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Deuterium NMR Investigation of Ether- and Ester-Linked Phosphatidylcholine Bilayers†

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ABSTRACT: Deuterium nuclear magnetic resonance (^{2}H NMR) spectra of specifically head-group- and chain-deuterated ester- and ether-linked phosphatidylcholine bilayers were studied as a function of temperature over the range −33 to 50 °C. Head-group-deuterated dihexadecylphosphatidylcholine ([α-$^2H_2$]DHPC) bilayers yield line shapes and spin–lattice relaxation times similar to those observed for its ester-linked counterpart, dipalmitoylphosphatidylcholine ([α-$^2H_2$]DPPC), in the high-temperature ripple and L_α bilayer phases. These results indicate the ether linkage has no effect on the dynamics or the orientational order at the α-C^2H_2 segment of the phosphocholine head group. At all temperatures, the ^{2}H NMR spectra of chain-deuterated 1,2[1′,1′-$^2H_2$]DHPC bilayers exhibit a reduced spectral width compared to 1,2[2′,2′-$^2H_2$]DPPC bilayers. The most *significant* feature of the deuterated alkyl chain spectrum of DHPC at 45 °C is the observation of *four* separate quadrupolar splittings from the α-methylene segments of the alkyl chains, in comparison to the *three* quadrupolar splittings reported previously from the α-methylene segments of the acyl chains of DPPC. Spin–lattice relaxation experiments performed on DHPC suggest an assignment of the two smaller and the two larger quadrupolar splittings to separate alkyl chains, respectively. Low-temperature ($T \leq -20$ °C) gel-phase spectra of deuterated head-group [α-$^2H_2$]DHPC remain an order of magnitude narrower than those observed for [α-$^2H_2$]DPPC. Line shapes of chain-labeled DHPC are invariant to temperature over the 40 °C range from 20 to −20 °C, whereas in DPPC, there is a substantial change in the line shape below 0 °C. These observations may be explained by the formation of an interdigitated DHPC bilayer gel phase in which axial diffusion of the lipid molecule persists down to temperatures at least 20 °C below the temperature at which axial diffusion of DPPC ceases ($T \sim -7$ °C).

Deuterium nuclear magnetic resonance (^{2}H NMR)[1] of lipids containing deuterated acyl chains has become the method of choice for the determination of molecular conformation and dynamics within the lipid bilayer of model and biological membranes (Seelig, 1977; Seelig & Seelig, 1980; Jacobs & Oldfield, 1981; Griffin, 1981; Davis, 1983). With the exception of the sphingolipids, such as cerebrosides (Huang et al., 1980; Skarjune & Oldfield, 1979, 1982) and sphingo-

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[1] Abbreviations: PC, phosphatidylcholine; ^{2}H NMR, deuterium nuclear magnetic resonance; DHPC, 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; DSC, differential scanning calorimetry; PA, phosphatidic acid; TLC, thin-layer chromatography; THF, tetrahydrofuran; TPS, 2,4,6-triisopropylbenzenesulfonyl chloride; [$^2H_{62}$]DPPC, 1,2-bis(perdeuteriopalmitoyl)-*sn*-glycero-3-phosphocholine.

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myelins (M. J. Ruocco, G. G. Shipley, and R. G. Griffin, unpublished results), *all* of the ^{2}H NMR studies of membranes to date have employed synthetically or biosynthetically labeled lipids whose fatty acyl chains are joined by *ester* linkages to a *glycerol* backbone. The most widely studied class of these lipids, and the most ubiquitous in biological membranes, are the glycerophospholipids whose diester glycerol moieties are coupled to either phosphocholine (PC), phosphoethanolamine (PE), phosphoglycerol (PG), phosphoserine (PS), or phosphate (PA) head groups.

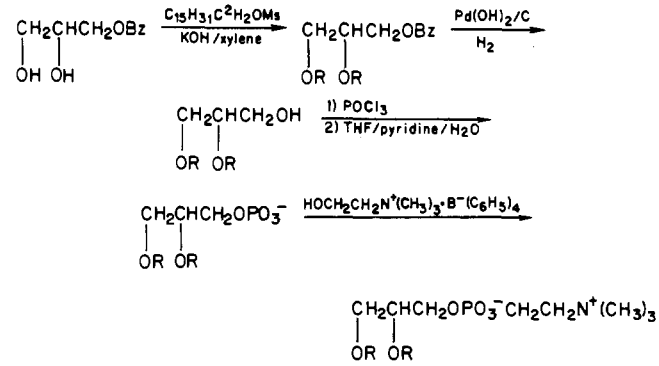
There are both historical and practical reasons for the predominance of ester-linked lipids in ^{2}H NMR studies of membranes. Ester-linked lipids were the first to be isolated and identified from biological sources, and after more than 50 years of study by biochemists and physical chemists, they are understandably the best characterized. Beginning in 1971 with the first application of ^{2}H NMR to the study of lipid bilayers (Oldfield et al., 1971; Seelig & Seelig, 1974), the original workers in the field devoted most of their efforts toward building a repertoire of reliable and facile synthetic techniques for the specific isotopic labeling of saturated and unsaturated fatty acids, and for the coupling of PC, PE, PG, or PS head groups to diacylglycerol moieties containing such specifically labeled fatty acids. On the other hand, after the initial discovery of ether lipids in marine organisms (Toyama, 1924), almost 30 years passed before reliable methods of analyzing and isolating ether lipids were developed, and 10 years more before the ether-linked analogue of PC was prepared (Eibl et al., 1967). It is not surprising, therefore, that ether-linked lipids have yet to be studied by ^{2}H NMR.

In this laboratory, DSC studies of DHPC bilayer dispersions (50 wt % water) demonstrated similar thermal behavior to that of DPPC bilayer dispersions over the range of temperatures from 35 to 50 °C (i.e., DHPC dispersions exhibit a pretransition and a main transition at 35 and 44 °C, respectively) (Ruocco et al., 1985). Furthermore, ^{31}P NMR and X-ray diffraction studies of DHPC in the ripple and L_α bilayer phases indicate that the dynamics of the phosphocholine head group and the structural characteristics of the bilayer are also very similar to those of DPPC (Ruocco et al., 1985; M. J. Ruocco, A. Makriyannis, D. J. Siminovitch, and R. G. Griffin, unpublished results). In the lamellar gel phases at $T < 35$ °C, however, a major structural alteration is observed for DHPC dispersions (50 wt % water). At these temperatures and this hydration level, an interdigitated lamellar phase is observed in contrast to the noninterdigitated $L_{\beta'}$ bilayer phase of DPPC dispersions. In addition, at low temperatures, DHPC undergoes a completely reversible gel → gel polymorphic transition which appears to involve only a two-dimensional hydrocarbon chain rearrangement (Ruocco et al., 1985), in contrast to the simultaneous changes in hydration *and* in hydrocarbon chain packing observed at the subtransition of DPPC bilayers (Ruocco & Shipley, 1982a,b). It would appear that the larger surface area per DHPC molecule in the interdigitated gel bilayers (78 Å^2) and the absence of carbonyl moieties at the bilayer interface allow rapid axial diffusion (on the ^{31}P NMR time scale) to persist to much lower temperatures than in DPPC bilayers. As a result, axially symmetric ^{31}P NMR spectra were observed at temperatures as low as −30 °C. In this paper, we will compare the effects of changes in the axial diffusion rate on the ^{2}H NMR gel-phase spectra of DHPC and DPPC which have been specifically labeled in the head-group or the hydrocarbon chain region.

First, we have prepared DHPC with deuterons at the α-C^2H_2 segment of the choline moiety in order to compare it with its ester-linked counterpart, also labeled at the α-C^2H_2 segment of the choline moiety. Measurements of the ^{2}H quadrupolar splittings and spin–lattice relaxation times in the liquid-crystalline (L_α) phase of each of these lipids indicate that the change in linkage at the glycerol backbone has little effect on the dynamics or the orientational order at the α-C^2H_2 segment of the choline head group. Furthermore, gel-phase spectra of these two molecules reveal only subtle differences at temperatures above ~0 °C. However, at much lower temperatures (below −20 °C), the spectra indicate that there are dramatic differences in the amount of motional averaging experienced by the α-C^2H_2 segment of DHPC and DPPC. Specifically, the breadth of the DHPC spectrum is invariant to temperature over the 80 °C range from −30 to 50 °C, whereas in DPPC, the breadth of the spectrum below −20 °C approaches the rigid-lattice limit. In the chain-labeled molecules, deuterons have been placed on the α-methylene segments of both hydrocarbon chains. Thus, we have prepared DHPC specifically deuterated at the C-1 position of both alkyl chains in order to compare it with its ester-linked counterpart labeled at the C-2 position of both acyl chains. In the liquid-crystalline phase, four separate quadrupole splittings are observed for bilayers of 1,2[1′,1′-$^2H_2$]DHPC. Spin–lattice relaxation experiments suggest that the four splittings observed arise from two pairs of inequivalent deuterons on chains which are most likely inequivalent due to different initial orientations of the alkyl chains near the bilayer interface. In the gel phase, the ^{2}H NMR line shape of 1,2[1′,1′-$^2H_2$]DHPC is invariant to temperature over the 40 °C range from 20 to −20 °C, whereas in 1,2[2′,2′-$^2H_2$]DPPC, there is a substantial change in the line shape below 0 °C, indicative of a reduction in the axial diffusion rate. These results, together with the gel-phase spectra of the head-group-labeled molecules, provide convincing evidence that in DHPC axial diffusion persists to at least −20 °C, whereas in DPPC, this type of motion is frozen out around 0 °C.

Experimental Procedures

Lipid Synthesis. All ether-linked phospholipids were synthesized in this laboratory as described below. Specifically head-group-deuterated DPPC was kindly supplied by Professor J. Seelig. The synthesis of 2[7′,7′-$^2H_2$]DPPC was carried out by Avanti Polar Lipids (Birmingham, AL) using [7,7-$^2H_2$]-palmitic acid prepared according to methods described by Das Gupta et al. (1982). 1,2-Di-*O*-[1′,1′-$^2H_2$]hexadecyl-*sn*-glycero-3-phosphocholine was synthesized as follows:



where R = $C_{15}H_{31}C^2H_2$–. [1,1-$^2H_2$]Hexadecyl methanesulfonate was obtained by using standard procedures (Hermetter & Paltauf, 1983) from [1,1-$^2H_2$]hexadecanol (80%) which was in turn obtained through the reduction of methyl hexadecanoate with $LiAl^2H_4$ (95%) (Aldrich Chemicals, Milwaukee, WI). The hexadecyl methanesulfonate was then used to alkylate commercially available L-α-benzylglycerol

(Sigma Chemical Co., St. Louis, MO) by refluxing with powdered KOH in xylene, using a slight modification of a procedure by Hermetter & Paltauf (1983) (60%). Debenzylation was then carried out by hydrogenolysis using 20% $Pd(OH)_2$ on charcoal (Pearlman, 1967). In our hands, and as reported by Kates et al. (1963), debenzylation proceeded sluggishly with the traditional catalyst, palladium on charcoal, while $Pd(OH)_2$ gave a smooth reaction with quantitative yields. Phosphorylation of the dialkylglycerol was accomplished by first reacting it with phosphorus oxychloride in trichloroethylene as described by Jahnig et al. (1979). The dialkylglycerol phosphoryl chloride obtained was then hydrolyzed to the corresponding phosphatidic acid by using a THF/pyridine/H_2O (8:1:1) mixture (72%). The final step in the reaction sequence was accomplished by condensing the dialkylphosphatidic acid with the choline tetraphenylborate salt (Harbison & Griffin, 1984), using TPS as a catalyst (85%).

Synthesis of head-group-labeled DHPC was carried out by phosphorylation of commercially available 1,2-dihexadecyl-*sn*-glycerol (R. Berchtold, Bern, Switzerland) using the [2,2-$^2H_2$]choline tetraphenylborate salt, as described above for the synthesis of chain-labeled DHPC. All lipids used for NMR experiments were shown to be >99% pure by thin-layer chromatography (TLC).

NMR Spectroscopy. Samples for the NMR experiments typically consisted of between 30 and 100 mg of lipid dispersed in an equal weight of ^2H-depleted water (Aldrich Chemicals, Milwaukee, WI), which were sealed under vacuum in 7-mm o.d. glass tubes. ^2H NMR experiments were performed at a frequency of 45.3 MHz using a home-built solid-state pulse spectrometer and a superconducting solenoid (6.8 T). ^2H NMR line shapes were obtained by using a two-pulse quadrupole echo sequence, $90°_x$–τ–$90°_y$ (Davis et al., 1976), with a 90° pulse length of 2.0–2.5 μs and a pulse separation of 40–60 μs. The decay of the quadrupole echo was measured by monitoring the echo amplitude as a function of the pulse spacing (τ) between the two 90° pulses. Spin–lattice relaxation experiments were performed by observing the recovery of magnetization following the application of a single 180° pulse, 4.5–5.0 μs in duration. The magnetization was sampled at various intervals (τ) following the inverting pulse using the quadrupolar echo, and the partially recovered spectra obtained by Fourier transformation of the echo signal were used to monitor the recovery of magnetization. All measurements of the spin–lattice relaxation times (T_1) were carried out in the liquid-crystalline phase of each lipid, where axially symmetric fast-limit powder pattern spectra with well-defined perpendicular edges are observed. Because the spin–lattice relaxation rate in the liquid-crystalline phase is independent of orientation (Brown & Davis, 1981), the intensity of the perpendicular edge, which is the most prominent spectral feature, was used to provide a measure of the recovery rate. The T_1 value was obtained by a least-squares fit to the amplitude data (Rance, 1981; MacDonald, 1980). Phase cycling and quadrature detection were used for all NMR experiments (Blume et al., 1982).

Results and Discussion

^2H NMR of Head-Group-Labeled PC. Figures 1 and 2 display ^2H NMR spectra of DPPC and DHPC, both specifically deuterated at the α-C^2H_2 segment of the choline moiety, at representative temperatures in the gel and liquid-crystalline phases. Previously, in the case of DPPC labeled at the C_α position of the choline head group, detection of the ^2H NMR signal below 37 °C was impossible (Gally et al., 1975), but with improvements in instrumentation and technique devoted to solid-state NMR (Griffin, 1981), principally the introduction of the quadrupole echo (Solomon, 1958; Davis et al., 1976), acquisition of high-fidelity NMR line shapes of spin 1 nuclei in the gel phase of lipid bilayers has become routine (Blume et al., 1982; Siminovitch et al., 1983). While the liquid-crystalline phase of head-group-labeled DPPC has been well characterized by ^2H NMR (Gally et al., 1975; Akutsu & Seelig, 1981), the spectra shown in Figures 1 and 2 represent the first detailed study of the temperature dependence of the ^2H NMR line shapes of the α-C^2H_2 segment of the PC head group in the gel phase. Above their respective gel to liquid-crystalline phase transition temperatures, T_c [T_c(DPPC) = 41.2 °C (Chapman et al., 1966; Lipka et al., 1984) and T_c(DHPC) = 43 °C (Vaughan & Keough, 1974)], the ^2H NMR line shapes are sharp, fast-limit powder patterns characteristic of the L_α bilayer phase. The temperature dependence and the magnitude of the quadrupole splittings are very similar for both lipids in the L_α phase. As Table I shows, the quadrupole splitting of DHPC remains constant from 45 to 73 °C, as does the quadrupole splitting of DPPC which

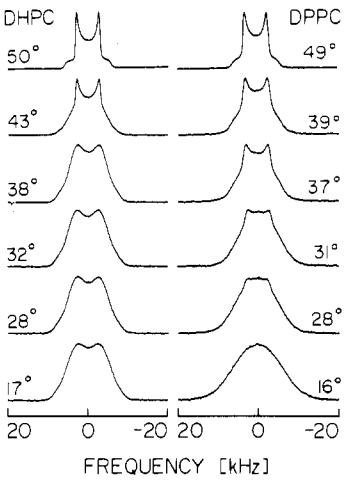


FIGURE 1: Temperature dependence of the ^2H NMR spectra of the α-C^2H_2 segment of the choline moiety in DHPC (left) and DPPC (right). Typically, 4000 scans were signal averaged to obtain these spectra.

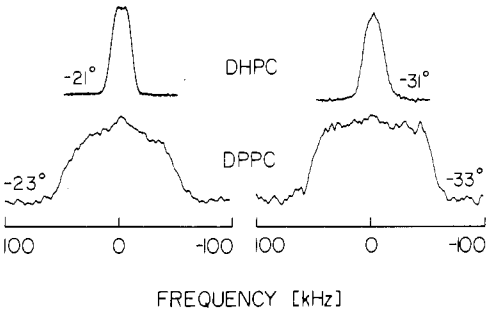


FIGURE 2: Representative low-temperature lamellar gel-phase spectra of head-group-labeled DHPC (top) and DPPC (bottom). Lipid dispersions were not annealed prior to the NMR experiments.

Table I: ^2H Quadrupolar Splittings and Spin–Lattice Relaxation Times for the α-C^2H_2 Segment of the Choline Moiety[a]

	temp (°C)	$\Delta\nu_Q$ (kHz)	T_1 (ms)
DHPC	45	5.83 ± 0.10	
	47	5.83	
	50	5.83	29.53 ± 0.75
	63	5.83	47.98 ± 0.87
	73	5.83	65.00 ± 0.96
DPPC	50	5.93 ± 0.10	29.7

[a] Value for T_1 at 50 °C in DPPC is taken from Akutsu & Seelig (1981).

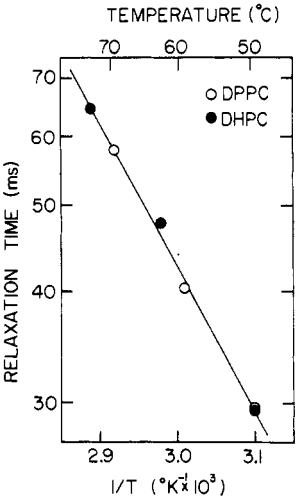


FIGURE 3: Arrhenius plot of ^{2}H spin–lattice relaxation times (given in Table I) of the α-C^2H_2 segment in the choline moiety of DHPC (closed symbols). For comparison, equivalent data for the α-C^2H_2 segment of DPPC (Akutsu & Seelig, 1981) are also plotted (open symbols). Estimated error in T_1 values for DHPC at all temperatures is less than 3%, which is always less than the symbol size.

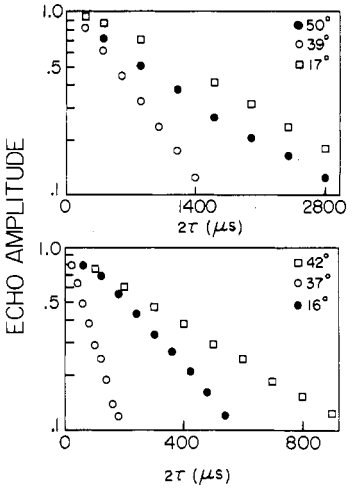


FIGURE 4: Temperature dependence of the quadrupole echo amplitude decay in [α-$^2H_2$]DHPC (top) and 2[7′,7′-$^2H_2$]DPPC (bottom). At each temperature, amplitudes are normalized to the echo amplitude observed at the shortest pulse spacing employed, typically 40 μs. The decay is plotted vs. twice the pulse spacing, so that if the decay is exponential, the slope gives the quadrupole echo decay rate, T_{2e}^{-1}.

remains constant from 41 to 70 °C (Gally et al., 1975). Within experimental error, and taking into account a slight increase in line broadening observable in the L_α DPPC spectra, the magnitudes of the quadrupole splittings for DHPC and DPPC are identical. The value measured for DPPC at 50 °C, 5.93 kHz, agrees well with the value of 5.95 kHz measured earlier by Akutsu & Seelig (1981).

The spin–lattice (T_1) relaxation times measured at 45.3 MHz for the α-C^2H_2 segment of the choline moiety in the liquid-crystalline phase of DHPC are given in Table I and plotted in Figure 3, together with equivalent data measured at 46.1 MHz for DPPC (Akutsu & Seelig, 1981). Within experimental error and neglecting any frequency dependence of the relaxation rates, the data for *both* lipids can be fit by the same straight line on an Arrhenius plot. The activation energy derived from this fit is 31 kJ mol^{-1}, which is in excellent agreement with the values of 31 kJ mol^{-1} for the α-C^2H_2 segment of DPPC (Akutsu & Seelig, 1981), 27 kJ mol^{-1} for the β-C^2H_2 segment of DPPC (Akutsu & Seelig, 1981), and 31 kJ mol^{-1} derived from the ^{14}N T_1 data for the C_β–N bond segment of the choline moiety in DHPC (Siminovitch et al., 1983).

Previous NMR studies of the PC head group of ester- and ether-linked lipids (Hauser, 1981; Hauser et al., 1981; Siminovitch et al., 1983; Ruocco et al., 1985), coupled with the results of the ^{2}H NMR experiments presented in this study, allow us to construct a complete profile in the liquid-crystalline phase of the conformational and dynamic changes in the phosphocholine head group induced by the change from diester to diether linkages. High-resolution ^{1}H NMR studies originally showed that the replacement of ester linkages by ether bonds had no significant effect on the average conformation and segmental motion of the PC head group as monomers in solution or in micelles (Hauser, 1981; Hauser et al., 1981). Solid-state ^{31}P and ^{2}H NMR studies of DHPC and DPPC in the L_α bilayer phase provide support for the idea that this is also true in liposomes. In the case of ^{31}P NMR, neither the line shapes nor the magnitudes of the chemical shift anisotropies are significantly different in the L_α phase (Hauser, 1981; Siminovitch et al., 1983; Ruocco et al., 1985), while the ^{2}H quadrupole splittings for the α-C^2H_2 segment are identical within experimental error. Only the ^{14}N quadrupole splittings for the C_β–N bond segment show any difference, and even then, the effect is small (≤2 kHz), most likely due to a small change in the average orientation of the C_β–N bond as a result of packing changes in the hydrocarbon chain region (Siminovitch et al., 1983). Finally, the change from ester to ether linkage has no discernible effect on the relaxation rates of the ^{2}H nucleus at the α-C^2H_2 segment or the ^{14}N nucleus of the C_β–N bond segment (Siminovitch et al., 1983), and therefore, the dominant relaxation mechanism in the choline head group must be the same for both lipids.

In Figure 4 (top panel), the decay of the quadrupole echo amplitude at representative temperatures in each of the L_α, $P_{\beta'}$, and interdigitated phases is shown for head-group-labeled [α-$^2H_2$]DHPC. We note that the decay is most rapid in the $P_{\beta'}$ phase (~39 °C), is slowest in the interdigitated lamellar phase (17 °C), and occurs at an intermediate rate in the L_α phase (50 °C). Exactly analogous results, i.e., $P_{\beta'} > L_\alpha > L_\beta$ (interdigitated), have been reported for the decay of the ^{14}N quadrupole echo amplitude in DHPC (Siminovitch et al., 1983), although the ^{14}N decay rates are uniformly somewhat greater at all corresponding temperatures. The fact that the decay of *both* ^{2}H and ^{14}N quadrupole echo amplitudes is most rapid in the $P_{\beta'}$ phase would suggest that the molecular explanation of this phenomenon must involve the entire choline moiety. In addition, we observe the same phenomenon for the decay of the ^{2}H quadrupole echo amplitudes in the $P_{\beta'}$ phase of 1,2[1′,1′-$^2H_2$]DHPC and 1,2[2′,2′-$^2H_2$]DPPC (data not shown), and in Figure 4 (bottom panel), we show echo decay data for 2[7′,7′-$^2H_2$]DPPC where again the decay rates are most rapid in the ripple phase. Finally, this behavior of echo amplitudes is not limited to spin 1 nuclei, however, since the decays of the ^{31}P Hahn echo in DPPC and DHPC (M. J. Ruocco, D. J. Siminovitch, and R. G. Griffin, unpublished results) and the ^{13}C Hahn echo in 2[1′-^{13}C]DPPC are also most rapid in this phase (Wittebort et al., 1981). An immediate practical consequence of this behavior is that it leads to spectra with significantly lower signal to noise ratios; i.e., these "echo distortions" lead to intensity losses in the spectra (Spiess & Sillescu, 1981; Blume et al., 1982). Using the data presented in Figure 4 (bottom panel) for 2[7,7-$^2H_2$]DPPC as an example, we see that for ^{2}H NMR spectroscopy with a 140-μs pulse spacing the echo amplitude in the $P_{\beta'}$ phase is only one-sixth of that observed in the L_α phase. More important, however, is the fact that these data require any microscopic

model of this phase to correctly predict not only the essential features of the line shapes observed but also the rapid decay of echo intensity. On the basis of ^{13}C NMR results for 2-[1′-^{13}C]DPPC in the $P_{\beta'}$ phase, Wittebort et al. (1981) have proposed that the $P_{\beta'}$ phase exhibits microscopic properties characteristic of both the $L_{\beta'}$ and L_α phases. If domains of gel- and liquid-crystalline-like molecules coexist in the $P_{\beta'}$ phase, then exchange between them may account for the rapid decay of echo intensity observed in both the head-group and the hydrocarbon chain regions. We are now reexamining this problem in light of our observations in DHPC and DPPC bilayers and will present a more detailed account of these matters elsewhere.

The gel-phase ^2H spectra of the DPPC and DHPC head groups shown in Figures 1 and 2 may have many features in common, but there are important differences, particularly at lower temperatures. Figure 1 shows that below their respective liquid-crystalline (L_α) → gel bilayer phase transitions, the ^2H NMR line shapes of the gel phase gradually lose the sharp spectral features characteristic of the L_α phase. Just below T_c, there is a definite broadening of the perpendicular edges observed in the liquid-crystalline spectra, but there is little change in the quadrupolar splitting. Furthermore, the spectra of both lipids continue to broaden as the temperature is lowered, but the total breadth of the spectra does not increase significantly. The changes in the ^2H NMR line shapes of the α-C^2H_2 segment as a function of temperature in the gel phase are very similar to the corresponding changes in the ^{14}N NMR line shapes of the C_β–N bond segment in the choline moieties of DPPC and DHPC (Siminovitch et al., 1983). Both NMR techniques show that, in comparison to the liquid-crystalline phase, the overall breadth of the head-group spectra in DHPC and DPPC does not increase significantly in the gel phase, as it does, for example, in the ^2H NMR spectra of the hydrocarbon chain region (vide infra). In addition, both the ^2H and ^{14}N NMR spectra of the $P_{\beta'}$ phase in DPPC and DHPC retain, in slightly broadened form, the perpendicular edges of the L_α phase. Thus, in common with earlier ^2H NMR studies of the PE head group (Blume et al., 1982) and ^{14}N NMR studies of PC head groups (Siminovitch et al., 1983), the total breadth of the NMR line shape in the L_α phase and in the L_β or $L_{\beta'}$ phase, at least above 0 °C, is roughly the same, approximately an order of magnitude smaller than the breadth expected for a rigid-lattice ^2H tensor. This suggests that whatever molecular motions are responsible for the L_α line shape, these motions persist in the gel phase, only at somewhat slower rates. The ^2H NMR line shapes of both lipids appear to be relatively insensitive to the pretransition, although as noted above, there is a marked increase in the decay rate of the quadrupole echo in the $P_{\beta'}$ phase. A careful inspection of the gel-phase line shapes between ~0 °C and the respective pretransition temperatures reveals the subtle differences between DPPC and DHPC. For example, as the temperature decreases in the gel phase, the central dip in the spectra of DHPC remains until at least 0 °C (spectrum not shown), whereas in DPPC, the corresponding spectral feature disappears around 28 °C, and by 16 °C, the DPPC spectrum is completely featureless. By comparing the DHPC line shape at 17 °C with the corresponding DPPC line shape at 16 °C, we see evidence of a trend which continues as the temperature decreases to 0 °C; namely, the DPPC spectrum becomes noticeably broader than the DHPC spectrum. These differences may be a reflection of the fact that DHPC forms interdigitated bilayers in which the surface area per molecule is 78 Å^2 (Ruocco et al., 1985) whereas DPPC bilayers are noninterdigitated and the molecular area per DPPC is ≤50 Å^2. At 0 °C, the most dramatic difference between the two lipids is not to be found in the line shapes, although the DPPC spectrum is somewhat broader than its ether-linked counterpart, but rather in the dramatic decrease in the intensity of the DPPC spectrum. Similar intensity losses are not observed in DHPC until ~−30 °C. This behavior is very similar to that reported earlier by Siminovitch et al. (1983) in a ^{14}N NMR study of DHPC and DPPC dispersions. In fact, the ^{14}N quadrupole echo decay in DPPC at 0 °C is so rapid that the signal is essentially lost, whereas in DHPC, the echo decay remains slow until ~−30 °C. Finally, the echo decay rate begins to dramatically increase, and the ^{14}N signal is lost around −40 °C in DHPC. Until the ^{14}N NMR signal is lost in each lipid, the breadth of the gel-phase spectra remains virtually identical with that observed at 40 °C. For DHPC this means that the spectrum does not change substantially over the 70 °C range from 40 to −30 °C. Spectra of [$^2H_{62}$]DPPC below 0 °C indicate that axial diffusion in the gel phase continues to decrease and stops on a ^2H NMR time scale at about −7 °C (Davis, 1979). This fact led Siminovitch et al. (1983) to conclude that the disappearance of the ^{14}N NMR signal in each lipid must be a result of a decrease in the rate of axial diffusion, an event which must occur at a significantly lower temperature in DHPC. This conclusion was supported by the observation of axially symmetric ^{31}P powder patterns, characteristic of rapid axial diffusion on the ^{31}P NMR time scale, down to −30 °C in DHPC interdigitated bilayers, whereas the line shapes of DPPC in the subphase below 0 °C are characteristic of reorientation of the phosphate moiety at an intermediate exchange rate (Ruocco et al., 1985). These marked differences in the dynamic behavior of the phosphocholine moiety of DHPC and DPPC at low temperatures lead to ^2H NMR spectra of the α-C^2H_2 segment which are no less dramatic, as Figure 2 demonstrates. At approximately the same temperature, there are striking differences between DHPC and DPPC in the breadth of the spectra observed. In DHPC, the spectra observed at −21 and −31 °C are much the same as those observed at higher temperatures in the gel phase. Thus, in agreement with previous ^{14}N (Siminovitch et al., 1983) and ^{31}P (Ruocco et al., 1985) NMR studies, the ^2H NMR gel-phase spectra of DHPC undergo no substantial change in shape or breadth over the 60 °C range from 30 down to −30 °C. Furthermore, the relative insensitivity of the DHPC head-group spectra to the low-temperature polymorphic "subtransition" at 4 °C is consistent with the insensitivity of the ^{31}P NMR spectra to this phase transition and with the suggestion that the subtransition in DHPC involves primarily a hydrocarbon chain packing rearrangement (Ruocco et al., 1985). On the other hand, in DPPC, by −23 °C there is a substantial increase in the breadth of the line shape, and at −33 °C, the breadth of the spectrum approaches that expected for a rigid-lattice ^2H tensor. Since axial diffusion has stopped on a ^2H NMR time scale around −7 °C in DPPC (Davis, 1979), it is not surprising to find the effects of this event clearly manifested in ^2H NMR spectra of the choline moiety at even lower temperatures.

^2H NMR of Chain-Labeled PC. Figure 5 shows the temperature dependence of the ^2H NMR spectra of 1,2[1′,1′-$^2H_2$]DHPC and 1,2[2′,2′-$^2H_2$]DPPC bilayer dispersions. The temperature dependence of the ^2H NMR gel-phase spectra of ester-linked lecithins has been well characterized by using chain-deuterated DPPC (Davis, 1979; Blume et al., 1982) and DMPC (Huang et al., 1980; Bienvenue et al., 1982), and so we will not dwell upon the line shapes of DPPC in any detail,

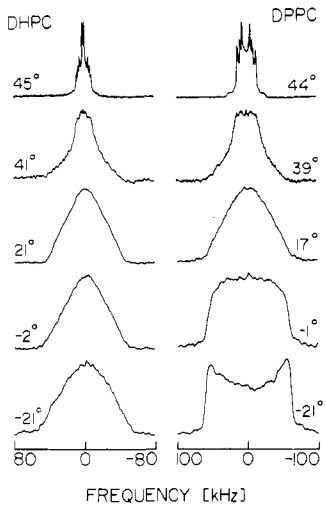


FIGURE 5: Temperature dependence of the ^{2}H NMR spectra of ester- and ether-linked phosphatidylcholine dispersions labeled at the α-methylene segments of both hydrocarbon chains. Left, 1,2[1′,1′-$^2H_2$]DHPC; right, 1,2[2′,2′-$^2H_2$]DPPC. Lipid dispersions were not annealed prior to the NMR experiments.

except to make the following points of comparison between DHPC and DPPC. As the temperature is lowered in DPPC, to $T \approx 0$ °C, there is a significant enhancement in the shoulders of the spectra, and by −21 °C, these shoulders, corresponding to the perpendicular edges of a rigid-lattice ^{2}H powder pattern, become very prominent. As noted earlier by Davis (1979), the enhanced intensity in the shoulders of the hydrocarbon chain spectra of DPPC observed around 0 °C indicates that at this temperature, a large fraction of the lipid molecules have ceased axial diffusion and by −20 °C the prominent shoulders of the spectra show that axial diffusion on the ^{2}H NMR time scale has stopped. In contrast, the line shapes of DHPC are invariant to temperature from 21 °C down to −21 °C, proving the axial diffusion persists in DHPC until at least −21 °C. When the temperature is further decreased to −40 °C, the shoulders of the DHPC spectrum become prominent features (spectrum not shown), indicating that axial diffusion has finally stopped. Even when the effects of axial diffusion are present in the line shapes of both lipids, a comparison of representative spectra in the $P_{\beta'}$ and lamellar gel phases, for example, clearly shows that although the line shapes are very similar, the breadth of the DHPC line shape is roughly 75% of that of the corresponding DPPC line shape. This may indicate either that there is more motional averaging of the quadrupolar interaction at the C-1 position of the hydrocarbon chains in DHPC than at the C-2 position of the hydrocarbon chains in DPPC or that there are orientational effects which contribute. The latter possibility is more likely, considering that the differences in quadrupolar splitting in the L_α phase are best explained by differences in C–^{2}H bond orientations (vide infra).

For both lipids, we observe that the line shapes of the $P_{\beta'}$ phase can be clearly distinguished from those of the lower temperature lamellar gel phase, unlike the corresponding spectra of the ^{2}H-labeled head groups (see Figure 1). Gel-phase spectra of 2[7′,7′-$^2H_2$]DPPC shown in Figure 6 demonstrate that the clear distinction between $P_{\beta'}$ and $L_{\beta'}$ line shapes is a general characteristic of chain-labeled spectra. In Figure 7, the pulse spacing dependences of the DPPC line shapes at two different temperatures in the gel phase are contrasted. At 18 °C, there is no difference between the line shape obtained with $\tau = 40$ μs and that obtained with $\tau = 150$ μs, in agreement with the observation of Davis (1983) that

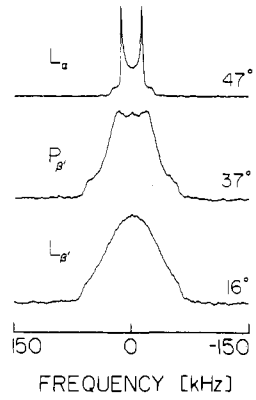


FIGURE 6: Representative ^{2}H NMR spectra of 2[7′,7′-$^2H_2$]DPPC in each of the L_α, $P_{\beta'}$, and $L_{\beta'}$ bilayer phases.

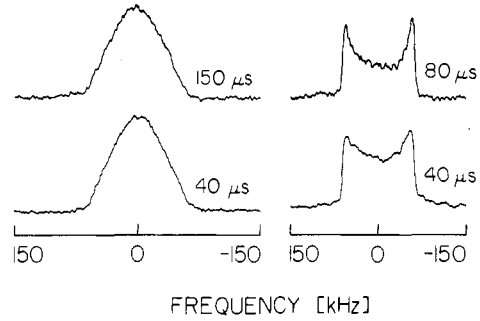


FIGURE 7: Pulse spacing dependence of the 1,2[2′,2′-$^2H_2$]DPPC ^{2}H NMR line shapes at 18 (left) and −21 °C (right).

at 0 °C the line shape of 1,2[4′,4′-$^2H_2$]DPPC does not change when the pulse spacing is increased from 40 to 150 μs. However, at −21 °C, not only is the lineshape very sensitive to pulse spacing but also the decay of the quadrupolar echo is very rapid. However, this is precisely the behavior expected when the motional correlation times are in the intermediate regime, i.e., when $\tau_c = 1/\Delta\nu_Q$. In the case of 3-fold axial jumps, the effect is to remove intensity from the center of the spectrum at a very rapid rate. The fact that we (1) observe these effects and (2) can simulate the τ dependence using a 3-fold jump model with a single correlation time suggests that the line shape at −20 °C is dominated by axial diffusion occurring at an intermediate rate. We draw a careful distinction between the rapid intensity losses observed at −20 °C and those suffered at higher temperatures in the $P_{\beta'}$ phase, noting that the intensity losses of the $P_{\beta'}$ phase are not accompanied by the echo distortions evident in the line shapes of Figure 7 at −20 °C. We believe that the intensity losses of the $P_{\beta'}$ phase documented above for the choline moiety and the hydrocarbon chains are largely determined by an *inter*molecular exchange between gel and liquid-crystalline molecules at an intermediate rate, whereas the echo distortions and intensity losses at much lower temperatures are the result of an *intra*molecular motion at an intermediate rate. A more detailed examination of the gel-phase line shapes in lecithins will be published elsewhere.

The respective L_α bilayer phase line shapes of both lipids are plotted in Figure 8 by using an expanded frequency axis together with spectral simulations using methods described elsewhere (Siminovitch, 1982). Simulation parameters are given in Table II, together with measurements of the T_1 relaxation times for each component included in the spectral simulations. The differences between DPPC and DHPC are clearly apparent in the L_α bilayer line shapes of Figure 8. For DPPC, three distinct quadrupolar splittings are observed, whereas there are four distinct splittings in the spectrum of DHPC. The multicomponent spectrum of DPPC labeled at

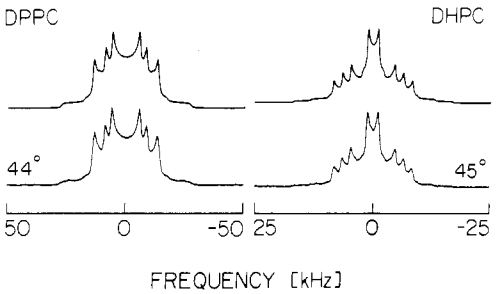


FIGURE 8: Representative ^2H NMR spectra in the L_α phase of phosphatidylcholine dispersions labeled at the α-methylene segments (bottom) together with spectral simulations (top). Simulation parameters are given in Table II. Left, 1,2[2′,2′-$^2H_2$]DPPC; right, 1,2[1′,1′-$^2H_2$]DHPC.

Table II: Simulation Parameters for the Simulations of Figure 8 and Corresponding Spin–Lattice Relaxation Times[a]

	rel intensity	$\Delta\nu_Q$ (kHz)	$\Delta\nu$ (Hz)	T_1 (ms)
DPPC	1	11.8	635	15.14 ± 0.10
	1	17.6	635	15.15 ± 0.15
	2	27.4	955	18.61 ± 0.16
DHPC	1	2.34	320	18.14 ± 0.19
	1	9.45	320	18.80 ± 0.28
	1	13.1	320	20.93 ± 0.37
	1	16.8	320	21.11 ± 0.49

[a] For the simulation parameters, $\Delta\nu_Q$ is the quadrupolar splitting and $\Delta\nu$ is the Lorentzian line width at half-height. T_1 values were measured at 46 °C.

the C-2 position of both hydrocarbon chains is now well understood. Seelig & Seelig (1974) first observed three quadrupolar splittings for the C-2 position of DPPC and subsequently, by labeling the chains individually, were able to assign the largest splitting to the C-2 position of the *sn*-1 chain and the other two splittings to the *sn*-2 chain (Seelig & Seelig, 1975). The inequivalence of the spectra for the *sn*-1 and *sn*-2 acyl chains was attributed to different average conformations of the chains in the region of the glycerol backbone, with the beginnings of the *sn*-1 and *sn*-2 chains oriented perpendicular and parallel, respectively, to the bilayer surface. This conclusion is in agreement with X-ray data for crystalline phospholipids (Hitchcock et al., 1974; Pearson & Pascher, 1979) and neutron diffraction studies of DPPC bilayers (Büldt et al., 1978; Zaccai et al., 1979; Büldt & Seelig, 1980). By stereospecifically deuterating the C-2 position of the *sn*-2 chain, Engel & Cowburn (1981) demonstrated unambiguously that the two components observed for the *sn*-2 chain of 2[2′,2′-$^2H_2$]DPPC arise from the magnetic inequivalence of the two deuterons. The multicomponent spectrum for the C-1 position of DHPC is remarkable because in addition to the *four* well-resolved components, instead of three observed for the C-2 position of DPPC, the magnitudes of the quadrupolar splittings for two of these components are less than any value observed at the C-2 position of DPPC. In simulating the spectrum of DHPC, we have assumed that there are four inequivalent deuterons contributing to the observed spectrum (see Table II). This assumption can only be justified at the present time by the more than satisfactory simulation of the experimental line shape shown in Figure 8. However, arguing by analogy from the relaxation behavior of the three components observed in the well-characterized spectrum of DPPC, we can at least assign the two smallest splittings to inequivalent deuterons on the same chain. The partially relaxed line shape of DPPC in Figure 9, together with the T_1 relaxation data given in Table II, clearly demonstrates that the inequivalent deuterons of the *sn*-2 chain relax at the same rate, different from that of the deuterons on the *sn*-1 chain. We observe in

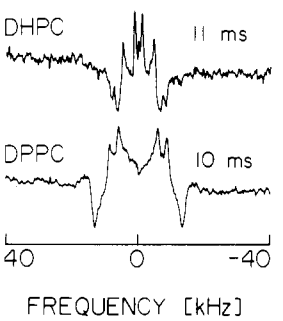


FIGURE 9: Partially recovered spectra of the chain-labeled phosphatidylcholine dispersions at the indicated τ values following a single inverting pulse.

the corresponding partially relaxed line shape of DHPC in Figure 9 that the two inner components appear to relax at the same rate, distinct from that of the two outer components. The relaxation times given in Table II confirm this observation and led us to the conclusion that the four splittings observed are due to two pairs of inequivalent deuterons. As in DPPC, the inequivalence of the chains in DHPC is most likely due to different initial orientations of the alkyl chains near the bilayer interface, which, if so, would suggest that chain inequivalence near the bilayer interface is a common feature of both ester- and ether-linked lipids. Since the C-1 or α-carbon has a different bonding orbital geometry (sp^3 vs. sp^2 for the acyl carbonyl carbon) and the first methylene segments of the alkyl chains in DHPC are closer to the glycerol backbone than the corresponding segments of the acyl chains in DPPC, they may have different average conformations in DHPC than in DPPC. It is undoubtedly this fact which explains the smaller quadrupolar splittings observed in DHPC.

It can be suggested that due to the absence of the sp^2 carbonyl groups, the initial orientation of the alkyl chains projecting from the glycerol backbone of DHPC may permit closer intramolecular chain contacts in contrast to the ester-linked acyl chains in DPPC. The closer intramolecular packing in conjunction with the absence of relatively bulky carbonyl groups, which project in a generally perpendicular orientation from the molecular long (rotation) axis, would yield a less irregular molecular surface (as projected down the molecular long axis).

The chemical structure of DHPC and a conformation as described above would further result in less in-plane, steric hindrance, hence, a lower activation energy for axial diffusion, and could partially account for the persistence of molecular rotation in the low-temperature interdigitated DHPC bilayers. Clearly, the enhanced surface area per head group, 78 Å^2, will also contribute to greater motional freedom of both the molecular long axis (e.g., chains) and the phosphocholine head group compared to the low-temperature, noninterdigitated DPPC bilayers.

Unfortunately, the molecular conformation of DHPC is yet to be defined by X-ray crystallography, neutron diffraction, and/or solid-state NMR methods. Further, it is recognized that, as in the case of the deuterated α-methylene position of DPPC acyl chains, individual labeling of the hydrocarbon chains, and possibly stereospecific labeling at the C-1 position, will be necessary to unequivocally assign the components observed in the L_α spectrum of 1,2[1′,1′-$^2H_2$]DHPC. We have recently modified the synthetic methods described above so that ether phospholipids with different alkyl chains can be prepared. This method, details of which will be published elsewhere, also allows us to prepare DHPC ^2H labeled in either of the alkyl chains. From these synthetic ether phosphati-

dylcholines, the question of conformation can be addressed through the application of one or more of the structural techniques mentioned above.

ACKNOWLEDGMENTS

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Registry No. DPPC, 63-89-8; DHPC, 36314-47-3; $C_{15}H_{13}C^2H_2OMs$, 56555-04-5; $HOCH_2CH(OH)CH_2Bz$, 56552-80-8; $C_{15}H_{31}C^2H_2OCH_2CH(OC^2H_2C_{15}H_{31})CH_2OBz$, 97352-39-1; $C_{15}H_{31}C^2H_2OCH_2CH(OC^2H_2C_{15}H_{31})CH_2OH$, 97352-40-4; $C_{15}H_{31}C^2H_2OCH_2CH(OC^2H_2C_{15}H_{31})CH_2OPO_3H_2$, 97352-41-5; $HOCH_2CH_2N^+(CH_3)_3 \cdot B^-(C_6H_4)_4$, 75667-82-2; $C_{15}H_{31}C^2H_2OCH_2CH(OC^2H_2C_{15}H_{31})CH_2OPO_3^-CH_2CH_2N^+(CH_3)_3$, 97352-42-6; head group labeled DHPC, 97352-43-7; 1,2-dihexadecyl-*sn*-glycerol, 67337-03-5; [2,2-$^2H_2$]choline tetraphenylborate, 94953-99-8.

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