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Effects of Replacement of the Hydroxyl Group of Cholesterol and Tocopherol on the Thermotropic Behavior of Phospholipid Membranes[†]

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ABSTRACT: The role of the hydroxyl groups of cholesterol and tocopherol in mediating their interaction with phospholipid bilayers has been a subject of considerable interest. We have examined this question by using derivatives of cholesterol and tocopherol in which the hydroxyl group is esterified to succinate. The hemisuccinate esters of cholesterol and α -tocopherol can be readily incorporated into phospholipid membranes and in fact can by themselves form closed membrane vesicles as demonstrated by the encapsulation of [³H]sucrose. The thermotropic behavior of mixtures containing each succinate ester and phospholipid was studied by differential scanning calorimetry. The effect of cholesteryl hemisuccinate on the thermotropic properties of dipalmitoylphosphatidylcholine and dimyristoylphosphatidylethanolamine is very similar to that of cholesterol. This indicates that the 3 β -OH is not required for the formation of a cholesterol-phospholipid complex. In mixtures of tocopherol acid succinate and phospholipids the peak transition temperature is progressively shifted to lower temperatures as the mole fraction of α -tocopherol succinate is increased, while the enthalpy of the transition is only slightly affected. At a tocopherol succinate/phospholipid molar ratio of 9/1 a phase transition is still detectable. A comparison between tocopherol succinate and tocopherol indicates that the substitution of the hydroxyl group reduces the interaction of tocopherol with phospholipids to a small but measurable extent. Thus, the hydroxyl group of tocopherol is more important than the hydroxyl group of cholesterol in influencing their interactions with phospholipids.

The biological functions attributed to cholesterol and α -tocopherol have stimulated a considerable number of studies on their physicochemical properties and their interactions with phospholipids. In the case of cholesterol, up to 50 mol % can be dissolved in phosphatidylcholine membranes (Ladbroke et al., 1968; Lecuyer & Dervichian, 1969). Below this limit cholesterol and lecithin form complexes of varying mole ratios that have been discussed in a number of recent reviews (Demel & de Kruijff, 1976; Presti et al., 1982). Although not as well studied, a complex between tocopherol and polyunsaturated phospholipids has been proposed by Diplock & Lucy (1973). Moreover, tocopherol, like cholesterol, can significantly broaden the gel-liquid-crystalline phase transition in phospholipid membranes (Massey et al., 1982) and, in the case of bilayers composed of unsaturated phospholipids, reduce the permeability of small molecules (Diplock et al., 1977).

The structural features of cholesterol that have been considered to be important for the formation of the phospholipid complex include the planar α face of the molecule, the acyl chain of between five and seven carbons, and the β -OH group which has been suggested to participate in a hydrogen bond with a polar component of the phospholipid (Brockerhoff, 1974; Huang, 1977; Presti et al., 1982). In the case of tocopherol the hydroxyl group has been suggested to form a hydrogen bond with one of the oxygen atoms of the phospholipid (Srivastava et al., 1983), while the phytanoyl chain has been considered to play a role in the tocopherol-unsaturated phospholipid interaction (Diplock & Lucy, 1973).

We had used derivatives of cholesterol and tocopherol modified at the hydroxyl group to prepare lipid vesicles which are destabilized at low pH (Ellens et al., 1984; M.-Z. Lai and F. C. Szoka, unpublished results) and became interested in the question of the role of the hydroxyl group in the interaction of these compounds with phospholipids. Reports in the literature concerning a number of hydrophilic substituents of the β -OH group of cholesterol indicated that such derivatives in phospholipid membranes behave in many respects like cholesterol (Lyte & Shinitzky, 1979; Shinitzky et al., 1979; Co-

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lombat et al., 1981; Demel et al., 1984). The increase in lipid membrane microviscosity and degree of order caused by cholesterylphosphocholine (Lyte & Shinitzky, 1979) and cholesteryl phosphate (Colombat et al., 1981) is close to that induced by cholesterol. Although the crystal structures of cholesteryl phosphate and cholesteryl sulfate have been elucidated (Pascher & Sundell, 1977, 1982), little additional information is available on the interaction between charged esters of cholesterol or α -tocopherol and phospholipids. In the present study, we have determined that cholesteryl hemisuccinate and α -tocopherol acid succinate are incorporated into phospholipid membranes and can in fact form membranes by themselves. In its influence on the thermotropic properties of phospholipids, cholesteryl hemisuccinate behaves much like cholesterol, which supports previous suggestions (Cadenhead & Muller-Landau, 1979; Demel et al., 1984) that a specific interaction between the β -OH group and the phospholipid is not necessary for the cholesterol-phospholipid complex to form. Replacing the hydroxy group of tocopherol with a succinate group affected the thermotropic properties of membranes composed of the tocopherol and phospholipid. Although the difference between the effects of TS and tocopherol is modest, the hydroxyl group appears to be a structural feature participating in the tocopherol-phospholipid interactions.

MATERIALS AND METHODS

Dipalmitoylphosphatidylcholine (DPPC)¹ and dimyristoylphosphatidylethanolamine (DMPE) were obtained from Avanti Polar Lipids (Birmingham, AL). Cholesteryl hemisuccinate (morpholine salt), α -tocopherol acetate, and α -tocopherol acid succinate were purchased from Sigma (St. Louis, MO). α -Tocopherol acid succinate was converted to its morpholine salt by mixing with equimolar morpholine (Sigma) in chloroform solution. α -Tocopherol was obtained from Supelco (Bellefonte, PA). All lipids were shown to be pure by thin-layer chromatography and were stored under nitrogen at -40°C . [^3H]Sucrose was obtained from Amersham (Arlington Heights, IL). All other chemicals were reagent grade or better.

Lipids (15 μmol total) were deposited onto the sides of a screw cap tube (13 \times 100 mm) by removing the organic solvent on a rotatory evaporator and then hydrated in 0.75 mL of 50 mM Tris-HCl buffer (pH 7.4, 100 mM NaCl) with constant N_2 flushing and intermittent vortexing for a least 1 h at a temperature 10°C above the transition temperature of the phospholipids. For DPPC/TS at 1/9 molar ratio, the dispersion was prepared by brief sonication (1 min) prior to vortexing. Lipid mixtures containing DMPE were hydrated in a buffer of pH 9.5 (carbonate-bicarbonate, 25 mM, and NaCl, 100 mM) and then neutralized to pH 7.6 by the addition of concentrated Tris-HCl buffer (300 mM, pH 7.0). The hydration at pH 9.5 was required for the thermograms of DMPE-TS mixtures to be reproducible. Direct hydration of DMPE-TS at pH 7.4 occasionally resulted in split peaks on the DSC scans. The reason is still unknown. The final solution contained 20 mM bicarbonate, 60 mM Tris-HCl, and 80 mM NaCl. Samples that were dialyzed against 100 mM NaCl-50 mM Tris-HCl, pH 7.4, buffer showed identical DSC scans.

¹ Abbreviations: CHEMS, cholesteryl hemisuccinate; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TS, α -d-tocopherol acid succinate.

Samples for DSC (100–200 μL) were concentrated by centrifugation in an Eppendorf centrifuge (12800g, 1 min), and the pellet was dispersed in 50 μL of buffer; 17 μL of the final dispersion was sealed in an aluminum sample pan. Routinely 0.7–1.3 μmol of lipid was added to the pan. The lipid content in the pan was increased for preparations with a lower phospholipid/TS or phospholipid/CHEMS ratio. DSC measurements were made with a Perkin-Elmer DSC-2 calorimeter operating at a sensitivity of 1 mcal/s and a scanning rate of $5^{\circ}\text{C}/\text{min}$. Selected compositions were scanned at 2.5 and $1.25^{\circ}\text{C}/\text{min}$ and gave similar results to those scanned at $5^{\circ}\text{C}/\text{min}$. At least three different samples were studied for each composition of the mixture except those of very low phospholipid content (two samples were studied for DPPC/TS and DMPE/TS at 3/7, 2/8, and 1/9 molar ratios). Three heating and two cooling runs were performed on each sample. The calorimetric scans were similar in these repetitive runs. The transition enthalpy was calculated by weighing cutouts of the peak area (heating scan) with indium as a standard. The phospholipid contents of the sample pan were determined by the method of Bartlett (1959). The content of α -tocopherol was measured by the absorbance in ethanol at 292 nm ($E = 3200 \text{ M}^{-1} \text{ cm}^{-1}$). The transition temperatures of DPPC and DMPE, as measured by extrapolation of the rising phase of the endothermic curve to the base line, were 41.7 ± 0.2 ($N = 4$) and $48.6 \pm 0.5^{\circ}\text{C}$ ($N = 6$), respectively. The enthalpy of transition was $8.7 \pm 0.5 \text{ kcal/mol}$ for the main transition of DPPC and $6.5 \pm 0.3 \text{ kcal/mol}$ for DMPE. The results agree very well with values in the literature (Silvius, 1982). However, due to the difficulty of measuring transition temperatures accurately on a broadened transition (Eliasz et al., 1976), the temperature at the maximum of the excess heat curve was used in the present study. The peak transition temperatures for DPPC and DMPE were 42.4 and 50.2°C , respectively.

The encapsulation volume of multilamellar liposomes was determined by [^3H]sucrose encapsulation. After preparation of the lipid dispersion, free [^3H]sucrose was removed by dialysis against Tris-HCl buffer (pH 7.4, 100 mM NaCl) for two days at room temperature with several buffer changes. The buffer volume was 500 times the sample volume. A small aliquot of sample was assayed for its phosphate content and radioactivity. The encapsulation ratio was calculated by comparing the radioactivity remaining to radioactivity in the original mixture.

RESULTS

Formation of Liposomes As Indicated by Sucrose Encapsulation. The succinate esters of cholesterol and α -tocopherol could be dissolved in phospholipid bilayers far beyond the limits of the parent compounds. Aqueous dispersions of mixtures of phospholipid with CHEMS or TS could be prepared easily. The measurement of [^3H]sucrose encapsulation of lipid mixtures indicated that the capture volumes were between 1 and 4 $\mu\text{L}/\mu\text{mol}$ of total lipid, similar to the entrapped volume of multilamellar vesicles of phospholipids (Szoka & Papahadjopoulos, 1980). Of particular interest is the capacity of DMPE-CHEMS and DMPE-TS to encapsulate sucrose, in contrast to the difficulties of preparing DMPE liposomes (Kolber & Haynes, 1979; Pryor et al., 1983). In addition, both CHEMS and TS alone were able to form liposomes with encapsulation volumes of 1.0 and 2.2 $\mu\text{L}/\mu\text{mol}$ of lipid, respectively.

Thermotropic Behavior of DPPC-CHEMS and DPPC-TS Mixtures. The partitioning of CHEMS and TS into phospholipid bilayers was studied by differential scanning calo-

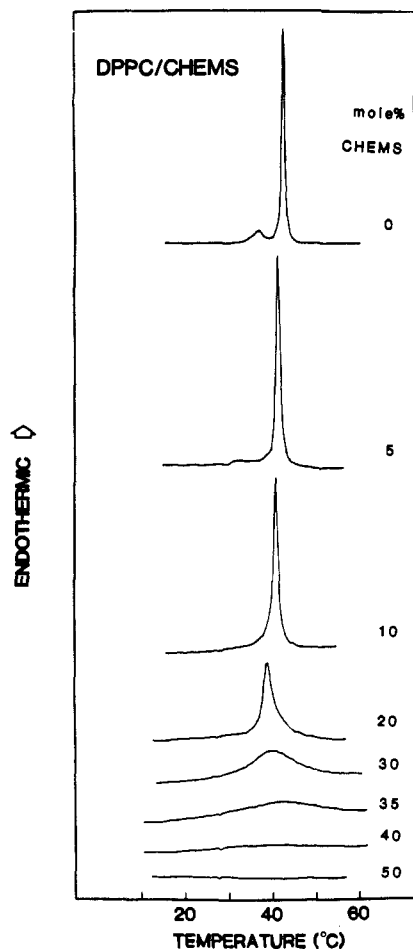


FIGURE 1: Differential scanning calorimetry thermograms of DPPC-CHEMS mixtures with 0, 5, 10, 20, 30, 35, 40, and 50 mol % of CHEMS.

rimetry. For these studies DPPC and DMPE were used as model phospholipids. The thermograms of DPPC-CHEMS mixtures are shown in Figure 1. The pretransition of DPPC was not completely abolished by the addition of 5 mol % cholesteryl hemisuccinate (Figure 1), rather it was shifted to a lower temperature as a shoulder in the endothermic peak. The shape of the main transition of DPPC was not changed by the addition of CHEMS up to 10 mol % (Figure 1). The addition of 20 mol % CHEMS broadened the half-height width of the excess heat curve of DPPC (Figure 1), while the curve shape became asymmetric with a sharp transition at the lower temperature and a shoulder at the higher temperature. The peak of the transition curve shifted continuously to lower temperature with the addition of CHEMS until 20 mol % (Figure 1). The sharp transition disappeared with the addition of 30 mol % CHEMS, the transition was further broadened, and the peak transition temperature shifted to a higher temperature (Figure 1). The transition curve of DPPC-CHEMS at 65/35 molar ratio was further broadened. Both the onset and the end of the transition were shifted away from the maximum of the transition as the concentration of CHEMS was increased. The phase transition of DPPC was barely detectable with 40 mol % CHEMS and was completely abolished at a 1/1 molar ratio of DPPC/CHEMS. The suppression of the endothermic transition is best demonstrated by the decrease in the transition enthalpy proportional to the content of CHEMS and its reduction to zero between 40 and 50 mol % CHEMS (Figure 2, circle).

DPPC-TS mixtures displayed a distinctive thermotropic behavior significantly different from DPPC/CHEMS mix-

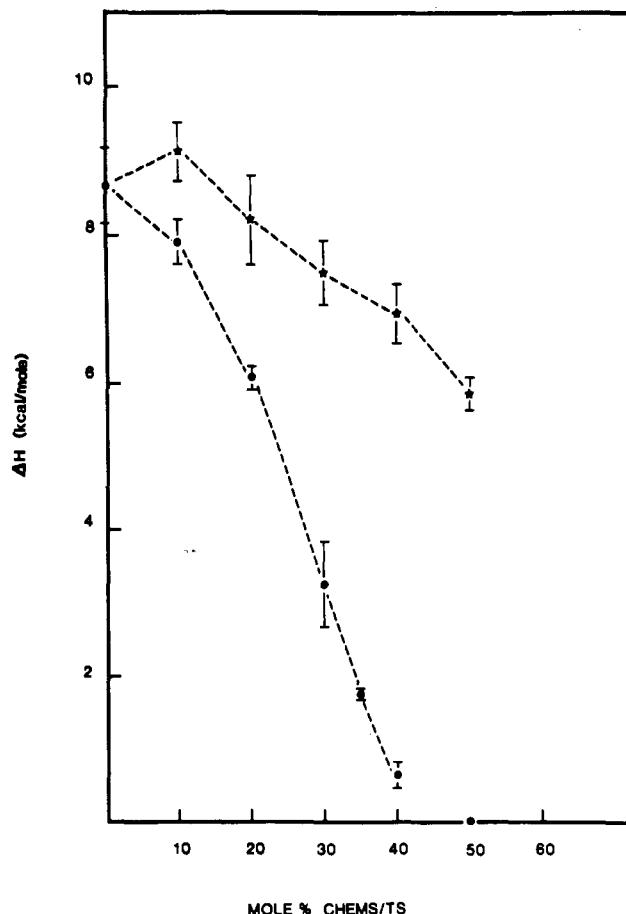


FIGURE 2: Plot of the total transition enthalpy (kilocalories per mole of DPPC) vs. the mole fraction of CHEMS (●) or TS (★).

tures. The incorporation of 5 mol % TS suppressed the pre-transition, broadened the main transition endotherm, and decreased the peak temperature of DPPC (Figure 3). A further increase in the total transition range of DPPC was observed with 10 and 20 mol % of TS. Incorporation of TS beyond 30 mol % showed little effect on the peak width of the excess heat curve (Figure 3). This is clearly demonstrated in the partial phase diagram of DPPC-TS (Figure 4). The transition curve remained asymmetric with a broad shoulder toward lower temperatures. With additional TS, the endotherm moved to lower temperatures (Figure 3 and 4). Only partial scans were performed on the mixtures containing more than 60 mol % TS, since the onset of the transition was lower than the temperature (about -10°C) that aqueous samples can be studied in the DSC without cryoprotectants. In our studies no cryoprotective agents were added to extend the thermoscan to lower temperatures. The temperature at the maximum of the endothermic curve at 80 mol % TS is about 30°C lower than that of DPPC (Figure 3). A phase transition in the mixture is still detectable at 90 mol % TS. Thus, even at a ratio of 9 to 1, TS is not able to completely abolish the transition of the mixture. The plot of enthalpy against mole percent TS added (Figure 2) shows only a relatively small decrease in transition enthalpy with the incorporation of α -tocopherol succinate. It should be noted that with TS dispersions no phase transition can be detected by DSC between -22 and 62°C .

We have reexamined the thermotropic behavior of DPPC/tocopherol. α -Tocopherol could be incorporated into DPPC bilayers up to 40 mol %. At 50 mol % tocopherol the lipid mixture was difficult to disperse and macroscopic phase separation occurred. The endothermic peak of DPPC was

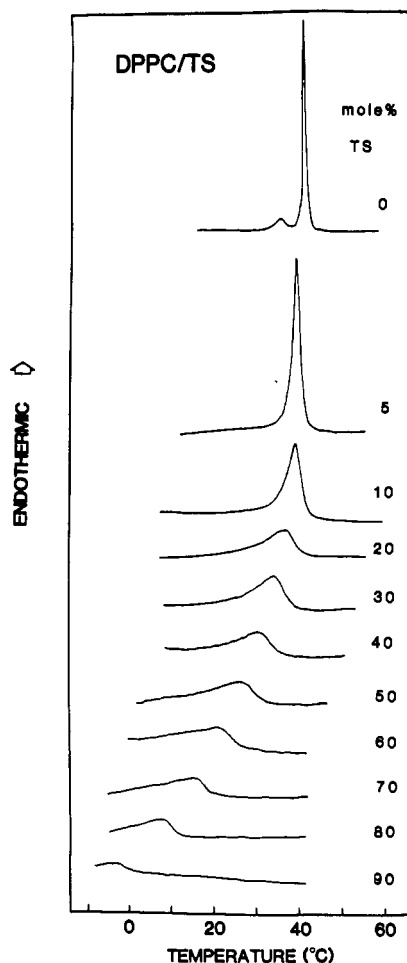


FIGURE 3: Thermograms of DPPC-TS mixtures with 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, and 90 mol % of TS.

significantly broadened with the incorporation of 5 mol % tocopherol (scan not shown); additional tocopherol further broadened the transition peak. The peak transition temperature of DPPC was shifted to a lower temperature: 39 ± 0.3 °C at 20 mol %, 37.3 ± 0.2 °C at 30 mol %, and 33.5 ± 1.5 °C at 40 mol % tocopherol. The incorporation of tocopherol also reduced the transition enthalpy of DPPC: 6.4 ± 0.2 kcal/mol at 30 mol % tocopherol and 4.8 ± 0.2 kcal/mol at 40 mol % tocopherol.

Thermotropic Behavior of DMPE-CHEMS and DMPE-TS Mixtures. To determine if the head group had a significant influence on the behavior of these compounds in membranes, the interactions between CHEMS and TS and DMPE were also studied by DSC. The addition of 5 mol % CHEMS to DMPE significantly increased the peak width of the excess heat curve (Figure 5). CHEMS at 10 mol % further broadened the endothermic curve, while at 20 mol % CHEMS the sharp transition disappeared. The addition of more CHEMS to DMPE led to further broadening of the transition curve. In addition to the increase in the temperature range of the transition, the maximum of the endothermic curve decreased continuously with the increase in molar ratio of CHEMS (Figure 6). At 50 mol % CHEMS the transition was no longer detectable (Figure 5).

DMPE-TS mixtures displayed thermotropic behavior similar to that of DPPC-TS. Upon incorporation of 10 mol % TS, the excess heat curve of DMPE was broadened and became highly asymmetric with the peak maximum at the high-temperature end (data not shown). Occasionally double peaks were observed in the thermograms of samples containing

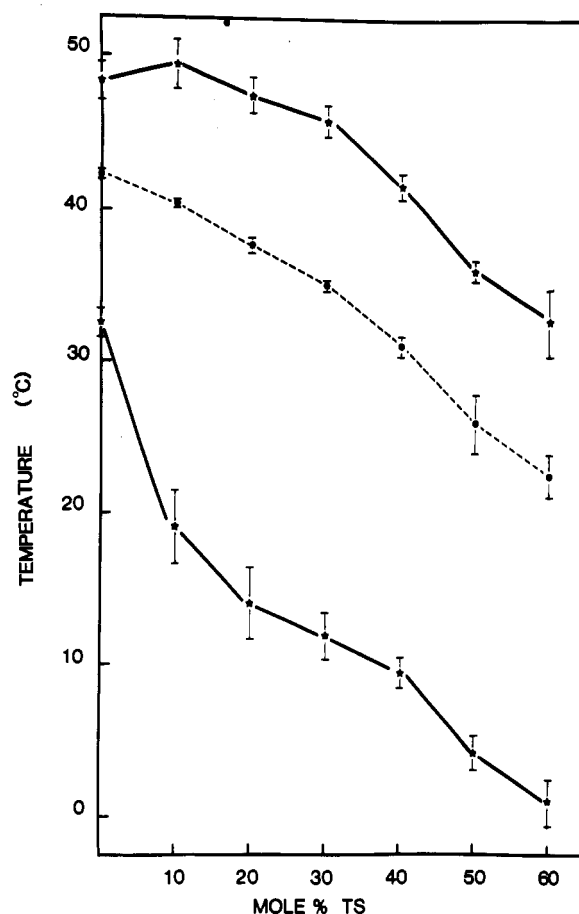


FIGURE 4: Partial phase diagram of DPPC-TS mixtures. (★) The beginning and the end of the phase transition; (●) the maximum of the excess heat curve. Each point represents the average value of four different samples.

10–20 mol % of TS, which suggests that phase separation during sample preparation might have been occurring. Little change in the endothermic peak width was observed with the incorporation of more than 20 mol % TS in DMPE. The transition curve was shifted toward lower temperatures as additional TS was incorporated. The shift in the peak temperature of the endothermic curve was greater for DMPE-TS than for DPPC-TS. At 80 mol % TS the temperature at the maximum of the transition curve was about 40 °C lower than that of DMPE. Similar to the DPPC-TS mixture, the phase transition of DMPE/TS at 1/9 molar ratio was still visible. However, the transition enthalpy of DMPE decreased only slightly with the addition of TS, whereas a plot of the transition enthalpy vs. mole ratio CHEMS in DMPE exhibited an almost linear decrease (Figure 7). The intercept of the extrapolated straight line at the abscissa (zero enthalpy) is around 42 mol % CHEMS.

DISCUSSION

Succinate Esters of Cholesterol and Tocopherol. Both cholesterol and α -tocopherol have limited solubility in phospholipid bilayers. Cholesterol can be incorporated into phospholipid membranes up to 50 mol %; beyond this limit cholesterol is separated from phospholipid bilayers (Ladbrooke et al., 1968; Lecuyer & Dervichian, 1969). Although the solubility of tocopherol in phospholipid membranes has not been established, we have found that lecithin liposomes containing more than 40 mol % tocopherol are difficult to prepare. The attachment of a charged ester greatly enhances the partitioning of cholesterol and tocopherol into phospholipid

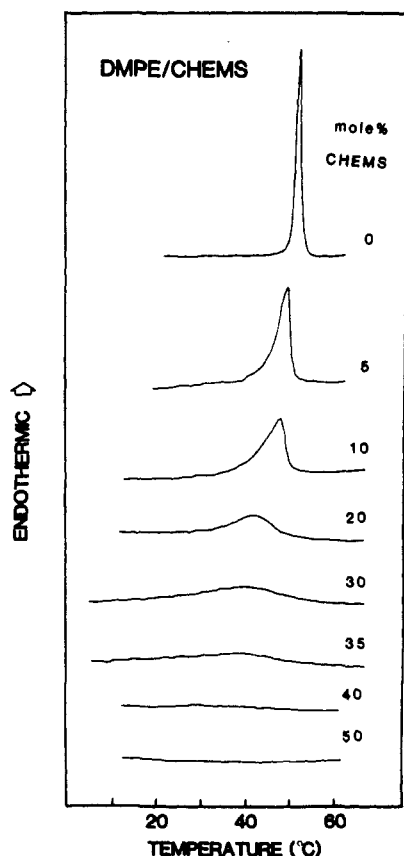


FIGURE 5: Thermograms of DMPE-CHEMS mixtures with 0, 5, 10, 20, 30, 35, 40, and 50 mol % CHEMS.

bilayers. Both CHEMS and TS can be dissolved in phospholipid membranes far beyond the limits of the parent compounds. The increased incorporation of CHEMS and TS does not affect the integrity of the phospholipid bilayers, as indicated by [^3H]sucrose encapsulation. Furthermore, the enhanced hydrophilicity of CHEMS and TS allows the formation of DMPE vesicles. In contrast to the instability of DMPE lipid vesicles at neutral pH (Stolley & Vail, 1977; Kolber & Haynes, 1979; Pryor et al., 1983), liposomes of DMPE can be prepared with 20 mol % CHEMS or TS. The ability to stabilize vesicles composed primarily of PE is a property CHEMS and TS have in common with other amphiphiles such as phosphatidylcholine (Kolber & Haynes, 1979).

In addition to enhanced solubility in phospholipid membranes, CHEMS and TS are able to form multilamellar vesicles by themselves. Sonication of these liposomes for 1 h (at 20 μmol of lipid/mL) resulted in formation of small unilamellar vesicles (electron micrograph not shown). The ease in the preparation of CHEMS vesicles is in contrast to other charged cholesterol esters such as cholesterylphosphocholine, cholesteryl sulfate, and cholesteryl poly(ethylene glycol). For these cholesterol esters, the addition of equimolar cholesterol is required for the formation of unilamellar vesicles (Brockerhoff & Ramsammy, 1982). The size difference in the charged head groups of these esters and CHEMS is likely to be the reason for this observation.

Role of the Hydroxyl Group in Cholesterol-Phospholipid Interactions. The widespread occurrence of cholesterol in mammalian cell membranes has been the impetus for a large number of physicochemical studies on the interaction of cholesterol with model phospholipid membranes [reviewed in Demel & de Kruijff (1976) and Presti et al. (1982)]. These studies have provided an understanding of many features of cholesterol-phospholipid interaction. However, the role of the

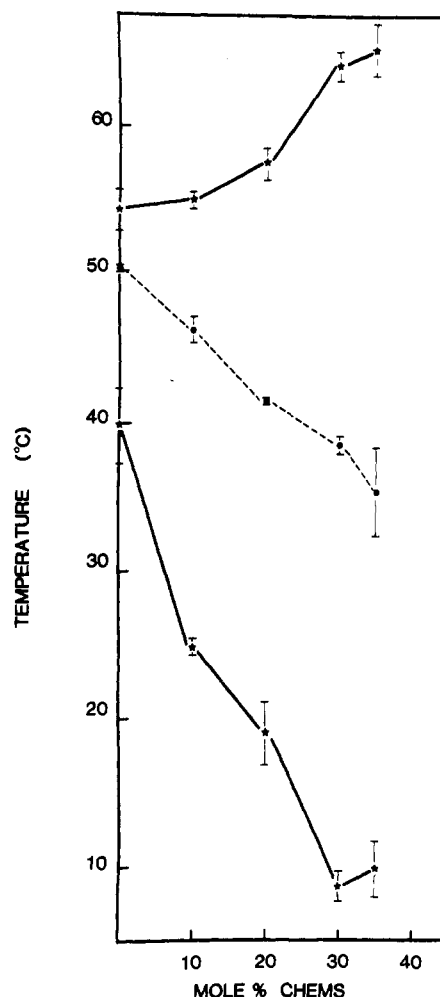


FIGURE 6: Partial phase diagram of DMPE-CHEMS mixtures. (★) The onset and end of the gel-liquid-crystalline phase transition deduced from the heating curve; (●) the maximum of the endothermic excess heat curves. The points are the average of three different preparations for each composition.

3β -OH group of the sterol remains controversial. It has been shown that neither the 3α -OH derivative, the 3-keto derivative, nor the 3-thiol derivative of cholesterol can mediate the membrane condensing effect (Hsia et al., 1972; Parkes et al., 1982) or decrease the permeability to glycerol and erythritol (Demel et al., 1972). These studies have led to the suggestion that the 3β -OH group of cholesterol forms a hydrogen bond with the carbonyl group (Brockerhoff, 1974; Huang, 1976, 1977) or with the glycerol oxygen (Presti et al., 1982) of the phospholipid. However, studies utilizing a variety of both physical techniques (Yeagle et al., 1975; Clejan et al., 1979; Bush et al., 1980) and chemical derivatives of phospholipids (de Kruijff et al., 1973; Fong et al., 1977; Tirri et al., 1977; Clejan et al., 1979; Bartholow & Geyer, 1982) have indicated that there is no direct hydrogen bond between the sterol and the phospholipid. An alternative approach is to work with derivatives of cholesterol with a modification at the 3β -OH position. We have used the succinate ester of cholesterol modified at the 3β -OH position as a model cholesterol compound and examined its effect on the thermotropic properties of phospholipid membranes using differential scanning calorimetry. The thermotropic behavior of the CHEMS mixtures closely resembles that of DPPC-cholesterol (Ladbrooke et al., 1968; Mabrey et al., 1978; Estep et al., 1978). Although the calorimeter used here is of lower sensitivity, many features of the DPPC-cholesterol mixtures revealed in high sensitivity DSC can be seen in the present studies. For example, the

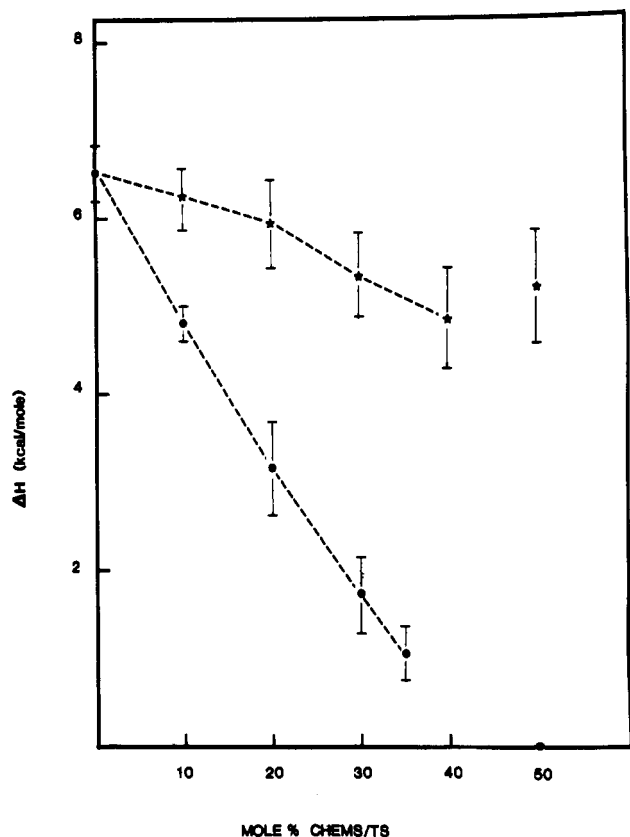


FIGURE 7: Plot of the total transition enthalpy (kilocalories per mole of DMPE) vs. mole fraction of CHEMS (●) and TS (★).

half-height width of the endothermic curve of DPPC is not changed with 10 mol % CHEMS (Figure 1) or cholesterol (Estep et al., 1978). The broadening of the transition peak was observed at 20 mol % for both CHEMS and cholesterol. Furthermore, like cholesterol, the endothermic peak of DPPC-CHEMS (80:20) is asymmetric. As CHEMS is increased from 20 to 30 mol %, the sharp transition at 38.5 °C disappeared and the shoulder at 40 °C became the maximum of a broad transition curve (Figure 1). The sharp transition observed with mixtures containing 20 mol % CHEMS (or less) has been interpreted to be due to pure DPPC domains (Mabrey et al., 1978; Estep et al., 1978; Presti et al., 1982). The abrupt broadening of the transition of DPPC as CHEMS is increased from 20 to 30 mol % supports the model of a 2:1 phospholipid-sterol complex originally proposed by Lecuyer & Dervichian (1969) and emphasized recently by Presti et al. (1982). Further addition of CHEMS or cholesterol broadens the transition curve until the gel-liquid-crystalline transition is completely suppressed. With CHEMS, the complete disappearance of the main transition occurs at a mole ratio between 40 and 50% CHEMS (Figure 2). Thus, CHEMS, within experimental error, is as effective in complexing with DPPC as is cholesterol, judging from the concentration of CHEMS required to abolish the sharp transition and to suppress the total transition.

The effects of CHEMS on the thermotropic behavior of a saturated phosphatidylethanolamine, DMPE, are also analogous to those of cholesterol (van Dijck et al., 1976; Blume, 1980). The addition of 5 mol % CHEMS (Figure 5) or cholesterol (Blume, 1980) broadens the endothermic peak of DMPE. Moreover, a shoulder is observed at the lower temperature region of the transition in DMPE mixtures containing a low molar ratio of CHEMS (Figure 5) or cholesterol (van Dijck et al., 1976; Blume, 1980). The phosphatidylethanolamines have a higher transition temperature than that of the

comparable phosphatidylcholine, an effect ascribed to an intermolecular hydrogen bond between adjacent PE molecules (Hitchcock et al., 1974; Chapman, 1975; Boggs, 1980). The incorporation of cholesterol into a PE membrane causes a downshift of the transition temperature (van Dijck et al., 1976; Blume, 1980). This is believed to be due to the intercalation of the cholesterol between PE molecules resulting in a disruption of the hydrogen-bonding network (Blume, 1980; Blume & Griffin, 1982). The extent of the lowering of the transition temperature (Figure 6) and the almost linear decrease of the transition enthalpy (Figure 7) with increasing amounts of CHEMS are, within experimental error, quantitatively the same as that observed with cholesterol (van Dijck et al., 1976; Blume, 1980). Moreover, the significant broadening of the transition at around 20 mol % CHEMS is the same as that observed with cholesterol (van Dijck et al., 1976; Blume, 1980). This implies that CHEMS may also form a 1:2 complex with DMPE.

The results here clearly demonstrate that CHEMS can form a complex with phospholipid as effectively as cholesterol. Shinitzky et al. (1979) have demonstrated that CHEMS increases the microviscosity of mammalian cell membranes as measured by diphenylhexatriene fluorescence polarization. Simmonds et al. (1984) showed the identical membrane ordering effects of CHEMS and cholesterol. The succinate esterification of the 3 β -OH group, therefore, does not affect the interaction between cholesterol and phospholipids.

This also seems to be true for other hydrophilic cholesterol esters. Lyte & Shinitzky (1979) measured the effect of cholesterylphosphorylcholine on the microviscosity of lipid vesicles composed primarily of phosphatidylcholine. They found the change to be similar to that caused by the same molar ratio of cholesterol. Colombat et al. (1981) arrived at a similar conclusion for the effect of cholesteryl phosphate. Although the increase in the microviscosity cannot be related to the ability of these derivatives for form a sterol-phospholipid complex, these studies suggest that replacing the 3 β -OH group with a hydrophilic ester does not affect the membrane ordering activity of cholesterol. More recently Demel et al. (1984) examined the interaction of a number of nonionic ether derivatives of cholesterol with phospholipids. They found that 3-cholesteryl 2-hydroxyethyl ether is as effective as cholesterol in the membrane-condensing effect, the reduction of glucose permeability, and the suppression of the gel-fluid transition of phospholipids. Demel et al. (1984) concluded that the 3 β -hydroxyl group is not necessary for the sterol effect to occur. Instead they proposed that the orientation of the sterol and the presence of the oxygen moiety at the interface are important for the sterol-phospholipid interaction; the latter may involve hydrogen bonding between the sterol head group and the bound water system.

These previous results with other cholesterol derivatives are complemented by our finding that the attachment of a succinate group to the 3 β -OH position of cholesterol does not significantly change the interaction with the two phospholipids studied here. Thus, it is unlikely that sterol effects involve a specific interaction between the 3 β -OH of cholesterol and the phospholipid. The role of the 3 β -OH group seems to be positioning the sterol ring near the aqueous interface so that the ring structure can maximize the van der Waals interactions with the acyl chains of the phospholipid, an argument that Cadenhead & Muller-Landau (1979) have previously postulated. The reduced ability of epicholesterol and 3-ketocholesterol in forming a complex with phospholipid can be explained by the geometry of the α -OH and keto group, which

position the sterol in an orientation not favorable for van der Waals interactions with the phospholipid (Cadenhead & Muller-Landau, 1979). A free 3β -OH group, however, is not necessary for the sterol-phospholipid interaction. Our results and those by Demel et al. (1984) suggest that the correct alignment of the sterol in phospholipid bilayers can be maintained if the 3β -OH group is substituted with a hydrophilic ester (such as succinate) or with an ether and additional 2-OH groups.

Role of the Hydroxyl Group in Tocopherol-Phospholipid Interactions. Tocopherol is a well-known biological antioxidant that has been proposed to have a structural function in membranes containing polyunsaturated fatty acyl groups (Diplock & Lucy, 1973). Despite its biological importance, surprisingly little is known about its behavior in model membranes. Its effect on the permeability of the bilayers is complex and depends on the acyl chain composition of the phospholipids and molar ratio of tocopherol in the bilayer. In egg phosphatidylcholine vesicles the addition of α -tocopherol at less than 20 mol % slightly decreases the permeability to small molecules such as glucose and chromate (Diplock et al., 1977; Fukuzawa et al., 1979; Stillwell & Bryant, 1983). However, at 25 mol % in egg phosphatidylcholine vesicles Pr^{3+} permeability increases 48-fold (Cushley & Forrest, 1977). In DPPC liposomes the addition of 16 mol % tocopherol increases the permeability to ascorbate 3-fold (Srivastava et al., 1983). Massey et al. (1982) have reported that the incorporation of tocopherol into lipid vesicles composed of a saturated phospholipid lower the transition temperature slightly (1–2 °C) and significantly broaden the phase transition as observed by DSC; the transition is abolished at 25 mol %. Broadening of the phase transition has also been observed by fluorescence polarization (Fukuzawa et al., 1980) and by ESR using the partitioning of the spin-label tetramethylpiperidyl-1-oxyl into bilayers containing tocopherol (Srivastava et al., 1983).

We have also observed that tocopherol broadens the phase transition of phospholipid (Fukuzawa et al., 1980; Massey et al., 1982; Srivastava et al., 1983). The broadening and suppression of the transition is proportional to the content of tocopherol. The studies here have also demonstrated that the peak transition temperature is significantly lowered by the addition of tocopherol and that a phase transition of the mixture is still detectable at 40 mol % tocopherol. Our results are thus substantially different from the DSC studies of Massey et al. (1982). Since DMPC was used in their studies, we have also examined the thermotropic behavior of DMPC-tocopherol mixtures. The incorporation of tocopherol significantly broadens the transition of DMPC, but at 40 mol % a phase transition is still detectable at a lower temperature (scan not shown).

Studies on the phase behavior of tocopherol acetate in phospholipid membranes have also generated conflicting results. Massey et al. (1982) have reported that tocopherol acetate at 25 mol % behaved almost exactly like tocopherol in abolishing the transition of DMPC. However, Schmidt et al. (1976) did not observe such an effect of tocopherol acetate in DPPC bilayers. Rather, they observed a broadening of the transition and a decrease in the onset transition temperature up to 40 mol % tocopherol acetate at which point the ester formed a separate phase. A similar result has been reported by Srivastava et al. (1983). They found that tocopherol acetate at 16 mol % depressed the transition temperature of DPPC to 28 °C while the sharpness of the transition was retained.

By comparing the effects of tocopherol and tocopherol acetate on the phase transition of DPPC and on the ^{13}C re-

laxation time and line width of the two compounds, Srivastava et al. (1983) proposed that the hydroxyl group of tocopherol might be involved in a hydrogen bond with one of the oxygen atoms of the phospholipid. However, their results do not differentiate between the disruption of a tocopherol-phospholipid interaction via a hydrogen bond and the displacement of tocopherol from the aqueous interface into the interior of the bilayer due to the increased hydrophobicity of the tocopherol acetate. Using the succinate derivative of tocopherol, which will be anchored at the aqueous surface, we can explore the role of the hydroxyl group in the tocopherol-phospholipid interaction. At 5 mol %, TS abolishes the pretransition of DPPC and broadens the peak width significantly. At 20 mol % TS the peak width of both DPPC and DMPE is further increased. Above this ratio there is little change in the endothermic curve shape, but the transition peak continues to shift to lower temperatures. This is in contrast to the progressively broadening of the transition by tocopherol. The reduced effect on the transition of the host lipid (DPPC or DMPE) can be clearly observed from the enthalpy of the transition (Figures 2 and 7), which decreases only 2 kcal/mol for DPPC and 1 kcal/mol for DMPE at 50 mol % TS. In comparison, the addition of 40 mol % α -tocopherol reduces the transition enthalpy of DPPC by 3.2 kcal/mol. Although the TS can be incorporated into phospholipids to a much higher ratio, a gel-liquid-crystalline transition remains detectable even with 90 mol % TS. This clearly shows that there are DPPC- or DMPE-rich domains in which a cooperative phase transition occurs. The lowering of the transition temperature with increasing TS in the bilayer may be explained by an impurity defect surrounding the phospholipid domains (Lee, 1977).

CONCLUSION

The use of the succinate analogues of cholesterol and tocopherol allows an appraisal of the role of the respective hydroxyl groups in their interactions with phospholipids. It appears that for cholesterol to exhibit its condensing effect on the phospholipid bilayer, the 3-position needs to be occupied by a hydrophilic group that permits the sterol ring to be positioned at the interface so that the van der Waals interactions with the acyl chains of the phospholipids can be maximized. This must be contrasted to tocopherol where succinylation of the hydroxyl group moderately reduces its interaction with phospholipids. Although a hydrogen bond to an adjacent phospholipid is one possibility, the exact nature of the tocopherol-phospholipid interaction must still be determined.

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Registry No. CHEMS, 1510-21-0; TS, 4345-03-3; DMPC, 13699-48-4; DMPE, 20255-95-2; DPPC, 2644-64-6; cholesterol, 57-88-5; α -tocopherol, 59-02-9.

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