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INDUCTION OF A RELATIVELY FAST TRANSBILAYER MOVEMENT OF PHOSPHATIDYLCHOLINE IN VESICLES

A ^{13}C NMR STUDY

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Summary

[N-$^{13}CH_3$]Phosphatidylcholines are introduced into the outer monolayer of phosphatidylcholine vesicles with the phosphatidylcholine exchange protein from bovine liver. The transbilayer distribution of the [N-$^{13}CH_3$]phosphatidylcholine is measured with ^{13}C NMR. The transbilayer movements of [N-$^{13}CH_3$]-dioleoyl phosphatidylcholine and [N-$^{13}CH_3$]dimyristoyl phosphatidylcholine at 30°C in vesicles composed of these phosphatidylcholines are extremely slow processes with estimated half-times of days. [N-$^{13}CH_3$]Dioleoyl phosphatidylcholine introduced into dimyristoyl phosphatidylcholine vesicles migrates from the outer to the inner monolayer with a half-time of less than 12 h. The data suggest that differential changes in the lateral packing of the two monolayers might be a driving force for transbilayer transport of phospholipids.

Introduction

Asymmetrical distributions of both proteins and lipids across biological membranes have been well established [1,2]. In understanding the mechanisms underlying this asymmetrical distribution it is essential to know whether molecules can migrate between the outer and inner half of the membrane. For biological membranes transmembrane movements of lipids have been reported with half-times varying from several hours to several days [3—5]. At present it is not known what controls the transmembrane movement. Sonicated liposomes

Abbreviations: NMR, nuclear magnetic resonance; DOPC, dioleoyl phosphatidylcholine (1,2-dioleoyl-*sn*-glycero-3-phosphorylcholine); DMPC, dimyristoyl phosphatidylcholine (1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine); [N-$^{13}CH_3$]DOPC, [N-$^{13}CH_3$]-1,2-dioleoyl-*sn*-glycero-3-phosphorylcholine; [N-$^{13}CH_3$]DMPC, [N-$^{13}CH_3$]-1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine. Tris, 2-amino-2-(hydroxymethyl)-propandiol-(1,3).

(single bilayer vesicles) of well-characterized phospholipids could possibly provide good models for studying this process in a systematic way. In the original observation of Kornberg and McConnell [6] the half-time of transbilayer movement of spin-labeled dipalmitoyl phosphatidylcholine in an egg phosphatidylcholine bilayer amounted to 6.5 h at 30° C. Subsequent studies where the rate of movement was measured for rat liver [^{32}P]phosphatidylcholine in egg phosphatidylcholine vesicles [7] and for [^{3}H]DOPC in DOPC vesicles [8], gave half-times of 4—11 days at 37° C.

In the latter studies phospholipid exchange proteins were used to exchange labeled phosphatidylcholine present in the outer vesicle monolayer for phosphatidylcholine of unlabeled rat liver mitochondria or erythrocyte ghosts. In the present study the phosphatidylcholine exchange protein from bovine liver has been used to introduce by a similar exchange mechanism both [*N*-$^{13}CH_3$]-DOPC and [*N*-$^{13}CH_3$]DMPC into DMPC vesicles as well as DOPC vesicles. The distribution of the *N*-$^{13}CH_3$-enriched phosphatidylcholine over the outer and inner monolayer of the vesicles was measured with ^{13}C NMR in the presence of a paramagnetic shift reagent. Recently this technique has been used to measure the transbilayer movement of [*N*-$^{13}CH_3$]lysophosphatidylcholine in the phosphatidylcholine vesicles [9].

Experimental

Materials. DOPC, DMPC, [*N*-$^{13}CH_3$]DOPC and [*N*-$^{13}CH_3$]DMPC were synthesized and purified as described before [9,10]. Of the two latter lipids one of the choline methyl groups was enriched with 90% ^{13}C. The choline moiety of [*N*-$^{13}CH_3$]DMPC was also ^{14}C labeled since a mixture of ^{13}C and ^{14}C methyl iodide was used in quaternisation of 1,2-dimyristoyl-*sn*-glycero-3-*N*,*N*-dimethylethanolamine (specific radioactivity of the product 52 000 dpm/μmol). [7α-^{3}H]Cholesterol oleate, ^{14}C-labeled egg phosphatidylcholine, egg phosphatidylcholine and phosphatidic acid were obtained as described previously [11]. Phosphatidylcholine exchange protein was purified from bovine liver [12]. The protein was stored at —20° C in 10 mM Tris · HCl/10 mM sodium acetate (pH 7.0) containing 50% (w/w) of glycerol. Dy_2O_3 was purchased from British Drug Houses (Poole, U.K.) and was converted to its chloride by HCl.

Preparation of vesicles. Single bilayer vesicles were prepared immediately before the NMR experiment in $^{2}H_2O$ containing 10 mM Tris · HCl/10 mM sodium acetate (p^{2}H 7.0) by ultrasonication of a lipid dispersion as described before [9]. DOPC and DMPC vesicles were prepared at 0 and 30° C, respectively.

Introduction of [N-$^{13}CH_3$]phosphatidylcholine into vesicles. The procedure is a modification of the exchange protein-mediated transfer of phosphatidylcholine between acceptor and donor phosphatidylcholine vesicles as previously published [11]. This method is based on the separation of acceptor and donor vesicles according to surface charge by DEAE-cellulose column chromatography. The acceptor vesicles which did not bind to the DEAE-cellulose, contained 98 mol % DOPC or DMPC and 2 mol % phosphatidic acid. The 2 mol % phosphatidic acid will give the vesicles a slight negative charge which will increase the stability of the vesicles due to the electrostatic repulsion between

the vesicles. Furthermore, the surface concentration of the paramagnetic cation added to shift the resonance from the outside facing molecules will be increased which makes it possible to work at low bulk concentrations of shift reagent. The donor vesicles which were retained by the DEAE-cellulose, contained 92 mol % [N-$^{13}CH_3$]DOPC and 8 mol % phosphatidic acid or 85 mol % [N-$^{13}CH_3$]DMPC and 15 mol % phosphatidic acid. In addition the donor vesicles contained as an internal standard a trace of [^{3}H]cholesterol oleate (0.01%, by weight). The ^{3}H label did not redistribute between donor and acceptor vesicles under the conditions used and serves as a marker for the binding of donor vesicles to the DEAE-cellulose. The incubation mixture consisted of 3 ml of donor vesicles (30 μmol of phosphatidylcholine) and 6 ml of acceptor vesicles (90 μmol of phosphatidylcholine), kept at 30° C. The transfer of phosphatidylcholine between the vesicles was started by the addition of 1 ml of exchange protein solution (100—115 μg of protein) and was continued for 1 h at 37° C. At the end of incubation donor and acceptor vesicles were separated on a DEAE-cellulose column at 30° C. This column (diameter of 2 cm) was prepared from 40 ml of a DEAE-cellulose slurry (20%, v/v) and washed with 40 ml of 10 mM Tris · HCl/10 mM sodium acetate (pH 7.0) followed by 10 ml of the same buffer in ^2H_2O. The incubation mixture (total volume of 10 ml) was applied to the column whereupon the column was eluted with 5 ml of the ^2H_2O buffer. The first 5 ml of the eluent which did not contain any acceptor vesicles, were discarded; the following 10 ml were collected and stored at 30° C. Chromatography was completed within 10—15 min. Recovery of the acceptor vesicles as measured by lipid phosphorus, amounted from 75 to 85%. The eluent contained also 1—4% (in most experiments 1—2%) of the ^{3}H label initially present in the donor vesicles. This label is indicative of the leakage of donor vesicles through the column. The amount of "donor" phosphatidylcholine introduced into the acceptor vesicles was determined by measuring radioactivity, fatty acid content and the intensity of the N-$^{13}CH_3$ signal. The values obtained by these three methods were in good agreement. After correcting for the donor vesicles which had leaked through the column, the amount of "donor" phosphatidylcholine transported by the exchange protein to the acceptor vesicles ranged from 8.7 to 13.8 μmol. In the absence of exchange protein no transport of phosphatidylcholine from donor to acceptor vesicles could be detected. As has been previously shown [11] the transport of phosphatidylcholine from donor to acceptor vesicles reflects an exchange process by which equal amounts of phosphatidylcholine are transported in the opposite direction.

Determination of outside-inside distribution of [N-$^{13}CH_3$]phosphatidylcholine. The percentage of [N-$^{13}CH_3$]phosphatidylcholine in the inner monolayer of vesicles was determined by first measuring the total intensity of the N-$^{13}CH_3$ signal followed by measuring the intensity of the unshifted resonance in the presence of the paramagnetic shift reagent dysprosium chloride ($DyCl_3$) [9]. Thus, the intensity of the shifted resonance was not used for the calculation of the outside-inside distribution, because the Nuclear Overhauser enhancement is reduced by the addition of the shift reagent [9]. Addition of 0.03 ml of 100 mM $DyCl_3$ to 1.5 ml of the N-$^{13}CH_3$-labeled vesicle solution obtained after DEAE-cellulose chromatography, was sufficient to shift the

N-$^{13}CH_3$ resonance of the outside facing phosphatidylcholine away from the resonance of the inside facing molecules. The equilibrium outside-inside distribution of [N-$^{13}CH_3$]phosphatidylcholine in these vesicles was obtained by resonication of a 1.5 ml aliquot. The vesicles were stable both in the absence and presence of $DyCl_3$ since the absorbance at 450 nm did not change significantly up to the maximal times in the experiments. This demonstrates that Dy^{3+} did not cause any aggregation or fusion of the vesicles. Furthermore the constant absorbance at 450 nm of the vesicles does demonstrate that over the length of the experiment no vesicle aggregation or fusion occurs. The intensity of the unshifted resonance was similar for vesicles which had been incubated for various times with $DyCl_3$, as compared to vesicles to which $DyCl_3$ was added immediately prior to the start of the data accumulation. From the intensity of the unshifted resonance it was further concluded that $DyCl_3$ did not penetrate the vesicles under the experimental conditions used. The percentage of [N-$^{13}CH_3$]phosphatidylcholine in the inner monolayer of the vesicles was obtained from the N-$^{13}CH_3$ signal intensity after correction for the contaminating [N-$^{13}CH_3$]phosphatidylcholine donor vesicles and for the intensity of the signal of the natural abundance ^{13}C nuclei in the $N(CH_3)_3$ groups in the acceptor vesicles. The percentage of phosphatidylcholine present in the inner monolayer of acceptor vesicles of DOPC and DMPC was determined to be 37 and 33%, respectively. In general, the sum of both corrections ranged from 9 to 12% of the total intensity of the observed N-$^{13}CH_3$ signal. The intensity of the signal of the natural abundance ^{13}C nuclei in the $N(CH_3)_3$ groups in the inside of the acceptor vesicles was 7—8% of the total intensity and was calculated from the measured phosphatidylcholine composition of the acceptor vesicle solution after the exchange. This amount of signal was subtracted from the inside signal determined after various times with $DyCl_3$. Since the signal intensity of the natural abundance nuclei in the vesicles might decrease as a consequence of the inside-outside flow of unlabeled and the outside-inside flow of ^{13}C-labeled phosphatidylcholine molecules, this correction will be a function of the rate of transbilayer movement and will decrease with increasing incubation times. The data presented therefore will give an upper limit to the transbilayer movement of phosphatidylcholine. The actual process will be (slightly) faster.

Nuclear magnetic resonance. All measurements were performed on a Bruker 360 WS spectrometer operating on ^{13}C at a frequency of 90.5 MHz as described before [9]. Accumulated free induction decays were obtained from up to 4000 transients with a 1.0 s interpulse time using a spectral width of 20 kHz and 16 000 data points. 1,4-Dioxane was used as an external reference. Peak intensities were measured with respect to the external reference using computer integration of the spectra or by cutting out and weighing the various peaks.

Analytical methods. Lipid phosphorus was measured after $HClO_4$ destruction of the lipids via the Fiske-SubbaRow procedure [13]. The fatty acid pattern of phosphatidylcholine extracted from an aqueous vesicle solution according to the Bligh and Dyer procedure [14] was determined by gas-liquid chromatography as described before [15]. The amount of ^{3}H and ^{14}C radioactivity in the vesicles was determined with a Packard-Tricarb scintillation instrument according to standard procedures.

Results

Transbilayer movement of [N-$^{13}CH_3$]DOPC in DOPC vesicles and [N-$^{13}CH_3$]-DMPC in DMPC vesicles

DOPC acceptor vesicles contained 9.7 mol % [N-$^{13}CH_3$]DOPC after incubation with [N-$^{13}CH_3$]DOPC donor vesicles and exchange protein. [N-$^{13}CH_3$]-DOPC is localized in the outside monolayer of the vesicle membrane (Table I). Incubation of the vesicles up to 40 h at 30° C results in a very slow translocation of [N-$^{13}CH_3$]DOPC to the inner monolayer (Table I). For comparison resonication of these vesicles translocates 37% of [N-$^{13}CH_3$]DOPC to the inside.

Transbilayer movement of [N-$^{13}CH_3$]DMPC was determined in DMPC vesicles which contained 15.3 mol % [N-$^{13}CH_3$]DMPC. At the earliest times measured approx. 7% of the [N-$^{13}CH_3$]DMPC introduced into the vesicles, was present in the inner monolayer (Table I). This translocation of [N-$^{13}CH_3$]-DMPC must have occurred during the incubation with donor vesicles and exchange protein and/or during the subsequent DEAE-cellulose column chromatography. Incubation of the DMPC vesicles at 30° C results in a slight increase of [N-$^{13}CH_3$]DMPC in the inner monolayer. After 25 h of incubation 14% of the [N-$^{13}CH_3$]DMPC is present on the inside as compared to 30% after resonication of these vesicles. From the data presented in Table I an accurate determination of the half-times of the transbilayer movements of DOPC and DMPC is difficult. Assuming an exponentional process the half-times can be estimated according to Kornberg and McConnel [6] to be in the order of days. It can be concluded that the rate of transbilayer movement of DMPC is larger than for DOPC.

Transbilayer movement of [N-$^{13}CH_3$]DMPC in DOPC vesicles and [N-$^{13}CH_3$]-DOPC in DMPC vesicles

[N-$^{13}CH_3$]DMPC introduced into the DOPC vesicles with the exchange pro-

TABLE I

TRANSBILAYER OF [N-$^{13}CH_3$]DOPC IN DOPC AND [N-$^{13}CH_3$]DMPC IN DMPC VESICLES

8.8 μmol [N-$^{13}CH_3$]DOPC and 13.8 μmol [N-$^{13}CH_3$]DMPC were introduced in DOPC and DMPC vesicles, respectively, by the phospholipid exchange protein. The vesicles were subsequently incubated at 30° C and after the indicated time intervals the percentage of [N-$^{13}CH_3$]phosphatidylcholine in the inside monolayer of the acceptor vesicles was determined. Dy^{3+} was added just prior to the data accumulation.

Vesicle			[N-$^{13}CH_3$]Phosphatidylcholine in inside monolayer of acceptor vesicle (%)
Donor	Acceptor	Time (h)	
[N-$^{13}CH_3$]DOPC	DOPC	2	0
		3.5	0
		18	0
		32	6
		40	3
		resonicated	37
[N-$^{13}CH_3$]DMPC	DMPC	1	8
		2.5	6
		4.5	9
		10.5	11
		25	14
		resonicated	30

tein, was present in the outer monolayer producing asymmetrical vesicles (Fig. 1a). In this case the outer monolayer consisted of 20 mol % [N-$^{13}CH_3$]-DMPC and 80 mol % DOPC, whereas the inner monolayer contained only DOPC. Incubation of the vesicles for up to 24 h at 30° C did not result in a translocation of [N-$^{13}CH_3$] DMPC to the inner monolayer. This shows that the heterogeneity in the phosphatidylcholine composition of the outer monolayer does not destabilize the bilayer to the extent that a translocation of [N-$^{13}CH_3$]-DMPC is induced. On the other hand it cannot be excluded that DOPC moved from the inner to the outer monolayer.

The exchange protein transfers [N-$^{13}CH_3$] DOPC from [N-$^{13}CH_3$] DOPC donor vesicles to DMPC acceptor vesicles. Immediately after the separation of acceptor from donor vesicles on DEAE-cellulose, 8% of the [N-$^{13}CH_3$] DOPC introduced into the DMPC vesicles was already present in the inner monolayer (Fig. 1b). This is a similar value to that found for the introduction of [N-$^{13}CH_3$] DMPC into DMPC vesicles (see Table I). Incubation of these vesicles at 30° C resulted in a translocation of [N-$^{13}CH_3$] DOPC to the inner monolayer. The [N-$^{13}CH_3$] DOPC translocation is clearly not an exponentional process (Fig. 1b); therefore a half-time cannot be calculated. From the data it can be estimated that half of the [N-$^{13}CH_3$] DOPC is translocated to the inner monolayer in less than 12 h. The equilibrium distribution of [N-$^{13}CH_3$] DOPC after 22 h was almost similar to the distribution of [N-$^{13}CH_3$] DOPC in resonicated vesicles.

Spectra of the vesicles immediately after DEAE-cellulose column chromatography and after 22 h of incubation are shown in Fig. 2. Prior to the data accumulation $DyCl_3$ was added to separate the N-$^{13}CH_3$ signal of the outer monolayer (12 ppm) from the signal of the inner monolayer (14 ppm). The resonance at 7 ppm arises from the Tris present in the solution. Comparison of the unshifted resonance from the inner monolayer with respect to the

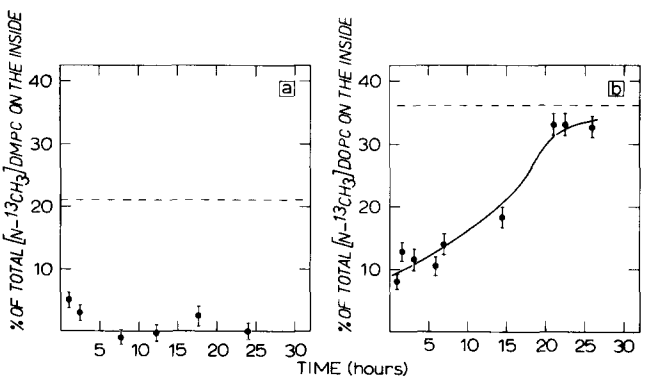

Fig. 1. Transbilayer movements of a, [N-$^{13}CH_3$] DMPC in DOPC vesicles and b, [N-$^{13}CH_3$] DOPC in DMPC vesicles. The incubation with the phospholipid exchange protein resulted in the incorporation of 11 μmol [N-$^{13}CH_3$] DMPC and 9—11 μmol [N-$^{13}CH_3$] DOPC in DOPC and DMPC vesicles, respectively. The vesicles were subsequently incubated at 30° C and after the indication time intervals the percentage of [N-$^{13}CH_3$] phosphatidylcholine in the inside monolayer of the acceptor vesicle was determined [8]. Dy^{3+} was added just prior to the data accumulation. The dotted lines represent the distributions of [N-$^{13}CH_3$]-phosphatidylcholine in the acceptor vesicles after resonication. The error bars indicate the accuracy of the N-$^{13}CH_3$ signal intensity determination.

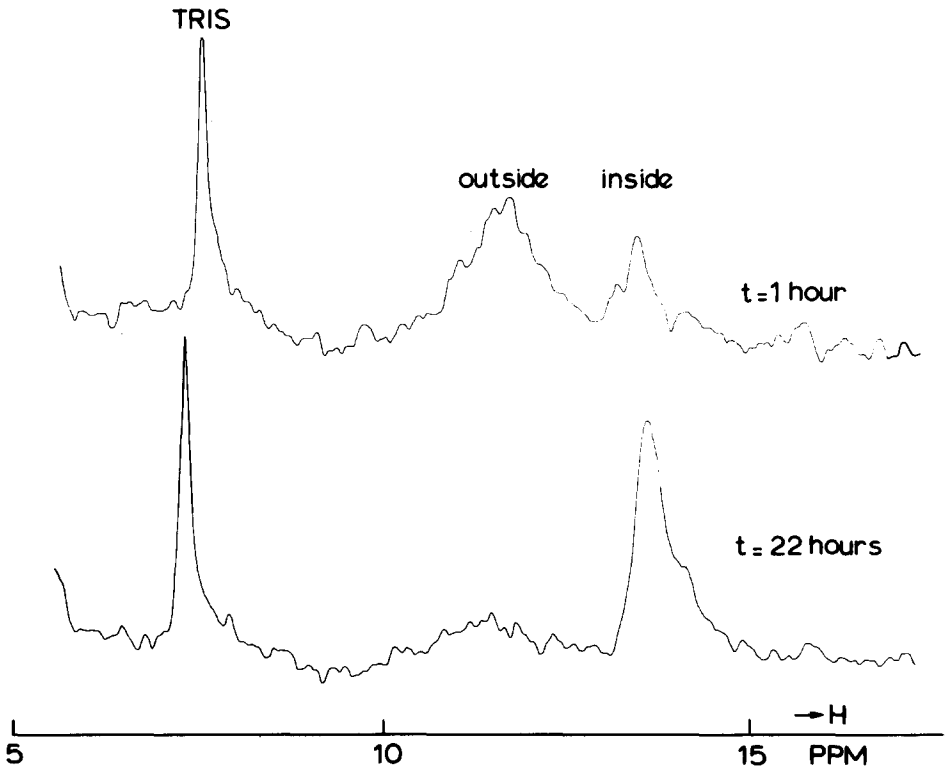

Fig. 2. 90.5 mHz ^{13}C NMR spectra of [^{13}C] DOPC containing DMPC vesicles. The spectra were recorded a, 2 h and b, 22 h after the introduction of 11 μmol [*N*-$^{13}CH_3$] DOPC in DMPC vesicles by the phospholipid exchange protein. Prior to the data accumulation $DyCl_3$ solution was added (2 mM). Shifts are up field from the resonance of 1,4-dioxane. The spectra were recorded using full proton decoupling.

resonance due to Tris indicates that after 22 h of incubation the inner monolayer of the vesicle was enriched in [*N*-$^{13}CH_3$] DOPC. The [*N*-$^{13}CH_3$] DOPC translocation is not accompanied by an inward flow of Dy^{3+} since the intensity, chemical shift and linewidth of the resonance from the inner monolayer was identical for vesicles which had been incubated for 22 h in the presence of Dy^{3+} and vesicles to which Dy^{3+} was added prior to recording the spectrum. The vesicles remain intact during the [*N*-$^{13}CH_3$] DOPC translocation because the absorbance at 450 nm does not change significantly with time till the end of the experiment. The relatively low intensity of the shifted resonance at 12 ppm is due to a partial loss of the nuclear overhauser effect upon interaction of the paramagnetic cation with the outside facing [*N*-$^{13}CH_3$] DMPC molecules [9].

To assess whether during the above [*N*-$^{13}CH_3$] DOPC translocation the outside-inside distribution of DMPC is affected the same experiment was repeated with [*N*-$^{13}CH_3$] DMPC vesicles into which 15 mol % DOPC was introduced. 1 h after the separation of the [*N*-$^{13}CH_3$] DMPC-DOPC vesicles by DEAE-cellulose chromatography 48% of the [*N*-$^{13}CH_3$] DMPC was present in the outer monolayer. Since 63% of the [*N*-$^{13}CH_3$] DMPC was present in the outer monolayer upon resonication of the vesicles it can be concluded that the exchange protein has replaced 15% of the [*N*-$^{13}CH_3$] DMPC for DOPC. After 12 h of incubation

the amount of [N-$^{13}CH_3$]DMPC present in the outer monolayer had increased to 52% suggesting that a transbilayer movement of DMPC from the inner to the outer monolayer may, in part, compensate for the movement of DOPC in the opposite direction in these vesicles. This experiment furthermore unambiguously demonstrates that the DOPC translocation is not accompanied by vesicle fusion because in that case an increase in the signal intensity of the unshifted [N-$^{13}CH_3$]DMPC resonance would be expected with time.

Discussion

In this paper a new method is described to measure in a direct way without perturbing probes the transmembrane movement of phosphatidylcholine in vesicles.

The present data demonstrate that transbilayer movements of [N-$^{13}CH_3$]-DOPC in DOPC vesicles and [N-$^{13}CH_3$]DMPC in DMPC vesicles at 30° C are extremely slow with minimal half-times of several days in agreement with data reported previously [7,8]. This shows that the two halves of single bilayer phosphatidylcholine vesicles at temperatures where the fatty acid chains are in the disordered state remain physically separated.

In this study we have also shown that the phosphatidylcholine exchange protein catalyzes the exchange of phosphatidylcholine between two vesicle populations prepared from different phosphatidylcholine molecular species. Since the phosphatidylcholine molecules are introduced into the outer monolayer, the vesicle bilayer becomes asymmetrical in phosphatidylcholine composition. In these vesicles there is a difference in the molecular packing of the two monolayers. The resulting imbalance in pressure between the outer and inner monolayer of the vesicle might induce the transbilayer movement of phospholipid molecules. The protein-mediated introduction of DOPC into the outer monolayer of DMPC vesicles will cause an expansion of the outer monolayer. Namely, the area per molecule of DOPC and DMPC are 78 and 60 $Å^2$, respectively, as measured in a monomolecular film at 20 dynes per cm [16]. When 20% of DMPC in the outer monolayer has been replaced with DOPC (Fig. 1b), the surface area increase amounts to approx. 5%. Under these conditions DOPC moves to the inner monolayer with a half-time of less than 12 h at 30° C which probably is accompanied by an inside-outside translocation of DMPC molecules.

Introduction of [N-$^{13}CH_3$]DMPC into the outer monolayer of DOPC vesicles will cause a shrinking of the outer monolayer with respect to the inner monolayer. This could possibly lead to an enhanced inside-outside flow of DOPC. Such a process, however, is not accompanied by a concomitant flow of DMPC molecules to the inner monolayer (Fig. 1a).

As yet, the molecular mechanism of the relatively fast movement of DOPC in DMPC vesicles is poorly understood. It is of interest to note that DMPC is the saturated phosphatidylcholine with the shortest fatty acid chains which can form stable bilayers [17,18]. Therefore, imbalance of monolayer pressure by introduction of other phospholipid molecules in these bilayers could more easily lead to a redistribution of phospholipid molecules.

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