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Deuterium NMR of Specifically Deuterated Fluorine Spin Probes*

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Deuterium nuclear magnetic resonance spectra (at 55.3 MHz) have been obtained of ^{19}F-^{2}H double-labeled phospholipids in pure lipid bilayers, and of ^{2}H-labeled lipid in a ^{19}F-labeled bilayer, as a function of concentration, to assess the perturbing influence of ^{19}F sites in lipid hydrocarbon chains. Order parameters of ^{2}H-labeled sites adjacent to C-8 myristic fluorine probes in pure lipid bilayers, and ^{19}F spin label order parameters themselves, are about 30% lower than those deduced from the use of nonperturbing ^{2}H probes. The effect is intramolecular rather than intermolecular and presumably represents increased *gauche* states due to the increased size of the ^{19}F label. This effect is consistent with the view that difluoromethylene fatty acyl chains function in a manner approximating that of unsaturated fatty acyl chains. The differences disappear in the presence of cholesterol at very high order parameters (S_{mol} ~ 0.8 to 0.9). These results represent the first attempt at elucidating the perturbing effects of a high sensitivity probe (^{19}F) and indicate that caution must be used when using spectroscopic probes to deduce the absolute magnitude of hydrocarbon chain order parameters.

There is currently a good deal of interest in applying a variety of physical methods to the determination of the structure of model and biological membranes (1–9). High sensitivity techniques, such as fluorescence (6, 10) and electron spin resonance spin-labeling (11, 12), have proven to be particularly popular and productive, while in the area of nuclear magnetic resonance spectroscopy, the highly sensitive NMR spin label fluorine-19 has also given interesting information on membrane structure (13–18). While the question of the possible "perturbing" effects of such high sensitivity probes has been frequently raised, there have, however, been no quantitative estimates of these effects. In many instances, for example in the case of probes used to determine phase diagrams (15, 19, 20), whether or not the probe influences the order (or motion) of its surroundings is irrelevant if the correct answer, in this case the phase diagram, is produced. For example, use of the nitroxide 2,2,6,6-tetramethylpiperidinoxy to determine lipid phase diagrams gives correct results due to the probes' preferential solubility in fluid bilayer regions, and whether lipid order is affected is of no consequence. On the other hand, the use of nitroxide-labeled fatty acids as probes of bilayer order must assume that neither intermolecular nor intramolecular perturbations are caused by the spin-labeled site, or at least they must always cancel. The problem of the existence of such perturbations may, in principle, be probed using a variety of spectroscopic techniques, but there are considerable problems associated with spectroscopic comparisons when widely different time scales must be employed (7, 21–23), and it is not clear whether more questions are answered or raised when such comparisons are attempted.

In this communication, we present results relatively free of such time scale difficulties which are relevant to the use of ^{19}F as a probe of molecular order in lipid bilayer systems. We have chemically incorporated ^{2}H nuclei into a ^{19}F-labeled phospholipid nuclear spin label and have recorded ^{2}H NMR spectra at sites close to and far removed from the ^{19}F-labeled site, both in the absence and presence of cholesterol, to assess intramolecular order perturbations, and for non-fluorine-labeled material as a function of fluorine probe concentration, to assess possible intermolecular perturbations. It is shown that ^{19}F effects are mainly intramolecular and, while significant, are not overwhelming.

EXPERIMENTAL PROCEDURES

[2,2,7,7,9,9-$^{2}H_6$-8,8-$^{19}F_2$] Tetradecanoic acid was prepared using a procedure similar to that of Prestegard and Grant (24), by ^{2}H exchange of 8-keto methyl myristate, followed by fluorination using MoF_6/BF_3 reagent. 8-Keto methyl myristate was prepared by the procedure of Cason and Prout (25), and was then deuterated at positions 2, 7, and 9 by reaction with a small amount of sodium in excess CH_3OD (three exchanges with fresh CH_3OD) at room temperature over 24 h. Deuterated 8,8-difluoromyristic acid was then prepared by the action of MoF_6/BF_3 in CH_2Cl_2 on the corresponding deuterated ketoester according to the method of Mathey and Bensoam (26), followed by hydrolysis of the ester group using alcoholic KOH. The resulting deuterated difluoromyristic acid was found by 600 MHz ^{1}H NMR spectroscopy to be more than 90% ^{2}H-labeled at positions 7 and 9, and approximately 25% deuterated at position 2.

Fluorinated DMPC[1] was prepared by acylation of lyso-DMPC with the fluorinated fatty acid using the method of Dahlquist *et al.* (27). The product was purified on a SilicAR CC-7 column (2 × 21 cm) by first eluting with pure $CHCl_3$ until the fractions were free of a fast moving contaminant, followed successively by elution with 9:1, 5:1, and 1.3:1 $CHCl_3$:MeOH (v/v) solutions. The desired deuterated fluorolipid was eluted in the 1.3:1 fractions. A faint yellow coloration was removed by trituration with hexane. The lipid obtained was a pure white solid and gave one spot on a heavily overloaded TLC plate ($CHCl_3$:MeOH:7 M NH_4OH, 230:90:15, v/v), using molybdenum phosphate-stain reagent (28).

Spectra were recorded on a "home-built" Fourier transform NMR spectrometer which consists of an 8.5 Tesla 3.5-inch bore high resolution superconducting solenoid (Oxford Instruments, Osney Mead,

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[1] The abbreviations used are: DF-DMPC, 1-myristoyl-2-[2′,2′,7′,7′,9′,9′-$^{2}H_6$-8′,8′-$^{19}F_2$]myristoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine.

Oxford, England) together with a variety of digital and radiofrequency electronics and a 400 kHz data acquisition system equipped with dual discs[2]. Spectra were recorded using an 800-μl sample volume and a quadrupole echo (29) pulse sequence, using a 90° pulse-width of between 3.5 and 7 μs. The spectrometer zero frequency was established using a 1% D_2O reference, the zero frequency for the lipid samples investigated being set at about 2 ppm upfield from this position. The deuterium resonance frequency was 55.273 MHz. Spectral simulations were carried out on the University of Illinois Digital Computer Laboratory's Control Data Corporation Cyber-175 system, which is interfaced to a Tektronix 4006 graphics terminal and interactive digital plotter (Tektronix, Beaverton, OR) in our laboratory, basically as described elsewhere (30). Samples for NMR spectroscopy were prepared as described previously (30).

RESULTS AND DISCUSSION

We show in Fig. 1 the 55.3 MHz ^2H NMR spectra of 1-myristoyl-2-[2′,2′,7′,7′,9′,9′-$^2H_6$-8′,8′-$^{19}F_2$]myristoyl-*sn*-glycero-3-phosphocholine in the presence and absence of unlabeled 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, at 33°C and in excess ^2H-depleted water. Note that the DF-DMPC contains ~ 25% ^2H at position 2′ but ~ 90% ^2H at positions C-7 and C-9.

The spectra of Fig. 1 contain two main components. The two intense outer resonances (marked *a* and *b* in Fig. 1*A*) are attributed to deuterons at C-7 and C-9, adjacent to the fluorine-label site at C-8. This assignment is based on the similarity in quadrupole splitting with that found for a C-8-labeled DMPC in its liquid crystal phase. The gel to liquid crystal phase transition temperature (T_c) for 1-myristoyl-2-[8′,8′-$^{19}F_2$]myristoyl-*sn*-glycero-3-phosphocholine is ~12°C[3] so the DF-DMPC of Fig. 1 is some 21°C above T_c. The two main splittings observed are 16 and 20 kHz (Fig. 1*A*). For [8′,8′$^2H_2$]-DMPC at the same reduced temperature (T_R = 0.07; T = 44°C), we have shown previously that $\Delta\nu_Q$ ~24 kHz (31). On the basis of quadrupole splittings and relative intensity with respect to the small features having the smaller splittings in Fig. 1*A*, the $\Delta\nu_Q$ = 16 kHz resonance is therefore assigned to C-9 and that having $\Delta\nu_Q$ ~20 kHz is assigned to C-7. These assignments are tentative, but it is not necessary to know them on a one-to-one basis for this study. The isotropic peak (*e*) at zero frequency arises from HO^{2}H, so the small shoulder at *c* (more clearly defined in Fig. 1*C*) and peak at *d* are therefore assigned to position 2 deuterons, but again not on a one-to-one basis.

The quadrupole splittings of position 7 and 9 deuterons are essentially independent of fluorolipid concentration in DMPC over the range 100% to 10 mol % DF-DMPC, being 20.5 ± 1 and 16 ± 1 kHz, respectively.

For purposes of comparison, we assume that the infinite dilution values of $\Delta\nu_Q$ for DF-DMPC in DMPC are the relevant spectral parameters with which to compare labeled and nonlabeled lipids. These values are ~21 ± 1 kHz (C-7) and ~16.5 ± 1 kHz (C-9). For [8′,8′-$^2H_2$]DMPC at 33°C, $\Delta\nu_Q$ = 27 kHz (C-8). Since odd-even alternations in $\Delta\nu_Q$ have not been observed in the analogous 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (32) or in ^2H-labeled *Acholeplasma laidlawii* B membranes (33), it is almost certain that the most appropriate estimate of the order parameter for C-8 in the fluorolipid, based on the C7 and C9 data, would be S_{mol} ~0.30. The corresponding value for DMPC at the same temperature is S_{mol} ~0.43.

These results strongly suggest, but do not prove, that $^{19}F_2$ label incorporation at position 8 of the myristic acid hydrocarbon chain causes a perturbation such as to increase the probability of *gauche* conformers occurring in this region of

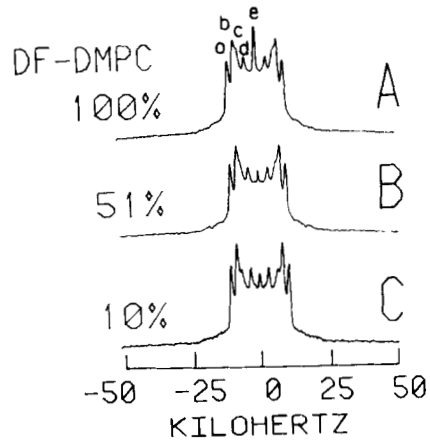

FIG. 1. **Deuterium Fourier transform NMR spectra of 1-myristoyl-2-[2′,2′,7′,7′,9′,9′-$^2H_6$-8′,8′-$^{19}F_2$]myristoyl-*sn*-glycero-3-phosphocholine-1,2-dimyristoyl-*sn*-glycero-3-phosphocholine mixed bilayers, at 33°C in excess ^2H-depleted H_2O.** The mole percentage of DF-DMPC in the bilayer is shown. Spectra were recorded at 55.3 MHz using a two-pulse echo sequence, spectrometer conditions being typically 100 kHz spectral width, 65 ms recycle time, 90° pulse widths = 9 μs, $\tau_1 = \tau_2 = 50$ μs, 4,096 data points, 100-Hz line broadening, and between 56,000 and 125,000 scans. Quadrupole splittings were obtained from spectral simulations which used a 400-Hz linewidth and a 30% deuteration of the 2′ segment relative to either C-7′ or C-9′.

the molecule, leading to decreases in $\Delta\nu_Q$ (34). In addition, a variety of other experimental evidence may be brought forth to support the notion that the F_2 label incorporation at C-8 decreases lipid order at the site of incorporation by ≈30%. For example, the average S_{mol} of position 8 of 8,8-difluoromyristate in a variety of systems (15, 16) is S_{mol} ~0.3, while that determined in a similar series of experiments but using ^2H NMR probes (33, 34) is S_{mol} ~0.45. More specifically, 8,8-F_2-myristate probe ^{19}F NMR-determined values of S_{mol} in egg yolk lecithin yield S_{mol} ~0.28 at 37° (16), while a value of S_{mol} ~0.46 is deduced when using a stearic acid probe (35, 36). It is likely, however, that this latter result is somewhat too high due to the condensing effects of the large amount of fatty acid probe used in these studies (23, 35, 36), a more accurate value, obtained for 1-chain deuterated 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine being S_{mol} ~0.4 (23). However, in both cases we find that the ^{19}F NMR order parameters are smaller than those determined on similar systems using ^2H NMR, a result consistent with the decreased S_{mol} values determined by ^2H NMR with the ^{19}F-^2H double-labeled DMPC system. Time scale problems (21–23) associated with comparing results from the different ^{19}F NMR and ^2H NMR resonance techniques are expected to be very small, and will clearly be insignificant compared with the difficulties associated with comparing ESR and ^2H NMR results (21–23), and in any case the ^2H NMR data on the ^{19}F-labeled lipid (Fig. 1) are independent of this type of complicating factor.

The resonances labeled as *c* and *d* in Fig 1*A*, which presumably arise from the 2-α and β deuterons, also show significant differences in quadrupole splitting compared with nonlabeled lipid. Infinite dilution values of $\Delta\nu_Q$ = 13 ± 1 and 6 ± 1 kHz may be determined from the results of Fig. 1 (and additional unpublished spectra at different mole fractions of F label), compared with the values $\Delta\nu_Q$ = 18 ± 1 and 12 ± 1 kHz obtained on 2′-labeled DMPC (at 33°C, in excess water, Ref. 31). These values are apparently in poor agreement, but as has been pointed out previously (37), even relatively large changes in $\Delta\nu_Q$ at position 2′ of the 2-chain may be due to small changes in average conformation due to the bent nature of the C-2′ segment. If the generally small $\Delta\nu_Q$'s observed at

[2] R. Jacobs, C. Reiner, and E. Oldfield, unpublished results.
[3] J. M. Sturtevant and C. Ho, unpublished results.

the C-2′ segment are due to a "magic-angle" effect, *i.e.* due to the C-^{2}H vectors being close to 54.7°, such that $(3\cos^2\theta - 1) \rightarrow 0$, then clearly very small changes in θ may cause large changes in $\Delta\nu_Q$ (37, 38).

The results presented above strongly suggest that the primary effect of ^{19}F substitution in the lipid hydrocarbon chain is intramolecular, causing a decrease in local order. These results are in agreement with those of Gent *et al.* (16), who have found that 8,8-F_2-labeled myristic acid readily incorporates into the phospholipids of an unsaturated fatty acid auxotroph of *Escherichia coli*. These results strongly suggest that the bacteria respond to the presence of a difluoromyristic acid in a manner similar to their response to a *cis* double bond containing fatty acid. If this view of a principally intramolecular effect is correct, then one must predict that the order of a specifically deuterated nonfluorine-labeled DMPC when determined at infinite dilution in a F-DMPC bilayer should be identical with that of the pure nonlabeled material determined at the appropriate reduced temperature, *i.e.* one related to the fluorolipid phase transition temperature.

We show in Fig. 2, ^{2}H Fourier transform NMR spectra, and their computer simulations, of DMPC labeled as $^2H_2$ at the C-8′ position of the 2-chain, as a function of concentration in a DF-DMPC bilayer. An additional point taken using 2-[8′,8′-F_2]DMPC as diluent is not shown. The results of Fig. 2 are tractable using computer simulation techniques, since the mole fractions and deuterium concentrations of each species are known. The ^{2}H resonance of 2-[8′,8′-$^2H_2$]DMPC is labeled as *A* in Fig. 2*B* (and its simulation) to illustrate that the DMPC resonance may be discerned relatively easily even in a complex spectrum. In Fig. 2*B*, *a* and *b* are the ^{2}H resonances of C-7 and C-9, and have the same quadrupole splittings as determined for *a* and *b* in Fig. 1 (20 ± 1, 16 ± 1 kHz). The *shoulder c* and *peak d* arise from the 2′ resonance (~25% labeled) and *e* arises from HO^{2}H. The results of Fig. 2 (and additional data using nondeuterated fluorinated lipid) give a value for the ^{2}H quadrupole splitting of 2-[8′,8′-$^2H_2$]DMPC, at infinite dilution in F-DMPC, of $\Delta\nu_Q$ ~24 ± 1 kHz. This result is in moderate agreement with the experimentally determined value $\Delta\nu_Q$ 27 ± 1 kHz for DMPC at 33°C, but is in excellent agreement with the value of 24 ± 1 kHz determined at 43°C (31). This temperature corresponds to the same reduced temperature (T_R = 0.069) for DMPC as F-DMPC at 33°C (the temperature of data acquisition) since as noted previously, the gel to liquid crystal phase transition temperature of 2-[8′,8′-F_2]DMPC is 12°C.[3]

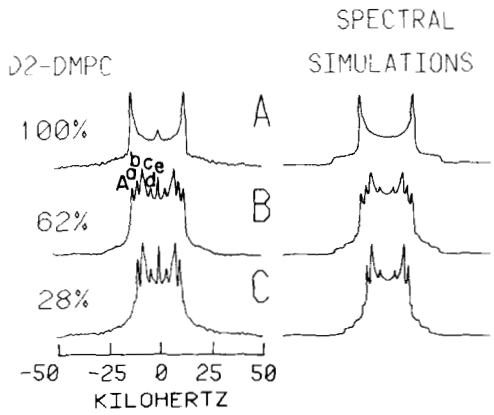

FIG. 2. **Deuterium Fourier transform NMR of 2-[8′,8′-$^2H_2$]-DMPC in the presence and absence of DF-DMPC.** Mixed bilayers were dispersed in ^{2}H-depleted H_2O, and spectra were recorded at 55.3 MHz and 33°C. The mole percentage of [^{2}H]DMPC in the bilayer is indicated. Spectral conditions were basically as in Fig. 1. Spectral simulations utilized the known mole fractions of lipids and their percentage deuteration, together with line broadenings (w) of ~600 Hz. Quadrupole splittings were as follows: *A*, $\Delta\nu_Q$ = 26.3 kHz; *B*, $\Delta\nu_Q$ = 25.3, 20.5, 16.0, 14.0, 7.0 kHz; C, $\Delta\nu_Q$ = 23.6, 20.5, 16.0, 14.0, and 7.0 kHz.

The observation that deuterated DMPC, when observed at infinite dilution in fluorinated DMPC (labeled as F_2 at the same position as the DMPC is deuterated), at the appropriate reduced temperature has the same quadrupole splitting as in a pure deuterated DMPC bilayer, supports (but does not prove) the idea that ^{19}F incorporation into the lipid causes principally an increase in *gauche* conformers near the position of label incorporation, with relatively little intermolecular effect on lipid organization, when corrected for possible changes in the phase transition temperature. Certainly, the intermolecular effects are not measurable using our techniques.

An additional prediction we may make is that any ^{19}F perturbation will be less "important" as the *all-trans* state is achieved, that is to say, order parameters will be relatively closer in the presence and absence of the ^{19}F label at high S_{mol}. This follows from the $P_2(\cos\theta)$ dependence of the quadrupole splitting (or order parameter) (31, 39) and is borne out experimentally.

Naturally occurring membranes usually contain unsaturated fatty acyl chains in position 2 of the glycerol backbone (40). Differential scanning calorimetric results[3] show that the T_c for 2-[8′,8′-F_2]DMPC of 12°C, as previously noted, is 11° less than the T_c of 23°C for DMPC, while Barton and Gunstone (41) have shown a difference of 58° for 1-octadecanoyl-2Δ^9-octadec-*cis*-enoyl-*sn*-glycero-3-phosphorylcholine compared to the saturated C-18 compound. The effect of introducing a *cis* double bond in the 2-acyl chain seems to be to perturb the bilayer in a manner analogous to introducing a difluoromethylene group, but the effect of the former is greater than that of the latter.

We have obtained ^{2}H NMR spectra of DF-DMPC and normal ^{2}H-labeled DMPC in the presence of equimolar quantities of cholesterol, at 33°C and in excess water, and typical results are shown in Fig. 3. As may be seen by comparison of Fig. 3, *A* and *B*, addition of cholesterol increases the $\Delta\nu_Q$ of both ^{19}F-labeled and nonlabeled DMPC. We obtain for 2-[8′,8′-$^2H_2$]DMPC cholesterol (1:1) $\Delta\nu_Q$ ~54 ± 1 kHz and for DF-DMPC $\Delta\nu_Q$ = 51, 28, and 15 kHz. The feature *a* and *b* in

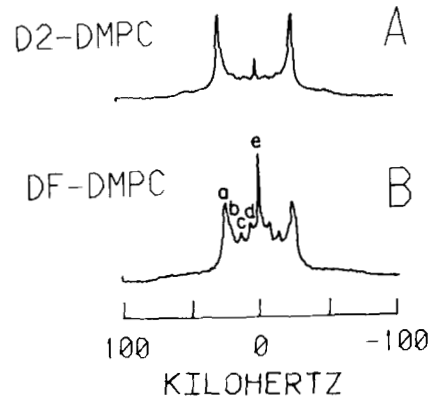

FIG. 3. **Deuterium Fourier transform NMR spectra of 2-[8′,8′-$^2H_2$]DMPC/cholesterol (1:1) and DF-DMPC/cholesterol (1:1) bilayers in excess ^{2}H-depleted water at 33°C.** Spectral conditions were 200 kHz spectral width, 0.4 s recycle time, 4K data points, 3 μs 90° pulse widths, $\tau_1 = \tau_2$ = 50 μs, 400 Hz line broadening, and 91K (*A*) or 93K (*B*) scans. Spectral stimulations yielded $\Delta\nu_Q$ (*A*) = 53.8 kHz and $\Delta\nu_Q$ (*B*) = 51.0, 27.5, and 15.0 ± 1 kHz. The spectral simulation linewidths were about 1000 Hz.

Fig. 3*B* arises from the C-7 and C-9 deuterons which have in this case the same $\Delta\nu_Q = 51 \pm 1$ kHz, obtained by spectral simulation. This value is in very good agreement with the value $\Delta\nu_Q \sim 54 \pm 1$ kHz for 2-[8′,8′-$^2H_2$]DMPC-cholesterol (Fig. 3*A*). We have not attempted to take into account phase transition temperature differences between normal and ^{19}F-labeled lipid since cholesterol is known to remove this transition in both cases.[4]

As expected, at high S_{mol} the effect of the $^{19}F_2$-label perturbation is quite small. For the 2α and 2β deuterons (*c* and *d* in Fig. 3*B*), $\Delta\nu_Q$ values of 28 and 15 ± 1 kHz are obtained, in close agreement with those determined previously with deuterated 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine-cholesterol at 43°C of 29, 18 ± 1 kHz (42).

The results presented in Figs. 1 to 3 strongly suggest at intermediate degrees of ordering ($S_{mol} \approx 0.4$) that the effect of $^{19}F_2$-label incorporation, at position 8 of a myristic acid fluorine probe, will be to cause an underestimation of the degree of order by ≈30% due to an intramolecular perturbation of the acyl chain conformation by the fluorine substituent. Such effects are less significant at high order, for example, in the presence of equimolar cholesterol. Our results emphasize the need for control experiments when "non-native" spectroscopic probes are used to estimate membrane order parameters. Since fluorine represents the smallest non-native probe currently in use, the validity with which other probes report on molecular order should undoubtedly be checked using other double-labeling experiments.

[4] R. W. K. Lee and E. Oldfield, unpublished data.

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