^{2}H NMR Studies of Isomeric ω3 and ω6 Polyunsaturated Phospholipid Membranes†

M. Alan McCabe,‡ George L. Griffith,‡ William D. Ehringer,§ William Stillwell,§ and Stephen R. Wassall*,‡

Department of Physics, Indiana University–Purdue University Indianapolis, 402 North Blackford Street, Indianapolis, Indiana 46202-3273, and Department of Biology, Indiana University–Purdue University Indianapolis, 723 West Michigan Street, Indianapolis, Indiana 46202-5132

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ABSTRACT: The properties of aqueous multilamellar dispersions of [$^2H_{31}$]16:0-α18:3 PC (1-[$^2H_{31}$]palmitoyl-2-*cis,cis,cis*-octa-9,12,15-trienoylphosphatidylcholine) and of [$^2H_{31}$]16:0-γ18:3 PC (1-[$^2H_{31}$]palmitoyl-2-*cis,cis,cis*-octa-6,9,12-trienoylphosphatidylcholine) were compared by broadline ^{2}H NMR spectroscopy. These isomeric phospholipids differ only in the location of the unsaturations in the *sn*-2 chain. The α18:3 chain has double bonds at Δ9, 12, and 15 positions whereas in the γ18:3 chain they are at positions Δ6, 9, and 12. Moment analysis of spectra recorded as a function of temperature reveals dramatically distinct phase behavior for the two isomers. The gel to liquid crystalline transition for [$^2H_{31}$]16:0-α18:3 PC membranes exhibits broad hysteresis which is characterized by a mid point temperature of −9 °C and −20 °C on heating and cooling, respectively. In contrast, the phase transition of [$^2H_{31}$]16:0-γ18:3 PC membranes does not exhibit hysteresis and occurs over a lower temperature range centred on −27 °C. Appreciably different molecular ordering also exists within the membranes in the liquid crystalline state. Average order parameters $\bar{S}_{CD}$ are smaller in [$^2H_{31}$]16:0-α18:3 PC than in [$^2H_{31}$]16:0-γ18:3 PC by 10% at the same temperature and by 20% at equal reduced temperature. Smoothed order parameter profiles generated from depaked spectra clarify the nature of the difference. There is an elevation in the order of the plateau region of approximately constant order in the upper portion (C2–C7) of the 16:0 *sn*-1 chain in [$^2H_{31}$]16:0-γ18:3 PC, while in the lower portion (C8–C16) of the chain the profiles gradually converge as order decreases toward the terminal methyl. Our results emphasize that the position of double bonds, not just their number, must be included in attempts to explain the unique biological role of PUFA (polyunsaturated fatty acids).

Considerable effort has been expended recently in the investigation of polyunsaturated membranes (Paddy *et al*, 1985; Ehringer *et al.*, 1990; Rajamoorthi and Brown, 1991). The objective is to develop a basic understanding of such systems at the molecular level. Ascertaining the origin of the nutritional advantages associated with PUFA (polyunsaturated fatty acid)[1] provides a rationale, while another motivation is elucidation of the role served by the high concentrations of PUFA found in certain membranes (Simopoulos *et al.*, 1986; Galli and Simopoulos, 1989). Of particular interest is the distinction in health benefits and biological function of ω3 as opposed to ω6 classes of PUFA (Popp-Snijders *et al.*, 1986; Muriana and Ruiz-Gutierrez, 1992). To address this issue, we apply ^{2}H NMR techniques to compare phase behavior and acyl chain order in phospholipid model membranes containing isomeric ω3 and ω6 fatty acids, thereby unambiguously determining the importance of the location of unsaturations.

Much less is known about polyunsaturated membranes than saturated membranes. Detailed phase diagrams have been documented for saturated phospholipids (Marsh, 1990), but for polyunsaturated phospholipids phase behavior is largely uncharacterized. In PCs (phosphatidylcholine) which possess the same saturated *sn*-1 chain, the introduction of the first two double bonds into the *sn*-2 chain progressively reduces the temperature T_m of the gel to liquid crystalline phase transition[2] (Coolbear *et al.*, 1983; Keough *et al.*, 1987). The presence of more double bonds, in contrast, does not cause much change thereafter. A similar relative insensitivity to the addition of subsequent double bonds into an already monounsaturated *sn*-2 chain of PC is exhibited by increases in molecular area measured from force-area characteristics for monolayers and by decreases in order within the bilayer observed by fluorescence polarization of DPH (1,6-diphenyl-1,3,5-hexatriene) (Demel *et al.*, 1972; Stubbs *et al.*, 1981). However, this apparent general trend is an oversimplification since it ignores differences in the properties of unsaturated phospholipid membranes brought about by changes in the position of double bonds. Calorimetry has demonstrated that the thermotropic behavior of positional isomers of monounsaturated PC is critically dependent upon the location of the double bond (Barton & Gunstone, 1975; Macdonald *et al.*, 1985). In 18:0-18:1 PC (1-stearoyl-2-*cis*-octadecenoylphosphatidylcholine) or 18:1–18:1 PC (1,2-*cis*-dioctadecenoylphosphatidylcholine) bilayers, the temperature of the main chain melting transition is a minimum when the double bond is at the 9 position in the middle of the chain and increases as the double bond is relocated toward each end. Work on 18:0-α18:3 PC (1-stearoyl-2-*cis,cis,cis*-octadeca-9,12,15-trienoylphosphatidylcholine) and 18:0-γ18:3 PC (1-stearoyl-2-*cis, cis,cis*-octadeca-6,9,12-trienoylphosphatidylcholine) has shown,

† Research supported by American Heart Association, Indiana Affiliate.

* Author to whom correspondence should be addressed.

‡ Department of Physics.

§ Department of Biology.

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[1] Abbreviations: PUFA, polyunsaturated fatty acid; PC, phosphatidylcholine; [$^2H_{31}$]16:0-α18:3 PC, 1-[$^2H_{31}$]palmitoyl-2-*cis,cis,cis*-octadeca-9,12,15-trienoylphosphatidylcholine; [$^2H_{31}$]16:0-γ18:3 PC, 1-[$^2H_{31}$]-palmitoyl-2-*cis,cis,cis*-octadeca-6,9,12-trienoylphosphatidylcholine; [$^2H_{31}$]16:0–16:0 PC, 1-[$^2H_{31}$]palmitoyl-2-palmitoylphosphatidylcholine.

[2] The gel to liquid crystalline phase transition temperature denoted by T_m is the temperature at which the endotherm reaches a maximum. It corresponds to the middle of the transition for a symmetric endotherm.

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moreover, that permeability and fusion differ for bilayers prepared from these positional isomers (Ehringer *et al.*, 1991). Specifically, permeability to erythritol as monitored by osmotic swelling is 20% higher in 18:0-α18:3 PC than in 18:0-γ18:3 PC, while fusion as observed by resonance energy transfer has a faster rate for the former phospholipid.

^{2}H NMR provides an ideal noninvasive means of probing the molecular properties of membranes (Davis, 1983; Bloom *et al.*, 1991). It is especially well suited to the study of phospholipid chain ordering. The order parameter S_{CD} of the C–^{2}H bond describes the degree of anisotropy for the reorientational motion of an individual segment with respect to the bilayer normal about which there is axial symmetry. The definition is

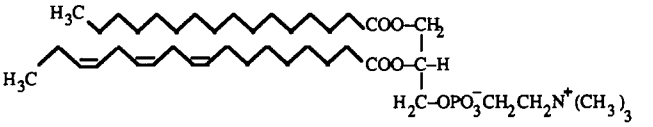
$$S_{CD} = \frac{1}{2}\langle 3\cos^2\beta - 1\rangle \qquad (1)$$

where β is the time-dependent angle between the C–^{2}H bond and the bilayer normal, and the angular brackets denote a time average over the ^{2}H NMR timescale ($<10^{-6}$ s). Values in the range $0 \leq |S_{CD}| \leq {}^1/_2$ are taken, the respective limits representing effective isotropic motion and fast axial rotation.

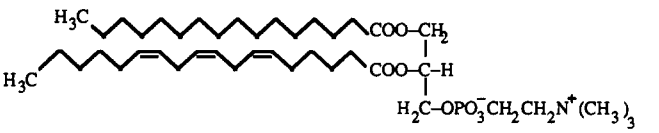
The dependence of order parameter on depth within saturated phospholipid bilayers was mapped in the seminal studies of Seelig and co-workers (Seelig, 1977). The profile obtained for 16:0–16:0 PC (1,2-dipalmitoylphosphatidylcholine) membranes is characteristic, consisting of a plateau region of almost constant order in the upper portion (C2–C9) of the chain followed by a gradual decrease in the lower portion (C10–C16) (Seelig & Seelig, 1977). Interpretation has been attempted at several levels of sophistication, most rigorously on the basis of mean field theory (Schindler & Seelig, 1975; DeLoof *et al.*, 1991). Assuming the first segment of a chain is parallel to the bilayer normal, subsequent segments are only allowed to be inclined at 0°, 60°, and 90° to the bilayer normal by rotational isomerism. The conformational state in each orientation may be *trans (t)* or *gauche (g)*. The plateau region of the order parameter profile is then explained by a predominance of *trans* segments oriented parallel to the bilayer normal (*t*, 0°), together with approximately constant probabilities for each conformation, in the upper portion of the chain. The loss in order in the lower portion of the chain is due to a reduction in the number of *t*,0° segments.

The impact of acyl chain unsaturation on membrane order and phase behavior has been investigated by ^{2}H NMR (Seelig & Waespe-Šarčevič, 1978; Paddy *et al.*, 1985; Baenziger *et al.*, 1991; Holte *et al.*, 1994). Most of the research has tended to focus on the number of double bonds, predominantly ignoring the crucial ramifications of their position. The studies on [$^2H_{31}$]16:0-22:6 PC (1-[$^2H_{31}$]palmitoyl-2-*cis,cis,cis,cis,cis,cis*-docosa-4,7,10,13,16,19-hexaenoylphosphatidylcholine) membranes by Brown and co-workers represent the most comprehensive work on a polyunsaturated system (Salmon *et al.*, 1987; Barry *et al.*, 1991). Greater disorder than within saturated [$^2H_{31}$]16:0-16:0 PC (1-[$^2H_{31}$]palmitoyl-2-palmitoylphosphatidylcholine) bilayers at the same temperature T was identified for the polyunsaturated bilayers. At equal reduced temperature $T_{red} = (T - T_m/T_m)$ where the two systems may be considered to be under equivalent thermodynamic conditions (Seelig & Browning, 1978), on the other hand, the polyunsaturated membrane is more ordered. ^{2}H NMR spectra have also been reported for 16:0-[$^2H_{12}$]22:6 PC (1-palmitoyl-2-[4,5,7,8,10,11,13,14,16,17,19,20-$^2H_{12}$]-docosahexaenoylphosphatidylcholine) membranes and 16:0-[$^2H_8$]20:4 PC (1-palmitoyl-2-[5,6,8,9,11,12,14,15-$^2H_8$]-*cis,cis,cis,cis*-eicosa-5,8,11,14-tetraenoylphosphatidylcholine) membranes (Dratz & Deese, 1986; Rajamoorthi & Brown, 1991). They are relatively narrow, indicating that the conformation of polyunsaturated chains differs appreciably from saturated chains. Definitive characterization awaits assignment of the multicomponent signals arising from vinyl perdeuteration.

A ^{2}H NMR study of [$^2H_{31}$]16:0-α18:3 PC (1-[$^2H_{31}$]-palmitoyl-2-*cis,cis,cis*-octadeca-9,12,15-trienoylphosphatidylcholine) membranes and [$^2H_{31}$]16:0-γ18:3 PC (1-[$^2H_{31}$]-palmitoyl-2-*cis,cis,cis*-octadeca-6,9,12-trienoylphosphatidylcholine) membranes is described here. By observing the perdeuterated *sn*-1 chain, our experiments intimately sense the consequences of the difference in position of the double bonds in the polyunsaturated *sn*-2 chain of these otherwise identical phospholipid molecules.



[$^2H_{31}$]16:0-α18:3 PC



[$^2H_{31}$]16:0-γ18:3 PC

EXPERIMENTAL PROCEDURES

Materials. Avanti Polar Lipids (Pelham, AL) was the source of [$^2H_{31}$]monopalmitoyl-phosphatidylcholine, while α-linolenic and γ-linolenic anhydrides were purchased from Nu Chek Prep (Elysian, MN). Synthesis of [$^2H_{31}$]16:0-α18:3PC and [$^2H_{31}$]16:0-γ18:3 PC was as outlined previously (Ehringer *et al.*, 1991). Deuterium-depleted water was obtained from Cambridge Isotope Laboratories (Woburn, MA).

Sample Preparation. The NMR samples consisted of aqueous multilamellar dispersions of 50% by weight [$^2H_{31}$]-16:0-α18:3 PC or [$^2H_{31}$]16:0-γ18:3 PC in 50 mM phosphate buffer (pH 7.0). Stock solutions of 100–120 mg of phospholipid in chloroform were initially dried under nitrogen, followed by vacuum pumping for 10–12 h to remove residual solvent. The appropriate volume of buffer was added to the dried phospholipids and multilamellar dispersions were prepared in the presence of excess (2 mL) deuterium-depleted water by vortex mixing at room temperature for several minutes. The pH was adjusted and three lyophilizations were then performed to reduce the ^{2}H NMR signal from natural abundance ^{2}HHO. To avoid oxidation of the polyunsaturated lipids, the various manipulations were carried out under a stream of nitrogen, the water was degassed, and exposure to direct light was minimized. The resultant samples were transferred to 5-mm NMR tubes and stored at −20 °C. They were always equilibrated at room temperature for at least 1 h prior to experimentation.

NMR Spectroscopy. ^{2}H NMR spectra were recorded on a homebuilt spectrometer operating at 27.6 MHz with a Nalorac 4.2 T superconducting magnet. The probe was constructed by Cryomagnet Systems, Inc. (Indianapolis, IN) and utilized a 5-mm transverse mounted coil. An Amplifier Research Model 200L radio frequency amplifier (Souderton,

PA) provided the high power pulses. The spectrometer was controlled by a Nicolet 1080 computer, which facilitated pulse programming with a Nicolet 293 I/O controller and acquired NMR signals *via* a Biomation 805 fast transient recorder. Data analysis was performed after transfer to a Zenith 386 computer. Sample temperature was regulated to an accuracy of ±0.5 °C by a Bruker B-VT-1000 temperature controller. The duration of equilibration immediately before data collection at each chosen temperature was about 20 min, while the rate at which temperature was adjusted to attain the desired temperature was approximately 1 °C/min.

The quadrupolar echo sequence $(90°_x–\tau_2–90°_y–\text{acquire–delay})_n$, which eliminates spectral distortion due to the receiver recovery time, was implemented to collect spectra (Davis *et al.*, 1976). The time delay τ_2 between pulses was usually 50 μs, the 90° pulse width was on the order of 2.5 μs, and the delay between pulse sequences was in the range 1.0 to 1.5 s. Phase alternation of the sequence canceled coherent receiver and pulse noise (Griffin, 1981). The experiments were conducted on resonance, and the "out of phase" channel was zeroed before Fourier transformation. The consequent spectra are thus reflected about the central resonant frequency. Spectral parameters were sweep width = ±250 kHz and ±100 kHz in the gel and liquid crystalline phases, respectively; line broadening = 125 and 50 Hz in the respective phases; data set = 2048; and the number of scans was 1024 unless otherwise stated.

Moments M_n were calculated from the symmetric spectra according to

$$M_n = \frac{\int_0^\infty \omega^n f(\omega)\, d\omega}{\int_0^\infty f(\omega)\, d\omega} \tag{2}$$

where ω is the frequency with respect to the central Larmor frequency ω_0, $f(\omega)$ is the line shape, and n is the order of the spectral moment (Davis, 1983). In practice, the integral is a summation over the digitized data. The temperature dependence of spectral moments maps phase behavior, while in the liquid crystalline state the first moment M_1 is related to average order $\bar{S}_{CD}$ of the perdeuterated 16:0 *sn*-1 chain *via*

$$M_1 = \frac{\pi}{\sqrt{3}}\left(\frac{e^2qQ}{h}\right)\bar{S}_{CD} \tag{3}$$

where $(e^2qQ/h) \cong 167$ kHz is the static quadrupolar coupling constant.

Numerical deconvolution of spectra by the depaking procedure was performed to elaborate upon membrane order (Sternin *et al.*, 1983). A version of the original computer program modified to run on our Zenith 386 computer was employed. Depaked spectra correspond to the spectra that would be obtained for a planar membrane aligned with its bilayer normal parallel to the applied static magnetic field. Their enhanced resolution reveals doublets with splittings $\Delta\nu(\theta)$ which equate to order parameters according to

$$\Delta\nu(\theta) = \frac{3}{2}\left(\frac{e^2qQ}{h}\right)|S_{CD}|P_2(\cos\theta) \tag{4}$$

where $\theta = 0°$ is the angle the membrane normal makes with the magnetic field and $P_2(\cos\theta)$ is the second-order Legendre polynomial. Construction of smoothed order parameter profiles is facilitated on the assumption that order varies monotonically along the acyl chain (Lafleur *et al.*, 1989).

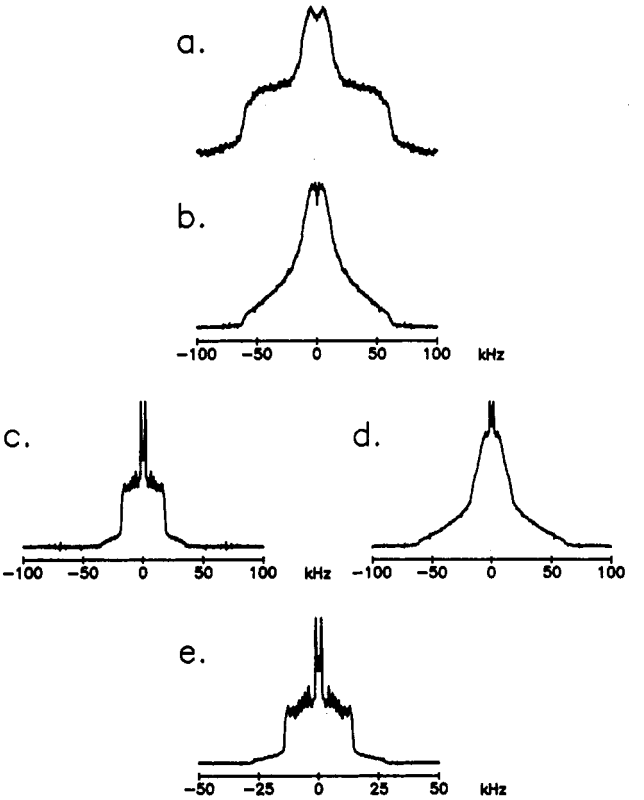


FIGURE 1: ^{2}H NMR spectra for [$^2H_{31}$]16:0-α18:3 PC (50% by weight aqueous multilamellar dispersion in 50 mM phosphate, pH 7.0): (a) −35 °C; (b) −25 °C; (c) −13 °C (cooling run); (d) −13 °C (heating run); (e) 8 °C. Spectral parameters are as described in the Experimental Procedures.

RESULTS

The dependence on temperature of ^{2}H NMR spectra recorded for multilamellar dispersions of 50 wt % [$^2H_{31}$]16:0-α18:3 PC and [$^2H_{31}$]16:0-γ18:3 PC in 50 mM phosphate buffer (pH 7.0) is compared in Figures 1 and 2. An appreciation of the experimental protocol is essential to the comparison. Prior to all experimentation, samples were equilibrated at room temperature ($T \cong 25$ °C). This constituted the starting point for data collection as a function of decreasing temperature, whereas cooling and further equilibration at $T \cong -40$ °C were performed before acquiring spectra as a function of increasing temperature. The two temperature extremes are, respectively, well above and below the gel to liquid crystalline phase transition of either phospholipid.

The spectrum presented in Figure 1 for [$^2H_{31}$]16:0-α18:3 PC at −35 °C (Figure 1a) is characteristic of the gel phase (Davis, 1983; Wassall *et al.*, 1986). Static methylenes are responsible for the broad component with edges at ±63 kHz, while the central component with a pair of peaks split by approximately 11 kHz is due to reorienting methylenes and methyls. A gel-state spectrum is similarly exhibited at −25 °C (Figure 1b). The smaller relative intensity of the broad component is consistent with greater acyl chain mobility upon increasing temperature. The spectra described so far are essentially independent of the direction in which temperature is changed. This is not true at somewhat higher temperature near the phase transition, as is illustrated by the spectra at −13 °C (Figure 1c and 1d). The much narrower spectrum collected on the cooling run (Figure 1c) is typical of the liquid crystalline phase (Davis, 1983; Wassall *et al.*, 1986). The sharp, well-defined sides at approximately ±15 kHz designate

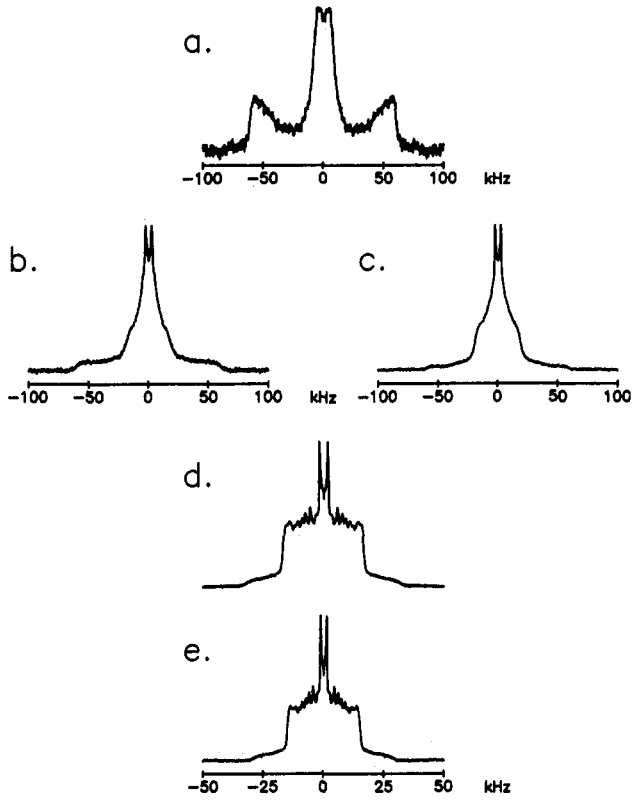


FIGURE 2: ^2H NMR spectra for [$^2H_{31}$]16:0-γ18:3 PC (50% by weight aqueous multilamellar dispersion in 50 mM phosphate, pH 7.0): (a) −35 °C; (b) −27 °C (cooling run); (c) −27 °C (heating run); (d) −12 °C; (e) 8 °C. Spectral parameters are as described in the Experimental Procedures.

a plateau region of virtually constant order in the upper portion of the phospholipid chain, while the peaks are produced by individual segments in the lower portion where order gradually decreases toward the terminal methyl. On the heating run, in contrast, an almost entirely gel membrane is indicated by a spectrum which contains only a very small liquid crystalline contribution superimposed upon the broad, major spectral component (Figure 1d). Thus, hysteresis in the phase behavior of [$^2H_{31}$]16:0-α18:3 PC membranes is implied. It disappears at higher temperature, where the spectra are liquid crystalline in form. The spectrum at 8 °C (Figure 1e) is an example.

A considerably different variation with temperature is shown in Figure 2 by the spectra for [$^2H_{31}$]16:0-γ18:3 PC. In particular, the temperature of the gel to liquid crystalline phase transition is lower and hysteresis is not discernible. The spectrum at −35 °C is gel-like (Figure 2a), in agreement with [$^2H_{31}$]16:0-α18:3 PC, but differs appreciably in shape. At −27 °C the spectra obtained on cooling and heating runs signify that the [$^2H_{31}$]16:0-γ18:3 PC membrane is in the region of the phase transition (Figures 2b and 2c). They are of mostly gel form, characteristically containing a component of intermediate width (approximately 50 kHz) associated with onset of the formation of liquid crystalline phase (Wassall *et al.*, 1993). This contrasts with [$^2H_{31}$]16:0-α18:3 PC which manifests a purely gel spectrum at comparable temperature (Figure 1b). Moreover, unlike the behavior of [$^2H_{31}$]16:0-α18:3 PC close to the phase transition (Figures 1c and 1d), the spectra for [$^2H_{31}$]16:0-γ18:3 PC membranes are identical within experimental uncertainty regardless of whether they were collected as a function of decreasing or increasing temperature. Confirmation that [$^2H_{31}$]16:0-γ18:3 PC becomes liquid crystalline at higher temperature is provided by

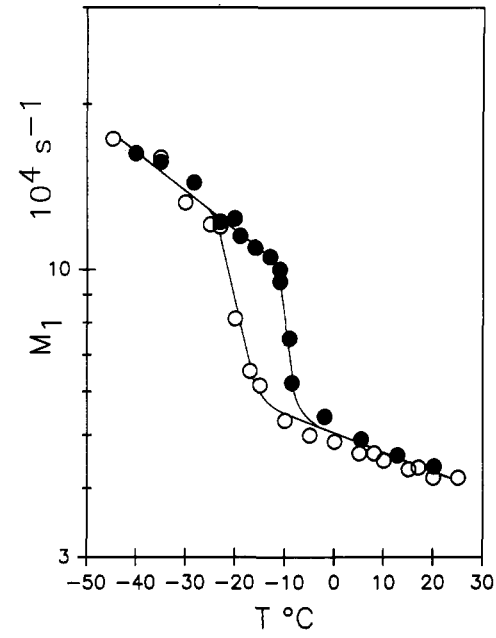


FIGURE 3: Variation of the first moment M_1 with temperature T for [$^2H_{31}$]16:0-α18:3 PC. Cooling (○) and heating (●) runs.

the spectra presented for −12 °C (Figure 2d) and 8 °C (Figure 2e).

Moment analysis elaborates upon the trends seen in the spectra. The substantial hysteresis in thermal behavior to which [$^2H_{31}$]16:0-α18:3 PC membranes are subject is clearly displayed by the first moments M_1 plotted against temperature in Figure 3. The moments describe the gel state ($M_1 \geq 12.0 \times 10^4$ s^{-1}) at low temperature ($T < -25$ °C), while at high temperature ($T > -5$ °C) liquid crystalline phase ($M_1 \leq 5.0 \times 10^4$ s^{-1}) applies. There is essentially no difference in the values calculated from heating or cooling runs in either temperature regime. At intermediate temperatures (-25 °C $< T < -5$ °C), deviation between the results from the respective runs demonstrates the hysteresis. Specifically, the drop in first moment from a gel to liquid crystalline value is centered at −9 °C and has a width of approximately 6 °C on the heating run, whereas the equivalent discontinuity on the cooling run has a mid point of −20 °C and is approximately 8 °C in width.

The graph of first moment M_1 *vs* temperature drawn in Figure 4 for [$^2H_{31}$]16:0-γ18:3 PC is very different. Corroboration that the temperature of the phase transition is lower and of the absence of hysteresis is immediately apparent. At low ($T < -30$ °C) and high ($T > -25$ °C) temperatures, the moments are symptomatic, respectively, of gel ($M_1 \geq 15.0 \times 10^4$ s^{-1}) and liquid crystalline ($M_1 \leq 6.0 \times 10^4$ s^{-1}) states. They are the same for spectra recorded when temperature was increased or decreased. The change between values ascribed to the two phases, furthermore, occurs over the same temperature interval on heating and cooling runs. The mid point of the transition is $T_m = -27$ °C, and it is approximately 5 °C in width.

Inspection of Figures 3 and 4 identifies another distinction between the isomeric membranes. The moments are about 5% higher for [$^2H_{31}$]16:0-γ18:3 PC when compared in the gel state at equal temperature. Greater value for the moments of [$^2H_{31}$]16:0-γ18:3 PC persists above the phase transition, suggesting a more ordered membrane in the liquid crystalline state. They are almost 10% larger than for [$^2H_{31}$]16:0-α18:3 PC at the same temperature.

Average order parameters $\bar{S}_{CD}$ may be calculated with eq 4 to quantify the situation in the liquid crystalline state. They

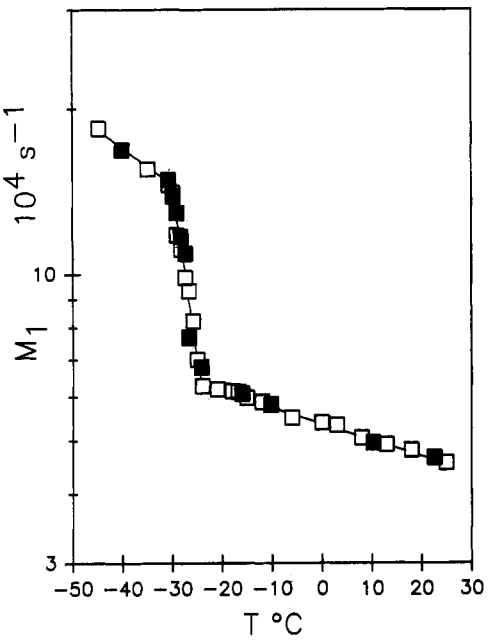


FIGURE 4: Variation of the first moment M_1 with temperature T for [$^2H_{31}$]16:0-γ18:3 PC. Cooling (□) and heating (■) runs.

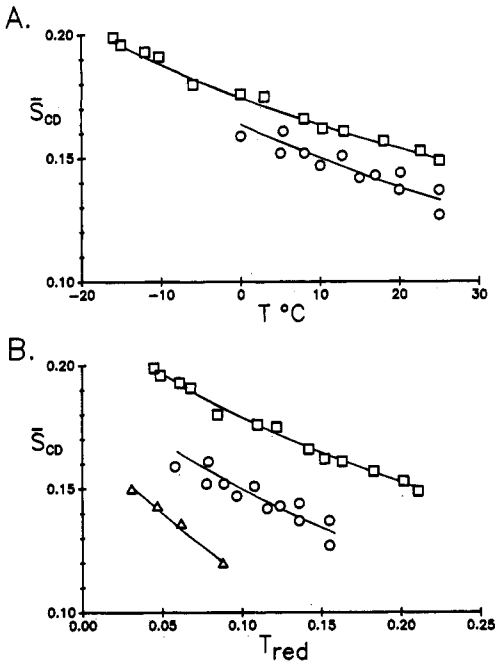


FIGURE 5: Average order parameter $\bar{S}_{CD}$ *vs* (A) temperature T and (B) reduced temperature T_{red} for [$^2H_{31}$]16:0-α18:3 PC (○), [$^2H_{31}$]-16:0-γ18:3 PC (□), and [$^2H_{31}$]16:0-16:0 PC (△).

are plotted as a function of temperature T and reduced temperature T_{red} in Figure 5. The latter parameter is commonly considered to represent a condition under which membranes are subject to equivalent average molecular forces, although doubts about its validity have been raised (Lafleur *et al.*, 1990). The graph of $\bar{S}_{CD}$ *vs* T (Figure 5A) is consistent with the deductions based on comparison of moments at equal temperature, illustrating that average order within [$^2H_{31}$]16:0-γ18:3 PC membranes is greater than that within [$^2H_{31}$]-16:0-α18:3 PC membranes. Qualitatively the same trend is followed in the plot of $\bar{S}_{CD}$ *vs* T_{red} (Figure 5B), except that the difference in molecular ordering between the polyunsaturated membranes is accentuated. In this graph, the transition temperature T_m = −15 °C utilized for [$^2H_{31}$]16:0-α18:3 PC is the mean of the mid point temperatures

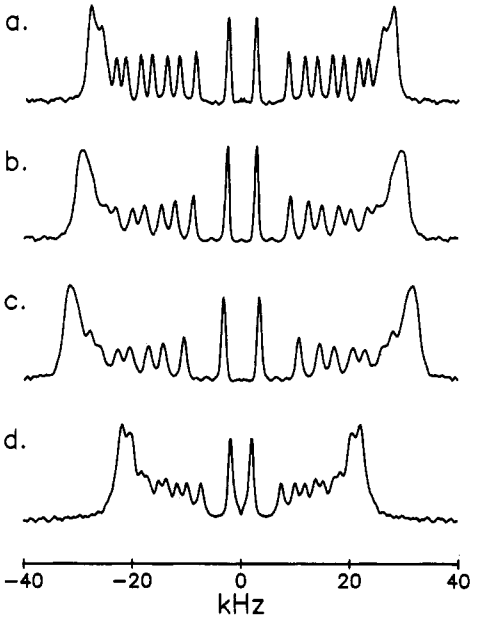


FIGURE 6: Depaked ^2H NMR spectra: (a) [$^2H_{31}$]16:0-α18:3 PC (50% by weight aqueous multilamellar dispersion in 50 mM phosphate, pH 7.0) at 8 °C; (b and c) [$^2H_{31}$]16:0-γ18:3 PC (50% by weight aqueous multilamellar dispersion in 50 mM phosphate, pH 7.0) at 8 °C and −6 °C, respectively; (d) [$^2H_{31}$]16:0-16:0 PC (50% by weight aqueous multilamellar dispersion in 20 mM Tris, pH 7.5) at 67 °C. The temperatures of a, c, and d correspond to the same reduced temperature T_{red} = 0.09. Spectral parameters are as described in the Experimental Procedures, except 2048 scans were collected. Typically, 10 iterations of the depaking program were performed.

measured in heating and cooling experiments. Data on [$^2H_{31}$]-16:0-16:0 PC is included to furnish an example of a saturated membrane for comparative purposes.

More detailed information on acyl chain order within liquid crystalline membranes becomes accessible after depaking the ^2H NMR spectra. Specifically, the conventional spectrum which consists of a superposition of powder patterns from individual chain segments in a random orientational distribution of membranes is replaced, by numerical deconvolution, with a corresponding superposition of doublets that is equivalent to the spectrum from a planar membrane aligned with the bilayer normal in the direction of the applied magnetic field (Sternin *et al.*, 1983). The superior resolution achieved is exemplified in Figure 6 by depaked spectra which compare [$^2H_{31}$]16:0-α18:3 PC and [$^2H_{31}$]16:0-γ18:3 PC at equal temperature T = 8 °C (Figures 6a and 6b) and, together with [$^2H_{31}$]16:0-16:0 PC, at equal reduced temperature T_{red} = 0.09 (Figures 6a, 6c, and 6d). They consist of 6–8 well-resolved doublets and an outermost composite doublet. The former doublets may be assigned sequentially with ascending splitting to the relatively disordered terminal methyl and adjacent methylenes in the lower portion of the chain, while the latter doublet may be ascribed to similarly ordered methylenes in the upper portion.

Profiles of order parameter *vs* chain position derived from the depaked spectra in Figure 6 are plotted in Figure 7. They are smoothed profiles that were generated by assigning equal integrated intensity to each methylene group in the 16:0 *sn*-1 chain and assuming order decreases monotonically toward the terminal methyl (Lafleur *et al.*, 1989). The application of this approach has previously yielded results which, apart from a few minor subtleties, agree well with those obtained in considerably more time consuming studies of selectively deuterated phospholipids. As demonstrated by Figure 7, the curves for [$^2H_{31}$]16:0-α18:3 PC, [$^2H_{31}$]16:0-γ18:3 PC, and

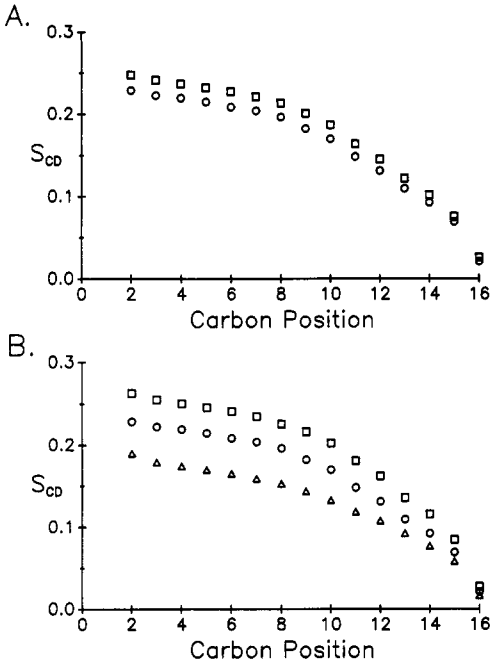


FIGURE 7: Order parameter profiles: (A) [$^2H_{31}$]16:0-α18:3 PC (○) and [$^2H_{31}$]16:0-γ18:3 PC (□) at the same temperature T = 8 °C; (B) [$^2H_{31}$]16:0-α18:3 PC (T = 8 °C) (○), [$^2H_{31}$]16:0-γ18:3PC (T = –6 °C) (□), and [$^2H_{31}$]16:0-16:0PC (T = 67 °C) (△) at the same reduced tempearture T_{red} = 0.09.

[$^2H_{31}$]16:0-16:0 PC membranes are qualitatively similar. Their shape typifies membranes in the liquid crystalline state, consisting of a plateau region of approximately constant order in the upper portion of the chain followed by a progressive reduction in order in the lower portion (Seelig, 1977; Bloom *et al.*, 1991). However, differences in magnitude and detail clearly exist between the profiles. They confirm the trend displayed by average order parameters $\bar{S}_{CD}$ (Figure 5). The confirmation is not merely qualitative, since average order parameters calculated from the smoothed profiles correspond with high precision (±1%) to the values determined from first moments *via* eq 3. At equal temperature (T = 8 °C), [$^2H_{31}$]-16:0-γ18:3 PC is more ordered than [$^2H_{31}$]16:0-α18:3 PC (Figure 7A). The differential is greater at equal reduced temperature (T_{red} = 0.09), and higher order than in saturated [$^2H_{31}$]16:0-16:0 PC is revealed throughout both polyunsaturated membranes (Figure 7B).

DISCUSSION

Broadline ^2H NMR techniques were utilized to investigate phase behavior and molecular ordering in [$^2H_{31}$]16:0-α18:3 PC and [$^2H_{31}$]16:0-γ18:3 PC membranes. These phospholipids are positional isomers that are identical apart from the location of a single double bond in the polyunsaturated *sn*-2 chain. Specifically, the double bonds are at positions Δ9, 12, and 15 in α18:3 while in γ18:3 they are at the Δ6, 9, and 12 positions. The number of carbons are equal and so are the number of double bonds. Comparison of the two molecules consequently offers a superb opportunity to monitor the manner in which solely the location of acyl chain unsaturation affects the properties of polyunsaturated phospholipid membranes.

The plots of first moment M_1 *vs* temperature presented in Figures 3 and 4 demonstrate that profoundly different phase behavior is exhibited by [$^2H_{31}$]16:0-α18:3 PC (Figure 3) and [$^2H_{31}$]16:0-γ18:3 PC (Figure 4) membranes. The gel to liquid crystalline transition of [$^2H_{31}$]16:0-α18:3 PC occurs at higher temperature than that for [$^2H_{31}$]16:0-γ18:3 PC and, moreover, possesses hysteresis. It is characterized by a mid point temperature of –9 °C when based on spectra recorded as a function of increasing temperature, whereas on the basis of spectra collected as a function of decreasing temperature the transition is centred on –20 °C. The transition width is approximately 6 °C and 8 °C, respectively, on heating and cooling runs. In contrast, hysteresis is not apparent with [$^2H_{31}$]-16:0-γ18:3 PC for which the center of the gel to liquid crystalline transition is at –27 °C on either heating or cooling runs. The width of the transition is 5 °C.

The phase behavior of neither 16:0-α18:3 PC nor 16:0-γ18:3 PC has been previously reported. The most closely related phospholipid studied to date is 18:0-α18:3 PC. It has a gel to liquid crystalline transition temperature of –13 °C (Coolbear *et al.*, 1983), but a value is yet to be published for 18:0-γ18:3 PC. Indeed, few studies of isomeric polyunsaturated phospholipids exist. Nevertheless, it is possible to reconcile our observation that [$^2H_{31}$]16:0-α18:3 PC undergoes phase transition at higher temperature than [$^2H_{31}$]16:0-γ18:3 PC with the results of earlier work. Calorimetric methods show a greater depression of the gel to liquid transition in 18:0–18:1 PC isomers when the double bond is located near the middle of the *cis* 18:1 *sn*-2 chain (Barton & Gunstone, 1975). The same general trend is followed by the melting points of *cis* 18:1 (octadecenoic acid) and *cis,cis* 18:2 (octadecadienoic acid) fatty acids (Gunstone & Ismail, 1967; Christie & Holman, 1967). Thus, the lower temperature seen here for the phase transition of [$^2H_{31}$]16:0-γ18:3 PC is consistent with the Δ6, 9, 12 combination of double bonds in the center of the 18:3 chain. Pressure–area curves for monolayer films also indicate that the area/molecule for isomeric polyunsaturated phospholipids depends upon the location of the double bonds (Evans & Tinoco, 1978). A larger molecular area would be expected to accompany less restricted packing in gel-state bilayers, which would facilitate chain "melting" at lower temperature. Langmuir trough experiments recently performed in our laboratories, in fact, demonstrate that the area occupied by 18:0-γ18:3 PC is 7% greater than that for 18:0-α18:3 PC (Ehringer *et al.*, unpublished results).

The presence *vs* absence of hysteresis in the thermal behavior of [$^2H_{31}$]16:0-α18:3 PC *vs* [$^2H_{31}$]16:0-γ18:3 PC is intriguing. The phenomenon has been widely documented for hydrated PC bilayers, although it is not universal. In saturated mixed chain PC, a metastable gel ($L_{\beta'}$) state in which chains interdigitate between the monolayers of the bilayer is responsible (Mattai *et al.*, 1987; Lin *et al.*, 1991). The state, which reverts to "crystalline" (L_c) phase over time, is formed on cooling but not heating. The precise nature of the process depends on the relative lengths of the *sn*-1 and -2 chains. The situation in saturated–unsaturated mixed chain PC is less well characterized (Keough *et al.*, 1987; Barry *et al.*, 1991). Hysteresis as defined by $\Delta T_m = T_m^{inc} - T_m^{dec}$ where T_m^{inc} and T_m^{dec} are the gel to liquid crystalline transition temperatures on heating and cooling runs, respectively, was measured by ^2H NMR for the homologous series [$^2H_{23}$]12:0-22:6 PC (1-[$^2H_{23}$]lauroyl-2-docosahexaenoylphosphatidylcholine), ΔT_m = 13.8 °C; [$^2H_{27}$]14:0-22:6 PC (1-[$^2H_{23}$]myristoyl-2-docosahexaenoylphosphatidylcholine), ΔT_m = 10.3 °C; [$^2H_{31}$]16:0-22:6 PC, ΔT_m = 9.7 °C; and [$^2H_{35}$]18:0-22:6 PC (1-[$^2H_{35}$]stearoyl-2-docosahexaenoylphosphatidylcholine), ΔT_m = 2.4 °C (Barry *et al.*, 1991). The data, as with saturated mixed chain PC, indicate that relative length of the *sn*-1 and -2 chains is an influential parameter. This suggests that the distinction between the hysteretic behavior of [$^2H_{31}$]16:0-

α18:3 PC and [$^2H_{31}$]16:0-γ18:3 PC may be associated with inequivalence in the effective length of the respective *sn*-2 chains. However, a close correlation between the thermotropic mesomorphism of saturated and polyunsaturated phospholipid membranes seems unlikely.

The phase transition temperatures measured in [$^2H_{31}$]-16:0-α18:3 PC and [$^2H_{31}$]16:0-γ18:3 PC membranes are over 50 °C less than that in [$^2H_{31}$]16:0-16:0 PC for which T_m = 39 °C. They illustrate a general trend. The constraints imposed upon acyl chain conformation by the presence of rigid *cis* double bonds disrupt molecular packing within gel-state polyunsaturated membranes, depressing the temperature of the gel to liquid crystalline transition with respect to saturated membranes (Keough *et al.*, 1987). The disparate shape of the spectra recorded for [$^2H_{31}$]16:0-α18:3 PC (Figure 1a) and [$^2H_{31}$]16:0-γ18:3 PC (Figure 2a) in the gel state, furthermore, reflects the different conformations of α18:3 and γ18:3 *sn*-2 chains which give rise to the difference in phase behavior observed for the isomeric phospholipids. Quantitative interpretation is not attempted here, since a theoretical treatment of ^2H NMR spectra from membranes in the gel phase is yet to be developed (Davis, 1983). Such spectra are characteristic of a non-zero asymmetry parameter η, in particular, so that to equate the first moment M_1 to average order $\bar{S}_{CD}$ *via* eq 4 is inappropriate.

Spectral analysis in the liquid crystalline phase, where η = 0 characterizes the powder patterns, is much more straightforward (Davis, 1983). Average order parameters $\bar{S}_{CD}$ calculated with eq 4 for [$^2H_{31}$]16:0-α18:3 PC and [$^2H_{31}$]16:0-γ18:3 PC membranes are plotted *vs* temperature and reduced temperature T_{red} in Figure 5. The graph against temperature shows that the latter ω6 membrane is about 10% more ordered than the former ω3 membrane when compared at the same temperature (Figure 5A). This observation disagrees with earlier fluorescence spectroscopy work which failed to detect a difference between steady-state depolarization for 9-anthroyloxy stearic acid probes incorporated into 18:0-α18:3 PC and 18:0-γ18:3 PC vesicles (Ehringer *et al.*, 1991). Perturbation problems associated with the bulky extrinsic fluorescent probe, as opposed to the essentially noninvasive ^2H NMR approach, are probably the major reason for the discrepancy. Inequivalent acyl chain order within the bilayer of multilamellar dispersions and sonicated unilamellar vesicles is another possibility (Parmar *et al.*, 1984). Our results emphasize the care that should be exercised in reaching global conclusions about the properties of broad classes of polyunsaturated membrane without due attention to the number of carbons and double bonds. ^2H NMR of [$^2H_{31}$]16:0-22:6 PC and [$^2H_{31}$]16:0-20:4 PC which are ω3 and ω6 phospholipids, respectively, found [$^2H_{31}$]16:0-22:6 PC to be more ordered (Rajamoorthi *et al.*, 1991). The difference is opposite to that seen here in the isomeric ω3 and ω6 polyunsaturated membranes.

The difference in ordering observed between [$^2H_{31}$]16:0-α18:3 PC and [$^2H_{31}$]16:0-γ18:3 PC at identical temperature is more pronounced when the membranes are considered at the same reduced temperature (Figure 5B). Specifically, the average order parameter $\bar{S}_{CD}$ is approximately 20% greater in the latter membrane. The inclusion of data for [$^2H_{31}$]16:0-16:0 PC, moreover, demonstrates that both polyunsaturated membranes possess higher order than the saturated membrane. This implies that the rigid C=C segments in the *sn*-2 chain are sensed in the adjacent *sn*-1 chain, reducing the available rotational isomeric states. Such a finding is consistent with the consensus of previous work, which generally indicates that unsaturated membranes are more ordered than saturated membranes under conditions of equal reduced temperature (Seelig & Seelig, 1977; Paddy *et al.*, 1985; Barry *et al.*, 1991). Hence, the original preconception that the introduction of double bonds "fluidizes" membranes clearly needs qualification. It was presumably based on the greater disorder seen in unsaturated membranes relative to saturated membranes at equal temperature (Seelig & Seelig, 1977; Stubbs *et al.*, 1981; Salmon *et al.*, 1987), a comparison which does not compensate for inequivalence in the temperature of the gel to liquid crystalline transition.

Elaboration on the trends identified in average order is provided in Figure 7 by the smoothed order parameter profiles constructed from depaked spectra (Figure 6). They confirm that [$^2H_{31}$]16:0-γ18:3 PC is more ordered than [$^2H_{31}$]16:0-α18:3 PC. The marked differential that exists between the isomeric polyunsaturated membranes at equal reduced temperature and their appreciably greater degree of ordering with respect to saturated [$^2H_{31}$]16:0-16:0 PC are distinctly displayed (Figure 7B). The profiles also establish that the differences are manifest as elevated order throughout the 16:0 *sn*-1 chain. The plateau region of almost constant order in the upper portion of the chain is slightly shortened in the polyunsaturated membranes, but not to the same extent reported for more highly unsaturated [$^2H_{31}$]16:0-22:6 PC (Salmon *et al.*, 1987). It extends from C2 to C8 in [$^2H_{31}$]16:0-16:0 PC, which contrasts with C2–C7 in [$^2H_{31}$]16:0-α18:3 PC and in [$^2H_{31}$]-16:0-γ18:3 PC. In the lower portion of the chain where order progressively decreases toward the terminal methyl group, however, there is a convergence of the variation described by each phospholipid, and the profiles become virtually superimposable at C16.

A discussion of our data in terms of the molecular modeling studies by Applegate and Glomset (1986, 1991) is worthwhile. They employed the MM2 molecular mechanics program, which is based on a force field developed for hydrocarbons (Allinger, 1977), to obtain the energy minimized structure for several diglycerides. The effect of acyl chain unsaturation, in particular, was investigated. An "angle iron" shaped conformation for methylene interrupted sequences of *cis* double bonds, in which the double bond directions are parallel and alternate double bond planes are perpendicular to one another, was found to be optimal. The groove, into which the saturated *sn*-1 chain can fit, formed by this structural motif in the polyunsaturated *sn*-2 chain of mixed saturated–polyunsaturated chain diglycerides facilitates a parallel, tightly packed arrangement. Although the relevance of such models to the situation inside the fluid interior of liquid crystalline phospholipid membranes remains to be proven, the minimal change in conformation of the polyunsaturated chain that was detected by Raman spectroscopy when 16:0-20:4 PC and 16:0-22:6 PC pass from gel to liquid crystalline phase is noted (Litman *et al.*, 1991).

The model presented for 18:0-α18:3 DG (1-stearoyl-2-α-linolenoylglycerol) has the methylene segments of the initial saturated portions of the two chains interdigitated. The chains are parallel and their configuration here resembles that in 18:0-18:0 DG (1,2-distearoylglycerol). Positions C6–C11 of the 18:0 *sn*-1 chain then fit into the groove formed by the "angle iron" adopted by the Δ9, 12, 15 combination of double bonds in the α18:3 *sn*-2 chain. Thus, the greater order seen for [$^2H_{31}$]16:0-α18:3 PC bilayers relative to [$^2H_{31}$]16:0-16:0PC is consistent with the constraint a groove in the *sn*-2 chain would be expected to impose upon the reorientational motion of the *sn*-1 chain. An analogous argument may be

invoked in the case of the γ18:3 isomer. Applying a somewhat naive extrapolation to 18:0-γ18:3 DG (1-stearoyl-2-γ-linolenoylglycerol), for which a model has not been published, the groove associated with the "angle iron" configuration of a Δ6, 9, 12 sequence of double bonds in the *sn*-2 chain would be shifted toward the beginning of the *sn*-1 chain by 3 carbons to coincide with positions C3–C8. Such a situation would be expected similarly to restrict the motion of the *sn*-1 chain within [$^2H_{31}$]16:0-γ18:3 PC bilayers. It is reasonable to further speculate that the shift of the groove into the plateau region of the order parameter profile correlates with the enhancement in membrane ordering with respect to [$^2H_{31}$]-16:0-α18:3 PC. We are currently initiating computer modeling studies and in our future work intend to comprehensively evaluate modeled structure in the context of membranes.

In conclusion, the results presented here for two positional isomers of an otherwise identical phospholipid molecule unequivocally establish that position of unsaturation is a critical determinant of phase behavior and molecular ordering in polyunsaturated membranes. The Δ9, 12, 15 combination of double bonds in [$^2H_{31}$]16:0-α18:3 PC produces a membrane which has a higher temperature for the gel to liquid crystalline transition and is less ordered in the liquid crystalline state than [$^2H_{31}$]16:0-γ18:3 PC where the double bonds are located at Δ6, 9, and 12 positions. Substantial hysteresis in the thermal behavior of the former isomer is another profound difference. Thus, our work demonstrates that in order to understand the biological function of PUFA at the molecular level, it is insufficient to focus on the number of double bonds without due regard to their location. A worthwhile next step would be to examine the positional isomers within a host bilayer, since the extent to which the differences identified in single component membranes would be observed in natural membranes of heterogeneous composition is an issue that ultimately deserves attention.

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