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Biochimica et Biophysica Acta 1279 (1996) 251–258

BBA Biochimica et Biophysica Acta

Interaction between α-tocopherol and heteroacid phosphatidylcholines with different amounts of unsaturation

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Received 19 July 1995; revised 13 October 1995; accepted 15 November 1995

Abstract

Differential scanning calorimetry was used to study the influence of α-tocopherol on the thermotropic properties of model membranes composed by a series of heteroacid phosphatidylcholines with different amount of unsaturation in the *sn*-2 chain. The effect of α-tocopherol on 1,2-distearoylglycerophosphocholine (18:0,18:0), 1-stearoyl-2-oleoylglycerophosphocholine (18:0,18:1), 1-stearoyl-2-linoleoylglycerophosphocholine (18:0,18:2), 1-stearoyl-2-linolenoylglycerophosphocholine (18:0,18:3) and 1-stearoyl-2-arachidonoylglycerophosphocholine (18:0,20:4) was determined. The addition of α-tocopherol perturbed the thermotropic gel to liquid-crystalline phase transition of these phospholipids. α-Tocopherol broadened the endotherm, lowered the transition temperature and decreased the associated enthalpy change. Partial phase diagrams showed the presence of fluid immiscibilities giving rise to lateral phase separation of domains containing different amounts of α-tocopherol. We suggest that, in these α-tocopherol-rich domains, the influence exerted by the vitamin on the phospholipids is strong enough to alter their thermotropic properties such that an additional endotherm appears in the thermogram, a characteristic not observed in homoacid phosphatidylcholines. α-Tocopherol caused a concentration-dependent removal of the detectable phase transition in all cases. The magnitude of the influence of α-tocopherol on phospholipid was dependent on the degree of unsaturation of the *sn*-2 acyl chain. These results are explained on the basis of the effect of α-tocopherol which will reduce the differences between gel and liquid crystalline states, the magnitude of these differences depending on the type of phospholipid considered, which are probably related to the change of molecular shape of phosphatidylcholines containing a polyunsaturated acyl chain.

Keywords: α-Tocopherol; Heteroacid phosphatidylcholine; DSC; Phosphatidylcholine

1. Introduction

α-Tocopherol is known to be an indispensable lipid component of biological membranes which provide for their structural and functional stability [1]. It is generally recognized that α-tocopherol functions as a potent antioxidant to protect membrane polyunsaturated lipids against peroxidation, presumably acting as an electron donor to free radicals [2,3]. The structural properties of α-tocopherol have implied a role for this vitamin in the stabilization of biological membranes. It has been suggested that α-tocopherol might restrict the molecular mobility of membrane components [4,5] and form complexes with potentially toxic unsaturated fatty acids [6,7] or lysophosphatidylcholine and related phospholipids [8].

Although the biological function of α-tocopherol is well known, its molecular mechanism of action is not fully elucidated. A number of physical techniques have been applied to study α-tocopherol and its influence on membranes; these included fluorescence spectroscopy [9–12] EPR [10,13,14], NMR [15–18], ultraviolet spectroscopy [19,20], Fourier transform-infrared spectroscopy [21,22] and differential scanning calorimetry [21,23–25]. Most of these experiments concluded that: (i) the chromanol group of α-tocopherol is located close to the aqueous interface, while the phytyl side chain extends towards the center of the membrane; (ii) the molecule increases order and decreases fluidity in liquid-crystalline membranes, and (iii)

Abbreviations: DSC, differential scanning calorimetry; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; SAPC, 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; SLPC, 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; SLnPC, 1-stearoyl-2-linolenoyl-*sn*-glycero-3-phosphocholine; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; T_c, onset temperature of the gel to liquid-crystalline phase transition.

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SSDI 0005-2736(95)00276-6

the phase transition temperature and enthalpy of saturated phosphatidylcholine are reduced.

Some studies have shown that not all phospholipids interact in the same way, or at least to the same extent, with α-tocopherol. In a previous study, we observed that, when α-tocopherol was incorporated into phosphatidylcholine bilayers, the broadening and displacement of the main transition to lower temperatures was quantitatively more important in phosphatidylcholines with longer acyl chains; that the effect of α-tocopherol on the phase transition of phosphatydylethanolamines was qualitatively different from that observed in phoshatidylcholines, and that, when incorporated in mixtures of different phospholipids, α-tocopherol preferentially partitioned into the most fluid phase [25]. We also observed that α-tocopherol was a strong hexagonal-H_{II}-phase promoting agent in systems composed of saturated or unsaturated phoshatidylethanolamines [17] and that α-tocopherol interacts with single-chain phospholipids stabilizing the bilayer phase [26]. In light of these findings, the diversity of lipid species, and different amounts of α-tocopherol in various membranes, require that details of lipid–α-tocopherol interactions be known for specific lipids or groups of lipids in order to properly appreciate the effect of α-tocopherol in diverse biological membranes.

Investigation of bilayer systems containing pure lipids have been done primarily with phospholipids of the homoacid type, that is, lipids with two identical chains. However, a striking fact about most biological membranes is that they contain phospholipids that have a saturated chain plus an unsaturated chain in which there are one or more double bonds. The study of the interaction of α-tocopherol with these phospholipids containing unsaturated acyl chains may be also relevant considering that unsaturated fatty acid are preferred target for both, the antioxidant and the stabilizing functions of α-tocopherol in membranes. In this study we have investigated by differential scanning calorimetry the effects of α-tocopherol on the gel to liquid-crystalline phase transition of a series of heteroacid phosphatidylcholines which have stearate (18:0) in the *sn*-1 position and one of a series of chains having zero (stearate, 18:0), one (oleate, 18:1, Δ9), two (linoleoate, 18:2, Δ9,12) three (linolenoate, 18:3, Δ9,12,15) or four (arachidonate, 20:4, Δ5,8,11,14) *cis*-double bonds. The results suggest that the gel to liquid-crystalline phase transitions of these lipids are more profoundly influence by α-tocopherol than are those of saturated homoacid phosphatidylcholines, and the results are discussed in the light of the possible mechanism of action of α-tocopherol.

2. Materials and methods

1,2-Distearoyl-*sn*-glycero-3-phosphocholine (18:0,18:0, DSPC), 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (18:0,18:1, SOPC), 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (18:0,18:2, SLPC), 1-stearoyl-2-linolenoyl-*sn*-glycero-3-phosphocholine (18:0,18:3, SLnPC) and 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (18:0,20:4, SAPC) were obtained from Avanti Polar Lipids (Birmingham, Alabama), α-tocopherol was from Sigma (Poole, UK). All other reagents were of the highest purity available. Before use, all lipids were analyzed by TLC and gas-liquid chromatography. All were pure by TLC and gave expected molar ratios of constituent fatty acids on gas chromatographic analysis.

The lipid mixtures for calorimetry measurements were prepared by combination of chloroform/methanol (1:1) solutions containing 5 μmol of the phospholipid and the appropriate amount of α-tocopherol when indicated. The organic solvents were evaporated under a stream of dry N_2, free of O_2, and the last traces of solvents were removed by a further 3 h evaporation under high vacuum. After the addition of 1 ml of 0.1 mM EDTA, 100 mM NaCl, 10 mM Hepes (pH 7.4) buffer, multilamellar vesicles were formed by mixing, using a bench-vibrator, always keeping the samples at a temperature above the gel to liquid-crystalline phase transition temperature of the lipid. The suspensions were centrifuged at 10000 rpm in a bench microfuge and the pellets were collected and placed into small aluminum pans. Pans were sealed and scanned in a Perkin-Elmer DSC-4 calorimeter. The heating and cooling rates were 4 C°/min. For the determination of the total phospholipid contained in a pan, this was carefully opened, the lipid was dissolved with chloroform/methanol (1:1) and the phosphorus contents were determined using the method of Böttcher et al. [27]. The instrument was calibrated using indium as standard. After DSC scans were completed, representative samples were assessed by TLC and fatty acid analysis. No appreciable degradation was observed in any sample under study.

In the phase diagrams, the solidus and fluidus points were determined from the beginning of the heating and cooling thermograms (i.e., onset temperatures) respectively. Heating and cooling thermograms were very similar.

In order to check the incorporation of α-tocopherol into phospholipid bilayers, α-tocopherol was extracted from the vesicles using n-pentane and its concentration was determined using an $\epsilon_{295} = 3200\ M^{-1}\ cm^{-1}$ in ethanol. In agreement with a previous report [21], it was found that, even for the most concentrated samples, more than 90% of the added α-tocopherol was incorporated into the bilayer.

3. Results

Fig. 1 shows the DSC thermograms of the gel to liquid-crystalline phase transitions for dispersions of samples of DSPC, SOPC, SLPC, SLnPC and SAPC. Thermodynamics data for the transitions of the dispersion of pure phosphatidylcholines are given in Table 1. Where there

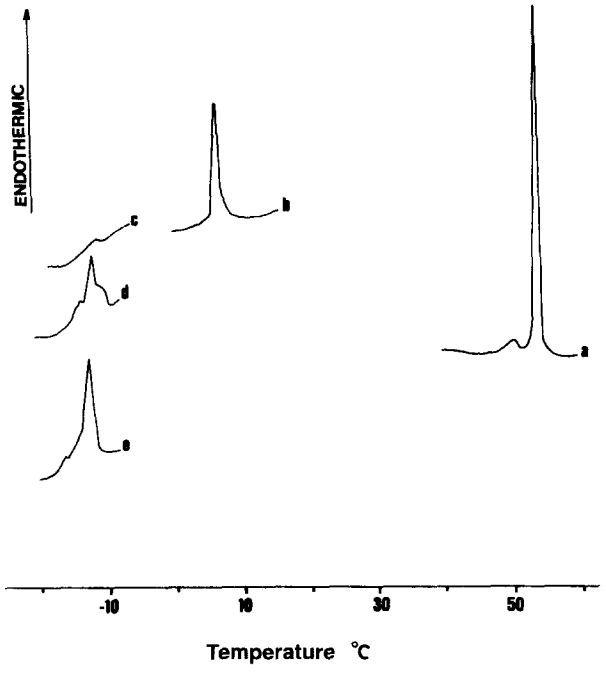


Fig. 1. The DSC calorimetric curves for various pure phosphatidylcholine systems. (a) DSPC (b) SOPC (c) SLPC (d) SLnPC and (e) SAPC. All thermograms were recorded at a sensitivity of 0.5 mcal/s, except for the DSPC sample which was at 2 mcal/s.

were overlapping multicomponent endotherms, all were included in the calculation of ΔH. Values are consistent with those found previously for these lipids [28]. As seen previously [29,30], the introduction of one double bond causes a large decrease in both T_c and ΔH, the presence of two double bonds additionally reduced T_c and ΔH. However, the presence of three double bonds produced a small increase in T_c and ΔH. The introduction of an additional double bond in the *sn*-2 chain (four in total) gave place to a small depression in T_c and an increase in ΔH. While the endotherms for DSPC and SOPC were fairly symmetrical and highly cooperative, the presence of two double bonds greatly affects the transition, and that of three and four double bonds (SLnPC and SAPC) produces broadened endotherms with one or two additional components. The presence of bimodal and trimodal transitions for heteroacid phosphatidylcholines has been previously described [28,29,31].

Table 1
Gel to liquid-crystalline transition temperatures and enthalpies for pure lipids

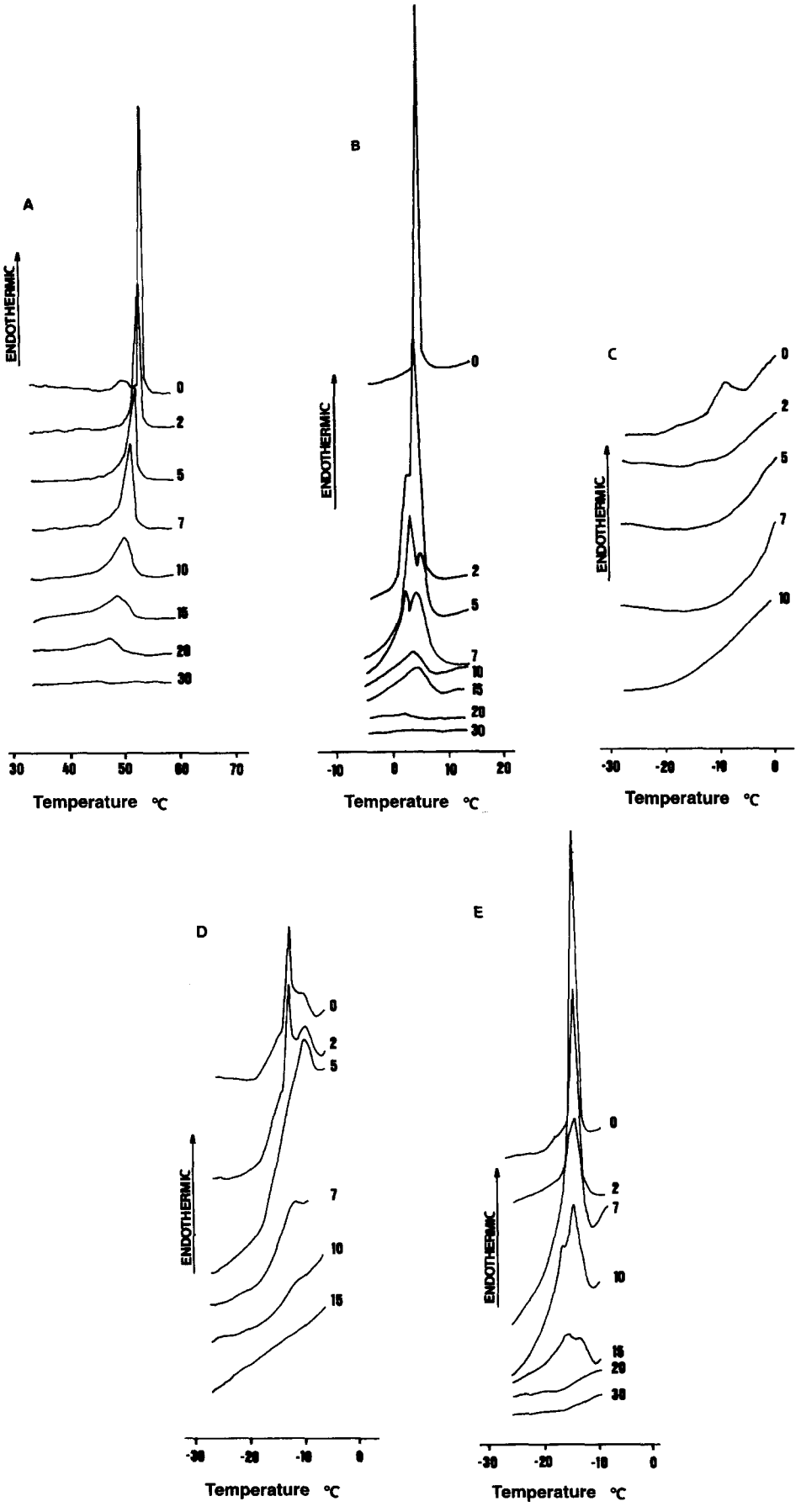
Phosphatidylcholine	T_c (°C)	ΔH (kcal mol^{-1})
DSPC (18:0,18:0)	52.7 ± 0.1	9.73 ± 0.40
SOPC (18:0,18:1)	4.1 ± 0.1	5.25 ± 0.18
SLPC (18:0,18:2)	− 16.7 ± 0.3	1.8 ± 0.35
SLnPC (18:0,18:3)	− 13.9 ± 0.2	5.39 ± 0.10
SAPC (18:0,20:4)	− 15.6 ± 0.3	7.49 ± 0.66

Values are means ± S.D. for three experiments.

Fig. 2 shows the DSC thermograms for the heteroacid phosphatidylcholines with different number of double bonds in the presence of increasing amounts of α-tocopherol. In agreement with previous reports [21,25], α-tocopherol produced the abolition of the pretransition and a broadening and shifting to lower temperatures of the main transition of DSPC (Fig. 2A). At 30 mol% α-tocopherol the transition of DSPC could not be observed. The presence of α-tocopherol also made the transition of SOPC (Fig. 2B) to broaden and shift to lower temperatures. However, the interaction between α-tocopherol with SOPC presented clear differences with respect to DSPC. First, the presence of even low concentrations of α-tocopherol produced the appearance of a second peak located at the lower temperature part of the thermogram, and second, the transition could not be observed at 20 mol%. It can be seen that even very low concentrations of α-tocopherol, such as 2 mol%, suppressed the phase transition of SLPC (Fig. 2C). Pure SLnPC (Fig. 2D) showed a trimodal transition, and the presence of α-tocopherol produced a broadening and a lowering of T_c which was less remarkable than in the case of DSPC and SOPC. The transition could not be observed at a α-tocopherol concentration as low as 15 mol%. α-Tocopherol, when incorporated into SAPC (Fig. 2E) gave rise to secondary peaks in the thermograms and a broadening and a shift of T_c to lower temperatures. The transition could not be detected at 20 mol% of α-tocopherol.

Fig. 3 shows the relative influence of α-tocopherol on ΔH of the transition of the phospholipids under study. Since each of the pure phospholipid has a different value of ΔH for its gel to liquid-crystalline phase transition, the values were normalized to 100% of the pure phospholipid in each case. For the calculations the whole areas under the curves were included. 0.23 mol fraction of α-tocopherol was necessary to totally remove the calorimetrically observable transition of DSPC, 0.17 mol fraction was necessary in the case of SOPC and only 0.02 mol fraction in the case of SLPC. However, 0.13 and 0.17 mol fraction were necessary to completely abolish the transition of SLnPC and SAPC, respectively.

Onset transition temperatures were obtained by extrapolation of the rising part of the thermograms to the baseline as described in [32] from heating and cooling experiments, and were used to construct partial phase diagrams corresponding to the different phospholipid/α-tocopherol systems (Fig. 4). The phase diagrams for all phosphatidycholines under study showed similar behavior. The solidus line displayed a near ideal behavior, the temperature decreasing as the α-tocopherol mol fraction increased. A fluidus immiscibility was observed, because the fluidus line keeps horizontal in the whole range of α-tocopherol concentration. Below the gel to liquid-crystalline phase transition temperature (solidus line) the system is orga-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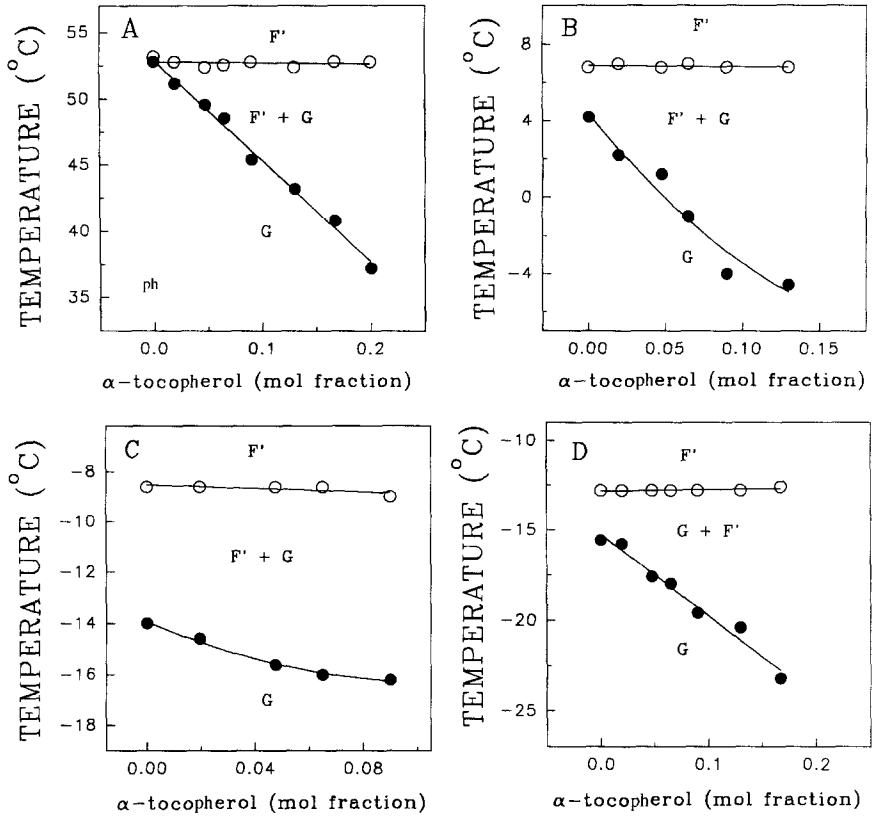

Fig. 4. Partial phase diagrams for mixtures of various phosphatidylcholines with α-tocopherol. (a) DSPC, (b) SOPC, (c) SLnPC and (d) SAPC. Filled circles correspond to the solidus line obtained from onset temperatures of heating scans, and open circles to the fluidus line obtained form onset temperatures of cooling scans. G indicates a single gel phase and F′ indicates that at least two different fluid phases coexist.

nized in a single gel phase (G in Fig. 4) composed by phospholipids interacting with α-tocopherol. In this G phase the thermotropic properties of the phospholipid are more perturbed when the presence of α-tocopherol is increased, producing a decrease of the onset of the transition with increasing α-tocopherol concentrations, as indicated by the solidus line. Above the liquid-crystalline to gel phase transition temperature (fluidus line), the system is organized in a state (F′ in Fig. 4) in which at least two different fluid phases coexist, one of them composed mostly by pure phospholipid (explaining why the fluidus line is not altered by the presence of α-tocopherol) and another one which is rich in α-tocopherol.

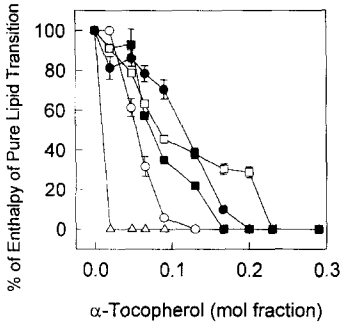

Fig. 3. The proportion of detectable enthalpy change as a function of α-tocopherol concentration for various phosphatidylcholine systems. (□) DSPC, (■) SOPC, (△) SLPC, (○) SLnPC, and (●) SAPC. Points represents means ± S.D. for three experiments.

The gel to liquid-crystalline phase transition of DSPC showed no significant hysteresis (Fig. 4A). The presence of one and three double bonds at the *sn*-2 chain produced a progressive hysteresis in the transition. The T_c for cooling experiments was 1.6 and 5.4°C lower than that of heating experiments respectively for SOPC and SLnPC (Fig. 4B and 4C). The presence of an additional fourth double bond as in the case of SAPC (Fig. 4D) diminished the hysteresis (2.8°C) compared to that of SLnPC.

Fig. 2. The DSC calorimetric curves for various phosphatidylcholines/α-tocopherol systems. (a) DSPC/α-tocopherol, (b) SOPC/α-tocopherol, (c) SLPC/α-tocopherol, (d) SLnPC/α-tocopherol and (e) SAPC/α-tocopherol. Molar percentages of α-tocopherol in phosphatidylcholine are indicated on the curves.

4. Discussion

This study was concerned with the effect of α-tocopherol on the calorimetrically observable gel to liquid-crystalline phase transition of model membranes composed of biologically relevant phospholipids with different unsaturations. The findings of this study were in general agreement with those observed in other systems containing homoacid saturated phospholipid (for example, [21,25]). We found that α-tocopherol broadened and shifted the transition to lower temperatures and ultimately it removed the observable gel to liquid-crystalline phase transition. The effect of α-tocopherol on the thermotropic behavior of these phospholipid is the expected behavior of a molecule which aligns itself principally with the prevailing direction of the phospholipid acyl chains, perturbing phospholipid acid chain packing and weakening the strong intermolecular forces produced by the *all trans* conformation of the acyl chains in the gel phase. Increasing the concentration of α-tocopherol continually disrupts the acyl chain packing so that the enthalpy of the transition is eventually eliminated. This general effect seems to be independent of the phospholipid headgroup [23,25] and, as shown in this study, it is also independent of the unsaturation of the acyl chain in the *sn*-2 position of phosphatidylcholines.

There were some significant differences between the thermotropic transitions of the phosphatidylcholine/α-tocopherol mixtures studied here and those of saturated phosphatidylcholines. These differences were seen both in the shapes of the endotherms, at a given α-tocopherol concentration, and in the concentrations of α-tocopherol at which the total enthalpy disappeared. It can be observed in Fig. 2 that the thermograms for mixtures of α-tocopherol with DSPC, in agreement with previous observations made with other saturated phosphatidylcholines [25], showed only one component. However, mixtures of α-tocopherol with heteroacid unsaturated phosphatidylcholines, at certain α-tocopherol concentrations, always showed secondary peaks in the thermograms. The presence of several endotherms suggests that there might be complex rearrangements taking place. The occurrence of more than one endotherm in unsaturated phosphatidylcholines is observed even for some pure phospholipids like SLnPC and SAPC (Fig. 1). Similar observations have been made previously by other authors using similar phospholipids [28,31]. These multicomponent transitions cannot be directly explained on the basis of analytical data or acyl chain composition; however, they are consistent with the microclustering of mixed-chain polyunsaturates reported by Litman et al. [33]. The presence of small peaks in these pure lipids is exacerbated by the incorporation of α-tocopherol, where clearly bigger peaks were detected. The presence of additional endothermic peaks in mixtures of α-tocopherol/phosphatidylethanolamines has been previously ascribed to the presence of different phases [25].

An important aspect of the systems studied in this work is related to the changes in ΔH of the transition. Fig. 3 showed that when the number of double bonds in the *sn*-2 chains increased from zero to two, the lipids became progressively more 'susceptible' to α-tocopherol. However, more α-tocopherol was required to remove the transition of SLnPC and SAPC than that of SLPC. The same type of influence have been seen previously for cholesterol [31]. This observation implies that the magnitude of the effect of α-tocopherol on the phospholipid is dependent upon the specific molecular species of phosphatidylcholines involved.

It has been shown that the presence of one and two double bonds may reduce the difference in the structural order between the gel and liquid-crystalline states [34,35]. However, the presence of three and four double bonds led to a more condensed acyl chain with restricted potential conformational states [36,37]. Therefore, less α-tocopherol will be necessary to suppress the phase transition of phospholipids where the gel and the fluid phases are more alike, as it is the case of SLPC. On the contrary, the highest amount of α-tocopherol was required to suppress the transition of DSPC where the differences between gel and fluid states were the greatest.

Very recently, Holte et al. [38] concluded that the presence of double bonds produced a change of molecular shape of the phospholipid, causing the acyl chains to occupy a slightly wedge-shaped space that loosens the packing at the interface and creates a free volume at the lipid/water interface between neighboring molecules that is greatest when *sn*-2 is polyunsaturated. This may explain our results at least partially, since those phosphatidylcholines with three or more double bonds will have a greater free volume at the lipid/water interface which will better accommodate α-tocopherol molecules, requiring higher concentrations of α-tocopherol to perturb the phospholipid structure.

Partial phase diagrams displayed in Fig. 4 clearly showed that mixtures of heteroacid unsaturated phosphatidylcholines with α-tocopherol present fluid immiscibilities. We believe that the different endotherms obtained at certain α-tocopherol concentrations represents different domains of the bilayer with different contents of α-tocopherol. Fluid immiscibilities were also found in mixtures of α-tocopherol with DSPC. The broadening of the transition observed in this homoacid saturated phosphatidylcholine may also reflects the formation of different domains. However, their thermotropic parameters are very similar, so that both phases undergo their transition under a unique broad endotherm. The presence of additional endotherms in heteroacid unsaturated phosphatidylcholines/α-tocopherol mixtures suggests that α-tocopherol exerts a stronger perturbation of the organization of these phospholipids than to that of DSPC. The perturbation exerted by α-tocopherol is so strong that the domains with high content in α-tocopherol undergo the transition with

thermotropic characteristics which are clearly different from that of the pure lipid and from the phospholipid rich phase, and they hence present two different endotherms. The presence of two distinct hydrophobic phases, one α-tocopherol poor and the other rich in α-tocopherol, has been previously suggested using EPR [10,39]. DSC experiments [40] and fluorescence studies [41] on phospholipid membranes have suggested an inhomogeneous distribution in membranes with a 'lateral clustering' of α-tocopherol. If the distribution of α-tocopherol is really not homogeneous, this may have important consequences not only in order to interpret the results obtained when studying its interaction with membranes, but also with respect to the biodispersion of the vitamin in biomembranes, where it may concentrate mainly in particular domains.

Previous DSC studies have reported hysteresis between heating and cooling experiments [28,38]; however, the explanation is not obvious. Theoretical models for lipid phase transitions proposed that at temperatures close to that of transition, domains of one phase will be present within a bulk matrix of the other phase [42]. Hysteresis will then follow because the free energy of a domain of liquid-crystalline lipid within a matrix of gel phase lipid will be different to that of a domain of gel-like lipid within a matrix of liquid-crystalline phase lipid. The modification of the hysteresis induced by the addition of double bonds in the phospholipids probably reflects the formation of different number of separate domains of one phase within the bulk second phase.

The data presented above suggest that there may be quantitatively, if not qualitatively, different interactions between α-tocopherol and various biological lipids of different degrees of unsaturation. This possibility should be kept in mind when considering potential effects of α-tocopherol in biological membranes. An interesting finding when considering the action of α-tocopherol in membranes, is that, as shown in this study, it presents immiscibilities, giving place to the formation of domains where its concentration will be specially high. This will facilitate its activity given the low concentration of α-tocopherol in membranes. It should be particularly remarked that these immiscibilities are observed in important classes of biological heteroacid phosphatidylcholines, and that these immiscibilities are present in the fluid state and this is very relevant given the normal fluid condition of biological membranes.

Acknowledgements

This work was supported by grants PB 92-0987 from Dirección General de Investigación Científica y Tecnológica, Spain, and PIB94/13 from Dirección General de Educación y Universidad, Comunidad Autónoma de Murcia, Spain.

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