# Fluorine-19 Nuclear Magnetic Resonance Investigation of Fluorine-19-Labeled Phospholipids. 1. A Multiple-Pulse Study†

Jan F. M. Post, Bruce W. Cook, Susan R. Dowd, I. J. Lowe, and Chien Ho*

ABSTRACT: A multiple-pulse nuclear magnetic resonance technique has been used to measure the order parameter, $S_{FF}$, at 40 MHz for dimyristoylphosphatidylcholine labeled with a difluoromethylene group at the 4-, 8-, or 12-position of the *sn*-2-acyl chain dispersed in water in the liquid-crystalline phase. The Carr–Purcell–Meiboom–Gill multiple-pulse sequence can resolve the homonuclear dipolar coupling between the two fluorine nuclei, thus making a direct determination of the order parameter, $S_{FF}$, for the F–F internuclear vector possible. Other interactions, such as the $^{19}F$ chemical shift anisotropy, heteronuclear dipolar couplings, and field inhomogeneity, which normally obscure the dipolar splitting, are effectively canceled. The order parameters obtained in this work compare well with those obtained by $^{19}F$ nuclear magnetic resonance line-shape analysis of the $^{19}F$-labeled phospholipids reported in the following paper [Dowd, S. R., Simplaceanu, V., & Ho, C. (1984) *Biochemistry* (following paper in this issue)] as well as comparable $S_{CD}$ order parameters, determined for the deuterium–carbon internuclear vector of deuterium-labeled phospholipids [Oldfield, E., Meadows, M., Rice, D., & Jacobs, R. (1978) *Biochemistry 17*, 2727–2740]. The present results clearly show the usefulness of using nuclear magnetic resonance spectroscopy to investigate lipid–lipid and protein–lipid interactions, especially for those systems containing a difluoromethylene group in the acyl chain of a phospholipid molecule.

For more than two decades, phospholipids have been the subject of intense investigation. These molecules can, when dispersed in water, form bilayers and other structures that are relevant to the structure and functioning of biological membranes. Nuclear magnetic resonance (NMR)[1] techniques have been shown to be fruitful to investigate the dynamic and structural features of phospholipids [for reviews, see Seelig (1977), Griffin (1981), Jacobs & Oldfield (1981), and Ho et al. (1984)].

Recently, $^{19}F$ has received attention as an attractive NMR probe for membrane studies (Longmuir & Dahlquist, 1976; Gent et al., 1978, 1981; Gent & Ho, 1978; Oldfield et al., 1980; Engelsberg et al., 1982; Macdonald et al., 1983; Dowd et al., 1984). $^{19}F$ NMR has certain distinct advantages for such studies. It has almost the same sensitivity as that of $^1H$ NMR. Furthermore, in a $CF_2$ group, several magnetic interactions play a role, such as the homonuclear dipolar coupling between the two fluorine nuclei, heteronuclear dipolar coupling between $^{19}F$ and nearby protons, and the $^{19}F$ chemical shift anisotropy (CSA) of the C–F bond. It has been shown that these interactions can be measured separately by using pulse NMR techniques (Post et al., 1982a,b). When the results are combined, a complete description of the average orientation of the labeled lipid segment can be given.

The Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence has been found to be an especially useful technique for investigating $CF_2$-labeled lipid systems. It cancels all the interactions except homonuclear dipolar couplings, making it possible to measure the average orientation of the F–F bond, described by the order parameter $S_{FF}$. The CPMG experiment has been used previously to measure the order parameters, $S_{FF}$, and to study the phase transitions in lyotropic liquid crystals formed by $^{19}F$-labeled potassium myristate (Post et al., 1982b). The effects of cholesterol and a membrane protein on $^{19}F$-labeled dimyristoylphosphatidylcholine (DMPC) were also studied by using the CPMG sequence (Post et al., 1981).

In this paper, we describe a series of measurements of the order parameter, $S_{FF}$, in the $^{19}F$-labeled DMPC–water system as a function of temperature, water content, cholesterol, and position of the $CF_2$ group along the acyl chain. The phospholipid was $^{19}F$-labeled with a $CF_2$ group at the 4-, 8-, or 12-position of the *sn*-2-acyl chain and the order parameters were measured in the liquid-crystalline phase.

## Experimental Procedures

*Materials.* Samples of phospholipids for NMR study were prepared by mixing them with $H_2O$ or $D_2O$ in a 4-mm tube and vortexing above the phase transition temperature. 1-Myristoyl-2-(4,4-[$^{19}F_2$]difluoromyristoyl)-*sn*-glycero-3-phosphocholine (2-[4,4-$^{19}F_2$]DMPC) and the corresponding 12,12-[$^{19}F_2$]difluoromethylene isomer (2-[12,12-$^{19}F_2$]DMPC) were synthesized by methods equivalent to those described previously for the preparation of 1-myristoyl-2-(8,8-[$^{19}F_2$]-difluoromyristoyl)-*sn*-glycerol-3-phosphocholine (2-[8,8-$^{19}F_2$]DMPC) (Engelsberg et al., 1982). $^1H$ NMR spectra of the phospholipids taken in deuteriochloroform solutions were consistent with the expected structures. Deuterium oxide was purchased from Bio-Rad. Cholesterol was purchased from Sigma and recrystallized twice from ethanol. Reagent-grade solvents were used unless otherwise stated.

*Methods.* The $^{19}F$ NMR experiments were carried out at 40 MHz, using a home-built spectrometer (Karlicek & Lowe, 1978). A Nicolet signal averager (Model 1270) was used to sample the NMR signal and to perform the Fourier transformation.

† From the Department of Biological Sciences, Carnegie-Mellon University, Pittsburgh, Pennsylvania 15213 (J.F.M.P., B.W.C., S.R.D., and C.H.), and the Department of Physics and Astronomy, University of Pittsburgh, Pittsburgh, Pennsylvania 15260 (I.J.L.). *Received February 14, 1984.* Supported by research grants from the National Science Foundation (PCM 82-08829) and the National Institutes of Health (GM-26874 and HL-24525).

[1] Abbreviations: NMR, nuclear magnetic resonance; CSA, chemical shift anisotropy; CPMG, Carr–Purcell–Meiboom–Gill; DMPC, dimyristoylphosphatidylcholine; $S_{FF}$, fluorine–fluorine internuclear vector order parameter; $S_{CD}$, carbon–deuterium internuclear order parameter; 2-[4,4-$^{19}F_2$]DPMC, 1-myristoyl-2-(4,4-[$^{19}F_2$]difluoromyristoyl)-*sn*-glycerol-3-phosphocholine; 2-[8,8-$^{19}F_2$]DMPC, 1-myristoyl-2-(8,8-[$^{19}F_2$]difluoromyristoyl)-*sn*-glycerol-3-phosphocholine; 2-[12,12-$^{19}F_2$]-DMPC, 1-myristoyl-2-(12,12-[$^{19}F_2$]difluoromyristoyl)-*sn*-glycerol-3-phosphocholine; AHT, average Hamiltonian theory.

0006-2960/84/0423-6138$01.50/0 

## Theoretical Section

Multiple-pulse NMR experiments on $^{19}$F-labeled lipids have been discussed by Post et al. (1982a). We shall give here a brief discussion of the CPMG experiment in terms of the average Hamiltonian theory (AHT). It has been shown that if a spin system is subjected to cyclic forces, it behaves as if it develops under a constant Hamiltonian when inspected in phase with the cycle (Mehring, 1983). This Hamiltonian can be calculated by using AHT. The time-dependent density matrix $\rho(t)$ can be written, at integral multiples $n$ of the cycle time $t_c$, as

$$\rho(nt_c) = L(nt_c)\rho(0)L^{\dagger}(nt_c) \quad (1)$$

where $L$ is the propagator operator for the cycle and $L^{\dagger}$ its Hermitian adjoint. $L$ can be written by using the Magnus formula (Haeberlen, 1976)

$$L(nt_c) = \exp[-int_c(\bar{\mathcal{H}}^0 + \bar{\mathcal{H}}^1 + \bar{\mathcal{H}}^2 + ...)] \quad (2)$$

where $\bar{\mathcal{H}}^0 = (1/t_c)\int_0^{t_c}\mathcal{H}(t)\,dt$ is the average Hamiltonian in the rotating frame over the cycle and $\bar{\mathcal{H}}^1$, $\bar{\mathcal{H}}^2$, etc., are higher order correction terms.

The spin Hamiltonian in the rotating frame for a system containing two dipolar coupled spin $^1/_2$ species I and S can be written as

$$\mathcal{H} = \mathcal{H}_{II} + \mathcal{H}_{IS} + \mathcal{H}_{CSA} \quad (3)$$

if spins I are observed. $\mathcal{H}_{II}$ denotes the truncated Hamiltonian for the homonuclear dipolar couping and has the form

$$\mathcal{H}_{II} \propto (\vec{I}_j\vec{I}_k - 3I_{jz}I_{kz}) \quad (4)$$

where $\mathcal{H}_{IS}$ and $\mathcal{H}_{CSA}$ are the Hamiltonians for the heteronuclear dipolar coupling and CSA, respectively, and are both of the form

$$\mathcal{H}_{IS}, \mathcal{H}_{CSA} \propto a_jI_{jz} + a_kI_{kz} \quad (5)$$

Note that $\vec{I}_j\vec{I}_k$ is invariant to rotation and that $\mathcal{H}_{II}$ is quadratic in $I_z$, while $\mathcal{H}_{IS}$ and $\mathcal{H}_{CSA}$ are linear functions of $I_z$. For a $90°_y-(\tau-180°_x-\tau)_n$ sequence, the cycle time is $4\tau$. A cycle consists of two 180° pulses. A 180° pulse can be described by a rotation operator $R = \exp(i\pi I_x)$. It is easy to see that

$$R^{\dagger}\mathcal{H}_{II}R = \mathcal{H}_{II} \quad (6a)$$

$$R^{\dagger}\mathcal{H}_{IS}R = -\mathcal{H}_{IS} \quad (6b)$$

$$R^{\dagger}\mathcal{H}_{CSA}R = -\mathcal{H}_{CSA} \quad (6c)$$

Thus, over the cycle $t_c = 4\tau$, $\bar{\mathcal{H}}^0 = \mathcal{H}_{II}$. In the case of rapid rotations around the C–C bonds, the $^{19}$F spins will be magnetically equivalent ($a_j = a_k$ in eq 5). It can be shown that all higher order correction terms $\bar{\mathcal{H}}^1$, $\bar{\mathcal{H}}^2$, etc., in the Magnus expansion (eq 2) vanish, because the Hamiltonians in eq 6 commute at all times (Mehring, 1983). Therefore, in contrast with other multiple-pulse experiments like the WAHUHA sequence, the cycle time $t_c$ does not influence the experimental resolution and the choice of $\tau$ is not critical. However, the convergence condition for eq 2 is roughly $t_c\|\mathcal{H}(t)\| \ll 1$, where $\|\mathcal{H}(t)\|$ is the magnitude of $\mathcal{H}(t)$. Therefore, in the CPMG experiment, only $\mathcal{H}_{II}$ is effective in the time evolution of the transverse magnetization. This makes a direct measurement of the order parameter $S_{FF}$ possible. $S_{FF}$ is defined as $(1/2)(3\cos^2\theta - 1)$ where $\theta$ is the average angle between the F–F vector and the bilayer normal. It should be mentioned that for the special case that $a_j = a_k$ and the Hamiltonian commutes with itself at all times, one does not need to use the Magnus expansion to justify the use of AHT, and for this case, there are no limitations on the value of $t_c$. For a discussion of the order parameters, see the Appendix and references cited therein.

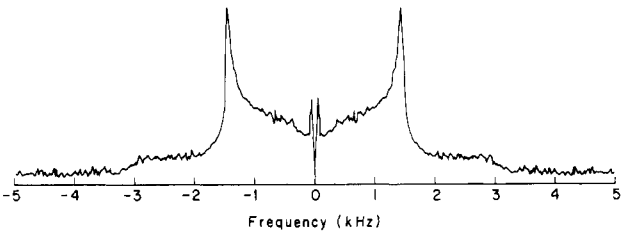


FIGURE 1: 40-MHz dipolar $^{19}$F NMR spectrum of 2-[8,8-$^{19}F_2$]DMPC in 30% $D_2O$ at 33.3 °C, obtained with the CPMG pulse sequence $90°_y-(\tau-180°_x-\tau)_n$: $\tau = 50$ μs; $n = 512$; 90° pulse = 1 μs; 1000 scans.

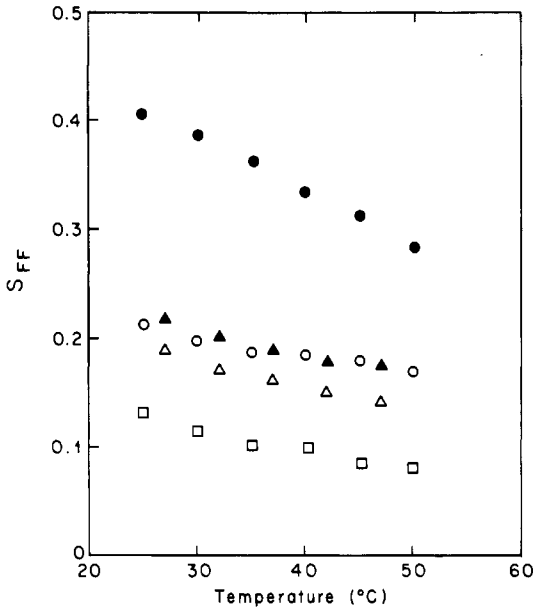


FIGURE 2: Order parameter $S_{FF}$ for $^{19}$F-labeled phospholipids as a function of temperature: (○) 2-[4,4-$^{19}F_2$]DMPC–$D_2O$ (30:70); (△) 2-[8,8-$^{19}F_2$]DMPC–$D_2O$ (30:70); (□) 2-[12,12-$^{19}F_2$]DMPC–$D_2O$ (30:70); (▲) 2-[8,8-$^{19}F_2$]DMPC–$D_2O$ (75:25); (●) 2-[8,8-$^{19}F_2$]DMPC plus 35 mol % cholesterol in excess $D_2O$.

## Results and Discussion

A typical $^{19}$F dipolar spectrum, obtained with the CPMG pulse sequence, is shown in Figure 1. The magnetization (echo peaks) is sampled between successive 180° pulses. Experimentally, only half of the spectrum is obtained, but for reasons of clarity, the whole (symmetrical) line shape is plotted. The spectrum obtained clearly shows the features of a Pake doublet expected for a dipolar coupled spin $^1/_2$ pair. Intermolecular line broadening is efficiently averaged out in liquid crystals by rapid lateral diffusion, so that we are, in fact, observing isolated spin $^1/_2$ pairs (Post et al., 1982a,b). The CSA, heteronuclear dipolar couplings, and field inhomogeneities, which normally obscure the F–F dipolar splitting, are efficiently canceled in the CPMG experiment. From the observed splitting Δ, the order parameter $S_{FF}$ can be calculated as $S_{FF} = \Delta/15.4$ kHz. The value of 15.4 kHz is the maximum theoretical splitting for a $CF_2$ group in $^{19}$F-labeled lipids.

Figure 2 shows the order parameters obtained in the temperature range 25–50 °C for DMPC in excess $D_2O$, labeled respectively at the 4-, 8-, or 12-position of the sn-2 chain. It can be seen that for all three phospholipids studied, the order parameters decrease with temperature. The order parameters are lower at positions deeper in the bilayer and are similar to the results of $^2$H NMR measurements on $^2$H-labeled DMPCs (Oldfield et al., 1978). For a discussion on the comparison between the $S_{FF}$ and $S_{CD}$ values of phospholipids, see the following paper by Dowd et al. (1984). The order parameters

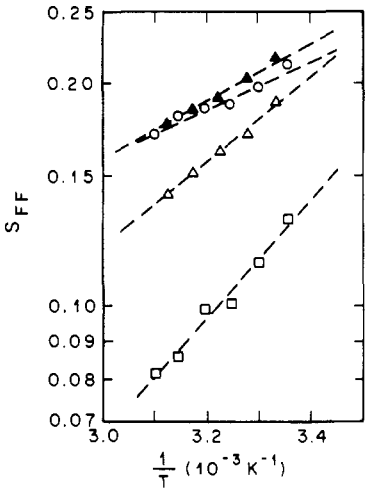


FIGURE 3: Order parameter $S_{FF}$ of $^{19}F$-labeled phospholipids as a function of reciprocal temperature: (○) 2-[4,4-$^{19}F_2$]DMPC-$D_2O$ (30:70); (△) 2-[8,8-$^{19}F_2$]DMPC-$D_2O$ (30:70); (□) 2-[12,12-$^{19}F_2$]-DMPC-$D_2O$ (30:70); (▲) 2-[8,8-$^{19}F_2$]DMPC-$D_2O$ (75:25).

are not very different for the 4- and 8-position, while the value for the 12-position is much lower. This observation is in good agreement with work on $^2H$-labeled dipalmitoylphosphatidylcholine (Schindler & Seelig, 1975), which has demonstrated a $S_{CD}$ order parameter "plateau" for roughly the first half of the chain.

The effects of cholesterol and a lower water content on the $S_{FF}$ are also shown in Figure 2. In both cases, there is a considerable increase in the values of $S_{FF}$, in agreement with other work (Post et al., 1981; Pope et al., 1981).

The interpretation of order parameters is not a straightforward matter and much work has been done to connect these order parameters with detailed models of molecular motion (Schindler & Seelig, 1975; Peterson & Chan, 1977; Pace & Chan, 1982; van der Ploeg & Berendsen, 1983). For our present work, simple statistical mechanical arguments suffice to obtain a qualitative understanding of our results. In an aliphatic chain, each C–C bond encounters three energy minima while rotating 360° and therefore exists in one of three conformations (Gruen, 1982). These are the trans ($t$) and two gauche ($g^+$ and $g^-$) conformers. The gauche states are higher in energy than the trans state by $E_g = 2.1 \pm 0.4$ kJ/mol. The weighted sum of configurations for a C–C bond is $[1 + 2 \exp[-E_g/(RT)]]$. For a polymer of $n$ bonds, the total weighted number of configurations $P$ is $P = [1 + 2 \exp[-E_g/(RT)]]^n$. In the temperature range of interest, $\exp[-E_g/(RT)] \simeq 0.5$ and thus $P \simeq 2^n$, which amounts to more than 8000 configurations for a C-14 chain. The measured order parameters reflect the rapid fluctuations between all possible configurations. For an isolated chain fixed at one end, each segment is expected to have a greater orientational freedom than the preceding one. If each segment is characterized by an order parameter, $S$, with respect to the preceding segment, then the order parameter, $S_n$, of the $n$th segment with respect to the symmetry axis of the system is given by $S_n = S^n$. Thus, one expects an exponential decrease in the order parameter along the chain. From our data, this is not the case: the decrease is less than exponential. This difference can be attributed to the steric repulsions between neighboring chains in the bilayers, which partly prevent the decrease of the orientational order along the chain.

It is interesting that the temperature dependence of the order parameters is reasonably well described by an Arrhenius curve, as shown in Figure 3, with "activation energies" of 6.3, 11.0, and 14.9 kJ/mol for the 4-, 8-, and 12-labeled compounds respectively. Note that the increase in activation energy is roughly linear instead of the exponential rise, which we would expect for a freely moving chain fixed at one end. This confirms that the acyl chains restrict each other in their motional freedom. Figure 3 also shows the Arrhenius plot for 2-[8,8-$^{19}F_2$]DMPC with lower $D_2O$ content. The "activation energies" are 11.0 and 9.4 kJ/mol for 70% and 25% $D_2O$, indicating that at lower water contents the chains are more motionally restricted than at higher water contents. The Arrhenius-type behavior indicates that the order parameter is related to the rate of the motions in the chain and to the thermal expansion of the bilayer (Nagle & Wilkinson, 1978). However, a complete explanation of this phenomenon would require the use of well-tested computer models of the lipid chain behavior in bilayers.

## Conclusions

The CPMG multiple-pulse technique was found to be a convenient way to accurately determine the order parameter, $S_{FF}$, of $^{19}F$-labeled phospholipids in liquid-crystalline bilayers. The observed order parameters decrease with increasing temperature, water content, or position in the acyl chain and can be understood in terms of steric repulsions between the phospholipid molecules. The present results as well as those presented in the following paper (Dowd et al., 1984) clearly show that $^{19}F$ labels (especially as a $CF_2$ group in an acyl chain) are an excellent choice for investigating both structural and dynamical properties of phospholipid dispersions. The presence of two $^{19}F$ atoms in a $CF_2$ group permits us to determine an order parameter, $S_{FF}$, directly by using the CPMG multiple-pulse sequence. This is a useful technique to investigate lipid–lipid, lipid–cholesterol, and lipid–protein interactions.

## Acknowledgments

We thank Dr. John F. Nagle for interesting discussions.

## Appendix

*Determination of Order Parameters.* Knowledge of the chemical shift tensor can be used to obtain all the order parameters for the $CF_2$ group. Unfortunately, this tensor is not known at present, but still useful conclusions can be drawn from the measured CSA. The chemical shift anisotropic line width can be written as

$$\Delta\sigma = \mathrm{Tr}(\sigma \boldsymbol{S})$$

where $\sigma$ is the chemical shift tensor, $\boldsymbol{S}$ is the order parameter tensor defined by Saupe (1964), and Tr denotes the trace. To a good approximation, $\boldsymbol{S}$ is diagonal in the frame where $x$ is in the F–F direction, $y$ is the direction dividing the F–C–F angle, and $z$ is perpendicular to the $CF_2$ plane (Post et al., 1982a). Now $\Delta\sigma$ can be expressed using $\mathrm{Tr}(\boldsymbol{S}) = 0$, as

$$\Delta\sigma = aS_{xx} + bS_{yy}$$

where $a$ and $b$ are constants, involving the elements of the chemical shift tensor. As can be seen in Figure 6 of Dowd et al. (1984), $\Delta\sigma$ is a linear function of $S_{xx}$, with slope $\Delta\sigma/S_{xx} = a + bS_{yy}/S_{xx}$. The slope is constant for all values of $S_{xx}$. It is known that at low temperatures the chains are in the all-trans configuration, rapidly rotating around their long molecular axis (Davis, 1983). In such a case, $S_{xx} = S_{yy} = 0.5$. As the ratio $S_{yy}/S_{xx}$ is to a good approximation constant over the temperature range studied, we can conclude that $S_{xx} \simeq S_{yy}$ for all temperatures. This means that there is no significant motional asymmetry around the molecular axis in the lipid chains.

*Estimation of Chemical Shift Tensor.* From the observed value $\Delta\sigma = \sigma_{\parallel} - \sigma_{\perp} = 166$ ppm (Dowd et al., 1984), one can estimate the principal elements of the chemical shift tensor for a $CF_2$ group in alkyl chains. The $CF_2$ plane is approximately a plane of symmetry and the least shielded component $\sigma_{11}$ is therefore in the $z$-direction perpendicular to the $CF_2$ plane. Thus, $\sigma_{11} = \sigma_{\perp} = -111$ ppm. Then $\sigma_{\parallel} = (1/2)(\sigma_{22} + \sigma_{33}) = 55$ ppm. In other compounds, it has been found that the most shielded component, $\sigma_{33}$, is oriented along the C–F bond. For the $CF_3$ group in $CF_3COOAg$, a value of $\sigma_{33} = 71$ ppm was found (Griffin et al., 1972). We estimate then for the chemical shift tensor in $CF_2$-labeled lipids $\sigma_{33} = 71$ ppm, $\sigma_{22} = 40$ ppm, and $\sigma_{11} = -111$ ppm.

**Registry No.** DMPC, 18194-24-6; 2-[4,4-$^{19}F_2$]DMPC, 92937-51-4; 2-[12,12-$^{19}F_2$]DMPC, 92937-52-5.

## References


Davis, J. H. (1983) *Biochim. Biophys. Acta 737*, 117–171.

Dowd, S. R., Simplaceanu, V., & Ho, C. (1984) *Biochemistry* (following paper in this issue).

Engelsberg, M., Dowd, S. R., Simplaceanu, V., Cook, B. W., & Ho, C. (1982) *Biochemistry 21*, 6985–6989.

Gent, M. P. N., & Ho, C. (1978) *Biochemistry 17*, 3023–3038.

Gent, M. P. N., Cottam, P. F., & Ho, C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 630–634.

Gent, M. P. N., Cottam, P. F., & Ho, C. (1981) *Biophys. J. 33*, 211–224.

Griffin, R. G. (1981) *Methods Enzymol. 72*, 108–173.

Griffin, R. G., Ellett, J. D., Mehring, M., & Waugh, J. S. (1972) *J. Chem. Phys. 57*, 2147–2155.

Gruen, D. W. R. (1982) *Chem. Phys. Lipids 30*, 105–120.

Haeberlen, U. (1976) *Adv. Magn. Reson., Suppl. 1.*

Ho, C., Dowd, S. R., & Post, J. F. M. (1984) *Curr. Top. Bioenerg. 14.*

Jacobs, R. E., & Oldfield, E. (1981) *Prog. Nucl. Magn. Reson. Spectrosc. 14*, 113–136.

Karlicek, R. F., & Lowe, I. J. (1978) *J. Magn. Reson. 32*, 199–225.

Longmuir, K. J., & Dahlquist, F. W. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2716–2719.

Macdonald, P. M., McDonough, B., Sykes, B. D., & McElhaney, R. N. (1983) *Biochemistry 22*, 5103–5111.

Mehring, M. (1983) *High Resolution NMR Spectroscopy in Solids, NMR Basic Principles and Progress*, 2nd ed., Vol. 11, Springer-Verlag Berlin, Heidelberg, and New York.

Nagle, J. F., & Wilkinson, D. A. (1978) *Biophys. J. 23*, 159–175.

Oldfield, E., Meadows, M., Rice, D., & Jacobs, R. (1978) *Biochemistry 17*, 2727–2740.

Oldfield, E., Lee, R. W. K., Meadows, M., Dowd, S. R., & Ho, C. (1980) *J. Biol. Chem. 255*, 11652–11655.

Pace, R. J., & Chan, S. I. (1982) *J. Chem. Phys. 76*, 4241–4247.

Peterson, N. O., & Chan, S. I. (1977) *Biochemistry 16*, 2657–2667.

Pope, J. M., Walker, L., Cornell, B. A., & Francis, G. W. (1981) *Biophys. J. 35*, 509–520.

Post, J. F. M., de Ruiter, E. E. J., & Berendsen, H. J. C. (1981) *FEBS Lett. 132*, 257–260.

Post, J. F. M., James, E., & Berendsen, H. J. C. (1982a) *J. Magn. Reson. 47*, 251–263.

Post, J. F. M., Kamman, R. L., & Berendsen, H. J. C. (1982b) *J. Magn. Reson. 47*, 264–281.

Saupe, A. (1964) *Z. Naturforsch., A 19A*, 161–171.

Schindler, H., & Seelig, J. (1975) *Biochemistry 14*, 2283–2287.

Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353–418.

van der Ploeg, P., & Berendsen, H. J. C. (1983) *Mol. Phys. 49*, 233–248.