Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 2669–2673, May 1984
Biochemistry

Phosphatidylserine functions as the major precursor of phosphatidylethanolamine in cultured BHK-21 cells

(membranes/phospholipids/metabolism/mitochondria)

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Communicated by Eugene P. Kennedy, December 27, 1983

ABSTRACT **Pulse–chase experiments with [^{3}H]serine provide evidence that significant amounts of phosphatidylserine turn over to form phosphatidylethanolamine in mammalian cells in tissue culture. Phospholipase C hydrolysis of [^{3}H]phosphatidylethanolamine synthesized from [^{3}H]serine by baby hamster kidney (BHK-21) cells demonstrates that nearly all of the radiolabel remains in the ethanolamine moiety. Uniform labeling experiments with [^{3}H]serine further demonstrate that the distribution of radiolabel in phosphatidylserine and phosphatidylethanolamine is nearly identical to the mass ratio of these lipids. Physiological concentrations of ethanolamine (20 μM) have only a marginal effect upon the ability of cells in culture to incorporate radiolabeled serine into either phosphatidylserine or phosphatidylethanolamine. These data provide compelling evidence that phosphatidylethanolamine synthesis via phosphatidylserine and phosphatidylserine decarboxylase contributes significantly to membrane biogenesis in mammalian cells.**

Phosphatidylethanolamine is the product of at least three enzyme reactions: (*i*) CDP-ethanolamine:1,2-diacylglycerol ethanolamine phosphotransferase (1); (*ii*) the back reaction of phosphatidylethanolamine:serine *O*-phosphatidyltransferase (2, 3) (this is the systematic name suggested on the basis of the recommendations in ref. 4 for the phosphatidylserine synthase of animal cells); and (*iii*) phosphatidylserine decarboxylase (2, 5). In general, the latter two enzymes are not thought to contribute significantly to phosphatidylethanolamine synthesis in animal cells (6, 7). However, few mammalian cells display any requirement for ethanolamine (8, 9), and virtually all tissue culture media are devoid of ethanolamine. The mechanisms for the net synthesis of ethanolamine or phosphatidylethanolamine under conditions of ethanolamine deprivation are via the enzyme phosphatidylserine decarboxylase (2, 5) and sphingosine kinase (10). The role of phosphatidylserine decarboxylase in mammalian cell membrane synthesis is considered in this manuscript. The results indicate that, under the appropriate conditions, most of the cellular phosphatidylethanolamine can be derived via phosphatidylserine decarboxylase. Several other laboratories have demonstrated that in mammalian cells phosphatidylserine synthase is localized to the endoplasmic reticulum and phosphatidylserine decarboxylase is localized to the mitochondria (5, 11). The segregation of these two enzymes in distinct organelle domains implicates phosphatidylserine movement between organelles as a necessary event in phosphatidylethanolamine synthesis. Thus, an understanding of the relative activity and the regulation of phosphatidylserine synthase and phosphatidylserine decarboxylase should prove useful in addressing problems of the intracellular movement of lipids.

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MATERIALS AND METHODS

Tissue Culture. Baby hamster kidney (BHK-21) cells were obtained from the American Type Culture Collection (ATCC CCL10). The cells were grown in Dulbecco's modified Eagle's medium supplemented with glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and fetal bovine serum (10%). The same medium devoid of serine was made in the laboratory from amino acids, vitamins, and salts. For pulse–chase experiments the cells were plated at a density of 10^6 per 60-mm dish 16–18 hr prior to the addition of radiolabeled compound. Cells were pulse-labeled in serine-free medium supplemented with [^{3}H]serine (specific activity, 16.8 Ci/mmol; 1 Ci = 37 GBq) at 2 μCi/ml for 2 hr. The cells were washed with phosphate-buffered saline and then incubated in complete medium supplemented to 2.5 mM serine for the duration of the chase period. Uniform labeling of cells with [^{3}H]serine was accomplished by seeding cells at a density of 0.5–1.0 × 10^5 cells per 60-mm dish in complete medium supplemented with [^{3}H]serine (final specific activity, 13.8 Ci/mol) at 5 μCi/ml and allowing growth to continue for 4 days. The effects of ethanolamine upon the short-term labeling of phosphatidylserine and phosphatidylethanolamine were determined under conditions similar to those of the pulse–chase experiments except that the cells were preincubated with ethanolamine at various concentrations for 2 or 24 hr prior to the addition of [^{3}H]serine. In these experiments the cells were kept in the [^{3}H]serine-supplemented medium for 4 hr and then harvested. The incorporations of [^{3}H]ethanolamine and [^{3}H]serine into phospholipid were compared under conditions similar to those described for the pulse–chase experiments except that the cells were labeled for 90 min in serine-free medium supplemented with 20 μM [^{3}H]serine or 20 μM [^{3}H]ethanolamine (final specific activity, 250 μCi/μmol). Cells from all experiments were routinely harvested in phosphate-buffered saline by using a rubber policeman. Protein was determined by the method of Lowry *et al.* (12). Unless otherwise indicated all points on graphs were acquired by averaging data from duplicate dishes within a given experiment; and at least two experiments were performed in each case.

Phospholipid Analysis. Lipids were extracted from cells by the procedure of Bligh and Dyer (13). Phospholipids were analyzed by using the following chromatography systems: A, chloroform/methanol/concentrated ammonia (65:25:5, vol/vol); B, chloroform/acetone/methanol/acetic acid/water (60:80:20:20:10, vol/vol); C (for descending paper chromatography), phenol/water (100:38, wt/vol). Where appropriate, authentic standards were added to samples prior to chromatography.

One-dimensional thin-layer chromatography was performed on silica gel H layers (Applied Sciences Laboratories, State College, PA). Two-dimensional thin-layer chromatog-

Abbreviations: PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine.

raphy was performed on Supelco Redi-Coat two-dimensional plates, using solvent systems A and B sequentially. Phospholipase C (Sigma type V) degradation was performed by using the procedure of Mavis *et al.* (14), except that 0.1 mM $ZnCl_2$ was included in the reaction. The extent of phospholipid degradation was monitored by using ^{32}P-labeled phospholipids isolated from cultured cells. Phosphoserine and phosphoethanolamine were chromatographically separated by using an 8-ml column of AG 1 X 8 anion exchanger (Bio-Rad) in the formate form. The phosphoethanolamine was eluted by using a gradient consisting of 100 ml of distilled water and 100 ml of 0.2 M formic acid. After completion of the gradient the column was washed with 24 ml of distilled water and a second gradient consisting of 100 ml of distilled water and 100 ml of 1 M citric acid was used to elute phosphoserine. Phospholipids were also subjected to mild alkaline hydrolysis (15), and the water-soluble components were analyzed by descending chromatography on Whatman no. 1 paper. Phospholipid phosphorus was determined by the method of Rouser *et al.* (16).

Phosphoethanolamine Determination. Approximately 1 ml of packed cells was obtained from nine 100-mm tissue culture dishes of BHK-21 cells. The cells were harvested in methanol/water (1:0.9, vol/vol). An internal recovery standard of [^{3}H]phosphoethanolamine was added to the cells. The cells were sonicated and subjected to a Bligh and Dyer extraction. The upper phase from the lipid extraction was lyophilized, resuspended in 1.5 ml, and dialyzed through 3500-dalton cutoff dialysis tubing against 20 ml of distilled water. The dialysate was adsorbed with charcoal, filtered, and applied to the anion-exchange column of AG 1 X 8 in the formate form. The phosphoethanolamine was eluted with a gradient of 200 ml of distilled water and 200 ml of 0.04 M formic acid. The phosphoethanolamine-containing fraction was concentrated and analyzed by paper chromatography. The phosphoethanolamine spot was eluted and the amount of phosphorus was determined (16).

RESULTS

Pulse–Chase Labeling of Phosphatidylserine and Phosphatidylethanolamine. BHK-21 cells were pulse labeled with [^{3}H]serine, and the incorporation of radiolabel into phosphatidylserine and phosphatidylethanolamine was determined. As shown in Fig. 1*A*, phosphatidylserine was efficiently labeled during the 2-hr pulse and then rapidly turned over. The radiolabel initially present in phosphatidylserine was quantitatively converted to phosphatidylethanolamine during the entire period of the chase. By 20 hr into the chase period the specific activity of phosphatidylethanolamine was equivalent to that of phosphatidylserine (Fig. 1*B*). Under the conditions of this experiment the doubling time of the cells was 20 hr. From the specific activity data in Fig. 1*B* the rate of synthesis of phosphatidylethanolamine from phosphatidylserine was determined to be 1.76 nmol/hr per mg of protein. This rate is sufficient to account for all of the phosphatidylethanolamine synthesis required for a cell doubling. Thus, the precursor–product relationship between phosphatidylserine and phosphatidylethanolamine is evident, and it indicates that substantial amounts of phosphatidylserine are converted to phosphatidylethanolamine by phosphatidylserine decarboxylase.

Degradative Analysis of Phospholipids. The [^{3}H]serine used as a label for phosphatidylserine can also label fatty acids, glycerol, and sphingolipids. To exclude the possibility that the radiolabel found in phosphatidylserine and phosphatidylethanolamine during the pulse–chase experiments was in fatty acids or the glycerol backbone, the total lipid extract from the 24-hr time point of the pulse–chase experiment was subjected to phospholipase C digestion and column chromatography. More than 95% of the radiolabel present in the total lipid extract as phosphatidylserine and phosphatidylethanolamine was recovered as phosphoserine and phosphoethanolamine after phospholipase C digestion. Phosphoserine and phosphoethanolamine were identified by using authentic ^{32}P-labeled standards and ion-exchange chromatography as shown in Fig. 2. The distribution of the radiolabel between the phosphoserine (22%) and phosphoethanolamine (78%) was in good agreement with the distribution of radiolabel between phosphatidylserine (28%) and phosphatidylethanolamine (72%) determined by the method of thin-layer chromatography. In preliminary experiments the labeled phospholipids were also analyzed by alkaline methanolysis and paper chromatography, and these results also demonstrated that most of the serine label that is incorporated into phosphatidylserine and phosphatidylethanolamine remains in the polar portions of the molecules.

Uniform Labeling of Phospholipids with [^{3}H]Serine. BHK-21 cells were labeled for five generations with [^{3}H]serine and the distribution of radioactivity among the various phospholipids was determined and compared to the distribution of

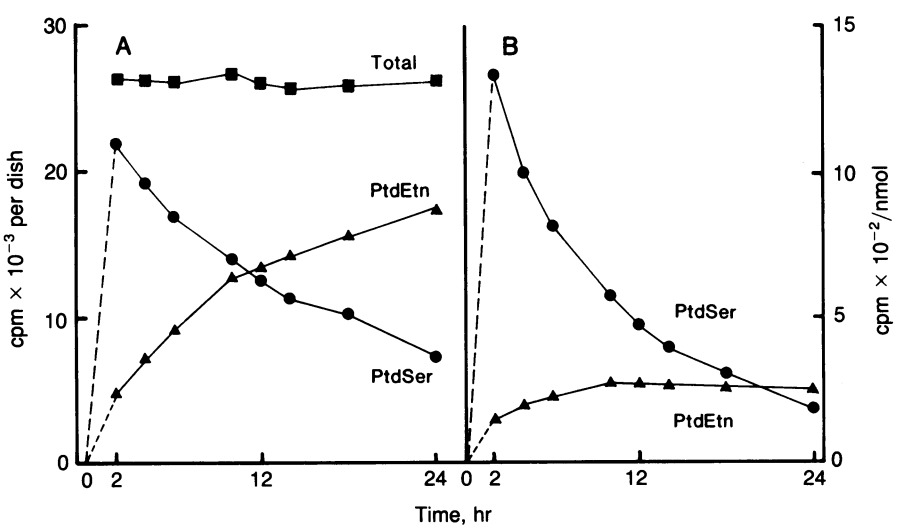

FIG. 1. Metabolism of phosphatidylserine to phosphatidylethanolamine in BHK-21 cells. The BHK-21 cells were pulsed with 8 μCi of [^{3}H]serine for 2 hr and then shifted to unlabeled medium supplemented to 2.5 mM serine. Triplicate dishes were harvested at each point. The results presented are from one of two experiments. The phospholipids were analyzed by thin-layer chromatography using solvent system A. PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine. "Total" refers to phosphatidylserine plus phosphatidylethanolamine.

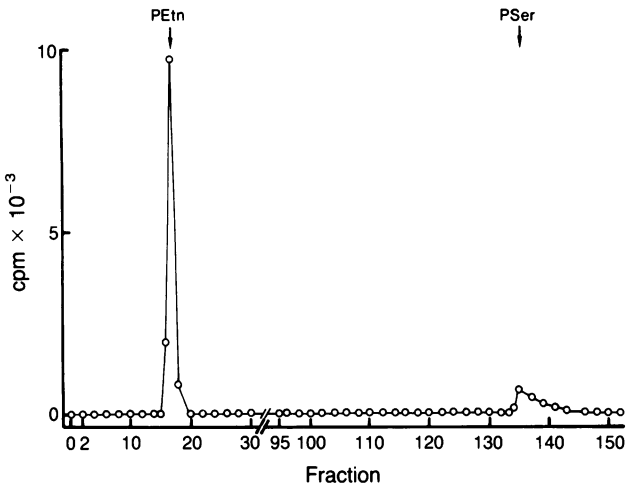

FIG. 2. Anion-exchange column chromatography of the water-soluble products of phospholipase C degradation. The total lipid extract from the 24-hr time point of the pulse–chase experiment (Fig. 1) was subjected to phospholipase C degradation. A portion (16,300 cpm) of the water-soluble reaction products was analyzed by ion-exchange chromatography. PEtn, phosphoethanolamine; PSer, phosphoserine.

chemical phosphorus (Table 1). The lipids were analyzed by two-dimensional thin-layer chromatography using solvent systems A and B. The radiolabel found in phosphatidylethanolamine, phosphatidylserine, and sphingomyelin accounted for 77% of the total. The ratio of [^{3}H]phosphatidylethanolamine to [^{3}H]phosphatidylserine, 2.13, was nearly identical to the ratio of chemical phosphorus, 1.88, for these two lipids. These data demonstrate that all of the phosphatidylethanolamine synthesized in BHK-21 cells can be derived from phosphatidylserine. The quantity of radiolabel found in sphingomyelin is approximately one-half that expected on the basis of chemical phosphorus. This may be due to the loss of ^{3}H from serine during the synthesis of 3-ketodihydrosphingosine or significant utilization of serum-derived sphingosine in sphingomyelin synthesis.

In a separate set of experiments the BHK-21 cells were subjected to a uniform labeling protocol with [^{3}H]serine. Phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine were preparatively isolated by two-dimensional thin-layer chromatography. The isolated lipids were subjected to phospholipase C digestion under conditions that yielded virtually complete degradation (as determined by ^{32}P-labeled internal standards). Analysis of the phospholipase C degradation products of the phospholipids demonstrated that 96% of the label in phosphatidylserine, 96% of the label in phosphatidylethanolamine, and 10% of the label in phosphatidylcholine was rendered water soluble. Thus even under the conditions of long-term radiolabeling the majority of the serine label in phosphatidylserine and phosphatidylethanolamine was confined to the polar portion of the molecule.

Table 1. Steady-state distribution of phosphorus and [^{3}H]serine label in phospholipids from BHK-21 cells

Lipid*	% phospholipid phosphorus	% uniform [^{3}H]serine label
Lyso-PtdCho	0.7 ± 0.7	3.5 ± 3.1
Cer*P*Cho	8.6 ± 0.9	11.9 ± 0.3
PtdCho	50.8 ± 4.4	10.7 ± 2.0
PtdSer	8.8 ± 0.7	20.8 ± 2.4
PtdIns	7.4 ± 0.8	0.8 ± 0.2
PtdEtn	16.5 ± 0.7	44.3 ± 1.6
Other†	7.3 ± 3.2	4.3 ± 1.1
Neutral lipids		3.8 ± 0.9

Values are mean ± SD for four determinations.
*IUPAC-IUB-recommended abbreviations (17).
†Other lipids include diphosphatidylglycerol, phosphatidic acid, lysophosphatidylinositol, and lysophosphatidylethanolamine. The recovery of phospholipid phosphorus was between 90% and 100% for all samples. The recovery of radiolabel was between 88% and 95% for all samples.

Effects of Ethanolamine upon Phosphatidylethanolamine Synthesis from [^{3}H]Serine. The effects of ethanolamine upon the incorporation of [^{3}H]serine into phosphatidylserine and phosphatidylethanolamine by cells in culture were examined. The results from this experiment are presented in Fig. 3. The addition of various amounts of ethanolamine to the growth media up to levels near physiological (20 μM) for rat serum (18) had only a slight inhibitory effect (19.8%) inhibition upon the incorporation of [^{3}H]serine into phosphatidylethanolamine. When cells were incubated with 20 μM ethanolamine for 24 hr prior to the addition of [^{3}H]serine, the identical results were obtained. Conversely, depriving cells of ethanolamine had little effect upon either phosphatidylserine or phosphatidylethanolamine synthesis. These results indicate that, even in the presence of ethanolamine, substantial amounts of phosphatidylserine are metabolized to form phosphatidylethanolamine.

When the cells were labeled with [^{3}H]ethanolamine and [^{3}H]serine at concentrations of 20 μM, the results shown in Fig. 4 were obtained. The apparent rate of labeling of phosphatidylethanolamine from [^{3}H]ethanolamine was 22 times higher than the rate observed for the labeling of this lipid by [^{3}H]serine. This result appeared inconsistent with that presented in Fig. 3. The high rate of labeling of phosphatidylethanolamine by free ethanolamine, however, appears to be a consequence of very low intracellular levels of ethanolamine. The intracellular levels of phosphoethanolamine were determined to be 4.8 μM (0.1 nmol/mg of protein). These intracellular levels would not be expected to significantly dilute the specific activity of the exogenous [^{3}H]ethanolamine. Assuming no radioisotopic dilution of precursor, the rate of phosphatidylethanolamine synthesis from [^{3}H]ethanolamine is 0.53 nmol/hr per mg of protein, which is 30% of the rate observed when phosphatidylserine is the precursor.

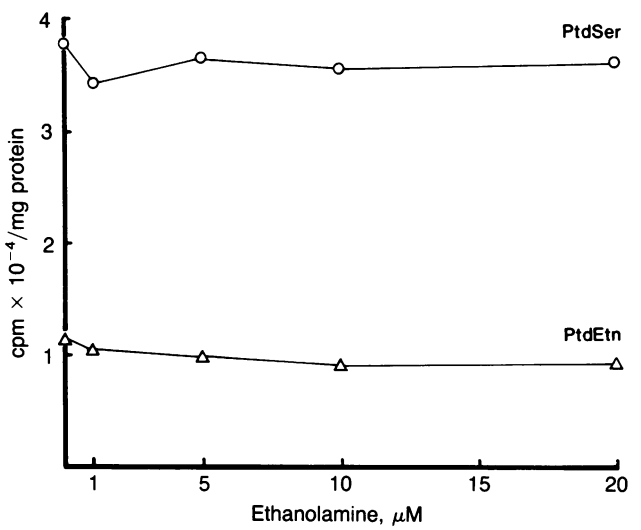

FIG. 3. Effect of ethanolamine upon the incorporation of [^{3}H]serine into phosphatidylserine and phosphatidylethanolamine. BHK-21 cells were incubated in the presence of ethanolamine at various concentrations for 2 hr and then the medium was exchanged for serine-free medium supplemented with 8 μCi of [^{3}H]serine and the indicated amounts of ethanolamine for 4 hr. The phospholipids were analyzed by thin-layer chromatography using solvent system A.

Therefore it appears that, even in the presence of ethanolamine, phosphatidylserine functions as the principal precursor of phosphatidylethanolamine.

DISCUSSION

The results from both pulse–chase and continuous labeling experiments demonstrate that, in mammalian cells in tissue culture, most of the cellular phosphatidylethanolamine is derived from phosphatidylserine. The analysis of degraded lipid products shows that the predominant label that appears in the glycerophospholipids is confined to the polar head group. There is no question that the synthesis of phosphatidylethanolamine via CDP-ethanolamine can contribute to phosphatidylethanolamine synthesis when free ethanolamine is available. However, the general consensus that mammalian phosphatidylserine decarboxylase does not contribute significantly to phosphatidylethanolamine synthesis is probably not correct for cells in culture. It may be argued that the phosphatidylserine–ethanolamine cycle does not yield net synthesis of a phospholipid molecule. This analysis is true if one focuses only upon the exchange reaction. However, if the reactions are written to include the reutilization of ethanolamine (denoted by asterisk),

$$\text{Ser} + \text{PtdEtn}^* \rightleftharpoons \text{PtdSer} + \text{Etn}^* \quad \text{[i]}$$

$$\text{Etn}^* + \text{ATP} + \text{CTP} + \text{diacylglycerol} \rightarrow \text{PtdEtn}^* + \text{ADP} + \text{CMP} + \text{PP}_i \quad \text{[ii]}$$

$$\text{PtdSer} \rightarrow \text{PtdEtn} + \text{CO}_2 \quad \text{[iii]}$$

$$\text{Ser} + \text{ATP} + \text{CTP} + \text{diacylglycerol} \rightarrow \text{PtdEtn} + \text{CO}_2 + \text{ADP} + \text{CMP} + \text{PP}_i\,, \quad \text{[I]}$$

it is apparent that only catalytic amounts of recycling phosphatidylethanolamine are necessary to achieve net synthesis of phosphatidylethanolamine from serine. The net reaction should be compared with the net reaction for synthesis of phosphatidylethanolamine from ethanolamine in eukaryotes and from serine in prokaryotes.

$$\text{Etn} + \text{ATP} + \text{CTP} + \text{diacylglycerol} \rightarrow \text{PtdEtn} + \text{ADP} + \text{CMP} + \text{PP}_i \quad \text{[II]}$$

$$\text{Ser} + \text{CTP} + \text{Ptd} \rightarrow \text{PtdEtn} + \text{CO}_2 + \text{CMP} + \text{PP}_i. \quad \text{[III]}$$

The net ATP requirement for phosphatidylethanolamine production in the higher eukaryotes is the same whether serine (reaction I) or ethanolamine (reaction II) is used. The higher eukaryotes require an additional mole of ATP (reactions I and II) to make phosphatidylethanolamine when compared to the prokaryotes (19) (reaction III). The results reported in this paper were obtained from work with BHK-21 cells, but the same experiments have also been performed with Chinese hamster ovary cells (CHO-K1), and the same results were obtained.

Ethanolamine appears to be a very poor isotopic trap for [^{3}H]serine incorporation into phosphatidylethanolamine. These observations provide strong evidence against the idea that the "decarboxylation cycle" (20) functions primarily to produce free ethanolamine, which is subsequently incorporated into phospholipid via CDP-ethanolamine. The results are more consistent with the idea that an ethanolamine–phosphatidylethanolamine cycle exists that permits serine to be metabolized to phosphatidylethanolamine. The results indicate that, even in the presence of ethanolamine, 70–80% of the phosphatidylethanolamine is synthesized from serine. The inability of ethanolamine to significantly inhibit serine incorporation into phosphatidylethanolamine also suggests

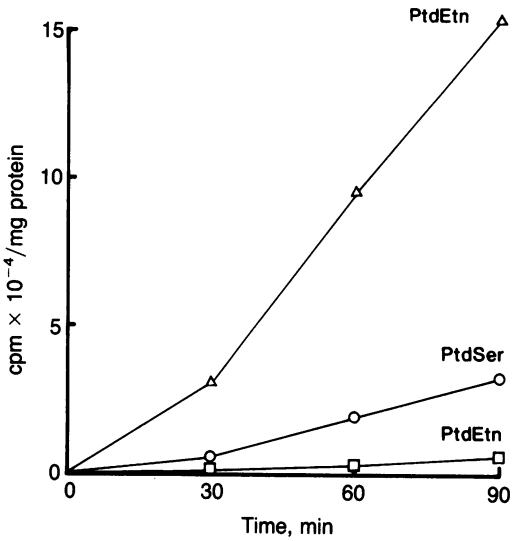

FIG. 4. Phospholipid synthesis from [^{3}H]ethanolamine and [^{3}H]serine precursors. BHK-21 cells were incubated in the presence of [^{3}H]serine or [^{3}H]ethanolamine (20 μM, specific activity 250 μCi/μmol). The cells were harvested at the indicated times and the phospholipids were analyzed by thin-layer chromatography using solvent system A. △, [^{3}H]Ethanolamine precursor; ○ and □, [^{3}H]serine precursor.

that there is neither short- nor long-term regulation of the activity of this enzyme by ethanolamine. The data indicate that phosphatidylserine decarboxylase is constitutively synthesized. In addition, these results also raise the possibility that, even in the presence of excess ethanolamine, mitochondrial phosphatidylethanolamine may be derived primarily from phosphatidylserine. Should this prove to be the case, it may explain the paradoxical difference observed for the equilibration rate of phosphatidylcholine and phosphatidylethanolamine between the microsomes (endoplasmic reticulum) and mitochondria (21).

Another interesting aspect of the present work is the demonstration that ethanolamine deprivation forces mammalian cells to make the bulk of their phosphatidylethanolamine via a mechanism that requires the transport of phosphatidylserine from the endoplasmic reticulum to the mitochondrion. This metabolic disposition may well serve as the basis for a genetic test of how phospholipids are transferred among subcellular membranes.

The author acknowledges the excellent technical assistance of Ms. Janet L. Frazier. This research was supported by Grants RR 05842 and GM 32453 from the National Institutes of Health.

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