Cholesterol versus α-Tocopherol: Effects on Properties of Bilayers Made from Heteroacid Phosphatidylcholines†

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ABSTRACT: The techniques of differential scanning calorimetry, fluorescence of merocyanine 540, fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene, proton permeability, and lipid peroxidation are used to compare the perturbations of cholesterol and α-tocopherol on lipid bilayer membranes composed of different phosphatidylcholines containing stearic acid in the *sn*-1 position and an unsaturated fatty acid (either oleic, α-linolenic, γ-linolenic, or docosahexaenoic acid) in the *sn*-2 position. It is concluded that the structural roles of cholesterol and α-tocopherol may be similar with membranes composed of some phosphatidylcholines but are clearly different with membranes composed of other related phosphatidylcholines. α-Tocopherol exerts a much larger effect than cholesterol on membranes rich in polyunsaturated fatty acids that have their initial double bond before the Δ9 position. Cholesterol interacts more favorably with fatty acids that do not have an double bond before the Δ9 position. The membrane structural effects are explained in terms of the larger size of the sterol ring structure of cholesterol compared to the smaller chromanol ring of α-tocopherol.

α-Tocopherol and cholesterol are polar organic molecules that are commonly found in membranes where they may perform some similar, essential functions. Both molecules posses a bulky ring component containing a polar hydroxyl moiety and have a hydrophobic, branched chain floppy tail. The ring hydroxyl locates both molecules near the aqueous interface with their tails extending perpendicular to the interface into the membrane hydrophobic interior. While the major function suggested for α-tocopherol is as an antioxidant (Machlin, 1980; Burton & Ingold, 1989; Gramms & Eakins, 1972), recent reports have suggested that cholesterol may also play a similar role in some membranes (Parassasi et al., 1995). Cholesterol has long been recognized as a major membrane stabilizing agent (Yeagle, 1988; McMullen et al., 1994; Lucy, 1972; Massey et al., 1982). Although less documented, α-tocopherol has also been proposed to play a membrane structural role, comparable to that of cholesterol (Tappel, 1972; Diplock & Lucy, 1973). Therefore, the two compounds have strikingly similar, overlapping effects on membranes.

Many of the similarities between cholesterol and α-tocopherol have been interpreted from differential scanning calorimetry studies on disaturated phosphatidylcholine (PC)[1] bilayer membranes (Massey et al., 1982; DeKruijff et al., 1974; Vist & Davis, 1990). Three distinct phases with cholesterol/1,2-dipalmitoyl-PC bilayers were reported by Vist and Davis; a liquid crystalline phase L_α, a gel phase, and a cholesterol-rich phase called β. At low levels, both cholesterol and α-tocopherol obliterate the pretransition of saturated bilayers (Massey et al., 1982; Ortiz et al., 1987), while at higher concentrations both broaden and decrease the temperature and reduce the enthalpy of the main transition (T_c) (Massey et al., 1982). Both compounds are reported to lower the onset and midpoint temperature of the main transition while having little effect on the completion temperature (Massey et al., 1982), implying these molecules preferentially partition into the liquid crystalline phase of the melting lipids where they disrupt packing of the acyl chains (Gomez-Fernandez et al., 1991). Both compounds therefore increase fluidity and decrease order in gel state and decrease fluidity and increase order in liquid crystalline state membranes (Stillwell et al., 1992; Wassall et al., 1986).

Parallel DSC experiments have been reported by Sanchez-Migallon et al. (1996) (for α-tocopherol) and by Hernandez-Borrell and Keough (1993) (for cholesterol) using similar sets of heteroacid PCs. Both groups report a maximal effect on the liquid crystalline−gel transition by either α-tocopherol or cholesterol for PCs with 18:2 in the *sn*-2 position. Diminished effects were reported as the number of double bonds in the *sn*-2 position of the PCs decreased or increased relative to linoleic acid. Above T_c cholesterol and α-tocopherol are both proposed to decrease the formation of *gauche* confomers (Gomez-Fernandez et al., 1991; Cortijo et al., 1980). Permeability of liquid crystalline state bilayers to ions and neutral solutes has been reported to be diminished by both cholesterol (Yeagle et al., 1977; Demel et al., 1972) and α-tocopherol (Diplock et al., 1977). The effect of α-tocopherol on membrane permeability has been contentious as accounts of the vitamin both increasing (Srivastava et al., 1983; Cushley & Forrest, 1977; Cushley et al., 1979;

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[1] Abbreviations: BCECF, 2′,7′-bis(2-carboxyethyl)-5-(and-6-)-carboxyfluorescein; DHA, docosahexaenoic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; LUV, large unilamellar vesicles; MC540, merocyanine 540; MLV, multilamellar vesicles; PC, phosphatidylcholine; PE, phosphatidylethanolamine; 18:0, 18:1 PC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; 18:0, α-18:3 PC, 1-stearoyl-2-α-linolenoyl-*sn*-glycero-3-phosphocholine; 18:0, γ-18:3 PC, 1-stearoyl-2-γ-linolenoyl-*sn*-glycero-3-phosphocholine; 18:0, 22:6 PC, 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine.

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Pohlmann & Kuiper, 1981) and decreasing (Diplock & Lucy, 1973; Stillwell & Bryant, 1983) permeability have appeared. Finally, complexes between various lipids and both cholesterol and α-tocopherol have been described (Massey et al., 1982; Diplock & Lucy, 1973; Stillwell et al., 1992; Erin et al., 1984).

While at a first approximation cholesterol and α-tocopherol seem to affect membranes in a similar fashion, important differences exist. With disaturated PCs more cholesterol than α-tocopherol is required to effect the same change in the phase transition properties (Massey et al., 1982). DSC studies have also shown that cholesterol prefers PC > PE (Van Dijck et al., 1976) while α-tocopherol prefers to interact with the lower melting lipid whether it is PC or PE (Ortiz et al., 1987). Cholesterol prefers interaction with monoenoic fatty acids while α-tocopherol prefers polyunsaturated fatty acids (Lucy, 1972; Diplock & Lucy, 1973). Low levels of α-tocopherol produce poorly understood multicomponent DSC curves (Sanchez-Migallon et al., 1994, 1996) while cholesterol simply diminishes most transitions. Taken together, these observations indicate that cholesterol and α-tocopherol affect membranes differently and therefore may segregate into separate lipid domains.

The concentration of cholesterol in membranes varies over a wide range (Green, 1977). This sterol is almost completely absent in plant, bacterial, and fungal membranes and is found in very low levels in some animal intracellular membranes such as the mitochondrial inner membrane. In contrast, cholesterol's concentration may exceed 50 mol % in animal plasma membranes. Although α-tocopherol's membrane concentration may also vary over a wide range, it tends to be found in much lower levels than cholesterol. Polyunsaturated fatty acid/α-tocopherol ratios have been reported to range between about 130/1 to more than 3000/1 (Tappel, 1980; Kelley et al., 1995; Evarts & Bieri, 1974; Greuger & Tappel, 1971; Trimenstein & Reed, 1989). Most model membrane studies which describe the membrane structural role of cholesterol and α-tocopherol have used very high, often unrealistic levels of these compounds (10−50 membrane mol %) (Stillwell et al., 1992). While large concentrations may be justified for modeling the role of cholesterol in animal plasma membranes, it does not address the role of cholesterol in the many membranes that lack significant levels of this sterol. Also, the very high levels of α-tocopherol which have been employed clearly are contradictory to the physiological levels actually found in biological membranes. The affinities of α-tocopherol and cholesterol for lipid "binding sites" (Massey et al., 1982) may result in lateral phase separation into domains that are cholesterol-rich and polyunsaturated fatty acid- and α-tocopherol-poor, and cholesterol-poor and polyunsaturated fatty acid- and α-tocopherol-rich. Years ago, Maggio et al. (1977) suggested that α-tocopherol may in fact be particularly important in membranes that have a large amount of polyunsaturated fatty acids and little cholesterol. Since complex heterogeneous distributions of the many membrane lipids may be the relevant factor in membrane structure/function, global membrane concentration averages, which are normally reported for cholesterol and α-tocopherol, may not present an accurate description of their actual local concentration in membranes. Much more work on lipid interactions is needed to grasp the fundamental principles behind lipid domain formation and function.

The obvious similarity in dynamic structural properties shared between cholesterol and α-tocopherol suggests the need for studies directly comparing the effect of these compounds on various membrane properties. To date, however, comparisons must be gleaned from experiments done with either cholesterol or α-tocopherol from various laboratories, using different phospholipids and different techniques. As a result, reports are often incomplete or contradictory. Here we attempt to clarify some of the ambiguities by side-by-side comparisons of cholesterol and α-tocopherol with biologically relevant, heteroacid, polyunsaturated phospholipids.

EXPERIMENTAL PROCEDURES

α-Tocopherol was purchased from Fluka BioChemica (Ronkonkoma, NY) and cholesterol from Sigma Chemical Co. (St. Louis, MO). The phospholipids DPPC; 18:0, 18:1 PC; 18:0, α-18:3 PC; and 18:0, 22:6 PC were purchased from Avanti Polar Lipids (Alabaster, AL). 18:0, γ-18:3 PC was synthesized in our laboratory as previously described (*36*). Merocyanine 540 was purchased from Sigma, and BCECF and DPH were from Molecular Probes (Eugene, OR).

Differential Scanning Calorimetry. Multilamellar vesicles (MLV) were made by hydrating overnight the appropriate phospholipid with or without cholesterol or α-tocopherol at 9−10 mg of lipid/mL in 10 mM sodium phosphate, pH 7.0. Prior to MLV formation, all organic solvents were carefully removed under nitrogen followed by 12 h under vacuum. Ultrapure Milli-Q water was used for all measurements. MLV were frozen and thawed 3 times in liquid nitrogen and then degassed for 20 min under vacuum. A 500 μL aliquot of the MLV suspensions was added to each of 3 chambers, with the 4th chamber containing 500 μL of the buffer. Scans were made at 5 °C/h in a Hart Scientific differential scanning calorimeter (Provo, UT). Only the cooling curves are presented.

MC540 Fluorescence. The fluorescent probe MC540 has been used to detect lipid packing in membranes (Williamson et al., 1983). The more efficiently the probe partitions into membranes, the higher is its fluorescence at 595 nm (Stillwell et al., 1993). The method employed is detailed in Stillwell et al. (1993). MLV were made by hydrating each of the following phospholipids, DPPC; 18:0, 18:1 PC; 18:0, α-18:3 PC; 18:0, γ-18:3 PC or 18:0, 22:6 PC, with either cholesterol or α-tocopherol at mol ratios of 10:0, 9:1, or 8:2, phospholipid/cholesterol or α-tocopherol in 10 mM Tris/10 mM sodium acetate, pH 7.4. The MLV were then extruded 10 times through 0.1 or 0.2 μm Nucleopore Filters using a temperature-controlled Extruder (Lipex, Vancouver, BC, Canada). The resultant large unilamellar vesicles (LUV) were then incubated with MC540 (10 μg/mL in 10 mM Tris/10 mM sodium acetate, pH 7.4) at a lipid/probe ratio of about 100/1 (mol/mol) for 30 min. Excitation was at 540 nm, and the emission spectra were recorded from 550 to 650 nm at 30 °C using a Perkin-Elmer MPF-66 fluorescence spectrophotometer.

Proton Permeability. The effect of cholesterol and α-tocopherol on permeability of protons to membranes composed of 18:0, α-18:3 PC and 18:0, 22:6 PC was measured using the fluorescence probe BCECF. BCECF fluorescence is highly pH dependent, and since the molecule carries 4−5 negative charges at physiological pH, it is impermeable to

membranes. For these reasons, BCECF has probably been used more than any other probe for determining intracellular pH (Jung et al., 1989). LUV (diameter 0.1 μm) were made in the presence of BCECF (2 μg/mL in 90 mM NaCl/10 mM Tris, pH 7.4 buffer) and the nonsequestered probe separated on a Sephadex G-50 column. LUV suspensions (about 0.5 mg of lipid/mL) were added to fluorescence cuvettes, and a pH gradient was established by adding a small volume of HCl to the mixing SUV. The initial pH was 7.4, and the final equilibrium pH, determined by the LUV-sequestered BCECF fluorescence, was between 4.9 and 5.2. The rate of fluorescence change (excitation at 500 nm and emission at 530 nm) was monitored on a Perkin-Elmer LS 50B fluorescence Spectrometer and represents the proton permeability rate of the membranes.

DPH Fluorescence. LUV were made by extruding MLV through 0.2 μm Nucleopore Filters as described above from either 18:0, 18:1 PC or 18:0, 22:6 PC that included either 0, 1, 3, 5, 10, 15, or 20 membrane mol % cholesterol or α-tocopherol. Prior to MLV formation DPH (1,6-diphenyl-1,3,5-hexatriene) was included into the lipid mixture at a 200:1 lipid to probe mole ratio. Anisotropies (A) were measured on a Perkin-Elmer MPF-66 fluorescence spectrophotometer at 37 °C as calculated by the following equation:

$$A = (I_{||} - GI_{\perp})/(I_{||} + 2GI_{\perp})$$

where $I_{||}$ and $I_{\perp}$ are the intensities of the horizontal and vertical components of the emitted light and $G = I_{||}/I_{\perp}$. Excitation was at 351 nm and emission at 430 nm. Results are expressed as relative anisotropies where the measured anisotropies are normalized to the cholesterol-free or α-tocopherol-free bilayers.

Lipid Peroxidation. Lipid peroxidation of SUV by Cu_2-SO_4/H_2O_2 in 50 mM Tris, pH 7.0, was determined by measuring conjugated diene formation spectrophotometrically (absorbance at 236 nm) with tandem cuvettes (Esterbauer et al., 1989; Vossen et al., 1993) over a period of up to 3 h. SUV were composed of an equimolar mixture of 18:0, 22:6 PC/18:0, 18:0 PC. The method is detailed in Vossen et al. (1993) and Zerouga et al. (1995).

RESULTS

Differential scanning calorimetry (DSC) was used to compare the effect of cholesterol (Figure 1, panel A) and α-tocopherol (Figure 1, panel B) on the phase behavior of phospholipid bilayers. A first experiment used 0−10 membrane mol % cholesterol or α-tocopherol on DPPC bilayers. In agreement with prior reports (Massey et al., 1982; DeKruijff et al., 1974), both compounds broadened the main transition (decreased cooperativity) and lowered the position of the initial and main transition while retaining the same completion temperature (Figure 1). In Figure 2 the main transition temperature (panel A) and cooperativity units, determined as described by Small (1986) (panel B), are plotted against the membrane mol % of α-tocopherol and cholesterol. While both compounds qualitatively affected the transition similarly, α-tocopherol exhibited a significantly greater influence on the transition of the disaturated PC than did cholesterol.

A second DSC experiment (Figure 3) compared the relative interaction of cholesterol and α-tocopherol with bilayers made from either 18:0, 18:1 PC or 18:0, 22:6 PC.

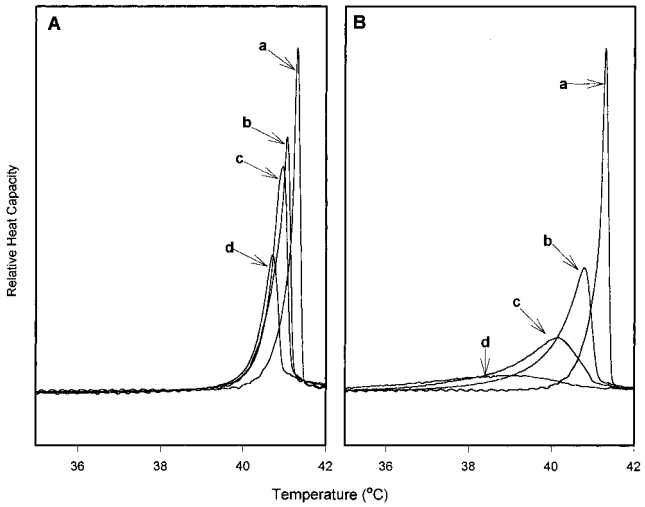

FIGURE 1: Panel A: Effect of 0 (curve a), 2 (curve b), 4 (curve c), and 10 (curve d) membrane mol % cholesterol on differential scanning calorimetry cooling scans of MLV made from DPPC. Panel B: Effect of 0 (curve a), 2 (curve b), 4 (curve c), and 10 (curve d) membrane mol % α-tocopherol on differential scanning calorimetry cooling scans of MLV made from DPPC.

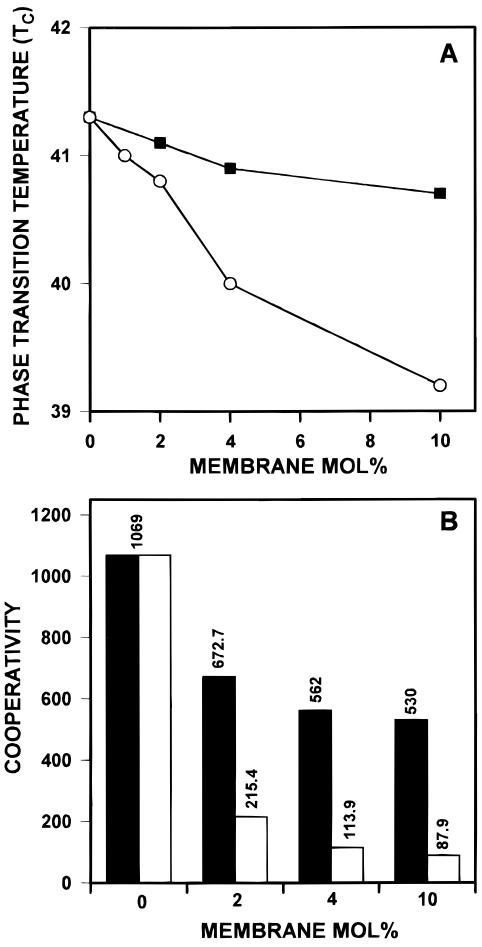

FIGURE 2: Effect of 0, 2, 4, and 10 membrane mol % cholesterol (■) and 0, 1, 2, 4, and 10 membrane mol % α-tocopherol (○) on the main transition temperature (T_c) (panel A) and cooperativity units (panel B, calculated as described in Small (1986)), of MLV made from DPPC. Values are obtained from the DSC curves presented in Figure 1.

These phospholipids were chosen because they contain fatty acids in the *sn*-2 position which represent the extremes of unsaturation found at realistic levels in biological membranes.

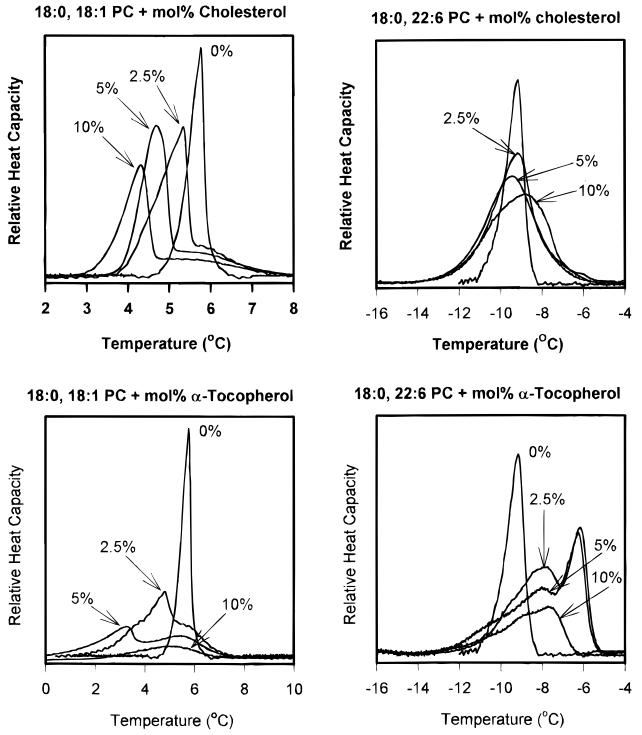

FIGURE 3: Effect of cholesterol and α-tocopherol on the phase transition of 18:0, 18:1 PC or 18:0, 22:6 PC bilayer membranes. Panel A: 0, 2.5, 5, and 10 membrane mol % cholesterol on 18:0, 18:1 PC membranes. Panel B: 0, 2.5, 5, and 10 membrane mol % cholesterol on 18:0, 22:6 PC membranes. Panel C: 0, 2.5, 5, and 10 membrane mol % α-tocopherol on 18:0, 18:1 PC membranes. Panel D: 0, 2.5, 5, and 10 membrane mol % α-tocopherol on 18:0, 22:6 PC membranes.

Most membrane phospholipids are heteroacids with the *sn*-1 chain being saturated (usually palmitic (16:0) or stearic (18:0) acid) and the *sn*-2 chain unsaturated (White, 1973). Oleic acid ($18:1^{\Delta 9}$) is an omega-9 fatty acid found in large amounts in most membranes while the omega-3 fatty acid docosahexaenoic acid (DHA $22:6^{\Delta 4,7,10,13,16,19}$) is the longest and most unsaturated fatty acid commonly found in membranes (Whitting et al., 1961). Oleic acid has its double bond at position Δ9 while the initial double bond in DHA is at position Δ4. 18:0, 18:1 PC has its main transition at +5.9 °C while 18:0, 22:6 PC has its main transition at −9.2 °C. Cholesterol broadens (decreases cooperativity) and decreases the temperature of the main transition of 18:0, 18:1 PC bilayers (Figure 3, panel A) but only broadens the transition without altering its temperature for bilayers made from 18:0, 22:6 PC (Figure 3, panel B). A slight shouldering centered around the unmodified 18:0, 18:1 PC (+5.9 °C) transition was also noted for the cholesterol/18:0, 18:1 PC bilayers. The effect of α-tocopherol is more complex than with cholesterol. Its presence produces very obvious bi- or multicomponent thermograms in both 18:0, 18:1 PC (Figure 3, panel C) and 18:0, 22:6 PC (Figure 3, panel D) bilayers. As with cholesterol, the major effect of α-tocopherol on 18:0, 18:1 PC bilayers is to broaden and decrease the main transition temperature. In contrast, the effect of α-tocopherol on 18:0, 22:6 PC bilayers is complex, producing multicomponent thermograms with increasing transition temperature. As was noted with cholesterol, a similar shouldering centered around the unmodified 18:0, 18:1 PC (+5.9 °C) transition was also noted for the α-tocopherol/18:0, 18:1 PC bilayers. Complex, multiple component DSC curves have been reported in previous α-tocopherol studies (Ortiz et al., 1987).

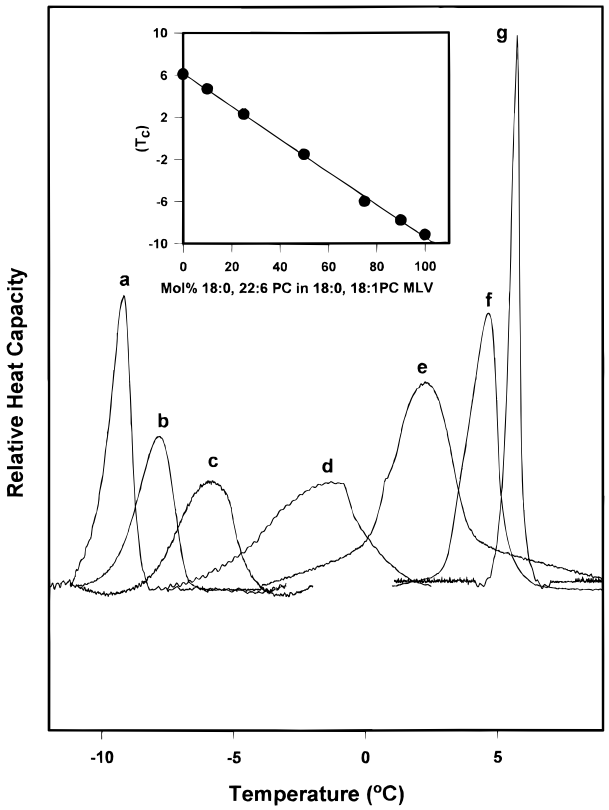

FIGURE 4: Differential scanning calorimetry cooling scans of MLV made from mixtures of 18:0, 18:1 PC/18:0, 22:6 PC. Curve a: 100 membrane mol % 18:0, 22:6 PC; curve b: 90 membrane mol % 18:0, 22:6 PC/10 membrane mol % 18:0, 18:1 PC; curve c: 75 membrane mol % 18:0, 22:6 PC/25 membrane mol % 18:0, 18:1 PC; curve d: 50 membrane mol % 18:0, 22:6 PC/50 membrane mol % 18:0, 18:1 PC; curve e: 25 membrane mol % 18:0, 22:6 PC/75 membrane mol % 18:0, 18:1 PC; curve f: 10 membrane mol % 18:0, 22:6 PC/90 membrane mol % 18:0, 18:1 PC; and curve g: 100 membrane mol % 18:0, 18:1 PC. Insert: Main transition temperatures (T_c) for the various 18:0, 18:1 PC/18:0, 22:6 PC mixtures presented in the main Figure.

Various mixtures of 18:0, 18:1 PC and 18:0, 22:6 PC exhibit single broad transitions between those demonstrated by the single component PCs (Figure 4). In fact, a plot of the main transition temperature (T_c) vs the mol % 18:0, 22:6 PC in 18:0, 18:1 PC bilayers produces a straight line (Figure 4, insert). Therefore, a measured transition temperature between +5.9 and −9.2 °C precisely defines the mole composition of an 18:0, 18:1 PC/18:0, 22:6 PC bilayer. At 10 membrane mol % both cholesterol and α-tocopherol greatly diminish the the transition of bilayers made from either 18:0, 18:1 PC or 18:0, 22:6 PC (Figure 3). Mixed phospholipid bilayer membranes made from equal molar mixtures of 18:0, 18:1 PC and 18:0, 22:6 PC exhibited a single transition at −1.5 °C. Upon the addition of increasing amounts of cholesterol to this mixed membrane, the measured transition temperature (T_c) was demonstrated to decrease (Figure 5). A cholesterol-induced decrease in T_c indicates that the membrane behaves as if it had a larger relative mol % of 18:0, 22:6 PC. One possible interpretation of this observation is that cholesterol associates more strongly with the 18:0, 18:1 PC component, leaving the bilayer enriched in free 18:0, 22:6 PC. In sharp contrast, α-tocopherol produces a two component curve in the 18:0, 18:1 PC/18:0, 22:6 PC (1:1. mol:mol) membranes (Figure 6).

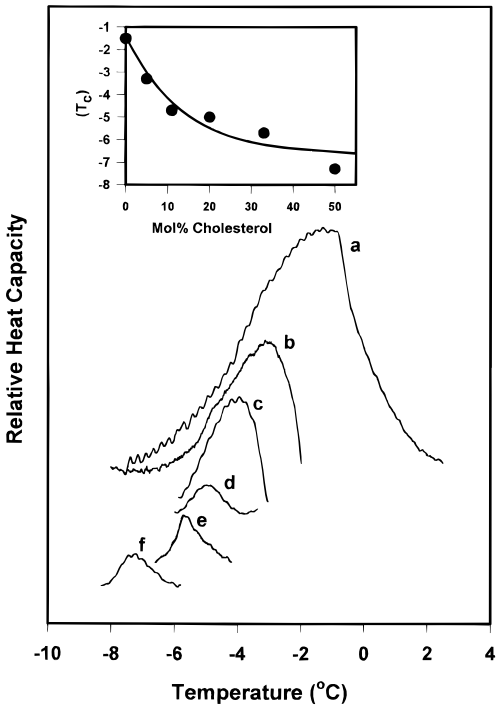

FIGURE 5: Differential scanning calorimetry cooling scans of MLV made from 18:0, 18:1 PC/18:0, 22:6 PC (1:1) to which (a) 0 membrane mol % cholesterol; (b) 5 membrane mol % cholesterol; (c) 11 membrane mol % cholesterol; (d) 20 membrane mol % cholesterol; (e) 33 membrane mol % cholesterol; and (f) 50 membrane mol % cholesterol are added. Insert: Main transition temperature for the 18:0, 18:1 PC/18:0, 22:6 PC/cholesterol mixed bilayers presented in the main figure.

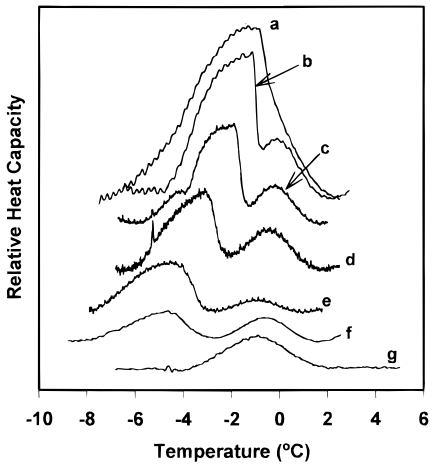

FIGURE 6: Differential scanning calorimetry cooling scans of MLV made from 18:0, 18:1 PC/18:0, 22:6 PC (1:1) to which (a) 0 membrane mol % α-tocopherol; (b) 0.3 membrane mol % α-tocopherol; (c) 1 membrane mol % α-tocopherol; (d) 3 membrane mol % α-tocopherol; (e) 5 membrane mol % α-tocopherol; (f) 7 membrane mol % α-tocopherol; and (g) 10 membrane mol % α-tocopherol are added.

Even at very low α-tocopherol levels (0.3 membrane mol %), the DSC curve, is split into a high temperature component (T_c about −0.5 °C) and a low temperature component that decreases in temperature and broadens with increasing levels of α-tocopherol. By 10 mol % α-tocopherol, the lower melting component is completely obliterated while the higher melting component remains intact. The effect of α-tocopherol on the low temperature component is similar to that of cholesterol on the 18:0, 18:1/18:0, 22:6 PC (1:1) bilayers reported in Figure 5.

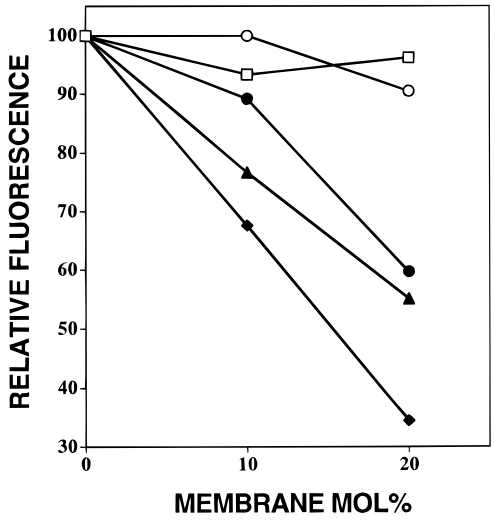

FIGURE 7: Effect of cholesterol incorporation, expressed as membrane mol %, into LUV made from 18:0, 22:6 PC (○), 18:0, γ-18:3 PC (□), DPPC (●), 18:0, α-18:3 PC (▲), and 18:0, 18:1 PC (♦) on the relative fluorescence of MC540 monitored at 598 nm.

The fluorescent probe MC540 was used to monitor the effect of cholesterol and α-tocopherol on packing of bilayer membranes made from different phospholipids. MC540 partitions between the aqueous bathing solution where its fluorescence is low and the membrane where its 595 nm fluorescence is greatly enhanced (Stillwell et al., 1993). Therefore, if either cholesterol or α-tocopherol increased bilayer packing density, MC540 would be excluded from the membrane and its fluorescence would decrease. Bilayers made of five phospholipids were tested including the three used in the DSC experiments, DPPC, 18:0, 18:1 PC, and 18:0, 22:6 PC. In addition, two PCs having 18:0 in the *sn*-1 position and either α-18:3 or γ-18:3 in the *sn*-2 position were also tested. The two linolenic acids have 18 carbons and three double bonds each and only differ in the location of the double bonds. α-18:3 ($18:3^{\Delta 9,12,15}$) is an omega-3 fatty acid with its first double bond in the Δ9 position while γ-18:3 ($18:3^{\Delta 6,9,12}$) is an omega-6 fatty acid with its first double bond in the Δ6 position. Early work from Ghosh and Tinoco (1971) and Demel et al. (1972) indicated that cholesterol condenses monolayers made from some phospholipids including DPPC, and heteroacid PCs containing 18:1 or α-18:3 in the *sn*-2 position. In contrast, heteroacid PCs containing 22:6 do not condense (Demel et al., 1972). We also demonstrated that MC540 could be successfully employed to monitor cholesterol condensation and obtained results parallel to those from pressure/area measurements on a Langmuir Trough (Stillwell et al., 1993). The MC540 experiments are reported in Figure 7 for cholesterol and Figure 8 for α-tocopherol. Because the addition of cholesterol to 18:0, γ-18:3 PC and 18:0, 22:6 PC did not alter MC540 fluorescence, we can conclude that cholesterol does not condense membranes made from these phospholipids. In contrast, DPPC, 18:0, α-18:3 PC, and 18:0, 18:1 PC exhibited a significant decrease in the 595 nm fluorescence, indicating that they are condensed by cholesterol. By comparison, α-tocopherol decreased the fluorescence for all five PCs tested and so may induce condensation for membranes composed of each lipid.

Upon membrane condensation by cholesterol, bilayer permeability rates are known to decrease (Demel et al.,

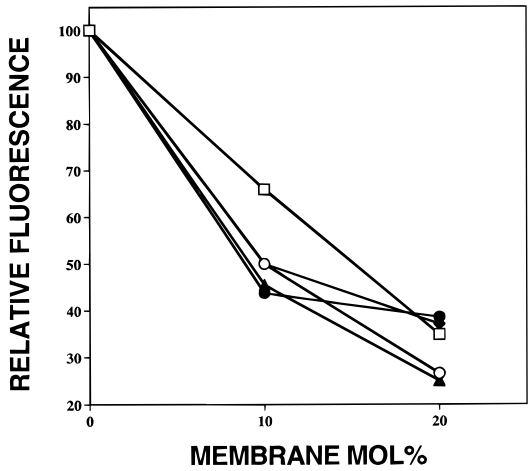

FIGURE 8: Effect of α-tocopherol incorporation, expressed as membrane mol %, into LUV made from 18:0, 22:6 PC (○), 18:0, γ-18:3 PC (□), DPPC (●), 18:0, α-18:3 PC (▲), and 18:0, 18:1 PC (♦) on the relative fluorescence of MC540 monitored at 598 nm.

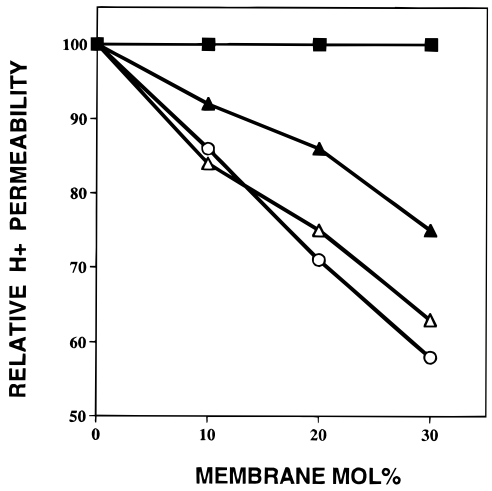

FIGURE 9: Effect of cholesterol, expressed as membrane mol %, on relative H^+ permeability rates of LUV made from 18:0, 22:6 PC (■) and 18:0, α-18:3 PC (▲). Effect of α-tocopherol, expressed as membrane mol %, on relative H^+ permeability rates of LUV made from 18:0, 22:6 PC (○) and 18:0, α-18:3 PC (△).

1972). To compare cholesterol with α-tocopherol, we made large unilamellar vesicles (LUV) by the extrusion method from either 18:0, α-18:3 PC or 18:0, 22:6 PC. Either cholesterol or α-tocopherol was incorporated into the LUV at 0, 10, 20, or 30 membrane mol %, and proton permeability rates were determined. Cholesterol was shown to significantly diminish H^+ permeability for the condensable 18:0, α-18:3 PC LUV but had no effect on permeability of the noncondensable 18:0, 22:6 PC LUV (Figure 9). In contrast, α-tocopherol diminished H^+ permeability of LUV made from both lipids.

DPH has been used more often than any other fluorescent membrane probe to monitor fluidity or order in the hydrophobic core of membranes. In Figure 10 we used this probe to compare the effect of cholesterol and α-tocopherol on membrane fluidity (expressed as relative anisotropies) to bilayers composed of either 18:0, 18:1 PC or 18:0, 22:6 PC. For membranes composed of 18:0, 18:1 PC both cholesterol and α-tocopherol slightly decreased fluidity (increase anisotropy) to about the same extent. By comparison, a large difference in the behavior of these compounds can be readily

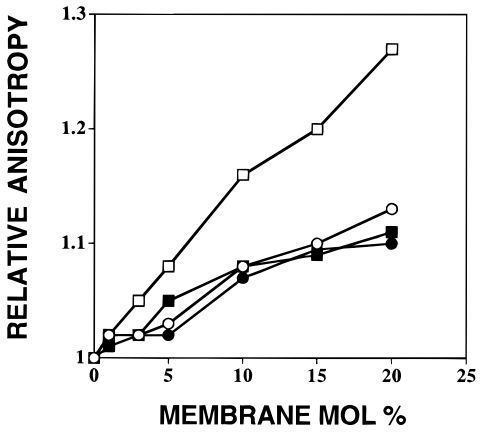

FIGURE 10: Effect of cholesterol and α-tocopherol, expressed as membrane mol %, on relative anisotropy of the fluorescent probe DPH in LUV composed of 18:0, 18:1 PC or 18:0, 22:6 PC. 18:0, 18:1 PC + cholesterol (●); 18:0, 22:6 PC + cholesterol (■); 18:0, 18:1 PC + α-tocopherol (○); and 18:0, 22:6 PC + α-tocopherol (□).

detected with membranes composed of 18:0, 22:6 PC. The presence of α-tocopherol in the highly unsaturated membranes was shown to substantially decrease fluidity (increase anisotropy) compared to cholesterol.

Although α-tocopherol's role as a membrane-bound antioxidant has been well-established (Machlin, 1980; Burton & Ingold, 1989; Gramms & Eakins, 1972), some reports have suggested that cholesterol may also prevent lipid oxidation (Parassasi et al., 1995). In the experiments presented here both cholesterol and α-tocopherol protected 18:0, 22:6 PC from oxidation; however, the modes of action for these compounds appear to be different. Upon initiation of oxidation by Cu_2SO_4/H_2O_2, the nonmodified 18:0, 22:6 PC/18:0, 18:0 PC control bilayers and the cholesterol-modified 18:0, 22:6 PC/18:0, 18:0 PC bilayers displayed an immediate increase in absorbance at 236 nm, indicating production of conjugated dienes. Both cholesterol and α-tocopherol significantly decreased the measured peroxidation rate of 18:0, 22:6 PC (Figure 11). For example, after 3000 s, 1.0 membrane mol % α-tocopherol decreased the extent of peroxidation by about 90% while cholesterol decreased peroxidation by 40%. The measured net peroxidation rates after long times with α-tocopherol, however, are misleading. With α-tocopherol, bimodal kinetics were observed. Initially the 18:0, 22:6 PC/18:0, 18:0 PC bilayers exhibited an α-tocopherol-dependent time delay before any oxidation could be measured (Figure 11, insert). During this time α-tocopherol totally prevented lipid peroxidation. Once all of the α-tocopherol was consumed, lipid peroxidation began, but at a diminished rate compared to cholesterol (results not shown). We propose that this reduced antioxidant function is due to α-tocopherol's structural role in membranes. α-Tocopherol, therefore, was a more effective antioxidant than was cholesterol and prevented oxidation by two distinct mechanisms, a free radical scavaging mechanism and a membrane structural mechanism.

DISCUSSION

The experiments presented here compare the effect of cholesterol to α-tocopherol in side-by-side measurements on identical phospholipid bilayer membranes. The results are in agreement with several reports that have indicated both

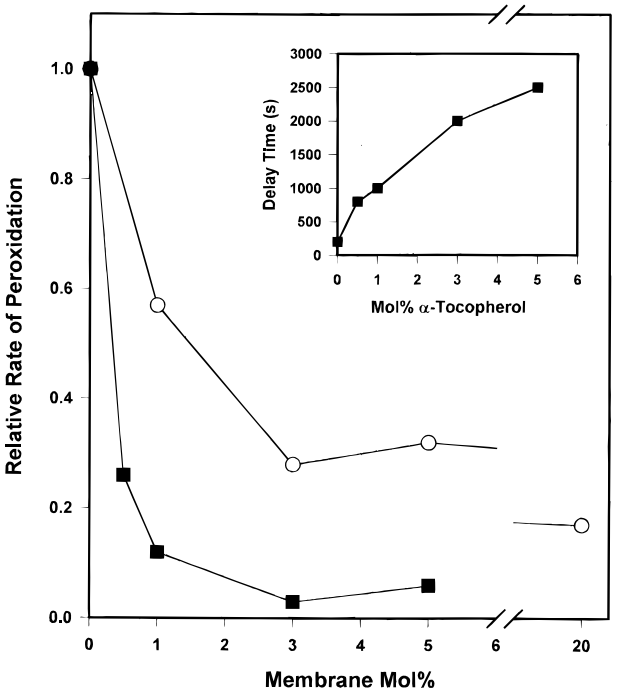

FIGURE 11: Effect of α-tocopherol (■) and cholesterol (○), expressed as membrane mol %, in preventing lipid peroxidation of SUV composed of equimolar mixtures of 18:0, 22:6 PC/18:0, 18:0 PC. Relative peroxidation rates were determined by the change in absorbance at 236 nm over 3000 s. Insert: Delay time before the initiation of peroxidation as a function of membrane mol % α-tocopherol.

cholesterol (Yeagle, 1988: McMullen et al., 1994; Lucy, 1972; Massey et al., 1982) and α-tocopherol (Tappel, 1972; Diplock & Lucy, 1973) may provide important structural roles in membranes. Both compounds may also have important antioxidant properties. Here we suggest that the functions of cholesterol and α-tocopherol may be similar with membranes composed of some PCs but are clearly different with membranes composed of other, more unsaturated PCs. We also hypothesize a relationship between the location of acyl chain double bonds and PC's association with α-tocopherol and cholesterol.

Both cholesterol and α-tocopherol have a similar molecular architecture with a polar hydroxyl anchoring the molecule to the aqueous interface, a series of rigid rings, and a flexible acyl tail. Both compounds are oriented perpendicular to the aqueous interface, but their precise depth in the bilayer remains contentious. Cholesterol (Van Echteld et al., 1981) and α-tocopherol (Salgado et al., 1993) have been shown to stabilize bilayers made from micelle-preferring, cone shaped lipids such as lyso PCs and PCs with one long chain and a second very short chain. The dynamic shape of cholesterol and α-tocopherol has been suggested to be complementary to the micelle-preferring lipids, resulting in cylindrical-shaped, bilayer-preferring complexes (Van Echteld et al., 1981). One obvious difference between cholesterol and α-tocopherol is in the size of the rigid rings. Cholesterol contains four rings while α-tocopherol only has two. We will interpret the results presented here in terms of the dynamic shape and ring size of cholesterol and α-tocopherol.

The differential scanning calorimetry experiments presented in Figures 1−6 demonstrate the paradox that both cholesterol and α-tocopherol may affect some bilayers in a very similar manner while exhibiting opposing effects with bilayers composed of related but different phospholipids. Both compounds obliterate the pretransition, decrease the temperature of the initial melting, and decrease the position and enthalpy of the main transition while having no effect on the completion temperature for bilayers composed of the disaturated phospholipid DPPC (Figure 1). α-Tocopherol has a larger influence on DPPC bilayers than does cholesterol (Figure 2). When incorporated into mixed heteroacid PC bilayers (18:0, 18:1 PC/18:0, 22:6 PC, 1:1), however, the effect of cholesterol (Figure 5) and α-tocopherol (Figure 6) can be very clearly distinguished. Low amounts of cholesterol first obliterate the transition of the higher melting 18:0, 18:1 PC component producing bilayers that appear to be enriched in 18:0, 22:6 PC. From this we conclude that cholesterol may exhibit a preference for 18:0, 18:1 PC over 18:0, 22:6 PC. The preference of cholesterol for the oleic acid-containing PC over the DHA-containing PC observed by DSC agrees with the lipid packing (Figures 7), proton permeability (Figure 9) and fluidity (Figure 10) experiments. When the identical DSC experiments were performed with α-tocopherol replacing cholesterol, a two component curve was generated. While these DSC scans and the other biophysical measurements may indicate that α-tocopherol has induced phase separation in the mixed PC membranes, the composition of each component is not certain. Also, other possibilities including the mixed PC bilayers undergoing two separate structural transitions cannot be ruled out. The lower melting component induced by α-tocopherol resembles that produced by cholesterol incorporation into the same mixed PC system. With increasing levels of α-tocopherol, the temperature and enthalpy of the main transition decreased. In contrast with cholesterol, α-tocopherol incorporation induced a second transition that remained at about −0.5 °C.

While multiple-component DSC curves, indicating phase separation, are easily obtained upon incorporation of α-tocopherol into unsaturated PC membranes, the molecular makeup of the phases is uncertain. The experiments monitoring membrane fluidity by DPH anisotropy (Figure 10), lipid packing (Figures 7 and 8), and proton permeability (Figure 9) may help in interpreting some of the DSC results. The anisotropy measurements (Figure 10) implicate a stronger association of α-tocopherol for the DHA-containing phospholipid (18:0, 22:6 PC) compared to the oleic acid phospholipid (18:0, 18:1 PC). Also, α-tocopherol induces a much larger decrease in fluidity in the 18:0, 22:6 PC membranes than does cholesterol (Figure 10). The effect of cholesterol and α-tocopherol on lipid packing in bilayers as detected by MC540 fluorescence (Figures 7 and 8) corroborates the fluidity results. Cholesterol excludes MC540 from bilayers composed of DPPC, 18:0, 18:1 PC, and 18:0, α-18:3 PC, resulting in diminished fluorescence, but has little effect on bilayers composed of 18:0, 22:6 PC and 18:0, γ-18:3 PC (Figure 7). In contrast, incorporation of α-tocopherol excludes MC540 from bilayers composed of all 5 PCs (Figure 8). The effect of cholesterol and α-tocopherol on proton permeability (Figure 9) further supports the DPH and MC540 conclusions. Cholesterol is shown to substantially decrease permeability of lipid vesicles made from 18:0, α-18:3 PC while having no effect on membranes made from 18:0, 22:6 PC. Again, α-tocopherol decreases permeability of both types of membranes.

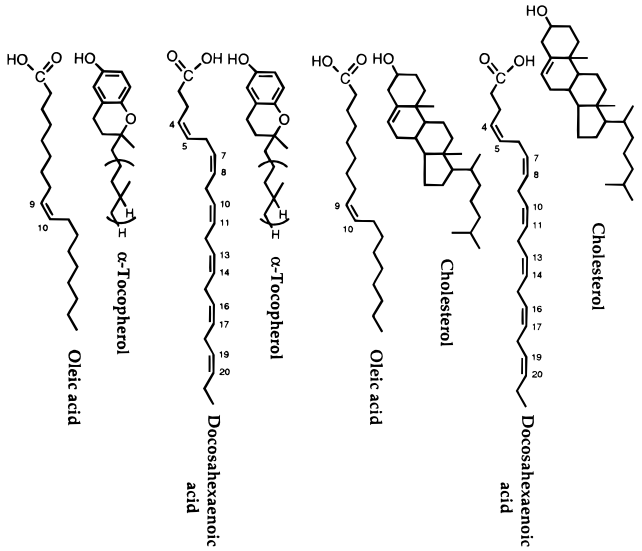

FIGURE 12: Diagram illustrating that α-tocopherol can fit into the Δ9 pocket of oleic acid and the Δ4 pocket of docosahexaenoic acid while the larger sterol ring prevents cholesterol from inserting into the Δ4 pocket of docosahexaenoic acid.

Results of the experiments reported here may be interpreted in terms of the larger size of the sterol ring component of cholesterol compared to the smaller chromanol ring of α-tocopherol. Huang (1977) has proposed that cholesterol fits into the Δ9 pocket in acyl chains which have their initial double bond at C9−10. Therefore, cholesterol could be accommodated into bilayers containing fatty acids with a Δ9 pocket (such as oleic and α-linolenic acid) while being excluded from bilayers containing fatty acids with smaller pockets like DHA (Δ4) and γ-linolenic acid (Δ6). In contrast, the smaller chromanol ring of α-tocopherol may allow this molecule to fit into the three different sized pockets (Δ4, Δ6, and Δ9) tested here, and so bilayers made from each of these 5 phospholipids may be affected. This hypothesis is demonstrated in Figure 12.

The reported measurements on DPH anisotropy, lipid packing, and proton permeability all indicate that α-tocopherol associates much more strongly than cholesterol with 18:0, 22:6 PC. An extension of this conclusion is that one of the two components observed in the DSC experiments with 18:0, 18:1 PC/18:0, 22:6 PC/α-tocopherol mixed bilayers (Figure 6) likely is composed of a domain that is rich in α-tocopherol and 18:0, 22:6 PC and poor in 18:0, 18:1 PC.

The conclusions from the experiments presented here, that α-tocopherol can associate much more strongly than cholesterol with the polyunsaturated fatty acid DHA and that cholesterol prefers the less unsaturated fatty acid oleic acid, agree with these compounds' well-established roles in membranes. As a major antioxidant, one might anticipate that α-tocopherol should accumulate in parts of the membrane that require antioxidant activity, i.e., regions that are enriched in polyunsaturated fatty acids. The selective interaction of cholesterol with oleic acid is consistent with its structural role in membranes as there is normally a large excess of oleic acid over DHA in most membranes. Perhaps cholesterol's limited antioxidant role is restricted to domains enriched in oleic acid and other less unsaturated acyl chains where α-tocopherol would be excluded. Clearly, cholesterol and α-tocopherol must function as antioxidants via different mechanisms. α-Tocopherol is effective at very low levels, implying participation of the chromanol moiety as a free radical scavenger, while cholesterol must be found in larger amounts to have a significant influence, implying a different, less efficient antioxidant mechanism. We believe the antioxidant mechanism for cholesterol is related to its well-established ordering effect on biological membranes. The biphasic kinetics of prevention of polyunsaturated fatty acid peroxidation by α-tocopherol implies that the chromanol ring may have two distinct modes of action. Initially, α-tocopherol is highly effective at preventing lipid peroxidation and remains so until the vitamin is totally oxidized. After this, α-tocopherol still serves as an antioxidant, but in a much diminished capacity, probably representing its structural role in membranes. The tighter membrane structure induced by cholesterol or α-tocopherol would decrease the availability of oxygen and create steric hindrance to the radical chain reactions, explaining how these molecules function as structural antioxidants.

If α-tocopherol is to be effective at preventing oxidation of lipids containing double bonds found at variety of membrane depths, the molecule must be able to rapidly alter its position in the membrane. The chromanol ring of α-tocopherol must be adjacent to the double bonds it is to protect while still being accessible to the bathing solution where it is regenerated by aqueous ascorbate or glutathione (Urano et al., 1993). Three possible membrane locations of α-tocopherol have been suggested (Fukuzawa et al., 1993). α-Tocopherol may be located at the aqueous interface (Srivastava et al., 1983), at a position 1 nm beneath the interface (Fragata & Bellemare, 1980), or may be located at a variety of depths dictated by the acyl chain double bonds (Bisby & Ahmed, 1989). It has been demonstrated that indeed α-tocopherol is not H-bonded to the phospholipid carbonyl or phosphate oxygens (Gomez-Fernandez et al., 1991, 1992; Salgado et al., 1993), in which case it would be held in a fixed position near the aqueous interface. α-Tocopherol's very rapid lateral mobility (reported to be about 270 times faster than PC (Aranda et al., 1989; Devaux et al., 1972)) also implies that the molecule is not held in one fixed location. In a previous report we demonstrated that α-18:3 and γ-18:3 protect α-tocopherol from acrylamide bleaching to the same extent whether the lipids were dissolved in ethanol or oriented into lipid bilayers (Stillwell et al., 1992). These earlier experiments also imply that α-tocopherol cannot be fixed in one position in membranes but must be free to migrate from the Δ9 pocket of α-linolenic acid or the Δ6 pocket of γ-linolenic acid to the aqueous interface. The smaller chromanol ring allows α-tocopherol to locate at different depths, depending on the nature of the unsaturated acyl chain. At all locations α-tocopherol will also provide a structural component to the membrane.

It is now recognized that biological membranes are not homogeneous mixtures of lipids and proteins but instead are composed of rapidly changing arrangements of different membrane patches called domains (Glaser, 1993). Formation of domains is in part the result of unequal affinities between different lipid species or between lipids and membrane proteins. Of particular interest here is the distribution of cholesterol and α-tocopherol as driven by the double bond position of membrane fatty acyl chains. It is now well-established that cholesterol is not randomly distributed in biological membranes but instead exists in transmembrane

and lateral domains (Schroeder et al., 1991; Hui, 1988; Schroeder & Nemecz, 1990). Subject to far less investigation has been the partitioning of α-tocopherol into membrane domains. Separation of α-tocopherol-rich and α-tocopherol-poor phases in phospholipid bilayers has been demonstrated by ESR (Severcan & Cannistraro, 1988), DSC (Sanchez-Migallon et al., 1994, 1996), and fluorescence (Kagan & Quinn, 1988). McMurchie and McIntosh (1986) have interpreted their DSC data as demonstrating nonhomogeneous lateral clustering of α-tocopherol in outer and inner leaflets of rat liver microsomes. Several reports of increasing α-tocopherol levels in membranes rich in polyunsaturated fatty acids have led to the proposal that α-tocopherol may accumulate in parts of the membrane where they are most needed for their antioxidant function (Maggio et al., 1977). Even the distribution of docosahexaenoic acid has been shown to be nonhomogeneous. Knapp et al. (1994) reported that DHA accumulates in PE of erythrocyte inner leaflets. Therefore, it has been documented for at least a few membranes that cholesterol, α-tocopherol, and DHA are heterogeneously distributed into lipid domains.

We propose that α-tocopherol will be located in DHA-rich domains that are poor in cholesterol. In most membranes these domains would comprise a relatively small percentage of the total lipid population and so to date have avoided scrutiny. We (Zerouga et al., unpublished results), and others (Knapp et al., 1994; Salem et al., 1986), have demonstrated that DHA accumulates in PE > PC, and PE has been shown by Van Dijck et al. (1976) to interact poorly with cholesterol. The poor interaction of cholesterol with both PE and DHA would result in it being excluded from these domains. In the cholesterol-poor domains, α-tocopherol would protect polyunsaturated fatty acids from oxidation and would provide a major stabilizing role to this portion of the bilayer. The remainder of the membrane would be rich in less unsaturated fatty acids such as oleic acid and would also be enriched in PC and cholesterol. In high amounts, cholesterol would help stabilize the major portion of the membrane bilayer while providing some antioxidant protection through its effect on membrane order.

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