Comparison of the Orientational Order of Lipid Chains in the Lα and HII Phases†

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ABSTRACT: The orientational order profile has been determined by using deuterium nuclear magnetic resonance (2H NMR) for POPE in the lamellar liquid-crystalline (Lα) and the hexagonal (HII) phases and is shown to be sensitive to the symmetry of the lipid phase. In the HII phase, as compared to the Lα phase, the acyl chains are characterized by a greater motional freedom, and the orientational order is distributed more uniformly along the lipid acyl chain. This is consistent with a change from a cylindrical to a wedge-shaped space available for the lipid chain. 2H NMR studies of POPE dispersions containing tetradecanol or decane, both of which can induce HII phase structure, show very different behavior. Tetradecanol appears to align with the phospholipid chains and experience the Lα to HII phase transition with a similar change in motional averaging as observed for the phospholipid chains themselves. In contrast, decane is apparently deeply embedded in the lipid structure and exhibits only a small degree of orientation. The Lα to HII phase transition for systems containing decane leads to a dramatic increase of the motional freedom of decane which is more pronounced than that observed for the lipid chains. This is consistent with a preferential partition of the decane molecules into a disordered environment such as the intercylinder spaces in the HII phase. The presence of decane in the HII phase structure does not modify the order of the lipid chains. However, the Lα phase of POPE is slightly disordered by the addition of 9 mol % decane whereas it can accommodate as much as 20 mol % tetradecanol without a significant change of order. Finally, the concept of a stretching vector associated with the lipid acyl chain has been introduced to analyze the orientational order profile obtained in the HII phase. With this model, the average order parameter of the HII phase has been calculated and found to be in good agreement with experiment.

The polymorphic phase tendencies of lipid systems are dependent on the molecular properties of the lipid species and can be modulated by a wide variety of factors. However, the molecular basis for lipid polymorphism remains poorly understood. Several lines of investigation suggest that lipid polymorphism and hydrocarbon order are sensitive to the same forces and are intimately related [see Lafleur et al. (1990) and references cited therein]. Here, we attempt to provide insight into the effects of the different lipid phases, or the polymorphic tendencies of the lipid, on the orientational order distribution in the acyl chain. Deuterium nuclear magnetic resonance (2H NMR) is well established as a suitable technique for the characterization of the orientational order of lipid chains, primarily in the liquid-crystalline lamellar phase (Lα). An important contribution of these studies has been to show the existence of a characteristic variation of orientational order along the lipid chain (Seelig & Seelig, 1974, 1980). Recently it has been shown that the orientational order profile probed with perdeuteriated tetradecanol is considerably different in the HII phase of a POPE lipid system than in the bilayer phase (Sternin et al., 1988). Instead of the relatively constant order in the methylene groups near the interface (the plateau region), which is characteristic of the Lα phase, a more uniform variation of the order along the tetradecanol chain is observed in the hexagonal (HII) phase. In order to verify whether this accurately reflects the behavior of the phospholipid acyl chains themselves, we have characterized the variation of the orientational order profile during the Lα to HII phase transition for the phospholipid chains using POPE in which the palmitoyl chain is fully deuterated (POPE-d31). Further, in a second part of this study, we have investigated the influence of certain HII phase promotors on the order profile of the phospholipid acyl chains in the Lα phase as well as in the HII phase. Tetradecanol and decane have been selected as HII phase inducers because their different molecular nature suggests different mechanisms of HII phase promotion. The response of the hydrocarbon order to the change of polymorphic tendencies is important for the understanding of the molecular events underlying a phase transition. In addition to examining lipid chain order, 2H NMR studies on deuteriated tetradecanol and deuteriated decane in nondeuteriated POPE systems have been used to compare their motional freedom with that of the lipid chains, providing information concerning their location in the lipid matrix. Finally, to conclude, an analysis of the order profile in the HII phase based on the stretching vector approach introduced by de Gennes (1974) is presented. Our results are well reproduced by this model taking in account some simple geometrical terms.

MATERIALS AND METHODS

1-Palmitoyl-2-oleoylphosphatidylethanolamine-d31 (POPE-d31) and 1-palmitoyl-2-oleoylphosphatidycholine-d31 (POPC-d31) and all other lipids were obtained from Avanti Polar Lipids.

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1 Abbreviations: ACS, anisotropic chemical shift; DPPC, di-palmitoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine.
Inc. (Birmingham, AL). The deuterated lipids showed a single spot on TLC analysis, and lipid chain analysis indicated an equimolar mixture of palmitoyl and oleoyl chains. The small peaks observed in the lipid spectra obtained in the L_α phase (e.g., see Figure 1; the small peaks correspond to the third and the fifth smallest quadrupolar splittings) suggest that acyl chain transmigration of about 20% has occurred during the synthesis of deuterated lipids. These small peaks arise because the sn-1 and sn-2 lipid chains are not equivalent (Paddy et al., 1985). Decane-d_{22} was obtained from Cambridge Isotope Laboratories (Woburn, MA). Tetradecanol and decane were purchased from Sigma Chemical Co. (St. Louis, MO).

For pure lipid samples, 40 mg of dry lipid was hydrated with approximately 700 µL of 20 mM Hepes buffer, 300 mM NaCl, and 5 mM EDTA, pH 7.4, in deuterium-depleted water (Sigma Chemical Co.). The samples were then mixed at a temperature above the gel to liquid-crystalline phase transition in order to achieve a fully hydrated dispersion. For the samples of POPE containing tetradecanol, both constituents were dissolved in chloroform, and after evaporation of the organic solvent, the samples were hydrated in the same manner as the pure lipid. The vapor pressure of decane is too high to use a similar procedure for the sample preparation. In this case, the required amount of decane was added directly to the fully hydrated lipid dispersion. The sample was then incubated above the gel to liquid-crystalline phase transition for at least 2 days.

All the spectra were obtained on a home-built 46-MHz ²H NMR spectrometer previously described (Davis, 1979; Sternin, 1985). The free induction decays were produced by a quadrupolar echo sequence with a τ value of 50 µs. After the second pulse, 2048 points were collected in quadrature with a dwell time of 5 µs, or 10 µs for spectra with narrow spectral width. The number of transients was between 42 000 and 100 000. The repetition time of the pulse sequence was at least 300 ms. The sample temperature was regulated by using a Bruker BV-T1000 temperature controller.

RESULTS

Comparison of Orientational Order of the Lipid Chains in the L_α and HII Phases. The first set of experiments was aimed at characterizing the influence of the lipid phase symmetry on the order profile of the lipid chains. The ²H NMR powder pattern and the dePaked spectra of POPE-d_{31} in the L_α phase (60 °C), the HII phase (75 °C), and when there is coexistence of both phases (68 °C) are shown in Figure 1. As can be seen, perdeuterated chains lead to complex ²H NMR spectra; the powder spectra of the lipid in a single phase are superpositions of 15 different powder patterns. No specific peak assignment can be made directly from these powder pattern spectra, particularly in the case of the HII phase for which the doublets assigned to the methylene groups are not resolved. However, since the signal arises from the deuterium nuclei uniformly distributed along the lipid chain, a smoothed function for the variation of the orientational order along the acyl chains can be defined by exploiting the dePaking method in a manner described in detail elsewhere (Sternin et al., 1988; Lafleur et al., 1989). Briefly, the numerical dePaking procedure (Bloom et al., 1981; Sternin et al., 1983) is first used to separate the information concerning the orientational order of the C-D bond from the dependence of the quadrupolar splittings on the angle between the lipid symmetry axis and the magnetic field direction. The dePaked spectra presented in this paper correspond by convention to spectra characteristic of a sample oriented parallel to the magnetic field (the shoulder of the

FIGURE 1: L_α to HII phase transition of POPE-d_{31} as experienced by the perdeuterated palmitoyl chain. (a) Powder pattern and (b) dePaked spectra of POPE-d_{31} in the L_α phase (60 °C), when the L_α and the HII phases coexist (68 °C), and in the HII phase (75 °C). The tick marks at the bottom of the dePaked spectra indicate the mean order parameters determined by the method described in the text.

powder spectra). The dePaked spectrum gives directly the probability distribution of the order parameters, and a mean value of the order parameter is calculated for each unit area associated with one methylene group (the quadrupolar splitting assigned to the terminal methyl is directly measured from the well-resolved innermost doublet). The tick marks at the bottom of the spectra represent these mean values. The order profiles presented in this paper are obtained from these order parameters, assuming a monotonic decrease of the order along the chain from the interface toward the middle of the bilayer (Sternin et al., 1988; Lafleur et al., 1989).

At 60 °C, the spectrum is typical of a phospholipid with a perdeuterated chain in the liquid-crystalline phase (Davis, 1979; Paddy et al., 1985). The dePaked spectrum of POPE-d_{31} at 60 °C exhibits the fluid bilayer signature. A large fraction of the signal is associated with the largest quadrupolar splittings and is assigned to the methylene groups near the interface. The several resolved doublets with a smaller quadrupolar splitting correspond to methylene positions in the more disordered terminal part of the chain (Davis, 1979). The shape of the spectrum obtained in the HII phase (at 75 °C) contrasts with that obtained for the L_α phase. First, the values of quadrupolar splittings of the deuterated lipid in the HII phase are smaller than those for the L_α phase, and, second, the distribution of order along the chain is modified by the change of the phase structure. In particular, the signal is distributed more uniformly along the acyl chain in the HII phase than in the L_α phase, though the relative intensity remains somewhat larger for the largest quadrupolar splittings. It should be noted that the free induction decay for POPE-d_{31} at 75 °C was recorded for a longer time (by using a dwell time of 10 µs instead of 5 µs) in order to enhance the spectral resolution. Even under these conditions, no well-resolved doublets of the type observed in the L_α phase were detected, except for the doublet assigned to the terminal methyl. The smoothed order profile obtained from such a spectrum, in the manner described above, is not affected by the resolution. At 68 °C, the spectrum is a combination of the L_α and the HII phase spectra, indicating that the lipid exchange between the two structures is slow on the NMR time scale.

These changes can be expressed in a quantitative way by calculating the moments of the spectra. As previously dem-
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| Table I: First and Second Moments, M<sub>i</sub> and M<sub>2</sub>, and the Relative Mean Square Deviation of the Distribution of Quadrupolar Splittings, ∆<sub>2</sub>, of POPE-d<sub>31</sub> |
|-----------------|--------|--------|----------------|
| temp (°C)       | M<sub>1</sub> (s<sup>-1</sup>) | M<sub>2</sub> (s<sup>-2</sup>) | ∆<sub>2</sub> |
| 50              | 5.4 × 10<sup>8</sup>       | 4.3 × 10<sup>9</sup>       | 0.08      |
| 60              | 5.2 × 10<sup>8</sup>       | 3.9 × 10<sup>9</sup>       | 0.09      |
| 68              | 2.3 × 10<sup>8</sup>       | 1.0 × 10<sup>9</sup>       | 0.50      |
| 75              | 1.7 × 10<sup>8</sup>       | 4.3 × 10<sup>8</sup>       | 0.22      |

The smoothed orientational order profiles for the L<sub>a</sub> and the H<sub>II</sub> phases obtained from these spectra are shown in Figure 2a. The largest value of quadrupolar splittings has been normalized to unity in order to highlight the difference in the shape of the distribution of quadrupolar splittings (Bloom et al., 1978; Davis, 1979). M<sub>1</sub> gives the mean of the order parameters, (S<sub>CD</sub>), M<sub>2</sub> gives the mean of the square order parameters, (S<sub>CD</sub><sup>2</sup>), etc. The values of M<sub>1</sub> and M<sub>2</sub> determined from the spectra of POPE-d<sub>31</sub> at various temperatures are shown in Table I. The increase of temperature by 10 °C in the L<sub>a</sub> phase leads to a decrease of M<sub>1</sub> by about 4%. M<sub>1</sub> decreases more drastically during the L<sub>a</sub> to H<sub>II</sub> phase transition. When all the lipids are in the H<sub>II</sub> phase, at 75 °C, the value of M<sub>1</sub> is 1.7 × 10<sup>4</sup> s<sup>-1</sup>; this is a reduction by a factor of 3 compared with the value calculated for the L<sub>a</sub> phase at 60 °C. The relative mean square deviation of the order parameter distribution, ∆<sub>2</sub>, is also influenced by the phase transition. In the L<sub>a</sub> phase, values around 0.09 are obtained; this is of the same order as results previously published for DPPC-d<sub>42</sub> (Davis, 1979). At 68 °C, ∆<sub>2</sub> reaches a maximum. As discussed by Davis (1979), this parameter is very sensitive to inhomogeneities in the sample. The coexistence of phases characterized by different quadrupolar splittings, such as the L<sub>a</sub> and H<sub>II</sub> phases, leads to a very wide distribution of order parameters. For example, a large value of ∆<sub>2</sub> is also observed for the coexistence of the L<sub>a</sub> and L<sub>a</sub> phases (Davis, 1979). Finally ∆<sub>2</sub> equals 0.22 at 75 °C, when POPE is in the H<sub>II</sub> phase, indicating that the distribution of quadrupolar splittings is wider in the H<sub>II</sub> phase than the L<sub>a</sub> phase.

The smoothest orientational order profiles for the L<sub>a</sub> and the H<sub>II</sub> phases obtained from these spectra are shown in Figure 2a. The largest value of quadrupolar splittings has been normalized to unity in order to highlight the difference in the shape of the distribution of the orientational order. The order profile along the acyl chains is affected by the symmetry of the lipid phase as has been already observed using perdeuterated tetradecanol as a probe (Sternin et al., 1988). For the L<sub>a</sub> phase, a relatively flat plateau region extending for about six carbon positions is observed, followed by a rapid decrease of the order toward the end of the lipid chain (Seelig & Seelig, 1974, 1980). By contrast, a more monotonic decrease of S(n) versus n is observed in the H<sub>II</sub> phase. The redistribution of the order has as a consequence the increase of ∆<sub>2</sub> mentioned above. The plateau in the L<sub>a</sub> phase corresponds to a substantial fraction of CD<sub>2</sub> groups having similar splittings, which gives rise to a reduced width of the order distribution. The more uniform distribution observed in the H<sub>II</sub> phase leads to an increased value of ∆<sub>2</sub>. The reduction in order parameter observed as a result of the L<sub>a</sub> to H<sub>II</sub> phase transition is shown in Figure 2b. The ratio of the order parameters in the L<sub>a</sub> phase to those of the H<sub>II</sub> phase is larger than 2 for every position of the chain and reaches a value of 3.5 near the end of the chain. This decrease of order is due mostly to a change in the lipid phase symmetry. There is also a reduction of order due to the increase of temperature necessary to induce the phase transition. That this does not contribute significantly to the change observed is shown by the results obtained for lamellar POPC-d<sub>31</sub>—the ratios measured over the same range of temperature in a single lipid phase are close to unity (Figure 2b).

FIGURE 2: Influence of the lipid phase symmetry on the order profile. (a) Normalized orientational order profile of POPE-d<sub>31</sub> in the L<sub>a</sub> phase at 60 °C (●) and in the H<sub>II</sub> phase at 75 °C (■). (b) Ratio of the order parameters S(n) in the L<sub>a</sub> phase (60 °C) to those in the H<sub>II</sub> phase (75 °C) for POPE-d<sub>31</sub> (●). The ratio for the same temperatures is plotted for POPC-d<sub>31</sub> (■) which forms an L<sub>a</sub> phase over the whole range of temperature.

Influence of Tetradeanol and Decane on Lipid Order. In the next part of this study, we investigated the influence of tetradeanol and decane, two H<sub>II</sub> phase inducers, on the orientational order profile of the lipid chain. The first step of this study was to characterize the effects of these agents on the polymorphism of POPE. The effects of tetradeanol and decane on the polymorphism of POPE-d<sub>31</sub> were determined by plotting the order parameter of the outermost doublet, S<sub>max</sub>, as a function of temperature. Figure 3 illustrates this plot for pure POPE-d<sub>31</sub> and for POPE-d<sub>31</sub> containing 20 mol % tetradeanol or 9 mol % decane. The vertical dashed lines represent temperature where the L<sub>a</sub> and the H<sub>II</sub> phases coexist for a given system, although the coexistence is not limited to that temperature. For pure POPE, there is a progressive decrease of the order parameter with increasing temperature due to the increase of the motion of the acyl chains. Around 68 °C, both bilayer and H<sub>II</sub> phases coexist, and at higher temperatures, POPE adopts the H<sub>II</sub> phase characterized by a small order parameter.

As shown in Figure 3, the addition of 20 mol % tetradeanol or of 9 mol % decane reduces the L<sub>a</sub> to H<sub>II</sub> phase transition temperature of POPE-
Thermotropism of pure POPE (●), POPE + 20 mol % tetradecanol (△), and POPE + 9 mol % decane (■) probed by using $S_{\text{max}}$, the order parameter determined for the outermost doublet of the perdeuteriated palmitoyl chain spectra.

$S_{\text{max}}$ by about 20 °C. It has been previously shown (Sternin et al., 1988) that the addition of 20% perdeuteriated tetradecanol induces a similar shift of the lamellar to hexagonal phase transition toward lower temperatures. Several other alkanes have also been shown to decrease the L₆ to H₇ phase transition temperature (Epand, 1985; Kirk & Gruner, 1985; Siegel et al., 1989; Sjölund et al., 1989).

An important question concerns how the shift of the transition temperature is reflected by the phospholipid chain order. It may be noted that $S_{\text{max}}$ is not significantly influenced by the presence of tetradecanol over the experimental temperature range (see Figure 3). The values obtained for the POPE/tetradecanol system in the L₆ and the H₇ phases are close to those observed for pure POPE or those which could be extrapolated from the data of pure POPE in the H₇ phase (T ≥ 68 °C). Figure 4 shows a comparison of the whole order profile of POPE and POPE + 20% tetradecanol (a) at 30 °C when both systems are in the L₆ phase and (b) at 75 °C for the H₇ phase. As one can see, the presence of tetradecanol does not significantly modify the distribution of the order along the chain in either phase. Previous studies have shown that the presence of 1-octanol (25 mol%) or 1-decanol (25 mol %) does not affect significantly the order profile of DPPC bilayers (Thewalt et al., 1985). Our results indicate that the same is true for a longer alcohol in a POPE liquid-crystalline bilayer or H₇ phase.

The order of the hydrophobic core of the POPE/tetradecanol system has been previously characterized by $^2$H NMR where the long-chain alcohol bore the deuterium nuclei (Sternin et al., 1988). Figure 5 shows the order profile obtained by $^2$H NMR for the system POPE/20 mol % tetradecanol in the L₆ and H₇ phases probed by the perdeuteriated palmitoyl chain of POPE or by perdeuteriated tetradecanol. Except for the chain terminal region, the general shape of the orientational order profiles for both phases is the same for both probes. An important difference between these probes is their chain length, the alcohol molecules being shorter than the palmitoyl chains by two carbon units. This is reflected in the profiles obtained with perdeuteriated lipid and perdeuteriated tetradecanol by the abrupt decrease of order at the end of the alcohol chain.

As can be seen in Figure 3, decane also shifts the L₆ to H₇ phase transition toward lower temperatures. The effects of this H₇ phase inducer on the order profile of POPE-$d_{19}$ are shown in Figure 4. In the L₆ phase, a small decrease in order of about 7% is observed in the presence of 9 mol % decane for all the positions along the acyl chains. In the H₇ phase, POPE accommodates 9 mol % decane without significant changes in the lipid chain order profile (Figure 5b).
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A new component with a very small quadrupolar splitting of 0.4 kHz is also observed. At 60 °C, when the spectrum of POPE-\( d_{31} \) indicates that all the lipids form an \( H_{II} \) phase, only the narrow components are detected for decane-\( d_{22} \). On the basis of the phase identification provided by the spectra of POPE-\( d_{31} \), this narrow signal is assigned to deuteriated decane in the \( H_{II} \) matrix of POPE. As observed for the bilayer, only one doublet is observed from the terminal methyls in the \( H_{II} \) phase. The rest of the deuterium nuclei give rise to a doublet with a quadrupolar splitting of 2 kHz. As previously observed for the lipid chains, the ratio of order parameters of deuteriated decane in the \( L_a \) over the \( H_{II} \) phase is significantly greater than 2. Measured at the maximum of intensity of each phase component at 50 °C when both phases coexist, the ratio is 3.1 for decane-\( d_{22} \); this is significantly larger than the same ratio measured for the palmitoyl chain at the same temperature which is 2.4. If the ratio \( S_{L_a}/S_{H_{II}} \) is measured using this time the spectra of decane-\( d_{22} \) recorded at 30 °C for the \( L_a \) phase and at 60 °C for the \( H_{II} \) phase, a value of 5.5 is obtained for the widest doublet of the decane spectra. This ratio is about 5.0 for the doublet which is partially resolved in the \( L_a \) phase.

It represents the additive effects of the phase transition and the temperature increase. Since a single phase is observed at these temperatures, the order profile can be obtained for the lipid chains. The ratio \( S(n)_{L_a}/S(n)_{H_{II}} \) are calculated as a function of \( n \), and the values obtained vary from 2.7 at the beginning of the lipid chain to 4.2 near its end (data not shown).

DISCUSSION

The orientational order profile of the lipid acyl chain is sensitive to the phase symmetry and is considerably different for the \( H_{II} \) than for the \( L_a \) phase. First, the absolute values of order parameters of the \( H_{II} \) phase are considerably smaller than expected if the lipid experiences similar motions to the \( L_a \) phase and diffuses around the \( H_{II} \) cylinders. The lipid diffusion around the cylinders causes extra motional averaging leading to a reduction of the order parameters by a factor of 2 (Cullis & de Kruijff, 1979; Seelig, 1978). This factor of 2 is expected because the lipid molecule diffuses around the cylindrical axes in a time short (\( \leq 10^{-2} \) s) on the NMR time scale, which projects the symmetry axis for the orientational order from the local surface normal to the axis of symmetry of the cylinder, which is at right angles to the surface normal. Then, the quadrupolar splittings are multiplied by \( [(3 \cos 90° - 1)/2] = 1/2 \). Since the radii of the hexagonally coordinated cylinders in the \( H_{II} \) phase are typically of order \( R \approx 2 \) nm (Seddon et al., 1984; Tate & Gruner, 1989) and the diffusion constants in fluid phases are of order \( D \geq 4 \times 10^{-12} \) m² s⁻¹, the correlation time for diffusion around the cylinder axes, \( \tau_c \leq R^2/2D = 10^{-6} \) s, is expected to be short on the NMR time scale. However, the change in order associated with the \( L_a \) to \( H_{II} \) phase transition is larger than this predicted factor of 2 for every position \( n \) along the lipid palmitoyl chain (Figure 2). This result indicates that the \( H_{II} \) phase is characterized by a more pronounced motional freedom of the lipid chain than observed in the \( L_a \) phase. Infrared spectroscopy has also shown that the proportion of gauche bonds over the whole chain is increased in the \( H_{II} \) phase as compared with the \( L_a \) phase (Mantsch et al., 1981). Second, the decrease of orientational order in the \( H_{II} \) phase cannot be expressed by a single scaling factor, but it is more pronounced toward the end of the chain. This leads to a more uniform distribution of order along the acyl chain in the \( H_{II} \) phase than in the \( L_a \) phase. This behavior is in good agreement with results previously obtained using specifically deuteriated POPE (Perly et al., 1985). These
changes are consistent with the change in geometry of the space available for the acyl chain motion resulting from the phase transition. In the bilayer structure, the cross-sectional hydrophilic area of the headgroups matches the cross-sectional hydrophobic area of the acyl chains. In the HII phase, the lipid chains have access to a "cone" or "wedge"-shaped space, leading to an increase of available space toward the end of the chain. This is consistent with the large decrease of order parameters observed here for the carbon positions in this region. In the next section, a simple model is proposed to describe the orientational order of the lipid chain in terms of the phase symmetry, and a theoretical relation between orientational order parameters in the LII and the HII phases will be discussed.

The order distribution along the palmitoyl chain is more uniform for the HII phase than for the LII phase. This is illustrated directly by the dePaked results (Figure 1b) or by the normalized order profile for which a continuous decrease of $S(n)$ versus $n$ is observed (Figure 2a). The plateau is reduced in the HII phase, and this causes an increase in the width of the distribution of order parameters (Table I). As discussed previously (Lafleur et al., 1989), the use of per-deuteriated saturated chains gives a smoothed shape of the profile since the assumption of a monotonic decrease of the order along the chain is made. Therefore, local variations that might exist in the profile are not reproduced. The plateau of the LII phase is actually a segment about six methylene groups long within which the order oscillates over a limited range (Seelig & Seelig, 1974). This is reflected in the smoothed order profile by a segment where $S(n)$ does not vary appreciably. For the HII phase, such a segment is not as obvious. However, as seen on the dePaked spectra of the HII phase, there is a larger intensity for the largest quadrupolar splitting, and this is expressed in the profile as a reduced slope of $S(n)$ versus $n$ near the interface. This may be interpreted as a small plateau region, as suggested by Sankarm and Marsh (1989).

All the conclusions obtained here for the deuterated lipid chain are in agreement with those obtained previously with perdeuteriated tetradeanol (Sternin et al., 1988). Tetradeanol also reflects the order in the bilayer since its chain aligns with the lipid chains. In order to examine the correlation between the two probes, it is of interest to compare the profiles obtained for the POPE/tetradeanol system using deuteriated lipid and deuteriated alcohol. However, certain comments should first be made about these profiles. As mentioned previously, the smoothed order profile does not describe the detailed structure of the order profile. The assumption of a monotonic decrease of the order may also introduce some systematic error in the order profile of the long-chain alcohol in a lamellar lipid matrix. For example, for decanol, the order parameter reaches a maximum value for carbon positions 4–5 (Thewalt et al., 1986). This is due to the fact that the hydroxyl group is situated at the interface in such a way that the first part of the chain is tilted to the bilayer normal or its motions are less restricted. The integration method smooths these variations to give a plateau followed by a rapid decrease of order (Sternin et al., 1988).

The remarkable coincidence of the profiles for the POPE/tetradeanol system obtained from the deuterated POPE and the deuteriated tetradeanol indicates that, except for local geometric variations, both molecular species experience in a very similar way the anisotropic forces inside the lipid matrix. This is demonstrated for the LII as well as the HII phase. The maximum order parameter values observed in both cases are the same (within 5%), and the order varies in a very similar way along the chain except for the sharper decrease at the end of the chain observed for tetradeanol due to its smaller chain length. This is in agreement with a previous study on decanol in DPPC bilayers (Thewalt et al., 1986). Similar values of order parameters were observed for this system no matter whether the deuterium nuclei were located on the alcohol or on the chain of DPPC.

In contrast to tetradeanol, deuteriated decane does not exhibit order parameters similar to those observed for the lipid chain. As previously shown for other alkanes (Jacobs & White, 1984a,b; Pope et al., 1984; Sjolund et al., 1987), the results obtained for decane suggest that the alkane molecules are deeply embedded into the lipid structure in the lamellar phase. The methylene groups of deuteriated decane show small quadrupolar splittings in the range of 9–12 kHz. There is an order gradient existing along the decane molecules as shown by the distinguishable doublets of the dePaked spectra of decane-$d_{22}$ in the LII matrix. The methylene groups at the center of the chain are likely the ones which correspond to the doublet with the largest splitting. The methyl groups show a single doublet, indicating that both halves of the decane molecules experience the same motional averaging. This is expected because of the reflection symmetry of the decane molecule and the short correlation time for the end-to-end flip-flops of the molecules (Pope et al., 1984). Though we cannot establish with certainty the location of decane on the basis of the quadrupolar splittings (we do not know whether the alkane molecules experience specific motions that average the NMR signal), the small splittings strongly suggest that they are mainly located near the end of the lipid chains which provide a more disordered environment.

As observed for the lipid acyl chains, there is a drastic decrease of the order parameters for the decane molecules during the LII to HII phase transition. Similar observations have been made for dodecane and hexadecane in a monomethylated DOPE matrix (Siegel et al., 1989) and for dodecane in DOPC (Sjolund et al., 1987). Three phenomena can give rise to the reduction of order: (i) the extra averaging caused by the rapid diffusion of decane around the HII cylinders; (ii) an increase of motion due to the change of phase symmetry; and (iii) a relocation of the decane molecules to a region allowing greater motion during the phase transition. On the basis of our results, it is difficult to determine to what extent each of these factors contributes to the reduction of the quadrupolar splittings. However, a new aspect of that question can be addressed, which concerns the relative change of order observed as a result of the LII to HII phase transition for the phospholipid and the long-chain alkane or alcohol under the same conditions. Here, it is shown that decane experiences an increase of motional freedom as a result of the LII to HII phase transition which is more pronounced than any position of the lipid chain. As a result, this change cannot be simply explained by a reorganization similar to that of the lipid chains where the decane molecules remain roughly aligned with the lipid chains, diffuse around the HII cylinders, and have more motional freedom due to the additional space available in the HII phase. It has been suggested that in the HII phase, decane partitions preferentially into interstitial spaces between the cylinders (Kirk & Gruner, 1985; Sjolund et al., 1987; Siegel et al., 1989). The drastic decrease of order that we observe for decane supports this suggestion. It is proposed that since the decane molecules partition preferentially into these intercylinder spaces, they release the packing tension and allow the intrinsic curling tendency of the lipid layer to be expressed without significant contribution of forces other than elastic.
Chain Order in the \( L_\alpha \) and \( H_{II} \) Lipid Phases

Under these conditions, the measurement of the radius of the \( H_{II} \) cylinders should correspond to the spontaneous radius of curvature of the lipid layer, \( R_0 \) (Kirk & Gruner, 1985; Gruner, 1985). Our results also indicate that the lipid chains of \( H_{II} \) phase POPE are not on average affected by the presence of decane. This observation and the small orientational order of the decane molecules themselves suggest the preferential partitioning of decane into "low constraint" spaces without influencing the \( H_{II} \) phase structure, supporting the use of alkane for the measurement of \( R_0 \).

The \( H_{II} \) phase inducers have different effects on the lipid chain order profile in the \( L_\alpha \) phase. The addition of decane leads to a small decrease of order, showing a direct effect of decane on the lipid bilayer structure. The increase in free energy associated with this destabilization of the bilayer structure can also contribute to the shift of the \( L_\alpha \) to \( H_{II} \) phase transition. In contrast, the lipid packing can accommodate 20 mol % tetradecanol without a significant change in order even though the presence of 20 mol % tetradecanol shifts the \( L_\alpha \) to \( H_{II} \) phase transition toward lower temperatures by about 20 °C. The presence of this \( H_{II} \) phase inducer produces, however, a change at the interface since the hydrophilic area occupied by the hydroxyl group of tetradecanol is relatively small and may lead to the destabilization of the bilayer. An X-ray diffraction study has shown that the distance between the centers of two adjacent cylinders at 80 °C decreases in the presence of 20 mol % tetradecanol in the POPE matrix to 62.5 Å from 69.5 Å, a change of about 10% (E. Eikenberry and S. Gruner, personal communication). We are presently investigating systems with a wider range of \( R_0 \) in order to verify the relationship between the order parameters, the curvature, and the polymorphic tendencies of lipids.

Theoretical Relationships between Orientational Order Parameters in the \( H_{II} \) and \( L_\alpha \) Phases. (i) Review of \( 31P \) NMR Results and Their Implications for \( ^2H \) NMR. It is customary in the analysis of \( 31P \) NMR data to assume that the \( 31P \) NMR anisotropic chemical shift (ACS) in the \( H_{II} \) phase is related to that in the \( L_\alpha \) phase by a factor \(-1/2\). This assumption is, in fact, normally quite well satisfied experimentally (Cullis & de Kruijff, 1976; Seelig, 1978). There is a well-defined theoretical basis for this factor of \(-1/2\). The packing of the polar headgroups of phospholipid molecules on the curved cylindrical surfaces of the \( H_{II} \) phase lipid-water interfaces is similar to that in the \( L_\alpha \) phase, so that the local orientational order is the same for both phases, and the diffusion of the lipid molecules around the \( H_{II} \) phase cylinders is fast on the NMR time scale, then the ACS is multiplied by \(-1/2\). As described above, the lipid diffusion around the water core of the \( H_{II} \) phase cylinders is fast enough to cause extra monolial averaging. The well-established result from \( 31P \) NMR that the local orientational order is identical in the two phases could not so easily have been anticipated, especially since the molecular motions near the cylinder surface should not be locally axially symmetric. Indeed, we are unaware of any explicit theoretical explanation of this rather simple and pleasing result.

Our observation that the \( ^2H \) NMR quadrupolar splittings on the acyl chains of the lipid chains do not scale by a factor of \( 1/2 \) (note that unlike \( 31P \) NMR ACS measurements, \( ^2H \) NMR quadrupolar splittings only give the magnitude and not the sign of the orientational order parameters) on going from the \( L_\alpha \) to the \( H_{II} \) phase is not surprising in view of the anticipated variation of the packing of the lipid chains in the interior of the hydrophobic region of the \( H_{II} \) phase. It would appear that the systematic variation with chain position \( q \) of the ratio of the order parameter \( S(n) \) in the \( L_\alpha \) phase to that in the \( H_{II} \) phase shown in Figure 2b can provide information on chain packing when a suitably detailed theory is developed. In the following section, a new theory of orientational order appropriate for this purpose is developed. Our theory is unconventional in that it relates the component \( S_{ij}(n) \) of the orientational order tensor for the \( n \)th carbon position to a local, position-dependent orientational order tensor component, \( S_j(r) \).

(ii) The Concept of a "Stretching Vector" for the Acyl Chains. An interesting approach to the problem of orientational order in chains, one that is independent of a detailed description of molecular conformational geometry, has been developed by de Gennes (1974). He defines a stretching vector \( J(r) \) at each position \( r \) in space by

\[
J(r) = \rho (\mathbf{R}_{n+1} - \mathbf{R}_n)
\]

where \( \rho \) is the monomer density of the methylene groups comprising the chain and \( \mathbf{R}_1, \ldots, \mathbf{R}_N \) are the locations of the successive monomers (defined as midway along the C-C bonds) for one chain. The broken brackets represent an average over all possible values of \( n \) which can be found at the position \( r \). de Gennes introduced the Ansatz that since "... in most of the hydrophobic region, there is no end group..." i.e., the end group \( \mathbf{R}_N \) is confined to a boundary layer of thickness \( e \approx N^{1/2}a \), where \( a \) is the monomer–monomer interval, \( J \) satisfies the same conservation law:

\[
\text{div } J = 0
\]

as does the electric field in a charge-free region. Although the vector \( J \) is a measure of local orientational order, it is not directly related to the second-rank tensor \( S(n) \) that is measured by \( ^2H \) NMR. Nevertheless, de Gennes was able to relate the plateau, i.e., the observed lack of variation of \( S(n) \) with \( n \) for the top half of the acyl chain in the \( L_\alpha \) phase with the constant value of \( J \) predicted by eq 2 for lamellar geometry, in analogy with the constant electric field obtained near an infinite, flat, uniformly charged plate. The argument is that if \( J \) is constant, so is the related second-rank tensor that is responsible for the nuclear electric quadrupolar splittings (de Gennes, 1974).

In order to relate \( J \) to our \( ^2H \) NMR measurements in the \( H_{II} \) phase, it is necessary to identify a second-rank tensor \( S_{ii}(r) \) from which our measurements of \( S(n) \) may be derived. In fact, a symmetric second-rank tensor associated with the vector \( J(r) \) may be defined in terms of the components \( J_i(r) \) as follows (see, e.g., Tinkham (1964)):

\[
S_{ii}(r) = (3/2)C[J_i(r)J_i(r) - \delta_{ij}(1/3)\sum_k [J_k(r)]^2]
\]

where \( C \) is a normalization constant.

The relatively rapid decrease of \( S(n) \) in the \( L_\alpha \) phase with \( n \) for the tail of the chain was then ascribed to the "end effects" neglected in the "derivation" of eq 2. It was suggested by de Gennes that a further test of eq 2, or an improved version of it, could be made in a different geometry. For example, in a system with cylindrical geometry, the solution to eq 2 is

\[
J(r) = \text{constant} \times r/\rho^2, \text{ i.e., } J = r^{-1}
\]

in the lipid region. In the spirit of de Gennes' Ansatz, we can guess that the effects of local cylindrical geometry may be expressed in terms of a power series of the form

\[
1/S(n) = a_0 + a_1n + a_2n^2 + ...
\]

In order to test this conjecture, we have fitted the ratio of \( S(n) \) in the \( L_\alpha \) phase to that in the \( H_{II} \) phase in Figure 2b to a linear function of \( n \) for values of \( n \) in the range \( 2 \leq n \leq n_{\text{max}} \), and

\[
\frac{1}{S(n)} = a_0 + a_1n + a_2n^2 + ...
\]
extrapolated to find the value of \( n = n_0 \) for which this ratio is 2.0 for different values of \( \eta_{\text{max}} \). As \( \eta_{\text{max}} \) was varied in the range \( 3 \leq \eta_{\text{max}} \leq 12 \), values in the range \( -1 \leq n_0 \leq -3 \) were obtained. Since this range corresponds to the polar headgroup data is consistent with the well-established results obtained by using acyl chain conformational averaging “stretching vector” model does provide a way of quantitatively

\[ S_{\text{w}} = S_1 = C(J^2 + J_0^2) \] (8)

where \( A \) is a constant determined by boundary conditions. This is valid (approximately) between \( r = R_a \) and values of \( r \) corresponding to the boundary of the hexagon formed by the bisectors of the vectors in the \((r, \theta)\) plane joining the cylinder axis at \( r = 0 \) with those of its nearest neighbors. Outside this hexagon, \( J(r) \) may be obtained from the translational symmetry properties of the HII phase. The principal axes of \( S_{\text{w}}(r) \), defined by eq 3, are along the \( u, v, \) and \( z \) directions where \( \hat{u} \) and \( \hat{v} \) are, respectively, parallel and perpendicular to \( J_\parallel + J_\perp \).

The \( u \) and \( v \) axes are in the \((r, \theta)\) plane and are rotated by an angle \( \Delta = \tan^{-1}(J_\parallel/J_\perp) \) from the \( r \) and \( \theta \) directions. The principal values of \( S_{\text{w}} \) are easily shown to be

\[ S_{\text{w}} = S_1 = C(J^2 + J_0^2) \]

As discussed earlier, we can identify the local orientational order parameter \( S_{\text{w}} \) in the immediate vicinity of the cylinder surface with that near the glycerol backbone in the L\(_{\alpha}\) phase. \( S_1(0) \). Thus, for \( (2R_a/d)^6 \ll 1 \), we make the approximation

\[ S_1(0) = S_{\text{w}}(r = R_a) = CA^2/R_a^2 \] (9)

This enables us to compare the predictions of the model described above for the average \(^3\text{H}\) NMR quadrupolar splitting in the HII phase, which is governed by \((S_{\text{w}})\), with that of the plateau in the L\(_{\alpha}\) phase. It should be noted that the average \((S_{\text{w}})\) over the volume occupied by the chains is identical with the average \((S_{\text{w}})\) over all chain positions \( n \). Again, neglecting terms of order \((2R_a/d)^6\) and average \( S_{\text{w}} \), over an assumed uniform distribution of lipid chains in the annular region between \( r = R_a \) and \( d/2 = R_a + 1.0 \), we obtain

\[
(S_{\text{w}}) \approx -CA^2 \left(1 + q \right) (1/\left(4R_a^2(2 + q)\right))
\]

\[
\approx -S_{\text{w}}(0) \left(1 + q \right) (1/\left(2 + q\right))
\] (10)

It may be seen that the correct average of \((S_{\text{w}})\) is \((-1/2)S_{\text{L}}(0)\) for \(^3\text{P}\) NMR is obtained by taking the limiting value of eq 9 as \( q \to 0 \).

Though measurements of \( q \) have not been carried out for POPE, data on \( R_a \) and \( d \) are available as a function of temperature for DOPE (Tate & Gruner, 1989). These measurements show that \( R_a \) varies between 23 and 17 \( \AA \) as the temperature is increased from 10 to 80 °C, while \((d/2) - R_a\) remains constant at about 16 \( \AA \). The value of \( d \) in DOPE at 45 °C is approximately the same as in POPE at 75 °C. At that temperature, \( q = 0.84 \) in DOPE, which gives a ratio \((S_{\text{w}})/S_{\text{L}}(0) \approx 0.26 \). This ratio may be compared with the value of 0.27 obtained by identifying \( S_{\text{L}}(0) \) with \( S_{\text{w}} \) at \( q \) = 0.20 (see Figure 3) and estimating \((S_{\text{w}})\) = 0.054 from the data of Figure 4. Though we are comparing different systems (POPE and DOPE), when these systems have identical values of \( d \), the close correspondence of their values of \( q \) obtained from two different types of measurements is impressive. It indicates that it may be possible to understand orientational order in the HII phase, and other liquid-crystalline phases as well, in terms of simple geometrical features of the structure and independent of detailed models of conformational averaging. This possibility should now be explored theoretically and experimentally.

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Chemiluminescence of the Mn$^{2+}$-Activated Ribulose-1,5-bisphosphate Oxygenase Reaction: Evidence for Singlet Oxygen Production

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ABSTRACT: Chemiluminescence has been observed during catalysis by Mn$^{2+}$-activated ribulose-bisphosphate carboxylase/oxygenase from spinach. The luminescence is ribulose 1,5-bisphosphate (RuBP) and O$_2$-dependent and is inhibited by 2-carboxyarabinitol 1,5-bisphosphate and high concentrations of bicarbonate; it is therefore ascribed to the RuBP oxygenase activity. The luminescence is inhibited by azide and enhanced in D$_2$O and in the presence of diazabicyclooctane. The emission maximum is between 620 and 690 nm. The initial rate of light emission is second order in enzyme concentration. The data strongly suggest that singlet oxygen is produced during turnover, that the observed chemiluminescence is due to dimol emission of singlet oxygen, and that this provides a basis for a highly sensitive assay for RuBP oxygenase.

The enzyme D-ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), as the name implies, catalyzes both the carboxylation and oxygenation of RuBP. These reactions are the initial steps in photosynthesis and photorespiration, respectively [for a review, see Miziorko and Lorimer (1983)]. Although the oxygenase activity competes for RuBP with the carboxylase activity and the resultant photorespiration appears to oppose photosynthesis, all RuBP carboxylases studied to date catalyze oxygenation. It has been proposed that the

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1 Abbreviations: CABP, 2-carboxyarabinitol 1,5-bisphosphate; cpm, counts per second; DABCO, diazabicyclooctane; MOPS, 3-(N-morpholino)propanesulfonic acid; O$_2$, singlet oxygen; O$_{2s}$, triplet oxygen; RuBisCO, D-ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, D-ribulose 1,5-bisphosphate; SOD, superoxide dismutase.

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