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Role of the Stereochemistry of the Hydroxyl Group of Cholesterol and the Formation of Nonbilayer Structures in Phosphatidylethanolamines†

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ABSTRACT: The phase behavior of mixtures of cholesterol or epicholesterol with phosphatidylethanolamine was studied by differential scanning calorimetry and by X-ray diffraction. Discrete domains of cholesterol are detected by X-ray diffraction in the L_α phase of phosphatidylethanolamine from egg yolk and synthetic dielaidoylphosphatidylethanolamine beginning at mole fractions of 0.35-0.4 cholesterol. Separate domains of crystalline epicholesterol can also be detected in the L_α phase of dielaidoylphosphatidylethanolamine by X-ray diffraction at as little as 0.16 mole fraction of epicholesterol. This is a result of poor miscibility of the epicholesterol with dielaidoylphosphatidylethanolamine. Epicholesterol does not alter the $L_\beta \rightarrow L_\alpha$ transition or bilayer spacing. Epicholesterol also has little effect on the diameter of the cylinders in the hexagonal phase. Formation of the inverted hexagonal phase is facilitated by addition of small amounts of cholesterol (mole fraction less than 0.2) in both egg phosphatidylethanolamine and dielaidoylphosphatidylethanolamine. However, at higher mole fractions of cholesterol, the stability of the liquid-crystalline phase is found to increase markedly for dielaidoylphosphatidylethanolamine but not for egg phosphatidylethanolamine, indicating the importance of the structure of the acyl chains in controlling the relative stability of the lamellar and nonlamellar phases in these systems. In contrast to cholesterol, epicholesterol markedly lowers the $L_\alpha \rightarrow H_{II}$ phase transition temperature at low mole fraction of sterol. This result demonstrates the importance of the orientation and motional properties of an additive in determining the $L_\alpha \rightarrow H_{II}$ transition temperature.

Although many phospholipids in aqueous dispersion spontaneously form bilayers, it has long been recognized that some of the major lipid components of biological membranes, e.g., phosphatidylethanolamine (PE),[1] spontaneously form nonbilayer phases in purified form and that nonbilayer structures may be important intermediates in membrane function (Cullis & De Kruijff, 1979; Cullis et al., 1980; Hui et al., 1981; Verkleij, 1984; De Kruijff, 1987). The polymorphism of natural and synthetic PE has been reported, and the phase diagrams are well characterized (Shipley, 1973; Seddon et al., 1983; Harlo & Eibl, 1980, 1981; Boggs et al., 1981). Extensive calorimetric studies of the interaction of sterols with synthetic

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[1] Abbreviations: EYPE, egg yolk phosphatidylethanolamine; DEPE, dielaidoylphosphatidylethanolamine; PE, phosphatidylethanolamine; L_β, lamellar-gel phase; L_α, lamellar-liquid-crystalline phase; H_{II}, hexagonal phase, type II; Tris, tris(hydroxymethyl)aminomethane; X(chol), mole fraction of cholesterol; X(epi), mole fraction of epicholesterol; PS, phosphatidylserine; PC, phosphatidylcholine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine.

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forms of PE have also been made (Epand & Bottega, 1987; Blume, 1980). However, similar data on natural PE are lacking. The importance of nonbilayer phases to biological systems is suggested by the observation that the microorganism *Acholeplasma ladilawii* adjusts the lipid composition of its surface membrane to maintain a balance between bilayer and nonbilayer phase formation (Wieslander et al., 1986) although not all strains of this microorganism exhibit this behavior (Bhakoo & McElhaney, 1988). It has also been shown that the activities of certain membrane-bound enzymes, including protein kinase C (Epand et al., 1988), Ca^{2+}-ATPase (Navarro et al., 1984), and dolichylphosphomannose synthase (Jensen & Schutzbach, 1988), are modulated in opposite directions by substances which favor bilayer vs nonbilayer phases in model systems.

The temperature of the phase transition between the bilayer (L_α) and inverted hexagonal phase (H_{II}) is particularly sensitive to the presence of certain additives in the membrane (Epand, 1985). A description of the molecular features which determine the position of the $L_\alpha \rightarrow H_{II}$ equilibrium is being developed. An algorithm based on a qualitative concept of "molecular shape" has been proposed by Israelachvili et al. (1980). More recently, Gruner (1985) has attempted to develop a more quantitative theory which includes the intrinsic radius of curvature, hydrocarbon packing constraints, and hydration. This theory still requires the use of empirically determined parameters. In the present work, we demonstrate the importance of the stereochemistry of an additive on its modulation of the $L_\alpha \rightarrow H_{II}$ transition. In contrast to cholesterol which has little effect on the $L_\alpha \rightarrow H_{II}$ transition temperature, epicholesterol greatly favors formation of the H_{II} phase (Gallay & De Kruijff, 1982). Epicholesterol differs from cholesterol only in the epimerization of the hydroxyl group at position 3. We also examine the separation of a pure sterol phase in these mixed lipid systems. Cholesterol has previously been found to be only partially soluble in phospholipid bilayers, the extent of solubility depending on the species of phospholipid present (Yeagle, 1985; Bach, 1984; Wachtel & Bach, 1987; Bach & Wachtel, 1989).

Materials and Methods

Materials. L-α-Dielaidoylphosphatidylethanolamine (DEPE) was obtained from Avanti Polar Lipids Inc., Birmingham, AL, egg yolk phosphatidylethanolamine (EYPE) was from Lipid Products, South Nutfield, U.K. (grade I), and epicholesterol was from Steraloids Inc., Wilton, NH. Cholesterol was the extrapure grade from Merck and was further purified by recrystallization from ethanol.

Sample Preparation. Phospholipid and sterol were dissolved in solutions of chloroform/methanol, 2/1. Appropriate volumes of phospholipid and sterol solutions were mixed to obtain a suitable range of concentrations. The solvent was evaporated with a stream of dry nitrogen gas, depositing the lipids as a film on the walls of a glass test tube. Samples were then kept for at least 3 h under 0.1 torr to remove any residual traces of solvent. The dried lipid film was suspended by vigorous vortexing at about 45 °C in either 20 mM PIPES, 150 mM NaCl, 0.02 mg/mL NaN_3, and 1 mM EDTA buffer at pH 7.40 [for differential scanning calorimetry (DSC) studies] or 10 mM Tris-HCl and 150 mM NaCl at pH 7.5 (for X-ray studies).

Differential Scanning Calorimetry. Lipid suspension (5 mg/mL) and buffer were degassed under vacuum and then loaded into the sample and reference cells, respectively, of a MC-2 high-sensitivity scanning calorimeter (Microcal Co., Amherst, MA). A heating scan rate of 39 K/h was employed, and transition temperatures and enthalpies were calculated by fitting the observed transitions to a single van't Hoff component. Repeated scans of the same sample showed good reproducibility.

X-ray Diffraction. The lipid dispersion (≈10 mg/mL) was incubated with frequent vortexing for 0.5 h at 45 °C for DEPE and at 4 °C for EYPE. This was followed by centrifugation for 10 min in an Eppendorf centrifuge. The precipitate in excess buffer was then loaded into the funnel of a 1.5-mm quartz X-ray capillary and centrifuged at 1000 rpm for 5 min. The capillary was then flamed-sealed or, in some cases, simply closed with parafilm. A modified Rigaku–Denki low-angle X-ray camera was used in conjunction with a one-dimensional position-sensitive detector of the delay line type (Reich et al., 1982). Copper radiation was obtained from a sealed-tube Philipis generator operating at 40 kV, 34 mA, and monochromated by a nickel filter and a single Franks mirror. Experiment times varied from 5 min to 16 h. Temperature control of the brass sample holder was provided by a circulating water/alcohol bath; the temperature was monitored by an iron/constantan thermocouple. Control was good to ±1 °C. Spacings of the X-ray reflections were measured directly from the histogrammed output of the detector and calibrated against the reflections of anhydrous cholesterol. Resolution was limited by the channel width to 0.0005 $Å^{-1}$. In the case of the gel (L_β) to liquid-crystalline (L_α) transition, the temperature of the midpoint was estimated from the plots of d spacing vs temperature, i.e., the temperature at which the transition-related compression of the bilayers reaches half its total value. This compression derives from the fact that in the L_β phase the acyl chains are well-ordered and extended and in the L_α phase they become more fluidlike and consequently are less extended (Engleman, 1970). In the case of the L_α to the hexagonal (H_{II}) transition, the presence of an extended biphasic region necessitated a different procedure. We used measurements of the peak height of the (11) reflection of the H_{II} lattice as a function of temperature in the biphasic region to obtain an estimate of the midpoint, T_M. The (11) reflection is strong, with apparently constant line width, and we assume it samples nearly the same molecular transform over the temperature range of interest. In this case, T_M is defined as the temperature at which the intensity is half the value it attains after completion of the phase transition.

In samples with large molar fractions of sterol, where the 34-Å reflection interferes with this measurement, we used instead the decrease in intensity of the $l = 1$ reflection of the L_α phase, and the measurements were made in the analogous way. This second procedure was tested on samples with less sterol, and the results for the two types of measurement were consistent within 2 °C of one another.

Results

Low-angle X-ray diffraction patterns were used to make phase assignments. In the case of the pure phospholipids, three phases were observed. They have previously been described as the gel (L_β) phase, the liquid-crystalline (L_α) phase, and the inverted hexagonal (H_{II}) phase (Shipley, 1973). Addition of increasing amounts of cholesterol broadened the $L_\beta \rightarrow L_\alpha$ transition region to the extent that the L_β phase could not be identified.

The characteristic low-angle X-ray diffraction patterns from EYPE–cholesterol in the L_α (Figure 1A) and the H_{II} (Figure 1B) phases are presented. The d spacings measured from a set of diffraction patterns are plotted as a function of temperature (Figure 2). The transition from the L_β to the L_α phase, resulting in a decrease of approximaaely 8 Å in the

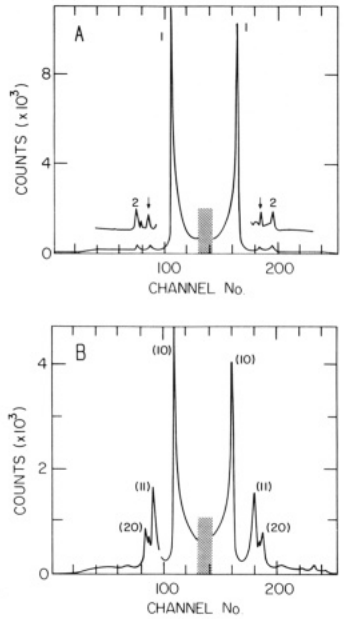

FIGURE 1: Characteristic low-angle X-ray diffraction patterns from the EYPE–cholesterol mixture, X(chol) = 0.45 at 22 °C (A) and at 41 °C (B). Depending on the phase, the numbers indicate either the order of the bilayer spacing or the Miller indexes of the two-dimensional hexagonal lattice, and the arrows indicate the reflections due to the presence of a separated cholesterol phase. The hatched region at the center of the pattern denotes the position of the beam stop.

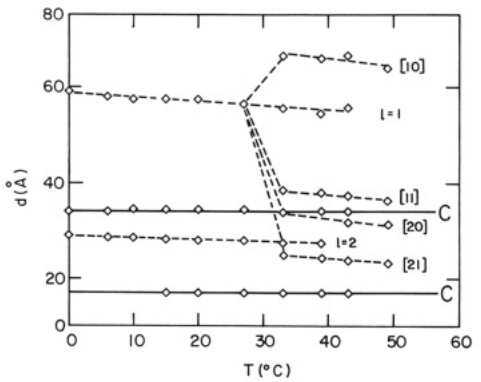

FIGURE 2: Temperature dependence of the d spacings of the low-angle X-ray reflections of the EYPE–cholesterol mixture with X(chol) = 0.44. The letter C marks the cholesterol reflections.

interbilayer spacing of pure EYPE, is not clearly observed for the EYPE–cholesterol mixture (Figure 2). The interbilayer spacing in L_α is larger by about 2 Å than for EYPE alone. Characteristic reflections of crystalline cholesterol (marked C) are observed throughout the temperature range of L_α. Such reflections are apparent for specimens with X(chol) approximately 0.40 and greater. The appearance of the cholesterol reflections was not a function of time and was reproducible in replicate experiments. Because of interference from the (11) and (20) reflections in the H_{II} phase, it is not clear if phase separation persists into H_{II} for low molar fractions of cholesterol. For X(chol) approximately 0.6 and above, phase separation does clearly persist in the H_{II} phase as characteristic reflections of crystalline cholesterol are readily observed.

Patterns very similar to those obtained with EYPE were also obtained for DEPE (Figure 3A). Addition of cholesterol at X(chol) = 0.35 and above produced reflections characteristic of a separate cholesterol phase (Figure 3B). However, when DEPE is mixed with epicholesterol, phase separation of epicholesterol is detected by X-ray diffraction beginning at a mole

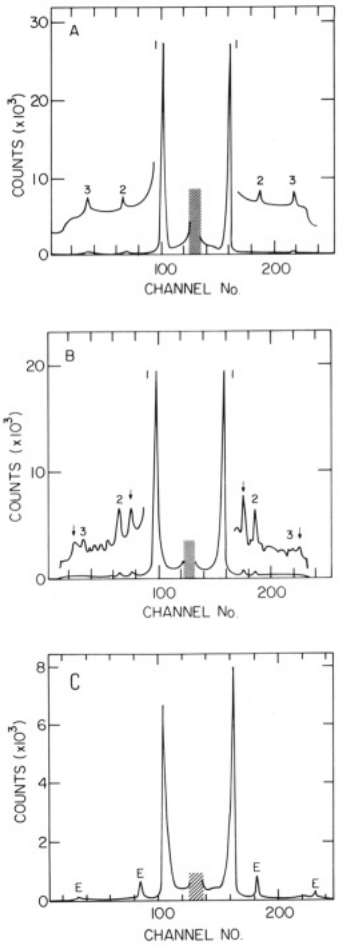

FIGURE 3: Characteristic low-angle X-ray diffraction patterns from (A) DEPE at 45 °C, (B) the DEPE–cholesterol mixture with X(chol) = 0.40 at 40 °C, and (C) the DEPE–epicholesterol mixture with X(epi) = 0.31 at 44 °C. E marks the epicholesterol reflections. Other details as in Figure 1.

fraction of epicholesterol X(epi) = 0.16 and higher (Figure 3C). The epicholesterol reflections (the first two reflections of epicholesterol appear at 34 and 17 Å; E. Wachtel, unpublished result) are much more prominent than the corresponding reflections of cholesterol (Figure 3B vs Figure 3C). The relative intensities of these reflections in the L_α phase, where they are unambiguously resolved from reflections of the phospholipid structures, are independent of temperature. The temperature dependence of the d spacings for DEPE–cholesterol at X(chol) = 0.40 (Figure 4A) and DEPE–epicholesterol at X(epi) = 0.31 (Figure 4B) is shown. Only in the case of epicholesterol (Figure 4B) can the $L_\beta \rightarrow L_\alpha$ phase transition still be observed by X-ray diffraction. In the L_β phase of epicholesterol–DEPE, the second order of the bilayer spacing approximately coincides with the first epicholesterol reflection and the fourth order with the second epicholesterol reflection, respectively. This overlapping of reflections limits the detection of phase separation in the L_β phase. However, since separated epicholesterol is observed in the L_α phase, it is reasonable to conclude that such phase separation exists in the L_β phase as well. Also, in the hexagonal H_{II} phase (within the temperature range examined), the (20) reflection is approximately coincident with the second epicholesterol reflection (Figure 4B). However, monitoring intensity as a function of temperature as the hexagonal phase develops indicates that phase-separated material persists into the hexagonal phase.

The dependence of the $L_\alpha \rightarrow H_{II}$ phase transitions on the mole fraction of sterol was determined. Representative DSC

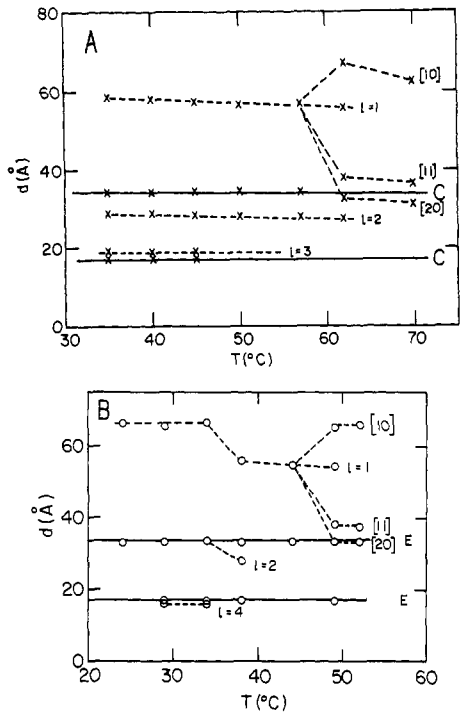

FIGURE 4: Temperature dependence of the measured d spacings for (A) the DEPE–cholesterol mixture with X(chol) = 0.40 and (B) the DEPE–epicholesterol mixture with X(epi) = 0.31. The letter C indicates reflections associated with cholesterol and the letter E those associated with epicholesterol.

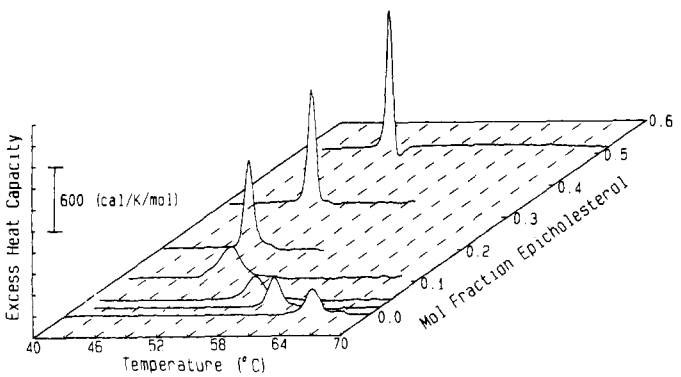

FIGURE 5: Representative DSC scans of the $L_\alpha \rightarrow H_{II}$ transition of DEPE with various mole fractions of epicholesterol. The epicholesterol mole fractions are 0.000, 0.026, 0.051, 0.118, 0.211, 0.348, and 0.516.

scans of mixtures containing DEPE and various mole fractions of epicholesterol are presented (Figure 5). In the parallel X-ray experiments, estimates of the transition temperatures were made as described under the Materials and Methods. The results of this analysis for mixtures of cholesterol with DEPE or with EYPE are shown in Figure 6. The temperature of this transition for DEPE–cholesterol mixtures as determined by DSC is in good agreement with the results from X-ray diffraction (Figure 6). Epicholesterol is a much better hexagonal phase promoter than is cholesterol (Figure 6).

Cholesterol affects the structure of the hexagonal phase of the two phosphatidylethanolamines to a different extent. The lattice constant, d, of the H_{II} phase at 69 °C for DEPE and at 40 °C for EYPE (Figure 7) is shown as a function of molar fraction of cholesterol. The temperatures were chosen in each case as being common to the H_{II} phases of all the samples of a given lipid studied. For DEPE, the lattice constant, a, decreases from 76 Å in the absence of cholesterol to about 71 Å at X(chol) = 0.64. On the other hand, similar behavior is not observed for EYPE, where only a small decrease in lattice

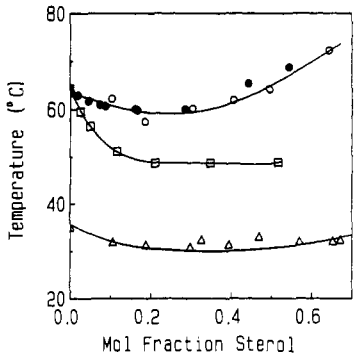

FIGURE 6: Dependence of the $L_\alpha \rightarrow H_{II}$ transition temperature on the mole fraction of sterol. DEPE–cholesterol (●, ○), DEPE–epicholesterol (□), and EYPE–cholesterol (△) as determined by X-ray diffraction (○, △) and by DSC (●, □).

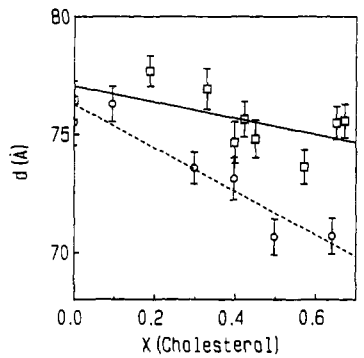

FIGURE 7: Lattice constant, d, of the H_{II} phase for (○) DEPE at 69 °C and (□) EYPE at 40 °C as a function of the molar fraction of cholesterol, X(chol). The error bars indicate the uncertainty derived from the channel width.

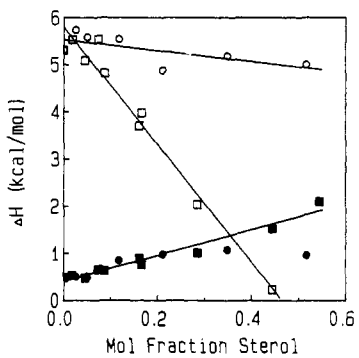

FIGURE 8: Transition enthalpy of DEPE with various mole fractions of epicholesterol (●, ○) or cholesterol (■, □) for the $L_\beta \rightarrow L_\alpha$ transition (□, ○) and the $L_\alpha \rightarrow H_{II}$ transition (■, ●).

constant may be discerned up to X(chol) = 0.67. However, the temperature in the case of EYPE is almost 30 °C lower than in the case of DEPE, and this may play a role in determining the structural stability. Structural changes in the H_{II} phase due to the presence of epicholesterol could not be detected by X-ray.

Epicholesterol increases the enthalpy of the $L_\alpha \rightarrow H_{II}$ transition of DEPE but achieves a maximum at about 0.23 mole fraction of sterol (Figure 8). The epicholesterol slightly broadens the transition at lower concentrations but at higher mole fractions (>0.20) it sharpens it dramatically, corresponding to an increased cooperativity of the transition. The cause of this increase of cooperativity is not known but may involve complex formation between DEPE and epicholesterol in the H_{II} phase. In comparison, cholesterol increases the enthalpy of the $L_\alpha \rightarrow H_{II}$ transition of DEPE while decreasing the cooperativity as evidenced by a broadening of the transition. This effect continues up to the highest mole fraction

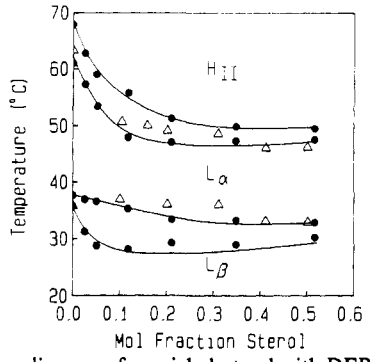

FIGURE 9: Phase diagrams for epicholesterol with DEPE, constructed from the onset and completion temperatures from DSC data (●). Also shown are the midpoints of the transitions as determined by X-ray diffraction (△).

of cholesterol tested (Figure 8). Epicholesterol does not significantly affect the enthalpy of the gel to liquid-crystalline transition of DEPE (Figure 8). However, cholesterol substantially lowers the enthalpy of this transition (Figure 8), similar to its well-known effects on phosphatidylcholine.

As is apparent from the phase diagram (Figure 9), the most striking effect of the addition of epicholesterol is the reduction of the range of stability of the L_α phase. This is accomplished by the downward shift of the onset of the H_{II} phase. The temperatures of the upper transition determined by X-ray diffraction are close to those measured by DSC; in the case of the lower transition, the temperatures tend to follow the liquidus line as determined by DSC. This latter effect may be related to the asymmetry of the melting peak.

Discussion

X-ray diffraction studies of the phase behavior of EYPE have been reported previously (Laggner, 1984), but no definitive structural assignments were made. We find that pure EYPE undergoes the $L_\beta \rightarrow L_\alpha$ transition at approximately 8 °C and the $L_\alpha \rightarrow H_{II}$ transition at 35 °C. The decrease of 30 °C in the $L_\alpha \rightarrow H_{II}$ transition temperature with respect to DEPE may be attributed to the increased unsaturated character of the natural lipids (Cullis & De Kruijff, 1979) and/or to the distribution of chain lengths (Cullis & De Kruijff, 1978). Both of these tend to favor increased acyl chain disorder and therefore promote formation of H_{II}. These two phosphatidylethanolamines differ considerably in the character of the hydrocarbon chains. DEPE has two equal C18 chains, each with a trans double bond at position 9, whereas the natural PE is a mixture of chain lengths from 16 to 22 carbons with varying amounts of cis double bonds. The most obvious difference between the two species of PE is apparent in the dependence of the $L_\alpha \rightarrow H_{II}$ transition on the molar fraction of cholesterol (Figure 6). Whereas with DEPE the L_α phase is first destabilized by the addition of cholesterol and then strongly stabilized by further addition of the sterol, in EYPE there is only a weak destabilization of L_α. T_m decreases about 3 °C for X(chol) = 0.1, and addition of more cholesterol has no further effect. Stabilization of the L_α phase at high cholesterol content is also much less pronounced in the case of POPE–cholesterol mixtures (Epand & Bottega, 1987). This fact lends some strength to the idea that there is a specific DEPE–cholesterol interaction which is promoted by the trans configuration of the double bond at the middle of each acyl chain. It has been suggested that the two angular methyl groups of a cholesterol molecule at C10 and C13 can be accommodated by unsaturated acyl chains in a hydrophobic pocket generated by a trans–gauche kink adjacent to a double bond at position 9 (Huang, 1977). Ayanoglu et al. (1988) have postulated that the cholesterol nucleus should fit between the carbonyl group of the acyl chain and this kink. The particular trans–gauche isomerism promoted by the double bond would thus determine how well cholesterol could fit into the bilayer.

The interbilayer spacing decreases from 64 Å in the L_β phase to 56 Å in the L_α phase. This decrease in spacing is thought to reflect the decrease in the order parameter of the hydrocarbon chains and the accompanying increased chain flexibility (Engleman, 1970). Upon addition of cholesterol, the L_β phase is no longer clearly observed, and the interbilayer spacing of the L_α phase increases as is also observed in the case of DEPE. We cannot separate the contributions of the water and the lipids to this parameter, but the additional 2 Å observed for the layer thickness of the L_α phase in the presence of cholesterol may be due to the restraining influence of the sterol on the conformational freedom of the PE molecule. This is in contrast to epicholesterol, which does not greatly modify the structural parameters of DEPE. The presence of phase-separated epicholesterol crystals in the L_α phase suggests that the solubility of epicholesterol in DEPE is very limited and is less than that of cholesterol. Cholesterol is more effective than epicholesterol in reducing the enthalpy of the $L_\beta \rightarrow L_\alpha$ transition of DEPE (Figure 8) as well as phosphatidylcholines (De Kruijff et al., 1972; Davis et al., 1986).

Despite the effects of cholesterol on the $L_\beta \rightarrow L_\alpha$ transition, cholesterol shows limited solubility in PE, with the onset of formation of cholesterol domains occurring at X(chol) = 0.35–0.4 for both phosphatidylethanolamines studied. As phase separation of cholesterol above X(chol) = 0.2 may be indicated by the invariant region for EYPE–cholesterol mixtures, it is possible that the true solubility limit in both cases is lower. The phase diagrams of cholesterol mixtures with DEPE (Epand & Bottega, 1987) show that the liquidus of the $L_\beta \rightarrow L_\alpha$ transition becomes invariant at X(chol) = 0.2. Because of the difficulty of resolving the cholesterol reflections in the H_{II} phase, we cannot say whether there is a change in solubility at temperatures above the $L_\alpha \rightarrow H_{II}$ transition. In previous X-ray studies, it was found that the zwitterionic phosphatidylcholine (PC) can solubilize cholesterol until a mole fraction X(chol) of approximately 0.5 (Yeagle, 1985). In the case of phosphatidylserine (PS), which bears one negative charge at neutral pH, the solubility is reduced, and a separate cholesterol phase is observed both by DSC and by X-ray diffraction already at X(chol) = 0.3 (Bach, 1984; Wachtel & Bach, 1987; Bach & Wachtel, 1989). It has recently been shown by the inverse contrast variation method in neutron scattering (Knoll et al., 1985) that X(chol) = 0.45 is the upper limit of cholesterol solubility in synthetic phosphatidylcholine bilayers. If we compare X-ray diffraction and DSC results on EYPE and DEPE with those on natural PS and PC, we find that the solubilizing capacity of the phosphatidylethanolamines is intermediate between the values found for these other lipids. Browning and Seelig (1980) have reported that PS has a more rigid headgroup than either PC or PE. In addition, PS bilayers are stabilized by intermolecular bonds, as indicated by the high temperature of melting. PE headgroups also form hydrogen bonds, and following Blume (1980), we may suggest that this fact is responsible for placing the solubility of cholesterol in PE bilayers midway between that in PS and PC.

Epicholesterol markedly depresses the $L_\alpha \rightarrow H_{II}$ transition temperature at low mole fractions. In this regard, it is more effective than cholesterol. The greater changes observed with epicholesterol can be explained in terms of the orientation and motion of the sterol in the lipid bilayer. There is no direct

experimental evidence for hydrogen bonding between the 3β-OH of cholesterol and phospholipid headgroups (De Kruijff et al., 1973). Instead, the cholesterol hydroxyl is thought to be hydrated at the surface of the bilayer (Demel et al., 1972; Murari et al., 1986). The hydration of the OH serves to orient the cholesterol molecule perpendicular to the bilayer surface and maximize favorable van der Waals interactions between the hydrocarbon region of cholesterol and the acyl chains of the phospholipids (Murari et al., 1986; Dufourc et al., 1984). The requirement for the rigid ring structure and tail regions of cholesterol for its characteristic condensing effect further emphasizes the importance of hydrophobic interactions between the phospholipid acyl chains and cholesterol (Yeagle, 1985; Demel et al., 1972). Epicholesterol may also be hydrated at the bilayer surface, but to achieve this, it must tilt its hydrocarbon region with respect to the normal to the surface of the bilayer (Murari et al., 1986; Dufourc et al., 1984). Deuterium NMR has shown that at physiological temperatures, cholesterol is oriented with its long axis perpendicular to the plane of the bilayer while epicholesterol is tilted with respect to the bilayer surface (Murari et al., 1986; Dufourc et al., 1984). The tilted orientation of the epicholesterol may also lead to unfavorable steric interactions in the hydrocarbon portion of the bilayer, thus increasing the effective hydrocarbon volume (Demel et al., 1972; Murari et al., 1986). This tilted orientation may lead to a more favorable packing arrangement with phospholipid in the H_{II} phase because both the phospholipid and the sterol would have a similar intrinsic radius of curvature. The fact that increasing amounts of epicholesterol appear to increase the cooperativity of the $L_\alpha \rightarrow H_{II}$ transition is rather unusual, and X-ray experiments at high angles to characterize the packing order of the acyl chains at temperatures just below the $L_\alpha \rightarrow H_{II}$ transition will be carried out. Addition of epicholesterol does not change the structure of the H_{II} phase. The center to center spacing of the rods in the H_{II} phase, which is determined by the length of two DEPE molecules and the water in the cylinder core, at a given temperature is roughly constant between X(epi) = 0.1–0.5. This is unlike the observation with DEPE–cholesterol mixtures where the center to center spacing at a given temperature decreases markedly with sterol content. The lattice constant in the hexagonal phase of DEPE decreases from 76 Å in the absence of cholesterol to about 71 Å for X(chol) = 0.64. In view of the straightening effect of cholesterol on fatty acid chains, such behavior has been attributed to progressive depletion of the water core with increasing cholesterol content (Gasset et al., 1988). However, this is not a general effect of cholesterol as the lattice constant in the hexagonal phase of EYPE is much less sensitive to the presence of cholesterol. This again suggests that epicholesterol and DEPE pack together in a stable arrangement in the hexagonal phase. This likely arises not just from the shape of the molecule but also from the motional properties of epicholesterol in the phospholipid environment.

The relative stability of the bilayer and hexagonal phases in the presence of additives has been considered in terms of the intrinsic radius of curvature, hydration, and hydrocarbon packing constraints (Kirk et al., 1984; Gruner, 1985). The present results show that simple epimerization of the hydroxyl group of cholesterol, through its influence on molecular orientation and motion, can affect bilayer stability. Thus, even though cholesterol and epicholesterol have exactly the same balance of polar and nonpolar groups, they produce markedly different changes in bilayer stability. The molecular properties of sterols have evolved to produce cholesterol, which despite its hydrophobic nature is not a potent hexagonal phase promoter in PE. The ability of epicholesterol to strongly promote hexagonal phase formation may partially explain its absence from biological membranes.

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Registry No. DEPE, 16777-83-6; cholesterol, 57-88-5; epicholesterol, 474-77-1.

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Quantitative Determination of Conformational Disorder in the Acyl Chains of Phospholipid Bilayers by Infrared Spectroscopy†

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ABSTRACT: A method is proposed and demonstrated for the direct determination of conformational disorder (trans-gauche isomerization) as a function of acyl-chain position in phospholipid bilayer membranes. Three specifically deuterated derivatives of dipalmitoylphosphatidylcholine (DPPC), namely 4,4,4′,4′-d_4-DPPC (4-d_4-DPPC), 6,6,6′,6′-d_4-DPPC (6-d_4-DPPC), and 10,10,10′, 10′-d_4-DPPC (10-d_4-DPPC), have been synthesized. The CD_2 rocking modes in the Fourier transform infrared (FT-IR) spectrum have been monitored as a function of temperature for each derivative. A method originally applied by Snyder and Poore [(1973) *Macromolecules 6*, 708-715] as a specific probe of hydrocarbon chain conformation in alkanes has been used to analyze the data. The rocking modes appear at 622 cm^{-1} for a CD_2 segment surrounded by a trans C-C-C skeleton and between 645 and 655 cm^{-1} for segments surrounded by particular gauche conformers. The integrated band intensities of these modes have been used to monitor trans-gauche isomerization in the acyl chains at particular depths in the bilayer. At 48 °C, above the gel-liquid-crystal phase transition, the percentage of gauche rotamers present is 20.7 ± 4.2, 32.3 ± 2.3, and 19.7 ± 0.8 for 4-d_4-DPPC, 6-d_4-DPPC, and 10-d_4-DPPC, respectively. The gel phase of the latter two molecules is highly ordered. In contrast, a substantial population of gauche rotamers was observed for the 4-d_4-DPPC. The conformational analysis yields a range of 3.6-4.2 gauche rotamers/acyl chain of DPPC above the phase transition. This range is in excellent accord with the dilatometric data of Nagle and Wilkinson [(1978) *Biophys. J. 23*, 159-175]. The significant advantages of the FT-IR approach are discussed.

Determination of the structure and dynamics of phospholipids, and the relationship of these quantities to the function of membrane proteins, is a long-sought goal of membrane biophysics. Toward this end, the arsenal of modern spectroscopic technology has been brought to bear. Each technique contributes information on its own time scale to a composite picture of phospholipid organization. Yet a quantitative picture of the contribution of any single motion to phospholipid dynamics is elusive. The reason for this is not difficult to understand. For example, the most powerful technique brought to bear on this problem to date is probably ^2H NMR spectroscopy [for a recent review, see Seelig and MacDonald (1987)]. The order parameters derived from this experiment incorporate all motions faster than about 10^{-5}-10^{-6} s, the characteristic time scale for the NMR measurement (Seelig & Seelig, 1974; Petersen & Chan, 1977). Thus, the contribution from any one of the possible motions (trans-gauche isomerization, acyl-chain librations, rigid body motions, etc.) to the spectrum is difficult to determine. Other commonly used spectroscopies, primarily fluorescence and ESR, are exquisitely sensitive, but in general require the use of probe molecules. These at best present difficulties in transferring the measured spectral properties of the probe to the physical properties of the phospholipid and at worst have the potential to perturb the order and dynamics of the system under investigation (Taylor & Smith, 1980).

Infrared and Raman spectroscopies, in principle, operate on a time scale that has the potential to sample directly the

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