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Low-Temperature ^{2}H NMR Spectroscopy of Phospholipid Bilayers Containing Docosahexaenoyl (22:6ω3) Chains†

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ABSTRACT: Polyunsaturated fatty acids are widely distributed components of biological membranes and are believed to be involved in many biological functions. However, the mechanisms by which they act on a molecular level are not understood. To further investigate the unique properties of ω3 polyunsaturated phospholipid bilayers, deuterium nuclear magnetic resonance (^{2}H NMR) studies have been made of the liquid–crystalline (L_α) and gel phases of a homologous series of mixed-chain phosphatidylcholines containing docosahexaenoic acid: (per-^{2}H-*n*:0)(22:6)PC, where n = 12, 14, 16, and 18. The moments of the ^{2}H NMR lineshapes have been evaluated, and from these the warming and cooling main phase transition temperatures were determined. The transition temperatures of the mixed-chain series were found to be significantly lower than those of the corresponding lipids in the disaturated series, di(per-^{2}H-*n*:0)PC, with hystereses ranging from 2 to 14 °C. Distinct effects of the docosahexaenoyl chain on bilayer order were found, though these effects varied across the mixed-chain series. In evaluating the moment data, an empirical method for normalizing the moments with respect to differences in temperature was applied, in addition to using the reduced temperature method. For the systems studied here, the method of normalization had no significant effect on the interpretation of the moment data.

Deuterium nuclear magnetic resonance (^{2}H NMR)[1] is uniquely suited to the study of membranes in that it yields information on both time-averaged conformations and molecular motions within lipid bilayers and biological membranes (Seelig, 1977; Davis, 1979; Seelig & Seelig, 1980; Griffin, 1981; Brown, 1982; Davis, 1983). Polyunsaturated ω3 phospholipids in bilayers are of particular interest because of widespread evidence suggesting their participation in many biological functions. Although metabolically costly to synthesize, long-chain polyunsaturated fatty acids are extraordinarily abundant in certain tissues. There is extensive evi-

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[1] Abbreviations: (per-^{2}H-12:0)(22:6)PC, 1-perdeuteriolauroyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine; (per-^{2}H-14:0)(22:6)PC, 1-perdeuteriomyristoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine; (per-^{2}H-16:0)(22:6)PC, 1-perdeuteriopalmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine; (per-^{2}H-18:0)(22:6)PC, 1-perdeuteriostearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine; di(per-^{2}H-12:0)PC, 1,2-perdeuteriolauroyl-*sn*-glycero-3-phosphocholine; di(per-^{2}H-14:0)PC, 1,2-perdeuteriomyristoyl-*sn*-glycero-3-phosphocholine; di(per-^{2}H-16:0)PC, 1,2-perdeuteriopalmitoyl-*sn*-glycero-3-phosphocholine; di-(per-^{2}H-18:0)PC, 1,2-perdeuteriostearoyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; ^{2}H NMR, deuterium nuclear magnetic resonance; DSC, differential scanning calorimetry; T_m, main (gel–liquid crystalline) phase transition temperature; T_{red}, reduced temperature; T_{rel}, relative temperature.

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dence connecting ω3 polyunsaturated fatty acids, docosahexaenoic acid in particular, to (i) visual dysfunction in rats (Anderson et al., 1974, 1976), monkeys (Neuringer et al., 1984, 1986), and humans (Converse et al., 1983; Howard-Williams et al., 1985; Anderson et al., 1987); (ii) proper function of the nervous system (O'Brien et al., 1965; Svennerholm, 1968; Lamptey & Walker, 1976; Holman et al., 1982); (iii) cardiovascular disease (Dyerberg & Bang, 1978, 1979; Saynor et al., 1984; Lee et al., 1985); and (iv) inhibited tumorigenesis (Karmali et al., 1984; Jurowski et al., 1985; Borgenson et al., 1989; Henderson et al., 1989). With ^{2}H NMR as a tool it is possible to investigate some of the unique physical properties that polyunsaturated phospholipids impart to membranes, as compared to those of their saturated counterparts. Once these properties are identified, we are closer to understanding the apparently essential niche that polyunsaturated fatty acids occupy in biological processes.

A number of investigators have applied ^{2}H NMR spectroscopy to study the differences in physical properties between saturated and unsaturated lipid bilayers. Deese and co-workers (1981) used ^{2}H NMR spectroscopy to measure the main phase transition temperature of a dispersion of the mixed-chain saturated–polyunsaturated phosphatidylcholine (C^2H_3-16:0)(22:6)PC and found it to be significantly lower than that in the corresponding disaturated phosphatidylcholine. Hysteresis, the difference in temperature at which a lipid dispersion undergoes the gel[2]-to-L_α phase transition as compared to the L_α-to-gel transition, was found to be very large in the mixed-chain lipid. Paddy and co-workers (1985) used ^{2}H NMR to compare the order in three multilamellar lipid dispersions with increasing degrees of unsaturation: (per-^{2}H-16:0)-(16:0)PC, (per-^{2}H-16:0)(16:1)PC, and (per-^{2}H-16:0)(22:6)PC. The data was analyzed in terms of the reduced temperature, defined as (Seelig & Browning, 1978)

$$T_{red} = \frac{T - T_m}{T_m} \quad (1)$$

where T is the temperature and T_m is the main phase transition temperature, on the absolute scale (K). At equivalent reduced temperatures, the authors concluded that six double bonds significantly increased bilayer order relative to complete saturation but that a single double bond had an even greater effect.

^{2}H NMR has also been used to compare randomly oriented multilamellar dispersions of the mixed-chain (per-^{2}H-16:0)-(22:6)PC with the disaturated phospholipid di(per-^{2}H-16:0)PC, in the lamellar liquid–crystalline (L_α) phase (Salmon et al., 1987). Again, at equivalent reduced temperatures, the palmitoyl *sn*-1 chain in the mixed-chain phospholipid was found to possess less orientational freedom and molecular area relative to that in the disaturated phospholipid; also, the motional constraints imposed by the tethered headgroups extended less far into the bilayer. This work has recently been extended to the homologous series of disaturated and mixed-chain saturated–polyunsaturated phosphatidylcholines in the L_α phase, di(per-^{2}H-*n*:0)PC and (per-^{2}H-*n*:0)(22:6)PC, where n = 12, 14, 16, and 18 (Brown et al., 1989; Dodd & Brown, 1989; Salmon & Brown, 1989). Differences similar to those for the saturated and polyunsaturated phospholipids with n = 16 were found to occur throughout the homologous series.

[2] The term "gel phase" is used in this work to refer to the phases or states existing below the main phase transition from the liquid–crystalline (L_α) phase, although there is some controversy as to whether the $L_{\beta'}$ and $P_{\beta'}$ states in phosphatidylcholines are true, thermodynamically stable phases (Serrallach et al., 1983).

This paper extends the work on the L_α phase of the mixed-chain (per-^{2}H-*n*:0)(22:6)PC series into the gel-phase region near and below the main (chain-melting) phase transition. The phase transition temperatures and bilayer order in the gel and L_α phases were examined in order to determine the effects of substitution of a saturated chain for a 22:6 chain as well as the effects of the saturated chain length. These results are discussed both within the context of the mixed-chain series itself and in comparison to those of the disaturated series.

Interpretation of ^{2}H NMR Spectra. The quadrupolar splitting in a powder pattern with a rigid, axially symmetric electric field gradient is

$$\Delta\nu_Q^{rigid} = \frac{3}{2}\frac{e^2qQ}{h}P_2(\cos\theta) \quad (2)$$

The e^2qQ/h term is the static quadrupolar coupling constant, equal for all C–^{2}H segments. The $P_2(\cos\theta)$ term is the second Legendre polynomial, equal to $^1/_2(3\cos^2\theta - 1)$, where θ is the orientation of the bilayer normal with respect to the static magnetic field direction. For bilayers oriented perpendicular to the magnetic field, $\theta = 90°$ and the splitting is

$$(\Delta\nu_Q)_\perp = \frac{3}{4}\frac{e^2qQ}{h} \quad (3)$$

The parallel orientation ($\theta = 0°$) gives a splitting $(\Delta\nu_Q)_\parallel$ 2 times that of $(\Delta\nu_Q)_\perp$. For a methylene quadrupolar coupling constant of about 168 kHz (Burnett & Müller, 1971), the static powder pattern spectrum has splittings of $(\Delta\nu_Q)_\perp \sim \pm 63$ kHz and $(\Delta\nu_Q)_\parallel \sim \pm 126$ kHz.

If there is rapid rotation of the chains about the lipid longitudinal axis (where rapid is defined on the ^{2}H NMR time scale of the inverse of the static quadrupolar interaction), then we must consider the angular fluctuation β of the C–^{2}H bond direction with respect to the bilayer normal. This angular dependence introduces an additional Legendre polynomial to the expression for $\Delta\nu_Q$ in eq 2, though this one is time-dependent:

$$\Delta\nu_Q = \frac{3}{2}\frac{e^2qQ}{h}P_2(\cos\theta)\langle P_2(\cos\beta(t))\rangle \quad (4)$$

The new Legendre polynomial is called the segmental bond *order parameter*, given by

$$S_{CD} = \frac{1}{2}\langle 3\cos^2\beta(t) - 1\rangle \quad (5)$$

which describes the time-averaged angular excursions experienced by the C–^{2}H segments. For an all-trans configuration of the saturated acyl chains rotating about the bilayer normal, $\beta = 90°$, $|S_{CD}| = 0.5$, and the corresponding powder pattern has splittings of $(\Delta\nu_Q)_\perp \sim \pm 31$ kHz and $(\Delta\nu_Q)_\parallel \sim \pm 63$ kHz. At higher temperatures, trans–gauche isomerizations about the carbon–carbond bonds lead to a mixture of gauche and trans configurations along the acyl chains, which reduces the absolute values of S_{CD} and the widths of the quadrupolar splittings.

To investigate the order *below* the chain-melting phase transition, moments of the spectral lineshapes are used to estimate the average C–^{2}H bond order parameter, $\langle|S_{CD}|\rangle$, since the broad gel state ^{2}H NMR lineshapes preclude the direct measurement of individual quadrupolar splittings. For a line symmetric about its center, the nth moment of half of a spectral line is expressed as

$$M_n = \frac{\int_0^\infty x^n F(x)\,dx}{\int_0^\infty F(x)\,dx} \quad (6)$$

where $F(x)$ is the distribution function for the line and $x = \omega - \omega_0$, the symmetric distribution about the resonance frequency, ω_0 (Davis, 1983). The nth moment can be related to the distribution of order parameters by

$$M_n = A_n\left(\frac{3\pi}{2}\frac{e^2qQ}{h}\right)^n \langle|S_{CD}|^n\rangle \quad (7)$$

where A_n is a constant that depends on n.

The first moment, M_1, is directly proportional to the mean of the order parameter, according to (Davis, 1979)

$$M_1 = \frac{\pi}{\sqrt{3}}\frac{e^2qQ}{h}\left\langle\left|S_{CD}\right|\right\rangle \quad (8)$$

where the values for S_{CD} are taken in this work as the absolute values. Similarly, the second moment, M_2, is

$$M_2 = \frac{9\pi^2}{20}\left(\frac{e^2qQ}{h}\right)^2 \langle|S_{CD}|^2\rangle \quad (9)$$

The first and second moments combined yield the relative mean square deviation of the distribution of order parameters, Δ_2 (Davis, 1979), given by

$$\Delta_2 = \frac{\langle|S_{CD}|^2\rangle - \langle|S_{CD}|\rangle^2}{\langle|S_{CD}|\rangle^2} = \frac{M_2}{1.35M_1^2} - 1 \quad (10)$$

The Δ_2 parameter represents the width of the quadrupolar splitting distribution; therefore, it varies from low to high to low upon passing from one phase through the transition into the new phase. The results of the moment analyses are plotted as a function of temperature in Figures 4, 5, and 6 for M_1, M_2, and Δ_2, respectively.

It is important to note that the use of lineshape analyses to describe the "order" in gel-phase lipids is an approximate technique that allows the relative freedom of motion in the deuterated chains of lipids to be compared qualitatively, as well as giving an indication of the extent of axial rotation below the phase transition. Lineshape analyses alone, however, do not quantitatively characterize order in gel-phase bilayers because they do not specifically describe the rates and types of motion in the chains.

Experimental Procedures

The saturated fatty acids were purchased commercially (Sigma, MO) and perdeuterated with ^2H gas using a 10% Pd–charcoal catalyst; the extent of deuteration was determined with mass spectrometry and proton NMR (Hsiao et al., 1974). The *cis*-4,7,10,13,16,19-docosahexaenoic acid was purchased from Nu Chek Prep, Inc. (Elysian, MN) and used without further purification. The disaturated phospholipids were synthesized from the fatty acid anhydrides and the cadmium chloride adduct of *sn*-glycero-3-phosphatidylcholine as described (Mason et al., 1981; Williams et al., 1985) and purified on silica gel columns. The mixed-chain phospholipids were synthesized by hydrolyzing the *sn*-2 chain from the disaturated lipids with snake venom phospholipase A_2 from *Crotalus adamanteus* (Sigma, MO). The resulting lysophospholipid was reacylated with docosahexaenoic acid as described (Salmon et al., 1987). All procedures involving docosahexaenoic acid were performed under argon with use of solvents containing butylated hydroxytoluene (BHT) as an antioxidant (in a molar ratio of about 1:1000 BHT to phospholipid). No migration of the acyl chains was detected (<2%) with gas–liquid chromatography.

The NMR samples consisted of 50 wt % multilamellar dispersions of 200–250 mg of lipid in 67 mM sodium phosphate buffer made with ^2H-depleted water. While not in use, the samples were stored under argon at −80 °C. The ^2H NMR spectra for (per-^2H-12:0)(22:6)PC, (per-^2H-16:0)(22:6)PC, and (per-^2H-18:0)(22:6)PC were acquired on a General Electric GN-500 spectrometer at a magnetic field strength of 11.7 T (^2H frequency of 76.78 MHz); the (per-^2H-14:0)-(22:6)PC spectra were acquired on a General Electric GN-300 spectrometer operating at a magnetic field strength of 7.0 T (46.13 MHz for ^2H). Both spectrometers were equipped with home-built, horizontal solenoid, high-power probes having a 10-mm coil diameter, an external fast digitizer (Nicolet 2090 digital oscilloscope), and a high-power radiofrequency amplifier (Henry Radio Tempo 2006). The free-induction decays (FID) were acquired with a phase-cycled quadrupolar echo pulse sequence, quadrature detection, a 6-μs 90° pulse, 40-μs pulse separation, recycle time of 0.5–1 s, and digitization rate (spectral width) of 500 kHz. The B-channel of the FID was zeroed before Fourier transforming.

For each lipid, two series of measurements were made over a range of temperatures: in the first series the lipid dispersion was cooled from the liqid–crystalline L_α (disordered) phase to the gel (ordered) phase; the second series of measurements was then made by warming from the gel phase. After each change in temperature, the sample was equilibrated for about 30 min. The temperature was measured with a thermistor and controlled with the variable temperature unit of the NMR instrument to ±0.02 °C. There were 1024 scans acquired in the L_α phase and 2048 scans made at the onset of the phase transition and below; the exception to this was (per-^2H-18:0)(22:6)PC, for which 4096 and 8192 acquisitions were made in the L_α and gel phases, respectively. The spectra were transferred by use of the NMR1 software (New Methods Research, Inc.) to a Digital Equipment Corporation MicroVax II minicomputer, where the moment analyses were performed.

Results

Representative ^2H NMR spectra in the region of the phase transition and extending into the gel phase are shown in Figure 1 for the mixed-chain saturated–polyunsaturated series and in Figure 2 for the disaturated series. The actual and reduced temperatures are given with each. Similar gel ^2H NMR spectra have been observed elsewhere, for example in di-(per-^2H-16:0)PC (Davis, 1979) and (16:0)(per-^2H-16:0)PC (Yue et al., 1988).

As the temperature is decreased below the phase transition, all spectra reflect an increasing proportion of the C–^2H segments in the trans configuration. Eventually all spectra show at least some intensity at ≈±63 kHz, as expected for the $\theta = 0°$ shoulders of the all-trans, *axially rotating* configuration. In some spectra, the intensity at ±63 kHz becomes pronounced, for example in (per-^2H-14:0)(22:6)PC at −46.5 °C and in di(per-^2H-16:0)PC at −31.7 °C (Figure 3). In these cases, the intensity at ±63 kHz originates either from the 90° orientation of rigid, *non*rotating lipids or from all-trans lipids with very slow axial rotation (on the ^2H NMR time scale). The corresponding $\theta = 0°$ shoulders are also present, visible out to about ±120 kHz. The signal intensity at these large spectral widths is reduced because of the power drop-off due to the relatively long 90° pulse width of 6 μs. In the gel phase, this results in a lower value for the moment, with the effect on the moment increasing as the temperature is decreased. However, since all spectra were acquired with the same probe and pulse length, the moments can still be compared to one another at equivalent reduced temperatures.

Of the eight lipids studied, all but the $n = 14$ and 18 members of the disaturated series were taken to low enough

Table I: Main Phase Transition Temperatures (in degrees Celsius) Determined from Plots of Moments versus Temperature for the Homologous Series of Mixed-Chain and Disaturated Phosphatidylcholines[a]

	sn-2 chain					
	per-^{2}H-*n*:0			22:6		
sn-1-per-^{2}H-	T_m^{dec}	T_m^{inc}	ΔT_m	T_m^{dec}	T_m^{inc}	ΔT_m
12:0	−0.6 ± 0.4	3.8 ± 0.4	4.4	−25.0 ● 0.4	−11.2 ● 0.6	13.8
14:0	19.5 ± 0.5	19.5 ± 0.5	0.0	−12.5 ± 0.7	−2.2 ● 0.5	10.3
16:0	37.1 ± 0.4	37.4 ± 0.4	0.3	−12.7 ± 0.5	−3.0 ± 0.5	9.7
18:0	50.0 ± 0.5	50.0 ± 0.3	0.0	−7.7 ● 0.4	−5.3 ± 0.7	2.4

[a] Both the heating (T_m^{inc}) and cooling (T_m^{dec}) transition temperatures are given. The hysteresis, $T_m^{inc} - T_m^{dec}$, is denoted by ΔT_m.

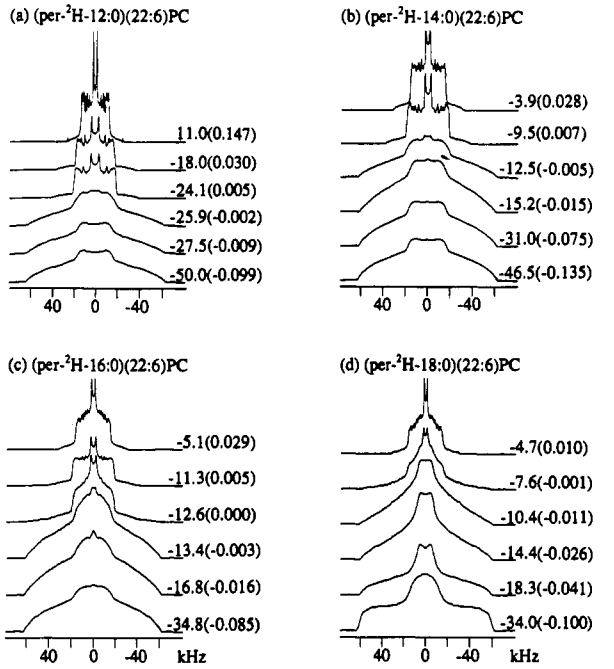

FIGURE 1: Representative ^{2}H NMR spectra of multilamellar dispersions of mixed-chain *saturated–polyunsaturated* phosphatidylcholines as a function of temperature: (a) (per-^{2}H-12:0)(22:6)PC, (b) (per-^{2}H-14:0)(22:6)PC, (c) (per-^{2}H-16:0)(22:6)PC, and (d) (per-^{2}H-18:0)(22:6)PC). With each spectrum the actual temperature (in degrees Celsius) is given followed in parentheses by the reduced temperature; the vertical scale is adjusted for clarity. The spectra in the gel state show spectral widths out to ~±60 kHz, corresponding to both the $\theta = 0°$ bilayer orientation of all-trans, axially rotating C^2H_2 groups and the $\theta = 90°$ orientation of rigid (nonrotating) or very slowing rotating C^2H_2 groups; the C^2H_3 groups contribute to the spectral center. A representative mixed-chain phosphatidylcholine spectrum showing the $\theta = 0°$ component of the nearly rigid spectrum is given in Figure 3a.

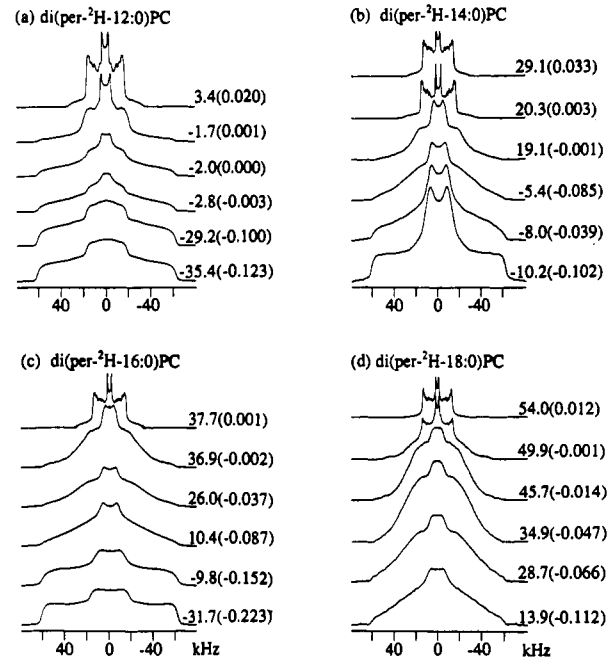

FIGURE 2: Representative ^{2}H NMR spectra of multilamellar dispersions of *disaturated* phosphatidylcholines as a function of temperature: (a) di(per-^{2}H-12:0)PC, (b) di(per-^{2}H-14:0)PC, (c) di(per-^{2}H-16:0)PC, and (d) di(per-^{2}H-18:0)PC. Each spectrum includes the actual temperature (in degrees Celsius), followed by the reduced temperature in parentheses; the vertical scale is arbitrary. Refer to Figure 3b for a spectrum of a disaturated phosphatidylcholine with the $\theta = 0°$ shoulders produced by rigid or nearly rigid methylene groups.

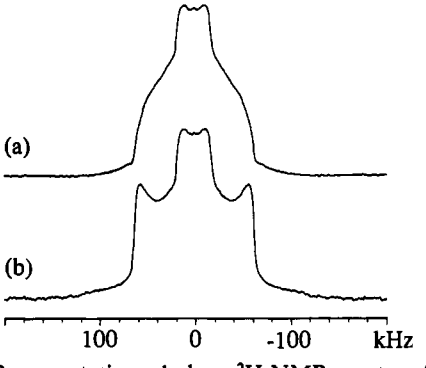

FIGURE 3: Representative gel-phase ^{2}H NMR spectra of (a) (per-^{2}H-14:0)(22:6)PC at −46.5 °C and (b) di(per-^{2}H-16:0)PC at −31.7 °C. The spectra possess very large distributions of quadrupolar splittings, including intensity out to ~±120 kHz due to the $\theta = 0°$ shoulders of either an all-trans, rigid configuration of the perdeuterated methylene groups or an all-trans configuration with very slow axial rotation. The wide range of quadrupolar splittings probably arises from variations in motion both between different acyl chains and along the acyl chains.

temperatures that the spectra exhibited intensity out to ±120 kHz. At reduced temperatures of about −0.1 °C, the ±120 kHz shoulders are present in the mixed-chain series and in di(per-^{2}H-12:0)PC but are not yet visible in the n = 14, 16, and 18 members of the disaturated series, indicating that the former lipids are more ordered than the latter. Although many of the spectra have intensity at ±120 kHz, *all* of the gel-phase spectra exhibit a wide range of quadrupolar splittings, including the narrow splittings corresponding to rotating methyl groups. Similar composite spectra are shown in Figure 8 of Davis (1979) for the gel phase of di(per-^{2}H-16:0)PC down to −7 °C. This author hypothesized that the broad distribution of splittings corresponds to a variable degree of order along the chains.

The L_α–gel phase transition temperatures, compiled in Table I, were derived from the M_1 plots as the point midway along the curve connecting the extrapolated base lines of the two phases. The transition temperatures are plotted as a function of the number of *sn*-1 acyl carbons in Figure 7. In the moment plots for di(per-^{2}H-16:0)PC (Figures 4c and 5c), there is an additional change in slope at about 9 °C, which may correspond to the transition to the crystalline L_c phase. This tem-

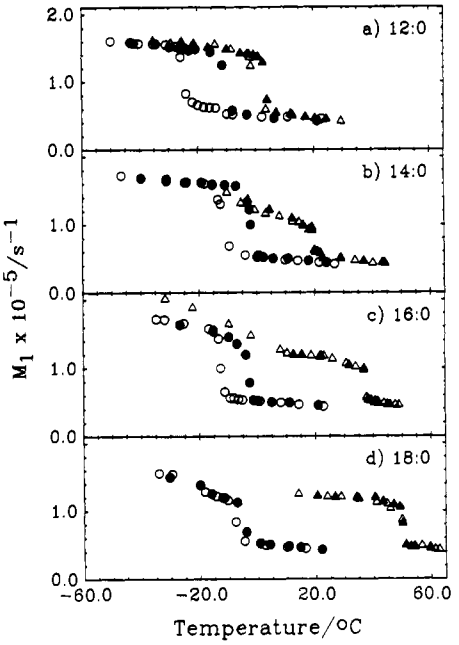

FIGURE 4: Plots of the first moment (M_1) of the ^2H NMR spectra as a function of both decreasing temperature (open symbols) and increasing temperature (filled symbols) for the (per-^2H-*n*:0)(22:6)PC series (circles) and di(per-^2H-*n*:0)PC series (triangles). The number of carbons (*n*) in the saturated (*n*:0) acyl chain is (a) 12, (b) 14, (c) 16, or (d) 18. The main phase transition is indicated by the abrupt change in the magnitude of the moments.

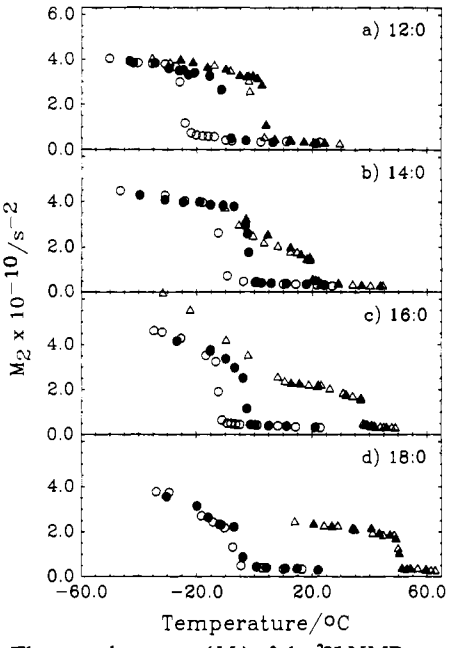

FIGURE 5: The second moment (M_2) of the ^2H NMR spectra for the (per-^2H-*n*:0)(22:6)PC and di(per-^2H-*n*:0)PC series as a function of temperature. Refer to the legend to Figure 4 for the definition of symbols.

perature is consistent with the pretransition temperature for protiated di(16:0)PC of about 13 °C (Serrallach et al., 1983), since the deuterium isotope shift for di(16:0)PC (discussed below) lowers the phase transition temperature by about 4 °C.

An earlier ^2H NMR measurement of 37 °C for the main transition temperature of di(per-^2H-16:0)PC (Davis, 1979) agrees well with the warming (T_m^{inc}) and cooling (T_m^{dec}) values of 37.4 and 37.1 °C in this work. Differential scanning calorimetry (DSC) warming curve transition temperatures for di(per-^2H-14:0)PC, di(per-^2H-16:0)PC, and di(per-^2H-18:0)PC of 20.2, 37.1, and 50.5 °C, respectively (Guard-Friar et al., 1985), are also close to the T_m^{inc} values in Table I. The ^2H NMR study of (C^2H_3-16:0)(22:6)PC (Deese et al., 1981) reported a warming curve value of −3 °C with an 8–9 °C hysteresis, in close agreement with the values of −3.0 and 9.7 °C, respectively, for the (per-^2H-16:0)(22:6)PC in this work.

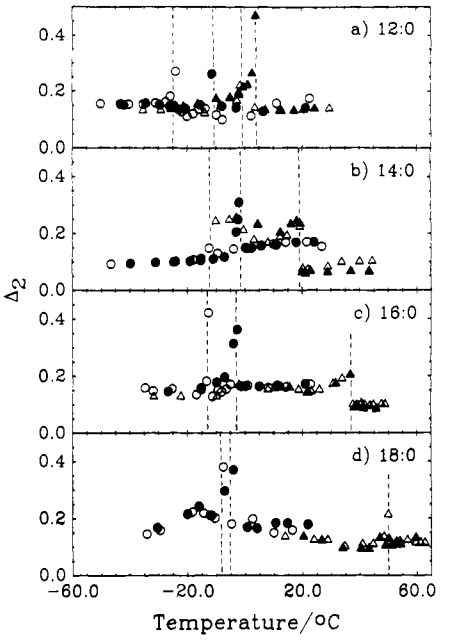

FIGURE 6: The parameter Δ_2 of the ^2H NMR spectra for the (per-^2H-*n*:0)(22:6)PC and di(per-^2H-*n*:0)PC series as a function of temperature, with the same symbol definitions used as in Figure 4. The discontinuities in the curves correspond to the increasing and decreasing phase transition temperatures, indicated by dotted lines.

For the *protiated* disaturated series, high-sensitivity DSC warming curves (Mabrey & Sturtevant, 1976; Lewis et al., 1987) show that chain perdeuteration decreases T_m by 4.4, 4.0, and 5.3 °C for n = 14, 16, and 18, respectively. Interestingly, the deuterium isotope effect for the n = 12 member of the series is reversed: the value for T_m^{inc} in di(per-^2H-12:0)PC was 5.6 °C *greater* than in the protiated lipid in Lewis et al. (1987). The hysteretic behavior observed with DSC in the protiated disaturated series agrees well with the results here, with little or no hysteresis occurring in the n = 14, 16, and 18 members, while a significant hysteresis was observed for n = 12 (about 2.5 °C with DSC, and 4.4 °C in the current work).

Normalizing the Data with Respect to Temperature. For the direct comparison of membrane properties at biological temperatures, the absolute temperature scale is the most relevant scale to use. In the current work, however, we wish to examine the relationship between bilayer order and molecular structure by comparing the order between lipids of systematically varying composition over a large range of temperatures. A problem inherent in comparing the physical properties of different lipid bilayers is that the temperatures at which the physical properties are measured vary depending on the phase transition temperatures of each lipid. For this type of analysis, then, temperature is a variable that must be accounted for in some way, depending on the type of information one wishes to extract from the data.

To simply compare bilayer properties at the same temperature relative to the phase transition temperature, one can employ a *relative* temperature defined as

$$T_{rel} \equiv T - T_m \tag{11}$$

This method places the phase transition temperature of all lipids at a common point but otherwise retains all information

Table II: First Moments (M_1) for the Homologous Series of Disaturated and Mixed-Chain Phosphatidylcholines, di(per-^{2}H-*n*:0)PC and (per-^{2}H-*n*:0)(22:6)PC, at Equivalent *Reduced* Temperatures in the Cooling Series of Measurements[a]

	sn-2 chain					
	per-^{2}H-*n*:0		22:6		M_1(22:6) − M_1(per-^{2}H-*n*:0)	
sn-1-per-^{2}H-	$M_1(L_\alpha)^b$	M_1(gel)^c	$M_1(L_\alpha)^b$	M_1(gel)^c	$L_\alpha{}^b$	gel^c
12:0	0.52 ± 0.01	[1.57 ± 0.03]	0.51 ± 0.01	1.57 ± 0.02	−0.01 ± 0.02	[0.00 ± 0.06]
14:0	0.48 ± 0.02	1.17 ± 0.02	0.53 ± 0.01	1.64 ± 0.02	0.05 ± 0.03	0.47 ± 0.04
16:0	0.46 ± 0.01	1.19 ± 0.03	0.53 ± 0.01	1.66 ± 0.03	0.07 ± 0.02	0.47 ± 0.07
18:0	0.43 ± 0.02	1.20 ± 0.03	0.48 ± 0.01	(1.35 ± 0.03)	0.05 ± 0.03	(0.15 ± 0.06)

[a] All moments are times 10^{-5} Hz. The reduced temperature is defined in eq 1. Brackets and parentheses denote that the enclosed values are affected by the unique behavior of di(per-^{2}H-12:0)PC and (per-^{2}H-18:0)(22:6)PC, respectively, as described in the text. [b] $T_{red} = 0.05$. [c] $T_{red} = -0.05$.

Table III: Normalized First Moments (M_1^{norm}) for the Homologous Series of Disaturated and Mixed-Chain Phosphatidylcholines, di(per-^{2}H-*n*:0)PC and (per-^{2}H-*n*:0)(22:6)PC, at Equivalent *Relative* Temperatures in the Cooling Series of Measurements[a]

	sn-2 chain					
	per-^{2}H-*n*:0		22:6		M_1^{norm}(22:6) − M_1^{norm}(per-^{2}H-*n*:0)	
sn-1-per-^{2}H-	$M_1^{norm}(L_\alpha)^b$	M_1^{norm}(gel)^c	$M_1^{norm}(L_\alpha)^b$	M_1^{norm}(gel)^c	$L_\alpha{}^b$	gel^c
12:0	0.58 ± 0.02	[1.53 ± 0.05]	0.54 ± 0.02	1.54 ± 0.03	−0.04 ± 0.04	0.01 ± 0.07
14:0	0.55 ± 0.02	1.09 ± 0.03	0.59 ± 0.02	1.58 ± 0.03	0.04 ± 0.04	0.49 ± 0.06
16:0	0.50 ± 0.02	1.14 ± 0.04	0.58 ± 0.02	1.57 ± 0.04	0.08 ± 0.04	0.43 ± 0.08
18:0	0.49 ± 0.03	1.16 ± 0.05	0.53 ± 0.02	(1.12 ± 0.05)	0.04 ± 0.05	(−0.04 ± 0.1)

[a] All moments are times 10^{-5} Hz. Relative temperature and normalized moment are defined in eqs 11 and 14, respectively. Brackets and parentheses denote that the enclosed values are affected by the unique behavior of di(per-^{2}H-12:0)PC and (per-^{2}H-18:0)PC, respectively, as described in the text. [b] $T_{rel} = 15.0$ °C. [c] $T_{rel} = -15.0$ °C.

on the effects of differences in temperature on the properties being studied. However, when one wishes to estimate the influences of lipid composition and structure on bilayer order *without* temperature as a variable, a commonly used method, also employed in the current work, is to analyze the data in terms of the *reduced* temperature in eq 1. The results of the moment analyses in terms of the reduced temperature are compiled in Table II and plotted in Figure 8.

Since one aim of this work is to determine the effects of chain length and unsaturation on bilayer properties, independent of the influences of temperature, we considered the theoretical basis for applying the reduced temperature concept to lipids. The origin of reduced temperature is the van der Waals equation of state describing the crucial point (T_c, P_c, V_c) on a vapor–liquid curve on a pressure–volume–temperature surface (Stanley, 1971). The law of corresponding states formulates the van der Waals equation of state in terms of the reduced variables, T_{red}, P_{red} and V_{red}, where reduced temperature is defined as

$$T_{red} \equiv \frac{T - T_c}{T_c} \tag{12}$$

for the condition that the absolute temperature T is close to the critical temperature T_c. When the concept of reduced temperature is applied to lipids, there are a number of assumptions being made: (1) the phase transition temperature T_m is close enough to the critical temperature T_c that eq 1 can be used in place of eq 12 as the definition of reduced temperature; (2) the law of corresponding states is obeyed; and (3) the lipids are at the same reduced pressure and volume. Depending on the validity of these assumptions, the use of reduced temperature in lipid systems may not entirely account for the influence of temperature.

Since it is uncertain whether or not the reduced temperature scale completely corrects for the effects of differences in temperature, we attempted to circumvent the theoretical unknowns by using a two-step method to eliminate the effect of temperature on order: first the temperatures are converted to a relative temperature scale (eq 11) and then the moments are normalized by empirically subtracting out the dependence of the moments on temperature in the vicinity of the phase transition. Using the relative temperature scale accounts for differences in the phase transition temperatures, while normalizing the moments approximately removes any remaining effects due to differences in temperature.

The normalization is based on a Taylor series expansion of the first moment as a function of temperature, $M_1(T)$, given by

$$M_1(T^*) = \left(\frac{\partial M_1}{\partial T}\right)(T - T^*) + \frac{1}{2!}\left(\frac{\partial^2 M_1}{\partial T^2}\right)(T - T^*)^2 + \ldots \tag{13}$$

where $M_1(T)$ is the moment at a temperature T and T^* is a reference temperature. If we make the first-order approximation to truncate the series after the second term and then assign the reference temperature as the transition temperature, T_m, eq 13 can be written as a normalized moment, M_1^{norm},

$$M_1^{norm} = M_1(T_m) = M_1(T) + \left(\frac{\partial M_1}{\partial T}\right)(T_m - T) \tag{14}$$

Since the function $M_1(T)$ has a discontinuity at the phase transition temperature, the L_α and gel phases must be analyzed separately. Therefore, $(\partial M_1/\partial T)$ corresponds to a slope in the moment plots in Figure 4 for a given phase. Values for the slopes were calculated empirically from linear regressions on the moment plots in Figure 4 for each phase of each lipid, and the normalized moments were calculated from eq 14. Values for the normalized moments at 15 °C above and below the phase transition were interpolated or extrapolated from these lines and compiled in Table III. The effects on the interpretation of the data when using normalized moments at equivalent relative temperatures as compared to unnormalized moments at equivalent reduced temperatures are discussed in the next section.

Another theory predicts that the degree of bending in polymer-like chains in the fluid state (or the fraction of gauche bonds in the case of lipids in the L_α phase) will be the same if compared at equivalent absolute temperatures (Flory, 1956; Nagle & Wiener, 1988). We tested this theory by extrapolating the moment data for the L_α phase to 80 °C, a temperature sufficiently high that all lipids were well in the L_α

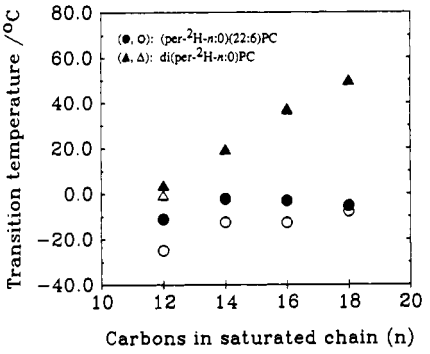

FIGURE 7: Dependence of the main order–disorder (gel–L_α) transition temperature (T_m) on the length of the saturated acyl chain in multilamellar phospholipid dispersions containing 50 wt % H_2O. Circles correspond to the transition temperatures of the disaturated phosphatidylcholines, while triangles denote those for the mixed-chain saturated–polyunsaturated phosphatidylcholines. Filled symbols represent increasing temperature, and open symbols represent decreasing temperature.

phase. The resulting values for M_1 varied from 0.04×10^5 to 0.30×10^5 Hz in the disaturated series and from 0.13×10^5 Hz to 0.23×10^5 Hz in the mixed-chain series, giving an overall mean value of 0.18×10^5 with a standard deviation of 0.09×10^5. These differences, while not great, are well outside the error limits of about $\pm 0.02 \times 10^5$ Hz for the L_α phase and are therefore statistically significant. The lipids in this work, then, do not become equally ordered in the L_α phase at high temperatures.

Discussion

Effects of Docosahexaenoic Acid on the Phase Transition Temperature. Substitution with docosahexaenoic acid (22:6) at the *sn*-2 position in place of a saturated acyl chain significantly depresses the main phase transition temperature (T_m) throughout the homologous series (see Figure 7 and Table I), consistent with previous results on (per-²H-16:0)(22:6)PC (Deese et al., 1981; Salmon et al., 1987). There is a pronounced dependence of T_m on the number of acyl carbons (n) in the disaturated series (see Figure 7). Substitution with a 22:6 chain, however, significantly reduces the dependence of T_m on n: the difference in T_m in the warming series of measurements from *sn*-1 = 12:0 to 18:0 is 46.2 °C in the disaturated phosphatidylcholines but only 5.9° in the mixed-chain polyunsaturated series. While the dependence of T_m on n is essentially linear in the disaturated phosphatidylcholines, T_m in the mixed-chain series varies as $T_m(12:0) < T_m(14:0) \approx T_m(16:0) < T_m(18:0)$. Hysteresis is absent within experimental error in the disaturated phosphatidylcholines except for di(per-²H-12:0), for which it is quite large (4.4 °C). Across the mixed-chain polyunsaturated series, however, the hysteresis is widely varied and very pronounced (except for the 18:0 member), following a trend of $\Delta T_m(12:0) > \Delta T_m(14:0) \approx \Delta T_m(16:0) > \Delta T_m(18:0)$.

The explanation for the trends in T_m and the hysteresis in the (n:0)(22:6)PC series may be connected to the length differences between the *sn*-1 and *sn*-2 chains. Applegate and Glomset (1986) estimated with computer-based molecular modeling that in a (18:0)(22:6) phospholipid the all-trans 18:0 chain is 4.5 Å *longer* than the 22:6 chain and in a (16:0)(22:6) lipid the 16:0 chain is about 1.9 Å *longer* than the 22:6. Extrapolating to the 12:0 and 14:0 analogues by subtracting 1.26 Å for each carbon–carbon bond projection (all-trans configuration), we estimate that the 14:0 chain is roughly 0.7 Å *shorter*, and the 12:0 chain about 3.3 Å *shorter*, than the 22:6 chain. In both (per-²H-16:0)(22:6)PC and (per-²H-14:0)(22:6)PC, then, the *sn*-1 chain is similar in length to the *sn*-2 chain. In the 12:0 and 18:0 members, the difference in length between the saturated and polyunsaturated chains is much greater. Therefore, the similarity between the 14:0 and 16:0 members in both the cooling and warming transition temperatures (and therefore also in the hysteresis) may be due to the similarity in length between the *sn*-1 and *sn*-2 chains since the relative length of adjacent chains influences the packing of the lipids (Lin et al., 1990). Packing in the bilayer in turn affects bilayer order, as well as the packing and unpacking of lipids that accompanies the formation and "melting" of the gel phase.

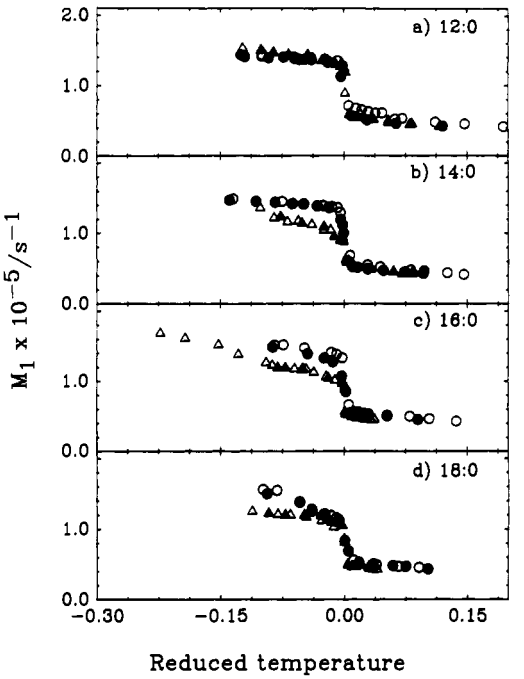

FIGURE 8: The first moment (M_1) of the ²H NMR spectra for the (per-²H-*n*:0)(22:6)PC and di(per-²H-*n*:0)PC series plotted on a *reduced* temperature scale, with the same symbols used as in Figure 4. The reduced temperature is defined as $(T - T_m)/T_m$.

Effects of Acyl Chain Length on Order. The first conclusion one can draw from Tables II and III is that the effects of acyl chain length on order are very subtle. (Two notable exceptions are di(per-²H-12:0)PC and (per-²H-18:0)(22:6)PC, which will be discussed later.) There are statistically significant differences, however, and by comparing the results from the reduced temperature method of normalization (Table II) to those from the empirical normalization (Table III), it can be seen that any effects of temperature on the reduced temperature results are rather small. For example, the lipids in the L_α phase of the disaturated series appear to become more disordered as the chain length increases, when compared at equivalent reduced temperatures (column 2, Table II). However, the longer the saturated acyl chain, the higher the temperature, at equivalent points relative to the phase transition (Figure 4). This poses the question of whether the observed increase in disorder at equivalent reduced temperatures is due in whole or part to the additional thermal energy present in the longer chained bilayers rather than to the additional carbon atoms. However, when the temperature was accounted for by empirically normalizing the moments and comparing them at equivalent relative temperatures (column 2, Table III), the same trend was observed. The same was true for both phases in all lipids studied in this work, although in some cases the empirical normalization had the effect of increasing the moments as chain length increased, relative to the reduced tem-

perature results, indicating that some residual effects of temperature may be present in the reduced temperature method. In the current work, however, these effects were not pronounced enough to alter conclusions regarding the effect of chain length on bilayer order.

In the gel phase of the disaturated series (column 3 of Tables II and III), bilayer order in the n = 14, 16, and 18 members increases very slightly as n increases, with both methods of normalization. The di(per-^{2}H-12:0)PC is significantly more ordered in the gel phase than the other disaturated lipids, consistent with the appearance of the spectra (Figure 2a); as a reminder of this, the moment values relating to the gel phase of this lipid are enclosed in square brackets in Tables II and III. In the L_α phase in the *mixed-chain* series (column 4, Tables II and III) the n = 14 and 16 members are equally ordered, with n = 12 and 18 slightly lower. The order in the *gel* phase of the n = 12, 14, and 16 members of the mixed-chain series follow the same trend (column 5 of Tables II and III). The behavior of the n = 18 member, however, is very unusual relative to all other lipids in this study: the degree of order at the onset of the gel phase is considerably lower than that of all other lipids in both series, yet it increases at an unusually rapid rate as the lipid is cooled. Yet well into the gel phase, for example at a reduced temperature of −0.1 °C, the order was close to but still lower than that of the other lipids in the series. Since the empirical method normalizes the moments to the value immediately following the phase transition, the exceptionally low order at this point causes the normalized moments at T_{red} = −0.5 °C and T_{rel} = −15 °C to be anomalously low. Therefore, the gel-phase moment values for this lipid are enclosed in parentheses in Tables II and III to indicate that they cannot be included in trends involving the other lipids. The unusual behavior of (per-^{2}H-18:0)-(22:6)PC in the gel phase is probably related to the small hysteresis observed in this lipid compared to the other mixed-chain lipids (Table I).

Effects of Docosahexaenoic Acid on Order. The *sn*-2 substituted docosahexaenoyl chain causes only a slight increase in order in the L_α phase as compared to the disaturated series (column 6 of Tables II and III). In the gel phase (column 7), the 22:6 chain *substantially* increases the order of the *sn*-1 chain in (per-^{2}H-14:0)(22:6)PC and (per-^{2}H-16:0)(22:6)PC relative to the disaturated lipids; the orders in (per-^{2}H-14:0)(22:6)PC and (per-^{2}H-16:0)(22:6)PC are equal within experimental error. Due to the significantly higher order exhibited in the gel phase of the di(per-^{2}H-12:0)PC, the gel-phase order of (per-^{2}H-12:0)(22:6)PC is equal to that of the disaturated lipid.

The significant amount of order induced by the 22:6 chain in the gel phase of (per-^{2}H-14:0)(22:6)PC and (per-^{2}H-16:0)(22:6)PC is consistent with (but does not prove) the conclusions of Applegate and Glomset (1986), who showed with computer molecular modeling studies of *sn*-1-*n*:0, *sn*-2-22:6 phospholipids that docosahexaenoic acid in an "angle iron" conformation can pack tightly with both saturated chains or with other 22:6 chains. The term angle iron was taken from the tool of the same name because alternating double bonds in the hexane string lie in two perpendicular planes, with the double bonds parallel to one another. When the lipids organize into an ordered packing array in the gel phase, the ability of iron angle shaped hexaenes to pack tightly with surrounding saturated and other hexaene chains could lead to a more highly ordered gel phase than a bilayer formed of only saturated chains (Applegate & Glomset, 1986), other factors permitting (such as headgroup size and the relative lengths of the *sn*-1 and *sn*-2 chains). In the L_α phase, where the chains are more loosely associated with one another compared to the regularly packed arrays characterizing the gel state, intermolecular interactions are too weak for this type of configurational information to be obtained. Therefore, on the basis of the current data alone, it cannot be ruled out that the 22:6 chain may change its average configuration during the L_α–gel phase transition, for example to include a preference for a helical configuration. However, a recent Raman study of (16:0)-(22:6)PC found that there were "minimal conformational reorganizations" in the 22:6 chain between the gel and L_α phases (Litman et al., 1991).

Summary

The moments of the ^{2}H NMR lineshapes for the homologous series of (per-^{2}H-n:0)(22:6) phosphatidylcholines, where n = 12, 14, 16, and 18, were determined as a function of temperature throughout the L_α and gel phases, both warming from the gel phase and cooling from the L_α phase. When compared to the phase transition temperatures of the corresponding disaturated phosphatidylcholines, the presence of docosahexaenoic acid (22:6) was found to (1) significantly decrease the main phase transition temperatures; (2) cause a substantial hysteresis between the warming and cooling transition temperatures; and (3) remove the approximately linear dependence of the transition temperature on chain length.

The (per-^{2}H-*n*:0)(22:6)PC series in the gel phase was found to have greater order than the disaturated series at the same reduced temperature, though there was little or no difference in order in the L_α phase. The one exception to this was di-(per-^{2}H-12:0)PC, which was much more highly ordered in the gel phase than the other disaturated lipids, with an order comparable to the mixed-chain series. Within the mixed-chain series, the (per-^{2}H-14:0)(22:6)PC and (per-^{2}H-16:0)(22:6)PC bilayers behaved almost identically in both phases at equivalent reduced temperatures, perhaps because in both lipids the *sn*-1 chain is similar in length to the *sn*-2 chain. The pair had nearly identical phase transition temperatures and hystereses and were approximately equal in order in both the L_α and gel phases. Also, the differences between (per-^{2}H-14:0)(22:6)PC and its disaturated counterpart were identical within experimental error to those between the n = 16 pair of lipids.

It is also important to remember in this study that perdeuterated saturatd chains are being used as probes of overall bilayer order in bilayers where half of the acyl chains are polyunsaturated. It is possible, particularly in the disordered L_α phase, that information from the perdeuterated saturated chain is dominated by the dynamics of this string of deuterated methylene groups, rather than adequately reporting on the contributions from the *polyunsaturated* chains also. This may be the reason that only small differences are seen between the disaturated and mixed-chain saturated–polyunsaturated bilayers in the L_α phase.

To test the applicability of reduced temperature for normalizing the moment data with respect to temperature, an alternate *empirical* method was used in which the lipids were compared by examining the order in the L_α and gel phases *at the phase transition temperature*. For the lipids in this study, a comparison of these two methods of normalization suggested that any residual effects of temperature left by the reduced temperature method were insignificant when the effects of acyl chain length and docosahexaenoic acid substitution on bilayer order were evaluated. The simple empirical method described here to remove the effects of temperature can also be applied to comparative studies of other lipid systems close to a phase

transition, as a prudent check on the applicability of reduced temperature for the system or systems being studied.

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