 3)</td>
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<tr>
<td>CDP-ethanolamine:1,2-diacylglycerolethanolamine-phosphotransferase</td>
<td>ND</td>
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**TABLE II**

Marker enzyme activities in subcellular fractions prepared from rat liver

Membrane fractions were prepared from rat liver and assayed for marker enzymes as described under "Materials and Methods." The units of enzyme activities are: NADPH:cytochrome c reductase, nmol of cytochrome c reduced/min/mg protein; cytochrome c oxidase, nmol of cytochrome c oxidized/min/mg protein; glucose-6-phosphate phosphatase, nmol of P, formed/min/mg protein; UDP-galactose:N-acetylglucosamine galactosyltransferase, nmol of [3H]galactose incorporated/min/mg protein; 5'-nucleotidase, nmol of P, formed/min/mg protein. All values are means ± S.D. The numbers in parentheses (n) give the numbers of individual subcellular fractionations. ND, not determined.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity in</th>
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<tr>
<td></td>
<td>Crude mitochondria</td>
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<tr>
<td>NADPH:cytochrome c reductase</td>
<td>0.46 ± 0.76</td>
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<td></td>
<td>(n = 13)</td>
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<tr>
<td>Cytochrome c oxidase</td>
<td>179.7 ± 10.8</td>
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<td></td>
<td>(n = 9)</td>
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<tr>
<td>Glucose-6-phosphate phosphatase</td>
<td>27.8 ± 3.5</td>
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<td></td>
<td>(n = 4)</td>
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<tr>
<td>UDP-galactose:N-acetylglucosamine galactosyltransferase</td>
<td>ND</td>
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<tr>
<td>5'-Nucleotidase</td>
<td>ND</td>
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</table>

* The specific activity of this enzyme in Golgi membranes prepared from rat liver by the method of Croze and Morré (1, 35) was 285 ± 68.0 (n = 3).

* In rat liver plasma membrane prepared by the method of Croze and Morré (1, 35), the specific activity of this enzyme was 118.3 ± 69.6 (n = 7).

The Capacity of Purified Mitochondria for Biosynthesis of Phospholipids—Although mitochondria prepared in the above manner were apparently not highly contaminated with endoplasmic reticulum membranes (the acknowledged source of PtdSer synthase and PtdEtn methyltransferase), it was important to purify the mitochondrial fraction further to determine whether or not these enzymes were indeed inherent to mitochondria. Thus, crude mitochondria were centrifuged on a Percoll gradient and a band of membranes, highly enriched in cytochrome c oxidase (approximately 5-fold compared with the homogenate), was recovered from the gradient. These purified mitochondrial membranes were assayed for several marker enzymes characteristic of other subcellular organelles (Table II). Assay of the enzyme NADPH:cytochrome c reductase was unaffected by the amount of Percoll that was present from the gradient. The activities of the phospholipid biosynthetic enzymes PtdSer synthase and PtdEtn methyltransferase in the purified mitochondria were very low (Table I) and were essentially absent when corrected for cross-contamina-
tein/g liver, from nine independent preparations) was exam-
ined for phospholipid biosynthetic enzyme activities and
their H content determined by liquid scintillation counting. \( \square = \text{PtdSer}; \blacktriangle = \text{PtdEtn}; \blacklozenge = \text{PtdCho}. \)

Isolation and Enzymatic Activities of Fraction X—Since the phospholipid enzymes present in crude mitochondrial membranes were removed by centrifugation on Percoll, the location of the “missing” enzymes was investigated. A second band of membranes was obtained from the Percoll gradient from immediately above the mitochondria. From this band a membrane fraction was obtained which has been designated “fraction X.” This membrane fraction (1.82 + 0.62 mg pro-

Several phospholipid biosynthetic enzymes were assayed in fraction X, microsomes, and purified mitochondria (Table I). The activity of PtdSer synthase was 2.70 nmol/h/mg protein in fraction X compared with 1.76 nmol/h/mg protein in microsomes. The properties of PtdSer synthase in fraction X and microsomes were indistinguishable. For example, in both membrane types ethanolamine and choline, which in addition to serine are substrates for PtdSer synthase, both inhibited the enzyme’s reaction with serine to a similar degree. Furthermore, in fraction X and microsomal membranes, the apparent \( K_a \) for serine was 0.1 mM, the pH optimum was 7.0

Fraction X also contained PtdEtn methyltransferase, cholinephosphotransferase, and ethanolaminephosphotransferase activities that were as high as, or higher than, those activities in microsomes (Table I). On the contrary, the specific activity of CTP:phosphocholine cytidylyltransferase in fraction X was only 17% of the microsomal activity. As expected from the low activity of cytochrome c oxidase in fraction X, PtdSer decarboxylase activity was very low in fraction X compared with mitochondria (0.04 versus 0.30 nmol CO\(_2\) released/min/mg protein) (Table I).

The quantitative significance of PtdSer synthase and PtdEtn methyltransferase activities in fraction X in relation to the total activities of these enzymes in the cell, cannot be accurately assessed. It is not possible to calculate the recovery of fraction X from the homog-
enate are the same, the total activity of PtdSer synthase recovered in fraction X is 23.5 ± 9.4% (\( n = 6 \)) of the total activity recovered in the microsomal membranes. (These data are calculated for each membrane as specific activity (nmol/h/mg protein) multiplied by the total amount of protein recovered in that fraction/g liver.) By a similar calculation, the total activity of PtdEtn methyltransferase recovered in fraction X is 18.5 ± 1.0% (\( n = 3 \)) of the total activity recovered in the microsomal fraction.

Although fraction X did not contain appreciable cyto-

Other Properties of Fraction X—The phospholipid com-
sitions of microsomes, mitochondria, and fraction X were compared (Table III). In agreement with other reports (43–45), microsomal and mitochondrial membranes had significantly different phospholipid compositions. In particular, in mitochondria the molar ratio of PtdCho/PtdEtn was 1.54,
there were significant differences in the two types of membranes (Fig. 3, A and B). There were also differences among the protein profiles of fraction X, and rough and smooth endoplasmic reticulum membranes (endoplasmic reticulum I and II respectively), although the proteins in fraction X were more similar to the proteins of the smooth, than the rough, endoplasmic reticulum (Figure 3B).

Pools of PtdSer-derived phospholipids are preferred for assembly into hepatic lipoproteins (12), and there is a selection for secretion of newly synthesized, rather than pre-existing, phospholipids (13, 14). One explanation for these observations is that there may be a coordination between the synthesis of the phospholipids and their assembly with apoproteins, so that PtdEtn and PtdCho newly made from PtdSer do not have the opportunity to mix with either phospholipid made from CDP-ethanolamine or with "old" phospholipids made from PtdSer. Since fraction X had high PtdSer synthesizing activity and appears to be associated with mitochondria, the source of PtdSer decarboxylase, it was possible that fraction X might be involved in providing some phospholipid used for lipoprotein assembly. Thus, the presence of VLDL apolipoproteins was investigated in fraction X. [3H]Leucine (2 mCi) was injected into the portal vein of a rat. After 20 min, the liver was removed, homogenized, and microsomes and fraction X were isolated. ApoB, apoE, and apoC in the microsomes and fraction X were immunoprecipitated with rabbit anti-rat VLDL antibody, the precipitated proteins were subjected to polyacrylamide gel electrophoresis and the protein bands corresponding to apoE, apoC, and the large and small forms of apoB were cut from the gel and counted for radioactivity (13). The results shown in Table IV demonstrate that all four apoproteins were indeed present in fraction X, at approximately the same levels as in microsomes.

Apart from its sedimentation with mitochondria, fraction X has many, but not all, of the properties of microsomes. Therefore, the presence of a membrane fraction equivalent to fraction X was investigated in the microsomal fraction. Microsomes and crude mitochondria were isolated and subjected to centrifugation on separate Percoll gradients. After centrifugation, fraction X from the mitochondrial fraction, and the band from the microsomal fraction from the position corresponding to fraction X on the gradient, were collected. From three similar experiments, the amount of protein in the fraction X band from the mitochondrial and the microsomal membranes was 1.8 ± 0.6 mg/g liver and 4.1 ± 1.0 mg/g liver, respectively. The specific activities of PtdSer synthase and PtdEtn methyltransferase were slightly higher in the mitochondrial than in the microsomal, fraction X. In contrast, the specific activity of cytidylyltransferase was much lower in fraction X from mitochondria (0.40 ± 0.15 nmol/min/mg).

### Table IV

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<tr>
<th>Apolipoprotein content of fraction X and microsomes</th>
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<td>A rat was intraportally injected with 2 mCi of [4,5-3H]leucine. After 20 min the liver was removed and fraction X, mitochondria, and microsomes were prepared. Each membrane (10 mg of protein) was immunoprecipitated with anti-VLDL antibody, and the precipitated proteins were separated by electrophoresis on a 3-15% gradient polyacrylamide gel containing 0.1% SDS. The protein bands corresponding to large and small molecular weight forms of apoB, apoE, and the C apoproteins were excised from the gel and radioactivity counted. The data are from one of two similar experiments.</td>
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<tr>
<td>ApoB&lt;sub&gt;large&lt;/sub&gt;</td>
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<tr>
<td><strong>dpm/mg protein</strong></td>
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<td>Mitochondria</td>
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<td>Microsomes</td>
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<td>Fraction X</td>
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than from the microsomal fraction X (1.51 ± 0.33 nmol/min/mg) and the NADPH:cytochrome c reductase activity in fraction X from mitochondria was approximately one-third that in the microsomal fraction X (11.0 ± 4.0 versus 32.0 ± 9.4 nmol/min/mg protein, respectively). Furthermore, the specific activity of glucose-6-phosphate phosphatase in fraction X from mitochondria was higher than in the equivalent fraction from microsomes (282.6 ± 5.4 versus 205.1 ± 25.0 nmol of P, formed/min/mg protein, respectively). Thus, fraction X from microsomes was not identical to fraction X isolated from the crude mitochondrial pellet.

**DISCUSSION**

The aim of the present study was to investigate whether or not there exists in rat liver an endoplasmic reticulum subfraction that is closely associated with mitochondria and that could explain the compartmentalization of phospholipids derived from PtdSer. A crude mitochondrial fraction isolated from rat liver contained enzymatic activities for PtdSer synthase, PtdEtn N-methyltransferase, and CDP-choline:diacylglycerol cholinephosphotransferase with specific activities similar to those activities in microsomes. When the crude mitochondrial fraction (containing fraction X plus mitochondria) was incubated with [3H]serine, labeled PtdSer, PtdEtn, and PtdCho were produced sequentially and rapidly. Thus, the membrane preparation apparently contained all ingredients necessary for the synthesis of PtdSer, for its transfer to mitochondria and decarboxylation therein, and for subsequent transfer of PtdEtn to the site of PtdEtn methylation by which PtdEtn was methylated to PtdCho. No additional protein factors or cofactors were required, other than Ca2+, S-adenosylmethionine, and dihydrothreitol. These results are in general agreement with the recent findings of Voelker (30) in which the translocation and decarboxylation of PtdSer was reconstituted in a rat liver cell-free system consisting of microsomes and mitochondria without any additional protein factors or cofactors. Voelker's experiments were interpreted to demonstrate that PtdSer transport to mitochondria, and the decarboxylation of PtdSer to PtdEtn, occurred via contact between the mitochondria and the endoplasmic reticulum (or vesicles derived from the endoplasmic reticulum).

**Properties of Fraction X**—When crude mitochondria were further purified on a Percoll gradient, they no longer possessed any of the phospholipid biosynthetic enzyme activities characteristic of the endoplasmic reticulum. However, from immediately above the mitochondrial band on the Percoll gradient a second membrane fraction (fraction X) was isolated that had the following properties. (i) Fraction X contained PtdSer synthase activity with specific activity 1.5-fold higher than that of microsomes. (ii) Fraction X contained PtdEtn methyltransferase, cholinephosphotransferase, and cholineaminephosphotransferase activities equivalent to those in microsomes. (iii) Fraction X contained only 15% of the specific activity of CTP:phosphocholine cytidylyltransferase of microsomes. (iv) Fraction X contained low levels of activity of the following marker enzymes relative to the activities in the organelles for which these enzymes are markers: NADPH:cytochrome c reductase (endoplasmic reticulum), galactosyltransferase (Golgii), cytochrome c oxidase (mitochondria), 5'-nucleotidase (plasma membrane), catalase or peroxidase A oxidase (peroxissomes), and acyl phosphatase (lysosomes). (v) The specific activity of glucose-6-phosphate phosphatase in fraction X was almost double that in microsomes. (vi) SDS-polyacrylamide gel electrophoresis revealed that the proteins of fraction X were quite different from those of mitochondria. Although the proteins of fraction X had some similarities to microsomes and rough and smooth endoplasmic reticulum, there were distinct differences. (The fraction isolated as smooth endoplasmic reticulum (39) has not been fully characterized but probably consists of true smooth endoplasmic reticulum as well as regions of rough endoplasmic reticulum from which ribosomes have been removed during isolation.)

Separation of a membrane fraction "equivalent to fraction X" from crude microsomes was attempted. Membranes were obtained from the Percoll gradient at the same density as that of fraction X derived from the mitochondria but, in contrast to fraction X, the activities of NADPH:cytochrome c reductase and cytidylyltransferase were as high as in the microsomes. Thus, the isolated microsomes did not contain a subtraction that was identical to the fraction X associated with mitochondria.

**Phospholipid Biosynthetic Enzymes in Mitochondria**—The presence of several phospholipid biosynthetic enzymes in liver mitochondria has been controversial. Clearly, certain enzymes, such as those involved in the biosynthesis of cardiolipin and phosphatidylglycerol, which are primarily mitochondrial phospholipids (5-7), are synthesized in situ in mitochondria. Similarly, the mitochondrial location of PtdSer decarboxylase has been firmly established (1, 3, 26). There are, however, conflicting reports on the subcellular distribution of cholinephosphotransferase which, although primarily microsomal (1, 4), has also been detected in mitochondria (46-48). In guinea-pig lung cholinephosphotransferase was found in mitochondria as well as in microsomes (46, 47) and the specific activity was highest in a membrane fraction intermediate in density between that of mitochondria and microsomes, which the authors concluded was outer mitochondrial membranes (47). Cholinephosphotransferase was also found in rat lung mitochondria (48) although the authors stated that "the basis for the unanticipated sedimentation of a considerable proportion of activities of enzymes normally recognized as endoplasmic reticulum constituents with the mitochondria remains unresolved." Other reports (1, 3, 49), however, have suggested that all cholinephosphotransferase activity in mitochondria is due to microsomal contamination. One crucial requirement in concluding that this enzyme is present in the mitochondria is that there should be accurate information about the degree of cross-contamination of the mitochondria by the endoplasmic reticulum.

PtdSer synthase is also primarily a microsomal enzyme (1, 3, 4), although PtdSer biosynthesis has been reported in mitochondria of Morris 7777 hepatoma cells at levels that could not be accounted for by contamination by microsomes, as measured by the activity of NADPH:cytochrome c reductase (50). Similarly, the Ca2+-dependent PtdSer synthase was detected in mitochondria of Tetrahymena pyriformis (51), and in mitochondria of Ehrlich ascites tumor cells (52).

A possible explanation for the inconsistent findings of cholinephosphotransferase and PtdSer synthase in mitochondria of animal cells is that these enzymes are not present in mitochondrial membranes but that the activities are due to contamination by other membranes tightly associated with the mitochondria, possibly fraction X, that do not have all the properties of microsomes. In the experiments reported in the present study, cholinephosphotransferase and PtdSer synthase activities in mitochondria can be attributed to fraction X membranes which are present only in the crude, but not in the highly purified, mitochondria.

**Evidence from Other Studies for an Endoplasmic Reticulum Subfraction Associated with Mitochondria**—The present data
support the concept that fraction X is a domain of the endoplasmic reticulum which is linked, perhaps through regions of membrane continuity, with the mitochondria when originally isolated, but that after Percoll centrifugation, the two types of membrane become separated from one another. Several previous reports support the idea of regions of contact between the endoplasmic reticulum and mitochondria. For example, the structural relationship between mitochondria and endoplasmic reticulum of rat and quail liver was examined in both biochemical and light and electron microscopy studies (53). Electron microscopy revealed that the mitochondria were held together by strands of endoplasmic reticulum which were continuous with the mitochondrial outer membrane. The authors found that approximately 30–40% of the total glucose-6-phosphate phosphatase activity of the liver homogenate was present in the mitochondrial fraction. On decreasing the ionic strength, the mitochondria were dissociated from the endoplasmic reticulum, and the latter membranes were isolated at a density lower than that of mitochondria (53).

In another study, a complex of endoplasmic reticulum closely associated with mitochondria was isolated from a rat liver homogenate (54). The isolated complex was dissociated by the addition of 0.6 M EDTA. The endoplasmic reticulum component had some properties of fraction X, for example the glucose-6-phosphate phosphatase activity was higher than in microsomes isolated from the postmitochondrial supernatant. In addition, a rapidly sedimenting endoplasmic reticulum fraction, that contained a high activity of glucose-6-phosphate phosphatase and that was associated with mitochondria, was isolated from rat liver (55). In contrast to classical microsomes, which were present in the form of vesicles, the rapidly sedimenting endoplasmic reticulum retained a structure closely resembling the endoplasmic reticulum of intact rat liver, and was not disrupted to form vesicles.

Several other investigations using microscopy have suggested a continuity between the mitochondrial outer membrane and endoplasmic reticulum in rat liver (56, 57) and cultured rat hepatocytes (58). Previously, many of these reports have been dismissed as being artifactual. Fraction X may be a previously uncharacterized region of the endoplasmic reticulum since it has many similar properties, but is not identical, to rough and smooth endoplasmic reticulum or total microsomes. Indeed, both rough and smooth endoplasmic reticulum membranes are probably heterogeneous. When rat liver smooth microsomes were subfractionated on a sucrose gradient (59) the enzymes glucose-6-phosphate phosphatase and NADPH-cytochrome c reductase were differentially distributed over the fractions of the gradient, suggesting that the smooth endoplasmic reticulum had a heterogeneous composition. More recently, Gierow and Jergil (61) subfractionated smooth endoplasmic reticulum from rat liver by counter-current distribution and obtained at least five distinct fractions which differed in their ratio of endoplasmic reticulum marker enzymes. Interestingly, glucose-6-phosphate phosphatase distributed in one manner and NADPH-cytochrome c reductase in another.

Potential Role of Fraction X—One objective of the present study was to propose a mechanism that would explain the compartmentalization of pools of PtdEtn and PtdCho derived from PtdSer (12, 14–16). If, as is suggested by the current work, fraction X membranes are intimately associated with mitochondria, this would provide a mechanism for the linked synthesis of PtdSer, PtdEtn, and PtdCho. Thus, pools of PtdSer- and PtdSer-derived phospholipids might be segregated from the bulk of the phospholipids of the endoplasmic reticulum. A potential destination of these pools of serine-derived phospholipids may be secretion (12). Preliminary evidence supporting the role of fraction X and mitochondria in providing phospholipids for lipoprotein secretion is the finding of apoB (both large and small forms), apoE, and apoC in fraction X membranes at similar levels to those apoproteins in microsomes. Moreover, the activity of glucose-6-phosphate phosphatase in fraction X was approximately twice as high as in microsomes. This finding would support a role of fraction X in the secretory process, since the function of glucose-6-phosphate phosphatase in the endoplasmic reticulum is to provide glucose for secretion.

Thus, evidence is accumulating that there may be a subfraction of the endoplasmic reticulum that is specifically associated with mitochondria. Fraction X may be this endoplasmic reticulum fraction, and the close physical association between the two types of membranes may be important for the transfer of lipid to and from the mitochondria. Such a juxtaposition may result in the generation of specific pools of phospholipids that are distinct from those in the bulk of the endoplasmic reticulum.

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