Plant Physiol. (1990) 93, 154–159
0032-0889/90/93/0154/06/$01.00/0

Received for publication October 20, 1989
and in revised form December 27, 1989

Phosphatidylethanolamine Synthesis by Castor Bean Endosperm[1]

Membrane Bilayer Distribution of Phosphatidylethanolamine Synthesized by the Ethanolaminephosphotransferase and Ethanolamine Exchange Reactions

Sungho Shin and Thomas S. Moore, Jr.*

Department of Botany, Louisiana State University, Baton Rouge, Louisiana 70803-1705

ABSTRACT

The bilayer distribution of phosphatidylethanolamine (PtdEtn) in the endoplasmic reticulum of castor bean (*Ricinus communis* L. var Hale) endosperm following synthesis by both the CDP-ethanolamine: 1,2-diacylglycerol ethanolaminephosphotransferase and ethanolamine base exchange reactions have been studied. Two chemical probes, 1-fluoro-2,4-dinitrobenzene (FDNB) and 2,4,6-trinitrobenzenesulfonic acid (TNBS), which covalently bind to the free amino groups, were utilized. The endoplasmic reticulum membranes were impermeable to TNBS at 4 and 25°C, but were permeable to FDNB at both temperatures. FDNB treatment of the PtdEtn from the base exchange reacted with 92% of the PtdEtn, while 80% of the lipid reacted with TNBS. Thus, at least 80% of the PtdEtn synthesized by the base exchange reaction was localized in the outer leaflet of the membrane, with about 12% occurring on the inner leaflet and about 8% being inaccessible. For PtdEtn formed by the CDP-ethanolamine pathway, 85% reacted with FDNB and 70% with TNBS, indicating that at least 70% was produced to the cytoplasmic face of the ER and 15% to the lumen side. The remainder was inaccessible to the probes. The sensitivity to trypsin of the two reactions also was tested. The ethanolamine base exchange enzyme, as well as that for L-serine exchange, retained activity following exposure to trypsin, but the activity of ethanolaminephosphotransferase disappeared after such treatment. This indicates that both base exchange enzymes are exposed to the lumenal side of the ER, while CDP-ethanolamine: 1,2-diacylglycerol ethanolaminephosphotransferase is exposed on the outer or cytoplasmic side. These results are discussed with respect to the final phospholipid distributions and the presumed sources of the water-soluble substrates.

In an accompanying paper (26), we demonstrated synthesis of PtdEtn[2] by an ethanolamine exchange reaction in the endoplasmic reticulum of castor bean (*Ricinus communis* L. var Hale) endosperm, and synthesis by ethanolaminephosphotransferase utilizing CDP-ethanolamine was described earlier for this same organelle (27). Numerous hypotheses have been offered to explain the significance of two reactions to provide the same phospholipid, but none has proven completely satisfactory. One possibility which has not been fully tested is that these two reactions provide PtdEtn to separate sides of the bilayer membrane, so that independent regulation might help control membrane properties of the opposite surfaces.

Several lines of evidence indicate an asymmetric distribution of phospholipids in the bilayers of biological membranes (7, 14, 23). For example, in castor bean endoplasmic reticulum 52% of the PtdCho and 65% of the PtdEtn have been found in the outer or cytoplasmic side of the membrane, but only 20% of the PtdCho and 15% of the PtdEtn exist in the inner. Such an uneven distribution of phospholipids in membranes has been observed in other plant organelles (5), human red blood cells (29), rat liver organelle membranes (22), bacterial cells (24), and viral membranes (25).

The origins of this asymmetry are not clearly defined. For example, it has been reported that large portions of the PtdEtn were located on the cytoplasmic side of rat microsomes (4). Key enzymes for the biosynthesis of PtdEtn were localized by utilizing protease treatments (3) and the ethanolaminephosphotransferase (EC2.7.8.1) was reported to be on the cytoplasmic side, while the base exchange enzyme was located on the lumenal side of rat microsomes. Recently, Corazzi *et al.* (6) reported that most of the PtdEtn formed by the base exchange reaction was in the outer leaflet of membranes in rat microsomes.

Here we report on an attempt to correlate the topographical relationships of enzymes involved in PtdEtn synthesis with the locations of their newly synthesized products. Trypsin treatment of ER vesicles and the reaction of PtdEtn molecules with chemical probes have been used in order to determine these relationships. Trypsin does not cross the membranes (6), and so reacts only with those portions of the enzymes exposed on the outer surface. The two probes were chosen because of their limited effects on membrane structure and the facts that one, FDNB, readily permeates the membranes while the other, TNBS, does not (6). Both chemical probes react with the amino groups of phosphatidylethanolamine

[1] This research was supported by grant DCB-8703739 from the National Science Foundation.

[2] Abbreviations: PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; TNBS, 2,4,6 trinitrobenzenesulfonic acid; FDNB, 1-fluoro-2,4-dinitrobenzene; DNP-PtdEtn, dinitrophenyl-phosphatidylethanolamine; TNP-PtdEtn, trinitrophenyl-phosphatidylethanolamine.

molecules to form derivatives which may be separated from unreacted PtdEtn by TLC. When the PtdEtn has been radiolabeled with pathway-specific precursors, the distribution of radioactivity between the derivatized and underivatized PtdEtn serves as an indicator of the availability of the product PtdEtn to the probes. PtdEtn reacting with TNBS can then be assigned to the outer membrane surface, while FDNB would react with the total available PtdEtn pool. The contents of the inner surface would be determined by subtraction.

MATERIALS AND METHODS

Material

[2-^{14}C]Ethanolamine hydrochloride (57.3 mCi/mmol) was from Amersham. [3-^{14}C]L-Serine (57 mCi/mmol) and [ethanolamine-1,2-^{14}C]CDP-ethanolamine (51 mCi/mmol) were from ICN. The FDNB, trypsin, and trypsin inhibitor were obtained from Sigma. TNBS was from Pierce and sucrose from E. M. Science. The TLC plates were from Analtech. All chemicals were of reagent grade or better.

Plant Material

Seeds of castor bean (*Ricinus communis* L. var Hale) endosperm were removed from their seed coats and sterilized in 10% bleach (5.25% hypochlorite) for 9 s. They were germinated and grown for 3 d at 30°C in moist vermiculite in the dark.

Homogenization of Tissue

Thirty endosperm halves were separated from the seedlings, rinsed in distilled water, and placed in a Petri dish on ice. They were chopped with a single razor blade for 15 min in 11 mL of homogenization medium containing 150 mM Tricine (pH 7.5], 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, and 13% (w/w) sucrose. The crude homogenate was filtered through two layers of Dacron cloth.

Density Gradient Centrifugation

This method was based on previously reported techniques for maintaining ribosomal association with castor bean ER, with the resultant vesicles maintaining their proper right-side-out orientation (16). Five mL of the filtered homogenate were applied to the top of a sucrose density gradient contained in a 37.5 mL centrifuge tube. The gradient was composed of 20 mL of sucrose solution decreasing linearly in concentration from 60 to 13% (w/w) over a 10 mL cushion of 60% sucrose. All gradient solutions contained 150 mM Tricine (pH 7.5), 10 mM KCl, 1 mM EDTA, and 1 mM $MgCl_2$. The gradients were centrifuged at 20,000 rpm with a Sorvall AH-629 rotor in a Sorvall OTD-65B ultracentrifuge for 3 h. These conditions have been reported to prevent the dissociation of membrane bound ribosomes (16), resulting in an increased density for the ER band, and our preliminary tests confirmed these results. The ER was collected from a 1.14g/cm^3 band.

Enzyme Assays

CDP-ethanolamine: 1,2 diacylglycerol ethanolaminephosphotransferase was assayed as previously described (27). For the base exchange reactions, ethanolamine incorporation was measured in 200 mM Hepes buffer (pH 7.8), 2 mM $CaCl_2$, and 0.01 mM [2-^{14}C]ethanolamine hydrochloride (2.4 mCi/mmol) (26), while for serine incorporation 40 mM Hepes buffer (pH 7.8), 2 mM $CaCl_2$, and 0.4 mM [3-^{14}C]L-serine (5 mCi/mmol) comprised the standard assay medium (20). All assay solutions were prepared in 35% (w/w) sucrose, which was iso-osmotic to the ER band on the gradients, in order to maintain vesicle integrity. The reaction was started by adding 100–200 μg of the ER fraction to the assay mixture and the tubes were incubated for 30 min at 30°C for the base exchange enzymes and 37°C for ethanolaminephosphotransferase. The reaction was stopped by adding 25 μL of 25 mM $CdCl_2$ (8).

Reaction with FDNB or TNBS

In most cases, after stopping the PtdEtn synthesis reaction a solution of FDNB or TNBS contained in 35% sucrose and 5% $NaHCO_3$ at pH 8.1 was added (18). The reaction was conducted for the times, concentrations and temperatures indicated in the figures and their legends. In some cases, 2% (v/v) Triton X-100 was added in order to disrupt the membranes and thereby estimate the total PtdEtn available for the reaction (4).

Trypsin Treatment

ER fractions (0.5 mg protein/mL) were incubated with trypsin (100 μg/mg ER protein) in 50 mM KCl, 35% sucrose, and 200 mM Hepes buffer (pH 7.8) at 30°C. The reaction was terminated by adding trypsin inhibitor (3 times the amount of trypsin present, w/w) (8). After a 30 min incubation the solutions were centrifuged at 105,000×g for 1 h and the resulting pellets assayed for enzyme activity. In order to determine the trypsin sensitivity of enzyme apparently on the lumenal side of the ER, the ER was incubated in the presence of 0.05% sodium deoxycholate, which increased accessibility of trypsin to the inner surface (15). This concentration of detergent had little effect on the enzyme activities.

Extraction and Analysis of Lipids

The lipids were extracted by a method based on that of Bligh and Dyer (1). The final chloroform extract was dried under nitrogen and the lipid residues were redissolved in chloroform-methanol (2:1, v/v) for application to 250 μm Silica Gel G plates. The phospholipids were separated with chloroform-methanol-7 N NH_4OH (70:30:5, v/v) in the first dimension and chloroform-methanol-acetic acid-acetone-water (70:15:15:30:7.5, v/v) in the second. DNP-PtdEtn and TNP-PtdEtn were identified by their yellow color and PtdEtn was visualized with iodine vapors. The spots of PtdEtn, DNP-PtdEtn, and TNP-PtdEtn were scraped from the plate for measuring radioactivity or the amount of phosphorus.

Analyses

Phosphorus was estimated by the method of Duck-Chong (9) and protein by the method of Bradford (2). Radioactivity generally was measured in 3 mL of Ready-Safe (Beckman) scintillation cocktail.

RESULTS

Distribution of Total PtdEtn in ER

The data in Figure 1 show that the maximum derivatization of total PtdEtn obtained in these experiments was obtained at approximately 4 mM FDNB, with 86% of the total membrane PtdEtn transformed into DNP-PtdEtn. The reaction of PtdEtn with TNBS approached a plateau at 3 mM, with about 75% of the total PtdEtn reacted to form TNP-PtdEtn. Thus, it appears that at least 75% of the total PtdEtn was located in the outer surface of the membranes, and at least 11% of the total PtdEtn occurred in the inner surface. The remaining 14% was not accessible to the probes. In the presence of detergent, 97% of the total PtdEtn reacted with each probe.

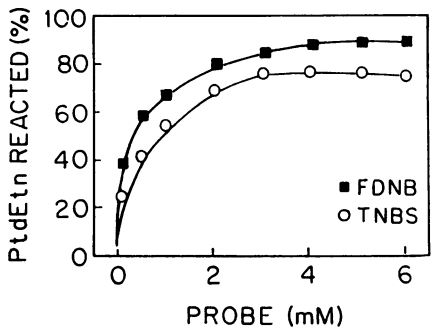

Figure 1. Reaction of total PtdEtn of ER membranes with FDNB and TNBS. Each point in this and the following figures represents the averages of 3 to 5 experiments.

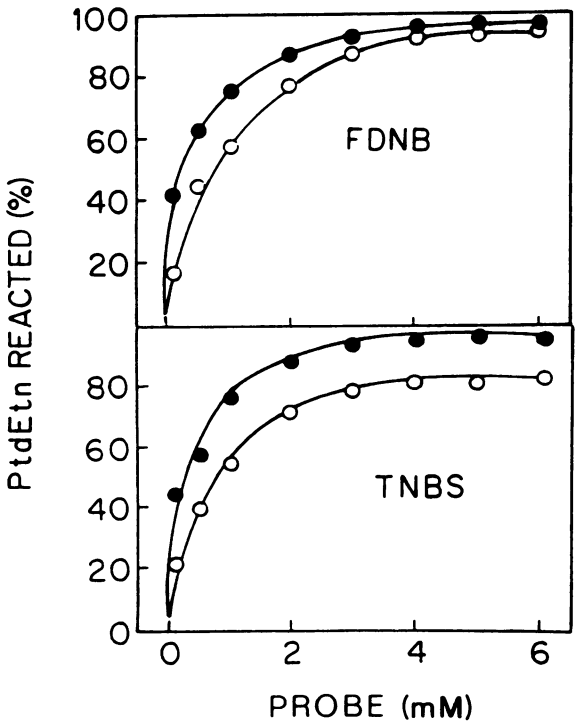

Figure 2. Reaction of varying FDNB (top panel) and TNBS (bottom panel) concentrations with PtdEtn in ER membrane fractions formed by the 1 h base exchange reaction. After the base exchange reaction with [^{14}C]ethanolamine, the ER was incubated for 1 h at 25°C with FDNB (top) or TNBS (bottom) and with (●) or without (○) Triton X-100.

PtdEtn Formed by Base Exchange

The results of reacting FDNB or TNBS with PtdEtn radiolabeled by base exchange with [^{14}C]ethanolamine are shown in Figure 2. The formation of DNP-PtdEtn depended on the initial concentration of FDNB and approached a plateau at about 4 mM. At this concentration, about 92% of the radiolabeled PtdEtn was derivatized by the probe. Similar results were obtained by treatment of membranes which had been disrupted with detergents.

The formation of TNP-PtdEtn (from TNBS; Fig. 2) increased with increasing concentrations of TNBS but saturated at about 3 mM, at which concentration about 80% of the radiolabeled PtdEtn had been transformed into TNP-PtdEtn. The maximum amount of PtdEtn transformed into TNP-PtdEtn with detergent-disrupted membranes also was reached at 3 mM TNBS with approximately 97% of the PtdEtn reacting.

PtdEtn Formed by the CDP-ethanolamine Pathway

The results of derivatization of membrane PtdEtn formed by the CDP-ethanolamine pathway with FDNB are contained in Figure 3. The reaction of PtdEtn with FDNB approached a plateau at 4 mM FDNB, with about 85% of the radiolabeled PtdEtn transformed into DNP-PtdEtn. The reaction of PtdEtn with TNBS to form TNP-PtdEtn fully saturated at 3 mM, with about 70% of the PtdEtn converted to TNP-PtdEtn. In detergent-treated membranes, both FDNB and TNBS reacted with 98% of the membrane PtdEtn formed by the CDP-ethanolamine pathway.

Time and Temperature Dependence of TNP-PtdEtn Formation

The time-dependence of derivatizing membrane PtdEtn with TNBS, the nonpenetrating probe, is shown in Figure 4. In the case of both the base exchange and nucleotide pathways, after 1 h the radiolabeled PtdEtn molecules reached a maximum with TNBS and did not change for up to 4 h. Figure 5 shows the temperature dependence of the reaction of membrane PtdEtn with TNBS. Similar responses were obtained with TNBS at both 4 and 25°C (Fig. 5), although the higher temperature experiments did have a slightly increased reaction rate. Thus, it appears that the ER membrane was stable under the conditions used.

Trypsin Treatment

The data contained in Table I demonstrate the sensitivity of the base exchange and ethanolaminephosphotransferase enzymes to trypsin. Treatment with trypsin completely eliminated the membrane-bound ethanolaminephosphotransfer-

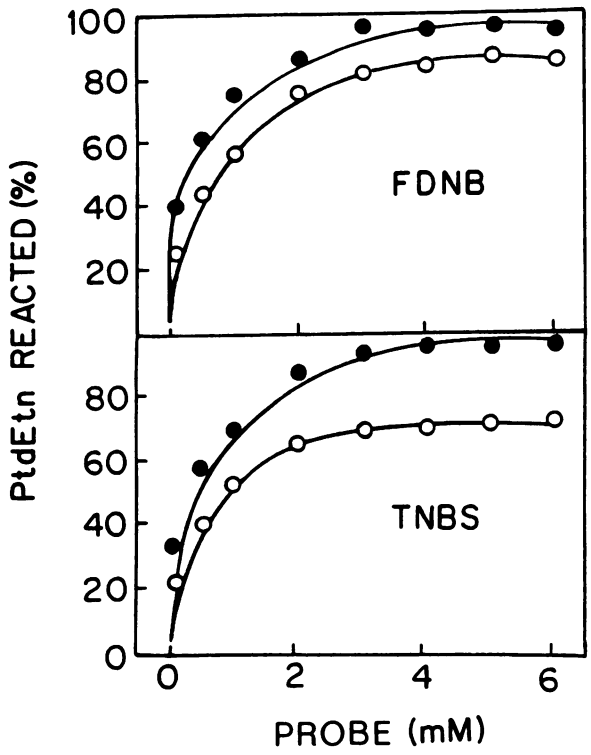

Figure 3. Reaction of varying FDNB (top panel) and TNBS (bottom panel) concentrations with PtdEtn in ER membrane fractions formed by the CDP-ethanolamine pathway. After the 30 min CDP-ethanolamine pathway reaction, the ER fractions were incubated for 1 h at 25°C with FDNB (top) or TNBS (bottom) and with (●) or without (○) Triton X-100.

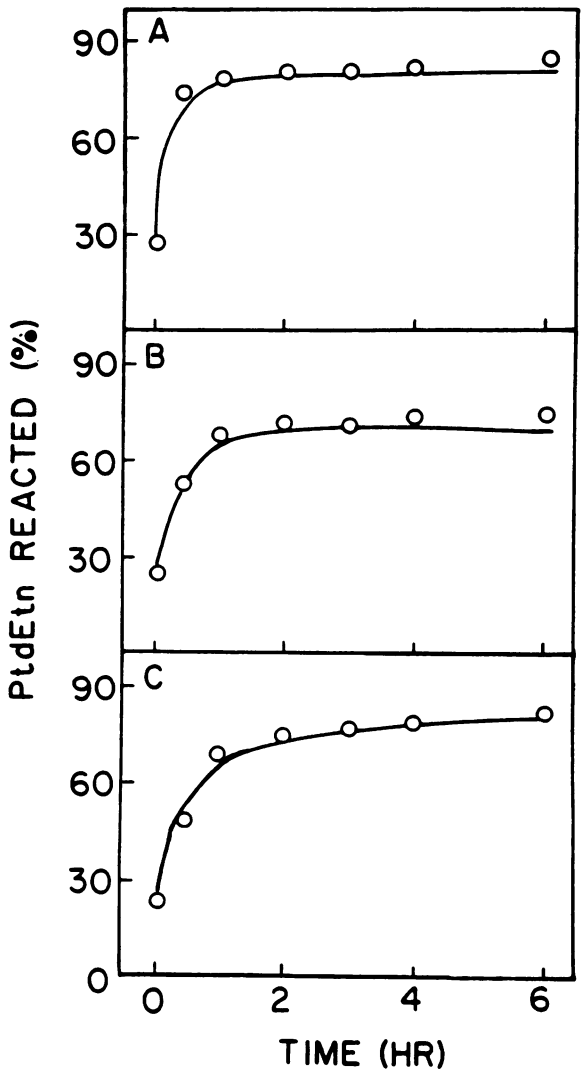

Figure 4. Time dependence of the formation of TNP-PtdEtn following the start of TNBS treatment, A, Base exchange reaction; B, CDP-ethanolamine pathway; C, total PtdEtn.

ase activity in intact ER, but both ethanolamine and serine base exchange activities were unaffected after such treatment. These two base exchange enzyme activities were reduced by trypsin treatment in the presence of sodium deoxycholate, most likely due to increased permeability of the membrane to the trypsin. The enzyme activities were not affected by the low concentrations of sodium deoxycholate used.

DISCUSSION

The localization of membrane PtdEtn formed by the nucleotide pathway and base exchange reactions has been determined via chemical probes. The PtdEtn synthesized by both pathways was highly asymmetric in its distribution, being concentrated in the outer leaflet, or cytoplasmic face, of the ER membrane. The fact that the total PtdEtn pool of the ER was asymmetrically distributed is in agreement with the previous results of Cheesbrough and Moore (5). These conclusions also are consistent with the data of Corazzi *et al.* (6), who observed that the PtdEtn formed by a base exchange reaction in rat brain microsomes was primarily localized in the cytoplasmic leaflet of the vesicles. This extensive asymmetry is achieved within two and one-half hours from the beginning of synthesis (assay plus probe treatment time), and most likely sooner since the distribution was so strongly skewed. It is not clear whether the PtdEtn is produced directly on the cytoplasmic surface or on the inner surface (in association with the trypsin-sensitive site), followed by rapid (within the duration of the experiment) translocation from the lumenal surface to the outer leaflet of the membranes (10, 11). The techniques used here coupled with the low radioactivities involved will not allow sufficient resolution of this question.

It should be emphasized that the data of Figures 4 and 5 indicate that the ER membrane was not penetrated by TNBS under our conditions. Therefore, the PtdEtn measured should be only that on the outer surface, except where a penetrating probe or detergent was used. Gordesky and Marinetti (11) also reported that TNBS was nonpenetrating in erythrocyte membranes. In addition, it has been demonstrated that mixed phospholipid vesicles were impermeable to TNBS at different temperature (17), and we have found this to be the case with our ER vesicles. In contrast, Rothman and Kennedy (24) reported that TNBS could not cross the membranes of *Bacillus megaterium* at 3°C, but slowly entered the cells at 15°C.

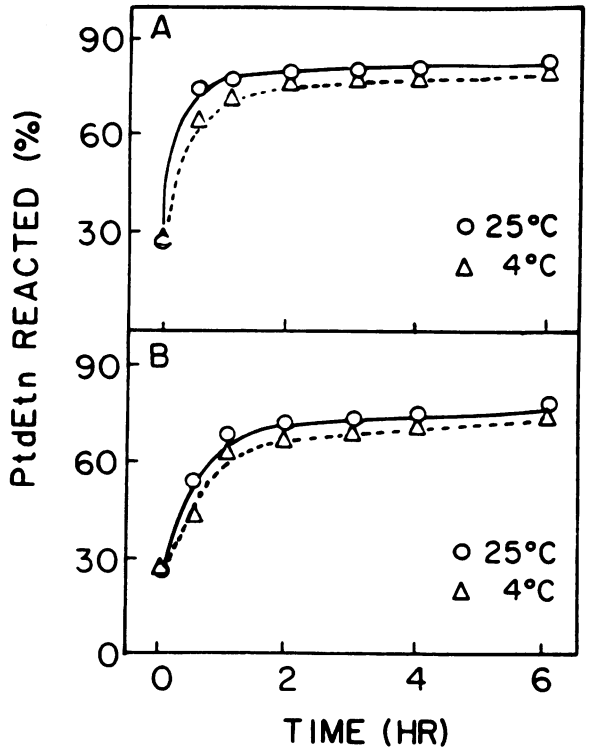

Figure 5. Temperature dependence of the formation of TNP-PtdEtn following the base exchange (A) and ethanolaminephosphotransferase (B) reactions. Temperatures for the chemical reaction were 4°C (Δ) and 25°C (O). Each point represents the average of 3 to 5 experiments.

Table I. *Effects of Preincubation of Castor Bean Endosperm Membranes with Trypsin on Ethanolamine and Serine Base Exchange Enzymes, and CDP-ethanolamine: 1,2-diacylglycerol Ethanolaminephosphotransferase*

Pretreatment was with trypsin for 30 min at 30°C. Sodium deoxycholate concentrations were 0.05%. Values are an average of three different experiments.

Enzyme	Control	Trypsin	Deoxycholate	Trypsin + Deoxycholate
			nmol h^{-1} (mg protein)$^{-1}$	
Exchange				
Ethanolamine	0.172	0.188	0.168	0.053
Serine	0.114	0.118	0.110	0.017
Phosphotransferase	3.061	0	3.015	0

The discrepancy over the membrane penetrating nature of TNBS very likely depends strongly on experimental conditions and membrane compositions.

A portion of the membrane PtdEtn is exposed to the probe only when the membrane is fully disrupted by the addition of detergent. In previous experiments similar results were obtained and interpreted as probable shielding of the PtdEtn molecules by proteins (4, 19, 28). Our results indicated that the shielding of PtdEtn formed by the CDP-ethanolamine pathway was twofold greater than that formed by the base exchange reaction (15% as compared to 8%, respectively). Earlier results from our laboratory indicated 20% of the PtdEtn (and 28% of the PtdCho) of the ER was inaccessible to the probes (5).

The location of the key enzymes within the ER membrane vesicles, as demonstrated by their sensitivities to trypsin, does not entirely correlate with the occurrences of their products. We found that ethanolaminephosphotransferase was strongly sensitive to treatment with trypsin, but neither the ethanolamine nor serine base exchange enzyme was affected without addition of detergent. Trypsin, along with controls containing a low concentration of sodium deoxycholate, has been used for determining the distribution of enzymes in membranes (8). Trypsin ordinarily does not penetrate the membranes, but can have access to the inner surface in the presence of concentrations of sodium deoxycholate sufficiently low to avoid disrupting the enzyme activities of the membranes (17). Following previous logic for such experiments (21), our results indicate that the ethanolaminephosphotransferase is located on the cytoplasmic side of the ER, while the two base exchange enzymes are in the lumenal side. These results are in agreement with those previously reporting the effects of trypsin on base exchange enzymes in rat microsomes (3).

If the above conclusion is correct, the question arises as to how substrates of the base exchange reactions are obtained by the enzymes. *In vivo* production of ethanolamine and serine is thought to be located in the cytoplasm (12), but the base exchange enzymes seem to be on an inside surface of a closed, semipermeable membrane which might limit accessibility. One possible resolution to this problem is that the enzymes do extend across the membrane, but that a trypsin insensitive portion of the molecule is exposed to the cytoplasmic surface (21). Buchanan and Kanfer (3) reported that microsomal membranes disrupted with phospholipase C treatment, which presumably was confined to the outer surface, influenced base exchange activities (13). These results would favor a portion of the enzyme occurring in the outer layer of the ER membrane. Thus, a likely model for the exchange enzymes is that they extend across the membrane with only the portion exposed to the lumen side being susceptible to trypsin attack. This, however, remains to be confirmed.

Finally, the hypothesis that the two pathways for PtdEtn exist in order to provide this phospholipid to the opposite sides of the bilayer seems unlikely in the face of our data. Other possible roles include production of specific subspecies of PtdEtn, synthesis within specific membrane sites (perhaps for interaction with different proteins as indicated by differences in the extent of shielding from the probes) or allowing for PtdEtn synthesis on a continuing basis when one of the pathways is deficient for some reason. All these remain as hypotheses to be explored in plant cells.

LITERATURE CITED

1. **Bligh EG, Dyer WJ** (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol **37:** 911–917
2. **Bradford MM** (1976) A rapid and sensitive methods for the quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254
3. **Buchanan AG, Kanfer JN** (1980) Topographical distribution of base exchange activities in rat brain subcellular fractions. J Neurochem **34:** 720–725

4. **Cheesbrough TM, Moore TS** (1980) Transverse distribution of phospholipids in organelle membranes from *Ricinus communis* L var Hale endosperm. Plant Physiol **65:** 1076–1080
5. **Cheesbrough TM, Moore TS** (1983) Peroxisome biogenesis. *In* Huang AHC, RN Trelase, TS Moore, eds, Plant Peroxisomes. Academic Press, New York, p 167
6. **Corazzi L, Binaglia L, Roberti R, Freysz L, Arienti G, Porcellati G** (1983) Compartmentation of membrane phosphatidylethanolamine formed by base-exchange reaction in rat brain microsomes. Biochim Biophys Acta **730:** 104–110
7. **Dawidowicz EA** (1987) Dynamics of membrane lipid metabolism and turnover. Annu Rev Biochem **56:** 43–61
8. **Depierre JW, Ernster L** (1977) Enzyme topology of intracellular membranes. Annu Rev Biochem **46:** 201–262
9. **Duck-Chong CG** (1979) A rapid sensitive method for determining phospholipid phosphorus involving digestion with magnesium nitrate. Lipids **14:** 492–497
10. **Etemadi AH** (1980) Membrane asymmetry. Biochim Biophys Acta **604:** 423–475
11. **Gordesky SE, Marinetti GV** (1973) The asymmetric arrangement of phospholipids in the human erythrocyte membrane. Biochem Biophys Res Commun **50:** 1027–1031
12. **Hanson AA, Scott NA** (1980) Betaine synthesis from radioactive precursors in attached water-stressed barley leaves. Plant Physiol **66:** 342–348
13. **Klein R, Miller N, Laser H** (1975) The activation of phospholipase C from *Clostridium welchii* by quinine. Chem Phys Lipids **15:** 15–26
14. **Kornberg RD, McConnell HM** (1971) Inside-outside transposition of phospholipids in vesicule membrane. Biochem **11:** 1111–1120
15. **Kreibich G, Debey P, Sabatini DD** (1973) Selective release of content from microsomal vesicles without membrane disassembly. J Cell Biol **58:** 436–462
16. **Lord JM, Kagawa T, Moore TS, Beevers H** (1973) Endoplasmic reticulum as the site of lecithin formation in castor bean endosperm. J Cell Biol **57:** 659–667
17. **Litman BJ** (1974) Determination of molecular asymmetry in the phosphatidylethanolamine surface distribution in mixed phospholipid vesicles. Biochemistry **13:** 2844–2848
18. **Marinetti GV, Love R** (1976) Differential reaction of cell membrane phospholipids and protein with chemical probes. Chem Phys Lipids **16:** 239–254
19. **Metcalfe JC, Bennett JP, Hesketh TR, Houslay MD, Smith CA, Warren GB** (1976) The lateral organization of lipids around a calcium transport protein: Evidence for a phospholipid annulus that modulates function. *In* Y Hatefi Ldjaradi-Bhaniance, ed, The Structural Basis of Membrane Functions. Academic Press, New York, pp 57–67
20. **Moore TS** (1975) Phosphatidylserine synthesis in castor bean endosperm. Plant Physiol **56:** 177–180
21. **Nilsson OS, Dallner G** (1977) Enzyme and phospholipid asymmetry in liver microsomal membranes. J Cell Biol **72:** 568–583
22. **Nilsson OS, Dallner G** (1977) Transverse asymmetry of phospholipids in subcellular membrane of rat liver. Biochem Biophys Acta **464:** 453–458
23. **Op den Kamp, JAF** (1979) Lipid asymmetry in membranes. Annu Rev Biochem **48:** 47–71
24. **Rothman JE, Kennedy EP** (1977) Asymmetrical distribution of phospholipids in membrane of *Bacillus megaterium.* J Mol Biol **110:** 603–618
25. **Rothman JE, Lenard J** (1977) Membrane asymmetry. Science **195:** 743–753
26. **Shin S, Moore TS** (1990) Phosphatidylethanolamine synthesis by castor bean endosperm. A base exchange reaction. Plant Physiol **93:** 148–153
27. **Sparace SA, Wagner LK, Moore TS** (1981) Phosphatidylethanolamine biosynthesis in castor bean endosperm. Plant Physiol **67:** 922–925
28. **Vale MGP** (1977) Localization of the amino phospholipid in sarcoplasmic reticulum membranes revealed by trinitrobenzene sulfonate and fluordinitro-benzene. Biochim Biophys Acta **471:** 39–48
29. **Verkleij AJ, Zwall RFA, Roelofsen B, Comfurus P, Kastelijin D, Van Deenen LLM** (1973) The asymmetric distribution of phospholipids in the human red cell membrane: a combined study using phospholipase and freeze-etch electron microscopy. Biochim Biophys Acta **323:** 178–193