

## Magnetic Resonance Studies on Membrane and Model Membrane Systems: Proton Magnetic Relaxation Rates in Sonicated Lecithin Dispersions\*

(PMR/nuclear relaxation/Fourier transform NMR)

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**ABSTRACT** We have used Fourier transform nuclear magnetic resonance methods to measure the spin-lattice and transverse relaxation times at 220 MHz of the choline *N*-methyl and the fatty-acid  $\alpha$ -carbonyl, allyl, vinyl, methylene, and methyl protons of sonicated egg-yolk lecithin. Over the temperature range investigated the  $T_1$  values were, in general, similar to, but different from, one another, suggesting that the relaxation rates of all of the fatty-acid protons are not determined solely by spin-diffusion to a heat sink. Arrhenius plots of the  $T_1$  data gave activation energies similar to those for the barriers to internal rotation in alkanes. The values of the transverse relaxation rate,  $T_2$ , showed a relatively large variation among the proton resonances; about 20% of the methylene protons had a  $T_2$  of 56 msec, while the remaining protons relaxed according to a distribution of values all shorter than 20 msec. Such a distribution of relaxation times is envisioned to arise from a distribution of correlation times stemming from complex motions in which extended angular excursions of the fatty acid chain are coupled to *trans*  $\rightarrow$  *gauche* conformational transitions.

Evidence is accumulating that many biological membranes contain, to a greater or lesser extent, regions of lipid bilayers (1-3). Since many conclusions drawn from models (4-8) of these systems may indeed be applicable to natural membranes, the structural and functional properties of lipid bilayers is an active area of research. Nuclear magnetic resonance (NMR) provides a powerful method for investigation of some structural and dynamic properties of such systems. In particular, the longitudinal,  $T_1$ , and transverse,  $T_2$ , nuclear relaxation times are explicitly and implicitly related to molecular motions (9). Recent advances in Fourier transform NMR spectroscopy provide the means for measurement of these relaxation times in complex spectra (refs. 10, 11, and Horwitz *et al.*, unpublished). Since sonication of aqueous dispersions of lecithin produces relatively reproducible and homogeneous vesicles that give rise to high-resolution NMR spectra (6-8, 12) we have chosen this system for our initial measurements of the nuclear relaxation times in aqueous dispersions of phospholipid bilayers.

### MATERIALS AND METHODS

Proton magnetic resonance (PMR) measurements were performed on a Varian HR-220 spectrometer, extensively modified for Fourier transform operation.  $^{31}\text{P}$  magnetic resonance measurements were performed at 24.3 MHz on an instrument

of our own design. Both spectrometers are interfaced with a computer system designed in this laboratory. The  $T_1$  values were measured by the method of Vold *et al.* (10), while the  $T_2$  values were measured by a variation of the Carr-Purcell method (13, 14). A Carr-Purcell sequence is established and terminated after the 1, 2, 3, . . . *n*th pulse in *n* experiments. In each experiment, the echo after the final pulse is Fourier transformed to yield the partially (transversally) relaxed spectrum. The decay of each individual line then establishes its value of  $T_2$ . The details of this method will be published elsewhere (Horwitz, A., Horsley, W. J., Salmeen, I. S., Klein, M. P., unpublished data). Nonspinning capillaries were used for the  $T_2$  measurements. The resonance assignments followed those of Chapman and Morrison (15).

Lecithin was prepared from hen egg yolks by the method of Singleton *et al.* (16), and was further purified by chromatography on silicic acid. Unsonicated lecithin dispersions were prepared with 30-60  $\mu\text{mol}$  of lipid/ml of  $\text{D}_2\text{O}$  by the method described by Demel *et al.* (17). The lipids were then sonicated for 15 min on ice in a Branson 185E sonicator and centrifuged at  $17,300 \times g$  for 30 min at  $4^\circ$ . All samples were prepared and stored under argon in 0.15 M KCl-0.1 mM EDTA. The lipid concentration was determined by phos-

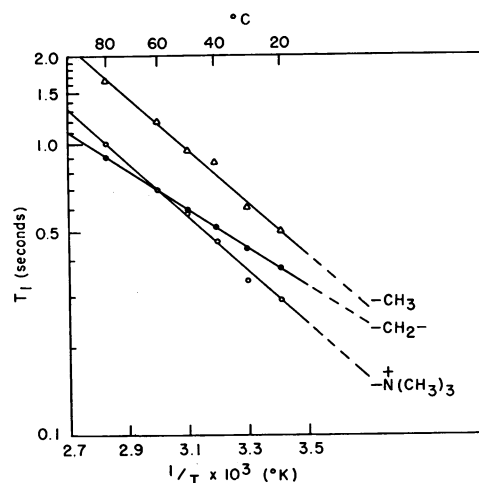


FIG. 1. Arrhenius plots of the spin-lattice relaxation time against temperature for some selected resonances in sonicated egg-yolk lecithin. The data presented were obtained from two different samples run on two different days.

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phate analysis (18), and the purity was checked by thin-layer chromatography on silica gel developed with chloroform-methanol-water 65:25:4, and by measurement of the oxidation index (19).

### RESULTS

In sonicated lecithin at 220 MHz, the choline *N*-methyl and the fatty-acid methylene, allyl, vinyl, methyl, and  $\alpha$ -carbonyl protons are among the resolved resonances (9, 15). Their  $T_1$  values are given in Table 1. Fig. 1 shows the temperature dependencies of the values of the spin-lattice relaxation times for three of these resonances. In the temperature region investigated, this plot reveals two important points: (a)  $T_1$  increases with temperature for each of these classes of protons, and (b) the  $T_1$  of the terminal methyl protons is clearly longer than that of the choline or methylene protons, which in turn appear to differ from each other. The fatty-acid methylene protons appear to be characterized by a single value of  $T_1$ , but a distribution of values cannot be excluded. The data presented in Table 1 indicate that the  $T_1$  values for the vinyl and  $\alpha$ -carbonyl protons are similar to, but different from, those of the methylene protons. We may treat the data of Fig. 1, as well as the data for the other resonances that have a similar dependence on temperature, as Arrhenius plots and derive the activation energies for the thermal relaxation processes (20). The values so derived are given in Table 1, and agree favorably with literature values for potential barriers to internal rotation in alkanes (21). Since our observations extended over a small temperature range, we may be unable to detect a distribution of activation energies for the methylene protons if such were to exist.

In Table 1 are also listed preliminary values of the transverse relaxation times for some selected resonances in sonicated egg lecithin. The *N*-methyl, methyl, and phosphorus nuclei each appeared to relax according to a single exponential. For each of these groups, the value of the transverse relaxation time that one estimates from the conventional linewidth,  $T_2^*$ , is less than or equal to our experimental  $T_2$  (12). The methylene protons exhibited a heterogeneity of  $T_2$  values. About 20% showed a single value of  $T_2$  of about 56 msec; the remaining 80% were much shorter and nonexponential, implicating a distribution of  $T_2$  values. Aside from the methyl and *N*-methyl protons, the remaining proton resonances exhibited relatively short values of  $T_2$ . Similar results have been obtained with dimyristoyl-*L*- $\alpha$ -lecithin (at temperatures above the transition point (22)).

### DISCUSSION

Because of the implicit complexity of relaxation processes in general, and in these systems in particular, a quantitative discussion lies outside the scope of this note (11, 20). Thus, the following comments on our relaxation results should be considered as suggestive. Let us assume, however, that the dominant relaxation results from modulation of the dipolar coupling to the nearest protons, e.g., the companion proton for the methylene group and the two companion protons on a methyl group.

For all proton resonances,  $T_1$  increases with increasing temperature, indicating that at 220 MHz we are in the short-correlation-time regime. The data in Table 1 reveal the close agreement between the activation energies for thermal relaxation and for internal rotation; they provide strong evidence that the dominant source of thermal relaxation derives from modulation of the intramolecular dipolar interactions by the internal rotations. In the short-correlation-time regime, theories based on isotropic motion predict that  $T_1 = T_2$ , a result at variance with our results that  $T_1 > T_2$ . Thus, we must conclude that for each and every methylene group there are at least two correlation times, one short that leads to  $T_1$  processes, and another long that leads to  $T_2$  processes. Two classes of motion may be suggested to account for the observations: (i) relatively small displacements due to rotations of individual methylene carbon atoms that occur at high frequencies, and (ii) relatively larger angular displacements of protons further down the chain that are a consequence of the high-frequency rotations. The former motions, which are roughly constant along the fatty-acid chains, would result in the longer, roughly constant values of  $T_1$ , in agreement with our observations. Manipulation of Corey-Pauling-Koltun (CPK) space-filling models indicates that motions of the latter class may involve large segments of the fatty-acid chains. That is, starting from the minimum energy, all *trans* conformation, rotation about a single C—C bond would result, for example, in a *gauche* (+) conformation. That segment of the molecule between the origin of the rotation and the methyl end would execute a large displacement; a simultaneous *gauche* (−) rotation about the bond  $\beta$  (toward the methyl end) from the first bond would virtually restore the original linear shape. Rotations about C—C bonds closer to the methyl end would lead to displacements requiring less volume, and would be less likely to lead to collisional encounters with neighboring fatty-acid chains, and thus would be more prob-

TABLE 1. Spin-lattice,  $T_1$ , and transverse,  $T_2$ , relaxation times and activation energies for some resonances of sonicated egg lecithin

	$-\overset{+}{N}(CH_3)_3$	$-\text{CH}_2-\overset{\text{O}}{\parallel}{\text{C}}-\text{O}-$	$-\text{CH}_2-$	$-\text{CH}_2-\overset{\text{H}}{\underset{\text{H}}{\text{C}}}=\overset{\text{H}}{\text{C}}-$	$-\text{HC}=\text{CH}-$	$-\text{CH}_3$	$^{31}\text{P}$
$T_1$ (sec)	$0.41 \pm 0.02$	$0.34 \pm 0.02$	$0.47 \pm 0.03$	$0.41 \pm 0.04$	$0.54 \pm 0.03$	$0.76 \pm 0.06$	$1.4 \pm 0.1$ (a) $8.5 \pm 0.7$ (b)
$T_2$ (sec)	0.075	0.008	$0.056$ (20%) $<0.02$ (80%)	0.015	0.020	0.036	0.110
$E_a$ (kcal/mol)	$4.3 \pm 0.3$	$2.8 \pm 0.4$	$3.0 \pm 0.2$	$2.7 \pm 0.2$	$3.2 \pm 0.3$	$4.2 \pm 0.3$	—

The  $T_1$  values were determined at 40°. For a given experiment the estimated error was within 10%, as indicated; however, for experiments performed on different days with different samples, the error sometimes exceeded this limit. The estimates of  $T_2$  were made at 20°; the text contains an explanation of the two relaxation times for the methylene protons. The phosphorus nuclear relaxation times were measured at 34° and are included here for completeness and will be discussed in a subsequent publication. (a) refers to dimyristoyl-*L*- $\alpha$ -lecithin and (b) refers to egg-yolk lecithin.

able and result in longer relaxation times. This is, of course, only one of many possible conformational transitions that could account for our observations. Such dynamics simultaneously maintain minimal displacements of large segments of the molecules, account for the observed activation energy for and nearly constant value of  $T_1$  for the methylene protons, and finally provide a mechanism for the abrupt increase in transverse relaxation times for the methyl protons and those methylene protons that are probably near the methyl terminus.

Simplistic estimates of the correlation times corresponding to the measured values of  $T_2$  lead to values in the range of  $10^{-11} < \tau_c < 10^{-8}$  sec (20). Since we have measurements at only 220 MHz, we do not assign a correlation time for the  $T_1$  processes, but may safely state that it is certainly less than  $10^{-9}$  sec. Since the minimum diameter for sonicated lecithin vesicles is about 25 nm (250 Å), for which the Debye correlation time at 20° is about  $10^{-6}$  sec (9)†, we may confidently rule out the tumbling of the vesicles as a significant source of motion contributing to nuclear relaxation.

A recent paper (23) reports values of  $T_1$  for the  $^{13}\text{C}$  NMR of sonicated lecithin bilayers that show that the  $^{13}\text{C}$  nuclei exhibit a distribution of thermal relaxation times. The shortest  $T_1$  values apply to the carbons at the polar end of the molecules, while the values for carbon atoms 3–13 are longer and nearly equal. The three remaining carbons, 14–16, show increasingly longer times, with the terminal methyl the longest. Although the relaxation processes or mechanisms for protons differ in detail from those for  $^{13}\text{C}$ , the relaxation times share a similar functional dependence on the correlation times. These  $^{13}\text{C}$  data imply a relatively long correlation time at the polar end, a shorter and nearly constant value for carbon atoms 3–13, and still shorter values of correlation times as the terminal methyl is approached. Although not stated by the authors, a reasonable interpretation of these data is that a large segment of the molecule executes relatively uniform motion at a high frequency that is significantly slower than that executed at the terminal methyl end of the molecule. This interpretation is in accord with that offered above to account for the proton  $T_1$  and  $T_2$  data.

Proton relaxation rates in lecithin have been measured and discussed by other authors (24–26). Their measurements, made by other methods, suggest that the protons of lecithin are characterized by a single value of  $T_1$ . By argument in analogy with results from studies on solid *n*-alkanes (27–29), a spin-diffusion mechanism has been proposed (30). The spin-diffusion mechanism for these molecules proposes that spin-spin flip-flops between pairwise adjacent protons propagate along the aliphatic chain toward the terminal methyl. Because of its relative freedom to reorient, the methyl has a shorter  $T_1$  than do the methylene protons, and thus serves as a heat sink at these low temperatures. The entire molecule is then characterized by a single  $T_1$ . If a spin-diffusion mechanism were operative in lecithin, as has been suggested, then the value of  $T_1$  would only provide information about the motion of the heat sink. In a recent paper, Chapman has tentatively proposed the choline headgroup as the heat sink (26).

The results from Table 1 show that there is not a single spin-lattice relaxation time characterizing the protons of

sonicated egg lecithin at 220 MHz. Further, the apparent existence of different relaxation times for protons along the methylene chain clearly excludes efficient coupling among all the methylene protons. If spin-diffusion contributes significantly to thermal relaxation in these molecules, it is likely restricted to short segments of the methylene chain. If so, one must inquire into the nature and location of the heat sinks.

The data in Table 1 suggest that the choline protons do not serve as a possible heat sink for the proposed spin-diffusion. Further evidence that the polar headgroups are magnetically isolated from the apolar regions derives from our observations on the effects of  $\text{Mn}^{++}$  ions added to the external aqueous phase of the dispersions. 0.1 mM  $\text{Mn}^{++}$  ions produced a marked effect on the width of the *N*-methyl protons, notably reduced their value of  $T_1$ , but had little effect on the parameters of the methylene or methyl protons. [In at least one experiment, the choline *N*-methyl protons exhibited two values of  $T_1$ , perhaps assignable to those groups on the inside and outside of the vesicles (31).] We conclude then that spin-diffusion toward the polar headgroup is not responsible for thermal relaxation of the apolar region of lecithin in sonicated bilayers.

#### NOTE ADDED IN PROOF

The conformational transitions described above would produce structures with interchain spacings consistent with those seen by x-ray diffraction (above the transition point) (3, 32).

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† For comparison, at 20°, for water  $\tau_c$  is about  $10^{-12}$  sec, and for hemoglobin  $\tau_c$  is about  $10^{-7}$  sec.

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