Biochimica et Biophysica Acta, 511 (1978) 105—115

BBA 78075

EFFECT OF THE PHASE TRANSITION ON THE TRANSBILAYER MOVEMENT OF DIMYRISTOYL PHOSPHATIDYLCHOLINE IN UNILAMELLAR VESICLES

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(Received December 27th, 1977)

Summary

Dimyristoyl phosphatidylcholine rapidly exchanges between vesicles at 37°C without vesicle fusion.

The rate of the transbilayer movement of dimyristoyl phosphatidylcholine in sonicated vesicles has been measured employing ^{13}C NMR using N-$^{13}CH_3$-labeled lipids which are introduced into the outer monolayer of non-labeled vesicles by a phosphatidylcholine exchange protein. The rate of transbilayer movement of dimyristoyl phosphatidylcholine shows a distinct maximum (half-time 4 h) in the temperature range at which the hydrocarbon phase transition occurs.

The activation energy of the flip-flop rate above the phase transition is 23.7 ± 2.0 kcal/mol.

Introduction

Transmembrane movements of lipids in biological membranes are in general faster than in unilamellar vesicles (for recent reviews on this topic see refs. 1 and 2). Halftimes of transbilayer movements of lipids in vesicles are reported to be on the order of days or more [3—9]. Recently, however, several conditions have been found in which the 'flip-flop' rate is significantly increased. Substitution of dimyristoyl phosphatidylcholine by dioleoyl phosphatidylcholine in the outer monolayer of dimyristoyl phosphatidylcholine vesicles by means of a phospholipid exchange protein resulted in a relatively fast inward movement of dioleoyl phosphatidylcholine [7]. Similarly, phosphatidic acid formed in the outer monolayer of egg phosphatidylcholine vesicles by phospholipase D is translocated to the inner monolayer with a halftime of 40 min or less [10]. Both observations indicate that a non-equilibrium trans-bilayer distribution of the lipid molecules can lead to transbilayer movements of lipids without loss of

barrier properties of the bilayer [7,10]. Further, the incorporation of glycophorin, a membrane-spanning protein from the human erythrocyte membrane, in phosphatidylcholine bilayers increased the rate of transbilayer movement of lysophosphatidylcholine by two orders of magnitude [11]. Finally, exchange experiments between vesicles and erythrocytes initially suggested that no flip-flop of cholesterol in vesicles occurred [12]. Recently however using very similar techniques and experimental conditions halftimes of less than 2 h were reported for the flip-flop of cholesterol in vesicles [13].

Gel to liquid-crystalline phase transitions have a profound effect on the properties of the lipid bilayer (for recent reviews see refs. 14 and 15). When lateral phase separation occurs, both in model [16—19,21] and *Escherichia coli* membranes [20—22] the permeability of small molecules has been found to be markedly increased. Also pancreatic phospholipase A_2 shows maximal activity at the phase transition temperature [23]. To test whether phase transitions have an effect on the transbilayer movements of lipids we investigated in this study the temperature dependence of the dimyristoyl phosphatidylcholine flip-flop in sonicated vesicles by ^{13}C NMR techniques [7]. It is found that the rate of transbilayer movement shows a distinct maximum in the temperature range of the gel to liquid-crystalline phase transition.

Experimental

Materials. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (14 : 0/14 : 0-phosphatidylcholine) and N-$^{13}CH_3$-labeled 14 : 0/14 : 0-phosphatidylcholine (one of the methyl groups is 90% ^{13}C-enriched) were synthesized as described before [7]. The latter lipid was also ^{14}C labeled in the choline methyl groups [7]. [7α-^{3}H]Cholesterol oleate and phosphatidic acid derived from egg phosphatidylcholine were obtained as described previously [7]. Phosphatidylcholine exchange protein was purified from bovine liver [24] and 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.

Procedures. Single bilayer vesicles were prepared by ultrasonication of a lipid dispersion in 10 mM Tris · HCl/10 mM sodium acetate (pH 7.0) with a tip sonicator at 30°C as described before [7].

N-$^{13,14}CH_3$-labeled 14 : 0/14 : 0-phosphatidylcholine was introduced in 14 : 0/14 : 0-phosphatidylcholine vesicles by means of the exchange protein in an identical way as described in detail before [7]. Briefly, the method is based on the separation of donor vesicles (prepared from the N-$^{13,14}CH_3$-labeled lipid) and acceptor vesicles (prepared from the unlabeled lipid) according to surface charge by DEAE-cellulose column chromatography. The acceptor vesicles, which did not bind to the DEAE-cellulose contained 1.75 mol% phosphatidic acid which gives the vesicles a slight negative charge to increase the stability of the vesicles. The donor vesicles contained 15 mol% phosphatidic acid and as a non-exchangeable marker a trace (0.01% by weight) of [^{3}H]cholesterol oleate. Transfer of phosphatidylcholine between donor vesicles (30 μmol phosphatidylcholine in 3.0 ml buffer) and acceptor vesicles (90 μmol phosphatidylcholine in 6.0 ml buffer) was initiated by the addition of 1 ml of exchange pro-

tein solution (100—115 μg protein) to the vesicles at 37°C and was, unless otherwise stated, continued for 1 h. Subsequently, the two vesicle populations were separated and the exchange protein was removed on a DEAE-cellulose column (diameter 2 cm, prepared from 40 ml of a 20% (v/v) slurry) at 30°C [7]. The vesicles were then incubated at the indicated temperatures. To determine the time course of the 14 : 0/14 : 0-phosphatidylcholine exchange between the vesicles, 500-μl aliquots of the incubation mixture were applied on to small columns (pasteur pipettes) prepared from 2 ml of the DEAE slurry and were washed with 250 μl of the buffer. The recovery of the acceptor vesicles as determined by phosphate analysis was 75—85%. The eluent contained 0—2% of the ^{3}H label initially present in the donor vesicles indicating a very small leak of donor vesicles through the column. This percentage was independent of the incubation time and the presence or absence of exchange protein. In the 1 h incubation with exchange protein 30—40% of the ^{14}C label (and therefore the ^{13}C label) initially present in the donor vesicles was recovered in the acceptor vesicles.

The transbilayer distribution of the ^{13}C-labeled lipids was determined by ^{13}C NMR using $DyCl_3$ as an impermeable shift reagent as described before [7,8]. The intensities of the total and inside (unshifted) choline methyl signal were corrected for the small amounts of contaminating donor vesicles (0—4% of the total signal). The remaining signal has two contributions: (1) from the N-$^{13}CH_3$-labeled 14 : 0/14 : 0-phosphatidylcholine (approx. 80% of the signal) and (2) from the natural abundance ^{13}C-labeled nuclei of the choline methyl groups of the unlabeled 14 : 0/14 : 0-phosphatidylcholine molecules (approx. 20% of the signal). As is shown in Appendix the halftime of the movement of the N$^{13,14}CH_3$-labeled 14 : 0/14 : 0-phosphatidylcholine to the inner monolayer of the 14 : 0/14 : 0-phosphatidylcholine vesicle can be obtained from the fraction of the signal arising from lipid molecules present in the inner monolayer at various times t and the distribution at $t = \infty$ (measured as the fraction of the signal in the inside monolayer of the vesicles after resonication).

The outside-inside distribution of 14 : 0/14 : 0-phosphatidylcholine in the acceptor vesicles was also measured by ^{31}P NMR using Nd^{3+} as a shift reagent [25].

Techniques. The ^{13}C NMR measurements were performed at a frequency of 90.5 MHz on a Bruker 360 WS spectrometer under the same conditions as described in detail before [7,8]. For the ^{31}P NMR experiments a Bruker WH 90 operating at 36.4 MHz for ^{31}P was used as described before [25]. The sample temperature in both spectrometers was 30 ± 1°C unless otherwise indicated.

^{3}H and ^{14}C radioactivity was determined with a Packard-Tricarb scintillation instrument using standard procedures. Lipid phosphorus was measured after perchloric acid destruction of the lipids via the Fiske-Subba Row procedure [26].

Results

Exchange of 14 : 0/14 : 0-phosphatidylcholine between vesicles

In order to find optimal conditions for the introduction of N-$^{13,14}CH_3$-labeled 14 : 0/14 : 0-phosphatidylcholine into the outer monolayer of 14 : 0/

14 : 0-phosphatidylcholine vesicles the time course of the exchange between donor and acceptor vesicle was studied with and without exchange protein. It was found that, at 37°C, in the absence of exchange protein 14 : 0/14 : 0-phosphatidylcholine molecules can move from the donor to the acceptor vesicles as is shown in Fig. 1. From the first part of the exchange curve a halftime of this exchange process of approx. 80 min can be calculated. The transfer of 14 : 0/14 : 0-phosphatidylcholine between the vesicles is an exchange process and does not involve vesicle fusion and subsequent vesicle separation as only 0—2% of the [^{3}H]-cholesterol oleate initially present in the donor vesicles was recovered in the acceptor vesicles (see Experimental). Similar rather fast exchange processes involving 14 : 0/14 : 0-phosphatidylcholine have been described previously [27,28]. In the presence of 100 μg of exchange protein the exchange rate of 14 : 0/14 : 0-phosphatidylcholine between the vesicles is faster (Fig. 1). Because of the limited number of data points in the first part of the curve (which is due to the time needed to separate the donor from the acceptor vesicles) only an approximate halftime for exchange of 31 min can be obtained from Fig. 1. From the known amounts of lipids in the donor and acceptor vesicles an estimate can be made of the fraction of ^{14}C-labeled 14 : 0/14 : 0-phosphatidylcholine remaining in the donor vesicle when an equilibrium situation has been reached. Using a value of 33% for the amount of 14 : 0/14 : 0-phosphatidylcholine present in the inner monolayer of the acceptor vesicle (ref. 7 and this study) and assuming a similar transbilayer distribution of 14 : 0/14 : 0-phosphatidylcholine in the donor vesicles it can be calculated for the experiment shown in Fig. 1 that 46% of the [^{14}C]-labeled 14 : 0/14 : 0-phosphatidylcholine should remain in the donor vesicle if exchange can only occur between the outer monolayers of the vesicles. On the other hand, if all the 14 : 0/14 : 0-phosphatidylcholine in both vesicle populations is exchangeable 33% of the ^{14}C-labeled 14 : 0/14 : 0-phosphatidylcholine should remain in the donor vesicle. The observed value of around 48% (Fig. 1) is in agreement with an exchange which occurs predominantly between the outer monolayers of the donor and acceptor vesicles. However a second, much slower exchange process appears to occur as the amount of the ^{14}C-labeled 14 : 0/14 : 0-phos-

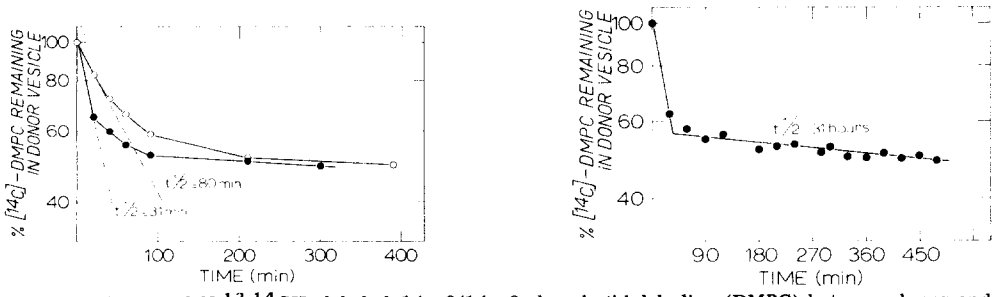


Fig. 1. Exchange of N-^{13,14}CH$_3$-labeled 14 : 0/14 : 0-phosphatidylcholine (DMPC) between donor and acceptor vesicles at 37°C in the absence (○) and presence of 100 μg exchange protein (●). See Experimental for details.

Fig. 2. Time course of the exchange of N-^{13,14}CH$_3$-labeled 14 : 0/14 : 0-phosphatidylcholine (DMPC) between donor and acceptor vesicles at 37°C in the presence of 100 μg of exchange proteins. See Experimental for details.

phatidylcholine remaining in the donor vesicles slowly decreases at longer times (see Fig. 2). From this second process, which also occurs in the absence of an exchange protein, a halftime of 31 h (at 37°C) can be calculated. It is tempting to speculate that this halftime is related to the transbilayer movement of 14 : 0/14 : 0-phosphatidylcholine in the donor and/or acceptor vesicles. However, because of the different chemical composition of the two vesicles and the rather indirect measurements a reliable analysis of this slower rate process in terms of a flip-flop rate in one of the vesicle populations is very difficult to give. In conclusion, the 1 h incubation period of the vesicles at 37°C with 100 μg exchange protein is sufficient to obtain a maximal incorporation of N-$^{13,14}CH_3$-labeled 14 : 0/14 : 0-phosphatidylcholine in the outer monolayer of the acceptor vesicles.

Transbilayer movements of 14 : 0/14 : 0-phosphatidylcholine

In order to ascertain whether the transbilayer distribution of 14 : 0/14 : 0-phosphatidylcholine is affected by the phase transition N-$^{13,14}CH_3$-labeled 14 : 0/14 : 0-phosphatidylcholine vesicles containing 1.75% phosphatidic acid were prepared at 30°C and then the transbilayer distribution was determined by ^{13}C NMR at 30 and 4°C. This is experimentally possible as only limited broadening of the N-$^{13}CH_3$ resonance occurs below the phase transition (linewidths of 16 and 31 Hz at 30 and 4°C, respectively). Percentages of 14 : 0/14 : 0-phosphatidylcholine in the inner monolayer of the vesicles of 34 and 35% were obtained at 30 and 4°C, demonstrating that the transbilayer distribution of 14 : 0/14 : 0-phosphatidylcholine is identical above and below the phase transition temperature.

The time course of the distribution of the [^{13}C]choline methyl signal over the outer and inner layer of the 14 : 0/14 : 0-phosphatidylcholine acceptor vesicles at 30°C after N-$^{13,14}CH_3$-labeled 14 : 0/14 : 0-phosphatidylcholine has been incorporated in the acceptor vesicles by the exchange protein is presented

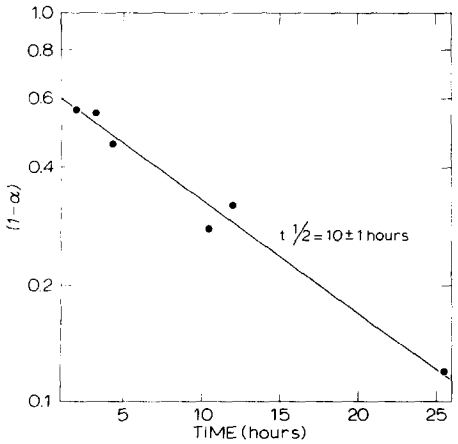
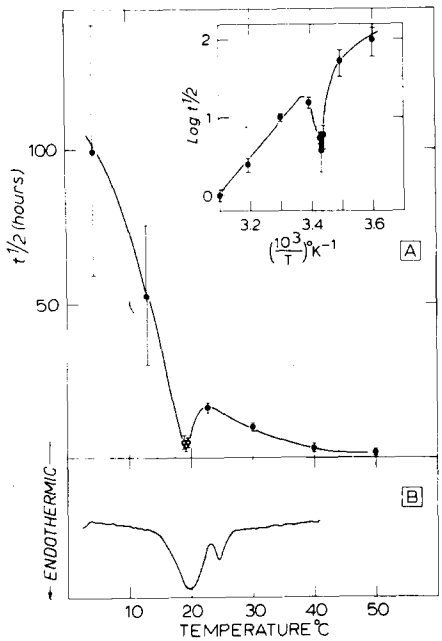


Fig. 3. Time course of the redistribution of the ^{13}C *N*-methyl resonance over the lipids on the outer and inner layer of the acceptor vesicles at 30°C. The indicated times are from the end of the 1 h incubation with exchange protein till the midpoint of the data accumulation. See text and Appendix for definition of α.

in Fig. 3 as a semilog plot of $1 - \alpha$ against time. As is shown in the Appendix α represents the fraction of the total number of labeled molecules (e.g. ^{13}C enriched and naturally abundant) in the inner monolayer divided by the fraction of the total number of molecules in the inner monolayer (which is identical to the distribution of the labeled molecules after resonication; this was measured as 0.33 ± 0.02). At time zero (time of the end of the 1 h incubation at 37°C with exchange protein) $1 - \alpha$ is 0.6 which indicates that 13% of the labeled molecules at that time are present in the inner monolayer. If all the N-$^{13,14}CH_3$-labeled 14 : 0/14 : 0-phosphatidylcholine molecules were present only in the outer monolayer it can be calculated that a signal intensity corresponding to 8% of the labeled molecules (due to the natural abundant [^{13}C]-methyl groups of 14 : 0/14 : 0-phosphatidylcholine molecules in the inner monolayer of the acceptor vesicles) would be present in the inner monolayer. The observed value of 13% shows that some N-$^{13,14}CH_3$-labeled 14 : 0/14 : 0-phosphatidylcholine molecules are already present in the inner monolayer of the acceptor vesicles after the exchange procedure which is in agreement with previous observations [7]. This most likely represents the inward movement of a fraction of the N-$^{13,14}CH_3$-labeled 14 : 0/14 : 0-phosphatidylcholine molecules during the 1 h incubation at 37°C. From the slope of the graph in Fig. 3 a halftime of 10 ± 1 h (S.D. obtained from least square analysis) can be obtained for the transbilayer movement of 14 : 0/ 14 : 0-phosphatidylcholine at 30°C. In a previous report we estimated the halftime at 30°C to be in the order of days [7]. However, in that study no correction was made for the fact that when labeled molecules move to the inner monolayer there are proportionally less molecules giving rise to the naturally abundant ^{13}C NMR signal. This led to an underestimate of the rate of the transbilayer movement in the previous work [7].

The temperature dependence of the 14 : 0/14 : 0-phosphatidylcholine flip-flop is presented in Fig. 4A. At 50°C the halftime is 1 h and increases with decreasing temperature ($t_{1/2}$ = 16 ± 1 h at 23°C). The activation energy of the flip-flop over the temperature range 50—30°C can be calculated from the Arrhenius plot of the data (see insert of Fig. 4A) to be 23.7 ± 2.0 kcal/mol. Between 13 and 22°C the flip-flop rate shows a relative maximum (4 ± 2 h at 18.5°C). Below 13°C the flip-flop rate strongly decreases. Fig. 4B shows the calorimetrically determined phase transition in sonicated 14 : 0/14 : 0-phosphatidylcholine vesicles. The transition is broad and ranges from 15 to 23°C [29—33]. The small sharper peak at 24°C represents the transition of a small fraction of larger vesicles present in the preparation [29—33]. It can be concluded that the maximum in the flip-flop rate occurs in the temperature range of the gel to liquid-crystalline phase transition occurring in the vesicles. To test whether the total transbilayer distribution of the lipids in the acceptor vesicles remained constant during the long incubations at different temperatures the experiment was repeated but the outside-inside distribution of 14 : 0/14 : 0-phosphatidylcholine was now measured by ^{31}P NMR after incubating the vesicles at different temperatures for various periods of time. As is shown in Fig. 5A the transbilayer distribution remained constant at all temperatures tested. This shows that the transbilayer movement of 14 : 0/14 : 0-phosphatidylcholine in these vesicles is a flip-flop process in which equal numbers of



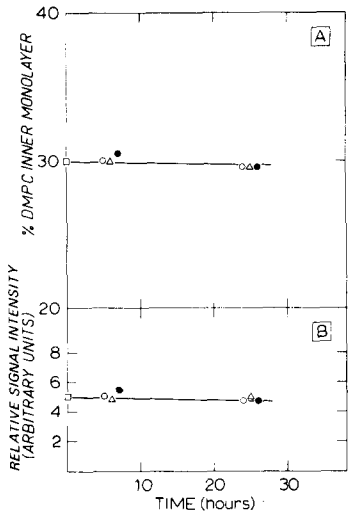


Fig. 4. (A) Temperature dependence of the transbilayer movement of 14 : 0/14 : 0-phosphatidylcholine. The error bars are the standard deviations in the halftime calculated from plots of log (1 — α) versus time as shown in Fig. 3. The insert shows the Arrhenius plot of the process. (B) Differential scanning calorimetry of sonicated 14 : 0/14 : 0-phosphatidylcholine vesicles. Data taken from ref. 29.

Fig. 5. (A) Outside-inside distribution of 14 : 0/14 : 0-phosphatidylcholine (DMPC) in acceptor vesicles as determined by ^{31}P NMR. (B) Intensity of the ^{31}P NMR signal of the vesicles before the addition of the shift reagent measured immediately after the DEAE-cellulose column chromatographic separation of the donor and acceptor vesicles (□). Part of the vesicles were incubated at 4°C (△), 19°C (○) and 40°C (●).

lipid molecules flow from the outer to the inner layer and vice versa. It can be further concluded that the vesicle size remains the same which argues against vesicle fusion. Other evidence against possible vesicle fusion is found in the observation that virtually no [^{3}H]cholesterol oleate is found in the acceptor vesicles during a 1 h incubation at 37°C with donor vesicles (see Experimental) and that the total intensity and linewidth (not shown) of the narrow ^{31}P NMR signal of the vesicles (measured before the addition of Nd^{3+}) remained constant at various temperatures for long times (Fig. 5B). Small changes in vesicle size have a profound effect on the ^{31}P NMR linewidths [34]. A further factor which may be expected to mitigate against fusion is the 1.75 mol% of phosphatidic acid present in the bilayer which gives the vesicles a negative charge thus discouraging aggregation.

Discussion

The transbilayer movement of 14 : 0/14 : 0-phosphatidylcholine in the temperature range 25—50°C measured in this study, is significantly faster than the transbilayer movement of dioleoyl phosphatidylcholine [4,7] and rat liver phosphatidylcholine [5]. This must be caused by the thinner bilayer of the

14 : 0/14 : 0-phosphatidylcholine vesicles and the more hydrophilic overall character of the molecule. This last property is clearly manifested in the relatively fast exchange of 14 : 0/14 : 0-phosphatidylcholine between vesicles as is shown in Fig. 1. The longer chain phosphatidylcholines do not display such a behaviour (refs. 4, 5 and 9, and de Kruijff, B. and van Zoelen, E.J.J., unpublished observations). That the flip-flop of 14 : 0/14 : 0-phosphatidylcholine is still a process which is severely restricted, can be seen from the high activation energy of 23.7 ± 2.0 kcal/mol in the 25—50°C temperature range. This high activation energy most likely represents the unfavourable translocation of the hydrophilic polar head group through the hydrocarbon core of the bilayer.

The rate of transbilayer movement of 14 : 0/14 : 0-phosphatidylcholine in sonicated vesicles shows a distinct maximum in the region of the phase transition (halftime of 4 ± 2 h at 18.5°C). This suggests that the interfaces between liquid and liquid-crystalline lipids in the bilayer must allow the 14 : 0/14 : 0-phosphatidylcholine molecules to pass relatively easy from on monolayer to the other. By analogy with previous observations that bilayer permeability [16—22] and pancreatic phospholipase A_2 activity [23] are maximal when both gel state and liquid-crystalline state lipids coexist in the bilayer we suggest that the increased lateral compressibility of the bilayer in the phase transition facilitates the transbilayer movements of lipids.

For bacterial membranes (notably the *E. coli* membrane) it has been demonstrated that several transport processes are greatly facilitated when the membrane lipids undergo phase separation (for review see ref. 22). Possibly the high flip-flop rate of phosphatidylethanolamine recorded in the *Bacillus megaterium* membrane is also (in part) caused by lateral phase separations occurring in the membrane, although protein-mediated mechanisms may also play an important role [35].

The dramatic increase in transbilayer movement of lysophosphatidylcholine in phosphatidylcholine bilayers caused by the incorporation of glycophorin [11] could possibly be explained by a similar mechanism. In analogy with the cytochrome oxidase containing bilayer [36] 'boundary' lipids around the protein (which are probably more rigid) coexist with the liquid-crystalline phosphatidylcholine molecules in the bilayer. This results in an area around the protein in which the lateral compressibility is increased possibly promoting the lysophosphatidylcholine flip-flop.

Appendix

Transbilayer movement of lipid molecules in an unilamellar vesicle can be described by means of first-order kinetics, in which the rate of transbilayer movement is proportional to the number of lipid molecules present in each monolayer. In the case of a homogeneous vesicle population this implies

$$\frac{dN_i}{dt} = -k_i N_i + k_o N_o = -(k_o + k_i)N_i + k_o N_t \qquad (1)$$

in which N_t is the total number of lipid molecules present, of which N_i are located in the inner and N_o in the outer monolayer. The constant k_i determines

the probability of a lipid molecule to move from the inner to the outer monolayer, and k_o of the reverse process. In a vesicle containing only one lipid species no net transport of lipid molecules will take place, making $dN_i/dt = 0$, and

$$k_i = -k_0 + k_0/\theta \tag{2}$$

in which θ is the fraction of the lipid molecules present in the inner monolayer ($\theta = N_i/N_t$).

If L_t of the lipid molecules carry a label, of which L_i are present in the inner monolayer, the rate of transbilayer movement of the labeled molecules is given by

$$\frac{dL_i}{dt} = -(k_0 + k_i)L_i + k_o L_t \tag{3}$$

It will be assumed that initially all label is present in the outer monolayer making $(L_i)_{t=0} = 0$.

Integration of the differential equation gives

$$L_i = L_t\theta(1 - e^{-k_o t/\theta}) \tag{4}$$

It can be seen from this equation that at $t = \infty$ the label has been equilibrated according to the distribution of the lipid molecules over the two monolayers ($L_i = \theta L_t$). A halftime for this process can be defined as that time after which half of this equilibration process has been accomplished, so $t = t_{1/2}$ when $L_i = \theta L_t/2$. Introduction in Eqn. 4 gives

$$t_{1/2} = \frac{\theta \ln 2}{k_o} \tag{5}$$

It will now be assumed that besides those molecules which have been introduced as labeled molecules also a fraction ϕ of the other lipid molecules contains the label (natural abundance label). The total number of labeled molecules (N_t^+) will then be given by:

$$N_t^+ = L_t + \phi(N_t - L_t) = (1 - \phi)L_t + \phi N_t \tag{6}$$

Similar equations can be given for the number of labeled molecules in the inner and outer monolayer. The rate of transbilayer movement of the labeled lipid molecules will be given by:

$$\frac{dN_i^+}{dt} = -(k_i + k_o)N_i^+ + k_o N_t^+ \tag{7}$$

If it is assumed that all introduced label is present in the outer monolayer at zero time, the inner monolayer will initially only have natural abundance labels, making

$$(N_i^+)_{t=0} = \phi N_i = \phi\theta N_t \tag{8}$$

Integration of Eqn. 7 and using Eqn. 8 gives

$$N_i^+/N_t^+ = \theta(1 - e^{-k_o t/\theta}) + \frac{\phi\theta N_t}{N_t^+} e^{-k_o t/\theta} \tag{9}$$

It can be seen from this equation that at zero time Eqn. 8 holds, and that at $t = \infty$ the total label has been equilibrated again according to the total lipid distribution over the bilayer (θ). The fraction of the number of labeled molecules in the inner monolayer divided by the fraction of the total number of molecules in the inner monolayer is now defined as

$$\alpha = N_i^*/N_t^*\theta \tag{10}$$

From combination of Eqns. 9 and 10 it follows

$$\ln(1-\alpha) = \frac{-k_o t}{\theta} + \ln\left(1 - \frac{\phi N_t}{N_t^*}\right) \tag{11}$$

Since k_o is related to the halftime of the process of transbilayer movement (see Eqn. 5) it follows

$$\ln(1-\alpha) = \left(\frac{-\ln 2}{t_{1/2}}\right) t + C \tag{12}$$

in which C is a constant. α can easily be measured from the NMR data, and $t_{1/2}$ can be determined by plotting $\ln(1-\alpha)$ versus time (see for instance Fig. 3). Since t appears in a linear form in Eqn. 12, it is possible to take any chosen time point t_0 as starting point for the measurements, which will only affect the interpretation of constant C.

Acknowledgements

The 90.5 MHz ^{13}C NMR measurements were carried out at the S.O.N. NMR facility in Groningen. We wish to thank Drs. J. Westerman and K.W.A. Wirtz for the kind gift of the phosphatidylcholine exchange protein, and Mrs. A.M.W. Lancée-Hermkens for the synthesis of 14 : 0/14 : 0-phosphatidylcholine. The stimulating discussions with the other members of the membrane group in Utrecht were very much appreciated. We thank Dr. P.R. Cullis for critically reading the manuscript.

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