Polymorphism of POPE/Cholesterol System: A ^{2}H Nuclear Magnetic Resonance and Infrared Spectroscopic Investigation

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ABSTRACT It is well established that cholesterol induces the formation of a liquid-ordered phase in phosphatidylcholine (PC) bilayers. The goal of this work is to examine the influence of cholesterol on phosphatidylethanolamine polymorphism. The behavior of 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE)/cholesterol mixtures was characterized using infrared and ^{2}H nuclear magnetic resonance (NMR) spectroscopy (using POPE bearing a perdeuterated palmitoyl chain in the latter case). Our results reveal that cholesterol induces the formation of a liquid-ordered phase in POPE membranes, similar to those observed for various PC/cholesterol systems. However, the coexistence region of the gel and the liquid-ordered phases is different from that proposed for PC/cholesterol systems. The results indicate a progressive broadening of the gel-to-fluid phase transition, suggesting the absence of an eutectic. In addition, there is a progressive downshift of the end of the transition for cholesterol content higher than 10 mol %. Cholesterol has an ordering effect on the acyl chains of POPE, but it is less pronounced than for the PC equivalent. This study also shows that the cholesterol effect on the lamellar-to-hexagonal (L_α-H_{II}) phase transition is not monotonous. It shifts the transition toward the low temperatures between 0 and 30 mol % cholesterol but shifts it toward the high temperatures when cholesterol content is higher than 30 mol %. The change in conformational order of the lipid acyl chains, as probed by the shift of the symmetric methylene C—H stretching, shows concerted variations. Finally, we show that cholesterol maintains its chain ordering effect in the hexagonal phase.

INTRODUCTION

Cholesterol is a major constituent of plasma membranes of mammalian cells, contributing to ~25 mol % of the lipid fraction in the human erythrocyte membrane, for example. It is now well established that cholesterol causes profound changes in the physical properties of membranes. Investigations on dipalmitoylphosphatidylcholine (DPPC)/cholesterol (chol) mixtures have shown the formation of a new phase at cholesterol concentrations above ~22 mol %, designated as a liquid-ordered phase (Ipsen et al., 1987; Vist and Davis, 1990; McMullen and McElhaney, 1995). This phase shares characteristics with liquid-crystalline and gel phases. It is characterized by axially symmetric motion and lateral diffusion, similar to those of the liquid-crystalline phase, but high orientational order of the acyl chains, similar to the gel phase. Such a liquid-ordered phase appears to exist over a wide temperature range in DPPC containing more than 22 mol % cholesterol (Ipsen et al., 1987; Vist and Davis, 1990) although some structural changes may occur upon heating (Reinl et al., 1992; McMullen and McElhaney, 1995). The formation of a similar liquid-ordered phase has been proposed for other phosphatidylcholines/cholesterol systems (Thewalt and Bloom, 1992; Linseisen et al., 1993). This phase generates a great interest because it is proposed to be more representative of plasma membrane organization.

Therefore, it is important to examine whether the formation of a liquid-ordered phase is a general trend of a phospholipid/cholesterol system or whether it is a singular behavior with phosphatidylcholines (PCs). As phosphatidylethanolamines (PEs) are the second most abundant phospholipid in animal cell membranes, we have investigated the influence of cholesterol on its polymorphism. Previous studies have examined the behavior of PE/chol mixtures by differential scanning calorimetry (DSC) (Blume, 1980; Ghosh and Seelig, 1982; Epand and Bottega, 1987; Cheetham et al., 1989; Takahashi et al., 1996; McMullen and McElhaney, 1997), solid-state nuclear magnetic resonance (NMR) spectroscopy (Brown and Seelig, 1978; Blume and Griffin, 1982; Ghosh and Seelig, 1982; Marinov and Dufourc, 1995; Marinov and Dufourc, 1996), and x-ray diffraction (Cheetham et al., 1989; Takahashi et al., 1996). Because of its molecular properties, unsaturated PE can generally form gel lamellar (L_β), liquid-crystalline lamellar (L_α), and inverted hexagonal (H_{II}) phases. In the lamellar phase, cholesterol is reported to disorder the L_β phase whereas it orders the phospholipids in the L_α phase (Brown and Seelig, 1978; Ghosh and Seelig, 1982; Blume and Griffin, 1982). The L_β-to-L_α phase transition is broadened and shifted toward low temperatures by cholesterol and is abolished for high cholesterol content for most PEs (between 30 and 50 mol %, depending on the phospholipid) (Brown and Seelig, 1978; Ghosh and Seelig, 1982; Blume and Griffin, 1982; Marinov and Dufourc, 1996; Takahashi et al., 1996; McMullen and McElhaney, 1997). For intermediate cholesterol proportions, the coexistence of a gel and a fluid phase has been reported below the L_β-to-L_α phase transition temperature (Tm) of the pure PE (Blume and Griffin, 1982; Takahashi et al., 1996). Cholesterol has also

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been shown to influence the formation of nonlamellar phases. Previous studies have reported that a limited amount of cholesterol leads to a decrease of the lamellar-to-hexagonal phase transition temperature (Th), but proportions of cholesterol higher than 30 mol % in PE reverse this effect and Th increases (Epand and Bottega, 1987; Cheetham et al., 1989; Takahashi et al., 1996). Cholesterol appears to reduce the intercylinder spacing in the H_{II} phase (Cheetham et al., 1989; Takahashi et al., 1996). Up to now, very little has been known about the molecular details of the changes induced by cholesterol in PE matrices.

In this study, we use Fourier transform infrared (FTIR) and ^{2}H-NMR spectroscopy to examine 1-palmitoyl-2-oleoyl-*sn*-phosphatidylethanolamine (POPE)/chol polymorphism. This phospholipid is particularly interesting as both Tm and Th are fairly accessible (Tm = 25°C and Th = 71°C; Epand and Bottega, 1987). In addition, a perdeuterated saturated acyl chain at the *sn*-1 position of the phospholipid constitutes a nice probe of the orientational order along the lipid chains. We have examined the effect of cholesterol on POPE acyl chain order and carbonyl hydration in the three phases and attempt to relate these molecular changes to the polymorphism. In addition, we have also addressed the question of whether cholesterol induces the formation of a liquid-ordered phase with POPE. These results lead us to propose a new temperature-composition diagram for the POPE/cholesterol system.

MATERIALS AND METHODS

POPE and 1-perdeuteriopalmitoyl-2-oleoyl-*sn*-phosphatidylethanolamine (POPE-d_{31}) were purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. Cholesterol was obtained from Sigma Chemical Co. (St. Louis, MO). Deuterium-depleted water was purchased from Aldrich (Milwaukee, WI).

Samples for FTIR were prepared as follows: 5 mg of POPE dissolved in a benzene/methanol (96:04) mixture was mixed with an appropriate volume of cholesterol in solution in the same solvent to yield the desired molar ratio. The solvent was removed by lyophilization. The powder was dispersed in 20 μl of 20 mM Hepes buffer containing 2 mM EDTA and 100 mM NaCl, pH 7.4. The sample was then shaken on a vortex mixer, heated to 40°C, and cooled down to liquid nitrogen temperature. This cycle was repeated at least three times to ensure sample homogeneity. The sample was put between two CaF_2 windows spaced out by a 5-μm Teflon spacer. The cell was mounted in a brass sample holder the temperature of which was computer controlled using thermopumps (Pézolet et al., 1983). The spectra were recorded on a Bio-Rad FTS-25 FTIR spectrometer equipped with a mercury-cadmium-telluride detector. For each spectrum, 100 scans were collected with 2-cm^{-1} nominal resolution.

To eliminate water contribution ($\nu_{O\text{-}H} \approx 3400\ cm^{-1}$) in the methylene stretching region, a polynomial was fitted to simulate the edge of the water band and then subtracted in the $\nu_{C—H}$ region. The cholesterol contribution in this region was also eliminated (Kodati and Lafleur, 1993). In the carbonyl region, correction for the contribution of the water deformation band was made by subtracting the buffer spectrum recorded at the same temperature. The Fourier self-deconvolution algorithm in the GRAMS software (Galactic Industries Corp., Salem, NH) was used to highlight the components of the carbonyl band.

Samples for ^{2}H-NMR spectroscopy were prepared the same way as for FTIR. However, the amount of lipid used was 30 mg and the lipid was perdeuterated on the palmitoyl chain. The buffer was prepared with deuterium-depleted water. ^{2}H-NMR spectra were acquired on a Bruker DSX-300 spectrometer with a probe equipped with a 5-mm solenoid coil. The quadrupolar echo sequence was used with a 90° pulse between 2.7 and 3.0 μs, an interpulse delay of 35 μs, and a recycling time of 0.5 s. After the second pulse, 8192 points were acquired in quadrature with a dwell time of 0.5 μs. The number of scans was at least 20,000. The sample temperature was regulated using a Bruker variable temperature controller. Spectral de-Pakeing was performed as described by Sternin et al. (1983). The quadrupolar splittings obtained from the ^{2}H-NMR spectra provide a measurement of the anisotropy of C—D bond motion. The order parameter of the C—D bond, $S_{C—D}$, is expressed by

$$S_{C-D} = \frac{\langle 3\cos^2\theta - 1\rangle}{2} = \frac{4}{3}\frac{\Delta\nu_q}{A_q},$$

where A_q is the static quadrupolar splitting constant (167 kHz for a C—D bond), $\Delta\nu_q$ is the quadrupolar splitting measured for a lipid oriented at 90° relative to the magnetic field, and θ is the angle between the C—D bond and the bilayer normal. The brackets represent the averaging over the NMR time scale. The de-Paked spectra were used to determine the smoothed orientational order profile, according to a procedure described elsewhere (Lafleur et al., 1989). This approach assumes a monotonic decrease of order along the chain and therefore reproduces only the smoothed features of the order variation (Lafleur et al., 1989).

RESULTS

Infrared spectroscopy

Thermotropism as probed by $\nu_{C—H}$

The variation of the position of the C—H stretching bands ($\nu_{C—H}$) of the methylene groups of the lipid acyl chain is a parameter that allows the characterization of lipid polymorphism in a straightforward way. From this parameter, the transitions from gel-to-liquid crystalline phase and lamellar-to-hexagonal phase are easily detectable (Umemura et al., 1980; Mantsch et al., 1981; Mantsch and McElhaney, 1991). The position of these bands has been mainly related to conformation order of lipid acyl chains, even though other phenomena may contribute to a band shift (Kodati et al., 1994). Fig. 1 shows the variation of the position of the symmetric $\nu_{C—H}$ band as a function of temperature for

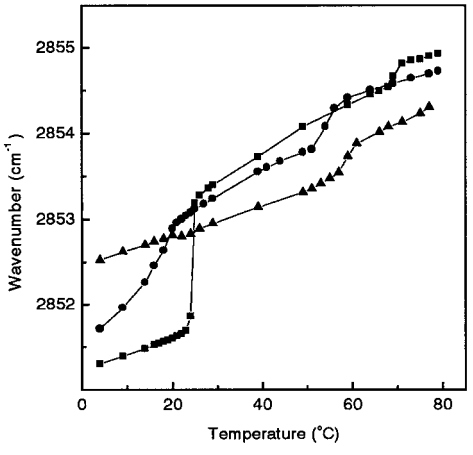

FIGURE 1 Variations of the symmetric $\nu_{C—H}$ band position as a function of temperature for pure POPE (■), POPE/20 mol % chol (●), and POPE/45 mol % chol (▲).

POPE and POPE containing 20 or 45 mol % cholesterol. Similar variations were observed from the antisymmetric $\nu_{C—H}$ band (data not shown). The gel-to-liquid crystalline phase transition of pure POPE is observed at 25°C as illustrated by the sharp increase of $\nu_{C—H}$ frequency by ~1.5 cm^{-1}. The lamellar-to-hexagonal transition occurring at 70°C is associated with the small upshift of the symmetric $\nu_{C—H}$ band; the amplitude of the shift is smaller than that observed for the gel-to-liquid crystalline phase transition because the acyl chain order is less perturbed during the lamellar-to-hexagonal phase transition (Mantsch et al., 1981). Cholesterol considerably modifies the polymorphism as studied by IR spectroscopy. First of all, cholesterol induces an increase of the frequency of the $\nu_{C—H}$ at temperatures below Tm whereas it causes a decrease of this frequency at temperature higher than Tm. This behavior is similar to that observed for PC bilayers (Umemura et al., 1980; Cortijq and Chapman, 1981). These changes lead to the reduction of the frequency shift during the transition, which is not detectable anymore when the cholesterol content is 45 mol %. It is also noted that, at 20 mol % chol, the transition is broaden and shifted toward lower temperatures; the steep frequency variation is over at ~21°C. These changes relative to Tm are in agreement with previous results obtained by DSC with unsaturated PE (Epand and Bottega, 1987; Takahashi et al., 1996; McMullen and McElhaney, 1997). The decrease in frequency shift amplitude is interpreted as a reduction of the conformational disorder introduced during the transition. This may be related to the decrease in the enthalpy variation during the main transition observed in the presence of cholesterol, this variation being mainly associated with the introduction of gauche conformers in the lipid acyl chains. Cholesterol also affects the lamellar-to-H_{II} phase transition. The transition temperature is shifted from 70°C for pure POPE to 55°C for POPE/20 mol % chol mixture. This shift is accompanied by an augmentation of the $\nu_{C—H}$ shift amplitude during this transition, increasing from 0.2 to 0.5 cm^{-1}. For 45 mol % cholesterol, Th increases back to 58°C and the $\nu_{C—H}$ shift amplitude is 0.4 cm^{-1}. It is interesting to note that this cholesterol content causes the abolition of the gel-to-fluid phase transition whereas the lamellar-to-H_{II} phase transition remains cooperative. Fig. 2 reports in more details the variations of Th and of the $\nu_{C—H}$ shift amplitude observed for POPE containing different cholesterol proportions. The effect is not monotonous, but for both parameters, there is an inversion of the trend at ~30 mol % chol. Between 0 and 30 mol % chol, Th decreases and the amplitude of the transition increases, whereas between 30 and 45 mol % cholesterol, Th increases and the shift amplitude decreases. The variation of Th observed in the presence of cholesterol is in agreement with DSC results obtained on POPE (Epand and Bottega, 1987) and 1,2-dielaidoyl-*sn*-phosphatidylethanolamine (DEPE) (Cheetham et al., 1989; Takahashi et al., 1996).

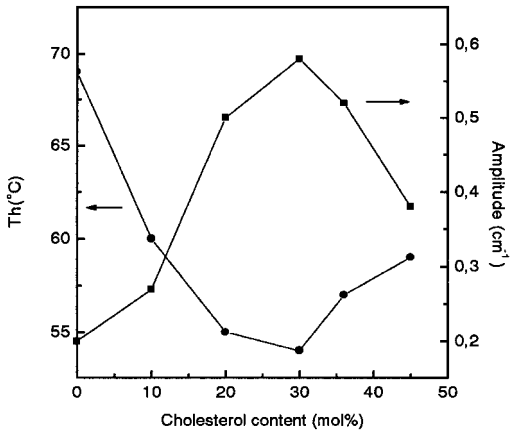

FIGURE 2 Influence of cholesterol on the lamellar-to-hexagonal phase transition temperature (±1°C; ●) and on the amplitude of the symmetric $\nu_{C—H}$ frequency shift during this transition (±0.03 cm^{-1}; ■).

Carbonyl bands

The region of the lipid carbonyl stretching ($\nu_{C=O}$) band is a sensitive probe of the interface hydration. It is well established that this band has two components. The high-frequency component observed at 1740 cm^{-1} is associated with free carbonyl groups whereas the low-frequency component observed around 1725 cm^{-1} is associated with carbonyl groups forming hydrogen bonds with water molecules (Blume et al., 1988; Lewis et al., 1994). Fig. 3 shows the self-deconvoluted lipid carbonyl bands for pure POPE and POPE containing 20 and 45 mol % cholesterol. In the gel phase (5°C), the band for pure POPE is dominated by the high-frequency component, indicative of the tight lipid packing. When cholesterol is added, there is an increase of the low-frequency component intensity, suggesting that the presence of cholesterol, at low temperature, leads to a looser lipid packing and a greater fraction of the carbonyl groups being exposed to water. In the fluid phase (40°C), the relative intensity of the low-frequency component increases significantly relative to that observed in the gel phase; this can be associated with the greater extent of water penetration at the interface level in the fluid phase. In the fluid phase, cholesterol has the opposite effect: the sterol leads to a decrease of the relative intensity of the low-frequency component. This can be associated with the ordering of the fluid phase leading to a reduced hydration of the interface. Actually, it turns out that heating POPE/45 mol % chol mixture from 5 to 40°C has very little effect in the $\nu_{C=O}$ region. It is consistent with the absence of phase transition reported from the $\nu_{C—H}$. In the H_{II} phase (78°C), the intensity of the low-frequency component of pure POPE is decreased relative to that in the fluid lamellar phase, indicating that the proportion of hydrogen-bonded carbonyl groups decreases. In the presence of cholesterol, there is a small decrease of the intensity of the low frequency component, suggesting a reduced hydration in the presence of cholesterol. A reduction of the hydration of POPE in the

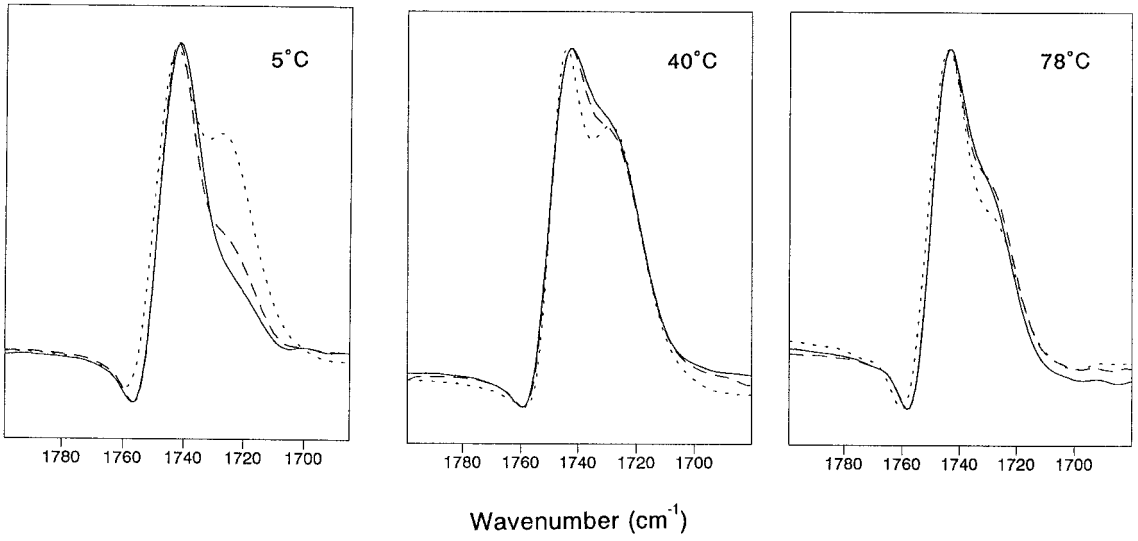

FIGURE 3 Carbonyl stretching regions for pure POPE (——), POPE/20 mol % chol (- - -), and POPE/45 mol % chol (· · ·) in the gel (5°C), the fluid lamellar (40°C), and the H_{II} (78°C) phase. The spectra have been corrected for water contribution and Fourier self-deconvoluted as described in Materials and Methods.

presence of cholesterol has been reported for the fluid lamellar and the H_{II} phases for low-hydration samples (15 water molecules per POPE) based on results obtained by ^{2}H-NMR of D_2O (Marinov and Dufourc, 1995).

NMR spectroscopy

^{2}H-NMR spectroscopy has been used to identify the different phases formed by the lipid systems. It is a powerful technique because the lamellar gel, the lamellar fluid, and the H_{II} phases provide distinct ^{2}H-NMR signatures. Fig. 4 displays typical spectra obtained from our samples. For pure POPE-d_{31} (bottom line), three different profiles are observed. At 5 and 15°C, broad and almost featureless spectra are obtained; these are typical of the gel phase where the chains are almost all-*trans* and the rotational diffusion is slow (Davis, 1983). At 32°C, the spectrum is typical of the fluid lamellar phase, where the fast rotation of the lipid along the bilayer normal (fast on the NMR time scale) gives rise to a set of powder patterns typical of axially symmetric systems, representative of the orientational order profile along the lipid acyl chains (Davis, 1983; Lafleur et al., 1989). At 70°C, the spectrum still indicates the axial symmetry of the lipid motion, but the width of the spectrum is reduced by a factor larger than two essentially due to the lipid diffusion around the H_{II} cylinder and the increased motional freedom (Lafleur et al., 1990b). The resolution of the powder patterns associated with the different CD_2 groups along the perdeuterated chain is reduced due to the more linear decrease of the order along the acyl chain (Lafleur et al., 1990b). The presence of cholesterol induces several changes in the spectra. At 5 and 15°C, cholesterol leads to the transformation of the broad gel-phase spectrum to a broad spectrum characteristic of lipids experiencing axially symmetric motion. Actually, at 45 mol % cholesterol, the spectra recorded at these temperatures are exclusively composed of a fluid component. At 5°C, the spectrum shows a $\Delta\nu_q$ of 50 kHz for the outermost doublet. A similar shape change of the NMR signal has also been observed for specifically labeled 2[4,4-^{2}H]dipalmitoylphosphatidylethanolamine (2[4, 4-^{2}H]DPPE) in the presence of cholesterol (Blume and Griffin, 1982). We observe that the transformation toward a spectrum typical of a fluid phase species appears to be completed at lower temperatures for higher cholesterol content (e.g., comparing 10 and 25 mol % at 15°C). At intermediate temperature and cholesterol content, the spectra show the coexistence of a gel-like and a fluid-like component (POPE/25 mol % chol mixture at 5°C and POPE/10 mol % chol mixture at 15°C, for example). The two components of such spectra can be isolated by spectral differences as previously shown for the DPPC/chol system (Vist and Davis, 1990). Fig. 5 illustrates an example of the process. The spectra of POPE-d_{31} containing 10 and 15 mol % cholesterol, recorded at 15°C, are composed of a gel and a fluid component; this is especially clear by looking at the central signal associated with the terminal methyls. The components obtained by spectral difference are displayed on the right side. From the subtractions, it is possible to estimate the cholesterol content corresponding to each component (Vist and Davis, 1990). At 15°C, our results indicate a cholesterol content of 5 and 20 mol % for the gel and the fluid component, respectively. These values should correspond to the borders of the coexistence region. This spectral difference approach was done at 5, 10, and 15°C.

The third column of Fig. 4 shows the spectra of the various POPE-d_{31}/chol mixtures recorded at 32°C. All of these are typical of lamellar-phase lipids experiencing axially symmetric motion. Cholesterol leads to an increase of

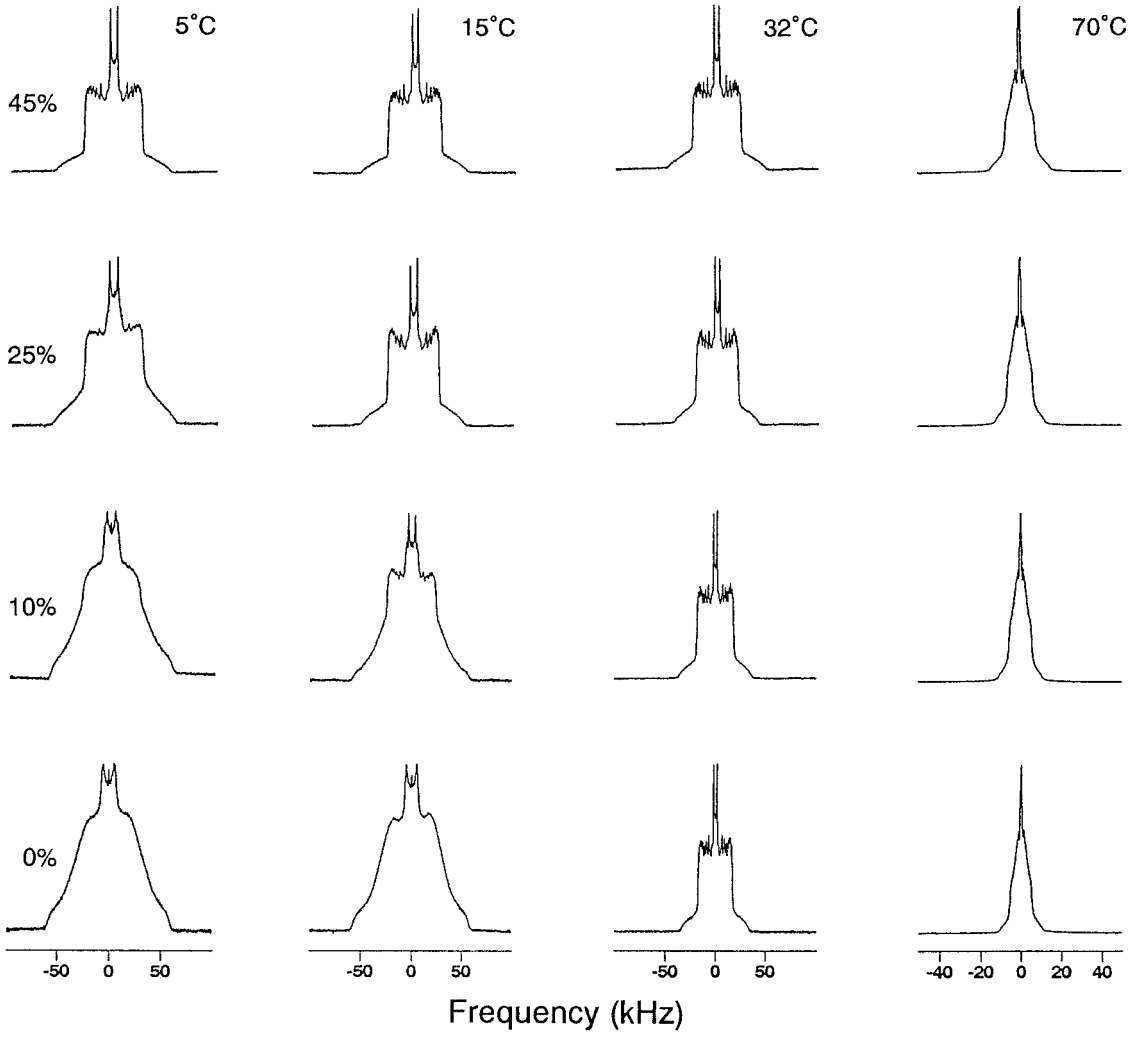

FIGURE 4 ^{2}H-NMR spectra of POPE-d_{31} containing various proportions of cholesterol (as indicated for each row), recorded at different temperatures (as indicated at the top of each column). Note that the frequency scale is different for the right column.

the quadrupolar splittings. For example, the quadrupolar splitting of the outermost doublet increases from 35 to 49 kHz when cholesterol content increases from 0 to 45 mol %. Finally, at 70°C, all of the spectra show a shape typical of the H_{II} phase. Similar to the behavior in the fluid lamellar phase, the presence of cholesterol leads to an increase of quadrupolar splittings as illustrated by the widening of the signal.

From the spectra in the fluid lamellar and the H_{II} phases, it is possible to extract smoothed order profiles that describe the variation of orientation order along the lipid acyl chains (Lafleur et al., 1989). The order profiles are shown for the fluid lamellar phase (32°C; Fig. 6 *A*) and the H_{II} phase (70°C; Fig. 6 *B*). The order profiles obtained at 32°C are typical of the lamellar phase, showing a plateau region where the order parameter does not vary considerably followed by a rapid decrease of the order toward the middle of the bilayer. The order profile of POPE-d_{31} is similar to that previously published (Lafleur et al., 1990a). As it has been observed for PCs (Vist and Davis, 1990; Lafleur et al., 1990a), cholesterol leads to an increase of $S_{C—D}(n)$ all along the chain of POPE-d_{31}. This result is in agreement with a previous study on 2[4,4-^{2}H]DPPE showing an increase of $\Delta\nu_q$ in the presence of cholesterol for a temperature greater than Tm of the phospholipid (Blume and Griffin, 1982). The change of overall order parameter of POPE-d_{31}, $\langle S_{C—D}\rangle$, as a function of cholesterol content is shown in Fig. 7 *A*. $\langle S_{C—D}\rangle$ represents the arithmetic average of $S_{C—D}(n)$ for $2 \leq n \leq 16$, $S_{C—D}(16)$ being linearly extrapolated from $S_{C—D}(14)$ and $S_{C—D}(15)$ to avoid the contribution of the additional motion of the terminal CD_3 methyl groups. Results obtained for POPC-d_{31} in the presence of cholesterol (Lafleur et al., 1990a) are reproduced for comparison. The order of pure POPE-d_{31} is greater than that observed for POPC-d_{31} due to the fact that the smaller effective size of the PE polar headgroup leads to a tight lipid packing and, as a consequence, restricted chain motions (Perly et al., 1985; Lafleur et al., 1990a). Cholesterol in-

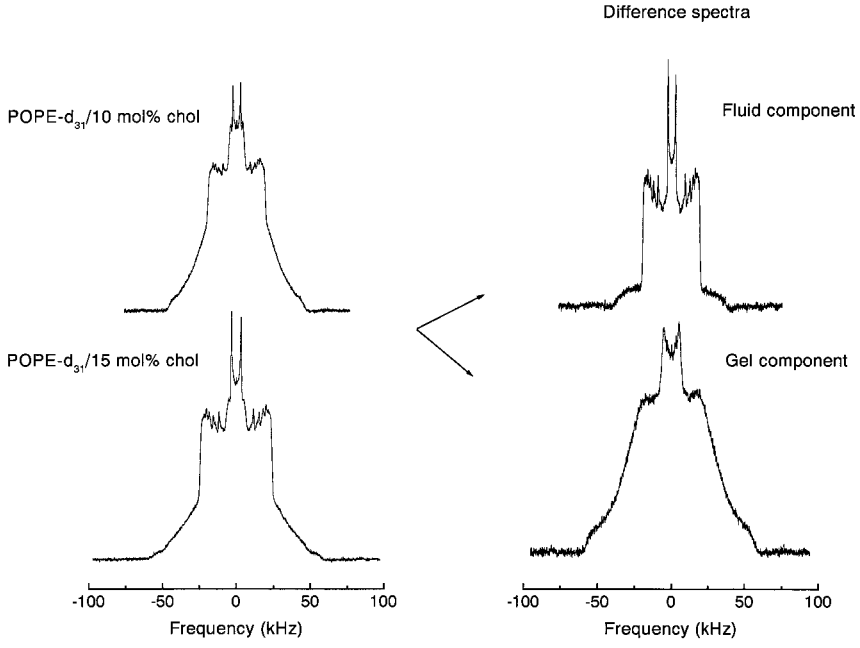

FIGURE 5 ^{2}H-NMR spectra of POPE-d_{31}/10 mol % chol and POPE-d_{31}/15 mol % chol at 15°C (left) and difference spectra (right). The gel component is obtained from the following subtraction: spectrum of POPE-d_{31}/10 mol % chol − 0.53 spectrum of POPE-d_{31}/15 mol % chol. The fluid component is obtained from the following subtraction: spectrum of POPE-d_{31}/15 mol % chol − 0.48 spectrum of POPE-d_{31}/10 mol % chol.

duces an ordering of the acyl chains for both phospholipids, but the magnitude of the increase of $\langle S_{C—D} \rangle$ is more pronounced for POPC-d_{31} than for POPE-d_{31}. From 0 to 45 mol % cholesterol, $\langle S_{C—D} \rangle$ increases by ~37% for POPE-d_{31} and by ~85% for POPC-d_{31}. Similarly, POPE-d_{31} and POPC-d_{31}/20 mol % chol have very similar orientational chain order at ~32°C, a value of $\langle S_{C—D} \rangle$ of ~0.215. The addition of an extra 10 mol % cholesterol to these two bilayers leads to a value of 0.232 and 0.250 for the POPE-containing and the POPC-containing bilayers, respectively. The chain order becomes very similar for both lipids when the cholesterol content is at ~45 mol %. In these conditions, $\langle S_{C—D} \rangle$ is ~0.29 and the order parameter at the plateau region is ~0.4, indicating very ordered lipid chains.

As can be seen in Fig. 6 *B*, the variation of order along the phospholipid acyl chain is more linear in the H_{II} phase, in

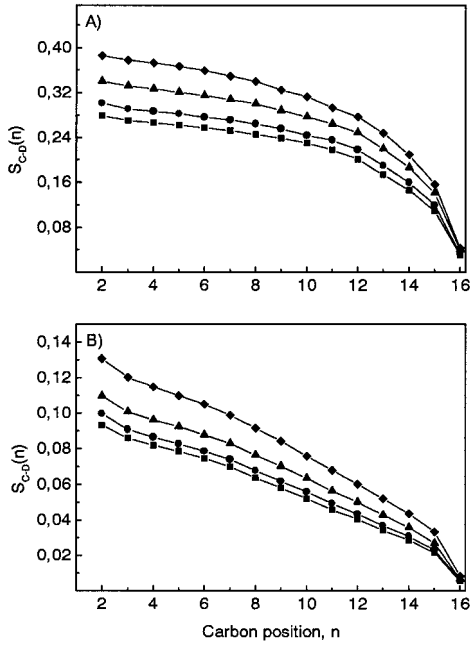

FIGURE 6 Smoothed orientational order profile obtained at 32°C (*A*) and 70°C (*B*) for POPE-d_{31} (■), POPE-d_{31}/10 mol % chol (●), POPE-d_{31}/25 mol % chol (▲), and POPE-d_{31}/45 mol % chol (♦).

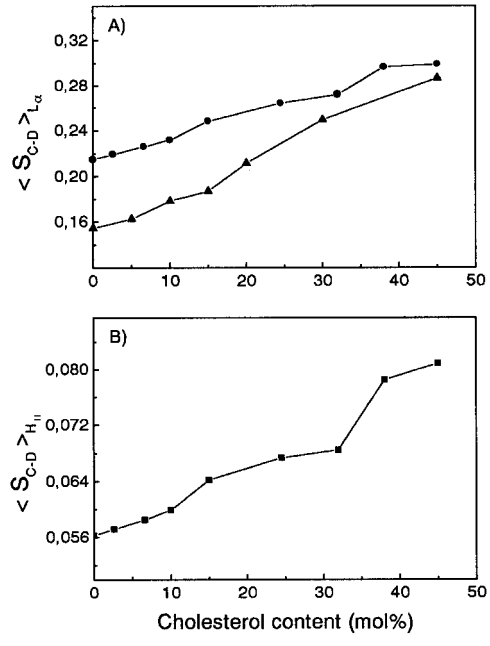

FIGURE 7 Variation of $\langle S_{C—D} \rangle$ as a function of cholesterol content in the lamellar phase (*A*) for POPE-d_{31} (32°C; ●) and POPC-d_{31} (▲) (from Lafleur et al., 1990a, at 30°C) and in the H_{II} phase (*B*) for POPE-d_{31} (70°C; ■).

agreement with previous observations (Lafleur et al., 1990b; Thurmond et al., 1990). Our results show that cholesterol maintains its ordering effect on the lipid acyl chains in the H_{II} phase. The increase of $S_{C—D}(n)$ is observed all along the chain. The addition of 45 mol % chol to POPE-d_{31} at 70°C, i.e., in the H_{II} phase, leads to an increase of $\langle S_{C—D} \rangle$ by 47%, from 0.056 to 0.081. The increase of $\langle S_{C—D} \rangle$ as a function of cholesterol content in the H_{II} phase is summarized in Fig. 7 *B*.

DISCUSSION

Lamellar phases

At temperatures below Tm of pure POPE, both IR and NMR results indicate that cholesterol perturbs the gel phase. By IR spectroscopy, the increase of $\nu_{C—H}$ frequency suggests an increase of conformational disorder whereas the $\nu_{C=O}$ region indicates an increased hydration at the carbonyl level. The ^{2}H-NMR results indicate that cholesterol increases the motion rate of the gel-phase lipids, giving rise to spectra associated with axially symmetric motions, similarly to previous observations on PC/chol systems (Vist and Davis, 1990; Thewalt and Bloom, 1992). At temperatures higher than Tm of pure POPE, our results indicate that cholesterol increases the order of the fluid-phase lipids. This is shown by the decrease of the $\nu_{C—H}$ frequency in the presence of cholesterol and the increase of orientational lipid chain order. This chain ordering effect causes also the reduction of the hydration of the carbonyl groups, in agreement with a previous study on low-hydration POPE samples (Marinov and Dufourc, 1996). Our results on POPE/chol system lead to the same conclusion as that proposed in the pioneer studies on the effect of cholesterol on PC bilayers (Umemura et al., 1980; Cortijq and Chapman, 1981): the sterol disorders the gel phase and orders the fluid phase.

These opposite effects lead to the abolition of the gel-to-liquid crystalline phase transition. In the IR data, the sharp shift of $\nu_{C—H}$ is no longer observed for POPE/45 mol % chol mixture, and in the NMR data, the spectra of this mixture show axial symmetry over a wide temperature range. The decrease of the transition amplitude and its eventual disappearance in the presence of cholesterol have been previously reported for various unsaturated PEs, using DSC (Epand and Bottega, 1987; Cheetham et al., 1989; McMullen and McElhaney, 1997), x-ray diffraction (Takahashi et al., 1996), and NMR (Ghosh and Seelig, 1982; Blume and Griffin, 1982). At high cholesterol content, the ^{2}H-NMR spectra indicate that the PE molecules in the mixture have axially symmetric motion relative to the normal of the bilayer, like in a fluid phase, but the values of the order parameters are high, indicating ordered chains. These characteristics are strikingly similar to those of the proposed liquid-ordered phase previously described for PCs (Vist and Davis, 1990; Thewalt and Bloom, 1992; Linseisen et al., 1993).

These findings are combined in the temperature-composition diagram shown in Fig. 8. Below Tm, three regions are defined: a gel phase, a fluid-ordered phase, and a coexistence region. The diagram differs from the one derived from DSC data (Epand and Bottega, 1987). This is somehow expected as, at high cholesterol concentration, the transition is abolished and, as a consequence, the calorimetry data provide little information. The lower part of the proposed temperature-composition diagram shows similar trends to the one proposed for the 1,2-dipalmitoyl-*sn*-phosphatidylethanolamine (DPPE)/chol system (Blume and Griffin, 1982). We have included in the temperature-composition diagram the end points obtained from spectral subtraction (as exemplified in Fig. 5) as well as symbols reporting the phase(s) present in the sample, as determined by visual inspection of the ^{2}H-NMR spectra. In general, both approaches are consistent. This good agreement is shown by the fact that the difference spectrum representative of one phase and the experimental spectrum of a sample with the corresponding amount of cholesterol were always very similar. For the liquidus line, there is a good agreement between the limits obtained from the spectral difference and the limits that can be inferred from the visual inspection of the spectra. For the solidus line, the subtraction of the spectrum of the gel component from an experimental spectrum from

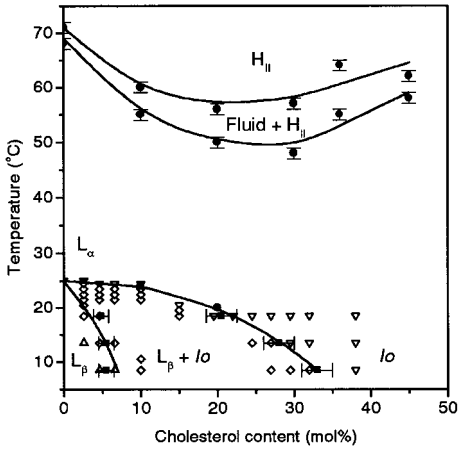

FIGURE 8 Proposed temperature-composition diagram for POPE/chol system. ●, limit of a transition as detected by IR spectroscopy; ■, obtained by spectral subtraction as illustrated in Fig. 5; △, conditions for which a spectrum typical of a gel phase is recorded; ▽, conditions for which a spectrum typical of a fluid phase is recorded; ◇, conditions for which a spectrum typical of the coexistence of a gel and a fluid phase is recorded, as determined by visual inspection. The temperatures associated to the NMR data were systematically increased by 3.5°C, the difference between the Tm of POPE and POPE-d_{31}, to include on the same figure the NMR and IR data. The error bars represent the composition uncertainty related to the determination of the subtraction factor. The lines on the diagram define regions over which the same phase(s) is(are) identified. These phase boundaries should be regarded with caution considering the limitations of the techniques used, as discussed in the paper; these include the impossibility of identifying the coexistence of fluid lamellar phases and the fact that the indirect information relative to the lamellar-to-H_{II} phase transition obtained from IR spectroscopy leads to a coexistence region that does not respect the phase rules, suggesting a more complex behavior.

a sample with the corresponding cholesterol content led to very little residual signal. However, a close inspection of the experimental spectra with cholesterol content defined by spectral subtraction indicates a slightly different position for the border between the L_β and the coexistence regions. The case for which the discrepancy was the most pronounced was for the gel end point at 15°C; the cholesterol content for the end point was estimated to be 5 mol % by spectral subtraction, but the experimental spectrum of the sample containing 5 mol % chol displayed a contribution of fluid lipids evaluated to be ~9% based on the relative area. This could be due to the fact that, at this temperature, the sample containing 15 mol % chol is close to the liquidus line; in such a situation, the fast lipid exchange between the phases may affect the signal shape, and the spectra close to the boundaries should be considered with caution (Blume and Griffin, 1982). This effect may lead to some deviations as discussed previously (Morrow et al., 1991; Linseisen et al., 1993).

Our results in the lamellar phase of the POPE/chol system reveal several features. First, at cholesterol content greater than ~35 mol %, POPE molecules form a fluid phase where the acyl chains are very ordered. From both the NMR and IR spectroscopy point of view, the organization of POPE bilayers containing ≥35 mol % chol is very similar to that observed with PCs. Inspired by recent papers on PC/chol systems (Vist and Davis, 1990; Thewalt and Bloom, 1992; Linseisen et al., 1993), this region of the temperature-composition diagram is referred to as a liquid-ordered phase. Therefore, the formation of such a phase does not seem to be exclusive to PC species, and this work extends this behavior to POPE. Second, our results suggest that the coexistence region of the gel and the liquid-ordered phases is different compared with that observed with PCs. The data do not suggest the presence of an eutectic, as proposed by Vist and Davis (1990) for the DPPC/chol system. The transition from a gel to a fluid phase of POPE-d_{31}, as probed by the shape of the ^{2}H-NMR spectra, is broadened progressively upon the addition of cholesterol. The transition occurs over 6°C when the sample contains as little as 3 mol % chol and over more than 10°C when the cholesterol proportion is 5 mol %. The proposed phase diagrams for several PC/chol systems (Ipsen et al., 1987; Vist and Davis, 1990; Thewalt and Bloom, 1992; Linseisen et al., 1993) also include a three-phase horizontal line representing the coexistence of the L_β, L_α, and liquid-ordered phases. This line stretches roughly from 5 to 20% chol. A similar line is proposed in the temperature-composition diagram of the DPPC/chol system proposed by McMullen and McElhaney (1995) except it begins at 0 mol % chol. Upon heating a sample, crossing this line is associated with the sharp peak observed by DSC. Our results do not suggest the presence of a similar horizontal line for the POPE/chol system. The completion of the gel-to-fluid phase transition is observed at lower temperatures when POPE contains more than 10 mol % chol. Therefore, a three-phase line is much shorter or absent in the POPE/chol temperature-composition diagram. This conclusion is supported by the DSC results obtained with unsaturated PE/chol systems (Epand and Bottega, 1987; Takahashi et al., 1996; McMullen and McElhaney, 1997). The addition of cholesterol to PE shifts the endotherm associated with the gel-to-fluid phase transition toward lower temperatures, and no sharp component is observed at the Tm of the pure phospholipid when cholesterol content is higher than 10 mol %. The liquidus boundary shows also a different shape for cholesterol proportion higher than 10 mol % compared with those in the proposed phase diagrams of PC/chol systems (Vist and Davis, 1990; Thewalt and Bloom, 1992; Linseisen et al., 1993). For the POPE/chol system, the proportion required to eliminate the gel-phase component from the spectrum is dependent on temperature; below Tm of the pure lipid, smaller proportions of cholesterol are needed to obtain exclusively a spectrum typical of a fluid phase at higher temperatures, as previously observed for the DPPE/chol system (Blume and Griffin, 1982). It is somehow different from the behavior reported for cholesterol-containing PC (Vist and Davis, 1990; Linseisen et al., 1993) for which a relatively constant cholesterol content is necessary to lead to a completely fluid (liquid-ordered) sample. This is clearly observed on the 1-stearoyl-2-elaidoyl-*sn*-glycero-3-phosphocholine/chol partial phase diagram (Linseisen et al., 1993) for which data were recorded 15°C below Tm.

Two comments should be added. First, the part of the proposed temperature-composition diagram at low cholesterol and around Tm of the pure phospholipid is simpler than those for PCs because POPE does not form a P_β (ripple) phase. Second, we have not included in the temperature-composition diagram regions where fluid phases coexist even though such regions must be present according to the phase rules. Such regions have been proposed for PC/cholesterol systems (Ipsen et al., 1987; Vist and Davis, 1990; McMullen and McElhaney, 1995). Because it is difficult experimentally to infer the existence of such regions, we make no attempt to define such regions in the POPE/chol temperature-composition diagram. Along the same line, coexisting liquid-ordered phases between which fast lipid exchange would occur cannot be detected by our approach.

From a molecular point of view, our results indicate that the ordering effect of cholesterol is less pronounced on POPE than on POPC. The weaker effect of cholesterol on the POPE matrix relative to the POPC one may be at the origin of the differences observed in the temperature-composition diagrams. It is interesting to note that both phospholipids converge toward similar orientational order at high cholesterol content; at 45 mol %, $\langle S_{C—D} \rangle$ is 0.29 and the orientational order in the plateau region is ~0.4, indicating a very ordered lipid. A 1-palmitoyl-2-oleoyl-phospholipid in a fluid bilayer should have, at a given temperature, an order parameter limit corresponding to a very tight chain packing. The $\langle S_{C—D} \rangle$ values calculated for POPE-d_{31} and POPC-d_{31} both containing 45 mol % cholesterol may approach this situation. Because of the relationship between

$S_{C—D}$ and bilayer thickness (Ipsen et al., 1990; Thurmond et al., 1991; Douliez et al., 1995), this implies also approaching a limit thickness for a fluid bilayer. Using the approach proposed by Douliez et al. (1995), we have estimated the effect of cholesterol on chain length for POPE and POPC, at 30°C. We have assumed that the molecular order parameter S_{mol} equals 1. The chain length of POPE-d_{31} is 14.4 and 16.1 Å for the pure lipid and the lipid containing 45 mol % chol, respectively; this corresponds to a 12% increase. In the case of POPC-d_{31}, the calculated chain length is 13.3 and 15.9 Å for the pure lipid and the lipid containing 45 mol % chol, respectively, an increase of 20%. These results indicate the more pronounced effect of cholesterol on POPC than on POPE, and the convergence toward a thick bilayer.

Lamellar-to-hexagonal phase transition

Cholesterol also has an influence on the lipid propensities to form nonlamellar phases. Between 0 and 30 mol % cholesterol, the Th of POPE, as detected by IR spectroscopy, is shifted toward lower temperatures whereas a larger amount of cholesterol leads to an increase of Th. This behavior has been already observed for various PEs by DSC (Epand and Bottega, 1987; Cheetham et al., 1989) and x-ray diffraction (Takahashi et al., 1996). These results are included in the temperature-composition diagram (Fig. 8). The symbols represent the beginning and the end of the more abrupt variation of $\nu_{C—H}$ as a function of temperature, this shift being associated with the lamellar-to-H_{II} phase transition. The NMR data confirm that, at 70°C, H_{II} phases are formed by POPE-d_{31} for cholesterol content varying from 0 to 45 mol %. The lines on the diagram should be considered as guidelines linking the points and do not necessarily correspond to the phase boundaries. The situation is likely more complex in this region of the diagram as the coexistence zone does not respect the phase rules. The present results reveal the influence of cholesterol on the lamellar-to-H_{II} phase transition of POPE and provide molecular details about this transition. First, the results reveal that cholesterol maintains its ordering effect of the phospholipid acyl chains; this is inferred from the increase of quadrupolar splittings observed by ^{2}H-NMR and from the shift of $\nu_{C—H}$ toward lower frequencies in the IR spectra. The variation in lipid chain length induced by the presence of cholesterol can be estimated from the order parameters, using a similar approach to that proposed in the L_α phase (Douliez et al., 1995), as the motions leading to the quadrupolar interaction averaging are similar in both phases. However, the order parameters measured in the H_{II} phase have to be multiplied by a factor of 2 to take into account the additional averaging introduced by the fast diffusion of the lipid molecules around the H_{II} cylinders (Thurmond et al., 1990; Lafleur et al., 1996). At 70°C, the chain length is estimated to be 12.5 and 13.4 Å for pure POPE-d_{31} and POPE-d_{31}/45 mol % chol mixture, respectively. The straightening effect of cholesterol is maintained in the H_{II} phase, but it is not as pronounced as in the L_α phase. The tighter lipid packing around the H_{II} cylinder is accompanied by a limited dehydration of the carbonyl region as observed by IR spectroscopy. A headgroup dehydration of POPE in the presence of cholesterol in the H_{II} phase has been also suggested by the ^{2}H-NMR of D_2O on low-hydration samples (Marinov and Dufourc, 1996). This region of the IR spectrum shows that, for pure POPE, there are fewer carbonyl groups participating in hydrogen bonds in the H_{II} phase than in the L_α as indicated by the weak relative intensity of the low-frequency component. This change during the lamellar-to-hexagonal phase transition is concomitant with a shift of the PO_2^- antisymmetric stretching band going from $\sim$1221 cm^{-1} in the lamellar phase to $\sim$1225 cm^{-1} in the H_{II} phase (data not shown). Actually, an abrupt increase of the frequency of this vibrational mode is observed during the lamellar-to-H_{II} phase transition, in agreement with a previous report on egg PE (Castresana et al., 1992). A shift toward high frequencies has been associated with weaker hydrogen bonding of the phosphate (Sen et al., 1988; Wong and Mantsch, 1988), which is consistent with the dehydration inferred from the carbonyl stretching region. It is therefore expected that cholesterol does not lead to a pronounced dehydration of this phase, which has already limited hydration.

The variation of Th as a function of cholesterol content is not monotonous. Two parameters will affect this transition. First, the presence of cholesterol shifts the amphiphilic balance as this molecule has a small headgroup and a considerable apolar core. In terms of the molecular shape model, cholesterol has a high packing parameter (Israelachvili et al., 1976). In terms of the curvature model, cholesterol should reduce the spontaneous radius of curvature of the lipid layer, R_0. This deduction has been verified with DEPE/chol systems, the x-ray diffraction data showing a decrease of the H_{II}-intercylinder spacing as a function of cholesterol content (Takahashi et al., 1996). This decrease is fairly linear over the cholesterol content varying from 0 to 40 mol %. This contribution should favor the formation of the H_{II} phase and seems to be the most significant to explain the decrease in Th observed in the presence of cholesterol. Second, we show here that cholesterol has an ordering effect of the phospholipid acyl chains in the H_{II} phase, despite the different geometry. It is generally accepted that the disordering of the acyl chains (with increasing temperature, introduced by the presence of unsaturations) favors the formation of the H_{II} phase by reducing R_0 (Rilfors et al., 1984; Lafleur et al., 1996). If an analogous interpretation is made, the ordering effect of cholesterol in the H_{II} phase should be unfavorable to its formation. This factor does not seem to contribute significantly for cholesterol content lower than 30 mol % as a decrease in Th is observed. It may, however, be involved in the increase of Th observed for a higher proportion of cholesterol. It is interesting to note that the amplitude of the chain order observed during the lamellar/H_{II} phase transition, as probed with the symmetric $\nu_{C—H}$, varies in a concerted manner with Th (Fig. 2). For low cholesterol content, the amplitude increases; this can be

rationalized on the basis that the lamellar phase becomes more ordered, and the structural change toward a phase for which the geometry allows considerable space for chain motions leads to the introduction of increasing chain disordering. For cholesterol content between 30 and 45 mol %, the amplitude of the $\nu_{C—H}$ shift during the lamellar/H_{II} phase transition decreases. The NMR spectra show that, in these conditions, the relative ordering of the phospholipid acyl chains in the H_{II} phase becomes more important than in the lamellar phase. This may be related to reaching a limit of chain packing in the lamellar phase whereas the cylindrical geometry of the H_{II} phase leaves some space for additional ordering. The variation of the enthalpy associated with the lamellar-to-H_{II} phase transition of POPE in the presence of cholesterol does not vary linearly between 0 and 45 mol % chol (Epand and Bottega, 1987), and it may be influenced by the extent of chain disordering during the transition. Finally, other factors, such as the influence of cholesterol on the curvature modulus Kc and cholesterol phase separation, should be examined. Additional characterization is being currently done.

The present work reports the characterization of the effect of cholesterol on PE acyl chains in the gel, the liquid-crystalline, and the H_{II} phases. The combined investigation of the phase transitions by IR spectroscopy and the phase characterization by ^{2}H-NMR leads to the conclusion that POPE forms a liquid-ordered phase in the presence of high cholesterol content. The formation of a lipid matrix with such properties is not related to a peculiar behavior of the PC species, and this conclusion reinforces the significance of this phase in biological membranes. It is significant to extrapolate the formation of liquid-ordered phase in the presence of cholesterol to PE matrices as PE is almost as abundant as PC species in certain membranes and it even constitutes the main phospholipid of the internal leaflet of the human erythrocyte plasma membrane. It should be noted, however, that this behavior is linked to the solubilization of a high concentration of cholesterol in fluid phospholipid membranes, and it is not clear that systems with limited cholesterol solubility (McMullen and McElhaney, 1997) would display the same trends. We also report that cholesterol maintains its acyl chain ordering effect in the H_{II} phase, and this phenomenon must be included in a detailed molecular description of the H_{II} cylinders formed by PE/cholesterol mixtures.

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