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DISTRIBUTION OF LYSOPHOSPHATIDYLCHOLINE IN SINGLE BILAYER VESICLES PREPARED WITHOUT SONICATION

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Summary

The cholate method originally introduced by Kagawa et al. (J. Biol. Chem. (1973) 248, 676—684) and further developed by Brunner et al. (Biochim. Biophys. Acta (1976) 455, 322—331) has been used to prepare single bilayer vesicles containing 5 mol% lysophosphatidylcholine embedded in a matrix of phosphatidylcholine. The distribution of lysophosphatidylcholine over outer and inner monolayer was found to be highly asymmetric (ratio 9 : 1), as determined by lysophospholipase treatment of the vesicles. This distribution is similar to the value found in sonicated vesicles.

Up to 20 mol% cholesterol could be incorporated in the vesicles by the cholate method. The method was succesfully used also for the preparation of single bilayer vesicles from total rat liver microsomal lipids, to which 5 mol% of 1-[1-^{14}C]palmitoyl lysophosphatidylcholine had been added. Surprisingly, almost 100% of lysophosphatidylcholine in the latter vesicles was directly available for hydrolysis by lysophospholipase. In contrast, only 70% of the lysophosphatidylcholine in sonicated vesicles of similar composition could be hydrolyzed by lysophospholipase.

Introduction

In a previous paper we described the distribution of lysophosphatidylcholine over inner and outer monolayer of sonicated egg phosphatidylcholine vesicles [1]. Lysophosphatidylcholine appeared to be distributed much more asymmetrically than phosphatidylcholine. Gel filtration demonstrated that these vesicles were rather inhomogeneous in size. This implies that the measured distribution is an average value for the whole vesicle population.

Recently, Brunner et al. [2] described a method for preparation of single bilayer vesicles by removal of cholate from cholate-phospholipid mixed mi-

celles. The vesicles prepared by this cholate method appeared to be more homogeneous than the sonicated vesicles.

In this paper we report on the possibility to incorporate lysophosphatidylcholine and cholesterol in phosphatidylcholine vesicles by the cholate method The distribution of lysophosphatidylcholine in both sonicated and cholate vesicles of various lipid composition has been examined by incubation with lysophospholipase.

Experimental Section

Materials

Egg phosphatidylcholine was prepared as described by Papahadjopoulos and Miller [3]. It was used to prepare lysophosphatidylcholine by phospholipase A_2 (*Crotalus adamanteus*) degradation and subsequent purification by silica gel chromatography.

Cholesterol was obtained from Merck (W. Germany) and sodium cholate from Fluka (Switzerland). Three batches of 1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine were prepared as described by van den Bosch et al. [4]. The specific activities are indicated in the legends of the figures.

Phosphatidyl[*Me*-^{14}C]choline was isolated from rat liver microsomes as described by van den Bosch et al. [5]. This product had a specific activity of 21 dpm/nmol. Synthetic 1,2-dipalmitoyl-*sn*-glycero-3-phospho[*Me*-^{14}C]choline with a specific activity of 50 nCi/nmol was purchased from New England Nuclear (lot Nr. 932-012).

[9,10-$^3H_2$]Palmitic acid, specific activity 500 nCi/nmol, was from The Radiochemical Centre, Amersham.

[2,4(*n*)-^{3}H]Cholic acid, specific activity 14 μCi/nmol, was from New England Nuclear (lot Nr. 853-176) and [1α,2α(*n*)-^{3}H]cholesterol, specific activity 43 μCi/nmol, was from The Radiochemical Centre, Amersham (batch Nr. 17).

Lysophospholipase II (EC 3.1.1.5) was purified to homogeneity from beef liver as described by de Jong et al. [6].

Methods

Preparation of sonicated vesicles. Lipids in chloroform solution were mixed and the solvent evaporated under low pressure using a rotary evaporator. The emulsion obtained after vortexing the dried lipid with buffer and glass beads was sonicated for 30 min with a Branson sonifier model B-12 at 70 W under nitrogen in an ice-cold bath. In order to remove large lipid aggregates and metal particles the sonicated preparation was centrifuged at 150000 $\times g$ for 1 h.

Preparation and analysis of non-sonicated vesicles. The procedure described by Brunner et al. [2] was followed. In a typical experiment to 3 ml of an emulsion containing 39 μmol phospholipid, obtained as described for the sonicated vesicles, 130 μl of sodium cholate (300 mg/ml) were added. The optically clear "solution" was then applied to a Sephadex G-50 (fine) column (2 $\times$ 55 cm) using downwards flow and a peristaltic pump in order to afford a constant flow of 12 ml/h at 4°C. The buffer employed in all steps was 10 mM Tris · HCl containing 0.1 M NaCl and 0.02% NaN_3 (pH 7.3). The vesicles obtained were used without further removal of the remaining cholate unless otherwise stated. Analysis concerning the size and homogeneity of the vesicles were performed by

means of Sepharose 4B gel partition chromatography through columns (2 × 45 cm or 2 × 42 cm) maintained at 4°C and operated at a constant flow rate of 12 ml/h. The eluate fractions (8 ml from the Sephadex columns, 4 ml from the Sepharose columns) were analysed for radioactivity by counting samples of 500 µl in 16 ml dioxane scintillation cocktail containing 10% naphthalene, 0.7% PPO and 0.03% dimethyl POPOP. A Packard Tricarb liquid scintillation spectrometer was used and values were corrected for quenching by means of the channel-ratio method.

Extraction of total lipids from rat liver microsomes. Rat liver microsomes were prepared at 4°C by centrifugation of a 10% (w/v) liver homogenate in buffered sucrose (0.25 M sucrose, 1 mM EDTA, 10 mM Tris · HCl, pH 7.3) for 15 min at 600 × *g*. The supernatant from this step was then centrifuged for 20 min at 15900 × *g*. A third centrifugation was carried out in this second supernatant for 1 h at 105600 × *g*. The resulting supernatant was discarded by vacuum aspiration with careful removal of the top-floating fat. The pellets were re-suspended in 25 ml buffer and the suspension extracted according to the method of Folch et al. [7].

Phosphorus assays. The amount of phosphorus in the egg phosphatidylcholine preparation and in the microsomal lipid extract was measured according to the method of Chen et al. [8] after ashing of the samples as described by Ames and Dubin [9].

Chemical analysis of the lipid extracts. Vesicles made of total lipids from rat liver microsomes and prepared by both methods were extracted according to Bligh and Dyer [10]. Aliquots from these extracts and from the microsomal extract were chromatographed two-dimensionally on thin-layer plates coated with silica gel HR (Merck) containing 2% (w/w) florisil (Merck), in order to determine the individual phospholipid composition of each lipid extract.

The eluent employed in the first dimension was chloroform/methanol/ammonia (25%)/water (90 : 54 : 5,5 : 5,5, v/v) and in the second chloroform/methanol/acetic acid/water (90 : 40 : 12 : 2, v/v) as described by Broekhuyse [11]. After lipid detection by iodine vapor the amount of phosphorus in each phospholipid spot was determined by the method of Rouser et al. [12].

Each extract was also analysed for cholesterol according to the method of Zlatkis and Zak [13].

Enzymatic hydrolysis of lysophosphatidylcholine. The hydrolysis of 1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine during incubation at 37°C with lysophospholipase II (EC 3.1.1.5) was assayed using [9,10-$^{3}H_2$]palmitic acid as internal standard and the amount of fatty acid released was calculated from the $^{14}C/^{3}H$ ratio in the palmitate in the heptane layer of the Dole extract as described by van den Besselaar et al. [14]. An alkaline hydrolysis of the initial vesicle preparation followed by evaporation of the volatile components and Dole extraction of the aqueous phase provided the $^{14}C/^{3}H$ ratio of the original vesicle preparation. This value was used to calculate the degree of enzymatic hydrolysis.

Results

Mixed micelles of phosphatidylcholine, lysophosphatidylcholine and sodium cholate (molar ratio 28.6 : 1.5 : 69.9) were filtered through a column of Sepha-

dex G-50. The elution patterns are given in Fig. 1. All lysophosphatidylcholine and phosphatidylcholine was eluted in the void volume of the column. The bulk of the cholate is separated from the phospholipids: only 0.8% of total cholate is included in the void volume and this represents approx. 2 mol% of the phospholipids. Sepharose 4B chromatography of the Sephadex G-50 void volume peak resulted in one symmetrical phospholipid peak, which indicates that a homogeneous population of single bilayer vesicles has been obtained (Fig. 2).

In order to study the action of lysophospholipase on these cholate vesicles a trace of [^{3}H]palmitic acid was incorporated as an internal standard [14]. Sephadex G-50 chromatography showed that 92% of this trace amount (0.02 mol% of total phospholipid) was associated with the vesicles. The remaining 8% eluted with the bulk of the cholate in the internal volume of the column. These vesicles were incubated with lysophospholipase II purified from beef

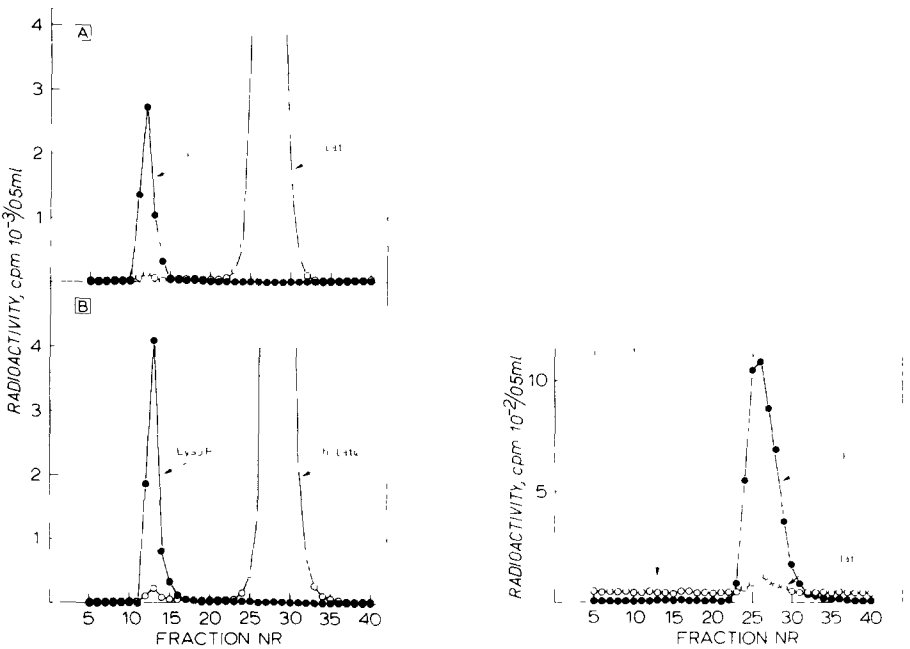

Fig. 1. Sephadex G-50 elution pattern of cholate-dispersed phospholipids (95 mol% phosphatidylcholine, 5 mol% lysophosphatidylcholine). In A a dispersion of 18.40 μmol [^{14}C]phosphatidylcholine from rat liver, 18.65 μmol egg phosphatidylcholine and 1.98 μmol egg lysophosphatidylcholine was prepared (see Experimental Section) using 130 μl of sodium cholate, 300 mg/ml, and a trace amount (0.2 nmol) of [^{3}H]cholic acid in a final buffer volume of 3.13 ml. In B the dispersion consisted of 37.08 μmol egg phosphatidylcholine, 1.76 μmol [^{14}C]lysophosphatidylcholine (specific activity 220 dpm/nmol) and 0.19 μmol egg lysophosphatidylcholine in 3.13 mg/ml, and a trace of [^{3}H]cholic acid. Fractions were monitored for ^{14}C (●———●) and ^{3}H (○———○) radioactivity. PC, phosphatidylcholine; lyso PC, lysophosphatidylcholine.

Fig. 2. Sepharose 4B chromatogram of vesicles prepared as described in the legend of Fig. 1B. Fractions eluting in the void volume of the Sephadex G-50 column were pooled and an aliquot was applied to a Sepharose 4B column. The elution pattern was obtained by measuring [^{14}C]lysophosphatidylcholine (lyso PC) (●———●) and [^{3}H]cholic acid (○———○) radioactivity in each sample. The void volume (V_o) of the column is indicated by an arrow.

liver [6]. The time course of lysophosphatidylcholine hydrolysis is shown in Fig. 3. Hydrolysis in zero time controls was 6% and this amount of fatty acid did not increase during a 3 h incubation of the vesicles in the absence of enzyme. The degree of enzymatic hydrolysis did not increase beyond 90%. Sepharose 4B chromatography of these vesicles after the 3 h incubation with lysophospholipase resulted in a narrow peak similar to the original vesicles (Fig. 4A). No aggregated structures were observed in the void volume. For comparison a similar experiment was carried out with sonicated vesicles. Lysophospholipase did not hydrolyze more than 85% of vesicular lysophosphatidylcholine, in agreement with our previous results [1]. The Sepharose 4B chromatogram of the incubated vesicles shows two peaks (Fig. 4B). A low but significant amount of radioactivity (4.9% of total) was observed in the void volume and this is to

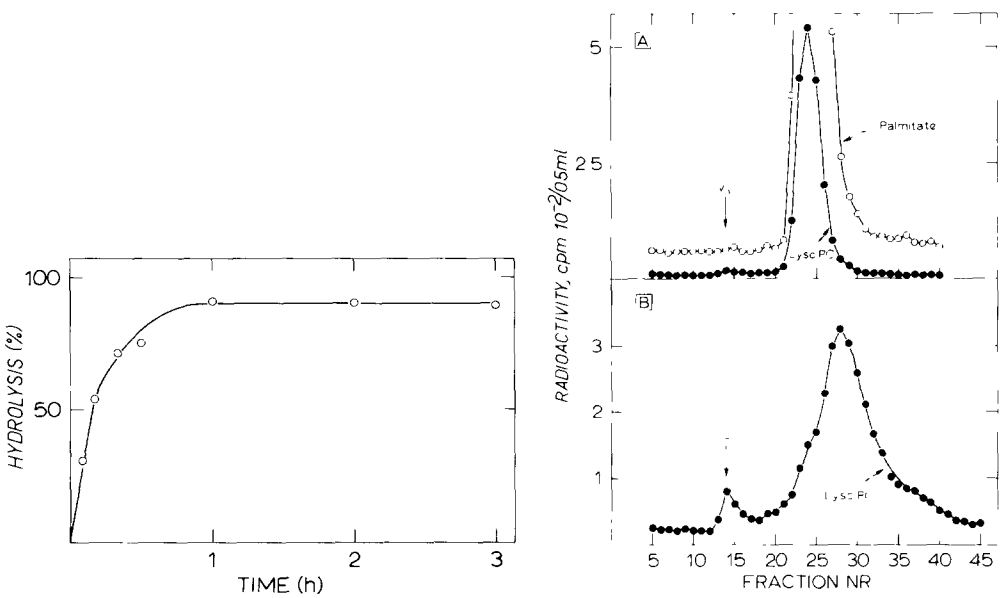

Fig. 3. Action of lysophospholipase II on vesicles made of 95 mol% egg phosphatidylcholine and 5 mol% [^{14}C]lysophosphatidylcholine. Vesicles were prepared from a dispersion of 37.08 μmol egg phosphatidylcholine, 1.95 μmol 1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (specific activity 220 dpm/nmol) and a trace amount (7 nmol) of [^{3}H]palmitic acid in 3 ml buffer to which 130 μl sodium cholate, 300 mg/ml, was added. A volume of 2.75 ml of this dispersion was applied to a Sephadex G-50 column and the fractions eluting in the void volume were pooled to yield a total volume of 45 ml. 12.8 ml of this vesicle preparation were incubated at 37°C in a total volume of 13.5 ml containing 4.6 mg lysophospholipase II. At the times indicated aliquots of 0.5 ml were taken to determine the extent of hydrolysis of the lysophosphatidylcholine.

Fig. 4. Sepharose 4B chromatograms of vesicles (95 mol% phosphatidylcholine, 5 mol% lysophosphatidylcholine), prepared by the detergent and sonication methods, after incubation with lysophospholipase II. A represents the chromatography of the vesicles prepared by the cholate method after a 3 h incubation with lysophospholipase II. For details concerning vesicle composition and incubation conditions see legend Fig. 3. In B sonicated vesicles were applied to the column after a 2 h incubation with the same enzyme, after which maximal hydrolysis had been obtained. Vesicles were prepared by sonication of 12.65 μmol egg phosphatidylcholine and 0.67 μmol 1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (specific activity 280 dpm/nmol) in 1.5 ml buffer (20 mM Tris · HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4). The incubation mixture was prepared with 600 μl of the vesicle preparation, 900 μl buffer and 700 μg lysophospholipase II. o———o, ^{3}H radioactivity and •———•, ^{14}C radioactivity. The arrow indicates the void volume (V_0) of the column. lyso PC, Lyso PC, lysophosphatidylcholine.

be attributed to aggregation of the vesicles. In a control experiment in which the sonicated vesicles were incubated without lysophospholipase no such aggregation was observed. The second peak containing most of the radioactivity is to be attributed to intact single bilayer vesicles. Analysis of this peak revealed that 85% of the radioactivity resides in the free fatty acid released by the lysophospholipase action. The remaining 15% of this radioactivity was recovered as intact lysophosphatidylcholine. This result is in agreement with our previous conclusion that about 15% of lysophosphatidylcholine is embedded in the inner monolayer of the sonicated vesicles and does not diffuse to the outer surface. Fig. 4 also shows that the cholate vesicles elute at a somewhat smaller volume than the average elution volume of the sonicated vesicles. Furthermore, the peak width of the cholate vesicles is smaller than that of the sonicated vesicles. This indicates that the cholate vesicles are larger and more homogeneous in size than the sonicated vesicles, in agreement with the results of Brunner et al. [2].

Cholesterol can be incorporated into sonicated phosphatidylcholine vesicles up to a molar ratio of 1 : 1. Cholate was not able to solubilize cholesterol/egg phosphatidylcholine mixtures with a molar ratio beyond 0.2 in agreement with the findings of Bourgès et al. [15]. A mixture of cholesterol/egg phosphatidylcholine/lysophosphatidylcholine/cholate in a molar ratio of 19 : 76 : 5 : 300 was optically clear and was filtered through a Sephadex G-50 column. The bulk of the cholesterol (99%), all phosphatidylcholine and lysophosphatidylcholine and only 0.8% of cholate were eluted in the void volume. Filtration of this void volume peak through Sepharose 4B showed that 3% of cholesterol appeared in the void volume of this column (Fig. 5) and the rest was associated with the vesicle peak. Relatively less phosphatidylcholine (0.8%, Fig. 6) and lysophosphatidylcholine (1%, Fig. 5) was observed in the void volume. Incubation of these vesicles with lysophospholipase resulted in a maximal hydrolysis comprising 84% of total lysophosphatidylcholine. Sepharose 4B chromatography of the incubated vesicles did not reveal any aggregation of the vesicles.

The cholate method was also used to prepare vesicles with a lipid composition similar to a natural membrane system in casu the microsomes of rat liver. Total lipid extracted from rat liver microsomes was dissolved in an aqueous cholate solution. Lysophosphatidylcholine (5 mol% of total phospholipid) was added. Radioactive tracers showed that all lysophosphatidylcholine and phosphatidylcholine and only 0.9% of cholate were present in the void volume of the Sephadex G-50 column (data not shown). Analysis of the phospholipids and cholesterol in the void volume fractions demonstrated that the major phospholipids were present in a ratio similar to the starting mixture (Table I). The void volume fractions of the Sephadex G-50 columns used to prepare the vesicles of the composition indicated in the legend of Fig. 7 (A and B) were pooled separately and dialyzed overnight against 30 volumes of elution buffer. This procedure reduced the cholate content of the vesicle preparations to about one-half, as could be deduced from the reduction in [^{3}H]cholate radioactivity of the vesicle population with labelled cholate. Part of these vesicle preparations were used for filtration through Sepharose 4B columns. The results indicate that some phosphatidylcholine (12%) was present in aggregated structures eluting in the void volume. This is believed to be related to the lipid composi-

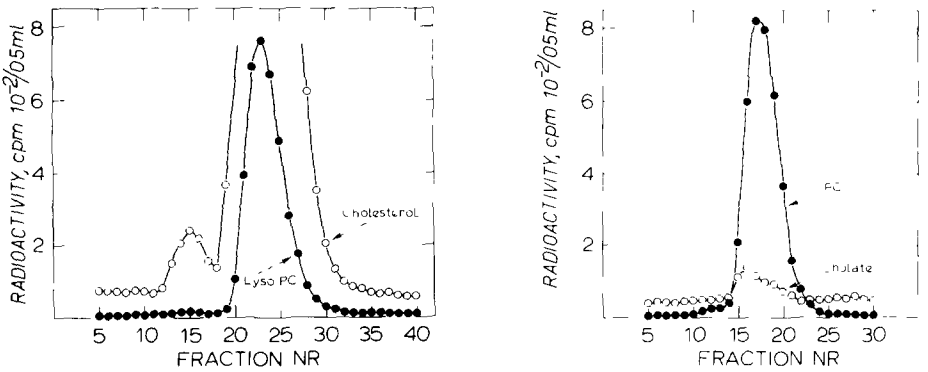

Fig. 5. Sepharose 4B elution pattern of vesicles prepared from a phospholipid dispersion containing initially 20 mol% cholesterol. 1.76 μmol [^{14}C]lysophosphatidylcholine (specific activity 220 dpm/nmol), 0.19 μmol egg lysophosphatidylcholine, 29.65 μmol egg phosphatidylcholine, 7.41 μmol cholesterol and a trace (0.07 nmol) of [^{3}H]cholesterol were dispersed in a final volume of 3.16 ml buffer containing 116.1 μmol sodium cholate. After Sephadex G-50 chromatography of 2.91 ml of this dispersion, the fractions eluting in the void volume were pooled and a sample applied to a Sepharose 4B column. The chromatography was followed by measuring ^{3}H (○———○) and ^{14}C (●———●) radioactivity. Lyso PC, lysophosphatidylcholine.

Fig. 6. Sepharose 4B chromatography of vesicles prepared from a phospholipid dispersion containing initially 1.95 μmol egg lysophosphatidylcholine, 18.40 μmol [^{14}C]phosphatidylcholine (specific activity 21 dpm/nmol), 11.25 μmol egg phosphatidylcholine, 7.41 μmol cholesterol, 116.1 μmol sodium cholate and a trace amount of [^{3}H]cholic acid in a final volume of 3.16 ml. Further details as in legend of Fig. 5. PC, phosphatidylcholine.

tion of the mixture as control experiments with vesicles prepared from phosphatidylcholine showed that no aggregation took place during dialysis. Lysophosphatidylcholine had a preference for incorporation into single bilayers since only 4% of this compound was recovered in the aggregated structures in the void volume (Fig. 7B) as opposed to the 12% of the phosphatidylcholine observed in that fraction. When the Sephadex G-50 void volume fraction of the lipid mixture containing [^{14}C]lysophosphatidylcholine (for composition see legend Fig. 7B) was incubated with lysophospholipase a similar curve as described in Fig. 3 was obtained, except that in 2 h 94% of the lysolecithin was

TABLE I

LIPID COMPOSITION OF CHOLATE AND SONICATED VESICLES PREPARED FROM TOTAL MICROSOMAL LIPIDS

Aliquots of microsomal extract and of lipid extracts from cholate and sonicated vesicles obtained after Sephadex G-50 chromatography were used to determine total phospholipid and cholesterol Phospholipid composition is expressed as percent of total phosphorus recovered after chromatography P = phosphatidyl, C = choline, E = ethanolamine, I = inositol, S = serine, SPH = sphingomyelin.

Lipid preparation	Phospholipid composition					Cholesterol phospholipid molar ratio
	PC	PE	PI	PS	SPH	
Microsomal extract	66	20	8	3	5	18
Cholate vesicles	71	15	6	1	7	22
Sonicated vesicles	69	19	6	trace	5	21

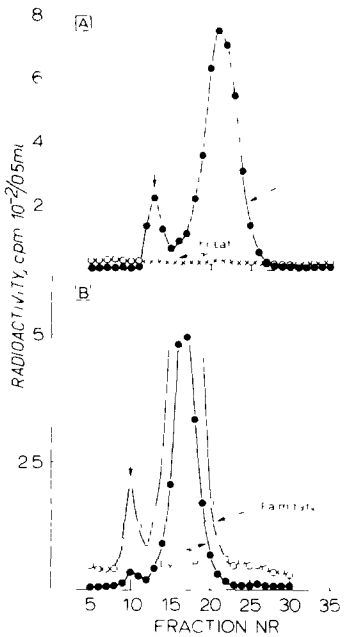

Fig. 7. Sepharose 4B chromatogram of vesicles prepared by the cholate method using total lipids from rat liver microsomes and 5 mol% lysophosphatidylcholine. In A the initial lipid-detergent dispersion consisted of 31.4 μmol total lipids from rat liver microsomes (expressed as phosphorus), a trace (6 nmol) of phosphatidyl[*Me*-^{14}C]choline (specific activity 50 nCi/nmol) and 1.53 μmol egg lysophosphatidylcholine in 1.65 ml buffer containing 116.1 μmol sodium cholate and a trace of [^{3}H]cholic acid. A sample of the vesicle preparation eluted from the Sephadex G-50 column was applied to a Sepharose 4B column and the ^{3}H (o———o) and ^{14}C (•———•) radioactivity was assayed in each fraction. In B the initial lipid-detergent dispersion had the following composition 31.4 μmol total lipids from rat liver microsomes (expressed as phosphorus), 1.53 μmol [^{14}C]lysophosphatidylcholine (specific activity 280 dpm/nmol) and a trace of [^{3}H]palmitic acid in 1.65 ml buffer containing 116.1 μmol sodium cholate. As in A a sample of the vesicle preparation eluted from the Sephadex G-50 column was applied to a Sepharose 4B column and the ^{3}H (o———o) and ^{14}C (•———•) radioactivity was measured in each fraction. The void volumes of the columns are indicated by arrows. PC, phosphatidylcholine, lyso PC, lysophosphatidylcholine.

degraded and that the extent of hydrolysis increased still further till 98.5% after 4 h incubation.

For comparison sonicated vesicles from microsomal lipids to which 5 mol% lysolecithin had been added were prepared. After high speed centrifugation (1 h at 150000 $\times g$) a pellet of lipid material was observed. The supernatant was analyzed for phospholipid and cholesterol and the composition turned out to be very similar to that of the original microsomal extract (Table I). The Sepharose 4B elution pattern of the supernatant showed some aggregated structures to be present in the void volume of the Sepharose column. Lysophospholipase treatment of the vesicle preparation after Sepharose chromatography lead to only 70% hydrolysis of the total lysophosphatidylcholine.

Discussion

This work demonstrates that it is possible to incorporate lysophosphatidylcholine in single bilayer vesicles prepared by the cholate method. Sepharose

chromatography showed that these vesicles were more homogeneous in size than their sonicated counterparts of similar chemical composition. Incubation of the cholate vesicles with lysophospholipase results in 90% hydrolysis of lysophosphatidylcholine associated with the vesicles. Since no aggregation took place during the incubation with enzyme (Fig. 4A) this result indicates that 90% of the lysophosphatidylcholine is present in the outer monolayer of the vesicle. A similar distribution is observed within sonicated vesicles. Sonicated vesicles aggregate to some extent during incubation with lysophospholipase (Fig. 4B). However, this aggregation is not the cause of incomplete lysophosphatidylcholine hydrolysis, since the non-aggregated vesicles still contained 15% of the initial lysophosphatidylcholine radioactivity as intact lysophosphatidylcholine, which is not accessible to the lysophospholipase. This finding is interpreted as 15% of lysophosphatidylcholine being present in the inner monolayer of the sonicated vesicle, and the movement of lysophosphatidylcholine from inner to outer monolayer being very slow. Although the size of the cholate vesicles is somewhat larger than the average size of the sonicated vesicles, no significant difference in the asymmetric distribution of lysophosphatidylcholine over inner and outer monolayer was observed. It should be noted that the distribution measured for the sonicated vesicles is the average of a rather inhomogeneous size population of vesicles.

Cholesterol can be incorporated into phosphatidylcholine vesicles by the cholate method up to about 20 mol%. About 84% of lysophosphatidylcholine in these vesicles is located in the outer monolayer, as determined by lysophospholipase action. This distribution is not very different from that found in vesicles without cholesterol. In sonicated vesicles containing 47 mol% cholesterol, only 60% of lysophosphatidylcholine was previously found to be exposed to the external medium [1,20]. This may be explained by the fact that the physical properties of sonicated phosphatidylcholine vesicles change rather abruptly when the cholesterol content exceeds 30 mol% [16–19].

Vesicles prepared from microsomal lipids by the cholate method have a composition similar to that of the original microsomal lipid extract. Lysophosphatidylcholine incorporated in these vesicles was almost completely available for enzymatic hydrolysis, in contrast to sonicated vesicles of similar composition in which only 70% of lysophospahtidylcholine could be hydrolyzed. Several possible explanations for the complete enzymatic hydrolysis in these cholate vesicles can be given. Firstly, lysophosphatidylcholine in the cholate vesicles is present in both monolayers but lysophosphatidylcholine movement from the inner layer to the outer is fast so that external lysophospholipase can hydrolyze ultimately all lysophosphatidylcholine. It is not likely that the small content of cholate which was not removed by the Sephadex chromatography facilitates this rapid movement since such an effect was not observed with the phosphatidylcholine vesicles, which also contained comparable amounts of cholate. The second possibility is that an extremely asymmetric distribution exists, with all lysophosphatidylcholine being located in the outer monolayer of the vesicles prepared from total lipid of rat liver microsomes. However, in the sonicated vesicles prepared from total lipid of rat liver microsomes only 70% of the lysophosphatidylcholine was accessible to the lysophospholipase, despite the similar composition of both vesicle preparations (Table I) with

respect to the major lipid components. It is possible that differences in the amounts of minor neutral lipid components exist between the cholate and sonicated vesicles which lead to the different distribution of lysophosphatidylcholine in those vesicles or that this different distribution is due to different mechanisms of vesicle formation. The third possibility for the complete accessibility of lysophosphatidylcholine in the vesicles prepared by the cholate method from total microsomal lipids is that these vesicles are leaky for lysophospholipase II (M_r = 60000) so that the enzyme can attack its substrate from both sides of the bilayer. Which of these possibilities holds is the subject of current investigations.

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