Magnetic alignment and orientational order of dipalmitoylphosphatidylcholine bilayers containing palmitoyllysophosphatidylcholine


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Mixed bilayers of 1-palmitoyl-sn-glycero-3-phosphocholine (palmitoyllysophosphatidylcholine; PaLPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (dipalmitoylphosphatidylcholine; DPPC) have been investigated by $^2$H-NMR and $^1$H-NMR spectroscopy. Binary phospholipid mixtures were studied in which the acyl chains of one or the other component were perdeuterated. At temperatures below the main order-disorder phase transition, the mixed PaLPC/DPPC bilayers appear to coexist with PaLPC micelles. The micelles disappear at temperatures above the phase transition, where mixed bilayers in the liquid-crystalline state are formed. The orientational order of the alkyl chains of the PaLPC component is essentially identical to that of the DPPC component in the mixed bilayers, both in the low temperature and liquid-crystalline phases. However, the presence of PaLPC perturbs the segmental ordering of DPPC as compared to the pure system. The order is increased in the low-temperature phase, where effective diffusion of the chains about their long axes occurs, but is decreased in the liquid-crystalline phase compared to pure DPPC bilayers. The mixed liquid-crystalline bilayers orient preferentially with their director axes perpendicular to the magnetic field. This alignment is easily observed in $^1$H- and $^2$H-NMR spectra, where the intensity of the perpendicular edges of the lineshapes is pronounced. One possible explanation of the magnetic alignment involves alteration of the curvature free energy of the DPPC bilayer due to incorporation of PaLPC in the mixed membranes.

Keywords: lysolecithin; DPPC; deuterium NMR spectroscopy; magnetic alignment.

Introduction

Lyso phospholipids, which can be found in most cellular membranes, are known to cause many unique effects in biological systems. They may induce morphological changes in cells [1], facilitate cell fusion [2], cause hemolysis [3], or affect the permeability properties of phosphatidylcholine liposomes [4]. Lysolecithins have physicochemical properties quite different from the corresponding diacylphosphatides. The high solubility in water and the ability to form micelles of lysolecithins can be rationalized on the basis of the smaller volume of the lipid hydrocarbon domain as compared to other phospholipids. At sufficiently high molar ratios, the inclusion of lysolecithins in membranes will lead to a destabilization of the membrane structure, and the formation of lipid aggregates with higher surface curvatures, i.e. hexagonal or micellar phases [5].

$^2$H-NMR measurements have been widely utilized to investigate the molecular properties of lipid bilayers [6—9]. In the anisotropic environment of a lipid membrane, in which the $^2$H-NMR spectrum comprises a quadrupolar powder
pattern, the frequency separation between the resonances yields directly the orientational order parameter for the symmetry axis of the electric field gradient tensor at the various deuterated sites. The moments of the spectra provide a means of characterizing the average orientation and the degree of orientational disorder induced by molecular motions of membrane lipids in different physical states, which in the liquid-crystalline phase can be described by a segmental order parameter. In the present article, $^2$H-NMR and $^{31}$P-NMR methods have been applied to investigate bilayers formed by mixtures of 1-palmitoyl-sn-glycero-3-phosphocholine (palmitoyllysophosphatidylcholine; PaLPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (dipalmitylphosphatidylcholine; DPPC). The main emphasis was to compare the physical properties of the mixed PaLPC/DPPC membranes to those of aqueous pure phosphatidylcholine dispersions. The wedge-like average molecular shape of PaLPC can be expected to perturb the molecular organization of the DPPC bilayers. One purpose of the study was to compare the physical properties of the mixed PaLPC/DPPC membranes to those of aqueous pure phosphatidylcholine dispersions. The wedge-like average molecular shape of PaLPC can be expected to perturb the molecular organization of the DPPC bilayers. It is not fully understood to what extent the magnitudes of the order parameters are affected by pure intermolecular influences, that is, the influences of the surrounding molecular environment on the orientational order of the lipid. Since the two lipids have quite different average shapes, a comparison of the orientational order of the lipids gives an insight into the intermolecular influences on the order profiles of the hydrocarbon chains. A further effect of the inclusion of PaLPC into DPPC bilayers is that it leads to magnetic alignment of the membranes perpendicular to the static magnetic field.

**Materials and Methods**

DPPC-$d_{62}$ and DPPC were prepared and characterized following a modification of the procedure of Mason et al. [10] as previously described [11]. The PaLPC-$d_{31}$ and PaLPC were prepared by deacylating the sn-2 chain with phospholipase $A_2$ from *Crotalus adamanteus* (EC 3.1.1.4) (Sigma, MO), following the procedure of Mason et al. [10]. Thin layer chromatography showed less than 1% impurities for all of the phospholipids studied. Samples of mixed phospholipids comprising 200—250 mg were prepared by dissolving the two lipids in 3:1 chloroform/methanol. The solvent was removed under high vacuum and the sample was lyophilized from cyclohexane/chloroform 20:1 (v/v), followed by addition of 200—250 mg of 0.067 M sodium phosphate buffer, pH 7, prepared from $^2$H-depleted water (Aldrich, WI) containing $10^{-4}$ M EDTA. The 50 wt% phospholipid dispersions were freeze-thawed to improve their homogeneity. The samples were then vortexed and centrifuged while above their main thermal phase transition temperature in 8-mm Pyrex tubes. Teflon plugs were inserted, the tubes were cut off and their edges were sealed with high-melting wax (Petrolite, OK). When not in use, the samples were stored at $-20^\circ$C.

The $^2$H-NMR spectra were acquired with a General Electric GN-300 spectrometer operating at a frequency of 46.13 MHz (magnetic field strength of 7.058 tesla). The spectrometer was equipped with a home-built horizontal solenoid, high-power probe, an external digitizer (Nicolet 2090 digital oscilloscope) and a radio frequency boost (Henry Radio Tempo 2006). A phase-cycled, quadrupolar echo pulse sequence was used [12], with a 6 $\mu$s 90° pulse, 40 $\mu$s pulse separation, recycle time of 0.5 s, and a digitization dwell time of 2 $\mu$s. The $^2$H-NMR spectra of the mixed PaLPC/DPPC system have been compared in some cases with $^2$H-NMR spectra of an aqueous dispersion of DPPC-$d_{62}$ (50 wt%) [13]. To ensure that any observed effects were not due to differences in the experimental conditions used to acquire the two sets of data (e.g. different NMR probe characteristics or different pulse parameters) [9], a DPPC-$d_{62}$ spectrum was collected at 25°C and compared to the data of Trouard et al. The difference in the first moments of the spectra (see below) was less than 3%, indicating the absence of systematic errors in contrasting the results. All $^{31}$P-NMR spectra were collected with a General Electric GN-500 spectrometer operating at 202.5 MHz (magnetic
field strength of 11.75 tesla), using a Hahn spin-echo with phase cycling and $^1H$ decoupling. The $^{31}P$-NMR chemical shifts were expressed relative to 85% $H_3PO_4$. The spectra were taken in order of increasing temperature. After each change in temperature, the samples were allowed to equilibrate at the new temperature for approximately 40 min prior to data acquisition.

A full description of the $^2H$-NMR method and its application to lipid systems can be found elsewhere [9,13,14]. Spectra were analyzed by using the method of moments. The moments of one half of a symmetric $^2H$-NMR spectrum are related to the moments of the distribution of segmental order parameters, $S_{CD}$, so that the first two moments $M_1$ and $M_2$ give the mean orientational order parameter and its mean square value, respectively. The first and second moments of the $^2H$-NMR spectra were calculated from the expression

$$M_n = A^{-1} \int_0^\infty d\omega \omega^n f(\omega)$$

in which

$$A = \int_0^\infty d\omega f(\omega)$$

where $n = 1,2$ and $f(\omega)$ is the spectral intensity at frequency $\omega$ relative the center of the spectrum at zero frequency. The fractional mean squared width of the distribution of order parameters, $\Delta_2$, is given by

$$\Delta_2 = \left( \frac{M_2}{1.35 M_2^2} \right) - 1$$

For lipids in liquid-crystalline phases the bilayer normal is an axis of motional averaging, and the shape of the $^2H$-NMR spectra corresponds to axially symmetric motion. In this case, the quadrupolar splitting, $\Delta \nu_Q$, between the two peaks is related to the orientational order parameter $S_{CD}$ by [13,14]

$$\Delta \nu_Q = 3/2 \left( e^2 qQ/h \right) P_2(\cos\theta) |S_{CD}|$$

In the above expression $(e^2qQ/h)$ denotes the static quadrupolar constant, with a value of 170 kHz for a C-$^2H$ bond, $P_2$ is the second Legendre polynomial, and $\theta$ is the angle between the bilayer normal (director axis) and the static magnetic field. The order parameter, $S_{CD}$, is defined by

$$S_{CD} = < P_2(\cos\theta) > < 3 \cos^2\theta - 1 >$$

where $\beta$ is the angle between the C-$^2H$ bond direction and the bilayer normal, and the brackets indicate a time or ensemble average.

Results

$^2H$-NMR spectroscopy

Two samples were investigated, one a 1:3 mixture of PaLPC-$d_{31}$ and DPPC in 50 wt% water, and the other a 1:3 mixture of PaLPC and DPPC-$d_{62}$ with the same water content. The $^2H$-NMR spectra were acquired for each of the samples over a temperature interval of 25—55°C in order of increasing temperature. Although a systematic study was not performed, an estimate showed that hysteresis of the phase transition of the mixed PaLPC/DPPC system was small and less than 1°C in magnitude. Representative $^2H$-NMR spectra of both systems are shown in Fig. 1 at some selected temperatures. Figure 2 depicts the spectra collected at 25°C and 55°C in more detail, together with reference spectra of a dispersion of pure DPPC-$d_{62}$ bilayers in 50 wt% water. The moments $M_1$ and $M_2$ and parameter $\Delta_2$ of the spectra are plotted as a function of temperature in Fig. 3. In the case of the DPPC-$d_{62}$ bilayers, the results are in good agreement with the more extensive previous $^2H$-NMR study of Davis [15].

Comparison of $^2H$-NMR spectra of DPPC-$d_{62}$ and PaLPC/DPPC-$d_{62}$

The change in the $^2H$ NMR lineshape for the mixed PaLPC/DPPC-$d_{62}$ system with temperature is shown in Fig. 1a. There are substantial differences in the $^2H$-NMR spectra obtained for the acyl chains of DPPC-$d_{62}$ in the mixed PaLPC/DPPC-$d_{62}$ system compared to the pure
DPPC-d_{62} bilayers originally investigated by Davis [15]. First, the $^3$H-NMR spectra of the low temperature phase of the PaLPC/DPPC-d_{62} system (Fig. 2a) show more structure as compared to the rather featureless methylene spectrum of the multilamellar dispersion of pure DPPC-d_{62} (Fig. 2c). A distinct splitting of $\approx 60$ kHz due to the $\theta = 90^\circ$ bilayer orientation is observed in the former spectra. The differences in the line-shapes of the low-temperature spectra of the PaLPC/DPPC-d_{62} and DPPC-d_{62} systems are also apparent from the first and second moments displayed in Figs. 3a and 3b. The $M_1$ and $M_2$ values are higher at temperatures below 37°C for the lipids in the mixed system as compared to the pure DPPC-d_{62} system. Since $M_1$ and $M_2$ are directly related to the mean orientational order of the lipid, this observation implies that the acyl chain ordering of DPPC is enhanced in the low temperature phase by the presence of lysophosphatidylcholine. From Eqn. (4) it is seen that a quadrupolar splitting of 63.75 kHz corresponds to the methylene groups of an all-trans chain ($S_{CD} = -1/2$) undergoing effective diffusion about its long axis. Likewise, a methyl splitting of 21.25 kHz is expected ($S_{CD} = -1/6$) which is close to the observed value (Fig. 2a). The DPPC acyl chains are thus all-trans in
the low temperature state of the PaLPC/DPPC bilayers, and undergoing effective axial rotation.

Secondly, although the onset of the phase transition is 37°C in both systems, the temperature interval is broader for the PaLPC/DPPC-d6 system than for the pure DPPC-d6 system. The difference in the phase transition behavior is evident from the change in the relative width of the distributions of quadrupolar splittings shown in Fig. 3c. Phase transitions of single component lipid systems are characterized by sharp maxima of $\Delta_2$ near their midpoint temperature [15]. (In Fig. 3c the data for the DPPC-d6 dispersion are lacking points in the transition region and the sharp maximum is omitted.) By contrast, the mixed PaLPC/DPPC-d6 system shows a broad maximum in $\Delta_2$ spanning over 6—7°C. It also appears that $M_1$ and $M_2$ may not change continuously with temperature, as seen from Figs. 3a and 3b. As the temperature is increased from 36°C and 37°C, a significant change in the lineshape can be observed, in which the spectra of the ordered phase are replaced by spectra containing both broad and narrow (liquid-crystalline) components (cf. Fig. 1a). The spectra containing mixed-phase features are retained over the temperature interval 38—43°C, where the $M_1$ and $M_2$ values are almost constant. Ultimately, when the temperature is increased to 45°C, pure liquid-crystalline spectra are obtained.

Thirdly, the lineshape of the liquid-crystalline $^2$H-NMR spectra of DPPC-d6 is different in the mixed PaLPC/DPPC-d6 and pure DPPC-d6 systems. In the latter case, distinct shoulders are observed for both the methylene group “plateau” and the smaller splitting due to methyl group deuterons (Fig. 2f). These shoulders correspond
to the quadrupolar splittings of those bilayers of the random distribution whose director axes are parallel to the static magnetic field ($\theta = 0^\circ$). By contrast, only traces of the shoulders can be observed in the PaLPC/DPPC-d$_{62}$ system (Fig. 2d). The reduction of the $\theta = 0^\circ$ shoulders in this case indicates that the bilayers are not randomly oriented, and tend to align in the magnetic field.

Finally, the magnitudes of the quadrupolar splittings are different in the PaLPC/DPPC-d$_{62}$ and the pure DPPC-d$_{62}$ systems in the liquid-crystalline state. A total of eight quadrupolar splittings, $\Delta \nu_Q$, can be resolved in the $^2$H-NMR spectra at 55°C and are displayed in Fig. 4. The quadrupolar splittings observed for the pure DPPC-d$_{62}$ dispersion are generally larger as compared to those of the mixed PaLPC/DPPC-d$_{62}$ bilayers. The largest splitting is 23.5 kHz for the DPPC-d$_{62}$ bilayers as compared to 20.2 kHz for the PaLPC/DPPC-d$_{62}$ system, corresponding to order parameters of 0.187 and 0.161, respectively. Although less apparent, the higher degree of orientational order in the liquid-crystalline phase of the pure DPPC-d$_{62}$ dispersion is also evident from the higher values of $M_1$ and $M_2$ observed at temperatures above the phase transition.

**Comparison of $^2$H-NMR spectra of PaLPC-d$_{31}$/DPPC and PaLPC/DPPC-d$_{62}$**

The most obvious difference between the $^2$H-NMR spectra of the PaLPC-d$_{31}$/DPPC and PaLPC/DPPC-d$_{62}$ systems is the presence of a large isotropic peak in the former spectra at temperatures below the phase transition (Fig. 2b). This peak disappears as the temperature is raised above the main order-disorder transition. Interestingly, the process is totally reversible, in that the isotropic peak reappears after cooling.
the sample below the phase transition temperature. It is a well-known phenomenon that isotropic peaks may appear in $^2$H-NMR spectra of lamellar systems due to formation of small vesicles during the sample preparation. Such peaks will, contrary to what is observed for the PaLPC-$d_{31}$ spectra, remain at temperatures above the phase transition, or disappear with time as the vesicles fuse. Hence, it is improbable that the reversible appearance and disappearance of the isotropic peak in the PaLPC-$d_{31}$/DPPC spectra with temperature (cf. Fig. 1b) can be rationalized by the presence of small vesicles in the system. On the other hand, the smaller isotropic peak observed in the PaLPC/DPPC-$d_{62}$ low temperature spectra (cf. Fig. 1a) may indeed be caused by small vesicles. In contrast to what is observed for the spectra of the PaLPC-$d_{31}$ component, the isotropic peak is also present in the liquid-crystalline phase spectra. Moreover, the intensity of the isotropic peak in the PaLPC/DPPC-$d_{62}$ spectrum was seen to decrease with time, which can be attributed to fusing of the vesicles with multilamellar liposomes.

If the isotropic peak is disregarded, a comparison of Figs. 2a and 2b shows that the $^2$H-NMR spectra of the PaLPC-$d_{31}$ and DPPC-$d_{62}$ components of the mixed PaLPC/DPPC systems are almost identical at 25°C. Consequently, $M_1$ and $M_2$ are nearly the same as is evident from Figs. 3a and 3b (the isotropic peak gives only a minor contribution to the moments). The presence of an isotropic peak in the spectrum of the PaLPC-$d_{31}$ component, together with the close resemblance between the low temperature spectra of the two lipids, suggests that phase separation occurs at temperatures below the thermal transition. Although the major fraction of the PaLPC molecules are mixed with the DPPC molecules, forming bilayers in the low temperature state, a smaller fraction of the PaLPC lipids are excluded from the lamellar phase and give rise to the isotropic peak. Integration of the $^2$H-NMR spectrum in Fig. 2b yields the fraction of PaLPC molecules confined within the isotropic phase to be approximately 0.25. Since this corresponds to a PaLPC concentration well above the critical micelle formation concentration [16], the isotropic phase probably corresponds to PaLPC-$d_{31}$ lipids in micelles.

Referring to the preceding section, one can conclude that in the low temperature state the PaLPC-$d_{31}$ chains in the mixed bilayers are all-trans and are undergoing effective axial diffusion. Thus the motions of both the PaLPC and DPPC components are similar. As the temperature is increased, the same type of change in $M_1$ and $M_2$ is found for the PaLPC-$d_{31}$/DPPC system as observed for the PaLPC/DPPC-$d_{62}$ system over the phase transition interval (Figs. 3a and 3b). However, the characteristic liquid-crystalline lineshape is obtained at a slightly lower temperature in the latter system (43°C versus 45°C; cf. Fig. 1). Since it is known that perdeuteration of alkyl chains decreases the transition temperature of lipids [15], the difference is probably ascribable to the fact that the main lipid component in the system, DPPC, is per-deuterated in the latter case.

In contrast to what is observed at the lower temperatures, no phase separation can be observed in the PaLPC/DPPC system at temperatures above the phase transition. Figure 4 shows that the quadrupolar splittings in the $^2$H-NMR spectra of the two lipid components are almost identical. The similarities in the $\Delta \nu_0$ values demonstrates that the orientational order of the PaLPC-$d_{31}$ and DPPC-$d_{62}$ lipids is almost the same in the mixed bilayer, implying that they form a completely mixed liquid-crystalline phase. Although the magnitudes of $\Delta \nu_0$ are identical, the lineshapes of the liquid-crystalline phase spectra for the two lipid components differ slightly. Figure 2 shows that the traces of the $\theta = 0^\circ$ shoulders found in the PaLPC/DPPC-$d_{62}$ spectra are not seen in the PaLPC-$d_{31}$/DPPC spectra. The observed difference in lineshape is probably due to the fact that the degree of orientation in the magnetic field is higher for the PaLPC-$d_{31}$/DPPC sample, which is also supported by the $^{31}$P-NMR data (see next section).

$^{31}$P-NMR spectroscopy

To investigate further the properties of the mixed 1:3 PaLPC/DPPC bilayers, $^{31}$P-NMR studies were also conducted. The $^{31}$P-NMR
investigations were performed with the same samples for which the $^1$H-NMR data were collected. The presence of small amounts of inorganic phosphorus from the sodium phosphate buffer (a 20:1 lipid/inorganic phosphorus ratio) yielded only a small contribution at the isotropic position in the $^3$P-NMR spectra, which did not affect the interpretation of the data.

The $^3$P-NMR data collected for the mixed 1:3 PaLPC/DPPC systems support the observations made from the $^1$H-NMR data regarding magnetic alignment of the bilayers. Figure 5 shows the $^3$P-NMR spectra of the PaLPC-$d_{31}$/DPPC, PaLPC/DPPC-$d_{62}$ and DPPC-$d_{62}$ samples at 55°C, respectively. The broader spectra collected for DPPC-$d_{62}$ is representative of multilamellar phospholipid dispersions in the $L_n$-phase at high magnetic field strengths. In contrast, the two mixed PaLPC/DPPC samples yield narrower spectra with single crystal-like lines, in which nearly all the intensity is at the perpendicular edge ($\theta = 90^\circ$) of the spectrum. It is also evident from Fig. 5 that the degree of orientation is somewhat higher in the PaLPC-$d_{31}$/DPPC sample as compared to the PaLPC/DPPC-$d_{62}$ sample. Although substantially oriented, the latter exhibits broader lines, implying that the orientation of the bilayers in the PaLPC/DPPC-$d_{62}$ sample is not complete. Leaving the sample in the magnetic field for 16 h did not change the lineshape of the spectrum, obtained after just a few minutes at the chosen temperature. The incomplete alignment of the sample is in agreement with the observation that $\theta = 0^\circ$ shoulders can be seen in the $^3$H-NMR spectra of the PaLPC/DPPC-$d_{62}$ sample, but not in the PaLPC-$d_{31}$/DPPC sample (cf. Fig. 2).

Three different peaks are observed in the $L_s$-phase $^3$P-NMR spectrum displayed in Fig. 5a; a small peak near 0 ppm corresponding to the inorganic phosphorus in the buffer, a peak at $\approx 2.7$ ppm, and the largest peak at $\approx 5.4$ ppm. Integration of the spectrum shows that the ratio of the integrals of the two larger peaks is approximately 1:3, which suggests that they may correspond to the PaLPC-$d_{31}$ and DPPC components, respectively. Figure 5 shows that the chemical shift difference between the largest perpendicular edge of the $^3$P-NMR spectra and the isotropic signal is smaller in the mixed PaLPC/DPPC systems as compared to the pure DPPC-$d_{62}$ sample. The perpendicular edge of the powder pattern of DPPC-$d_{62}$ is found to be near $\sigma_z = 15$ ppm at 55°C, as compared to $\sigma_z = 54$ ppm in the mixed systems. Since the $^3$P rigid-lattice chemical shift tensors of PaLPC and DPPC are essentially the same [5], the difference most likely represents a change in the average phosphocholine headgroup orientation in the PaLPC/DPPC system relative to the pure DPPC bilayer.

Fig. 5. $^3$P-NMR spectra of aqueous dispersions of (a) 1:3 PaLPC-$d_{31}$/DPPC, (b) 1:3 PaLPC/DPPC-$d_{62}$ and (c) DPPC-$d_{62}$, acquired at a magnetic field strength of 11.75 tesla and a temperature of 55°C. The samples contained 0.033 M sodium phosphate buffer, pH 7. Peaks exclusively due to inorganic phosphorus are indicated by an asterisk.
Figure 6 shows $^{31}$P-NMR spectra of the 1:3 PaLPC-d$_3$/DPPC at 33°C, 43°C and 47°C, collected in order of increasing temperature. The absence of a significant isotropic peak is, furthermore, compatible with the coexistence of bilayers in the ordered state and a fraction of PaLPC molecules confined within micelles, as suggested by the $^2$H-NMR data. Increasing the temperature to 43°C, i.e. into the mixed phase temperature region, leads to a substantial alignment of the PaLPC-d$_3$/DPPC bilayers. The alignment occurs within a few minutes after the temperature has been increased. Interestingly, the isotropic peak still remains in the spectrum at 43°C. A further increase in the temperature to 47°C leads to the disappearance of the isotropic peak (the small peak remaining is due to the presence of inorganic phosphorus). It thus appears that all lipids are confined within the bilayers in the liquid-crystalline phase.

Discussion

Magnetic alignment of PaLPC/DPPC membranes

The occurrence of magnetic alignment of the PaLPC/DPPC bilayers at temperatures above the phase transition has been detected by means of both $^{31}$P-NMR and $^2$H-NMR spectroscopy. The $^2$H-NMR and $^{31}$P-NMR powder patterns generally observed for randomly oriented phospholipid dispersions arise from the orientation dependence of the quadrupolar splitting or the chemical shift, and are in these systems replaced with comparatively sharp, single crystal-type spectra. In the case of the $^2$H-NMR spectra, the intensity at the perpendicular edges ($\theta = 90^\circ$) of the powder pattern arises from phospholipid lamellae in which the symmetry axis of the residual electric field gradient tensor, i.e. the director axis normal to the bilayer surface, is perpendicular to the magnetic field. Similar observations of substantial magnetic ordering of hydrated lipid bilayers have been made in earlier studies. The first report was that by Seelig et al. [17], who showed that membranes composed of Escherichia coli lipids and a mixture of 4:1 phosphatidylethanolamine and phosphatidylglycerol oriented perpendicular to the magnetic field. Speyer et al. [18] have also reported the same phenomenon for 3:2 mixtures of N-palmitoyl sphingomyelin and dimyristoylphosphatidylcholine.

The origin of the magnetic orientation may be traced back to the diamagnetic anisotropy of the lipids in the bilayer, $\Delta \chi = \chi_4 - \chi_1$, which is the
difference between the magnetic susceptibilities parallel and perpendicular to the molecular symmetry axis (the bilayer normal). The value of $\Delta \chi$ for single crystals of DPPC has been estimated to be $-68 \times 10^{-6} \text{ EMU/mol}$ by Sakurai et al. [19]. The negative value indicates that lipids will orient with their long axes perpendicular to the magnetic field. Since the molar anisotropy of crystalline stearic acid has been reported to be $-26 \times 10^{-6} \text{ EMU/mol}$ [20], it appears that most of the contribution to $\Delta \chi$ comes from the alkyl chains. The tendency to orient in a magnetic field is small for a single isolated lipid, but is enhanced for lipids in bilayers where diamagnetically anisotropic molecules are packed parallel to each other. Moreover, by comparing the values of $\Delta \chi$ obtained for a single DPPC crystal, $-68 \times 10^{-6} \text{ EMU/mol}$, with that found for egg phosphatidylcholine in the less ordered $L_\alpha$ phase, $-2 \times 10^{-6} \text{ EMU/mol}$ [21], it appears that the magnitude of $\Delta \chi$ is sensitive to the degree of order of the hydrocarbon chains in the bilayer. Taking these observations into account, the magnitude of $\Delta \chi$ can be anticipated to be smaller for the mixed 1:3 PaLPC/DPPC bilayer as compared to a pure DPPC bilayer for two different reasons. First, the number of hydrocarbon chains per molecule is less in the former case, and second, the orientational order of the lipids in the liquid-crystalline state is slightly lower in the mixed PaLPC/DPPC system. Since pure DPPC bilayers do not orient appreciably at the magnetic field strengths employed for this study, it appears that the magnitude of $\Delta \chi$ alone does not determine if a sample will orient in the magnetic field. The absence of magnetic alignment of the PaLPC/DPPC bilayers below the phase transition temperature, where the degree of molecular order is much higher, also supports this conclusion. The reason why the bilayers orient when one out of four DPPC molecules is replaced with a PaLPC molecule may be related to an increased curvature elasticity of the membrane, i.e. a decrease in bending rigidity [22], caused by inclusion of the PaLPC lipids. The process of alignment may involve deformation of the liposomes towards an ellipsoidal or cylindrical geometry [18], in which the work done by the system opposes the reduction in free energy associated with orientation in the magnetic field [22]. The spontaneous membrane curvature [22] may also play a role in governing the shape or size of the liposomes. In either case, a change in the curvature free energy of the DPPC membrane due to the presence of PaLPC could alter the balance of forces yielding magnetic alignment.

In previous investigations of magnetic alignment of lipid bilayers by Seelig et al. [17] and Speyer et al. [18], it was found that magnetic ordering was only possible when the membranes were in the liquid-crystalline state. Once the orientation was obtained in the $L_\alpha$-phase it was retained when the temperature was decreased below the phase transition. Interestingly, the $^{31}$P-NMR data in Fig. 6 indicate that the bilayers of PaLPC-d$_{31}$/DPPC orient to some extent in the mixed-phase region. Thus it appears that formation of discrete liquid-crystalline regions in the liposomes is sufficient to enable magnetic alignment of the bilayers. Since the degree of orientation was found to be slightly different in the two samples examined (traces of $\theta = 0^\circ$ shoulders could be seen in the $^2$H-NMR spectra of the PaLPC/DPPC-d$_{62}$ system, but not in the spectra of the PaLPC-d$_{31}$/DPPC system), it appears that the orientation may not only be determined by the composition of the sample, but also the sample preparation procedure. One factor which can affect the ability of membranes to orient is the average liposome size produced by the sample preparation. One can anticipate that it requires more free energy to deform small liposomes in order to obtain a high degree of magnetic alignment than for larger liposomes. More data are necessary, however, to draw any firm conclusion about the influence of the sample preparation on magnetic alignment of the liposomes.

**Temperature dependence of molecular organization of the PaLPC/DPPC system**

The presence of a sufficiently high molar ratio of PaLPC to DPPC in membranes will lead to their disruption and formation of
micelles. A convenient tool to rationalize the formation of different kinds of lipid aggregate shapes is the concept of a packing parameter, \( \frac{v}{a_0/l_0} \), where \( v \) is the hydrocarbon volume, \( a_0 \) the average or optimal head group area, and \( l_0 \) is related to the average length of the extended lipid, as introduced by Israelachvili et al. [23]. Different values of the packing parameter are associated with different average shapes of the lipid aggregates. The large cross-sectional surface area and the small hydrophobic volume of PalLPC correspond to a low value of the packing parameter and a preference for the formation of micellar aggregates. By contrast, the packing parameter is about twice as high for DPPC and packing of lipids into bilayers is preferred. For mixtures of two lipids the packing parameter characterizing the system can be defined as a weighted average of the two components. The critical molar ratio of PalLPC up to which the bilayer structure is maintained has been shown to be \( \approx 0.3 \) [5]. The molar ratios of PalLPC are less in the present experiments, which suggests that the PalLPC/DPPC system favors a bilayer geometry, in agreement with the \(^2\)H-NMR spectra collected at the higher temperatures. At temperatures below the phase transition, however, the \(^2\)H-NMR and \(^3\)P-NMR results indicate the coexistence of PalLPC/DPPC bilayers with PalLPC micelles. It thus appears that a larger amount of PalLPC can be incorporated in bilayers in the liquid-crystalline state as compared to the low temperature ordered state. Expressed in terms of the packing parameter, it cannot be reduced to the same extent in the ordered state as in the liquid-crystalline state and still retain a pure bilayer system. Intuitively, this is what one could expect, as it should be more difficult to accommodate a PalLPC molecule with a wedge-like average shape in the more rigid ordered phase as compared to the liquid-crystalline phase. Van Echteld et al. concluded from a study of PalLPC/DPPC systems that the bilayer organization was retained up to a higher concentration of PalLPC in the ordered state than in the liquid crystalline state [5]. The apparent discrepancy between this observation and the results reported here may be due to the fact that the phase separation does not lead to disruption of the bilayers.

From the change in the first and second moments of the \(^2\)H-NMR spectra and the distribution of quadrupolar splittings shown in Fig. 3, it can be seen that the temperature interval of the phase transition is substantially broader for the mixed PalLPC/DPPC system (6—8°C) than for the pure DPPC-d6 dispersion (1°C—2°C). It has been shown for several other mixed lipid bilayers that the melting ranges are broader than for single-lipid systems. From \(^2\)H-NMR investigations, it has been estimated that the melting ranges for 1:4 mixtures of phytanic acid and DPPC [24] and 1:4 mixtures of palmitic acid and DPPC [25] are 11°C and 7°C, respectively. In all these broad transitions, the \(^2\)H-NMR spectra show characteristic features from both coexisting phases, whose relative proportions change with temperature. However, the \(^2\)H-NMR spectra of the PalLPC/DPPC system show features that are not present for the other mixed lipid systems. Instead of showing a continuous decrease of \( M_1 \) and \( M_2 \) with temperature, the moments appear to change in a less gradual fashion. Although it is not possible to determine the origin of the specific features of the PalLPC/DPPC phase transition from these data only, it is reasonable to assume they are related to the phase separation observed at lower temperatures. In contrast to the phytanic acid/DPPC and palmitic acid/DPPC systems, two different changes in the molecular organization of the PalLPC/DPPC system are possible at the phase transition. These include a fusion of the PalLPC micelles with the bilayers, as well as “melting” of the hydrocarbon chains. Thus, the absence of an isotropic phase below the phase transition temperature [24,25] may explain why the phytanic acid/DPPC and the palmitic acid/DPPC systems behave differently in this aspect.

*The phospholipid studied in Ref. 24 comprises palmitic acid/stearic acid chains in a 3:1 ratio at the sn-1 position and palmitic acid-d6 at the sn-2 position. The properties closely resemble DPPC and for simplicity we refer to the mixture in terms of its predominant constituent.
Enthalpy data from differential scanning calorimetry experiments have indicated that PaLPC and DPPC form a liquid-crystalline phase up to 30 wt% PaLPC [26]. The quadrupolar splittings of the two lipid components in Fig. 4 show that order parameters as a function of chain position are almost identical in the mixed L_α-state. The same observation has been made for the 1:4 palmitic acid/DPPC system, where the order profile of the perdeuterated palmitic acid was found to fall midway between those observed for the two perdeuterated chains DPPC-d_62 [25]. The fact that two different lipids in a mixture have the same order parameter profile is not self-evident. The anisotropy of the local motions of the C—^3H bond vector in the alkyl chain, e.g. rotations of the bond due to trans-gauche isomerizations or torsions of the alkyl chain, could also depend on intramolecular properties, such as the sizes of the lipid head group and the hydrophobic domain. An influence of intramolecular properties on the order parameter profiles has been suggested from applications of the so-called “two-step” model to the frequency dependence of NMR relaxation times in surfactant systems [27,28]. For example, the order parameters estimated for a specific surfactant in different molecular environments, such as sodium dodecyl sulfate in micellar and hexagonal phases [29] or dodecyltrimethylammonium chloride in micellar and cubic phases [30], have been found to be very similar. Moreover, it has been shown in a mixed micellar system of decyl dimethylammoniopropane sulfonate and lithium dodecyl sulfate that different order parameters are obtained for the two surfactants, with S ≈ 0.3 and S ≈ 0.2 at the surface of the micelle for the zwitterionic and ionic surfactants, respectively (M. Jansson and P. Li, unpublished results). With these results in mind, it is an interesting observation that the segmental order profiles of the PaLPC and DPPC components appear similar in the mixed liquid-crystalline bilayers. Even in the low-temperature ordered phase, the two components behave similarly in that the chains are all-trans and undergo effective axial diffusion.

**Orientational order in mixed PaLPC/DPPC bilayers compared to other mixed DPPC systems**

As discussed by Davis [15], the first moment of a spectrum is directly related to the mean order parameter of the deuterons in an alkyl chain. To systematize the influences of single-chain lipids on the orientational order of DPPC, M_1 values from ^2H-NMR spectra of DPPC in some different mixed lipid systems are compared in this section. The following systems are considered; 1:3 PaLPC/DPPC [M_l(lyso)], 1:4 palmitic acid/DPPC [M_l(palm)] from Ref. 25, and 1:4 phytanic acid/DPPC [M_l(phyt)] from Ref. 24 (see previous footnote). Since it is known that the moments of ^2H-NMR spectra are affected by experimental parameters, such as the pulse length or time to echo [9], one has to be prudent when comparing the different sets of data. However, all the data sets have been independently compared to moments obtained for the corresponding DPPC dispersions. Hence, although it may not be valid in view of experimental uncertainties to compare directly the absolute magnitudes of M_1 for the different systems, a comparison of the M_1 values relative to those obtained for DPPC with perdeuterated acyl chains is appropriate.

The M_1 values in the low-temperature phase at 25°C were found to decrease in the order

\[ M_1(\text{lyso}) > M_1(\text{palm}) \approx M_1(\text{DPPC}) > M_1(\text{phyt}) \]

Since the head group of PaLPC is larger than the carboxylate head group, and the volume of the branched alkyl chain of phytanic acid is larger than that of the other chains, the packing parameter, v/a_d, increases in the series PaLPC < palmitic acid < phytanic acid. Hence, a higher value of M_1 in the ordered state is correlated with a lower value of the packing parameter of the perturbing single chain molecule; i.e. the more wedge-shaped the perturbing lipid is, the higher the orientational order of the lipid. As mentioned above, the enhanced order in the presence of PaLPC is associated with all-trans chains rotating about their long axes. An oppos-
ite trend is found when comparing the $M'_1$ values of the liquid-crystalline state at 55°C:

$$M'_1(\text{phyt}) = M'_1(\text{palm}) > M'_1(\text{DPPC}) > M'_1(\text{lyso})$$

At temperatures above the phase transition there is instead a correlation between higher values of $M'_1$ and higher values of the packing parameter; that is the presence of DaLPC in the bilayers now decreases the orientational order of the DPPC lipids. Since specific molecules can cause opposite effects on the orientational order of DPPC in the low-temperature and liquid crystalline phases, which is the case for both PaLPC and phytanic acid, it is evident that the packing conditions of the lipids in the bilayer are quite different above and below the phase transition.

It is noteworthy that X-ray diffraction data obtained for mixed lysophosphatidylcholine/phosphatidylcholine liposomes in the L$_\alpha$-phase have shown a significant decrease in the average bilayer thickness with increased lysolecithin concentration [31]. Since a thinner bilayer is associated with a lower average order parameter of the alkyl chain deuterons [14], the X-ray analysis is in qualitative agreement with the conclusion from $^2$H-NMR spectroscopy that PaLPC decreases the orientational order of the DPPC acyl chain segments in the liquid-crystalline state. The above results are also consistent with previous $^2$H-NMR studies of PaLPC/DPPC mixtures [32]. The differences in the $^{31}$P-NMR spectra of DPPC-d$_{62}$ and the mixed PaPLC/DPPC systems, in which the position of the perpendicular edge changes from $\approx$ 15 ppm to $\approx$ 5.4 ppm at 55°C (corresponding to a change in apparent chemical shift anisotropy, $\Delta\sigma$, from $\approx -45$ ppm to $\approx -16$ ppm) (cf. Fig. 5), suggest that variations in the ordering at the level of the phosphocholine moiety may also be present [33]. It is possible that a change in the average phosphocholine head group conformation occurs in the PaLPC/DPPC mixtures relative to pure phosphatidylcholine dispersions [34]. Moreover, whole molecule motions could lead to a reduction of the orientational order of both the acyl chain and head group segments, and contributions from other factors [35] may be involved.

Finally, it is interesting that a correlation between increased ion permeability of lipid membranes and a decrease in the orientational order of the lipid acyl chains is found in membrane systems [36]. Cholesterol, which decreases the permeability of membranes, substantially increases the ordering of lipid acyl chain segments [37]. By contrast, the local anesthetic tetracaine increases the membrane permeability but decreases the orientational order of the lipids [38]. As lysolecithins are known to increase the permeability of lipid systems [4], the decrease in the order parameters of DPPC-d$_{62}$ in the L$_\alpha$-phase observed upon addition of PaLPC is in general agreement with this trend. One explanation of these phenomena is that the thickness of the paraffin core, which is directly related to the average order parameter, is important for the bilayer to act as an effective permeability barrier [31]. Another possible explanation of the increased permeability with decreased lipid order is that the lateral compressibility of the membrane is increased in the more disordered bilayer system. It has been suggested that the increase in permeability is associated with increased fluctuations of the cross-sectional area of the lipids, which is directly related to the lateral compressibility of the membrane [39].

**Conclusions**

The presence of palmitoyllysosphatidylcholine (PaLPC) in mixed bilayers with dipalmitoylphosphatidylcholine (DPPC) leads to their alignment in relatively high magnetic fields. The membranes do not orient significantly in the ordered state, but rather in the broad temperature interval defining the phase transition, and in the liquid-crystalline state. The magnetic alignment may be related to alteration of the curvature free energy of the DPPC bilayer due to incorporation of PaLPC. At temperatures below the main phase transition the PaLPC/
DPPC bilayers appear to coexist with PaLPC micelles. The micelles disappear above the transition temperature where mixed bilayers in the liquid-crystalline state are formed. The orientational order of the acyl chain segments of PaLPC is essentially identical to that of DPPC in the mixed bilayers, both in the low temperature and liquid-crystalline phases. However, the presence of PaLPC perturbs the orientational order of the DPPC molecules relative to pure DPPC membranes. It appears that PaLPC causes opposite effects on the segmental ordering of the DPPC acyl chains in the ordered and liquid-crystalline states. The order is increased in the low-temperature phase, where effective diffusion of the chains about their long axes occurs, but is decreased in the L°-phase.

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