# Nuclear Magnetic Resonance Line-Shape Analysis of Fluorine-19-Labeled Phospholipids†

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ABSTRACT: The application of a fluorine-19 probe to the problem of motions present in the hydrophobic region of phospholipid dispersions and biological membranes has been extended to the study of phospholipids labeled with fluorine-19 in the 8 position and with deuterium in the 2, 7, and 9 positions of the 2-acyl chain. 1-Myristoyl-2-(8,8-difluoro[2,2,7,7,9,9-$^2H_6$]myristoyl)-*sn*-glycero-3-phosphocholine and its nondeuterated analogue have been investigated by $^{19}F$ nuclear magnetic resonance at 282.4 MHz. Spectra obtained from macroscopically oriented bilayers exhibit Pake doublets from which order parameters can be obtained. The spectra obtained from nonoriented liposomes of the phospholipids can be explained in a satisfactory manner as a random superposition of doublets broadened by heteronuclear magnetic dipole–dipole interactions. From an analysis of the data, several conclusions about the motional state of the hydrocarbon chains in the liquid-crystalline phase can be drawn. The present results show that appropriate fluorine-19 probes in the acyl chains of phospholipids can be used to investigate the structure and dynamics of model and biological membranes.

In recent years, nuclear magnetic resonance (NMR)[1] techniques have been used extensively to study the various dynamical processes and structural changes that take place in the phospholipid bilayer on both sides of the gel to liquid-crystalline phase transition [for recent reviews, see Griffin (1981) and Jacobs & Oldfield (1981)]. The $^{19}F$ nucleus is of considerable interest as an NMR probe in the study of phospholipid bilayers. Earlier work from this laboratory has shown the utility of a difluoromethylene group substituted in the hydrocarbon chain of a fatty acid in gaining useful information about the local motional states present in the hydrophobic region of the acyl chains in both model and biological membranes (Gent et al., 1978, 1981; Gent & Ho, 1978). In addition, our recent results also show that the substitution of a $CH_2$ group by a $CF_2$ group in the acyl chain of a phospholipid molecule produces only a minor perturbation of the acyl chain (Oldfield et al., 1980).

The large magnetic moment of $^{19}F$, which has a nuclear spin of $^1/_2$, makes it especially attractive for NMR studies, owing to the good sensitivity and an inherently large signal-to-noise ratio that can be attained. Since the $^{19}F$ nucleus has no quadrupole moment, the analysis of the line shape by a second moment formalism is simplified by the presence of only magnetic dipole–dipole interactions. Furthermore, the relatively large chemical shifts encountered in $^{19}F$ NMR enable one to probe the motional state of the molecule not only through the averaging of the magnetic dipole–dipole interaction, but also through the effect of motion upon the anisotropy of the $^{19}F$ chemical shift in high magnetic fields.

Although the motional averaging of the magnetic dipole–dipole interaction affects the line width, the interaction involves the coupling among many nuclei. In principle, however, the presence of a fluorine pair in a methylene group should permit one to assign unambiguously changes in the line shape to motion occurring at a definite site in the molecule. This is made possible by the observation of a resolved Pake doublet (Pake, 1948). Although the doublet is normally not resolved in *nonoriented* fluorinated phospholipid multilayers, the use of a multiple-pulse sequence, which averages out the chemical shift anisotropy and the heteronuclear dipolar interactions, has recently permitted the observation of a classical powder pattern corresponding to the Pake doublet associated with a pair of fluorine nuclei (Post et al., 1982).

Conventional Fourier-transform $^{19}F$ NMR spectra of macroscopically oriented phospholipid multilayers, prepared from both 1-myristoyl-2-(8,8-difluoro[2,2,7,7,9,9-$^2H_6$]myristoyl)-*sn*-glycero-3-phosphocholine (2-[$^2H_6$,8,8-$^{19}F_2$]DMPC) and its nondeuterated analogue (2-[8,8-$^{19}F_2$]DMPC), and of the corresponding nonoriented liposomes have been obtained at 282.4 MHz. The NMR spectra of the oriented multilayers, which are free of the broadening effect of the $^{19}F$ chemical shift anisotropy, clearly display directly the doublet structure without resorting to additional resolution enhancement techniques. Furthermore, from a study of the $^{19}F$ NMR spectra of oriented multilayers a reasonably good understanding of the spectra found in nonoriented liposomes has been attained.

In the liquid-crystalline phase, the motional state of the hydrocarbon chain at the fluorinated site can be adequately described by an order parameter that is a measure of the degree of anisotropy of the motion. The variation of the order parameter as a function of temperature was determined from the splittings in the doublet structure and was unambiguously assigned to the motion of the fluorine–fluorine internuclear vector. Moreover, the overall changes in the line shape as a function of temperature, both in the oriented multilayers for various angles and in the nonoriented liposomes, have also been investigated and related to the motional behavior of the long hydrocarbon chains.

## Experimental Procedures

*Materials.* Monomyristoylphosphatidylcholine was prepared according to the method of Oldfield et al. (1978) by the action of *Crotalus adamanteus* phospholipase $A_2$ on dimyristoylphosphatidylcholine. 8,8-Difluorotetradecanoic acid, prepared

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[1] Abbreviations: NMR, nuclear magnetic resonance; 2-[$^2H_6$,8,8-$^{19}F_2$]DMPC, 1-myristoyl-2-(8,8-difluoro[2,2,7,7,9,9-$^2H_6$]myristoyl)-*sn*-glycero-3-phosphocholine; 2-[8,8-$^{19}F_2$]DMPC, 1-myristoyl-2-(8,8-difluoromyristoyl)-*sn*-glycero-3-phosphocholine; TLC, thin-layer chromatography; FID, free-induction decay; $S_{FF}$, the order parameter associated with the $^{19}F$–$^{19}F$ internuclear vector; $S_{CD}$, the order parameter associated with the C–$^2H$ internuclear vector.

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as previously described (Gent & Ho, 1978), was converted to its acid anhydride and used to acylate monomyristoylphosphatidylcholine according to the method of Gupta et al. (1977). The product, 1-myristoyl-2-(8,8-difluoromyristoyl)-*sn*-glycero-3-phosphocholine, was isolated by column chromatography on SilicAR CC-7 silica gel by elution with 2:1, 1.4:1.0, and 1.2:1.0 $CHCl_3$–$CH_3OH$ solutions. Final purification was achieved by crystallization from acetone–chloroform (95:5) (Mason et al., 1981). The desired product gave a single spot on thin-layer chromatography (TLC) ($CHCl_3$–$CH_3OH$–7 N $NH_4OH$, 230:90:15 v/v), which was visualized with a molybdenum phosphate–stain reagent (Dittmer & Lester, 1964). 8,8-Difluoro[2,2,7,7,9,9-$^2H_6$]tetradecanoic acid, prepared as previously described (Oldfield et al., 1980), was used to acylate monomyristoylphosphatidylcholine as described above. The crystallized phospholipid product gave a single spot on TLC.

$^1H$ NMR spectra of the two phospholipids taken as deuteriochloroform solutions were consistent with the desired structures (Plückthun & Dennis, 1982) and showed >90% of deuterium incorporation for the deuterated compound. Treatment of the phospholipids with phospholipase $A_2$ showed >95% 8,8-difluoromyristic acid to be present in the 2-acyl position by gas chromatography.

Dimyristoyl-L-$\alpha$-phosphatidylcholine and *C. adamanteus* phospholipase $A_2$ were purchased from Sigma. SilicAR CC-7 silica gel was purchased from Mallinckrodt. Silica gel 60 F-254 precoated TLC plates and sheets were obtained from MC/B Manufacturing Chemists, Inc. Deuteriochloroform was purchased from Aldrich. Deuterium oxide (99.6%) was purchased from Bio-Rad. Reagent-grade solvents were used unless otherwise stated.

*Preparation of Samples.* Nonoriented liposome (powder) samples were prepared by weighing appropriate quantities of phospholipid and $D_2O$ into a 5-mm NMR tube and vortexing the resulting mixture vigorously above the phase transition temperature. Samples were sealed with caps and stored at −20 °C. Periodically, small amounts were removed to monitor the sample purity by TLC. Samples were vortexed at 40 °C before an NMR run. No changes in the spectra were observed over a 6-month time period. Observed under a microscope, the liposomes were highly heterogeneous in size.

Oriented samples were prepared by first weighing the phospholipid and $H_2O$ (25% by weight) into a test tube that was constricted in the middle. The tube was then sealed, and the contents were well mixed by centrifugation. The translucent material was streaked onto a 7.5 mm × 22 mm glass coverslip. An identical coverslip was placed on top with gentle pressure and moved so as to spread and align the material. Individual sandwiches were kept in a closed vial at 40 °C until examination under a polarizing light microscope showed the sample to be >90% oriented. The sandwiches were stacked (~14 sandwiches) and placed in a rectangular glass cell, which could be rotated to the desired angle in the probe coil. The oriented samples were stored at −20 °C until use. The 54.7° orientation was checked before each use to ensure that the samples remained ordered.

*Methods.* The $^{19}F$ NMR spectra were obtained at 282.4 MHz on a Bruker WH-300 spectrometer interfaced with an Aspect 2000A computer and operated in the Fourier-transform mode, with home-built 5- and 13-mm probes. All materials used in building the probes were fluorine free, coaxial cables included. The $^{19}F$ NMR spectra were obtained by using a coil arrangement based on a design by Karlicek & Lowe (1978). We extended the concept to achieve a high-volume, high-Q, tuned array resonating at 282.4 MHz. The array used for oriented samples was 13 mm in diameter and 30-mm long. With 36-W input, a 90° pulse was 14 $\mu$s. The probe is compatible with the Bruker VT-1000 temperature control system. The uncertainty in temperature is 1 °C. A detailed description of the tuned array is being presented elsewhere (Cook & Lowe, 1982). The oriented bilayer samples were positioned in the field in a rotation device that enabled control of the angle with respect to the magnetic field to ±2°. The $^{19}F$ NMR spectra of the oriented phospholipid bilayers were taken with the following parameters so as to suppress a background signal that arose from fluoride present in the glass: sweep width 166 kHz, filter width 250 kHz, 37° pulse width of 5.7 $\mu$s, acquisition time 3 ms, and repetition time 0.6 s.

The spectra of nonoriented liposomes were acquired in the quadrature mode, with 512 or 1024 scans, an acquisition time of 3 or 6 ms, and a pulse width of 2 $\mu$s. The free-induction decay (FID) was Fourier transformed with 300-Hz line broadening.

## Theory

*Dipolar Interactions and Chemical Shift Anisotropy.* The $^{19}F$ NMR line shapes observed for the phospholipid samples can be understood on the basis of dipolar interactions (homonuclear and heteronuclear) and an anisotropic $^{19}F$ chemical shift. For the oriented multilayers in the liquid-crystalline phase, the broadening effect of the anisotropic chemical shift is removed, and the line shape can be understood, in principle, on the basis of dipolar interactions alone. For the nonoriented liposomes at a frequency of 282.4 MHz, however, the anisotropy of the $^{19}F$ chemical shift plays an important role as a broadening mechanism and cannot be neglected (Gent et al., 1978; Gent & Ho, 1978).

*Magnetic Dipole–Dipole Interactions.* For an isolated pair of fluorine nuclei in an external magnetic field $\vec{H}_0$, an absorption spectrum consisting of two lines is expected. If the pair of nuclei is rapidly reorienting about an axis and the angle between the internuclear vector $\vec{r}$ and the rotation axis is denoted by $\psi_{FF}$, the frequency separation of $\Delta\nu_{FF}$ between the two lines can be shown to be given by (Slichter, 1980)

$$\Delta\nu_{FF} = (3/2)\frac{\gamma_F^2\hbar}{2\pi r^3}(3\cos^2\theta - 1)\frac{3\cos^2\psi_{FF} - 1}{2} \quad (1)$$

where $\gamma_F$ is the gyromagnetic ratio of the fluorine nuclei and $\theta$ is the angle between the rotation axis and the external magnetic field, $\vec{H}_0$. In general, in the liquid-crystalline phase, because of the presence of some degree of disorder, the angle $\psi_{FF}$ varies with time. The splitting ($\Delta\nu_{FF}$) then becomes proportional to the average value, $(1/2)\langle 3\cos^2\psi_{FF} - 1\rangle = S_{FF}$, which defines an order parameter associated with the fluorine–fluorine internuclear vector. Thus, one obtains from eq 1 the following relationship between the frequency separation of the doublet and the order parameter $S_{FF}$:

$$\Delta\nu_{FF} = (3/2)\frac{\gamma_F^2\hbar}{2\pi r^3}(3\cos^2\theta - 1)S_{FF} \quad (2)$$

Since the pair of fluorines substituted into a methylene group of a hydrocarbon chain is not really an isolated pair, the magnetic dipole–dipole interactions between the fluorine nuclei and other nuclei (protons or deuterons) in the chain must also be taken into account. The broadening of each member of the doublet by heteronuclear intramolecular dipolar interactions can be examined by considering the second moment. The analysis leading to eq 1 can be extended to include dipolar interactions among many spins (Wennerström, 1973). This

yields the following expression for the contribution to the second moment of the observable $^{19}$F NMR line shape from heteronuclear interactions:

$$\langle \Delta\nu_F^2 \rangle_{\text{hetero}} = \frac{1}{4\pi^2}(1/3)\gamma_F^2\hbar^2(3\cos^2\theta - 1)^2[\gamma_H^2 S_H(S_H + 1)\sum_H (1/2) r_{FH}^{-6}\langle 3\cos^2\psi_{FH} - 1\rangle^2 + \gamma_D^2 S_D(S_D + 1)\sum_D (1/2) r_{FD}^{-6}\langle 3\cos^2\psi_{FD} - 1\rangle^2] + \langle \Delta\nu_F^2 \rangle_{\text{intermolecular}} \quad (3)$$

where the sums are extended over all hydrogen atoms (and, if necessary, all deuterium nuclei) in the hydrocarbon chains of the phospholipid molecule. $\gamma_H$ and $\gamma_D$ are the gyromagnetic ratios, and $S_H$ and $S_D$ are the nuclear spins of protons and deuterons, respectively. The internuclear vectors joining one of the fluorine nuclei in the pair with protons or deuterons are denoted by $\vec{r}_{FH}$ or $\vec{r}_{FD}$, and the corresponding angles between $\vec{r}_{FH}$ or $\vec{r}_{FD}$ and the axis of rotation are denoted by $\psi_{FH}$ or $\psi_{FD}$. The rapid reorientation about the long molecular axis, which in the liquid-crystalline state is approximately coincident with the normal to the bilayer, causes the intramolecular contribution to the second moment to vary as $(3\cos^2\theta - 1)^2$. Thus, for the "magic angle", i.e., $\theta = 54.7°$, the line width is determined solely by intermolecular spin–spin interactions.

*$^{19}$F Chemical Shift Anisotropy.* The rapid reorientation about the long molecular axis also has a marked effect upon the anisotropy of the $^{19}$F chemical shift. The normal to the bilayer becomes the principal axis of an axially symmetric chemical shift tensor and the resonance frequency $\nu(\theta)$ is given by (Seelig, 1978)

$$\nu(\theta) = \nu(54.7°) - \frac{\gamma_F H_0}{2\pi}\frac{\Delta\sigma}{3}(3\cos^2\theta - 1) \quad (4)$$

where $\theta$ denotes, as before, the angle between the external magnetic field $\vec{H}_0$ and the normal to the bilayer, $\nu(54.7°)$ is the resonance frequency at the magic angle, and $H_0$ is the external magnetic field strength.

In eq 4, $\Delta\sigma = \sigma_\parallel - \sigma_\perp$ and is the difference between the principal values of the averaged uniaxial shielding tensor. Although $\Delta\sigma$ is also related through order parameters to the principal values of the static shielding tensor (Seelig, 1978), the exact determination of the order parameters from eq 4 is hampered by the fact that the orientation of the principal axes of the static shielding tensor in the molecular frame is seldom known accurately. Furthermore, two order parameters rather than one must be specified, in general, to determine $\Delta\sigma$. In spite of that, the observation of the anisotropy of the $^{19}$F chemical shift, which has a marked effect upon the line shape of nonoriented liposomes, is a rather sensitive test to detect variations from the simple behavior predicted by eq 4.

## Results and Discussion

Figure 1A shows the $^{19}$F NMR line shapes obtained for the oriented multilayers of 2-[$^2$H$_6$,8,8-$^{19}$F$_2$]DMPC at a temperature of 27 °C as a function of the orientation of the normal to the bilayer with respect to the magnetic field. Except for $\theta = 54.7°$, the line shapes exhibit a resolved doublet pattern. As predicted by eq 3, the line shape is very anisotropic. The line width corresponding to $\theta = 54.7°$ is only 450 Hz while that for $\theta = 0°$ is 12.8 kHz. The result implies that, according to eq 3, $\langle \Delta\nu_F^2 \rangle_{\text{intermolecular}}$ must be greatly reduced in the liquid-crystalline phase due to the rapid lateral diffusion of the molecules. Part of the residual width at $\theta = 54.7°$ is produced by an inhomogeneity of the magnetic field (~250 Hz) over the sample volume. Slight misalignment of the bilayers may also have caused some small additional line broadening. The angular dependence of the position of the resonance lines is

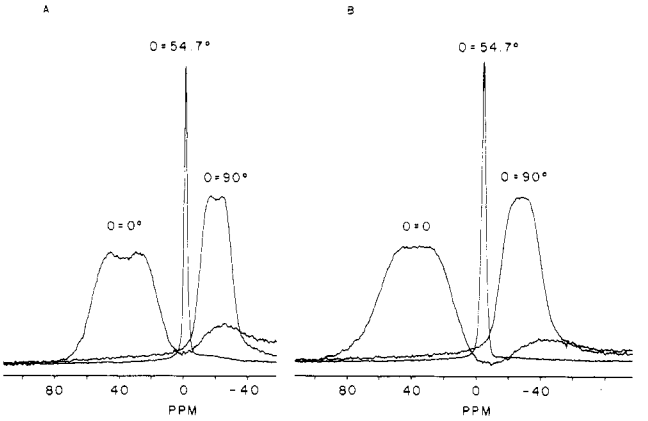


FIGURE 1: 282.4-MHz $^{19}$F NMR spectra of oriented $^{19}$F-labeled phospholipids in $H_2O$ at 27 °C: (A) 2-[$^2$H$_6$,8,8-$^{19}$F$_2$]DMPC at $\theta$ = 0, 54.7, and 90°; (B) 2-[8,8-$^{19}$F$_2$]DMPC at $\theta$ = 0, 54.7, and 90°. The origin of the $^{19}$F chemical shift (i.e., 0 ppm) is set at the isotropic value, namely, at the magic angle (54.7°) value. The broad, weaker signal at ~−20 ppm is due to (i) a background $^{19}$F signal from fluoride in the glass used for orienting the bilayers and (ii) a small percentage of nonoriented sample presumably present around the edges of the glass cover slips.

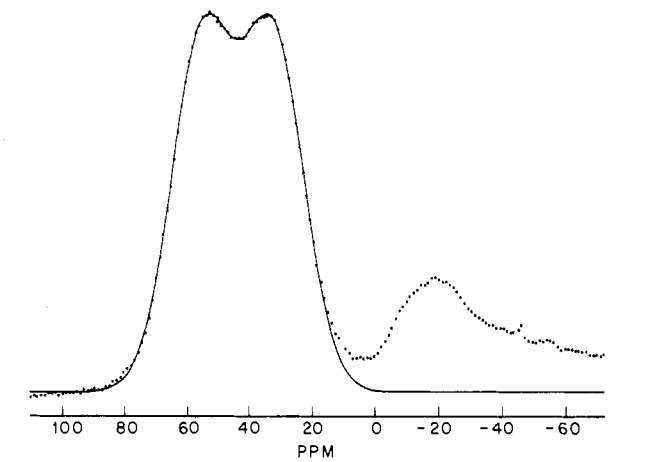


FIGURE 2: 282.4-MHz $^{19}$F NMR spectra of 2-[$^2$H$_6$,8,8-$^{19}$F$_2$]DMPC oriented at $\theta = 0°$ and 27 °C with a best-fit Gaussian function: (···) experimental spectrum; (—) simulated spectrum. For comments on the broad, weaker signal at ~−20 ppm, refer to Figure 1.

well described by eq 4, confirming the assumption of a uniaxial average chemical shift tensor with its symmetry axis directed along the normal to the bilayers. The spectra obtained for oriented multilayers of 2-[8,8-$^{19}$F$_2$]DMPC in $H_2O$ at 27 °C as a function of $\theta$ = 0, 54.7, and 90° are shown in Figure 1B. The Pake doublet is not as well resolved as that in the deuterated sample. From the position of the centers of $\theta$ = 0 and 90° resonances, the chemical shift anisotropy for each sample was determined as 58 ppm for 2-[$^2$H$_6$,8,8-$^{19}$F$_2$]DMPC and 64 ppm for 2-[8,8-$^{19}$F$_2$]DMPC at 27 °C.

Figure 2 shows a plot of the $^{19}$F line shape of oriented multilayers of 2-[$^2$H$_6$,8,8-$^{19}$F$_2$]DMPC for $\theta = 0°$ at 27 °C and a best fit to the line shape obtained by superimposing two Gaussian functions centered at each of the peaks in the doublet. From the frequency separation ($\Delta\nu_{FF}$) between the two peaks obtained by this fit, the order parameter ($S_{FF}$) is obtained by using eq 2 with a value of $r$ = 2.18 Å (Gent & Ho, 1978). The frequency separation is determined for both the 0 and 90° orientations. In accordance with theory, the values for the $\theta$ = 90° are half those for $\theta = 0°$. The broad, weak signal (around −20 ppm) observed in Figure 1 is identified as both a background $^{19}$F signal from fluoride in the glass used for orienting the bilayers and a small percentage of nonoriented

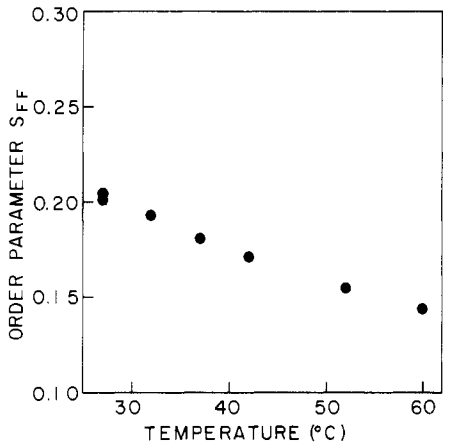


FIGURE 3: Order parameter $S_{FF}$ vs. temperature for 2-[$^2H_6$,8,8-$^{19}F_2$]DMPC in $H_2O$.

sample presumably present around the edges of the glass cover slips. A flat base line was obtained with the empty probe.

Figure 3 shows a plot of the order parameter $S_{FF}$ as a function of temperature for the 2-[$^2H_6$,8,8-$^{19}F_2$]DMPC sample. Previous work (Oldfield et al., 1980) gave an estimate of 0.15 for the order parameter $S_{FF}$. This value was estimated by extrapolation from the two order parameters $S_{CD}$ found for the deuterated 7′ and 9′ positions of 2-[$^2H_6$,8,8-$^{19}F_2$]DMPC by 55.6-MHz $^2H$ NMR at 33 °C. The extrapolated $S_{FF}$ is 20% lower than the $^{19}F$ NMR determined value of 0.19 at 33 °C. It is interesting to note that $S_{FF} = 0.19$ for position 8 of the acyl chain compares well with the order parameter $S_{CD}$ value of 0.21 determined for dimyristoylphosphatidylcholine substituted with two deuteriums at the same position and reduced temperature (Oldfield et al., 1978). The implication seems to be that the $CF_2$ group has little additional perturbing effect in the lipid bilayer. The temperature behavior of 2-[$^2H_6$,8,8-$^{19}F_2$]DMPC liposomes in excess $D_2O$ is shown in Figure 4. As the temperature increases, the line shape narrows and moves toward the isotropic resonance position.

The $^{19}F$ NMR line shapes in nonoriented liposomes can be explained in a satisfactory manner as resulting from a random orientation of the bilayers. Figure 5 shows such a line shape for 2-[$^2H_6$,8,8-$^{19}F_2$]DMPC at 27 °C. The $^{19}F$ NMR spectra of nonoriented samples containing ≥70% water were found to be very reproducible over long periods of time with different sample preparations. Also shown is a simulated spectrum obtained by an integral of the form

$$f(\nu) = C\int_0^{\pi/2} [g_1(\nu,\theta) + g_2(\nu,\theta)][f_1(\theta)]^{-1/2} \sin\theta \, d\theta \quad (5)$$

where

$$f_1(\theta) = \delta_0^2 + \delta_1^2(3\cos^2\theta - 1)^2$$

$$g_1(\nu,\theta) = \exp\left(-\frac{1}{2}\left[\nu + \left(\frac{2}{3}\Delta\sigma - \Delta F\right)(3\cos^2\theta - 1)\right][f_1(\theta)]^{-1}\right)$$

and

$$g_2(\nu,\theta) = \exp\left(-\frac{1}{2}\left[\nu + \left(\frac{2}{3}\Delta\sigma + \Delta F\right)(3\cos^2\theta - 1)\right][f_1(\theta)]^{-1}\right)$$

The factor $C$ in eq 5 is a normalization constant, and $f_1(\theta)$ is the orientation-dependent second moment, while $g_1(\nu,\theta)$ and $g_2(\nu,\theta)$ are the two Gaussian functions that represent the Pake doublet; $\delta_0^2 = \langle\Delta\nu_F^2\rangle_{residual}$ and $\delta_1^2(3\cos^2\theta - 1) = \langle\Delta\nu_F^2\rangle_{hetero}$, where $\Delta\sigma$ is the anisotropy of the uniaxial, motionally averaged

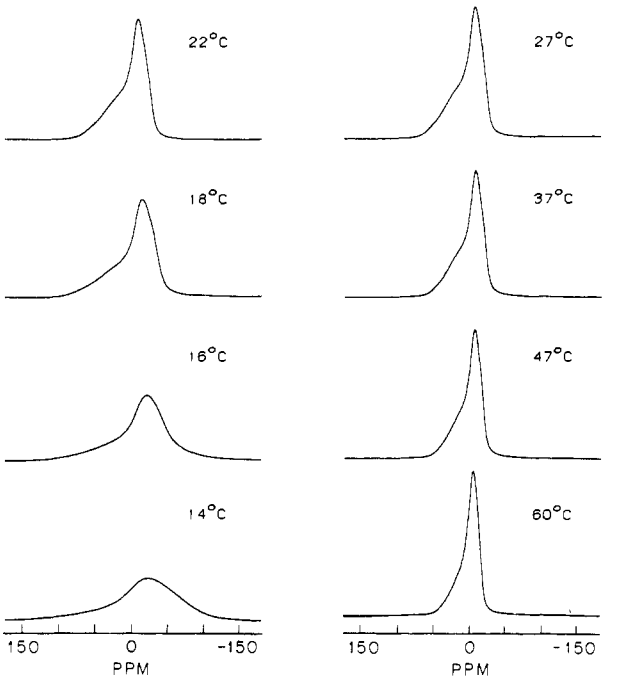


FIGURE 4: 282.4-MHz $^{19}F$ NMR spectra of 2-[$^2H_6$,8,8-$^{19}F_2$]DMPC liposomes in $D_2O$, (30:70 w/w) as a function of temperature. The origin of the $^{19}F$ chemical shift is set at the isotropic value.

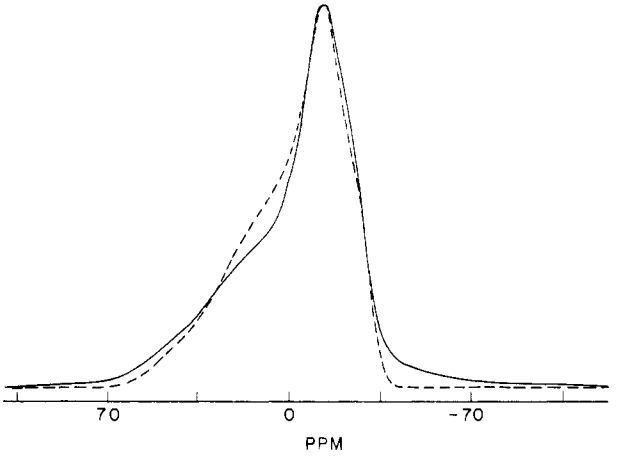


FIGURE 5: 282.4-MHz $^{19}F$ NMR spectra of nonoriented 2-[$^2H_6$,8,8-$^{19}F_2$]DMPC dispersed in $D_2O$ (30:70 w/w) at 27 °C simulated with a Gaussian function: (—) experimental spectrum; (--) simulated spectrum. The origin of the $^{19}F$ chemical shift is set at the isotropic value.

chemical shift tensor and the term $\Delta F$ is related to the frequency separation $\Delta\nu_{FF}$ between the members of the doublet through $\Delta\nu_{FF} = \Delta F(3\cos^2\theta - 1)$. The width parameters for the simulation of Figure 5 were obtained from the spectrum of oriented bilayers at the same temperature, and the chemical shift anisotropy constant $\Delta\sigma$ was adjusted to yield the best fit to the experimental spectrum.

From the simulated spectra obtained from eq 5 by varying the parameter $\Delta F$, one can determine the way in which the presence of a resolved doublet affects the line shape of nonoriented liposomes. As the parameter $\Delta F$ is increased and the doublet becomes better resolved, the steep side of the spectrum becomes more curved. In the absence of a resolved doublet, this part of the spectrum is steeper and almost straight as is observed, for example, in some $^{31}P$ spectra of nonoriented phospholipid liposomes (Rajan et al., 1981). The spectrum of Figure 5 exhibits instead a noticeable curvature in the region −14 to −35 ppm. The simulated spectrum corresponds quite

well to the experimental spectrum. However, the correspondence is not sufficient enough to allow an exact value for $S_{FF}$ to be determined from the lipid dispersions. The small deviation in the region 15–35 ppm seems to indicate that the experimental spectrum is either less isotropic, i.e., more ordered, than eq 5 assumes or the initial dead time in the acquisition of the FID could have distorted the line shape. More work is needed to resolve these two possibilities. Spectra obtained for both deuterated and nondeuterated samples dispersed in water were found to have practically identical line shapes. Liposomes containing only 25% water were found to have line shapes somewhat broader than those from liposomes containing 70% water (results not shown). The high sensitivity of the $^{19}F$ probe to the water content of the liposomes is being investigated further. We are currently also examining the 12,12-difluoro- and 4,4-difluorophospholipid analogues by $^{19}F$ NMR spectroscopy. Additionally, computer programs taking into account other motions that could affect the dipolar interaction and the chemical shift anisotropy are being investigated.

*Conclusions.* The high-field $^{19}F$ NMR spectra of oriented and nonoriented phospholipids specifically labeled in the 2-acyl chain have shown fluorine to be a sensitive probe for motion occurring in the hydrocarbon portion of a model lipid membrane. It will be of interest to examine the effect of incorporating cholesterol and proteins into model bilayers containing the difluoromethylene group at various positions along the 2-acyl chain.

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# Resonance Raman Spectroscopic Studies of Axial Ligation in Oxyhemoglobin, Oxymyoglobin, and Nitrosylmyoglobin†

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ABSTRACT: Raman intensity measurements for the Fe–$O_2$ stretching band of $HbO_2$ (Hb = hemoglobin) have been used to construct an excitation profile, which shows that resonance enhancement occurs mainly via the B and Q transition; no contribution is detectable from an out-of-plane charge-transfer transition. Direct coupling of $\nu_{Fe-O_2}$ to the porphyrin $\pi$–$\pi^*$ transitions is explained on the basis of competition between the $\pi^*$ orbitals of porphyrin and $O_2$ for Fe $d_\pi$ electrons. The RR spectrum of MbNO (Mb = myoglobin) at pH 8.4 is due solely to six-coordinate heme–NO, but lowering the pH to 5.8 converts the RR spectrum to one characteristic of five-coordinate heme–NO, consistent with Fe–ImH (ImH = imidazole) dissociation via protonation. The Fe–NO stretching frequencies are at 553 and 596 $cm^{-1}$ for the high- and low-pH forms, as expected, but the low-pH form shows an additional $^{15}NO$-sensitive band, at 573 $cm^{-1}$, which is assigned to Fe–N–O bending in the five-coordinate complex. The RR spectrum of $MbO_2$ shows a shoulder at $\sim$270 $cm^{-1}$, which shifts down by $\sim$3 $cm^{-1}$ upon $^{18}O_2$ substitution, and is suggested to contain the Fe–ImH stretching mode. The weakness of $\nu_{Fe-ImH}$, relative to $\nu_{Fe-O_2}$, is attributable to the lack of ImH involvement in the heme $\pi$ bonding.

Resonance Raman (RR) spectroscopy is currently being applied as a probe of structure and dynamics in heme proteins (Spiro, 1981, 1983; Asher, 1982; Rousseau et al., 1979). There has been special interest in the signatures of the axial ligation, particularly for $O_2$ and NO complexes. The Fe–$O_2$ stretching mode, $\nu_{Fe-O_2}$, was identified via its $^{18/16}O_2$ isotope shift in the oxyhemoglobin ($HbO_2$) RR spectrum, in an early study by Brunner (1974), and it has naturally been of great interest as a probe of the Fe–$O_2$ bond strength (Nagai et al., 1980a; Tsubaki et al., 1980; Walters et al., 1980). In this study we evaluate the scattering mechanism for $\nu_{Fe-O_2}$ via its excitation

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