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THE INTERACTION OF PARAMAGNETIC IONS AND SPIN LABELS WITH LECITHIN BILAYERS

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SUMMARY

1. In dipalmitoyllecithin vesicles in ^2H_2O, partially overlapping proton NMR resonances are obtained from inward and outward facing $-\overset{+}{N}Me_2$ groups. These resonances can be completely resolved by the addition of Eu^{3+}, Nd^{3+} or UO_2^{2+}.

2. The ratio of metal ion binding sites on the external surface of the vesicles to outward facing lecithin molecules is less than 0.13:1 and more than 0.03:1.

3. The binding of the ions has only small effects in reducing the spin lattice relaxation times (T_1) of the $-\overset{+}{N}Me_3$ protons, but there is a marked increase in the temperature of the lamellar liquid crystalline phase transition, as detected by changes in the linewidths of the proton NMR spectra and by changes in the binding of the spin label TEMPO.

4. Addition of spin labelled lecithin and fatty acids causes a reduction in T_1 values of the protons of the lipid $-\overset{+}{N}Me_3$ groups and fatty acid chains. Lateral diffusion of the lipid molecules in the surface of the bilayer is fast enough to average the T_1 values of all the lipids. Both the lecithin spin label data and the T_1 relaxation measurements are consistent with small differences in the packing of the inward and outward facing $-\overset{+}{N}Me_3$ groups.

INTRODUCTION

We have shown elsewhere[1–5] that ^1H and ^{13}C NMR can be used to study the relative motions of atoms and groups within lecithin molecules incorporated into bilayers. Since, in general, the NMR signals from lipids on the inside and outside of the bilayer overlap, it is normally only possible to obtain information about the average behaviour of the lipids in the two sides of the bilayer. In simple lipid vesicle structures in the absence of proteins, it is expected that any differences in the number and packing of the lipids in the inner and outer layers will be determined primarily by the difference in the radius of curvature for the two surfaces. This difference is appreciable for small sonicated vesicles of approx. 500 Å outer diameter, and results in the significant differences in the ESR spectra of spin labelled lecithins located in the inner and outer halves of the bilayer described by Kornberg and McConnell[6]. Their data for the proportion of lecithin spin labels exposed on the

exterior surface of the vesicles, and a recent study[7] using the paramagnetic Eu^{3+} ion to discriminate the two surfaces of the lecithin vesicle, both indicate that the lipids are distributed in the approximate ratio of two lecithins on the exterior surface to one on the internal surface. This difference in proportion and the difference in packing of the lipids in the two surfaces is expected to become negligible as the size of the vesicle increases, and similarly it is expected that different chemical classes of phospholipids will be present in very similar proportions in the two surfaces. In biological membranes, however, there is the obvious possibility that the membrane proteins may impose an asymmetric distribution of the various phospholipid classes in the two surfaces of the membrane as shown for cholesterol in myelin by Casper and Kirschner[8] and suggested, for example, by Bretscher[9]. It is also possible that the total number of lipids in the two parts of the bilayer may differ.

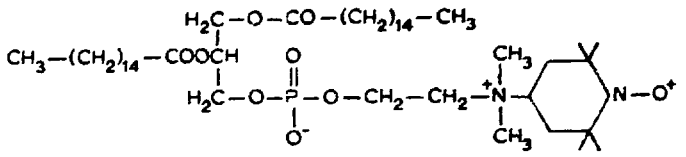
The experiments described here show that the accessibility of solvated paramagnetic ions to lipid headgroups in lipid vesicles can be detected by NMR shift and relaxation measurements, which provide a potential method for examining the distribution of lipid classes in the surface of biological membranes. Inaccessibility of the ions to the lipids when exposed to one surface of biological membranes would imply that either the lipids are protected from interaction with the ions by other membrane components, or that the lipids are not located in that surface of the membrane. In this paper we characterise the interaction of paramagnetic ions with the lecithin vesicle as a model system for comparison with biological membranes to be described in a future paper. The approximate concentration of binding sites for the ions is estimated and their effects on the proton nuclear magnetic relaxation times of $-\overset{+}{N}Me_3$ groups of accessible lecithin molecules have been measured. This group provides a useful marker since it is shown to be particularly sensitive to the packing of the lecithin molecules and its resonance is readily detected in the ^{13}C spectra of biological membranes[3,4,10]. The effect of paramagnetic ions on ^{13}C and ^{1}H NMR resonances from microsomal sarcoplasmic reticulum[10], and microsacs from innervated membrane of the electroplax of *Electrophorus electricus* which also give useful spectra (Changeux, J.-P, Weber, M., Birdsall, N. J. M., Lee, A. G. and Metcalfe, J. C., unpublished) will be reported elsewhere.

The interaction of paramagnetic nitroxide spin labels with lecithin vesicles has also been examined. Their effects on the T_1 relaxation times of the lecithin resonances are consistent with lateral diffusion fast enough to average the T_1 values of all the lecithin $-\overset{+}{N}Me_3$ headgroups in the surface of the vesicle. These results are generally consistent with similar conclusions based on linewidth measurements by Kornberg and McConnell[11].

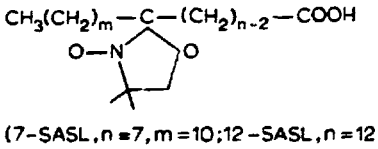
EXPERIMENTAL

Samples

Dipalmitoyllecithin was obtained from Koch Light. Hen egg lecithin was prepared by the method of Dawson[13] and gave a single spot on thin-layer chromatography in two solvent systems [chloroform–methanol–7 M NH_4OH (690:270:45, by vol.) and light petroleum–ether–glacial acetic acid (60:40:1, by vol.)]. The headgroup spin label



was prepared by the method of Kornberg and McConnell[6] and the stearic acid spin labels



(7-SASL, n = 7, m = 10; 12-SASL, n = 12, m = 5)

were prepared by the method of Keith *et al.*[14].

Lanthanum and europium chlorides and neodymium nitrate were obtained from Koch Light and uranyl nitrate from B.D.H. Concentrations of solutions of rare earth ions were determined by back titration of standard EDTA with copper sulphate at neutral pH using 1-(2-pyridazo)-2-naphthol as indicator.

^2H_2O phosphate buffer (45 mM NaCl, 30 mM sodium acetate, 5 mM sodium phosphate; p^2H 7.8) was used in the spin labelling experiments and a Tris buffer (7.5 mM Tris, 50 mM NaCl; p^2H 7.8) was used in the ion binding experiments. Dipalmitoyllecithin was sonicated at 50 °C in deoxygenated buffer in glass vials under nitrogen in a Dawes 1130A sonicating bath until the sample was translucent and the residual light scattering was minimized. Using this sonication procedure no detectable amounts of lysolecithin were produced; sonication with a probe inserted into the lecithin suspension has been reported to cause considerable decomposition[15].

Sonicated dispersions of dipalmitoyllecithin in pure ^2H_2O (p^2H 9.4) were found to have a p^2H of about 7.4, and dispersions in Tris and phosphate buffers to have p^2H values of 7.1 and 7.8, respectively. Metal ions in Tris buffer were added to the sonicated dispersions of lecithin keeping the total volume of the samples constant at 0.5 ml: the lecithin concentration in the final sample was 110 mM. The p^2H values of the lecithin samples changed on addition of metal ion from 6.6 at 4 mM ions to 6.0 at 20 mM ions. Lowering the p^2H by 0.5 did not affect the measured chemical shifts.

NMR measurements

Chemical shift measurements were made on a Varian HA-100 NMR spectrometer operating at 100 MHz for protons, and locked onto internal ^2H_2O. Spin-lattice (T_1) relaxation measurements were made on a Varian XL-100 spectrometer operating at 100 MHz for protons and equipped with a VFT-100 Fourier transform accessory. A 180°-t-90° pulse sequence (inversion recovery) was used where t is a suitable delay, followed by Fourier transformation of the free induction decay[16]. Typically 25 measurements of the transient signal S_t were made, alternating with an equal number of measurements of S_∞, the thermal equilibrium magnetization. A delay of at least five times the longest T_1 in the sample was introduced between the measurements of S_t and S_∞ to allow equilibrium to be attained within the sample. The deviation from equilibrium, $S_\infty - S_t$, was then displayed directly, and in this

way the effects of slow drift of spectrometer gain or homogeneity were minimized. Plots of log $(S_\infty - S_t)$ against t are generally straight lines, the slopes of which give T_1.

RESULTS AND DISCUSSION

Interaction of metal ions with dipalmitoyllecithin.

Separation of inward and outward facing $-\overset{+}{N}Me_3$ groups

Coarse aqueous dispersions of dipalmitoyllecithin can be sonicated above the temperature of the lamellar liquid crystalline phase transition (approx. 43 °C) to give vesicles which give high resolution proton NMR spectra above 40 °C. The proton signal due to the $-\overset{+}{N}Me_3$ groups shows a well developed shoulder on the high field side (Fig. 1a) which becomes more pronounced as the temperature of the sample is lowered, as described previously[2].

On addition of increasing quantities of Nd^{3+} to the bulk aqueous phase, this shoulder is resolved from the larger peak, as the larger peak moves downfield: there is a smaller upfield shift of the smaller peak corresponding to the original shoulder (Fig. 1a). Both shifts are measured relative to internal H^2HO. Eu^{3+}, whose *g*-value anisotropy is of opposite sign to that of Nd^{3+}, causes proton shifts in the opposite direction[17]. Thus, on addition of increasing quantities of Eu^{3+} to the bulk aqueous phase, the upfield shoulder first disappears and then reappears on the low field side as the larger peak moves upfield (Fig. 1b).

We assign the larger peak to the outward facing $-\overset{+}{N}Me_3$ groups, and the smaller peak, appearing as a shoulder on the high field side in the absence of paramagnetic ions, to inward facing $-\overset{+}{N}Me_3$ groups. The small upfield shift of the inward facing groups is consistent with an increase in the bulk magnetic susceptibility of the sample on addition of paramagnetic ions. At all concentrations of Nd^{3+} and Eu^{3+}, the ratio of the areas of the resolved peaks is approx. 1.8:1. If we assume that the vesicle consists of a bimolecular spherical shell of thickness 50 Å and external diameter 500 Å. then if there is an equal distribution of lipids per unit area on the inside and the outside, 60% of the lipids will be facing outwards. The observed area ratio is therefore consistent with rapid exchange of metal ions between binding sites distributed fairly uniformly over the surface of the vesicle so that all the external $-\overset{+}{N}Me_3$ groups are equivalent. This exchange rate has to be faster than the separation, δ, of the proton chemical shifts of the bound and free $-\overset{+}{N}Me_3$ groups; that is faster than approx. 30 per s, as shown below.

Plots of the separation, δ, of the two $-\overset{+}{N}Me_3$ resonances of dipalmitoyllecithin as a function of concentration of added metal ion are shown in Fig. 2. At 52 °C, Nd^{3+} causes larger shifts than equivalent concentrations of Eu^{3+}. No changes in the measured shifts were detected after maintaining the sample for 48 h at 52 °C in the presence of the ions. On lowering the temperature to 35 °C, the $-\overset{+}{N}Me_3$ shifts for dipalmitoyllecithin caused by Nd^{3+} increased considerably (Fig. 2) while only small increases (approx. 3 Hz) were observed with Eu^{3+}. The observation of a split $-\overset{+}{N}Me_3$ resonance at 35 °C confirms that the vesicles remain

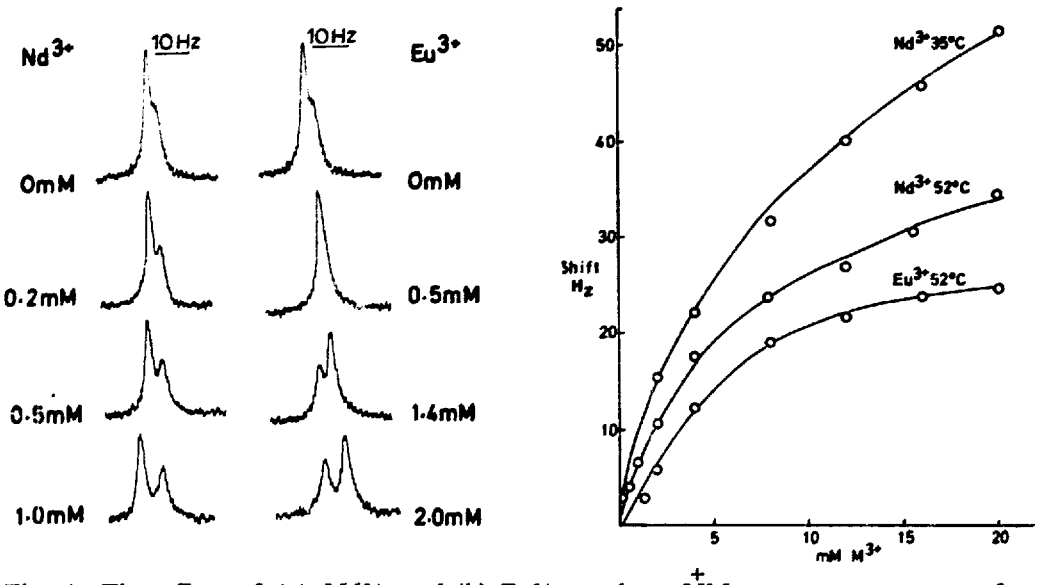


Fig. 1. The effect of (a) Nd^{3+} and (b) Eu^{3+} on the $-\overset{+}{N}Me_3$ proton resonance of sonicated aqueous dispersions of dipalmitoyllecithin at 52 °C.

Fig. 2. The chemical shift separation (Hz) of inward and outward facing $-\overset{+}{N}Me_3$ proton resonances of sonicated aqueous dispersions of dipalmitoyllecithin as a function of metal ion concentration and temperature.

as closed shells of unaltered size below the transition, in spite of the aggregation of the vesicles described previously.

With egg lecithin, which contains a variety of fatty acid chains, there is sometimes an indication of asymmetry of the $-\overset{+}{N}Me_3$ peak, but the shoulder is never as well resolved as for dipalmitoyllecithin. The observation of distinguishable resonances from the inward and outward facing $-\overset{+}{N}Me_3$ groups suggests a difference in chemical environment that could be attributed to differences in packing characteristics. The addition of 20 mM Nd^{3+} to egg lecithin vesicles (10%, w/w) caused a 28 Hz splitting of the $-\overset{+}{N}Me_3$ resonance, and this splitting was approximately constant over the temperature range 25–52 °C. The splitting caused by 20 mM Eu^{3+} increased slightly from 17.4 Hz at 25 °C to 21.3 Hz at 52 °C. The differences in behaviour between dipalmitoyllecithin and egg lecithin on lowering the temperature are attributed to a change in the conformation of the headgroup of dipalmitoyllecithin at the thermal transition[2-4].

The splitting of the dipalmitoyllecithin $-\overset{+}{N}Me_3$ resonance caused by Nd^{3+} and Eu^{3+} is unaltered by the addition of Ca^{2+} up to a Ca^{2+}/M^{3+} ratio of 50. The diamagnetic UO_2^{2+} ion causes an upfield shift of the exterior $-\overset{+}{N}Me_3$ resonance of 6 Hz at a concentration of 10 mM at 52 °C, whereas no splitting is caused by the diamagnetic La^{3+}. Addition of UO_2^{2+} to dipalmitoyllecithin containing Nd^{3+} or Eu^{3+} causes a considerable reduction in splitting even at a UO_2^{2+}/M^{3+} ratio of 0.1.

Number of ion binding sites and affinity

The chemical shift data can be used to estimate the upper limit of the concentration of ion binding sites on the outside surface of the bilayer. Assuming that

the observed $-\overset{+}{N}Me_3$ peak separation, δ, is proportional to the fraction of binding sites occupied, then

$$\delta = \frac{[\alpha]_B}{[\alpha]_T}\delta_B \tag{1}$$

where $[\alpha]_B$ and $[\alpha]_T$ are the concentrations of bound and total binding sites, respectively, and δ_B is the separation of the $-\overset{+}{N}Me_3$ resonances of free and bound lecithin molecules. It is also assumed that δ_B is independent of $[m]_T$, the total metal ion concentration. If the ion binding sites are regarded as separate and independent, then we can define an affinity constant, K, from the law of mass action as

$$K = \frac{[\alpha]_B}{([m]_T - [\alpha]_B)([\alpha]_T - [\alpha]_B)} \tag{2}$$

where $[m]_T$, is the total metal ion concentration. Following Nakano *et al.*[18] we can combine Eqns 1 and 2 in the form

$$\frac{[m]_T}{\delta} = \frac{1}{\delta_B}\left([\alpha]_T + [m]_T - \frac{\delta}{\delta_B}[\alpha]_T\right) + \frac{1}{K\delta_B} \tag{3}$$

and set up an iterative procedure to calculate both the affinity constant K and δ_B for a given concentration of binding sites $[\alpha]_T$. The maximum value of $[\alpha]_T$ which yields meaningful values of K and δ_B compatible with the experimental data is about 8 mM for both Nd^{3+} and Eu^{3+}, when the total concentration of external $-\overset{+}{N}Me_3$ groups is 60 ± 10 mM.

A limit on the value of $[\alpha]_T$ compatible with the experimental data is in fact implicit in Eqn 1, since positive K values are only obtained if

$$[m]_T \geqq [\alpha]_B \tag{4}$$

$$[m]_T \geqq \frac{\delta}{\delta_B}[\alpha]_T \tag{5}$$

since $\delta/\delta_B \leqslant 1$, an upper limit can be set to $[\alpha]_T$, which is determined by the lowest concentration of ions producing a measurable shift in the experimental system. For example, δ is $\simeq$ 4 Hz at a value of $[m]_T = 0.5$ mM Nd^{3+} and δ_B is $\geqslant$30 Hz; it follows that $[\alpha]_T \leqslant 7.5$ mM, which is consistent with the estimate obtained by the iterative procedure.

As the value of $[\alpha]_T$ is increased up to the limit of 8 mM, the calculated affinity constant is found to increase so as to maintain the fraction of sites occupied ($[\alpha]_B/[\alpha]_T$) at the constant value (δ/δ_B). However, for any given value of $[\alpha]_T$, the affinity constant calculated for Nd^{3+} is always considerably smaller than that calculated for Eu^{3+} (Table I). The chemical shift data for dipalmitoyllecithin in the presence of Nd^{3+} also show that for a given value of $[\alpha]_T$, both the affinity and the value of δ_B increase on lowering the temperature. The affinity constant increases by about 20% while δ_B increases by at least 50% at 35 °C.

The above chemical shift data indicates that the affinity of ions for dipalmi-

TABLE I

COMPARISON OF AFFINITY CONSTANTS OF DIPALMITOYLLECITHIN VESICLES AT 52 °C FOR Nd^{3+} AND Eu^{3+} FOR VALUES OF $[\alpha]_t$ CORRESPONDING TO THE MAXIMUM AND MINIMUM CONCENTRATIONS OF BINDING SITES ESTIMATED FROM THE NMR DATA (SEE TEXT)

The concentration of outward facing $-\overset{+}{N}Me_3$ groups is 60 ± 10 mM (see text).

Metal ion	$[\alpha]_t$ (*mM*)	*Affinity constant* K ($l \cdot mole^{-1}$)	δ_B (*Hz*)
Nd^{3+}	2	120	46
Nd^{3+}	8	1000	38
Eu^{3+}	2	284	30
Eu^{3+}	8	9400	24.5

toyllecithin is in the order $UO_2^{2+} > Eu^{3+} > Nd^{3+} > Ca^{2+}$ and that the concentration of ion binding sites is, at most, only 0.12 per lecithin molecule. This is consistent with other data available for ion binding to bilayers. At physiological pH, dipalmitoyllecithin is a zwitterion and a variety of techniques have shown that there is only weak absorption of Ca^{2+} onto phosphatidylcholine monolayers[19]. The concentration of binding sites for Fe^{3+} on bilayers as suggested by MacDonald and Thompson[20] is less than 0.01 per lecithin which is an order of magnitude lower than the upper limit from the chemical shift data. If this lower value were to apply also to Eu^{3+} and Nd^{3+}, then the shifts of the $-\overset{+}{N}Me_3$ groups of lecithins directly interacting with the ions must be very large ($\geqslant$500 Hz) to account for the observed chemical shifts of the averaged $-\overset{+}{N}Me_3$ resonance. To observe an averaged $-\overset{+}{N}Me_3$ resonance with such large chemical shifts necessitates a fast rate of exchange in the surface of the bilayer: it seems likely that the averaging process could be achieved through a sequential exchange of lecithins liganded to the Eu^{3+} at the surface of the bilayer, without the necessity for complete dissociation of the ion from the vesicle surface.

Effect of ions on line width

The line widths of the $-\overset{+}{N}Me_3$ resonances at half height increase progressively with the addition of rare earth ions. The width of the shifted external resonance increases from 3.6 Hz at 4 mM Eu^{3+} to 5.0 Hz at 20 mM Eu^{3+}, while the corresponding widths of the unshifted (internal) resonance are 2.5 Hz and 3.6 Hz. The effect of Nd^{3+} is greater: the line width of the shifted resonance is 4.0 Hz at 2 mM and 8.0 Hz at 20 mM while the unshifted resonance line widths are 3.5 Hz and 5.0 Hz, respectively. The broadening caused by both ions increases considerably below the thermal transition, and measurement of the line widths is only possible with well separated peaks which requires high Nd^{3+} concentrations. The half widths of the shifted and unshifted $-\overset{+}{N}Me_3$ resonances at 35 °C in the presence of 20 mM

Nd^{3+} are 28 Hz and 18 Hz, respectively, compared with 13.5 Hz for the composite peak in the absence of Nd^{3+}.

Sheard[21] has reported that the line widths of the $-\overset{+}{N}Me_3$, $-(CH_2)_n-$ and $-CH_3$ proton resonances in sonicated aqueous dispersions of egg lecithin are field dependent. We have confirmed this observation for sonicated dispersions of dipalmitoyllecithin. The line widths (Hz) uncorrected for instrumental broadening at 100 and 220 MHz at 52 °C are: $-\overset{+}{N}Me_3$, 4.5 and 7.3; $-(CH_2)_n-$, 25 and 38; $-CH_3$, 18 and 20. For the $-\overset{+}{N}Me_3$ resonance part, at least, of this field dependence must be due to the difference in chemical shifts of the inward and outward facing lipids. The observed $-\overset{+}{N}Me_3$ resonance at 100 MHz in the absence of paramagnetic ions can be simulated by combining two Lorentzian lines with a chemical shift difference of approx. 2.5 Hz, one of relative amplitude 2 and a half-width of 2.0 Hz, and the other of relative amplitude 1 and a half-width of 3.0 Hz.

The effect of ions on the thermal transition of dipalmitoyllecithin

The thermal transition of the hydrocarbon chains of dipalmitoyllecithin in bilayers is shifted to higher temperatures in the presence of Eu^{3+}, Nd^{3+} or UO_2^{2+}.

The transition was followed both by the change in linewidth of the $-\overset{+}{N}Me_3$ resonance and the disappearance of the resonance signals from the hydrocarbon chains, described elsewhere[1,2,4]. If the vesicles of dipalmitoyllecithin are formed by sonication in solutions containing Eu^{3+} or Nd^{3+}, only a single shifted resonance is observed indicating that the concentrations of rare earth ions are very similar on both sides of the vesicle. The transition temperature in the presence of 50 mM Eu^{3+} or 6 mM UO_2^{2+} is increased by about 5 °C as judged from the line-width changes of the $-\overset{+}{N}Me_3$ resonance (Fig. 3). The onset of broadening and the disappearance of the hydrocarbon chain resonances also occurs at about 5 °C higher than in the absence of these metal ions. The liquid crystalline phase transition of the hydrocarbon chains can also be followed by ESR from the large increase in

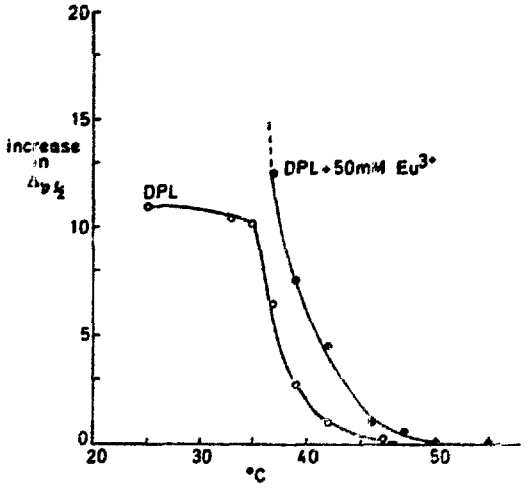


Fig. 3. The effect of 50 mM Eu^{3+} on the increase in the linewidth ($\Delta\nu_{1/2}$) of the $-\overset{+}{N}Me_3$ resonance of sonicated aqueous dispersions of dipalmitoyllecithin as a function of temperature. The linewidth increases are measured relative to the values at 55 °C for both samples.

the partition of TEMPO into the bilayer through the transition temperature. Again a 5 °C increase in the transition temperature was detected in the presence of 50 mM Eu^{3+}, 50 mM Nd^{3+}, or 6 mM UO_2^{2+}, on both sides of the vesicle.

Effect of metal ions on relaxation times (T_1)

Measurements of spin lattice relaxation times (T_1) were made for the $-\overset{+}{N}Me_3$, $-(CH_2)_n-$ and $-CH_3$ protons of dipalmitoyllecithin vesicles using a (π-t-$\pi/_2$) pulse sequence, where t is the delay between the π and $\pi/_2$ pulses. The decay of the signal due to the $-\overset{+}{N}Me_3$ protons with t is linear in the range $t=10$–750 ms. It is not possible to make accurate measurements on the height of the upfield shoulder, but it is clear that there are no large differences between the relaxation times of the inside and outside groups. A single exponential decay is also observed for the $-(CH_2)_n-$ and $-CH_3$ protons. We have shown elsewhere that this does not imply that all the $-(CH_2)_n-$ protons have a single T_1 and that a range of T_1 values may appear experimentally as a single exponential.

TABLE II

^1H T_1 (s) FOR DIPALMITOYLLECITHIN (110 mM) AT 52 °C IN TRIS BUFFER

	$-\overset{+}{N}Me_3$ T_1 (s)	
	Shifted	*Unshifted*
Dipalmitoyllecithin	—	0.49±0.01
Dipalmitoyllecithin+20 mM Eu^{3+} on outside	0.46±0.02	0.40±0.10
Dipalmitoyllecithin+50 mM La^{3+} on both sides	—	0.45±0.01

In order to make more accurate comparisons of the inside and outside facing $-\overset{+}{N}Me_3$ groups, it is necessary to resolve the resonances by the addition of Eu^{3+}, and Table II shows T_1 data obtained in the Tris buffer, which was used because of the general insolubility of the lanthanide phosphates. It is clear that the presence of Eu^{3+} on the outside of the vesicles causes a reduction in T_1, with the surprising result that the reduction is somewhat greater for the inward facing $-\overset{+}{N}Me_3$ groups. This suggests that the interaction of the Eu^{3+} with the phosphate groups of the outward facing lipid may "tighten" the outer lipid layer, and so cause tighter packing of the inner lipid layer. Evidence in favour of tighter packing of the inner lipid layer has been obtained from spin label experiments and is discussed below. The addition of diamagnetic La^{3+} to both sides of the vesicles also causes a slight reduction in the T_1 value of the $-\overset{+}{N}Me_3$ groups (Table II).

The relaxation measurements in Tris buffer suggest that there is some interaction between the Tris cations and the lipid phosphate groups, since the T_1 values for the $-\overset{+}{N}Me_3$ group are slightly but consistently lower than in phosphate buffer. Variation of p^2H between 1.4 and 7.4 in unbuffered ^2H_2O had no significant effect

on the $-\overset{+}{N}Me_3$ T_1 (0.52–0.54 s). The presence of choline chloride also had no measurable effect on the relaxation times. Ca^{2+} causes a small reduction in T_1, and Tl^+, which resembles K^+ in its properties, except that being more polarisable, it binds more strongly to groups such as phosphate, causes a larger reduction in T_1 to 0.44 s (Table III).

The results suggest that there are no large differences between the inside and outside of the bilayers although small differences could not be detected by this technique because of the perturbation caused by the metal ions. This conclusion is important in interpreting the relaxation times of lecithin $-\overset{+}{N}Me_3$ resonances from biological membranes. The most likely cause of the small differences in T_1 observed between the two sides of the bilayer is a tighter packing of the inside lipids, which can be examined by incorporating spin labels into the bilayer.

The data also set a lower limit to the fraction, f, of lecithins which form the ion binding sites. For intramolecular dipolar relaxation, the lowest possible value of T_1 for a proton pair is 0.097 s. For a methyl group we then expect a minimum possible value of about 0.05 s. The relaxation times T_{1B} of the lecithin $-\overset{+}{N}Me_3$ groups which form the ion binding sites must therefore be greater than 0.05 s. If T_{1B} had the minimum value, then for the relaxation time T_{1F} of the unbound lecithin $-\overset{+}{N}Me_3$ groups to be averaged with T_{1B}, the dissociation rate constant must be greater than (1/0.05) s^{-1}, *i.e.*>20 s^{-1}. For any longer value of T_{1B} a slower rate constant would be sufficient for averaging. Since the observed chemical shift of the averaged $-\overset{+}{N}Me_3$ resonances implies a dissociation rate of at least 30 s^{-1}, this condition is satisfied. It follows that the observed T_1 value, T_{1OBS}, is given by

$$T_{1\,OBS} = fT_{1\,P} + (1-f)T_{1\,F} \tag{6}$$

For T_{1B} to be >0.05 s implies

$$f \geqq (T_{1\,F} - T_{1\,OBS})/(T_{1\,F} - 0.05) \tag{7}$$

Taking values of T_{1F} and T_{1OBS} for dipalmitoyllecithin $\pm$ M^{3+} from Table II implies $f \geqslant 0.1$. Since the concentration of lecithins on the exterior surface of the vesicles is 60 ± 10 mM, this implies that the concentration of lecithin molecules

TABLE III

^1H T_1 (s) FOR DIPALMITOYLLECITHIN (110 mM) AT 52 °C IN ^2H_2O

Conditions	T_1 (*s*)
In phosphate buffer (p^2H 8.0)	0.54 ± 0.01
In ^2H_2O (p^2H 2.8)	0.52 ± 0.01
In ^2H_2O (p^2H 1.4)	0.52 ± 0.01
+20 mM choline chloride: phosphate buffer	0.52 ± 0.01
+150 mM Ca^{2+} in ^2H_2O	0.49 ± 0.01
+25 mM Tl^+ in ^2H_2O	0.44 ± 0.02

forming the binding site is $\geqslant$6 mM. It is probable that an M^{3+} ion can interact directly with not more than 3 lecithin molecules, which sets a lower limit of 2 mM on the concentration of binding sites.

It is also of interest that lanthanides increase the electrical resistance of the bilayer[22]. We attribute the reduction in T_1 of the headgroups and the increase in the transition temperature in the presence of these ions to increased packing of the lecithin molecules. This is consistent with the more general observation that the permeability of lecithin vesicles to solutes can be correlated directly with the tightness of chain packing, as expressed in the ^{13}C relaxation times of the chains when the structure of the lecithin molecule is varied (Lee, A.G., Birdsall, N. J. M., Levine, Y. K. and Metcalfe, J. C., unpublished).

Effect of spin labels on relaxation times

Reduction of T_1 values

Incorporation of the headgroup spin label into vesicles of dipalmitoyllecithin causes a considerable reduction in the T_1 values of all three resonances. The $-(CH_2)_n-$ and $-CH_3$ resonances are again characterised by a single exponential decay but for the $-\overset{+}{N}Me_3$ resonance at 52 °C there is a slight non-linearity (Fig. 4). The T_1 values, estimated approximately from the slopes of the two parts of the curve are very similar (0.14 s and 0.18 s at 52 °C). At lower temperatures, the resonances are broader and the decay curves were linear within experimental error; only the average T_1 values are given in Table IV.

The effect of the nitroxide spin label can be characterised by a time T_{1SL} given by

$$T_{1SL}^{-1} = T_{1OBS}^{-1} - T_{1O}^{-1} \qquad (3)$$

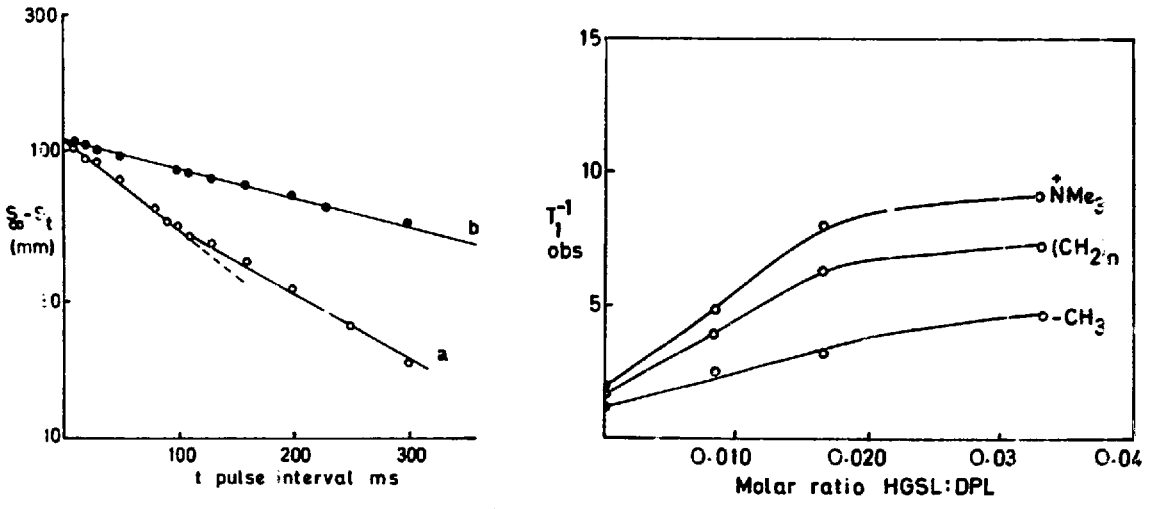


Fig. 4. Intensities ($S_\infty - S_t$ mm) of the $-\overset{+}{N}Me_3$ proton resonance in a sonicated aqueous dispersion of dipalmitoyllecithin containing headgroup spin label (dipalmitoyllecithin:headgroup spinlabel = 60:1) as a function of t at 52 °C. a, Experimental; b, theoretical, in the absence of surface diffusion.

Fig. 5. Variation of T_{1obs}^{-1} for resonances from dipalmitoyllecithin containing headgroup spin label as a function of headgroup spin label:dipalmitoyllecithin ratio.

TABLE IV

OBSERVED RELAXATION TIMES (T_{1OBS} ms)) OF DIPALMITOYL LECITHIN RESONANCES IN THE PRESENCE OF HEADGROUP SPIN LABEL (MOLAR RATIO 1:60)

Values at 52 °C in the absence of spin label are given in brackets.

Temp. (°C)	$-\overset{+}{N}Me_3$	$-(CH_2)_n-$	$-CH_3$
62	202±23	132±5	384±40
52	153±6 [570±50]	128±5 [450±10]	315±63 [940±60]
47	128±6	116±3	172±24
44	116±3	106±3	166±9

TABLE V

RELAXATION TIMES (T_{1OBS} ms)) OF DIPALMITOYLLECITHIN RESONANCES IN THE PRESENCE OF SASL (1:60 MOLAR RATIO)

Values at 52 °C in the absence of spin labels are given in brackets.

Temp. (°C)	*7-SASL*			*12-SASL*		
	$-\overset{+}{N}Me_3$	$-(CH_2)_n-$	$-CH_3$	$-\overset{+}{N}Me_3$	$-(CH_2)_n-$	$-CH_3$
52	120±5 [570±50]	98±3 [450±10]	152±12 [940±60]	190±10	82±2	87±6
45	105±3	90±2	157±20	158±10	61±1	72±5
40	87±5	79±4		137±10	42±3	

where T_{1OBS} and T_{10} are the observed relaxation times in the presence and absence of the spin label respectively. The paramagnetic effect T_{1SL}^{-1} increases linearly with spin label concentration for all three resonances up to a molar ratio of 1:60 (spin label:lipid) beyond which the effect begins to level off (Fig. 5). The paramagnetic effect is temperature dependent, and decreases with increasing temperature in the range 44–62 °C for all three resonances (Table IV).

Incorporation of the stearic acid spin labels (7-SASL and 12-SASL) into dipalmitoyllecithin vesicles also causes a reduction in T_1 values (Table V) but now the decay of all three resonances is exponential within experimental error. The paramagnetic effect caused by the 12-SASL increases linearly with concentration up to a molar fraction of 1:60 for all three resonances and decreases with increasing temperature with both 12-SASL and 7-SASL at this concentration.

Analysis of relaxation data

Proton relaxation in the absence of the nitroxide spin label is largely determined by other protons within the same or adjacent lipid molecules. In the presence of nitroxide, however, because the magnetic moment of the unpaired electron is over 600 times greater than that of the proton, intermolecular inter-

action of the proton with an adjacent nitroxide is very important. The protons on the spin-labelled molecule itself are not seen because they are severely broadened by the nitroxide. For isotropic rotational motions, the relaxation time T_{1HE} of a proton a distance τ from an unpaired electron is given by[23]

$$T_{1HE}^{-1} = \frac{1}{10}\hbar^2\gamma_H^2\gamma_E^2\left[\frac{\tau_C}{1+(\omega_H-\omega_E)^2\tau_C^2}+\frac{3\tau_C}{1+\omega_H^2\tau_C^2}+\frac{6\tau_C}{1+(\omega_H+\omega_E)^2\tau_C^2}\right] \quad (9)$$

where γ_H and ω_H are the magnetogyric ratio and angular frequency of the proton; γ_E and ω_E are the same quantities for the electron and τ_C is the electron–proton correlation time.

Since $\omega_E \simeq 5\cdot 10^{11}$ in the NMR field and $\tau_C > 10^{-11}$ we can neglect terms containing the product $\omega_E^2\,\tau_C^2$ and Eqn 9 then reduces to

$$T_{1HE} = \frac{3}{10}\frac{\hbar^2\gamma_H^2\gamma_E^2}{\tau^6}\left(\frac{\tau_C}{1+\omega_H^2\tau_C^2}\right) = B/\tau^6 \quad (10)$$

It is generally assumed that τ_C depends on temperature according to the relation $\tau_C = \tau_C^\circ \exp(\Delta E/RT)$ where ΔE is the activation energy of the relaxation process and τ_C° is a constant of motion having the dimension of time. In the limit $\omega_H^2\tau^2 \ll 1$ (which can still satisfy the inequality $\omega_E^2\tau_C^2 \ll 1$), T_{1HE}^{-1} is proportional to τ_C and therefore decreases with increasing temperature. In all the experiments described we have observed a decrease in the paramagnetic effect (Tables IV and V) and thus T_{1HE}^{-1} is determined by the product $\tau_C\,\tau^{-6}$.

According to Eqn 10 the maximum possible reduction in T_{1HE}^{-1} is observed when $\omega_H^2\,\tau_C^2 \simeq 1$ and thus when $\tau_C \simeq 10^{-9}$. In the limit $\omega_H^2\,\tau_C^2 \ll 1$ and in the absence of any slow motion which will contribute to T_2 and not to T_1, we would expect that $T_{1SL} = T_{2SL}$ and thus we might expect T_{2SL} to be equivalent to the increase in linewidth caused by the spin label. That is, we might try to put

$$T_{1SL}^{-1} \simeq \pi\Delta\nu_{\frac{1}{2}SL} \quad (11)$$

In fact the observed line broadening caused by headgroup spin label (*e.g.* 15 Hz at a molar ratio of lecithin: headgroup spin label of 120:1 at 52°C) is very much larger than the paramagnetic effect on T_1 (Table IV). Similar observations have been made by Kornberg and McConnell[11] who noted that the greater line broadening was consistent with a correlation time $\tau_C = 4.7\cdot 10^{-9}$ s, for which $\omega_H^2\,\tau_C^2 > 1$ in which case it is expected that $T_2 > T_1$. Our data on the temperature variation in T_1 in the absence of spin labels strongly suggests that τ_C has a value such that $\omega_H^2\,\tau_C^2 < 1$. In fact we have shown elsewhere that the linewidths of the proton resonances in dipalmitoyllecithin bilayers cannot be explained in terms of isotropic intramolecular dipolar relaxation processes alone, because the linewidths indicate that $T_2 \ll T_1$ and this has been confirmed by direct measurement of T_2 by Horwitz *et al.*[12]. We concluded that for T_2 to be much shorter than T_1 under the condition of fast exchange ($\omega_H^2\tau_C^2 < 1$), there must be some relatively slow motion affecting T_2 but not T_1. This might be attributable to some relatively slow anisotropic motion within the lipid molecules, but there is no *a priori* reason for neglecting intermolecular dipole–dipole relaxation, and comparison of the ^1H and ^{13}C relaxation times

for the lipid chains suggest that such intermolecular effects are probably very important for ^1H relaxation. This can be tested experimentally by studying relaxation in vesicles of lecithin and perdeuterated lecithin.*

Diffusion in the surface of the bilayer

If the headgroup spin label and lipid molecules are fixed in the surface of the bilayer, the protons will exhibit a range of T_1 values, since for a random distribution of molecules in the surface there will be a range of values for the nuclear–electron separation. Protons of lipid molecules close to the spin label will have short T_1 values, but those at a distance of more than approx. 30 Å will be virtually unaffected (about 50% at a molar ratio of headgroup spin label:lipid of 1:60). The effect that the distribution of relaxation times will have on the observed decay of the signal due to the $-\overset{+}{N}Me_3$ protons can be calculated using the binomial distribution as used by Kornberg and McConnell[11]

$$A(t) = (1-c)^N e^{-t/T_{10}} + \frac{Nc(1-c)^{N-1}}{\pi(r_1 - r_0)^2} \int_{r_0}^{r_1} 2\pi e^{-t(1/T_{10} + B/\tau_6)} r_1 d\tau_1 + \ldots \qquad (12)$$

where A(t) is the amplitude of the decay curve at time t, c is the molar fraction of nitroxide molecules and N is the number of nitroxide molecules in a surface area $\pi\tau_1^2$ where each molecule occupies 65 Å^2 (ref. 24); τ_1 is the centre to centre separation of the nitroxide and a lecithin molecule and τ_0 is the limiting value of τ_1 of about 8 Å. The value of B is calculated from Eqn 10. The results calculated for $\tau_c \sim 10^{-9}$ s, which is the most effective correlation time for the relaxation processes, and for $\tau_1 = 30$ Å (at distances greater than 30 Å the paramagnetic effect is very small), at molar ratio of headgroup spin label:lecithin of 1:60 are shown in Fig. 4 at the same values of t as were used experimentally. Terms higher than the second on the right hand side of Eqn 12 were found to be very small and were neglected in the calculation. From this line a value of 0.4 s is calculated for T_1 for the $-\overset{+}{N}Me_3$ protons. The reason for the relatively long T_1 value is that the slope of the line is determined mainly by the $-\overset{+}{N}Me_3$ groups a long way from the spin label; those close to the spin label have very short T_1 values and have already decayed at very short t values. The much shorter T_1 values observed in the presence of the nitroxide spin label indicate that fast diffusion must be occurring in the surface of the bilayer to average out the environment of the $-\overset{+}{N}Me_3$ groups.

The non-exponential decay observed for the $-\overset{+}{N}Me_3$ resonance at 52 °C in the presence of headgroup spin label (Fig. 4) could be due to an intrinsically non-exponential relaxation process. It could also be caused by the summation of two exponential decays characterized by slightly different T_1 values. In the latter case, the two different decays could be associated with lipids on the inside and outside surfaces of the bilayer with slightly different packing characteristics. ESR data

* This has now been done, and the experiments show that ^1H intermolecular relaxation is of major importance in lipid bilayers. The relaxation can be treated in terms of translational motion, to give a coefficient of self diffusion of lipid molecules in the surface of the bilayer of approx. $1 \cdot 10^{-8}$ cm^2/s (Lee, A.G., Birdsall, N. J. M. and Metcalfe, J. C., unpublished).

suggest that there may be two populations of headgroup spin label, one strongly immobilized and one weakly immobilized[6]. The shoulder on the $-\overset{+}{N}Me_3$ resonance in the absence of spin label could then also be due to slightly different packing characteristics in the two surfaces of the bilayer. This chemical shift difference could also arise from effects of anisotropic magnetic susceptibility if the vesicles were non-spherical. Attwood and Saunders[25] have interpreted their data on light scattering from lecithin vesicles in terms of non-spherical shells, but Tinker[26] has recently shown that the experimental results are also consistent with completely spherical shells.

Relative effects of spin labels

The effects of the fatty acid spin labels on the relaxation times are consistent with the expected positions of the nitroxide groups in the bilayer. The 7-SASL has a larger effect on the $-\overset{+}{N}Me_3$ protons than 12-SASL, but the effect on T_1 is reversed for the terminal $-CH_3$ group. The effect on the $-(CH_2)_n$ -protons is more difficult to estimate, since only a weighted average relaxation time is obtained for these protons. The 12-SASL would be expected to have a larger effect on the protons nearer the terminal methyl end of the chain, which are the protons expected to have the longer T_1 values, and the $-(CH_2)_n-$ relaxation time is lower in the presence of 12-SASL than 7-SASL. A similar effect of these spin labels on the ^{13}C T_1 relaxation times of the lipid chains has been observed[4]. In contrast to the similar chemical and physical environment expected for the nitroxide group in 7-SASL and 12-SASL, the environment of the nitroxide group in headgroup spin label is substantially different. A comparison of the relative effects of headgroup spin label and SASL on the proton relaxation times then requires detailed knowledge of the effective relaxation mechanisms for protons in lipid bilayers which is not available at present.

CONCLUSIONS

1. Confirmation of the assignment of the two partially overlapping $-\overset{-}{N}Me_3$ resonances to inward and outward facing $-\overset{+}{N}Me_3$ groups is obtained from the relative shifting of the peaks in the presence of the paramagnetic ions Eu^{3+} and Nd^{3+}.

2. The chemical shift data indicate that the affinity of ions for dipalmitoyllecithin is in the order $UO_2^{2+} > Eu^{3+} > Nd^{3+} > Ca^{2+}$. The chemical shift data and the relaxation data set upper and lower limits to the number of binding sites available to the ions. The upper limit is probably determined by charge repulsion from the occupied binding sites.

3. The relaxation data indicate that none of the above ions has a very large effect on the observed T_1, but the reduction in the T_1 value of the $-\overset{+}{N}Me_3$ groups of lecithins directly bonded to the ions must be substantial, since the exchange rate between ion binding sites is shown to be sufficiently fast to average the T_1 values of free and bound lecithin resonances, and the proportion of bound lecithins is known to be small.

4. The reduction in T_1 values of the headgroup resonances produced by the ions is consistent with tighter packing of the lecithin molecules causing a reduction in permeability and an increase in the thermal transition temperature.

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