Newly Made Phosphatidylserine and Phosphatidylethanolamine Are Preferentially Translocated between Rat Liver Mitochondria and Endoplasmic Reticulum*

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The translocation of: (i) phosphatidylserine (PtdSer) from its site of synthesis on microsomal membranes to its site of decarboxylation in mitochondrial membranes and (ii) phosphatidylethanolamine (PtdEtn) from the mitochondria to its site of methylation to phosphatidylycholine on microsomal membranes has been reconstituted in cell-free systems consisting of rat liver mitochondria and microsomes. Two types of systems have been reconstituted. In one, the translocation of newly made PtdSer or PtdEtn was examined by incubation of microsomes and mitochondria with [3-3H]serine. In the other, membranes were prelabeled with radioactive PtdSer or PtdEtn, and the transfer of these two lipids between mitochondria and microsomes was monitored. For the transfer of both PtdSer from microsomes to mitochondria and PtdEtn from mitochondria to microsomes, newly made phospholipids were translocated much more readily than pre-existing phospholipids. The data suggest that with respect to their translocation between these two organelles, the pools of newly synthesized PtdSer and PtdEtn were distinct from the pools of "older" phospholipids pre-existing in the membranes.

Transfer of neither phospholipid in vitro depended on the presence of cytosolic proteins (i.e. soluble phospholipid transfer proteins) or on the hydrolysis of ATP, although there was some stimulation of PtdSer transfer by ATP and several other nucleoside mono-, di-, and triphosphates. The data are consistent with a collision-based mechanism in which the endoplasmic reticulum and mitochondria come into contact with one another, thereby effecting the transfer of phospholipids. The proposal that there is contact between the endoplasmic reticulum and mitochondria is supported by the recent isolation of a membrane fraction having many, but not all, of the properties of the endoplasmic reticulum, but which was isolated in association with mitochondria (Vance, J. E. (1990) J. Biol. Chem. 265, 7248–7256).

Although the mechanism of targeting and assembly of proteins into eukaryotic cell membranes is becoming well understood at the molecular level, our understanding of how lipids are assembled into membranes remains limited. Moreover, how the asymmetrical distribution of phospholipids in different subcellular membranes and within the two leaflets of the membrane bilayer is established and maintained is not clear. The majority of phospholipids must be transported from their sites of synthesis on the cytosolic surfaces of the endoplasmic reticulum (1, 2) and Golgi (3) membranes to other subcellular organelles such as the mitochondria, nucleus, peroxisomes, lysosomes, and plasma membrane. It seems unlikely that most phospholipids are sufficiently water-soluble that they can flow unaided through the aqueous milieu between membranes. Several plausible mechanisms have been proposed for the interorganelle trafficking of lipids, some of which are supported by experimental evidence (4). For example, lipids may be transported intracellularly via mediator proteins, the so-called phospholipid exchange/transfer proteins. Alternatively, or in addition, phospholipids may move within cells via a vesicle-mediated transfer whereby vesicles bud from the donor membrane and fuse with the acceptor membrane, resulting in the net transfer of lipid. There is also some evidence, mostly morphological, that flow of membrane lipid may occur as a result of physical contact and/or fusion between two membranes in close juxtaposition with one another. Perhaps all three of these mechanisms occur, but the relative importance of each for net membrane biogenesis has not yet been determined.

Although the majority of membrane phospholipid is synthesized on the endoplasmic reticulum, it is becoming apparent that a significant proportion of PtdEtn\(^1\) (>80% in some cell types (5, 6)) is synthesized on the outer aspect of the inner mitochondrial membranes via the action of PtdSer decarboxylase (7, 8). In animal cells, PtdEtn can also be synthesized from ethanolamine via the CDP-ethanolamine pathway (9). Since most PtdSer is made on endoplasmic reticulum membranes (3, 10), the formation of PtdEtn from PtdSer requires the obligatory transport of PtdSer from these membranes to the mitochondria. The mechanism of translocation and decarboxylation of PtdSer has recently been studied in three systems: in baby hamster kidney cells (11), in a reconstituted system consisting of rat liver microsomes and mitochondria (12), and in permeabilized Chinese hamster ovary cells (13). These studies suggested that PtdSer translocation from the endoplasmic reticulum to the mitochondria occurred in the absence of cytosolic proteins. In addition, ATP stimulated the transfer in intact and permeabilized cells, but not in the reconstituted system of isolated microsomes plus mitochondria. The data were consistent with a collision-

\(^1\) The abbreviations used are: PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; PtdCho, phosphatidylcholine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylendiaminetetraacetic acid]; App(NH)\(_2\), adenylyl-5'-yl imidodisphosphate.
based mechanism for the import of PtdSer into mitochondria. In liver, the methylation of PtdEtn apparently provides ~20% of the PtdCho, the remainder being synthesized from the CDP-choline pathway (14). The enzyme catalyzing the three successive methylations of PtdEtn is the endoplasmic reticulum enzyme PtdEtn N-methyltransferase (15); the mitochondrial contains essentially no PtdEtn methyltransferase activity (3). If PtdSer-derived PtdEtn were to be methylated to PtdCho, translocation of PtdEtn from the mitochondria to the endoplasmic reticulum would be required. The mechanism of this movement of PtdEtn has not previously been investigated in hepatocytes, although a study in yeast suggested that the transport of PtdEtn from the mitochondria to the endoplasmic reticulum is dependent on metabolic energy (16).

In this study, an *in vitro* reconstituted system of rat liver mitochondria and microsomes has been developed in which the concerted synthesis of PtdSer, PtdEtn, and PtdCho occurs (Fig. 1). The sequence of reactions involves two interorganelle translocation steps in which PtdSer is transferred from the microsomes to the mitochondria, and PtdEtn is transferred in the reverse direction: from the mitochondria to the endoplasmic reticulum. The results of the study demonstrate that newly synthesized PtdSer is transferred from the microsomes to the mitochondria and decarboxylated more readily than is pre-existing PtdSer. Similarly, the data suggest that newly made, rather than "old," PtdEtn is prefered for translocation from the mitochondria to the endoplasmic reticulum. In addition, the transport of PtdEtn from the endoplasmic reticulum to the mitochondria and of PtdEtn from the mitochondria to the endoplasmic reticulum can occur in the absence of cytosolic phospholipid exchange proteins.

**MATERIALS AND METHODS**

Chemicals—Boehringer Mannheim supplied NADPH and S-adenosylmethionine. Cytochrome c (type III), phospholipase C (type IX from Clostridium welchii), all nucleotides, glucose 6-phosphate, dithiothreitol, and serine were purchased from Sigma. [3-3H]Serine (specific activity of 23 Ci/mmol) and [1-3H]ethanolamine (specific activity of 19 Ci/mmol) were obtained from Amerham Corp. Thin-layer chromatography plates (Silica Gel G 60, 0.25-mm thickness) were purchased from BDH Chemicals. Percoll was from Pharmacia LKB Biotechnology Inc. The standard phospholipids, PtdSer, PtdEtn, and PtdCho were from Avanti Polar Lipids, Inc. (Birmingham, AL). All other chemicals were from Sigma or Fisher.

**Preparation of Subcellular Membrane Fractions from Rat Liver—** Female Sprague-Dawley rats (~200 g) were provided with standard diet and drinking water *ad libitum*, but were fasted overnight before being killed by decapitation. Livers were quickly removed and immersed in ice-cold homogenization medium (250 mM mannitol, 5 mM HEPES, pH 7.4, 0.5 mM EGTA, 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 600 × g for 5 min to remove large debris and nuclei. The supernatant was centrifuged for 10 min at 10,000 × g to pellet the crude mitochondrial fraction. The resultant mitochondrial fraction was centrifuged at 100,000 × g for 1 h in a Beckman Ti-70 rotor to pellet the microsomes. The supernatant from this centrifugation was designated as cytosol. The mitochondria were further purified by centrifugation of the mitochondrial pellet by hand homogenization in ~4 ml of homogenization medium. The supernatant was centrifuged at 24,000 × g for 10 min, and the mitochondria were resuspended in 2 ml of buffer R and centrifuged at 100,000 × g for 1 h. The mitochondrial pellet was washed twice in homogenization medium at 70 °C and centrifuged at 100,000 × g for 1 h. The resulting mitochondrial pellet was resuspended in homogenization medium and stored at ~70 °C. Membrane fractions were examined for the following marker enzyme activities by standard procedures: NADPH-cytochrome c reductase (endoplasmic reticulum) (17), glucose-6-phosphate dehydrogenase (endoplasmic reticulum) (18), and cytochrome c oxidase (mitochondria) (19). Phosphatidylserine synthase (exchange enzyme) was assayed as described (19).

**Preparation of Radiolabeled Subcellular Membrane Fractions—** Microsomes containing [3H]serine-labeled PtdSer were prepared by incubation of microsomes (50 μg of protein), prepared as described above, with [3H]serine (2.0 mM, 44 Ci/mmol) for 1 h at 37 °C in 3 ml of 25 mM HEPES, pH 7.4, containing 10 mM CaCl₂, after which the sample was diluted with 10 ml of buffer R (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, and 0.1 mM phenylmethylsulfonyl fluoride). The microsomes were centrifuged at 100,000 × g for 45 min at 37 °C, 70.1 rotor, and the pellet was washed twice with buffer R. The radiolabeled microsomal pellet (typically containing 0.5–1.0 × 10⁶ dpm in PtdSer/mg of protein) was resuspended in buffer R and frozen at ~70 °C for future use.

Mitochondria labeled with [3H]PtdEtn were prepared by injection of either [1-3H]ethanolamine (300 μCi) or [3-3H]serine (500 μCi) into the portal vein of a rat. After 10 min, the liver was rapidly removed and placed on ice. Mitochondria, labeled with either [1-3H]ethanolamine or [3-3H]serine, were immediately isolated as described above, and the incorporation of label into the phospholipids was determined. From the labeling injection of [3H]ethanolamine ( typically 0.5–1.0 × 10⁶ dpm/mg of protein, whereas there was only a small amount of label in PtdCho (25 × 10⁶ dpm/mg of protein). From the labeling with [3H]serine, PtdSer, PtdEtn, and PtdCho contained 0.28 × 10⁶, 2.72 × 10⁶, and 1.68 × 10⁶ dpm/mg of protein in the head group moieties, respectively.

**In Vitro Incubations of Isolated Mitochondria and Microsomes—** Two alternative systems of reconstitution of lipid translocation were used. In one system, isolated microsomes (typically 100 μg of protein) and mitochondria (typically 500 μg of protein) were incubated at 37 °C in a total volume of 200 μl with buffer A (25 mM HEPES, pH 7.4, 0.2 mM dithiothreitol, 0.2 mM S-adenosylmethionine, and 10 mM CaCl₂) containing 1–5 μCi of [3H]serine (0.4 mCi). In some translocation experiments, 0.3 mM sucrose was included in buffer A with identical results. In the alternative reconstituted system, microsomes, preslabeled as described above with [3H]PtdEtn (typically 100 μg of protein, 1.6 × 10⁶ dpm), were incubated with mitochondria (typically 500 μg of protein) in buffer A in a total volume of 200 μl at 37 °C. Both systems of incubation were terminated by the addition of 5 ml of chloroform/methanol (2:1, v/v) and 1 ml of water. The lipids were extracted; and PtdSer, PtdEtn, and PtdCho were isolated by thin-layer chromatography in the solvent system chloroform/methanol/acetone/formic acid/water (70:30:12:4:2, v/v). The phospholipids were visualized under ultraviolet light after the plates had been sprayed with Primulin (21). The bands corresponding to PtdSer, PtdEtn, and PtdCho were scraped from the plates into scintillation vials, and radioactivity was counted. Phospholipase C digestion (22) and acetic acid/formic acid/water (7:5:2, v/v) was used to hydrolyze the phospholipid head groups.
Analysis of Water-soluble Metabolites of PtdSer and PtdEtn—Thin-layer chromatography of water-soluble products of the [3H]PtdSer membrane incubations was performed in the solvent system 28% NH$_4$OH, methanol, 0.6% NaCl (1:10:10, v/v). The plate was sprayed with 0.25% ninhydrin in acetone, and the metabolites were visualized after the plate had been heated to 100 °C. In this solvent system, ethanolamine, phosphoethanolamine, phosphoserine, glycerophosphoserine, and CDP-ethanolamine were well separated. However, glycerophosphoethanolamine and serine cochromatographed. The spot containing these latter two compounds was scraped from the plate and subjected to acid hydrolysis in 1 N HCl for 16 h at 100 °C. The HCl was neutralized by the addition of solid AgCO$_3$ until the evolution of CO$_2$ ceased. The precipitate was removed by filtration, and the supernatant was evaporated to dryness. The product was redissolved in a small volume of methanol/H$_2$O (1:1, v/v) and subjected to thin-layer chromatography in the solvent system 28% NH$_4$OH, methanol, 0.6% NaCl (1:10:10, v/v). Radioactivity in serine and ethanolamine (derived from glycerophosphoethanolamine) was measured.

RESULTS

Reconstitution of PtdSer Transport from Microsomes to Mitochondria and Decarboxylation therein—In an attempt to reconstitute the movement of PtdSer from the endoplasmic reticulum to the mitochondria, purified mitochondria and microsomes were isolated from rat liver. Contamination of the mitochondria by the endoplasmic reticulum was 3.2 ± 1.3% according to the endoplasmic reticulum marker enzyme NADPH-cytochrome c reductase and 15.2 ± 2.7% according to the endoplasmic reticulum marker enzyme glucose-6-phosphate phosphatase. Contamination of microsomes by mitochondrial membranes was 9.0 ± 2.7% according to the mitochondrial marker enzyme cytochrome c oxidase. These membranes (typically 100 μg of microsomal protein and 500 μg of mitochondrial protein) were mixed and incubated with [3-3H]serine (0.4 mM, 2 μCi) for up to 1 h at 37 °C in buffer A. The products of the reaction were extracted with chloroform/methanol (2:1, v/v), and PtdSer and PtdEtn were isolated by thin-layer chromatography. The incorporation of radioactivity into PtdSer and PtdEtn was shown in Fig. 2A. [3H]Serine was rapidly incorporated into PtdSer, presumably via PtdSer synthase (exchange enzyme), which is present in microsomal membranes (3). After a 20-min incubation, the radioactivity in PtdSer declined gradually with a concomitant increase in radioactivity in PtdEtn. Phospholipase C digestion of PtdEtn (22) confirmed that >98% of the radioactivity in PtdEtn resided in the ethanolamine moiety, with essentially no radioactivity in the diacylglycerol portion of the molecule. After a 1-h incubation, the distribution of radioactivity between PtdSer and PtdEtn was 64.1% in PtdEtn and 35.9% in PtdSer. There was also some radioactivity in PtdCho (see below). Thus, apparently PtdSer had been synthesized in microsomal membranes and had been efficiently translocated from the endoplasmic reticulum to the mitochondria, where the PtdSer was decarboxylated to PtdEtn. The extent of formation of PtdSer, PtdEtn, and PtdCho was identical whether or not 0.3 M sucrose was included in the incubation mixture. Thus, the rapid translocation of newly made PtdSer (and PtdEtn) was not dependent upon the low osmolality of the incubation media. The formation of PtdEtn increased with time (Fig. 2A) and was dependent upon the amount of mitochondrial membranes (Fig. 2B). Since PtdSer synthase activity requires the presence of Ca$^{2+}$ (optimum Ca$^{2+}$ concentration is 10 mM (3)), the formation of PtdSer (and also of PtdEtn) depended upon the presence of Ca$^{2+}$ in the incubation.

A second reconstitution system for PtdSer transfer from the endoplasmic reticulum to the mitochondria and subsequent decarboxylation to PtdEtn was developed. Microsomes were isolated from rat liver and incubated with [3-3H]serine for 1 h. The membranes, which were labeled with [3H]PtdSer, were resiolated by centrifugation and washed free of serine. Greater than 95% of the radiolabeled lipid was PtdSer, with only a small amount of labeled PtdEtn. Since contamination of the microsomes with mitochondrial membranes was estimated to be 9.0% according to the mitochondrial marker enzyme cytochrome c oxidase, the small amount of labeled PtdEtn could be accounted for by PtdSer decarboxylase activity present in contaminating microsomal membranes. The [3H]PtdSer-labeled microsomal membranes were incubated with purified mitochondria at 37 °C under the same conditions as those used for the experiment depicted in Fig. 2, except that [3H]serine was omitted, but the incubation contained 0.4 mM unlabeled serine. The radioactivity in PtdSer and PtdEtn after various incubation times is shown in Fig. 3 (A and B). Although the formation of [3H]PtdEtn increased with time of incubation (Fig. 3, A and B) and there was a decrease in radioactivity in PtdSer (Fig. 3A), after a 1-h incubation, only 15% of the disintegrations/minute were in PtdEtn, whereas 85% were in PtdSer. The extent of conversion of microsomal PtdSer into PtdEtn by the mitochondria was therefore markedly less than for the reconstitution experiment shown in Fig. 2A. Thus, the transfer of PtdSer to and the decarboxylation of PtdSer in the mitochondria were far more efficient for newly synthesized PtdSer than for PtdSer that was already present in the microsomal membranes.

Although the incorporation of prelabeled microsomal [3H] PtdSer into PtdEtn was stimulated slightly by increased amounts of mitochondrial protein (Fig. 3C), the dependence of PtdEtn formation on mitochondrial concentration was not as great as for the experiment in which newly synthesized PtdSer was converted into PtdEtn (Fig. 2B).
apparent discrepancy as the disintegrations/minute in PtdCho from such an incubation were only ~10% of the total. As a means of distinguishing between possibilities i and ii, water-soluble metabolites of the incubation were analyzed by thin-layer chromatography. After a 1-h incubation, there was very little radioactivity in ethanolamine, phosphoethanolamine, glycerophosphoserine, or phosphoserine; but after a 1-h incubation, ~30,000 dpm were in a spot on the silica gel corresponding to either serine or glycerophosphoethanolamine, which cochromatographed. To determine in which compound the radioactivity resided, the mixture was subjected to acid hydrolysis to convert glycerophosphoethanolamine to ethanolamine, and the product was rechromatographed in the same solvent system. Acid hydrolysis, under the conditions used, quantitatively converted glycerophosphoethanolamine into ethanolamine. Essentially all the radioactivity was present in serine, whereas ethanolamine was unlabeled. Thus, the disappearance of radioactivity from PtdSer which was not accounted for by the disintegrations/minute in PtdEtn (Fig. 3A) could be accounted for by the degradation of PtdSer to serine, with a small amount of PtdEtn being converted into PtdCho.

**Effect of Cytosolic Protein on PtdSer Translocation and Decarboxylation**—One proposed mechanism for interorganelle movement of phospholipids involves carrier proteins (the phospholipid exchange/transfer proteins), several of which are known to be present in the cytosol and have been characterized (23). At least one of these proteins (24) is capable of transferring PtdSer between liposomes and biological membranes. Thus, the effect of the addition of cytosol (the source of soluble phospholipid exchange proteins) on the translocation and decarboxylation of newly synthesized and pre-existing PtdSer was examined. In the first experiment, microsomes and mitochondria were mixed with [3-3H]serine in the presence of different amounts of cytosolic protein (up to 1.0 mg/200 μl of incubation mixture). The incorporation of H into PtdSer and PtdEtn was monitored. The addition of cytosol did not increase the formation of 3H-labeled PtdEtn (data not shown). In the second experiment, purified mitochondria were incubated in the presence of different amounts of cytosolic protein and microsomal membranes that had been prelabeled with [3H]PtdSer. Cytosol did not stimulate the formation of labeled PtdEtn from pre-existing [3H]PtdSer of microsomal membranes (data not shown). Apparently, PtdSer movement from microsomes to mitochondria and the decarboxylation of PtdSer to PtdEtn proceeded equally well in the presence or absence of cytosolic protein.

**Effect of Nucleoside Phosphates on PtdSer Translocation and Decarboxylation**—One alternative to protein-mediated phospholipid transfer between the endoplasmic reticulum and mitochondria is a vesicle-mediated transport mechanism in which vesicles containing phospholipids bud from the donor membrane and fuse with the acceptor membrane, thereby resulting in the net transfer of lipid from one membrane to the other. Such a membrane budding process would likely be dependent upon a supply of energy. The effect of exogenous ATP (0–2.0 mM) on the concerted synthesis of PtdSer and PtdEtn from [3-3H]serine is shown in Fig. 4A. ATP, at a physiological concentration (2.0 mM) (26), stimulated the conversion of PtdSer to PtdEtn by ~2-fold, compared to the reaction in the absence of exogenously added ATP. When microsomes prelabeled with [3H]PtdSer were incubated with mitochondria, the conversion of PtdSer to PtdEtn was not altered by the addition of exogenous ATP (Fig. 4B). Consequently, the transfer and decarboxylation of de novo synthesized PtdSer were stimulated by ATP, whereas the formation...
of PtdEtn from pre-existing PtdSer was unaffected by ATP.

The nucleoside specificity of the stimulation of the conversion of PtdSer to PtdEtn was examined. Several nucleoside mono-, di-, or triphosphates were incubated with the two types of reconstituted systems (i.e. using either newly synthesized (Table I) or preformed (Table II) PtdSer). The concentration of all nucleotides was 2.0 mM, except that GTP was tested at both 2.0 and 0.2 mM. The physiological concentration of ATP in hepatocytes/cultured cells is ~2.0 mM (25), whereas other nucleotides are present at much lower concentrations, probably one-tenth that of ATP (26). All nucleotides examined stimulated the concerted synthesis of PtdEtn from [3H]serine, compared with the reaction in the absence of nucleotides, and many were more potent activators than was ATP (Table I). Surprisingly, UDP (2.0 mM) had the greatest effect, increasing PtdEtn formation from PtdSer by more than three times as much as did ATP. The nonhydrolyzable ATP analog App(NH)p also stimulated the reaction but to a smaller degree than did ATP (Table I).

The effects of the nucleotides on the conversion of premade [3H]PtdSer to PtdEtn (Table II) were less than for the synthesis of PtdEtn from newly made PtdSer, although UDP caused the greatest stimulation.

Reconstitution of Transfer of Newly Synthesized PtdEtn from Mitochondria to Microsomes and Methylation to PtdCho—The enzyme PtdEtn N-methyltransferase is primarily located in the endoplasmic reticulum (3). There is virtually no enzymatic activity for PtdEtn methylation in purified mitochondria (3). When PtdSer-derived PtdEtn is methylated to PtdCho, an obligatory transfer of PtdEtn from the mitochondria to the endoplasmic reticulum must occur (see Fig. 1). Reconstitution of the transfer of mitochondrial PtdEtn to microsomes was examined in experiments similar to those described above for PtdSer transfer. Purified mitochondria and microsomes were mixed and incubated with [3-3H]serine (0.4 mM, 2 μCi) at 37°C in buffer A. After various times of incubation, PtdEtn and PtdCho were extracted and purified by thin-layer chromatography. Transfer of PtdSer-derived PtdEtn from mitochondria to microsomes occurred because the formation of [3H]PtdCho was observed (Fig. 5A). With increasing time, there was a steady increase in formation of labeled PtdCho (and PtdEtn). After 1 h the distribution of radioactivity between PtdEtn and PtdCho was 68.7% in PtdEtn and 31.3% in PtdCho. Previous studies (22) have established that in cultured rat hepatocytes, [3-3H]serine is incorporated into both the polar head group and the diacylglycerol portions of PtdCho. That the incorporation of [3H]serine to PtdCho was virtually identical in both mitochondria and mitochondria (diacylglycerol) moiety was confirmed by phospholipase C degradation (22); >98% of the radioactivity in PtdCho was in the choline head group moiety. Since decarboxylation of PtdSer to PtdEtn occurs only in mitochondria and PtdEtn methyltransferase activity is restricted to microsomes, the formation of labeled PtdCho implied that the sequential transport of PtdSer from microsomes to mitochondria and of PtdEtn from pre-existing PtdSer was unaffected by ATP.

A. Microsomes (100 μg of protein) and mitochondria (500 μg of protein) were incubated for 30 min with [3-3H]serine in the presence of various concentrations of ATP (0–2.0 mM). Incorporation of radioactivity into PtdSer (6) and PtdEtn (7) was measured. B. Microsomes (100 μg of protein) in which PtdSer had been prelabeled with [3-3H]serine were mixed with mitochondria (500 μg of protein) and incubated for 30 min with various concentrations of ATP. The incorporation of radioactivity into PtdSer (6) and PtdEtn (7) was measured.

**TABLE I**

Effect of nucleoside phosphates on formation of PtdSer, PtdEtn, and PtdCho from [3H]serine

| Nucleotide added | PtdSer | PtdEtn | PtdCho |
|------------------|--------|--------|--------|
| None             | 18.7 ± 2.6 | 11.1 ± 1.2 | 6.9 ± 0.3 |
| ATP              | 16.3 ± 0.9 | 16.6 ± 0.6 | 7.5 ± 0.2 |
| GTP (2.0 mM)     | 24.0 ± 4.4 | 19.9 ± 1.9 | 6.3 ± 0.6 |
| GTP (0.2 mM)     | 17.9 ± 2.4 | 12.5 ± 1.7 | 6.2 ± 0.7 |
| UTP              | 18.4 ± 1.7 | 22.8 ± 1.9 | 4.9 ± 0.5 |
| CTP              | 22.6 ± 0.1 | 12.8 ± 1.6 | 5.8 ± 0.5 |
| ADP              | 21.6 ± 1.6 | 22.8 ± 0.9 | 5.9 ± 0.6 |
| GDP              | 14.9 ± 1.2 | 25.4 ± 1.2 | 7.2 ± 1.3 |
| UDP              | 13.5 ± 1.9 | 41.5 ± 2.7 | 9.0 ± 0.2 |
| CDP              | 15.9 ± 1.5 | 15.6 ± 2.7 | 6.8 ± 0.5 |
| CMP              | 12.0 ± 0.9 | 14.0 ± 1.4 | 5.6 ± 0.6 |
| AMP              | 18.7 ± 2.8 | 11.3 ± 0.3 | 6.6 ± 1.5 |
| App(NH)P         | 22.3 ± 0.7 | 14.1 ± 2.0 | 7.5 ± 0.5 |

**TABLE II**

Effect of nucleoside phosphates on formation of PtdEtn and PtdCho from prelabeled [3H]PtdSer

| Nucleotide added | PtdSer | PtdEtn | PtdCho |
|------------------|--------|--------|--------|
| None             | 26.1 ± 2.1 | 2.4 ± 0.1 | 2.7 ± 0.2 |
| ATP              | 24.9 ± 2.8 | 2.7 ± 0.1 | 2.8 ± 0.6 |
| GTP (2.0 mM)     | 23.3 ± 0.4 | 3.2 ± 0.2 | 2.9 ± 0.2 |
| GTP (0.2 mM)     | 27.3 ± 1.6 | 2.2 ± 0.1 | 2.7 ± 0.2 |
| UTP              | 21.1 ± 4.7 | 3.5 ± 0.1 | 2.5 ± 0.2 |
| CTP              | 28.3 ± 3.7 | 2.4 ± 0.1 | 2.4 ± 0.2 |
| ADP              | 22.7 ± 2.5 | 3.0 ± 0.2 | 2.1 ± 0.1 |
| GDP              | 21.6 ± 2.8 | 2.7 ± 0.1 | 2.8 ± 0.4 |
| UDP              | 17.2 ± 2.3 | 4.4 ± 0.5 | 2.1 ± 0.1 |
| CDP              | 22.8 ± 4.0 | 2.3 ± 0.2 | 2.0 ± 0.6 |
| CMP              | 17.9 ± 2.3 | 2.1 ± 0.2 | 2.7 ± 0.1 |
| AMP              | 25.5 ± 1.1 | 2.1 ± 0.2 | 2.7 ± 0.1 |

S.D. of three measurements.
PtdEtn from mitochondria to microsomes had occurred.

In separate experiments, PtdSer-labeled microsomes were incubated with unlabeled mitochondria, and the conversion of PtdSer to PtdEtn was measured. In this type of reconstituted system with prelabeled PtdSer, although the conversion of PtdSer to PtdEtn (Fig. 3, A and B) was much less efficient than for the concerted synthesis of PtdSer and PtdEtn from [3H]serine (Fig. 2A), some conversion of PtdSer to PtdEtn did occur. Fig. 5B demonstrates that PtdEtn, newly made from pre-existing PtdSer, was rapidly converted into PtdCho, presumably by the microsomal PtdEtn methyltransferase. After 1 h, the distribution of radioactivity between PtdEtn and PtdCho was 57.7% in PtdEtn and 42.3% in PtdCho.

The translocation of PtdSer-derived PtdEtn and its methylation to PtdCho were not influenced by the addition of cytosolic protein (up to 1 mg) to the incubation mixture. Although 1 mM ATP stimulated PtdCho formation from PtdEtn (by ~70%) compared to incubation in the absence of ATP, the stimulation most likely reflected the increased production of the substrate, PtdEtn. Eleven other nucleotides, individually added to the incubation mixture, had effects similar to those of ATP on PtdCho formation from PtdEtn (Tables I and II). Any apparent stimulation was probably due to the increased supply of PtdEtn. Thus, neither exogenous ATP nor the other nucleotides tested significantly stimulated the translocation and methylation of PtdEtn.

Reconstitution of PtdEtn Transport and Methylation to PtdCho Using Mitochondrial Membranes Prelabeled with Ethanolamine-derived PtdEtn—Since the transport of PtdSer from microsomes to mitochondria occurred more readily with nascent PtdSer than with pre-existing membrane PtdSer, the difference between the transport/methylation of newly made versus preformed mitochondrial PtdEtn also was assessed. A rat was injected intraportally with 300 μCi of [1-3H]ethanolamine. After 10 min, the liver was removed, and mitochondria were isolated. Both PtdEtn and PtdCho from the mitochondrial membranes were labeled with [3H]ethanolamine. However, the majority of label was in PtdEtn (505.1 × 10^3 dpm/mg of protein); a small amount of label was in PtdCho (24.7 × 10^3 dpm/mg of protein). As a measure of the transfer of pre-existing mitochondrial PtdEtn to microsomes and methylation to PtdCho, mitochondria containing the ethanolamine-labeled PtdEtn were incubated with unlabeled microsomes in buffer A. Essentially no conversion of PtdEtn to labeled PtdCho was observed (Fig. 6). This result is in contrast to the experiments depicted in Fig. 5 (A and B), in which newly made PtdEtn was efficiently methylated to PtdCho. The formation of [3H]PtdCho was not stimulated by the addition of cytosol (up to 1.0 mg of protein) or ATP (2.0 mM) or by increasing the pH of the incubation to pH 9.2, the pH optimum of PtdEtn methyltransferase (15). Thus, nascent PtdEtn was efficiently translocated and methylated to PtdCho, whereas old PtdEtn, already present in mitochondrial membranes, was not.

A possible explanation for the different results obtained from the two experiments shown in Figs. 5 and 6 may be that different biosynthetically derived pools of PtdEtn were used for the two types of experiments. In the experiment with prelabeled mitochondrial PtdEtn (Fig. 6), the PtdEtn was made from [3H]ethanolamine presumably via the CDP-ethanolamine pathway. In the experiments in which the coordinated synthesis of PtdEtn and PtdCho was observed (Fig. 5, A and B), the PtdEtn was made from [3H]PtdSer. Possibly, PtdEtn from the two different biosynthetic origins behaved differently in its transport from mitochondria to microsomes. Hence, mitochondrial membranes were labeled with PtdSer-derived PtdEtn by injection of [3H]serine (500 μCi) into the portal vein of a rat. After 10 min, the mitochondria containing PtdSer, PtdEtn, and PtdCho labeled with [3H]serine were isolated. The labeled mitochondria were incubated with unlabeled microsomes for various times up to 1 h, and the PtdEtn and PtdCho produced were degraded with phospholipase C (22) so that incorporation of 3H into the ethanolamine and choline head group moieties was measured. After a 1-h incubation with microsomes, there was no measurable conversion of PtdSer-derived PtdEtn to PtdCho (Fig. 7). The addition of cytosol (up to 1.0 mg) or ATP (2.0 mM) had no effect on the conversion of prelabeled, serine-derived PtdEtn to PtdCho.

Calcium and other divalent cations have been shown to stimulate the enzyme PtdEtn N-methyltransferase; for ex-

Fig. 5. Transfer of serine-labeled PtdEtn from mitochondria to microsomes and methylation to PtdCho. A, microsomes (100 μg of protein) and mitochondria (500 μg of protein) were incubated in buffer A for the indicated times with [3-3H]serine, and the incorporation of radioactivity into PtdEtn (C) and PtdCho (D) was measured. B, microsomes (100 μg of protein) in which PtdSer had been prelabeled with [3-3H]serine were incubated with mitochondria (500 μg of protein). At the indicated times, PtdEtn (C) and PtdCho (D) were isolated, and radioactive incorporation was measured.

Fig. 6. Attempted in vitro reconstitution of translocation and methylation of mitochondrial PtdEtn labeled with [1-3H]ethanolamine. Mitochondria were labeled in vivo by injection of [1-3H]ethanolamine (300 μCi) into the portal vein of a rat. Labeled mitochondria were isolated, and 500 μg of mitochondrial protein was incubated with unlabeled microsomes (100 μg of protein) in buffer A. After various incubation times, the radioactivity in PtdEtn (C) and PtdCho (D) was determined.
The radioactivity in PtdSer, PtdEtn, and PtdCho was 0.38 µCi by injection of [3-3H]serine (500 µCi) into the portal vein of a rat. The radioactivity in PtdSer, PtdEtn, and PtdCho was 0.38 × 10^3, 2.72 × 10^3, and 1.68 × 10^3 dpm/mg of protein in the labeled, serolamine, and choline moieties, respectively. The labeled mitochondria (500 µg of protein) were incubated with unlabeled microsomes in buffer A. PtdEtn and PtdCho were isolated from the incubation mixture and purified by thin-layer chromatography. The lipids were eluted from the silica gel and hydrolyzed with phospholipase C as previously described (22). The incorporation of radioactivity into the ethanolamine and choline moieties of PtdCho (7) and PtdEtn (33) was determined.

In summary, isolated mitochondrial membranes containing PtdEtn prelabeled with either serine or ethanolamine did not provide PtdEtn for methylation to PtdCho in a reconstituted system with microsomes, whereas newly synthesized PtdEtn was apparently readily transported from mitochondria to microsomes and efficiently methylated to PtdCho.

**DISCUSSION**

Lipid movement within cells can be a very rapid process (27-29). For example, in Chinese hamster ovary cells, the half-time for transfer from 3H-serine to the plasma membrane has been estimated to be 2 min (27). Similarly, PtdEtn, formed from [3H]ethanolamine on the endoplasmic reticulum, reached the plasma membrane with a half-time of 10 min (28). It is possible that much of the phospholipid trafficking between the endoplasmic reticulum (the major site of phospholipid synthesis), the Golgi apparatus, and the plasma membrane is vesicle-mediated (29) and requires ATP (28, 29). However, the rate of interorganelle phospholipid movement appears to depend on the organelle, the type of phospholipid, and the biosynthetic origin of the phospholipid (30-33). In a study of baby hamster kidney cells, the half-time for transfer of PtdCho from the endoplasmic reticulum to the cytosolic, surface of the membrane bilayer and are consequently in a pool that is available for transfer to other membranes. In contrast, older molecules of these phospholipids may have rapidly undergone transbilayer movement to the inner, or luminal, surface of the bilayer and therefore may no longer be so readily accessible for intermembrane transport. An alternative explanation may be that the translocation of PtdSer and PtdEtn is linked to their synthesis. Simbeni et al. (38) have recently reported that in yeast, PtdSer and phosphatidylinositol translocation from the outer to the inner mitochondrial membrane is driven by synthesis. These authors also found that PtdEtn, newly synthesized in the inner membrane, was preferentially exported from the inner to the outer mitochondrial membranes without mixing with the pool of old PtdEtn in the inner membrane.

A possible explanation for the efficient in vitro transfer of PtdSer between microsomes and mitochondria could be that the high Ca^2+ concentration used in the incubations (10 mM) causes a fusion of the membranes and thus a direct and artificial transfer of PtdSer which would not occur under physiological conditions. A calcium concentration of 10 mM was found to be optimal for PtdSer synthesis (3). Therefore, this concentration was selected for studies on the concerted synthesis of PtdSer, PtdEtn, and PtdCho from serine, as depicted in Fig. 2. For meaningful comparison, 10 mM Ca^2+ was included even in experiments not involving de novo PtdSer synthesis, for example those experiments depicted in Fig. 3. Since there was a dramatically reduced transfer of pre-existing PtdSer, compared to newly made PtdSer, even in the presence of a high Ca^2+ concentration, a fusion event mediated by calcium is most likely not responsible for the efficient in vitro transfer of new PtdSer to the mitochondria.

In this study, contact of the membranes of the mitochondria and microsomes by collision alone is not sufficient for maximal phospholipid transfer. A unique property of newly made PtdSer and PtdEtn makes these phospholipids more competent than old phospholipids for translocation. Whether or not newly synthesized PtdSer and PtdEtn are preferentially translocated in vivo is not yet established. However, recent...
experiments suggest that newly made phospholipids may be required for assembly into lipoproteins (36, 37) and that newly made PtdEtn is preferred over old PtdEtn for methylation to PtdCho (37, 39).

Effect of Cytosolic Proteins and Nucleotides on PtdSer and PtdEtn Translocation—All the translocation reactions occurred equally well in the presence or absence of cytosolic proteins, suggesting that soluble phospholipid exchange/transfer proteins were not required either for the transfer of PtdSer from the microsomes to the mitochondria or for the transfer of PtdEtn from the mitochondria to the microsomes. This conclusion is in agreement with recent experiments of Voelker on PtdSer translocation using both a reconstituted rat liver membrane system (12) and permeabilized Chinese hamster ovary cells (13).

The synthesis of PtdEtn from [3-^3H]serine was stimulated 2-fold by a physiological concentration of ATP (2 mM). However, several other nucleotides, including a nonhydrolyzable ATP analog, stimulated the process at least as well as did ATP. Indeed, UDP was a particularly potent stimulator of the process. Thus, the effect of ATP appeared to be nonspecific and is probably not related to a requirement for hydrolysis of ATP as an energy source. Neither ATP nor the other nucleotides tested significantly stimulated the translocation and decarboxylation of preformed PtdSer. Moreover, none of the nucleotides, including ATP, stimulated the translocation/methylation of PtdEtn.

Recent studies in intact baby hamster kidney cells (11) and in permeabilized Chinese hamster ovary cells (13) showed that ATP was required for PtdSer translocation, although in the latter study, other nucleotides also stimulated the reaction to various degrees, depending on the nucleotide. On the other hand, in a study (12) using a reconstituted rat liver system with microsomal membranes prelabeled with PtdSer, the translocation of PtdSer from microsomes to mitochondria was not affected by ATP. One explanation proposed by Voelker (12) for the apparently conflicting data on the ATP requirement was that the translocation of PtdSer was a two-step process. ATP would be required in the first step to place microsomal PtdSer in a position that would allow formation of a collision complex between the endoplasmic reticulum and mitochondria; this step was by-passed in the reconstituted system, but was required in intact or permeabilized cells. The second step, which would not require ATP, would be the actual transfer of PtdSer to the mitochondria (12, 13). The results of those experiments (12, 13) agree well with this study.

Support for Collision-based Mechanism for PtdSer and PtdEtn Translocation between Mitochondria and Microsomes—The vesicle-mediated movement of proteins from the endoplasmic reticulum via the Golgi apparatus to the plasma membrane is well documented. Proteins targeted to the endoplasmic reticulum, Golgi apparatus, lysosomal and plasma membranes, and proteins destined for secretion are synthesized on ribosomes attached to endoplasmic reticulum membranes (40) and move through this pathway via vesicular transport. Recent calculations (29) have estimated that ~50% of the phospholipids of the endoplasmic reticulum may leave these membranes every 10 min and travel to the plasma membrane via the Golgi apparatus by a vesicle-mediated process. In contrast, proteins destined for mitochondria are synthesized on free ribosomes in the cytosol (41). There is no evidence that proteins travel to the mitochondria via vesicles. Movement of proteins to mitochondria apparently does not require the concomitant movement of lipids.

One attractive alternative mechanism for the movement of lipids between the endoplasmic reticulum and mitochondria is that soluble, cytosolic phospholipid exchange/transfer proteins could mediate the process. However, neither this study nor the recent studies of Voelker (12, 13) support such a mechanism. A similar conclusion was reached by Yaffe and Kennedy (31) in their investigation of the movement of phospholipids between the endoplasmic reticulum and mitochondria of baby hamster kidney cells.

A third possible mechanism for the transfer of phospholipids between the mitochondria and endoplasmic reticulum is a process of physical contact and fusion of mitochondrial and endoplasmic reticulum membranes. In support of this mechanism, we have recently isolated a unique endoplasmic reticulum membrane fraction from rat liver (42). The membrane fraction, which sediments with mitochondria, has many (but not all) properties of microsomal membranes. For example, the specific activity of the endoplasmic reticulum marker enzyme NADPH:cytochrome c reductase was less than one-third that in microsomal or endoplasmic reticulum membranes. In addition, the membranes contained high activities of several phospholipid biosynthetic enzymes, in particular PtdEtn synthase and PtdEtn methyltransferase. Several previous biochemical studies (43, 44) have suggested that neither the rough nor the smooth endoplasmic reticulum is homogeneous with respect to protein composition, and many morphological studies (45–47) have indicated that there may be regions of continuity between the endoplasmic reticulum and mitochondria. Possibly this unique membrane fraction is a domain of the endoplasmic reticulum that in vivo is in close juxtaposition with the mitochondria or has a special affinity for mitochondrial membranes and may play a role in the transfer of phospholipids between the two organelles.

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