Interaction of 7-Ketocholesterol with Two Major Components of the Inner Leaflet of the Plasma Membrane: Phosphatidylethanolamine and Phosphatidylserine[†]

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ABSTRACT: 7-Ketocholesterol is one of the major forms of oxidized cholesterol found in vivo. Several toxic effects of this sterol have been documented, and it is suggested to have a role in atherosclerosis. We have studied how this oxysterol modifies the physical properties of bilayers composed of the major lipid components of the cytoplasmic leaflet of the plasma membrane. 7-Ketocholesterol is much less effective in promoting the formation of the H_{ii} phase in phosphatidylethanolamines than cholesterol. This is likely due to the fact that 7-ketocholesterol is more polar than cholesterol and hence would be located closer to the membrane interface. However, in ternary mixtures of dipalmitoleoylphosphatidylethanolamine with low concentrations of both sterols, the effect of 7-ketocholesterol on lowering T_H is enhanced. Both cholesterol and 7-ketocholesterol are very soluble in bilayers of phosphatidylethanolamine, particularly with 1-palmitoyl-2-oleoylphosphatidylethanolamine. There is, however, a much greater solubility of 7-ketocholesterol in bilayers of 1-stearoyl-2-oleoylphosphatidylserine than is the case for cholesterol. In ternary mixtures of 1-stearoyl-2-oleoylphosphatidylserine with both sterols, it appears that the solubility of cholesterol is enhanced by the presence of 7-ketocholesterol. It is thus to be expected that several of the biophysical properties of a membrane would change as a result of the oxidation of cholesterol to 7-ketocholesterol.

7-Ketocholesterol (Scheme 1) is a derivative of cholesterol produced by nonenzymatic oxidation at the C7 position (*1*). This compound has many pathological effects. In atherosclerotic lesions, 7-ketocholesterol comprises 50% of the oxysterols (*2*). Oxysterols are believed to play an important role in atherosclerosis, and they are found in elevated concentrations in relation to cholesterol in arterial plaques compared with normal tissue or plasma (*3*). Plasma oxysterol concentrations have also been shown to be higher in individuals with coronary arterial disease, independent of their LDL[1] cholesterol levels (*4*). 7-Ketocholesterol is one of the oxysterols that is pro-oxidant and pro-inflammatory (*5*). It is detected also in neurodegenerative diseases (*6*). 7-Ketocholesterol causes apoptosis in various cells, e.g., cultured human vascular smooth cells (*7*), and in human macrophages (*8*), perhaps by activating the mitochondrial pathway of apoptotic death (*5, 9*). It has been shown that 7-ketocholesterol promotes the production of reactive oxygen species (*10*). It is possible that this is a consequence of the destabilization of the inner mitochondrial membrane, resulting in a breakdown of the proton and electrochemical gradients. As a consequence, instead of ATP being produced from electron transport, reactive oxygen species are produced. In addition, 7-ketocholesterol induces retinal degeneration (*11*). 7-Ketocholesterol interacts with the protein caveolin-1 (*12*). This compound is also an inhibitor of membrane-bound enzymes and probably also modifies membrane permeability (*13*). It is possible that at least some of the pathological effects of 7-ketocholesterol stem from the changes which it causes in the biophysical properties of the cell membranes.

Due to the complexity of cell membranes, investigation of the effect of 7-ketocholesterol has been performed on simpler model phospholipid membranes, primarily the phosphatidylcholines. Egli et al. (*14*) investigated the influence of 7-ketocholesterol alone or in mixtures with cholesterol on the thermotropic properties of dipalmitoylphosphatidylcholine (DPPC). A progressive decrease in the enthalpy of melting was detected. Using FTIR and Raman spectroscopies, Rooney et al. (*15*) showed that 7-ketocholesterol condenses the acyl chains of DPPC in the fluid state less

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[1] Abbreviations: LDL, low-density lipoprotein; DPPC, dipalmitoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; diPoPE, dipalmitoleoylphosphatidylethanolamine; SOPS, 1-stearoyl-2-oleoylphosphatidylserine; SAXS, small angle X-ray diffraction; DSC, differential scanning calorimetry; T_M, main transition temperature; T_H, lamellar to hexagonal phase transition temperature; ΔH_M, main transition enthalpy; ΔH_H, lamellar to hexagonal phase transition enthalpy; Lβ, gel phase; Lα, liquid crystalline phase; H_{ii}, inverse hexagonal phase.

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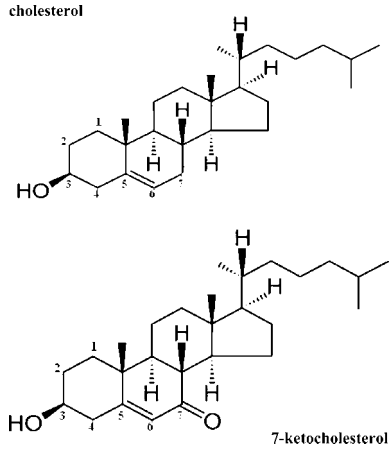
Scheme 1: Molecular Structures of Cholesterol and 7-Ketocholesterol



than cholesterol but orders the headgroups of the phospholipids more than cholesterol. The presence of cholesterol in the bilayers enhances the effect of 7-ketocholesterol. Phillips et al. (*16*) showed that at high concentrations, 7-ketocholesterol forms crystalline domains in POPC bilayers. The formation and stability of ordered domains in DPPC and DOPC bilayers induced by 7-ketocholesterol were monitored by measuring the solubility in Triton X-100 and by fluorescence quenching (*17*). The domains were as thermally stable as those of cholesterol but less tightly packed. The formation of ordered domains by 7-ketocholesterol in DOPC/DPPC bilayers and in mixtures with sphingomyelin was investigated by Massey and Pownall (*18*). These authors also found that 7-ketocholesterol is less effective than cholesterol in the formation of ordered domains but decreases the degree of hydration less than cholesterol. In the liquid ordered phase of phosphatidylcholine, both cholesterol and 7-ketocholesterol would be expected to lie quasi perpendicular to the interface (*18*). Wang et al. (*17*) found that in mixtures with DPPC, 7-ketocholesterol stabilizes the liquid ordered phase, which has been associated with raft formation, just as cholesterol does, but the packing of the hydrocarbon chains was not as tight as with cholesterol. In fact, whether 7-ketocholesterol will actually promote or inhibit formation of the liquid ordered phase depends on the phospholipid composition (*18*). Introducing 7-ketocholesterol reduced the level of formation of detergent resistant membranes by 45% for the 1:1 DOPC/DPPC mixture. The difference between the effects of cholesterol and 7-ketocholesterol is consequently much greater in the DOPC/DPPC mixture than in DPPC only, and this difference grows with increasing total sterol content. In ternary mixtures including cholesterol, no synergism has been found between cholesterol and 7-ketocholesterol. Massey and Pownall (*18*) suggest that in the liquid disordered phase, both oxygen-containing groups of 7-ketocholesterol are at the water−membrane interface. In that case, one would expect the sterol to expand the interface and to minimize interaction with the acyl chains. In addition, the keto group can in principle form hydrogen bonds with the phospholipid headgroup.

Here we focus on the interaction between 7-ketocholesterol and three phospholipids: diPoPE (1,2-dipalmitoleoyl-*sn*-glycero-3-phosphoethanolamine), POPE (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine), and SOPS (1-stearoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine). The effect of 7-ketocholesterol on the properties of phosphatidylethanolamine or phosphatidylserine membranes has not yet been investigated. These are the major lipids of the cytoplasmic monolayer of the plasma membrane that is intimately involved in many signal transduction pathways. For these lipid molecules, particular structural features that may modulate interaction with sterols include an unsaturated C−C bond at positions 9 and 10, the possibility of interheadgroup hydrogen bonding, and, in the case of SOPS, a negatively charged headgroup. Ternary mixtures in which both cholesterol and 7-ketocholesterol are present were also examined. Phosphatidylethanolamine is a lipid that is known to readily convert into nonlamellar phases. The tendency to form inverse phases is further promoted by cholesterol (*19–22*). In this work, we will determine the consequence of the conversion of cholesterol to one of its common oxidized forms, 7-ketocholesterol, on the lamellar−hexagonal phase transition.

MATERIALS AND METHODS

Materials. SOPS, POPE, and diPoPE were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol was from NuChek (Elysian, MN). 7-Ketocholesterol was either from Sigma or from Steraloids (Newport, RI). No difference was observed in the X-ray diffraction profiles using samples of 7-ketocholesterol from either of these two sources.

Preparation of Lipid Films for X-ray Diffraction. Phospholipids or sterols were individually dissolved in a 2:1 (v/v) chloroform/methanol solution and the solutions mixed at the appropriate ratios to obtain the desired mole fraction of sterol. The solvents were driven off using a stream of nitrogen, and the samples were then kept for 3 h under high vacuum to remove the last traces of solvent.

Small and Wide Angle X-ray Scattering (SAXS and WAXS, respectively). For X-ray diffraction experiments, the dry lipid films of phospholipids or phospholipid/sterol mixtures were dispersed in either 0.15 M NaCl (diPoPE and POPE) or 0.5 M NaCl (SOPS) in 0.01 M Tris-HCl buffer (pH 7.4) to give a final lipid concentration of approximately 10 mg/mL. The higher ionic strength is necessitated by the fact that SOPS, a negatively charged phospholipid, will form well-ordered multilayers only in the presence of ≥0.5 M NaCl at full hydration (*23*). The thermodynamic properties of natural PS bilayers at pH 7.4 are only marginally influenced in the presence of 0.5 M as compared to 0.15 M NaCl: T_m increases 1–2 °C (*23, 24*) while the enthalpy of chain melting is not at all affected (*24*). Mole fraction total sterol is calculated as (mole cholesterol + mole 7-ketocholesterol)/(mole cholesterol + mole 7-ketocholesterol + mole phospholipid). The dispersions were incubated for 0.5 h at 50 °C with frequent vortexing. In the case of SOPS, this procedure was then followed by centrifugation for 15 min in an Eppendorf centrifuge, and the resulting pellet was loaded into X-ray capillaries. In the case of the phosphatidylethanolamines, the material adhered to the wall of the glass vials, necessitating scraping to transfer the lipids to the X-ray capillaries.

Small angle X-ray diffraction measurements of the aqueous dispersions of pure phospholipids and phospholipid/sterol mixtures were performed as a function of temperature as described by Bach et al. (*25*). In the lamellar phases, generally two diffraction maxima could be observed, with

reciprocal space positions related by a ratio of 1:2. For each of the three lipids studied here, we verify the lamellar phase assignments of the pure materials by comparison with the literature. In the two-dimensional hexagonal phases, at least three diffraction maxima were observed with positions related in the ratio 1:$\sqrt{3}$:2. Linear regression, including all maxima, with the added constraint of passing through the origin, was used to determine the lattice constant. Onset of sterol crystallization in the phospholipid/sterol mixtures was determined by the appearance of a small angle diffraction peak with the characteristic *d* spacing, 34 Å for cholesterol and 35 Å for 7-ketocholesterol. The 34 Å reflection of cholesterol is due to the 010 planes in the triclinic anhydrous crystal (low-temperature form) (*26*) or to the 001 planes in the triclinic monohydrate form (*27*). At 37 °C, the anhydrous crystal undergoes a polymorphic phase transition to a high-temperature form with a larger unit cell (*28*), but the 34 Å peak is unchanged. As first reported by Dorset (*29*), we found that crystalline mixtures of the two sterols display only a single diffraction peak, which shifts from the lower to the higher spacing with increasing amounts of 7-ketocholesterol. Nevertheless, Dorset (*29*) shows that the mixture deviates from ideal with a high content of 7-ketocholesterol and there is only partial cosolubility.

Wide angle X-ray diffraction data were recorded on imaging plates (Fuji) using a Searle camera equipped with double Franks optics and affixed to the generator described by Bach et al. (*25*). Calcite powder was used as the calibrating material and coated the capillary wall. The sample temperature was controlled with a homemade Peltier-based controller. The imaging plates were scanned with a He-Ne laser (JDS Uniphase, Santa Rosa, CA) in conjunction with a homemade reader based on an Optronics (Chelmsford, MA) densitometer and interfaced with a personal computer. Images were analyzed using the public domain program ImageJ (W. Rasband, NIH, Bethesda, MD) and POLAR (D. Fang, State University of New York, Stony Brook, NY).

Differential Scanning Calorimetry (DSC). Measurements were taken using a Nano II differential scanning calorimeter (Calorimetry Sciences Corp., Lindon, UT). The scan rate was 2 °C/min with a delay of 5 min between sequential scans in a series to allow for thermal equilibration. The features of the design of this instrument have been described previously (*30*). DSC curves were analyzed by using the fitting program DA-2 provided by Microcal Inc. (Northampton, MA) and plotted with Origin, version 5.0. Dry lipid films were suspended in 20 mM PIPES, 1 mM EDTA, 150 mM NaCl, and 0.002% NaN_3 (pH 7.40) by vortexing above the phase transition temperature of the lipid to form multilamellar vesicles (MLVs). The dispersions were then incubated for 0.5 h at 50 °C with frequent vortexing. The concentration of MLV in the samples studied was maintained at 2.5 mg/mL. The cell volume was 340 μL.

RESULTS

POPE/Sterol Mixtures. Due to the periodic nature of bilayer stacking in multilamellar lipid vesicles or sheets, small angle X-ray scattering (SAXS) allows determination of the distance between bilayers comprising the stack. This distance includes the thickness of the bilayer and that of the interbilayer water. In multilamellar sheets of POPE, the lamellar repeat (*d*) is approximately 63 Å in the gel phase (Lβ) below T_m [25 °C (*19*)] and 52.5 Å in the liquid crystal phase (Lα). These values are in good agreement with those reported by Wang and Quinn (*21*). This pronounced decrease in *d* spacing is considered a marker for melting of the lipid (*21*), i.e., the thermally induced change in the conformation of the acyl chains with an increase in the number of gauche conformers. As a result, the thickness of the bilayer decreases. Our wide angle diffraction measurements (WAXS) confirm this conformational change (data not shown). In the gel state at 3 °C, the reflection due to the packing of the acyl chains, located at $d = 3.9$ Å, has a full width at half-height of 0.4°, while the corresponding values at 30 °C are 4.4 Å for *d* and ~2° for line width. Since the line width is inversely related to the extent and quality of ordered domains, these results provide direct evidence of thermally induced disordering.

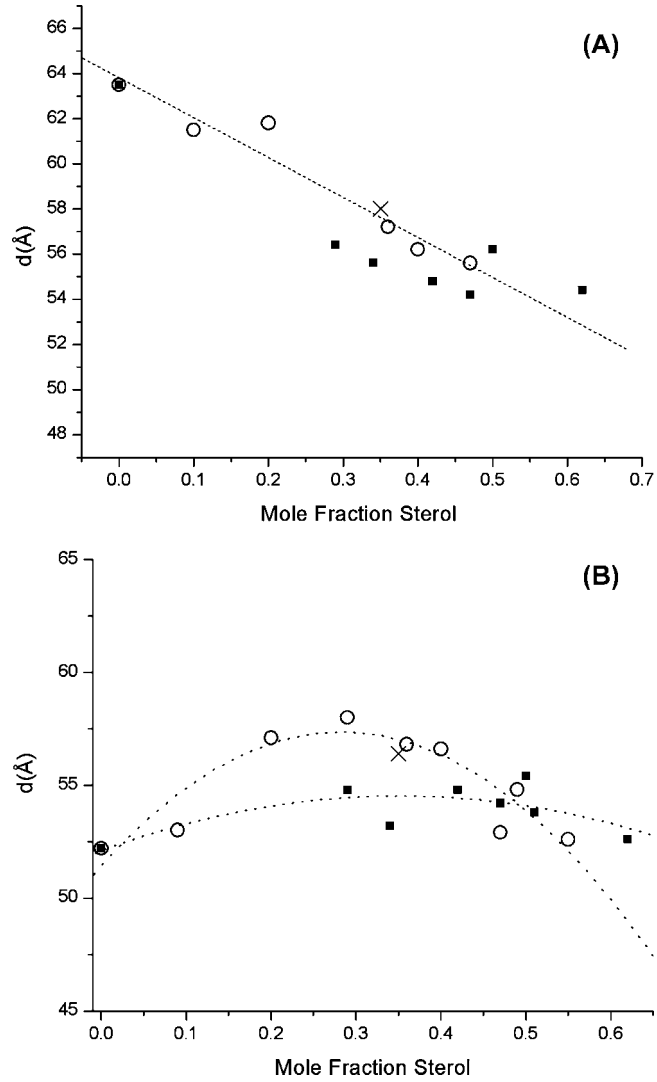


FIGURE 1: Dependence of the lamellar repeat (*d*) of POPE bilayers on the mole fraction of added cholesterol (■), 7-ketocholesterol (○), or a ternary system with a 3.7:1 cholesterol:7-ketocholesterol ratio, plotted at mole fraction total sterol 0.35 (×): (A) in the gel phase and (B) in the liquid crystalline phase.

Introduction of 7-ketocholesterol or cholesterol into the gel state POPE bilayers also causes a reduction in the lamellar repeat. Figure 1A shows a monotonic decrease in the *d* spacing to 56 Å in the presence of 0.47 mole fraction 7-ketocholesterol. Data were measured at approximately 7

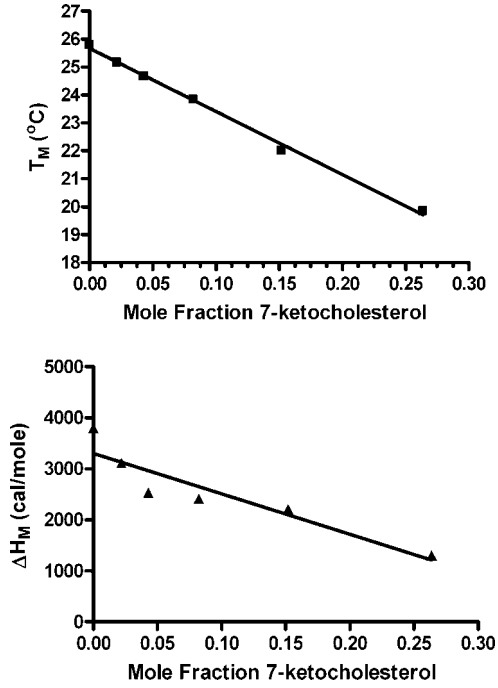


FIGURE 2: Shift in the temperature (T_M) and enthalpy (ΔH_M) of the gel to liquid crystalline phase transition of POPE with added 7-ketocholesterol as determined by DSC.

°C. Similar behavior is observed when cholesterol is the added sterol at ~14 °C or when cholesterol is mixed with 7-ketocholesterol in a molar ratio of 3.7:1, with mole fraction total sterol equaling 0.35 in the ternary mixture. It is suggested that the portion of the sterol molecule which extends into the hydrophobic region of the POPE bilayer prevents the hydrocarbon chains of the phospholipid from assuming their maximally ordered conformation. Support for this idea is found in the results of wide angle diffraction measurements. At 5 °C and in the presence of 0.1 mole fraction 7-ketocholesterol and 0.3 mole fraction cholesterol, the position of the chain packing reflection shifts from 3.9 to 4.2 Å and the line width increases to ~2° (data not shown). As described above, this provides direct evidence of the disordering caused by the sterol molecules.

In the liquid crystal state at ~30 °C, on the other hand, the lamellar repeat increases with increasing amounts of 7-ketocholesterol, until at 0.3 mole fraction, it reaches a maximum value of 58 Å (Figure 1B). Beyond this point, the *d* spacing decreases with an increase in 7-ketocholesterol content, independent of temperature, down to 52.5 Å, approximately the same spacing that is observed in the absence of sterol. A weaker effect is observed when cholesterol is added in place of 7-ketocholesterol at ~38 °C, with the largest *d* spacing reaching only 54–55 Å. A similar weak dependence was observed for POPE/cholesterol mixtures at 40 °C (*21*). We also used DSC to monitor the effects of 7-ketocholesterol on the Lβ to Lα transition of POPE (Figure 2). The oxidized sterol lowers the T_M of POPE to a smaller extent than we had found with cholesterol (*19*). The transition enthalpy is also lowered by both 7-ketocholesterol (Figure 2) and cholesterol (*19*). This is consistent with what would be expected if both sterols disorder the gel state and order the liquid crystalline state. The ternary mixture behaves more like the 7-ketocholesterol/POPE binary mixture in SAXS, in spite of the fact that there is more cholesterol.

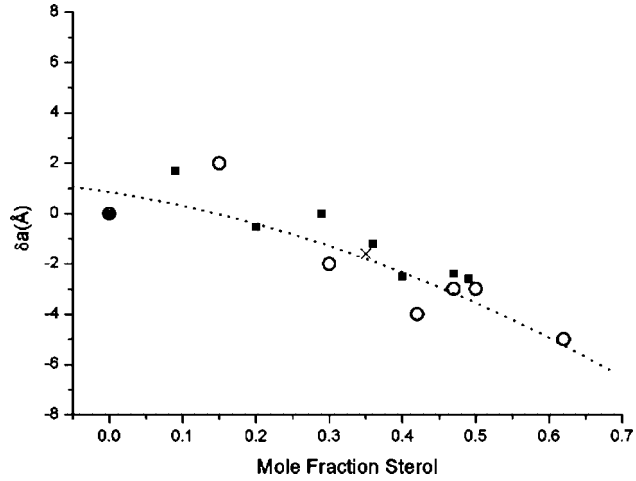


FIGURE 3: Change in the cylinder diameter *a* in the H_{ii} phase of POPE as a function of mole fraction of added cholesterol (○) at 71 °C or 7-ketocholesterol (■) at 72 °C. One ternary system with a 3.7:1 cholesterol:7-ketocholesterol ratio is also plotted at a mole fraction of total sterol of 0.35 (×).

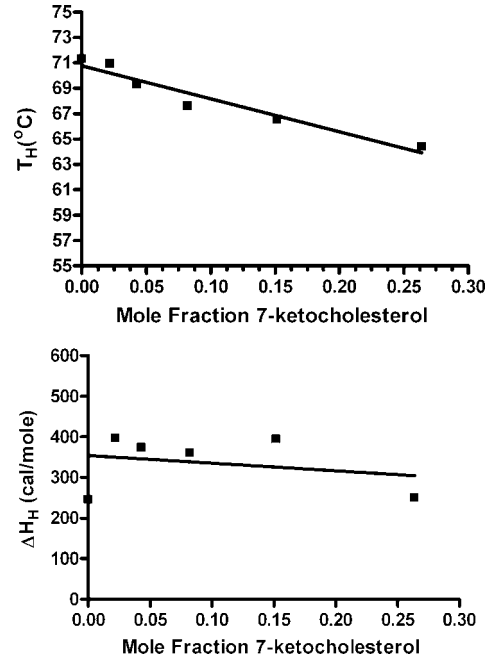


FIGURE 4: Shift of the temperature (T_H) and enthalpy (ΔH_H) of the liquid crystalline to hexagonal phase transition of POPE with added 7-ketocholesterol as determined by DSC.

When POPE is heated above 71 °C, it undergoes a transition to the inverse hexagonal phase, H_{ii}. Figure 3 shows that in the H_{ii} phase, addition of 7-ketocholesterol acts to progressively increase the curvature of the inverse cylindrical micelle. The POPE cylinder diameter (71–72 Å) decreases 4 Å in the presence of 0.5 mole fraction 7-ketocholesterol. As one can see in the graph, this behavior is very similar to that observed when cholesterol is present in binary mixtures with POPE (*31*). [It should be noted that we did not observe diffraction peaks characteristic of cubic phases in the presence of 0.1–0.2 mole fraction cholesterol as reported by Wang and Quinn (*21*).] At low mole fractions of 7-ketocholesterol, DSC studies indicate a lowering of the transition temperature, consistent with X-ray data (Figure 4). The bilayer to hexagonal phase transition temperature is the temperature at which the stabilities (chemical potential) of

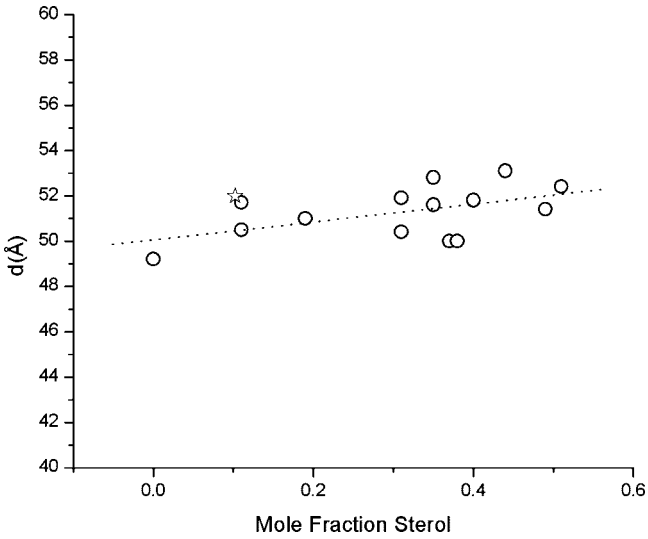


FIGURE 5: Dependence of the lamellar repeat (*d*) in the liquid crystalline phase of diPoPE at 5 °C on the mole fraction of added 7-ketocholesterol (○). A ternary system with a 0.8:1 cholesterol: 7-ketocholesterol ratio is also plotted at a mole fraction of total sterol of 0.1 (☆).

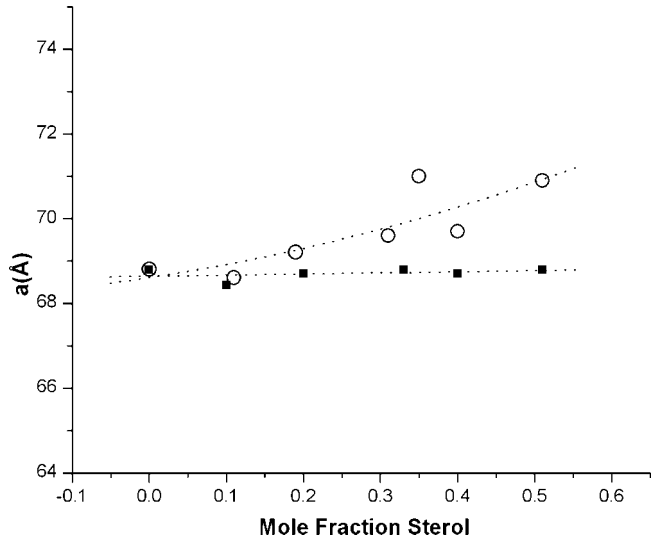


FIGURE 6: Dependence of cylinder diameter *a* in the H_{ii} phase of diPoPE at 45 °C on the mole fraction of added cholesterol (■) or 7-ketocholesterol (○).

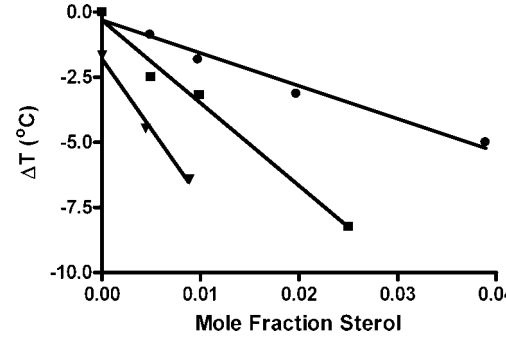


FIGURE 7: Shift in the T_H of diPoPE with an increase in the mole fraction of cholesterol (■), 7-ketocholesterol (●), and 7-ketocholesterol and 1 mol % cholesterol (▼).

the two phases are equal. As the temperature is increased, the hexagonal phase will become more stable relative to the lamellar phase until the stabilities of the two are equal, at which point the transition will occur. Since less heating of the lamellar phase in the presence of 7-ketocholesterol is required to reach the transition temperature, it indicates that this oxysterol stabilizes the hexagonal phase relative to the lamellar phase. The transition from lamellar to hexagonal phase is driven by increased negative curvature stress in the lamellar phase. Thus, the results indicate that the sterol increases the negative curvature of the lipid. A somewhat more pronounced decrease in this transition temperature is also observed with cholesterol (*19*), but in this case, there are multiple peaks that have been interpreted as a consequence of the formation of cubic phases (*21*). Up to 0.27 mole fraction 7-ketocholesterol there appears to be no change in the bilayer to hexagonal transition enthalpy (Figure 4), while for mixtures of POPE with comparable amounts of added cholesterol, the enthalpy was observed to increase (*19*). The cylinder diameter for the ternary system (mole fraction of total sterol of 0.35) is also shown. To within the scatter of the data points, the diameter of the hexagonal phase cylinder does not differ significantly from the result obtained for the binary systems in the presence of comparable amounts of sterol. As described above, SAXS measurements can also detect the presence of phase-separated crystalline sterol. In binary mixtures of liquid crystalline phase POPE with 7-ketocholesterol, diffraction at 35 Å is first definitely observed at 0.55 mole fraction, whereas for the binary mixtures with cholesterol, definite observation at 34 Å is first made at 0.62 mole fraction. Obviously, both sterols are highly soluble in POPE bilayers.

diPoPE/Sterol Mixtures. Due to the fact that the gel−liquid crystalline transition of diPoPE occurs below 0 °C, we studied only the liquid crystalline (Figure 5) and hexagonal (Figure 6) phases of this phospholipid. The liquid crystalline−hexagonal phase transition of diPoPE occurs at 42 °C. With increasing amounts of 7-ketocholesterol, the lamellar repeat of diPoPE measured at ~5 °C increases monotonically from approximately 50 Å to approximately 52 Å (linear regression) at 0.5 mole fraction 7-ketocholesterol. The zero sterol value is in good agreement with that measured by Colotto and Epand (*32*) at 11 °C, i.e., 50 Å. It was not possible to study diPoPE/cholesterol mixtures in the liquid crystalline state with a mole fraction of cholesterol of >0.1, since larger amounts of this sterol promote the formation of the hexagonal phase at temperatures as low as 5 °C. In the hexagonal H_{ii} phase at 45 °C, the cylinder diameter *a* is observed to increase weakly from approximately 69 to 71 Å at 0.5 mole fraction 7-ketocholesterol (Figure 6). At the same temperature, in the absence of sterol, Colotto and Epand (*32*) found a value of 71 Å. Surprisingly, cholesterol has no effect on *a*. Both cholesterol and 7-ketocholesterol strongly lower the bilayer to hexagonal phase transition temperature of diPoPE (Figure 7). Cholesterol is more effective than 7-ketocholesterol in lowering this transition temperature, and a combination of the two sterols is synergistic. The decrease in T_H when adding a certain mole fraction of 7-ketocholesterol to the 0.01 mole fraction cholesterol is greater than that produced by an equivalent amount of 7-ketocholesterol alone and similar to that observed for an equivalent amount of cholesterol alone (Figure 7). The shifts in T_H measured by DSC suggest that the sterols increase the negative curvature of diPoPE as discussed above for POPE. It is therefore surprising that 7-ketocholesterol weakly increases the diameter of the hexagonal phase cylinders while cholesterol

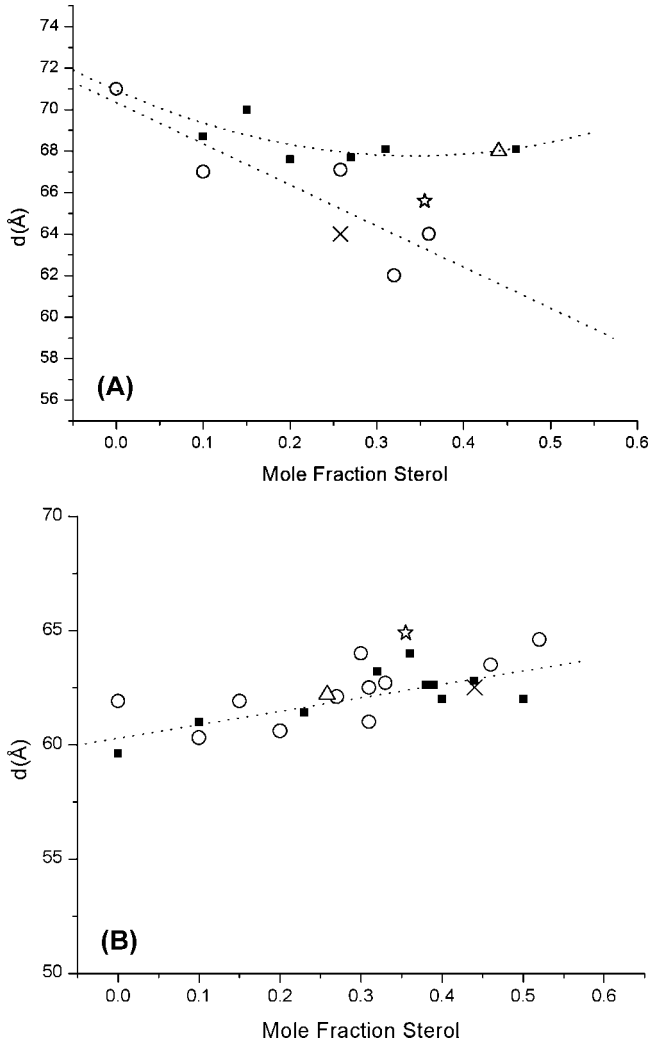


FIGURE 8: Dependence of the lamellar repeat (*d*) of SOPS on the mole fraction of added 7-ketocholesterol (○) or cholesterol (■): (A) in the gel phase and (B) in the liquid crystalline phase. The *d* spacings for the ternary mixtures with mole fractions of total sterol of 0.285 (☆), 0.35 (×), and 0.44 (△) as described in the text are also included.

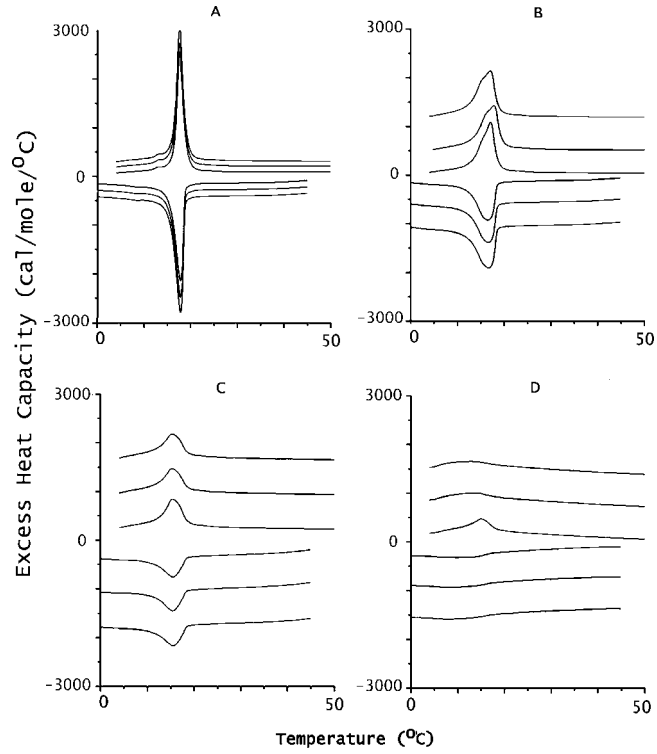


FIGURE 9: DSC scans of SOPS alone (A) and in the presence of 10 (B), 20 (C), or 30 mol % 7-ketocholesterol (D). The lipid concentration was 2.5 mg/mL in 20 mM PIPES, 1 mM EDTA, 150 mM NaCl, and 0.002% NaN_3 (pH 7.40). The scan rate was 2°/min. The top three curves are heating scans and the bottom three curves cooling scans. Curves have been displaced along the *y*-axis for presentation. Excess heat capacity is expressed per mole of SOPS.

appears to leave it unchanged. We discuss this unusual behavior below. Phase separation of crystalline sterol was observed in the liquid crystalline phase at approximately 0.31–0.35 mole fraction 7-ketocholesterol. The ternary system containing a molar ratio of cholesterol to 7-ketocholesterol equal to 0.8 (0.1 mole fraction total sterol) behaves like 7-ketocholesterol only. The ternary system containing a molar ratio of cholesterol to 7-ketocholesterol of 1.1:1 (0.18 mole fraction total sterol) was biphasic ($L\alpha/H_{ii}$) at 5 °C, indicating a synergistic effect; i.e., the phase behavior is similar to that promoted by cholesterol alone.

SOPS/Sterol Mixtures. Comparison with ref *33* shows that 7-ketocholesterol is much more effective than cholesterol in reducing the lamellar repeat in the gel state of SOPS (Figure 8A). In the case of cholesterol, the decrease levels off at ~68 Å with a mole fraction of sterol of approximately 0.3 (*33*). On the other hand, with 7-ketocholesterol one observes a decrease in *d* spacing that is proportional to the amount of added 7-ketocholesterol up to a mole fraction of 0.4. Low-temperature (3–5 °C) WAXS data show a marked increase in the line width of the chain packing from 0.5° (with d = 4.2 Å) to 3° (with d = 4.4 Å), when 0.32 mole fraction 7-ketocholesterol is added (data not shown). As in the case of POPE/sterol mixtures, this is evidence of the disordering effect of sterol on the hydrocarbon chains of the phospholipids. Phase separation of crystalline 7-ketocholesterol, as detected by the appearance of the characteristic diffraction peak at ~35 Å, occurs at ~0.4 mole fraction, much higher than the 0.2 mole fraction determined previously for cholesterol (*33*). In ternary systems, with molar ratios of cholesterol to 7-ketocholesterol of 6:1, 3.6:1, and 2.2:1 (mole fractions of total sterol of 0.44, 0.36, and 0.29, respectively), the first behaves like the binary mixture with cholesterol only and the last behaves like the binary mixture with 7-ketocholesterol only. The *d* spacing for the second mixture lies intermediate between those expected for the two binary systems. In the liquid crystalline phase at approximately 32 °C, addition of either 7-ketocholesterol or cholesterol (*33*) up to a sterol mole fraction of 0.5 leads to a monotonic increase in *d* spacing from approximately 60 to 62.5 Å (by linear regression). The ternary systems do not display any distinguishing behavior. In DSC, addition of up to 20 mol % 7-ketocholesterol broadens the melting transition of SOPS, and by 30 mol %, the enthalpy associated with the transition has been reduced by approximately 82% (Figure 9). Separation of crystallites of 7-ketocholesterol cannot be detected by DSC. Separation of crystallites of cholesterol is detected either by the polymorphic transition of anhydrous crystallites at 35 °C or by dehydration of the monohydrate crystalline form at approximately 100 °C (*34*). However, DSC thermograms of mixtures of both cholesterol and 7-ketocholesterol do show that 7-ketocholesterol may facilitate the solubili-

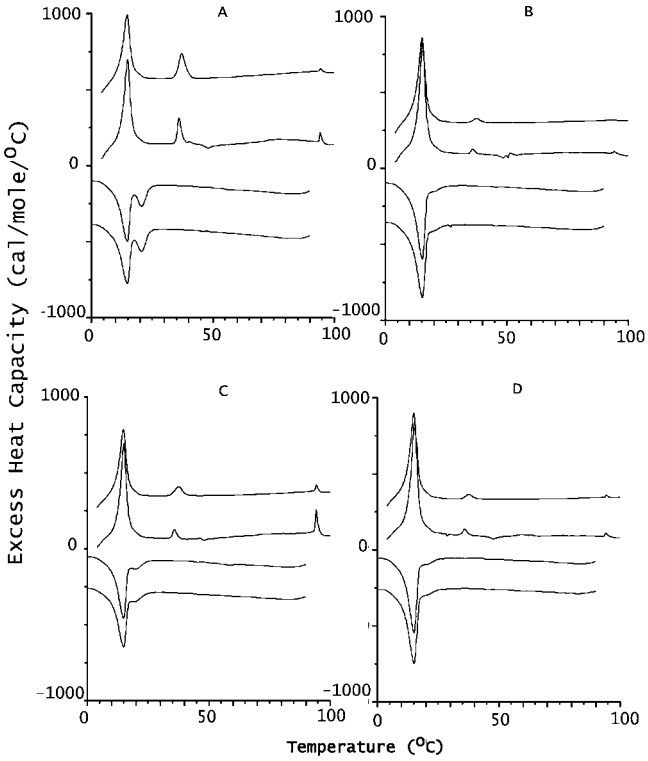


FIGURE 10: DSC scans of 6:4 SOPS/cholesterol mixtures with 0 (A), 5 (B), 10 (C), or 15 mol % 7-ketocholesterol (D). The lipid concentration was 2.5 mg/mL in 20 mM PIPES, 1 mM EDTA, 150 mM NaCl, and 0.002% NaN_3 (pH 7.40). The scan rate was 2°/min. The top two curves are heating scans and the bottom two curves cooling scans. Curves have been displaced along the *y*-axis for presentation. Excess heat capacity is expressed per mole of SOPS.

Table 1: Enthalpy (calories per mole of cholesterol) of Cholesterol Crystallites in Mixtures of SOPS and Cholesterol (6:4) with Added 7-Ketocholesterol

7-ketocholesterol (mol %)	ΔH (cal/mol of cholesterol)[a]
0	435
5	190
10	190
15	85

[a] Taken from data shown in Figure 10.

zation of cholesterol in bilayers of SOPS (Figure 10). This is detected by the marked decrease in the enthalpy associated with the endothermic peak at ~35 °C in DSC heating scans with an increase in 7-ketocholesterol content (Figure 10 and Table 1). The transition displays hysteresis upon cooling (*35*) (Figure 10). It must be noted, however, that if cholesterol and 7-ketocholesterol form mixed crystals in the bilayer, then the presence of the 7-keto form may serve to suppress the polymorphic phase transition of the anhydrous cholesterol crystals. In some of these scans, there is also a peak at 95 °C. As noted above, this phenomenon has been previously described for the dehydration transition of crystalline cholesterol monohydrate (*34*).

DISCUSSION

We have studied the interaction of 7-ketocholesterol with two of the major lipid components of the cytoplasmic leaflet of the plasma membrane, phosphatidylethanolamine and phosphatidylserine. The behavior was compared with that of cholesterol as well as, in a few cases, sterol mixtures. Phosphatidylethanolamine is not only an abundant lipid component of biological membranes but also an example of a lipid that readily converts to inverted phases. One of the forms of phosphatidylethanolamine that we used is POPE. POPE is a phospholipid with one oleoyl and one palmitoyl chain and a zwitterionic ethanolamine headgroup. Aqueous dispersions of POPE display three thermotropic phases: a gel phase below approximately 26 °C, a liquid crystalline phase between 26 and 71 °C, and an inverse hexagonal phase, H_{ii}, above 71 °C. Both cholesterol and 7-ketocholesterol are highly soluble in POPE bilayers. As detected by SAXS, phase separation of the crystalline sterol does not occur until the molar ratio is ~1:1 or higher. This high solubility is also displayed by cholesterol in POPC (*36*). In the gel phase of POPE, 7-ketocholesterol is as effective as cholesterol in reducing the lamellar repeat from 64 to 55 Å when the sterol content increases from 0 to 0.5 mole fraction. That this effect derives from the introduction of gauche conformers is confirmed by the marked increase in the line width of the hydrocarbon chain packing reflection at $d \sim 4$ Å upon addition of sterol. In the liquid crystalline phase, for a sterol mole fraction of <0.35, 7-ketocholesterol is marginally more effective than cholesterol in increasing the lamellar repeat. The maximum *d* spacing for 7-ketocholesterol in this phase is approximately 57 Å, while for cholesterol, it is approximately 54.5 Å. With a sterol mole fraction of >0.35, the *d* spacing in both cases decreases to 52.5 Å, i.e., almost equal to the *d* spacing of the liquid crystalline bilayers in the absence of sterol (~52 Å). According to our earlier DSC study of POPE/cholesterol mixtures, the enthalpy of the gel−liquid crystalline transition extrapolates to zero in the vicinity of 0.35 mole fraction cholesterol (*19*) while for 7-keto the extrapolation to zero enthalpy is at 0.42 mole fraction sterol. The POPE/sterol mixture giving zero enthalpy would exhibit little difference in acyl chain conformation between high and low temperatures. In this sense, it would be similar to a liquid ordered state in which the acyl chain conformation is fully extended. It is also true that cholesterol more strongly promotes the liquid crystalline phase than 7-ketocholesterol since cholesterol decreases T_M much more strongly, yet the enthalpy of the transition is approximately the same. In the H_{ii} phase, the increase in curvature caused by the presence of 7-ketocholesterol is similar to that caused by cholesterol (Figure 3). Chen and Rand (*22*) also found that cholesterol was potent in promoting negative curvature in phosphatidylethanolamine. One would anticipate that as the more hydrophobic sterol, cholesterol would penetrate further into the bilayer and hence would promote more negative curvature than 7-ketocholesterol. This is in agreement with the DSC measurements that show that cholesterol, at least at low mole fractions, decreases T_H (*19*) more than 7-ketocholesterol does (Figure 4). The shift in T_H determined by DSC is a measure of the relative stability of the lamellar and hexagonal phases. This criterion has been shown to be an indication of how biological activities can be sensitive to lipid composition (*37*). However, no difference is observed between these two sterols in the diameter of the H_{ii} cylinders (Figure 3), and at low mole fractions of sterol, the diameter actually increases slightly. The other phosphatidylethanolamine studied was diPoPE. This phospholipid has two 16-carbon acyl chains, each having a double bond at the C9

and C10 positions. DiPoPE has a T_H at 43 °C, much lower than that of POPE and one closer to physiological temperatures. However, the gel state forms below the freezing point of water and was not studied in this work. Above 0 °C, bilayers of diPoPE in an aqueous dispersion are in the liquid crystalline phase. In the liquid crystalline phase at 5 °C, 7-ketocholesterol up to 0.5 mole fraction is able to straighten hydrocarbon chains as indicated by the increased *d* spacing (Figure 5). Phase separation of crystalline 7-ketocholesterol occurs in the liquid crystalline phase at 0.3–0.35 mole fraction sterol as indicated by the formation of crystallites detected by SAXS. An estimate of the solubility of cholesterol in diPoPE cannot be made since cholesterol is such a strong promoter of the H_{ii} phase even at 5 °C that no cholesterol crystals are formed prior to the conversion to the H_{ii} phase. 7-Ketocholesterol, as also cholesterol, has much greater solubility in POPE than in diPoPE. This is likely to be a consequence of the presence of a saturated acyl chain, palmitic acid, in POPE that has a stronger tendency to interact with sterol ring systems and hence increase sterol solubility.

The temperature of the transition from the liquid crystalline phase to the hexagonal H_{ii} phase of diPoPE, measured by DSC, is significantly lowered by very small mole fractions of sterol, particularly cholesterol (Figure 7). Therefore, cholesterol is a stronger promoter of the H_{ii} phase of diPoPE than 7-ketocholesterol. SAXS shows that at mole fractions of cholesterol above 0.1, the transition to the H_{ii} phase is lowered to below 5 °C. An additive that increases the volume of the hydrocarbon chain region promotes the formation of the H_{ii} phase, as seen by the decrease in T_H. Cholesterol has a much stronger effect in lowering the T_H of diPoPE than of either dielaidoyl-PE ($18:1_t$) (*20*) or POPE (*19*). This may be a consequence of diPoPE having cis double bonds in the center of both acyl chains, while for dielaidoyl-PE, the double bonds have the trans configuration and therefore are less kinked and fit better with the planar sterol ring system, as does the saturated palmitoyl group on C1 of glycerol in POPE. Surprisingly, in the H_{ii} phase at 45 °C, addition of cholesterol has no effect on the curvature of the hexagonal phase cylinders. This is also true at 7 °C (data not shown). This lack of an effect may be the result of a balance between an increase in the volume of lipid chains as a consequence of the addition of cholesterol and a decreased radius caused by the promotion of negative curvature. In addition, there is the factor of the elastic bending modulus, which if it is high will reduce the change in cylinder radius as a consequence of changes in curvature. 7-Ketocholesterol, being more hydrophilic than cholesterol, sits closer to the headgroup region; both oxygen-containing groups of 7-ketocholesterol are at the water−membrane interface (*18*). Therefore, 7-ketocholesterol increases the surface area at the water−phospholipid interface and also may increase the degree of hydration as compared with cholesterol. This may explain the observed increase in the cylinder diameter with increasing amounts of 7-ketocholesterol. It should be noted, however, that the increase in area is quite small. With regard to ternary systems, it is interesting that low concentrations of cholesterol have a synergistic effect on 7-ketocholesterol with respect to formation of the H_{ii} phase.

The other major lipid component of the cytoplasmic leaflet is phosphatidylserine. This is an anionic lipid that is known to have low miscibility with cholesterol (*38*). The PS investigated here, SOPS, has one oleoyl and one stearoyl acyl chain. The transition from the gel to liquid crystalline phase occurs at 18 °C. In the gel state, 7-ketocholesterol is more effective than cholesterol in disordering the hydrocarbon chains. This can be due at least in part to the earlier onset of phase separation of cholesterol (0.2 mole fraction) from SOPS bilayers (*33*). There is little effect on the lamellar repeat above this amount of cholesterol, likely because of the lack of miscibility. On the other hand, added 7-ketocholesterol continues to reduce the lamellar repeat up to the highest content measured, which was 0.36 mole fraction The enthalpy of melting is reduced by approximately 82% at 0.3 mole fraction. Phase separation of crystalline 7-ketocholesterol occurs at ~0.4 mole fraction. In ternary mixtures, 7-ketocholesterol may facilitate the solubilization of cholesterol crystallites (Figure 10 and Table 1). The gel phase *d* spacing of a ternary mixture rich in cholesterol is similar to those of binary mixtures with cholesterol, while mixtures rich in 7-ketocholesterol behave like binary mixtures with 7-ketocholesterol. In the liquid crystalline phase, cholesterol and 7-ketocholesterol have similar effects on the lamellar repeat, i.e., increasing it with increasing sterol content.

Thus, oxidation of cholesterol to form one of the major oxidation products found in vivo, i.e., 7-ketocholesterol, causes significant changes in the biophysical properties of the lipid bilayers. 7-Ketocholesterol is more polar than cholesterol and hence is located closer to the membrane interface. From this, we can understand, in the case of PE, that 7-ketocholesterol is much less effective in promoting the formation of the H_{ii} phase and, in the case of POPE, also in promoting chain melting. The lower hydrophobicity of 7-ketocholesterol results in it being closer in polarity to phospholipids compared with the more hydrophobic cholesterol. This likely contributes to its greater solubility in SOPS bilayers and weaker tendency to segregate into crystallites. It is thus to be expected that several of the biophysical properties of a membrane would change as a result of the oxidation of cholesterol to 7-ketocholesterol. These changes in biophysical properties of the membrane bilayer would also have consequences for membrane proteins, in part through alterations in the curvature properties of the membrane as well as in the lateral segregation of components into domains in the plane of the membrane.

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