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OUTSIDE-INSIDE DISTRIBUTION AND TRANSLOCATION OF LYSOPHOSPHATIDYLCHOLINE IN PHOSPHATIDYLCHOLINE VESICLES AS DETERMINED BY ^{13}C-NMR USING (N-$^{13}CH_3$)-ENRICHED LIPIDS

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Summary

1. The outside-inside distribution of palmitoyl lysophosphatidylcholine and dioleoyl phosphatidylcholine in mixed sonicated vesicles is measured with (N-$^{13}CH_3$)-labelled lipids using ^{13}C NMR and Dy^{3+} as an impermeable shift reagent.

2. Palmitoyl lysophosphatidylcholine is preferentially localised in the outside layer of the vesicle membrane. Incorporation of cholesterol in the vesicle diminishes the extent of lysophosphatidylcholine asymmetry.

3. Palmitoyl lysophosphatidylcholine added to dioleoyl phosphatidylcholine vesicles is incorporated in the outer monolayer of the vesicle. Even after 40 h less than 2% of the lysophosphatidylcholine could be detected in the inner monolayer. Since in the cosonicated vesicles 17% of the lysophosphatidylcholine is present in the inner monolayer it can be concluded that the transmembrane movement of lysophosphatidylcholine across the lipid bilayer of these vesicles is an extremely slow process.

Introduction

In recent years the trans-membrane distribution and movements of lipids in both biological and model membranes have been studied extensively. Using chemical labelling [1,2], phospholipases [3], exchange techniques [4—6] and NMR [7—10] the distribution of several classes of phospholipids and cholesterol over the halves of various membranes have been established. In model membrane systems consisting of sonicated aqueous lipid dispersions (vesicles), it was found that the outside-inside distribution of phospholipids was dependent upon the size and charge of the polar headgroup [8,9], the fluidity of the fatty acyl chains [10,11] and the presence of cholesterol [10,12].

In general, trans-membrane movements of both diacyl phospholipids [4,13] and cholesterol [6] in these systems were found to be very slow.

The outside-inside distribution and translocation of lysophospholipids in biological and model membranes is not known. Of the various possible techniques only the use of pure lysophospholipases (accompanying paper, [14]) or NMR are likely to provide information about this subject. ^{1}H and ^{31}P NMR which have been used successfully for elucidating the trans-membrane distribution of phospholipids over vesicle membranes cannot be used since lysophospholipids have no signals from the polar headgroup different in chemical shift from the signals of the corresponding phospholipids.

Recently, ^{13}C NMR on (N-$^{13}CH_3$)phosphatidylcholine vesicles has been used to study the distribution of phosphatidylcholines over the vesicle membrane [11,15]. The possibility of specifically labelling the (N-CH_3)-carbon atoms with ^{13}C thus provides a method for investigating the trans-membrane distribution and translocation of lysophosphatidylcholine in phosphatidylcholine vesicle membranes.

Experimental

Lipids

1,2-Dioleoyl-*sn*-glycero-3-phosphorylcholine ($18:1_c/18:1_c$-phosphatidylcholine) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (16:0/16:0-phosphatidylcholine) were synthesized as described before [16]. (N-$^{13}CH_3$)-labelled $18:1_c/18:1_c$-phosphatidylcholine and (N-$^{13}CH_3$)-labelled 16:0/16:0-phosphatidylcholine were synthesized by the quaternization of the respective *N,N*-dimethyl amino compounds with [^{13}C]methyliodide. The above phosphatidylcholines were demethylated by refluxing 4 mmol phosphatidylcholine with 20 mmol diazobicyclooctane in 30 ml dimethylformamide under nitrogen for 5 h (Stoffel, W., personal communication). After evaporation of the dimethylformamide the residue was dissolved in ether and washed three times with 2 M HCl and 3 times with water. The resulting *N,N*-dimethyl phosphatidylcholines were pure and were used directly for the methylation procedure [17]. To 3 mmol *N,N*-dimethyl phosphatidylcholine 1 g [^{13}C]methyliodide in 50 ml methanol and 20 ml tetrahydrofuran was added. The mixture was stirred at room temperature for 4 h and thereafter cooled to 0°C. 75 ml 0.1 M HCl was added and the lipids were extracted twice with 200 ml chloroform. The combined chloroform phases were washed with 100 ml 0.2 M $NaHCO_3$ and twice with 50 ml H_2O. The (N-$^{13}CH_3$)phosphatidylcholines were purified with a final yield of 50—65% over a silicagel column eluting with a gradient of increasing amounts of methanol in chloroform.

1-Palmitoyl-*sn*-glycero-3-phosphorylcholine (16:0-lysophosphatidylcholine) and (N-$^{13}CH_3$)-labelled 1-palmitoyl-*sn*-glycero-3-phosphorylcholine (16:0-(N-$^{13}CH_3$)lysophosphaticylcholine) were obtained by hydrolysis of the corresponding phosphatidylcholines by pancreatic phospholipase A_2. 1-[1-^{14}C]-palmitoyl-*sn*-glycero-3-phosphorylcholine (16:0-[^{14}C]lysophosphatidylcholine) was prepared as described by van den Bosch et al. [18].

Chemicals

[^{13}C]Methyliodide was obtained from Prochem (London, U.K.), Dy_2O_3 was purchased from British Drug Houses (Poole, U.K.) and was converted to its chloride by HCl. Yb $(NO_3)_3$ was bought from Wilmad (Buena, N.J.). Deuterium oxide was obtained from Merck, Sharp and Dome (Munich, G.F.R.). All other chemicals were analytical grade.

Preparation of lipid vesicles

20—50 μmol phosphatidylcholine with or without cholesterol or lysophosphatidylcholine was dried from a chloroform solution and was dispersed by vortexing at room temperature in 1.5—4.0 ml of ^2H_2O containing 25 mm Tris · HCl p^{2}H 7.0 and 0.2 mM EDTA. The resulting lipid dispersion was sonicated under nitrogen with a Branson tip sonicator, power setting 4, till clearness which usually took 3—6 min. The sonication vial was kept in ice-water during the sonication procedure. Metal particles from the probe and any residual unbroken lipid aggregates were removed by centrifugation for 30 min at 30 000 × *g* at 0°C. The opalescent vesicle solutions were used directly for the NMR experiment. It was found that during sonicating the p^{2}H of the solution decreased to 6.6—6.8. The vesicles were stable, also in the presence of the shift reagent, for the time of the NMR measurements as indicated by the constant E_{450} of the vesicle solution. Micellar lysophosphatidylcholine solutions were prepared in the same buffer as used for the vesicles.

Gel filtration

Gel filtration was performed on a Sepharose 4B column (Pharmacia) (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 utilized throughout was 150 mM NaCl, 1 mM EDTA, 20 mM Tris · HCl, pH 7.6. 200 μl samples from the eluate fractions were counted in 15 ml dioxane scintillation liquid (10% naphtalene, 0.7% PPO, 0.03% dimethyl POPOP) using a Packard Tricarb liquid scintillation spectrometer. Phosphorus in the eluate was measured according to Chen et al. [19] after destruction of the sample as described by Ames and Dubin [20].

Nuclear magnetic resonance

Most measurements were performed on a Bruker 360 WS spectrometer operating at ^{13}C in the fourier transform mode at a frequency of 90.5 MHz. The spectrometer was equipped with broadband proton noise decoupling, deuterium lock, quadrature detection and temperature control unit and was interfaced with a 20 K Nicolet BNC-12 computer. Accumulated free induction decays were obtained from up to 2000 transients with a 1.5—2.0 s interpulse time. Typically a spectral width of 20 kHz was used with 16 K data points. Dioxane or benzene in a central capillary were used as external references. Typically 1,2 ml samples were used in 10 mm tubes. Teflon plugs were used to avoid vortexing of the sample during spinning. The sample was always placed in the probe at an identical height. Nuclear Overhauser enhancements were measured using gated decoupling. To shift the resonance arising from the outside facing molecules of the vesicle aliquots of a 100 mM $YbCl_3$, $Nd(NO_3)_3$ or

$DyCl_3$ solutions were added. Intensities of the shifted and unshifted resonances were measured with respect to the intensities of the external reference using computer integration of the spectra or by cutting out and weighing the various peaks. The intensities were corrected for the small dilution caused by the addition of the shift reagent. The error in the peak intensity determination is estimated as 10%.

For some experiments a Varian CFT-20 ^{13}C spectrometer was used. ^{31}P-NMR measurements on vesicles were performed on a Varian XL-100 spectrometer operating at 40.4 MHz under conditions as described before [10]. All spectra were recorded at 28—30° C.

Results and Discussion

Effect of paramagnetic ions on the ^{13}C spectrum of 16:0-(N-$^{13}CH_3$)lysophosphatidylcholine micelles and (N-$^{13}CH_3$)-labelled 18:1_c/18:1_c-phosphatidylcholine vesicles

The principle of determining the outside-inside distribution of lipids in vesicles with NMR is that impermeable paramagnetic ions when added to the vesicles only can interact with the polar headgroups of the lipids in the outer monolayer of the membrane. The resonance of these groups will be shifted and/or broadened dependent upon the ion used. The most commonly used shift reagents in ^1H and ^{31}P NMR studies on vesicles are Eu^{3+}, Pr^{3+}, Nd^{3+} and $K_3Fe(CN)_6$. Recently, it was reported that these shift reagents at concentrations which only give incomplete resolution of the signals from the outside and inside of (N-$^{13}CH_3$)egg phosphatidylcholine vesicles already cause precipitation of these vesicles [15]. This we could confirm with (N-$^{13}CH_3$)-labelled 18:1_c/18:1_c-phosphatidylcholine vesicles. Yb^{3+} was reported to give complete separation of the outside and inside resonance of (N-$^{13}CH_3$)egg phosphatidylcholine vesicles at Yb^{3+} to phospholipid molar ratios as low as 0.06 [15]. With (N-$^{13}CH_3$)-labelled 18:1_c/18:1_c-phosphatidylcholine vesicles however, still no complete separation (shift outside resonance 1.0 ppm upfield) could be obtained at a Yb^{3+}/phosphatidylcholine ratio of 1.5. This discrepancy is as yet not understood, but it has to be realised that low concentrations of negatively charged lipids will affect the surface concentration of the shift reagent and therewith the actual shift observed. Since in the study cited above the (N-$^{13}CH_3$)egg phosphatidylcholine was prepared via egg phosphatidic acid the possibility exists that trace amounts of egg phosphatidic acid which are difficult to detect and to remove were present in the vesicles. In this respect we noticed that by the incorporation of only 2 mol% egg phosphatidic acid in (N-$^{13}CH_3$)-labelled 18:1_c/18:1_c-phosphatidylcholine vesicles the concentration of shift reagent (in this case $DyCl_3$) needed to produce a certain shift was reduced by a factor of 2.5.

In a search for a suitable shift reagent in our system we tested Dy^{3+} because this cation is reported to cause the largest shifts of the various lanthanides [21]. In Fig. 1 the 90.5 MHz ^{13}C spectrum of (N-$^{13}CH_3$)-labelled 18:1_c/18:1_c-phosphatidylcholine vesicles is shown. Besides for the Tris resonance at 7.6 ppm only the resonance of the (N-$^{13}CH_3$)-carbon atoms is observed in the spectrum. The addition of Dy^{3+} to the vesicles causes a broadening and a down-

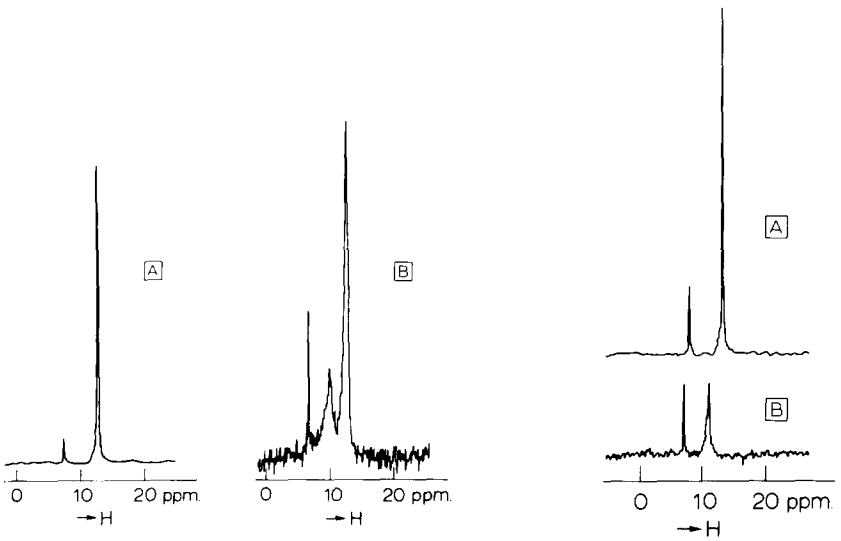

Fig. 1. 90.5 MHz ^{13}C spectrum of a 16 mM (N-$^{13}CH_3$)-labelled 18:1_c/18:1_c-phosphatidylcholine vesicle solution (A) in the absence and (B) in the presence of 10 mM $DyCl_3$. Chemical shifts upfield from 1.3-dioxane.

Fig. 2. 90.5 MHz ^{13}C spectrum of 10 mM 16:0-(N-$^{13}CH_3$)lysophosphatidylcholine micelles (A) in the absence and (B) in the presence of 6 mM $DyCl_3$.

field shift of part of the intensity of this signal. The shifted resonance arises from the molecules present in the outside layer of the vesicle. As is immediately apparent from the spectrum the intensity of the shifted resonance is less than the intensity of the unshifted resonance. This was noticed also in the 20 MHz ^{13}C spectrum. In general the ratio of the number of molecules present in the outside layer to the number in the inside layer ($R_{o/i}$) of phosphatidylcholine vesicles is about 2 [7—12]. Several effects can account for the relatively low intensity of the shifted resonance. (a) The vesicles are (partially) multilayered. In view of our previous observations on 18:1_c/18:1_c-phosphatidylcholine vesicles [10] and because of the method of vesicle preparation (high speed centrifugation after the sonication), this seems unlikely. Furthermore, a large quantity of multilayered vesicles must be present in the sample to account for the low outside resonance intensity. (b) Not all of the (N-$^{13}CH_3$) groups in the outside layer of the vesicles are accessible to Dy^{3+}. In Fig. 2 the ^{13}C spectrum of a 16:0-(N-$^{13}CH_3$)lysophosphatidylcholine solution is shown. Because lysophosphatidylcholines form micelles all polar headgroups are exposed to the solution. Dy^{3+} addition causes a downfield shift of all the (N-$^{13}CH_3$) resonances, demonstrating that all the polar headgroups are accessible to Dy^{3+} (Fig. 2b). Furthermore, the intensity of the (N-$^{13}CH_3$)-signal decreased by 44% due to the addition of Dy^{3+} which implicates that the shift reagent causes a loss in signal intensity. (c) Part of the outside (N-$^{13}CH_3$) resonances are broadened by Dy^{3+} beyond detection. (d) The intensity of the ^{13}C signal is unlike the intensity in ^1H NMR not only determined by the number of nuclei, but also by the nuclear Overhauser enhancement that results from proton decoupling [15]. A partial loss of of nuclear Overhauser enhancement by Dy^{3+} could account for the low intensity of the outside

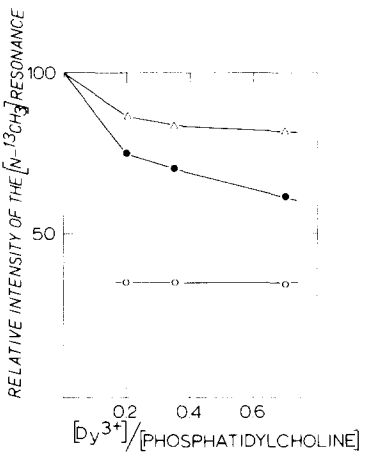

Fig. 3. Effect of Dy^{3+} on the intensity of the ($N\text{-}^{13}CH_3$) resonances in ($N\text{-}^{13}CH_3$)-labelled $18{:}1_c/18{:}1$-phosphatidylcholine vesicles at 90.5 MHz. To a 10 mM ($N\text{-}^{13}CH_3$)-labelled $18{:}1_c/18{:}1_c$-phosphatidylcholine vesicle solution increasing amounts of $DyCl_3$ were added and the intensity of the inside ($N\text{-}^{13}CH_3$) resonance (○———○) and the sum of the inside and outside ($N\text{-}^{13}CH_3$) resonance (●———●) was determined using proton decoupling. The intensity of the sum of the inside and outside ($N\text{-}^{13}CH_3$) resonance was also recorded using gated proton decoupling (△———△). The intensity of the sum of the inside and outside ($N\text{-}^{13}CH_3$) resonance in the absence of Dy^{3+} was arbitrarily set at 100%.

resonance. In agreement with this interpretation are the following observations. (1) The loss of intensity of the 16:0-($N\text{-}^{13}CH_3$)lysophosphatidylcholine signal by Dy^{3+} (Fig. 2) was only 22% when the spectrum was recorded using gated decoupling (decoupler on during the 0.5 s aquisition time and off for the following 2.0 s) to reduce the nuclear Overhauser enhancement. (2) The intensity of the unshifted resonance of the ($N\text{-}^{13}CH_3$)labelled $18{:}1_c/18{:}1_c$-phosphatidylcholine vesicles is independent upon Dy^{3+} concentration whereas the intensity of the shifted resonance decreased by increasing Dy^{3+} concentration (Fig. 3). This decrease in intensity was much less when the spectra were recorded using gated decoupling (Fig. 3).

In view of these effects it was decided to omit the use of the intensity of the shifted resonance for the outside-inside measurements. The percentage of ($N\text{-}^{13}CH_3$) lipids present in the inner monolayer of the vesicles was determined by first measuring the intensity of the ($N\text{-}^{13}CH_3$)-signal in the absence of shift reagent and then by measuring the intensity of the inside resonance in the presence of a Dy^{3+} concentration high enough to shift the outside signal sufficiently away from the inside signal. Both measurements were performed using full proton decoupling. In this way $R_{o/i}$ of ($N\text{-}^{13}CH_3$)-labelled $18{:}1_c/18{:}1_c$-phosphatidylcholine vesicles (Fig. 3) was found to be 1.75 ± 0.10 in close agreement with the value of 1.75 reported for $18{:}1_c/18{:}1_c$-phosphaticylcholine vesicles using ^{31}P NMR [10].

Outside/inside distributions in mixed lysophosphatidylcholine-phosphatidylcholine vesicles

Fig. 4A shows the 90.5 MHz spectrum of 15 mol% 16:0-($N\text{-}^{13}CH_3$)lysophosphatidylcholine containing $18{:}1_c/18{:}1_c$-phosphatidylcholine vesicles in the presence of Dy^{3+}. From a comparising of Fig. 1B it is immediately clear that in

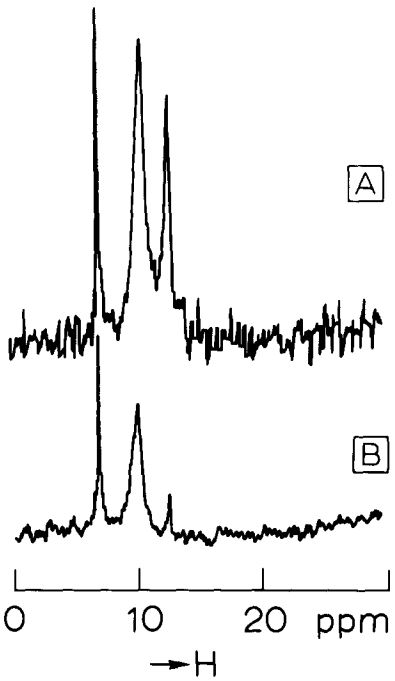

Fig. 4. 90.5 MHz ^{13}C spectra of (A) cosonicated 15 mol% (N-$^{13}CH_3$)-labelled 16:0-lysophosphatidylcholine containing 18:1_c/18:1_c-phosphatidylcholine vesicles (26 mM phosphatidylcholine) in the presence of 8 mM $DyCl_3$. (B) A mixture of 3.5 mM 16:0-(N-$^{13}CH_3$)lysophosphatidylcholine and 26 mM 18:1_c/18:1_c-phosphatidylcholine vesicles (molar ratio 15 : 85) which have been incubated at 30°C for 20 h and to which prior to the data accumulation 8 mM $DyCl_3$ was added.

these mixed vesicles lysophospatidylcholine prefers more strongly the outside layer than does 18:1_c/18:1_c-phosphatidylcholine. For the outside-inside determination the intensity of the inside 16:0-(N-$^{13}CH_3$)lysophosphatidylcholine signal is corrected for the amount of natural abundance ^{13}C atoms in the $N(CH_3)_3$ groups of the 18:1_c/18:1_c-phosphatidylcholine present in the inside layer of the vesicle. This correction was done, with identical results, both by calculating this intensity from the outside-inside distribution of 18:1_c/18:1_c-phosphatidylcholine in the mixed vesicle (8.1% of the signal arises from the inside 18:1_c/18:1_c-phosphatidylcholine molecules) or by measuring the intensity of the inside N-$(CH_3)_3$ signal in 15 mol% 16:0-lysophosphatidylcholine containing 18:1_c/18:1_c-phosphatidylcholine vesicles under exactly the same conditions as done in the experiment described in Fig. 4A. The distribution of phosphatidylcholine in 15 mol% 16:0-lysophosphatidylcholine containing vesicles was measured using (N-$^{13}CH_3$)-labelled 18:1_c/18:1_c-phosphatidylcholine. The quantitative data are presented in Fig. 5. Lysophosphatidylcholine is preferentially localised in the outside monolayer of the vesicle. Lysophosphatidylcholine contains next to the polar part only one fatty acid chain. The cross-sectional area of the fatty acid chain is smaller than the cross-sectional area of the phosphorylcholine group which will give the molecule a wedge shape. From geometrical considerations a molecule with a wedge shape will fit more easily in the outer monolayer than in the inner monolayer of the

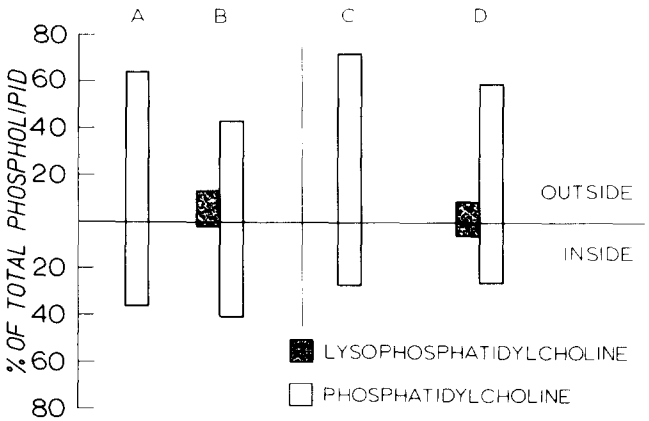

Fig. 5. Outside-inside distributions in mixed vesicles. The amount of lysophosphatidylcholine or phosphatidylcholine on either side of the vesicle membrane is given as a percentage of the total phospholipid. (A) 18:1_c/18:1_c-phosphatidylcholine vesicles; (B) 16:0-lysophosphatidylcholine/18:1_c/18:1_c-phosphatidylcholine (15 : 85) vesicles; (C) 18:1_c/18:1_c-phosphatidylcholine-cholesterol (1 : 1) vesicles; (D) 16:0-lysophosphatidylcholine/18:1_c/18:1_c-phosphatidylcholine/cholesterol (8 : 46 : 46) vesicles.

vesicle bilayer. The disposition of the large percentage of 16:0 lysophosphatidylcholine in the outside layer of the vesicle is counter-balanced by a lower amount of 18:1_c/18:1_c-phosphatidylcholine in that layer.

Lysophosphatidylcholines are believed to play a role in membrane fusion [22]. Extending our observations to biological membranes would imply that in highly curved membrane regions, which are thought to occur during membrane fusion or phagocytosis, lysophospholipids would be preferentially enriched in the outside curved monolayer, and depleted in the inside curved monolayer. This process could then possibly facilitate fusion because the outside layer which has to interact with the other membrane will be more destabilised by the lysophospholipid.

In view of the various observations [23,24] that lysophosphatidylcholine does interact with cholesterol in model membranes forming an equimolar complex we thought it of interest to see whether cholesterol incorporation affects the transmembrane distribution of 16:0 lysophosphatidylcholine. Vesicles were prepared from (N-$^{13}CH_3$)-labelled 16:0-lysophosphatidylcholine, 18:1_c/18:1_c-phosphatidylcholine and cholesterol (8/46/46, molar ratios). The outside-inside distribution of lysophosphatidylcholine was measured with ^{13}C NMR and the outside-inside distribution of the sum of 16:0-lysophosphatidylcholine and 18:1_c/18:1_c-phosphatidylcholine was measured with ^{31}P NMR using Nd^{3+} as a shift reagent [10]. From these measurements the transmembrane distribution of both phospholipids could be determined (Fig. 5). The extent of 16:0-lysophosphatidylcholine asymmetry in these vesicles is reduced by the incorporation of 50 mol% cholesterol. This can be explained in two ways. First, it is known that the incorporation of 50 mol% cholesterol increases the vesicle size [12,25,26]. The difference in curvature of both monolayers will be less thus giving a more symmetrical distribution of the membrane components. That this is not necessarily the case was shown recently for mixed cholesterol/phosphatidylcholine (1 : 1) vesicles. Despite the larger size of the vesicle more

phosphatidylcholine is present in the outside layer (ref. 12, see also Fig. 5). The size of the $18{:}1_c/18{:}1_c$-phosphatidylcholine/cholesterol (1 : 1) vesicles is not much affected by the incorporation of 15 mol% 16:0-lysophosphatidylcholine (based on total phospholipid), because the ^{31}P resonance linewidth of the vesicles which is a sensitive indicator for the vesicle size [12], was not much different from the control vesicle (20.8 and 20.2 Hz, respectively). The linewidth of the ^{31}P resonance of $18{:}1_c/18{:}1_c$-phosphatidylcholine vesicles at 40.4 MHz was 9.3 Hz.

The second possibility is that there is a relatively high affinity of lysophosphatidylcholine for cholesterol. Because cholesterol in cholesterol/$18{:}1_c$/$18{:}1_c$-phosphatidylcholine (1 : 1) vesicles is enriched in the inner monolayer [12], more lysophosphatidylcholine can be incorporated on that side of the membrane. Whether the incorporation of lysophosphatidylcholine affects the cholesterol distribution is not known.

The measured inside-outside distributions of lysophosphatidylcholine in vesicles are in good agreement with the results obtained with lysophospholipase [14].

Trans-membrane movement of 16:0-lysophosphatidylcholine in $18{:}1_c/18{:}1_c$-phosphatidylcholine vesicles

By measuring the appearance of an inside ($N\text{-}^{13}CH_3$) resonance after the addition of ($N\text{-}^{13}CH_3$)-labelled 16:0-lysophosphatidylcholine to $18{:}1_c/18{:}1_c$-phosphatidylcholine vesicles an estimate of the rate of translocation of 16:0-lysophosphatidylcholine from the outside to the inside layer of the vesicle will be obtained. Several experimental conditions have to be fullfilled for such an approach. (1) The vesicles have to remain intact after the addition of 16:0-lysophosphatidylcholine. The light scattering of a vesicle solution is a very sensitive measure for aggregation, fusion and lysis of the vesicles. Absorbance (at 450 nm) of a 20-mM $18{:}1_c/18{:}1_c$-phosphatidylcholine vesicle solution did not change (less than 5%) over a 6-h period after the addition of 15 mol% 16:0-lysophosphatidylcholine. Furthermore, the intensity of the inside resonance in ($N\text{-}^{13}CH_3$)-labelled $18{:}1_c/18{:}1_c$-phosphatidylcholine vesicles was not affected by the addition of 15 mol% 16:0-lysophosphatidylcholine in the presence of Dy^{3+} (Fig. 6A), which unambigiously demonstrates that the permeability barrier of the vesicles remains intact upon the addition of lysophosphatidylcholine. (2) A significant fraction of the 16:0-lysophosphatidylcholine has to be incorporated in the $18{:}1_c/18{:}1_c$-phosphatidylcholine vesicles. Sepharose 4B chromatography of a mixture of $18{:}1_c/18{:}1_c$-phosphatidylcholine vesicles incubated with 15 mol% 16:0-[^{14}C]lysophosphatidylcholine micelles gives a single radioactivity peak (Fig. 7). This peak coincides with the lipid phosphorus peak of the vesicles. 84% of the lipid phosphorus and 61% of ^{14}C-radioactivity were recovered in the eluate. This indicates that a minor part of lysophosphatidylcholine is not incorporated in the vesicles. This part is probably bound to the Sepharose since pure 16:0-lysophosphatidylcholine is very poorly eluted from the column.

In Fig. 4B the 90.5 MHz ^{13}C spectrum is shown of vesicles which have been incubated at 30°C for 20 h with 15 mol% 16:0($N\text{-}^{13}CH_3$)lysophosphatidylcholine and to which prior to the measurement Dy^{3+} was added. Although a

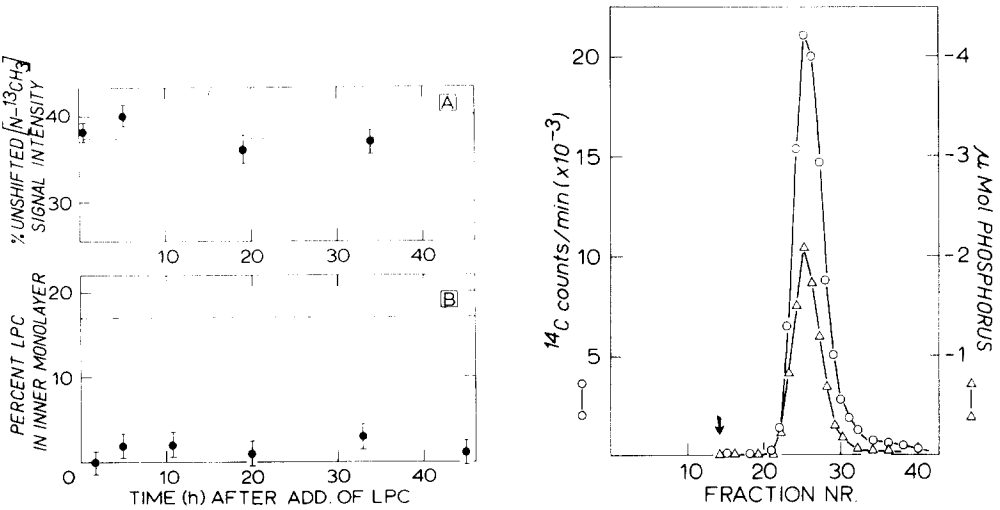

Fig. 6. (A) Percentage of the unshifted (N-$^{13}CH_3$)-signal intensity of 16 mM (N-$^{13}CH_3$)-labelled 18:1_c/18:1_c-phosphatidylcholine vesicle which have been incubated for various times at 30°C with 15 mol% 16:0-lysophosphatidylcholine micelles in the presence of 10 mM $DyCl_3$. The dotted line represents the percentage of phosphatidylcholine present in the inside layer of 18:1_c/18-1_c-phosphatidylcholine vesicle in the absence of 16:0-lysophosphatidylcholine. (B) Percentage of total 16:0-lysophosphatidylcholine (LPC) present in the inside layer of 18:1_c/18:1_c-phosphatidylcholine vesicles (26 mm) which have been incubated with 3.5 mM 16:0-(N-$^{13}CH_3$)lysophosphatidylcholine. After certain times 8 mM $DyCl_3$ was added to 1.5-ml aliquots of this mixture and the 90.5 MHz ^{13}C spectrum was recorded immediately. The indicated times are the midpoints of the half-hour data accumulation. The percentage of lysophosphatidylcholine present on the inside of the membrane was calculated from the measured inside intensity after correcting for the intensity of the natural abundance ^{13}C $N(CH_3)_3$ resonance intensity of the 18:1_c/18:1_c-phosphatidylcholine molecules present on the inside of the vesicle. The dotted line represents the percentage of 16:0-lysophosphatidylcholine present in the inner layer of a cosonicated 15 mol% (N-$^{13}CH_3$)-labelled 16:0-lysophosphatidylcholine containing 18:1_c/18:1_c-phosphatidylcholine vesicle.

Fig. 7. Sepharose 4B chromatography of 18:1_c/18:1_c-phosphatidylcholine vesicles mixed with 15 mol% 16:0-lysophosphatidylcholine micelles. 12.7 μmol 18:1_c/18:1_c-phosphatidylcholine vesicles were mixed with 2.24 μmol 16:0-[^{14}C]lysophosphatidylcholine micelles and incubated for 30 min at 37°C. The mixture was chromatographed at 4°C. The void volume of the column is indicated by an arrow.

small inside resonance is observed the intensity is much less than in the case where both lipids were cosonicated (Fig. 4A). This directly demonstrates that no equilibrium is reached in the trans-membrane distribution of 16:0-lysophosphatidylcholine in that time. After correcting the intensity of the observed inside resonance for the intensity of the natural abundance ^{13}C in the $N(CH_3)_3$ group of 18:1_c/18:1_c-phosphatidylcholine the percentage of 16:0-(N-$^{13}CH_3$)-lysophosphatidylcholine present in the inner monolayer can be obtained (Fig. 6B). Virtually all the 16:0-lysophosphatidylcholine is present in the outer monolayer of the vesicle even after 45 h of incubation. In the cosonicated mixture (Fig. 5) 17 mol% of the total lysophosphatidylcholine is present in the inner monolayer. This demonstrates that the translocation of 16:0-lysophosphatidylcholine over the vesicle membrane is an extremely slow process with a half time of several days as was reported for various diacyl phospholipids [4,5,13]. A similar conclusion could be derived from lysophospholipase digestion of lysophosphatidylcholine in phosphatidylcholine vesicles [14]. The

outer monolayer of the $18{:}1_c/18{:}1_c$-phosphatidylcholine contains at least 12.8 mol% lysophosphatidylcholine (61% of the lysophosphatidylcholine added) and should therefore expand which respect to the inner monolayer. Our findings demonstrate that the vesicle membrane can overcome such a mass imbalance of the two sides of the membrane and that this does not lead to loss of membrane integrity nor is it compensated by a rapid translocation of lipid molecules across the membrane.

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