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BIOGENESIS OF ENDOPLASMIC RETICULUM PHOSPHATIDYLCHOLINE

TRANSLOCATION OF INTERMEDIATES ACROSS THE MEMBRANE BILAYER DURING METHYLATION OF PHOSPHATIDYLETHANOLAMINE

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Summary

Phosphatidylethanolamine of rat liver microsomes is rapidly methylated by *S*-adenosyl[*methyl*-^{14}C]methionine to produce phosphatidyl-*N*-monomethylethanolamine, phosphatidyl-*N*,*N*-dimethylethanolamine and phosphatidylcholine. Using phospholipase C as a probe, on both opened (0.4% taurocholate or French pressure cell treatment) and unopened microsomes, it is demonstrated that phosphatidylcholine is labelled in the inner leaflet of the bilayer and, to a greater extent, in the outer leaflet. Phosphatidyl-*N*,*N*-dimethylethanolamine is labelled in the outer leaflet and in a pool sequestered from phospholipase C in open and closed vesicles. Phosphatidyl-*N*-monomethylethanolamine is labelled in a similarly sequestered pool. When microsomes containing labelled phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine were incubated with unlabelled *S*-adenosylmethionine, these phospholipids were methylated to produce phosphatidylcholine in the outer leaflet. This metabolism was inhibited by *S*-adenosylhomocysteine.

Trypsin treatment of unopened microsomes inhibited 95% of the incorporation of $^{14}CH_3$ into the outer leaflet of the bilayer with no effect on incorporation into sequestered phosphatidyl-*N*-monomethylethanolamine, phosphatidyl-*N*,*N*-dimethylethanolamine and phosphatidylcholine. Therefore, sequestered phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine are apparently synthesised by enzymes located at the inner surface of the microsomal membranes.

These observations suggest that initial methylation of phosphatidylethanolamine takes place at the innner surface of the microsomes and that phosphatidyl-*N*-monomethylethanolamine is transferred to the outer leaflet to produce phosphatidylcholine. However, phosphatidyl-*N*-monomethylethanolamine is also methylated at the inner leaflet to produce phosphatidylcholine which does not equilibrate with that of the outer leaflet. Phosphatidylcholine of both the inner and outer bilayer leaflets is uniformly labelled by injection of [^{14}C]-methionine, in vivo.

Introduction

The proteins of all biological membranes which have been investigated exhibit a transverse asymmetry [1—3]. The phospholipids of red blood cell membranes have also been shown to have an asymmetric distribution [3]. Studies of the phospholipids of intracellular membranes have been fewer and in some cases only preliminary. However, most studies suggest that asymmetry of phospholipids is a general property of biological membranes. We have recently demonstrated, using phospholipase C as a probe, that the phospholipids of liver endoplasmic reticulum are arranged asymmetrically with phosphatidylcholine concentrated in the cytoplasmic leaflet of the bilayer and phosphatidylethanolamine in the cisternal leaflet [4]. The endoplasmic reticulum is the major site of synthesis of phospholipid in the rat hepatocyte [5] and is therefore the probable source of most of the phospholipid of other membranes. It is important, therefore, to determine how phospholipid asymmetry is produced during the formation of endoplasmic reticulum in order to understand both the mechanism of biogenesis of this membrane and its relationship with other cellular membranes.

Phosphatidylcholine is the major phospholipid of endoplasmic reticulum membranes and 70—75% of this lipid is located in the outer leaflet of microsomal vesicles, corresponding to the cytoplasmic leaflet of the endoplasmic reticulum membrane in situ [4]. Phosphatidylcholine is synthesised predominantly by two pathways in rat liver [6]. One pathway involves the transfer of phosphorylcholine from CDP-choline to diglyceride and is catalysed by choline phosphotransferase. We have recently demonstrated that choline incorporated either in vivo from [*methyl*-^{14}C]choline or in vitro from CDP-[*methyl*-^{14}C]-choline is mainly located in the outer leaflet of the endoplasmic reticulum bilayer, suggesting an asymmetric biogenesis by this pathway [7]. The second pathway involves sequential transfer of three methyl groups from *S*-adenosylmethionine to phosphatidylethanolamine. Hirata et al. [8] have recently demonstrated that in bovine adrenal medulla this sequence of reaction is catalysed by two enzymes, the first producing phosphatidyl-*N*-monomethylethanolamine, and the second transferring two methyl groups to produce phosphatidyl-*N*,*N*-dimethylethanolamine, followed by phosphatidycholine. In the red blood cell membrane, these enzymes appear to be asymmetric, the first enzyme being located at the inner surface of the membrane and the second at the outer surface [9]. We have now investigated the site of methylation of phosphatidylethanolamine in endoplasmic reticulum membranes using

phospholipase C to determine the site of incorporation of $^{14}CH_3$ groups into phospholipids of each side of the bilayer. The observations reported here indicate that, in endoplasmic reticulum, there is an asymmetric distibution of methyltransferases and that translocation of methylated intermediates across the membrane bilayer takes place.

Methods

General methods. These were described as previously [4,7].

Prepation of total microsomes. Livers of male Sprague-Dawley rats (150—200 g) were removed and homogenized in 0.25 M sucrose to yield 20% homogenates. These were centrifuged at 12 500 rev./min (10 000 $\times g_{av}$) for 20 min in the SW 40 rotor of a Beckman L65 centrifuge. The supernatant was then centrifuged at 40 000 rev./min (105 000 $\times g_{av}$) for 45 min. The pellet was resuspended in the appropriate buffer and used without storage. Microsomes prepared in this way have a very low contamination with either plasma membrane or Golgi membranes [10]. Based on determination of 5′-nucleotidase and galactosyltransferase, the contributions of plasma membrane and Golgi membranes to the microsomal fraction are less than 4 and 2%, respectively. Microsomal pellets were resuspended in the buffer used for subsequent treatment (see below) using a Potter Elvejhem homogenizer by hand.

Incorporation of $^{14}CH_3$ groups from methionine in vivo. L-[*methyl*-^{14}C]-Methionine (spec. act., 50 mCi/mmol) (The Radiochemical Centre, Amersham), 5 μCi/100 g body wt., was injected intraperitoneally 1 h prior to killing of rats and the preparation of microsomes.

Incorporation of $^{14}CH_3$ groups from S-adenosylmethionine in vitro. Methyl groups were incorporated into microsomal phospholipids using conditions similar to those described by Hirata et al. [8,9] for assay of both methyl transferases. The first methylation step was assayed at 37°C in a medium containing 4 μM *S*-adenosyl-L-[*methyl*-^{14}C]methionine (The Radiochemical Centre, Amersham) diluted to a specific activity of $2—5 \cdot 10^7$ cpm/μmol with unlabelled *S*-adenosylmethionine (Sigma Chemical Co.), 10 mM $MgCl_2$, 0.1 mM sodium EDTA, 50 mM sodium acetate buffer, pH 6.5 (low pH buffer). Complete methylation was assayed at 37°C in a medium containing 1 mM *S*-adenosyl-L-[*methyl*-^{14}C]methionine (spec. act. $2—5 \cdot 10^6$ cpm/mol), 10 mM $MgCl_2$, 50 mM Tris-HCl buffer, pH 8.5, in 0.87% NaCl (high pH buffer).

Treatment of microsomes with phospholipase C. After incorporation of methyl groups into microsomal phospholipid in vitro, phospholipase C (10 units, type 1 from *Clostridium welchii*, Sigma Chemical Co.), 0.1 M Tris-HCl, pH 7.4, containing 2 mM $CaCl_2$ was added to each incubation. After 15 min the phospholipids were extracted as described below. Aliquots of the total lipid extract were removed for determination of phospholipid phosphorus or total radioactivity. The remainder was applied to thin layer plates and separated (see below). Hydrolysis of phospholipids was determined by comparison of the phospholipase C-treated microsomes with those incubated without phospholipase C.

Microsomes labelled in vivo were resuspended in 0.1 M Tris-HCl, pH 7.4, and treated with phospholipase C as above.

Extraction and analysis of phospholipids. Phospholipid markers were purchased from Lipid Products, Nutfield Nurseries, Crab Hill Lane, South Nutfield, Surrey, U.K., and from Sigma Chemical Co. and were used on all plates.

Phospholipids were extracted as described previously except that $CHCl_3$/CH_3OH/HCl (2 : 1 : 0.02, v/v) [9], was used as an extraction medium. Phospholipids were separated on thin layers of silica gel (Merck 60F 254) using either $CHCl_3$/CH_3OH/glacial CH_3CO_2H/H_2O (60 : 50 : 1 : 4, v/v) to separate phosphatidylcholine, sphingomyelin, phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine and neutral lipids, or *n*-propyl alcohol/propionic acid/$CHCl_3$/H_2O (3 : 2 : 2 : 1, v/v) to separate phosphatidylcholine, phosphatidyl-*N*,*N*-dimethylethanolamine, phosphatidyl-*N*-monomethylethanolamine and phosphatidylethanolamine. In the latter system, phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine were resolved but were not fully separated from phosphatidylinositol and phosphatidylserine. However, phosphatidylinositol and phosphatidylserine are not labelled and only the distribution of radioactivity in phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine was determined. These phospholipids are produced in very small amounts in these experiments and it is not possible to determine their specific activities. In some experiments, phospholipids containing spots were scraped directly into vials, scintillation fluid added, and the radioactivity counted. In other experiments, phospholipids were eluted from silica gel with $CHCl_3$/CH_3OH (1 : 1, v/v) and the specific activity of the lipid determined. Over 85% of the phospholipid phosphorus and over 90% of the radioactivity applied to the thin layer plates were recovered in the fractions.

Treatment of microsomes with trypsin. Microsomes were suspended in 0.25 M sucrose, 0.05 M Tris-HCl, pH 7.4, and incubated with trypsin (1 mg/6—8 mg microsomal protein) (Boehringer Mannheim) for 30 min at 37°C. At the end of the incubation period trypsin inhibitor (1 mg/mg trypsin, Boehringer Mannheim) was added. In control experiments, the inhibitor was added with trypsin at the beginning of the incubation.

Treatment of microsomes with phospholipase D. Microsomes were incubated with phospholipase D (10 U/ml, Sigma Chemical Co.) in 0.05 M acetate buffer, pH 5.7, 50 mM $CaCl_2$ for a range of times. The reaction was stopped by addition of 20 vol. of $CHCl_3$/CH_3OH (2 : 1, v/v). Lipids were extracted and phospholipids separated and assayed as described previously [4].

Determination of latency of mannose-6-phosphatases. Microsomes were suspended in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose with and without 0.4% taurocholate at 0°C for 30 min. 0.1 M mannose-6-phosphate (1 mM, Sigma Chemical Co.) was added and the microsomes incubated at 37°C for a range of times. The reaction was stopped by addition of 1% trichloroacetic acid, the precipitate removed by centrifugation, and phosphate release determined on the supernatant. Latency is the percentage of activity not expressed, except by addition of taurocholate [11].

Preparation of opened microsomal vesicles. Microsomes were opened by treatment with 0.4% taurocholate in Tris-HCl buffer (0.05 M, pH 7.4) in 0.25 M sucrose for 30 min on ice, or by suspension in the same buffer at a concentration of 1—5 mg/ml and passage through a French pressure cell at 20 000

lb/inch^2. Both these procedures cause loss of mannose-6-phosphatase latency and loss of labelled protein contents [4].

Results

Incorporation of methyl groups from S-adenosyl[methyl-^{14}C]methionine into microsomal phospholipid

Hirata et al. [8] have reported that methylation of phosphatidylethanolamine to yield phosphatidylcholine is catalysed by two enzymes. The first catalyses transfer of one methyl group, has a pH optimum of 6.5, a low K_m value for *S*-adenosylmethionine and an absolute requirement for Mg^{2+}. The second catalyses two successive methylations, has a pH optimum of 10 and a high K_m value for *S*-adenosylmethionine. Consistent with this report we found that, at pH 8.5, with high concentrations of *S*-adenosylmethionine (1 mM), phosphatidylcholine is the major phospholipid labelled, although small amounts of label are found in the mono- and dimethylated precursors (Fig. 1). At pH 6.5 with low concentrations of *S*-adenosylmethionine (4 μM), the labelled precursors tend to accumulate indicating that the second and third methyl transfer steps are slower under these conditions (Fig. 2). However, unlike Hirata et al. [8], we did not find that phosphatidyl-*N*-monomethylethanolamine was the predominant labelled species, except at early time points.

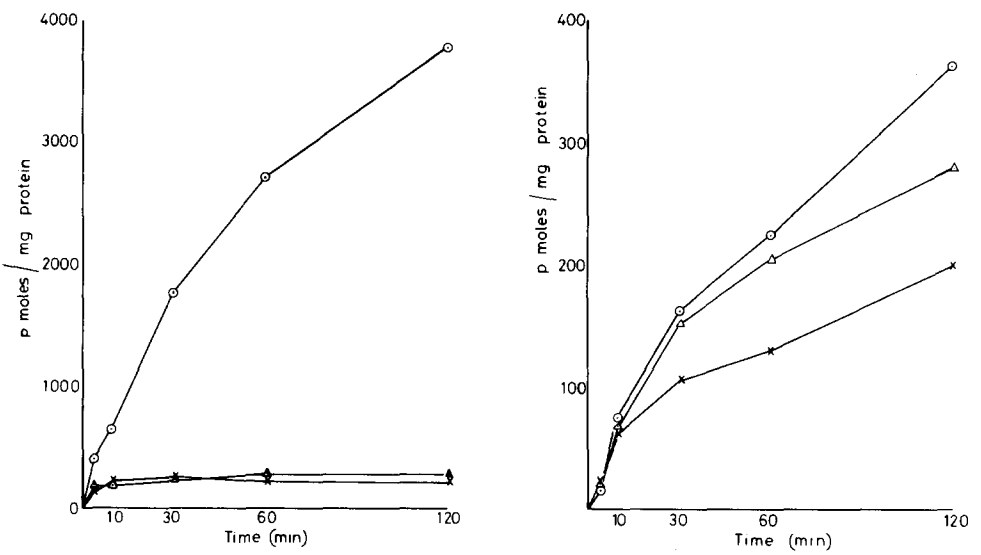

Fig. 1. Microsomes (5 mg protein) were incubated with *S*-adenosyl[*methyl*-^{14}C]methionine (1 mM) in high pH buffer. Phospholipids were extracted and separated as described in Methods. Values of pmol $^{14}CH_3$ groups incorporated into phpspholipid per mg microsomal protein are plotted against time of incubation. ○———○, phosphatidylcholine; △———△, phosphatidyl-*N*,*N*-dimethylethanolamine; X———X, phosphatidyl-*N*-monomethylethanolamine.

Fig. 2. Microsomes (4 mg protein) were incubated with *S*-adenosyl[*methyl*-^{14}C]methionine (4 μM) in low pH buffer. Phospholipids were extracted and separated as described in Methods. Values of pmol $^{14}CH_3$ groups incorporated into phospholipid per mg microsomal protein are plotted against time of incubation. ○———○, phosphatidylcholine; △———△, phosphatidyl-*N*,*N*-dimethylethanolamine; X———X, phosphatidyl-*N*-monomethylethanolamine.

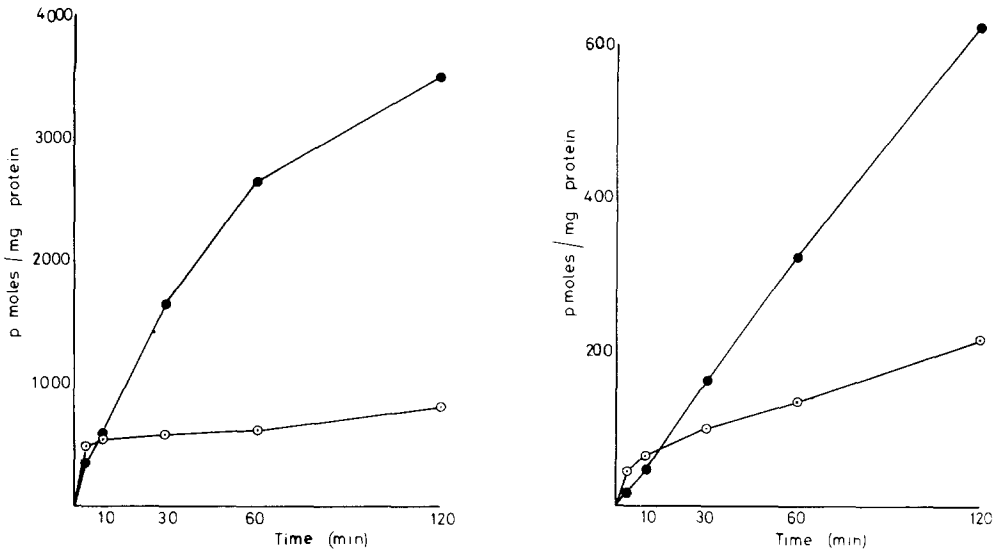

Fig. 3. Microsomes (5 mg protein) were incubated with *S*-adenosyl[*methyl*-^{14}C]methionine (1 mM) in high pH buffer. At different times phospholipase C was added and the distribution of labelled phospholipid in hydrolysed and unhydrolysed pools was determined as described in Methods. Values of pmol $^{14}CH_3$ groups incorporated into phospholipids unhydrolysed (○——○) and hydrolysed (●——●) are plotted against time of incubation.

Fig. 4. As Fig. 3 except that initial incubation was with *S*-adenosyl[*methyl*-^{14}C]methionine (4 μM) in low pH buffer.

Availability of labelled phospholipids for hydrolysis by phospholipase C

Under both conditions described above, $^{14}CH_3$ groups were initially incorporated to the greatest extent into a pool of phospholipids not hydrolysed by phospholipase C. However, with increased incubation time, label was incorporated rapidly into hydrolysed phospholipids (Figs. 3 and 4).

Label incorporated into the unhydrolysed pool of phosphatidylcholine remained fairly constant, while that incorporated into the hydrolysed pool increased during the 120 min incubation (Figs. 5 and 6). In a typical experiment, the specific activity of unhydrolysed phosphatidylcholine was 4100 cpm/μmol at 2 min and 4300 cpm/μmol at 60 min, while that of the total phosphatidylcholine of the membrane was 2912 cpm/μmol at 2 min and 9805 cpm/μmol at 60 min. By calculation, the specific activity of the phosphatidylcholine of the outer pool is 2516 cpm/μmol at 2 min and 11 653 cpm/μmol at 60 min. Hydrolysed and unhydrolysed pools of phosphatidylcholine, therefore, do not equilibrate and are labelled separately.

Labelled phosphatidyl-*N*-monomethylethanolamine was mainly unhydrolysed by phospholipase C (Figs. 5 and 6). Labelled phosphatidyl-*N*,*N*-dimethylethanolamine occurred in both hydrolysed and unhydrolysed pools, but was most abundant in the former. The results illustrated are from experiments using 4 μM *S*-adenosylmethionine. However, similar labelling patterns were obtained when microsomes were incubated with 1 mM *S*-adenosylmethionine, although incorporation into phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine was low.

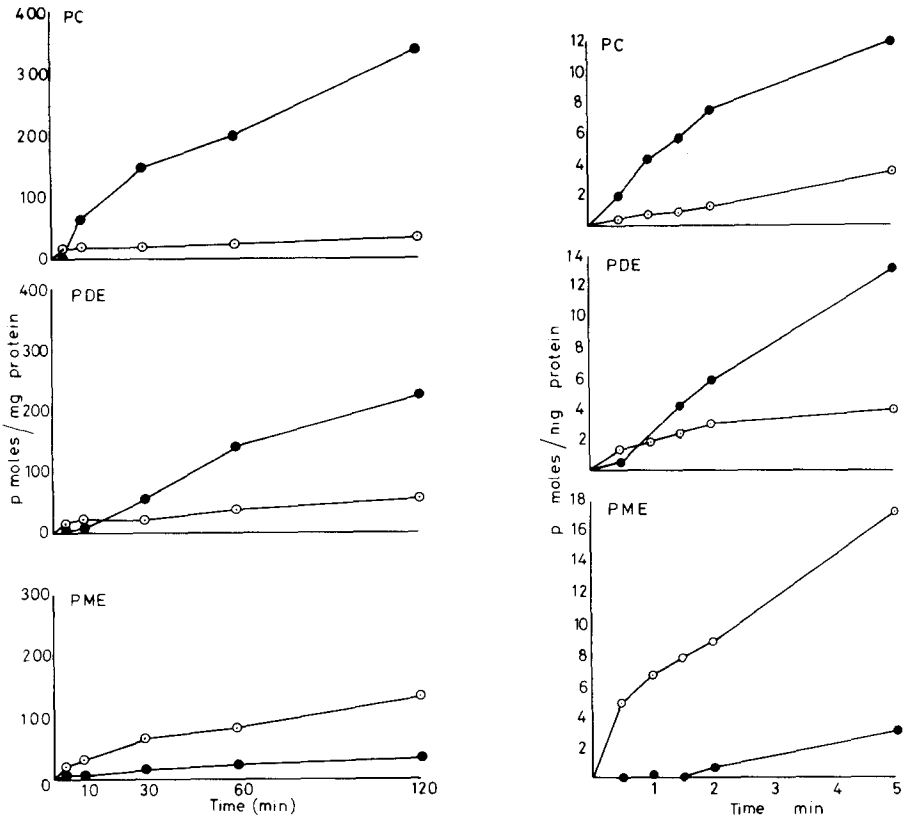

Fig. 5. As Fig. 4, except that distribution of incorporation of label into individual phospholipids in the hydrolysed pool (●———●) and the unhydrolysed pool (○———○) is plotted. PC, phosphatidylcholine; PDE, phosphatidyl-*N*,*N*-dimethylethanolamine; PME, phosphatidyl-*N*-monomethylethanolamine.

Fig. 6. As Fig. 5 except that short incubation times were used.

Our previous observations have suggested that phospholipids hydrolysed by phospholipase C are in the outer leaflet of the membrane bilayer and those not hydrolysed in the inner leaflet [4]. In the present experiments, a distribution of phospholipids about the bilayer was found similar to that reported previously [4]. However, the labelled phospholipid represents a very small pool and may not behave as the bulk of the membrane phospholipid. The above results could therefore be interpreted in two ways. Labelled phospholipids in the unhydrolysed pool may be in the inner leaflet of the bilayer, or, alternatively, may be sequestered possibly by the methyltransferases; in either case, the hydrolysed phospholipids are in the outer leaflet of the bilayer. When microsomes were opened with taurocholate, or a French pressure cell, after incorporation of labelled methyl groups before treatment with phospholipase C, phosphatidyl-*N*-monomethylethanolamine remained unhydrolysed, phosphatidyl-*N*,*N*-dimethylethanolamine hydrolysis was not increased, and hydrolysis of phosphatidylcholine was increased (Table I). Hydrolysis of total phosphatidylcholine and labelled phosphatidylcholine

TABLE I

HYDROLYSIS BY PHOSPHOLIPASE C OF LABELLED PHOSPHOLIPIDS OF MICROSOMAL VESICLES OPENED WITH TAUROCHOLATE OR A FRENCH PRESS CELL

Microsomes (30 mg protein in 5 ml) were incubated with *S*-adenosyl[*methyl*-^{14}C]methionine in low pH buffer for 2 min. The incorporation was stopped by addition of *S*-adenosylhomocysteine and 5 ml of Tris-HCl buffer, pH 7.4, in 0.25 M sucrose were added. 5 ml of the suspension were treated with a French pressure cell, 2.5 ml were treated with 0.4% taurocholate on ice for 30 min, and 2.5 ml were held on ice without further treatment. Aliquots of each group of samples were incubated with and without phospholipase C as described in Methods. % hydrolysis of labelled phospholipids was determined. Results given are means of four determinations. PC, phosphatidylcholine; PDE, phosphatidyl-*N,N*-dimethylethanolamine; PME, phosphatidyl-*N*-monomethylethanolamine.

	% hydrolysis		
	PC	PDE	PME
Taurocholate-opened vesicles	61.4	42.7	0
French pressure cell-opened vesicles	64.3	44.0	0
Closed vesicles	39.7	44.1	0

was not complete, however, the specific activity of the hydrolysed phosphatidylcholine was the same as that not hydrolysed. In open vesicles, therefore, phosphatidylcholine at the outer and inner leaflets is equally available for hydrolysis by phospholipase C. Phosphatidylcholine is, therefore, labelled on both sides of the membrane bilayer, but unhydrolysed phosphatidyl-*N,N*-dimethylethanolamine and phosphatidyl-*N*-monomethylethanolamine are sequestered in the membrane, and from these experiments it is not possible to assign them to either side of the bilayer.

Translocation of sequestered phospholipids to the outer leaflet of the bilayer

In order to determine whether labelled precursors move from the unhydrolysed pool to the outer leaflet of the bilayer, a series of experiments were performed in which microsomes were 'pulsed' with *S*-adenosyl[^{14}C]methionine, isolated, and 'chased' with unlabelled *S*-adenosylmethionine.

When microsomes were incubated with *S*-adenosyl[^{14}C]methionine for 2 min, cooled by addition of buffer at 0°C, isolated by centrifugation, washed and reincubated in either high pH buffer or low pH buffer with or without unlabelled *S*-adenosylmethionine, there was a considerable increase in the label incorporated. *S*-Adenosylmethionine becomes bound to the microsomal vesicles during the initial short incubation and continues to be utilized after centrifugation and reincubation. Hirata and Axelrod [9] reported a similar finding and incubated red cell ghosts overnight at 0°C with labelled *S*-adenosylmethionine in order to introduce the substrate into the interior of the ghosts. In some of our experiments, *S*-adenosylhomocysteine was added during the chase period to prevent further incorporation of bound substrate.

After incubation of microsomes at low pH for 2 min followed by centrifugation, phosphatidyl-*N*-monomethylethanolamine, phosphatidyl-*N,N*-dimethylethanolamine and phosphatidylcholine were labelled (Table II). The distribution of label in hydrolysed and unhydrolysed pools of these phospholipids was not the same as that at 2 min in Figs. 5 and 6, when phosphatidyl-*N*-

TABLE II

TRANSLOCATION OF SEQUESTERED PHOSPHOLIPIDS WITHIN THE BILAYER

Microsomes (7 mg protein) were incubated with *S*-adenosyl[*methyl*-^{14}C]methionine (4 μM) in low pH buffer for 2 min. Ice-cold buffer was added, the microsomes isolated by centrifugation and suspended in high pH buffer. In A, microsomes were treated immediately with phospholipase C as described in Methods. In B, *S*-adenosylhomocysteine (1 mg/ml) was added and the samples incubated for 60 min before addition of phospholipase C. In C, unlabelled *S*-adenosylmethionine (1 mM) was added and the samples incubated for 60 min before addition of phospholipase C. The labelled phospholipid hydrolysed by phospholipase C and not hydrolysed was determined, as described in Methods. Averages of four determinations are given. Abbreviations as in Table I.

		pmol $^{14}CH_3$ groups incorporated per mg protein	
		Inner leaflet	Outer leaflet
(A)	PC	2.53	6.62
	PDE	4.03	2.36
	PME	4.10	2.42
(B)	PC	2.73	6.41
	PDE	4.15	2.16
	PME	4.15	2.87
(C)	PC	2.03	21.46
	PDE	0.39	0
	PME	0.56	0

monomethylethanolamine was the predominant labelled species and restricted to the inner leaflet. However, it is probable that slow metabolism continued during the centrifugation and resuspension steps. When the microsomes were reincubated at 37°C with unlabelled *S*-adenosylmethionine (1 mM) in high pH buffer, labelled phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine were methylated to produce labelled phosphatidylcholine in the outer leaflet of the bilayer (Table II), while phosphatidylcholine of the inner leaflet remained unchanged.

When microsomes labelled by incubation with *S*-adenosyl[^{14}C]methionine were reincubated in the presence of *S*-adenosylhomocysteine to inhibit further incorporation of bound substrate, no movement of labelled intermediates to the outer leaflet occurred. Translocation of phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine is therefore dependent on methylation.

A variety of different conditions were investigated in this group of experiments. Essentially, the same results were found when initial incorporation of label was in high pH buffer with high concentrations of *S*-adenosylmethionine or if the chase incubations were at low pH.

Integrity of microsomal vesicles during pulse-chase experiments

As repeated experimental manipulation may result in loss of the integrity of microsomal vesicles, the latency of mannose-6-phosphatase at each stage of the experimental steps was determined. Latency was 87% in untreated microsomes, 96% after short incubation at low pH followed by centrifugation, and 81% after the first 60 min of incubation with unlabelled *S*-adenosyl-

methionine. After incubation for 120 min latency fell to 60%, suggesting that the vesicles are partly opened. During our experimental procedure, however, microsomal vesicles retain their integrity. It is not possible to determine mannose-6-phosphatase latency after phospholipase C treatment, as a combination of taurocholate and phospholipase treatment inhibits this enzyme. However, we have shown previously that phospholipase C-treated microsomes have the same mannose-6-phosphatase activity as untreated microsomes, suggesting that the enzyme is still latent [7]. Consistent with this, phospholipase C treatment does not open microsomal vesicles to allow leakage of protein contents [4].

Effect of trypsin treatment of microsomes on methylation of phosphatidylethanolamine

In order to investigate directly the site of enzymes involved in methylation of phosphatidylethanolamine, microsomes were treated with trypsin. Incorporation of methyl groups into phospholipids was inhibited more than 80%, and 95% of the incorporation of label into phospholipids of the outer leaflet was inhibited (Table III). Incorporation of label into phosphatidyl-*N*-monomethylethanolamine, and phosphatidyl-*N*,*N*-dimethylethanolamine, not available for hydrolysis by phospholipase C, was unaffected by trypsin treatment of microsomes while incorporation of label into phosphatidylcholine was reduced approx. 50%. Mannose-6-phosphatase activity remained 90% latent during this treatment, suggesting that the vesicles remained unopened.

When microsomes were opened by taurocholate or a French pressure cell, before trypsin treatment, incorporation of $^{14}CH_3$ groups into phosphatidyl-*N*-monomethylethanolamine, phosphatidyl-*N*,*N*-dimethylethanolamine and phosphatidylcholine was decreased to a similar extent (Table IV). All methyltransferases of opened vesicles are uniformly available to trypsin therefore, and it appears that phosphatidyl-*N*-monomethylethanolamine and phospha-

TABLE III

INHIBITION OF METHYLTRANSFERASES BY TRYPSIN TREATMENT

Microsomes (15 mg protein) were incubated with trypsin (2 mg) as described in Methods. Trypsin inhibitor (2 mg) was added after 30 min incubation or at the beginning of the incubation. Aliquots (3 mg protein) were removed and incubated with *S*-adenosyl[*methyl*-^{14}C]methionine (1 mM) in high pH buffer for 30 min. Phospholipase C was added and the phospholipids hydrolysed and unhydrolysed determined as described in Methods. Averages of four determinations are given. Abbreviations as in Table I.

	pmol $^{14}CH_3$ group incorporated per mg protein	
	Inner leaflet	Outer leaflet
No treatment		
PC	59.3	497.3
PDE	27.6	65.6
PME	70.4	0
Trypsin-treated		
PC	23.4	22.9
PDE	23.2	11.8
PME	65.9	0

TABLE IV

INHIBITION BY TRYPSIN OF INCORPORATION OF $^{14}CH_3$ GROUPS FROM *S*-ADENOSYL-METHIONINE INTO PHOSPHOLIPIDS OF OPEN AND CLOSED MICROSOMAL VESICLES

Microsomes (10 mg protein/ml) in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose were treated with a French pressure cell or with 0.4% taurocholate for 30 min at 0°C or held on ice for 30 min. Trypsin (1 mg/5 mg protein) was added to half the samples, which were incubated at 37°C for 30 min. At the end of the incubation, trypsin inhibitor (1 mg/5 mg protein) was added. Aliquots (2 mg protein) were removed and incubated with *S*-adenosyl[*methyl*-^{14}C]methionine (4 μM) in low pH buffer for 30 min. Phospholipids were extracted and separated as described in Methods. Results are averages of four determinations ± S.D. Abbreviations as in Table I.

	pmol incorporated per mg protein	% reduction incorporated label
No treatment		
PC	475.6 ± 17.6	
PDE	85.9 ± 2.5	
PME	63.9 ± 3.0	
Trypsin on closed vesicles		
PC	40.3 ± 2.5	91.2
PDE	30.3 ± 1.3	64.7
PME	57.2 ± 0.7	10.5
Trypsin on vesicles opened with taurocholate		
PC	97.9 ± 0.3	78.6
PDE	3.3 ± 4.7	84.7
PME	18.0 ± 9.8	71.8
Trypsin on vesicles opened with French pressure cell		
PC	50.3 ± 4.2	89.0
PDE	19.2 ± 2.0	77.6
PME	17.2 ± 8.1	73.1

tidyl-*N*,*N*-dimethylethanolamine sequestered from hydrolysis by phospholipase C are synthesised by enzymes located at the inner surface of the microsomal membrane.

Site of incorporation of [^{14}C]methionine into the microsomal bilayer in vivo

Liver microsomal phosphatidylcholine was labelled after injection of [^{14}C]-methionine in vivo. The specific activity of this phospholipid of the inner leaflet of the bilayer was 996.6 ± 51.8 cpm/μmol, while that of the total microsomal pool was 1017.7 ± 30.7 cpm/μmol in four determinations. The *S*-adenosyl-methionine pathway, therefore, uniformly labels phosphatidylcholine on both sides of the membrane bilayer in vivo. This is in contrast to the CDP-choline pathway, which preferentially labels phosphatidylcholine of the outer leaflet [7].

Hydrolysis of microsomal phospholipids by phospholipase D

Hydrolysis of microsomal phospholipids by phospholipase D reached a plateau at approx. 50% after 120 min incubation. 72% of the phosphatidylcholine, 68% of the sphingomyelin, 28% of the phosphatidylethanolamine and 27% of the phosphatidylserine were hydrolysed. During incubation the vesicular protein contents, labelled with [^{3}H]leucine in vivo [5], were retained suggesting that the vesicles remained intact. Hydrolysis by phospholipase

was increased, by opening the vesicles using a French pressure cell, to 84% of the phosphatidylcholine, 75% of the sphingomyelin, 72% of the phosphatidylethanolamine and 75% of the phosphatidylserine. Assuming that in unopened vesicles only phospholipids of the outer leaflet of the membrane bilayer are hydrolysed and that in opened vesicles all the phospholipids are available, these results suggest a asymmetric distribution of phospholipids similar to those indicated by studies using phospholipase C [4].

Phospholipase D was not selected for further investigations because the incubation conditions caused microsomes to aggregate, which might interfere with subsequent experimental treatment. However, these observations provide independent support for our results with phospholipase C.

Discussion

The use of phospholipases as probes of membranes architecture is subject to many potential criticisms [2]. In our experiments the assumption that phospholipase C hydrolyses only the outer leaflet of microsomal membranes is based on several observations. In intact microsomes, approximately half of the phospholipid is not available for hydrolysis. This pool consists of approximately one-quarter of the phosphatidylcholine and sphingomyelin and three-quarters of the phosphatidylethanolamine and phosphatidylserine [4]. After phospholipase C treatment, the microsomal vesicles retain their protein contents and the activity of mannose-6-phosphatase, a latent enzyme, is not increased [4,7]. The microsomal vesicles, therefore, appear to remain closed during treatment. This is supported by their appearance in the electron microscope (Higgins, J.A., unpublished observations). However, if the microsomal vesicles are opened by mechanical means (French pressure cell), detergents (deoxycholate, lysolecithin) or pH changes (0.1 M NH_4OH) before treatment with phospholipase C, phospholipid hydrolysis increases and this is mainly due to a greater hydrolysis of phosphatidylethanolamine and phosphatidylserine [4]. If microsomal phospholipids are extracted and dispersed by sonication, these are completely hydrolysed by phospholipase C with the exception of phosphatidylinositol [4]. The most probable explanation for these observations is that the phospholipids hydrolysed in unopened vesicles are in the outer, available, leaflet of the bilayer, and that when vesicles are opened phospholipids of both sides of the bilayer are available for hydrolysis. Experiments with phospholipase D suggest a asymmetric distribution of phospholipids similar to those found with phospholipase C. Investigations of the distribution of phospholipid in microsomes would be greatly benefited by the preparation of inside-out vesicles. However, attempts to prepare such vesicles in other laboratories [13] and by ourselves have not been successful. Controls in which vesicles are opened by a variety of techniques provide the presently available alternative.

There have been two investigations, in addition to ours, of the transverse distribution of phospholipids across the microsomal membrane. Nilsson and Dallner [13,14] used phospholipase A_2 to probe the outer leaflet of microsomes and concluded that phosphatidylcholine and phosphatidylethanolamine are located mainly in the outer leaflet of the bilayer. Sundler et al. [15] also

used phospholipase A_2 and observed that this enzyme completely hydrolysed the phospholipids of microsomal vesicles. However, before the vesicles opened, there was no selective hydrolysis of phospholipids suggesting that these are not asymmetrically distributed. We have recently shown that resuspension of microsomal vesicles in 10 mM EDTA at 0°C or buffered sucrose followed by centrifugation will induce a relocation of phospholipids, although the vesicles retain mannose-6-phosphatase latency. Such vesicles exhibit no phospholipid asymmetry when probed with phospholipase [17]. It is possible, therefore, that phospholipase A_2 treatment before opening the microsomal vesicle will induce a similar translocation of phospholipids to produce a random distribution. Lysolecithin, one of the products of phospholipase A activity, is a known lytic and membrane-perturbing agent, and may induce a micellar structure in the membrane bilayer prior to its disruption [16]. Nilsson and Dallner [13, 14] found that phospholipase A, purified by the method of Cremona and Kearny [17], did not open microsomal vesicles. This is in conflict with the observation of Sundler et al. [15], who used a similar purification method, and ourselves [4]. Recently, Krebs et al. [18] have reported that phospholipase A purified in this way contains a lytic factor. This group has also demonstrated, using immunological methods, labelling with fluorescamine and using phospholipase A purified by the method of Deems and Dennis [19], that the inner mitochondrial membrane is asymmetric with phosphatidylcholine being predominantly at the cytoplasmic side and phosphatidylethanolamine at the inner surface. These results are in direct conflict with those of Nilsson and Dallner [13,14] as are ours on microsomal membranes.

Phosphatidylcholine in each leaflet of the endoplasmic reticulum bilayer has a similar specific activity when labelled with [*methyl*-^{14}C]methionine in vivo. However, methyl groups from *S*-adenosylmethionine are incorporated to the greatest extent into the outer leaflet phosphatidylcholine in vitro. It is possible that in vivo, some control mechanisms exist which regulate the methylation pathway and which are inoperative in vitro. Phosphatidylcholine synthesised by the CDP-choline phosphotransferase pathway is predominantly located in the outer leaflet of the endoplasmic reticulum bilayer, whether labelled by choline in vivo or by CDP-choline in vitro [7], therefore it appears that the two pathways for phosphatidylcholine synthesis contribute to different extents to the two sides of the bilayer, CDP-choline phosphotransferase being located mainly in the outer leaflet and the methylation pathway contributing phosphatidylcholine to both leaflets of the bilayer.

The labelling patterns of microsomal phosphatidyl-*N*-monomethylethanolamine, phosphatidyl-*N*,*N*-dimethylethanolamine and phosphatidylcholine, the specific activity of the pools of phosphatidylcholine, unavailability for hydrolysis, and the chase experiments could all be explained by two models. Initial synthesis of phosphatidyl-*N*-monomethylethanolamine may take place on the inner leaflet of the bilayer, followed by translocation and methylation to produce phosphatidylcholine at the outer leaflet. Alternatively, unhydrolysed phospholipids in intact vesicles may be sequestered and thus protected from phospholipase C. Hirata and Axelrod [9] have demonstrated that phosphatidyl-*N*-monomethylethanolamine synthesised by inside-out erythrocyte vesicles is not available to phospholipase C, and our observations also suggest

that the labelled phosphatidyl-*N*-monomethylethanolamine and part of the labelled phosphatidyl-*N*,*N*-dimethylethanolamine are sequestered in microsomes. However, treatment of intact microsomes with trypsin suggests that sequestered phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine are synthesised by enzymes located at the inner surface of the membrane. Incorporation of $^{14}CH_3$ groups into phospholipids not available to phospholipase C was unaffected by trypsin, while 95% of the incorporation into the outer leaflet was inhibited. If sequestered phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine were synthesised by enzymes located at the outer leaflet, trypsin treatment would be expected to inhibit synthesis of these phospholipids, or to modify the labelling pattern of phospholipids remaining unhydrolysed.

Our observations are consistent with a modification of the model suggested by Hirata and Axelrod [9] for the methylation of phosphatidylethanolamine in the red blood cell membrane. Thus, the inner leaflet of the microsomal membrane appears to have enzymes for complete methylation of phosphatidylethanolamine to phosphatidylcholine, although the phosphatidylcholine pool of the inner leaflet is rapidly saturated in vitro. Phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine are sequestered after synthesis and are translocated and methylated to produce phosphatidylcholine by enzymes in the outer leaflet. The microsomal membrane differs from the red blood cell membrane in that enzymes catalysing the second and third methyltransferase steps are located on both sides of the membrane bilayer. However, the red blood cell is a final stage in differentiation and net synthesis of phospholipid does not take place in these membranes in vivo, although enzymes which modify phospholipids are retained. In contrast, the endoplasmic reticulum is the major site of phospholipid synthesis in liver and is continually turned over even in the resting cell. The differences between our results and those of Hirata and Axelrod [9] could, therefore, be accounted for by differences in the function of the membranes investigated. However, an asymmetric localization of methyltransferases and translocation of intermediates may be common in the synthesis of phosphatidylcholine and phosphatidylethanolamine in a variety of membranes.

Rothman and Kennedy [20] have demonstrated that there is a rapid translocation of phosphatidylethanolamine from the inner surface of the membrane in *Bacillus megaterium* to the outer surface during membrane assembly. Studies with phospholipid-exchange protein have also suggested that under some circumstances there is a rapid translocation of phosphatidylcholine across the microsomal membrane [21,22]. In our experiments, however, the phosphatidylcholine pools on each side of the microsomal membrane do not equilibrate and only phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine cross the membrane. This translocation is rapid, but is enzyme linked. It differs therefore from the systems above, in which translocation is not linked to the metabolism of the phospholipid.

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