Biogenesis of Membrane Lipids: Mutants of *Escherichia coli* with Temperature-Sensitive Phosphatidylserine Decarboxylase*

(membrane function/phosphatidylethanolamine/*psd* mutants/phospholipid)

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ABSTRACT   Phosphatidylserine decarboxylase cata-lyzes the last step in the pathway leading to phosphatidyl-ethanolamine, the principal membrane lipid of *E. coli*. Mutants of *E. coli* have now been isolated in which this enzyme is thermolabile. The structural gene for phos-phatidylserine decarboxylase (*psd* gene) is closely linked to the *purA* locus at about 83 min on the standard map of the *E. coli* chromosome. When a mutant with thermolabile decarboxylase is incubated at 42°, growth ceases, but only after a substantial fraction (20–40%) of the total phospho-lipid of the cell has been replaced by phosphatidylserine. Examination of such mutants with altered content of phospholipids may shed light on the role of specific phos-pholipids in membrane function.

Phospholipids are major structural components of biological membranes throughout nature and are also thought to play important functional roles in certain membrane-localized processes. One approach to the study of specific lipid functions is to isolate mutants defective in a single step of the biogenesis of membrane lipids, thus giving rise to cells with a specifically altered pattern of membrane lipids.

Various classes of bacterial mutants deficient in some phase of lipid synthesis have been isolated and have proven useful in studying certain aspects of the metabolism and function of phospholipids (for reviews see refs. 1 and 2). Until recently, the only phospholipid mutants available were affected early in the pathway at the level of phosphatidic acid synthesis (3–7). Reports have recently appeared, however, describing the isolation of mutants of *Escherichia coli* defective in some early stage of the formation of phosphatidylethanolamine, the predominant phospholipid in *E. coli* (8–10).

This report describes the isolation of mutants with tem-perature-sensitive phosphatidylserine decarboxylase, the mapping of the structural gene for the decarboxylase (*psd* gene), and a preliminary characterization of the phenotype of some of these mutants.

MATERIALS AND METHODS

Bacterial and Phage Strains. *E. coli* strains (described in Table 1) used in this study are all K-12 derivatives. Phage P1c was used for generalized transduction. Phage P1CM*clr*100 (11) was obtained from Dr. A. L. Taylor. Transduction was performed essentially according to the method of Lennox (12).

Preparation of Extracts. Single colony isolates were grown in 5 ml of L-broth (13) at 30° to a density of 5 to 9 × 10⁸ cells per ml. The culture was centrifuged at 3500 × *g* for 30

min and the cells were immediately suspended in 2 ml of a solution of lysozyme (100 μg/ml) (Worthington, 11,800 units/ mg) in 25 mM Tris·HCl buffer (pH 8.2) containing 2.5 mM Na₂EDTA at 4°. After approximately 10 min at room tem-perature the suspensions visibly cleared and the lysates were stored at 4° until the time of assay.

Assay for Phosphatidylserine Decarboxylase. The assay mix-ture contained 0.1 M potassium phosphate buffer (pH 7.0), 0.1% Triton X-100, 0.2 mM phosphatidyl[1-¹⁴C]serine (10,000 cpm/μmol), and enzyme in a final volume of 0.5 ml. Normally the enzyme extract was incubated in the assay mixture in the absence of the substrate for 30 min at the ap-propriate temperature and the reaction was started by the addition of 0.1 ml of 1 mM phosphatidyl[1-¹⁴C]serine. After 20 min, 0.5 ml of 1 N H₂SO₄ was added and the amount of radioactive CO₂ liberated was determined (14). One unit of activity is the amount of enzyme which forms 1 nmol of product per min at 25°.

TABLE 1. *Bacterial strains*

Strain	Relevant characteristics	Source or construction
A324	F⁻, *lacI, pro, thi, strA*	S. Luria
EH36	*psd-1*, contains a sec-ond, unidentified mutation that pre-vents growth at 42°	Derived from A324 after mutagenesis
EH102	*psd-1*, normal growth at 42°	Transductant of EH36 from A324
EH103	Same as EH102	Same as EH102
KG20	F⁺, *purA, ampA1, his, argH, pro, thi, strA*	K. G. Eriksson-Grennberg
ES4	F⁻, *purA*	CGSC*
KLF17/KL132	F117/*pyrB, thi, pro, his, recA, thy, rel, thr, leu, strA*	CGSC*
EH140	F117 *purA*⁺/ES4 *purA* (P1CM*clr*100 lysogen)	Episome transfer; KLF17/KL132 × ES4, followed by infection with P1CM*clr*100
EH150	*psd-2*₍ₜₛ₎, *purA*⁺	Transductant of ES4 (see *text*)
EH180	Revertant of EH150 that grows normally at 42°	Spontaneous revertant

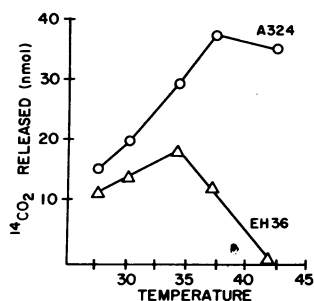* *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

FIG. 1. Temperature profile of phosphatidylserine decarboxylase activity in extracts of *E. coli* A324 and EH36. Bacteria were grown at 30° to 5 to 6 × 10⁸ cells per ml in L-broth. Extracts were incubated for 12 min in the assay mixture in the absence of the substrate (phosphatidyl[*1*-¹⁴C]serine) at the temperatures indicated. Substrate was then added, and decarboxylase activity was measured as the release of $^{14}CO_2$ during 20 min further incubation at the same temperature. (○) A324 extract; (△) EH36 extract.

Materials. DL-[*1*-¹⁴C]serine, DL-[*3*-³H]serine, and [³²P]phosphoric acid were products of the New England Nuclear Co., Boston. Phosphatidyl[*1*-¹⁴C]serine was prepared enzymatically as previously described (15).

RESULTS

On the assumption that the phosphatidylserine decarboxylase is essential for the continued growth of *E. coli*, we examined a number of conditional lethal mutants, unable to grow at 42°, for a temperature-sensitive decarboxylase. One such strain (EH36) was indeed found to have an altered decarboxylase. However, this alteration was not responsible for the observed temperature-sensitivity of growth, since the mutant strain did not accumulate phosphatidylserine *in vivo*. EH36 is, therefore, a double mutant, with a lesion in the structural (*psd*) gene for the decarboxylase, and a second mutation, as yet unidentified, responsible for the inability to grow at 42°. The mapping of the *psd* mutation, however, made it possible to select other mutants, in which phosphatidylserine accumulates *in vivo* at 42°, preventing growth at that temperature.

Isolation of Strain EH36. Mutagenesis was performed on A324 cells according to the method of Adelberg *et al.* (16) with 300 μg/ml of *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine in Tris-maleate buffer (pH 6.0) containing 0.2% glucose for 30 min at 37°. Approximately 2% of the survivors, plated out on L-broth agar at 30°, were unable to grow on similar plates at 42°, as revealed by replica plating. Of 92 temperature-sensitive isolates examined, one (EH36 *psd-1*) had less than 1% of wild-type phosphatidylserine decarboxylase activity in extracts at 42°.

As shown in Fig. 1, an extract prepared from EH36 cells has an altered temperature profile with near normal levels of activity at the low temperatures. Extracts of the mutant did not inhibit the decarboxylase activity observed in extracts of A324 at 42°, arguing against the existence of a heat-activated inhibitor or phospholipase.

Characterization of Altered Phosphatidylserine Decarboxylase in the psd-1 Mutant. Recombinants of EH36 were obtained by P1 transduction, which were able to grow at 42°, but which nevertheless exhibited the same thermolabile decarboxylase activity observed in EH36. Clearly the altered decarboxylase is not the reason for the failure of EH36 to grow at 42°.
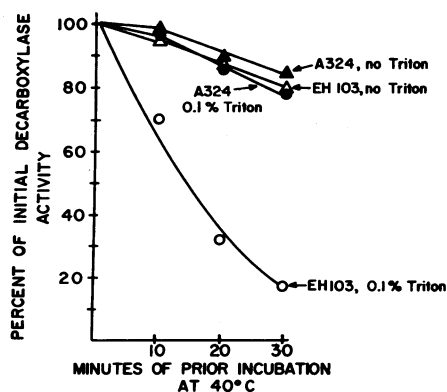

FIG. 2. Thermolability of the phosphatidylserine decarboxylase activities in extracts of strains A324 and EH103. Sonicated extracts of A324 and EH103 cells grown at 37° were incubated in phosphate buffer in the presence or absence of 0.1% Triton X-100 at 40°. At the indicated times, samples were removed and their initial rates of decarboxylase activity were determined at 25°. The data are plotted relative to unheated samples (= 100%). (●) A324 extract + 0.1% Triton; (▲) A324 extract, no Triton; (○) EH103 extract + 0.1% Triton; (△) EH103 extract, no Triton.

The thermal lability of the mutant decarboxylase was examined in one such *psd-1* recombinant in the experiment of Fig. 2. The mutant enzyme is rapidly inactivated by treatment at 40° in the presence of 0.1% Triton X-100, but not in its absence. The same concentration of Triton X-100 has little or no effect on the wild-type enzyme. However, at a higher concentration of Triton (1.1%) even the wild-type enzyme is inactivated at 40°, as shown in Fig. 3. Thus, as a result of the *psd-1* mutation, the enzyme has acquired a higher sensitivity to the combined treatment with detergent and heat. The mutant enzyme is protected to a considerable degree against
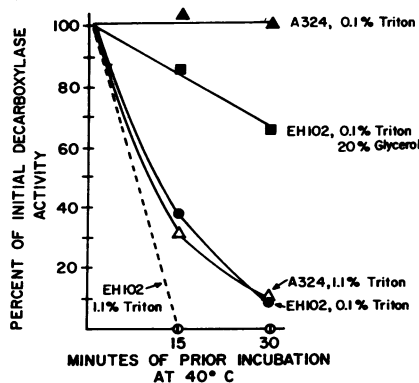

FIG. 3. Triton-sensitivity of phosphatidylserine decarboxylase activities in extracts of A324 and EH102 at 40°. Triton-extracted enzyme fractions (Fraction 2, Table 2) of A324 and EH102 cells were incubated in phosphate buffer at two different Triton concentrations (0.1% and 1.1%) at 40°. One sample of EH102 extract was in addition incubated in the presence of both 0.1% Triton and 20% glycerol at 40°. At the indicated time intervals, samples were removed and their initial rates of decarboxylase activity were determined at 25°. The data are plotted relative to unheated samples (= 100%). (▲) A324 extract + 0.1% Triton; (△) A324 + 1.1% Triton; (●) EH102 extract + 0.1% Triton; (○) EH102 extract + 1.1% Triton; (■) EH102 extract + 0.1% Triton + 20% glycerol.
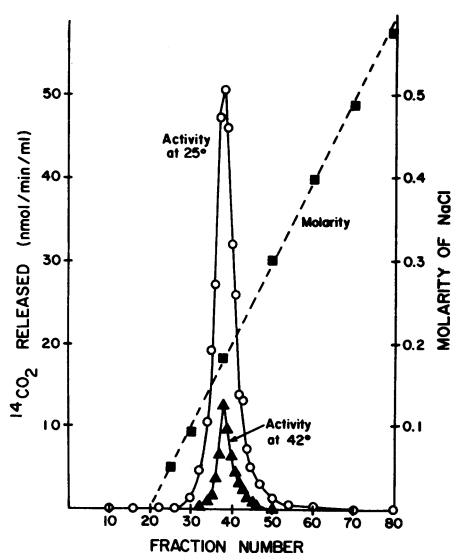
FIG. 4. DEAE-cellulose chromatography of a combined extract of A324 and EH102 cells. Enzyme extracts of A324 and EH102 cells (Fraction 3, Table 2) were mixed to produce a ratio of mutant to wild-type enzyme of 3.3:1. This combined extract was applied to a DEAE-cellulose column (1.3 cm² × 12 cm; Whatman DE-52) at 25° in 10 mM potassium phosphate buffer (pH 7.4) containing 1% Triton X-100, 10% glycerol, and 10 mM 2-mercaptoethanol. After the column was washed with this buffer, the enzyme was eluted with a linear gradient of NaCl in the same buffer. Decarboxylase activity was determined at 25° and at 42° as described in *Materials and Methods*. Both peaks of activity were eluted at 0.18 M NaCl. (■) Molarity of NaCl; (O) phosphatidylserine decarboxylase activity at 25°; (▲) phosphatidylserine decarboxylase activity at 42°.

this combined treatment by the presence of 20% (v/v) glycerol (Fig. 3).

Purification of the Decarboxylase from a psd-1 Strain. The purification of the thermosensitive decarboxylase was found to be identical with that of the wild-type enzyme through at least the first four steps (Table 2 and Fig. 4). At each stage the mutant decarboxylase retained its characteristic thermosensitive behavior, exhibiting less than 1% of wild-type activity at 42° under the standard assay conditions. When preparations of wild-type and mutant enzyme were mixed and chromatographed, the peak of the thermosensitive activity was eluted in the same fractions as wild-type decarboxylase (Fig. 4). The psd-1 mutation thus leads to the production of a slightly altered enzyme with reduced thermal stability in

TABLE 2. *Purification of mutant and wild-type phosphatidylserine decarboxylase*

	Specific activity (Units/mg of protein)		Yield (%)	
Fraction	A324	EH102	A324	EH102
1. Cell-free extract	18	10	(100)	(100)
2. Triton extract	48	32	76	69
3. Acetone treatment	61	39	57	45

A324 and EH102 *psd-1* cells were grown and phosphatidylserine decarboxylase was purified essentially as described previously (14).

TABLE 3. *Failure of EH102 psd-1 to accumulate phosphatidylserine in vivo*

	Incorporation of DL-[1-¹⁴C]serine into lipid (cpm/ml of culture)		Incorporation of DL-[3-³H]serine into lipid (cpm/ml of culture)	
Strain	30°	42°	30°	42°
A324 *psd⁺*	457	288	7,047	2,721
EH102 *psd-1*	289	330	6,388	2,416

Bacterial cultures (6 ml) were grown in minimal medium at 30° to a cell density of about 5 × 10⁸/ml. At this point half of each culture was shifted to 42°. After 15 min further incubation DL-[1-¹⁴C]serine and DL-[3-³H]serine was added at a final concentration of 0.05 mM. The specific activity of the DL-[1¹⁴-C]serine was 5 × 10⁶ cpm/μmol and that of the DL-[3-³H]serine was 10⁷ cpm/μmol. Labeling was terminated after 30 min by the addition of an equal volume of 1 N HCl and the immediate chilling of the suspension. Carrier cells were added and the suspensions were centrifuged at 8000 × *g* for 30 min at 4°. The pellets were extracted with 3 ml of methanol for 15 min at 55°. After the extracts were cooled to room temperature, chloroform (6 ml) was added with thorough mixing. The lipid phase was washed twice with 10 ml of distilled water. A portion (2 ml) of the chloroform layer was dried and its radioactivity was measured.

the presence of detergent, but with other properties closely similar to those of wild-type enzyme.

Activity of Phosphatidylserine Decarboxylase In Vivo in the psd-1 Mutant. Strain EH102, bearing the *psd-1* mutation, grows at 42° at the same rate as wild-type. The experiments of Figs. 2 and 3 explain this finding, since the membrane-bound mutant enzyme is almost as stable as wild-type enzyme, and its altered stability is detected only in the presence of added detergent.

Phosphatidylserine is present only in trace amounts in normal cells of *E. coli* (15), since it is decarboxylated almost as rapidly as it is formed. To test the function of the decarboxylase in the *psd-1* mutant *in vivo*, mutant EH102 and control parental cells were labeled with a mixture of [1-¹⁴C]-serine and [3-³H]serine at 30° and at 42° (Table 3).

The incorporation of [3-³H]serine into phospholipid should reflect the amount of synthesis of phosphatidylethanolamine plus phosphatidylserine. The incorporation of [1-¹⁴C]serine

TABLE 4. *Three-factor cross between psd, purA, and ampA markers*

Donor	Recipient	Selection	Distribution of unselected markers	
			Type	No.
EH102 (*psd-1*, *ampA⁺*, *purA⁺*)	KG20 (*psd⁺*, *ampA1*, *purA*)	Pur⁺	*ampA1*, *psd⁺*	12
			ampA⁺, *psd-1*	5
			ampA⁺, *psd⁺*	0
			ampA1, *psd-1*	12

A P1 lysate of strain EH102 *psd-1* was prepared and used for generalized transduction with KG20 *ampA1*, *purA* as recipient. Selection was for Pur⁺ recombinants, which occurred with a frequency of approximately 40/10⁶ plaque-forming units. Twenty-nine recombinants were isolated, tested for ampicillin sensitivity, and assayed for thermolabile phosphatidylserine decarboxylase activity.

should in turn reflect the amount of accumulated phosphatidylserine, since 1-^{14}C-radioactivity will be lost upon decarboxylation to phosphatidylethanolamine. Only traces of phosphatidylserine accumulate in EH102 or in wild type, at either 30° or 42° (Table 3).

Genetic Mapping of the psd Locus. Preliminary mapping of the *psd-1* mutation in EH36 was accomplished by standard conjugation mapping techniques (to be described in detail elsewhere). Final localization was achieved by transduction with phage P1. The *psd-1* locus is 59–72% cotransducible with *purA* and approximately 6% cotransducible with *melA*. No cotransduction was observed with *pyrB*. The *psd* locus is thus placed at about 83 min on the Taylor and Trotter map (17). The gene order in this region was determined by a triple cross performed again by P1 transduction. As shown in Table 4, the least frequent recombinant class is *purA*$^+$, *ampA*$^+$, *psd*$^+$, and if this is presumed to require a quadruple cross-over, the gene order in this region is *ampA-psd-purA*.

Isolation of Conditional Lethal Phosphatidylserine Decarboxylase Mutants. To obtain decarboxylase mutants that are expressed *in vivo*, as well as *in vitro*, a strategy was developed, using a variation on the theme of localized mutagenesis (18). It should be generally applicable for any genetic locus with a known high-frequency cotransducing marker.

Strain EH140 (Table 1) contains the F117 episome (19) with the *psd*$^+$ gene closely linked to the *purA*$^+$ locus. Another copy of the *psd*$^+$ gene lies on the chromosome; thus a temperature-sensitive mutation in the episomal *psd* gene should not prevent the growth of the strain at higher temperatures. It is also lysogenic for phage P1CM*clr*100 (11) so that high titer lysates can easily be produced.

This strain was subjected to mutagenesis with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine. A P1 lysate was prepared (13) and used for the transduction of the episomal *purA*$^+$ gene with its closely linked *psd* locus to an unmutagenized *purA* recipient (ES4). The *purA*$^+$ recombinants, grown out at 30°, were tested for growth at 42° by replica-plating. Temperature-sensitive mutants thus isolated should result from mutations cotransduced with the *purA*$^+$ gene. From the cotransduction frequency of *psd* and *purA*, it is apparent that about 60% of the *purA*$^+$ recombinants must also have received their *psd* genes via P1 transduction from the mutagenized EH140 cells. Thus, some of the temperature-sensitive mutants should be caused by mutations in the *psd* locus.

Of approximately 45,000 *purA*$^+$ recombinants examined, 11 were unable to grow on L-broth plates at 42°, and were shown not to be lysogenic for phage P1CM*clr*100. Of the 11, three have a defective phosphatidylserine decarboxylase as measured under standard assay conditions. In addition, all three of these mutants accumulate phosphatidylserine *in vivo* at 42° and are thus of the phenotype sought for studies of membrane function.

One such mutant (EH150 *psd-2*(ts)) was compared with a revertant, EH180, with recovered ability to grow at 42°. The lipid composition of the two strains was measured in the experiment of Fig. 5. Only traces of phosphatidylserine were found in either strain at 30°. However, 4 hr after the shift to 42° (approximately three doubling times) 25% of the phospholipid of the mutant EH150 was in the form of phosphatidylserine, whereas there was no accumulation of this lipid in the revertant EH180. In other experiments with all
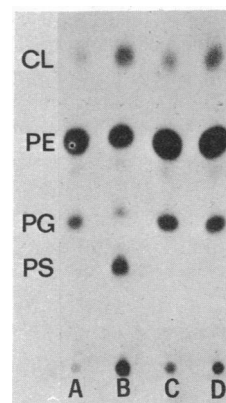

FIG. 5. Thin-layer chromatography of phospholipids extracted from EH150 *psd-2*(ts) and EH180 cells. Bacterial cultures were grown for three generations in medium 56-LP (20) in the presence of [^{32}P]phosphate (11 Ci/mol) at 30° to a density of approximately 7 × 10^7 cells per ml. Half of each culture was then shifted to 42°. After 4 hr at 42° samples of the bacterial cultures (2.4 ml) were added to 9 ml of methanol:chloroform (2:1, v/v), and thoroughly mixed. Three milliliters of 2 M KCl containing 2.5 mM Na$_2$EDTA were added followed by an additional 3 ml of chloroform. The lower phase was washed twice with 9 ml of 2 M KCl containing 2.5 mM Na$_2$EDTA. A portion (1 ml) of the chloroform layer was dried and the lipids were redissolved in 0.05 ml of chloroform:methanol (2:1, v/v). Half of this amount was applied to a silica-gel sheet (Baker-flex silica gel 1B) and developed in chloroform:methanol:water (65:25:4, v/v). The positions of radioactive phospholipids were determined by radioautography and the percentage of total isotope present in each spot was ascertained by cutting out and determining the radioactivity of the spots. The abbreviations are: CL, cardiolipin; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; and PS, phosphatidylserine. *Lane A*, EH150 *psd-2*(ts) culture maintained at 30°; *Lane B*, EH150 culture shifted to 42°; *Lane C*, EH180 revertant culture maintained at 30°; *Lane D*, EH180 culture shifted to 42°.

three mutants, accumulation of phosphatidylserine has ranged from 20 to 40%.

The mutant enzyme in EH150 *psd-2*(ts) is very labile, since no activity can be detected under standard assay conditions even at 30°. However, when 20% glycerol or 20% sucrose was added to the assay mixture, about 20% of wild-type activity could be detected at 30° in the mutant extracts. This activity was completely abolished by prior heating of the extracts at 42°. Thus, the mutant decarboxylase of EH150 can be shown to be thermolabile *in vitro*, as well as *in vivo*.

Only a few revertants of EH150 *psd-2*(ts) with restored ability to grow at 42° have so far been examined. All of these contain a decarboxylase apparently more thermostable than that of EH150 *psd-2*(ts), but less than that of wild-type cells. Extracts of these revertants are similar to extracts of the *psd-1* mutant; that is, the decarboxylase is active at 30° under standard assay conditions, but not at 42°. Reversion of these strains thus appears to be caused by intragenic suppression rather than true back-reversion. Like EH36 *psd-1*, the revertants do not accumulate phosphatidylserine at 42° (as shown in Fig. 5).

DISCUSSION

The altered properties of the phosphatidylserine decarboxylase caused by the *psd-1* mutation offer a striking illustration of

the stabilization of an enzyme by its association with the cell membrane. While membrane-bound, the mutant enzyme is little affected by heating at 40°, but in the presence of the detergent Triton X-100 it is completely inactivated. It is possible that many potentially lethal mutations in membrane-bound enzymes may be phenotypically silenced by the unique stabilizing environment of the membrane. This may be especially significant in the search for temperature-sensitive mutants, since hydrophobic interactions are strengthened at higher temperatures.

The decarboxylase from the *psd-1* mutant is active at 30° under standard assay conditions in the presence of Triton X-100. The *psd-2*$_{(ts)}$ mutation leads to the production of an enzyme that is inactivated at 42° *in vivo* and displays no activity *in vitro* in the presence of Triton even at 30°; it is thus much less stable than the enzyme produced in the *psd-1* mutant. Significantly, the limited number of temperature-resistant revertants so far studied are similar to the *psd-1* mutant in that the revertant enzymes are apparently less thermostabile than true wild-type decarboxylase. Either the *psd-2*$_{(ts)}$ mutant, or these revertants, may represent double-mutant alleles of the wild-type gene.

The isolation of conditional lethal mutants, unable to grow at a temperature at which the phosphatidylserine decarboxylase does not function *in vivo*, confirms the initial assumption of the essential nature of the decarboxylase to the continued growth of *E. coli* and supports the proposed metabolic pathway for the synthesis of phosphatidylethanolamine in this organism (15). Cessation of growth in these mutants could result from the lack of sufficient phosphatidylethanolamine to participate in some vital membrane function for which other phospholipid species cannot substitute. On the other hand, the accumulation of phosphatidylserine may itself adversely affect membrane function and prove lethal to the cell. The resolution of such questions obviously requires a detailed study of membrane function under conditions in which the decarboxylase is inoperative.

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