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DIFFERENTIAL SCANNING CALORIMETRY AND ^{31}P NMR STUDIES ON SONICATED AND UNSONICATED PHOSPHATIDYLCHOLINE LIPOSOMES

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SUMMARY

1. Phase transitions in sonicated (vesicles) and unsonicated liposomes composed of various synthetic phosphatidylcholines are monitored using differential scanning calorimetry and ^{31}P NMR.

2. The temperature (T_c), heat content and width of the phase transition are comparable in both vesicles and liposomes prepared from 1,2-dipalmitoyl phosphatidylcholine and 1,2-dimyristoyl phosphatidylcholine. In vesicles composed of a (1 : 1) mixture of 1,2-dipalmitoyl phosphatidylcholine and 1,2-dioleoyl phosphatidylcholine phase separation occurs as in the bilayers of the unsonicated liposomes.

3. The linewidth of the ^{31}P resonances in vesicles is not greatly dependent upon the fatty acid composition when the lipids are in the disordered liquid crystalline state (above T_c). When the lipids are in the gel state (below T_c), however, there is a marked increase in linewidth, demonstrating a reduction in motion of the phosphate group.

4. The ratio of the amounts of phosphatidylcholine present in the outside and inside monolayer of the vesicle membrane was determined with ^{31}P NMR using Nd^{3+} as a non-permeating shift reagent.

5. The outside/inside ratio is dependent upon the hydrocarbon chain length. Increasing chain length gives a lower outside/inside ratio and a larger vesicle. Introduction of *cis* or *trans* double bonds in the chain influences the outside/inside ratio slightly.

6. The incorporation of cholesterol decreases the outside/inside ratio and increases the size of 1,2-dimyristoyl phosphatidylcholine vesicles. The cholesterol concentration in the outside and inside monolayer is approximately the same. The size of the 1,2-dioleoyl phosphatidylcholine vesicles is also increased by cholesterol incorporation but the outside/inside distribution is also increased, especially between 30 and 50 mol % cholesterol. In these vesicles cholesterol is asymmetrically distributed and strongly prefers the inside monolayer of the vesicle.

INTRODUCTION

Phospholipid vesicles, produced by sonication of aqueous dispersions of lipids (liposomes) are extensively employed as model systems to further the understanding of biological membranes [1–11]. Because of the high curvature of the vesicle bilayer it is questionable whether the physical properties of the vesicle membrane are comparable to the properties of the much larger liposomal or biological membranes [3]. The transition temperature and heat content (ΔH) of the gel \rightarrow liquid crystalline phase transition in lipid bilayers are very sensitive to the packing properties of the molecules in the membrane. In the case of 1,2-distearoyl phosphatidylcholine for example, the introduction of a *cis* double bond in the acyl chains to produce 1,2-dioleoyl phosphatidylcholine increases the area per molecule at the air-water interface, decreases the transition temperature by approx. 80 °C and reduces the heat content by 30 % [12]. It might be expected that any gross change in lipid packing in going from liposomes to vesicles would also appreciably change these physical parameters. As part of this study we have therefore compared the calorimetric properties of phosphatidylcholine vesicles and liposomes.

It has recently been observed that ^{31}P NMR spectra of dipalmitoyl phosphatidylcholine vesicles are sensitive to effects associated with the phase transition of the hydrocarbon chains [13, 14]. We have extended these studies to vesicles composed of different defined phosphatidylcholine species, and the results were compared with differential scanning calorimetry measurements.

Recently several reports have indicated that asymmetrical distributions of lipids across vesicle bilayers are obtained in vesicles prepared from mixtures of lipids [15–17]. In particular, asymmetric distributions of lipids with different polar head groups were observed, where the packing properties of these lipids appeared to be crucial to the asymmetry. In these studies, however, the fatty acid composition of the various lipids used was complex and different for the various lipid classes. Since the fatty acid composition strongly influences the packing of lipid molecules [12, 18, 19], we have investigated, using ^{31}P NMR, the outside/inside distribution of well defined phosphatidylcholine molecular species over the vesicle membrane.

MATERIALS AND METHODS

Lipids. 1,2-dilauroyl-*sn*-glycero-3-phosphorylcholine (12 : 0/12 : 0 phosphatidylcholine), 1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine (14 : 0/14 : 0 phosphatidylcholine), 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (16 : 0/16 : 0-phosphatidylcholine), 1,2-distearoyl-*sn*-glycero-3-phosphorylcholine (18 : 0/18 : 0-phosphatidylcholine), 1,2-dioleoyl-*sn*-glycero-3-phosphorylcholine (18 : 1c/18 : 1c-phosphatidylcholine), 1,2-dielaidoyl-*sn*-glycero-3-phosphorylcholine (18 : 1t/18 : 1t-phosphatidylcholine) and 1,2-dipalmitoleyl-*sn*-glycero-3-phosphorylcholine (16 : 1c/16 : 1c-phosphatidylcholine) were synthesised as described previously [20]. 1-Palmitoyl-*sn*-glycero-3-phosphorylcholine (1-16 : 0-lysophosphatidylcholine) was prepared by phospholipase A₂ degradation of 16 : 0/16 : 0-phosphatidylcholine. Egg phosphatidylcholine was isolated from hen eggs according to established procedures. Essential phospholipid (phosphatidylcholine isolated from soya bean), was generously supplied by Dr H. Eikermann of Natterman and Cie, Köln, Germany. Cholesterol was

obtained from Fluka (Buchs, Switzerland) and was recrystallised twice from absolute ethanol. Essential phospholipid contained approx. 3% of lysophosphatidylcholine, but all other lipids were at least 99% pure as indicated by thin-layer chromatography.

Chemicals. $^2\text{H}_2\text{O}$ was obtained from Ryvan, Southampton, U.K. and neodymium nitrate from Koch Light, Colnbrook, Bucks, England. All other chemicals were analar grade.

Preparation of liposomes and vesicles. Liposomes were prepared by dispersing 50 μmol of phosphatidylcholine in 1.6–1.8 ml $^2\text{H}_2\text{O}$ containing 25 mM Tris/acetic acid ($\text{p}^2\text{H} = 7.0$) and 0.2 mM EDTA and vortexing above the transition temperature of the lipid. Vesicles were formed by sonicating the liposome dispersion until complete clearness (3–6 min) with a DAWE soniprobe, power setting 2, under a stream of N_2 . The sonication vial was immersed in a water bath held at 0 °C in the case of the unsaturated lipids or at a temperature approx. 5 °C above the transition temperature in the case of the other lipids. The sonicated vesicle preparation was subsequently centrifuged at $20\,000 \times g$ for 10 min in order to remove titanium (introduced by the sonication probe) and any residual liposomes. The resulting translucent vesicle preparation was then immediately used in the differential scanning calorimetry or NMR experiments.

Phosphorus determinations [21] showed the final phosphatidylcholine concentration to be 27–35 mM. The lipids did not show any decomposition during sonication and subsequent differential scanning calorimetry and NMR experiments as evidenced by thin-layer chromatography on silica gel G using chloroform/methanol/water (65 : 35 : 4, v/v) as solvent.

Differential scanning calorimetry. Thermal transitions in liposomes and vesicles were measured as described previously [22] on a Perkin-Elmer DSC-2B calorimeter, using heating and cooling rates of 4 °C/min and sensitivity ranges 0.2 or 0.5. When the sample pan was put in the sample holder great care was taken to prevent the sample pan from touching any of the liquid nitrogen cooled parts of the sample holder, otherwise the sample could freeze which induces vesicle aggregation. At least three heating and three cooling curves were recorded which were superimposable, except for a small shift of the transition to lower temperatures in the cooling curves (see Fig. 2), possibly caused by undercooling.

Nuclear magnetic resonance. Two ^{31}P NMR spectrometers were employed in this investigation, both of which were interfaced with Nicolet B-NC-12 computers and operated in the Fourier transform mode. The lower frequency (36.4 MHz) machine was a Bruker WH-90 spectrometer equipped with temperature control, broad band proton decoupling and field stabilisation via a deuterium lock. The higher frequency 129 MHz instrument was built in this laboratory [23] and was similarly equipped except for proton decoupling. Accumulated free induction decays were obtained from up to 1500 transients with a 4-s interpulse time. Triphenylphosphine (in chloroform), in a central capillary insert in the NMR sample tube, was used as an external standard.

As previously reported [13, 14] the 36.4 MHz ^{31}P NMR spectra of 16 : 0/16 : 0-phosphatidylcholine vesicles above the transition temperature show two resonances separated by about 0.15 ppm which correspond to 16 : 0/16 : 0-phosphatidylcholine molecules on the inside and outside layer of the vesicle bilayer membrane (Fig. 1a). The resolution between these resonances may be considerably enhanced

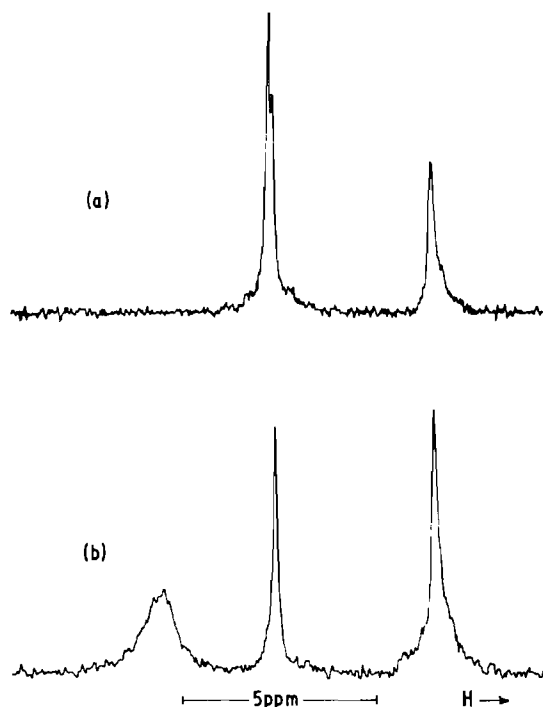


Fig. 1. ^{31}P NMR spectra of 16 : 0/16 : 0-phosphatidylcholine vesicles at 55 °C before (a) and after (b) the addition of 4 mM Nd^{3+} . The peak at high field is from the external reference triphenylphosphine.

by the addition of the shift reagent $\text{Nd}(\text{NO}_3)_3$ to the vesicle solution [24], which causes a downfield shift of the outside resonance (Fig. 1b). In such situations, a computer integration of the spectra gives a measure of the intensity of the outside resonance relative to that of the inside resonance. When saturation effects can be neglected (i.e. when the interpulse time is greater than $5T_1$) the outside/inside signal intensity ratio gives a measure of the ratio of the number of phospholipid molecules on the outside to that on the inside of the vesicle. We found that proton decoupling introduces a systematic error in the measured values of the outside/inside signal intensity ratio by enhancing the relative intensity of the unshifted resonance. This was interpreted as due to a nuclear Overhauser enhancement [25] of the unshifted resonance. For the shifted resonance the paramagnetic cation provides the dominant mode of relaxation of the phosphorus nucleus and such an enhancement would not be expected. Under our experimental conditions (interpulse time 4 s) it was found that the measured outside/inside signal intensity ratio in the presence of proton decoupling should be increased by a factor of 1.25 to obtain the true outside/inside ratio $R_{o/i}$.

RESULTS

Phase transitions in phosphatidylcholine vesicles

Sonication of 16 : 0/16 : 0-phosphatidylcholine liposomes does not signifi-

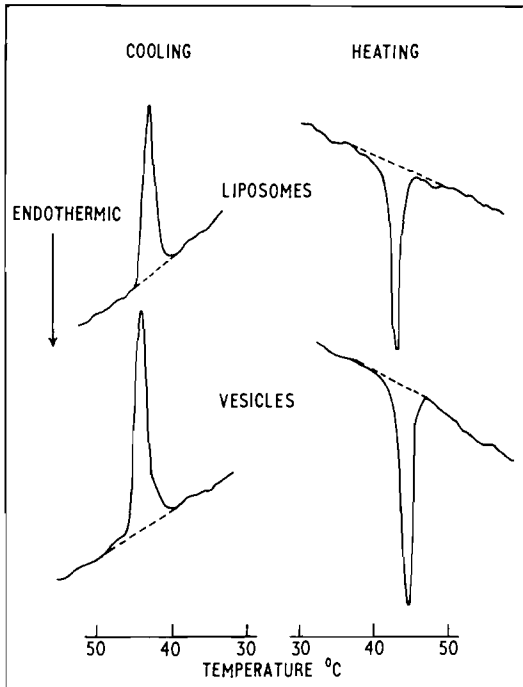


Fig. 2. Differential scanning calorimetry of 16 : 0/16 : 0-phosphatidylcholine liposomes and vesicles. Both cooling and heating scans are presented.

cantly affect the gel \rightarrow liquid crystalline phase transition as detected by calorimetry (see Fig. 2). The melting and freezing of the chains in the liposomes and vesicles occurs at the same temperature and has the same heat content e.g. 8.6 ± 0.2 kCal/mol for the liposomes and 8.9 ± 0.3 kCal/mol for the vesicles. Previously reported values for the heat content of the phase transition in 16 : 0/16 : 0-phosphatidylcholine liposomes are 8.7 [34], 9.7 [35], 8.5 [36], 9.0 [37] and 8.6 kCal/mol [38]. Under the conditions of these experiments (which are the same conditions as those used for the NMR experiments described later), no pronounced pretransition could be detected in the liposomes and vesicles which might be caused by the relative low lipid concentration (compare ref. 36). Preparation of the liposomes and vesicles in the presence of 50 % (v/v) ethylene glycol, in order to prevent freezing of water, did not alter the calorimetric behaviour. As shown in Fig. 3, the temperature and width of the transition of 14 : 0/14 : 0-phosphatidylcholine liposomes and vesicles are also very similar.

The amount of sample used in the calorimetric experiment is too small for quantitative analysis of possible vesicle aggregation. However, no aggregation (increased turbidity) was seen in the sample after the scans. Quantitative data about possible aggregation of vesicles during heating and cooling the sample was obtained by ^{31}P NMR of 16 : 0/16 : 0-phosphatidylcholine vesicles.

Vesicles prepared and measured at 55 °C have a ^{31}P NMR spectrum as in Fig. 1a with a relative intensity of the sum of both the resonances coming from the outside and inside layers of 100. Cooling the sample at a rate of 4 °C/min to 10 °C

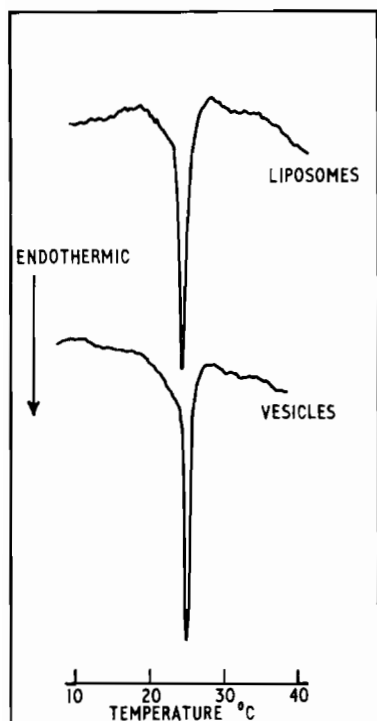


Fig. 3. Differential scanning calorimetry of 14 : 0/14 : 0-phosphatidylcholine liposomes and vesicles. Only heating scans are shown.

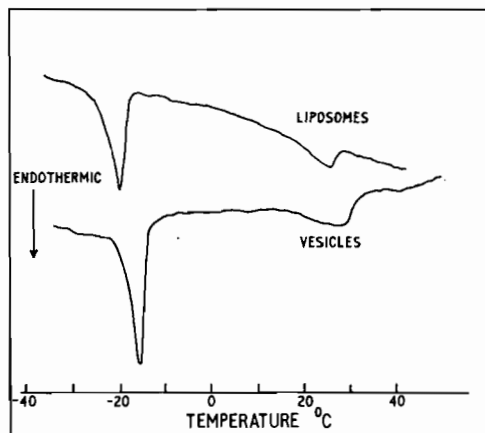


Fig. 4. Differential scanning calorimetry of 16 : 0/16 : 0-phosphatidylcholine-18 : 1c/18 : 1c-phosphatidylcholine (1 : 1) liposomes and vesicles. The liposomes and vesicles were prepared in the presence of 50 % (v/v) of ethylene glycol. Only heating scans are shown.

and heating again at the same rate to 55 °C (as is done in the calorimetric experiment) had no effect on the width and intensity of the signal. Repeating the cooling and heating of the sample for three times only decreased the relative intensity, measured at 55 °C to 96 % whereas the linewidth did not change significantly. Freezing and thawing the sample causes a strong increased turbidity due to vesicle aggregation. The relative intensity of the ^{31}P signal decreased to 19 % demonstrating the formation of large aggregates (liposomes) which ^{31}P NMR signal is too broad to be detected under the conditions used. The residual 19 % of the intensity probably arises from non-aggregated vesicles. This experiment demonstrates that under the conditions of the calorimetric experiment no significant aggregations occur.

Liposomes of equimolar amounts of 18 : 1c/18 : 1c-phosphatidylcholine and 16 : 0/16 : 0-phosphatidylcholine show phase separation [33] as the difference between the transition temperatures of 18 : 1c/18 : 1c-phosphatidylcholine (−20 °C) and 16 : 0/16 : 0-phosphatidylcholine (40 °C) is too large to allow cocrystallization of the fatty acid chains. In such a situation, two phase transitions are observed (Fig. 4). The phase transition of 16 : 0/16 : 0-phosphatidylcholine, in the mixture, is broadened and shifted to lower temperatures because of interactions with 18 : 1c/18 : 1c-phosphatidylcholine (compare with Fig. 2) which remains in the liquid-

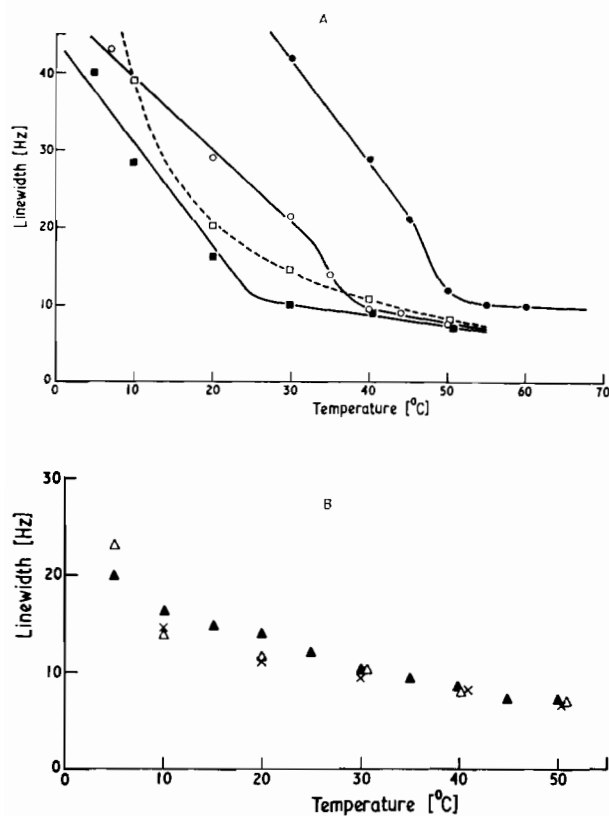


Fig. 5. Temperature dependence of the 36.4 MHz ^{31}P NMR linewidths of phosphatidylcholine vesicles with varying fatty acid compositions: (a) vesicles of the saturated phosphatidylcholines: ●, 18:0/18:0-phosphatidylcholine; ○, 16:0/16:0-phosphatidylcholine; ■, 14:0/14:0-phosphatidylcholine; □, 12:0/12:0-phosphatidylcholine; (b) vesicles of the unsaturated phosphatidylcholines: △, 18:1t/18:1t-phosphatidylcholine; ▲, 18:1c/18:1c-phosphatidylcholine; ×, 16:1c/16:1c-phosphatidylcholine. The indicated linewidths are the full widths at half height of the composite spectrum arising from the resonances of the outside and inside phosphatidylcholine molecules. The estimated error in the linewidth is 5%, except for 18:0/18:0-phosphatidylcholine below 50 °C where visible aggregation occurs. The transition temperatures of 18:0/18:0-phosphatidylcholine, 16:0/16:0-phosphatidylcholine, 14:0/14:0-phosphatidylcholine and 18:1t/18:1t-phosphatidylcholine, measured with calorimetry are: 58 °C [12], 40 °C (fig. 2), 24 °C (fig. 3) and 5 °C [27].

crystalline state down to -20 °C. Sonication of these liposomes produced vesicles which also clearly demonstrated phase separation (Fig. 4). The 18:1c/18:1c-phosphatidylcholine transition in these vesicles occurs at a somewhat higher temperature than in the liposomes.

The temperature dependence of the 36.4 MHz ^{31}P NMR linewidths of vesicles prepared from various phosphatidylcholines is shown in Figs 5a and 5b. Above the possible phase transitions the linewidths are comparable for all the species of vesicles except 12:0/12:0-phosphatidylcholine. These vesicles showed significant visible aggregation as reported previously [26], which results in broader ^{31}P

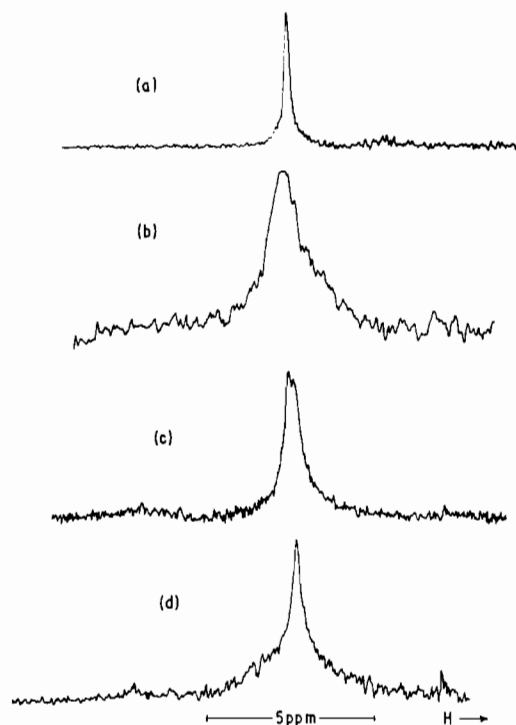


Fig. 6. 36.4 MHz ^{31}P NMR spectra at 10 °C of (a) 18 : 1c/18 : 1c-phosphatidylcholine vesicles, (b) 16 : 0/16 : 0-phosphatidylcholine vesicles, (c) 16 : 0/16 : 0-phosphatidylcholine-18 : 1c/18 : 1c-phosphatidylcholine (1 : 1) vesicles and (d) 16 : 0/16 : 0-phosphatidylcholine-18 : 1c/18 : 1c-phosphatidylcholine vesicles in the presence of 6 mM Co^{2+} .

NMR resonance lines. Below the respective phase transition temperatures (see legend of Fig. 5) the ^{31}P spectra of 14 : 0/14 : 0-phosphatidylcholine, 16 : 0/16 : 0-phosphatidylcholine and 18 : 0/18 : 0-phosphatidylcholine show a significant increase in linewidth, indicating that the motion of the phosphate group becomes progressively restricted. It was noted that above the phase transition temperature the chemical shift difference between the 'outside' and 'inside' phospholipid resonances (see Materials and Methods) was independent of the fatty acid composition. Below the transition temperature resonances from the outside and inside phosphatidylcholines could not be resolved.

Effects corresponding to lateral phase separation in vesicles composed of mixed phospholipid species may also be observed with ^{31}P NMR. Fig. 6 shows the ^{31}P NMR spectra of 18 : 1c/18 : 1c-phosphatidylcholine (Fig. 6a), 16 : 0/16 : 0-phosphatidylcholine (Fig. 6b) and 16 : 0/16 : 0-phosphatidylcholine-18 : 1c/18 : 1c-phosphatidylcholine (1 : 1) vesicles (Fig. 6c) at 10 °C. The spectrum of the 16 : 0/16 : 0-phosphatidylcholine-18 : 1c/18 : 1c-phosphatidylcholine (1 : 1) vesicles is composed of a broad line, which is ascribed to 16 : 0/16 : 0-phosphatidylcholine molecules in the gel state and two narrower components, presumably due to the liquid crystalline 18 : 1c/18 : 1c-phosphatidylcholine molecules on the inside and outside of the vesicle (compare with Fig. 4). The outside resonances can be broadened beyond detection

TABLE I

DISTRIBUTION OF PHOSPHATIDYLCHOLINE ON THE OUTSIDE AND INSIDE LAYERS OF PHOSPHATIDYLCHOLINE VESICLES

$$R_{o/i} = \frac{\text{amount of phosphatidylcholine outside monolayer}}{\text{amount of phosphatidylcholine inside monolayer}}$$

All $R_{o/i}$ measurements at 30 °C, except for the 16 : 0/16 : 0-phosphatidylcholine and 18 : 0/18 : 0-phosphatidylcholine vesicles where $R_{o/i}$ was measured at 50 and 60 °C, respectively. Error in $R_{o/i}$ is 0.05.

Vesicle composition	$R_{o/i}$	distribution of phosphatidylcholine (%)		Membrane thickness (Å)*	Calculated vesicle outer radius (r_o) (Å)
		Outside	Inside		
12 : 0/12 : 0-Phosphatidylcholine	*	—	—	—	—
14 : 0/14 : 0-Phosphatidylcholine	2.65	72.5	27.5	32	84
16 : 0/16 : 0-Phosphatidylcholine	2.2	69	31	37	112
18 : 0/18 : 0-Phosphatidylcholine	1.7	63	37	42	180
16 : 1c/16 : 1c-Phosphatidylcholine	1.8	64.5	35.5	28	110
18 : 1c/18 : 1c-Phosphatidylcholine	1.75	63.5	36.5	32	131
18 : 1t/18 : 1t-Phosphatidylcholine	2.0	67	33	36	123
Egg phosphatidylcholine	2.0	66.5	33.5	35	120
Essential phospholipid	2.0	67	33	35	118
16 : 0/16 : 0-Phosphatidylcholine					
16 : 1c/16 : 1c-Phosphatidylcholine (1 : 1)	1.9	65.5	34.5		—
18 : 0/18 : 0-Phosphatidylcholine					
16 : 1c/16 : 1c-Phosphatidylcholine (1 : 1)	1.85	65	35		—
16 : 0/16 : 0-Phosphatidylcholine					
1-16 : 0-lysophosphatidylcholine (1 : 10)	2.4**	70.5	29.5	—	107

* See text.

** Distribution of the sum of 16 : 0/16 : 0-phosphatidylcholine and 1-16 : 0-lysophosphatidylcholine.

by the addition of Co^{2+} [13]. The resultant spectrum of the inside resonances at 10 °C, as shown in Fig. 6a is composed of a broad and a narrow line, indicating the occurrence of phase separation in the inside monolayer of this vesicle. Fig. 6 also demonstrates that the chemical shift difference between the outside and inside resonances of the narrow component is much larger than the chemical shift difference between the outside and inside resonances of 18 : 1c/18 : 1c-phosphatidylcholine vesicles. This was also observed for other mixtures which showed phase separation e.g. 16 : 0/16 : 0-phosphatidylcholine-16 : 1c/16 : 1c-phosphatidylcholine (1 : 1) and 18 : 0/18 : 0-phosphatidylcholine-16 : 1c/16 : 1c-phosphatidylcholine (1 : 1).

Outside/inside distributions in phosphatidylcholine vesicles

In Table I the ratio of the number of phosphatidylcholine molecules on the outside of the vesicle to the number on the inside of the vesicle ($R_{o/i}$) are presented for various fatty acid compositions. The data on 16 : 0/16 : 0-phosphatidylcholine and egg phosphatidylcholine vesicles agree reasonably well with previous results. The ratio $R_{o/i}$ of 16 : 0/16 : 0-phosphatidylcholine vesicles has been reported using proton NMR as 2.2 [10] and 1.8 [28] whereas in proton NMR and ^{31}P NMR studies

$R_{o/i}$ values of 2.2 [10], 1.85–1.95 [17], 2.31 [26], 1.9 [29] and 1.85–1.98 [24] have been observed for egg phosphatidylcholine vesicles. The results from phosphatidylcholine vesicles with saturated chains show that $R_{o/i}$ decreases with increasing chain length (see Table 1). For 12 : 0/12 : 0-phosphatidylcholine vesicles $R_{o/i}$ could not be determined as these vesicles are permeable to shift reagents. Immediately after the addition of $\text{Nd}(\text{NO}_3)_3$ only one downfield shifted resonance was observed. This instability of the 12 : 0/12 : 0-phosphatidylcholine vesicle bilayer is in agreement with earlier work [26]. As also shown in Table I, the introduction of a *cis* double bond in the chains tends to decrease $R_{o/i}$, whereas the presence of a *trans* double bond slightly increases $R_{o/i}$. Vesicles which are composed of more than one molecular species have outside/inside distributions which are intermediate between the $R_{o/i}$ values of vesicles consisting of the individual species. Incorporation of 10 % 1-16 : 0-lysophosphatidylcholine in 16 : 0/16 : 0-phosphatidylcholine vesicles increases $R_{o/i}$. 16 : 0/16 : 0-phosphatidylcholine vesicles with 20 mol % 1-16 : 0-lysophosphatidylcholine were found to be very unstable, for although sonication produced a clear solution, all lipids separated out of solution within minutes after the sonication.

If the membrane thickness is known, the outer radius of the vesicle can be calculated from the measured $R_{o/i}$ values assuming identical packing densities in the outside and inside layers of the vesicle. This assumption seems justified by the calorimetric experiments as the width of the phase transition in vesicles is as narrow as that observed for the liposomes. The phase transition in the outside and inside monolayer must therefore occur at approximately the same temperature, which suggests that the packing densities are similar on both sides of the vesicle membrane. The thickness of the 16 : 0/16 : 0-phosphatidylcholine bilayer has been reported [30] as 37 Å from which the thickness of 14 : 0/14 : 0-phosphatidylcholine and 18 : 0/18 : 0-phosphatidylcholine bilayers were extrapolated to be 32 and 42 Å, respectively. A membrane thickness of 35 Å has been reported for egg phosphatidylcholine [31]. Using these data the 16 : 1c/16 : 1c-phosphatidylcholine, 18 : 1c/18 : 1c-phosphatidylcholine, 18 : 1t/18 : 1t-phosphatidylcholine and essential phospholipid bilayer thickness were estimated to be 28, 32, 36 and 35 Å, respectively. The calculated outer radius (r_o) of the egg phosphatidylcholine vesicle (Table I) is close to the value of 125 Å reported by Huang [2]. The 18 : 0/18 : 0-phosphatidylcholine vesicle is calculated to be the largest and decreasing chain length gives smaller vesicles. The unsaturated phosphatidylcholine vesicles of 18 carbon atoms chain length have a somewhat smaller r_o than the vesicles of the saturated analog.

Cholesterol is a rigid molecule and can be expected to influence the vesicle size and/or $R_{o/i}$ when incorporated into the vesicle. Gel filtration [11] and sedimentation experiments [8] have demonstrated that the size of essential phospholipids and egg phosphatidylcholine vesicles increases by about 60 % when 50 mol % cholesterol is incorporated. Furthermore it was shown recently with freeze etch electron microscopy of egg phosphatidylcholine-cholesterol vesicles that at cholesterol concentrations up to 50 mol% the vesicles remain spherical (Forge, A., Knowles, P. F. and Marsh, D., unpublished observations). An increase in the size of phosphatidylcholine vesicles due to the incorporation of cholesterol is also shown by ^{31}P NMR. The 129 MHz ^{31}P NMR spectra of 50 mol% cholesterol containing 14:0/14:0-phosphatidylcholine and 18:1c/18:1c-phosphatidylcholine vesicles were approx. 12 times broader than at 36.4 MHz. This can be compared to the ^{31}P NMR spectra of pure

TABLE II
EFFECT OF CHOLESTEROL UPON THE DISTRIBUTION OF PHOSPHATIDYLCHOLINE ON THE OUTSIDE AND INSIDE
LAYERS OF PHOSPHATIDYLCHOLINE VESICLES

$R_{o/i}$ measurements were done at 30 °C.

Vesicle composition	$R_{o/i}$ of phosphatidylcholine	Distribution of phosphatidylcholine (%)		Calculated composition* of			
		Outside	Inside	Outer layer (mol %)		Inner layer (mol %)	
				Phosphatidylcholine	Cholesterol	Phosphatidylcholine	Cholesterol
14 : 0/14 : 0-phosphatidylcholine	2.65	72.5	27.5				
14 : 0/14 : 0-phosphatidylcholine + 50 mol % cholesterol	1.90	66.0	34.0	57	43	41	59
18 : 1c/18 : 1c-phosphatidylcholine	1.75	63.5	36.5				
18 : 1c/18 : 1c-phosphatidylcholine † 15 mol % cholesterol	1.75	63.5	36.5				
18 : 1c/18 : 1c-phosphatidylcholine † 30 mol % cholesterol	2.15	68.5	31.5				
18 : 1c/18 : 1c-phosphatidylcholine † 50 mol % cholesterol	2.95	75.0	25.0	67	33	28	72

* In order to obtain the distribution of cholesterol and 14 : 0/14 : 0-phosphatidylcholine and 18 : 1c/18 : 1c-phosphatidylcholine over the vesicle membrane, literature data on egg phosphatidylcholine and egg phosphatidylcholine-cholesterol (1 : 1) bilayers was used. The membrane thickness of the phosphatidylcholine-cholesterol (1 : 1) bilayer is 45 Å [8] and the outer radius of the phosphatidylcholine-cholesterol (1 : 1) vesicles is 220 Å [8]. The areas of the outer and inner layer of the vesicle can be calculated from these data. The number of cholesterol and phosphatidylcholine molecules in the outer and inner layer are then obtained using the areas per molecule of cholesterol (38 Å) [19] and phosphatidylcholine in the absence (72 Å) or presence of 50 mol % cholesterol (46.7 Å) [31].

16 : 0/16 : 0-phosphatidylcholine vesicles which are about five times broader at 129 MHz than at 36.4 MHz [14]. The stronger field dependence of the ^{31}P NMR signal of the cholesterol-containing vesicles is interpreted as due to a larger contribution of the chemical shift anisotropy relaxation mechanism to the linewidth, which results from the longer vesicle tumbling time for larger vesicles [14]. Larger phospholipid vesicles will obviously show smaller $R_{o/i}$ values, as there is less difference between the areas of the inner and outer bilayer surface. For the enlarged cholesterol-containing vesicles therefore a decreased $R_{o/i}$ value would be expected. This is observed for the 14 : 0/14 : 0-phosphatidylcholine-cholesterol (1 : 1) vesicles (Table II). However, for 50 mol % cholesterol containing 18 : 1c/18 : 1c-phosphatidylcholine vesicles, $R_{o/i}$ increases which can only result from an asymmetric distribution of cholesterol and 18 : 1c/18 : 1c-phosphatidylcholine over the vesicle membrane, with a disproportionately greater amount of cholesterol on the inside. The extent of asymmetry in these vesicles can be calculated (Table II) and it is found that there is almost three times as much cholesterol as phosphatidylcholine on the inner surface of the vesicle. At lower (less than 30 mol %) cholesterol concentrations the asymmetry is much less since $R_{o/i}$ (Table II) and the size of these vesicles [8, 11] are much less affected. The calculated asymmetry in the 14 : 0/14 : 0-phosphatidylcholine-cholesterol (1 : 1) vesicles is small (Table II) and is possibly due to using the data on the egg phosphatidylcholine-cholesterol membranes for the calculation.

DISCUSSION

It has been suggested that the fatty acid chains in the curved vesicles are more disordered than in unsonicated liposomes, which would result in different properties of membranes of vesicles and liposomes. In this study we demonstrated that width and heat content of the gel \rightarrow liquid crystalline phase transition of 16 : 0/16 : 0-phosphatidylcholine and 14 : 0/14 : 0-phosphatidylcholine liposomal bilayers as measured by calorimetry are not significantly affected by sonication. Furthermore, phase separation occurs in mixed vesicles of 16 : 0/16 : 0-phosphatidylcholine-18 : 1c/18 : 1c phosphatidylcholine (1 : 1) as in the unsonicated bilayers. These results are supported by recent fluorescence measurements on 14 : 0/14 : 0-phosphatidylcholine vesicles which also demonstrated that the phase transition occurs at a similar temperature as the transition in 14 : 0/14 : 0-phosphatidylcholine liposomes [9]. These data clearly suggest that the packing in the vesicle membrane is not significantly different from the packing in the unsonicated liposomal membrane. The fatty acid packing in the outside and inside layer must be comparable because of the small width of the phase transition. Since the ^{31}P resonances from the outside and inside layer shows a small chemical shift difference (Fig. 1a), packing of the polar groups on both sides of the vesicles might be slightly different. The packing in the outside and inside layer must be comparable because of the small width of the phase transition. The reason for the discrepancy between these results and the dilatometry result of Sheetz and Chan [3] in which a broadening and a shift of the phase transition to lower temperature was observed in 16 : 0/16 : 0-phosphatidylcholine vesicles is not understood.

Vesicles composed of various well defined phosphatidylcholines differing in chain length and degree of unsaturation were studied to investigate the influence of the fatty acyl residue on the ^{31}P line-width, the occurrence of phase separation and

the outside/inside distribution of the phosphatidylcholine molecules over the membrane.

The ^{31}P NMR linewidths of 14 : 0/14 : 0-phosphatidylcholine, 16 : 0/16 : 0-phosphatidylcholine and 18 : 0/18 : 0-phosphatidylcholine vesicles, are affected by the occurrence of the gel \rightarrow liquid crystalline phase transition (Fig. 5) as previously reported for 16 : 0/16 : 0-phosphatidylcholine vesicles [13, 14]. The phase transition temperatures implied by these linewidth studies are slightly below the phase transition temperature as measured with calorimetry. This could indicate that the phase transition measured with ^{31}P NMR is associated with the pretransition temperature [35].

In the liquid crystalline state the linewidths of the various vesicles are comparable. These results demonstrate that the motion of the phosphate group in the gel state is more restricted as compared with the motion of the phosphate group in the liquid crystalline state [14]. From ^{31}P NMR measurements at 129 MHz of various synthetic and natural phosphatidylcholines in the liposome form a more quantitative description of the motions of the phosphate group can be obtained (Cullis P. R. and de Kruijff, B., unpublished).

^{31}P NMR can also monitor phase separation in mixed vesicles of 16 : 0/16 : 0-phosphatidylcholine and 18 : 1c/18 : 1c-phosphatidylcholine. Lateral phase separation can be observed separately in the outside and inside monolayer (Fig. 6) by using the appropriate shift or broadening reagent, a possibility which techniques such as calorimetry cannot provide. ^{31}P NMR can also be employed to decide whether in a biological membrane the motion of the polar part of the lipid molecules is affected by the phase transition. In this regard we are presently investigating the temperature dependence of the ^{31}P NMR resonances of membranes of *Acholeplasma laidlawii* cells grown on various fatty acids.

The ratio of the number of phosphatidylcholine molecules on the outside of the vesicle to the number on the inside of the vesicle is dependent upon the chain length of the phosphatidylcholine molecule (Table 1). Longer chains produce a vesicle with a smaller $R_{o/i}$ and larger size. It would appear that 18 : 0/18 : 0-phosphatidylcholine vesicles are larger than 14 : 0/14 : 0-phosphatidylcholine vesicles because of the possibility of more van der Waals interaction between the 18 : 0 chains in a larger vesicle. The presence of double bonds only influences $R_{o/i}$ slightly and the calculated size of the vesicles is somewhat decreased as for the shorter chain phosphatidylcholines.

Since the size of the vesicle is dependent upon the fatty acid composition, care must be taken in interpreting the results of outside/inside distributions in vesicles composed of lipids with complex fatty acid compositions [15-17].

The incorporation of cholesterol in vesicles of 14 : 0/14 : 0-phosphatidylcholine and 18 : 1c/18 : 1c-phosphatidylcholine increases the vesicle size and influences $R_{o/i}$ of phosphatidylcholine. The effect on $R_{o/i}$ is, however, different for 14 : 0/14 : 0-phosphatidylcholine and 18 : 1c/18 : 1c-phosphatidylcholine vesicles. In 14 : 0/14 : 0-phosphatidylcholine vesicles $R_{o/i}$ decreases, demonstrating that cholesterol is more or less equally distributed on both sides of the vesicle. In 18 : 1c/18 : 1c-phosphatidylcholine-cholesterol (1 : 1) vesicles, however, $R_{o/i}$ is increased, which implies that cholesterol prefers the inside monolayer of the vesicle. A similar phenomenon was noted in a proton NMR study of Huang et al. [10] for 16 : 0/16 : 0-phosphatidylcholine-cholesterol and egg phosphatidylcholine-cholesterol vesicles. Only for the

egg phosphatidylcholine-cholesterol vesicles did $R_{o/i}$ increase. This strongly suggests a difference in the cholesterol-phosphatidylcholine interaction between saturated and unsaturated phosphatidylcholine molecules, especially for cholesterol concentrations of more than 30 mol %. The inside monolayer of the 18 : 1c/18 : 1c-phosphatidylcholine-cholesterol (1 : 1) vesicle is calculated to contain about three times as much cholesterol as 18 : 1c/18 : 1c-phosphatidylcholine (Table II). This demonstrates the possibility of incorporating more than 50 mol % cholesterol in one monolayer of a bilayer without disrupting that bilayer. These vesicles were very stable as 16 h (at 30 °C) after the addition of Nd^{3+} all inside resonances were as sharp as in the absence of Nd^{3+} , demonstrating that the permeability barrier of these vesicles is intact. In the myelin membrane cholesterol is asymmetrically distributed [32]. The possibility of generating model membranes which have an asymmetric cholesterol distribution therefore offers interesting possibilities for studying the effect of this asymmetry on membrane properties.

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