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HIGH RESOLUTION PROTON RELAXATION STUDIES OF LECITHINS

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SUMMARY

1. Spin-lattice (T_1) proton relaxation data have been obtained for lecithins in solution in methanol, in micelles in [^{2}H]chloroform, and in bilayers in ^2H_2O. The relaxation times are characteristic of the type of structure formed, and reflect molecular motion within the lecithin molecule in each structure.

2. The data are not consistent with a single T_1 for all the protons in the lecithin bilayer in ^2H_2O and spin diffusion is not a dominant relaxation mechanism.

3. Sonicated aqueous suspensions of dipalmitoyl lecithin show a striking set of spectral changes through the transition temperature at 43° in which the fatty acid chain resonances disappear completely and the $-N(CH_3)_3^+$ resonance undergoes a sharp increase in linewidth. These spectral changes through the transistion are entirely reversible and do not involve a significant change in vesicle size.

4. The T_1 values for the major resonances of lecithin in bilayers are consistent with a structure, based on ^{13}C T_1 measurements, in which the bilayer is most tightly packed at the glycerol region of the lecithin molecules, with increasing freedom of motion both towards the terminal methyl of the alkyl chain and the $-N(CH_3)_3^+$ group at the surface of the bilayer.

5. Cholesterol perturbs the bilayer structure and produces changes in the T_1 values of the lecithin resonances, consistent with the effect on the permeability of the lecithin vesicle.

INTRODUCTION

Proton nuclear magnetic resonance (NMR) studies of lipid bilayers and membranes[1–5] have raised a number of theoretical problems of spectral interpretation which have tended to obscure the value of NMR relaxation measurements in defining the dynamic properties of membrane components. For example, the mechanism by which a high resolution proton spectrum is obtained on sonicating aqueous dispersions of lecithin remains controversial[2], and several features of the spectra themselves are poorly understood. Thus the linewidths of the resonances are field-dependent[2] and their shapes are non-Lorentzian. The most surprising observation however is that two recent reports[5,6] suggest that all the protons in the lecithin molecule in bilayers have the same spin-lattice (T_1) relaxation times, and in both instances this was attributed to a spin diffusion mechanism by which all the protons

have the same spin temperature. CHAN *et al.*[5] were undecided whether spin diffusion was to the terminal methyl of the alkyl chain or 'to some methylene protons further up the chain'. SALSBURY *et al.*[6] interpreted BARRATT's results to imply spin diffusion from the chain to the quaternary choline methyls across the non-magnetic O–P–O group. If these observations are correct, it implies that spin diffusion is the dominant relaxation mechanism in the bilayer, and that proton relaxation times are unable to give information about molecular motion in the bilayer.

This is certainly not true for other magnetic nuclei. The measurements of ^{19}F relaxation times for monofluorostearic acids in lecithin bilayers[7] and ^{13}C relaxation times of unmodified lecithins[8] both indicate a gradation in relaxation times increasing towards the terminal methyl, implying a large increase in molecular motion along the chain. Spin diffusion cannot be a relaxation mechanism for the ^{13}C nuclei since the majority of the molecules contain only one ^{13}C nucleus. The increased resolution and narrow linewidths obtained from the ^{13}C nuclei of lecithin in bilayers indicate that this is the nucleus of choice for these systems, but it remains important to establish the consistency of the data from all appropriate nuclei. The mechanism of line broadening of proton resonances and other nuclei in bilayer structures remains an outstanding problem in defining molecular motion from relaxation measurements in membranes.

The spin echo techniques frequently used to measure proton T_1 relaxation times suffer from the disadvantage that the measured relaxation time is generally an averaged value for all the protons in the sample and the measurements are inherently insensitive to heterogeneous relaxation times. In this work we have used a Fourier Transform technique employing a $(\pi - t - \pi/2)$ pulse sequence recently described by FREEMAN AND HILL[9] which allows the separate measurement of the T_1 values of all the resolvable signals in the spectrum. We have measured T_1 values for lecithin molecules in three different structures, including the bilayer, to determine whether the relaxation times reflect molecular motion of the molecules in each structure, or whether spin diffusion is the dominant relaxation mechanism. We have also measured the linewidths of the resonances to compare the T_1 values with the apparent T_2 values estimated from the linewidths, and it is quite clear that there are additional contributions to the linewidths apart from spin–spin relaxation in the bilayer structure. The linewidth changes are particularly marked through the thermal transition of dipalmitoyl lecithin, indicating a constraint on the conditions under which proton resonances are likely to be observed in membranes.

EXPERIMENTAL

Samples

Dipalmitoyl lecithin was obtained from Koch Light. Hen egg lecithin was prepared by the method of DAWSON[21] 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–ethanol–glacial acetic acid (60:40:1, by vol.)). Samples of egg lecithin were agitated under nitrogen in a ^2H_2O buffer (45 mM NaCl; 30 mM sodium acetate; 5 mM sodium phosphates, p^{2}H 7.8), and were then sonicated in glass vials at 30° under nitrogen until the sample was translucent and the residual light scattering was minimised. Aqueous dispersions of dipalmitoyl lecithin were sonicated

at 50°. Samples were transferred under nitrogen to 5-mm NMR tubes and thoroughly deoxygenated with nitrogen. Previous experiments had shown that the results obtained on such samples were identical with those degassed by repeated freeze–pump–thaw cycles. Samples in ^2H_2O were 230 mM, in [^{2}H]chloroform were 290 mM, and in [$^2H_4$]methanol were 140 mM.

Nuclear relaxation measurements

Spectra were obtained by the Fourier transform technique on a Varian XL 100-15 spectrometer, locked onto deuterium in the solvent (^2H_2O, [^{2}H]chloroform or [$^2H_4$]methanol), and accumulated in a Varian 620i computer. A pulse method employing a $(\pi - t - \pi/2)$ pulse sequence was used to measure T_1 relaxation times, where t is the delay in seconds between the π and $\pi/2$ pulses. A $\pi/2$ pulse had a duration of 52 μsec. Rf pulses were generated by a Varian VFT-100 Fourier transform accessory. A fast analog-to-digital converter sampled 8192 points on each free induction decay for a sweep width of 1000 Hz, and typically 50 such transients were accumulated. The spectrum acquisition time was generally 4 sec; reduction to 0.5 sec reduced the resolution of the spectrum but had no effect on the measured T_1 values.

T_1 measurements are usually analysed in terms of the simple expression developed[10] for relaxation among pairs of magnetic dipoles.

$$\frac{1}{T_1} = K\left(\frac{\gamma^4\hbar^2}{\omega_0}\right) I(I+1)\sum_j r_{ij}^{-6}\cdot\left\{\frac{\omega_0\tau_c}{1+(\omega_0\tau_c)^2} + \frac{4\omega_0\tau_c}{1+(2\omega_0\tau_c)^2}\right\}$$

Here γ is the magnetogyric ratio of the nuclear spin I, ω_0 is the angular frequency of the applied radiofrequency field, r_{ij} is the distance between magnetic nuclei, K is a constant that depends on the process of molecular reorientation and τ_c is the correlation time for this motion. It is commonly assumed that τ_c depends on temperature according to the relation

$$\tau_c = \tau_c^0 \exp(\Delta E/RT)$$

where ΔE is the activation energy of the relaxation process and τ_c^0 is a constant of the system having the dimensions of time. In the limit $\omega_0^2\tau_c^2 \ll 1$, $1/T_1$ is proportional to τ_c, and hence $1/T_1$ decreases with temperature. Further, in this limit, $T_1 = T_2$ unless there is some other slow motion present which can affect T_2 but not T_1. The linewidths observed are then equal to $1/\pi T_2$ *plus* any line broadening contributions[11]. ESR spectra were obtained on a Varian E3 spectrometer.

RESULTS

Sonicated dipalmitoyl lecithin

Above 40°, sonicated dipalmitoyl lecithin gives a high resolution proton NMR spectrum in which the $-N(CH_3)_3^+$, $-(CH_2)_n-$ and $-CH_3$ protons are well-defined resonances. As the temperature falls below 40°, however, the spectra undergo striking changes (Fig. 1). The spectral changes are completely reversible through repeated temperature cycles. At 43° solid dipalmitoyl lecithin containing approx. 20% water undergoes a transition to a lamellar liquid crystalline phase[6]. Coarse aqueous disper-

sions of dipalmitoyl lecithin can be sonicated above this temperature to give translucent solutions. On cooling below 40°, such solutions become cloudy, but on rewarming again become translucent. Evidence from ESR experiments described below show that there is no significant change in size of the vesicles during these transformations. On cooling from 67° to 35° the peak width at half height ($\Delta\nu_{\frac{1}{2}}$) of the $-N(CH_3)_3^+$ protons increases considerably (Fig. 2) while the area of the peak remains constant. Over the transition, a shoulder appearing on the side of the $-N(CH_3)_3^+$ peak

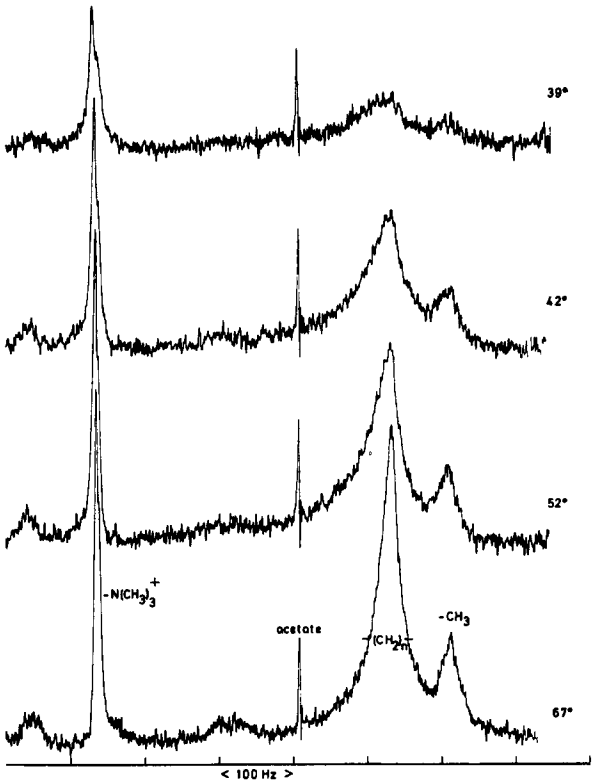


Fig. 1. Proton NMR spectra of sonicated aqueous dispersions of dipalmitoyl lecithin (230 mM) as a function of temperature.

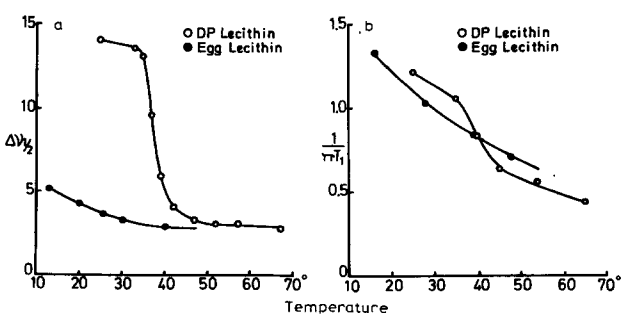


Fig. 2. (a) linewidths $\Delta\nu_{1/2}$ (Hz) and (b) $1/\pi T_1$ (sec^{-1}) for the $-N(CH_3)_3^+$ protons of sonicated aqueous dispersions of dipalmitoyl (○) and egg (●) lecithins as a function of temperature.

TABLE I

T_1 VALUES FOR PROTONS IN SONICATED DIPALMITOYL LECITHIN

Temp.	T_1		
	$-N(CH_3)_3^+$	$-(CH_2)_n-$	$-CH_3$
65°	0.73 ± 0.03	0.57 ± 0.04	0.83 ± 0.05
54°	0.57 ± 0.02	0.53 ± 0.04	0.84 ± 0.08
45°	0.50 ± 0.04	0.38 ± 0.02	0.57 ± 0.08
40°	0.38 ± 0.03	0.32 ± 0.02	0.42 ± 0.03
35°	0.30 ± 0.01	(0.35 ± 0.1 *	0.35 ± 0.1 *)
25°	0.26 ± 0.02	—	—

* Value for the broad peak corresponding to both the residual $-(CH_2)_n-$ and $-CH_3$ protons observed in the spectrum.

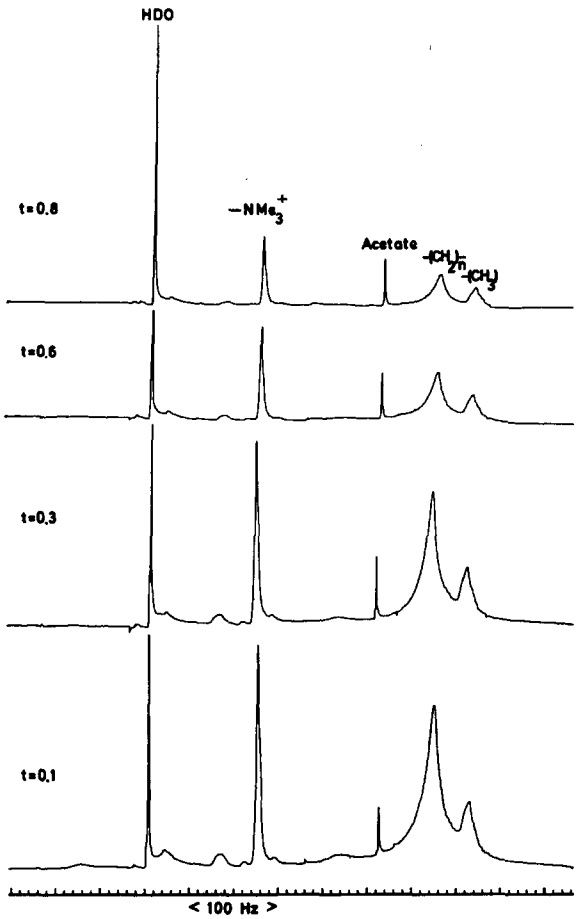


Fig. 3. Proton NMR spectra of sonicated aqueous dispersions of dipalmitoyl lecithin at 54° with a pulse sequence $(\pi - t - \pi/2 - t_2 - \pi/2)$, where t is the delay in secs between the π and $\pi/2$ pulses and t_2 is a delay which is long compared with the T_1 values to be measured.

becomes more pronounced (Fig. 1). This change is included in measurements of peak width at half height (Fig. 2); measurements at 3/4 height do not include this shoulder but change with temperature in a similar way.

From 67° to 42°, the peak width of the $-(CH_2)_n-$ resonance increases from 13.5 to 34 Hz and the area of the resonance remains constant, but at 39° the area has more than halved and has halved again by 37°, and is barely detectable at 35°. The terminal methyl group increases in linewidth at constant area from 14.5 Hz at 67° to 17 Hz at 42°, which is much less than the corresponding change in the $-(CH_2)_n-$ protons. Below 39°, however, the signal due to the terminal methyl group also disappears from the spectrum.

Measurements of T_1 have been made over the transition temperature (Table I). Fig. 3 shows typical spectra obtained with a pulse sequence $(\pi - t - \pi/2)$ where t is the delay between the π and $\pi/2$ pulses. With this pulse sequence, the shorter the T_1 value for a nucleus, the faster the decrease in amplitude of its resonance as t increases. Figs. 4a and 4b show plots of peak intensities for the $-N(CH_3)_3^+$, $-(CH_2)_n-$ and $-CH_3$ protons against pulse delay t. The decay of all three resonances with increasing t values appears to be completely uniform, and there were no significant changes in peak width or shape, or of the separation of the $-(CH_2)_n-$ and $-CH_3$ peaks with t.

The observation of a single exponential decay for the $-(CH_2)_n-$ protons (Fig. 4b) does not necessarily imply that all these protons are characterised by a single T_1, thus a range of T_1's from approx. 0.1 to approx. 1.0 sec, the majority having the longer T_1's, would appear experimentally as a single T_1. From the slopes of these lines, the values of T_1 given in Table I were calculated.

The variation of $1/\pi T_1$ with temperature for the $-N(CH_3)_3^+$ protons shows exactly the same discontinuity at approx. 40° as observed for the linewidth measure-

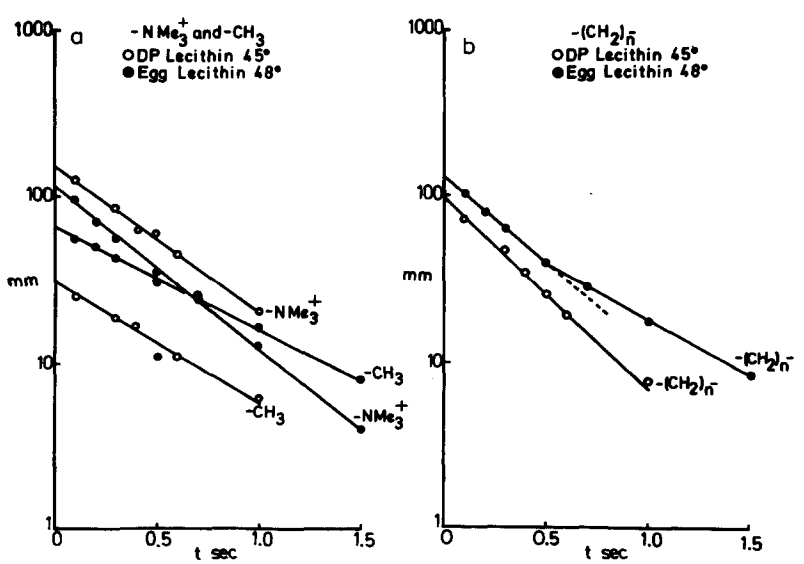


Fig. 4. Intensities (mm) of the resonances of sonicated aqueous dispersions of dipalmitoyl lecithin at 45° (○) and egg lecithin at 48° (●) as a function of t. (a) $-N(CH_3)_3^+$ and $-CH_3$ protons. (b) $-(CH_2)_n-$ protons.

ments (Fig. 2b). $1/\pi T_1$ values for the $-(CH_2)_n-$ and $-CH_3$ protons also show a sudden sharp increase below approx. 50° and then a steady increase down to 35°, below which temperature no signal could be detected. It is clear that for all three resonances, $\Delta\nu_{\frac{1}{2}}$ is substantially greater than $1/\pi T_1$ and that the linewidth also increases considerably more than $1/\pi T_1$ through the transition.

The activation energies of the relaxation of the $-N(CH_3)_3^+$ protons calculated from plots of ln T_1 against 1/K where K is the absolute temperature are shown in Table II. Above and below the transition, the activation energy is 3–4 kcal/mole, but from 45° to 35° it increases to 10 kcal/mole. The data in Table I indicate that the activation energies for the spin-lattice relaxation of the $-(CH_2)_n-$ and $-CH_3$ protons also undergo a considerable increase at temperatures below approx. 50°.

Sonicated egg lecithin

The corresponding T_1 values for the $-N(CH_3)_3^+$, $-(CH_2)_n-$ and $-CH_3$ protons in samples of sonicated egg lecithin are given in Table III. Results obtained for 20 % and 5 % vesicle suspensions (w/w) agreed within experimental error. Plots of peak intensities against pulse delay t at 48° (Figs. 4a, 4b) show a linear decay for the $-CH_3$ and $-N(CH_3)_3^+$ protons, but a significant non-linearity in the decay of the $-(CH_2)_n-$ protons. This appears to be a result of the distribution of chain lengths and double bonds in egg lecithin, since this effect was not observed with dipalmitoyl lecithin

TABLE II

ACTIVATION ENERGIES FOR RELAXATION PROCESSES.

	(kcal/mole) $-N(CH_3)_3^+$ *protons*
Sonicated dipalmitoyl lecithin from 65 to 45°	3.9
Dipalmitoyl lecithin from 45 to 35°	10
Dipalmitoyl lecithin from 35 to 25°	3
Sonicated egg lecithin	3.4

TABLE III

T_1 VALUES FOR SONICATED EGG LECITHIN SYSTEMS

Systems	*Temp.*	T_1		
		$-N(CH_3)_3^+$	$-(CH_2)_n-$	$-CH_3$
Sonicated egg lecthin	48°	0.45 ± 0.01	0.53 ± 0.02 *	0.72 ± 0.02
	40°	0.39 ± 0.01	0.47 ± 0.02 **	0.54 ± 0.02
	28°	0.31 ± 0.02	0.38 ± 0.02	0.36 ± 0.02
	16°	0.24 ± 0.05	0.25 ± 0.06	0.28 ± 0.06
Sonicated egg lecithin + 4:1 cholesterol	48°	0.45 ± 0.07	0.35 ± 0.05	0.40 ± 0.03
	40°	0.40 ± 0.01	0.31 ± 0.01	0.31 ± 0.01
	28°	0.29 ± 0.01	0.31 ± 0.02	0.34 ± 0.01
Sonicated egg lecithin + 2:1 cholesterol	48°	0.41 ± 0.02	0.18 ± 0.01	0.22 ± 0.02
	40°	0.35 ± 0.01	0.14 ± 0.03	0.19 ± 0.02
	28°	0.21 ± 0.01	0.08 ± 0.02	0.12 ± 0.01

* Average of 0.43 ± 0.02 and 0.57 ± 0.01.
** Average of 0.42 ± 0.02 and 0.53 ± 0.04.

(see above). The results are similar at 40°, but at 28° and lower, the decay was linear within experimental error.

All the relaxation times increase with temperature (Table I) so that $\omega_0^2\tau_c^2 \ll 1$ and thus $1/T_1 \propto \tau_c$ (as for dipalmitoyl lecithin). Linear plots are obtained for ln T_1 against $1/K$, so that the activation energies remain constant for all three resonances over this temperature range (Table II), and the $-N(CH_3)_3^+$ value of 3.4 kcal/mole is similar to that for dipalmitoyl lecithin either side of the transition. The thermal transition in egg lecithin occurs at approx. −10°.

Again, changes in linewidth cannot be attributed to changes in $1/\pi\ T_1$. The linewidth of the $-N(CH)_3)_3^+$ protons increased from 2.8 Hz at 40° to 4.7 Hz at 16°, whereas the change in $1/\pi\ T_1$ over this temperature range was only 0.5 Hz. There appears to be considerable variation in the linewidth for the $-(CH_2)_n-$ protons from one sample of egg lecithin to another. The increase in linewidth from 40 to 25°, how-however, is fairly constant at 3 Hz. The change in $1/\pi\ T_1$ over this temperature range is 0.2 Hz.

The effects of cholesterol

There are significant differences between the NMR spectra obtained from sonicated egg lecithin with and without cholesterol, some of which have been reported previously[12]. Cholesterol slightly broadens the peak due to the $-N(CH_3)_3^+$ protons. T_1 values for these protons at 1:4 and 1:2 cholesterol: lecithin (C:L) ratios are given in Table III. It is clear that at all temperatures T_1 is smaller than in the absence of cholesterol, so that motion of the $-N(CH_3)_3^+$ is more restricted. The activation energy for the $-N(CH_3)_3^+$ proton is significantly increased at 1:2 C:L to 5–8 kcal/mole. The $-N(CH_3)_3^+$ linewidth with 1:2 C:L is 3.8 Hz at 39° and 4.9 Hz at 29°, as compared with linewidths of 2.9 and 3.3 Hz, respectively, in the absence of cholesterol. Again the linewidth changes cannot be attributed solely to changes in T_1, since for 1:2 C:L ($1/\pi T_1$) increases by only approx. 0.6 Hz over this temperature range. It is also clear that the line broadening effect of cholesterol is more marked at the lower temperature.

The most marked effect of cholesterol, however, is on the peaks due to the $-(CH_2)_n-$ and $-CH_3$ protons. On addition of cholesterol these broaden and reduce in intensity. The $-(CH_2)_n-$ peak has more than doubled in width at a 1:2 C:L ratio, and at 1:1 ratio, the $-(CH_2)_n-$ peak is extremely broad. Since cholesterol itself has protons which appear in this region of the spectrum, the interpretation of these results is uncertain. However, the similarity to the effect of cooling sonicated dipalmitoyl lecithin below the transition temperature is marked, in that addition of cholesterol broadens the $-N(CH_3)_3^+$ peak and broadens the $-(CH_2)_n-$ peak eventually to the point where it is no longer visible. T_1 values for the $-(CH_2)_n-$ and $-CH_3$ peaks are given in Table III. The T_1 values are an undefined average for the lecithin and cholesterol protons. It certainly seems however that there is a reduction in T_1 value for the lecithin-$(CH_2)_n-$ protons. If, for example, the lecithin $-(CH_2)_n-$ protons had the same T_1 value as in the absence of cholesterol, then T_1 values for the cholesterol protons would have to be less than 0.1 sec to yield the observed average T_1 values for the composite signal. Since it can be calculated that this mixture of T_1 values would appear as a non-exponential decay of the composite resonance, whereas a single exponential decay is observed for all temperatures (see, for example, Fig. 5), it can

be concluded that the T_1 values for the $-(CH_2)_n-$ component of the resonances is substantially reduced.

These results therefore suggest that cholesterol causes a considerable reduction in the degree of freedom of motion of the chain protons and a much smaller reduction for the $-N(CH_3)_3^+$ protons. The change in T_1 for the chain protons is again quite inadequate to explain the change in linewidth caused by addition of the cholesterol.

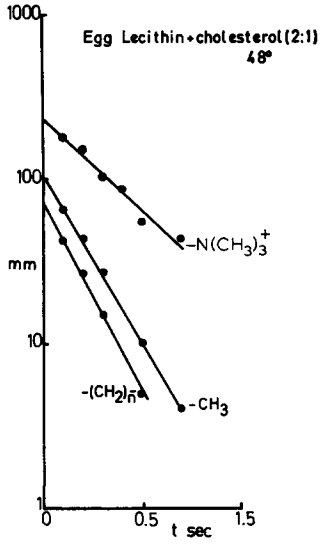
Lecithin in organic solvents

We have also examined lecithin in methanol and chloroform solutions to determine whether the T_1 relaxation times observed in the bilayer structure are characteristic of that structural arrangement.

Egg lecithin exists as a monomer in methanol at the boiling point and the molecular weight at lower temperatures corresponds to a trimer[13,14]. The linewidth at 40° due to the $-N(CH_3)_3^+$ protons is 1.7 Hz, consistent with ^{14}N nuclear quadrupole coupling. The linewidth due to the $-(CH_2)_n-$ protons is 2.3 Hz and the terminal methyl protons appear as a distorted triplet of total linewidth approx. 11 Hz. A single exponential decay is again observed for the $-(CH_2)_n-$ protons (for example, see Fig. 6).

In chloroform solution, ^{13}C relaxation data suggest the formation of a micellar structure with the polar headgroups restricted in motion by being at the centre of the micelle with the alkyl chains exposed to the [^{2}H]chloroform solvent. The T_1 values from chloroform solutions of dipalmitoyl lecithin are listed in Table IV. The increase in T_1 with increasing temperature again shows that $\omega_0^2\tau_c^2 \ll 1$. There is a general decrease in motion for all the protons relative to methanol solution, although that for the $-N(CH_3)_3^+$ protons is significantly greater.

Activation energies calculated for the relaxation of all these protons, in both methanol and chloroform solution, are in the range 3–4 kcal/mole.



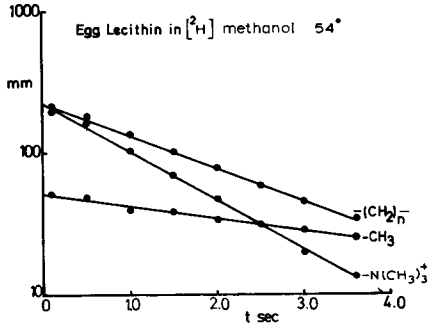
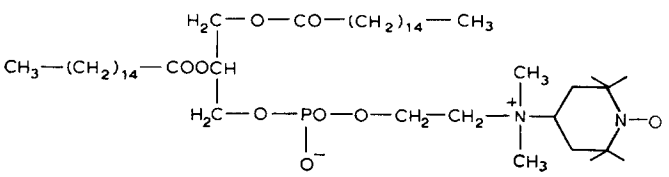


Fig. 5. Intensities (mm) of the $-N(CH_3)_3^+$, $-(CH_2)_n-$ and $-CH_3$ proton resonances of sonicated aqueous dispersions of egg lecithin: cholesterol (2:1), at 48° as a function of t.

Fig. 6. Intensities of the $-N(CH_3)_3^+$, $-(CH_2)_n-$ and $-CH_3$ protons for a solution of 140 mM egg lecithin in [$^2H_4$]methanol at 54° as a function of t.

ESR

Spin-label experiments suggest that sonicated vesicles of dipalmitoyl lecithin retain their size and state of sonication after being cycled repeatedly through the transition temperature of the lipid. The size and number of shells present in a suspension of liposomes can be estimated by determining the distribution of a nitroxide-labelled dipalmitoyl lecithin molecule (I)[22]



(I)

between the outside facing surface of the liposomes and the interior. As the vesicles decrease in diameter, the difference in surface area of the inside and outside of the bilayer causes an asymmetry in the number of labels on each side. The outside facing nitroxides may be reduced instantaneously by the addition of ascorbic acid at 0°, leaving a residual spectrum due to nitroxides either facing inwards or incorporated into smaller vesicles inside. It was found that only 12 % of the spin label was instantaneously reducible in unsonicated dipalmitoyl lecithin, suggesting that the vesicles are large and contain many shells. When the vesicles were sonicated to clearness and maintained above the transistion temperature, 61 % of the labels were instantly reducible. On cooling the same sample below the transition temperature, the same percentage of spin labels remained reducible, in spite of the large increase in light scattering described previously. Further temperature cycles over 2 h did not alter the distribution of reducible labels. If it is assumed that the labels distribute equally over a given area of lipid, then 61 % of labels facing outwards corresponds to vesicles with an average outside diameter of approx. 500Å, assuming that all the shells are single spheres and that the lipid bilayer is 50 Å thick. Any reversion to the unsonicated state with the formation of larger vesicles would cause the proportion of outside facing spin labels to decrease. One sample of the sonicated preparation of spin labelled vesicles was maintained at 18° overnight, and the amount of label on the outside surface was then reduced to 55 %. Another sample was subjected to eleven cycles of freezing and thawing and the amount of outside label was reduced from 60 to 52 %. This suggests that partial reversion to the unsonicated state occurs after these harsh treatments.

TABLE IV

T_1 VALUES FOR LECITHIN PROTONS IN ORGANIC SOLVENTS

Systems	*Temp.*	T_1		
		$-N(CH_3)_3^+$	$-(CH_2)_n-$	$-CH_3$
Egg lecithin in [$^2H_4$]methanol	54°	1.24 ± 0.02	1.84 ± 0.02	4.93 ± 0.30
	40°	0.91 ± 0.01	1.37 ± 0.01	3.74 ± 0.12
Dipalmitoyl lecithin in [^{2}H]chloroform	54°	0.18 ± 0.01	1.06 ± 0.02	3.23 ± 0.11
	40°	0.15 ± 0.01	0.89 ± 0.03	3.29 ± 0.30
	28°	0.10 ± 0.01	0.64 ± 0.03	2.03 ± 0.30

DISCUSSION

The resolution and sensitivity of the T_1 measurements by the Fourier transform technique of FREEMAN AND HILL[9] used in these studies clearly demonstrated that in all the structural forms of the lecithin molecules examined, the T_1 values for the three major resonances are not equal. Both the magnitudes of the T_1 values and the relative values in each solvent are characteristic of the type of structure formed and provide information about the molecular motion and interactions of the lecithin molecules.

In the simplest system of lecithin dissolved in methanol, the T_1 values are larger than in [^{2}H]chloroform or ^2H_2O, and consistent with very rapid molecular motion of a paucimeric structure (τ_c approx. 10^{-11} sec). The relatively small T_1 value for the $-N(CH_3)_3^+$ protons could well be caused by strong solvation of the polar headgroup by methanol, resulting in reduced mobility. The T_1 values for the $-(CH_2)_n-$ and terminal $-CH_3$ are similar to the reported values for dodecanol[15] of 1.7 sec at 32°, extrapolated to infinite dilution.

The T_1 values in [^{2}H]chloroform are all shorter than in methanol, with the largest decrease for the $-N(CH_3)_3^+$ protons. This agrees with the structure based on ^{13}C relaxation data with the $-N(CH_3)_3^+$ groups packed at the centre of a micelle in [^{2}H]chloroform. In such an inverted micelle with the hydrocarbon chains rather strongly immobilised at the headgroup end, chain motion will be cumulative towards the relatively free terminal methyl, giving these protons the longer T_1 values observed. The T_1 values for the terminal methyl in the micelle structure are considerably shorter than in methanol, so that chain motion is clearly more restricted in the polymeric micelle. As explained previously, the proton T_1 data for the $-(CH_2)_n-$ chain can be insensitive to a distribution of correlation times within the chain, but a gradation of increasing T_1 values towards the terminal methyl in [^{2}H]chloroform is obtained by ^{13}C relaxation measurements[8].

On reorganising dipalmitoyl or egg lecithin into a bilayer in ^2H_2O, the proton T_1 values again decrease for both chain resonances, indicating strong steric interaction through lateral packing of the chains. The $-N(CH_3)_3^+$ resonance however has an increased T_1 when the $-N(CH_3)_3^+$ group is on the surface of the bilayer and exposed to ^2H_2O, compared with the inverted micelle structure in [^{2}H]chloroform. Although the three T_1 values in the bilayer are more closely grouped than in the other two solvents, they are generally quite distinct, contrary to the data of BARRATT[6] and CHAN *et al.*[5]. The proton T_1 values are consistent with a structure which is strongly immobilised at the carboxyl end of the fatty acid chains, with increasing molecular motion both towards the terminal methyl and the polar $-N(CH_3)_3^+$ group, based on ^{13}C data for the same system [8]. Spin diffusion to either the terminal methyl or the $-N(CH_3)_3^+$ protons as the dominant relaxation mechanism is ruled out, and the differences in the proton T_1 values reflect motional differences within the bilayer.

The experimental observation of a single T_1 for the chain $-(CH_2)_n-$ protons does not necessarily imply a single T_1 value for all these protons, since a gradation in T_1 values can appear experimentally as a single T_1. Our results show that spin diffusion does not equalize all the proton T_1 values in the sample; there will clearly be dipole–dipole coupling between nearest neighbour protons, and this will tend to equalize the T_1 values of these protons, and so tend to make motional differences appear smaller than in reality they are. The ^{13}C relaxation studies do not suffer from

this difficulty, since the probability of two ^{13}C nuclei being nearest neighbours is negligible (< 0.001), and a clear gradation in T_1 values is found for the ^{13}C nuclei in the $-(CH_2)_n-$ chain.

In some circumstances differences in T_1 values for a single composite resonance can be detected as non-exponential relaxation, for example for the $-(CH_2)_n-$ chains in egg lecithin. This probably arises from the presence of a wide range of chain lengths of different degrees of saturation, rather than gradations in T_1 within individual chains, since a single exponential relaxation is observed in the homogeneous dipalmitoyl lecithin bilayer, as described above. It is also of interest that the average $-(CH_2)_n-$ T_1 is consistently shorter than T_1 for the $-N(CH_3)_3^+$ in the dipalmitoyl lecithin bilayer, but longer in egg lecithin bilayers, perhaps reflecting a greater fluidity in the polydisperse chain system. We will report shortly on systematic proton and ^{13}C T_1 measurements for simple mixtures of lecithins of defined structure (*e.g.* dipalmitoyl + dioleyl lecithins), in which the relaxation times for the separate lipids can be determined simultaneously. The effect on the thermal transition observed in pure dipalmitoyl lecithin is of interest and should provide experimental evidence about concerted motion of chains within the bilayer as suggested by the tilted chain model of McFARLAND AND McCONNELL[23], and about the nature of heterogeneous lipid interactions which are a universal feature of biological membranes.

The increase in molecular motion from the glycerol group both towards the terminal methyl and the $-N(CH_3)_3^+$ implied by the ^1H and ^{13}C T_1 data, suggests that the lipids molecules are most tightly packed together in a bilayer somewhere in the region of the glycerol group. This is also consistent with evidence from spin labelled lecithin studies[24]. The molecular motion of the lipids and therefore their packing is clearly sensitive to perturbation as shown by the effects of cholesterol which produces a large decrease in T_1 for the fatty acid chain resonances. Cholesterol also reduces the permeability of the vesicles to small molecules and ions (J. R. HOULT, unpublished data). Since there is good evidence that the partition of a number of solutes into the bilayer decreases with increased chain packing, it is probable that the tightly packed region at the glycerol group constitutes the main permeability barrier in a bilayer. The perturbations in molecular motion induced by cholesterol in this region of the bilayer may be directly relevant to their effects on permeability.

The broadening and disappearance of the fatty acid chain resonances of dipalmitoyl lecithin through the thermal transistion demonstrate considerable changes in the proton environment and coincide with other well-defined physical changes in the structure of the bilayer. Above the thermal transistion temperature, X-ray diffraction data show that the hydrocarbon chains are in a relatively liquid condition, with considerable thermal motion, but that below the transition the chains are crystalline and motion is very restricted. The X-ray diffraction data also suggest that over the transition, there are regions in the lamellae in which the chains are liquid whereas in other regions they are crystalline[16,17].

The decrease in area of the signals due to the $-(CH_2)_n-$ and $-CH_3$ protons in the NMR spectrum below 42°, can be attributed to the formation of regions in which the chains are frozen. In such regions, extreme signal broadening will cause the signals to become undetectable.

The observation of a signal from the $-N(CH_3)_3^+$ protons below the transition, shows that considerable motion must be allowed for these protons even when the

hydrocarbon chains are frozen. The inflection in both the linewidth and the T_1 curves occuring at the transition may reflect some configurational change for the $-N(CH_3)_3^+$ group (perhaps from a gauche conformation of the headgroup below the transition to some straightened form above it). Relaxation in both structures will be governed by reorientation of the $-N(CH_3)_3^+$ groups which are at least partially exposed to the ^2H_2O solvent, and it is consistent that the activation energy of viscosity of ^2H_2O is 3.94 kcal/mole, comparable to the activation energies found for the relaxation of the $-N(CH_3)_3^+$ protons either side of the transition region (Table II).

In contrast to the T_1 data which can be interpreted at least semiquantitatively in terms of the molecular motion of lecithin in each solvent system, the linewidth data are much harder to interpret. For lecithin in methanol, the linewidths (allowing for coupling) correspond to the values of $1/\pi\ T_1$, implying that the linewidths are determined by T_2 (as defined by ABRAGAM[11]) and that $T_1 \simeq T_2$.

In ^2H_2O, the linewidths are considerably greater than $1/\pi\ T_1$ with the largest differences for the chain resonances in the bilayer structure. Since the temperature dependence indicates that the extreme narrowing condition holds ($\omega_0^2\ \tau_c^2 \ll 1$), one might expect $T_1 \simeq T_2$. It therefore follows that either there is some slow motion present which will contribute to T_2 and not to T_1 (so that $T_2 < T_1$) or that there is some further line broadening mechanism (or, of course, a mixture of these two possible effects). Possible causes of further line broadening are magnetic anisotropy and dipole–dipole broadening. The non-Lorentzian shape of the peaks, which increases as the temperature is lowered, would suggest a significant contribution from these additional line broadening mechanisms. What is required is T_2 values measured independently of the linewidths, so that the additional line broadening and the relative T_1:T_2 values can be estimated. We defer further discussion of the mechanism of line broadening until independent T_2 data are available for lecithins specifically labelled with ^{13}C and ^{19}F nuclei. We simply note here that proton linewidth measurements cannot be used alone as a quantitative measure of motional changes since $1/\pi\ T_1$ is generally $\ll \Delta\nu_{\frac{1}{2}}$.

It is, however, important to understand the mechanism of line broadening in the lecithin vesicle bilayer, since all the experimental methods of measuring T_1 for individual resonances depend on being able to observe the resonances. The disappearance of the proton (and ^{13}C) fatty acid resonances below the transition temperature is relevant to attempts to observe NMR spectra in isotopically labelled membranes. For example, *Mycoplasma laidlawii* membranes grown on a medium containing palmitic acid incorporate up to 80 % palmitic acid in the membrane phospholipids[25], and these membranes show a thermal transition in these lipids virtually identical with lipid vesicles made from the separated membrane lipids[17]. There is substantial evidence from spin labelling experiments that the membrane proteins in Mycoplasma membranes have only weak effects on lipid–lipid interactions within the bilayer[18], so that we would expect the same line broadening mechanisms to cause the spectra to disappear in the membrane below the thermal transition, as in the present model system experiments.

Although the data suggest that cholesterol has qualitatively similar effects on the lecithin spectra as the thermal transition, other experiments have been reported which suggest that cholesterol is not entirely responsible for the absence of a high resolution proton spectrum from the lipids in erythrocyte membranes[20]. The con-

clusions from the present experiments are that in extending them to biological membranes, a membrane should be selected containing little cholesterol, in which the protein has little effect on the packing of the lipids in the bilayer and the thermal transition in the lipids should be below approx. 40°.

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