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Bilayers of Arachidonic Acid Containing Phospholipids Studied by ^2H and ^{31}P NMR Spectroscopy†

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ABSTRACT: The configurational properties and dynamics of the arachidonic acyl chains of phospholipid bilayers have been investigated for the first time by solid-state ^2H NMR techniques, with the goal of achieving a better understanding of the biological roles of polyunsaturated phospholipids. Vinyl perdeuterated arachidonic acid ($20:4^{\Delta5,8,11,14}$-d_8) was prepared from eicosatetraynoic acid (ETYA) and was esterified with 1-palmitoyl-*sn*-glycero-3-phosphocholine to yield 1-palmitoyl-2-vinylperdeuterioarachidonoyl-*sn*-glycero-3-phosphocholine [(16:0)(20:4-d_8)PC]. ^{31}P NMR spectra of aqueous dispersions of (16:0)(20:4-d_8)PC as well as 1-perdeuteriopalmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine [(per-^2H-16:0)(20:4)PC] were characteristic of the lamellar liquid-crystalline state. The dispersions had similar ^{31}P chemical shift anisotropies, with little apparent motional averaging of the lineshapes due to macroscopic reorientation of liposomes or lateral diffusion of phospholipids about their curved surfaces. Comparison to other phosphatidylcholines indicated that both samples comprised the fully hydrated L_α phase plus excess water. However, the dispersion of (16:0)(20:4-d_8)PC yielded relatively narrow powder-type ^2H NMR spectra, compared to (per-^2H-16:0)(20:4)PC in the liquid-crystalline state. The differences in the ^2H NMR powder patterns thus reflect differences in the configurational properties of the polyunsaturated *sn*-2 arachidonic acyl chain compared to the saturated *sn*-1 palmitic chain. When the powder-type ^2H NMR spectra of the (16:0)(20:4-d_8)PC bilayer were dePaked ($\theta = 0°$), they showed three kinds of deuterons upon integration: one with a large splitting (≈25-35 kHz), two with intermediate splittings (≈10-15 kHz), and the remainder with smaller splittings (≈0.3-5 kHz). The residual quadrupolar couplings of the vinylic >C=C< deuterons were less than those of most C^2H_2 groups of the saturated acyl chain. It is concluded that some of the >C=C< segments are inequivalent and exhibit differences along the arachidonic acyl chain, as found previously for the C^2H_2 groups of the polymethylene chains of saturated phospholipid bilayers. In addition, the ^2H spin-lattice relaxation rates, R_{1Z}, of the vinylic >C=C< deuterons of the *sn*-2 arachidonic acyl chain were larger relative to their quadrupolar splittings than for most C^2H_2 segments of the saturated *sn*-1 chain. Simple models for interpretation of the findings are briefly mentioned and discussed. The results indicate clearly that the structural and dynamic properties of polyunsaturated acyl chains differ from those of saturated and monounsaturated chains in phospholipid bilayers.

^2H NMR[1] spectroscopy provides a useful and general means of obtaining information regarding the orientational order and motion of individual segments of the hydrocarbon chains of lipid bilayers in the liquid-crystalline state (Seelig & Seelig, 1977; Seelig & Browning, 1978; Brown et al., 1979, 1983; Brown, 1979, 1982, 1984; Paddy et al., 1985; Salmon et al., 1987; Thurmond et al., 1991). At present, ^2H NMR spectroscopy has been used mainly for investigations of bilayers containing saturated and monounsaturated acyl chains (Seelig & Seelig, 1974; Seelig & Waespe-Šarčević, 1978; Rance et

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[1] Abbreviations: BHT, butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; ETYA, eicosatetraynoic acid; GC, gas-liquid chromatography; GC-MS, gas chromatography-mass spectrometry; HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; ppm, parts per million; TLC, thin-layer chromatography; (per-^2H-16:0)(20:4)PC, 1-perdeuteriopalmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; (16:0)(20:4-d_8)PC, 1-palmitoyl-2-vinylperdeuterioarachidonoyl-*sn*-glycero-3-phosphocholine; (16:0)(22:6-d_{12})PC, 1-palmitoyl-2-vinylperdeuteriodocosahexaenoyl-*sn*-glycero-3-phosphocholine; (18:1)(18:1-d_2)PC, 1-oleoyl-2-vinylperdeuteriooleoyl-*sn*-glycero-3-phosphocholine.

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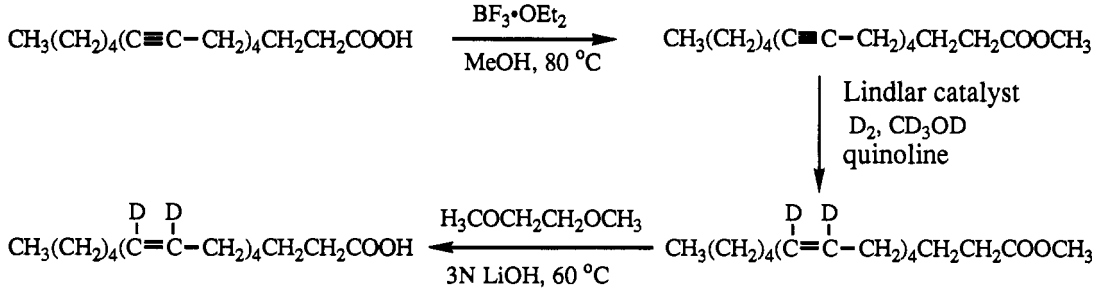
Scheme I



al., 1980). A biosynthetic approach has also been described involving preparation of a specifically deuterated diunsaturated fatty acid for membrane ^{2}H NMR studies (Baenziger et al., 1987, 1988). However, ^{2}H NMR methods have not yet been applied extensively to phospholipids containing more highly polyunsaturated acyl chains. Chemical synthesis and ^{2}H labeling of polyunsaturated fatty acids can be achieved by preparation of polyynoic acids, followed by stereospecific reduction of the acetylenic groups to yield cis double bonds (Kunau, 1971a–c; Taber et al., 1982). Such an approach has been used earlier to prepare 1-palmitoyl-2-vinylperdeuteriodocosahexaenoyl-*sn*-glycero-3-phosphocholine, (16:0)(22:6-d_{12})PC (Dratz & Deese, 1986). Since the analysis of a maximum of 12 superimposable and potentially narrow powder patterns is complex, we decided to synthesize vinyl perdeuterated arachidonic acid having only eight deuterons (20:4$^{\Delta 5,8,11,14}$-d_8) and to conduct ^{2}H NMR studies of aqueous dispersions of 1-palmitoyl-2-vinylperdeuterioarachidonoyl-*sn*-glycero-3-phosphocholine, (16:0)(20:4-d_8)PC, in the liquid-crystalline state.

Arachidonic acid is a normal constituent of phospholipids in many tissues; it is liberated from membranes by the action of phospholipase A_2 and is known to function as a second messenger, e.g., in activating potassium channels in cardiac (Kim & Clapham, 1989) and smooth muscle (Ordway et al., 1989) cells. It serves as a precursor for the biosynthesis of prostaglandins, thromboxanes, and leukotrienes (Lagarde et al., 1989; Stinson, 1989), which modulate cellular functions such as platelet aggregation, smooth muscle contraction, and neural excitation (Needleman et al., 1986). Arachidonic acid and its oxidative metabolites also regulate pathological pain, fever, and inflammation. They are among the most common eicosanoids, and their metabolic interactions are of current interest, especially in blood and vascular cells, where multiple cell types may interact during function (Lagarde et al., 1989). Moreover, arachidonic acid is generally representative of ω6 fatty acids, which seem to have different biological roles compared to ω3 fatty acids (Neuringer et al., 1986; Barcelli, 1988). Studies of bilayers of arachidonoyl phospholipids employing fluorescent probes have been reported previously (Straume & Litman, 1987a,b). Yet at the molecular level direct knowledge of the behavior of phospholipids containing arachidonic acid is currently lacking. Such *direct* studies of the configurational properties and dynamics of polyunsaturated chains are needed to gain insight into their special roles in membrane functions (cf. Crawford et al., 1977; Racker, 1987; Wiedmann et al., 1988).

One hypothesis is that the configurational properties and dynamics of arachidonic acid chains in phospholipid membranes differ intrinsically from those of less unsaturated acyl groups, e.g., due to the *polyallylic structural motif* that may enable *coiled* or *helical* chain configurations (Kunau, 1976; Applegate & Glomset, 1986; Salmon et al., 1987). As a result, selective hydrolysis of arachidonic acid from membrane phospholipids may occur by the action of phospholipase A_2 (cf. Bills et al., 1977). Here we report the first ^{2}H and ^{31}P NMR studies of 1-palmitoyl-2-vinylperdeuterioarachidonoyl-*sn*-glycero-3-phosphocholine, (16:0)(20:4-d_8)PC, and a comparison to parallel studies of 1-perdeuteriopalmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, (per-^{2}H-16:0)(20:4)PC. The results suggest that the >C=C< segments of the polyunsaturated 20:4 chains of the (16:0)(20:4-d_8)PC bilayer, in the liquid-crystalline state, are inequivalent in their motional and configurational behavior, as found previously for the acyl C^2H_2 groups of saturated phosphatidylcholines (Seelig & Seelig, 1974). Moreover, differences in the properties of arachidonic acyl chains in membranes relative to both saturated and monounsaturated phosphatidylcholines are evident.

Materials and Methods

Palmitic and arachidonic acids (>99% purity) were obtained from Sigma (St. Louis, MO) and Nu Chek Prep (Elysian, MN), respectively; eicosatetraynoic acid (ETYA) was procured from Merck & Co. (Teterboro, NJ) as a generous gift; and chain-perdeuterated palmitic acid-d_{31} was prepared as described (cf. Williams et al., 1985). Deuterium gas (99.5%) was purchased from MSD Isotopes (Montreal, Canada). High-pressure liquid chromatography (HPLC) was carried out on a Spectra-Physics (San Jose, CA) SP-8700 system equipped with a Waters (Division of Millipore, Milford, MA) 7.8 mm × 30 cm μBondapak phenyl semipreparative column with MeOH/H_2O/CH_3CN solvent gradients. ^{1}H NMR spectra were acquired with a General Electric GN-500 spectrometer (Fremont, CA), and mass spectra were recorded on a Hewlett Packard model HP-5988A mass spectrometer. Methyl esters of the fatty acids were prepared as described (cf. Williams et al., 1985) and were analyzed with a Hewlett Packard (Palo Alto, CA) model 5880 gas–liquid chromatograph (GC) equipped with a flame ionization detector. A capillary column, 30 m × 0.25 mm packed with fused silica Supelco SPB-1, was used with helium as the carrier gas (0.8 mL/min), the column oven temperature was programmed from 220 to 280 °C, and the injector and detector temperatures were 230 and 300 °C, respectively. Figure 1 shows gas–liquid chromatograms obtained for the various fatty acid methyl esters. It is noteworthy that the retention time of the protiated palmitic acid methyl ester (part a) is longer than that of the perdeuterated palmitic acid methyl ester (part b), as observed previously (Patton et al., 1981).

Synthesis of Vinyl Perdeuterated Arachidonic Acid (20:4-d_8). Arachidonic acid labeled with deuterium at the vinylic positions was synthesized from its tetraacetylene precursor, eicosatetraynoic acid (ETYA), according to Scheme I. The procedure of Taber et al. (1982) was followed except that the reduction was carried out by using the Lindlar catalyst (Kunau, 1971c). First, ETYA was esterified with methanol in the presence of boron trifluoride etherate under a nitrogen

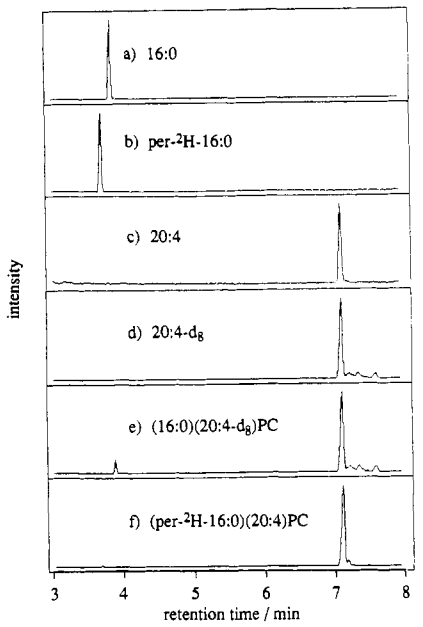


FIGURE 1: Gas–liquid chromatograms of methyl esters of (a) protiated palmitic acid (16:0), (b) perdeuterated palmitic acid (per-^{2}H-16:0), (c) authentic protiated arachidonic acid (20:4), (d) vinyl perdeuterated arachidonic acid (20:4-d_8), (e) fatty acid(s) hydrolyzed from the *sn*-2 position of (16:0)(20:4-d_8)PC by treatment with phospholipase A_2, and (f) fatty acid(s) hydrolyzed from *sn*-2 position of (per-^{2}H-16:0)(20:4)PC by phospholipase A_2. Note that the retention time of the methyl ester of protiated palmitic acid (trace a) is greater than that of the perdeuterated palmitic acid (trace b). The protiated 20:4 arachidonic acid methyl ester (trace c) has about the same retention time as the deuterated 20:4-d_8 acid methyl ester (trace d). Trace d includes approximately 5% impurities due to over or incomplete reduction of ETYA, yielding longer retention times compared to arachidonic acid. In trace e a minor peak is evident near 3.9 min, which corresponds to protiated palmitic acid (trace a), indicating about 6% acyl migration in the product (16:0)(20:4-d_8)PC after completion of the experiments. Trace f reveals on vertical expansion a minor peak near 3.7 min, which is similar to perdeuterated palmitic acid, evincing the occurrence of less than 1% acyl migration in the (per-^{2}H-16:0)(20:4)PC product.

atmosphere and then hydrogenated in $C^2H_3O^2H$ with the Lindlar catalyst in the presence of quinoline and deuterium gas. The crude methyl ester of vinyl perdeuterated arachidonic acid was chromatographed on a column of 70–230 mesh silica gel 40 (E. Merck, Darmstadt, West Germany), with 1.5% ethylacetate in petroleum ether. It was then hydrolyzed to vinyl perdeuterated arachidonic acid in 1,2-dimethoxyethane with 3 N aqueous LiOH. Finally, it was purified by high-pressure liquid chromatography. The purity of the products at every step was checked by ^{1}H NMR spectroscopy and gas chromatography–mass spectrometry (GC–MS). Comparison of ^{1}H NMR spectra of an authentic sample of arachidonic acid and the vinyl perdeuterated arachidonic acid (Figure 2) revealed almost 90% deuterium incorporation at the vinylic positions. The GC of the methyl ester of vinylperdeuterated arachidonic acid had almost the same retention time (part d of Figure 1) as its protiated arachidonic acid counterpart (part c), and it gave a molecular ion of MH^+ equal to 327 in the mass spectrum. The GC in part d of Figure 1 indicates approximately 5% of impurities, which may be due to over or incomplete reduction of ETYA.

Preparation of (16:0)(20:4-d_8)PC and (per-^{2}H-16:0)-(20:4)PC. The symmetric disaturated phospholipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine was first synthesized from the anhydride of palmitic acid and the cadmium chloride adduct of *sn*-glycero-3-phosphocholine (cf. Williams et al., 1985). It was then treated with snake venom phospholipase

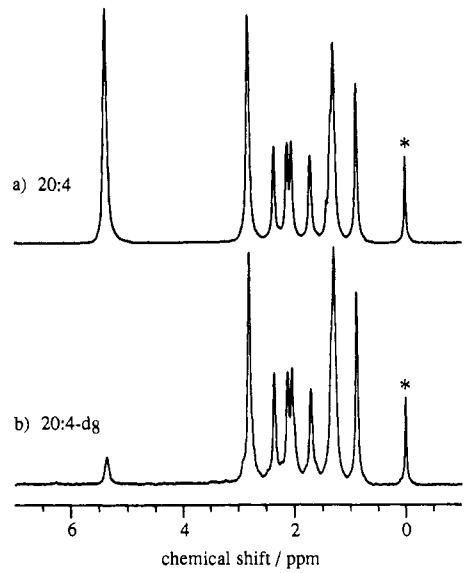
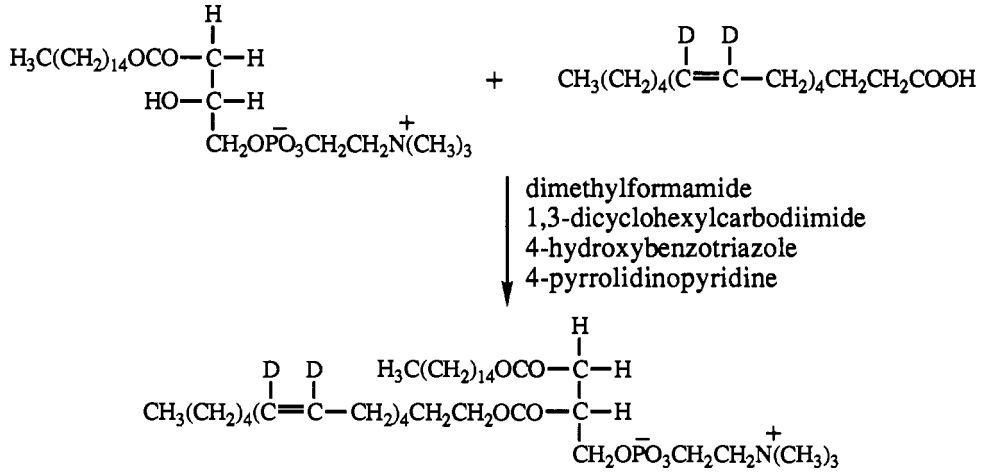


FIGURE 2: Comparison of ^{1}H NMR spectra of (a) an authentic sample of protiated arachidonic acid and (b) vinyl perdeuterated arachidonic acid (20:4-d_8) in C^2HCl_3. The chemical shifts are relative to tetramethylsilane (*). Note that the intensity of the vinylic protons at approximately 5.4 ppm in spectrum b is decreased substantially due to deuterium incorporation as compared to that in spectrum a. Integration of spectrum b revealed almost 90% incorporation of deuterium into arachidonic acid at the vinylic positions. The carboxyl proton resonance that appears near 12.5 ppm in both cases is not shown.

A_2 from *Crotalus adamanteus* (Sigma, MO) to stereospecifically hydrolyze the *sn*-2 palmitoyl chain, yielding the corresponding lysophospholipid, 1-palmitoyl-*sn*-glycero-3-phosphocholine, as described (Mason et al., 1981). Hydrolysis of the starting phosphatidylcholine was essentially complete (>99%), indicating only the natural L stereoisomer. The lysophosphatidylcholine was reacylated with the vinyl perdeuterated arachidonic acid following the procedure of Liman and O'Brien (1988) in dimethylformamide, in the presence of 1,3-dicyclohexylcarbodiimide, 4-hydroxybenzotriazole, and 4-pyrrolidinopyridine, to yield the mixed-chain phosphatidylcholine (16:0)(20:4-d_8)PC as shown in Scheme II. The method of Liman and O'Brien (1988) precludes the additional step of preparing the acid anhydride (Mason et al., 1981) and works very well even for quantities of a few milligrams of arachidonic acid. The yield is 32% with respect to the fatty acid, compared to only 12% with the anhydride procedure. Similarly, (per-^{2}H-16:0)(20:4)PC was prepared from the corresponding saturated phospholipid, 1,2-diperdeuteriopalmitoyl-*sn*-glycero-3-phosphocholine, by reacylation of the lysophosphatidylcholine with protiated arachidonic acid. The final phosphatidylcholines were then purified by column chromatography on silicic acid (Bio-Sil A, 110–200 mesh; Bio-Rad, Richmond, CA) with a $CHCl_3$/MeOH solvent gradient containing butylated hydroxytoluene (BHT) (1 mg per 1000 mL). The purity was checked by thin-layer chromatography (TLC) with $CHCl_3$/MeOH/H_2O (65/35/5), followed by charring the TLC plate with 40% H_2SO_4 in ethanol. The isomeric purity of the (16:0)(20:4-d_8)PC and (per-^{2}H-16:0)(20:4)PC products was determined by treatment with snake venom phospholipase A_2 from *C. adamanteus*, followed by transesterification of the *sn*-2 hydrolyzed fatty acids with BCl_3/MeOH (Supelco, PA) and their identification by gas–liquid chromatography (cf. Figure 1). The results of gas–liquid chromatography for the (16:0)(20:4-d_8)PC sample after completion of the experiments (part e of Figure 1) indicated approximately 6% acyl migration. In the case of

Scheme II



(per-^{2}H-16:0)(20:4)PC less than 1% acyl migration was found (part f).

NMR Spectroscopy. Approximately 200 mg of (16:0)-(20:4-d_8)PC were dispersed in an equal weight of 20 mM Tris buffer, pH 7.5, prepared from ^{2}H-depleted ^1H_2O (Aldrich, WI) and containing 10^{-4} M EDTA. The sample was subjected to freeze–thaw–vortex cycles under argon to ensure complete dispersion of the lipid; it was vortexed less than a minute each time to minimize the formation of small liposomes or vesicles. Similarly, a sample of (per-^{2}H-16:0)(22:6)PC was prepared in 67 mM sodium phosphate buffer, pH 7.1. ^{2}H NMR spectra were acquired with a General Electric GN-300 spectrometer (Fremont, CA), operating at 46.13 MHz (magnetic field strength of 7.058 T) for deuterium and equipped with a home-built high-power transverse solenoid probe. The 90° pulse length was typically 6 μs. A phase-cycled quadrupolar echo pulse sequence (Davis et al., 1976) was employed with a pulse spacing of 40 μs. Data were acquired beginning at 30 μs after the second pulse and were Fourier transformed carefully beginning at the top of the echo. The pulse sequence was repeated every 500 ms, and the spectra were acquired with a digitization rate (spectral width) of 500 kHz. Apodization of the echo decays corresponded to a 100-Hz line broadening in the frequency domain. The ^{2}H NMR spectra were then transferred to a Digital Equipment Corporation Microvax II computer and deconvoluted numerically to obtain subspectra corresponding to the $\theta = 0°$ orientation of the bilayer normal relative to the main magnetic field, by using the dePakeing algorithm of Bloom et al. (1981). Further data reduction was carried out by employing the NMR1 software program (New Methods Research, Inc., Syracuse, NY). Spin-lattice relaxation experiments were performed by using a phase-cycled composite-pulse quadrupolar echo sequence (Bloom et al., 1980; Freeman et al., 1980; Williams et al., 1985). The spin-lattice relaxation times of each of the resolved splittings of the dePaked spectra were obtained with the NMR1 program, which employs a three-parameter decaying exponential function. Proton-decoupled ^{31}P NMR spectra were recorded with the commercial high-resolution broad-band probe of the General Electric GN-300 spectrometer operating at 121.65 MHz, with a phase-cycled Hahn spin echo sequence (cf. Rance & Byrd, 1983). A pulse spacing of 20 μs was employed, and the data were Fourier transformed beginning at the top of the echo with a line broadening of 50 Hz; the recycle delay for the pulse sequence was 1 s.

Results and Discussion

NMR Spectral Lineshapes of Arachidonoyl Phospholipids in the Liquid-Crystalline State.

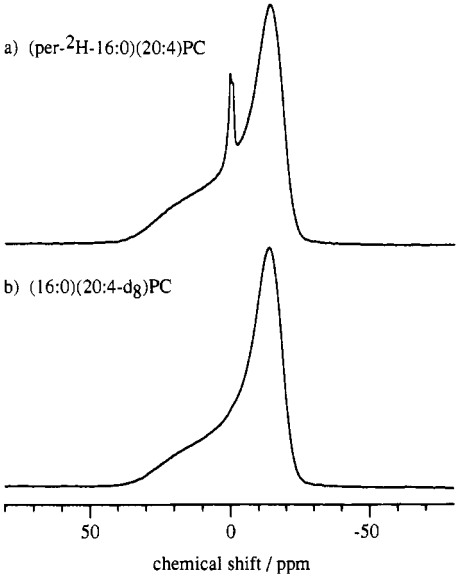


FIGURE 3: ^{1}H-decoupled ^{31}P NMR spectra of aqueous dispersions of (a) (per-^{2}H-16:0)(20:4)PC and (b) (16:0)(20:4-d_8)PC in the liquid-crystalline state at 25 °C. The sample in part a contained 50 wt % of 67 mM phosphate buffer at pH 7.1, whereas that in part b contained 50 wt % of 20 mM Tris buffer at pH 7.5. The relatively narrow isotropic peak near the center of spectrum a arises from the phosphate buffer and does not affect interpretation of the results. In both cases the observed chemical shift anisotropy ($\Delta\sigma$) is indicative of the lamellar liquid-crystalline state. Motional averaging of the ^{31}P NMR powder patterns by macroscopic reorientation of liposomes is small or negligible, and multiple spectral components are largely absent.

Figure 3 shows a comparison of representative ^{31}P NMR spectra of aqueous dispersions of (per-^{2}H-16:0)(20:4)PC and (16:0)(20:4-d_8)PC, each containing 50 wt % water at 25 °C. The ^{31}P NMR spectra of both phosphatidylcholines are very similar in shape and are characteristic of the lamellar liquid-crystalline state (Seelig, 1978). The narrow peak near 0 ppm in part a of Figure 3 arises from inorganic phosphorus in the buffer and does not affect interpretation of the results. The residual phosphorus chemical shift anisotropy ($\Delta\sigma$) can be obtained directly from the edges of the axially symmetric powder patterns and is about $\Delta\sigma \approx$ 45 ppm in both cases. Comparison to results for other phospholipid dispersions (Seelig, 1978) indicates that both samples comprise the fully hydrated liquid-crystalline L_α phase plus excess water (two-phase region with no swelling). No evidence for multiple spectral components, aside from the buffer peak, is found, consistent with the results of ^{2}H NMR

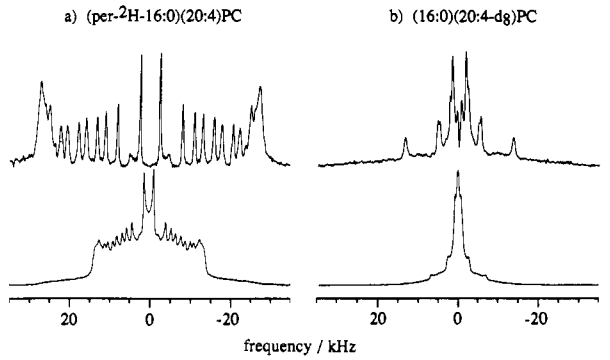


FIGURE 4: Comparison of ^2H NMR spectra of aqueous multilamellar samples of (a) (per-^2H-16:0)(20:4)PC and (b) (16:0)(20:4-d_8)PC in the liquid-crystalline state at 15 °C. The dispersion in part a included 50 wt % of 67 mM phosphate buffer at pH 7.1 and was identical to that in Figure 3. The sample in part b contained 50 wt % of 20 mM Tris buffer at pH 7.5. In each case, the experimental powder-type ^2H NMR spectra are given at bottom and the corresponding dePaked spectra ($\theta = 0°$) at top on the same frequency scale. The ^2H NMR spectra of the *sn*-1 palmitic and *sn*-2 arachidonic acyl groups reflect differences in the configurational properties of the saturated and polyunsaturated chains of the membrane phospholipids.

spectroscopy (vide infra). In addition, the ^{31}P NMR spectra suggest that motional averaging of the lineshapes due to liposome reorientation is minimal in each of the two samples (cf. Burnell et al., 1980; Larsen et al., 1987). The observed chemical shift anisotropy ($\Delta\sigma$) sets a lower limit for the correlation time due to macroscopic reorientation of liposomes and/or diffusion about their curved surfaces, which is about $\tau_c > 1/\Delta\sigma \cong 10^{-4}$ s in each case.

By contrast, ^2H NMR spectra of aqueous dispersions of the two samples are dramatically different. Typical results for randomly oriented multilamellar dispersions of (per-^2H-16:0)(20:4)PC and (16:0)(20:4-d_8)PC, each containing 50 wt % H_2O in the liquid-crystalline state at 15 °C, are shown at the bottom in parts a and b of Figure 4, respectively. In each case, the ^2H NMR spectra comprise a series of overlapping powder patterns arising from the various deuterated chain segments. For a random distribution of bilayers, the sharp spectral features or edges correspond to the $\theta = 90°$ orientation of the bilayer normal relative to the magnetic field direction, and the less well-defined shoulders with smaller intensity represent the $\theta = 0°$ orientation. The powder patterns are indicative of axially symmetric motion of the C–^2H bonds of the acyl chain segments about a preferred direction, which is known from studies of other phospholipid systems to represent the macroscopic bilayer normal (director axis). The ^2H NMR spectrum of the (per-^2H-16:0)(20:4)PC bilayer in part a of Figure 4 is representative of mixed-chain polyunsaturated phosphatidylcholines in the liquid-crystalline state (cf. Salmon et al., 1987). By comparison, the ^2H NMR spectrum of the dispersion of (16:0)(20:4-d_8)PC in part b of Figure 4 shows a relatively narrow powder pattern. The rather narrow breadth of the powder pattern of (16:0)(20:4-d_8)PC, which contains a vinyl perdeuterated *sn*-2 arachidonic acyl chain, may be due to various factors. First of all, one can rule out the possibility of significant motional averaging of the ^2H NMR lineshape of (16:0)(20:4-d_8)PC or, less likely, of the sample being in a nonlamellar state. Rather, the ^{31}P NMR spectrum of the same sample is indicative of the lamellar liquid-crystalline state (cf. part b of Figure 3) and is similar to that of (per-^2H-16:0)-(20:4)PC. The essentially identical ^{31}P chemical-shielding anisotropies of both phosphatidylcholines imply that the differences in their ^2H NMR powder-type spectra arise mainly from differences in the configurational properties of the *sn*-1 palmitoyl and *sn*-2 arachidonic acyl chains. It is conceivable that the narrow ^2H NMR spectrum of the polyunsaturated arachidonic acyl chain of (16:0)(20:4-d_8)PC represents a larger angular range of statistical fluctuations of the >C=C< segment orientations, that is compared to the C^2H_2 groups of the saturated palmitoyl chain. But other explanations are possible, including the influences of geometric effects (cf. Brown & Seelig, 1977; Seelig & Waespe-Šarčevič, 1978). For example, given an energy-minimized angle-iron or helical-shaped configuration of the arachidonic acyl chain (cf. Applegate & Glomset, 1986; Salmon et al., 1987), it could be that some of the vinylic C–^2H bonds are fluctuating rapidly about preferred average orientations near the magic angle (54.7°).

Table I: Deuterium Quadrupolar Splittings and Corresponding Spin-Lattice Relaxation Times of (per-^2H-16:0)(20:4)PC and (16:0)(20:4-d_8)PC in the Liquid-Crystalline State at 30 °C

(per-^2H-16:0)(20:4)PC		(16:0)(20:4-d_8)PC	
$\Delta\nu_Q$ (kHz)[a]	T_{1Z} (ms)[b]	$\Delta\nu_Q$ (kHz)[a]	T_{1Z} (ms)[b]
47.7	23.1 ± 0.3	24.2	14.8 ± 1.6
44.3	23.8 ± 0.2	11.7	26.1 ± 1.2
42.2	23.5 ± 0.3	4.52	33.2 ± 0.6
37.1	26.5 ± 0.7	0.85	31.7 ± 1.0
33.7	29.1 ± 0.5		
28.6	32.9 ± 0.5		
24.9	41.6 ± 0.5		
20.5	50.8 ± 0.6		
16.8	72.4 ± 1.2		
12.3	105 ± 5		
3.54	346 ± 5		

[a] Residual deuterium quadrupolar splittings measured from de-Paked ^2H NMR spectra ($\theta = 0°$). [b] Spin-lattice relaxation times obtained from de-Paked partially relaxed ^2H NMR spectra at a magnetic field strength of 7.06 T. The spin-lattice relaxation rate $R_{1Z} \equiv 1/T_{1Z}$, where T_{1Z} is the spin-lattice relaxation time.

In order that the differences between the ^2H NMR spectra of the two liquid-crystalline phosphatidylcholines are clearly demonstrated, they are also displayed as dePaked spectra ($\theta = 0°$) at the top in parts a and b of Figure 4. In the case of the (per-^2H-16:0)(20:4)PC bilayer, the segmental order parameters, S_{CD}, of the *sn*-1 palmitoyl chain derived from the dePaked splittings (not shown) are similar to those of other mixed-chain polyunsaturated phosphatidylcholines (cf. Salmon et al., 1987). However, the residual splittings of the majority of the vinylic deuterons of the *sn*-2 arachidonic acyl chain are substantially smaller than those of most deuterons of the saturated *sn*-1 chain (cf. Figure 4 and representative data in Table I). For the $\theta = 0°$ orientation, Figure 4 indicates that at 15 °C the maximum residual coupling observed for the (per-^2H-16:0)(20:4)PC bilayer is ≈55 kHz, whereas it is only ≈25 kHz for (16:0)(20:4-d_8)PC. Upon integration, the de-Paked ($\theta = 0°$) ^2H NMR spectrum of (16:0)(20:4-d_8)PC reveals three types of deuterons: one showing the largest splitting (≈25 kHz), two deuterons in the intermediate range of splittings (≈10 kHz), and the remainder with smaller splittings (≈0.3–5 kHz). ^2H NMR spectra obtained as a function of temperature for (16:0)(20:4-d_8)PC in the liquid-crystalline state over the range from −10–40 °C are depicted in Figure 5. The experimental powder-type spectra are indicated at left (a), and the corresponding dePaked ^2H NMR results are shown at right (b). In general, the observed quadrupolar splittings decrease with increasing temperature, except possibly those nearest the spectral center. Recall that ^{31}P NMR spectra obtained over the same range of temperatures (cf. Figure 3) indicate only a single spectral component with an axially symmetric powder pattern.

The quadrupolar splittings obtained from the dePaked ^2H NMR spectra for the vinyl perdeuterated arachidonyl chain

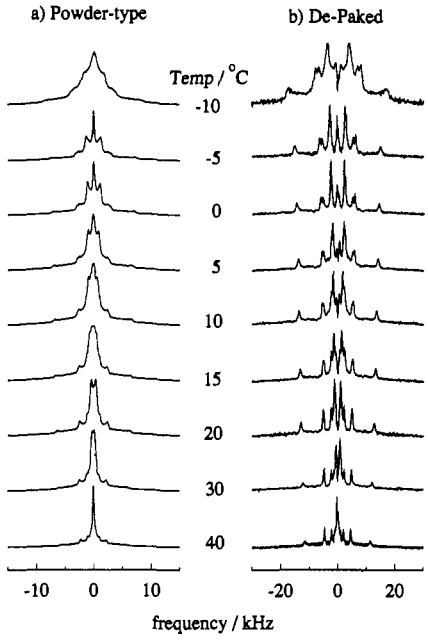


FIGURE 5: ^{2}H NMR spectra of an aqueous (16:0)(20:4-d_8)PC dispersion, containing 50 wt % of 20 mM Tris buffer at pH 7.5, as a function of temperature in the liquid-crystalline state. The experimental powder-type ^{2}H NMR spectra of the aqueous dispersion of randomly oriented bilayers are depicted at left (a) and the corresponding dePaked ^{2}H NMR spectra at right (b). The temperatures (°C) are indicated in the figure. A number of individual quadrupolar splittings are evident that decrease as the temperature is increased, with the possible exception of those nearest the spectral center.

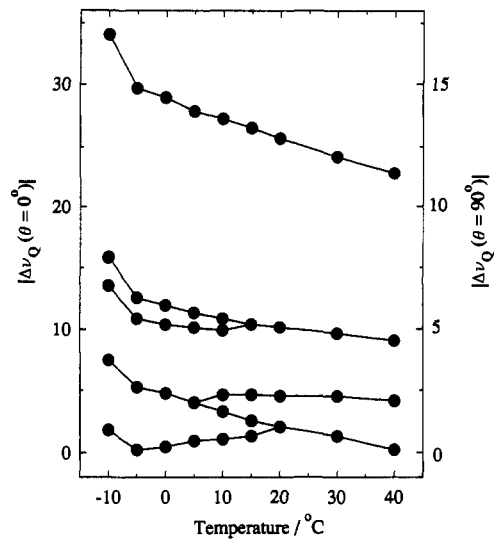


FIGURE 6: Plot of quadrupolar splittings as a function of temperature for an aqueous dispersion of (16:0)(20:4-d_8)PC, containing 50 wt % of 20 mM Tris buffer at pH 7.5. The left-hand axis indicates the splittings obtained from the dePaked ^{2}H NMR spectra due to the $\theta = 0°$ orientation of the director axis relative to the main magnetic field. The right-hand axis refers to the splittings calculated for the $\theta = 90°$ orientation by using the relation $|\Delta\nu_Q(\theta)| = |\Delta\nu_Q(\theta = 0°)P_2(\cos\theta)|$, where P_2 is the second Legendre polynomial and corresponds to results typically measured in ^{2}H NMR spectroscopy. One of the splittings is relatively large in absolute value and is well separated from the remainder closer to the spectral center; most splittings decrease with increasing temperature.

of the (16:0)(20:4-d_8)PC bilayer are summarized as a function of temperature in Figure 6. Such plots of the quadrupolar splittings versus temperature generally resemble previous results for the vinylic >C=C< deuterons of phosphatidylcholines containing monounsaturated and diunsaturated acyl chains (Seelig et al., 1981; Baenziger et al., 1987). At all temperatures, a component with a relatively large splitting is evident in the dePaked ^{2}H NMR spectra ($\Delta\nu_Q \approx 25$–35 kHz), together with contributions from other deuterons whose splittings are smaller and are clustered near the spectral center ($\Delta\nu_Q \approx 0$–15 kHz). The above values are similar to those observed for systems containing oleoyl chains deuterated at the cis-Δ^9 double bond, viz., 18:1$^{\Delta 9}$-d_2 (Seelig & Waespe-Šarčevič, 1978; Rance et al., 1980; Seelig et al., 1981), as well as phosphatidylcholines with isolinoleoyl chains deuterated at the cis-Δ^9 double bond, 18:2$^{\Delta 6,9}$-d_2 (Baenziger et al., 1987), or vinyl perdeuterated docosahexaenoyl chains, 22:6$^{\Delta 4,7,10,13,16,19}$-$d_{12}$ (Dratz & Deese, 1986). However, differences are also evident, for example the observed splittings (cf. Figure 6) do not match exclusively those obtained for phospholipids containing either oleoyl or isolinoleoyl chains over the indicated range of temperatures. Rather, the ^{2}H NMR spectra (cf. Figures 4 and 5) appear to represent a superposition of contributions resembling both the monounsaturated and diunsaturated acyl chains of phosphatidylcholines in the liquid-crystalline state.

From the above experimental results, one can draw some general conclusions regarding the average orientations of the arachidonyl cis double bonds. First, it should be remarked that the ^{2}H NMR spectra are very sensitive to geometrical effects, in addition to statistical fluctuations described in terms of a molecular order parameter (cf. Brown & Seelig, 1977; Seelig & Waespe-Šarčevič, 1978; Baenziger et al., 1987). Only the natural L stereoisomer of the glycerol backbone is present, so that one can rule out the possibility that enantiomers with different absolute configurations at the glycerol *sn*-2 chiral center yield different residual couplings (cf. Olsson et al., 1990). The dePaked ^{2}H NMR spectrum of the (16:0)(20:4-d_8)PC bilayer in part b of Figure 4 shows up to six splittings, in which the differences between some of the adjacent peaks vary from ≈1 to 3 kHz. It is likely that the various splittings are quadrupolar in origin, since the direct ^{2}H–^{2}H and ^{2}H–^{1}H dipolar couplings of vicinal nuclei are in the range of only 0.01–0.1 kHz in liquid crystals (Diehl & Tracey, 1975; Emsley, 1985) and in the liquid-crystalline state of lipid bilayers (Jarrell et al., 1987). The static (maximum) ^{2}H–^{2}H and ^{2}H–^{1}H dipolar coupling constants are calculated to be 0.181 and 1.18 kHz, respectively, assuming a fixed internuclear distance of $r = 2.5$ Å (cf. Diehl & Khetrapal, 1969). If it is supposed that the various couplings are quadrupolar in origin, then the presence of up to six splittings in the dePaked ^{2}H NMR spectra of (16:0)(20:4-d_8)PC (cf. part b of Figure 4) suggests that the two deuterons of some of the four cis double bonds are inequivalent in their average orientations. Otherwise a maximum of four splittings would be seen. Because each vinylic segment has two deuterons, it follows that at least three of the cis double bonds are oriented somewhat differently with respect to the bilayer normal, on average, to explain the presence of up to six splittings. Thus (i) three of the four >C=C< groups are inequivalent and have different average tilt angles relative to the bilayer normal, each giving rise to two quadrupolar splittings (inequivalent deuterons). Alternatively (ii), all four >C=C< groups are inequivalent, two differing in their average tilt angles, each giving rise to two splittings, and two with average parallel orientations resulting in single splittings (equivalent deuterons). The vinylic >C=C< segments may thus exhibit differences in their order parameters along the polyunsaturated arachidonic acyl chain, as found previously for the C^2H_2 groups of the polymethylene chains of saturated phospholipid bilayers (Seelig & Seelig, 1974).

Beyond the above, little more can be said at present with certainty regarding the average orientations of the cis double bonds of the arachidonic acyl chains of the (16:0)(20:4-d_8)PC

bilayer. Further information is needed to assign the residual couplings to specific deuterons of the arachidonic acyl chain and to define completely the ordering tensors of the individual cis double bonds. The latter in turn require determination of additional order parameters due to the C_{2v} symmetry of the vinylic segments (cf. Seelig & Waespe-Šarčevič, 1978) and necessitate additional experimental studies. However it is useful to compare the observed quadrupolar splittings to those currently available for monounsaturated (Seelig & Waespe-Šarčevič, 1978; Rance et al., 1980; Seelig et al., 1981) and diunsaturated (Baenziger et al., 1987) systems. The observation of a splitting in the range of ≈25–35 kHz in the dePaked ^{2}H NMR spectrum ($\theta = 0°$), apparently corresponding to a single deuteron (cf. Figure 6), suggests that one of the cis double bonds may have an average orientation resembling the cis-Δ^9 double bond of the monounsaturated *sn*-2 oleoyl chain, 18:1$^{\Delta 9}$-d_2, of phosphatidylcholines (cf. Seelig & Waespe-Šarčevič, 1978; Seelig et al., 1981). The spin-lattice (R_{1Z}) relaxation rate is also larger for this splitting (vide infra). The remaining segments of the polyunsaturated chain may then have average orientations similar to the second cis-Δ^9 double bond of the *sn*-2 isolinoleoyl chain, 18:2$^{\Delta 6,9}$-d_2, of diunsaturated phosphatidylcholines [cf. Figure 3 of Baenziger et al. (1987)].[2] One can hypothesize that the presence of a single double bond in an unsaturated chain may disrupt the packing of the bilayer hydrocarbon region. The other >C=C< segments of a polyunsaturated chain may then adopt average orientations that reflect to a greater degree the polyallylic rhythm of alternating vinyl and methylene groups. This is consistent with the observation that the physical properties of phosphatidylcholines change very little with introduction of more than a single cis double bond into the acyl chains (cf. Demel et al., 1972). It is noteworthy that a polyallylic acyl chain comprising cis double bonds is stereochemically analogous to a polypeptide chain with trans amide linkages, such as polyglycine (cf. Dickerson & Geis, 1969). Consequently, the formation of preferred regular structures such as helices is possible (cf. Salmon et al., 1987).

Deuterium Spin-Lattice Relaxation Rate Studies. Information regarding the molecular dynamics of phospholipids can be obtained from analysis of their spin-lattice relaxation rates, R_{1Z}, which include contributions from the types, rates, and amplitudes of their motions. We have also carried out R_{1Z} studies of (per-^{2}H-16:0)(20:4)PC and (16:0)(20:4-d_8)PC in the liquid-crystalline state. Table I includes representative values of the spin-lattice relaxation times, T_{1Z}, where $R_{1Z} \equiv 1/T_{1Z}$. In general, the magnitudes of the ^{2}H R_{1Z} rates of the C^2H_2 and >C=C< groups are similar to those of phosphatidylcholines with saturated and monounsaturated acyl chains, respectively, at comparable magnetic field strengths (Brown et al., 1979; Williams et al., 1985). The behavior of the >C=C< groups of the polyunsaturated *sn*-2 arachidonyl acyl chain differs from the C^2H_2 segments of the saturated *sn*-1 chain in that the R_{1Z} rates tend to be somewhat larger relative to their quadrupolar splittings (Table I). Similar observations have been made previously for the vinylic segments of the oleoyl chains of (18:1)(18:1-d_2)PC in the liquid-crystalline state (Brown et al., 1979) and also in microbial membranes (cf. Smith, 1985). The spin-lattice relaxation rate, R_{1Z}, decreases (that is T_{1Z} increases) with increasing temperature in the case of the vinylic deuterons of the arachidonic acyl chain of (16:0)(20:4-d_8)PC (not shown), as observed for other phospholipids (Brown et al., 1983). In addition, R_{1Z} is observed to decrease (T_{1Z} increases) with decreasing quadrupolar splitting, both for the (per-^{2}H-16:0)(20:4)PC bilayer containing a perdeuterated *sn*-1 palmitoyl chain as well as for the polyunsaturated vinyl perdeuterated *sn*-2 arachidonic acyl chain of the (16:0)(20:4-d_8)PC bilayer (Table I). It is possible that the R_{1Z} values of the >C=C< groups decrease along the polyunsaturated arachidonyl chain, as observed for saturated phosphatidylcholines in the L_α phase (Brown et al., 1979).

In order for the relaxation data and their variation with the quadrupolar splittings of the (16:0)(20:4-d_8)PC bilayer in the liquid-crystalline state to be further explained, models for the acyl chain dynamics need to be introduced. A detailed discussion is beyond the scope of this paper, but some general observations can be made by considering three possible relaxation models available at present (Brown, 1982, 1990; Brown & Söderman, 1990). If (i) the relaxation is described in terms of fast effective *segmental motions* (cf. Brown et al., 1979; Brown, 1982), e.g., torsional oscillations, trans–gauche isomerizations, molecular librations etc., that modulate the *static* electric field gradient tensor, this suggests that the >C=C< segments would have somewhat larger ^{2}H relaxation rates compared to the C^2H_2 groups on account of their larger size and hindrance to rotational isomerization of the polyvinylic chain (cf. Brown, 1979; Brown et al., 1979). It follows that a profile of the rates of motion of the >C=C< groups would exist along the arachidonic acyl chain. Alternately (ii) one can consider a model in which the relaxation is described in terms of order fluctuations due to molecular motions (*noncontinuum model*; cf. Brown, 1982; Brown & Söderman, 1990). Long-axis rotation and wobbling or tilting of the acyl chains would then modulate the *residual* electric field gradient tensor, i.e., quadrupolar coupling, remaining after preaveraging by faster segmental motions that do not contribute strongly to the relaxation. An inherent feature of such a picture is that separation of time scales into relatively fast and slow motions is assumed (Brown, 1982). The slow motions then involve rotations about molecular principal axes, for which the moments of inertia are reduced by preaveraging over the coordinates of more rapid segmental fluctuations (Brown & Söderman, 1990). Finally (iii) one can also consider an analysis in which relatively slow-order fluctuations are formulated for simplicity in terms of motion of a local director axis (*continuum model*; cf. Brown, 1982; Marqusee et al., 1984; Brown et al., 1990). This model shares features in common with the above molecular picture in that it is the *residual* tensor remaining from faster motions that is modulated by slower motions leading to the relaxation. As recently suggested (Bonmatin et al., 1988; Jarrell et al., 1988; Brown & Söderman, 1990), such a treatment may constitute an oversimplification and may not suffice to fully explain the relaxation of the acyl chains in membranes. Either of the latter two models predicts that similar motions govern the ^{2}H relaxation in the case of both the >C=C< and C^2H_2 segments: any variations would primarily represent differences in the residual interaction tensors modulated by the slow motions. Since the breadth of the ^{2}H NMR spectra of the >C=C< segments is much less than for most of the C^2H_2 groups (cf. Figure 4), it is plausible that the residual tensors governing the relaxation are proportionately smaller in the former case. This in turn would suggest a *smaller* ^{2}H R_{1Z} relaxation rate due to order fluctuations. But experimentally one observes a *larger* relaxation rate for the >C=C< segments versus those C^2H_2 groups having comparable quadrupolar splittings. Hence

[2] Comparative studies of phospholipids containing isomers of octadecadienoic acid deuterated at the cis-Δ^9 double bond, viz., linoleic acid, 18:2$^{\Delta 9,12}$-d_2, and isolinoleic acid, 18:2$^{\Delta 6,9}$-d_2, could resolve this question (cf. Baenziger et al., 1987).

it is possible that a greater contribution from local motions exists in the former case; geometrical effects may also be important (cf. Brown, 1982).

Conclusions

The current studies demonstrate the utility of ^{2}H NMR methods for direct investigation of the configurational properties and dynamics of polyunsaturated chains in model and biological membranes and thus open up a new avenue for characterizing highly unsaturated lipids. Our initial ^{2}H NMR investigations of (16:0)(20:4-d_8)PC evince that the structural and dynamical properties of the arachidonic acyl chain are different from those of saturated and monounsaturated acyl chains. Compared to saturated phosphatidylcholines, the smaller quadrupolar splittings of the arachidonic acyl chain could reflect geometrical factors or statistical fluctuations, whereas the somewhat larger R_{1Z} relaxation rates versus the residual couplings may indicate hindered rotations of the chain due to the larger vinylic segments (Brown et al., 1979). It is concluded that some of the >C=C< groups are inequivalent in their motional behavior, leading to differences along the polyunsaturated acyl chain. A more detailed interpretation rests upon further investigations to determine the double bond orientations and molecular order parameters. Such studies will enable detailed comparison of the properties of bilayers of polyunsaturated lipids to those of saturated and less unsaturated systems and thus contribute to understanding their special biological roles.

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Sequential Assignments of the ^1H NMR Resonances of $Zn(II)_2$ and $^{113}Cd(II)_2$ Derivatives of the DNA-Binding Domain of the GAL4 Transcription Factor Reveal a Novel Structural Motif for Specific DNA Recognition†

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ABSTRACT: The DNA-binding domain of the GAL4 transcription factor, consisting of the 62 N-terminal amino acid residues and denoted GAL4(62*), contains a novel $Zn(II)_2Cys_6$ or $Cd(II)_2Cys_6$ binuclear cluster [Pan, T., & Coleman, J. E. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2077]. Specific DNA recognition requires residues located within as well as C terminal to this binuclear cluster. ^1H NMR sequential assignments have been carried out on $Zn(II)_2$- and $^{113}Cd(II)_2$GAL4(62*) by using DQF-COSY, relayed COSY, double-relayed COSY, and NOESY. The ligands of the two tetrahedral metal-binding sites have been identified as Cys^{11}, Cys^{14}, Cys^{21}, and Cys^{31} to one metal ion and Cys^{28}, Cys^{38}, Cys^{21}, and Cys^{31} to the other metal ion with Cys^{21} and Cys^{31} as ligands shared between the two metal ions. No α-helices can be found within the GAL4(62*) structure, which consists of a series of turns to accommodate the metal cluster, followed by irregular loops and turns from residues 42 to 60, the "specificity region", whose sequence contributes importantly to specific DNA recognition. Long-distance NOE's are observed between residues forming the binuclear cluster and several residues within the specificity region, indicating that the latter is folded compactly onto the metal cluster. The requirement of the $Zn(II)_2Cys_6$ binuclear cluster and the specificity region for binding to DNA reveals GAL4 as a member of a class of specific DNA-binding proteins using a new structural motif for the recognition of specific DNA sequences. Specific DNA binding by this class of proteins is achieved by use of turns and loops that enclose a $Zn(II)_2Cys_6$ binuclear cluster, instead of α-helices or β-strands as observed in specific DNA-binding proteins described previously.

The GAL4 protein is a transcription factor from *Saccharomyces cerevisiae* required for the transcriptional activation of the genes encoding the galactose-metabolizing enzymes [for a review, see Johnston (1987a)]. Although the intact GAL4 protein consists of 881 amino acid residues, its DNA-binding domain has been located within the 62 N-terminal residues by limited proteolysis (Pan & Coleman, 1990a,b). The DNA-binding domain of GAL4 contains a Cys^{11}-X_2-Cys^{14}-X_6-Cys^{21}-X_6-Cys^{28}-X_2-Cys^{31}-X_6-Cys^{38} motif, conserved among a group of eleven fungal transcription factors (Andre, 1990). The cysteine cluster in GAL4 has been proposed (Johnston, 1987b) and was shown to bind Zn(II) (Pan & Coleman, 1989). The binding of Zn(II) is required for specific DNA recognition (Pan & Coleman, 1989). Cd(II) can also efficiently restore specific DNA binding to the apo GAL4 protein (Pan & Coleman, 1989). Contrary to prediction, two rather than one Zn(II) have been found to bind tightly to the DNA-binding domain of GAL4 (Pan & Coleman, 1989, 1990a). Both ^{113}Cd NMR and phase-sensitive ^1H COSY show unambiguously that only the six highly conserved cysteine residues act as ligands to Zn(II) or Cd(II) (Pan & Coleman, 1990a,b). ^1H-^{113}Cd coupling patterns have identified two cysteine residues in which the $-S^-$ is shared between the two bound metal ions (Pan & Coleman, 1990b).

On the basis of these observations, we have proposed that GAL4 forms a $Zn(II)_2Cys_6$ or $Cd(II)_2Cys_6$ binuclear cluster

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