# EFFECT OF CHAIN LENGTH ON THE STABILITY OF LECITHIN BILAYERS

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SUMMARY: The shift reagent $NdCl_3$ was added to vesicles of synthetic, saturated ($DiC_{10}$-$C_{16}$) lecithins and egg lecithin and the accessibility of the $N(CH_3)_3$ groups to $Nd^{3+}$ ions was studied by NMR. Long chain lecithins, e.g. dipalmitoyl and egg lecithin form bilayers "stable" on the time scale of our experiments and practically impermeable to cations. Short chain lecithins on the other hand form short-lived vesicles surrounded by unstable bilayers which are not effective cation barriers. Ion transport across the latter lecithin bilayers may involve, besides passive diffusion, collision-induced transient rupture and resealing of bilayers coupled with ion movement.

The stability of phospholipid bilayers is a subject of considerable relevance to the study both of lipid-protein interactions and of the ion permeability of biological membranes.

It is now well established that phospholipid bilayers such as those of egg lecithin and ox brain phosphatidylserine are extremely impermeable to cations (1,2,3). Changes in permeability through variations of polar head groups (4) and of fatty acid chain length and unsaturation have received considerable attention (5). Van Deenen and his collaborators have shown that lecithin bilayers become increasingly permeable to glycerol and glucose as the chain length decreases or the unsaturation of the hydrocarbon chains increases (5,6,7).

The results presented below are relevant to the problem of measuring intrinsic bilayer permeabilities and throw light on some kinetic properties of phospholipid bilayers.

## MATERIALS AND METHODS:

$DiC_{10}$ and $DiC_{12}$PC were prepared by acylation of L-α-glycerylphosphorylcholine cadmium chloride with the appropriate acid chloride (8). $DiC_{14}$ and $DiC_{16}$PC were purchased from Koch Light Ltd., and if necessary purified according to Dawson (9).

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Abbreviations:

$DiC_{10}$PC = Dicapryl lecithin: $DiC_{12}$PC = Dilauroyl lecithin: $DiC_{14}$PC = Dimyristoyl lecithin: $DiC_{16}$PC = Dipalmitoyl lecithin: EYL = Egg yolk lecithin: NMR = Nuclear magnetic resonance.

For NMR experiments 1% (w/v) phospholipid dispersions were prepared by adding $^2H_2O$ (at room temperature for $DiC_{10}$–$C_{14}PC$ and at ~ 45°C for $DiC_{16}PC$) to the weighed amount of the dry lipid; the dispersion was shaken for about 15 min and 1 ml of it was sonicated for 6 min with an MSE 150 Watt Ultrasonic Disintegrator (Measuring and Scientific Equipment, Crawley, U.K.) using a microtip at low power level (nominal frequency 20 KHz). Sonication did not cause any significant chemical decomposition of the lecithins. The lecithin samples for gel filtration and electron microscopy were dispersed in buffer ($10^{-2}$M Tris pH 8.6, $10^{-3}$M EDTA, 0.02% sodium azide) as described above. NMR spectra were obtained on a Varian XL–100 spectrometer operating at 100 MHz for protons. All experiments were carried out at room temperature except that with $DiC_{16}PC$ which was done at 45°C. Analytical gel filtration on Sepharose 4B and electron microscopy are described elsewhere (16).

RESULTS:

Fig. 1 shows high resolution spectra of sonicated lecithins and the downfield shift of part of the $N(CH_3)_3$ signal introduced after adding $10^{-2}$M $NdCl_3$ (dotted line). Not shown in Fig. 1 are the downfield shifts of part of the $CH_2N$ and $CH_2OP$ (choline) groups which are also affected in the presence of $Nd^{3+}$. However no chemical shift changes of the signals from the hydrocarbon chains were observed. With $DiC_{10}PC$ a single, broadened $N(CH_3)_3$ peak shifted downfield by 4.5 Hz was observed, with $DiC_{12}PC$ a single, broadened peak shifted downfield by 6.3 Hz with a shoulder at high field, and with $DiC_{14}PC$ and $DiC_{16}PC$ two distinct, sharp peaks about 7.5 Hz apart were observed. Part of the EYL $N(CH_3)_3$ signal was also shifted downfield (10,12,15,21). With $DiC_{14}PC$, $DiC_{16}PC$ and EYL the relative areas of the two peaks of the $N(CH_3)_3$ group depended on the intensity and duration of the sonication, e.g. with single shelled EYL vesicles of 250 Å diameter obtained under sonication conditions described before (11) the average intensity ratio was 2.3:1. Exactly the same figures were obtained for the ratio of EYL molecules on the outer and inner layer of the bilayer derived from the titration of single shelled vesicles with $Mn^{++}$ (13). It was demonstrated that the downfield peak of the $N(CH_3)_3$ signal in Fig. 1 originated from the accessible outer surface of the bilayer by adding increasing amounts of $Mn^{++}$; this progressively broadened and at $Mn^{++}$ concentrations of $10^{-3}$ – $10^{-2}$M completely abolished the more intense low field peak. Above a certain threshold concentration of $Nd^{3+}$ the intensity ratio of the two $N(CH_3)_3$ signals remained constant while the downfield shift of the more intense peak increased with increasing $Nd^{3+}$ concentration approaching asymptotically a saturation value. Also shown in Fig. 1 (lines labelled a) are the $N(CH_3)_3$ signals obtained 18 – 19 hours after adding $Nd^{3+}$. While

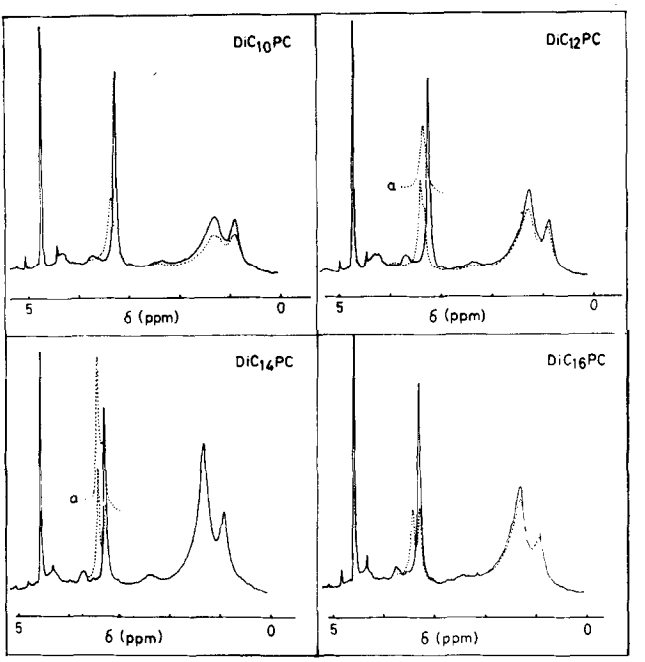


Fig. 1. 100 MHz proton spectra of $DiC_{10}PC$, $DiC_{12}PC$, $DiC_{14}PC$ and $DiC_{16}PC$ (1% w/v) dispersions in $D_2O$ - solid lines; 4–8 minutes after addition of $10^{-2}$M $NdCl_3$ - dotted lines; 18–19 hours after addition of $10^{-2}$M $NdCl_3$ - dotted lines marked 'a'. Spectra were obtained using a Fourier transform accessory - 100 transients, acquisition time - 2 seconds, pulse width - 50 μsec.

over this period of time no change was observed with $DiC_{10}PC$ and $DiC_{16}PC$, the shoulder of the $N(CH_3)_3$ signal of $DiC_{12}PC$ disappeared and the low field peak of the $N(CH_3)_3$ signal of $DiC_{14}PC$ grew at the expense of the highfield peak.

Fig. 2a shows the Sepharose 4B elution profile of a $DiC_{12}PC$-dispersion sonicated for 6 min. The first peak at the void volume $V_o$ = 58 ml represents particles of Stokes radius $r \gtrsim 280$ Å and the broad peak centred at an elution volume $V_e$ = 80 ml represents particles with a Stokes radius of about 140 Å. Turbidity measurements confirm that both large, multilamellar particles and small vesicles were present. In contrast to longer chain ($C_{16}$) lecithins (2,14) no radioactivity was associated with the two peaks containing the $DiC_{12}PC$ indicating that the $DiC_{12}PC$ bilayers are unable to retain trapped $^{22}Na^+$ during the gel filtration experiment. When the intensity and/or the duration of the sonication was increased no significant reduction in the size of the $DiC_{12}PC$ particles was observed (Fig. 2b) and as before no $^{22}Na^+$ radioactivity was detected in the regions of the phospholipid peaks. Fig. 3a is an electron micrograph of a

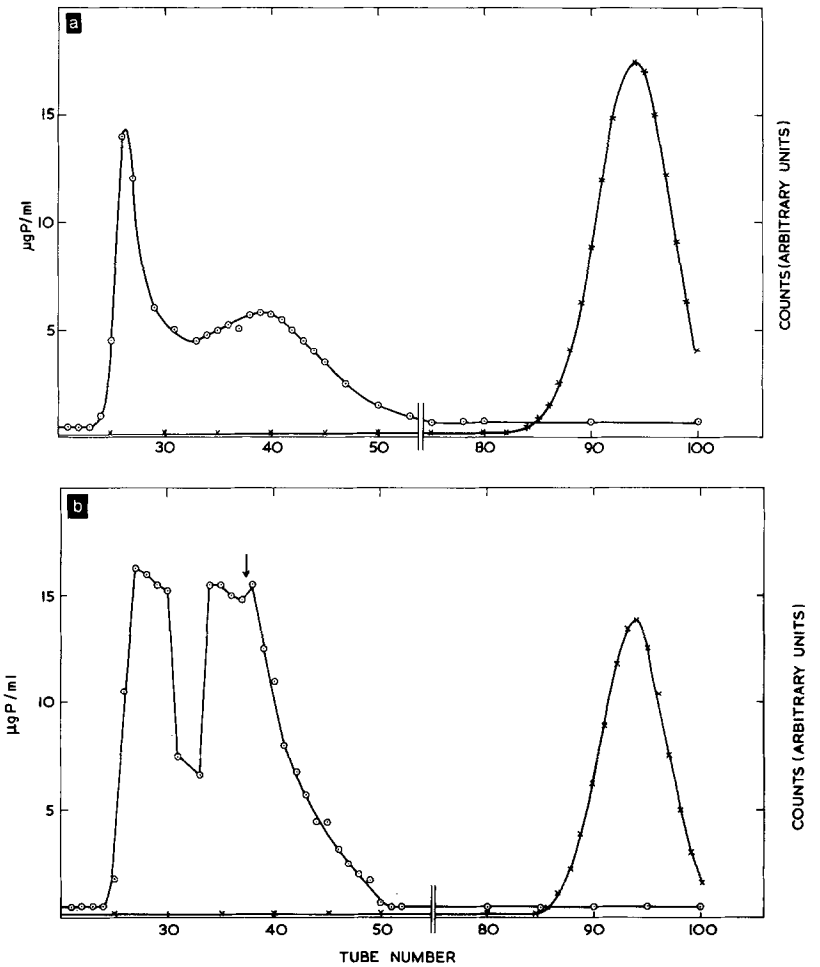


Fig. 2. Analytical gel filtration on Sepharose 4B (column: 33 x 2.5 cm). Elution was carried out with buffer ($10^{-2}$M Tris pH 8.6, $10^{-3}$M EDTA, 0.01% sodium azide) at 4°C and 2 ml fractions were analysed for phosphorus (O—O) and $^{22}Na^{+}$ content (X—X). The 1% (w/v) $DiC_{12}PC$ dispersions in Fig. 2a and b were prepared as described in the text, except that $^{22}NaCl$ (10µCi/ml) was added to the buffer and that the dispersion (volume ~ 3 ml) of Fig. 2b was sonicated for 20 min.

negatively stained preparation of particles present in the second peak (see arrow Fig. 2b). This confirms the lack of single shelled vesicles and the prevalence of particles larger than ~ 300 Å consisting of more than 1 bilayer and usually containing negative stain in the internal compartments. This is quite distinct from EYL or $DiC_{16}PC$ which under similar sonication conditions gave single shelled vesicles (2,13) (diameter ~ 250 Å and 450 Å, respectively) devoid of negative stain in the internal compartment (Fig. 3b).

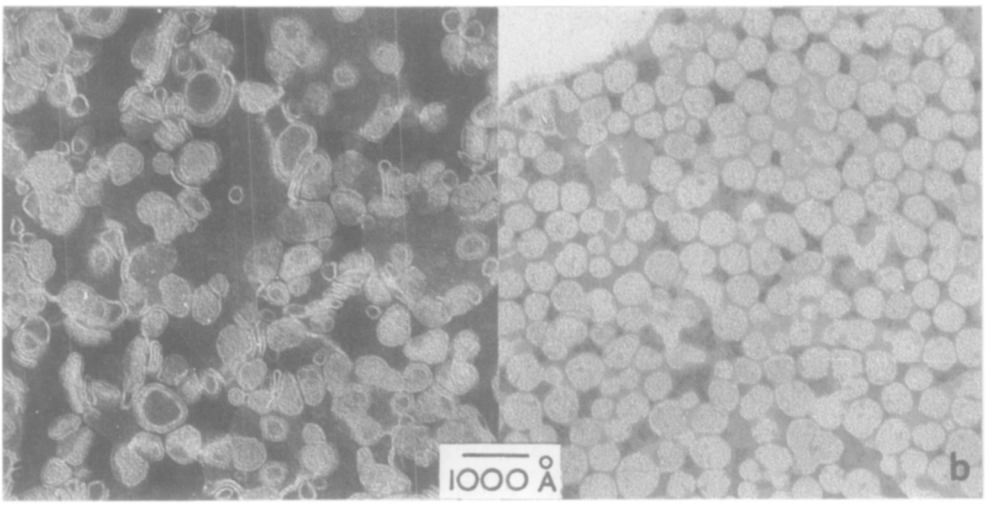


Fig. 3. Electron micrographs of negatively stained (potassium phosphotungstate, pH 7) specimens of
a) A sonicated $DiC_{12}PC$ dispersion which was chromatographed on Sepharose 4B [the fraction eluted at $V_e$ = 75 ml (see arrow in Fig. 2b) was used] .
b) A $DiC_{16}PC$ dispersion sonicated under the same conditions as the $DiC_{12}PC$ sample.

DISCUSSION:

It has been shown (10,15) that the chemical shifts of the choline groups of lecithin molecules move towards low fields when these groups are exposed to the lanthanide ion $Nd^{3+}$. Fig. 1 shows that for the longer chain lecithins the lanthanide ion is not accessible to all of the $N(CH_3)_3$ groups whereas with the shorter chain lecithins a single $N(CH_3)_3$ signal is observed indicating that all of the choline groups are accessible to the lanthanide ion. The difference in behaviour between lecithins of different chain lengths on the addition of the shift reagent, and the time dependence of the effect considered in isolation could be explained in terms of differences in cation permeability (5-7).

Analytical gel filtration of $^{22}Na^+$ labelled $DiC_{12}PC$ particles (Fig. 2a) however showed that neither the large multilamellar aggregates nor the small single-shelled vesicles were capable of retaining the $^{22}Na^+$ ions. Attempts to produce a sample containing only single-shelled vesicles, by increasing the time and/or intensity of sonication failed (Fig. 2). Electron microscopy (Fig. 3a) confirmed that the majority of the particles present in sonicated $DiC_{12}PC$ dispersions were aggregated and multilamellar.

When the NMR results are considered together with the gel filtration and electron microscopy data, an explanation alternative to cation permeability becomes apparent. Although coarse dispersions of short-chain

lecithins may be completely clarified by low-intensity sonication in 1-2 minutes, it is apparent that the vesicles formed are less stable than those from long-chain lecithins. It seems likely that although they form vesicles more readily in an ultrasonic field, the vesicles so formed tend to aggregate and coalesce through collisions induced by Brownian motion, thus resulting in the formation of larger multilamellar particles. This mechanism involving the collision of particles and rupture of bilayers would be enhanced at shorter chain lengths where the interaction energy between the lecithin molecules in the bilayer is lower (17,18,19). The process of rupturing and resealing of bilayers as described would facilitate the release of trapped ions or non electrolytes from internal compartments or conversely allow external ions to reach internal sites.

Thus the transport of ions across bilayers may well take place by two separate mechanisms, i.e. diffusion across the bilayer and collision-induced rupture of bilayers coupled with exchange of ions. The relative contributions of the two mechanisms cannot be separated at this stage. The collision-induced mechanism has been observed previously for egg lecithin and phosphatidylserine (3). The results presented above indicate that this process is chain length dependent and enhanced at shorter chain lengths. Another possible consequence of the reduced stability of short chain lecithin bilayers is an enhanced flip-flop movement (14) of lecithin molecules within the bilayer which exposes molecules from both inner and outer layer of the bilayer to $Nd^{3+}$. This enhanced flip-flop mechanism could also contribute to the increased ion transport across bilayers. The difficulties of measuring intrinsic permeabilities of phospholipid bilayers have been discussed previously (2,3). From this and the current work it would appear that permeability measurements *per se* are only meaningful when any other mechanism but simple passive diffusion can be excluded.

Invoking the collision-induced mechanism explains several experimental observations.

(1) Sonicated dispersions of $DiC_{10}$ and $C_{12}$ lecithins are less stable than those of longer chain lecithins as evident from a significant turbidity increase during storage.

(2) $DiC_{12}PC$ dispersions sonicated under conditions which yield single-shelled vesicles with $DiC_{16}PC$ or EYL, consist mainly of larger, and usually multilamellar particles (Figs. 2 and 3). Electron microscopy shows that the number of single shelled vesicles decreases and concomitantly the number of multilamellar aggregates increases as the time elapsed between sonication and taking the electron micrograph increases.

(3) Sonication of $DiC_{12}PC$ dispersions under conditions which with EYL produced vesicles giving ~ 100% $N(CH_3)_3$ signal (13) gave high resolution spectra in which the intensity of the $N(CH_3)_3$ signal varied greatly, but never reached 100%.

(4) The time dependence (Fig. 1) indicates that $DiC_{16}PC$ bilayers are stable on the time scale of our experiments. This is consistent with the finding of Phillips et al. (19, 20) that in the liquid crystalline state the interaction energy of $DiC_{14}PC$ and higher homologues is sufficient[$\geq$ 6 kcal/mole $\geq$ 10 kT] to warrant a stable association of molecules in the bilayer.

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