Effect of Unsaturation on the Chain Order of Phosphatidylcholines in a Dioleoylphosphatidylethanolamine Matrix

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ABSTRACT The properties of phosphatidylcholines (PCs) having a perdeuterated stearic acid, $18:0_{d35}$, in the sn-1 position and the fatty acid 18:0, 18:1ω9, 18:2ω6, 18:3ω3, 20:4ω6, 20:5ω3, or 22:6ω3 at the sn-2 position were investigated in a matrix of dioleoylphosphatidylethanolamine (DOPE) by ^{2}H and ^{31}P NMR spectroscopy. At a mole ratio of DOPE/PC = 5:1, the lipids form liquid crystalline lamellar phases below 40°C and coexisting lamellar, inverse hexagonal (H_{II}), and cubic phases at higher temperatures. The sn-1 chain of the PCs in a DOPE matrix is appreciably more ordered than in pure PCs, corresponding to an increase in the hydrophobic bilayer thickness of approximately 1 Å. Distearoylphosphatidylcholine in the DOPE matrix has a higher sn-1 chain order than the unsaturated PCs. We observed distinct differences in the lipid order of upper and lower sections of the hydrocarbon chains caused by changes of temperature, unsaturation, headgroups, and ethanol. Unsaturation lowers chain order, mostly in the lower third of the hydrocarbon chains. By contrast, the increase in chain order caused by the DOPE matrix and the decrease in order with increasing temperature have a constant magnitude for the upper two-thirds of the chain and are smaller for the lower third. Addition of 2 M ethanol reduced order parameters, in effect reversing the increase in chain order caused by the DOPE matrix.

INTRODUCTION

The biological significance of non-bilayer-preferring lipids in membrane systems is now largely recognized. It has been proposed that bilayers containing lipids, such as phosphatidylethanolamines (PEs), that are predisposed to adopting an inverted hexagonal phase (H_{II}) are under a curvature-associated lateral stress if packed in a lamellar phase (Gruner, 1992). A detailed understanding of the relationship of lipid headgroup composition and unsaturation of lipid hydrocarbon chains to this intrinsic curvature-related stress in membranes is needed at a molecular level.

Phase changes in phospholipid dispersions are easily monitored using ^{31}P NMR or x-ray diffraction and have been investigated extensively for mixtures of PE and PC (Cullis et al., 1978; Hui et al., 1981). The phase behavior of dioleoylphosphatidylethanolamine (DOPE) as a function of hydration (Gawrisch et al., 1992; Rand and Fuller, 1994), and the phase diagrams of fully hydrated saturated PC/PE mixtures (Arnold et al., 1981) and of DOPC/DOPE mixtures (Tate and Gruner, 1989; Rand et al., 1990) have been reported. Mixtures of PC and PE may form inverted cubic phases or micelles that give isotropic ^{31}P NMR signals (Hui et al, 1981; Tilcock et al., 1982). The existence of inverted micelles has been related to the existence of transition structures between lamellar and H_{II} phases (Siegel, 1986).

Mixtures of DOPE with saturated and monounsaturated PCs undergo a lamellar-to-H_{II} transition at lower temperatures with increasing chain length PCs (Tate and Gruner, 1987). Not only the chain length, but also the degree of unsaturation affects the bilayer-to-hexagonal transition temperature, with small amounts of polyunsaturated chains significantly lowering the transition temperature (Dekker et al., 1983). Furthermore, addition of alkanes facilitates the transition to nonlamellar phases (Kirk and Gruner, 1985; Gruner et al., 1986; Sjolund et al., 1989).

The effect of DOPE on the order profile of chain perdeuterated dimyristoyl-PC at the onset of the H_{II} phase transition has been studied by ^{2}H NMR spectroscopy (Fenske et al., 1990), and the results have been interpreted as an increase in the lateral pressure over the entire length of the hydrocarbon chain brought about by the non-bilayer-preferring PE. The order profile of palmitoyl$_{d31}$oleoyl-PE in both the lamellar and H_{II} phases has been determined (Lafleur et al., 1990), with the H_{II} phase characterized by greater motional freedom and the absence of a well-defined plateau region in the order parameter profile of lipid hydrocarbon chains. Similar results were obtained for perdeuterated tetradecanol in hydrated palmitoyl-oleoyl-PE dispersions (Sternin et al., 1988).

Fluorescence depolarization techniques have been used to determine the molecular order and reorientational dynamics of aligned DOPE dispersions in the lamellar and H_{II} phases (van Langen et al., 1989). Fluorescence studies with labeled PE in PC membranes indicate that the presence of PE increases the stress near the center of the bilayer and that this stress is reduced by converting from the lamellar to the H_{II} phase (Cheng et al., 1994).

In a previous study on unsaturated PCs containing from one to six double bonds in the sn-2 chain (Holte et al., 1995), we attributed the effect of sn-2 unsaturation on membrane properties to changes in the effective molecular

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shape of lipid hydrocarbon chains. We have extended this analysis to DOPE/PC mixtures containing the same mixed-acid PCs to probe the effect of the smaller PE headgroup on lipid packing.

^{31}P NMR was used to monitor the phase state of fully hydrated DOPE/PC (5:1) mixtures where the PC contains a perdeuterated stearoyl ($18:0_{d35}$) sn-1 chain and a variable degree of unsaturation of the sn-2 chain. A DOPE/PC ratio of 5:1 was chosen so as to observe a nonlamellar phase transition around 50°C. Measurements were performed over a temperature range of −20°C to +70°C.

^{2}H NMR order parameter profiles and the sn-1 chain length were determined for the PC in the bilayer phase of the DOPE/PC dispersions and compared to the results for the corresponding pure PC multilamellar dispersion. In addition, the effect of ethanol on these parameters was examined.

MATERIALS AND METHODS

Materials

PCs, containing perdeuterated stearic acid ($18:0_{d35}$) at the sn-1 position with 18:0, 18:1ω9, 18:2ω6, 18:3ω3, 20:4ω6, 20:5ω3, or 22:6ω3 at the sn-2 chain, and DOPE were purchased from Avanti Polar Lipids (Alabaster, AL). Deuterium-depleted water was obtained from Sigma Chemical Co. (St. Louis, MO). Lipid purity and oxidation were checked by analytical high-performance liquid chromatography (Holte et al., 1990).

Sample preparation

The NMR samples consisted of hydrated multilamellar dispersions of DOPE/PC (5:1) 50% by weight in water. Samples were prepared from stock solutions of PC, 5 mg/ml in cyclohexane/ethanol (1:1) for 18:0, 18:1ω9, 18:2ω6, 18:3ω3, and 20:4ω6 or methylene chloride for 20:5ω3 and 22:6ω3, and DOPE, 20 mg/ml in methanol. The DOPE/PC ratio was verified by integration of the ^{31}P NMR solution state spectra. The antioxidant butylated hydroxytoluene (BHT) in methanol was added to give a final lipid-to-BHT ratio of 250:1. After mixing, the lipids were dried under a stream of argon gas and in vacuum. After the removal of organic solvents, the lipids were dispersed in 0.5 ml degassed water and transferred under argon to a 5-mm o.d. sample tube. The lipids were pelleted by spinning samples on a benchtop centrifuge. Most of the clear supernatant was removed, and the pellet was lyophilized overnight. Typically lipid samples weighed 25 mg, and 25 μl of degassed deuterium depleted water was added to the dry powder. Under argon, the samples were vortex mixed, centrifuged, and flame sealed to a length of about 2 cm. The hydrated dispersions were then centrifuged back and forth to ensure adequate mixing and freeze-thawed several times before storage at −20°C. For the ethanol study the DOPE/PC mixture was suspended in degassed 2 M ethanol solution, made by using deuterium depleted water. The lipid was pelleted by centrifugation, the pellet flame sealed in a 5-mm sample tube under argon, vortex mixed, freeze-thawed, and centrifuged to concentrate the sample at the bottom of the tube.

NMR spectroscopy

^{2}H and ^{31}P NMR spectra were recorded on a Bruker MSL 300 NMR spectrometer (Billerica, MA), using a high-power probe with a 5-mm solenoidal sample coil. The sample temperature was regulated to within ±0.5°C, using a Bruker B-VT-1000 temperature controller. ^{2}H NMR measurements were performed at 46.1 MHz, using a quadrupolar echo sequence (Davis et al., 1976) with a 3-μs, 90° pulse, a 30-μs time delay between pulses, and a repetition time of 0.5 or 1.0 s. The carrier frequency was placed at the center of the spectrum, and a spectral width of 125 kHz was used with 4096 data points in the time domain. Ten to twenty thousand scans were accumulated. The free induction decays were left shifted to begin at the top of the echo and multiplied with an exponential window function equivalent to a line broadening of 100 Hz. Proton-decoupled ^{31}P experiments were carried out at 121.5 MHz using a Hahn echo sequence with a 1.6-μs, 90° pulse, a 30-μs echo time, and a delay between pulse sequences of 1.0 or 2.0 s. A sweep width of 72 kHz, 2048 data points, and a 100-Hz line broadening were used, and 512 scans were routinely acquired.

The lamellar-H_{II} phase transition temperature was determined from the ^{31}P spectra and calculation of the first moment of the ^{2}H spectra according to

$$M_1 = \frac{\int_0^\infty \omega f(\omega)\, d\omega}{\int_0^\infty f(\omega)\, d\omega}, \quad (1)$$

where ω is the frequency with respect to the central Larmor frequency ω_o, and $f(\omega)$ is the lineshape (Davis, 1983). The temperature dependence of the spectral first moment, M_1, parallels the lipid phase behavior.

^{2}H NMR powder spectra were dePaked according to the method of Sternin et al. (1983), a numerical deconvolution procedure that results in spectra that would be obtained for an aligned membrane with its bilayer normal parallel to the magnetic field. This enhances the resolution and results in doublets with splittings $\Delta\nu_Q$ that relate to the order $S(n)$ according to

$$\Delta\nu_Q = \frac{3}{4}\frac{e^2qQ}{h} S(n), \quad (2)$$

where e^2qQ/h = 167 kHz is the static quadrupolar coupling constant for a C-^{2}H bond. Smoothed order parameter profiles were calculated by assuming that the order varies monotonically along the acyl chain (Lafleur et al., 1989). The integral of the dePaked methylene peaks was divided into 16 segments corresponding to each of the -CD_2 groups in the 18:0 chain. Order is assumed to be lowest at the end of the chain and highest at the glycerol backbone. The narrow splitting from the terminal methyl can be assigned unequivocally, but to calculate the sn-1 chain length, a tail-end methylene order parameter, $S(18)$, was extrapolated from the order parameters $S(16)$ to $S(17)$ (Lafleur et al., 1989). The order parameters were summed and then divided by the number of deuterated carbons in the chain to give the average order parameter, $\langle S\rangle$.

Utilizing a lattice model, it has been demonstrated that in the liquid-crystalline phase, the effective length of a saturated sn-1 chain is proportional to the average order parameter, $\langle S\rangle$ (Seelig and Seelig, 1974; Schindler and Seelig, 1975; Bloom et al., 1991; Nagle, 1993). The average length, $\langle L\rangle$, projected on the bilayer normal can be calculated from the following relation:

$$\langle L\rangle = 1(0.5 + |\langle S\rangle|), \quad (3)$$

where l is the projected length of an all-*trans* chain and is equal to 1.27 × n Å, 1.27 Å being the distance between two carbon atoms in the all-*trans* state (Nagle, 1993), and n is the number of C-C bonds in the sn-1 chain.

RESULTS

At ambient temperature the ^{31}P NMR spectra had a chemical shift anisotropy of −41 ppm, typical for DOPE in a lamellar phase. Nonlamellar components (hexagonal and isotropic) were discernible at 40 ± 5°C and higher. The

shoulders on the high field side of the lamellar spectra (Fig. 1) are caused by the chemical shift anisotropy of the PCs, which is about −45 ppm. The gel-to-liquid crystalline phase transition temperatures of the DOPE/PC mixtures are in the range from −10 to +10°C. The transition range of DOPE/distearoylphosphatidylcholine (DSPC) mixtures was somewhat broader. Trace amounts of the saturated DSPC in the gel phase could be detected up to temperatures of +20°C. From ^{31}P NMR, the onset of the lamellar to H_{II} transition was approximately 40 ± 5°C for all DOPE/PC (5:1) mixtures. The transition was broad, and the higher temperature ^{31}P NMR spectra included fluid phase lamellar, hexagonal, and isotropic components (Fig. 1 *B*), the isotropic most likely being due to a cubic phase or micellar structures (Tilcock and Cullis, 1987). The graph of first moment of ^{2}H NMR spectra versus temperature indicated that the midpoint of the chain transition from a liquid crystalline lamellar to nonlamellar phase was in the range 50 ± 5°C. More unsaturated PCs had a tendency of conversion to nonlamellar phases at slightly lower temperature. However, we may not exclude the possibility that the differences have been caused by small variations in the PE/PC ratio. The relative proportions of each lipid phase depended on the thermal history of the sample. Once a nonlamellar component developed, it increased in intensity with the length of time that the sample was kept at constant temperature.

Adding ethanol stabilized the bilayer phase, with no evidence of a hexagonal component up to 70°C. An isotropic peak representing a few percent of the total lipid, most likely a cubic phase, appeared at 50°-60°C (Fig. 1 *C*).

Deuterium NMR spectra of aqueous dispersions of DOPE/PC (5:1) at 30°C are shown in Fig. 2. As the unsaturation increases for the sn-2 chain, the quadrupolar splittings of the sn-1 chain are reduced (see dePaked spectra in Fig. 3). Profiles of order parameter versus chain position derived from the dePaked spectra are plotted in Fig. 4. The sn-1 chain length was calculated by deriving $\langle S \rangle$ from the ^{2}H NMR spectra of the lipid in the fluid-lamellar phase and using Eq. 3. After the addition of the first double bond, there is a reduction of the average chain order, corresponding to a decrease in the sn-1 chain length, $\langle L \rangle$, of approximately 0.7 Å. A further reduction in $\langle L \rangle$ of about 0.2 Å is observed as the number of double bonds is increased, even though the sn-2 chain may contain an additional two or four carbon atoms. For comparison, the order profiles of the pure PC bilayers acquired at 30°C by Holte et al. (1995) were subtracted from those of the DOPE/PC dispersion incorporating the same unsaturated PC (Fig. 5). In the DOPE matrix the unsaturated PCs show an increase in quadrupolar splitting of several kilohertz for the carbons closer to the glycerol backbone, with a tendency toward a slightly bigger increase around carbon atom 12, whereas the terminal methyls display little change. Clearly, PCs in the DOPE/PC

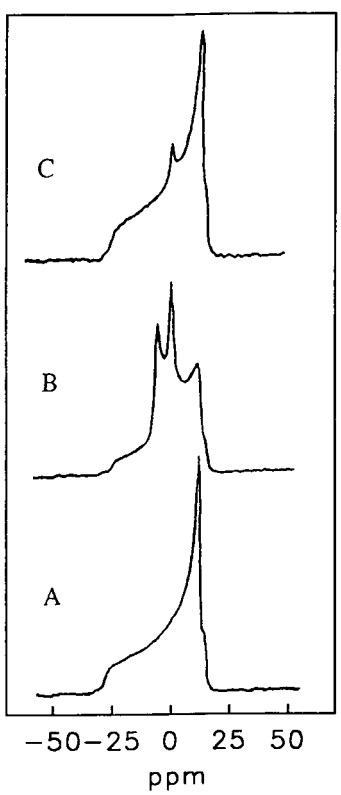

FIGURE 1 ^{31}P NMR spectra for the DOPE/(18:0_{d35})(18:1ω9)PC dispersion. (*A*) At 30°C in the liquid crystalline lamellar phase; (*B*) at 60°C, depicting coexisting lamellar, H_{II}, and isotropic (cubic) phases; (*C*) with 2 M ethanol in water at 60°C, consisting of a mainly lamellar spectrum with a small isotropic component.

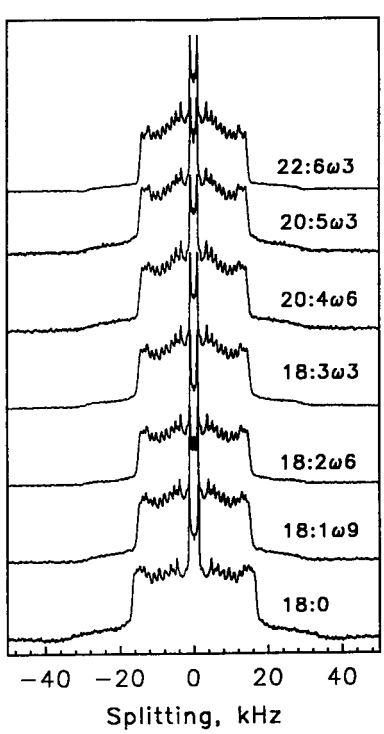

FIGURE 2 ^{2}H NMR spectra for DOPE/(18:0_{d35})(sn-2)PC dispersions recorded at 30°C. The sn-2 chain of the PC is indicated to the right of the spectra.

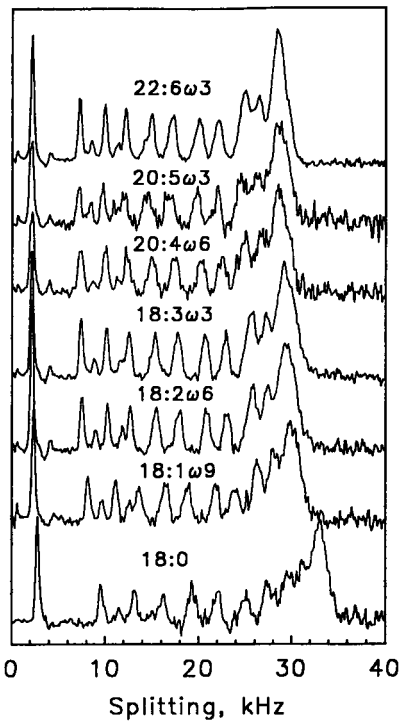

FIGURE 3 DePaked ^{2}H NMR half-spectra of DOPE/(18:0_{d35})(sn-2)PC dispersions at 30°C. The sn-2 chain is indicated above the spectra.

mixtures have order of larger magnitude, consistent with an increase in the sn-1 PC chain length, $\langle L \rangle$, of about 0.5 Å. The smaller PE headgroup appears to have an ordering effect on the PC, resulting in a thicker bilayer.

The effect of increasing the number of double bonds in the sn-2 chain on the order parameter profile of the carbons in the sn-1 chain can be examined by subtracting the order parameter profile of the PC with the 18:0 sn-2 chain from those of the unsaturated PCs obtained at the same temperature (Fig. 6). The reduction of quadrupolar splitting in the upper part of the chain is about a factor of 2 smaller than the decrease in order after the plateau region from about the 10th to 15th carbon (5–8 kHz). Analogous results were obtained in the temperature range 20–50°C. This parallels the results seen for pure PC systems (Holte et al., 1995).

All lipids displayed a decrease in sn-1 chain length as the temperature was increased. As a rough estimate of the sensitivity of $\langle L \rangle$ to temperature, a linear approximation gave an average decrease of -0.018 ± 0.007 Å per degree rise, with 18:0 and 22:6ω3, respectively, being the most and least sensitive to temperature. This decrease in hydrocarbon chain length as a function of temperature rise is roughly half the value deduced by Holte et al. (1995) for the pure PC systems and is presumably due to the presence of DOPE as the major component. Extrapolating back to 0°C resulted in an sn-1 chain length $\langle L_0 \rangle$ of 16.0 ± 0.1 Å for 18:0; 15.5 ± 0.1 Å for 18:1ω9; and 15.2 ± 0.1 Å for 18:2ω6, 18:3ω3, 20:4ω6, 20:5ω3, and 22:6ω3 sn-2 chain PCs in DOPE/PC (5:1), suggesting that after the first two double bonds, unsaturation has little effect on sn-1 chain length. The decrease in chain length with increasing temperature is the result of a decrease of order for the upper two-thirds of the hydrocarbon chain.

The addition of ethanol to DOPE/PC (5:1) dispersions reduced the quadrupolar splitting of the PCs, in effect reversing the influence of the DOPE. The order parameter profiles for DOPE/(18:0_{d35})(18:1ω9)PC and DOPE/(18:0_{d35})(22:6ω3)PC dispersions with and without ethanol are presented in Fig. 7 *A*. By subtracting the order profile of the hydrated dispersion from that of the dispersion with ethanol obtained at the same temperature, it can be seen that the PC with a 22:6ω3 acyl chain is less affected by the presence of ethanol (Fig. 7 *B*). For the dispersion containing the 22:6ω3 fatty acid, the quadrupolar splitting is reduced by 1–2 kHz along the entire chain because of the presence of ethanol. In the case containing an 18:1ω9 chain, the splitting is reduced by 2–4 kHz. The reduction of order corresponds to a reduction in the sn-1 chain length for the DOPE/(18:0_{d35})(18:1ω9)PC dispersion by 0.5 Å in the temperature range from 0 to 30°C, essentially to values that are close to data for a pure PC system (Holte et al., 1995). For DOPE/(18:0_{d35})(22:6ω3) PC the reduction in $\langle L \rangle_n$ after the addition of ethanol was only 0.2 Å. When compared at the same temperature in the range 20–50°C, the ethanol reduces the sn-1 chain length to essentially the same value, independently of the number of double bonds in the sn-2 chain. The sensitivity of $\langle L \rangle$ to temperature is unchanged, at -0.02 Å per degree rise.

DISCUSSION

The addition of 16.6 mol% PC to DOPE raised the lamellar-hexagonal phase transition temperature from 5 ± 5°C for pure DOPE (Gawrisch et al., 1992) to about 50 ± 5°C for the DOPE/PC mixtures. Traces of a phase with an isotropic ^{31}P NMR signal, most likely a cubic phase, have been observed near the phase transition. At a molar ratio PE/PC of 5:1, differences in the degree of unsaturation of the PCs had a negligible influence on transition temperature.

In previous investigations the molecular order of lipid hydrocarbon chains in the lamellar phase has been compared at the same reduced or absolute temperature. The reduced temperature scale is correcting for differences in the main phase transition temperature of lipids (see, e.g., Seelig and Seelig, 1977; McCabe et al., 1994; Holte et al., 1995). The question of which temperature scale to use becomes an issue, especially when one of the lipids in the series has a phase transition temperature that is much different from that of the others, as in the case of DSPC and unsaturated PCs. Introduction of a single double bond into the sn-2 chain of PCs with a chain length of 18 carbons or longer results in a decrease of the gel-to-liquid crystalline phase transition temperature of more than 50°C (Keough, 1986; Niebylski and Salem, 1994; Holte et al., 1995). If compared at the absolute temperature of 55°C, at which

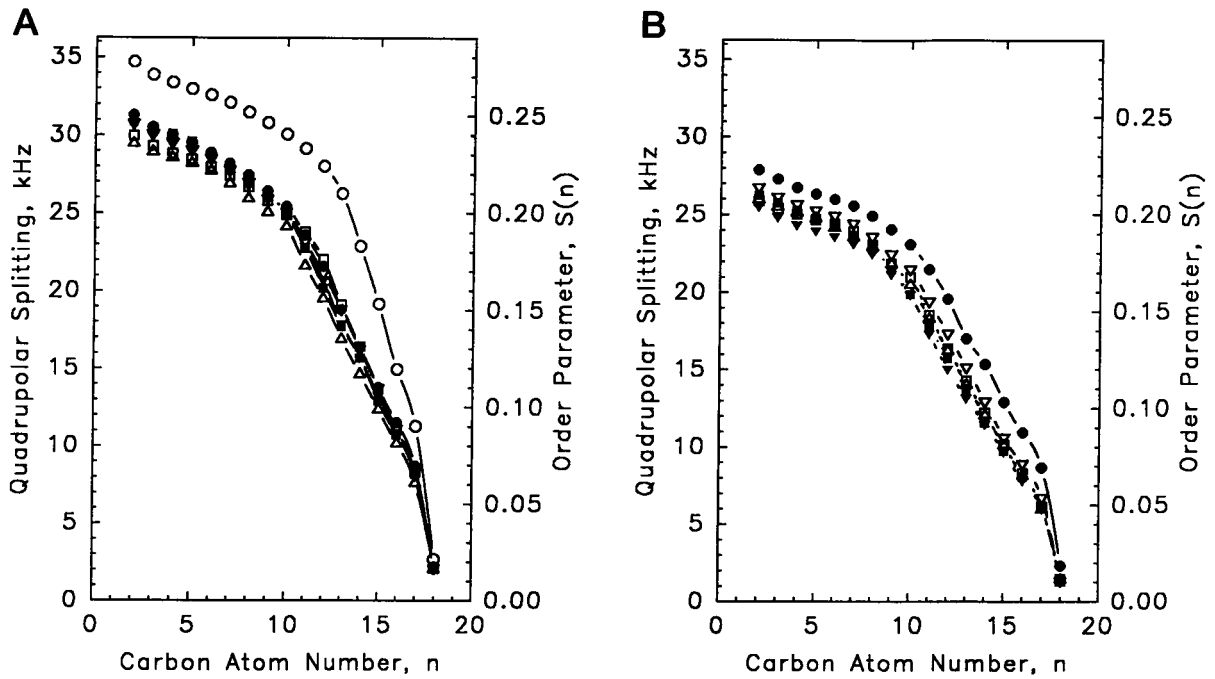

FIGURE 4 (*A*) Order parameter profiles of the dePaked spectra of Fig. 3 for DOPE/(18:0_{d35})(sn-2)PC at 30°C. Methods for deriving the order profile are described in Materials and Methods. (*B*) For comparison, order parameter profiles of the unsaturated PCs without the DOPE matrix are given (data from Holte et al., 1995). ○, (18:0_{d35}) (18:0)PC; ●, (18:0_{d35}) (18:1ω9)PC; ▽, (18:0_{d35}) (18:2ω6)PC; ▼, (18:0_{d35}) (18:3ω3)PC; □, (18:0_{d35}) (20:4ω6)PC; ■, (18:0_{d35}) (20:5ω3)PC; △, (18:0_{d35}) (22:6ω3)PC.

DSPC has just entered the L_α phase, DSPC has significantly higher order parameters than unsaturated PCs. But when compared on a reduced temperature scale, just the opposite is true—DSPC has the lowest order (Holte et al., 1995).

When the same series of PCs is included at low concentrations in a DOPE matrix, the gel-to-liquid crystalline phase transition temperature of the mixture is mainly determined by DOPE. As a result, absolute and reduced temperature scales are now almost identical. Under these conditions, DSPC does in fact have higher order than any unsaturated lipid in the series studied. The higher order of DSPC in the DOPE matrix is not a result of phase separation of both lipids for the following reasons: 1) DSPC is in the liquid crystalline phase at temperatures far below the gel-to-liquid crystalline phase transition temperature of pure DSPC. 2) The L_α-H_{II} phase transition temperatures of all

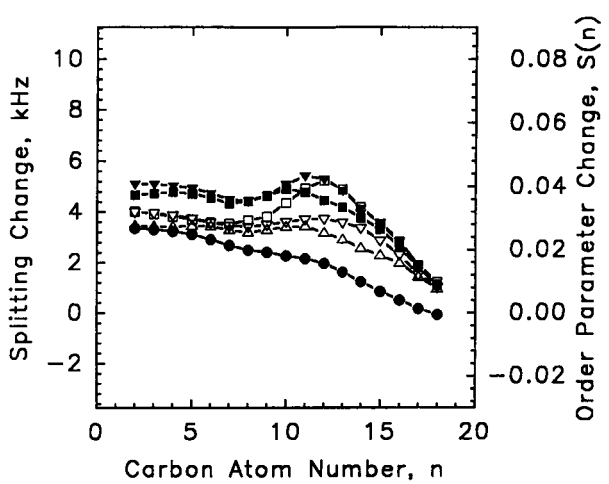

FIGURE 5 A comparison of the order parameter profiles at 30°C for mixed DOPE/PC (5:1) and plain 18:0_{d35}(sn-2)PC bilayers obtained by subtraction of the pure PC order parameter profile from that of the DOPE/PC dispersion incorporating the same PC. Symbols are the same as in Fig. 4.

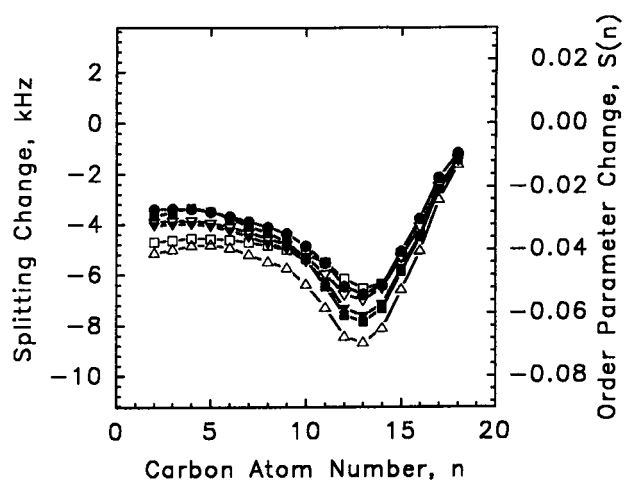

FIGURE 6 A comparison of the order parameter profiles for DOPE/PC (5:1) bilayers obtained by subtraction of the DOPE/(18:0_{d35})(18:0)PC order parameter profile from that of the unsaturated DOPE/(18:0_{d35})(sn-2)PC dispersions. Spectra were acquired at 30°C. Symbols are the same as in Fig. 4.

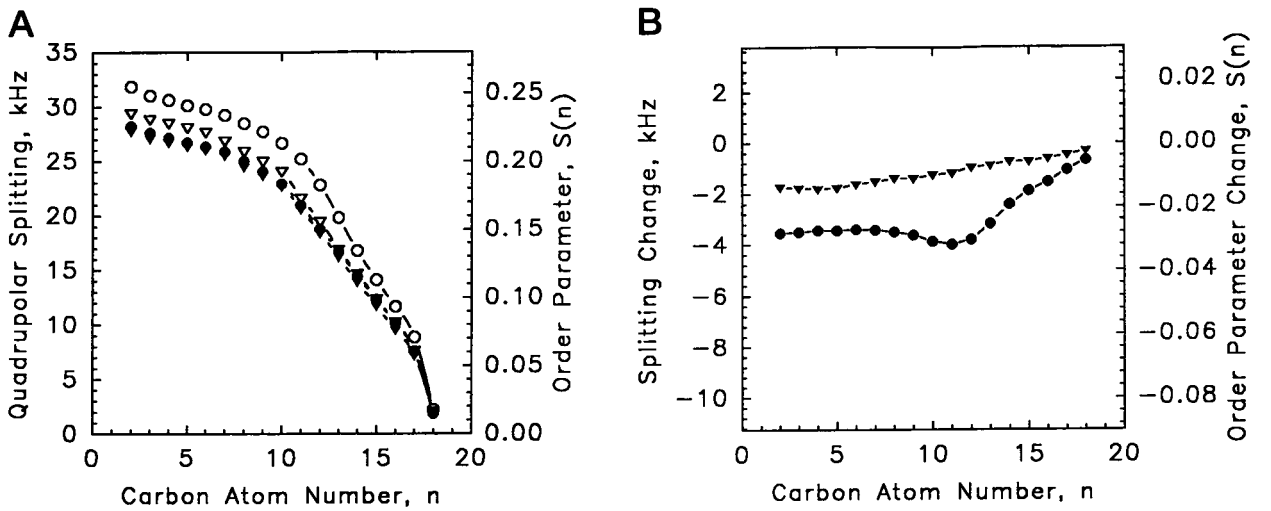

FIGURE 7 (*A*) The order parameter profiles for DOPE/(18:0_{d35})(18:1ω9)PC (5:1) (○, ●) and DOPE/(18:0_{d35})(22:6ω3)PC (5:1) (▽, ▼) at 30°C, with (●, ▼) and without (○, ▽) 2 M ethanol. (*B*) A subtraction of the order profiles without ethanol from those with ethanol for the lipid dispersions shown in *A*. The negative values indicate a disordering effect due to the presence of ethanol.

DOPE/PC mixtures are around 50°C compared to a value of 5°C for DOPE water dispersions. 3) The order of DSPC in a DOPE matrix is higher than the order of pure DSPC immediately after the phase transition to the liquid crystalline lamellar phase.

DOPE, with its smaller headgroup, results in a tighter packing at the lipid-water interface of the incorporated saturated, monounsaturated, and polyunsaturated PC molecules, which translates into higher chain order. The increase in chain order of PCs in DOPE translates into an increase in the hydrophobic thickness of membranes of about 1 Å. Higher chain order in PEs compared to PCs has been observed previously by NMR (Marsh et al., 1983; Perly et al., 1985; Fenske et al., 1990; Thurmond et al., 1991). The DOPE matrix reduces the sensitivity to temperature of the mixed-acid PC by almost a factor of 2 compared to a pure PC dispersion (Holte et al., 1995), to an approximately −0.02 Å decrease in $\langle L \rangle$ per degree increase in temperature.

Regardless of their degree of unsaturation, sn-1 chain order of all PCs indicates a tighter packing of lipids in a DOPE matrix. The influence of PC sn-2 chain unsaturation on sn-1 chain order may reflect a short-range interaction between hydrocarbon chains within PC molecules. Additional experiments are necessary to determine whether 16.6 mol% of PCs with different degrees of unsaturation will result in changes of bilayer hydrophobic thickness as large as 2 Å (DSPC versus (18:0)(22:6ω3)PC).

At 30°C the effect of PC unsaturation is seen in the reduction of sn-1 chain length by 0.75 Å with the addition of the first double bond, and a further reduction of 0.1 to 0.25 Å for two to six double bonds. The quadrupolar splitting is reduced by 3–5 kHz in the upper part of the chain, with the largest reduction (5–8 kHz) occurring around the 12th carbon (Fig. 6). It appears that the "disordering" effect of unsaturation is larger toward the middle of the bilayer, as already seen for pure mixed-acid PC dispersions by Holte et al. (1995). By contrast, the increase in chain order as a result of incorporation of PCs into a DOPE matrix and the decrease in order due to increasing the temperature have a constant magnitude for the upper two-thirds of the chain and are smaller for the lower third. This illustrates that the structural properties of polyunsaturated membranes are not equivalent to properties of less unsaturated membranes that are investigated at a higher temperature.

Ethanol has been added to the lipid-water dispersion because of its ability to decrease lipid chain order and to increase the lamellar-to-hexagonal phase transition temperature of PEs (Hornby and Cullis, 1981). After the addition of 2 M ethanol to the DOPE/PC mixtures, no global transition to a nonlamellar phase state has been observed up to temperatures of 70°C, the highest temperature investigated. Between 50 and 60°C a few percent of the lipid dispersion converted to a phase with an isotropic ^{31}P NMR signal—most likely the cubic phase.

Ethanol reverses the increase in PC-chain order caused by the DOPE matrix. The reduction in order is larger for the (18:0_{d35})(18:1ω9) PC compared to (18:0_{d35})(22:6ω3) PC (Fig. 7 *A*). After the addition of ethanol, the sn-1 chain order parameter profile and chain length are identical for both lipids. The decrease in chain order in the presence of ethanol is most likely the result of increased lipid spacing at the lipid-water interface (Barry and Gawrisch, 1994, 1995). This view is strongly supported by the observed increase in the L_α-to-H_{II} phase transition temperature in the presence of ethanol. Lipid headgroups like PE reduce the area per molecule at the lipid-water interface and promote the formation of nonlamellar lipid phases. The ethanol negates the effect of the DOPE, and no hexagonal phase lineshape is observed up to 70°C by ^{31}P NMR spectroscopy (Fig. 1 *C*). The unsaturated PC occupies a larger area per molecule, even

without ethanol, and is therefore less vulnerable to the disordering influence of ethanol.

Slater et al. (1993) interpret changes of lipid order in the presence of ethanol as a result of a perturbation of membrane surface hydrogen bonds. It is possible that the ethanol is taking the place of water molecules of hydration at the lipid-water interface (Barry and Gawrisch, 1994, 1995).

Changes in area per molecule at the interface may be estimated by calculating the hydrocarbon volume and dividing it by the chain length $\langle L \rangle$ derived from the NMR measurements (Holte et al., 1995). This approach neglects chain upturns and interdigitation of chains from opposing bilayers (Nagle, 1993). From Table 1 it can be seen that at 30°C, the sn-1 chains are about 0.5 Å longer and the area per PC-molecule 2.0 $Å^2$ smaller in the DOPE/PC mixtures. This appears reasonable in light of x-ray diffraction results (Fenske et al., 1990), which show a decrease in the area per molecule with increasing PE content in DOPE-dimyristoyl PC bilayers. The DOPE/PC mixtures decrease the chain length and increase the area per molecule as the temperature is increased up to the point where the dispersions undergo a nonlamellar phase transition. The addition of 2 M ethanol to the mixed lipid dispersion results in an increase in the PC area by 1–3 $Å^2$, close to values of the pure PC dispersion.

Lipid monolayers may lower their free energy by adapting to a certain curvature that brings headgroup and chain areas closer to optimal values. When lipid monolayers are bent, the change in the area per molecule is smallest in the hydrocarbon region just below the lipid glycerols (Rand and Fuller, 1994). This region acts like a hinge region for adjustment to different curvatures. In a bilayer arrangement without curvature, lipids are packed in volumes of cylindrical shape. A differential increase in headgroup or chain repulsion cannot be released by bending of the two monolayers and will result in an increase of the area per lipid molecule (Sadoc and Charvolin, 1986). In bilayers unsaturation may be a means by which the PE lipid compensates for the tighter packing at the lipid-water interface caused by the PE headgroups (Slater et al., 1994). However, headgroup and chain interactions have an opposite influence on spontaneous monolayer curvature because they act above and below the hinge region, respectively. As a result, membranes composed of small PE headgroups and polyunsaturated hydrocarbon chains are under curvature stress.

In some lipid-protein systems curvature stress appears to control protein function. Keller et al. (1993) have shown that increasing the concentration of PE in DOPC/DOPE mixtures increases the probability of the existence of higher conductive states of the alamethicin channel. It has been demonstrated that the metarhodopsin I-II structural transition is favored by lipids with PE headgroups and polyunsaturated hydrocarbon chains (Brown, 1994). The hydrolysis of mixed dilinoleoylphosphatidylethanolamine/1-palmitoyl-2-oleoylphosphatidylcholine dispersions by porcine phospholipase A_2 is controlled by curvature stress (Sen et al., 1991). The activity of protein kinase C is modulated by PC unsaturation as well as the PC/PE ratio and appears to correlate with spontaneous membrane curvature (Slater et al., 1994).

The chains of polyunsaturated PC molecules in a DOPE matrix are compressed over their entire length, independently of their degree of unsaturation, as in the results obtained by Fenske et al. (1990) for saturated DMPC in a DOPE matrix. The decrease in area per molecule is significantly larger for the saturated DSPC compared to unsaturated PCs. This parallels the difference in thermal expansivity of saturated and unsaturated PCs. Polyunsaturated PCs change their area per molecule significantly less with temperature than saturated and monounsaturated PCs (Holte et al., 1995).

Chain order parameter profiles of polyunsaturated membranes differ significantly from profiles of saturated and monounsaturated membranes. The question arises: How much does order change in the presence of intrinsic membrane proteins? According to recent measurements by Thurmond et al. (1994), membrane proteins have little effect on the packing of lipid molecules. By using a perdeuterated acyl chain as a reporter molecule, they found only slight to undetectable differences between order or membrane thickness of intact *Acholeplasma laidlawii* membranes and extracted lipids. The organism may regulate properties, such as membrane curvature energy, that are related to lipid packing through lipid composition. These parameters can be studied in mixed lipid dispersions.

Seelig and Seelig (1977) were the first ones to report detailed information on the lowering of hydrocarbon chain order by a single *cis* double bond. Our data support the premise that the largest changes in membrane organization by unsaturation are observed after incorporation of the first two double bonds per hydrocarbon chain. When the unsaturation of the PC sn_T2 chain is increased to six double bonds and the number of carbons rises from 18 to 22, a further change as a result of an increase in the area per PC molecule can be expected, even though the sn-1 chain length is not changing. The DOPE matrix has resulted in a higher order

TABLE 1 $\langle L \rangle$, sn-1 chain length, and cross-sectional area for mixed-acid phosphatidylcholines in hydrated dispersions of pure PCs and in DOPE/PC mixtures (5:1, mol/mol) at 30°C

sn-2 chain	$\langle L \rangle$ PC(Å)	$\langle L \rangle$ (Å) DOPE/PC	Area ($Å^2$) PC	Area ($Å^2$) DOPE/PC
18:0	14.85*	15.40	65.4*	63.1
18:1ω9	14.40	14.65	66.6	65.5
18:2ω6	14.05	14.55	67.3	65.0
18:3ω3	14.00	14.50	66.6	64.3
20:4ω6	13.80	14.55	70.6	66.9
20:5ω3	13.90	14.55	69.1	66.0
22:6ω3	14.00	14.40	71.6	69.6

The area was calculated by dividing the hydrophobic chain volume V_{chain} by the sn-1 chain length $\langle L \rangle$. V_{chain} was calculated according to $V_{chain} = n \cdot V_{CH} + n' \cdot V_{CH2} + V_{CH3}$, where $V_{CH} = 20.5$ $Å^3$, $V_{CH2} = 27.0$ $Å^3$, and $V_{CH3} = 54.0$ $Å^3$ (Marsh, 1992). The chain volume of stearic acid is 486 $Å^3$. *For the (18:0_{d35}) (18:0) PC dispersion, $\langle L \rangle$ was taken at 53°C, immediately above the gel-fluid phase transition temperature.

parameter corresponding to about a 1 Å increase in bilayer thickness in comparison to a pure PC bilayer, which translates into a decrease of the area per molecule at the lipid-water interface on the order of 2.0 $Å^2$. The effect of PE on the acyl chain order involves the hydrocarbon chain over its entire length. Increasing the number of double bonds has a more selective effect and preferentially reduces the order in the lower part of the hydrocarbon chains close to the methyl group. The greater changes in order toward the center of the bilayer with increasing unsaturation suggest a change in the effective shape of the sn-1 hydrocarbon chain (Holte et al., 1995). Forces at the hydrocarbon-water interface may be modulated by lipid headgroup structure and chain unsaturation, resulting in optimum conditions for membrane function.

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