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^{13}C Nuclear Magnetic Resonance Relaxation Measurements of Synthetic Lecithins and the Effect of Spin-Labeled Lipids†

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ABSTRACT: The ^{13}C spin–lattice relaxation times of dipalmitoyllecithin in bilayers have been measured above the thermal transition, below which the fatty acid chain resonances disappear. The T_1 values above the transition show that molecular motion in the bilayer increases from the glycerol carbons toward both the terminal methyl of the chains and the N^+Me_3 polar head group. The structure is most tightly packed at the glycerol group which probably constitutes the main permeability barrier in the structure. The T_1 values are characteristic of both the chemical structure of the lecithin, and the steric interactions between the molecules in different solvents. The N^+Me_3 resonance from the choline phosphate head group can be observed in the bilayer below the transition, and appears to undergo a conformation change which is coupled to the crystallization of the fatty acid chains. The reversible aggregation of the vesicles which occurs below the transition is attributed to a structure in which the choline phosphate dipoles lie in the plane of the vesicle surface; above the transition the dipoles are in a more extended conformation with the N^+Me_3 groups forming the extreme surface of the vesicle. The effects of nitroxide-labeled lipids incorporated into the bilayer on ^{13}C relaxation times are interpreted qualitatively in terms of the localization of the nitroxide group within the structure.

Preliminary measurements of ^{13}C spin–lattice relaxation times (T_1)[1] of dipalmitoyllecithin (DPL) in sonicated aqueous suspensions have shown that detailed information about the molecular motion of the lipids can be obtained (Metcalfe *et al.*, 1971). Here, the relaxation times in DPL bilayers have been measured for six of the fatty acid chain carbons, and all

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[1] Abbreviations used are: DPL, dipalmitoyllecithin; LSL, lecithin spin label; SASL, stearic acid spin label; T_1 is the spin–lattice relaxation time.

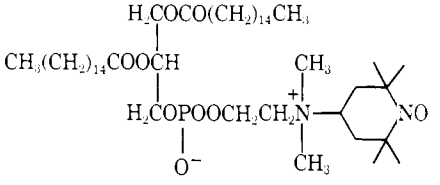
of the glycerol and choline carbons. In principle, T_1 values can be obtained for every carbon nucleus in the structure by specific ^{13}C enrichment, and a further advantage of the technique is that the data refer to a bilayer structure unperturbed by chemical modification.

To examine the sensitivity of the relaxation measurements to the interactions between lipid molecules in the bilayer, T_1 has been measured for dioleyllecithin, which contains a double bond in the fatty acid chains (Δ^9), and for an analog of DPL labeled with a single fluorine nucleus (on C_7) in each chain. The fluorine nuclei did not cause a detectable perturbation of the bilayer, but the introduction of a double bond in dioleyllecithin produced well-defined relaxation changes. In both lipids additional chain resonances were observed from carbons near the fluorine nuclei or the double bonds. The relaxation times of lecithin in $CDCl_3$ and CD_3OD were also found to be characteristic of the structural organization of the molecules in these solvents.

Below the thermal transition, the chain resonances from DPL broaden and eventually disappear, indicating a constraint on the conditions under which ^{13}C spectra are likely to be observed from lipids in biological membranes. A further requirement of the study of biological membranes is for a technique sensitive to the spatial organization of membrane components. The effect of the nitroxide group in producing differential T_1 changes for the carbon nuclei in DPL bilayers suggests that such changes may be useful in estimating the proximity of nitroxide-labeled membrane proteins to ^{13}C-enriched lipids.

Materials and Methods

Dipalmitoyllecithin was obtained from Koch-Light and egg lecithin was prepared as described previously (Birdsall *et al.*, 1971). Di-7-fluoropalmitoyllecithin was synthesized from 7-fluoropalmitic acid (Birdsall, 1971) and dioleyllecithin was synthesized by the method of Robles and Van den Berg (1969). The lecithin spin label (I) was prepared as described



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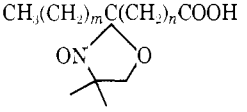
by Kornberg and McConnell (1971) and the stearic acid spin labels (SASL)



with the nitroxide group attached at C_7 or C_{12} were prepared by the method of Keith *et al.* (1968).

Dipalmitoyl- and dioleyllecithin samples (230 mM) were sonicated at 50° in deoxygenated D_2O buffer (45 mM NaCl–30 mM sodium acetate–5 mM sodium phosphate, pD 7.4) in glass vials under nitrogen until the sample was translucent and the residual light scattering was minimized. Samples were transferred under nitrogen to 12-mm nuclear magnetic resonance (nmr) tubes. Samples in $CDCl_3$ (290 mM) and in CD_3OD (140 mM) were thoroughly deoxygenated with nitrogen; previous experiments (Metcalfe *et al.*, 1971) have shown that ^{13}C spin–lattice relaxation times (T_1) obtained on such samples were identical with those from samples degassed by repeated freeze-pump-thaw cycles.

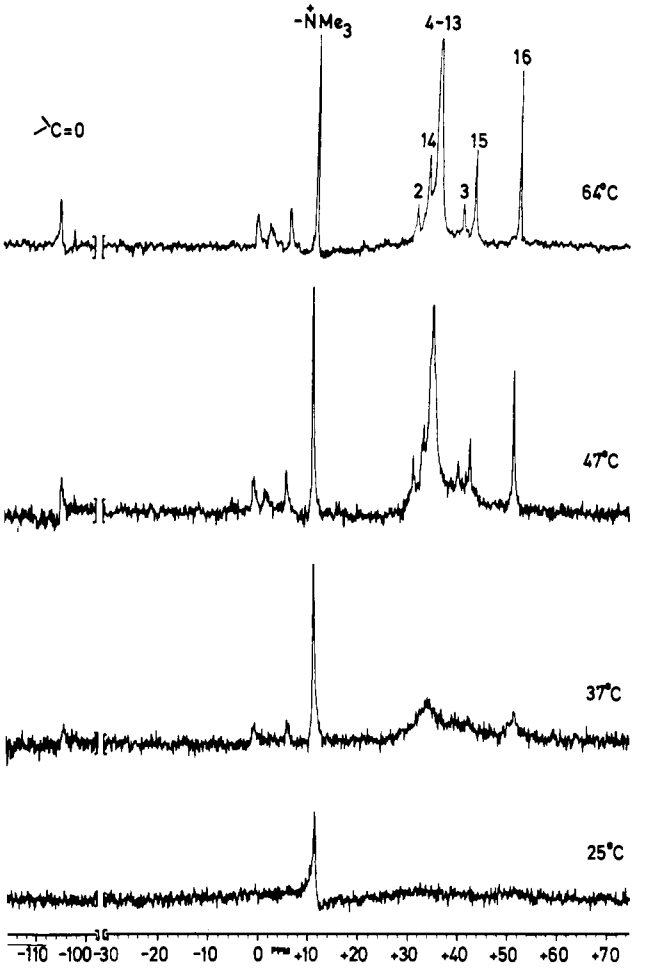


FIGURE 1: ^{13}C nmr spectra of sonicated DPL (230 mM) in D_2O buffer as a function of temperature. Chemical shifts are corrected to dioxane as internal reference.

The T_1 relaxation measurements were made on proton-decoupled ^{13}C spectra by the Fourier transform technique on a Varian XL-100-15 spectrometer locked on solvent deuterium, and employing a pulse sequence ($\pi - t - \pi/2$) as described by Freeman and Hill (1971), where t is the delay in seconds between the π and $\pi/2$ pulses. For technical details, see Lee *et al.* (1972).

In all experiments the decrease in amplitude of each resonance with increasing t followed an exponential curve characterized by a single T_1 relaxation time, and there was no evidence of heterogeneous relaxation times.

Results

Thermal Transition in DPL. Above the thermal transition at ~40°, sonicated vesicles of DPL exhibit sharp resonances from the carbons of fatty acid chains and the N^+Me_3 head group (Figure 1); the remaining choline and glycerol carbons are also assigned in Figure 2a (Birdsall *et al.*, 1972). Resolved resonances are observed from the fatty acid chains for the carbonyl carbons (C_1), the terminal methyls (C_{16}), and for carbons-2, -3, -14, and -15, in addition to the main methylene

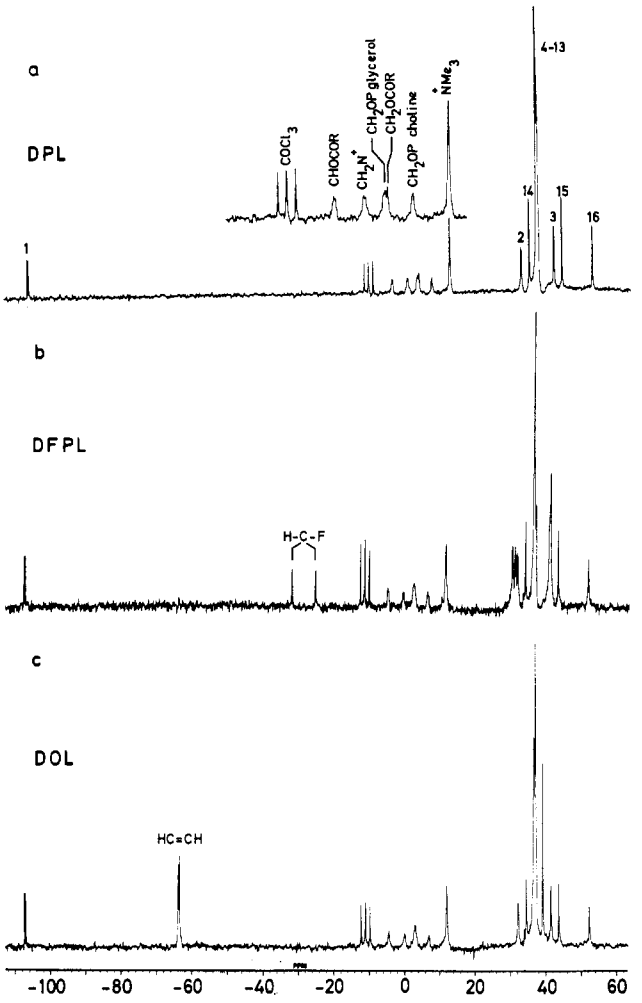


FIGURE 2: Spectra of synthetic lecithins in $CDCl_3$ at 52°. (a) DPL, (b) di-7-fluoropalmitoyllecithin, and (c) dioleyllecithin.

envelope containing carbons-4 to -13. Two resonances are resolved for carbons-1 and -2 for DPL in $CDCl_3$ corresponding to the α- and β-fatty acid chains (Figure 2a), but these resonances are not resolved in the broadened spectra from the DPL bilayer in D_2O. In $CDCl_3$, the integrated intensity of each resolved resonance due to a protonated carbon is proportional to the number of nuclei in the molecule within experimental error (±20%) so that differences in any Overhauser effects are small (Kuhlmann *et al.*, 1970). Similar intensity ratios were obtained for the resonances in the bilayer structure which could be measured to the same accuracy (C_{16} and N^+Me_3).

The T_1 relaxation times for the lecithin molecule in the bilayer at 52° increase from the glycerol carbons toward both

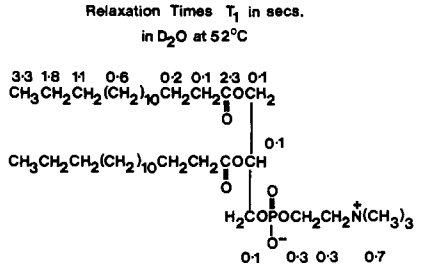


FIGURE 3

TABLE I: T_1 Relaxation Times[a] of Sonicated Dipalmitoyllecithin in D_2O.

Carbon		T_1 (s) 52°	T_1 (s) 65°
Choline	$N^+(CH_3)_3$	0.70 ± 0.03	1.15 ± 0.07
	N^+CH_2	0.32 ± 0.08	
	CH_2OP	0.27 ± 0.04	
Glycerol	CH_2OP	0.11 ± 0.01[b]	
	CH_2O		
	CHO	⩽0.1	
Chain	C_1	2.24 ± 0.05	
	C_2	0.10 ± 0.02	0.29 ± 0.04
	C_3	0.22 ± 0.03	0.36 ± 0.03
	C_{4-13}	0.53 ± 0.01[c]	0.63 ± 0.01[c]
	C_{14}	1.13 ± 0.18	1.28 ± 0.06
	C_{15}	1.81 ± 0.08	2.29 ± 0.18
	C_{16}	3.34 ± 0.25	5.31 ± 0.30

[a] The errors quoted are the standard deviations on the slopes of the lines through up to 12 points: the T_1 values are reproducible to within 10%. [b] These two resonances are not sufficiently resolved to determine their relaxation times separately (see Figure 2a). [c] These values are unspecified averages for all the carbons-4 to -13 in the composite methylene envelopes. The observed decay of the resonance with increasing t could not be distinguished from a single exponential curve, although the component carbons clearly have different T_1 values.

the N^+Me_3 group and the terminal methyl carbons, with the exception of the nonprotonated carbonyl nuclei (Figure 3). It should be noted that the T_1 values for all the chain carbons are the average values for the two chains, and that the T_1 value for the methylene envelope (C_4–C_{13}) is an unspecified average of the component resonances calculated from the exponential decay of the composite resonance as a function of t.

On lowering the temperature through the phase transition to 37° the resonances from the fatty acid chains broaden and reduce in intensity and at 25° are very broad and weak compared to the N^+Me_3 resonance (Figure 1). The N^+Me_3 group itself is only slightly affected by the decrease in temperature with a small increase in half-width ($\Delta\nu_{1/2}$) of the resonance by <5Hz from 47 to 25°, without a significant loss in integrated intensity. These spectral changes with temperature were completely reversible over 5 hr. The T_1 relaxation times of all the alkyl carbon chains decrease with temperature down to the transition (Table I) below which they could not be measured. The temperature dependence of T_1 for the N^+Me_3 resonance can be followed through the transition (Figure 4) and there is probably a small inflection at the transition similar to the well-defined inflection in the corresponding proton curve (Lee *et al.*, 1972). Specific ^{13}C enrichment of DPL will be necessary for a detailed analysis of the temperature dependence of T_1 and the activation energies of relaxation.

Unsonicated suspensions of DPL above the transition also exhibit ^{13}C nmr spectra (Metcalfe *et al.*, 1971) in which both the N^+Me_3 and terminal methyl resonances are fairly narrow ($\Delta\nu_{1/2} \backsimeq 25$ Hz at 52°), though the methylene carbon envelope is considerably broadened compared to the reso-

TABLE II: T_1 Relaxation Times[a] of Sonicated Lecithins in D_2O at 52°.

Carbon		T_1(s) DPL	Di-7-fluoropalmitoyllecithin	Dioleyllecithin
Choline	$N^+(CH_3)_3$	0.70 ± 0.03	0.66 ± 0.07	1.06 ± 0.06
	N^+CH_2	0.32 ± 0.08		0.49 ± 0.15
	CH_2OP	0.27 ± 0.04		0.26 ± 0.06
Glycerol	CH_2OP / CH_2O	0.11 ± 0.05		0.11 ± 0.03
	CHO	⩽0.1		⩽0.05
Chain	C_1	2.24 ± 0.05		2.61 ± 0.50
	C_2	0.10 ± 0.02		0.17 ± 0.01
	C_3	0.22 ± 0.03		0.26 ± 0.12
	$(CH_2)_n$	0.53 ± 0.01 (C_{4-13})	0.55 ± 0.09[a]	0.73 ± 0.03[a]
	$CH_2CH_2CH_3$	1.13 ± 0.18 (C_{14})	1.15 ± 0.50 (C_{14})	1.38 ± 0.16 (C_{16})
	CH_2CH_3	1.81 ± 0.08 (C_{15})	1.84 ± 0.02 (C_{15})	2.26 ± 0.11 (C_{17})
	CH_3	3.34 ± 0.25 (C_{16})	3.10 ± 0.08 (C_{16})	3.88 ± 0.37 (C_{18})
	HC=CH			0.80 ± 0.04 (C_{9-10})
	CH_2			0.75 ± 0.07[b]

[a] Average value for the main methylene envelope. [b] Unassigned methylenes resolved from the main methylene envelope due to the presence of the double bonds.

nance from sonicated vesicles (⌒300 Hz, *cf.* 40 Hz at 52°). However, to obtain spectra from unsonicated DPL comparable to spectra from the same concentration of sonicated DPL it was necessary to accumulate more transients. It is possible that only the outer layers of the unsonicated multishell liposomes give rise to the high-resolution spectrum. Below the transition, only a broad resonance (⌒100 Hz) for the N^+Me_3 group is observed.

Comparison of Lecithins in Bilayers. The substitution of a single fluorine nucleus at the C_7 position in both fatty acid chains of DPL results in the separation of additional carbon resonances from the main methylene envelope (Figure 2b). It was not possible to measure satisfactorily the T_1 values of these additional resonances in the bilayer structure, but the resonances from DPL and from the fluorinated DPL analog with the same chemical shifts had similar T_1 values (Table II). We conclude that the ^{19}F nuclei do not cause a large perturbation of the DPL bilayer structure and that ^{19}F nmr relaxation measurements of ^{19}F-substituted DPL (Birdsall *et al.*, 1971) will provide data relevant to the bilayer structure.

The introduction of double bonds (Δ^9) into the C_{18} chains of dioleyllecithin also results in the separation of new resonances from the methylene envelope from carbon nuclei near the olefinic carbons (Figure 2c). The double bonds cause a consistent increase in the T_1 values of the chain carbons compared to DPL, and a large increase in T_1 for the N^+Me_3 group (Table II). The T_1 value of the methylene carbons displaced from the methylene envelope by the olefinic group is the same as that of the remaining methylene carbon envelope itself.

Lecithin in Organic Solvents. Lecithin was examined in methanol and chloroform to determine whether the T_1 relaxation times already described for the sonicated vesicles are characteristic of a bilayer structure.

Egg lecithin is monomeric in methanol at the boiling point but the molecular weight at lower temperatures corresponds

TABLE III: T_1 Relaxation Times of Lecithins in Different Solvent Systems: 52 ± 1°.

Carbon	T_1(s) D_2O (DPL)	$CDCl_3$ (DPL)	CD_3OD (Egg Lecithin)
$N^+(CH_3)_3$	0.70 ± 0.03	0.22 ± 0.03	0.94 ± 0.19
C_2	0.10 ± 0.02	0.27 ± 0.04	0.71 ± 0.06
C_3	0.22 ± 0.03	0.50 ± 0.10	1.06 ± 0.06
C_{4-13}	0.53 ± 0.01	1.24 ± 0.03	1.75 ± 0.07[a]
$CH_2CH_2CH_3$	1.13 ± 0.18	2.65 ± 0.12	4.8 ± 0.6
CH_2CH_3	1.81 ± 0.08	3.75 ± 0.16	5.9 ± 1.0
CH_3	3.34 ± 0.25	4.29 ± 0.33	5.8 ± 1.5

[a] Main methylene envelope.

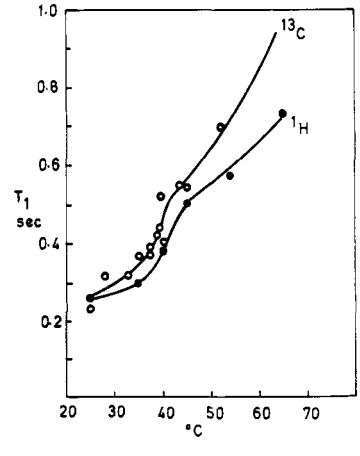


FIGURE 4: The temperature dependence of T_1 for the ^{13}C and proton nuclei of the $N^+(CH_3)_3$ group in sonicated DPL in D_2O.

TABLE IV: Effect of Spin Labels on ^{13}C T_1 Values of Sonicated DPL in D_2O at 52° (Spin Label:Lecithin, 1:60).

Carbon	DPL Control	DPL + LSL	DPL + 7SASL	DPL + 12SASL
$N^+(CH_3)_3$	0.70 ± 0.03	0.42 ± 0.03	0.47 ± 0.04	0.58 ± 0.03
C_{4-13}	0.53 ± 0.01	0.35 ± 0.01	0.41 ± 0.03	0.32 ± 0.01
C_{15}	1.81 ± 0.08	1.05 ± 0.20		0.66 ± 0.03
C_{16}	3.34 ± 0.25	1.77 ± 0.25	1.17 ± 0.07	0.85 ± 0.05

to a trimer (Price and Lewis, 1929; Elworthy and Macintosh, 1961). Because of the heterogeneity of the fatty acid chain composition, the chain resonances of egg lecithin are described by their positions relative to the carboxyl group and the terminal methyl in Table III, since the chemical shifts of these resolved resonances are independent of chain length for fatty acids containing more than nine carbons (unpublished data). In $CDCl_3$, lecithin exists as spherical micelles containing 60–70 molecules.

The T_1 relaxation times of the chain resonances of egg lecithin in methanol are consistently longer than T_1 values for the corresponding nuclei of DPL in $CDCl_3$, which are also generally longer than in DPL bilayers in D_2O (Table III). In both organic solvents there is a marked increase in T_1 from the carbonyl carbon group toward the terminal methyls although there are quantitative differences in the gradation of the changes along the chains. The N^+Me_3 T_1 value is similar to that of the C_2 nucleus in both organic solvents, whereas in the bilayer structure in D_2O the N^+Me_3 T_1 is much longer than the C_2 value.

Effect of Spin-Labeled Lipids on T_1 in DPL Bilayers. The head-group spin-labeled lecithin (LSL) and the two stearic acid nitroxide analogs (7SASL and 12SASL) were incorporated into sonicated DPL vesicles at a molar ratio of 1 spin label:60 lecithin molecules. The T_1 values of the resonances which were still measurable were generally reduced in the presence of the spin labels (Table IV). The relative effectiveness of the spin labels in reducing T_1 of the N^+Me_3 group was in the order LSL > 7SASL > 12SASL, and the order was reversed for the terminal methyl carbons. The effect of the nitroxides on the methylene envelope (C_4–C_{13}) is clearly complex because of the differing contributions to the observed relaxation time from the component resonances. T_1 values for C_2, C_3, and C_{14} and the glycerol carbons could not be obtained with sufficient accuracy in the presence of the nitroxides. The presence of the nitroxide spin labels caused no large changes in the peak intensities compared to samples of DPL vesicles of the same concentration in the absence of nitroxides.

Discussion

The spectral intensity changes in the fatty acid chains of DPL observed through the transition indicate a gradual crystallization or melting of the chains within the vesicles, with coexisting crystallized and fluid regions changing in proportion through the transition. This is in accord with X-ray diffraction data (Luzzati, 1968; Engelman, 1970), and proton nmr studies of the same transition (Lee *et al.*, 1971). In membranes which show a well-defined thermal transition for the lipids (*e.g.*, mycoplasma membranes), it should be possible to estimate the proportion of the lipids in the membrane which are organized in a bilayer structure from the change in intensity through the thermal transition. The absence of high resolution resonances from the fatty acid chains below the transition is probably due to broadening by ^{13}C-proton dipolar interactions in the crystalline matrix rather than a large increase in the rotational correlation time, since there is evidence that fatty acid chains packed hexagonally in the fully extended conformation retain considerable motional freedom about their long axes below the transition temperature (Luzzati, 1968; Hubbell and McConnell, 1971; van Putte, 1970). Qualitatively similar spectral changes are observed in unsonicated lecithin through the transition, which suggests that the broadening of the fatty acid chain resonances above the transition in unsonicated lecithin is due to chemical shift differences within the multishell vesicles, rather than residual ^{13}C-proton dipolar broadening.

The size of the individual vesicles of sonicated DPL does not change during the transition as judged by the accessibility of solvent to their outer surfaces (Lee *et al.*, 1971), although the striking increase in light scattering indicates that aggregation of the vesicles must occur. Both the light-scattering changes and nmr spectral changes through the transition are reversible. The aggregation of the vesicles below the transition temperature is inhibited by the addition of polyvalent cations (*e.g.*, Eu^{3+}), suggesting that the aggregation results from a change in the charge distribution of the polar surfaces (unpublished observations). A simple explanation is that above the transition the zwitterionic choline phosphate group is in a conformation with the N^+Me_3 group extended from the glycerol group so that it dominates the surface charge of the vesicle. Below the transition the choline phosphate dipole may lie parallel to the surface of the bilayer, so that the surface is a mosaic of dipoles with no net charge. Aggregation of the vesicles would then occur by lateral dipole–dipole interactions in the surfaces of the vesicles. These interactions would be sensitive to cations binding strongly to the phosphate group, leaving the surface with a net positive charge, and hence preventing aggregation. The inflection in the proton and ^{13}C T_1 curves at the transition may reflect this conformation change in the head group. Since the conformation change clearly depends on the crystalline to liquid-crystalline transition of the chains, the two processes are probably cooperative, and it would be expected that perturbation of the head-group conformation, for example, by strong cation binding to the phosphate, would also alter the transition temperature of the chains.

The ratios of the integrated intensities of the ^{13}C resonances in organic solvents and in D_2O are found to be proportional to the number of nuclei in the lecithin molecule. This is consistent with a dominant relaxation mechanism from dipolar interactions between the carbon nuclei and directly bonded protons (Kuhlmann *et al.*, 1970). Thus for uniform motions of a set of carbon nuclei, the relaxation times of the carbon nuclei will be inversely proportional to the number of directly bonded protons. This accounts for the long re-

laxation time of the nonprotonated >C=O nucleus. Relaxation of this carbon could be due either to dipolar interaction with protons on adjacent carbon nuclei (C_2), or to anisotropic shielding, or to a combination of these mechanisms. It is not possible at present to determine which mechanism is dominant.

The T_1 relaxation times for the fatty acid chains indicate that in each structure the molecular motion of the chains increases steeply toward the terminal methyl, since uniform T_1 values would be expected for the methylene carbons if the chain motion was determined by the tumbling of the molecule as a whole. In general the relaxation time will depend on the molecular tumbling of the molecule with a correlation time τ_r and internal motion within the chain with a correlation time τ_g, so that for isotropic motions: $T_1 \propto \tau_r^{-1} + \tau_g^{-1}$.

The internal motions detected by the increasing T_1 values along the chain may be due to any oscillations about C–C bonds within a conformation, or to conformational changes of the chain, which result in the reorientation of the CH vectors with respect to the magnetic field. For isotropic motions the two correlation time τ_r and τ_g can be evaluated, but any realistic model of chain motion involves varying anisotropic motion along the chain and the appropriate correlation times cannot be calculated simply. The problem of the summation of motion along a series of bonds is discussed by Wallach (1967) and the application of this treatment to lipid chain motion will be described elsewhere.

The T_1 values for the choline head group and the glycerol carbons provide information about the interactions of the lecithin molecules in each structure. The short T_1 value for the N^+Me_3 carbons in CD_3OD may imply a strong solvation of the head group by alcohol or it may be due to ionic interactions between two lipid head groups; a similar effect was observed in proton T_1 measurements of the same system (Lee *et al.*, 1972). The N^+Me_3 T_1 value is also relatively short in $CDCl_3$ compared to the methylene envelope, and probably results from tight packing of the headgroups in the inverted micellar structure. In the bilayer structure, the T_1 values of protonated carbon nuclei increase from the glycerol carbons toward the N^+Me_3 group, which is relatively mobile. The packing of the bilayer structure as a whole therefore appears to be determined by the close packing of the chains at the glycerol group which is also likely to provide the main permeability barrier of the bilayer. In principle, the activation energies for each group in the structure should provide additional evidence for the packing of the lecithin molecules, but we defer this analysis until more accurate values for the activation energies are available from specifically enriched DPL lecithins. Measurement of ^{13}C coupling constants along the chain together with T_1:T_2 ratios should also enable a critical test to be made of the bent chain model for packing within the bilayer proposed by McFarland and McConnell (1971). The general description of chain motion within the bilayer is in agreement with the spin-label studies of Hubbell and McConnell (1971).

The preferential relaxation changes in the terminal methyl and adjacent carbons induced by the 12SASL compared to the 7SASL analog, and their marked differential effects on the N^+Me_3 group are consistent with their expected localization in the structure. It is clear that the nitroxides can give qualitative evidence of their proximity to ^{13}C nuclei, but internuclear distances will only be defined by this technique when a quantitative description of nitroxide-induced relaxation is obtained. A detailed description of the effects of nitroxides on proton and ^{13}C relaxation as a function of temperature and concentration will be published elsewhere.

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