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Effects of unsaturation on ^{2}H-NMR quadrupole splittings and ^{13}C-NMR relaxation in phospholipid bilayers

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Motional order and motional rates in unsonicated phospholipid bilayers were assessed as a function of unsaturation of the phospholipid. A measurement sensitive to motional order was obtained using ^{2}H-NMR of 18:1, 18:1-phosphatidylcholine labelled at positions 9 and 10 with deuterium and included as a probe in phospholipid bilayers of interest at 10 mole percent. Spin lattice relaxation times from magic angle spinning ^{13}C-NMR spectra of phospholipid dispersions of interest were used as a measure of motional rates. Measurements were made of phospholipid bilayers containing from 0 to 8 double bonds per molecule. No large effect of an increase in unsaturation was noted for the ^{2}H-NMR quadrupole splittings or for the ^{13}C-NMR spin lattice relaxation rate.

Introduction

Unsaturation of membrane lipid hydrocarbon chains is a ubiquitous structural element of biological membranes. Fatty acids containing from one carbon-carbon double bond to six carbon-carbon double bonds per molecule are contained in membrane lipids. For example, the bovine rod outer segment disk membrane contains in excess of 40 mole percent 22:6 in its membrane lipids. What roles such highly unsaturated membrane lipids might play in cell membrane structure and function is not known.

A common hypothesis is that unsaturation increases membrane 'fluidity' because the gel-to-liquid crystal phase transition temperature is reduced by the addition of a double bond to a saturated hydrocarbon chain. The well-defined parameters that might relate to this hypothesis are membrane lipid motional order and motional rates (i.e., rate of *gauch-trans* isomerizations about carbon-carbon bonds). This study is designed to test whether unsaturation in a phospholipid bilayer causes a significant decrease in membrane order and whether unsaturation has any significant effect on the rates of carbon-carbon bond rotation in the phospholipid hydrocarbon chains in the bilayer.

Materials and Methods

14 : 0, 14 : 0-Phosphatidylcholine, 16 : 0, 16 : 0-phosphatidylcholine, 16 : 0, 18 : 1-phosphatidylcholine, 18 : 1, 18 : 1-phosphatidylcholine, 18 : 2, 18 : 2-phosphatidylcholine, 20 : 4, 20 : 4-phosphatidylcholine were obtained in pure form from Avanti Biochemicals. 18 : 1, 18 : 1-Phosphatidylcholine (*d*9,10), specifically deuterated at positions 9 and 10, was obtained from Cambridge

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Isotope Laboratories. Deuterium-depleted water was obtained from Aldrich.

Phospholipid dispersions were made by drying the lipid into a thin film out of chloroform, having mixed lipids in chloroform. The thin film was hydrated in 10 mM histidine (pH 7)/1 mM EDTA buffer, and dispersed with limited vortexing. For the ^{1}H-NMR measurements, the sample was hydrated in a buffer made from deuterium-depleted water and subsequently introduced into a 10 mm NMR tube under argon.

^{2}H-NMR measurements were obtained at 41.4 MHz on a JEOL FX270 multinuclear NMR spectrometer, in the quadrupole echo mode. A sweep width of 50 kHz was used and 2K data points were collected in the time domain. Free induction decyas were transformed from the top of the echo.

The ^{13}C-NMR spectra were obtained on a Nicolet NT-150 spectrometer at a carbon frequency of 37.735 MHz with a homebuilt magic-angle spinning unit, including the probe. The decoupling field was 45 kHz. The spinner system is a modified version of Wind et al. (1983), with a sample volume of 0.3 cm^3. The samples were spun at 3200 rps. 4K points were collected in the Block-decay mode (pulse and acquire) with a spectral width of 20 kHz and an acquisition time of 104 ms. The repetition time was 5 s. Chemical shifts are relative to external tetramethylsilane, with hexamethylbenzene as a secondary standard (methyl signal at 17.35 ppm).

A lyophilized lipid sample was dispersed in 10 mM histidine buffer (pH 7.0) and transferred to a Teflon sleeve. A simicone rubber plug was inserted into the sleeve, which was then pressed into the Kel-F rotor body. Nonviscous air-sensitive liquids have been spun with this arrangement with no liquid leaking out and no air linking in. The rotor was spun with dry nitrogen gas which had passed through a heat-exchange coil immersed in an ice bath. The sample temperature was 15°C except when noted otherwise.

Longitudinal relaxation data were collected with a modified inversion-recovery pulse sequence [3] that results in peak intensities decaying exponentially to zero with increasing delay time. The experiment was modified to incorporate a composite pi pulse [4] and proton decoupling was gated on during acquisition. The cross-polarization version of this approach [5] is attractive because it suppresses contributions from the Teflon and Kel-F rotor materials, but the extreme sensitivity of these highly mobile systems to drift in the Hartmann-Hahn power level match results in unacceptable scatter in the peak intensities [6]. Delay times (12 to 20 values) were sequenced randomly and varied from 1 ms to 5 s, with typical spacing of a factor of 1.5 [7]. The peak height vs. delay time data were fitted with a nonlinear least-squares routine in the Nicolet 1180 NMR program (DEXPN), and selected sets were also treated graphically to verify the fit. The T_1 values are reliable to about $\pm 10\%$, estimated from replicate experiments and scatter within each experiment. The first and last delay times were both 1 ms in order to check for drift in spectrometer performance or change in the sample; no significant changes were detected.

Results and Discussion

Unsaturation and motional order

The purpose of this study was to determine the effects of unsaturation in phospholipid hydrocarbon chains on the motional order and motional rates experienced by the phospholipid hydrocarbon chains in the lamellar phase. To examine motional order, ^{2}H-NMR spectra of specifically deuterated lipids was employed. In anisotropic media, such as a membrane, the quadrupolar interaction between the electric field gradient and the deuterium nucleus is manifest in the ^{2}H-NMR spectra as a quadrupole splitting. Three such splittings are observed from the 18:1,18:1-phosphatidylcholine (*d*9,10) used here. As reported previously [8], two of these splittings arise from position 10 on each of the two chains and one of the splittings arises from position 9.

The quadrupole splittings observed are related directly to the motional order characterizing the segment to which the deuteriums are attached.

$$D_q = \tfrac{3}{4}(e^2qQ/h)S_{C^2H}$$

However, the determination of the order parameter characterizing the labelled segment is not that simple. There is also an orientational parameter which scales the observed order parameter, related to the angle θ between the C-^{2}H

bond and the director characterizing the rotation of the hydrocarbon chain as a whole. In this case, the observed quadrupole splittings are scaled by the factor $(3\cos^2\theta - 1)$. This scaling is in addition to the scaling by the order parameter. Such an orientational effect is most prominent in the ^2H resonance from position 10, which results in small quadrupole splittings [8]. This concept has been reinforced by a more analytical approach to the cyclopropane substituent on the chain. Here the orientation problem can be solved more precisely. It was subsequently shown that, although the ^2H quadrupole splittings are small, the order parameter is not dramatically reduced. Rather there is a dominant orientational effect [11]. Therefore, the ^2H-NMR resonance from position 9 was employed to reflect any significant changes in motional order.

We employed the (9,10-^2H)-labelled phospholipid as a probe for three reasons. One is that the most important region of the bilayer to examine is the region in which the double bonds are found and where the probe is labelled. Another is that an adequate representation of the ^2H-NMR spectra of this labelled lipid is obtained with the 50 kHz sweep width of the FX270. Thirdly, using one probe for all these systems has a distinct advantage. It was necessary to study a variety of phospholipids with different chain lengths and extent of unsaturation. Using one labelled lipid in all the environments avoided the problem of adequately comparing the quadrupole splittings observed from one lipid to another each with different orientation and order parameters. Furthermore the task of synthesizing specifically labelled versions of all the phospholipids studied here was avoided. The deuterium-labelled lipid was used at 10 mole percent in all the measurements reported here.

The ^2H-NMR spectra of this deuterated phospholipid in phosphatidylcholine bilayers containing from no double bonds per molecule to 8 double bonds per molecule are presented in Fig. 1. Shorter saturated chains were not examined because of possible mismatch between the chain length of the probe and the dominant phospholipid. Fig. 1 shows a broadening of the spectra in the case of the more unsaturated phospholipids. This may reflect an increase in the intensity of low

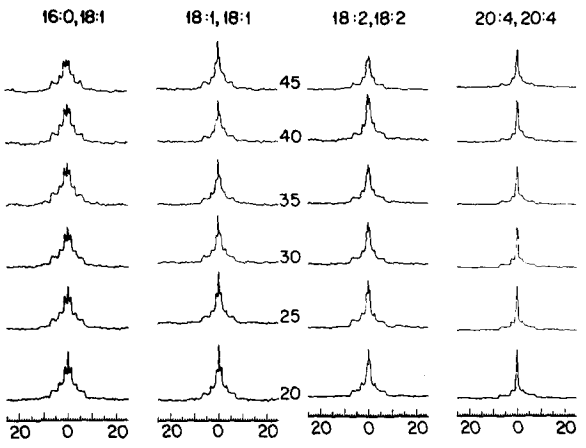

Fig. 1. 41.4 MHz ^2H-NMR spectra of 18:1,18:1-phosphatidylcholine (d9,10) incorporated at 10 mole percent into multilamellar dispersions of the indicated phosphatidylcholines as a function of temperature as indicated. Spectra were obtained in deuterium-depleted water. Total phospholipid concentration was 100 mg/ml.

frequency motions in the system from the increase in unsaturation.

The temperature dependence of the quadrupole splittings from each of the three positions for the specifically deuterated phosphatidylcholine (incorporated at 10 mole percent) in several different phospholipid dispersions are graphed in Figs. 2a–2d. A modest decrease in the quadrupole splittings with an increase in temperature is noted. This is expected because of the reduction in motional order due to an increase in temperature. Such a temperature dependence has been observed previously for this labelled lipid [9].

At any temperature, no significant decrease in the deuterium quadrupole splitting can be observed as a function of an increase in unsaturation of the phospholipids. This could possibly be due to a complete phase separation of the labelled lipid, in which case the labelled lipid is simply measuring itself. However, this is unlikely, since all the phospholipids are phosphatidylcholine, and all but one of the phospholipids are in the liquid-crystal state. Close inspection of the data reveals there may be a small increase in quadrupole splitting with increase in unsaturation. The quadrupole splitting from position 9 in 20:4,20:4-phosphatidylcholine is about 0.8–1.0 kHz larger than in 16:0,18:1-phosphatidylcholine at all tempera-

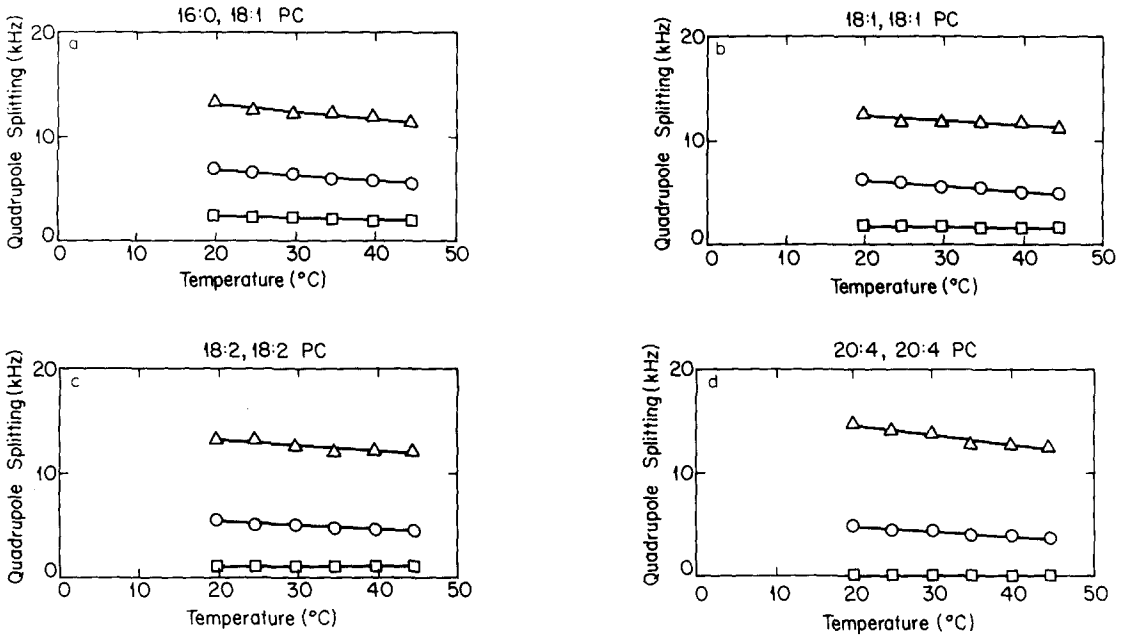

Fig. 2. Plot of quadrupole splittings as a function of temperature for the three distinguishable resonances observed in Fig. 1 for 18:1,18:1-phosphatidylcholine incorporated at 10 mole percent into (a) 16:0,18:1-phosphatidylcholine; (b) 18:1,18:1-phosphatidylcholine; (c) 18:2,18:2-phosphatidylcholine; (d) 20:4,20:4-phosphatidylcholine multilamellar dispersions. (△) is position 9; (○) is position 10 of chain 1; and (□) is position 10 of chain 2.

tures. The simplest explanation of the data is that the motional order in the region of the double bonds is not decreasing significantly as a function of a radical change in the number of double bonds per molecule. Alternatively there could be a concerted change in order and orientation to fortuitously produce a constant quadrupole splitting. This seems unlikely, but cannot be ruled out.

One other interesting observation is the effect of unsaturation on the orientation of the C-10 deuterium of chain 2. As the extent of unsaturation increases, the quadrupole splitting observed decreases, as can be seen in Fig. 1. Thus the average orientation of the C-^{2}H bond at position 10 of the probe molecule changes as a function of the unsaturation of the phospholipid matrix. In the diarachidonyl phosphatidylcholine, the splitting decreases nearly to zero. In this case, the average orientation of the C-^{2}H vector (for C-10 of the 2-chain of the labelled phosphatidylcholine) is at the magic angle with the bilayer normal.

Unsaturation and rates of motion

^{13}C-NMR spin lattice relaxation was used to assess the rates of motion of the segments of the phosphatidylcholine hydrocarbon chains, as a function of unsaturation. ^{13}C-NMR was chosen because of the ability to observe a number of

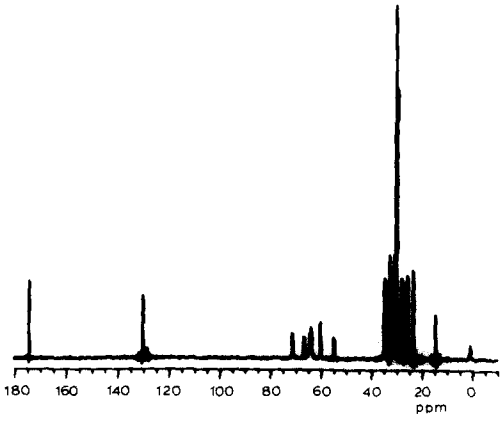

Fig. 3. Magic-angle spinning (MAS) ^{13}C-NMR spectrum of unsonicated 16:0,18:1-phosphatidylcholine dispersions 100 mg/ml, obtained at 37.7 MHz. Spinning rate was 3200 rps. A 45 kHz decoupling field (gated) was employed. A spectra width of 20 kHz was used.

positions within the phospholipid. For proper comparison with the ^{2}H-NMR measurements, ^{13}C-NMR measurements were required on the same unsonicated phospholipid dispersions. This goal required high resolution ^{13}C-NMR spectra of the unsonicated phospholipid dispersions to see the behavior of several individual carbon atoms in the lipid hydrocarbon chains.

High-resolution ^{13}C-NMR spectra of these unsonicated aqueous dispersions of phospholipids requires magic-angle spinning (MAS) of the fully hydrated samples. Normally such dispersions do not produce spectra as highly resolved as the NMR spectra obtained from sonicated vesicles due to strong dipolar interactions that increase linewidths. Rotational diffusion for sonicated vesicles is sufficiently rapid to motional average much of the dipolar interactions produce relatively high resolution resonances. As can be seen in Fig. 3, ^{13}C-MAS are obtained of greater resolution than is observed from sonicated phospholipid vesicles with normal ^{13}C-NMR [10], as has been reported previously [12,13].

With the high resolution seen in Fig. 3, T_1 for individual carbon atoms on the phospholipids can be measured. This relaxation parameter is then scaled by the number of hydrogen atoms attached to the carbon in question, to produce NT_1, since

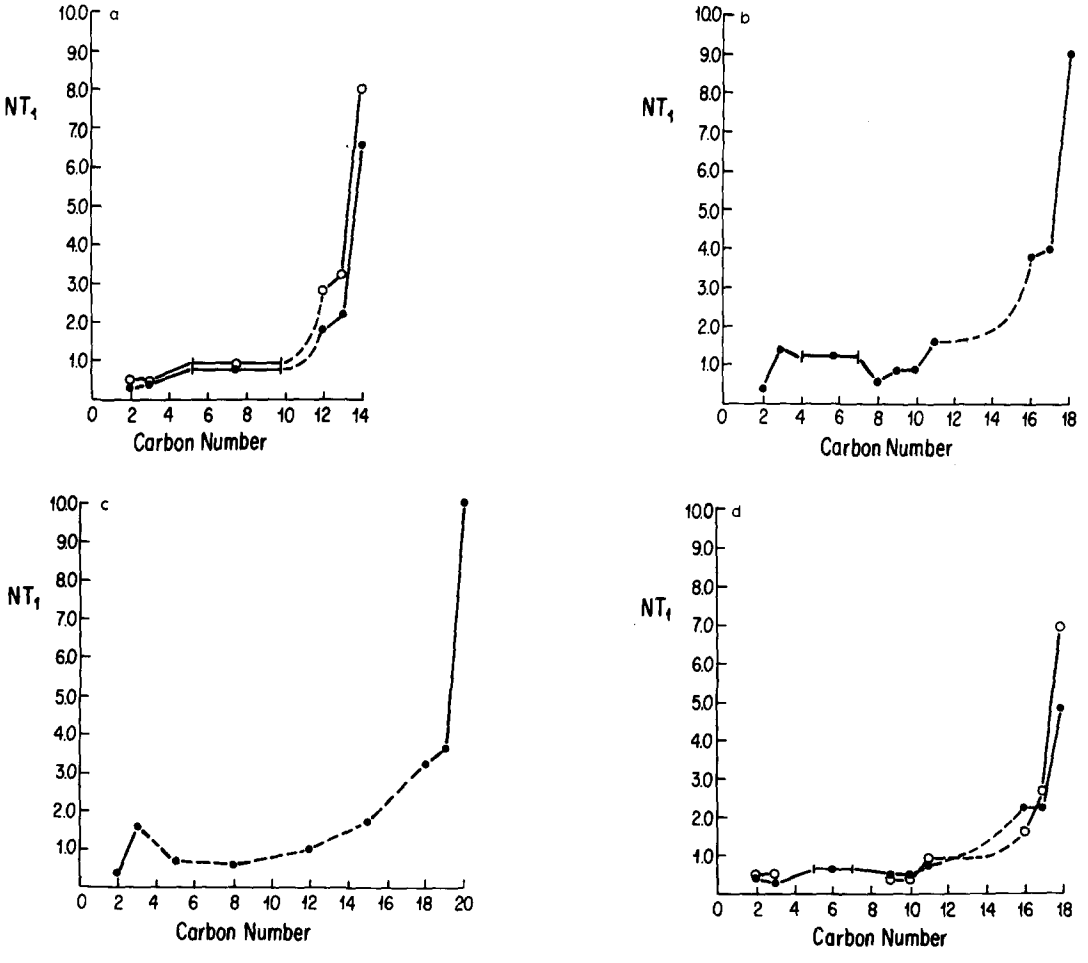

Fig. 4. T_1 relaxation values obtained from magic-angle spinning (MAS) ^{13}C-NMR spectra of unsonicated dispersions of phosphatidylcholines. (a) 14:0,14:0-phosphatidylcholine at (●) 15°C and (○) 25°C. (b) 18:2,18:2-phosphatidylcholine. (c) 20:4,20:4-phosphatidylcholine. (d) 16:0,18:1-phosphatidylcholine pure (○), and with 20 mole percent cholesterol (●).

the relaxation rate is proportional to the number at directed bonded hydrogens. It is NT_1 which is plotted in Fig. 4 as a function of the position in the phospholipid hydrocarbon chain. In each case the spin lattice relaxation rate is relatively fast and flat across the portion of the hydrocarbon chain encompassing carbons 2 through about 10, depending on the overall chain length. At the terminus of the chain, the spin lattice relaxation rate is long, reflecting relatively fast motion of the segments of the lipid chains in the center of the bilayer.

The remarkable result from these spectra is that large changes in the extent of unsaturation produce no dramatic change in the rates of motion in the system across the profile of the membrane. This conclusion is limited to motions with time scales within 1 or 2 orders of magnitude of the Larmor frequency. (Note that the Larmor frequencies of the ^{2}H-NMR measurements and the ^{13}C-NMR measurements are similar here, due to the different field strengths employed). Theories have been advanced for the motions modulating spin lattice relaxation in phospholipid bilayers. Models have been advanced that include collective modes of motion based on a $W^{1/2}$ dependence of the spin lattice relaxation [14]. However, more recent measurements, made over several orders of magnitude frequency (as compared to about 1 order of magnitude previously [14]) do not show the $W^{1/2}$ dependence [16]. The model advanced on that data proposes three main motional modes for high frequency. They are axial diffusion, time dependence of the orientation of the director, and segmental motion [16]. A similar model was recently applied to cholesterol motion [15].

Nonetheless, the most important conclusions of this work are model independent. Extent of unsaturation of phospholipid hydrocarbon chains does not significantly influence either the profile of motional rates across the phospholipid bilayer, or the magnitude of the rates themselves. Concurrently, motional order in the membrane is not profoundly affected by unsaturation either. Yet to be examined are the effects of unsaturation on low frequency motions in the membrane.

Acknowledgments

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