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Membrane Assembly: Movement of Phosphatidylserine Between the Cytoplasmic and Outer Membranes of *Escherichia coli*

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Phosphatidylserine, normally a trace phospholipid in *Escherichia coli*, accumulates at high levels in temperature-sensitive phosphatidylserine decarboxylase mutants at nonpermissive temperatures. The intracellular localization of this phospholipid has now been determined. All of the accumulated phosphatidylserine is membrane bound and is distributed about equally between the inner and outer membrane fractions of *E. coli* as determined by isopycnic sucrose gradient fractionation. Phosphatidylserine is therefore effectively translocated from the inner to the outer membrane. Furthermore, this movement is bidirectional. Outer membrane phosphatidylserine can return to the inner membrane, as shown by the complete conversion of accumulated radioactive phosphatidylserine to phosphatidylethanolamine by inner membrane phosphatidylserine decarboxylase during chase periods. Pulse-chase experiments indicated that newly made phosphatidylserine appears first in the inner membrane and then equilibrates between the inner and outer membranes with a half-time of 12 to 13 min.

In recent years, extensive progress has been made in studies of the genetic regulation of the phospholipid composition of *Escherichia coli* membranes. The fatty acid components and the polar head group can now be altered within certain limits by genetic manipulation (27, 33). The study of these phospholipid mutants permits analysis of the functional role of specific phospholipids in biological membranes and is proving useful in elucidating the mechanisms of membrane biogenesis.

In this study we report the intracellular location of phosphatidylserine in temperature-sensitive, conditionally lethal phosphatidylserine decarboxylase (*psd*) mutants of *E. coli*. Phosphatidylserine decarboxylase catalyzes the formation of phosphatidylethanolamine, the major *E. coli* lipid (16), from phosphatidylserine. Phosphatidylserine is normally present only as a rapidly decarboxylated trace membrane component in *E. coli* (<1% of total phospholipid) (1), but it accumulates at high levels at the nonpermissive temperatures in *psd* mutants blocked in this terminal step of phosphatidylethanolamine biosynthesis (11–13).

Studies on the localization and biogenesis of components of the *E. coli* envelope have been made possible by the availability of techniques for separating the inner (cytoplasmic) and outer membranes of the envelope (23, 25). The inner membrane contains all of the enzymes of phospholipid biosynthesis in *E. coli*, including the phosphatidylserine decarboxylase (4, 20, 27, 35). Since phosphatidylethanolamine, and phosphatidylglycerol as well, are found in both the inner and outer membranes (23), a means must exist for their transfer from the site of biosynthesis, the inner membrane, to the outer membrane. In the case of phosphatidylethanolamine, such transfer has been experimentally demonstrated by pulse-chase studies. Newly made phosphatidylethanolamine appears first in the inner membrane and is then rapidly equilibrated between the inner and outer membranes (9, 26). In addition, the transfer appears to be reversible. Jones and Osborn (15), by an approach involving liposome fusion with the outer membrane, have shown that phospholipids can move from the outer to the inner membrane in *Salmonella typhimurium*.

A goal of the present study was to shed light on the mechanism and specificity of phospholipid transfer by examining the incorporation of the "foreign" lipid phsophatidylserine into the inner and outer membranes of the temperature-sensitive *psd* mutants. The fate of radioactively labeled phosphatidylserine during the chase has also been investigated to obtain further information about the possible reversibility of phospho-

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lipid transfer between the two cellular membranes. Finally, we report pulse-chase studies showing that newly made phosphatidylserine in a *psd* mutant behaves similarly to phosphatidylethanolamine in its rate of transfer from the inner membrane to the outer membrane.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* K-12 strains EH470 *psd-4* and EH450 *psd-2* were used in these studies. Both were constructed by P1 transduction of the prototropic strain W3110 (12). They are temperature sensitive for growth, the nonpermissive temperature being 42°C, and synthesize temperature-sensitive phosphatidylserine decarboxylase (13). At semipermissive (33 or 37°C) or nonpermissive (42°C) temperatures, phosphatidylserine accumulates at the expense of phosphatidylethanolamine (13).

Specific culture conditions, including medium LP56 (6), L broth (18), and PPBE (25), are given below. L broth or PPBE was frequently included in otherwise defined medium because this results in improved separation of inner and outer membranes (25). Doubling times were between 40 and 60 min. Cell growth was monitored by absorbance at 650 nm. In general, growth was exponential to cell densities of about 3×10^9 cells per ml and slowly declined in rate thereafter.

Membrane fractionation. The total membrane fraction was prepared, and isopycnic sucrose gradient centrifugation for separation of the inner and outer membranes was performed essentially as described by Osborn and Munson (25). Spheroplasts were lysed sonically, and subsequent steps were performed in the presence of hydroxylamine (10 mM), an inhibitor of phosphatidylserine decarboxylase (29, 32). Fractions of about 0.45 ml were collected from the sucrose gradients. Outer membrane (H), inner membrane (L1 and L2), and intermediate density (M) bands were located on the basis of radioactivity, and fractions were combined to yield total outer membrane and total inner membrane pools. Cyanide-sensitive NADH oxidase activity (23) was used as an inner membrane marker. In all cases, the outer membrane pool contained less than 10% of the total NADH oxidase activity. Recoveries of phospholipid in the procedure were 70 to 80% relative to the sonically prepared lysates.

Phospholipid analysis. Phospholipids were extracted from membrane fractions, or from intact cells, as follows. Bovine serum albumin (0.25 mg/ml, final concentration) was added, followed by trichloroacetic acid (5%, wt/vol, final concentration) (31), and the precipitate, containing protein and lipid, was collected by centrifugation. Extraction of lipid was then accomplished by a modification of the Bligh and Dyer (5) procedure, as described previously (31), yielding organic (chloroform) and aqueous phases. The aqueous phase (usually 1.9 ml) was extracted with an additional 0.5 ml of chloroform. The combined chloroform phases (usually 1.5 ml) were in turn washed with 2.85 ml of methanol-water (1:0.9, vol/vol; chloroform saturated) and dried in a stream of N_2, and the phospholipid composition was determined by thin-layer chromatography on plastic-backed silica gel thin-layer plates (20 by 20 cm; polygram Sil G; Brinkmann Instruments, Inc., Westbury, N.Y.). Except where indicated otherwise, chloroform-methanol-water (65:25:4, vol/vol) was used as the developing solvent. Radioactive phospholipids were located on thin-layer plates by autoradiography as described previously (31), and radioactivity was measured by liquid scintillation counting of silica gel scraped from the plastic backing.

Chase of radioactivity from uniformly labeled [^{3}H]phosphatidylserine. A culture of strain EH470 *psd-4* in medium LP56 containing glycerol (0.004%, wt/vol), [2-^{3}H]glycerol (9.5 Ci/mmol; 7.5 μCi/ml), casein hydrolysate (0.5%, wt/vol), and $^{32}P_i$ (0.5 μCi/ml) was grown at 33°C from an initial cell density of about 2×10^7 cells per ml. After 5 h, when the cell density had reached about 7×10^8 cells per ml, a chase of the labeled glycerol was begun by the addition of unlabeled glycerol to a final concentration of 0.5% (wt/vol). During the chase, samples (1 ml) were removed at various times and analyzed with respect to ^{3}H and ^{32}P in the various species of phospholipid.

Chase of radioactivity from pulse-labeled [^{3}H]phosphatidylserine. A culture (11 ml) of strain EH470 *psd-4* in medium LP56 containing glycerol (0.025%, wt/vol), casein hydrolysate (0.5%, wt/vol), L broth (0.05%, vol/vol), and $^{32}P_i$ (0.5 μCi/ml) was grown at 33°C from an initial cell density of about 1.5×10^7 cells per ml. After 4 h, at a density of 5×10^8 cells per ml, the cells were harvested by centrifugation, washed with 2 ml of the original medium lacking glycerol, and suspended in 11 ml of the latter. (The presence of glycerol during the initial 4-h period of growth was designed to induce proteins of the *glp* operon [19] and bring about efficient uptake and utilization of [2-^{3}H]glycerol during the subsequent pulse-labeling.) After 10 min of additional growth, [2-^{3}H]glycerol (9.5 Ci/mmol) was added to a final concentration of 9 μCi/ml. Fifty seconds later, a sample (1 ml) was removed for extraction and analysis of phospholipid, unlabeled glycerol was added to a final concentration of 0.5% (wt/vol), and the culture was filtered (0.45-μm type HA filters; Millipore Corp., Bedford, Mass.); the cells were washed on the filter with 1 ml of the medium containing unlabeled glycerol and suspended in 10 ml of the latter. Samples (1 ml) were then removed at various times for extraction of phospholipid and determination of radioactivity in the various species of phospholipid.

Chase of radioactivity from [^{3}H]phosphatidylserine in inner and outer membranes. A culture of strain EH470 *psd-4* was grown as described for Fig. 1 (below), except that the medium also contained [2-^{3}H]glycerol (9.5 Ci/mmol; 9 μCi/ml). When the culture had reached a cell density of about 7×10^8 cells per ml, a portion (30 ml) was removed for separation of the inner and outer membranes; the remainder was filtered, and the cells were washed on the filter with 10 ml of medium lacking [2-^{3}H]glycerol and containing unlabeled glycerol at a concentration of 0.5% (wt/vol). The cells were suspended in the latter medium (80 ml), and growth was continued. At 15, 50, and 102 min after the filtration, portions (30, 20, and 15 ml, respectively) were removed for separation of the inner and outer membranes. Growth continued exponentially during this period. Once inner and outer membranes were separated for the various culture samples, phosphatidylserine was isolated from pooled fractions by extraction and silica gel thin-layer chromatography; the region of the thin-layer chromatograms containing phosphatidylserine was scraped, and the phosphati-

dylserine was eluted from the silica gel by successive treatments with 1 ml each of chloroform-methanol (2:1, vol/vol), chloroform-methanol (1:2, vol/vol), and methanol. The phosphatidylserine was deacylated (8), and the resulting glycerophosphorylserine was analyzed for $^{3}H/^{32}P$ ratio after paper chromatography (Whatman 43 paper; developing system ethanol–1 M ammonium acetate, pH 7.4 [7:3, vol/vol]) and elution from the paper (with water as eluting solvent). Glycerophosphorylserine spots were located on the paper chromatogram by autoradiography. Recoveries of radioactivity after the deacylation and paper chromatography were about 40 to 50%.

Localization of [^{3}H]phosphatidylserine in the inner and outer membranes during the chase period after short pulse-labeling with [2-^{3}H]glycerol. A culture (150 ml) of strain EH450 *psd-2* was grown in PPBE (25) medium containing glycerol (0.2%, wt/vol), $MgSO_4$ (20 mM), and $^{32}P_i$ (0.5 μCi/ml) at 42°C. After 3 h, at a density of 11 × 10^8 cells per ml, the cells were harvested by centrifugation, washed twice with medium (5 ml; 42°C) lacking glycerol, resuspended in the latter medium (150 ml), brought to 37°C within 5 min, and labeled with [2-^{3}H]glycerol (10 Ci/mmol; 10 μCi/ml). After 1 min of labeling, unlabeled glycerol was added to a final concentration of 0.5% (wt/vol). At various times during the chase, portions (20 ml) were removed for separation of inner and outer membranes. Phosphatidylserine was extracted and isolated (by silica gel thin layer chromatography with chloroform-methanol-acetic acid [7:3:1, vol/vol] as the developing solvent) from pooled fractions and analyzed with respect to $^{3}H/^{32}P$ ratio.

Radiochemicals. [^{32}P]phosphate and [2-^{3}H]glycerol were obtained from New England Nuclear Corp., Boston, Mass. The radiochemical purity of the [2-^{3}H]glycerol, as judged by paper chromatography with 1-butanol–acetic acid–water (5:4:1, vol/vol) as the developing solvent, was greater than 90%.

RESULTS

Localization of phosphatidylserine in mutant strains having elevated phosphatidylserine levels. Although all of the phospholipid normally present in *E. coli* is localized in the cell envelope, the possibility remained that the high levels (up to 50 mol% of total phospholipid) of phosphatidylserine found in the temperature-sensitive *psd* mutants (11, 13) may not be readily assimilated into the envelope. To test for this possibility, experiments were performed with cultures of the mutant strain EH470 *psd-4* grown at the intermediate temperature of 37°C. At this temperature, phosphatidylserine accumulates at an intermediate, steady-state level (10 to 30 mol% of total phospholipid; phosphatidylethanolamine is 40 to 60 mol%); presumably the decarboxylase is partially inactivated, i.e., the mutation is leaky in the sense that substantial phosphatidylethanolamine can still be made, but phosphatidylserine accumulates at much higher steady-state levels than in wild-type cells. The growth of EH470 *psd-4* is normal at 37°C. Therefore, possible complications from cell death are minimized, but there is sufficient phosphatidylserine present for analysis. Cell viability, as measured by colony-forming ability at 30°C, was reduced up by to 30% in these mutant cultures as compared to the wild-type strain. However, some or all of this apparent reduced viability may be explained by the fact that the *psd-4* strain forms filaments under these conditions. Unseparated cells would thus decrease the apparent viable count per unit of turbidity.

It was initially observed in these cultures that essentially all (>95%) of the phosphatidylserine remained in association with cells, as determined by low-speed centrifugation and membrane filtration. Inspection by light and electron microscopy failed to reveal any unusual extracellular structures. Thus, no evidence was obtained for excretion or loss of phosphatidylserine into the culture medium.

In addition, membrane association of the accumulated phosphatidylserine could be demonstrated. Cells were disrupted by osmotic lysis or sonic irradiation of lysozyme-induced spheroplasts (25). Upon high-speed centrifugation of the lysates (25), all phosphatidylserine was recovered in the membrane-containing pellet fraction.

A further fractionation, yielding inner and outer membranes, was accomplished by the method of Osborn and Munson (25). Uniform labeling of membrane phospholipids and lipopolysaccharide was achieved by growth of cultures for several generations in the presence of [^{32}P]phosphate. Upon isopycnic centrifugation of the total membrane fraction, four peaks of label were obtained in the sucrose gradient (Fig. 1). The profiles thus obtained were similar to those reported by Osborn and co-workers (23, 25) for *S. typhimurium* and by McIntyre and Bell (21) for *E. coli*. No significant differences were observed between the membrane profiles obtained for *psd* mutant cultures and those obtained for wild-type cultures. Using the conventional nomenclature (23, 25), the peak of label designated H contains the dense outer membrane fraction of the cell envelope. The M fraction is presumably unresolved material, whereas fractions L1 and L2 contain the inner, cytoplasmic membrane as evidenced by activity of the marker enzyme NADH oxidase.

Pooled fractions from the sucrose gradient were analyzed for phospholipid content and composition. As expected (23), approximately equal amounts of phospholipid were found in the inner and outer membrane fractions. (The larger amount of total ^{32}P label evidently present in the H peak relative to the L peaks for the sucrose gradient of Fig. 1 reflects the fact that lipopolysaccharide, which contains phosphate moieties

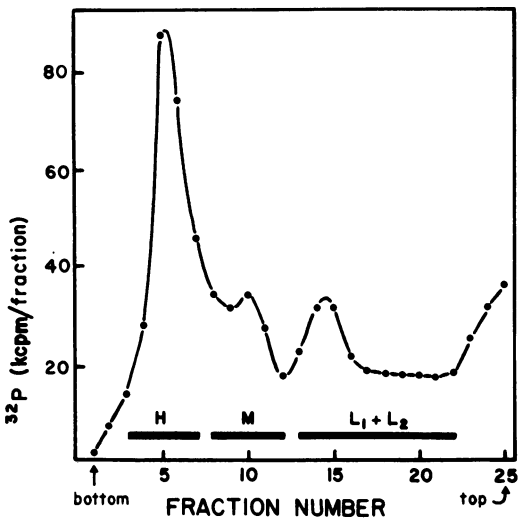

FIG. 1. Separation of the inner and outer membranes of strain EH470 *psd-4* by sucrose gradient fractionation. Culture medium (30 ml) LP56 containing glycerol (0.025%, wt/vol), casein hydrolysate (0.5%, wt/vol), $^{32}P_i$ (0.5 μCi/ml), and 0.05 volumes of L broth was inoculated with a sample (0.02 ml) from an overnight culture of strain EH470 *psd-4*. Cells were harvested by centrifugation after 5 h of incubation at 37°C at a cell density of about 8×10^8 cells /ml. The inner and outer membrane fractions were isolated by the sucrose gradient method of Osborn and Munson (25). Fractions of about 0.45 ml were collected from the gradients. A portion (0.025 ml) of each fraction was removed for determination of radioactivity by liquid scintillation counting. Fractions were pooled as indicated.

[26], is located primarily in the outer membrane [23, 25].) In this experiment, phosphatidylserine made up 23% of the outer membrane phospholipid and 18% of the inner membrane phospholipid, suggesting an even distribution between the two membrane fractions. (Compositional values for other phospholipids were as follows: outer membrane, 9% phosphatidylglycerol, 50% phosphatidylethanolamine, 2% cardiolipin, 16% unidentified; inner membrane, 15% phosphatidylglycerol, 56% phosphatidylethanolamine, 2% cardiolipin, 9% unidentified.) Although the membrane fraction cross-contamination (as judged by NADH oxidase activity distribution) was minimal, we cannot rule out the possibility that an individual membrane component, such as phosphatidylserine, could undergo redistribution during sonication and fractionation. However, in similar experiments with a diglyceride kinase (*dgk*) mutant strain, Raetz and Newman (30) found no evidence for such redistribution of diglyceride or phospholipid. The slightly lower % phosphatidylserine value for the inner membrane relative to the outer membrane is thought to reflect a selective loss of phosphatidylserine from the inner membrane fraction during membrane isolation. Such a loss is most probably due to decarboxylation by residual phosphatidylserine decarboxylase, which is localized exclusively in the inner membrane (4, 35). Although all operations were performed in the presence of 10 mM hydroxylamine, an inhibitor of phosphatidylserine decarboxylase (29, 32), the membrane separation requires a lengthy centrifugation during which some conversion of phosphatidylserine to phosphatidylethanolamine by a trace amount of uninhibited enzyme may take place. Two results are consistent with this interpretation. (i) In cases where the level of phosphatidylserine was substantially less in the inner membrane fraction than in the outer membrane fraction, less than complete recovery of the phosphatidylserine applied to the gradient was obtained, and the phosphatidylserine level for the outer membrane fraction was always the same as that for the unfractionated total membrane pellet. When the separation of membranes was performed in the absence of hydroxylamine, phosphatidylserine was identified in the outer membrane fractions, but was completely absent from the inner membrane. (ii) To further test the proposal that inner membrane phosphatidylserine is subject to decarboxylation in the membrane preparations of this mutant, an experiment was performed with a suspension of separated but unresolved inner and outer membranes again prepared by the Osborn procedure. A total membrane fraction uniformly labeled with [^{32}P]phosphate was obtained from strain EH470 *psd-4* grown at 37°C, and the phosphatidylserine content was found to be 22 mol%. This membrane suspension containing both the inner and outer membranes was then incubated at 28°C, and the decline in radioactive phosphatidylserine was followed. Within 12 h, 46% of the phosphatidylserine initially present had been decarboxylated. During an additional 15 h essentially no further decarboxylation of phosphatidylserine occurred since 51% of the initial radioactive phosphatidylserine remained in suspension. A value of 50% decarboxylation is of course the expected value if (i) phosphatidylserine is evenly distributed between both membranes, (ii) phosphatidylserine decarboxylase is localized solely in the inner membrane, and (iii) the membrane-bound decarboxylase cannot act on the phosphatidylserine in a different membrane bilayer.

It is thus clear that phosphatidylserine, a phospholipid not normally present in large amounts in *E. coli*, can be efficiently incorporated into the outer membrane and appears, in fact, to be evenly distributed between the two envelope membranes.

Fate of accumulated radioactive phosphatidyl-

serine during the chase. The above results strongly suggest that phosphatidylserine can move from its site of synthesis to the outer membrane. (Experiments to be presented below enable a more precise estimate of the rate of such movement.) Can phosphatidylserine also return from the outer membrane to the inner membrane? An examination of the metabolic fate of phosphatidylserine in experiments with a *psd* mutant provides some insight into this question (Fig. 2). A culture of strain EH470 *psd-4* was grown at 33°C such that phosphatidylserine and phosphatidylethanolamine accumulated to levels of 11 and 51%, respectively, of the total phospholipid. The cells were uniformly labeled with both [2-^{3}H]glycerol and $^{32}P_i$. A chase with excess unlabeled glycerol was begun; within four generations of growth, 90% of the [^{3}H]phosphatidylserine previously accumulated at 33°C was converted to phosphatidylethanolamine (Fig. 2). Ultimately, more than 98% of the [^{3}H]phosphatidylserine was decarboxylated. In terms of ^{3}H radioactivity, values for phosphatidylserine, phosphatidylethanolamine, and total phospholipid were 32.7, 138.9, and 295.5 kcpm/ml of cell culture, respectively, at the start of the chase period. When values for [^{3}H]phosphatidylserine plus [^{3}H]phosphatidylethanolamine at subsequent times during the chase were normalized to that at the start of the chase, to correct for variability in phospholipid recovery, it was clear that ^{3}H counts lost from phosphatidylserine during the chase were quantitatively recovered as phosphatidylethanolamine; the actual values obtained for [^{3}H]phosphatidylserine plus [^{3}H]phosphatidylethanolamine ranged between 171.6 and 199.1 kcpm/ml of cell culture. Cell growth continued exponentially (doubling time, 60 min) for the first 1.5 h of the chase and then slowed gradually (apparent doubling time about 120 min over next 3.5 h). Note that $^{32}P_i$ remained present with constant specific radioactivity throughout the [^{3}H]glycerol labeling and the chase. This enabled monitoring of the actual pool sizes of various phospholipids during the experiment. The pool size of phosphatidylserine declined from its initial value of 11% of the total phospholipid to a final value of 4.5%, presumably because the declining growth rate led to a declining rate of phosphatidylserine synthesis relative to decarboxylation. The phosphatidylethanolamine pool size increased from its initial value of 51% of the total phospholipid to a final value of 58%. In this and subsequent experiments involving uniform labeling with [2-^{3}H]glycerol, no label was found in the fatty acid-containing fraction after deacylation of phospholipid by the procedure of Dittmer and Wells (8).

Results similar to those of Fig. 2 were obtained for experiments in which cells were uniformly labeled with $^{32}P_i$ only, and the ^{32}P label was then chased by harvesting of the cells, followed by suspension and growth in nonradioactive medium; more than 98% of accumulated [^{32}P]phosphatidylserine was converted to phosphatidylethanolamine during the chase (further data not shown).

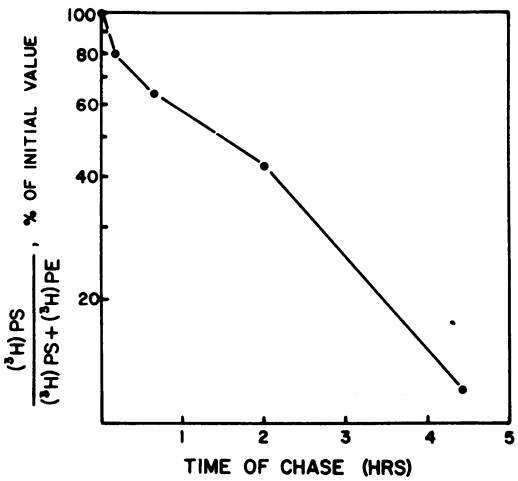

FIG. 2. Loss of uniformly-labeled [^{3}H]phosphatidylserine in strain EH470 *psd-4* during the chase. See the text for details.

Thus, the accumulation of phosphatidylserine in temperature-sensitive *psd* mutants under partially nonpermissive conditions is fully reversible. Phosphatidylserine accumulated in the outer membrane appears to be capable of movement back to the inner membrane where it can be decarboxylated.

A puzzling aspect of the chase experiment of Fig. 2 is the rather slow rate of decarboxylation of the [^{3}H]phosphatidylserine. In this experiment, the [^{3}H]phosphatidylethanolamine/[^{3}H] phosphatidylserine ratio was about 5 after uniform labeling, and the cell doubling time was 1 h; if the phosphatidylserine were behaving as a homogeneous pool available for decarboxylation, one would expect the label to be lost much more rapidly than was observed, since phosphatidylserine is the sole precursor of phosphatidylethanolamine.

We have considered a number of possibilities to explain the apparent slow decarboxylation. (i) Conceivably, incorporation of radioactivity into phosphatidylserine could continue during the chase as a result of poor chase of the ^{3}H label or as a result of the so-called diglyceride salvage pathway (27). Both considerations are unlikely because the sums of radioactive count in phosphatidylserine and phosphatidylethanolamine remained constant during the chases. (ii) A nonmetabolizing population of cells in the bacterial

culture could accumulate phosphatidylserine but not decarboxylate it. To test this possibility we labeled cells uniformly with $^{32}P_i$ and [2-^{3}H]glycerol and chased with unlabeled glycerol (for about 50 min) as in the experiment of Fig. 2. Penicillin G (2.5 mg/ml) was then added for a period of 2 h to bring about lysis of actively metabolizing, growing cells. A supernatant fraction, containing membranous material from lysed cells, and a pellet fraction, presumably containing nonmetabolizing cells and some envelope material from lysed cells, were isolated by centrifugation (15 min at 3,000 × *g*). The supernatant fraction contained one-third of the total lipid ^{32}P, but the $^3H/^{32}P$ ratio for phosphatidylserine was identical in both fractions. This result suggests that no sizeable population of non-metabolizing cells was present in the cultures. (iii) Individual cells could contain segregated pools of phosphatidylserine which are metabolized at different rates; the majority of phosphatidylserine would be segregated in a slowly metabolized pool that exchanges slowly with a small, more rapidly decarboxylated pool (serving as precursor of phosphatidylethanolamine). If the latter type of pool existed, and if it were labeled by a short pulse with radioactive precursor, the label should be rapidly lost during a subsequent chase period. This was indeed the case (Fig. 3); after a 50-s pulse-labeling with [2-^{3}H]glycerol, label from phosphatidylserine was rapidly incorporated into phosphatidylethanolamine during the chase. In terms of ^3H radioactivity, values for phosphatidylserine, phosphatidylethanolamine, and total phospolipid were 6.8, 0.8, and 20.0 kcpm/ml of cell culture, respectively, at the start of the chase.

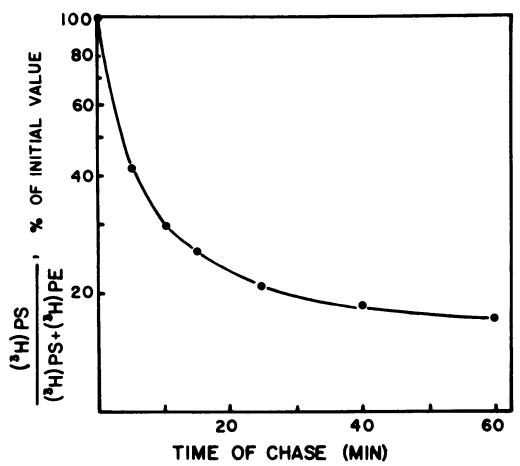

FIG. 3. Loss of label from phosphatidylserine in strain EH470 *psd-4* during the chase after a short pulse-labeling with [^{3}H]glycerol. See the text for details.

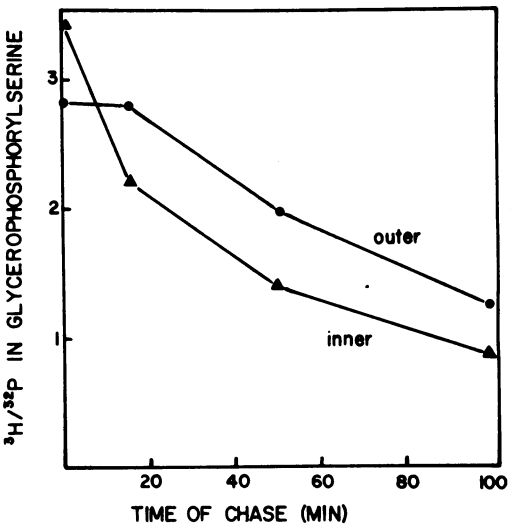

FIG. 4. Loss of uniformly labeled [^{3}H]phosphatidylserine from inner and outer membranes of strain EH470 *psd-4* during the chase. See the text for details.

The value for [^{3}H]phosphatidylserine plus [^{3}H]phosphatidylethanolamine increased to 18.3 kcpm/ml of cell culture during the first 10 min of the chase, presumably due to inefficiency of chase of the [2-^{3}H]glycerol, and remained at 19 to 21 kcpm/ml of cell culture thereafter. Growth continued exponentially during the chase. $^{32}P_i$ was included in the medium for the reason mentioned above in connection with Fig. 2. The phosphatidylserine and phosphatidylethanolamine levels remained at 12.3 ± 1.5 and 56.3 ± 5.0%, respectively, during the pulse and chase periods. The halftime of approximately 3 to 4 min for phosphatidylserine turnover in this experiment contrasts sharply with that observed during the chase after uniform labeling (Fig. 2). Thus, slowly and rapidly metabolized pools of phosphatidylserine appear to coexist in the cell envelope. The slowly metabolized pool may be physically segregated; however, it does not represent phosphatidylserine in the outer, as opposed to the inner, membrane since labeled phosphatidylserine disappears slowly from both of these membranes during a chase after uniform radioactive labeling (Fig. 4). For the experiment of Figure 4, it should be noted that ^3H radioactivity values for glycerophosphorylserine from the inner and outer membrane fractions were 10.4 and 11.2 kcpm, respectively, at the start of the chase. The pool sizes for phosphatidylserine and phosphatidylethanolamine, as determined by ^{32}P radioactivity in the various species of phospholipid, were 14.5 ± 2.8 and 53.8 ± 2.9%, respectively, for the inner membrane and 19.4 ± 3.6 and 48.8 ± 1.1%, respectively, for the outer membrane during the course of the experiment.

Movement of pulse-labeled phosphatidylserine from inner to outer membrane during the chase. Data presented above indicate that phosphatidylserine moves reversibly from the inner to outer membrane, but do not show the rate of such movement. To estimate the rate, we performed a pulse-chase experiment (Fig. 5). After a 1-min period of pulse-labeling with [2-^{3}H]glycerol, inner and outer membranes were separated at various times during the chase (Fig. 5), and the distribution of pulse-labeled phosphatidylserine between the two was measured. $^{32}P_i$ remained present in the medium throughout the experiment as a uniform label. Therefore, the $^3H/^{32}P$ ratios represent specific radioactivity values for the pulse-labeled phosphatidylserine. The $^3H/^{32}P$ ratios are modified values in that incremental ^{32}P radioactivity that accumulated during the chase (as calculated from growth rate) was subtracted from the ^{32}P counts per minute total at each point. Growth continued exponentially during the chase. Phosphatidylserine and phosphatidylethanolamine comprised 55 and 10%, respectively, of the total ^{32}P-labeled phospholipid. Initial values for ^3H radioactivity recovered in phosphatidylserine were 30.4 and 8.4 kcpm for the inner and outer membrane fractions, respectively. Complications caused by decarboxylation of labeled phosphatidylserine during the chase were reduced by the use of strain EH450 *psd-2* in these experiments; in the presence of sufficient Mg^{2+}, this strain continues to grow at the usually nonpermissive temperature of 42°C and accumulates phosphatidylserine at extremely high levels at the expense of phosphatidylethanolamine (13; see above). It is evident from Fig. 5 that pulse-labeled phosphatidylserine appears first in the inner membrane and can then move rapidly to the outer membrane in this strain. The apparent half-time for equilibration of label between the two membranes is 12 to 13 min; this may be a slight underestimate since some of the labeled phosphatidylserine (about 15%) was lost from the inner membrane by decarboxylation rather than by movement to the outer membrane.

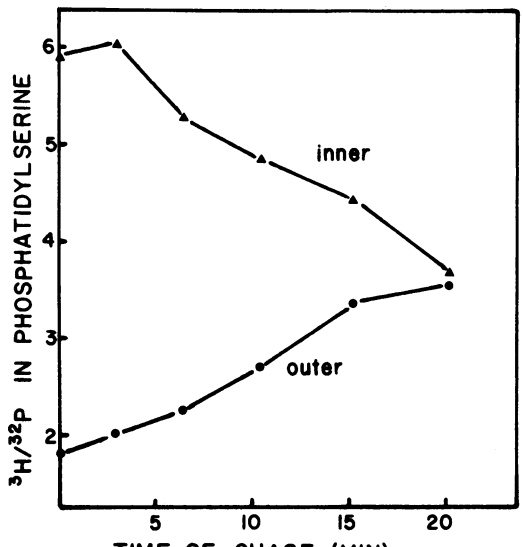

FIG. 5. Movement of [^{3}H]phosphatidylserine from the inner membrane to the outer membrane in strain EH450 *psd-2* during the chase after a short pulse-labeling with [2-^{3}H]glycerol. See the text for details.

DISCUSSION

The incorporation of phosphatidylserine into the membranes of *E. coli* provides further evidence for the high level of adaptability displayed by this organism with respect to its membrane composition. Considerable variation in lipopolysaccharide content (17), outer membrane protein content (14), and phospholipid headgroup (27) and fatty acid (33) composition can all be accomodated with little or no apparent physiological effect. Electron microscopy, performed on freeze-fracture preparations from the *psd-4* mutant membrane containing levels of phosphatidylserine described in this paper (L. Margaritas, unpublished observations), indicated no striking morphological alterations in either the inner or outer membrane fracture faces. Thus, the accumulation of high levels of phosphatidylserine appears to produce a minimal effect on the basic membrane morphology.

The results reported here indicate that not only is phosphatidylserine effectively incorporated into the cell membrane of the *psd-4* mutant, but also, it becomes evenly distributed between the inner and outer membranes. Since the enzymes involved in phosphatidylserine synthesis are not associated with the outer membrane, the demonstration of this normally trace phospholipid in the outer membrane fraction suggests a lack of specificity in whatever mechanism is involved in phospholipid translocation from the inner membrane to the outer membrane. This view is supported by studies, with appropriate mutants of phospholipid synthesis, on the accumulation and localization of other phospholipids normally present in low or trace amounts. For example, a mutant in phosphatidylserine synthesis accumulates cardiolipin to high levels in both the inner and outer membranes (28), and a mutant in CDP-diglyceride synthesis accumulates phosphatidic acid to a level of 1.7% of the total phospholipid, such that 2% of the inner membrane phospholipid is phosphatidic acid, and 0.5% of the outer membrane phospholipid is phosphatidic acid (10). The major *E. coli* phospholipids in wild-type cells, phosphatidylethanolamine and phosphatidylglycerol,

are also found in both membranes of the envelope, although the phosphatidylglycerol distribution is weighted more toward the inner membrane (23).

The results of our chase studies after uniform radioactive labeling indicate that phosphatidylserine translocation between membranes is reversible in the *psd-4* mutant. Under chase conditions, essentially all of the accumulated radioactive phosphatidylserine undergoes conversion. Since no other major degradative pathway is known, the implication is that phosphatidylserine in the outer membrane migrates to the inner membrane where it is decarboxylated. Alternatively, the presence of a small amount of phosphatidylserine decarboxylase in the outer membrane could account for the results. However, the fact that in a membrane suspension containing both the inner and outer membranes only 50% of the phosphatidylserine undergoes decarboxylation in vitro argues against this possibility. If, in fact, all of the phosphatidylserine decarboxylase remains localized in the inner membrane, then our results indicate that phosphatidylserine, a major membrane phospholipid in these experiments, is capable of bidirectional movement between the inner and outer membranes. Jones and Osborn (15) have shown that phosphatidylserine, and other phospholipids as well, can move from the outer to the inner membrane in *S. typhimurium*.

The rate of reverse translocation of phosphatidylserine to the inner membrane is not clear from our uniform labeling-chase studies because accumulated phosphatidylserine does not appear to behave as a homogeneous pool in terms of availability for decarboxylation; the accumulated phosphatidylserine, in both the inner and outer membranes, is decarboxylated much more slowly than expected for maintaining observed levels of phosphatidylethanolamine. In other words, it appears that some (slow) step other than the translocation step itself is rate determining in the loss, during the chase, of previously accumulated radioactive phosphatidylserine.

Regarding translocation of phosphatidylserine from the inner to the outer membrane, the rate must be at least sufficient to allow translocation of an amount of phosphatidylserine equivalent to half that accumulated per generation. Our direct rate measurement by pulse-chase analysis in the *psd-2* mutant suggests a half-time of a few minutes for equilibration of pulse-labeled phosphatidylserine between the membranes. This is similar to rates of phosphatidylethanolamine equilibration observed by Osborn et al. (26) and by Donohue-Rolfe and Schaechter (9). The initial predominance of pulse-labeled phosphatidylserine in the inner membrane also provides some support for the idea that phosphatidylserine synthetase is located in the inner membrane; evidence has been reported recently that the tendency of the synthetase to associate with ribosomal fractions (29) is artifactual, and that the inner membrane is the true in vivo location of the enzyme (20).

Our results are consistent with two types of models for phospholipid translocation. Translocation may be mediated by transfer factors operating between physically separate membranes, i.e., factors similar to the phospholipid exchange proteins identified in mammalian systems (36). Alternatively, translocation may occur through sites of membrane fusion of the inner and outer membranes. This has been suggested previously (15, 24) based on Bayer's observations of morphological zones of adhesion (3), of which there are 200 to 400 per cell. If either of these mechanisms were applicable, our results would indicate that it must also be capable of acting on phosphatidylserine.

In considering mechanisms of envelope assembly, it is instructive to mention studies of the synthesis, translocation, and localization of components other than phospholipids. In *S. typhimurium*, lipopolysaccharide is rapidly and irreversibly (15, 24) translocated to the outer membrane after synthesis at the inner membrane, and the zones of adhesion have been implicated in this process (22). Like lipopolysaccharide, several proteins become localized in the outer membrane exclusively (7). They may first be secreted through the inner membrane and then incorporated into the outer (7). However, at least one outer membrane matrix protein is located initially at zones of adhesion during its biosynthesis (34). On the other hand, many proteins of course remain localized in the inner membrane. The same is true of the lipid diglyceride, accumulated at high levels in diglyceride kinase (*dgk*) mutants (30).

Our finding of unexpectedly slow turnover of uniformly labeled phosphatidylserine in the *psd-4* mutant deserves some final comment. Pulse-labeled phosphatidylserine, in contrast, turns over much more rapidly and, if anything, turns over more rapidly than expected (Fig. 3). It thus appears that newly made phosphatidylserine is more readily decarboxylated, and that "older" phosphatidylserine becomes segregated into pools that are less metabolically active. (Data for two other anionic lipids, phosphatidylglycerol [2] and phosphatidic acid [10], can be interpreted similarly, i.e., longer labeling periods result in slower turnover during a subsequent chase period.) As mentioned above, it seems most likely that much of the phosphatidylserine is metabolically inactive owing to a physical segregation in some pool, other than simply the outer membrane.

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