Fluorine-19 Nuclear Magnetic Resonance Investigation of Fluorine-19-Labeled Phospholipids. 2. A Line-Shape Analysis†

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ABSTRACT: Fluorine-19 nuclear magnetic resonance spectra at 282.4 MHz of dimyristoylphosphatidylcholine specifically labeled with a difluoromethylene group at the 4-, 8-, or 12-position of the *sn*-2-acyl chain and dispersed in excess water show the characteristic powder-pattern line shapes associated with an anisotropic axially symmetric chemical shift tensor, altered by the presence of the homonuclear dipolar interaction of the fluorine nuclei and of heteronuclear dipolar interactions between fluorine and nearby protons. Values for the anisotropy of the fluorine-19 chemical shift and for the fluorine–fluorine internuclear vector order parameter, S_{FF}, as a function of temperature have been determined for the phospholipid dispersions with and without cholesterol. An increased mobility is evidenced in both cases as the temperature is raised. For the phospholipid dispersions in water, the values of S_{FF} parallel quite well the behavior of the carbon–deuterium internuclear vector order parameter, S_{CD}, as determined by deuterium nuclear magnetic resonance spectroscopy for the same labeled position. The effect of adding cholesterol is seen as a restriction of the chain mobility and the eventual disappearance of the phase transition. These new experiments provide a value of 166 ppm for the anisotropy of the axially symmetric chemical shift tensor of a difluoromethylene group in a phospholipid acyl chain. They also demonstrate the feasibility as well as the advantages of using a difluoromethylene group as a probe for molecular motions in the phospholipid bilayers.

The fluorine-19 nucleus as a nuclear magnetic resonance (NMR)[1] probe of the motions present in membrane systems continues to hold promise as a useful and informative tool (Langmuir & Dahlquist, 1976; Gent & Ho, 1978; Gent et al., 1978, 1981; Oldfield et al., 1980; Esfahani et al., 1981; Post et al., 1981; Engelsberg et al., 1982; Macdonald et al., 1983). For a recent review on ^{19}F NMR investigations of membranes, see Ho et al. (1984). Recent work from this laboratory has shown that, above the phase transition temperature, bilayers of dimyristoylphosphatidylcholine (DMPC) labeled with a difluoromethylene group in the 8-position of the 2-acyl chain give rise at high magnetic fields to NMR powder-pattern line shapes clearly dominated by the chemical shift anisotropy (Engelsberg et al., 1982). From spectral simulations both order parameter and chemical shift anisotropy (CSA) values can be derived. ^{19}F NMR spectra of macroscopically oriented bilayers show a partially resolved Pake doublet due to the homonuclear dipolar interaction between the two fluorine nuclei. The order parameter, S_{FF}, for the CF_2 group can be determined directly from the splitting of the doublet, and the anisotropy of the averaged, axially symmetric ^{19}F chemical shift tensor can also be determined by measuring the position of the doublet at various orientations of the bilayers with respect to the magnetic field.

Further studies extending the model bilayer system to include DMPC samples with a CF_2 group at position 4 or 12 of the 2-acyl chains are reported here. De-Paking, a technique developed by Bloom and co-workers (Bloom et al., 1981; Sternin et al., 1983), has been applied to the ^{19}F NMR spectra of ^{19}F-labeled phospholipid dispersions in order to extract both order parameter and CSA values. The studies on the temperature behavior of the phospholipids in the presence of various amounts of cholesterol resulted in a wide range of order parameter values. Although restricting the mobility of the acyl chains, as evidenced by the overall broadening of the ^{19}F NMR spectra, the presence of cholesterol preserves the axial symmetry of the averaged chemical shift tensor. A value of 166 ppm was found experimentally for the maximum CSA of the CF_2 group in the DMPC acyl chain in the presence of cholesterol.

Experimental Procedures

Materials. 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 as 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.

Preparation of Samples. Multilayered liposomes of phospholipid were prepared by vortexing a weighed amount of phospholipid and H_2O or D_2O in a 5-mm NMR tube (3.6 cm in length), above the phase transition temperature of the phospholipid. Samples were checked for purity by thin-layer chromatography using $CHCl_3$–CH_3OH–7 N NH_4OH (230:90:15 v/v) as the solvent and visualized by using a molybdenum phosphate stain reagent.

Samples containing phospholipid and cholesterol were prepared by weighing the desired quantities into a small round-bottomed flask (25 mL), dissolving the mixture in 5 mL of benzene–chloroform (90:10 v/v), and lyophilizing. The flask

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[1] Abbreviations: NMR, nuclear magnetic resonance; DMPC, dimyristoylphosphatidylcholine; S_{FF}, fluorine–fluorine internuclear vector order parameter; S_{CD}, carbon–deuterium internuclear vector order parameter; S_{HH}, proton–proton internuclear vector order parameter; CSA, chemical shift anisotropy; 2-[4,4-$^{19}F_2$]DMPC, 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; FID, free induction decay; CPMG, Carr–Purcell–Meiboom–Gill; $T_R = [(T - T_c)/T_c] \times 10^3$, reduced temperature.

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was then pumped at room temperature for 48 h under high vacuum. The sample was dispersed in water by vortexing at a temperature above the phase transition and transferred by pipetting into an NMR tube.

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 a home-built, fluorine-free, 5-mm probe (Cook & Lowe, 1982; Engelsberg et al., 1982). A fast, broad-band preamplifier (Stoll, 1981) using passive limiters in order to prevent amplifier saturation was built by using Avantek UTO-544 modular amplifiers and UTL-1002 thin film limiters; the dead time of the cascade is less than 1 μs. The spectrometer interface was modified so as to generate a shorter receiver blanking pulse, only 2 μs longer than the transmitter pulse. With these changes, meaningful data can be taken 6–9 μs after the pulse, as compared to 15–18 μs in the standard configuration of the Bruker spectrometer.

Typically 1024 scans were acquired in the quadrature mode by using a spectral width of 166.667 kHz, a pulse width of 2 μs (30–45° rotation angle, depending on the temperature), a relaxation delay of 1 s, and an acquisition time of 3 or 6 ms corresponding to 1024 or 2048 time domain data points.

The Aspect 2000A computer was used for Fourier transformation and for phase correction of the spectra. A line broadening of 300 Hz was applied as an exponential multiplication of the free induction decay (FID) in order to improve the signal-to-noise ratio. Some FIDs were back-extrapolated to correct the initial two or three points distorted due to the instrument dead time. For these cases, the first 10–20 points were used in a polynomial least-squares fit and extrapolation procedure. When only the first two points had to be replaced in the FID, the Fourier transformation of either the original or the corrected FID resulted in essentially the same spectrum, except for a tilt and offset of the base line, which was easily corrected with the standard application software.

The de-Paking of the ^{19}F NMR spectra and subsequent least-squares line fitting calculations, as well as the spectral simulations, were carried out on a remote VAX-11/780 computer using Fortran programs. The real part of the original spectra was transferred from the Aspect 2000A to the VAX computer via a RS-232 line.

Theoretical Section

The observed ^{19}F NMR spectrum of the ^{19}F-labeled DMPC liposomes can be readily described as a powder-pattern line shape arising from the anisotropic ^{19}F chemical shift and from the homo- and heteronuclear dipolar interactions of the pair of fluorine nuclei and between the fluorine and its nearby protons (Gent & Ho, 1978; Engelsberg et al., 1982). In the approximation of fast reorientation about the long molecular axis, the intramolecular interactions are scaled as $(3\cos^2\theta - 1)/2$, where θ denotes the angle between the axis of reorientation (in this case also the normal to the bilayer) and the direction of the static magnetic field. There is also an angle-independent component of the line broadening mainly due to magnetic field inhomogeneity and intermolecular interactions. This term is generally small, as evidenced by studies on oriented samples (Engelsberg et al., 1982). The line shape is well described by the equation[2]

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

[2] It should be noted that a factor of $^1/_2$ and a power of 2 were inadvertently omitted in the printed definition of $g_1(\nu,\theta)$ and $g_2(\nu,\theta)$ in an earlier paper (Engelsberg et al., 1982).

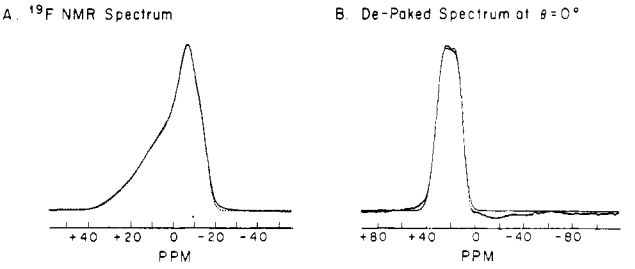

FIGURE 1: 282.4-MHz ^{19}F NMR spectra of 2-[12,12-$^{19}F_2$]DMPC dispersed in H_2O (30:70 w/w) at 27 °C: (A) experimental (—) and simulated (···) spectrum; (B) spectrum de-Paked at $\theta = 0°$ (---) and fitted with a Gaussian doublet (—).

where C is a normalization constant and $f_1(\theta) = \delta_0 + \delta_1(3\cos^2\theta - 1)^2$ represents dipolar broadening explicitly showing the orientation independent broadening, δ_0, while

$$g_1(\nu,\theta) = \exp[-(1/2)[(\nu - \nu_0) - (1/2)[(2/3)\Delta\sigma - \Delta F] \times (3\cos^2\theta - 1)]^2[f_1(\theta)]^{-1}]$$

and

$$g_2(\nu,\theta) = \exp[-(1/2)[(\nu - \nu_0) - (1/2)[(2/3)\Delta\sigma + \Delta F] \times (3\cos^2\theta - 1)]^2[f_1(\theta)]^{-1}]$$

are the two components of the Pake doublet, separated by $2\Delta F$ when $\theta = 0°$. The quantity $\Delta\sigma$ is the effective value of the anisotropy of the motionally averaged axially symmetric ^{19}F chemical shift tensor (in frequency units) and ν_0 is the frequency position of the isotropic spectrum.

The Pake splitting, ΔF, is also an effective, motionally averaged value, and the fluorine–fluorine internuclear vector order parameter, S_{FF}, can be defined as

$$S_{FF} = \Delta F/F_0 \quad (2)$$

where ΔF_0 = 15.4 kHz and represents the static dipolar coupling of the isolated fluorine atom pair (Post et al., 1982).

Results and Discussion

Powder NMR spectra can be simulated by using eq 1 and values can be obtained for all the relevant parameters, δ_0, δ_1, $\Delta\sigma$, and ΔF, on the basis of a best fit. However, such an operation is time consuming when carried out for many spectra. A new technique known as de-Paking proves to be very useful in obtaining S_{FF} and $\Delta\sigma$. De-Paking is a mathematical procedure that, assuming that the interactions governing the NMR spectrum scale as $(3\cos^2\theta - 1)/2$, is able to recover the spectrum of an oriented system from the spectrum of a superposition of randomly oriented domains (Bloom et al., 1981). This is exactly the case for phospholipid bilayers (liposomes) obtained by dispersing a phospholipid in water.

A Fortran program written by Sternin (1982) has been used with some minor modifications to de-Pake the ^{19}F NMR spectra. The program determines the position of the isotropic peak, taken as the frequency origin, as the point around which the first moment of the absorption is zero. The resulting "oriented" spectrum corresponds to $\theta = 0°$. Therefore, the anisotropy of the chemical shift is determined as being $^3/_2$ the distance from this point to the center of the Pake doublet (see Figure 1), while the Pake splitting is taken as half the peak separation of the members of the resolved doublet. For greater accuracy, a least-squares fit is performed on the oriented spectrum by using a pair of Gaussian functions. The dipolar broadening results from the width of the Gaussian lines, but in order to determine δ_0 and δ_1, the line-shape simulation with $\Delta\sigma$ and ΔF determined by de-Paking would have to be used especially when δ_0 is not negligibly small, for example, at low

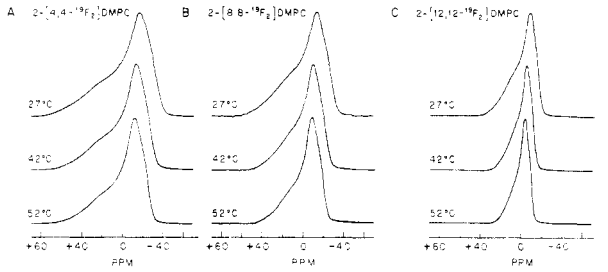

FIGURE 2: 282.4-MHz ^{19}F NMR spectra of ^{19}F-labeled phospholipids dispersed in H_2O (30:70 w/w) as a function of temperature: (A) 2-[4,4-$^{19}F_2$]DMPC; (B) 2-[8,8-$^{19}F_2$]DMPC; (C) 2-[12,12-$^{19}F_2$]DMPC.

temperatures. It is very important that the original spectrum be free of distortions and correctly phased. Any base-line imperfections, such as those due to transients at the beginning of the FID, will contribute to errors in the results. The most adversely affected parameter is the CSA, given the manner in which the frequency origin is determined.

^{19}F NMR spectra have been obtained from DMPC samples selectively labeled with a difluoromethylene group in the 4-, 8-, or 12-position of the 2-acyl chain, over a range of temperatures above their phase transition temperatures as shown in Figure 2. The values determined for $\Delta\sigma$ and S_{FF} by de-Paking were then used to simulate the spectra and led to well-fitting simulations as can be seen in Figure 1 (see also Figure 5).

The decrease in S_{FF} values observed in Figure 3 with increasing temperature is consistent with there being less order and greater mobility of the probe, which means that the distance between the chains increases and/or the probability of gauche conformations is greater. There is only a small difference in the slopes of the S_{FF} values for the 4- and 8-position of the acyl chain even though the phase transition is 26 °C for 2-[4,4-$^{19}F_2$]DMPC and 14 °C for 2-[8,8-$^{19}F_2$]-DMPC as determined by differential scanning calorimetry (J. M. Sturtevant, personal communication). A similar slight difference in the slope of the S_{CD} order parameter values is observed between the 4- and 8-position of DMPC labeled with deuterium (Oldfield et al., 1978). This implies that the portion of the acyl chain encompassing these positions experiences similar motions, though the 4,4-difluoro seems to be closer in behavior to the 8,8-difluoro than the 4,4-dideuterio is to the 8,8-dideuterio isomer.

The S_{FF} values obtained for the 12,12-difluoro-labeled lipid are considerably smaller than those for the middle chain segments, which is consistent with greater motion toward the end of the chain as reported in a number of other studies [see, for example, Hubbell & McConnell (1971), Seelig & Seelig (1974), Stockton et al. (1976), and Oldfield et al. (1978)].

Examination of Figure 3 shows that the values of S_{FF} parallel the S_{CD} values quite well as the temperature is changed, but are always somewhat smaller than S_{CD} at the same reduced temperature. For the 8,8-labeled isomers, S_{FF} is 15% smaller than S_{CD} at the same reduced temperature, T_R = 45 (around room temperature). Earlier, Higgs & McKay (1977) found that the order parameter, S_{HH}, for an acyl chain CH_2 group should be very close in value to S_{CD}. Similar results have been reported by Peterson & Chan (1977), who, on the basis of computer simulations of the chain motions, found that the acyl chain S_{HH} can be up to 15% smaller than the corresponding S_{CD}. This result compares very well with ours, suggesting that the perturbation introduced by the bulkier CF_2 group cannot be significant.

To investigate the behavior of the ^{19}F-labeled phospholipids in the presence of cholesterol, samples for each positional

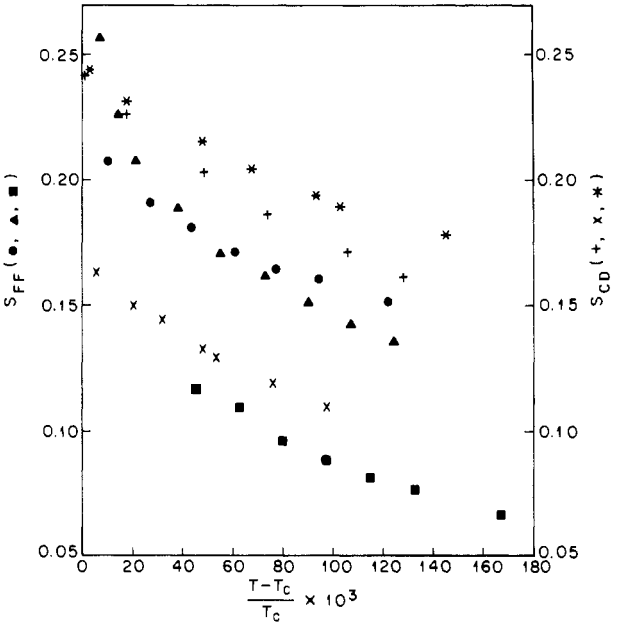

FIGURE 3: A comparison of S_{FF} and S_{CD} of phospholipids as a function of reduced temperature (T_R): (●) 2-[4,4-$^{19}F_2$]DMPC in D_2O; (▲) 2-[8,8-$^{19}F_2$]DMPC in D_2O; (■) 2-[12,12-$^{19}F_2$]DMPC in D_2O; (*) 2-[4,4-$^2H_2$]DMPC in H_2O; (+) 2-[8,8-$^2H_2$]DMPC in H_2O; (×) 2-[12,12-$^2H_2$]DMPC in H_2O. ^{19}F-Labeled phospholipids were dispersed in H_2O or D_2O (30:70). The data for S_{CD} were taken from Oldfield et al. (1978).

isomer were prepared with various mole percentages of cholesterol. The presence of larger amounts of cholesterol broadens the transition temperature region until a transition is no longer seen. As the spectra gradually broaden with lower temperatures, they preserve the powder-pattern line shape, until at temperatures lower than −20 °C, they become virtually indistinguishable from the spectrum of solid fluorinated myristic acid (results not shown). Figure 4 shows the S_{FF} and CSA values for the three isomers alone and with various amounts of cholesterol over a temperature range from −8 to +62 °C. In all cases, the cholesterol molecule restricts the mobility of the chains, as shown by the increased values of S_{FF} and of the CSA, but does not impede the fast motions responsible for the axial symmetry of the ^{19}F–^{19}F interactions. These results are similar to those for ^2H-labeled DMPC with cholesterol (Stockton et al., 1976; Oldfield et al., 1978; Jacobs & Oldfield, 1979) and nitroxide spin-labeled phospholipids (Shimshick & McConnell, 1973) in that an ordering effect on the acyl chains and a large smoothing out of the phase transition are observed. As illustrated by Figure 5, the powder-pattern line shape or bilayer structure is maintained to at least −3 °C for the 4,4-difluoro isomer with 35 mol % cholesterol. Deconvoluting and fitting of this spectrum give an S_{FF} order parameter of 0.46, indicating an almost all-trans configuration, and an effective chemical shift anisotropy of −158 ppm. Similar values were obtained for the 8,8-difluoro and 12,12-difluoro isomers.

The measured anisotropy of the ^{19}F chemical shift has the same dependence on temperature as the order parameter, S_{FF} (see Figure 4). A graphical representation of the CSA vs. the order parameter, S_{FF} (Figure 6), demonstrates an excellent linear correlation. A least-squares fit gives a value of 166 ± 6 ppm for the anisotropy of the axially symmetric chemical shift tensor of an isolated CF_2 group in an acyl chain having a rigid all-trans configuration and undergoing fast reorientations around the axis of the chain, which is assumed to be parallel to the magnetic field. It is interesting to note that the extrapolated value of −166 ± 6 ppm is very close to a CSA

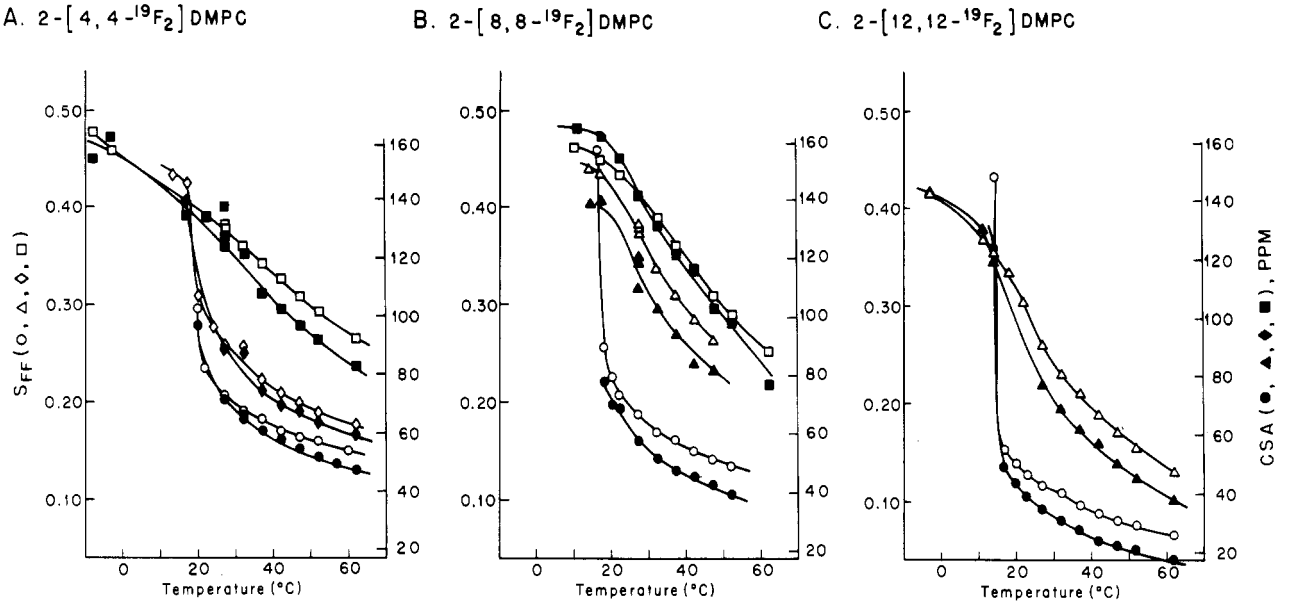

FIGURE 4: Effects of cholesterol on S_{FF} and CSA of the CF_2 group of ^{19}F-labeled phospholipids dispersed in H_2O (30:70) as a function of temperature: (A) 2-[4,4-^{19}F]DMPC; (B) 2-[8,8-$^{19}F_2$]DMPC; (C) 2-[12,12-$^{19}F_2$]DMPC. The open symbols represent S_{FF} and solid symbols represent CSA values: (○, ●) no cholesterol; (◇, ◆) 10 mol % cholesterol; (△, ▲) 23.6 mol % cholesterol; (□, ■) 35 mol % cholesterol. The ordinate scales have been slightly offset to avoid superposition of S_{FF} and CSA symbols.

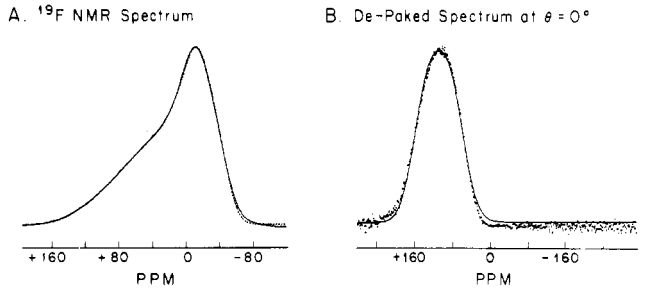

FIGURE 5: 282.4-MHz ^{19}F spectra of 2-[4,4-$^{19}F_2$]DMPC plus 35 mol % cholesterol at −3 °C: (A) experimental (—) and simulated (- - -) powder spectrum; (B) spectrum de-Paked at $\theta = 0°$ (···) and fitted with a Gaussian doublet (—).

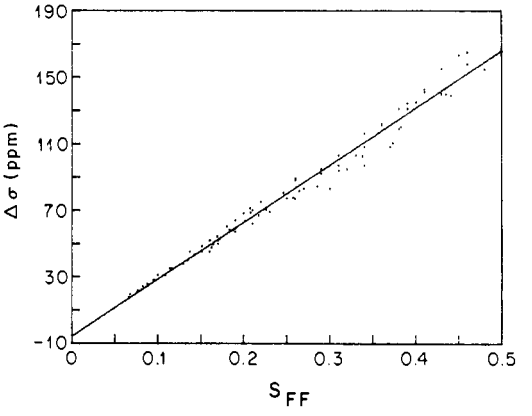

FIGURE 6: Effective value of the anisotropy of the motionally averaged axially symmetric ^{19}F chemical shift tensor ($\Delta\sigma$) vs. the S_{FF} order parameter for 2-[4,4-$^{19}F_2$]DMPC, 2-[8,8-$^{19}F_2$]DMPC, and 2-[12,12-$^{19}F_2$]DMPC. The points represent the results for all the measurements of S_{FF} and CSA shown in Figure 4. The equation of the straight line results in $\Delta\sigma = (-5.6 \pm 0.9) + (342.5 \pm 3.6)S_{FF}$.

value of −171 ± 15 ppm for the difluoromethylene group in polytetrafluoroethylene derived from broad-line NMR experiments (Wilson, 1962).

Recently, Macdonald et al. (1983) have reported a ^{19}F NMR study of monofluoro fatty acids incorporated into *Acholeplasma laidlawii* B membranes. In their determination of the relevant order parameter, S_{mol}, the authors had to estimate the value of the ^{19}F CSA for the monofluoromethylene group from the available data on the CF_2 group. Using the static shielding tensor elements for Teflon (Mehring et al., 1971), they derived a value of −82.2 ppm for the CSA in the liquid-crystalline bilayer.[3] However, this value is dependent on the particular orientation chosen for the static shielding tensor with respect to the molecular frame. For example, if the most shielded component is chosen to be parallel to the molecular axis [as in Post et al. (1982)], a CSA of −120 ppm results.

When the order parameter profile of palmitic acid enriched membranes of *A. laidlawii* B obtained with monofluorinated palmitic acids was compared with a similar study by Stockton et al. (1977) using dideuteriopalmitic acid, though the slopes were similar, the S_{mol} for the CHF group was 50% lower for positions 5–10 of the acyl chain. The authors concluded that a smaller value for the ^{19}F CSA would provide better agreement. Unfortunately, at this point the authors misinterpreted the experimentally observed, motionally averaged CSA value of −64 ppm for 2-[8,8-$^{19}F_2$]DMPC and −58 ppm for its tetradeuterated isomer at 27 °C, which we reported for oriented samples (Engelsberg et al., 1982), as being maximum CSA values as defined in their own paper. Since these values are clearly not maximum values, it would appear that a determination of the CSA for each individual system is necessary.

In conclusion, the line-shape analysis of ^{19}F-labeled phospholipids has been extended successfully to additional acyl chain ^{19}F-labeled isomers and to mixtures with cholesterol. The method of de-Paking the powder-pattern spectra has been found to be very convenient in obtaining the values of the order parameter, S_{FF}, and, in conjunction with line-shape simulation, of the CSA. Using cholesterol mixtures and lower temperatures, we have obtained an estimate of the maximum CSA without needing to know the elements and orientation of the static shielding tensor. Measurements performed on the three fluorinated isomers using the Carr–Purcell–Meiboom–Gill

[3] Gent & Ho (1978) reported a value of 118 ppm for the ^{19}F CSA of a CF_2 group in a phospholipid using the same coordinate system. The earlier calculations when corrected for a mathematical error give a value of 82.2 ppm as reported by Macdonald et al. (1983).

(CPMG) multiple-pulse sequence (Post et al., 1984) yielded S_{FF} values in excellent agreement with those reported here, demonstrating the accuracy and reliability of both methods. The utility of incorporating a difluoromethylene group into the acyl chain of a lipid lies in the ability to determine easily the S_{FF} values by either de-Paking the normal spectrum or by a multiple-pulse technique such as the CPMG pulse sequence. The linear correlation found between S_{FF} and CSA reinforces an earlier assumption that the ^{19}F chemical shift and dipolar intractions scale in the same manner (Gent & Ho, 1978). The new data presented in this work offer a better understanding of the phospholipid bilayers and prove the usefulness of fluorine as a probe for their study.

Acknowledgments

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Registry No. DMPC, 18194-24-6; cholesterol, 57-88-5.

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