Correlation Between Lipid Plane Curvature and Lipid Chain Order

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ABSTRACT The 1-palmitoyl-2-oleoyl-phosphatidylethanolamine:1-palmitoyl-2-oleoyl-phosphatidylcholine (POPE:POPC) system has been investigated by measuring, in the inverted hexagonal (H_{II}) phase, the intercylinder spacings (using x-ray diffraction) and orientational order of the acyl chains (using ^2H nuclear magnetic resonance). The presence of 20 wt% dodecane leads to the formation of a H_{II} phase for the composition range from 0 to 39 mol% of POPC in POPE, as ascertained by x-ray diffraction and ^2H nuclear magnetic resonance. The addition of the alkane induces a small decrease in chain order, consistent with less stretched chains. An increase in temperature or in POPE proportion leads to a reduction in the intercylinder spacing, primarily due to a decrease in the water core radius. A temperature increase also leads to a reduction in the orientational order of the lipid acyl chains, whereas the POPE proportion has little effect on chain order. A correlation is proposed to relate the radius of curvature of the cylinders in the inverted hexagonal phase to the chain order of the lipids adopting the H_{II} phase. A simple geometrical model is proposed, taking into account the area occupied by the polar headgroup at the interface and the orientational order of the acyl chains reflecting the contribution of the apolar core. From these parameters, intercylinder spacings are calculated that agree well with the values determined experimentally by x-ray diffraction, for the variations of both temperature and POPE:POPC proportion. This model suggests that temperature increases the curvature of lipid layers, mainly by increasing the area subtended by the hydrophobic core through chain conformation disorder, whereas POPC content affects primarily the headgroup interface contribution. The frustration of lipid layer curvature is also shown to be reflected in the acyl chain order measured in the L_α phase, in the absence of dodecane; for a given temperature, increased order is observed when the curling tendencies of the lipid plane are more pronounced.

INTRODUCTION

It is now well established that biological membranes contain a significant proportion of lipids which, individually, form nonlamellar phases in aqueous dispersion. This finding has generated considerable interest in lipid polymorphism, because an understanding of the structural properties of lipids may lead to an enhanced understanding of the roles that different lipids play in membranes. In this regard, x-ray diffraction and ^2H nuclear magnetic resonance (NMR) provide fundamental information characterizing lipid structure. X-ray diffraction has proved to be useful in characterizing the curvature properties of lipid layers and associated polymorphic tendencies. As shown in Fig. 1 *b*, the x-ray diffraction patterns of lipid dispersions in water are characteristic of their phase symmetry. The ratios of spacing of the peaks observed for the lamellar phase are 1, 1/2, 1/3, 1/4 . . . , whereas the ratios are 1, $1/\sqrt{3}$, 1/2, $1/\sqrt{7}$, 1/3 . . . for the inverted hexagonal (H_{II}) phase. In addition to providing a way to identify the phase symmetry, x-ray diffraction allows the measurement of the unit cell dimensions. In the lamellar phase, the bilayer spacing, *d* (Fig. 1 *a*), is determined directly from the diffraction pattern. In the H_{II} phase, the basis vector length *d* corresponds to the distance between the centers of two adjacent cylinders.

^2H NMR is also a powerful technique for characterizing lipid structure because it provides a measurement of the orientational order of the acyl chains of the lipid molecules. This chain order has been shown to be sensitive to the phase symmetry adopted by the lipids (Perly et al., 1985; Lafleur et al., 1990a; Thurmond et al., 1990, 1993). As can be seen in Fig. 1 *c*, characteristic ^2H NMR spectra are obtained for the L_α and the H_{II} phases. The quadrupolar splittings in the H_{II} phase are more than a factor of 2 smaller than those of the L_α phase because of the combined effects of lipid diffusion around the H_{II} cylinders and the increased orientational freedom in the H_{II} phase. In addition, the order varies more uniformly along the lipid chain in the H_{II} than in the L_α phase. Thus, in addition to providing information for the identification of the phase symmetry, the order parameters also allow characterization of the motional freedom of the lipid acyl chains. Studies on lipid chain order have revealed that chain motions are sensitive to various factors, such as temperature, polar headgroup composition, and cholesterol content (see Lafleur et al., 1990b, for example).

So far, two approaches have given insight into the physical basis of lipid polymorphism. First polymorphic tendencies have been associated with the molecular shape of the lipidic constituents (Cullis and de Kruijff, 1979; Israelach-

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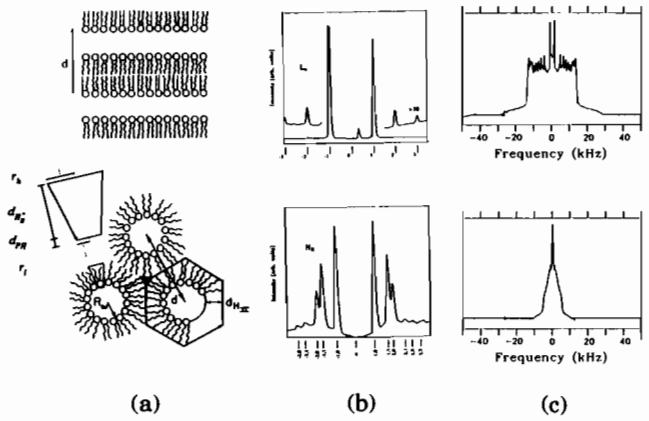

FIGURE 1 Characterization of the L_α and H_{II} phases by x-ray diffraction and ^2H NMR. (*a*) Illustration of the lamellar and hexagonal phases, showing the parameters that describe the structure (see text). (*b*) Typical x-ray diffraction patterns of the lamellar and hexagonal phase lipids. (*c*) Typical ^2H NMR spectra of the lamellar and hexagonal phase lipids.

vili et al., 1980; Cullis et al., 1986). According to the shape concept, the phase formed by the lipid molecules is dictated by the ratio of the area subtended by the headgroup at the lipid-water interface to the area subtended by the acyl chains in the hydrophobic core. This simple model can qualitatively rationalize most of the factors dictating lipid polymorphism. However, up to now, quantitative development of this model has been difficult because the lipid shape, as we will discuss, varies with the experimental conditions, and there is no established method to estimate the overall shape of a complex mixture.

An alternative approach to considering the lipid molecules individually is to relate lipid polymorphism to the elastic properties of a lipid monolayer (Gruner, 1985; Kirk and Gruner, 1985). The resulting lipid phase is the one that minimizes the free energy associated with the packing stress and the curvature of the lipid layer. First, the packing stress reflects the tension induced by the stacking of opposed lipid layers. If two lipid layers are opposed to form a bilayer, the stress due to the packing is small. On the other hand, an H_{II} phase formed by the stacking of lipid cylinders would lead to the formation of relatively difficult-to-reach triangular spaces between the cylinders (Fig. 1), provoking a stress that is unfavorable in terms of free energy. Second, the curvature of a lipid monolayer can be characterized by the spontaneous radius of curvature, R_0, which is the radius of curvature adopted by the lipid layer when the intrinsic curling tendency is expressed, free from other constraints. One difficulty in describing lipid polymorphism using R_0 is in the measurement of this quantity. X-ray diffraction data on the "relaxed" hexagonal lattice as a function of water content has been used to determine R_0. To release the packing stress, hydrophobic molecules are added to the lipid dispersions. It has been suggested (Gruner, 1985; Kirk and Gruner, 1985) that the hydrophobic molecules partition preferentially into the intercylinder spaces, and thus, in an excess of hydrophobic solute, the lipid layer can freely express its intrinsic curvature. Then, using the method proposed by Luzatti and Husson (Luzatti and Husson, 1962; Luzatti, 1968), the dimensions of the lipid and water regions can be calculated from the measurement of the basis vector length, thereby providing an estimate of R_0. The preferential partitioning of alkane into the intercylinder spaces has been demonstrated by neutron diffraction (Turner et al., 1992).

It is likely that the curvature properties of lipid films are reflected in lipid chain order. A model has been proposed recently, based on the analysis of pure 1-palmitoyl-d_{31}-2-lauroyl-phosphatidylethanolamine (PLPE-d_{31}), which relates chain order in the H_{II} phase and membrane curvature (Thurmond et al., 1990, 1993). In this paper, we examine the relationship between the curvature of lipid layers and the orientational order of the phospholipid acyl chains, and propose a unified view of these two parameters. The 1-palmitoyl-2-oleoyl-phosphatidylcholine:1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPC:POPE) system has been studied by x-ray diffraction to obtain information about the long-range order and by ^2H NMR to obtain information about the molecular order. The molar proportion of POPC:POPE and the temperature have been varied to modulate the polymorphic properties of the system.

MATERIALS AND METHODS

1-Palmitoyl-d_{31}-2-oleoyl-phosphatidylethanolamine (POPE-d_{31}), 1-palmitoyl-d_{31}-2-oleoyl-phosphatidylcholine (POPC-d_{31}), and all other lipids were purchased from Avanti Polar Lipids (Birmingham, AL). The lipids showed a single spot by thin-layer chromatography. For the deuterated lipids, a chain analysis revealed an equimolar mixture of palmitoyl and oleoyl chains. As previously discussed (Lafleur et al., 1989), approximately 20% acyl chain transmigration is suspected.

The lipid mixtures were first dissolved in a benzene:methanol 96:4 (v/v) mixture and then freeze-dried. The lipid was then hydrated with a 5 mM HEPES buffer, 100 mM NaCl and 2 mM EDTA, pH 7.4. The final phospholipid concentration was approximately 40 mg/ml. For samples containing dodecane, 20 wt% dodecane was added to the lipid dispersions, and then the samples were equilibrated for at least 24 h at room temperature.

A home-built 46-MHz ^2H-NMR spectrometer described previously (Davis, 1979; Sternin, 1985) was used. The powder pattern spectra were produced by using a quadrupolar echo pulse sequence with a pulse spacing of 50 μs. The 90° pulse length was 4 μs, and the free induction decays were acquired in quadrature collecting 4096 points with a dwell time of 5 or 10 μs. The time between successive pulse sequences was at least 300 ms. The number of scans was at least 36,000. DePaked spectra were obtained using the iterative method previously presented (Bloom et al., 1981; Sternin et al., 1983) and were smoothed on five points. Smoothed order profiles have been determined from the powder pattern spectra, using a method discussed in detail elsewhere (Lafleur et al., 1989). The spectra were recorded as a function of decreasing temperature.

Small-angle x-ray diffraction was carried out to determine the lipid phase and unit cell dimensions of samples as a function of temperature. Lipid samples were placed in 1-mm-diameter glass or quartz capillaries (Blake Industries), which were sealed with epoxy. For diffraction analysis, specimens were placed in a temperature-controlled copper block in an evacuated sample chamber. The x-ray diffraction source was a Rigaku RU200 generator equipped with a copper anode and microfocus cathode and operated at 40 kV, 60 mA. X-rays were focused by double Frank's mirror optics. Two-dimensional powder diffraction patterns were recorded

with a Princeton SIT area detector, using a specimen-to-detector distance of 267 mm (Tate and Gruner, 1989), or another comparably instrumented beamline. Matched pairs of images, each with an exposure time of 400 s to 600 s, were recorded at each temperature. The temperature was ramped up or down in 5°C steps with a 10-min equilibration period after each step and before x-ray exposure. The temperature range was −20 to +90°C. The matched pairs of images were corrected for distortion, flat field, and spurious cosmic ray events and analyzed by azimuthal integration along an arc ±15° from the equator. Bragg peaks characteristic of the H_{II} phase were fitted to a single lattice to determine the lattice spacing, and hence the basis vector length of the hexagonal structure, d, as defined in Fig. 1. Lead stearate (lattice spacing = 47.46 Å) was used to calibrate the patterns. Some of the data were taken at temperatures that were not integer multiples of 5°C. Those data were interpolated to multiples of 5°C using a linear interpolation on the reciprocal of the spacings:

$$\frac{1}{d_i} = \frac{1}{T_1 - T_2}\left(\frac{T_i - T_2}{d_1} + \frac{T_1 - T_i}{d_2}\right),$$

where d_i is the interpolated result at temperature T_i, and d_1 and d_2 are measured spacings at temperatures T_1 and T_2, respectively. Empirically, this function is observed to model these data closely.

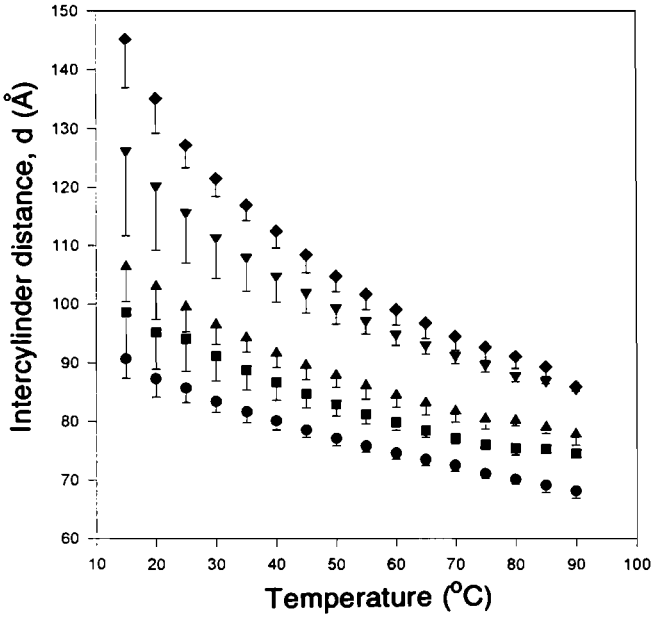

FIGURE 2 Intercylinder spacing, d, of the H_{II} phase formed by mixtures of POPE and POPC in the presence of 20 wt% dodecane as a function of temperature and composition, as determined by x-ray diffraction. The data shown represent the averages of three to six separate determinations at each composition and were recorded for descending temperatures. ●, 100 (mol)% POPE; ■, 91 (mol)% POPE; ▲, 82 (mol)% POPE; ▼, 68 (mol)% POPE; ♦, 61 (mol)% POPE. The balance of the material in each specimen was POPC. Only the lower half of the standard deviation error bar is shown for clarity.

RESULTS

A characterization of the phase diagram of the POPE:POPC system in the presence of 20 wt% dodecane and an excess of aqueous buffer was carried out. X-ray diffraction measurements were made for a wide range of lipid compositions, at both pH 7.0 and pH 7.4, using the standard buffer described above, over the temperature range 90–0°C. The data are summarized in Fig. 2. In this work, the efforts were focused on the part of the phase diagram where the lipids form an H_{II} phase in the presence of dodecane. Under these conditions, the x-ray diffraction measurement provides a quantitative description of the curvature properties of the lipid layers. The hexagonal phase was observed for lipid mixtures containing from 0 to 39 mol% POPC, and again at high temperatures for pure POPC, all in the presence of 20 wt% dodecane. The formation of reversed hexagonal phase in the POPC-alkane-water system is in agreement with previous results (Sjölund et al., 1989). The lamellar-to-hexagonal phase transition temperature increases slowly over the 0 to 39% POPC range from about 5 to 15°C. The H_{II} phase has been found to be stable under these conditions over the temperature range of 20 to 80°C. When the sample contains a larger proportion of POPC or when the temperature is below 20°C, the system forms only a lamellar phase, despite the presence of dodecane. Below the lamellar-to-hexagonal phase transition temperature, the lipids in the presence of dodecane were in the gel phase, and the L_α phase exists on a very restricted range of temperature, if at all. Similar observations have been made for the 1-,2-oleoyl-phosphatidylethanolamine:1-,2-oleoyl-phosphatidylcholine (DOPE:DOPC) system (Kirk and Gruner, 1985). Such lamellar gel-to-hexagonal phase transitions have also been observed for long-chain lipid (Cullis and de Kruijff, 1978; Seddon et al., 1983). It was observed that the data for pure POPE and the data at higher temperatures were highly reproducible, as shown by the small error bars in Fig. 2. However, as conditions moved toward the limits of stability of the hexagonal phase, either lower temperatures or higher fractions of POPC, the system exhibited instability, in that it became sensitive to unknown factors (including probably small variations in pH and salt concentration). These factors introduced variability into the size of the H_{II} structure, as demonstrated in the wider error bars toward the upper left of Fig. 2. It was only when all of the data were examined together that this pattern of unstable behavior became apparent. Thus, Fig. 2 shows the average of all measurements at the various compositions. Another manifestation of this instability was in the approach to equilibrium spacing after a temperature change. It was not feasible to devote more than 10 min to this equilibration, but kinetic experiments were performed to characterize the process. From a low starting temperature, the sample was shifted to a target temperature, e.g., 35°C, and monitored by x-ray diffraction for a few hours; the sample was then brought briefly to a high temperature and again shifted to the target temperature and monitored. It was found that the spacing approached an asymptotic value more rapidly with descending temperature than with ascending temperature; i.e., the H_{II} tubes could more easily take up water than expel it. Hence, the data shown in Fig. 2 are all taken from the descending temperature data, although the ascending temperature data are generally quite consistent with those shown, except near the

low-temperature transition between the lamellar and H_{II} phases.

As can be seen in Fig. 2, the intercylinder distance, *d*, varies considerably, from 70 to 145 Å, over the portion of the phase diagram of the POPE:POPC:dodecane system where the H_{II} phase is formed. Two main trends can be inferred: *d* increases when the temperature is decreased and when the proportion of POPC is increased. This is similar to previous observations on the DOPE:DOPC system (Kirk and Gruner, 1985).

The first ^2H NMR experiment presented here shows the effect of the addition of 20% dodecane on the lipid acyl chain orientational order in the H_{II} phase. Dodecane has been added to POPE-d_{31}, which spontaneously forms an H_{II} phase at 75°C (the L_α-to-H_{II} phase transition temperature of POPE-d_{31} is about 68°C; Lafleur et al., 1990a). The smoothed order profiles obtained at 75°C for POPE-d_{31} with and without dodecane are characteristic of the H_{II} phase (Fig. 3). As can be seen, the mean order parameter is decreased by about 9% in the presence of 20 wt% dodecane. This decrease is reflected all along the acyl chain.

The temperature effect on the order parameters of the phospholipid acyl chain has been characterized for the mixture POPE-d_{31}:POPC 82:18 (Figs. 4 and 5). The temperature has been varied from 70 to 20°C, and the order profiles have been determined for the mixture (Fig. 4 *a*) in the absence and (Fig. 4 *b*) in the presence of dodecane. As mentioned previously, the sample without dodecane forms an L_α phase, whereas in the presence of dodecane, the lipids adopt an H_{II} phase. The change in order distribution observed as a result of the addition of dodecane reflects clearly the change of phase symmetry, the values of $S(n)$ decreasing more linearly in the H_{II} than in the L_α phase. For the L_α as well as for the H_{II} phase, an increase in temperature leads to a decrease in the order parameters, $S(n)$. This decrease is observed all along the lipid palmitoyl chain. To illustrate the effect of temperature on the chain order, the mean order parameter over all *n*, $\langle S \rangle$, has been determined as a function of temperature (Fig. 5 *a*). As can be seen, the values of $\langle S \rangle$ are different for the L_α and the H_{II} phases, and the difference in symmetry of the lipid phase is the origin of this change in order. When the difference in the phase symmetry is taken into account by normalizing the largest value of the order parameter (the one obtained at 20°C) to unity, the variation of lipid chain order observed as a function of temperature is equivalent for the L_α and the H_{II} phases, i.e., $\langle S \rangle_{L_\alpha}/\langle S \rangle_{H_{II}}$ is independent of temperature (Fig. 5 *b*). This ratio of the average order in the L_α phase and the H_{II} phase for POPE:POPC 82:18 is 2.93 ± 0.04 over the whole temperature range studied. Actually, $S(n)_{L_\alpha}/S(n)_{H_{II}}$ is independent of temperature for all *n* (data not shown). However, $\langle S \rangle_{L_\alpha}/\langle S \rangle_{H_{II}}$ is dependent on the proportion of POPC in the mixture; it is 3.11 for pure POPE and 2.61 for POPE:POPC 68:32. The temperature dependence of orientational order has been plotted from the variation of $\ln\langle S \rangle$ with 1/temperature, for the different lipid mixtures investigated (Fig. 6). Each set of points can be described by a linear fit. The slopes of the least-squares fits are similar for the various lipid compositions, and for both the L_α and H_{II} phases. The average value of the slope is −885 K.

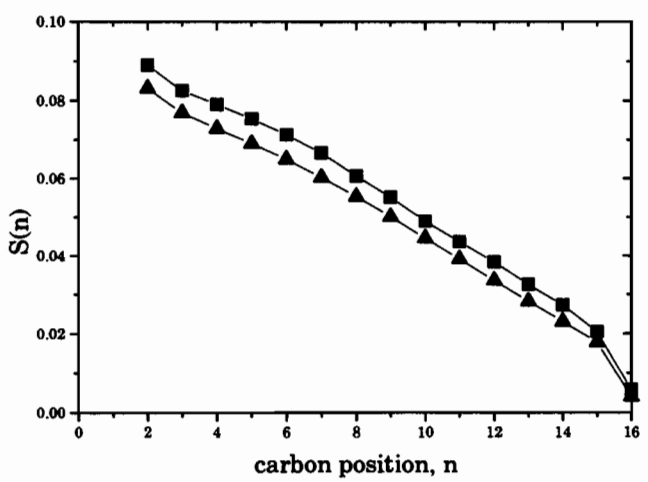

FIGURE 3 Orientational order profile obtained at 75°C for POPE-d_{31} with (▲) and without (■) dodecane.

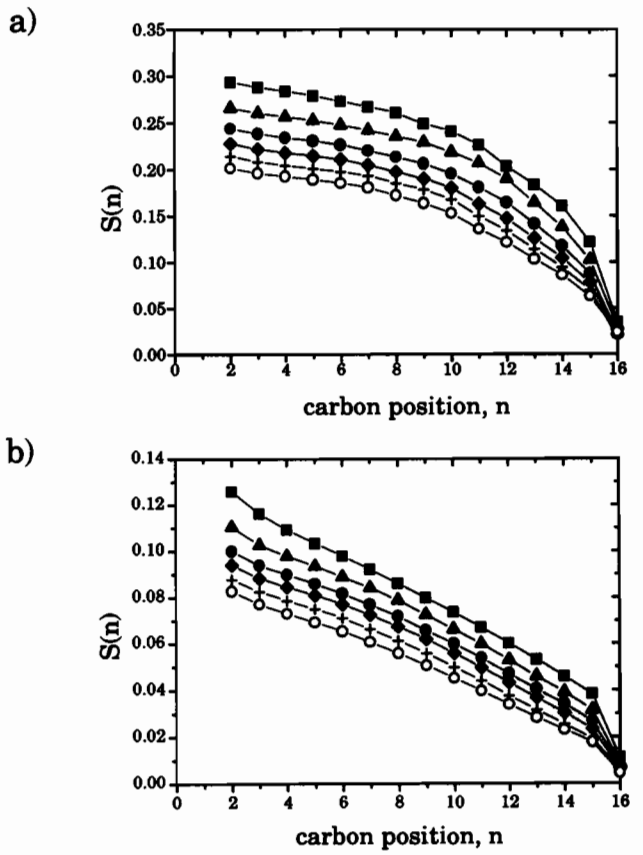

FIGURE 4 Effect of temperature on the orientational order profile of the POPE-d_{31}:POPC 82:18 mixture (*a*) in the L_α (without dodecane) and (*b*) in the H_{II} phase in the presence of 20 wt% dodecane. ■, 20°C; ▲, 30°C; ●, 40°C; ◆, 50°C; +, 60°C; ○, 70°C.

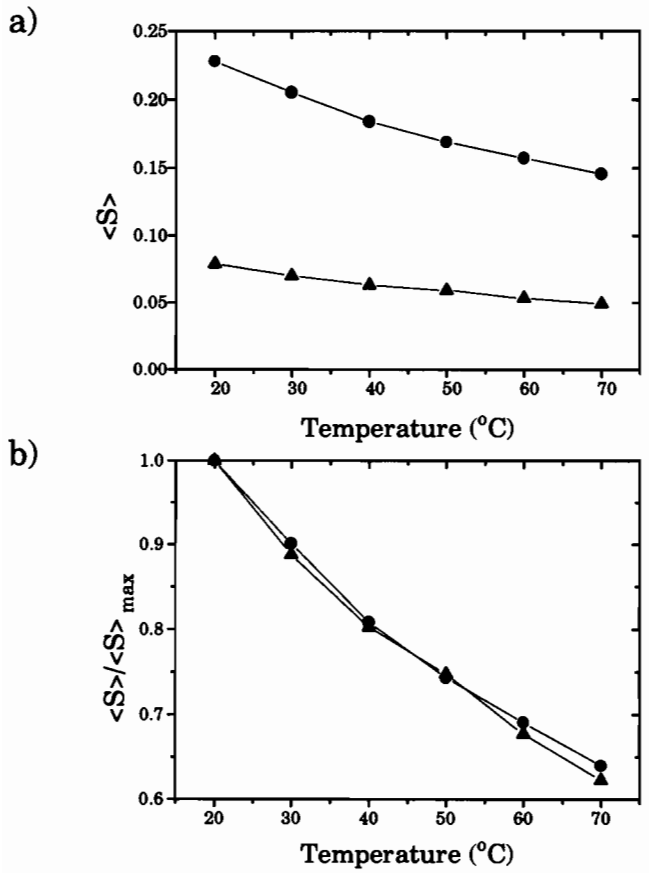

FIGURE 5 Effect of temperature on $\langle S \rangle_{L_\alpha}$ (in the absence of dodecane) (●) and $\langle S \rangle_{H_{II}}$ (in the presence of dodecane) (▲) for POPE-d_{31}:POPC 82:18. (*a*) Absolute values of $\langle S \rangle$ and (*b*) when the largest has been normalized to unity.

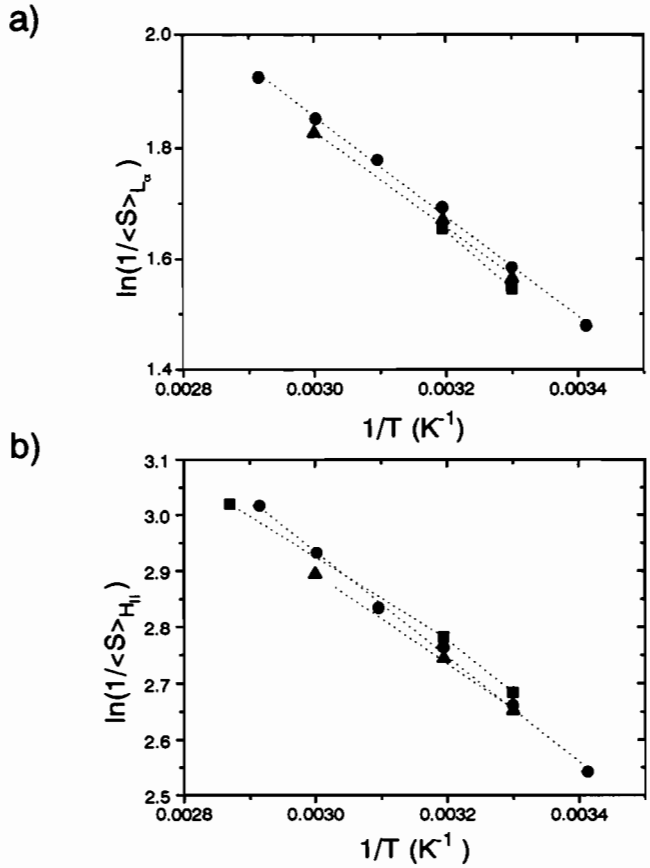

FIGURE 6 Temperature dependence of orientational order for POPE-d_{31} (■), POPE-d_{31}:POPC 91:9 (▲), and POPE-d_{31}:POPC 82:18 (●), (*a*) in the lamellar phase (without dodecane) and (*b*) in the H_{II} phase (with 20 wt% dodecane).

We have also investigated how the lipid chain order is influenced by the proportion of POPC present, another factor affecting the spontaneous curvature of the lipid monolayers. The order profiles obtained at 40°C in *a*) the absence and *b*) the presence of dodecane for mixtures with different POPC content are given in Fig. 7. A striking feature of the variation of the lipid composition is its rather limited effect on $S(n)$. For example, in the H_{II} phase (i.e., in the presence of dodecane), the average order parameter varies by only 3% when 32 mol% POPC is added to POPE at 40°C. In the absence of dodecane, in the L_α phase, the change in $\langle S \rangle_{L_\alpha}$ induced by a change of the lipid composition is also limited: a variation of 5% in $\langle S \rangle_{L_\alpha}$ is observed when 32 mol% POPC is added to POPE at 40°C. The order parameters of the lipid chains decrease progressively when the proportion of POPC is increased, moving toward the order parameters obtained for pure POPC-d_{31} (Fig. 5 *a*). This is in agreement with previous results showing that in PE:PC mixtures, order parameters intermediate to those observed for the pure components are obtained (Cullis et al., 1986; Lafleur et al., 1990b). In these POPC:POPE mixtures, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) molecules experience the same orientational order (Lafleur et al., 1990b).

DISCUSSION

Influence of alkane on the phase behavior and chain order of lipids

It has previously been shown that the addition of alkane to phospholipids favors the formation of an H_{II} phase (Kirk and Gruner, 1985). The results obtained for POPE:POPC systems are in agreement with this finding. POPE:POPC mixtures whose POPC content varies from 0 to 39% undergo a transition from a lamellar to an H_{II} phase when an excess of dodecane is added. In the present investigation, a proportion of 20 wt% dodecane has been used because this amount should exceed the amount of hydrophobic material needed to fill the intercylinder spaces for H_{II} structures (Rand et al., 1990). This shift of the lamellar to hexagonal phase transition temperature is rationalized on the basis that the alkane molecules partition preferentially into the intercylinder spaces (Turner et al., 1992) and, thus, release the packing stress. Studies using deuterated alkanes have shown

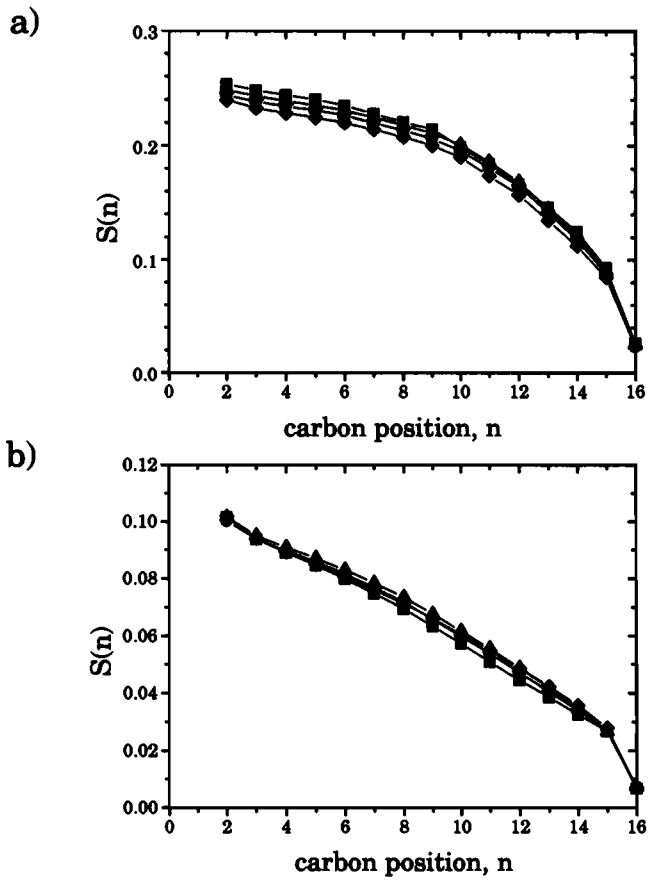

FIGURE 7 Order profile obtained at 40°C for different lipid compositions for the POPE-d_{31}:POPC system (*a*) in the absence and (*b*) in the presence of 20 wt% dodecane. ■, 0 (mol)% POPC; ▲, 9 (mol)% POPC; ●, 18 (mol)% POPC; ♦, 32 (mol)% POPC.

that the quadrupolar splittings of alkane molecules embedded in a lipid H_{II} phase are small, about 2 kHz (Siegel et al., 1989; Lafleur et al., 1990a). This indicates that their location allows a great deal of motional freedom and is consistent with partitioning into the intercylinder spaces.

The promotion of H_{II} phase formation by the addition of alkanes has been suggested to arise primarily from a reduction of the unfavorable packing associated with fitting intercylinder spaces. The curling tendency of the lipid film appears not to be significantly affected by the presence of alkane. This is demonstrated by the fact that the addition of alkanes does not greatly influence the radius of curvature of systems in excess water that form the H_{II} phase in the absence of alkanes (Kirk and Gruner, 1985). Because alkane is added to POPE:POPC system to induce the H_{II} phase, it is of interest to examine its effect on the chain order. The results obtained show that the lipid chain order in the H_{II} phase structure is reduced by about 9% when 20 wt% dodecane is added to POPE-d_{31} (Fig. 3). This observation may be interpreted by the curvature model. In the absence of dodecane, the lipid chains would have to stretch to fill the intercylinder spaces; this stretching corresponds to an ordering of the lipid acyl chain relative to chains oriented along the planes containing the cylinder centers. If one assumes that the alkane molecules added to the phospholipids partition preferentially in the intercylinder spaces, the phospholipid chains should show a decrease in order because the lipid chains do not have to stretch as much in these conditions. This is a possible interpretation for the ^2H NMR results showing such a decrease in order. However, such a decrease in order could arise from other sources, including increased motion due to direct interactions between the phospholipids and alkanes. It should be noted that the observed effect is rather modest, considering the large amount of alkane added. This is consistent with a previous study (Lafleur et al., 1990a), which has shown that the presence of 9 mol% (2 wt%) decane in POPE does not significantly influence the order profile of the lipid chain.

Correlation between R_0 and lipid order measured in the H_{II} phase

Measurements of the lipid chain order and basis vector length have been made on the structures in which the packing stress was relieved. The influence of POPC content and temperature on the order parameters, $S(n)$, and on the intercylinder distance, d, has been investigated. The curvature tendency of these stress-relieved structures has been characterized in this study by x-ray diffraction for different phospholipid compositions and temperatures. The conclusions are consistent with those obtained for various PE:PC systems (Kirk and Gruner, 1985; Tate and Gruner, 1989). First, an increase in the POPC content leads to an H_{II} phase structure with a larger d. This can be rationalized by the fact that POPE monolayers have a greater tendency to curl than POPC monolayers, as illustrated by the formation of an H_{II} phase by pure POPE above 70°C. Thus, the incorporation of POPE in POPC increases the overall curling tendency of the lipid leaflet and results in the formation of smaller H_{II} cylinders in the presence of dodecane. Second, when temperature is increased, d decreases. This dependence on temperature has been observed for several lipid systems and is readily understood in terms of thermal thinning of lipid monolayers (Tate and Gruner, 1989).

What is the concomitant change in lipid acyl orientational order? The order parameter profiles determined for the POPE:POPC system in the presence of dodecane (i.e., in the H_{II} phase) indicate that $S(n)$ is decreased for every n when temperature is increased, as is also observed in the L_α phase. This is explained, as for the L_α phase, by the increase in motional averaging of the interactions giving rise to the quadrupolar effects at higher temperatures.

In the case of the L_α phase, an empirical relationship between the mean order parameter of the lipid chain and its length has been proposed (Seelig and Seelig, 1974; Salmon et al., 1987; Ipsen et al., 1990). This is expressed in the form of a linear relationship:

$$\langle L \rangle = L_0(a_L |\langle S \rangle_{L_\alpha}| + b_L), \quad (1)$$

where $\langle L \rangle$ is the average length of the lipid chain, L_0 is the length of an all-*trans* chain, and a_L, b_L are constant coefficients for the L_α phase with the constraint $0.5a_L + b_L = 1$ (Ipsen et al., 1990). Equation 1 is based on the facts that in the L_α phase, the axis of symmetry for the fast motions of the lipid molecule is perpendicular to the bilayer interface, and that the quadrupolar interactions are essentially averaged by *trans-gauche* isomerization and the rotation of the lipid around its long axis. Because similar conditions exist for the H_{II} phase, we explore the possibility that an equivalent relationship between $d_{H_{II}}$ and $\langle S \rangle_{H_{II}}$ exists. An equivalent approach has been used previously for pure PLPE-d_{31} (Thurmond et al., 1990, 1993). In the H_{II} phase symmetry, a factor of 2 times the mean order parameter should be introduced, because the lipid diffusion around the cylinder causes an extra averaging and leads to the rotation of the axis of symmetry of the fast motion of the lipid by 90° (Seelig, 1978; Lafleur et al., 1989). In this case, Eq. 1 becomes

$$d^*_{H_{II}} = L_0(2\, a_H \langle S \rangle_{H_{II}} + b_H). \qquad (2)$$

The notation $d^*_{H_{II}}$ is used here to express the average hydrocarbon thickness as measured by ^{2}H NMR. This is not strictly equivalent to $d_{H_{II}}$ measured by x-ray diffraction. In the latter case, this corresponds to the length of the lipid section, from the water/lipid interface to the middle of two adjacent cylinders. The value of $d^*_{H_{II}}$ corresponds to the average thickness of the area sampled by the deuterium nuclei borne by the phospholipid palmitoyl chain. The coefficients a_H and b_H should be positive and satisfy the condition $0.5a_H + b_H = 1$ (Ipsen et al., 1990). Despite the different geometry of the lipid phase, they are taken, as a first guess, as equal to those used in the L_α phase, 1 and 0.5 for a_H and b_H, respectively. The average order of the POPE-d_{31}:POPC/dodecane 82:18 mixture varies from 0.0787 at 20°C to 0.0490 at 70°C. If we estimate L_0 to be 18.7 Å, corresponding to 15 *trans* C-C bonds, the values of $d^*_{H_{II}}$ vary from 12.3 to 11.2 Å over this temperature range, corresponding to a decrease of 1.1 Å. A decrease in $d^*_{H_{II}}$ was indeed expected because an increase in temperature should lead to a higher probability of *gauche* rotamers.

The values of d obtained for POPE or POPE:POPC mixtures are significantly larger than those reported for DOPE or DOPE:DOPC mixtures (Kirk and Gruner, 1985; Tate and Gruner, 1989). This is associated with a more pronounced curvature of systems containing more acyl chain unsaturation. However, the dependence on temperature and lipid composition is similar for both systems, and a comparison between the two systems seems justified. The DOPE:DOPC system has been investigated in detail (Tate and Gruner, 1989), and the contributions of the water core radius, R_w, and of the thickness of the lipid layer, $d_{H_{II}}$, have been calculated. It has been shown that the reduction in d is mainly associated with a change in the radius of the water core, R_w, whereas the thickness of the lipid layer, $d_{H_{II}}$, has been found to decrease by only about 0.7 Å, from 20 to 70°C (Tate and Gruner, 1989). The x-ray diffraction measurements indicate that the value of $d_{H_{II}}$ for DOPE varies from 16.2 to 15.5 Å over this temperature range. These values are consistent with those of $d^*_{H_{II}}$ derived from the ^{2}H NMR measurement for the POPE:POPC system (12.3 to 11.2 Å), the difference in absolute value being on the order of the distance between the first deuterium-labeled methylene group of the *sn*-1 chain and the water/lipid interface (Büldt et al., 1978). This agreement supports the validity of the relationship between order parameter and chain length in the H_{II} phase. If this relationship turns out to be generally valid, it could provide a new way to estimate the size of the hydrocarbon region in the H_{II} phase, using ^{2}H NMR. This method would be appealing, because it is based on a well-defined and straightforward measurement and, coupled with the x-ray diffraction technique, would lead to a detailed description of the H_{II} structure.

This change of $d^*_{H_{II}}$ by about 1 Å over 50°C accounts for only a small part of the change in d. Over the same temperature range, d determined for a POPE:POPC 82:18 mixture varies from 103.0 to 81.7 Å. The reduction of the H_{II} lattice dimension is then clearly related to a change in the size of the water core, R_w, and $d^*_{H_{II}}$ accounts for less than 6% of the reduction. A similar observation is made for the change in d induced by a variation in lipid composition. The value of d increases by about 25% when the proportion of POPC is increased from 0 to 32 mol%. However, $\langle S \rangle_{H_{II}}$ is decreased by only 3% for the same range of the concentration—this is at the limit of the experimental error. Thus, it is clear that these changes in d correspond primarily to a change in R_w, similar to the results of the DOPE:DOPC system (Tate and Gruner, 1989).

Changes in R_w reflect the modifications of the curvature of the lipid layer. The curvature is a consequence of many interactions occurring within a single lipid layer (Seddon, 1990). It is likely that these interactions are reflected at the molecular level. Because it is possible to describe molecular details of the H_{II} phase using ^{2}H NMR, the next question we addressed was whether the orientational order can be quantitatively related to the curvature properties of the lipid layer via the thickness of the H_{II} monolayer. It is clear from the results presented here that temperature and lipid composition affect the hydrocarbon order in a way that is different from the way in which they affect curvature. For the POPE:POPC systems, higher temperature leads to smaller R_0 values and decreased order, whereas increased POPC content leads to larger R_0 values and has little effect on the lipid chain order. Therefore, changes in curvature are not uniquely related to the orientational order of the lipid acyl chain. A relationship between lipid leaflet curvature and lipid chain order may be postulated only if an additional parameter is introduced.

Tate and Gruner (1989) pointed out that, because of the two-dimensional geometry of the H_{II} phase, knowledge of any two of the three parameters of monolayer thickness, interfacial area, and lipid molecular volume completely determines the dimensions of the phase and that very small

changes in interfacial area or monolayer thickness can result in large changes in R_w. X-ray diffraction was used to determine the parameters. Thurmond et al. (1990, 1993) showed how NMR could be used to estimate the monolayer thickness and the H_{II} phase dimensions. In this paper, we have investigated a similar geometrical model using an NMR determination of the monolayer thickness, along with the assumption of linear additivity of interfacial areas of different lipids, to estimate the size of the H_{II} tubes in excess water dispersions of lipid mixtures. The average shape of a lipid molecule in the H_{II} phase is approximated by a frustum of a right circular cone, and the H_{II} cylinders are formed by the stacking, side by side, of these truncated cones (see Fig. 1). This close packing does not fill completely the volume of the H_{II} phase, but this approximation was used to simplify the problem to two dimensions. The molecular volume (V) can be expressed as

$$V = \frac{\pi}{3}(d^*_{H_{II}} + d_{PH})(r_i^2 + r_i r_h + r_h^2). \quad (3)$$

The parameters r_i and r_h are the segment length defined by the projection, on the plane perpendicular to the cylinder axis, of the area at the interface and at the wide base of the frustum, respectively. The projected length of the frustum is the sum of the contributions of the chain, $d^*_{H_{II}}$, as determined by the ^2H NMR measurements (see Eq. 2), and of the polar headgroup, d_{PH}, approximated as 2.6 Å; this value provided the best calculated d values and is comparable to previous results obtained from neutron diffraction studies (Büldt et al., 1978).

The value of r_i is obtained from the average molecular area of the lipid at the interface, A_i, estimated by a linear combination of the molecular area occupied by the polar headgroup of each lipid component:

$$\pi r_i^2 = A_i = x_{POPC} A_{POPC} + x_{POPE} A_{POPE}, \quad (4)$$

where x represents the molar fraction, and A_{POPE} and A_{POPC} the molecular interfacial area of each lipid species in the H_{II} phase. Although these areas have not been measured, for the sake of calculation we assume 1) that these areas may be approximated by the areas of DOPE and DOPC, respectively, and 2) that the molecular areas and volumes do not change significantly with temperature. The measured interfacial H_{II} area of DOPE is about 48 Å^2, with only 4% variation over a 70°C span, and the change in volume is comparably small (Tate and Gruner, 1989). Because these changes are considerably smaller than the change in order of the phospholipid acyl chains in the H_{II} phase, which is about 35% over 50°C, it is a reasonable approximation to take the molecular interfacial areas and volumes as constant over the temperature range of our study. The interfacial area of DOPC in the H_{II} phase has not been directly measured, but it has been estimated by extrapolation of measurements in DOPE:DOPC mixtures (Tate, 1987; Tate and Gruner, 1989) to be 72 Å^2, using the assumption that the molecular areas are additive in mixtures. Hence, we will use the values of 48 and 72 Å^2 for A_{POPE} and A_{POPC}, respectively.

These molecular areas are considered as additive in the mixtures and independent of temperature. The linear variation of the quadrupolar splittings as a function of POPC molar proportion in POPE:POPC system in the lamellar phase (Lafleur et al., 1990b) supports the validity of the linear combination expressing A_i. Moreover, the lipid packing factor of a lipid mixture has been proposed to be evaluated, to a first approximation, by the weighted average of individual molecular packing contribution (Hui and Sen, 1989). Our estimation of the interfacial contribution is an important difference with the model proposed by Thurmond et al. (1990, 1993); in that model, the interfacial area is considered to be same in both the L_α and H_{II} phases and is calculated from the order parameters measured in the lamellar phase. When temperature is considered, this approach is no longer valid, because $\langle S \rangle_{L_\alpha}$, used to measure the interfacial area in the H_{II} phase, is strongly dependent on temperature, whereas the interfacial area is not (Tate and Gruner, 1989).

The molecular volume is approximated as 1144 Å^3; this parameter value is consistent with the values obtained for DOPE, DOPE:DOPC 5.07:1 mixtures, and DPPC (Nagle and Weiner, 1988; Tate and Gruner, 1989; Rand and Fuller, 1994). Using these parameters, it is straightforward to calculate r_h for each sample at each temperature, using Eq. 3. Then, the lipid layer curvature can be expressed in geometrical terms by the ratio of the projection of the interface and hydrophobic areas on the plane perpendicular to the axis of the H_{II} cylinder. In this case,

$$\frac{r_h}{r_i} = \frac{R_w + d^*_{H_{II}} + d_{PH}}{R_w}. \quad (5)$$

For the bilayer structure, r_h equals r_i, implying that $R_w \gg (d^*_{H_{II}} + d_{PH})$, as expected. For the H_{II} phase, $r_h/r_i > 1$ (Israelachvili et al., 1980; Cullis et al., 1986).

From Eq. 5, R_w is calculated, and the intercylinder spacing, d, is estimated using

$$d = 2(R_w + d^*_{H_{II}} + d_{PH}). \quad (6)$$

The agreement between the values of intercylinder spacing calculated from the chain order measurements (Eq. 6) and those measured by x-ray diffraction is very good (Table 1 and Fig. 8); the data points represent various POPC:POPE mixtures recorded at several temperatures. This simple model relates the average order parameter of the lipid molecule to the dimensions of the unit cell of the H_{II} phase. In other words, it demonstrates that the curling tendency of the lipid layer can be expressed in terms of the thickness of the lipid layer determined from the lipid chain order (Eq. 2) and the interfacial area. Both temperature and POPC content effects are successfully described by the model. The model is based on several assumptions that may be valid only in first approximation, and uses parameters that can be refined. For example, A_{POPC}, which we derived from measurements

TABLE 1 Numerical data used for the comparison between the intercylinder distance derived from NMR and x-ray diffraction

	$\langle S \rangle_{H_{II}}$	$d^*_{H_{II}}$ (Å)	A_i (Å^2)	d as calculated by NMR (Å)	d as measured by x-ray diffraction (Å)
POPE; 30 °C	0.0683	11.9	48	82.3	83.4
POPE; 40 °C	0.0619	11.7	48	79.1	80.1
POPE; 75 °C	0.0488	11.2	48	72.9	71.1
POPE:POPC (91:09); 30 °C	0.0705	12.0	50.16	89.3	91.2
POPE:POPC (91:09); 40 °C	0.0643	11.8	50.16	85.7	86.7
POPE:POPC (91:09); 60 °C	0.0554	11.4	50.16	80.7	79.8
POPE:POPC (82:18); 20 °C	0.0787	12.3	52.32	102.1	103.0
POPE:POPC (82:18); 30 °C	0.0699	12.0	52.32	95.7	96.5
POPE:POPC (82:18); 40 °C	0.0631	11.7	52.32	90.7	91.7
POPE:POPC (82:18); 50 °C	0.0588	11.5	52.32	88.3	87.9
POPE:POPC (82:18); 60 °C	0.0533	11.3	52.32	85.0	84.5
POPE:POPC (82:18); 70 °C	0.0490	11.2	52.32	82.4	81.7
POPE:POPC (68:32); 40 °C	0.0680	11.9	55.68	107.0	104.8

in DOPE:DOPC mixtures, appears to be somewhat large compared to the reported values for phosphatidylcholines in the L_α phase (Marsh, 1990), and a validated value would be useful for improving the model. A_{POPC}, V, and d_{PH} are strongly covariant, and the set of near-best-fit values shown here represent a shallow minimum of χ^2 in parameter space; other sets of values fit the data almost as well.

In addition to fitting the data, the model proposes insights into the different molecular mechanisms associated with a change in curvature. It suggests that temperature and POPC content variations both modulate lipid plane curvature, but the molecular origins of these changes are different. Temperature modulates mainly the area subtended by the hydrophobic core through the conformational order. The motions of the lipid acyl chains are more important at high temperature with, as a consequence, a larger area subtended by the lipid acyl chain. On the other hand, POPC content affects essentially the headgroup interface contribution. Similar conclusions were found in the DOPE:DOPC study of Tate and Gruner (1989). In that case, it was shown that the ratio of molecular interfacial area to volume was temperature independent but varied with the DOPE/DOPC ratio. At a fixed composition, the change in curvature of the H_{II} tubes with temperature was shown to come almost entirely from the thermally induced changes in the thickness of the lipid monolayer.

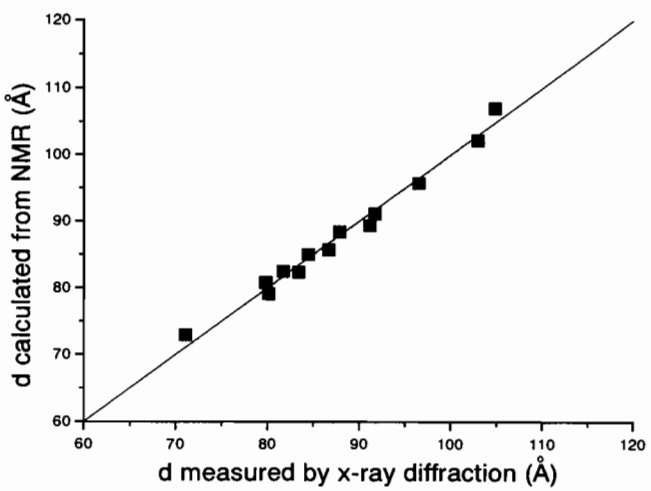

FIGURE 8 Comparison between the intercylinder distance derived from the chain order measurements and the values obtained by x-ray diffraction. These data were obtained, in the presence of dodecane, with POPE (30, 40, and 75°C), POPE:POPC 91:9 (30, 40, and 60°C), POPE:POPC 82:18 (20, 30, 40, 50, 60, and 70°C), and POPE:POPC 32:68 (40°C). The solid line represents perfect agreement.

Lipid chain order in the L_α phase

Another major goal of this study was to determine the influence of the lipid layer curvature on the lipid chain order in the L_α phase, when the explicit expression of the curling tendency is unfavorable from a free energy point of view. As for the H_{II} phase, temperature and POPC content have different effects on the lipid chain order in the L_α phase. An increase in temperature induces a large decrease in the orientational order of the lipid chains for both the L_α and the H_{II} phases. The absolute values of order parameters are indeed affected by the phase symmetry, as previously reported (Sternin et al., 1988; Lafleur et al., 1990a). Surprisingly, the evolution of the mean orientational order of the lipid acyl chain as a function of temperature is similar for the L_α and H_{II} phases when a scaling factor is introduced to account for the change of symmetry (Fig. 5). The ratio of the mean order parameter observed in the L_α phase to that of the H_{II} phase appears to be characteristic for a given lipid composition and independent of temperature. For all of the lipid compositions studied, this ratio is always larger than 2; it varies from 3.11 for pure POPE-d_{31} to 2.61 for POPE-d_{31}:POPC 68:32.

A change in POPC proportion is also reflected in the orientational order in the L_α phase, but to a lesser extent. When the curling tendency of the lipid plane is increased by a higher proportion of POPE, $S(n)$ increases for every n in the L_α as a consequence of the lateral compression exerted on the acyl chains to compensate the intrinsic curvature of POPE, in agreement with previous results (Perly et al.,

1985; Fenske et al., 1990; Lafleur et al., 1990b; Thurmond et al., 1991). This lipid packing tension in the L_α phase increases when the POPE proportion increases. Because the acyl chain composition does not change, it is clear that the change in A_i is the origin of this lateral compression determined by the variation of order. The proportion of POPE to POPC affects the orientational order, but also affects $\langle S \rangle_{L_\alpha} / \langle S \rangle_{H_{II}}$. This ratio increases with increased POPE content. This can be associated with the tension present in the L_α phase, which is released when dodecane is added and an H_{II} phase is formed. Thus, as the tension in the lamellar phase increases, the ratio also increases.

The orientational order observed in the L_α and the H_{II} phases includes two contributions. First, the thermal contribution illustrates the dependence of the order on temperature; our results suggest that this is relatively independent of the lipid phase and lipid composition. The change in order likely originates from a common process, the slopes describing temperature dependence of orientational order (Fig. 6) being similar for the different lipid compositions and phase symmetry. This is presumably related to the Boltzmann factors for the conformational excitations. Second, the geometrical contribution depends on intrinsic parameters of the lipid mixture. It relates the order observed for the L_α and the H_{II} phases, and describes the primary effect of the lipid headgroup composition. A better understanding of this latter contribution would be important in comprehending the effect of curvature in the L_α phase in detail.

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