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Average Structural and Motional Properties of a Diunsaturated Acyl Chain in a Lipid Bilayer: Effects of Two Cis-Unsaturated Double Bonds†

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ABSTRACT: Isolinoleic acid ($18:2^{\Delta 6,9}$) deuterated at 10 different positions was esterified to form 1-palmitoyl-2-isolinoleoyl-*sn*-glycero-3-phosphocholine (PiLPC), and the average structural and motional properties of the diunsaturated chain, in aqueous dispersions of PiLPC, were examined by ^2H NMR spectroscopy. For each sample, ^2H spectra were acquired over a temperature range of 1-40 °C and the quadrupolar splittings interpreted in terms of carbon-deuterium bond order parameters, S_{CD}. Furthermore, definition of the average orientation of the C8 methylene unit with respect to the bilayer normal [Baenziger, J. E., Smith, I. C. P., Hill, R. J., & Jarrell, H. C. (1988) *J. Am. Chem. Soc. 110*, 8229-8231] provided sufficient information to calculate both the average orientations and the molecular order parameters, S_{mol} (which reflects the amplitudes of motion), for the C6-C7 and the C9-C10 double bonds. The results indicate that both the motional freedom (reflected in the order profile) and the average structure (reflected in the orientation of carbon segments with respect to the bilayer normal) are strongly affected by the presence of two cis-unsaturated double bonds. The data were interpreted in terms of two possible models whereby, in each case, the chain adopts a conformation consistent with the low-energy conformation of 1,4-pentadiene [Applegate, K. R., & Glomset, J. A. (1986) *J. Lipid Res. 27*, 658-680] but undergoes a two-site jump between the conformations. The jump motion arises mainly from rotations about the C7-C8 and the C8-C9 single bonds that disorder the C8 and the C9-C10 segments (S_{mol} = 0.15 and 0.08, respectively) but leave the C6-C7 double bond relatively immobile (S_{mol} = 0.55; all at 40 °C). It is suggested that acyl chains containing three or more double bonds could not undergo a similar jump motion and therefore would be highly ordered and not "fluid" as is generally thought.

Polyunsaturated fatty acyl chains are common components of most mammalian cell membranes and are concentrated at high levels at postsynaptic neurons, in the rod outer segment, and in other excitable cells (Nielson et al., 1970; Stone et al., 1979; Crawford et al., 1977). Considerable evidence also indicates that highly unsaturated fatty acids, such as docosahexaenoic acid (DHA,[1] $22:6^{\Delta 4,7,10,13,16,19}$), play an essential role in the biological function of these tissues (Lamptey & Walker, 1976; Wheeler et al., 1975; Neuringer et al., 1984; Wiedmann et al., 1988). Therefore, defining the physicochemical properties of polyunsaturated lipids in bilayers is an important step to gaining a complete understanding of membrane structure and function.

It has been proposed that the role of polyunsaturated lipids is to increase and perhaps even modulate the "fluidity" of membranes. This hypothesis arose from calorimetry experiments (DSC) which showed that saturated lecithin bilayers exist in the relatively rigid gel phase, at physiological temperatures, whereas bilayers containing monounsaturated acyl chains exist in the liquid crystalline state (Chapman et al., 1966). This observation has been extrapolated to suggest that there is a correlation between the number of double bonds and the temperature of the gel-to-liquid crystal phase transition and a direct relationship between the degree of unsaturation and the "fluidity" of a lipid bilayer. In contrast, recent DSC and NMR studies have shown that bilayers composed of highly unsaturated lipids have phase transition temperatures that are similar to, or even higher than, those composed of their less unsaturated counterparts (Coolbear et al., 1983; Deese et al., 1981; Drat & Deese, 1986). Furthermore, only subtle differences in the order and dynamics of lipid probes in bilayers of varying degrees of unsaturation have been observed (Stubbs et al., 1981; Straume & Litman, 1987; Deinum et al., 1988; Yeagle et al., 1987). However, the probe studies are only sensitive to those motions that occur over a limited range of

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[1] Abbreviations: DHA, *cis,cis,cis,cis,cis,cis*-4,7,10,13,16,19-docosahexaenoic acid; DSC, differential scanning calorimetry; FID, free induction decay; iLPPC, 1-isolinoleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine; NMR, nuclear magnetic resonance; PC, *sn*-glycero-3-phosphocholine; PiLPC, 1-palmitoyl-2-isolinoleoyl-*sn*-glycero-3-phosphocholine; PLPC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; T_c, gel-to-liquid crystal transition temperature.

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frequencies, and in the case of fluorescence, the probes are rigid structures that may not be sensitive to all types of lipid motion. Therefore, more detailed studies are required in order to gain a complete understanding of the physical properties of polyunsaturated lipids and thus their biological function.

^2H NMR spectroscopy can provide detailed information on the structural and motional properties of lipids in membranes (Smith, 1989). The NMR data are generally interpreted in terms of time-averaged structural parameters, such as order parameters (Davis, 1983), but more recently ^2H relaxation and line-shape analyses have been utilized to gain insight into the nature and time scales of the molecular motions occurring in lipid bilayers (Meier et al., 1986; Auger et al., 1990). Unfortunately, the application of ^2H NMR to polyunsaturated lipids has been retarded due to difficulties in the synthesis of specifically ^2H-labeled polyunsaturated acyl chains. We have synthesized an 18-carbon diunsaturated fatty acid (18:2$^{\Delta 6,9}$; referred to as isolinoleic acid) labeled with deuterium at 10 different positions along the chain (Baenziger et al., 1987, 1990) and present here a detailed analysis of the time-averaged structural and motional properties of the isolinoleoyl chain in model bilayers of 1-palmitoyl-2-isolinoleoyl-PC (PiLPC). The results indicate that both the average structure and the motions of an acyl chain containing two double bonds differ dramatically from those of saturated and monounsaturated acyl chains and suggest that polyunsaturated acyl chain are relatively rigid rather than "fluid" structures.

Materials and Methods

Sample Preparation. [4,4-$^2H_2$]-, [5,5-$^2H_2$]-, [6-^2H]-, [7-^2H]-, [8,8-$^2H_2$]-, [9,10-$^2H_2$]-, [11,11-$^2H_2$]-, [14,14-$^2H_2$]-, and [18,18,18-$^2H_3$]-*cis,cis*-octadeca-6,9-dienoic (isolinoleic) acids, synthesized as described previously (Baenziger et al., 1987, 1990), were esterified to form 1-palmitoyl-2-isolinoleoylphosphatidylcholine (PiLPC) by the method of Perly et al. (1984). Each deuterated lecithin was then purified on a Bio-Sil column (Pierce); the unesterified fatty acid was eluted with $CHCl_3$ and the pure product with $CHCl_3/CH_3OH/H_2O$, 45/25/2.5 (v/v/v). Column fractions were collected under argon in small vials, and the appropriate fractions were pooled and analyzed for purity by thin-layer chromatography (TLC) using two different solvent systems [see Baenziger et al. (1987) for details]. All chromatograms were charred with 10% $CuSO_4$ (w/v) (Bitman & Wood, 1982) in 8% H_3PO_4/H_2O (v/v) and some with Dragendorff's reagent (Kates, 1969) to test whether the catalyst *N,N*-dimethylaminopyridine was completely removed from the sample. Each deuterated PC was then dried, dissolved in pure $CHCl_3$, and passed through a small column containing Celite (Fisher Scientific) to remove any silica gel from the sample.

The purified 1-palmitoyl-2-isolinoleoyl-PCs were dried under a stream of argon, lyophilized to remove all traces of $CHCl_3$, and dispersed in excess deuterium-depleted H_2O (samples contained at most 35 mg of lipid in 200 μL of H_2O). Each sample was subjected to, on the average, five freeze–thaw–vortex cycles to ensure complete dispersion of the lipid and was sealed in a 5-mm NMR tube. All manipulations were carried out under argon, and the NMR samples were stored in the dark at −20 °C in order to prevent oxidation of the lipid. Reproducible NMR spectra were obtained months after the initial NMR experiments, and subsequent analysis by TLC showed no detectable levels of oxidation.

A small portion of each sample was also analyzed for acyl chain migration. In each case, the phospholipid was treated with phospholipase A_2 (*Crotalus adamanteus*; Keough & Davis, 1979), and the free fatty acids liberated from the *sn*-2 position were isolated by preparative TLC and identified by gas chromatography (GC). The GC analysis was carried out on an HP 5890A gas chromatograph equipped with a Supelcowax 10 (30 m × 0.25 mm) capillary column, a flame-ionization detector, and a Waters 745 integrator.

NMR Spectroscopy. ^2H NMR spectra were recorded either at 30.7 MHz on a custom-build spectrometer or at 46.06 MHz on a Bruker MSL-300 spectrometer. Spectra were acquired by using a modified quadrupolar echo pulse sequence (Rance et al., 1980) with full phase cycling of the radio-frequency pulses. The 90° pulse lengths were generally 2.3 μs (5-mm solenoid coil) or 4.6 μs (10-mm solenoid coil) in length, and the echo pulse spacing was 60 μs. Recycle times were ≥100 ms (always $>5 \times T_1$), and the spectra were generally acquired with a sweep width of 100 kHz. The sample temperature was monitored and maintained to within ±0.5 °C of the desired value with a custom-built variable temperature unit. Proton-decoupled spectra (MSL-300) were acquired with WALTZ-16 decoupling (Shaka et al., 1983) during acquisition of the free induction decay (FID). The ^1H decoupling was performed with 90° pulses of 60 μs at a decoupler power of 6 W. The time of decoupling (the acquisition time) was never allowed to exceed 2% of the recycle time in order to avoid sample overheating.

The ^2H NMR spectra were analyzed on a Nicolet 1280 data processor. The FIDs were left-shifted to the top of the echo before Fourier transformation of the trailing edge (Davis, 1983), and the transformed spectra were then dePaked by the method of Bloom et al. (1981) to give the 90° oriented-sample spectra. The quadrupolar splittings were measured from the dePaked spectra.

Results and Discussion

Liquid Crystalline Phase Spectra. ^2H spectra were acquired over a temperature range of 0–40 °C from each of the nine dispersions of deuterated 1-palmitoyl-2-isolinoleoylphosphatidylcholine (PiLPC). A representative spectrum, for each sample, is shown in Figure 1. All are indicative of lipids undergoing rapid ($>10^5$ s^{-1}) axially symmetric motions in the bilayer (Seelig, 1977) except for those acquired from [8′,8′-$^2H_2$]PiLPC. In contrast, these spectra consist of a single, relatively narrow peak that could arise if the lipid was organized in small vesicles or micelles that undergo rapid isotropic tumbling. However, the sample was a homogeneous opaque dispersion and gave a broad ^{31}P NMR powder pattern with a chemical shift anisotropy of 48 ppm (Baenziger, 1989), indicating that the lipids are organized in large multilamellar vesicles. Acquiring spectra with either increased digital resolution or varied pulse spacings had no effect on the spectral line shapes, but proton decoupling narrowed the ^2H line widths and spectra indicative of restricted axially symmetric motions were observed (Figure 2). The spectra consist of two distinct overlapping powder patterns that collapse at 15 °C to give a single isotropic line, indicating that the C8 methylene unit adopts an average orientation in which each C–^2H bond is at the "magic angle" (54.7°) with respect to the macroscopic director of motional averaging. In addition, a third, less intense, ^2H powder pattern was observed that was attributed to deuterated 1-isolinoleoyl-2-palmitoyl-PC (iLPPC; see below). Although previous work has suggested that proton–deuterium dipolar couplings have no effect on the deuterium powder spectra of deuterated acyl chains (Seelig & Seelig, 1974; Kang et al., 1979), they are large enough for [8′,8′-$^2H_2$]PiLPC to cause a severe distortion of the quadrupolar powder pattern line shape. The salutory effect of acquiring ^2H spectra with proton decoupling is clearly illustrated by comparing the

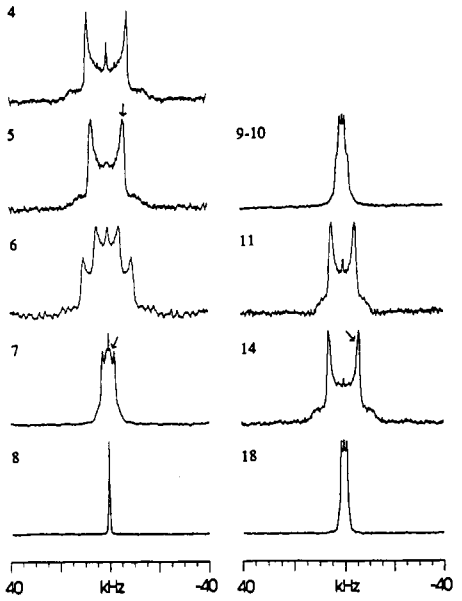

FIGURE 1: Proton-coupled ^2H powder spectra acquired from aqueous dispersions of PiLPC deuterated at the designated location of the isolinoleoyl chain. All spectra were acquired at 30 °C and at 30.7 MHz and for presentation purposes were folded about the center of the pattern to increase the signal-to-noise ratio by a factor of $\sqrt{2}$. The arrows indicate peaks arising from iLPPC. Note: no additional features were introduced by the spectral symmetrization.

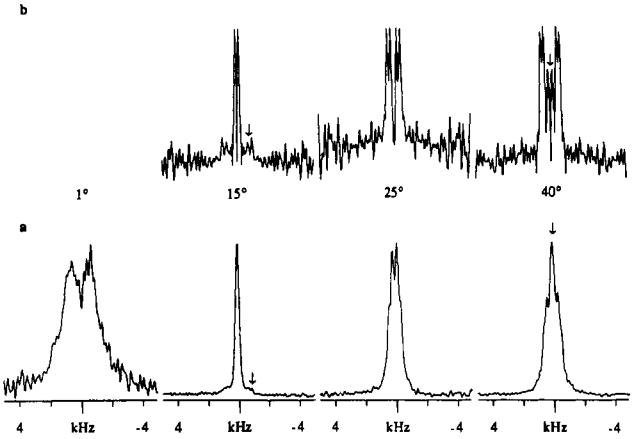

FIGURE 2: (a) Proton-decoupled ^2H spectra of aqueous dispersion of [8′,8′-$^2H_2$]PiLPC acquired at 46.064 MHz with a sweep width of 50 kHz, 2K data points, and a recycle time of 2 s. Spectra are not folded. (b) The oriented sample spectra obtained by dePaking the spectra shown in (a). The powder spectrum acquired at 1 °C was too noisy to obtain useful information from the dePaked spectrum.

spectra of [8′,8′-$^2H_2$]PiLPC in Figures 1 and 2.

Several of the ^2H spectra consist of at least two overlapping powder patterns, as is evident in those acquired from [9′,10′-$^2H_2$]- and [6′-^2H]PiLPC (Figures 1 and 3). For [9′,10′-$^2H_2$]PiLPC, the two signals are of equal intensity and are likely due to the inequivalence of the deuterium atoms attached to the C9 and the C10 carbon atoms of the double bond. In contrast, for [6′-^2H]PiLPC the "second" powder pattern was assigned to deuterated 1-palmitoyl-2-linoleoyl-PC (PLPC) as a small amount (~5%) of deuterated linoleic ($18:2^{\Delta 9,12}$) acid was formed during the biosynthesis of deuterated isolinoleic ($18:2^{\Delta 6,9}$) acid (Baenziger et al., 1990). Comparison of the relative intensities of the two ^2H powder patterns to the relative intensities of the methylene ([6,6-$^2H_2$]linoleic acid) and alkene ([6-^2H]isolinoleic acid) peaks in the high-resolution ^2H spectrum of the NMR sample (data not shown) indicates that the powder pattern with the larger splitting arises from PLPC. Although the sample contains only

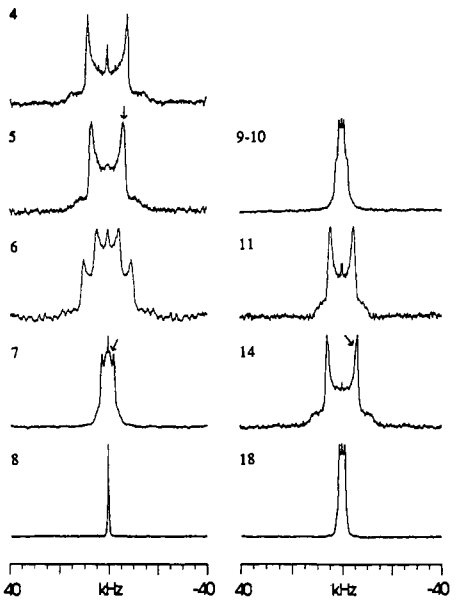

FIGURE 3: DePaked spectra of PiLPC deuterated at the indicated positions of the isolinoleoyl chain. Spectra were acquired as indicated in Figure 1 and were dePaked by the method of Bloom et al. (1981). The proton-coupled ^2H spectra of [8′,8′-$^2H_2$]PiLPC were not dePaked because of the nonaxially symmetric spectral line shape (see text), but those obtained from spectra acquired with proton decoupling are shown in Figure 2. Arrows indicate peaks arising from iLPPC.

Table I: Comparison of Relative Intensities of the ^2H Signals Arising from iLPPC and PiLPC (Both Deuterated along the Isolinoleoyl Chain) to the Amount of Acyl Chain Migration in the Samples

position of label on PiLPC	% powder pattern assigned to iLPPC	% acyl chain migration
C5	27	29
C7	15	20
C8	23	18
C9–C10	–[a]	15
C14	25	23
C18	–[a]	22

[a] No signal arising from iLPPC was observed.

a small amount of [6′,6′-$^2H_2$]PLPC, the lipid has twice as many deuterons per molecule and is likely deuterated to a much higher level (Baenziger et al., 1990), thus giving rise to a relatively intense deuterium signal. The presence of PLPC in the NMR sample is not expected to have a significant effect upon the ^2H results presented below but does provide the opportunity to gain some insight into the average structural properties of linoleoyl chains in lipid bilayers (see Implications of This Study).

^2H spectra of PiLPC deuterated at the C5, C7, C8, and C14 positions also contain a second (or a third in the case of the C8-labeled sample) powder pattern (Figures 1–3), but in each case it represents only a small proportion of the total signal and the value of its quadrupolar splitting is close to that (those) measured for the more intense powder pattern(s). The additional signals could arise from symmetrization of the spectra, but unsymmetrized spectra acquired at a greater digital resolution and to a higher signal-to-noise ratio clearly show the additional signals. For each sample, the relative intensity of the two ^2H powder patterns was estimated by spectral simulations (Baenziger, 1989) and in each case corresponded to the amount of acyl chain migration that occurred during the esterification procedure (Table I). Therefore, the less intense powder patterns were assigned to 1-isolinoleoyl-2-palmitoylphosphatidylcholine (iLPPC) with the deuterium labels located

at the corresponding position of the *sn*-1 position isolinoleoyl chain.

It has previously been reported that, under the esterification conditions used, little (<7 ± 3%) chain migration occurs (Perly et al., 1984; Dufourc et al., 1983). In our case the reactions were performed in the presence of an antioxidant, butylated hydroxytoluene, which may have had an effect upon the reaction. The presence of iLPPC in the samples could, in principle, affect the ^2H results by altering the gel-to-liquid crystal phase transition temperature of each individual lipid preparation. Such an effect on the transition temperatures of bilayers has been demonstrated for other lipid stereoisomers (Keough & Davis, 1979), but in general the differences only occur when the *sn*-1 and *sn*-2 acyl chains differ substantially in their length. The difference in chain length between the palmitoyl (16 carbons) and isolinoleoyl (18 carbons with two cis-unsaturated double bonds) chains is likely very small, and as a consistent level of acyl chain migration is evident, the presence of iLPPC is not expected to affect the analysis presented below.

Chain Order. For a deuterated lipid undergoing axially symmetric motion in a lipid bilayer, the separation between the two intense peaks in the deuterium powder spectrum, the quadrupolar splitting ($\Delta\nu_Q$), is defined as (Seelig, 1977; Davis, 1983)

$$\Delta\nu_Q = \frac{3}{4}\frac{3e^2qQ}{h}S_{CD} \qquad (1)$$

where e^2qQ/h is the quadrupolar coupling constant (170 or 175.3 kHz for deuterium in saturated or unsaturated C–^{2}H bonds, respectively) and S_{CD} is the carbon–deuterium bond order parameter. The order parameter S_{CD} has been used extensively as a quantitative measure of the conformational freedom of the methylene carbons of saturated acyl chains and, assuming *axially symmetric ordering*, is defined as

$$S_{CD} = \left(\frac{3\cos^2\beta - 1}{2}\right)\left(\overline{\frac{3\cos^2\alpha - 1}{2}}\right) \qquad (2)$$

The term involving the angle α is referred to as S_{mol}, the molecular order parameter, and it is a quantitative measure of the amplitudes of fluctuation of the C–^{2}H bond about its average orientation (the segmental order; the line denotes a time average). The term involving the angle β is a geometric factor (referred to here as S_{geo}) that describes the average orientation of the C–^{2}H bond vector with respect to the director of motion (usually the bilayer normal). Therefore, the quadrupolar splitting and thus the order parameter S_{CD} are sensitive to both the order and the average orientation with respect to the bilayer normal of a deuterated acyl chain segment. However, the quadrupolar splittings cannot be interpreted explicitly in terms of either the average orientation or the order without additional experimental observables (Seelig, 1977). Both equations assume that the electric field gradient is axially symmetric about the C–^{2}H bond direction (Seelig, 1977).

The temperature dependence of the deuterium quadrupolar splittings, measured from the 90°-oriented sample (dePaked) spectra (Figures 2 and 3b), is summarized in Figure 4. As expected, the splittings generally decrease with increasing temperature, indicating that the chain becomes less ordered (i.e., S_{mol} decreases in value). In contrast, the splittings observed for the two deuterons attached to the C8 methylene segment and for the deuteron attached to the C9 carbon of the C9–C10 double bond actually increase with increasing temperature, suggesting that the average orientation of both

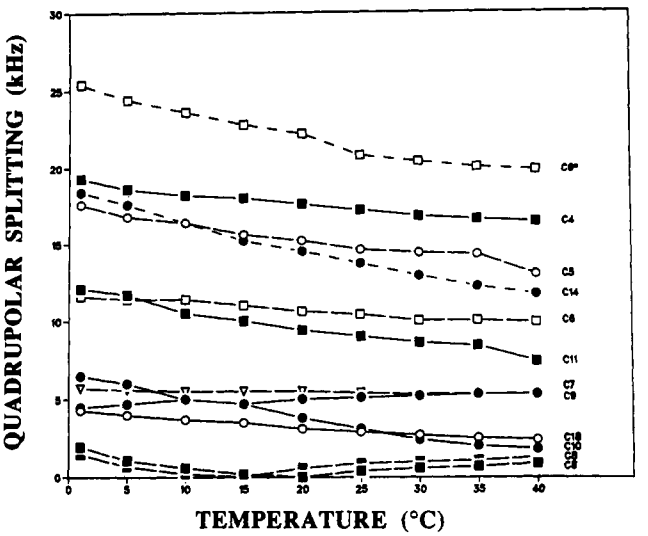

FIGURE 4: Summary of the values of the quadrupolar splittings for each deuterated position of the isolinoleoyl chain of PiLPC as a function of temperature. The quadrupolar splitting data obtained from the powder pattern assigned to [6′,6′-$^2H_2$]PLPC are also shown (C6*). The two splittings arising from [9′,10′-$^2H_2$]PiLPC were assigned to either the C9 or the C10 deuterons on the basis of the calculations performed in the Appendix.

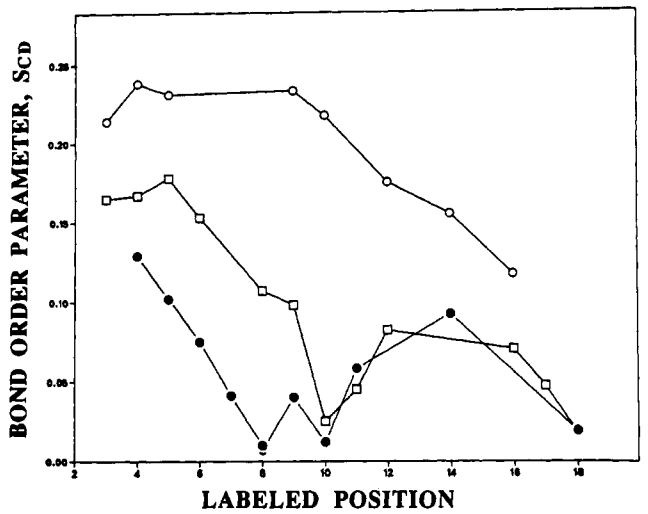

FIGURE 5: Plot of bond order parameters, S_{CD}, versus labeled position for the isolinoleoyl chain of PiLPC, the oleoyl chain from oleate-enriched bilayers of *A. laidlawii* (Rance et al., 1980), and palmitoyl chains of DPPC (Seelig & Seelig, 1974), all at 40 °C. For PiLPC, S_{CD} values were determined from the quadrupolar splittings as indicated in the text.

segments changes with temperature (i.e., there is a change in S_{geo}).

S_{CD} order parameters were calculated for each of the deuterated PiLPCs and are plotted as a function of labeled position in Figure 5. Similar profiles obtained previously for saturated and monounsaturated acyl chains are also plotted at the same absolute temperature for comparison. The ordering profile deviates substantially from those observed for either saturated (Seelig & Seelig, 1974; Stockton et al., 1977) or monounsaturated (Seelig & Seelig, 1977; Rance et al., 1980) acyl chains, the most dramatic effect being the large drop in S_{CD} values for the methylene carbons in the vicinity of the two double bonds and for the four alkene carbons themselves (carbons 5–11). Note that a drop in the ordering profile occurs at the C9–C10 double bond of oleoyl chains (Figure 5), and this has been attributed to a structural as opposed to a motional effect (Seelig & Waespe-Šarčevič, 1978; Dufourc et al., 1983). When the average conformation of the oleoyl chain is taken

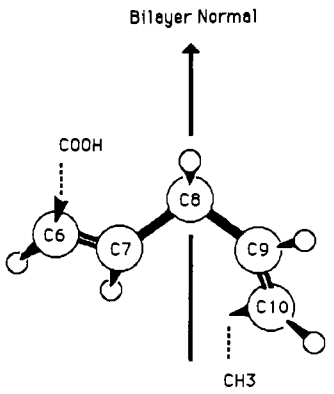

FIGURE 6: Average conformation of the isolinoleoyl chain in bilayers of PiLPC at 40 °C. The C8 methylene is oriented such that, on average, the bilayer normal essentially bisects the H–C–H tetrahedral angle; the C6–C7 double bond is rotated out of the plane of the paper by an angle of ±60°; and the C9–C10 double bond is rotated out of the plane of the paper by an angle of ±53°.

into account (i.e., S_{geo}), the resulting profile of S_{mol} (which reflects the amplitudes of motion without any geometrical contribution) versus labeled position is very similar to that obtained for saturated acyl chains (Seelig & Waespe-Šarčevič, 1978; Browning, 1981). Similarly, in our case, the unusual order profile could reflect a unique acyl chain conformation as opposed to a difference in the amplitudes of motion occurring at this location in the bilayer. Therefore a comparison of the ordering of the diunsaturated chain to the ordering of saturated and monounsaturated chains is only possible when molecular order parameters, S_{mol}, are used. As discussed above, this requires more experimental observables.

In a previous publication, spectra of oriented bilayers of [8′,8′-$^2H_2$]PiLPC were acquired with proton decoupling. By spectral simulation, both the relative sign (relative to the signs of the quadrupolar splittings) and the magnitude of the dipolar coupling between the two geminal C8 deuterons were determined (Baenziger et al., 1988), and this provided the additional spectral information necessary to calculate the average orientation of the C8 methylene segment with respect to the bilayer normal (see Appendix 1 for details). On the basis of this previously defined structure, the average orientation of the C6–C7 and the C9–C10 double bonds was defined (Figure 6; Appendix 2), and on the assumption of axially symmetric ordering, the molecular order parameters, S_{mol}, for the corresponding carbon segments were also determined (eq 2). The resulting ordering profile (Figure 7) indicates that the amplitudes of motion occurring along the isolinoleoyl chain are dramatically different from those occurring at equivalent positions of saturated and monounsaturated acyl chains. Furthermore, the results were unexpected. The double bonds are rigid units, and any rotation that brings about motion of a double bond should also cause a large displacement in the parallel packing of the chain. One would therefore expect the amplitudes of motion in the vicinity of the two double bonds to be restricted and the observed order parameters, S_{mol}, to be relatively large. At 40 °C, the order parameter assigned to the C6–C7 double bond is 0.55, reflecting restricted motion. In contrast, for the C8 methylene segment and the C9–C10 double bond, the calculated order parameters are 0.15 and 0.08, respectively, indicating considerable motional freedom. An internal motion arising from rotations about the C7–C8 and the C8–C9 single bonds therefore must lead to an increase in the mobility of the C8 and C9–C10 segments and the observed low molecular order parameters.

The average conformation of the isolinoleoyl chain was also unexpected. Computer modeling studies suggest that highly

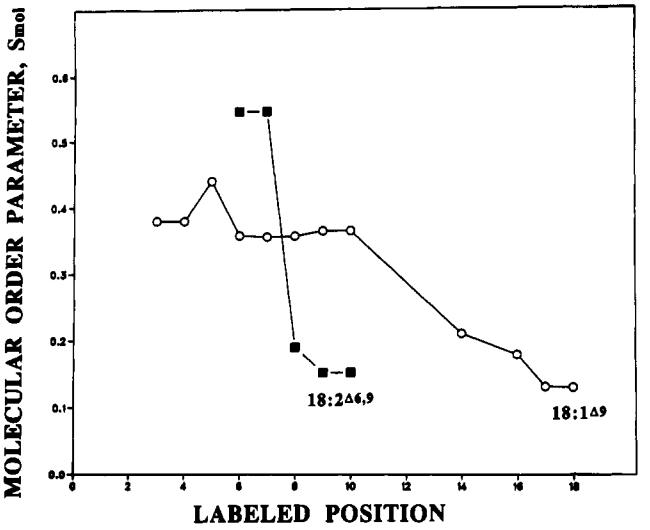

FIGURE 7: Comparison of the molecular order parameter, S_{mol}, profile obtained for the isolinoleoyl chain of PiLPC with those determined for oleoyl chains [data compiled by Browning (1918) from Seelig and Waespe-Šarčevič (1978) and Rance et al. (1980)] at the same reduced temperature, θ.

unsaturated docosahexaenoyl chains adopt an "angle-iron" and "helical" structure in which all six double bonds are aligned parallel to the bilayer normal and each successive double bond plane is at a 90° angle with respect to the previous double bond plane (Applegate & Glomset, 1986; Dratz & Deese, 1986). It was also suggested that the helix and angle-iron are compact structures that would pack relatively well into lipid bilayers and that the packing properties of these structures might account for the important role of polyunsaturated lipids in photoreceptor and neural membranes (Applegate & Glomset, 1986). The average structure defined for the isolinoleoyl chain (Figure 6) is not consistent with an angle-iron or helical structure (note that the distinction between the angle-iron and helix conformations proposed by Applegate and Glomset cannot be made with a fatty acyl chain containing only two double bonds). However, the isolinoleoyl chain can adopt two different conformations consistent with the angle-iron or helix (Figure 8a). If the chain were to adopt both conformations (Figure 8a) but undergo a jump between the two, then our defined conformation (Figure 6) would reflect the average of the two structures as opposed to each individual state. Furthermore, jumping between two conformations could possibly reduce the molecular order parameters to the low values observed for the C8 and C9–C10 segments. An internal two-site jump may therefore explain both the defined average conformation (Figure 6) and the unusual ordering profile of the isolinoleoyl chain. Note also that the low-energy conformation proposed by Applegate and Glomset (1986) for the 1,4-pentadiene segments of docosahexaenoyl chains has a relatively broad minimum compared to the localized minima calculated for the gauche and trans conformations of *n*-pentane. Furthermore, the torsional energy barriers along pathways between conformations were lower for the 1,4-pentadiene segment than for *n*-pentane, indicating that conversion between the two conformations, suggested above, is a likely possibility.

The value of the order parameter assigned to the C6-C7 double bond corresponds to values assigned to the whole body fluctuations of saturated phospholipids in membranes (Meier et al., 1986; Lange et al., 1985; Kar et al., 1985). This implies that the local motions involving the C6–C7 double bond are essentially absent (i.e., this would lead to further averaging of the quadrupolar tensors and thus lower order parameters, S_{mol}). Therefore, an alternative interpretation is that the

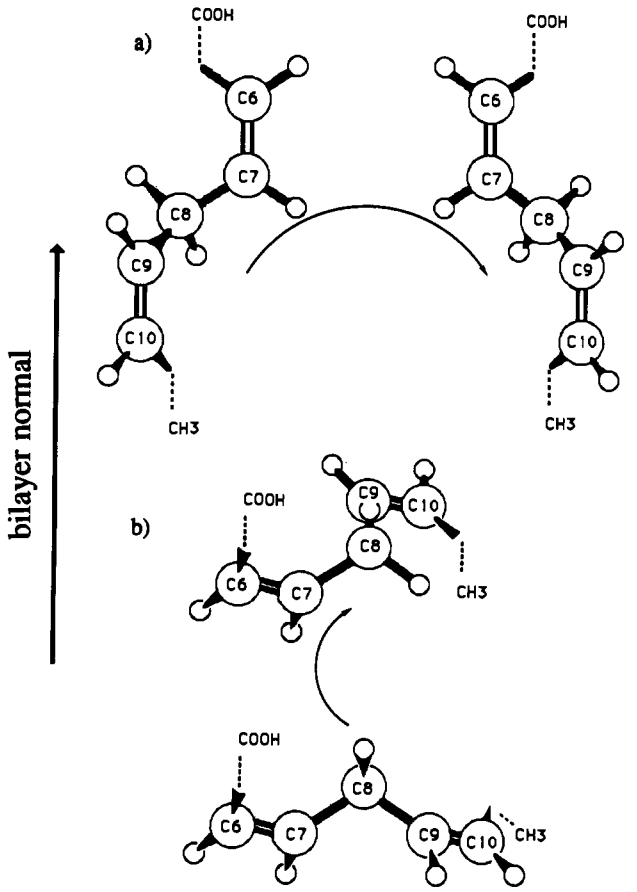

FIGURE 8: Two possible models for the motions of the isolinoleoyl chain. (a) The chain adopts two essentially identical conformations in which both double bond directions are parallel to the bilayer normal and the two double bond planes are at 90° with respect to each other, but interconverts, at a fast rate ($>10^5$ s^{-1}), between the two conformations. Note that both conformations are consistent with the angle-iron and helical structures proposed for docosahexaenoyl acyl chains by Applegate and Glomset (1986). (b) The 1,4-pentadiene segment of the chain adopts the same two conformations, but in each case the whole segment is tilted such that the C6–C7 double bond is oriented as defined in Figure 6. The chain still interconverts between the two states, but in this case the C6–C7 double bond does not contribute to the two-site jump whereas in the former model, the double bond is involved in the local motion.

1,4-pentadiene unit of the isolinoleoyl chain may adopt both low-energy conformations shown in Figure 8a but with the double bonds and intervening methylene segment tilted away from the bilayer normal such that the C6–C7 double bond is oriented as defined in Figure 6 (see Figure 8b). Even if the motions of the C6–C7 double bond are attenuated, the low order parameters observed for the C8 and C9–C10 segments could still arise from an internal jump between the two low-energy conformations (Figure 8b), occurring, as discussed above, via rotations about the C7–C8 and C8–C9 bonds. Although neither of the two conformations described in Figure 8b is consistent with either the angle-iron or helical structures suggested for docosahexaenoyl chains, Applegate and Glomset (1986) indicated that the cross-sectional surface area of a choline-containing phospholipid is too large to accommodate the compact angle-iron or helix structure. A tilt of both double bond axes relative to the bilayer normal (as shown in Figure 8b) would increase the cross-sectional surface area of the chain perhaps sufficiently to match that of the larger headgroup. If the chain were attached to a phospholipid with a smaller headgroup, it might adopt a conformation consistent with the structures proposed for highly unsaturated acyl chains (Applegate & Glomset, 1986).

Preliminary line-shape simulations indicate that both two-site jump models (Figure 8) can account for the residual quadrupolar splittings and the unusual temperature dependences of the splittings (Figure 4) observed for the C8 methylene segment. In order to distinguish between the two models, a more complete ^2H line-shape and relaxation study is clearly required. Such an approach will also provide information on the time scales upon which these motions occur and is currently under way in our laboratory.

Comparison with the Ordering of Saturated and Monounsaturated Acyl Chains. Both saturated and monounsaturated chains are relatively ordered for the first 8–10 carbon segments (the "plateau" region) but gradually become more disordered toward their methyl termini (the center of the bilayer). In addition, when the profiles are compared at the same reduced temperature θ, where $\theta = (T_{observe} - T_c)/T_c$, the corresponding methylene segments of both types of chains have almost identical order parameters (Seelig & Browning, 1978). The quantitative and qualitative similarities in the ordering of saturated and monounsaturated chains in a variety of different model and biological lipid systems have led to the suggestion that the order profile is a characteristic of the hydrophobic interior of lipid bilayers in general. In addition, several statistical models that account for the observed order profiles, transition temperatures, and surface areas of phospholipids containing two saturated acyl chains suggest that the hydrophobic interior of bilayers is a homogeneous region of highly flexible or fluid hydrocarbon chains (Marcelja, 1974; Gruen, 1980; Schindler & Seelig, 1975). The flexibility of the chains may be important for accommodating rigid molecules such as cholesterol and proteins (Israelachvili et al., 1980; Seelig et al., 1982).

In order to facilitate a comparison of the ordering of the isolinoleoyl chain with the ordering of other acyl chains, the transition temperature of PiLPC bilayers was determined by both ^2H and ^{31}P NMR to be approximatly −15 °C (data not shown). Even at the same reduced temperature θ, the chain is significantly more ordered at the C6–C7 position, and more disordered at the carbons C8 and C9–C10, than the corresponding segments of monounsaturated acyl chains (Figure 7). [Similar data for saturated acyl chains are not available for this reduced temperature, but the molecular order parameters observed for the corresponding carbon segments of saturated and monounsaturated acyl chains are similar at the same θ (Seelig & Browning, 1978).] Therefore, the molecular order profiles defined previously for saturated and monounsaturated chains are clearly not universal, and the conclusions derived from experiments using saturated acyl chains are not necessarily applicable to membranes rich in polyunsaturated lipids. In addition, although there is still uncertainty as to which motional model accounts for the observed order parameter profile, a jump between two low-energy conformations appears to be a reasonable explanation. If the chain contained three or more double bonds, it is unlikely that such a two-site jump could occur. This motion would cause a large displacement of a polyunsaturated chain parallel to the bilayer surface, which is inconsistent with the constraints imposed by the packing of acyl chains in bilayers. If the dominant motion occurring in this region of the isolinoleoyl chain is the two-site jump, then highly unsaturated acyl chains, which cannot undergo the jump motion, should be highly ordered, not "fluid", as is usually assumed. This contrasts with the notion that the flexibility of the acyl chains in the hydrophobic region of the bilayer is important for accommodating rigid molecules such as proteins and cholesterol. Polyunsaturated acyl chains may

be rigid structures and therefore their interactions with other lipids and with proteins should differ substantially from the interactions between saturated acyl chains and other lipids and proteins.

Implications of This Study. The results presented above represent the first detailed analysis of the average structural and motional properties of a specifically deuterated acyl chain containing more than one double bond. ^{2}H NMR investigations of specifically deuterated polyunsaturated lipids have been limited to a preliminary report of bilayers composed of 1-palmitoyl-2-docosahexaenoyl-PC in which the docosahexaenoyl chain was perdeuterated at all double bond carbons (12 deuterated sites) (Dratz & Deese, 1986). It was suggested that the observed spectrum is compatible with a helical structure, such as proposed by Applegate and Glomset (1986), but a thorough analysis of the data has not yet been published. Our results do not necessarily support the presence of such a structure for the isolinoleoyl chain but they do support the existence of particular low-energy conformations for the 1,4-pentadiene segments of polyunsaturated acyl chains. The presence of six double bonds in the docosahexaenoyl chain will clearly have a strong effect on its average conformation in the bilayer, and therefore the angle-iron or helical structures are both strong possibilities for highly unsaturated acyl chains.

Bilayers composed of phospholipids with *sn*-1 perdeuterated palmitoyl chains and *sn*-2 protiated docosahexaenoyl chains have also been examined by ^{2}H NMR, and the conformational freedom of the *sn*-1 chain has been compared to the same properties of the *sn*-1 position acyl chain in 1,2-dipalmitoyl-PC (DPPC) bilayers (Salmon et al., 1987; Paddy et al., 1985). At the same temperature, the palmitoyl chains in the polyunsaturated bilayer were found to have increased motional freedom relative to the palmitoyl chains in DPPC bilayers. This increase is evident in the lower order parameters, S_{CD}, observed for each deuterated segment along the chain, and in a shortening of the plateau region of high order. It is thought to arise due to an increase in the surface area of the phospholipid relative to the saturated PCs (Salmon et al., 1987). However, at the same *reduced temperature*, the order parameters for each segment in the polyunsaturated bilayers were larger than those observed for the saturated bilayers, indicating that the hexaunsaturated fatty acyl chain restricts the conformational freedom of the *sn*-1 saturated chain.

Fluorescence and ^{2}H NMR analyses of the ordering and motional rates of lipid probes in membranes of increasing levels of unsaturation have also been performed (Straume & Litman, 1987; Deinum et al., 1988, Stubbs et al., 1981; Yeagle et al., 1987). Although there is disagreement about the details of the effects of unsaturation on probe motion, all the techniques show only subtle changes in bilayers containing acyl chains with two to six double bonds. If the presence of three or more double bonds attenuates most of the acyl chain motion, then one would not expect large changes in the motions of NMR or fluorescence probes under increased levels of unsaturation. The data are consistent with our hypothesis that polyunsaturated chains are rigid structures, and therefore their interactions with proteins and with other lipids should differ from those involving saturated lipids.

It is of interest to note that the value of the splitting observed for [6′,6′-^{2}H$_2$]PLPC (the sample of [6′-^{2}H]PiLPC was found to contain a small amount of [6′,6′-^{2}H$_2$]PLPC; see Figures 1 and 4) and its temperature dependence correspond closely to the same parameters for oleoyl chains deuterated at the C6 position in membranes of *Acholeplasma laidlawii* (Rance et al., 1980). Both are saturated at this position and appear to undergo similar amplitudes of motion (note that both systems have similar phase transition temperatures). Thus, the two double bonds of the linoleoyl chain (18:2$^{\Delta 9,12}$), which are located closer to the center of the bilayer, appear to have essentially no effect on the order parameters observed in the plateau region of high order (i.e., at the C6 position). In contrast, the two double bonds of the isolinoleoyl chain (18:2$^{\Delta 6,9}$) are closer to the ester linkage and have a dramatic effect on the order parameters and thus the motions occurring in the plateau region. Highly unsaturated acyl chains, such as docosahexaenoic acid, also have double bonds close to their carboxyl termini, and it is possible that the presence of double bonds and the corresponding effects on the ordering of motions occurring in the plateau region may be important for their biological function. An unambiguous interpretation of the data obtained for [6′,6′-^{2}H$_2$]PLPC in terms of both the conformation and the molecular order profile of the isolinoleoyl chain, and similar data for other polyunsaturated acyl chains, is clearly required in order to investigate this hypothesis.

Conclusions

We present here the first detailed ^{2}H NMR analysis of a specifically deuterated acyl chain containing more than one double bond. The results clearly indicate that both the average structural and the motional properties of a diunsaturated acyl chain differ substantially from the same properties of saturated and monounsaturated acyl chains. Polyunsaturated lipids may also have unique physical properties in bilayers.

The diunsaturated acyl chain appears to adopt two conformations consistent with the low-energy structures proposed for each of the overlapping 1,4-pentadiene segments of polyunsaturated acyl chains (Applegate & Glomset, 1986). Our data therefore lend support to the hypothesis that polyunsaturated acyl chains adopt unique structures in bilayers that contribute to their important role in membrane function. The data also suggest that the isolinoleoyl chain undergoes a two-site jump between the low-energy conformations. Acyl chains containing three or more double bonds could not undergo similar types of local motion and therefore should be relatively ordered and inflexible. They may interact in a different manner with proteins and other rigid lipids, such as cholesterol, than do saturated and monounsaturated acyl chains which are relatively flexible. This hypothesis clearly requires more detailed analysis with deuterated acyl chains containing three or more double bonds.

Appendix 1

Orientation of the C8 Methylene Segment. Following the approach of Seelig (1977) and Seelig and Waepse-Šarčvič (1978), a right-handed Cartesian coordinate system was attached to the C8 methylene segment as shown in Figure 9. The irreducible tensor components of the electric field gradient tensor for each deuteron, $V^{(2,0)}$, were defined in the axis system X–Y–Z and then trasnformed to an axis system X'–Y'–Z' where the Z' axis is coincident with the bilayer normal. The transformation leads to the following expression for the S_{CD} bond order parameters:

$$S_{CD} = \left(\frac{3\cos^2\beta - 1}{2}\right)S_{33} \pm (\sin 2\beta)S_{13} + \frac{(\sin^2\beta)(2S_{11} - S_{33})}{2} \quad (3)$$

where the angle $\beta = 54.7°$ and the terms S_{11}, S_{22}, and S_{33} are order parameters describing the fluctuations of the three segment-fixed axes X, Y, and Z, respectively, in relation to the bilayer normal. Note, eq 2 (text) assumes axially sym-

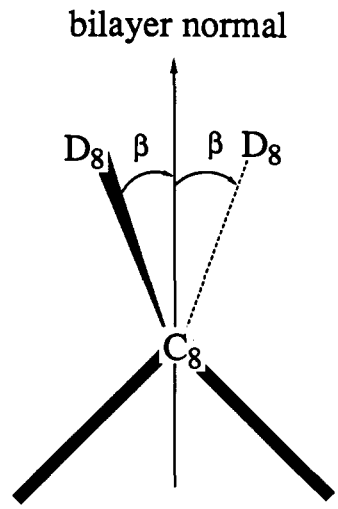

FIGURE 9: X–Y–Z coordinate system attached to the C8 methylene segment. The Z and Y axes are in the plane of the paper, and the X axis is projected straight out of the plane of the paper. Z bisects the ^2H–C–^2H dihedral angle.

metric ordering ($S_{11} = S_{22}$; $S_{13} = 0$).

The dipolar coupling between the two C8 deuterons is defined as (Emsley & Lindon, 1975)

$$D_{DD} = \frac{\gamma_D{}^2\mathbf{h}}{4\pi\mathbf{r}^3} S_{mol}\left(\frac{3\cos^2\eta - 1}{2}\right)\left(\frac{3\cos^2\theta' - 1}{2}\right)\frac{\mu_0}{4\pi} \quad (4)$$

where the terms involving the angle η describe the average orientation of the ^2H–^2H internuclear vector with respect to the bilayer normal and μ_0 is $4\pi \times 10^{-7}$ kg m s^{-2} ($\theta' = 0°$). The order parameter S_{DD} is defined as

$$S_{DD} = S_{mol}\left(\frac{3\cos^2\eta - 1}{2}\right) \quad (5)$$

and it describes the fluctuations of the deuterium internuclear vector that is also coincident with the X axis (Figure 9). S_{DD} therefore can be referred to as S_{11}.

At 40 °C, two S_{CD} order parameters and S_{DD} can be calculated directly from the spectra presented in a previous publication (Baenziger et al., 1988); S_{CD} (a) = 0.010, S_{CD} (b) = 0.008, and $S_{DD} = S_{11} = -0.062$, or the reverse sign for all three values. Substitution of all three values into eq 2 leads to definition of the complete ordering tensor for the C8 segment ($S_{11} + S_{22} + S_{33} = 0$):

$$S = \begin{pmatrix} -0.062 & 0 & 0.002 \\ 0 & -0.089 & 0 \\ 0.002 & 0 & 0.150 \end{pmatrix}$$

which can be diagonalized by rotations about the Euler angles α' and β' to give

$$S^* = \begin{pmatrix} -0.089 & 0 & 0 \\ 0 & -0.062 & 0 \\ 0 & 0 & 0.150 \end{pmatrix}$$

The calculated orientation of the C8 methylene unit with respect to the bilayer normal at 40 °C is shown in Figure 6 and arises by a rotation of the X–Y–Z axis system by an angle $\beta' = \pm 0.40°$ about the Y axis, followed by a rotation of $\alpha' = \pm 90.0°$ about the Z axis. The parameter S_{mol} quoted in the text refers to the order parameter, S_{33} (in this case $S_{33} = 0.150$).

APPENDIX 2

Average Orientation of the C6–C7 and C9–C10 Double Bonds. On the basis of the defined average orientation of the C8 methylene unit (Appendix 1), the average orientation of both the C6–C7 and the C9–C10 double bonds can also be defined. In principle, the C6–C7 and the C9–C10 double bonds can adopt any orientation within the bilayer. However, within the constraints of the specified orientation of the C8 methylene segment, only average orientations brought about by rotations of the C7–C8 and the C8–C9 carbon–carbon single bonds, respectively, are possible. In addition, since the molecular order parameters, S_{mol}, for the C6 and the C7 deuterons, and for the C9 and the C10 deuterons, are equivalent (i.e., in each case they are both attached to the same rigid segment and therefore undergo the same molecular fluctuations described by S_{mol}), the ratios of their quadrupolar splittings are specified only by the ratios of their geometric factors (S_{geo}). Only rotations about the respective carbon–carbon single bonds that yield geometric factors consistent with the observed quadrupolar splitting ratios are possible. Therefore, by calculation of all possible rotations about the C7–C8 and the C8–C9 single bonds that satisfy the splitting ratios, the average orientation of the each double bond can be defined.

One complicating factor arises when the splittings for the C6 and the C10 deuterons are examined. If standard bond geometries are assumed, the C6–^2H and the C10–^2H bond vectors are parallel to the C7–C8 and C8–C9 bond vectors, respectively, and at 15 °C the latter two are oriented at the "magic angle" with respect to the axis of motional averaging (Baenziger et al., 1988). Consequently, the C6–^2H and the C10–^2H bonds must also be oriented at the magic angle, and therefore both should give rise to spectra with no measurable quadrupolar splittings. The observation at 15 °C of two powder spectra with splittings of 11.0 and 4.7 kHz for the C6 and C10 deuterons, respectively, suggests that either the orientation of the C8 methylene unit is specified incorrectly or the bond angles of the two double bonds deviate significantly from "normal". X-ray crystallography has shown that the bond angles of the two double bonds of linoleic acid deviate by as much as 8.1° from normal (Ernst et al., 1978), and therefore the latter conclusion seems likely. All calculations were therefore performed by using the bond angles of the 1,4-pentadiene segment of linoleic acid (18:2$^{\Delta 9,12}$) assigned to the corresponding bond angles of the 1,4-pentadiene segment of isolinoleic acid (18:2$^{\Delta 6,9}$). Unfortunately, the angle between the carbon–deuterium bond vectors and their double bond axes was not defined in the crystal structure so each Cx–^2H bond was assumed to bisect the corresponding (Cx − 1)–Cx–(Cx + 1) double bond angle. This assumption was made soley from geometrical arguments, but its validity was tested by allowing the angles to vary over a range of 60–66°, and the variations had little effect on either the calculated double bond orientations or the respective molecular order parameters shown below (Baenziger, 1989). Also, the X-ray data for linoleic acid suggest that the double bonds deviate slightly from planarity, but such deviations had no significant effects upon the results (Baenziger, 1989).

A right-handed Cartesian coordinate system (X–Y–Z) was attached to both the C6–C7 and the C9–C10 double bonds as shown in Figure 10 for the C9–C10 double bond. The C–^2H vectors, Px, of all four deuterons in their respective axis system were defined as

$$\text{P6} = (0, \cos\phi 6, \sin\phi 6); \quad \phi 6 = \psi 6 - 52.4°$$
$$\text{P7} = (0, \sin\phi 7, \cos\phi 7); \quad \phi 7 = \psi 7 - 37.6°$$
$$\text{P9} = (0, \sin\phi 9, \cos\phi 9); \quad \phi 9 = \psi 9 - 38.1°$$
$$\text{P10} = (0, \cos\phi 10, \sin\phi 10); \quad \phi 10 = \psi 10 - 51.9°$$

The angles ψ are between the respective carbon–deuterium

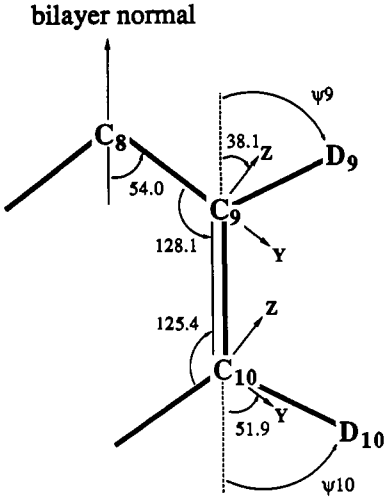

FIGURE 10: Two X–Y–Z axes systems attached to the C9–C10 double bond. In both cases, the Z and Y axes are in the plane of the double bond, the X axes are projected straight out of the plane of the paper, and the two Y axes are parallel to the C8–C9 carbon–carbon single bond. All bond angles are from the crystal structure of linoleic acid (18:2$^{\Delta 9,12}$) (Ernst et al., 1979) except for $\phi 9$ and $\phi 10$, which were calculated as described in the text. The X–Y–Z axes systems are transformed to an X'–Y'–Z' axis system where Z' is coincident with the bilayer normal by rotating about X by an angle of $\eta = 36.0°$. The bilayer normal bisects the C7–C8–C9 dihedral angle as specified in Appendix 1.

bond vectors and their double bond axes ($\psi 6 = 63.5°$, $\psi 7 = 63.8°$, $\psi 9 = 64.1°$, and $\psi 10 = 62.7°$; see below), and the angles ϕ were calculated, as shown above, from the bond angles determined for the 1,4-pentadiene segment of linoleic acid (Ernst et al., 1978).

Each double bond was then allowed to adopt any orientation consistent with the specified average orientation of the C8 methylene segment with respect to the bilayer normal by rotating each bond vector Px by an angle θ about either the C7–C8 or the C8–C9 single bonds, respectively (this corresponds to a rotation about the Y axis in each coordinate system). All four deuteron coordinates were then referred to a new axis system X'–Y'–Z' by rotating the old axis X–Y–Z counterclockwise about the X axis by an angle $\eta = 36.0°$. This rotation brings the Z axis parallel to the bilayer normal. Both rotations were accomplished by using transformation matrices for the C9–^{2}H vector, P9:

$$\begin{pmatrix} 1 & 0 & 0 \\ 0 & \cos\eta & \sin\eta \\ 0 & -\sin\eta & \cos\eta \end{pmatrix} \begin{pmatrix} \cos\theta & 0 & -\sin\theta \\ 0 & 1 & 0 \\ \sin\theta & 0 & \cos\theta \end{pmatrix} \begin{pmatrix} 0 \\ \sin\phi 9 \\ \cos\phi 9 \end{pmatrix}_{P9} =$$

$$\begin{pmatrix} -\sin\theta\cos\phi 9 \\ \cos\eta\sin\phi 9 + \sin\eta\cos\theta\cos\phi 9 \\ -\sin\eta\sin\phi 9 + \cos\eta\cos\theta\cos\phi 9 \end{pmatrix}_{P'9}$$

In X'–Y'–Z', the Z' component of each vector P'x corresponds to the cosine of the angle between the respective Cx–^{2}H bond vector and the Z' axis or bilayer normal (angle β, see eq 2), and therefore the geometric factors for each deuteron can be obtained directly from the coordinate vectors. At 40 °C, the geometric factors must satisfy the following:

$$\frac{3\cos^2\beta_9 - 1}{3\cos^2\beta_{10} - 1} = \pm 3.3 \qquad \frac{3\cos^2\beta_6 - 1}{3\cos^2\beta_7 - 1} = \pm 2.0$$

where the angles β are between the respective Cx–^{2}H bond vectors and the axis of motional averaging (assumed to be the bilayer normal). For each double bond, substituting the two Z components into the appropriate equation leads to a new equation that can be solved to yield all possible rotations θ (and thus all possible double bond orientations) consistent with the quadrupolar splitting ratios observed for the two deuterons of each double bond. The biologically relevant solutions are in boldface in Table II. Due to the ambiguity in the assignment of the quadrupolar splittings recorded for [9′,10′-$^2H_2$]PiLPC to the C9 or the C10 deuteron, additional solutions for the orientation of the C9–C10 double bond, and its molecular order parameter, are possible. Only the solutions achieved when the larger splitting, observed at temperatures above 15 °C, is assigned to the C9 deuteron are shown. The rationale for this assignment will be presented in a future publication.

Table II: Average Orientation of the C6–C7 and C9–C10 Double Bonds at 40 °C

double bond	rotation angle θ (deg)	$\beta 6^a$ or $\beta 9$ (deg)	$\beta 7$ or $\beta 10$ (deg)	S_{mol}
C6–C7	**±120**	**131**	**128**	**0.546**
	±113	130	123	0.708
C9–C10	**±59.3**	**82.5**	**119**	**0.075**
	±104	115	128	0.155

[a] See eq 2 for definition of angle β.

Note that the molecular order parameter obtained for the C9–C10 double bond at 40 °C is 0.08, which is reasonably close to the order parameter S_{33} (usually referred to as S_{mol}) of 0.15 obtained for the C8 methylene unit at the same temperature (Baenziger et al., 1988, and Appendix 1), thus lending support to the validity of the calculations. The order parameters calculated for the C6–C7 double bond may be overestimated due to the proximity of both C–^{2}H bond vectors to the magic angle of 54.7°, or 180° − 54.7° = 125.3° (Table II).

Registry No. PiLPC, 130614-06-1; iLPPC, 130614-07-2; isolinoleic acid, 28290-77-9.

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