Biochimica et Biophysica Acta, 470 (1977) 342—356

BBA 77826

ASYMMETRY OF THE PHOSPHOLIPID BILAYER OF RAT LIVER ENDOPLASMIC RETICULUM

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(Received March 10th, 1977)

Summary

The phospholipids of intact microsomal membranes were hydrolysed 50% by phospholipase C of *Clostridium welchii*, without loss of the secretory protein contents of the vesicle, which are therefore not permeable to the phospholipase. Phospholipids extracted from microsomes and dispersed by sonication were hydrolysed rapidly by phospholipase C-*Cl. welchii* with the exception of phosphatidylinositol. Assuming that only the phospholipids of the outside of the bilayer of the microsomal membrane are hydrolysed in intact vesicles, the composition of this leaflet was calculated as 84% phosphatidylcholine, 8% phosphatidylethanolamine, 9% sphingomyelin and 4% phosphatidylserine, and that of the inner leaflet 28% phosphatidylcholine, 37% phosphatidylethanolamine, 6% phosphatidylserine and 5% sphingomyelin. Microsomal vesicles were opened and their contents released in part by incubation with deoxycholate (0.098%) lysophosphatidylcholine (0.005%) or treatment with the French pressure cell. Under these conditions, hydrolysis of the phospholipids by phospholipase C-*Cl. welchii* was increased and this was mainly due to increased hydrolysis of those phospholipids assigned to the inner leaflet of the bilayer, phosphatidylethanolamine and phosphatidylserine. Phospholipase A_2 of bee venom and phospholipase C of *Bacillus cereus* caused rapid loss of vesicle contents and complete hydrolysis of the membrane phospholipids, with the exception of sphingomyelin which is not hydrolysed by the former enzyme.

Introduction

There is considerable evidence indicating an asymmetric distribution of phospholipids across the bilayer of the erythrocyte membrane [1—9]. This has been

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demonstrated by digestion of the membrane phospholipids of whole cells and ghosts with phospholipases [1—5] and by the use of penetrating and non-penetrating probes, which react with those phospholipids having free amino groups, phosphatidylethanolamine and phosphatidylserine [6—9]. There have also been reports that a similar asymmetry exists in influenza virus membranes [10], rod outer segment membranes [11], liver microsomes [12], mitochondria [13] and *Escherichia coli* [9], although these studies have been less detailed and often more preliminary than those on erythrocyte membranes. However, as erythrocytes are atypical cells, it is of considerable importance that the pattern of distribution of phospholipids of other membranes be investigated. It is also important to elucidate the organisation of phospholipids in a membrane responsible for their biogenesis, a major emphasis of the laboratory of one of the authors (J.A.H.) [14—16]. For these reasons we have now examined the distribution of phospholipids across the bilayer of rat liver endoplasmic reticulum.

On homogenisation of the liver, the endoplasmic reticulum fragments and reseals to form small vesicles, which are isolated in the microsomal fraction [17]. Golgi membranes and plasma membranes form similar small vesicles and these may also be isolated in this fraction. However, it is possible to prepare rat liver microsomes in a yield of 37—47% of the original endoplasmic reticulum with a low contamination by other membranes calculated by determination of the specific activity of appropriate marker enzymes (less than 4% of the protein contributed by plasma membrane and less than 2% by Golgi membranes [16]).

It has been demonstrated by cytochemical localisation of glucose-6-phosphatase [18] and cytochrome b_5 [19] that all microsomal vesicles have the same orientation as the original endoplasmic reticulum with the cytoplasmic side of the membrane outside and the cisternal side of the membrane inside. This is also indicated, with rough endoplasmic reticulum, by the finding that bound ribosomes are always on the outside of the microsomal vesicles. Rat liver microsomes prepared under controlled conditions therefore consist of a population of closed vesicles, the orientation of which is established with respect to their original position in the cell. These, therefore, provide a suitable system on which to investigate phospholipid asymmetry.

Materials and Methods

Materials

Phospholipase A_2 of bee venom, (EC 3.1.1.4) (specific activity 1300 units/mg) phospholipase C of *Clostridium welchii* (EC 3.1.4.3) (specific activity 10 units/mg) and fatty acid-free albumin were purchased from Sigma Chemical Co. Phospholipase C of *Bacillus cereus* (EC 3.1.4.3) (specific activity 400 units/mg) was purchased from Boehringer Mannheim. The amount of enzyme used is expressed in international units.

General methods

Protein was determined by the method of Lowry et al. [20] using bovine serum albumin as a standard. Phospholipid phosphorus was determined by the method of Bartlett [21] after digestion of the lipid with perchloric acid.

Preparation of microsomes

Rat livers were removed and homogenized using a Potter Elvejhem homogenizer in ice-cold sucrose, 0.25 M, to yield 20% (w/v) homogenates, which were centrifuged at 12 500 rev./min (10 000 × *g* average) for 20 min in the 40 rotor of the Spinco ultracentrifuge. The supernatants were removed and recentrifuged at the same speed to ensure removal of large granules [16]. Total microsomes were isolated from the supernatant by centrifugation at 40 000 rev./min (105 000 × *g* average) for 45 min. The pellets were resuspended in the buffer used for incubation with phospholipase.

Measurement of leakage of microsomal content

To label the secretory protein contents of the microsomal vesicles, rats were given intraperitoneal injections of [^{3}H]leucine in distilled water (5 μCi/100 g body weight) 30 min before sacrifice [22]. To determine loss of labelled content after experimental manipulation microsomes were isolated by centrifugation (105 000 × *g* average for 30 min). The pellets and aliquots of untreated microsomes were dissolved in Soluene 350 (Packard Inst. Co.) and 15 ml of scintillation fluid added to the solution before counting. Aliquots of the supernatant were counted in 15 ml of scintillation fluid. Leakage of contents was expressed as the % of counts in the supernatant over those recovered in both pellet and supernatant.

Incubation conditions

Microsomes were incubated with phospholipase A_2 or phospholipase C from *Cl. welchii* or *B. cereus* in 1 ml of 0.87% NaCl, 0.25 mM $CaCl_2$ adjusted to pH 7.4 with sodium bicarbonate.

Lipid extraction and analysis

The incubation mixtures with phospholipase A_2 were extracted by the method of Reed et al. [23]. Incubation mixtures with phospholipase C were extracted with 20 ml of chloroform/methanol (2 : 1, v/v) and the phases separated by addition of 5 ml of 0.05 M $CaCl_2$. Aliquots of the upper phase were taken for determination of the production of water-soluble organic phosphate and the lower phase for determination of loss of phospholipid phosphate. Aliquots of the lipid extracts from both phospholipase A_2 and phospholipase C experiments were taken to dryness under vacuum, the lipid dissolved in chloroform/methanol (1 : 1, v/v) and aliquots used for separation of lipid classes.

Lipids were separated on thin layers of Silica Gel (Merck, 60 F 254) using either a one-dimensional system, chloroform/methanol/acetic acid/water, (25/15/4/2, by vol.) or a two-dimensional system, chloroform/methanol/0.88 M ammonia, (65/35/5 by vol.) in the first dimension and chloroform/methanol/acetone/acetic acid/water, (10/2/4/2/1 by vol.) in the second dimension. The one-dimensional procedure separated the major phospholipid classes and the two-dimensional procedure separated the phospholipids and their lysoderivatives. Lipid-containing spots were detected by iodine vapour, were scraped into test tubes and digested with 1.5 ml of perchloric acid for approximately 30 min at 230°C. Water (7.6 ml) was added and the tubes centrifuged to remove the silica gel. Phosphate was determined on the supernatant.

Results

Loss of microsomal vesicle content by treatment with phospholipases

Secretory proteins released into the cisternae of the endoplasmic reticulum are trapped inside the microsomal vesicle during homogenization of the liver. These may be labelled specifically, with no significant labelling of the membrane proteins, by injection of [^{3}H]leucine 30 min prior to sacrifice of the animal [22]. Loss of the labelled contents into the incubation medium during treatment of microsomes with phospholipases is therefore a measure of the formation of holes in the microsomal membrane large enough to allow passage of proteins of a molecular weight range 50 000—100 000. Treatment of microsomal vesicles with phospholipase A_2 at a concentration of 5 units of enzyme per mg protein caused rapid release of up to 80% of the contents. This was reduced by lowering the enzyme concentration, but, if hydrolysis of phospholipids occurred, loss of microsomal content was always rapid and significantly above that of the control incubated in buffer alone (Fig. 1). Addition of fatty acid-free albumin (50 mg/ml) to the medium caused a more rapid loss of labelled contents, which reached the same final level. Incubation of microsomal vesicles with phospholipase C-*B. cereus*, also caused rapid loss of contents. As with phospholipase A_2 loss could be reduced by using less enzyme, but if phospholipid hydrolysis took place loss of contents always occurred. This was not complete suggesting that not all vesicles were leaking or that some of the content proteins were retained. In contrast, leakage of vesicular contents

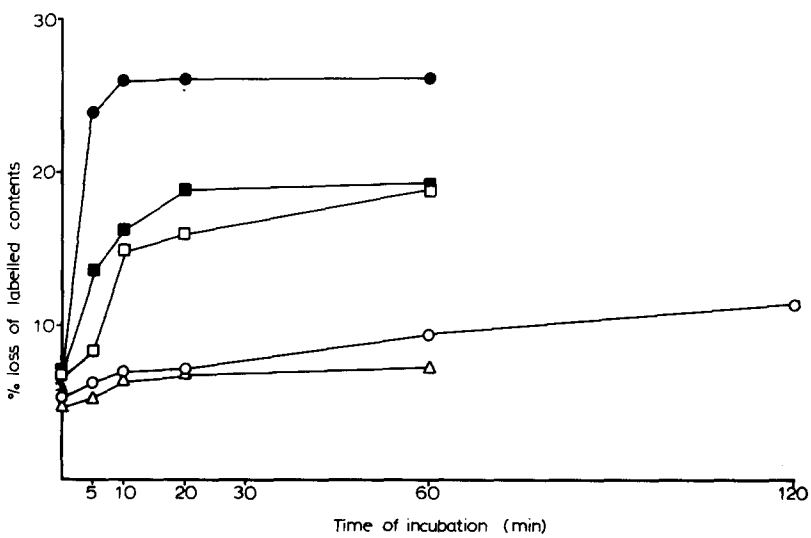

Fig. 1. Loss of microsomal vesicle contents by treatment with phospholipases. Microsomes (5 mg protein in 1 ml) were incubated with phospholipases at 37°C under the conditions described in Materials and Methods. The reaction was stopped at the appropriate time by addition of the incubation buffer (10 ml) containing EDTA (200 mM). Microsomes were isolated by centrifugation (105 000 × *g* for 30 min) and the leakage of contents into the incubation medium determined as described in Materials and Methods. % loss of content is plotted against time of incubation: △———△, no enzyme; ○———○, phospholipase C-*Cl. welchii* (1 unit/ml); □———□, phospholipase A_2, (0.65 units/ml); ■———■, phospholipase A_2, (0.65 units/ml) plus 50 mg fatty acid-free albumin per ml; ●———●, phospholipase C-*B. cereus*, (1 unit/ml).

during treatment with phospholipase C-*Cl. welchii* under conditions in which hydrolysis of the membrane phospholipids takes place, was similar to that of the control for the first 20 min of incubation and only slightly elevated on incubation for up to 2 h. It is unlikely, therefore, that phospholipase C-*Cl. welchii* (M_r approximately 50 000, ref. 24) can gain access to the interior of the microsomal vesicle initially; however, a small percentage of the microsomal vesicles may become permeable to the enzyme on prolonged incubation.

Loss of microsomal vesicle content by treatment with deoxycholate, lysolecithin or the French pressure cell

Treatment of microsomal vesicles (4—5 mg protein) with deoxycholate (0.098%, ref. 22) caused loss of 49% of the labelled contents (Table I). This was increased to 56% by increasing the concentration of deoxycholate to 0.49%; however, at this concentration some membrane phospholipids are also lost into the medium [22]. Lysophosphatidylcholine was more effective in opening vesicles and caused loss of 85% of the vesicle contents at a concentration of 0.005%. The French pressure cell used at 20 000 lb. per sq. in. released 83% of the labelled content (Table I). Deoxycholate and lysophosphatidylcholine probably act as detergents in disrupting the structure of the phospholipid bilayer, while the French pressure cell breaks upon the vesicles mechanically. These methods for opening vesicles therefore differ in any potential effects on the phospholipase used to probe the membrane phospholipid structure.

Treatment of microsomal vesicles by phospholipase C of Cl. welchii

Hydrolysis of total microsomal phospholipid by phospholipase C-*Cl. welchii* proceeded until approximately 50% of the lipid phosphate was removed after 30 min incubation (Fig. 2). Only slightly more hydrolysis took place and in five separate determinations hydrolysis at 60 min was 49.24 ± 1.79% S.D. of the total phospholipid. The amount of phospholipid hydrolysed in 25 min increased linearly with microsomal concentration; however, the percentage remained constant (Fig. 3) suggesting that the enzyme does not have access to

TABLE I

EFFECT OF DEOXYCHOLATE, LYSOLECITHIN OR TREATMENT WITH THE FRENCH PRESSURE CELL ON LOSS OF MICROSOMAL CONTENTS

Microsomes (4—5 mg protein) were incubated in 0.87% NaCl, 0.25 mM $CaCl_2$ adjusted to pH 7.4 with sodium bicarbonate containing deoxycholate or lysophosphatidylcholine in the concentrations indicated, at room temperature for 30 min. Microsomes at the same concentration were equilibrated at 20 000 lb. per sq. in. pressure for 5 min in the French pressure cell and the cell opened. The microsomes were isolated by centrifugation and the % loss of content determined as described in Materials and Methods.

Treatment		% Loss of content
Deoxycholate	0.049%	45
	0.098%	49
	0.186%	49
	0.490%	56
Lysolecithin	0.005%	85
French pressure cell		83

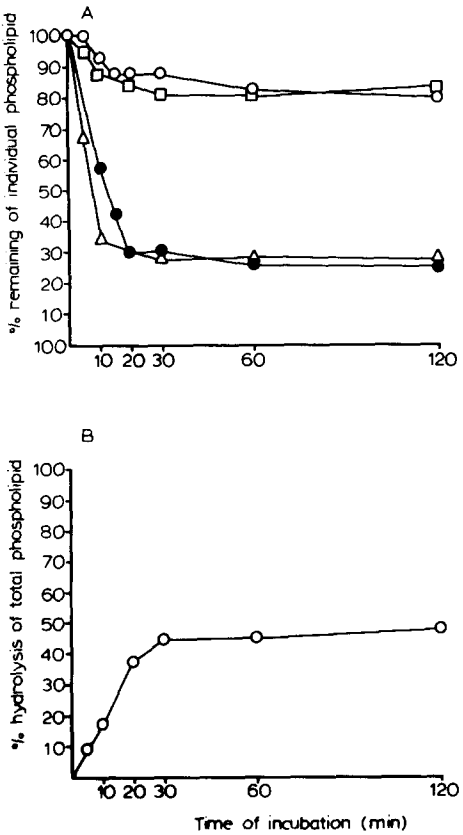

Fig. 2. Hydrolysis with time of microsomal phospholipids by phospholipase C-*Cl. welchii*. Microsomes (5 mg protein) were incubated for different times with phospholipase C-*Cl. welchii*. Lipids were extracted and the loss of total phospholipid and individual phospholipids determined as described in Materials and Methods. Hydrolysis of total phospholipid is plotted against time of incubation in B, and loss of individual phospholipids is plotted against time of incubation in A. ○———○, phosphatidylethanolamine; ●———●, phosphatidylcholine; □———□, phosphatidylserine; △———△, sphingomyelin.

all the membrane phospholipids, or, that accumulation of diglyceride inhibits further hydrolysis. It should be noted that in these experiments hydrolysis was just short of completion, as incubation times of 25 min were used. This probably accounts for the lower hydrolysis at low microsomal protein concentration, as substrate may have been limiting at these concentrations of membrane phospholipid. However, with time, hydrolysis proceded to 50%. In subsequent experiments, protein and phospholipid concentrations were constant at 5 mg of membrane protein or the equivalent amount of phospholipid and incubations were up to 60 min.

Phospholipids extracted from microsomes and dispersed by sonication were rapidly hydrolysed by phospholipase C-*Cl. welchii* (Fig. 4) with the exception of phosphatidylinositol, which was unaffected. Over 90% of each phospholipid component was hydrolysed in 10 min and hydrolysis was complete in 60 min. This contrast to the observations of other [4,5] who have reported that phosphatidylserine is not hydrolysed by phospholipase C-*Cl. welchii*. The

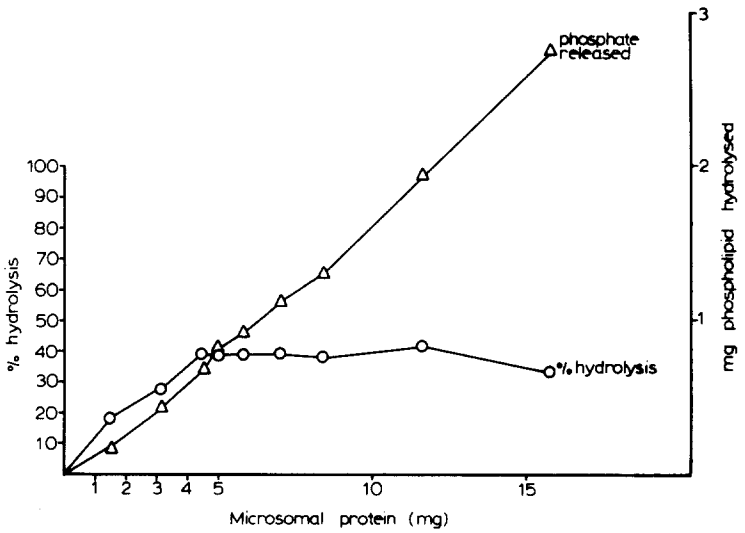

Fig. 3. Hydrolysis of microsomal phospholipid by phospholipase C-*Cl. welchii* over a range of microsomal protein concentrations. Microsomes were incubated with phospholipase C-*Cl. welchii* (1 unit/ml) for 25 min. The reaction was stopped by addition of chloroform/methanol (2 : 1, v/v) the lipid extracted and the loss of phospholipid determined as described in Materials and Methods. Hydrolysis is expressed as % loss of lipid phosphate as a % of that at zero time and is plotted against the concentration of microsomes in mg protein.

conditions of incubation in these experiments were identical with those using microsomal vesicles. The limited hydrolysis of microsomal phospholipid is therefore not due to enzyme specificity or to inhibition by accumulation of products. It appears that in intact microsomal vesicles there are two pools of phospholipid, which are approximately equal in quantity, only one of which is normally available to phospholipase C-*Cl. welchii*. As the vesicles remain impermeable during phospholipase treatment, it seems highly probable that the phospholipid pool hydrolysed is that of the outer leaflet of the bilayer and that unhydrolysed is that of the inner leaflet.

All phospholipids of intact microsomes, with the exception of phosphatidylinositol, were hydrolysed by phospholipase C-*Cl. welchii*. Loss of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin was rapid for 15—20 min after which hydrolysis was level to 30 min (Fig. 2). On prolonged incubation, there was a slight increase in microsomal permeability and also a small increase in hydrolysis of phospholipids (Fig. 2). When microsomes were incubated with phospholipase C-*Cl. welchii* for 30 min, in four determinations averages of 72% of the phosphatidylcholine, 18% of the phosphatidylethanolamine, 70% of the sphingomyelin and 16% of the phosphatidylserine were hydrolysed. The composition of microsomal phospholipid measured on eight separate preparations was 56.0 ± 3.4 phosphatidylcholine, 19.1 ± 1.9% phosphatidylethanolamine, 6.4 ± 1.7% sphingomyelin, 4.6 ± 1.8% phosphatidylserine and 12.9 ± 2.3% phosphatidylinositol. Assuming that only phospholipids on the outer leaflet of the bilayer are available for hydrolysis, the distribution on each side of the bilayer is shown in Table II. Thus, the outer leaflet of the bilayer is predominantly phosphatidylcholine and the inner leaflet

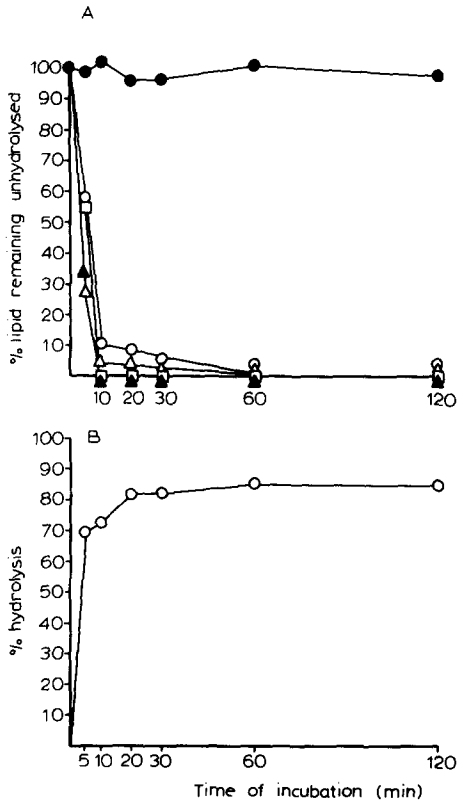

Fig. 4. Hydrolysis of phospholipids extracted from microsomes by phospholipase C-*Cl. welchii*. Phospholipids were extracted from microsomes and dispersed by sonication in the incubation buffer. Aliquots (2 mg phospholipid equivalent to 5 mg of microsomal protein) were incubated with phospholipase C-*Cl. welchii* for a range of times. Lipids were extracted and loss of total phospholipid and individual phospholipids determined, as described in Materials and Methods. Hydrolysis of total phospholipids is plotted against time of incubation in B. Loss of individual phospholipids is plotted against time of incubation in A. ○———○, phosphatidylethanolamine; △———△, phosphatidylcholine; □———□, phosphatidylserine; ▲———▲, sphingomyelin; ●———●, phosphatidylinositol.

has approximately equal amounts of phosphatidylcholine and phosphatidylethanolamine. Phosphatidylinositol is not hydrolysed by phospholipase C-*Cl. welchii*, however, the hydrolysed lipid accounts for 50% of the total phospho-

TABLE II

DISTRIBUTION OF PHOSPHOLIPIDS ON EITHER SIDE OF THE BILAYER OF MICROSOMAL MEMBRANES

Microsomes were incubated with phospholipase C-*Cl. welchii* for 30 min and the lipids extracted and separated as described in Materials and Methods. The distribution of the phospholipids on either side of the bilayer was calculated in terms of the composition of the total phospholipids. The average distribution of each lipid is given ± standard deviation.

	Phosphatidyl-choline	Phosphatidyl-ethanolamine	Phosphatidyl-serine	Sphingomyelin
Outer leaflet (%)	84.8 ± 2.7	7.8 ± 1.6	0.9 ± 0.07	9.1 ± 0.8
Inner leaflet (%)	28.4 ± 2.7	33.5 ± 1.6	8.9 ± 0.07	4.7 ± 0.8

lipid and for this reason, the bulk of the phosphatidylinositol is tentatively assigned to the inner leaflet of the bilayer.

Hydrolysis of phospholipids of open microsomal vesicles by phospholipase C of Cl. welchii

Low concentrations of deoxycholate or lysophosphatidylcholine, or treatment with the French pressure cell opened the microsomal vesicles (Table I) and should therefore make the interior surface of the membrane available to phospholipases in the incubation medium. Hydrolysis by phospholipase C of the phospholipid of microsomal vesicles opened in this way was increased to the greatest extent by lysolecithin (to 75%) or by treatment with the French press (to 68%) (Fig. 5). Hydrolysis in the presence of deoxycholate was also increased over the control (to 62%), but less than that with the alternative two treatments. However, theoretically hydrolysis should have been approximately 80%; presumably, therefore, no treatment irreversibly opens all the vesicles.

Hydrolysis of phosphatidylethanolamine in open vesicles was more rapid than that of closed vesicles. With vesicles treated with the French pressure cell 67% of the phosphatidylethanolamine was hydrolysed in 60 min compared with 19% hydrolysis of this lipid in the untreated vesicles (Table III). Similarly, inclusion of deoxycholate or lysophosphatidylcholine in the incubation medium resulted in an increased hydrolysis of phosphatidylethanolamine to 45% and 58% respectively. Hydrolysis of phosphatidylserine was more rapid in the open vesicles and showed a similar pattern to phosphatidylethanolamine. Hydrolysis phosphatidylcholine was more rapid in vesicles treated with the French pressure cell or lysophosphatidylcholine, althought the final level of hydrolysis reached was only slightly increased compared with the control. Hydrolysis of sphingomyelin was increased in a similar way to phosphatidyl-

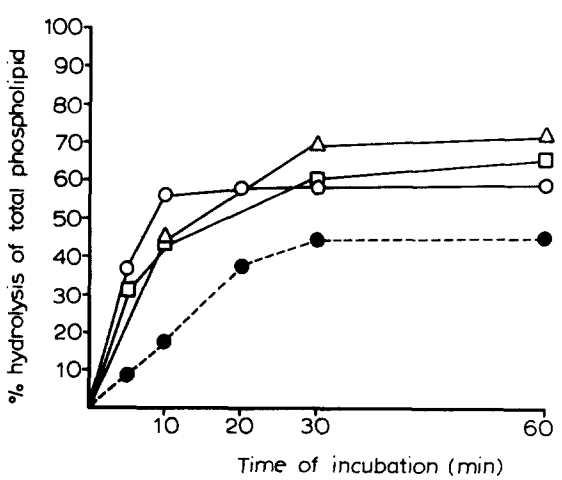

Fig. 5. Hydrolysis of phospholipids of opened microsomal vesicles by phospholipase C-*Cl. welchii*. Microsomes were incubated with phospholipase C-*Cl. welchii* in the presence of deoxycholate (0.098%), (○———○); lysophosphatidylcholine (0.05%), (△———△); or after treatment with the French pressure cell (equilibrated for 5 min at 20 000 lb. per sq. in.), (□———□); or with no treatment, (●- - - - -●). Lipids were extracted at the appropriate times and the % hydrolysis of total phospholipids determined as described in Materials and Methods.

TABLE III

HYDROLYSIS OF INDIVIDUAL PHOSPHOLIPIDS IN OPENED MICROSOMAL VESICLES BY PHOSPHOLIPASE C

Microsomes were incubated with phospholipase C-*Cl. welchii* under the conditions in Fig. 6. Lipids were extracted at appropriate times and separated as described in Materials and Methods.

	% Hydrolysis		
	10 min	30 min	60 min
Phosphatidylcholine			
Untreated	43	71	74
Plus deoxycholate (0.098%)	48	76	78
Plus lysophosphatidylcholine (0.005%)	82	86	86
French press-treated	87	87	89
Phosphatidylethanolamine			
Untreated	7	18	19
Plus deoxycholate (0.098%)	16	34	45
Plus lysophosphatidylcholine (0.005%)	39	46	58
French press-treated	30	46	67
Sphingomyelin			
Untreated	56	71	73
Plus deoxycholate (0.098%)	58	77	76
Plus lysophosphatidylcholine (0.005%)	66	90	90
French press-treated	71	76	84
Phosphatidylserine			
Untreated	12	18	19
Plus deoxycholate (0.098%)	16	37	35
Plus lysophosphatidylcholine (0.005%)	35	43	50
French press-treated	51	71	78

choline. These results are consistent with those on intact vesicles and suggest that a large percentage of the phosphatidylethanolamine and phosphatidylserine is only available at the inner surface of the microsomal vesicle, while over 70% of the phosphatidylcholine and sphingomyelin are available on the external surface.

Treatment of microsomal vesicles with phospholipase A_2

Hydrolysis of microsomal phospholipid by phospholipase A_2 was almost complete except for sphingomyelin, which was unhydrolysed (Table IV). Lowering the enzyme concentration resulted in a slower hydrolysis but this was continuous and there was no selective hydrolysis of any of the individual phospholipids. Lysophospholipids produced by partial hydrolysis remained associated with the microsomes and were recovered in the pellet, when these were isolated by centrifugation. Addition of fatty acid-free albumin to the incubation medium caused a slightly more rapid hydrolysis of the phospholipid and hydrolysis was still complete.

Treatment of microsomal vesicles with phospholipase C of B. cereus

Hydrolysis of microsomal phospholipid by phospholipase C-*B. cereus*, was almost complete (Table IV). As with the phospholipase A_2, lowering the enzyme concentration reduced the rate of hydrolysis; however, there was no selective hydrolysis of individual phospholipids.

TABLE IV

HYDROLYSIS OF PHOSPHOLIPIDS OF MICROSOMAL VESICLES BY PHOSPHOLIPASE A_2 OR PHOSPHOLIPASE C-*B. CEREUS*

Microsomes were incubated with phospholipase A_2 or phospholipase C-*B. cereus* and the lipids extracted and separated as described in Materials and Methods. PC, phosphatidylcholine; PE, phosphatidylethanolamine; Sph, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol.

	% Hhydrolysis in 60 min				
	PC	PE	Sph	PS	PI
Phospholipase A_2					
0.65 units/ml	84	81	0	64	70
26 units/ml	89	98	0	98	99
Phospholipase C					
1 unit/ml	88	93	94	60	97

Discussion

In order for an investigation of the transverse asymmetry of the lipid bilayer of the lipid bilayer of microsomes by phospholipases to be valid it is necessary that the enzyme used fulfil two major criteria. (1) The enzyme should not have access to the interior of the microsomal vesicle unless the latter is opened experimentally and should not have a lytic component as impurity, and (2) the specificity of the enzyme should be broad so that all of the membrane phospholipids are hydrolysed, if these are freely accessible. In the present study, no phospholipase investigated completely fulfilled these conditions. However, phospholipase C-*Cl. welchii* did not gain access to the interior of the vesicle and, with the exception of phosphatidylinositol, caused complete hydrolysis of all of the microsomal phospholipids when these were presented to the enzyme in the form of a dispersion. This enzyme is therefore satisfactory for these investigations and provides information concerning the distribution of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin. Unfortunately, with the present study, it is not possible to locate directly the site of phosphatidylinositol. Those enzymes which hydrolysed this lipid also caused leakage of the vesicle content and complete hydrolysis of the membrane phospholipids. Attempts were made to investigate the distribution of phosphatidylinositol with a phospholipase C which is specific for this lipid and which occurs in the post-microsomal supernatant of liver [25]. However, this enzyme did not hydrolyse phosphatidylinositol of microsomal membranes, either intact or open, under conditions in which hydrolysis of the phosphatidylinositol of extracted and dispersed microsomal phospholipids took place. Low and Finean [26] also found the phosphatidylinositol specific phospholipase C of lymphocytes did not act on phosphatidylinositol of rat liver microsomes. The site of phosphatidylinositol may be tentatively determined indirectly by calculation of the distribution of other phospholipids assuming that the inner and outer leaflet of the membrane bilayer are equal in weight, and that there is an equal distribution of non-phospholipid lipid components on either side of the bilayer. On this basis we

tentatively assign the bulk of the phosphatidylinositol to the inner side of the microsomal vesicle.

Phospholipase A_2 and phospholipase C-*B. cereus* caused rapid leakage of the contents of microsomal vesicles and also caused complete hydrolysis of the phospholipids. These enzymes therefore do not fulfil condition 1 above and are not satisfactory for an investigation of the transverse asymmetry of phospholipids of microsomal membranes. The reason for this is not clear; however, it is possible that lysis of microsomal vesicles is caused by impurities, rather than a direct effect on the phospholipids. Nilsson and Dallner have reported preliminary studies of the asymmetry of microsomal vesicles using phospholipase A_2 of *Naja naja* [12]. In our hands, phospholipase A_2 from this source caused rapid leakage of vesicle contents (unreported observations) and hydrolysis of phospholipids by this enzyme from bee venom was continuous, and complete, and accelerated by addition of albumin, in contrast to their findings that hydrolysis levelled off at 60% and that albumin protected the vesicles against becoming leaky. In addition, we found that small amounts of lysophosphatidylcholine, a product of the action of phospholipase A_2, caused leakage of vesicle contents. It would appear, therefore, that opening of the vesicles is an unavoidable result of their treatment with this enzyme and not due to differences in the incubation conditions used.

A major potential criticism of experiments designed to investigate the transverse asymmetry of the phospholipid bilayer of membranes is that the experimental treatment used as a probe may result in a rearrangement or perturbation of the structure. This is extremely difficult to rule out. However, movement of phospholipids across the bilayer of liposomes and membranes is extremely slow [27—31] and would probably only take place if the membrane structure were sufficiently disturbed to cause a leakage of contents. In the present study, we have used the retention of content as an index of the maintenance of structural integrity. The loss of vesicle contents during phospholipase A_2 and phospholipase C-*B. cereus*, treatment was paralleled by hydrolysis of all the membrane phospholipids. This was also found in experimentally opened microsomal vesicles whether these were opened chemically or mechanically. We consider therefore that treatment of intact microsomal vesicles with phospholipase C-*Cl. welchii* does not cause a loss of structural integrity of the membrane or a shift in the site of phospholipid molecules. Lenard and Singer [32] demonstrated that removal of phospholipids of red cell membranes with phospholipase C-*Cl. welchii* does not alter the circular dichroism and optical rotary dispersion spectra of the membrane proteins. In addition, loss of free amino acid from squid axon membranes occurs on treatment with phospholipase A_2 but not phospholipase C.-*Cl. welchii* [35]. Coleman et al. [33] have demonstrated the appearance of dense spots, presumed to be diglyceride, in the membranes of red cells treated with phospholipase C-*Cl. welchii*. However, the rest of the red cell membrane examined in the electron microscope retains the trilammellar structure with the same dimensions as the untreated membrane. These observations suggest that phospholipase C-*Cl. welchii* does not cause a marked disruption of the membrane structure.

A number of investigators have used phospholipase C-*Cl. welchii* in studies of

membranes. This enzyme was found to hydrolyse the phospholipids of red cells and ghosts [4,5,32,33], rat liver plasma membranes [34], virus membranes [10] and squid axons [35]. In contrast to our observations with liver microsomal vesicles, phospholipase C-*Cl. welchii* caused lysis of red cell membranes and hydrolysis of up to 85% of the phospholipid. However, this enzyme selectively hydrolysed the outer surface phospholipids of inside-out vesicles of red cell membranes and demonstrated transverse asymmetry consistent with that found in the intact red cell using other phospholipases [4]. The lytic action of phospholipase C appears therefore to depend on the form of the membrane used. Phospholipase C-*Cl. welchii* has also been used to investigate asymmetry of the influenza virus membrane and apparently does not gain access to the inner surface of the virus membrane [10].

Studies of model systems have indicated that mixed phospholipid liposomes exhibit an asymmetric distribution of different phospholipids across the bilayer [36—40]. Thus, vesicles prepared from phosphatidylcholine mixed with phosphatidylethanolamine, phosphatidylserine or phosphatidylinositol have a greater concentration of phosphatidylcholine in the outer leaflet of the bilayer [37,38], while vesicles prepared from mixtures of phosphatidylcholine and phosphatidylglycerol have a greater concentration of the former in the inner leaflet [36]. The distribution of phosphatidylserine in such mixed lipid liposomes is pH dependent and this lipid is less concentrated in the inner leaflet at low pH [38]. There is also a tendency for cholesterol to be distributed in the inner leaflet of the liposome bilayer, when this lipid is present at a concentration above 30 mol% [39]. Under these conditions phosphatidylcholine molecules having *cis*-unsaturated fatty acids concentrate in the outer leaflet of the bilayer [39]. These results suggest that the size and charge of the phospholipid molecule is important in the packaging of these molecules in mixed bilayers [38]. The relationship of these observations to biological membranes is not clear. The liposomes used were extremely small vesicles, 200—300 Å in diameter, and therefore the packaging of the phospholipids is subject to steric and geometric restraints not found in large vesicles or flat sheets of membranes. However, there are parts of most biological membranes which have small radii of curvature, for example the ends of the cisternae of the endoplasmic reticulum, and which may therefore be subject to the restrains governing packaging of the phospholipids in liposomes. It is interesting that all biological membranes investigated including the endoplasmic reticulum in the present study have a higher concentration of phosphatidylcholine in the outer leaflet of the bilayer.

The existence of an asymmetric distribution of phospholipids across the bilayer of liver microsomes raises questions concerning the biogenesis of these membranes. The endoplasmic reticulum has the enzymic machinery for the synthesis of its phospholipid components [40]. The possibilities are, therefore, that phospholipids are synthesised asymmetrically, or that there is a transfer of the complete molecule or some intermediate in its synthesis across the bilayer. Cytochemical localisation of the acyl CoA-*sn*-glycerol-3-phosphate acyltransferases, which catalyse the first steps in the synthesis of glycerophospholipids, suggest that these enzymes are on the inner surface of the endoplasmic reticulum membrane [14,16]. There is also an asymmetric incorporation of

fatty acids at the inner surface of the red cell membrane [31]. These observations tentatively suggest that phospholipids are synthesised at least partly on one side of the membrane and that some mechanism for the transport of phosphatidic acid, diglyceride or the phospholipid molecule across the bilayer must exist. As the 'flip-flop' of phospholipid molecules is extremely slow [27—31], such a postulated mechanism must be selective and energy dependent.

The microsomal membranes used in these studies are derived predominantly from the endoplasmic reticulum [16] and the orientation of these is with the cytoplasmic side of the membrane outwards. The distribution of phospholipids across the bilayer of the microsomal membrane suggested by the present investigation, is similar to that described for red cell membranes and for influenza virus membranes derived from the plasma membrane of the host kidney cells [4]. However, in terms of the topography of the cell the outer surface of the microsomal membrane corresponds to the inner surface of the plasma membrane. Our results are somewhat unexpected therefore, and might suggest that the bilayer of the endoplasmic reticulum does not contribute to that of the plasma membrane, or, that significant modification of the phospholipids takes place during movement of vesicles of the endoplasmic reticulum membrane via the Golgi complex to the plasma membrane or of the plasma membrane in situ. Nilsson and Dallner [14] have reported a completely reversed distribution of phospholipids to our findings. These would be more in keeping with the concept of a simple functional continuity between endoplasmic reticulum, Golgi membranes and plasma membrane. However, these membranes clearly differ in composition and function, and the relationship between these is complex. The transverse asymmetry of the Golgi membrane and plasma membrane of liver is currently under investigation in this laboratory to examine this relationship.

Acknowledgements

J.A.H. is the recipient of career development award 5 K04 GM 70729 of the National Institutes of Health. This research was supported by grant 5 R01 GM 20001 from the National Institutes of Health. During the preparation of this paper J.A.H. was supported by a Faculty Scholar Award for 1976-1977 from the Josiah Macy Jr. Foundation.

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