Biochimica et Biophysica Acta, 465 (1977) 454—465

BBA 77641

TRANSBILAYER DISTRIBUTION AND MOVEMENT OF LYSOPHOSPHATIDYLCHOLINE IN LIPOSOMAL MEMBRANES

A.M.H.P. VAN DEN BESSELAAR, H. VAN DEN BOSCH and L.L.M. VAN DEENEN

Biochemistry Laboratory, Padualaan 8, De Uithof, Utrecht (The Netherlands)

(Received August 23rd, 1976)

Summary

Single bilayer vesicles were prepared by sonication of 5 mol% 1-palmitoyl lysophosphatidylcholine and 95 mol% egg phosphatidylcholine. Incubation with lysophospholipase results in a fast hydrolysis of 80—90% of lysophosphatidylcholine. The remaining lysophosphatidylcholine is only very slowly hydrolysed. There results are interpreted as lysophosphatidylcholine being asymmetrically distributed over the two halves of the bilayer. The slow phase of lysophosphatidylcholine hydrolysis sets an upper limit to the rate of transbilayer movement of lysophosphatidylcholine. The half time of this process at 37°C is estimated to be about 100 h. Incorporation of cholesterol in the vesicles reduces the distributional asymmetry of lysophosphatidylcholine to the extent of an outside-inside ratio of 60 : 40. [^{14}C]Lysophosphatidylcholine introduced into the outer monolayer of such vesicles by intervesicular transfer of lysophosphatidylcholine remains virtually completely available for hydrolysis by lysophospholipases, corroborating the interpretation that transbilayer movement of lysophosphatidylcholine in these vesicles is an extremely slow process.

In handshaken liposomes consisting of 5 mol% 1-palmitoyl lysophosphatidylcholine and 95 mol% egg phosphatidylcholine 15—20% of lysophosphatidylcholine is readily available for exogenous lysophospholipase. This pool may represent lysophosphatidylcholine in the outer monolayer of the liposomes.

Introduction

Lysophosphatidylcholine occurs in many biological membranes usually at relatively low concentrations. It is an important intermediate in the metabolism of phosphatidylcholine, one of the most abundant membrane phospholipids. In the endoplasmic reticulum of eukaryotic cells an active deacylation-reacylation cycle adjusts the fatty acid composition of phosphatidylcholine [1].

Apart from its metabolic role lysophosphatidylcholine has been suggested to influence many membrane properties. Several membrane-bound enzyme activities are affected by lysophosphatidylcholine for example galactosyltransferase in rat liver microsomes [2] and alkaline phosphatase in HeLa cells [3]. Eytan et al. [4] showed that lysophosphatidylcholine facilitates the incorporation of several membrane proteins into preformed liposomes. Blume et al. [5] demonstrated that relatively small amounts of a lysolecithin analog have a pronounced influence on the phase transition of pure lipids and on the miscibility of different molecular species of phospholipids. The possibility that lysophosphoglycerides are involved in membrane fusion processes has been suggested by many investigators. This idea emerged from the observation that lysophosphoglycerides are able to induce cell fusion [6].

In recent years considerable attention has been directed to the transverse distribution of diacylphospholipids in both biological and artificial membranes. Transmembrane compositional asymmetries of diacylphosphoglycerides have been described for erythrocytes [7,8], rat liver microsomes [9] and influenza virus [10]. Transbilayer asymmetries of diacylphosphoglycerides in artificial membranes, in casu sonicated single walled liposomes, may well be caused by the small radius of curvature which is characteristic for these vesicles [11]. The problem of transmembrane asymmetry of lipids is closely related to the rate of their transverse movements. It was estimated that the half time of phosphatidylcholine flip-flop in phosphatidylcholine vesicles is in excess of 11 days at 37°C [12]. Roseman et al. [13] estimated the half time of phosphatidylethanolamine movement across the vesicle bilayer to be at least 80 days at 22°C.

In a previous paper we studied the action of two different lysophospholipases from bovine liver on membrane-bound lysophosphatidylcholine [14].

In this paper we report the use of purified lysophospholipases as a tool to explore the distribution and dynamics of lysophosphatidylcholine incorporated in liposomal membranes.

Experimental section

Materials

Lysophospholipases I and II were purified to homogeneity from beef liver as described by de Jong et al. [15]. Cholesterol was obtained from Merck. Isolation of phospholipids has been described in the previous paper [14].

[1α,2α(n)-^{3}H]cholesterol oleate was kindly donated by Dr. K.W.A. Wirtz from this laboratory and had a specific activity of 10^5 dpm/nmol. Thin-layer chromatography on silica plates (solvent system: petroleum ether/diethyl ether/formic acid, 60 : 40 : 1.5, v/v/v) showed one major radioactivity peak. Only 2.% of the radioactivity cochromatographed with free cholesterol.

Phosphatidyl-[*Me*-^{14}C]choline was isolated from rat liver microsomes as described by van den Bosch et al. [16]. This product had a specific activity of 21 000 dpm/μmol. 1-[9,10-$^3H_2$]stearoyl-*sn*-glycero-3-phosphorylcholine and 1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine were synthesized according to the procedure by van den Bosch et al. [17]. The specific activities were about 10^3 dpm/nmol and 10^2 dpm/nmol, respectively.

Methods

Preparation of liposomes and vesicles. Lipids were mixed in chloroform solution. After evaporation of the chloroform in vacuo the lipid was suspended in buffer solution by shaking with glass beads. For the preparation of single bilayer vesicles the liposomes were sonicated for 30 min at 0° C under nitrogen with a Branson sonifier (energy output, 70 W). The sonicated mixture was centrifuged for 60 min at 150 000 × *g* for removal of particles from the sonifier tip and larger lipid aggregates.

Lysophospholipase assay. Lysophospholipase activity (EC 3.1.1.5) was assayed as described earlier [14] using 1-[1-^{14}C]palmityol-*sn*-glycero-3-phosphorylcholine as a substrate and [9,10-$^{3}H_2$]palmitic acid as an internal standard. The degree of deacylation was calculated from the ^{14}C/^{3}H-ratio of the palmitate in the heptane layer of the Dole-extract.

Gel filtration. Gel partition chromatography was performed on Sepharose 2B and 4B columns (2 × 45 cm), using upward flow and a peristaltic pump to afford constant flow. Column void volume was determined by elution of Dextran blue 2000. The buffer always contained 150 mM NaCl. Samples (100 μl or 200 μl) from the eluate fractions were counted in 15 ml dioxane scintillation liquid (10% napthalene, 0.7% PPO, 0.03% dimethyl POPOP) using a Packard Tricarb Liquid Scintillation Spectrometer.

Introduction of [^{14}C]lysophosphatidylcholine into pre-existing vesicles by means of exchange. Sonicated vesicles composed of 47 mol% egg phosphatidylcholine, 47 mol% cholesterol, 5 mol% egg lysophosphatidylcholine and 1 mol% egg phosphatidic acid (total amount of lipid was 100 μmol) were incubated with sonicated vesicles consisting of 5 mol% 1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine, 15 mol% egg phosphatidic acid, 80 mol% egg phosphatidylcholine and a trace (0.03 mol%) of [^{3}H]cholesterol oleate (total amount of lipid was 10 μmoles) in a total volume of 7 ml buffer (10 mM 2-mercapto ethanol, 12,5 mM phosphate pH 7.0) for 2 h at 37° C. After the incubation the mixture was applied to a DEAE-cellulose (DE 52, Whatman) column (about 10 ml bed volume) and eluted with about 7 ml buffer (10 mM 2-mercapto ethanol, 12,5 mM phosphate pH 7.0). Aliquots (500 μl) of the eluate were used for incubation with 15 μg lysophospholipase I.

Introduction of [^{14}C]lysophosphatidylcholine into pre-existing handshaken liposomes by means of exchange. Handshaken liposomes prepared from 5 μmol egg lysophosphatidylcholine and 97 μmol egg phosphatidylcholine were incubated with 9.4 μmol sonicated vesicles consisting of 5 mol% 1-[1-^{14}C]palmitoyl lysophosphatidylcholine, 95 mol% egg phosphatidylcholine and a trace of [^{3}H]-cholesterol oleate (0.06 mol%) in a total volume of 4 ml buffer (150 mM NaCl, 10 mM 2-mercapto ethanol, 0.5 mM EDTA, 20 mM phosphate pH 7.0) for 1 h at 37° C. The mixture was centrifuged at 50 000 × *g* for 20 min at 4° C. The supernatant was decanted and the pellet was resuspended in 8 ml buffer. The suspension was again centrifuged at 50 000 × *g* for 30 min at 4° C. After decantation the pellet was resuspended in 4 ml buffer using glass beads. Aliquots (200 μl) of this suspension were used for incubation with 6 μg lysophospholipase I.

Results

Sonication of 95 mol% egg phosphatidylcholine and 5 mol% 1-palmitoyllysophosphatidylcholine results in the formation of small sized vesicles consisting of a single phospholipid bilayer. No indications for the presence of multilayered structures were obtained by negative staining electron microscopy. Sepharose 4B chromatography showed that [^{3}H]lysophosphatidylcholine was associated with the vesicles (Fig. 1). A single radioactivity peak with a leading edge was obtained. This leading edge has been ascribed by Andrews et al. [18] to be the result of heterogeneity in the size of the unilaminar vesicle population. As can be seen from Fig. 1 the ratio of lysophosphatidylcholine to phosphatidylcholine is slightly higher in the larger vesicles. However, from fraction 26 on, i.e. in the fractions comprising the majority of the vesicle preparation, the ratio reaches a constant value.

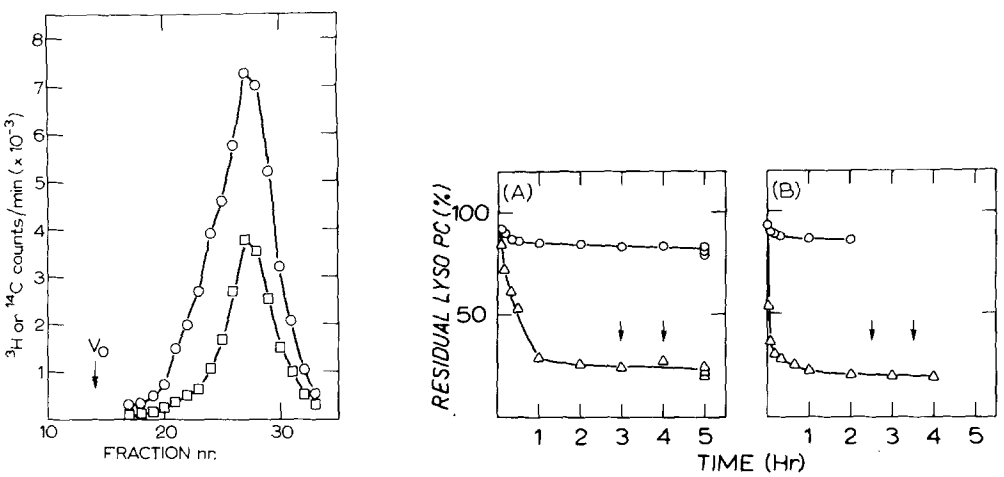

Fig. 1. Sepharose 4B chromatography of 5 mol% lysophosphatidylcholine, 95 mol% phosphatidylcholine vesicles. Vesicles were prepared by sonication of 5.2 μmol egg phosphatidylcholine, 3.7 μmol of rat liver phosphatidyl-[*Me*-^{14}C]choline, 0.22 μmol of egg lysophosphatidylcholine and 0.23 μmol 1-[9,10-$^3H_2$]-stearoyl lysophosphatidylcholine in 3 ml 150 mM NaCl, 10 mM 2-mercapto ethanol, 0.5 mM EDTA, 20 mM phosphate pH 7.0. Phosphatidylcholine and lysophosphatidylcholine were traced by counting ^{14}C (□———□) and ^{3}H (○———○) radioactivity, respectively. The void volume of the column is indicated by an arrow. The ^{14}C/^{3}H ratio of the preparation before the chromatography was equal to the ^{14}C/^{3}H ratio of the eluted peak, indicating that all lysophosphatidylcholine has been incorporated in the vesicles.

Fig. 2. (A) Time course of lysophosphatidylcholine (LYSO PC) hydrolysis by lysophospholipase I: 5 mol% lysophosphatidylcholine, 95 mol% egg phosphatidylcholine sonicated vesicles (20 nmol of lysophosphatidylcholine) were incubated with 10 μg of enzyme I (△———△). 5 mol% lysophosphatidylcholine, 95 mol% egg phosphatidylcholine handshaken liposomes (20 nmol of lysophosphatidylcholine) were incubated with 10 μg of enzyme I (○———○). At the times marked by arrows an additional amount of 10 μg of enzyme I was added. The effect of these additions was measured at t = 5 h. Incubation medium: 150 mM NaCl, 10 mM 2-mercapto ethanol, 20 mM Tris · HCl, pH 7.4. Temperature: 37°C. (B) Time course of lysophosphatidylcholine hydrolysis by lysophospholipase II: 5 mol% lysophosphatidylcholine, 95 mol% egg phosphatidylcholine handshaken liposomes (80 nmol of lysophosphatidylcholine) were incubated with 300 μg of enzyme II (○———○). 5 mol% lysophosphatidylcholine, 95 mol% egg phosphatidylcholine sonicated vesicles (6 nmol of lysophosphatidylcholine) were incubated with 75 μg of enzyme II (△———△). These vesicles were obtained after fractionation through a Sepharose 2B column. At the times marked by arrows an additional amount of 75 μg of enzyme II was added. The effect was measured 30 min after the extra addition. Buffer system: 150 mM NaCl, 20 mM Tris · HCl, pH 7.4. Temperature: 37°C.

Incubation of sonicated vesicles containing 5 mol% lysophosphatidylcholine with lysophospholipase gives at first a relatively fast hydrolysis of lysophosphatidylcholine. The initial rate of this first phase is directly proportional to the enzyme concentration and the mol fraction of lysophosphatidylcholine [14]. After 80—90% of the original lysophosphatidylcholine has been degraded, the rate of hydrolysis is greatly reduced. This is not due to denaturation of the enzyme, since addition of a fresh amount of enzyme does not increase the extent of hydrolysis. Both lysophospholipases I and II gave essentially the same result (Fig. 2). Since it has previously been shown [14] that the enzymes do not penetrate the lipid bilayer, these data are interpreted as indicating that 80—90% of the lysophosphatidylcholine is located in the outer monolayer of the lipid vesicle. This value may represent the average of distributions in the total vesicle population. Essentially the same quantitative results were obtained when vesicles chromatographed on Sepharose 2B were used. This strongly asymmetric distribution of lysophosphatidylcholine over inner and outer monolayer of the vesicle was confirmed by NMR data [28].

In sonicated vesicles containing lysophosphatidylcholine the initial enzymatic lysophosphatidylcholine hydrolysis increases with the mol fraction of lysophosphatidylcholine in the vesicles [14]. The initial rates were obtained under saturating conditions with respect to vesicle concentration. In sonicated vesicles containing lysophosphatidylcholine, phosphatidylcholine and cholesterol the initial enzymatic lysophosphatidylcholine hydrolysis is slower than in vesicles without cholesterol but with the same lysophosphatidylcholine, phosphatidylcholine ratio (Table I). However, also in the cholesterol-containing vesicles a biphasic enzymic hydrolysis is observed. About 60% of lysophosphatidylcholine in 5 mol% 1-palmitoyl lysophosphatidylcholine, 47.5 mol% egg phosphatidylcholine, 47.5 mol% cholesterol vesicles is hydrolysed in the first phase of the incubation (Figs. 3 and 4). These data suggest that in the cholesterol-containing vesicles the lysophosphatidylcholine is more equally distributed over inner and outer monolayers, a finding completely corroborated by NMR data [28]. The observation that about 40% of the lysophosphatidylcholine is not available for hydrolysis by the lysophospholipase makes it very unlikely that the biphasic hydrolysis is caused by the existence of both single and multilayer vesicles, especially since it has recently been shown that incorporation of 50 mol% cholesterol in phosphatidylcholine vesicles does not give rise to formation of multilayer structures [29].

TABLE I

EFFECT OF CHOLESTEROL ON INITIAL RATE OF LYSOPHOSPHATIDYLCHOLINE HYDROLYSIS IN SONICATED VESICLES

Vesicle composition (mol %)			V_{sat}, Enzyme I (nmol · min^{-1} · mg^{-1})	V_{sat}, Enzyme II (nmol · min^{-1} · mg^{-1})
Lysophosphatidylcholine	Egg phosphaticylcholine	Cholesterol		
2	98	—	193	39
4	96	—	320	75
2	49	49	109	15

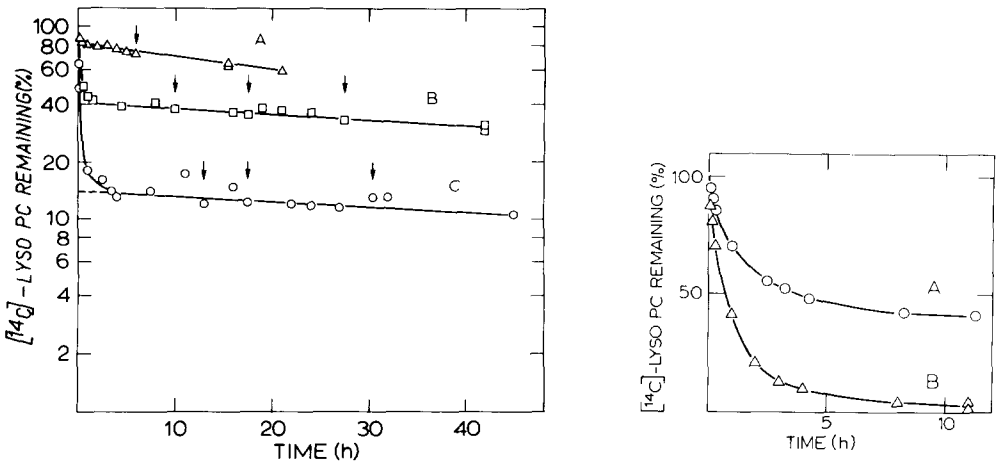

Fig. 3. Time course of lysophosphatidylcholine (LYSO PC) hydrolysis by lysophospholipase I. (A) 5 mol% lysophosphatidylcholine, 95 mol% egg phosphatidylcholine handshaken liposomes (25 nmoles of lysophosphatidylcholine) were incubated with 6 μg of enzyme I. Incubation medium: 150 mM NaCl, 10 mM 2-mercapto ethanol, 0.5 mM EDTA, 20 mM phosphate, pH 7.0. Temperature: 37°C. (B) 5 mol% lysophosphatidylcholine, 47.5 mol% egg phosphatidylcholine, 47.5 mol% cholesterol vesicles (15 nmol of lysophosphatidylcholine) were incubated with 7.5 μg of enzyme I. Before incubation these vesicles were fractionated by Sepharose 4B chromatography. (C) 5 mol% lysophosphatidylcholine, 95 mol% egg phosphatidylcholine vesicles (10 nmol of lysophosphatidylcholine) were incubated with 7.5 μg of enzyme I. At the times marked by arrows additional amounts of enzyme were added.

Fig. 4 (A) Action of lysophospholipase I on vesicles where the labelled lysophosphatidylcholine (LYSO PC) was introduced into the original sonicated mixture. Original vesicle composition: 5 mol% lysophosphatidylcholine, 47 mol% egg phosphatidylcholine, 47 mol% cholesterol, 1 mol% egg phosphatidic acid. Medium: 10 mM 2-mercapto ethanol, 12.5 mM phosphate pH 7.0. Temperature: 37°C. (B) Action of lysophospholipase I on vesicles where the labelled lysophosphatidylcholine was introduced into vesicles (population I) by a previous incubation with vesicles (population II) containing [^{14}C]lysophosphatidylcholine (see text).

The slow phase of lysophosphatidylcholine hydrolysis is thought to monitor the appearance of lysophosphatidylcholine in the outer monolayer by transbilayer movement from the inner monolayer. More accurate data on the hydrolysis of lysophosphatidylcholine in this slow phase were obtained by extending the incubation times up to 45 h. All data were corrected for the hydrolysis of lysophosphatidylcholine in the absence of enzyme. This hydrolysis increased linearly with time and after 45 h amounted to only 2.8% of the total lysophosphatidylcholine present. If the enzymic lysophosphatidylcholine hydrolysis in the slow phase is a first order process, it is justified to represent the results in a semilogarithmic plot (Fig. 3). The slope of the slow phase hydrolysis was determined by least squares analysis of the data points. (Half times were calculated according to $t_{1/2} = -\log 2/\text{slope}$). The results are given in Table II.

Incubation with exogenous lysophospholipase generates a nonequilibrium distribution of lysophosphatidylcholine over the bilayer, which constitutes the driving force of lysophosphatidylcholine transposition from inside to outside. This net movement of lysophosphatidylcholine should be distinguished from lysophosphatidylcholine transbilayer exchange. The latter process does not abolish concentration gradients of lysophosphatidylcholine over the bilayer

TABLE II

HALF TIMES OF SLOW PHASE HYDROLYSIS AS DETERMINED BY LEAST SQUARES ANALYSIS

Liposome type	Composition (mol %)			$t_{1/2}$ (h) *
	Lysophosphatidylcholine	Egg phosphatidylcholine	Cholesterol	
Single bilayer	5	47.5	47.5	93 ± 12
Single bilayer	5	95	—	100 ± 30
Multilayer	5	95	—	46 ± 2

* Data points corresponding to the slow phases in Fig. 3 were used. Standard errors were derived from the regression equation.

since movement of a molecule in one direction is counterbalanced by the movement of a second molecule in the opposite direction.

Lysophosphatidylcholine transbilayer exchange was studied in the following experiment. One vesicle population (I) containing 5 mol% unlabelled lysophosphatidylcholine was incubated with a second vesicle population (II) containing 5 mol% [^{14}C]lysophosphatidylcholine in order to exchange lysophosphatidylcholine between the populations. Population I acquires an isotopic gradient over the bilayer: the inside contains unlabelled molecules while the outside is labelled. This gradient can decay by a transbilayer exchange process. This exchange can be monitored by incubation with external lysophospholipase, since only labelled lysophosphatidylcholine molecules at the outside can be detected by enzymic hydrolysis. Before incubation of vesicle population I with lysophospholipase, vesicle population II has to be removed. This can be done by giving population II a relatively high negative surface change (15 mol% phosphatidic acid). During passage of the mixture through an anion exchange column population II is bound while population I is not [19,20].

In the experiment of Fig. 4 a ten-fold molar excess of vesicles consisting of 5 mol% egg lysophosphatidylcholine, 47 mol% egg phosphatidylcholine, 47 mol% cholesterol and 1 mol% egg phosphatidic acid were incubated with vesicles consisting of 5 mol% 1-[^{14}C]palmitoyl lysophosphatidylcholine, 15 mol% egg phosphatidic acid, 80 mol% egg phosphatidylcholine and a trace (0.03 mol%) of [^{3}H]cholesterol oleate for 2 h at 37°C. After this incubation the mixture was applied to a DE-52 column. The eluate contained 4% of the total ^{3}H and 54% of the total ^{14}C radioactivity. Obviously, considerable exchange of lysophosphatidylcholine between the vesicle populations had occurred. Cholesterol probably also redistributed between the two vesicle populations, but due to the large excess of the vesicles containing cholesterol, the cholesterol content of these vesicles could only slightly be decreased. Cholesterol oleate does not partition spontaneously between vesicles and can be considered as a marker for the 15 mol% phosphatidic acid vesicles [12,19]. The fact that still 4% of ^{3}H is recovered in the eluate may be attributed to incomplete binding to the column, vesicle fusion or hydrolysis of the marker. The eluate of the column was incubated with lysophospholipase I 2.5 h after mixing of the original vesicle populations. Ultimately, some 97% of [^{14}C]lysophosphatidylcholine was hydrolysed

(Fig. 4). As mentioned before, vesicles of the same chemical composition but containing the labelled lysophosphatidylcholine in equilibrium distribution over the bilayer, expose only 60% of their [^{14}C]lysophosphatidylcholine to the exterior. From this it can be concluded that in the time preceding the incubation with lysophospholipase no appreciable transbilayer exchange of lysophosphatidylcholine had occurred. An analogous experiment was done with 5 mol% lysophosphatidylcholine, 95 mol% egg phosphatidylcholine vesicles. Here too, transbilayer exchange of lysophosphatidylcholine could not be detected within 3 h.

Handshaken multilayer liposomes, in which 5 mol% 1-palmitoyl lysophosphatidylcholine and 95 mol% egg phosphatidylcholine were homogeneously distributed, were incubated with lysophospholipase I and II. Lysophosphatidylcholine hydrolysis occurred in two phases. Some 15 or 20% of total lysophosphatidylcholine was hydrolysed rather rapidly. The remaining lysophosphatidylcholine was hydrolysed much slower (Fig. 2). Prolonged incubation (up to 21 h) gave more accurate data on the slow phase of lysophosphatidylcholine hydrolysis. A semi-logarithmic plot of lysophosphatidylcholine hydrolysis as a function of time yields a straight line for the slow phase. The half time of the slow phase hydrolysis was 46 h (Table II).

An attempt was made to create handshaken liposomes with [^{14}C]lysophosphatidylcholine at the outer surface only. A ten-fold molar excess of handshaken liposomes consisting of 5 mol% egg lysophosphatidylcholine and 95 mol% egg phosphatidylcholine were incubated with sonicated vesicles containing 5 mol% 1-[1-^{14}C]palmitoyl lysophosphatidylcholine, 95 mol% egg phosphatidylcholine and a trace (0.06 mol%) of [^{3}H]cholesterol oleate. After 1 h incubation at 37°C the liposomes were separated from the vesicles by high speed centrifugation. About 50% of the total ^{14}C-radioactivity and only 4% of the total ^{3}H-radioactivity were recovered in the liposomal pellet. Incubation of the resuspended liposomes with lysophospholipase I, 2.5 h after the mixing of vesicles and liposomes, resulted in about 70% hydrolysis of [^{14}C]lysophosphatidylcholine within 5 h (Fig. 5).

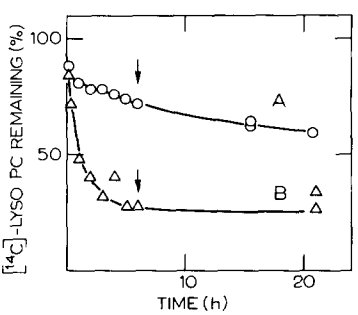

Fig. 5. A. Action of lysophospholipase I (6 μg) on handshaken liposomes (5 mol% lysophosphatidylcholine (LYSO PC), 95 mol% egg phosphatidylcholine) where the labelled lysophosphatidylcholine was present at the formation of the multilayers. These data are also shown in Fig. 2A. (B) Action of lysophospholipase I (6 μg) on handshaken liposomes where the labelled lysophosphatidylcholine was introduced into the liposomes by a previous incubation with sonicated vesicles containing [^{14}C]lysophosphatidylcholine (see text). At the time marked by arrows an additional amount of 6 μg of lysophospholipase I was added.

If lysophosphatidylcholine transbilayer exchange were negligible during the liposome-vesicle incubation and the subsequent lysophospholipase incubation, one would expect that all exchanged [^{14}C]lysophosphatidylcholine was still present at the outer surface of the multilayers and consequently directly available for enzymic attack. One explanation for the experimental results is that part of the [^{14}C]lysophosphatidylcholine has moved to the inner layers by transbilayer exchange. When the liposomes were preincubated for 8 h at room temperature (before the lysophospholipase incubation was started) slightly less [^{14}C]lysophosphatidylcholine (60% instead of 70%) was hydrolysed after 15 h incubation with lysophospholipase at 37°C. A second explanation is that during the separation of vesicles from liposomes some rearrangements of the multilayers have taken place due to the mechanical agitations. Anyway, lysophosphatidylcholine transbilayer exchange is not so fast that all [^{14}C]lysophosphatidylcholine has become homogeneously distributed over all bilayers of the liposome during the time before the lysophospholipase action had attained its slow phase.

Discussion

In this paper we have presented evidence that lysophosphatidylcholine in sonicated egg phosphatidylcholine vesicles is present in two distinct pools, one of which is readily available for hydrolysis by exogenous lysophospholipase and the other of which is hydrolysable only very slowly. We propose that the readily available pool of lysophosphatidylcholine is present in the outer surface of the vesicle and that the second pool is present in the inner monolayer. This interpretation implies that the rate at which the second pool is hydrolysed is controlled by the rate of lysophosphatidylcholine movement from inside to outside. However, other processes may also contribute to the overall rate of enzymatic hydrolysis. Firstly, lysophospholipase permeation through the bilayer might contribute to the overall rate. Secondly, vesicle fusion might be responsible for mixing the original inner and outer membrane halves. Rousselet et al. [21,22] showed that fusion of vesicles with erythrocytes and mitochondrial inner membranes introduces the vesicle phospholipids on both sides of these natural membranes. In any way, the half time of lysophosphatidylcholine transbilayer movement equals or exceeds the half time of hydrolysis in the slow phase. This half time was found to be about 100 h (Table II).

An asymmetrical distribution of lipid components in sonicated single bilayer vesicles has been detected by many investigators. The total number of lipid molecules in the outer monolayer always exceeds the total number in the inner monolayer, due to the larger surface of the outer layer when compared with the inner. When the vesicles are composed of at least two different lipid species, a preference of one component for the outer layer may be observed when compared with the other species present. Several causes for this differential distribution have been suggested. Berden et al. [23] discussed the effect of polar head group size and charge on the preferential distribution. Head group size appeared to be the most important factor in determining head group asymmetry. Recently, Yeagle et al. [24] showed that in mixed phosphatidylcholine vesicles the species with increasing fatty acyl unsaturation are preferentially

located in the outer monolayer. The ratio of outer and inner phosphatidylcholine molecules in egg phosphatidylcholine vesicles is 1.9 as determined by ^{1}H- and ^{31}P-NMR techniques [25,26]. Johnson et al. [27] determined the pool size of the outer surface of rat liver phosphatidylcholine vesicles using phospholipid exchange protein and reported a value of 61—65% of total phosphatidylcholine. In dioleoyl phosphatidylcholine vesicles 70% of phosphatidylcholine is in the outside layer [12]. Our results indicate that 80—90% of 1-palmitoyl lysophosphatidylcholine in 5 mol% lysophosphatidylcholine, 95 mol% egg phosphatidylcholine vesicles is present in the outer monolayer. Similar results were obtained by ^{13}C-NMR experiments as presented in the accompanying paper by de Kruyff et al. [28]. This more asymmetrical distribution of lysophosphatidylcholine when compared with phosphatidylcholine may be due to the more tapered shape of the lysophosphatidylcholine molecule or to a different (smaller) size of the vesicles containing lysophosphatidylcholine. In 5 mol% lysophosphatidylcholine, 47 mol% egg phosphatidylcholine, 47 mol% cholesterol vesicles about 60% of total lysophosphatidylcholine is at the outside (Figs. 3 and 4). This reduction in asymmetry when compared with the 95 mol% egg phosphatidylcholine vesicles may in part be due to the larger size of the 47 mol% cholesterol vesicles [30]. Another explanation could be a specific interaction between cholesterol and lysophosphatidylcholine, since it has been shown that cholesterol is less asymmetrically distributed across the bilayer than phosphatidylcholine [29].

Incorporation of cholesterol decreases the initial rates of lysophosphatidylcholine hydrolysis by lysophospholipases I and II approximately to the same extent (Table I). This suggests that cholesterol inhibits at substrate level rather than at enzyme level. Moreover, it is unlikely that lysophospholipases interact specifically with cholesterol. Cholesterol may dilute the surface density of lysophosphatidylcholine and thereby decrease the lysophospholipase activity according to the kinetic interpretation given in our previous paper [14]. However, the effect of cholesterol is too large as may be expected from the surface area per molecule of cholesterol. A partial explanation may be the reduced lysophosphatidylcholine pool in the outer monolayer when compared with vesicles without cholesterol. Yeagle and Martin [31] suggested that cholesterol forms a hydrogen bond to the α-carbonyl ester group of phosphatidylcholine in phosphatidylcholine/cholesterol vesicles. Possibly cholesterol can also form a hydrogen bond with the ester carbonyl group of lysophosphatidylcholine. This interaction might hinder the lysophospholipase action. Cholesterol does not have much of an effect on the rate of transbilayer movement of lysophosphatidylcholine (Table II).

Several findings indicate that the transbilayer movement of lysophosphatidylcholine in multilayer liposomes is not faster than its slow phase hydrolysis by exogenous lysophospholipase. The possibility that transfer of lysophosphatidylcholine between the lamellae of the liposome is the rate limiting step in the slow phase is not very likely. [^{14}C]lysophosphatidylcholine introduced in a preformed liposome is not readily distributed in a homogenous way over all lamellae (Fig. 5). Furthermore, we have shown that the exchange of lysophosphatidylcholine between two populations of sonicated vesicles and between vesicles and multilayer liposomes is fast when compared with lysophosphatidyl-

choline transbilayer exchange. Therefore, the lysophosphatidylcholine pool in handshaken liposomes that is readily available for hydrolysis is likely to represent the outer monolayer of this multilayer structure. This pool comprises 15—20% of the total lysophosphatidylcholine. Bangham et al. [32] estimated that the outer surface area of an egg phosphatidylcholine liposome is about 10% of the possible total monolayer area.

The slow transbilayer movement of phospholipid bilayer components in sonicated vesicles may be imposed by the high curvature of the vesicle surface [13]. It was suggested that transbilayer movement in planar bilayers of large area may be more rapid, since the temporary mass imbalance caused by the transposition of single phospholipid molecule from one side to the other can be accomodated more easily, when compared with the small vesicles. However, our experiments with handshaken liposomes indicate that the transbilayer movement of lysophosphatidylcholine in this system is not very much faster than in sonicated vesicles (Table II). This comparison between transbilayer movement in vesicles and liposomes is somewhat hampered by the unknown contribution of other possible processes to the overall hydrolysis rate.

Translocation rates of (phospho)lipids in artificial bilayer systems and biological membranes have been measured by a number of different techniques. Chemical labelling [13], ESR-techniques [21—23], protein mediated exchange of phospholipids [10,12,22,27,34] and degradation by phospholipase A_2 [35] have been used for this purpose. Methods which disturb membrane structure in a minimal way are likely to give the most significant results. In their study of phosphatidylcholine flip-flop in rat erythrocyte ghosts Bloj and Zilversmit [34] concluded that the transposition of phosphatidylcholine through natural membranes is much faster than through a bilayer of pure phosphatidylcholine liposomes. In this respect it would be interesting to study the transbilayer movement of lysophosphatidylcholine in biological membranes.

References

1 Van den Bosch, H. (1974) Ann. Rev. Biochem. 43, 243—277
2 Mookerjea, S. and Yung, J.W.M. (1974) Biochem. Biophys. Res. Commun. 57, 815—822
3 Hung, S.C. and Melnykovych, G. (1976) Biochim. Biophys. Acta 429, 409—420
4 Eytan, G., Matheson, M.J. and Racker, E. (1975) FEBS Lett. 57, 121—125
5 Blume, A., Arnold, B. and Weltzien, H.U. (1976) FEBS Lett. 61, 199—202
6 Poole, A.R., Howell, J.I. and Lucy, J.A. (1970) Nature 277, 810—813
7 Bretscher, M.S. (1972) J. Mol. Biol. 71, 523—528
8 Verkleij, A.J., Zwaal, R.F.A., Roelofsen, B., Comfurius, P., Kastelijn, D. and van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 323, 178—193
9 Nilsson, O. and Dallner, G. (1975) FEBS Lett. 58, 190—193
10 Lenard, J. and Rothman, J.E. (1976) Proc. Natl. Acad. Sci. U.S. 73, 391—395
11 Michaelson, D.M., Horwitz, A.F. and Klein, M.P. (1973) Biochemistry 12, 2637—2645
12 Rothman, J.E. and Dawidowicz, E.A. (1975) Biochemistry 14, 2809—2816
13 Roseman, M., Litman, B.J. and Thompson, T.E. (1975) Biochemistry 14, 4826—4830
14 Van den Besselaar, A.M.H.P., Verheijen, J.H. and van den Bosch, H. (1976) Biochim. Biophys. Acta 431, 75—85
15 De Jong, J.G.N., van den Bosch, H., Rijken, D. and van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 369, 50—63
16 Van den Bosch, H. Aarsman, A.J. and van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 348, 197—209
17 Van den Bosch, H., Aarsman, A.J., Slotboom, A.J. and van Deenen, L.L.M. (1968) Biochim. Biophys. Acta 164, 215—225

18 Andrews, S.B., Hoffman, R.M. and Borison, A. (1975) Biochem. Biophys. Res. Commun. 65, 913—920
19 Hellings, J.A., Kamp, H.H., Wirtz, K.W.A. and van Deenen, L.L.M. (1974) Eur. J. Biochem. 47, 601—605
20 Van den Besselaar, A.M.H.P., Helmkamp, Jr., G.M. and Wirtz, K.W.A. (1975) Biochemistry 14, 1852—1858
21 Rousselet, A., Guthmann, C., Matricon, J., Bienvenue, A. and Devaux, P.F. (1976) Biochim. Biophys. Acta 426, 357—371
22 Rousselet, A., Colbeau, A., Vignais, P.M. and Devaux, P.F. (1976) Biochim. Biophys. Acta 426, 372—384
23 Berden, J.A., Barker, R.W. and Radda, G.K. (1975) Biochim. Biophys. Acta 375, 186—208
24 Yeagle, P.L., Hutton, W.C., Martin, R.B., Sears, B. and Huang, C.H. (1976) J. Biol. Chem. 251, 2110—2112
25 Bystrov, V.F., Dubrovina, N.I., Barsukov, L.I. and Bergelson, L.D. (1971) Chem. Phys. Lipids 6, 343—350
26 Bystrov, V.F., Shapiro, Yu.E., Viktorov, A.V., Barsukov, L.I. and Bergelson, L.D. (1972) FEBS Lett. 25, 337—338
27 Johnson, L.W., Hughes, M.E. and Zilversmit, D.B. (1975) Biochim. Biophys. Acta 375, 176—185
28 De Kruyff, B., van den Besselaar, A.M.H.P. and van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 465, 443—453
29 De Kruyff, B., Cullis, P.R. and Radda, G.K. (1976) Biochim. Biophys. Acta 436, 729—740
30 Gent, M.P.N. and Prestegard, J.H. (1974) Biochemistry 13, 4027—4033
31 Yeagle, P.L. and Martin, R.B. (1976) Biochem. Biophys. Res. Commun., 69, 775—780
32 Bangham, A.D., de Gier, J. and Greville, G.D. (1967) Chem. Phys. Lipids, 1, 225—246
33 Kornberg, R.D. and McConnell, H.M. (1971) Biochemistry 10, 1111—1120
34 Bloj, B. and Zilversmit, D.B. (1976) Biochemistry 15, 1277—1283
35 Renooy, W., van Golde, L.M.G., Zwaal, R.F.A. and van Deenen, L.L.M. (1976) Eur. J. Biochem. 61, 53—58