Let us now consider $|\mathbf{D}_{i,j}|$, the determinant of the matrix defined as

$$\mathbf{D}_{i,j} = \begin{cases}\text{matrix formed from } \mathbf{D} \text{ by striking} \\ \text{row } i \text{ and column } j\end{cases} \quad \text{(A14)}$$

We shall evaluate this determinant in two ways, once by expansion along the "jth row", then again down the "ith column"; by "jth row" we mean the row whose elements are d_{j1}, $d_{j2}, \ldots$, though this will actually occur in row $j + 1$ of $\mathbf{D}_{i,j}$ if $j > i$, and similar comments apply to the "ith column". In the first case we get

$$|\mathbf{D}_{i,j}| = \sum_{\substack{k=1 \\ (k \neq j)}}^{N} (-1)^{\sigma_{kj}+\sigma_{ji}} d_{jk} |\mathbf{D}_{ij,jk}| \text{ (along "}j\text{th row")} \quad \text{(A15a)}$$

and in the second

$$|\mathbf{D}_{i,j}| = \sum_{\substack{k=1 \\ (k \neq i)}}^{N} (-1)^{\sigma_{ki}+\sigma_{ij}} d_{ki} |\mathbf{D}_{ik,ji}| \text{ (down "}i\text{th column")} \quad \text{(A15b)}$$

Now from the symmetry ($d_{ij} = d_{ji}$) of $\mathbf{D}$ and the obvious symmetries

$$|\mathbf{D}_{ij,jk}| = |\mathbf{D}_{jk,ij}| = |\mathbf{D}_{kj,ij}|, |\mathbf{D}_{ik,ji}| = |\mathbf{D}_{ki,ji}| \quad \text{(A16)}$$

of the definition (A10), one can recognize from (A13a) and (A15a) that

$$A_{ij}|\mathbf{D}_j| = (-1)^{\sigma_{ij}+\sigma_{ji}+1}|\mathbf{D}_{i,j}| \quad \text{(A17a)}$$

and from (A13b) and (A15b) that

$$A_{ji}|\mathbf{D}_i| = (-1)^{\sigma_{ji}+\sigma_{ij}+1}|\mathbf{D}_{i,j}| \quad \text{(A17b)}$$

from which it follows that

$$A_{ij}|\mathbf{D}_j| = A_{ji}|\mathbf{D}_i| \quad \text{(A18)}$$

which, in view of (A7), is the desired result.

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(10) The detailed computational procedure for obtaining MO's in the LCBO framework (e.g., the construction of unitary matrices relating AO's to BO's, and subsequent transformation of the SCF equations into the BO basis) will be described elsewhere. In the present application, idealized geometries and orbital hybridizations were employed throughout, and contributions from antibonding BO's were ignored (as suggested by ref 9), but the qualitative conclusions should be insensitive to these specific choices.

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(12) Professor R. Hoffmann has kindly indicated (private communication) how the overlap phase effects can be derived in higher orders of perturbation theory. Perturbative expansions of c_i^2/c_j^2 can also be developed directly from eq 10 (J. Diamond, private communication), showing that the phase effects contribute in 3d, 5th, and successively higher orders.

^{19}F Nuclear Magnetic Resonance Studies of Lipid Bilayer Systems. 1

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Received June 28, 1975

Abstract: A fluorinated lipid, 1-palmitoyl-2-8,8-difluoropalmitoyl-*sn*-glycero-3-phosphorylcholine, has been synthesized and the dynamic properties of lipid bilayer systems containing this molecule have been studied using fluorine-19 NMR. Spin-lattice relaxation rates and nuclear Overhauser effects have been measured over a range of temperatures and the results have been interpreted in terms of correlation times for specific motions involving the *gem*-difluoromethylene group. The correlation times are shown to be consistent with ^{1}H and ^{13}C relaxation data of similar lipid bilayer systems. The data, however, prove to be particularly valuable in characterizing a motion on the time scale of translational diffusion.

Introduction

In recent years much effort has been devoted to the characterization of the hydrocarbon chain mobility of phospholipids in bilayer membranes. The subject is of interest because the bilayer is an indigenous component of virtually all biological membranes.[2] The phospholipid motions are intimately linked to the activity of proteins and the transport of metabolites within and through the membrane.[2] Characterization of the anisotropic motion in the liquid crystalline lipid bilayer has also proven to be a challenging problem in physical chemistry.

EPR nitroxide spin-label studies[3-6] and nuclear magnetic resonance relaxation studies[7-10] have yielded valuable results concerning the segmental rotational motions and translational diffusion of the phospholipids, but both methods have been subjected to some criticism. Spin labels can perturb the structure of the bilayer. This is shown by the fact that in identical lipid bilayer systems, even after correction for time

scale differences, the order parameter for a nitroxide probe, measured by EPR, is different than that for the C-D bond, measured by deuterium NMR.[11] In addition, the methylene resonance in ^1H and ^{13}C NMR does not correspond to a single defined methylene group, but to an envelope of resonances from different methylenes with a distribution of correlation times.[12] Thus, the NMR relaxation cannot be interpreted in terms of the motion and magnetic interactions of one specific nucleus. Because of the small contribution to ^1H relaxation rates from diffusional motions, and because of experimental problems with the deuterium dilution technique used to resolve this contribution, the diffusion constants measured by ^1H NMR have low accuracy.

^{19}F NMR relaxation experiments can be designed to avoid some of the deficiences of the techniques mentioned above. First, fluorine can be inserted into a specific position of the phospholipid so that the motion of a single defined nucleus is represented in the relaxation behavior. Second, experiments can be designed to be sensitive to specific types of motion, such as translational diffusion. Finally the CF_2 group is similar to the CH_2 group in terms of its size, geometry, and physical characteristics.[13,14] Thus fluorine containing lipids will not greatly perturb the structure of the bilayer membrane. Because of these advantages we have studied the ^{19}F NMR relaxation of 1-palmitoyl-2-8,8-difluoropalmitoyl-*sn*-glycero-3-phosphorylcholine (8,8F-PC) incorporated into lipid bilayer membranes.

In order to use ^{19}F relaxation measurements to determine the rates of the various molecular motions of the phospholipids, all the relaxation mechanisms or the contributions to relaxation from different magnetic interactions must be resolved. Because of the complexity of molecular motion, the results are not easy to interpret. Previous ^{19}F relaxation studies of amphipathic molecules in bilayer membranes assumed that the similar gyromagnetic ratios of ^{19}F and ^1H nuclei and the similar structures of the CF_2 and CH_2 groups should lead to the same relaxation mechanisms for ^1H and ^{19}F nuclei.[14,15] In fact, similar relaxation rates and activation energies are observed for ^1H and ^{19}F nuclei incorporated into the methylenes of hydrocarbon chains in membranes.[14,15] However, a careful consideration of the contributions to ^{19}F relaxation shows that the relation between ^1H and ^{19}F relaxation is more complicated than it appears to be.

Proton NMR studies of phospholipids in lipid bilayer membranes show that the relaxation is due to dipole-dipole interactions modulated by molecular motions. Interactions with the geminal-bonded hydrogen make a large contribution,[7,10] but intermolecular effects account for 20-40% of the relaxation.[9] The temperature dependence of the spin-lattice relaxation rate, R_1, indicates a correlation time faster than 10^{-9} s.[7] However $R_1 \neq R_2$, so this fast motion must be anisotropic. The spin-spin relaxation, R_2, is due to slower isotropic averaging of the residual dipolar interactions.[10]

For several reasons the ^{19}F relaxation could be due to very different interactions than those that govern ^1H relaxation. The mean squared value of the geminal-bonded dipolar interaction is a factor of four smaller for the CF_2 group than for the CH_2 group. This difference is due to the longer C-F bond, 1.35 Å, relative to the C-H bond, 1.1 Å, and the slightly smaller fluorine magnetogyric ratio. There are minor chemical differences between protons and fluorine that could change the rate of motion of the CF_2 group and cause more efficient relaxation. Fluorine-induced changes in the C-C bonds and the steric hindrance of the slightly larger CF_2 group compared to the CH_2 group could affect the motion of the methylene. Magnetic field fluctuations due to chemical shift anisotropy and spin-rotation interactions are much larger for fluorine than for protons and could make a large contribution to relaxation. Even if the ^{19}F relaxation is only due to the nuclear magnetic dipole-dipole mechanism, there are many possible interactions that could contribute to relaxation. These include dipolar interactions with the geminal nucleus, with vicinal and other intrachain nuclei, and with nuclei on adjacent chains. There is a $J(\omega_H - \omega_F)$ term in the H-F spin-lattice relaxation equation that is not present in either the H-H or F-F relaxation equations. This term indicates that dipolar interactions modulated by slow motions, such as diffusion motions with $\tau_c \approx 5 \times 10^{-8}$ s, will be much more efficient in causing ^{19}F relaxation than ^1H relaxation.

In order to gain information about the interactions that cause relaxation, and thus to determine how much of each of the above considerations affect the results, several experiments can be done. These include studies of the H-F nuclear Overhauser enhancement, (NOE) $(\eta + 1)$, of the temperature dependence of relaxation, and of the effect of deuterium dilution.

It is possible to experimentally separate the H-F relaxation due to intermethylene and interchain dipolar interactions from contributions due to F-F geminal interactions by examination of the H-F NOE. The NOE is a measure of the increase in intensity of the fluorine resonance due to saturation of the proton resonances.[16] It reaches a maximum value, the magnitude of which depends on the correlation time of the interaction causing relaxation,[17] if all the fluorine relaxation is due to H-F dipolar interactions. At a resonance frequency of 84 MHz for fluorine, it can be calculated that the H-F NOE maximum varies from 1.532 for $\tau_c \ll 1 \times 10^{-10}$ s to -0.034 for $\tau_c > 1 \times 10^{-7}$ s.[17] The equation to predict the NOE if there are several contributions to relaxation is

$$\text{NOE} - 1 = \sum_i (\text{NOE}_{\max} - 1)(R_{1i}/R_{1\,\text{total}}) \qquad (1)$$

where R_{1i} is the spin-lattice relaxation contribution due to the ith H-F interaction modulated at a rate described by τ_{ci}. If the correlation time for ^{19}F spin lattice relaxation is faster than 10^{-10} s, which holds true for ^1H and ^{13}C relaxation; then the amount of relaxation due to H-F interactions is given by

$$\sum_i R_{1i} = R_{1\,\text{total}} \frac{\text{observed NOE} - 1}{0.532} \qquad (2)$$

If the correlation times are slower than 10^{-10} s, then the separation of relaxation contributions is not straight forward. However, the calculation of the NOE using eq 1 is a useful experimental check of any postulated set of relaxation contributions.

The temperature dependence of the spin-lattice relaxation and of the NOE can be used to characterize the motions causing relaxation. If $\tau_c < 10^{-9}$ s then the NOE will increase or the R_1 will decrease with temperature, but for $\tau_c > 10^{-9}$ s R_1 will increase with temperature. The activation energy extracted from the temperature dependence is also useful in determining which molecular motions cause relaxation.

That part of the H-F spin-lattice relaxation due to diffusional motions can be measured by diluting the fluorine labeled lipids with lipids having highly deuterated hydrocarbon chains. Since the F-D dipolar interactions are small due to the small magnetic moment of deuterium, the measured R_1 will decrease in proportion to the interchain contribution to R_1.[9]

Once the contributions to relaxation have been separated by the methods described above, the rate of molecular motions can be calculated. Equations have been developed to relate the relaxation rate to the magnitude of magnetic interactions and the correlation time for the motions that modulate the interactions.[18] These equations can be expressed as

$$\text{F-F} \quad R_1 = H^2(\tfrac{6}{15}J(\omega_F) + \tfrac{24}{15}J(2\omega_F)) \qquad (3)$$

$$\text{H-F} \quad R_1 = H^2(\tfrac{2}{15}J(\omega_H - \omega_F) + \tfrac{6}{15}J(\omega_F) + \tfrac{12}{15}J(\omega_H + \omega_F)) \qquad (4)$$

Similar equations hold for R_2 relaxation. H^2 is the mean-squared strength of the magnetic fields modulated by the molecular motion. For dipole–dipole interactions modulated by rotational motion[18]

$$H^2 = \frac{\gamma_I^2 \gamma_S^2 \hbar^2 S(S+1)}{r^6} \quad (5)$$

for nucleus S relaxed by nucleus I at a distance r. For dipole–dipole interactions modulated by translational motions, if the distance of closest approach, d, is much smaller than the jump distance, r_J,[19]

$$H^2 = \frac{4\pi}{3} \frac{\gamma_I^2 \gamma_S^2 N_I \hbar^2 S(S+1)}{d^3} \quad (6)$$

for the nucleus S relaxed by nucleus I with a spin density N_I. All of the quantities in H^2 can be evaluated independently of the measured relaxation. They depend on known physical constants and on molecular dimensions that can be evaluated from molecular models. Since H^2 is known for each possible interaction causing relaxation, the $J(\omega)$ can be evaluated. For isotropic rotational motions $J(\omega)$ is related to the correlation time by[18]

$$J(\omega) = \tau_c/(1 + \omega^2\tau_c^2) \quad (7)$$

For translational motions with $d^2 \ll r_J^2$ the same equation holds with $\tau_c = 1/8 \langle r_J^2 \rangle / D$.[19]

For anisotropic motion $J(\omega)$ cannot be expressed in such a simple form.[20] However, in the interest of simplicity, we have chosen in this work to rely on the above isotropic and translational models for extraction of correlation times from R_1 contributions. It can be argued that the values of τ_c derived do not differ significantly from those which would be obtained from more sophisticated models. For example, a model for lipid methylene motion which reproduces proton and carbon R_1 and R_2 data well, is one in which the geminal proton internuclear vector is allowed to reorient rapidly within a sector of revolution having limits 60 and 120°, as well as undergo a slower isotropic reorientation (manuscript in preparation). Predicted correlation times for the fast and slow motions of a vesicle sample at 50 °C are then 5×10^{-11} and 1×10^{-7} s, respectively. The correlation time extracted from the proton R_1 data using a purely isotropic model would be 4.7×10^{-11} s. The similarity of the pure isotropic model τ_c and the restricted intrachain reorientation τ_c for this case is partially the result of having a rather slow and inefficient isotropic relaxation and partially the result of allowing the chain to sample a rather large range of angles (±30°) at the faster time. The correlation may degrade if the slow isotropic reorientation becomes faster and the range of angles is restricted. Since fluorinated lipids execute motions similar to normal lipids we expect the purely isotropic τ_c values derived here to be closely related to correlation times for fundamental lipid motions.

Methods

1-Palmitoyl-2-8,8-difluoropalmitoyl-*sn*-glycero-3-phosphorylcholine (8,8F-PC) was synthesized via a keto acid intermediate. This procedure is based on a recently described synthesis of fluorine compounds that requires only mild reaction conditions. The synthesis uses MoF_6 to convert a ketone group into a *gem*-difluoromethylene.[21]

Cyclooctane and bromooctane were condensed via a Grignard reaction to form 1-octyl-1-cyclooctanol.[22] The tertiary alcohol was dehydrated by refluxing in dilute H_2SO_4 for 12 h, resulting in an isomeric mixture of olefins. These were subjected to ozonolysis in 2:1 CH_2Cl_2:CH_3OH and reduced to give, among other products, 8-ketohexadecanal. This was oxidized to 8-ketopalmitic acid with CrO_3 in glacial acetic acid.[22] The percent theoretical yield based on the amount of cyclooctanone used was 30%. Methyl-8-ketopalmitate was fluorinated using Fluoreze-M, the MoF_6 reagent supplied by P.C.R., Gainsville, Fla., according to the method of Mathey and Bensoam.[21] A crude product, distilled from the reaction mixture at <2 mmHg pressure and 180–220 °C, was hydrolyzed with 1 N NaOH solution. The acid was partially purified by dissolving in petroleum ether, in which the keto acid is less soluble. It was further purified on a silicic acid column using a petroleum ether:ether gradient. The yield was ~20%.

Synthesis of 8,8F-PC. 8,8F-PC was synthesized from lysolecithin and 8,8-difluoropalmitic acid.[23] The lysolecithin was prepared by degradation of egg yolk lecithin with phospholypase A in ether solution.[3] 8,8-Difluoropalmitic anhydride was formed by reaction with half the stoichiometric amount of dicyclohexylcarbodiimide in CCl_4.[24] The lysolecithin and fatty acid anhydride in a 1:4 mol ratio were mixed with 5 mg of Na_2O and sealed in a flask under N_2. The mixture was slowly rotated in an oil bath at 80 °C for 2 days. The resulting 8,8F-PC was purified on silicic acid using a $CHCl_3$:CH_3OH gradient. The yield based on the amount of fluorinated acid was 15%. The synthetic lipid migrated like egg yolk PC on silica gel G. TLC plates were developed with $CHCl_3$:CH_3OH:H_2O 65:25:4. The fluorine compound turns brown with age. It can be efficiently purified on an aluminum column eluted with $CHCl_3$:CH_3OH 9:1. The ^{19}F NMR spectrum in $CHCl_3$ showed a pentet with an F–H coupling constant of 16 Hz. The chemical shift was ~23 ppm upfield from trifluoroacetic acid in D_2O buffer at pH 7.

Isolation of Other Lipids. Egg yolk PC was isolated from fresh egg yolks by the method of Singleton et al.[25] Partially deuterated phosphatidylethanolamine (PE) was extracted from the membranes of wild type *E. coli* grown in D_2O. The membranes were a gift of D. M. Engleman, Yale University. The lipids were extracted with $CHCl_3$:CH_3OH 1:1. The PE was purified on a silicic acid column using a $CHCl_3$:CH_3OH gradient. ^1H NMR analysis indicated that the PE hydrocarbon chains were 80 ± 5% deuterated.

Sample Preparation. 8,8F-PC in micelle form was obtained by dissolving it to 10% w/v in $CHCl_3$. Aqueous dispersions of lipids were prepared by first weighing the lipids and dissolving them in $CHCl_3$, then taking them to dryness under vacuum. They were kept in vacuo at 50 °C for an additional 2 h to remove all traces of solvent. Vesicle dispersions were prepared from mixtures of 6% w/v 8,8F-PC and 6% w/v egg yolk PC or from 4% w/v 8,8F-PC and 6% w/v *E. Coli* PE. Multilayer dispersions were prepared from 30% w/v 8,8F-PC or 12% w/v 8,8F-PC and 18% w/v *E. coli* PE. The buffer solution for the aqueous samples contained 0.01 M Tris, 0.10 M KCl, and 0.02% NaN_3 in 99.8% D_2O titrated to pH 7.5 with concentrated HCl. The multilayer dispersions were made by adding the buffer to the mixed lipids and then vortexing repeatedly at 50 °C. The samples were degassed and capped under nitrogen. Vesicle samples were prepared from the multilayer dispersions by sonication to clearness in a Branson Model E bath sonicator at 30 ± 5 °C. The vesicle dispersions were stable for several weeks at temperatures above 30 °C.

NMR Measurements. ^{19}F NMR relaxation studies were carried out on an extensively modified Bruker HFX-90 single coil pulsed Fourier transform spectrometer at a ^{19}F frequency of 84.67 MHz. Modifications unique to this experiment included reconstruction of the transmitter/receiver insert to remove fluorine-containing parts, the use of orthogonal transmitter coils for ^1H decoupling, and addition of selective filters to remove interference between the decoupler and the ^{19}F receiver channel. A deuterium lock was used for field-frequency stabilization. The proton resonances were continuously decoupled with a noise modulated frequency at 10 W of power. The temperature of the sample was controlled to ±1 °C.

The spin–spin relaxation rate, R_2, was determined from the

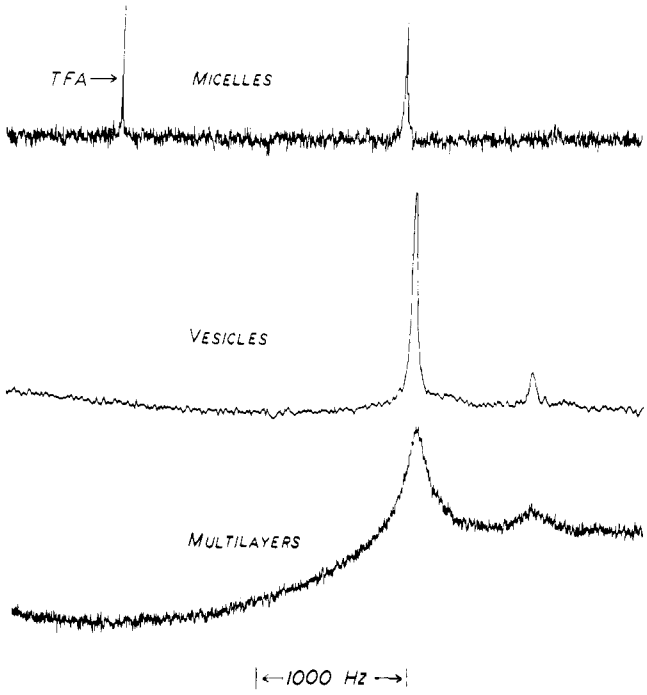

Figure 1. ^{19}F NMR spectra of 8,8F-PC in micelle, vesicle, and multilayer dispersions at 52 °C. The resonance frequency is 84 MHz with proton decoupling at 90 MHz. The micelles are 10% w/v 8,8F-PC in $CHCl_3$ with TFA in D_2O buffer contained in a coaxial capillary tube. The vesicles contained 6% w/v 8,8F-PC and 6% w/v egg yolk PC in D_2O buffer. The multilayers are 25% w/v 8,8F-PC in D_2O buffer. The small resonance on the right is due to a fluorinated lipid impurity.

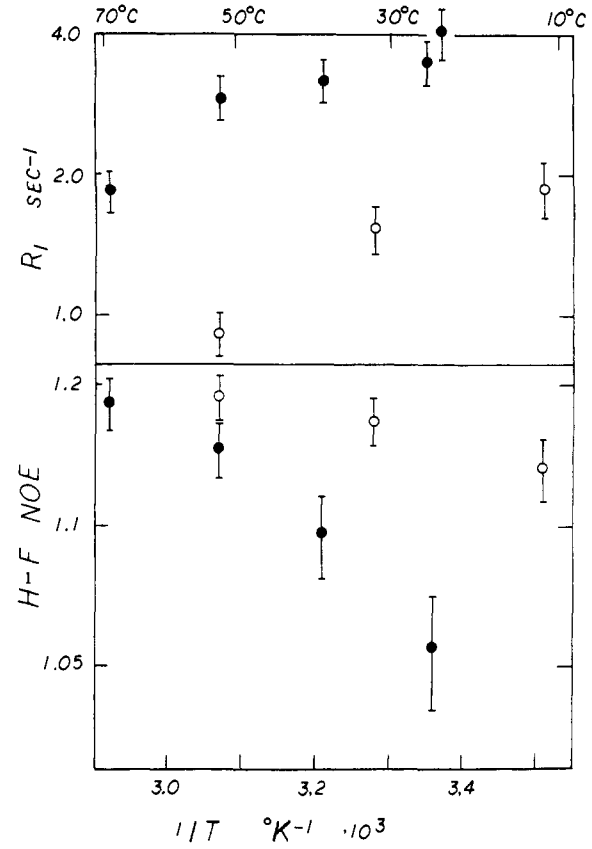

Figure 2. Activation energy plots of the observed ^{19}F spin-lattice relaxation of the 8,8F-PC resonance in micelles and vesicles. The data are taken at 84 MHz under proton decoupling. ○, 8,8F-PC as micelles in $CHCl_3$. ●, vesicles containing 6% w/v 8,8F-PC and 6% w/v egg yolk PC in D_2O buffer.

line width of proton-decoupled spectra obtained from Fourier transformation of the FID, $R_2 = \pi\nu_{1/2}$. For vesicle dispersions, 300–600 scans were taken using a 95 μs data acquisition delay and a 1250 Hz data acquisition rate for 1024 points. For multilayer dispersion, 100–2000 scans were taken using a data acquisition delay of 30 μs and an acquisition rate of 10 kHz for 4096 points.

The spin-lattice relaxation rate, R_1, was measured using a $(180°\text{-}\tau\text{-}90°\text{-}t\text{-})_N$ pulse sequence,[26] with t at least five times T_1 and with continuous noise decoupling of the proton frequencies. R_1 is evaluated from the slope of the line resulting from a semilog plot of peak heights $(h_\infty - h_\tau)$ vs. τ for 10 to 12 values of τ. The R_1 is accurate to ±15%.

The ^1H-^{19}F NOE was measured by a gated decoupling technique. For the full NOE the proton resonances were continuously irradiated. To eliminate the NOE, the proton decoupler was turned on only during acquisition and then turned off during the long delay, five times T_1, between 90° pulses in order to allow the proton magnetization to come to equilibrium. The experiment was repeated three times or until agreement within 10% for the intensity ratios of three experiments was achieved. The accuracy is ±0.03.

Results

Proton-decoupled ^{19}F NMR spectra of 8,8F-PC in micelle, vesicle, and multilayer dispersions are presented in Figure 1. The resonance in $CHCl_3$ solution is a symmetric pentet of 16 Hz splitting that is collapsed to a singlet of ~4 Hz width by proton decoupling. The ^1H decoupled resonance line width in a vesicle dispersion is much broader. It predicts $R_2 = 100 \pm 15$ s^{-1} at 50 °C. This is similar both to the value of R_2 measured by Birdsall et al.[15] for 7-fluoropalmitate incorporated into vesicles, 90 s^{-1}, and to the ^1H R_2 value in egg yolk PC vesicles,[9] 90 s^{-1}. The similarity is coincidental since the gem-bonded dipolar interaction that governs the ^1H R_2 is much larger for the CH_2 group than for the CF_2 group. The ^{19}F R_2 shows an activation energy of 5 ± 0.5 kcal/mol, which is substantially different than the 3.0 kcal/mol seen for ^1H R_2.[9] These facts suggest that the ^{19}F spin-spin relaxation is caused by different magnetic interactions than ^1H relaxation. One such interaction that contributes to the ^{19}F R_2 is chemical shift anisotropy. This may not be averaged by anisotropic motions that occur in bilayers.

The ^{19}F R_2 for 8,8F-PC in a multilayer dispersion is 1400 ± 200 s^{-1}. This is three times less than the value of 4100 s^{-1} measured for the ^1H R_2 in egg yolk PC multilayers at 50 °C (unpublished results). The ^1H R_2 in multilayers has been shown to be due to static local magnetic fields due to nonaveraged geminal-bonded dipolar interactions.[10] The ^1H $R_2/^{19}F$ R_2 ratio postulated from this relaxation mechanism is two. The narrower ^{19}F resonance is experimentally advantageous because it leads to a factor of three improvement in the ^{19}F NMR sensitivity.

Both the vesicle and multilayer samples show a minor fluorine resonance due to an impurity. Both the R_1 and R_2 relaxation for the minor resonance are the same as for the major one, so the impurity is probably a lipid dissolved in the bilayer. Its identity is unknown.

In contrast to R_2, the spin-lattice relaxation is relatively insensitive to the state of the bilayer. The relaxation behavior for micelles in chloroform is shown in Figure 2. R_1 decreases with temperature. This implies that the correlation time for the motions causing relaxation are faster than 10^{-9} s, $1/\omega_F$. The ^{19}F R_1, 0.90 s^{-1} at 52 °C, is similar to the ^1H R_1 in the same system, 0.95 s^{-1}. Since the CF_2 dipolar interaction is four times smaller than that for the CH_2 group, these results suggest the presence of extra contributions to the fluorine relaxation. The NOE increases slightly with temperature. This is consis-

tent with a lower activation energy for the H-F than for the F-F relaxation.

The temperature dependence of the relaxation of the 8,8F-PC resonance for a vesicle dispersion is similar to that for micelles (Figure 2). This shows that rapid motions still govern the R_1 relaxation. However the R_1 is three times larger in vesicles than micelles. It is substantially larger than the ^1H R_1 in the same system; ^{19}F $R_1 = 2.9\ s^{-1}$, ^1H $R_1 = 2.0\ s^{-1}$ at 52 °C. Either the motion of the CF_2 group is slower than the motion of the CH_2 group or contributions to relaxation other than that due to the geminal-bonded dipolar interaction have become even more important. The temperature dependence of the H-F NOE is surprisingly steep, which suggests unusual behavior for some aspect of the H-F relaxation.

The intermolecular H-F contribution to R_1 can be estimated by diluting the 8,8F-PC with deuterated lipids and observing the decrease in the relaxation rate. The R_1 for a vesicle dispersion prepared from a mixture of 40% 8,8F-Pc and 60% *E. coli* PE, 80% deuterated in the hydrocarbon chains, is $2.65 \pm 0.15\ s^{-1}$, while an analogous fully protonated sample has $R_1 = 2.90 \pm 0.12\ s^{-1}$. These figures suggest that at 52 °C less than $1.2\ s^{-1}$ of the relaxation is due to translational diffusion motions. A single NOE measurement of the dueterated sample gave a value of 1.26 ± 0.10 at 52 °C, which is marginally larger than the value of 1.14 ± 0.03 measured for the protonated sample. This suggests that the H-F intermolecular contribution to R_1 has a low maximum NOE value and the correlation time for this relaxation is slower than 10^{-9} s.

The ^{19}F R_1 relaxation rate in multilayers is exactly the same as that in vesicles. This is true for the partially deuterated samples also. This implies that the motions and the contributions to the R_1 relaxation are identical in the two-lipid bilayer systems.

Discussion

The discussion will center on ^{19}F spin-lattice relaxation in lipid bilayers, and comparison with ^1H and ^{13}C results. A comparison of the spin-spin relaxation will not be attempted. Motions in the bilayer are both complex and anisotropic, so the relationship between R_2 and the rate of molecular motion is more difficult to analyze than that for R_1.

The results definitely show that the ^{19}F R_1 is much larger than would be expected from the observed ^1H R_1 if all the relaxation is due to dipolar interactions modulated by identical fast anisotropic motions. This behavior can be explained either by assuming that the CF_2 group moves more slowly than the CH_2 group or by assuming that there are magnetic interactions that contribute to the ^{19}F relaxation, but not to the ^1H relaxation. We believe that both of these effects occur in the relaxation of the ^{19}F resonance of 8,8F-PC in lipid bilayers.

Two mechanisms that can contribute to the ^{19}F, but not to the ^1H, relaxation are chemical shift anisotropy and spin-rotation interactions. Chemical shift anisotropy causes relaxation by modulating the applied field through the different electronic shielding for various orientations of the CF_2 group.[18] For the CF_2 group in Teflon, this shielding differs by 200 ppm for fields in the plane of and perpendicular to the F-C-F plane.[27] At a fluorine resonance frequency of 84 MHz, the mean-squared interaction strength for the chemical shift anisotropy versus the F-F dipole-dipole interaction in the CF_2 group is $H^2_{CSA}/H^2_D = 7.55 \times 10^8\ s^{-2}/3.03 \times 10^9\ s^{-2}$.

If the R_1 in bilayers can be interpreted in terms of fast and apparently isotropic motion of the CF_2 group, then only 20% of the geminal-bonded contribution to relaxation will come from the chemical shift anisotropy interaction.

Spin rotation is another possible relaxation mechanism for ^{19}F resonances that could make contributions as large as $1\ s^{-1}$ to the relaxation rate. These large values only occur near the R_1 maximum for spin rotation.[28] Thus, if a large spin rotation contribution to relaxation exists for 8,8F-PC, then an anomolously low apparent activation energy should be seen. Since this is not observed, we suspect that this contribution is also small.

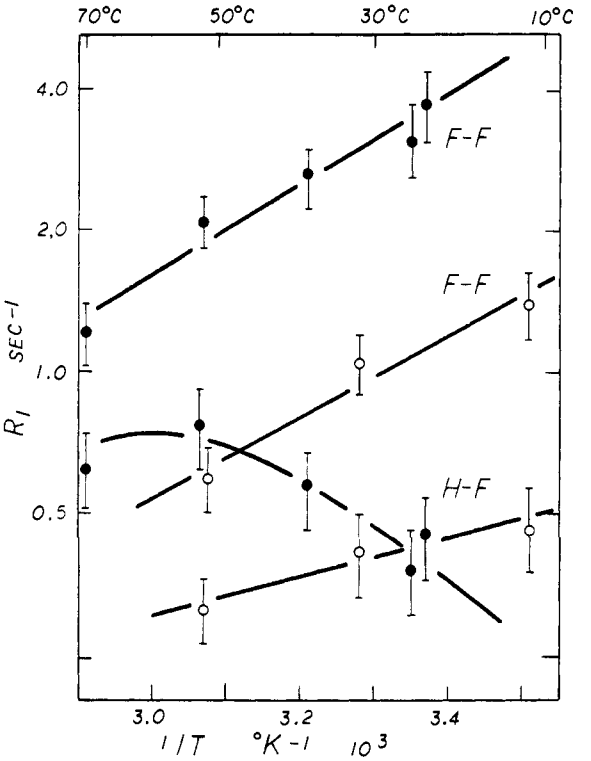

Figure 3. Activation energy plots for the separated ^{19}F-^{19}F and ^1H-^{19}F spin-lattice relaxation of 8,8F-PC in micelles and vesicles. The separation of the contributions to relaxation is described in the text. ○, 8,8F-PC as micelles in $CHCl_3$. ●, vesicles containing 6% w/v 8,8F-PC and 6% w/v egg yolk PC in D_2O buffer.

The efficient ^{19}F relaxation must, therefore, be due to dipole-dipole interaction contributions. One such contribution is due to the geminal-fluorine interaction. The coupled gauche carbon-carbon bond isomerizations, which are the predominant means of modulating this interaction, can be slowed down due to the steric interactions of the slightly larger CF_2 group compared to the CH_2 group. A measure of these effects can be derived from the relaxation data of 8,8F-PC for micelles in chloroform.

The separation of relaxation due only to F-F interactions can be accomplished by examining the H-F NOE. Because of the fluidity of the hydrocarbon chains and the small size of the micelles,[29] all of the relaxation should be due to interactions modulated by motions faster than $1/\omega_F$. Under these conditions, the maximum value for the NOE of 1.532 can be assumed. Equation 2 can be used to separate F-F from H-F relaxation contributions. The results have been plotted as a function of temperature in Figure 3. The temperature dependence of both the F-F and H-F relaxation contributions are consistent with a correlation time faster than 5×10^{-10} s. The activation energy from motions of the CF_2 group is 4.2 kcal/mol, while the activation energy for longer range interactions is only 2.5 kcal/mol. The F-F R_1 of $0.59\ s^{-1}$ at 52 °C predicts a correlation time for the CF_2 group of 7.7×10^{-11} s when both dipole-dipole and chemical shift anisotropy interactions are accounted for using an isotropic model.

This value can be compared to the correlation time for the CH_2 group calculated from ^{13}C relaxation. The ^{13}C R_1 is due only to geminal-bonded ^1H-^{13}C dipolar interactions,[12] so it can be used as a measure of the motional time scale of single methylene group reorientation. The ^{13}C $R_1 = 0.80\ s^{-1}$ at 52° and 25 MHz[9] implies a correlation time of 2.0×10^{-11} s when

isotropic motion is assumed.[12] If we assume that the motions executed by a CH_2 group and a CF_2 group are similar in their degree of anisotropy and that the assumption of isotropic motion leads to proportional deviations from true correlation times in both cases, we conclude that the motion dominating spin-lattice relaxation is approximately four times slower for the CF_2 group than for the CH_2 group. Combined with the chemical shift anisotropy contribution to relaxation, this explains the fast ^{19}F R_1 spin-lattice relaxation observed.

Comparison with ^1H relaxation for egg yolk PC in micelles in chloroform suggests that the separation performed above is a reasonable one. The correlation time for the CH_2 group of 2.0×10^{-11} s predicts a R_1 contribution from geminal-bonded dipolar interactions of 0.49 s^{-1} to the ^1H relaxation. The H-F relaxation contribution is due to nongeminal dipolar interactions. If the interaction distances and correlation times are the same for the analogous H-F and H-H interactions, then the ratio of H-H R_1/H-F R_1 contributions is 1.70 for $\tau_c < 10^{-10}$ s. Thus, the contribution to the ^1H R_1 from nongeminal dipolar interactions is predicted to be 0.53 s^{-1} at 52 °C. The total ^1H R_1 predicted 1.02 s^{-1} agrees remarkably well with the observed value, 0.95 s^{-1}.

It is not as easy to separate the contributions to the R_1 relaxation of the 8,8F-PC resonance in vesicles. This is because some of the motions causing relaxation are slow enough to change the maximum value of the NOE. The contributions to relaxation, separated using eq 2, which assumes a maximum NOE of 1.53, are shown in Figure 3. The anomolous temperature dependence of the H-F relaxation contribution is immediately obvious. The negative slope observed at low temperatures is inconsistent with the assumption of a fast correlation time used to achieve the separation of relaxation contributions. This problem can be resolved by postulating H-F relaxation contributions with slow correlation times.

For dipolar interactions modulated slower than 10^{-10} s the NOE (maximum) is dependent on the correlation time. The theoretical NOE maxima calculated for $\omega_F/2\pi = 84.67$ MHz and a series of correlation times are presented in Table I. These calculations are based on the treatment of Kuhlmann et al.[17] The predicted ^1H $R_1/^{19}F$ R_1 ratios calculated from eq 3 and 4 for interactions with the same internuclear distance and correlations times are also shown in Table I. Suitable choices for τ_{ci} and R_{1i} in eq 1 will produce a relaxation temperature dependence in line with that observed. Part of the H-F dipolar interaction must be modulated by a motion slower than 10^{-9} s. The extent of this contribution to R_1 will also be reflected in the ^1H $R_1/^{19}F$ R_1 ratio.

The fact that the NOE increases from 1.14 ± 0.03 to 1.26 ± 0.10 when 8,8F-PC is diluted with deuterated chains indicates that the slow correlation time is associated with intermolecular interactions. The diffusion constant for phospholipids in bilayer membranes is 4×10^{-8} cm^2/s from recent EPR/NMR measurements.[30] Assuming that the translational diffusion step is 6 Å, then the correlation time would be $\sim 1 \times 10^{-8}$ s. Motions on this time scale would give an NOE (maximum) of 0.14. Thus, translational diffusion could give rise to the observed NOE's and their temperature dependence.

The ^{19}F results demonstrate the presence of a slow motion contribution to H-F relaxation. Evaluation of the extent of this contribution or accurate characterization of τ_c require comparison with proton and carbon relaxation data. It is intuitively obvious that slow translational diffusion would not be the only contribution to H-F relaxation. There are strong intramolecular H-F interactions modulated by chain rotation and β-coupled isomerizations. Even within the intermolecular contributions, the H-F dipolar interactions will be modulated by fast rotational motions of adjacent chains in addition to the slower translational steps. The existence of the fast motions can be verified experimentally. If all the intermolecular contributions to ^1H and ^{19}F relaxation were due to motions on a slow time scale (10^{-8} s) as suggested by some authors,[9] the H-H R_1/H-F R_1 ratio would be only 0.2 due to the additional $J(\omega_H - \omega_F)$ term in ^{19}F relaxation. The observed ratio (0.5) clearly demonstrates that the slow motion contribution is a minor one to the ^1H relaxation.

Table I. The Dependence of the H-F NOE and the ^1H $R_1/^{19}F$ R_1 Ratio on the Correlation Time for the Motion Causing Relaxation[a]

τ_c, s	Maximum H-F NOE	H-H R_1/H-F R_1
1.0×10^{-11}	1.531	1.695
1.0×10^{-10}	1.528	1.690
4.0×10^{-10}	1.481	1.627
1.0×10^{-9}	1.302	1.406
2.0×10^{-9}	1.011	1.079
4.0×10^{-9}	0.600	0.651
1.0×10^{-8}	0.142	0.198
2.0×10^{-8}	0.013	0.0828
4.0×10^{-8}	−0.024	0.0436

[a] These values apply for resonance frequencies of $\omega_H/2\pi = 90.0$ MHz and $\omega_F/2\pi = 84.67$ MHz.

Intermolecular contributions are very difficult to treat quantitatively, since they involve both distance and angular modulation of interactions. The fact that at least two correlation times are involved in a case where equivalent pairs of nuclei interact suggests that the motions involved are not isotropic.

An approximate contribution from fast motions, such as those from β-coupled isomerization or rotation of adjacent chains, can be calculated using the equations of Kruger[19] if we assume that the change in dipole interaction on jumping from a distance of closest approach of 2.4 Å to a distance of 9.6 Å, as one would in rotating the chain 180°, is approximately equal to the change on jumping from closest approach to infinity. This assumption in fact reproduces the measured value of 0.5–0.8 s^{-1} for the intermolecular contribution to proton relaxation when a correlation time of 5×10^{11} s is used. A similar assumption predicts a contribution of 0.3 s^{-1} to H-F relaxation.

An additional contribution to the H-F R_1 of ≤ 0.7 s^{-1} due to the fact that the adjacent protons do not jump to infinity at a fast time scale, but are further modulated by slow intermolecular motions is then within the experimental limits set by the intermolecular relaxation contribution observed by deuterium dilution experiments. This will not lead to abnormally low H-H R_1/H-F R_1 ratios.

Although the intramolecular contributions to relaxation are modulated by motions faster than 10^{-9} s, the ^{19}F contributions are again more efficient than the ^1H contribution. By subtracting the intermolecular contribution, values of ~ 2.0 s^{-1} for ^{19}F and ~ 1.4 s^{-1} for ^1H are found. Since a large part of the intramolecular relaxation is due to gem-bonded dipolar interactions, which are four times smaller for the CF_2 group than the CH_2 group, the larger ^{19}F value must be due to slower motion of the CF_2 group. In micelles, it was found that the CF_2 group moves four times slower than the CH_2 group. If the same phenomenon is assumed to occur in vesicles, then the observed values can be explained. From the ^{13}C relaxation for the CH_2 group of phospholipids in vesicles[12] a correlation time for assumed isotropic rotational motion can be calculated, $R_1 = 1.8$ s^{-1}, $\tau_c = 4 \times 10^{-11}$ s. This motion predicts a contribution to the ^1H R_1 of 1.0 s^{-1}, and with $\tau_c = 1.6 \times 10^{-10}$ s it predicts contributions to the ^{19}F R_1 of 1.2 s^{-1}. The residual relaxation is due to longer range intramolecular interactions.

The validity of the various assumptions used to calculate the individual contributions to the ^1H and ^{19}F spin-lattice relax-

Table II. One Possible Separation of the Contributions of ^1H and ^{19}F Spin-Lattice Relaxation That Agrees with the Experimental Results[d]

	70 °C			52 °C			26 °C		
	$\tau_c \times 10^{11}$ s	R_1, s^{-1}	NOE	$\tau_c \times 10^{11}$ s	R_1, s^{-1}	NOE	$\tau_c \times 10^{11}$ s	R_1, s^{-1}	NOE
				Predicted ^{19}F Relaxation[c]					
Geminal-bonded dipole ^{19}F–^{19}F	8.2	0.62		12.0	0.91		26.0	1.96	
Chemical shift anisotropy									
Nongeminal-bonded intramolecular	5.7	0.80	1.200	7.0	0.98	1.195	10.0	1.38	1.165
Intermolecular (fast)	2.7	0.22	1.055	3.3	0.26	1.052	4.6	0.39	1.047
Intermolecular (slow)	370.	0.47	0.918	500	0.51	0.893	900.	0.66	0.875
Total predicted		2.11	1.173		2.66	1.147		4.39	1.087
Observed[a]		1.85	1.188		2.90	1.145		4.10	1.055
				Predicted ^1H Relaxation[c]					
Gem-bonded dipole ^1H–^1H	3.0	0.74		4.0	0.99		7.0	1.72	
Nongeminal-bonded intramolecular	1.6	0.38		2.0	0.48		2.8	0.67	
Intermolecular (fast)	2.7	0.41		3.3	0.50		4.6	0.70	
Intermolecular (slow)	370.	0.30		500.	0.25		900.	0.14	
Total predicted		1.83			2.22			3.23	
Observed[b]		1.55			1.90			3.10	

[a] The resonance frequency for the measurement was $\omega_H/2\pi$ = 90.0 MHz and $\omega_F/2\pi$ = 84.67 MHz. [b] The resonance frequency for the measurement was $\omega_H/2\pi$ = 100 MHz. Data were taken from Lee et al.[7] for the relaxation of egg yolk PC vesicles. [c] The following mean-squared interaction strengths are used in the calculations: geminal-bonded F–F, r = 2.18 Å, 3.03×10^9 s^{-2}; Nongeminal-bonded intramolecular H–F, 8 protons, 2.5 Å, 1.05×10^{10} s^{-2}; Intermolecular (fast) H–F, d = 2.6 Å, $N = 6 \times 10^{22}$ protons/cm^3, 6.0×10^9 s^{-2}; Intermolecular (slow) H–F, d = 6.0 Å, $N = 6 \times 10^{22}$ protons/cm^3, 5.0×10^8 s^{-2}; Geminal-bonded H–H, r = 1.805 Å, 1.23×10^{-10} s^{-2}; Nongeminal-bonded intramolecular H–H, 8 protons, 2.5 Å, 1.2×10^{10} s^{-2}; Intermolecular (fast) H–H, d = 2.4 Å, $N = 6 \times 10^{22}$ protons/cm^3, 7.6×10^9 s^{-2}; Intermolecular (slow) H–H, d = 6.0 Å, $N = 6 \times 10^{22}$ protons/cm^3, 7.2×10^8 s^{-2}. [d] The values are for a methylene of a phospholipid hydrocarbon chain in a vesicle dispersion.

ation can be checked by calculating the total R_1 and the predicted H–F NOE at several temperatures and comparing these with observation (Table II). The predicted NOE is very sensitive to the time scale and the fractional contribution of the slow intermolecular motion.

The correlation time for translational diffusion measured using the ^{19}F relaxation data and an appropriately averaged distance of closest approach (6 Å), $\tau_c = 5.0 \times 10^{-9}$ s, predicts a diffusion constant $D = 9 \times 10^{-8}$ cm^2/s at 52 °C, which is close to the value of 4×10^{-8} cm^2/s predicted from EPR studies[30] at 40 °C.

The other numbers in Table II are consistent with the correlation times predicted from the available ^{13}C and ^1H R_1 data for phospholipids in bilayers. One exception is that the CF_2 group is assumed to move 3.5 times slower than the analogous CH_2 group. The activation energies assumed to do the calculations at several temperatures are 4.5 kcal/mol for the CH_2[31] and 5.0 kcal/mol for the CF_2 geminal-bonded interactions, 5.0 kcal/mol for intermolecular diffusion,[32] and 2.5 kcal/mol for all other motions. These activation energies also agree with those predicted by previous studies.

It should be stressed that the motions causing spin-lattice relaxation are very similar in vesicle and multilayer dispersions. This is shown by the identical ^{19}F R_1's observed in both systems, and is further supported by the fact that the ^{13}C R_1's are also the same in vesicles and multilayers.[31] Therefore the correlation times predicted in Table II, including those for diffusional motion, apply to multilayers, and presumably similar rates of molecular motion will be found in biological membranes.

Summary

We believe that ^{19}F NMR will be a useful technique for studying biological membranes. It combines high sensitivity with a single resonance due to a nucleus incorporated into a specific location in the membrane. The purpose of this paper is to lay the ground work for the interpretation of the relaxation behavior seen in biological systems.

Acknowledgment. This work was supported by a grant from the National Institute of General Medical Sciences, U.S. Public Health Service GM 19035.

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