THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 270, No. 19, Issue of May 12, pp. 11190–11198, 1995
Printed in U.S.A.

Evidence That Phosphatidylserine Is Imported into Mitochondria via a Mitochondria-associated Membrane and That the Majority of Mitochondrial Phosphatidylethanolamine Is Derived from Decarboxylation of Phosphatidylserine*

(Received for publication, December 8, 1994)

Young-Ji Shiao, Gabriella Lupo, and Jean E. Vance‡

From the Lipid and Lipoprotein Research Group and the Department of Medicine, University of Alberta, Edmonton, AB, Canada T6G 2S2

Phosphatidylserine is synthesized in both the endoplasmic reticulum and a unique membrane fraction, the mitochondria-associated membrane (MAM) (Vance, J. E. (1990) *J. Biol. Chem.* 265, 7248–7256). In Chinese hamster ovary cells labeled with [^{3}H]serine or [^{3}H]ethanolamine, we found that the majority of mitochondrial phosphatidylethanolamine was derived from phosphatidylserine decarboxylation. Essentially no mitochondrial phosphatidylethanolamine, especially that in the inner membrane, was imported from the endoplasmic reticulum. We tested the hypothesis that phosphatidylserine made in the endoplasmic reticulum is delivered via the MAM to mitochondria for decarboxylation to phosphatidylethanolamine. Cells were pulse-labeled with [^{3}H]serine and subsequently incubated either in the presence of hydroxylamine (for inhibition of phosphatidylserine decarboxylation) or under conditions for which cellular ATP had been depleted (for inhibition of phosphatidylserine import into mitochondria). In hydroxylamine-treated cells, within 2 h, the amount of radiolabeled phosphatidylserine in the MAM and mitochondria, but not microsomes, was greater than in untreated cells. Moreover, in ATP-depleted, but not in control, cells the amount of radiolabeled phosphatidylserine in the MAM approximately doubled by 3 h. These observations are consistent with the hypothesis that newly synthesized phosphatidylserine normally traverses the MAM *en route* to mitochondria.

In eukaryotes, the final stages in the biosynthesis of the majority of phospholipids occur on endoplasmic reticulum membranes (Vance and Vance, 1988). The other membranes in the cell (*e.g.* mitochondria, nucleus, plasma membrane) are therefore assembled from lipids synthesized in, and exported from, the endoplasmic reticulum. An exception to this generalization is phosphatidylethanolamine which is synthesized in mitochondria by the decarboxylation of PtdSer[1] (Dennis and Kennedy, 1972), as well as in the endoplasmic reticulum from the CDP-ethanolamine pathway (Jelsema and Morré, 1978; Vance and Vance, 1988). An additional, minor route of PtdEtn biosynthesis is the base exchange pathway which also occurs on the endoplasmic reticulum (van Golde *et al.*, 1974; Vance and Vance, 1988).

Compared to other cellular membranes, mitochondrial membranes are enriched in PtdEtn. For example, in rat liver the inner and outer mitochondrial membranes contain 39 and 35%, respectively, of their total phospholipids as PtdEtn, whereas in the endoplasmic reticulum only 20% of membrane phospholipids consist of PtdEtn (Colbeau *et al.*, 1971). Correspondingly, in the inner and outer mitochondrial membranes the molar ratio of PtdEtn:phosphatidylcholine is 0.96 and 0.71, respectively, whereas in the endoplasmic reticulum the ratio is only 0.33.

An aim of our study was to determine the biosynthetic origin of PtdEtn in mitochondrial membranes. One possibility was that all mitochondrial PtdEtn was synthesized *in situ* from the decarboxylation of PtdSer, which would imply that transfer of PtdEtn synthesized in the endoplasmic reticulum from CDP-ethanolamine to the mitochondria was restricted. A second possibility was that mitochondrial PtdEtn was derived from both CDP-ethanolamine and from PtdSer decarboxylation and that PtdEtn synthesized from these two sources equilibrated rapidly between the endoplasmic reticulum and mitochondria. A third possibility was that PtdEtn in mitochondrial membranes was synthesized exclusively in the endoplasmic reticulum from CDP-ethanolamine and that PtdEtn synthesized in mitochondria from PtdSer decarboxylation was rapidly exported to other cellular membranes. Although the most likely source of mitochondrial PtdEtn would intuitively appear to be the decarboxylation of PtdSer, our investigation of the origin of mitochondrial PtdEtn was prompted by two reports that PtdEtn made from PtdSer on the inner mitochondrial membrane rapidly flowed to the outer membrane without mixing with PtdEtn in the inner mitochondrial membrane (Simbeni *et al.*, 1990; Hovius *et al.*, 1992). We report that in CHO-k1 cells pulse-labeled with either [1-^{3}H]ethanolamine or [3-^{3}H]serine, mitochondrial PtdEtn, especially that in inner membranes, was mainly derived *in situ* from decarboxylation of PtdSer, even when ethanolamine was supplied in the culture medium. Very little ethanolamine-derived PtdEtn was detected in either inner or outer mitochondrial membranes.

Since mitochondrial PtdEtn is derived primarily from PtdSer decarboxylation, and PtdSer is not synthesized in mitochondria (van Golde *et al.*, 1974; Vance and Vance, 1988; Vance, 1990) we also investigated the mechanism by which PtdSer is translocated from its site(s) of synthesis to mitochondria. This interorganelle movement of PtdSer is believed to occur by a mechanism that does not involve cytosolic phospholipid transfer

* This work was supported by a grant from the Medical Research Council of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Lipid and Lipoprotein Research Group, 315 Heritage Medical Research Center, University of Alberta, Edmonton, AB, Canada T6G 2S2. Tel.: 403-492-7250; Fax: 403-492-3383.

[1] The abbreviations used are: PtdSer, phosphatidylserine; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; MAM, mitochondria-associated membrane; PtdEtn, phosphatidylethanolamine.

proteins (Voelker, 1989; Vance, 1991a). Recent experiments have suggested that the most likely mechanism of transfer involves a direct contact between mitochondria (the site of PtdSer decarboxylation) and the membranes that synthesize PtdSer (Voelker, 1993). The concept that such regions of contact exist between endoplasmic reticulum and mitochondria is supported by our recent isolation of a mitochondria-associated membrane, the MAM (originally called fraction X) (Vance, 1990). The MAM was discovered during its co-isolation with rat liver mitochondria. Although the MAM has many properties characteristic of endoplasmic reticulum, distinct differences are apparent between the two types of membranes. For example, the specific activity of the endoplasmic reticulum marker enzyme, NADPH:cytochrome *c* reductase in the MAM isolated from rat liver is only 30% of that in the endoplasmic reticulum. Moreover, the specific activities of several lipid biosynthetic enzymes (*e.g.* PtdSer synthase, diacylglycerol acyltransferase, and acyl-CoA:cholesterol acyltransferase) in the MAM are 2–4-fold higher than in the endoplasmic reticulum (Vance, 1990; Rusiñol *et al.*, 1994). In addition, several other phospholipid biosynthetic enzymes, including choline- and ethanolamine-phosphotransferases and phosphatidylethanolamine methyltransferase, have been detected in the MAM with similar specific activities to those in the endoplasmic reticulum.

In an attempt to elucidate the role of the MAM in the import of newly synthesized PtdSer into mitochondria we inhibited either the decarboxylation of PtdSer (with hydroxylamine) or the import of PtdSer into mitochondria (by energy depletion) and observed an accretion of PtdSer in the MAM.

EXPERIMENTAL PROCEDURES

Materials—Chinese hamster ovary cells (CHO-k1) were obtained from the American Type Culture Collection. The radiochemicals [1-^{3}H]ethanolamine and [3-^{3}H]serine were purchased from Amersham, Oakville, Ontario, Canada. Silica gel G thin-layer chromatography plates, 0.25 mm thickness, were from BDH Chemicals. Fetal bovine serum, Dulbecco's modified Eagle's medium and minimum essential medium were obtained from Life Technologies Inc. L-Serine, ethanolamine, and hydroxylamine were purchased from Sigma. Primaria culture dishes (100 mm) were from Becton-Dickinson, Oxnard, CA. Phospholipid standards were obtained from Avanti Polar Lipids, Birmingham, AL. Percoll was from Pharmacia Fine Chemicals, Uppsala, Sweden. The reagent for ATP bioluminescence constant light signal was obtained from Boehringer Mannheim. All other reagents were from Sigma or Fisher.

Cell Culture—CHO-k1 cells were routinely maintained in 100-mm culture dishes either in DMEM or in Ham's F-12/DMEM (1:1, v/v) supplemented with 10% fetal bovine serum, 40 units/ml of penicillin, and 40 μg/ml of streptomycin at 37 °C in an atmosphere containing 5% CO_2. Half-confluence was attained after 1 day of culture and confluence after 3 days, as judged by visualization under the light microscope. Viability of the cells was estimated by the ability of the cells to exclude trypan blue and by the lack of release of lactate dehydrogenase into the medium (Vance, 1991b).

Pulse-Chase Experiments—For labeling experiments with [1-^{3}H]ethanolamine, CHO-k1 cells were washed twice with DMEM. The medium was removed and cells were incubated with 3 ml/dish of serum-free DMEM containing 1 μCi/ml of [1-^{3}H]ethanolamine for 1 h (pulse). For confluent cells, radioactive medium was removed and replaced with 5 ml of DMEM containing 400 μM serine, with or without 1 mM ethanolamine. For actively growing cells, after the pulse, the medium was replaced with Ham's F-12/DMEM supplemented with 10% fetal bovine serum and 400 μM serine, with or without 1 mM ethanolamine.

For labeling experiments with [3-^{3}H]serine, CHO-k1 cells were washed twice with serine-free minimum essential medium. The cells were then incubated in 3 ml/dish of serum-free, serine-free minimum essential medium containing 5 μCi/ml of [3-^{3}H]serine for 1 h. The medium was removed and replaced with 5 ml of DMEM containing 1 mM serine, with or without 1 mM ethanolamine for confluent cells, or with Ham's F-12/DMEM supplemented with 10% fetal bovine serum and 1 mM serine, with or without 1 mM ethanolamine, for experiments with half-confluent cells. In some experiments, as indicated, 1 mM hydroxylamine was added to the chase medium. In experiments in which energy was depleted, 20 mM potassium fluoride and 3 mM potassium cyanide were added to the chase medium (Shiao and Vance, 1993) which reduced the cellular ATP level by approximately 90% after 2 h.

After defined chase periods, media were collected, cells were harvested by scraping them into phosphate-buffered saline, and subcellular fractions were prepared as described below. Lipids were extracted from each fraction by addition of 3 volumes of chloroform/methanol (2:1, v/v). Individual phospholipids were isolated by thin-layer chromatography in the solvent system chloroform/methanol/acetic acid/formic acid/water (70:30:12:4:2) after addition of unlabeled carrier phospholipids. The phosphorus content of PtdSer and PtdEtn was determined from a parallel group of unlabeled cells (Rouser and Fleischer, 1967), and specific radioactivity was calculated.

Double Labeling Experiment—For double labeling experiments with [2-^{14}C]ethanolamine and [1-^{3}H]ethanolamine, confluent CHO-k1 cells were incubated in 3 ml/dish of serum-free DMEM containing 1 μCi/ml of [2-^{14}C]ethanolamine for 5 h. The medium was removed, and cells were harvested by trypsinization and divided into duplicate cultures. The cells were incubated in Ham's DMEM/F-12 medium supplemented with 10% fetal bovine serum and 400 μM serine for an additional 18 h. The medium was removed and replaced with 3 ml/dish of serum-free DMEM containing 1 μCi/ml of [1-^{3}H]ethanolamine for 1 h. Radioactive medium was removed and replaced with medium containing unlabeled ethanolamine (1 mM). The cells were harvested by scraping them into phosphate-buffered saline, and subcellular fractions were prepared as described below. Radiolabeled metabolites in each fraction were extracted and separated for measurement of radioactivity. For double labeling experiments with L-[U-^{14}C]serine and [3-^{3}H]serine, the procedure was as for the above double labeling experiment with ethanolamine except that the initial labeling was with 15 μCi/dish of L-[U-^{14}C]serine for 5 h, and the second labeling was for 1 h with 15 μCi/dish of [3-^{3}H]serine. For the simultaneous measurement of ^{3}H and ^{14}C the channel windows of the scintillation counter were set at 0–350 for ^{3}H and 450–670 for ^{14}C.

Preparation of Subcellular Fractions—Culture dishes were placed on ice and cells were scraped from dishes into 5 ml/dish of phosphate-buffered saline at 4 °C. Cellular material from four dishes was combined. Cells were pelleted by centrifugation at 500 × *g* for 5 min, and the pellet was resuspended in 8 ml of homogenization buffer (0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 100 μM leupeptin, 100 μM phenylmethylsulfonyl fluoride). The cell suspension was transferred to a minibomb cell disruption chamber (Kontes) and subjected to a pressure of 500 p.s.i. for 10 min at 4 °C. Cells were disrupted by rapidly subjecting the cells to atmospheric pressure. The pressure treatment in the minibomb was repeated resulting in disruption of essentially all cells as determined by visualization in the light microscope.

Microsomes, mitochondria, and the MAM were isolated from the homogenate by a procedure similar to that described previously for rat liver (Vance, 1990). The homogenate was centrifuged at 600 × *g* for 10 min to remove nuclei and cell debris. The supernatant was centrifuged at 10,300 × g_{max} for 10 min to pellet crude mitochondria. The resulting supernatant was centrifuged at 100,000 × g_{max} for 1 h in a Beckman Ti 70.1 rotor to pellet microsomes, and the pellet was resuspended in homogenization buffer. Mitochondria were further purified as follows. The crude mitochondrial pellet was suspended by hand homogenization in 300 μl of isolation medium (250 mM mannitol, 5 mM HEPES (pH 7.4), 0.5 mM EGTA, and 0.1% bovine serum albumin), and the suspension was layered on top of 8 ml of Percoll medium (225 mM mannitol, 25 mM HEPES (pH 7.4), 1 mM EGTA, 0.1% bovine serum albumin, and 30% (v/v) Percoll) in a 10 ml of polycarbonate ultracentrifuge tube. The tube was centrifuged for 30 min at 95,000 × g_{max}. A dense band, consisting of purified mitochondria, was recovered from approximately ¾ down the tube. Mitochondria were removed with a Pasteur pipette, diluted with isolation medium, and washed twice by centrifugation at 6,300 × g_{max} for 10 min to remove the Percoll. The mitochondrial pellet was resuspended in homogenization buffer. The MAM was isolated from the Percoll gradient as a diffuse white band located above mitochondria. The band was removed, diluted with isolation medium, and centrifuged at 6,300 × g_{max} for 10 min. The resulting supernatant was centrifuged at 100,000 × g_{max} for 1 h in a Beckman Ti 70.1 rotor. The pellet (MAM) was resuspended in homogenization buffer.

In some experiments, mitochondria were further fractionated into inner and outer membranes by a method similar to that described by Hovius *et al.* (1992). The mitochondrial pellet was suspended in buffer H which consisted of 220 mM mannitol, 70 mM sucrose and 2 mM HEPES (pH 7.4), and 0.5 mg/ml bovine serum albumin/20 mg mitochondrial protein/ml. An equal volume of digitonin (6 mg/ml) in buffer H was added, and the sample was incubated at 0 °C for 15 min. The digitonin

TABLE I

Specific activity of marker enzymes in CHO-k1 cell membranes

Microsomes, MAM and mitochondria were prepared from CHO-k1 cells. The units of enzyme activity are: NADPH:cytochrome c reductase, nmol of cytochrome *c* reduced per min/mg of protein; cytochrome *c* oxidase, nmol of cytochrome *c* oxidized per min/mg of protein; succinate cytochrome *c* reductase, nmol of cytochrome *c* reduced per min/mg protein; PtdSer synthase, nmol PtdSer synthesized per h/mg protein. The abbreviations are: ER, endoplasmic reticulum; IMT, inner mitochondrial membrane; MAM, mitochondria-associated membrane. Data are averages ± S.D. of three subcellular fractionations.

Fraction	Specific activity			
	NADPH: cytochrome *c* reductase[a]	Cytochrome *c* oxidase[b]	Succinate: cytochrome *c* reductase[b]	PtdSer synthase[c]
Homogenate	0.26 ± 0.24	11.32 ± 8.79	18.01 ± 15.31	1.90 ± 0.39
Mitochondria	0	101.7 ± 14.2	117.6 ± 31.3	1.11 ± 1.13
MAM	0.49 ± 0.28	36.36 ± 0.95	60.25 ± 19.50	14.48 ± 3.04
Microsomes	5.01 ± 1.61	3.90 ± 3.10	3.33 ± 1.21	5.69 ± 1.51

[a] Marker for ER.
[b] Marker for IMT.
[c] Marker for ER/MAM.

reaction was terminated by dilution of the mixture with an equal volume of a solution of 5 mg/ml of bovine serum albumin in buffer H. The sample was centrifuged at 12,000 × *g* for 10 min and mitochondrial outer and inner membranes were obtained from the supernatant and pellet, respectively.

Membrane fractions were assayed for the following marker enzymes by published procedures: NADPH:cytochrome *c* reductase (Mahler, 1955), cytochrome *c* oxidase (Mahler, 1955), NADH:cytochrome *c* reductase, and succinate cytochrome *c* reductase (Sottocasa *et al.*, 1967). PtdSer synthase activity was measured as described previously except that Triton X-100 was omitted from the buffer (Vance and Vance, 1988).

Other Methods—The protein content of cells was determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Cellular ATP levels were measured by a bioluminescence method (Martin and Pagano, 1987; Shiao and Vance, 1993).

RESULTS

Isolation and Purity of Subcellular Fractions from CHO-k1 Cells—The relative contributions of the PtdSer decarboxylation and CDP-ethanolamine pathways to mitochondrial PtdEtn were evaluated by subcellular fractionation of CHO-k1 cells pulse-labeled with either [^{3}H]serine or [^{3}H]ethanolamine. Three subcellular fractions were isolated: mitochondria, microsomes, and a mitochondria-associated membrane, MAM (Vance, 1990). Purity of the membrane fractions was assessed by measurement of activities of marker enzymes for endoplasmic reticulum (NADPH:cytochrome *c* reductase) and mitochondria (cytochrome *c* oxidase and succinate:cytochrome *c* reductase) (Table I). Cross-contamination of the microsomal preparation by mitochondria, and of mitochondria by endoplasmic reticulum, were each 4% or less. The specific activity of the endoplasmic reticulum marker enzyme, NADPH:cytochrome *c* reductase, in the MAM was only 10% of that in microsomes.

The specific activities of the mitochondrial marker enzymes cytochrome *c* oxidase and succinate:cytochrome *c* reductase in the MAM were 35 and 51%, respectively, of those in mitochondria. The presence of this relatively high degree of contamination of the MAM by mitochondrial membranes was not unexpected, because we (Vance, 1990) and others (Ardail *et al.*, 1993) have suggested that a physical linkage may exist between the MAM and mitochondria, and fragments of mitochondria may have been co-isolated with the MAM. The presence of some mitochondrial membranes in the MAM fraction does not, however, significantly affect the studies on the subcellular distribution of labeled newly synthesized phospholipids for two reasons. First, mitochondrial contamination of the microsomes and *vice versa* is very low. Second, the phospholipid:protein ratio of the mitochondrial preparation (3.2 nmol of phospholipid/mg of protein) is low compared with that of the MAM or microsomal fractions (14.1 nmol of phospholipid/mg of protein). Therefore, although the MAM contains some mitochondrial protein, we estimate that less than 10% of the MAM phospholipids are the result of contamination by mitochondrial membranes.

The activity of PtdSer synthase, an enzyme which we have previously shown is enriched in the MAM compared with the bulk of the endoplasmic reticulum from rat liver (Vance, 1990), was also measured (Table I). In agreement with our studies with rat liver (Vance, 1990), the specific activity of PtdSer synthase in the MAM from CHO-k1 cells was approximately 2.5-fold higher than that in microsomes. The specific activity of PtdSer synthase in mitochondria from CHO-k1 cells was only 7.6% of that in the MAM, indicating that mitochondria were minimally contaminated by MAM.

Distribution of [^{3}H]Serine-labeled PtdSer and PtdEtn among Subcellular Fractions—The incorporation of [^{3}H]serine into PtdSer and PtdEtn of subcellular fractions of confluent CHO-k1 cells was examined in pulse-chase experiments. As shown in Fig. 1*A* the specific radioactivity of [^{3}H]serine-labeled PtdSer was initially highest in the microsomal and MAM fractions and declined slowly thereafter. At all times, the lowest specific radioactivity of PtdSer was in mitochondria, the site of PtdSer decarboxylation. Since the loss of radioactive PtdSer was largely reflected in the increased radioactivity in PtdEtn (Fig. 1*C*), the decline in specific radioactivity of PtdSer in microsomes and the MAM throughout the chase period is most likely the result of [^{3}H]PtdSer being exported to mitochondria for decarboxylation.

At the start of the chase period, the specific radioactivity of PtdEtn was highest in mitochondria (Fig. 1*B*), but [^{3}H]serine-derived PtdEtn gradually equilibrated throughout all three fractions as the chase period progressed.

These data show that some PtdEtn generated in mitochondria from PtdSer decarboxylation was retained in mitochondria whereas some was exported to other subcellular organelles. Similar results were obtained in parallel pulse-chase studies with nonconfluent cells (data not shown), indicating that the distribution of PtdSer-derived PtdEtn among subcellular fractions was not dependent upon whether or not the cells were actively dividing.

Distribution of [^{3}H]Ethanolamine-labeled PtdEtn among Subcellular Fractions—The MAM and microsomes both have the capacity to synthesize PtdEtn from the CDP-ethanolamine pathway, since both membranes contain CDP-ethanolamine:diacylglycerol ethanolaminephosphotransferase activity (Vance, 1990). The contribution of ethanolamine-derived PtdEtn to mitochondrial membranes was assessed in an experiment in which confluent CHO-k1 cells were pulse-labeled for 1 h with [^{3}H]ethanolamine, and the distribution of labeled PtdEtn in subcellular fractions was determined. Fig. 2*A* demonstrates that labeled PtdEtn was distributed mainly in the microsomal and MAM fractions, but was not significantly imported into mitochondria, even after 23 h. Moreover, in another experiment in which the cells were incubated with [^{3}H]ethanolamine for 24 h, the specific activity of PtdEtn in mitochondria was only approximately 1/10 of that in the MAM and microsomes (data not shown). A similar result was obtained for growing cells (Fig. 2*B*). In both dividing and quiescent cells, ethanolamine (1 mM) was added to the chase medium because: (i) a lack of ethanolamine in the medium might have restricted the synthesis of PtdEtn by the CDP-ethanolamine pathway, and (ii) we wished that no further incorporation of radioactivity into PtdEtn would occur after the end of the pulse. The decline in specific radioactivity of PtdEtn in the MAM and microsomes over the 23-h period reflects the dilution of labeled PtdEtn by

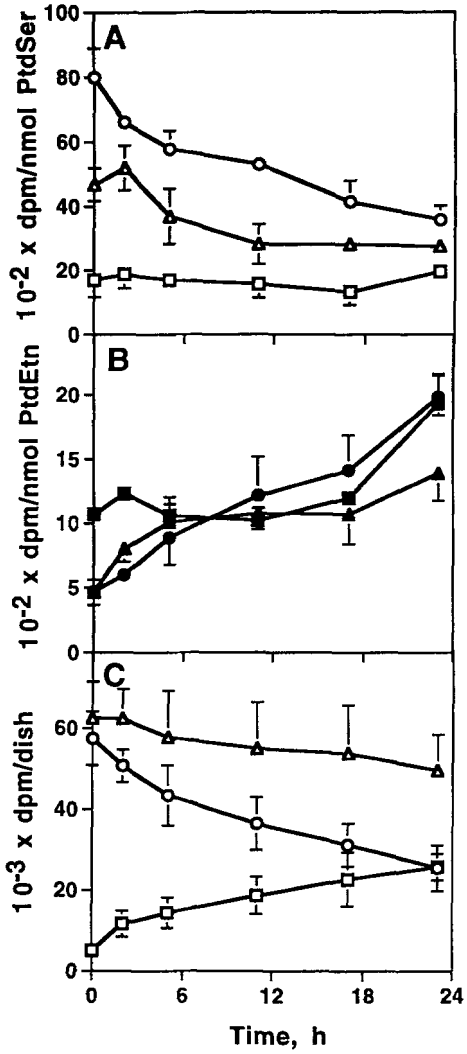

FIG. 1. **Distribution of [3-^{3}H]serine-labeled PtdSer and PtdEtn among subcellular fractions of CHO-k1 cells.** Confluent monolayer cultures of CHO-k1 cells were pulse-labeled with 5 μCi/ml of [3-^{3}H]serine for 1 h at 37 °C. The pulse medium was removed, and cells were further incubated in medium containing 1 mM serine for 2–23 h. At the indicated times, cells from four dishes combined were harvested and disrupted by nitrogen cavitation. Three membrane fractions, mitochondria (*squares*), MAM (*triangles*), and microsomes (*circles*) were prepared and lipids isolated. The content of [^{3}H]PtdSer and [^{3}H]PtdEtn was measured. Parallel dishes were used to determine the phosphorus content for determination of specific radioactivities of PtdSer (*A*) and PtdEtn (*B*). In *C* the dpm/dish in PtdSer (*circles*) and PtdEtn (*squares*) are presented. Total incorporation of [^{3}H]serine into phospholipids (*PtdSer + PtdEtn*) (*triangles*) is also shown. All data are averages ± S.D. of three independent experiments. In some instances *error bars* are too small to be visible.

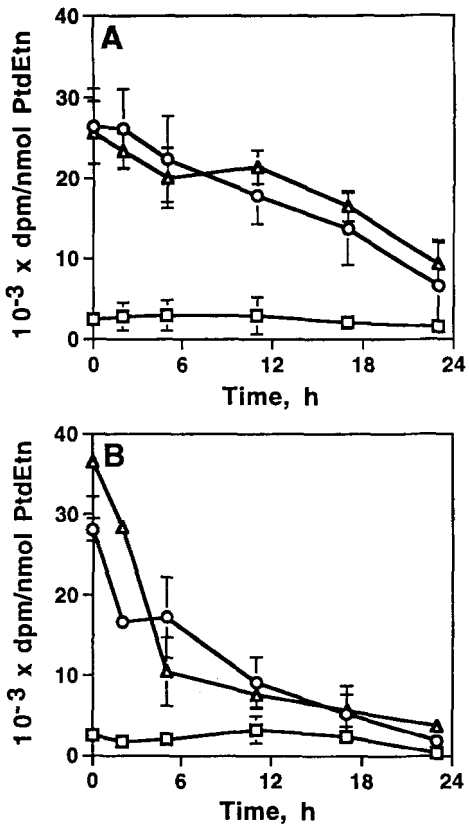

FIG. 2. **Distribution of [1-^{3}H]ethanolamine-labeled PtdEtn among subcellular fractions of quiescent and actively dividing CHO-k1 cells.** *A*, confluent monolayer cultures of CHO-k1 cells were pulse-labeled with 1 μCi/ml of [1-^{3}H]ethanolamine for 1 h at 37 °C. The pulse medium was removed, and cells were incubated in medium containing 1 mM unlabeled ethanolamine for 2–23 h. At indicated times, cells from four dishes were harvested, combined, and disrupted by nitrogen cavitation. Three membrane fractions, mitochondria (*squares*), MAM (*triangles*), and microsomes (*circles*) were prepared. Lipids were extracted and radioactivity in PtdEtn was measured. Parallel dishes were used for determination of the phosphorus content of PtdEtn for calculation of specific radioactivity. Data are averages ± S.D. of three independent experiments. In some instances *error bars* are too small to be visible. *B*, half-confluent monolayer cultures of CHO-k1 cells were pulse-labeled, incubated, and subfractionated as described for *A*, except that chase medium contained 1 mM unlabeled ethanolamine. Symbols are as for *A*. All data are averages ± S.D. of three independent experiments. In some instances *error bars* are too small to be visible.

the continual catabolism and synthesis of PtdEtn using the abundant supply of unlabeled ethanolamine in the medium.

PtdEtn in the Inner Mitochondrial Membrane Is Largely Derived from Decarboxylation of PtdSer, Not from CDP-ethanolamine—Previous studies in yeast (Simbeni *et al.*, 1990) and rat liver (Hovius *et al.*, 1992) have suggested that PtdEtn synthesized from PtdSer in inner mitochondrial membranes is rapidly exported to outer mitochondrial membranes without equilibrating with the PtdEtn pool in inner membranes. We measured the distribution of PtdEtn, derived from its two biosynthetic routes, between inner and outer mitochondrial membranes. CHO-k1 cells were pulse-labeled with either [^{3}H]serine or [^{3}H]ethanolamine. Two submitochondrial fractions, one enriched in inner, the other in outer, membranes were isolated from purified mitochondria after solubilization of the outer membranes with digitonin. The purity of the membrane fractions was assessed by measurement of activities of marker enzymes for outer mitochondrial membranes (NADH: cytochrome *c* reductase) and inner mitochondrial membranes (succinate:cytochrome *c* reductase) (Table II). Contamination of the outer membrane preparation by inner membranes was less than 14% and of the inner membrane preparation by outer membranes was only 2%.

The incorporation of [^{3}H]serine into PtdSer and PtdEtn in the inner and outer membranes was examined in pulse-chase experiments in confluent CHO-k1 cells. As shown in Fig. 3 (*upper panel*), the specific radioactivity of [^{3}H]serine-labeled PtdSer was higher in outer, than in inner, membranes throughout the chase period. The PtdSer pools in the inner and outer membranes did not equilibrate, even after 23 h. In contrast, the specific radioactivity of serine-derived PtdEtn in the inner membranes was at least double that in the outer membranes (Fig. 3, *middle panel*). Fig. 3 (*lower panel*) also shows that the specific radioactivity of ethanolamine-derived PtdEtn in inner membranes was very low compared with that in outer mem-

TABLE II
Specific activity of marker enzymes in submitochondrial membranes of CHO-k1 cells

Mitochondrial outer and inner membranes were prepared from CHO-k1 cells by digitonin solubilization. Units of enzyme specific activity are nmol of cytochrome *c* reduced per min/mg of protein. The abbreviations are: IMT, inner mitochondrial membranes; OMT, outer mitochondrial membranes. Data are averages ± S.D. of three subcellular fractionations.

Fraction	Specific activity	
	NADH: cytochrome *c* reductase[a]	Succinate: cytochrome *c* reductase[b]
Mitochondria	137 ± 10	117 ± 31
OMT	137 ± 15	5 ± 2
IMT	19 ± 2	248 ± 14

[a] Marker for OMT.
[b] Marker for IMT.

branes. Indeed, some radiolabeling of inner membranes is likely to be the result of contamination of inner membranes by outer membranes (Table II). Moreover, the small amount of [^{3}H]ethanolamine-derived PtdEtn in outer membranes may be partially, at least, due to contamination of outer membranes with the MAM, because the specific radioactivity of ethanolamine-derived PtdEtn in the MAM was 5–10 times higher than in mitochondrial membranes (Fig. 2).

The combined results presented in Figs. 1–3 indicate that the CDP-ethanolamine pathway does not serve as a quantitatively significant source of PtdEtn in inner or outer mitochondrial membranes of CHO-k1 cells, even when the cells are growing and require large amounts of phospholipid for membrane expansion. The data suggest that decarboxylation of PtdSer provides the major source of mitochondrial PtdEtn. Moreover, PtdSer-derived PtdEtn is efficiently exported from mitochondria and becomes distributed throughout extramitochondrial membranes, such as the MAM and endoplasmic reticulum.

Newly Synthesized PtdSer Is Preferred for Decarboxylation to PtdEtn—In a double labeling pulse-chase experiment, we compared the utilization of newly made PtdSer, and pre-existing PtdSer, for decarboxylation to PtdEtn. PtdSer of CHO-k1 cells was labeled with [^{14}C]serine for 23 h, then [^{3}H]serine was added for 1 h, and radioactivity was chased for an additional 23 h. Radioactivity in [^{14}C]PtdSer (Fig. 4, *upper panel*) and [^{14}C]PtdEtn (Fig. 4, *lower panel*) remained almost constant throughout the 23-h chase period. However, during this period, radioactivity in newly made PtdSer ([^{3}H]serine-labeled) (Fig. 4, *upper panel*) decreased by approximately 30%, reflecting the conversion of [^{3}H]PtdSer to PtdEtn (Fig. 4, *lower panel*). The ratio of disintegrations/min in [^{3}H]PtdEtn/[^{14}C]PtdEtn increased as time progressed (Fig. 4, *lower panel, inset*). New PtdSer was therefore selected over old PtdSer for decarboxylation to PtdEtn. If newly made PtdSer had not been preferentially selected for decarboxylation, the radiolabel in PtdEtn from each of ^{14}C and ^{3}H would have increased proportionately, and the ratio of ^{3}H/^{14}C in PtdEtn would have remained constant. These data support the idea that a pool of newly made, rather than pre-existing, PtdSer is preferentially channeled into decarboxylation.

Although very little CDP-ethanolamine-derived PtdEtn was present in mitochondrial membranes (only approximately 3–4% of that in the cell), we examined whether or not there was a preference for transport of new or pre-existing ethanolamine-derived PtdEtn to mitochondria. We therefore performed a double labeling experiment using the same protocol as for the experiment depicted in Fig. 4, except that "old" PtdEtn was labeled with [2-^{14}C]ethanolamine and "new" PtdEtn was labeled for 1 h with [1-^{3}H]ethanolamine. In contrast to the results for PtdSer-derived PtdEtn, small amounts of newly made

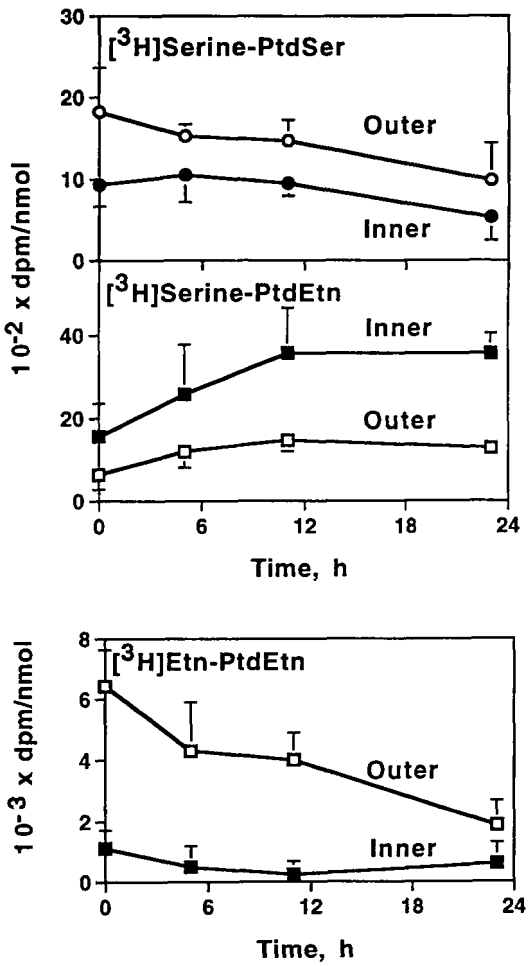

FIG. 3. **Distribution of [^{3}H]serine-labeled PtdSer and PtdEtn between outer and inner mitochondrial membranes.** *Upper* and *middle panels*, confluent monolayer cultures of CHO-k1 cells were pulse-labeled with 5 μCi/ml of [^{3}H]serine for 1 h at 37 °C, then cells were incubated with medium containing 1 mM serine for 5–23 h. At indicated times, cells from 10 dishes combined were harvested, disrupted by nitrogen cavitation, and mitochondria were isolated. The outer and inner mitochondrial membranes were separated by centrifugation after digitonin solubilization of whole mitochondria. *Lower panel*, half-confluent CHO-k1 cells were incubated with medium containing 1 μCi/ml of [^{3}H]ethanolamine for 1 h at 37 °C, then incubated with medium containing 1 mM ethanolamine for 5–23 h. Mitochondria were isolated and fractionated, and radioactivity in lipids was determined as described for experiments depicted in *upper panels*. In all panels, the specific radioactivity of PtdSer (*circles*) and PtdEtn (*squares*) was determined. *Open symbols*, outer mitochondrial membranes; *closed symbols*, inner mitochondrial membranes. All data are averages ± S.D. of three independent experiments. In some instances *error bars* are obscured by symbols.

and pre-existing PtdEtn were equally selected for transport from the endoplasmic reticulum to mitochondria (*i.e.* the ratio of ^{14}C/^{3}H remained constant (data not shown).

The MAM Mediates PtdSer Import into Mitochondria—PtdSer that is utilized for decarboxylation is not synthesized in mitochondria but is imported from its sites of synthesis, presumably in the endoplasmic reticulum and the MAM (Vance, 1990). The mechanism of PtdSer import into mitochondria has not yet been clearly defined. Because of the high activity of PtdSer synthase in the MAM, and indications that the MAM and mitochondria might be physically linked, we (Vance, 1990) and others (Ardail *et al.*, 1993; Voelker, 1993) have proposed that the MAM mediates the import of PtdSer into mitochondria.

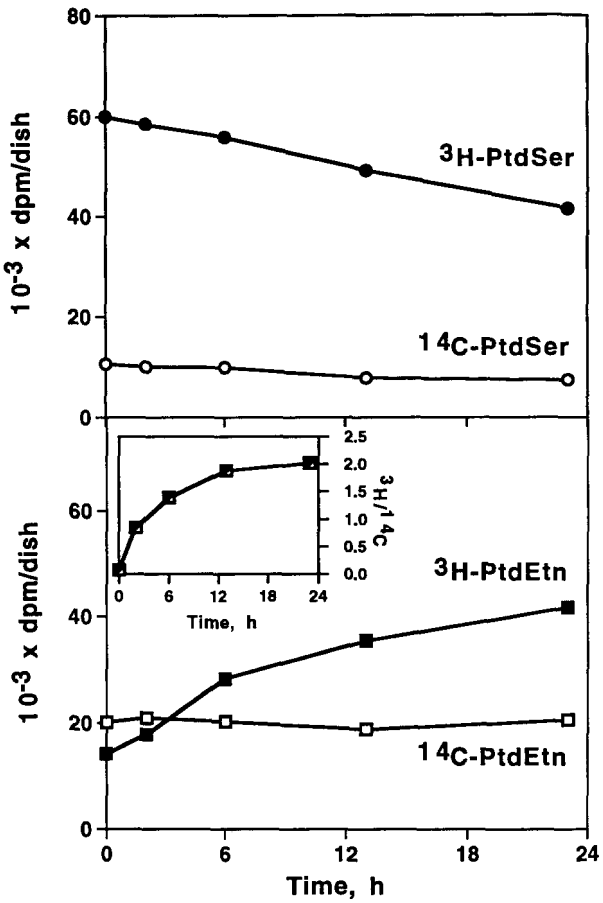

FIG. 4. **Newly synthesized PtdSer is preferred for decarboxylation to PtdEtn.** Confluent CHO-k1 cells were incubated with 15 μCi/dish of [U-^{14}C]serine for 5 h. Radioactive medium was removed, then cells were split into duplicate cultures and incubated for 18 h, after which 15 μCi/dish of [3-^{3}H]serine was added for 1 h. The medium was removed and cells were incubated for up to 23 h with medium containing 1 mM unlabeled serine. The incorporation of ^{14}C (*open symbols*) and ^{3}H (*closed symbols*) into PtdSer (*upper panel*) and PtdEtn (*lower panel*) was measured. The *inset graph* in the *lower panel* depicts the ratio of disintegrations/dish in PtdEtn from [^{3}H]serine (*i.e.* newly made PtdSer) and [^{14}C]serine (*i.e.* older PtdSer). The experiment was repeated twice with similar results.

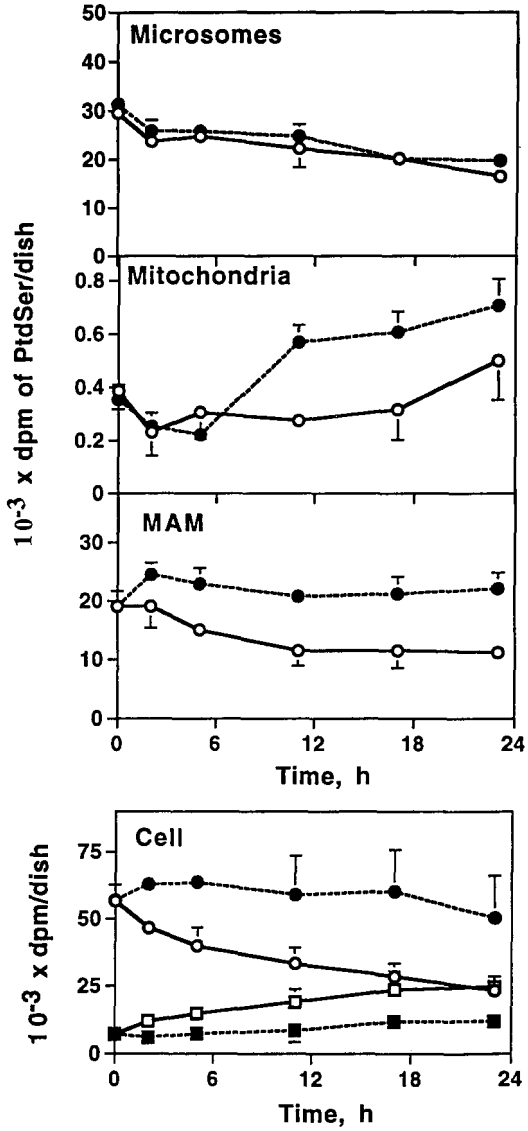

FIG. 5. **Hydroxylamine causes an accumulation of [^{3}H]PtdSer in both the MAM and mitochondria, but not in microsomes.** Confluent monolayer cultures of CHO-k1 cells were pulse-labeled with 5 μCi/ml of [3-^{3}H]serine for 1 h at 37 °C. Pulse medium was removed and cells were incubated in chase medium containing 1 mM serine with (*closed symbols*) or without (*open symbols*) 1 mM hydroxylamine for 2 to 23 h. For experiments depicted in the *lowest panel*, cells were harvested at indicated times, lipids were extracted and radioactivity in PtdSer (*circles*) and PtdEtn (*squares*) was determined. For experiments depicted in the *three upper panels*, at the indicated times, cells from four dishes combined were harvested and disrupted by nitrogen cavitation. Three membrane fractions, mitochondria, MAM, and microsomes, were isolated. Lipids were extracted and radioactivity in PtdSer was measured. All data are averages ± S.D. of three independent experiments. In some instances *error bars* are too small to be visible.

One strategy for testing this hypothesis in intact cells was the use of hydroxylamine as an inhibitor of PtdSer decarboxylase. In mammalian cells this enzyme is pyruvoylated. Consequently, its action is inhibited by hydroxylamine (Snell *et al.*, 1977; Ardail *et al.*, 1991; Voelker *et al.*, 1993). If the MAM were indeed an intermediate in the translocation of PtdSer from endoplasmic reticulum to mitochondria, inhibition of PtdSer decarboxylation might be expected to cause an accumulation of newly made PtdSer at, and immediately prior to, the site of decarboxylation. We therefore pulse-labeled CHO-k1 cells with [^{3}H]serine, and radioactivity was subsequently chased in medium that either contained or lacked 1 mM hydroxylamine. Fig. 5 (*lowest panel*) shows that hydroxylamine inhibited the conversion of PtdSer to PtdEtn by approximately 70% and caused a concomitant accumulation of newly synthesized PtdSer in the cells. Viability of the cells was not compromised by the presence of 1 mM hydroxylamine. Subcellular fractions (mitochondria, microsomes, and MAM) were isolated from these cells, and radioactivity in PtdSer (dpm/dish) was determined. We detected no difference between treated and untreated cells in the recovery of any of the subcellular fractions. In microsomes, the labeling pattern of PtdSer was very similar in cells incubated with or without hydroxylamine (Fig. 5, *top panel*); over the 23-h chase period, the amount of radiolabeled PtdSer in microsomes gradually declined by approximately 30% (Fig. 5). In the MAM isolated from hydroxylamine-treated cells, however, within 2 h of the end of the pulse period, the pool of [^{3}H]-labeled PtdSer in the MAM isolated from hydroxylamine-treated cells increased by approximately 25% and the level of radiolabeling remained almost constant for the remainder of the 23-h chase period (Fig. 5). In cells not treated with hydroxylamine, the [^{3}H]PtdSer pool gradually declined. In mitochondria from hydroxylamine-treated cells the radioactivity in PtdSer increased to approximately twice that in mitochondria from untreated cells by 10 h (Fig. 5) and by the end of the 23-h chase period was approxi-

mately 75% higher than at the end of the pulse period. The extent of recovery of the MAM from the cellular homogenate cannot be determined at this time, because no marker protein is known for the MAM of CHO cells. (Phosphatidylethanolamine methyltransferase 2, the marker protein for the MAM in rat liver, is not detectable in CHO-k1 cells.) We were therefore unable to correlate quantitatively the loss of disintegrations/min from PtdSer of microsomes with the increase of disintegrations/min in PtdSer in the MAM. However, these studies imply that since PtdSer synthesis from [^{3}H]serine ceased at the end of the pulse period, the increment in [^{3}H]PtdSer in the MAM and mitochondria from hydroxylamine-treated cells was the result of the import of [^{3}H]PtdSer from elsewhere, most likely from the endoplasmic reticulum.

As a second strategy for examining whether or not PtdSer made in microsomes traversed the MAM *en route* to mitochondria, [^{3}H]PtdSer-labeled CHO-k1 cells were incubated for up to 8 h with energy poisons (20 mM potassium fluoride and 3 mM potassium cyanide) which depleted the cellular ATP level by approximately 90% after 2 h and 95% after 8 h. The viability of the cells and the recovery of subcellular fractions were not significantly affected by the energy poisons. The import of PtdSer into mitochondria has been shown to be an ATP-dependent process (Voelker, 1989). Consequently, depletion of cellular ATP would be expected to result in an accumulation of [^{3}H]PtdSer at a site immediately prior to the site at which transport was disrupted.

The distribution of radiolabeled PtdSer was determined in the MAM, microsomes, and mitochondria at various times after addition of energy poisons. Fig. 6 (*lowest panel*) shows that upon energy depletion, the conversion of PtdSer to PtdEtn almost completely ceased and [^{3}H]PtdSer accumulated in the cells. Within 3 h after the end of the pulse period, the amount of radiolabeled PtdSer in the MAM from ATP-depleted cells had doubled (Fig. 6), whereas in the MAM from untreated cells the amount of [^{3}H]PtdSer gradually declined over the 8-h course of the experiment. The amount of radiolabeled PtdSer was less in mitochondria of energy-depleted cells than in untreated cells, presumably because import of PtdSer into mitochondria had been disrupted by ATP depletion. In microsomes, the level of [^{3}H]PtdSer decreased by 20–30% over the 8-h chase period, and the labeling pattern was the same in untreated, and ATP-depleted, cells. As was the case for the experiment described above using hydroxylamine, we were unable to estimate the recovery of the MAM from the homogenate and therefore could not calculate whether or not the increase in dpm in PtdSer in the MAM corresponded to the loss of disintegrations/min from microsomes. However, the accumulation of [^{3}H]PtdSer in the MAM from energy-depleted cells indicates that after the end of the pulse period, [^{3}H]PtdSer was imported into the MAM from another membrane source, most likely from endoplasmic reticulum, but that the export of [^{3}H]PtdSer from the MAM to mitochondria was inhibited because of depletion of ATP.

In conclusion, the results from the experiments with hydroxylamine and energy poisons support the hypothesis that the MAM is an intermediate compartment through which PtdSer traverses during its movement from microsomes to mitochondria.

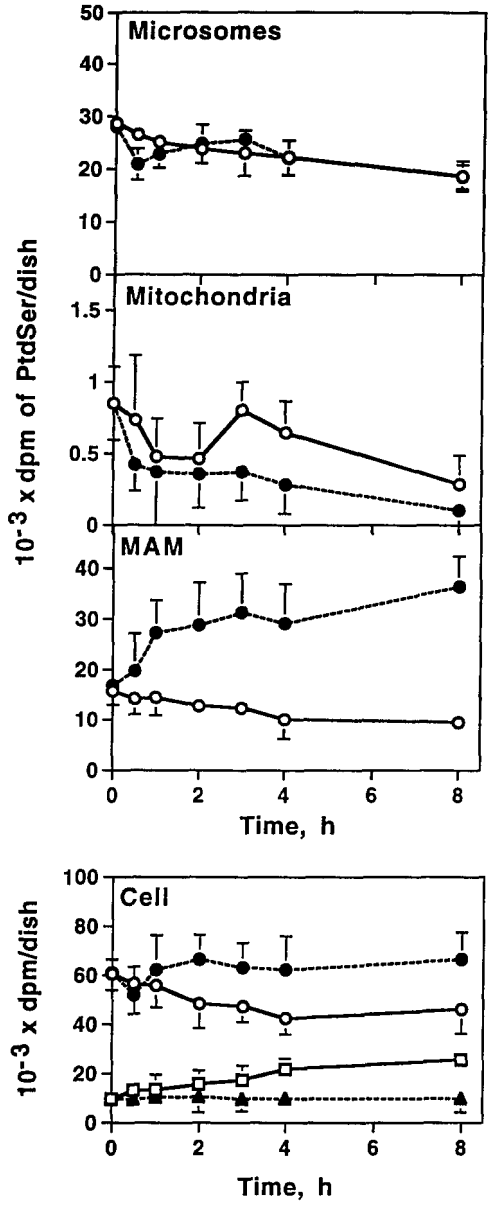

FIG. 6. **[^{3}H]Serine-derived PtdSer accumulates in the MAM upon depletion of cellular ATP.** Confluent monolayer cultures of CHO-k1 cells were pulse-labeled with 5 μCi/ml of [^{3}H]serine for 1 h at 37 °C. Pulse medium was removed, and cells were incubated in medium containing 1 mM serine with (*closed symbols*) or without (*open symbols*) the energy poisons potassium fluoride (20 mM) and potassium cyanide (3 mM) for 1–8 h. For the experiment depicted in the *lowest panel*, cells were harvested at indicated times and lipids were extracted. Radioactivity in PtdSer (*circles*) and PtdEtn (*squares* and *triangles*) was measured. For experiments depicted in the *three upper panels*, at the indicated times, cells from four dishes combined were harvested and disrupted by nitrogen cavitation. Mitochondria, microsomes, and MAM were isolated, lipids were extracted, and radioactivity in PtdSer was measured. All data are averages ± S.D. of three independent experiments. In some instances *error bars* are hidden by symbols.

DISCUSSION

In these studies we report three novel findings. First, we show that the bulk of PtdEtn of both inner and outer mitochondrial membranes from CHO-k1 cells is synthesized in mitochondria from PtdSer decarboxylation, rather than from the CDP-ethanolamine pathway in the endoplasmic reticulum. Second, we provide evidence that newly made PtdSer, rather than the bulk of membrane PtdSer, is preferred for decarboxylation to PtdEtn. Third, we show that when PtdSer decarboxylation is inhibited by treatment of CHO-k1 cells with hydroxylamine, PtdSer accumulates in the MAM and mitochondria, but not in microsomes. Similarly, when cellular ATP levels are depleted, PtdSer accumulates in the MAM. These findings provide support for the hypothesis that the MAM mediates the import of PtdSer into mitochondria.

The mechanisms involved in the intermembrane transport of

lipids are not clearly understood, although evidence is accumulating that not a single, unifying mode of lipid transfer between organelles exists (Voelker, 1991a). Rather, the mechanism of transport for each type of lipid must be considered individually, as must the type of donor and acceptor membranes involved in the process. In the present studies we have investigated the transport of PtdSer, which is synthesized in the endoplasmic reticulum and related membranes (Vance and Vance, 1988), to mitochondria, where PtdSer is decarboxylated to PtdEtn (Dennis and Kennedy, 1972). Translocation of PtdSer from its site of synthesis to its site of decarboxylation on the inner mitochondrial membrane (Zborowski *et al.*, 1983) does not require the participation of soluble, cytosolic phospholipid transfer proteins. This conclusion was based on studies in intact CHO-k1 cells (van Heusden *et al.*, 1990), permeabilized CHO-k1 cells (Voelker, 1989; Voelker, 1990) and reconstituted systems of rat liver mitochondria and microsomes (Vance, 1991a). The translocation step is ATP-dependent (Voelker, 1989) and is inhibited by adriamycin, a reagent which inhibits protein import into mitochondria (Voelker, 1991b). These observations suggest that translocation of PtdSer to mitochondria occurs by a collision-based mechanism, either one mediated by vesicles or one in which the membranes that synthesize PtdSer come into direct contact with the membranes that perform the decarboxylation. Two experimental results are inconsistent with a vesicle-mediated transfer. First, a 45-fold dilution of cytosol from [^{3}H]PtdSer-labeled, permeabilized, CHO-k1 cells did not reduce the efficiency of transfer and decarboxylation of PtdSer (Voelker, 1990). Second, in CHO-k1 cells that had been permeabilized then sheared, a process which mechanically destroys most organized cellular structures but leaves the mitochondria intact, PtdSer translocation/decarboxylation could only be reconstituted when donor and acceptor membranes were derived from the same population of cells. When mitochondria from one population of cells were used with PtdSer from a heterologous population of cells, the heterologous PtdSer was not decarboxylated (Voelker, 1993). These data indicate that newly synthesized PtdSer is transported to mitochondria via a channeling mechanism, perhaps one involving direct membrane contact.

In support of the concept of a direct contact between the membranes that synthesize and decarboxylate PtdSer, we have isolated a membrane fraction, the MAM, from rat liver that might fulfill the role of a membrane "bridge" that would function in the import of lipids into mitochondria (Vance, 1990). Although there is no direct evidence that the MAM performs this role, several observations support this concept. The MAM has many, but not all, properties characteristic of endoplasmic reticulum, but is co-isolated with mitochondria during centrifugation. The specific activities of several lipid biosynthetic enzymes in the MAM are as high as, or higher than, those in endoplasmic reticulum. In particular, the activity of PtdSer synthase is enriched in the MAM compared with the bulk of endoplasmic reticulum (Vance, 1990; Rusiñol *et al.*, 1994). A specific marker protein for the MAM in rat liver, phosphatidylethanolamine *N*-methyltransferase 2, that is absent from other organelle membranes (endoplasmic reticulum, mitochondria, plasma membrane, nucleus, and Golgi) has recently been identified (Cui *et al.*, 1993). Immunoelectron microscopy studies with rat liver slices, using affinity-purified antibody specific for phosphatidylethanolamine methyltransferase 2, have revealed that the membranes containing this methyltransferase are located in defined clusters in hepatocytes that do not correspond to the bulk of endoplasmic reticulum but are, for the most part, localized in the vicinity of mitochondria (Cui *et al.*, 1993). Because the MAM appears to be associated with mitochondria and has high lipid synthesizing capacity, we have proposed that the MAM might be involved in the transport of phospholipids to mitochondria.

The idea of regions of membrane continuity between endoplasmic reticulum and mitochondria is not novel. Several electron microscopy and biochemical studies have detected contact zones between these organelles (Franke and Kartenbeck, 1971; Shore and Tata, 1977; Pickett *et al.*, 1980; Katz *et al.*, 1983; Meier *et al.*, 1981). In addition, Ardail *et al.* (1993) have recently isolated from mouse liver a membrane fraction that is enriched in contact sites between inner and outer mitochondrial membranes. This contact site preparation is also associated with an endoplasmic reticulum-like membrane that has properties similar to the MAM (Ardail *et al.*, 1993). A membrane fraction which is enriched in PtdSer synthase activity, and which might be similar to the MAM from mammalian cells, has also been isolated from the yeast *Saccharomyces cerevisiae* (Zinser *et al.*, 1991).

Several interesting questions concerning the translocation of PtdSer remain unresolved. One is: what is the nature of the linkage, if any, between the MAM and mitochondria? The decarboxylase is located on the outer aspect of the inner mitochondrial membrane (Zborowski *et al.*, 1983). Consequently, once newly synthesized PtdSer has been transferred from its biosynthetic membrane to the outer mitochondrial membrane, PtdSer presumably undergoes transbilayer movement, then is either transferred from the inner leaflet of the outer membrane and across the intermembrane space to the outer aspect of the inner membrane for decarboxylation, or the decarboxylase on the inner membrane has direct access to PtdSer on the inner leaflet of the outer membrane. One way in which this sequence of events might be accomplished is via the contact site membrane fraction described by Ardail *et al.* (1993) in which the MAM appears to be in contact with the outer mitochondrial membrane at the same site at which the outer and inner mitochondrial membranes are contiguous. One should note, however, that PtdSer decarboxylase is apparently not concentrated at the contact sites between inner and outer mitochondrial membranes (Hovius *et al.*, 1992).

Another unresolved question is: are phospholipids exported from mitochondria to endoplasmic reticulum via the MAM? Certainly, the efficient export of PtdSer-derived PtdEtn to the plasma membrane of intact hepatocytes occurs (Vance, 1988), by a mechanism that is insensitive to brefeldin A (Vance *et al.*, 1991). In addition, the intracellular transport of mitochondria-derived PtdEtn, followed by its methylation to phosphatidylcholine (presumably in the endoplasmic reticulum or the MAM) has been demonstrated in intact hepatocytes (Vance and Vance, 1986; Vance, 1988). However, the mechanism of transfer of lipids from mitochondria to other organelle membranes has not been delineated. We have so far been unable to reconstitute the export of PtdSer-derived PtdEtn to the endoplasmic reticulum either in permeabilized hepatocytes or with isolated organelles from rat liver.[2]

Although newly-made PtdSer is efficiently transferred to mitochondria and converted into PtdEtn, translocation of PtdSer does not, apparently, require the active synthesis of PtdSer, because when the synthesis of PtdSer was inhibited by several methods, the conversion of PtdSer to PtdEtn was unaffected (Voelker, 1990). However, several reports suggest that a pool of newly synthesized PtdSer is preferred over pre-existing PtdSer for decarboxylation to PtdEtn (Bjerve, 1985; Vance, 1991a). The preferential use of new, rather than old, PtdSer for decarboxylation was confirmed in the present studies in a

[2] J. E. Vance, unpublished data.

double labeling experiment.

We have also investigated the biosynthetic origin of PtdEtn of mitochondrial membranes. In CHO-k1 cells (Kuge *et al.*, 1986) and baby hamster kidney cells (Voelker, 1984) PtdSer decarboxylation is the major source of PtdEtn, even when the cells are supplemented with ethanolamine, the precursor of PtdEtn synthesis from CDP-ethanolamine. In the present study, we have found that PtdEtn derived from the CDP-ethanolamine pathway did not equilibrate rapidly among microsomes, mitochondria, and the MAM, even in the presence of excess unlabeled ethanolamine in the culture medium. Indeed, very little ethanolamine-labeled PtdEtn was imported into mitochondria. In contrast, PtdEtn of mitochondria was rapidly labeled from [^{3}H]serine, and the PtdSer-derived PtdEtn was also rapidly distributed to the MAM and microsomes. From these data we conclude that mitochondrial PtdEtn is, for the most part, synthesized *in situ* in mitochondria via PtdSer decarboxylation. Moreover, an efficient mechanism of export of PtdSer-derived PtdEtn from mitochondria exists. However, the import of CDP-ethanolamine-derived PtdEtn into mitochondria occurs only very slowly. These studies agree with the report of Yaffe and Kennedy (1983) who showed that transfer of ethanolamine-derived PtdEtn from the endoplasmic reticulum to mitochondria of baby hamster kidney cells occurred with a half-life 20–80 times that of phosphatidylcholine. McMurray and Dawson (1969) reached a similar conclusion from their studies in an *in vitro* reconstituted system of rat liver microsomes and mitochondria.

An unexpected conclusion regarding the origin of PtdEtn in inner mitochondrial membranes was drawn from two recent studies. Simbeni *et al.* (1990) found that newly synthesized PtdSer-derived PtdEtn in yeast was located mainly in the outer mitochondrial membrane and did not mix with the pool of PtdEtn in inner membranes. In other studies, Hovius *et al.* (1992) reconstituted the decarboxylation of PtdSer *in vitro* using isolated rat liver mitochondria and radiolabeled PtdSer delivered from donor vesicles. The PtdSer was efficiently imported and decarboxylated to PtdEtn, but radiolabeled PtdEtn was enriched in outer, compared with inner, membranes. The implication of both these studies was that PtdEtn in inner mitochondrial membranes was, for the most part, not derived from newly made PtdSer but was supplied from an alternative source, possibly the CDP-ethanolamine pathway. However, in neither of these studies was the biosynthetic origin of PtdEtn in inner mitochondrial membranes examined directly. Our experiments show that over the 23-h period subsequent to pulse labeling, mitochondrial membranes as a whole contained only small amounts of CDP-ethanolamine-derived PtdEtn. In addition, we found that the specific radioactivity of PtdEtn derived from CDP-ethanolamine in inner membranes was only one-fifth to one-tenth of that in outer membranes. In contrast, the specific radioactivity of PtdSer-derived PtdEtn in inner mitochondrial membranes was approximately double that in outer membranes. Therefore, we conclude that in inner membranes the large majority of PtdEtn is derived from PtdSer. The observations of Simbeni *et al.* (1990) that PtdEtn of yeast outer mitochondrial membranes was enriched in PtdSer-derived PtdEtn compared with that in inner membranes might be explained by contamination of outer membranes with the yeast equivalent of the MAM, because in our studies the specific radioactivity of PtdEtn derived from PtdSer in the MAM was similar to that in mitochondria. We have no satisfactory explanation for why our findings differ from those of Hovius *et al.* (1992), who showed that PtdSer-derived PtdEtn was enriched in outer, compared to inner, membranes. One difference in methodology between our studies and those of Hovius *et al.* (1992) is that the latter delivered PtdSer to the outer membranes from liposomes, whereas in our experiments PtdSer was endogenously synthesized.

REFERENCES

Ardail, D., Gasnier, F., Lermé, F., Simonot, C., Louisot, P., and Gateau-Roesch, O. (1993) *J. Biol. Chem.* **268,** 25985–25992

Bjerve, K. S. (1985) *Biochim. Biophys. Acta* **833,** 396–405

Colbeau, A., Nachbaur, J., and Vignais, P. M. (1971) *Biochim. Biophys. Acta* **249,** 462–492

Cui, Z., Vance, J. E., Chen, M. H., Voelker, D. R., and Vance, D. E. (1993) *J. Biol. Chem.* **268,** 16655–16663

Dennis, E. A., and Kennedy, E. P. (1972) *J. Lipid Res.* **13,** 263–267

Franke, W. W., and Kartenbeck, J. (1971) *Protoplasma* **73,** 35–41

Hovius, R., Faber, B., Brigot, B., Nicolay, K., and de Kruijff, B. (1992) *J. Biol. Chem.* **267,** 16790–16795

Jelsema, C. L., and Morré, D. J. (1978) *J. Biol. Chem.* **253,** 7960–7971

Katz, J., Wals, P. A., Golden, S., and Raijman, L. (1983) *Biochem. J.* **214,** 795–813

Kuge, O., Nishijima, M., and Akamatsu, Y. (1986) *J. Biol. Chem.* **261,** 5790–5794

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275

Mahler, H. R. (1955) *Methods Enzymol.* **2,** 553–559

Martin, O. C., and Pagano, R. E. (1987) *J. Biol. Chem.* **262,** 5890–5898

McMurray, W. C., and Dawson, R. M. C. (1969) *Biochem. J.* **112,** 91–108

Meier, P. J., Spycher, M. A., and Meyer, U. A. (1981) *Biochim. Biophys. Acta* **646,** 283–297

Pickett, C. B., Montisano, D., Eisner, D., and Cascarano, J. (1980) *Exp. Cell Res.* **128,** 343–352

Rouser, G., and Fleischer, S. (1967) *Methods Enzymol.* **10,** 385–406

Rusiñol, A. E., Cui, Z., Chen, M. H., and Vance, J. E. (1994) *J. Biol. Chem.* **269,** 27494–27502

Shiao, Y.-J., and Vance, J. E. (1993) *J. Biol. Chem.* **268,** 26085–26092

Shore, G. C., and Tata, J. R. (1977) *J. Cell Biol.* **72,** 714–725

Simbeni, R., Paltauf, F., and Daum, G. (1990) *J. Biol. Chem.* **265,** 281–285

Snell, E. E. (1977) *Trends Biol. Sci.* **2,** 131–135

Sottocasa, G. L., Kuylenstierna, B., Ernster, L., and Bergstrand, A. (1967) *J. Cell Biol.* **32,** 415–438

van Golde, L. M. G., Raben, J., Batenburg, J. J., Fleischer, B., Zambrano, F., and Fleischer, S. (1974) *Biochim. Biophys. Acta* **360,** 179–192

van Heusden, G. P. H., Bos, K., Raetz, C. R. H., and Wirtz, K. W. A. (1990) *J. Biol. Chem.* **265,** 4105–4110

Vance, J. E. (1988) *Biochim. Biophys. Acta* **963,** 10–20

Vance, J. E. (1990) *J. Biol. Chem.* **265,** 7248–7256

Vance, J. E. (1991a) *J. Biol. Chem.* **266,** 89–97

Vance, J. E. (1991b) *J. Lipid Res.* **32,** 1971–1982

Vance, J. E., and Vance, D. E. (1986) *J. Biol. Chem.* **261,** 4486–4491

Vance, J. E., and Vance, D. E. (1988) *J. Biol. Chem.* **263,** 5898–5909

Vance, J. E., Aasman, E. J., and Szarka, R. (1991) *J. Biol. Chem.* **266,** 8241–8247

Voelker, D. R. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 2669–2673

Voelker, D. R. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 9921–9925

Voelker, D. R. (1990) *J. Biol. Chem.* **265,** 14340–14346

Voelker, D. R. (1991a) in *Biochemistry of Lipids, Lipoproteins and Membranes* (Vance, D. E., and Vance, J. E., eds) pp. 489–523, Elsevier Science Publishers, Amsterdam

Voelker, D. R. (1991b) *J. Biol. Chem.* **266,** 12185–12188

Voelker, D. R. (1993) *J. Biol. Chem.* **268,** 7069–7074

Yaffe, M. P., and Kennedy, E. P. (1983) *Biochemistry* **22,** 1497–1507

Zinser, E., Sperka-Gottlieb, C. D. M., Fasch, E.-V., Kohlwein, S. D., Paltauf, F., and Daum, G. (1991) *J. Bacteriol.* **173,** 2026–2034

Zborowski, J., Dygas, A., and Wojtcak, L. (1983) *FEBS Lett.* **157,** 179–182