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ASYMMETRIC SYNTHESIS FOLLOWED BY TRANSMEMBRANE MOVEMENT OF PHOSPHATIDYLETHANOLAMINE IN RAT LIVER ENDOPLASMIC RETICULUM

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Studies with phospholipase C have indicated that two-thirds of the phosphatidylethanolamine of rat liver endoplasmic reticulum is located in the inner leaflet of the membrane bilayer. Phosphatidyl[^{14}C]ethanolamine is synthesised in microsomes incubated with CDP[^{14}C]ethanolamine. Using phospholipase C as a probe we have observed that the labelled phospholipid is initially (1–2 min) concentrated in the 'outer leaflet' of the membrane bilayer. The specific activity of this pool of phosphatidylethanolamine was 3.5 times that of the inner leaflet. If, however, the microsomes were opened with 0.4% taurocholate or the French pressure cell to make both sides of the bilayer available to phospholipase C, the phosphatidylethanolamine behaves as a single pool for hydrolysis. On longer incubation, up to 30 min, with CDP[^{14}C]ethanolamine the specific activity of the outer leaflet phosphatidylethanolamine becomes close to that of the inner leaflet. In chase experiments, in which microsomal phosphatidylethanolamine was labelled by incubation with CDP[^{14}C]ethanolamine for 1 min, the reaction stopped by addition of calcium, and the microsomes isolated by centrifugation and reincubated, labelled phosphatidylethanolamine was transferred from the 'outer leaflet' to the 'inner leaflet', so that both were equally labelled. These observations suggest that phosphatidylethanolamine is synthesised at the cytoplasmic leaflet of the endoplasmic reticulum and subsequently transferred across the membrane to the cisternal leaflet of the bilayer. Transmembrane movement is apparently temperature-dependent and independent of continued synthesis of phosphatidylethanolamine.

Introduction

During homogenization of rat liver the endoplasmic reticulum fragments to form small vesicles which, by choice of appropriate centrifugation conditions, can be isolated in the microsomal fraction with low contamination by either Golgi membranes or plasma membranes [1]. Microsomal vesicles have been demonstrated by electron microscopy, including examination of sections or freeze-fracture preparations [2–5], and by cytochemical experiments [6,7] to consist of vesicles having the same orientation in which the outer surface of the vesicle corresponds to the cytoplasmic surface of the endoplasmic reticulum membrane and the inner surface to the cisternal side of the membrane. We have demonstrated using phospholipase C from *Clostridium perfringens* and phospholipase D that there is an asymmetric distribution of phospholipids about the bilayer of microsomal vesicles [8–10]. Approximately three quarters of the phosphatidylcholine and sphingomyelin are located in the outer or cytoplasmic leaflet of the membrane and approximately two-thirds of the phosphatidylethanolamine and phosphatidylserine are located in the inner or cisternal leaflet.

The endoplasmic reticulum is the major site of

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synthesis of phospholipids and therefore plays a central role in its own biogenesis and the biogenesis of other cellular membranes. In order to understand the interrelationship between membranes of the hepatocyte it is therefore important initially to determine the mechanism by which asymmetry is produced at the site of synthesis of membrane phospholipid. Using phospholipase C as a probe of the outer leaflet, we have observed that phosphatidylcholine synthesised by the choline phosphotransferase pathway is preferentially located in the outer leaflet of the microsomal membrane, when choline is incorporated in vivo or in vitro [9]. Synthesis of phosphatidylcholine by the methylation of phosphatidylethanolamine is more complex [11]. The initial methylation step apparently takes place at the inner leaflet of the bilayer. Transfer of mono- and dimethylated derivatives across the bilayer occurs and methylation is completed at the outer leaflet. Complete methylation also occurs at the inner leaflet, resulting in two pools of phosphatidylcholine synthesised by this pathway, one in the inner leaflet and one in the outer leaflet, which in vitro do not equilibrate with each other [11]. phosphatidylethanolamine is the second major lipid of the endoplasmic reticulum accounting for 20% of the total phospholipid [8]. The enzyme catalysing synthesis of this phospholipid by transfer of phosphorylethanolamine from CDPethanolamine to diacylglycerol is apparently located at the cytoplasmic side of the membrane [12]. As two-thirds of the endoplasmic reticulum phosphatidylethanolamine is located at the inner leaflet and this is methylated and transferred to the outer leaflet as phosphatidylcholine, there must exist a mechanism for the incorporation of newly synthesised phosphatidylethanolamine into the inner leaflet of the membrane bilayer. In the present investigation we have used phospholipase C as a probe of the site of synthesis of phosphatidylethanolamine and its subsequent location within the bilayer. The results suggest that there is a transmembrane movement of this phospholipid after its synthesis at the outer leaflet.

Methods

General methods were as described previously [8,9].

Preparation of microsomes. Microsomes were prepared from livers of male Sprague-Dawley rats (150–200 g) as described previously [1,11]. This preparation contains approx. 40% of the endoplasmic reticulum of the total liver, measured by assay of glucose-6-phosphatase, and has less than 4% of the plasma membrane and less than 2% of the Golgi membranes based on determination of 5′-nucleotidase and galactosyltransferase, respectively.

Incorporation of [^{14}C]ethanolamine in vivo. [2-^{14}C]Ethan-1-ol-2-amine (spec. act. 40–60 mCi per μmol) (The Radiochemical Centre, Amersham) 5 μCi/100 g body weight was injected intraperitoneally 1 h prior to sacrifice of rats, removal of livers and preparation of microsomes.

Incorporation of phosphoryl[^{14}C]ethanolamine into microsomal phospholipids in vitro. Cytidine diphospho[1,2-^{14}C]ethanolamine (CDPethanolamine) was purchased from ICN (spec. act. 100 mCi/μmol) and diluted with unlabelled CDPethanolamine (Sigma Chemical Co.) to a specific activity of 10000–20000 dpm/μmol. Microsomes (50 mg protein) were incubated with CDP[^{14}C]ethanolamine 0.4 mM in 0.5 or 0.25 ml of a 0.05 M sodium phosphate buffer (pH 7.4) containing 0.14 M NaCl, 1.0 mM EGTA and 15 mM $MgCl_2$ at 37°C. At the end of the incubation in some experiments the microsomal lipid was extracted immediately by addition of chloroform/methanol/conc. HCl (2:1:0.02, v/v) while in others ice cold buffer (11 ml) was added and the microsomes isolated by centrifugation. In some experiments $CaCl_2$ (final concentration 4 mM) was added with the buffer to arrest incorporation of labelled ethanolamine. The microsomal pellets were washed with ice-cold buffer to ensure removal of labelled substrate before resuspension and subsequent treatment.

Treatment of microsomes with phospholipase C. After labelling in vivo microsomes were resuspended to give a final protein concentration of 5 mg/ml in 0.05 M phosphate buffer as above. 1 ml aliquots were either extracted with chloroform/methanol/HCl immediately or incubated with 4 mM $CaCl_2$ and 10 units of phospholipase C (Type 1 from *Cl. perfringens,* Sigma Chemical Co.) at 37°C for 10 min before extraction. EDTA (10 mM) was added to the incubation medium at the

same time as chloroform/methanol to ensure inhibition of phospholipase C during extraction of the lipids. In some experiments chromatographically purified phospholipase C (Sigma Chemical Co. Type X) was used. The same results were obtained with either preparation.

Microsomal pellets isolated from in vitro incubations were resuspended in 0.05 M phosphate buffer to give a final protein concentration of 5 mg/ml and treated with phospholipase C in the same way as those labelled in vivo.

Extraction and separation of lipids. After treatment with phospholipase C the microsomal lipids were extracted with chloroform/methanol/HCl (2:1:0.02, v/v) (20 vol.). The phases were separated by addition of 0.05 M $CaCl_2$ (4 vol.). The solvent was removed from the lower phase and aliquots taken for radioassay and for determination of total phosphate. Aliquots were separated into phospholipid classes on thin layers of silica gel (Merck 60F 254) using a one dimensional system chloroform/methanol/glacial acetic acid/water (60:50:1:4, v/v). The phospholipid containing spots were detected with iodine vapour, which was allowed to fade before these were scraped into counting vials and counted. In some experiments the phosphatidylethanolamine or phosphatidylcholine spots were removed, extracted with chloroform/methanol (1:1, v/v) followed by methanol and the specific activity of the phospholipid determined.

Preparation of opened microsomal vesicles. Microsomes were opened by treatment with 0.4% taurocholate added to the appropriate incubation medium or by passage through a French pressure cell at 20000 lbs/inch^2. Either of these procedures cause loss of mannose-6-phosphatase latency and loss of labelled protein contents [8].

Determination of the latency of mannose-6-phosphatase was as described previously [11].

Results

Incorporation of phosphoryl[^{14}C]ethanolamine from CDPethanolamine into microsomal phospholipid in vitro

Rate of reaction and effect of calcium ions. Phosphoryl[^{14}C]ethanolamine was rapidly incorporated into microsomal phospholipid and more than 90% of the incorporated label was in phosphatidylethanolamine. Synthesis of labelled phosphatidylethanolamine was linear for 5 min and was linear with protein concentration to 10 mg. Addition of calcium ions to the incubation medium, after initiation of the reaction, completely inhibited further incorporation of label into microsomal phospholipid.

Effect of pretreatment of microsomes with phospholipase C. Ethanolamine phosphotransferase catalyses the transfer of phosphorylethanolamine from CDPethanolamine to diacylglycerol. However, diacylglycerol was not added to the incubation medium in these investigations, as this is not water soluble and must be added in a dispersion with a detergent such as Tween. This would perturb the membrane structure and open the microsomal vesicles. In the present studies, therefore, the small amount of diacylglycerol present in mi-

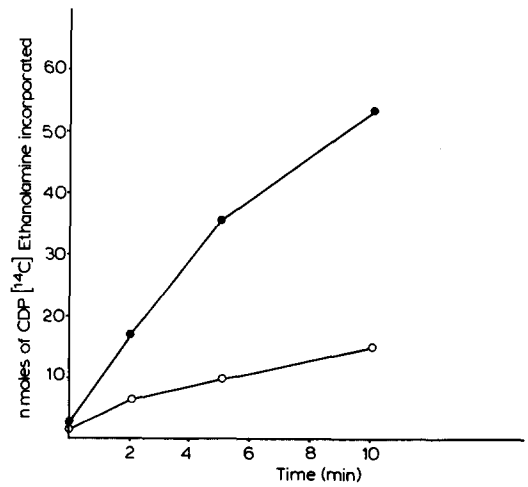

Fig. 1. Microsomes (5 mg protein) were treated with phospholipase C (10 units) in a volume of 1 ml for 2 min. Ice cold buffer (11 ml) was added and the microsomes isolated by centrifugation. These were resuspended in buffer and incubated with CDP[^{14}C]ethanolamine as described in Methods. The reaction was stopped at a range of times and the lipids extracted by addition of chloroform/methanol/HCl (2:1:0.02, v/v). nmol of [^{14}C]ethanolamine incorporated into total lipids (of phospholipase C-treated microsomes and untreated control microsomes) are plotted against time. O——O, untreated microsomes; ●——●, phospholipase C-treated microsomes. Results plotted are of at least two determinations and similar patterns were obtained from repeated experiments.

crosomes was used as an endogenous acceptor for phosphorylethanolamine.

In experiments in which microsomes were incubated with CDP[^{14}C]ethanolamine and subsequently treated with phospholipase C, without isolation by centrifugation to remove the labelled substrate, it was observed that even after addition of calcium ions incorporation of phosphoryl[^{14}C]ethanolamine was stimulated. To examine this more closely microsomes were treated for 2 min with phospholipase C to increase diacylglycerol in the outer leaflet of the membrane bilayer [20]. This treatment stimulated incorporation of the labelled substrate into phosphatidylethanolamine (Fig. 1), suggesting that diacylglycerol is a limiting factor in synthesis of phosphatidylethanolamine. These observations also indicate that the outer leaflet of the bilayer is the site of ethanolamine phosphotransferase. The observations of Coleman and Bell [12,13] and Vance et al. [14] of the latency of this enzyme and its accessibility to trypsin in the external medium are also consistent with the conclusion that ethanolamine phosphotransferase is located at the cytoplasmic surface of the microsomal membranes.

Effect of opening microsomes with French pressure cell. When microsomal vesicles are treated with the French pressure cell at 20000 lbs/inch^2 mannose-6-phosphatase latency is abolished and the vesicles lose their protein contents [8,11]. In the electron microscope the membranes remain as flattened vesicles, suggesting that these are opened by holes forming in the membrane rather than by the vesicles rupturing to form sheets. Opening microsomal vesicles by French pressure cell treatment did not increase incorporation of phosphoryl[^{14}C]ethanolamine into microsomal phospholipid. The substrate, therefore, has access to the enzyme in unopened vesicles, suggesting that the site of synthesis of phophatidylethanolamine is the outer leaflet of the membrane bilayer. This is consistent with the observations above.

Site of incorporation of phosphoryl[^{14}C]-ethanolamine into microsomal phosphatidylethanolamine

In all experiments in which the site of newly synthesised phosphatidylethanolamine was investigated, microsomes were isolated from the incubation medium by centrifugation and washed to remove residual CDPethanolamine. The microsomes were then resuspended and treated with phospholipase C to investigate the availability of phosphatidyl[^{14}C]ethanolamine for hydrolysis. Labelled phospholipids appeared in both hydrolysed and unhydrolysed pools (Fig. 2). The label was initially slightly greater in the hydrolysed pool, although rapidly increased in the unhydrolysed pool. At all times investigated hydrolysis of the total phospholipid was approx. 50%. The difference in % label hydrolysed is not a reflection of total hydrolysis therefore. In the experiment illustrated in Fig. 2 the reaction was stopped by addition of ice-cold buffer prior to centrifugation. In a second series of experiments calcium was added with the buffer. Similar levels and patterns of incorporation were found.

The specific activity of phosphatidylethanolamine in the total pool was greater than that

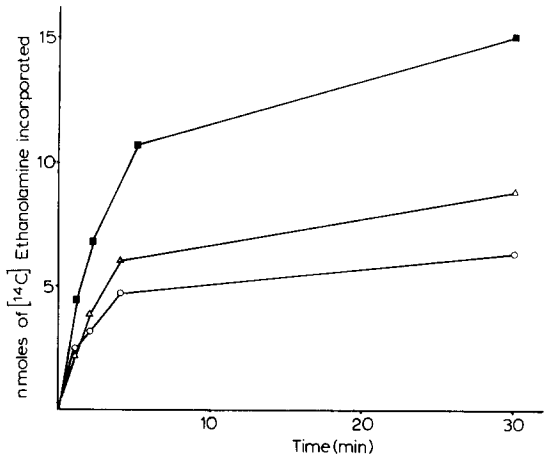

Fig. 2. Microsomes were incubated with CDP[^{14}C]ethanolamine as described in Methods for a range of times. Ice cold buffer (11 ml) was added and the microsomes isolated by centrifugation. The pellets were rinsed with buffer to remove labelled substrate, resuspended in buffer, and aliquots taken for incubation with and without phospholipase C for 10 min. The lipids were extracted as described in Methods. The nmol of CDP[^{14}C]ethanolamine incorporated into total phospholipids (■), into the pool not available to phospholipase C for hydrolysis (△) and, by difference, into the hydrolysed pool (○) are plotted against incubation time. Points plotted are of duplicate or triplicate determinations on at least two microsome preparations. Data from several experiments are included and similar results were obtained in repeated experiments.

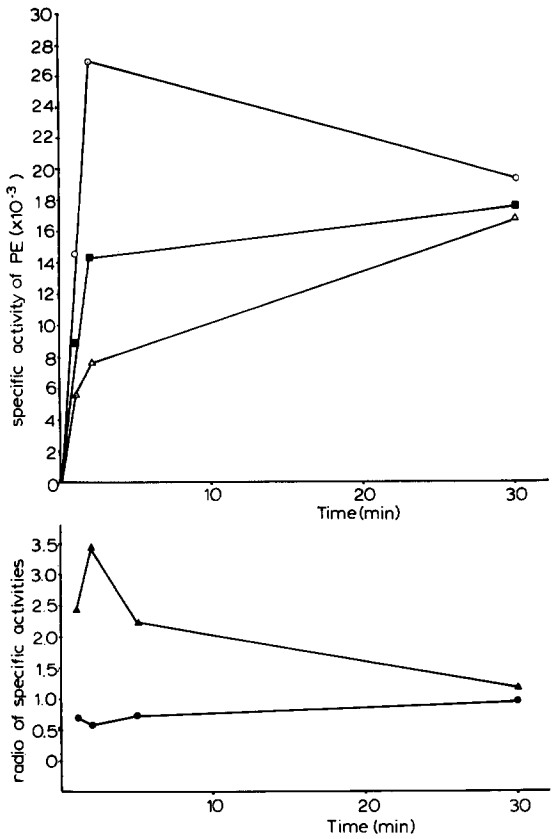

Fig. 3. Microsomes were incubated with CDP[^{14}C]ethanolamine, isolated by centrifugation, and treated with phospholipase C as described in the legend to Fig. 2. The phospholipids were extracted, separated by thin-layer chromatography and the specific activity of phosphatidylethanolamine determined as described in Methods. The specific activities of phosphatidylethanolamine of the total pool (■), that not hydrolysed by phospholipase C (△) and, by calculation, that hydrolysed by phospholipase C (○) are plotted against time of incubation. Points plotted are of duplicate or triplicate determinations. Results of one experiment are plotted as the specific activity varies between experiments. However, similar results were obtained from three repeated experiments.

unhydrolysed initially, although after continued incubation for 30 min the unhydrolysed and total pools had similar specific activities (Fig. 3). Taking into account the amount of phosphatidylethanolamine in the total and hydrolysed pools of phospholipid the specific activity of hydrolysed phosphatidylethanolamine can be calculated. This was greater than that unhydrolysed by a factor of 3.5 after 2 min incorporation. The specific activity of the hydrolysed pool subsequently fell while that of the unhydrolysed pool rose, so that after 30 min the two pools were close in specific activity (Fig. 3).

Our previous observations are consistent with the assumption that phospholipids hydrolysed by phospholipase C are in the outer leaflet of the microsomal membrane bilayer, while those unhydrolysed are in the inner leaflet [8,10,20]. The newly synthesised labelled phospholipid is only a small fraction of the total phosphatidylethanolamine, however, and may not behave in the same way as the total pool. Microsomal vesicles were therefore treated with 0.4% taurocholate or the French pressure cell after incubation with CDP[^{14}C]ethanolamine for 1 min prior to phospholipase C. Both of these treatments cause loss of labelled protein contents and loss of mannose-6-phosphatase latency, suggesting that the vesicles are opened. Under these conditions the specific activities of the total and unhydrolysed pools were more uniform (Table I). Selective hydrolysis of labelled phosphatidylethanolamine in intact microsomes is therefore reduced by making both sides of the membrane available to the phospholipase C. These observations suggest that the hydrolysed labelled phospholipid is in the outer leaflet of the bilayer, while that unhydrolysed is in the inner leaflet. The pool of phospholipid hydrolysed by phospholipase will therefore be indicated by 'outer leaflet' and that unhydrolysed by 'inner leaflet' in the remaining text.

The ratio of the specific activities of phosphatidylethanolamine in the 'outer leaflet' of the microsomal membrane bilayer to that in the 'inner leaflet' was 2.4 at 1 min, rose to 3.4 at 2 min and then fell to 1.15 after 30 min. Synthesis of phosphatidylethanolamine and its movement from a pool accessible to phospholipase C to one inaccessible are not parallel, therefore, during the initial rapid synthesis of phosphatidylethanolamine. However, the specific activity of the 'inner leaflet' never exceeds that of the 'outer leaflet', suggesting that the total phosphatidylethanolamine pools on each side of the bilayer are in equilibrium. If newly synthesised labelled phosphatidylethanolamine is initially sequestered, equilibrates with that of the total 'outer leaflet' at a slower rate than synthesis, and moves from the 'outer leaflet' to the 'inner leaflet', these kinetics would be expected.

TABLE I

SPECIFIC ACTIVITY OF PHOSPHATIDYL[^{14}C]ETHANOLAMINE IN POOLS OF PHOSPHATIDYLETHANOLAMINE OF 'UNOPENED AND OPENED' MICROSOMAL VESICLES

Microsomes were incubated with CDP[^{14}C]ethanolamine for 1 min as described in Methods. Ice cold buffer was added, the microsomes isolated by centrifugation, washed to remove labelled substrate, and resuspended. Two separate groups of experiments were performed in which half of the microsomal suspension was treated with either the French pressure cell or 0.4% taurocholate to open the vesicles, as described in Methods. Untreated microsomes and opened microsomes were then incubated with or without phospholipase C. The lipids were extracted and the specific activities of total, unhydrolysed and hydrolysed pools of phosphatidylethanolamine determined as described in Methods. Results given are the averages of triplicate determinations.

	Specific activity of phosphatidylethanolamine (dpm/μmol)		
	Total	Unhydrolysed	Hydrolysed
Group I			
No treatment	8649	7631	10688
Taurocholate treated	8703	8666	8777
Group II			
No treatment	13407	11518	17185
French pressure cell treated	13311	13721	12491

Translocation of phosphatidyl[^{14}C]ethanolamine across the microsomal membrane bilayer

To determine whether labelled phosphatidylethanolamine in the 'outer leaflet' of the membrane bilayer is indeed transferred to the 'inner leaflet', microsomes were incubated with CDP[^{14}C]ethanolamine for 1 min to label the 'outer leaflet'. These were isolated by centrifugation and reincubated for a range of times prior to treatment with phospholipase C. The labelled phospholipid in the 'outer leaflet' fell rapidly from 66% of the total to 36%, while that in the 'inner leaflet' rose by a corresponding amount (Fig. 4). Movement of labelled phosphatidylethanolamine from the 'outer' to the 'inner leaflet' was almost completed in 5 min and incubation for 120 min did not result in any

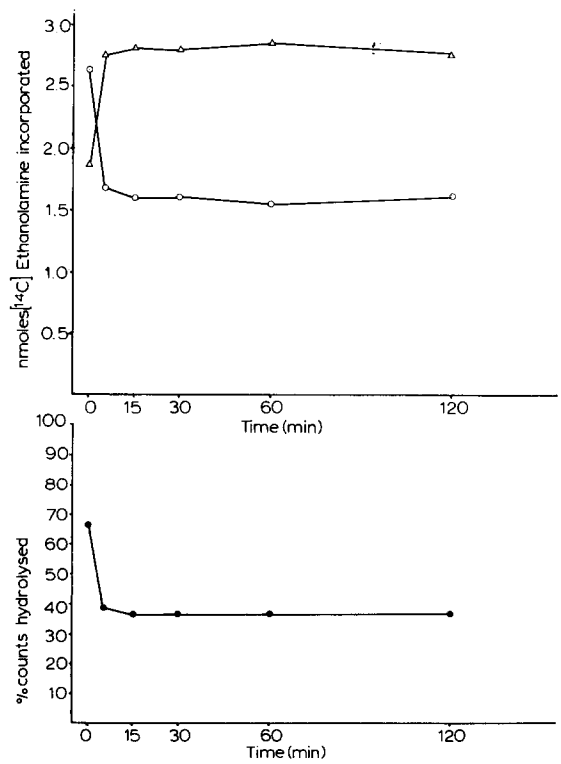

Fig. 4. Microsomes were incubated with CDP[^{14}C]ethanolamine for 1 min as described in Methods. Ice cold buffer was added and the microsomes isolated by centrifugation. The pellets were rinsed with buffer, resuspended and reincubated for a range of times before addition of $CaCl_2$ (4 mM) and phospholipase C (10 units/ml). The incorporation of CDP[^{14}C]ethanolamine into phospholipids available (○) and unavailable (△) for hydrolysis by phospholipase C was determined at each time point. nmol of [^{14}C]ethanolamine in each pool is plotted against time of incubation. In the lower graph the % of the incorporated label in the hydrolysed pool is plotted against time. Points plotted are averages of duplicate or triplicate determinations on at least two microsomal preparations. Data from several experiments are included and similar results were obtained in repeated experiments.

further movement (Fig. 4). Transfer of phosphatidylethanolamine from one pool accessible to phospholipase C to a second inaccessible pool is rapid. However, as hydrolysis with phospholipase C takes minimally several minutes to reach completion it is not possible to pinpoint zero-time. In addition it is not known at what level of hydrolysis of the phospholipid transfer of phosphatidylethanolamine is inhibited. It is likely that transfer of phosphatidylethanolamine continues during at least a part of the incubation with phospholipase C. It is possible therefore that a greater proportion of labelled phosphatidylethanolamine is in the 'outer leaflet' of the bilayer initially and that zero-time represents the distribution of label at the shortest time which can be determined under these experimental conditions. These observations indicate, however, that transmembrane movement is rapid and reaches equilibrium.

In a typical experiment the specific activity of the total phosphatidylethanolamine was 2343 dpm/mol, that of the 'inner leaflet' at the beginning of the incubation was 1341 dpm/mol and that calculated for the 'outer leaflet' was 4347 dpm/mol. After 120 min incubation the specific activity of the phosphatidylethanolamine of the 'inner leaflet' was 2219 dpm/mol and that of the 'outer leaflet' was 2591 dpm/mol. Thus, the ratio of the specific activities of phosphatidylethanolamine of the 'outer' to the 'inner leaflet' fell from 3.24 to 1.17. Similarly the specific activities of the phosphatidylethanolamine of the 'inner leaflet' to the total pool rose from 0.57 to 0.98. Labelled phosphatidylethanolamine is therefore transferred across the bilayer to the 'inner leaflet'; however, equilibration occurs and the 'inner leaflet' does not become more labelled than the 'outer' even on prolonged incubation. The transfer of labelled phosphatidylethanolamine is independent of further synthesis of phosphatidylethanolamine, as this occurs in the absence of CDPethanolamine.

Integrity of microsomal vesicles during experimental manipulation

In experiments in which phospholipase C is used to probe the distribution of phospholipid about the microsomal bilayer it is essential that the vesicles remain closed. After incubation with CDPethanolamine, isolation by centrifugation, reincubation and treatment with phospholipase C, the mannose-6-phosphatase latency of the microsomes was 90%. This was similar to the latency of the vesicles prior to the experiment and after each step in the experimental procedures and indicates that the microsomes remain sealed during experimental manipulation.

Site of incorporation of [^{14}C]ethanolamine into microsomal phospholipids in vivo.

[^{14}C]Ethanolamine injected intraperitoneally

TABLE II

INCORPORATION OF [^{14}C]ETHANOLAMINE INTO MICROSOMAL PHOSPHOLIPID IN VIVO

[^{14}C]Ethanolamine was injected intraperitoneally into rats 60 min before sacrifice and preparation of liver microsomes as described in Methods. In one group of experiments the lipids were separated by thin-layer chromatography and the distribution of incorporated counts into the individual phospholipids determined. In a second group of experiments the microsomes were treated with phospholipase C and the specific activity of total, unhydrolysed and hydrolysed phosphatidylcholine and phosphatidylethanolamine determined as described in Methods. Data from one experiment are given, because the specific activity varied between animals. Similar results were obtained in repeated experiments.

	% distribution of incorporated label	specific activity (dpm/μmol)		
		Total	Unhydrolysed	Hydrolysed
Sphingomyelin	0.1			
Phosphatidylcholine	8.3	1018	1040	974
Phosphatidylserine	2.6			
Phosphatidylinositol	1.1			
Phosphatidylethanolamine	86.0	21467	15085	34231

was incorporated into microsomal phospholipid. Approx. 86% of the incorporated label was in phosphatidylethanolamine and 8% in phosphatidylcholine (Table II). On treatment of the labelled microsomes with phospholipase C the specific activity of total, hydrolysed and unhydrolysed pools of phosphatidylcholine were similar (Table II). Our previous observations have indicated that phosphatidylcholine labelled by injection of methionine in vivo has a similar specific activity in the inner and outer leaflets of the membrane bilayer [11]. As this pathway involves methylation of phosphatidylethanolamine it is consistent with the present observations that phosphatidylcholine labelled with [^{14}C]ethanolamine has an even distribution.

In contrast to phosphatidylcholine, the specific activity of phosphatidylethanolamine in the hydrolysed pool was greater than that in the unhydrolysed pool by a factor of two. 60 min after injection of [^{14}C]ethanolamine, therefore, the 'outer leaflet' is still preferentially labelled. This may be related to the pool size of [^{14}C]ethanolamine, and the rate of its utilization. If [^{14}C]ethanolamine continues to be used during the whole post-injection period then the 'outer leaflet' of the bilayer would have a higher specific activity than the 'inner leaflet'. Transmembrane movement of phosphatidylethanolamine is very slow at 0°C and hence would not occur to a significant extent during isolation of microsomes. We have observed, however, that if micosomes labelled in vivo are incubated at 37°C the labelled phosphatidylethanolamine does move across the bilayer so that both leaflets reach a similar specific activity. Investigation of the kinetics of incorporation of labelled precursors into the microsomal membrane and their subsequent fate are in progress to examine this further. However, these observations are consistent with those in vitro and indicate that ethanolamine is initially incorporated into the outer leaflet of the membrane bilayer.

Discussion

Several of the observations reported here suggest that phosphorylethanolamine is incorporated from CDPethanolamine into the 'outer leaflet' of the microsomal bilayer. (1) At short times after incubation, labelled phosphatidylethanolamine in the 'outer leaflet' has a specific activity of more than three times that of the 'inner leaflet', and more than 65% of the incorporated label is at this site, although only one third of the total phosphatidylethanolamine is in the outer leaflet. (2) If the microsomal vesicles are opened to allow CDPethanolamine access to the inner surface, synthesis of phosphatidylethanolamine is not increased. (3) Hydrolysis of the 'outer leaflet' of the membrane bilayer by phospholipase C which increases the diacylglycerol at this site stimulates synthesis of phosphatidylethanolamine. These pieces of evidence all support the conclusion that phosphatidylethanolamine is initially located in the 'outer leaflet' of the microsomal membrane bilayer. Investigation of the site of ethanolaminephosphotransferase using proteases [12–14] or using mercury dextran, an impermanent inhibitor [15] have also suggested that the enzyme is accessible at the outer surface of the microsomal membrane.

Phosphatidylethanolamine is synthesised at the 'outer surface' of the microsomal membrane and yet has a high concentration in the 'inner leaflet'. A mechanism must exist, therefore, for the movement of this phospholipid from the 'outer leaflet' of the bilayer to the 'inner leaflet'. Our observations indicate that such transmembrane movement does occur. After 1–2 min incubation with CDP[^{14}C]ethanolamine, labelled phosphatidylethanolamine is concentrated in the 'outer leaflet' of microsomes. However, on incubation of these membranes in the absence of substrate the labelled phosphatidylethanolamine decreases in the 'outer leaflet' and increases in the 'inner leaflet', so that the specific activities of the two pools become constant. Equilibration occurs rapidly at 37°C and on prolonged incubation the specific activity of the inner pool does not exceed that of the outer pool. The factors involved in the transmembrane movement of phosphatidylethanolamine are under investigation. However, this is independent of continued synthesis of phosphatidylethanolamine and is temperature-dependent as equilibration does not take place during centrifugation at 4°C.

The assignment of labelled phosphatidylethanolamine to the inner or outer leaflet of the microsomal bilayer is based on our previous ob-

servations using phospholipase C as a probe [8,10]. There have been conflicting reports of the distribution of phospholipids about the microsomal membrane bilayer from different laboratories using different probes [16–18]. However, we have observed that phospholipase C hydrolyses approx. 50% of the membrane phospholipid, without loss of either labelled protein contents from the vesicles or loss of mannose-6-phosphatase latency [8,9]. The microsomes, therefore, apparently remain closed after hydrolysis of the phospholipid by phospholipase C. If the vesicles are opened using the French pressure cell, lysolecithin, taurocholate, deoxycholate or high pH, the phospholipids are hydrolysed uniformly and to a greater extent, suggesting that the hydrolysed pool of intact vesicles is in fact the outer leaflet and that if the vesicles are opened both sides of the bilayer become accessible for hydrolysis [8–10]. It is possible that hydrolysis by phospholipase C might cause rearrangement of the membrane phospholipids without the vesicles becoming leaky. However, this does not appear to occur in studies of red blood cell membranes in which both inside-out and outside-out vesicles have been subjected to phospholipase C treatment and yielded consistent results of the distribution of phospholipids about the membrane bilayer [19]. In addition, we have demonstrated that the bilayer structure is retained in phospholipase C-treated microsomes examined both in conventional sections and in freeze-fracture preparations [20]. The available evidence, therefore, indicates that phospholipase C is a valid probe for the distribution of phospholipids about the membane bilayer. These investigations would benefit from the availability of a method for the preparation of inside-out microsomal vesicles. We have investigated a number of experimental procedures in attempts to prepare such vesicles; however, to date these methods have been unsuccessful. One problem in using phospholipase C as a probe of phospholipid movement within the transverse plane of the membrane is the need to incubate microsomes with phospholipase C for at least several minutes during which phospholipids may move. However, for these studies the phospholipids rather than the protein components must be probed. At present only phospholipase or chemical labelling of aminophospholipids are available for such studies and both require incubation to either label or hydrolyse the newly synthesised phospholipid.

The reasons for the difference between reports of the distribution of phospholipids about the microsomal membrane bilayer are not clear. Sundler et al. [18] observed that phospholipase A_2 completely hydrolysed all of the phospholipids of microsomal vesicles. However, before the vesicles opened there was no selective hydrolysis of individual phospholipids. They interpreted these observations as indicating that the microsomal membrane is not asymmetric with respect to phospholipids. However, in these experiments it is not possible to exclude a rearrangement of the membrane prior to the vesicles becoming leaky. Lysophosphatidylcholine, one of the products of phospholipase A_2 activity is a lytic agent and may cause a rearrangement of phospholipids in the membrane. Nilsson and Dallner [16,17] used phospholipase A_2 as a probe and concluded that the phosphatidylethanolamine is located mainly in the outer leaflet. However, we have attempted to repeat these experiments and find that in our hands phospholipase A causes complete hydrolysis of the phospholipids of microsomal vesicles.

Using phospholipid exchange proteins it has been demonstrated that up to 95% of the phospholipids of microsomal membranes are exchangeable [22,23]. This suggests a rapid transmembrane movement of phospholipids in these membranes, although they retain an asymmetric distribution of phospholipid when probed with phospholipase C [24]. Rothman and Kennedy [21] have demonstrated using trinitrobenzene sulphonic acid as a probe, that 65% of the phosphatidylethanolamine is located on the cytoplasmic leaflet of the membrane of *Bacillus megaterium*. This phospholipid is apparently synthesised at the cytoplasmic side of the membrane bilayer and transferred to the outer leaflet so that equilibrium is reached. Our results show similar events take place in the biosynthesis of endoplasmic reticulum phosphatidylethanolamine. All of these observations riase questions concerning the mechanism involved in maintenance of membrane asymmetry if transmembrane movement of phospholipid takes place.

Our previous observations have suggested that

synthesis of phosphatidylcholine by the CDPcholine pathway occurs at the cytoplasmic side of the endoplasmic reticulum either in vivo or in vitro and that the phospholipid synthesised by this pathway remains concentrated in the outer leaflet of the bilayer [9]. In contrast, during synthesis of phosphatidylcholine by methylation of phosphatidylethanolamine, the first methylation step apparently occurs at the inner surface of the membrane and translocation of partly methylated intermediates across the membrane bilayer takes place so that phosphatidylcholine at both sides of the bilayer becomes labelled [11]. These observations, together with those reported in this paper, indicate that both asymmetric synthesis of phospholipids and transmembrane movement of phospholipids takes place during biogenesis of the endoplasmic reticulum. However, the membrane has an asymmetric distribution of phospholipids, which is retained even when the membrane function is modulated, for example after phenobarbital treatment [10]. The transmembrane movement of phospholipids must, therefore, occur in a controlled manner. The complex interrelationships in phospholipid synthesis and reorganization which have been indicated by our observations, however, could provide fine controls which allow adjustment of the distribution of membrane phospholipids during formation of the membrane bilayer and its turnover.

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