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The interaction of α-tocopherol with phosphatidylserine vesicles and calcium

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Abstract

The interaction of α-tocopherol with dimyristoylphosphatidylserine (DMPS) has been studied in the presence and in the absence of Ca^{2+} by using differential scanning calorimetry (DSC), Fourier transform infrared spectroscopy (FT-IR) and $^{45}Ca^{2+}$-binding. In the absence of Ca^{2+}, DSC showed that α-tocopherol decreases the temperature of the lamellar gel to lamellar liquid crystalline phase transition as well as it decreases ΔH of this transition. Two different peaks were detected at 10 mol% of α-tocopherol and probably one of the peaks correspond to pure or nearly pure DMPS and the other to DMPS incorporating most of the α-tocopherol. The phase transition was totally abolished at 30 mol% of α-tocopherol. In the presence of Ca^{2+} this L_β to L_α phase transition of DMPS was even more perturbed by α-tocopherol, so that it was totally abolished by only 7 mol% of α-tocopherol, at Ca^{2+} concentrations which were clearly non-saturating, like those giving DMPS/Ca^{2+} molar ratio of 4:1 and 10:1. Furthermore, the transition of the DMPS/Ca^{2+} complex observed at 91.6°C was perturbed by the presence of α-tocopherol, indicating a change in the structure of the crystalline complex. The FT-IR analysis of the effect of α-tocopherol on DPMS phase transition confirmed the decrease in the phase transition temperature of the phospholipid, and also that α-tocopherol increases the number of gauche isomers in the gel state but has no effect in the liquid crystalline state. The binding of $^{45}Ca^{2+}$ was also affected by the presence of α-tocopherol, so that the number of binding sites was decreased, and this may be interesting for situations in which phosphatidylserine and Ca^{2+} are simultaneously implicated in biological functions, such as membrane fusion and enzyme activation.

Keywords: α-Tocopherol; Phosphatidylserine; DSC; FT-IR; Calcium-45 ion binding

1. Introduction

α-Tocopherol (vitamin E) has been proposed to have several function in biological membranes, such as antioxidant, membrane stabilizer and enzyme modulator [1]. The antioxidant function is by far the most documented one [2,3]. Its role in the stabilization was suggested long ago [4,5] but it is not yet so convincingly established as the antioxidant role. In order to test this membrane stabilization role, it is interesting to systematically study the molecular interaction of α-tocopherol with phospholipid membranes. In this way, it has been shown that α-tocopherol modulates the L_β to L_α phase transition of phosphatidylcholines, inducing a decrease in T_c and ΔH [6–10], and it also affects the L_α to H_{II} phase transition of phosphatidylethanolamines, inducing a decrease in T_H [11].

It is also interesting that α-tocopherol stabilizes the lamellar phase when associated with asymmetric phospholipids bearing only one fatty acyl chain [12]. However, although the interaction of α-tocopherol with zwitterionic phospholipids has been extensively studied, almost nothing is known about its interaction with phospholipid bearing electrical charge at neutral pH. This work is precisely addressed to investigate the interaction of α-tocopherol with membranes of phosphatidylserine, both in the presence and in the absence of Ca^{2+}. Phosphatidylserine is one of the most important phospholipid in animal membranes, and its electrical charge may be decisive to understand the electrostatic interaction with the membrane of proteins and ions such as Ca^{2+}.

Our results show that α-tocopherol modulates the phase transition of DMPS in a qualitatively similar way to that

Abbreviations: DMPS, 1,2-dimiristoyl-*sn*-glycero-3-phosphoserine; DSC, differential scanning calorimetry; FT-IR, Fourier transform infrared spectroscopy; T_c, onset temperature of the gel to liquid-crystalline phase transition.

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previously described for phosphatidylcholines, but it is interesting that it affects to the interaction of DMPS with Ca^{2+}.

2. Materials and methods

2.1. Materials

Dimiristoylphosphatidylserine (DMPS) was obtained from Avanti Polar Lipids (Birmingham, AL, USA), α-tocopherol from Sigma (Poole, Dorset, UK), and ionophore A23187 from Boehringer-Mannheim (Germany).

2.2. Differential scanning calorimetry

The lipid mixtures for calorimetry measurements were prepared by combination of chloroform solutions containing 5 μmol of DMPS and the appropriate amount of α-tocopherol when indicated. In the case of measurements in the presence of calcium the appropriate amount of ionophore A23187 in ethanol to give a phospholipid/ionophore ratio of 500:1, was also added. The organic solvents were evaporated under a stream of dry nitrogen, 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 phospholipid. Calcium containing samples were prepared in 1 ml of 100 mM NaCl, 10 mM Hepes, pH 7.4 buffer, to which a specific volume of calcium-containing buffer was added in order to obtain the specific DMPS:Ca^{2+} molar ratio required. The

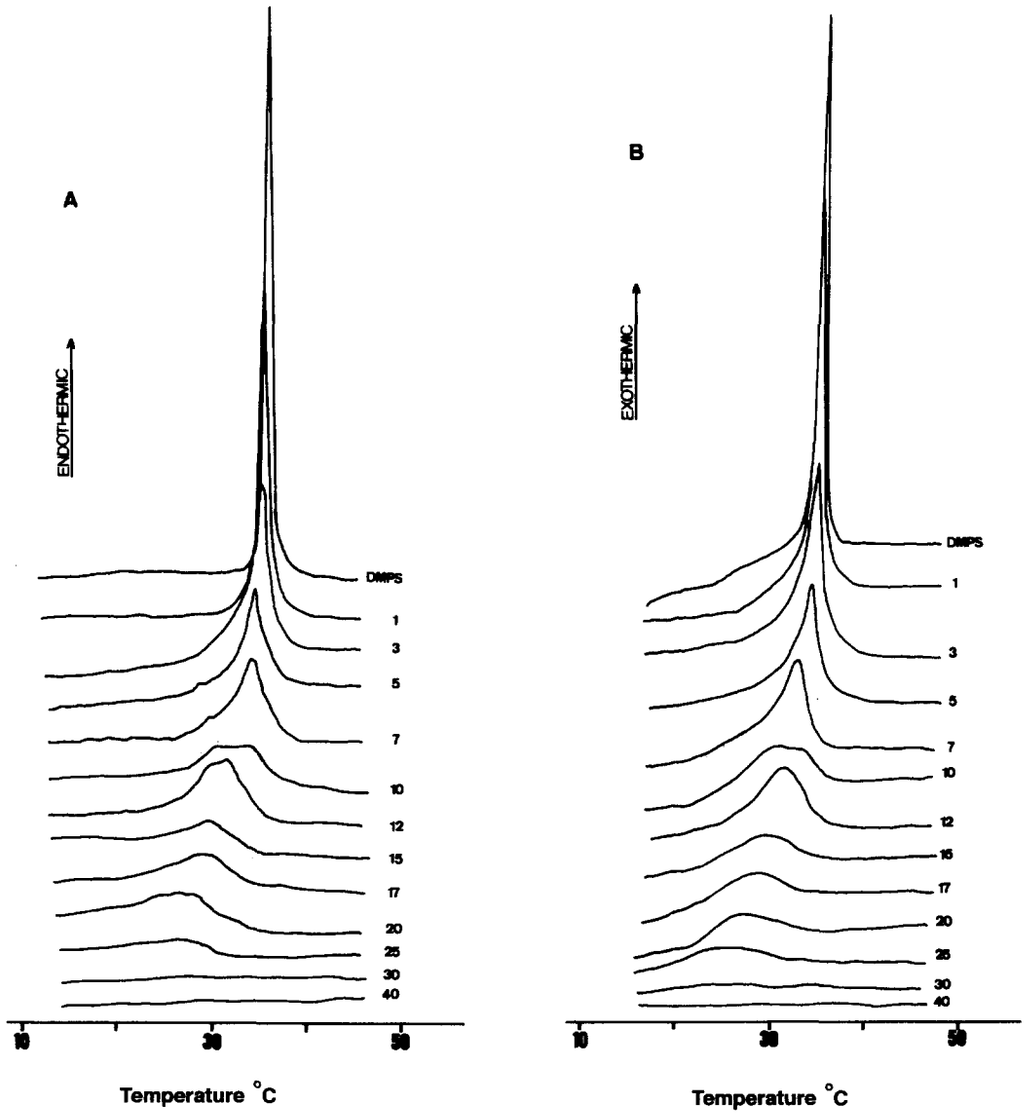

Fig. 1. DSC thermograms for mixtures of DMPS and α-tocopherol. The concentration of α-tocopherol in the membrane (molar percentage) is expressed on the curves. The profiles correspond to heating (A) and cooling (B) scans.

suspensions were centrifuged at 10000 rpm in a bench microfuge and the pellets were collected and placed into aluminum pans. Pans were sealed and scanned in a Perkin-Elmer DSC-4 calorimeter at a heating/cooling rate of 4°C/min. For the determination of the total phospholipid content of a pan, the pan was carefully opened, the lipid was dissolved with chloroform/methanol (1:1) and the phosphorus content was determined using the method of Bötcher et al. [13]. The instrument was calibrated using indium as standard. In the partial phase diagrams, the solidus and fluidus points were determined from the onset temperatures of transition in the heating and cooling thermograms, respectively [14]. Heating and cooling thermograms were very similar.

2.3. Fourier transform infrared spectroscopy

Samples for FT-IR measurements were prepared as described above and the obtained pellets were suspended in a 40 μl of final buffer volume. Infrared spectra were obtained in a Philips PU9800 Fourier transform infrared spectrometer. Samples were examined in a thermostated Specac 20710 cell equipped with CaF_2 windows and using 25 μm teflon spacers (all from Specac, Kent, UK). Each spectrum was obtained by collecting 100 interferograms with a nominal resolution of 2 cm^{-1} and triangular apodization using a sample shuttle accessory in order to average background spectra between consecutive sample spectra over the same time period. Samples were scanned between 20 and 50°C at 1°C intervals with 5 min delay between each consecutive scan with a water bath interfaced to the spectrometer computer. Subtractions from buffer was performed interactively using Spectra-Calc software (Galactic Industries, Salem, MA, USA). Band frequencies were calculated as described before [15].

2.4. $^{45}Ca^{2+}$ binding experiments

For $^{45}Ca^{2+}$ binding experiments, the calcium buffer contained 580 cpm/nmol Ca^{2+}. Suspensions of multilamellar vesicles containing different proportions of α-tocopherol were centrifuged at 11000 rpm for 15 min in a bench microfuge. The resulting pelleted vesicles were resuspended and different Ca^{2+} concentrations were added. The samples were incubated 1 h at 50°C, i.e., above the phase transition temperature of the phospholipid, followed by 1 h at room temperature. Finally, the samples were centrifuged at 13000 rpm for 45 min at 4°C. Supernatants were measured for $^{45}Ca^{2+}$ in a Beckman LS1701 liquid scintillation counter.

As it has been described before [15], the concentration of ionophore used to allow a rapid equilibration with Ca^{2+} in the multilamellar vesicles did not affect the phase transition of pure DMPS as observed by DSC. In order to check the incorporation of α-tocopherol into phospholipid vesicles, 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 [7] 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

3.1. Effect of α-tocopherol on DMPS thermotropic phase transition

The effect of incorporating different amounts of α-tocopherol on the thermotropic phase transition of DMPS in the absence of Ca^{2+} is shown in Fig. 1. Fig. 1a depicts the heating thermograms corresponding to mixtures of DMPS containing up to 40 mol% of α-tocopherol. A considerable broadening of the peak was observed at already low concentrations such as 3 mol% of α-tocopherol. This broadening was asymmetric so that the T_c was depressed down to 32°C. At 5 mol% the broadening was larger and a small peak was seen at 27.2°C. At 10 mol% of α-tocopherol, two peaks were overlapped but clearly discernible, so that one of them was still centered at 36.5°C, like pure DMPS, whereas another one was centered at a lower temperature (31°C). At 12 mol% of α-tocopherol the peak centered at the same temperature than pure DMPS (36.5°C) had completely disappeared, and another peak very broad, centered at 31.5°C, was observed. At 15 and 17 mol% of α-tocopherol a very broad peak centered at 29°C remained. At 20 mol% of α-tocopherol the very broad peak was centered at 26.5°C. At 30 mol% no transition was observed.

Fig. 1b shows the cooling exotherms of the same samples studied above. The patterns were essentially similar to the heating ones with a progressive broadening of the transitions and the whole of the peak appearing at lower temperatures as the concentration of α-tocopherol was increased.

It is remarkable that the temperature of the L_α to L_β transition is not very much modified until about 10–12

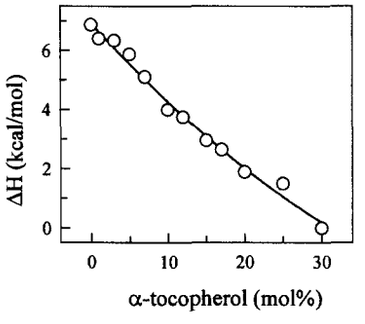

Fig. 2. The enthalpy change (ΔH) for the gel to liquid-crystalline phase transition of mixtures of DMPS/α-tocopherol at different α-tocopherol molar percentage.

mol% of α-tocopherol, in clear contrast with the heating thermograms where T_c decreased already at very low concentrations. It should be also remarked that, at 10 mol%, the components were seen in a pattern similar to that of the heating thermogram at the same concentration of α-tocopherol, so that the component appearing at the higher temperature has T_c identical to pure DMPS.

The molar enthalpies of the endothermic transitions presented in Fig. 1a are plotted in Fig. 2 versus the molar percentage of α-tocopherol. It can be observed that ΔH progressively decreased with increasing concentrations of α-tocopherol.

The onset temperatures of the heating and cooling thermograms were used to construct a partial phase diagram, according to what has been previously described [14] and this is shown in Fig. 3. It can be seen that at very low α-tocopherol concentrations the onset cooling temperature did not appreciably change, the onset heating temperature clearly decreased from very low concentrations of α-tocopherol. This can be taken as an indication of fluid immiscibility at low α-tocopherol concentrations.

The effect of α-tocopherol on the thermotropic phase transition of DMPS was further characterized by FT-IR. The phase transition of phospholipids can be conveniently monitored through the shift in frequency of the CH_2 stretching modes, being characteristic of the acyl chain movement and conformation [16–18]. The CH_2 stretching frequencies reflect mainly conformational disorder and they increase with the introduction of *gauche* bonds in the fatty acyl chains [19]. We have used a deuterated form of DMPS to specifically follow the transition of the phospholipid avoiding any hypothetical contribution of the phytilic chain of α-tocopherol. Fig. 4 shows the dependence with temperature of the symmetric CD_2 stretching band frequency. The phase transition of pure DMPS was detected at 34°C, the difference with the T_c obtained by DSC (36.5°C) may be attributed to a general decrease in phase transition temperatures for lipids with deuterated chains. Clearly the presence of increasing concentrations of α-tocopherol produces a shift of T_c to lower temperatures

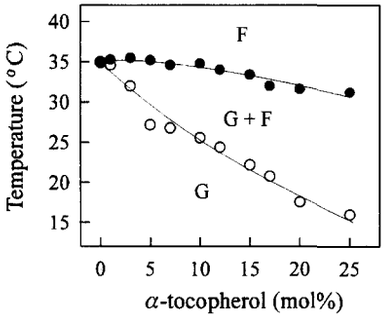

Fig. 3. Partial phase diagram for the mixture of DMPS/α-tocopherol. Open circles are obtained from DSC heating scans and black circles from DSC cooling scans. G indicates a gel phase and F a liquid-crystalline phase.

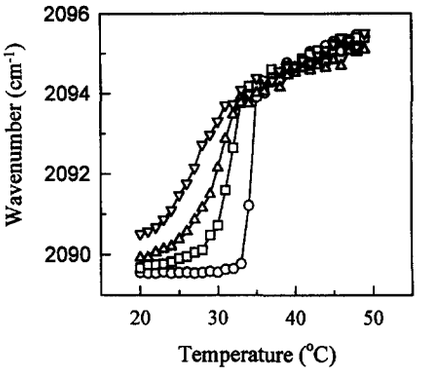

Fig. 4. Frequency of the CD_2 symmetric stretching mode of deuterated DMPS versus temperature, for pure DMPS (○) and mixtures of DMPS/α-tocopherol containing 5 mol% (□), 10 mol% (△) and 30 mol% (▽) of α-tocopherol.

and a broadening of the transition. An interesting effect is that the presence of α-tocopherol produced an increase of the frequency of the band below the phase transition but did not produce any significant change in the frequency above the transition, indicating that in the gel state there is an increase in *gauche* isomers and thus an increase in fluidity, and that α-tocopherol did not alter in an appreciable way the fluidity of the system in the liquid crystalline state.

Another interesting feature is that those samples showing two peaks in DSC, like the sample containing 10 mol% of α-tocopherol, present a broad transition with the onset located well below that of pure DMPS. This indicates that either DMPS was present in both phases, although perhaps in different proportions or that both transitions are produced by a single phase. Note that only one transition can be observed through FT-IR and not two like DSC, but it is well known that DSC has a much better capacity for resolutions of broad transitions occurring at close temperatures.

3.2. Effect of Ca^{2+} on DMPS / α-tocopherol systems

DSC experiments were carried out using particular DMPS/Ca^{2+} molar ratios in the absence of α-tocopherol and the results are shown in Fig. 5a, up to a DMPS/Ca^{2+} molar ratio of 1:10, i.e., saturating or nearly saturating Ca^{2+} concentrations. The phase transition of pure DMPS was broadened as the DMPS/Ca^{2+} was decreased, so that the transition was no longer observed at a molar ratio of DMPS/Ca^{2+} of 2:1. This is in agreement with previous observations which concluded that the phosphatidylserine/Ca^{2+} binding stoichiometry is 2:1 (mol/mol) [20], as it was also confirmed by later measurements of Ca^{2+} binding between PS lamellae [21] and infrared spectroscopy [15].

The DSC pattern which was found for DMPS/α-tocopherol mixtures in the presence of Ca^{2+} was different from either pure DMPS in the presence of Ca^{2+} or

DMPS/α-tocopherol mixtures in the absence of Ca^{2+}. Fig. 5b shows the DSC thermograms of different DMPS/α-tocopherol mixtures at a DMPS/Ca^{2+} molar ratio of 10:1, i.e., at subsaturating Ca^{2+} concentrations. It can be appreciated that the phase transition of DMPS/Ca^{2+} was clearly affected by α-tocopherol, even at very low concentrations

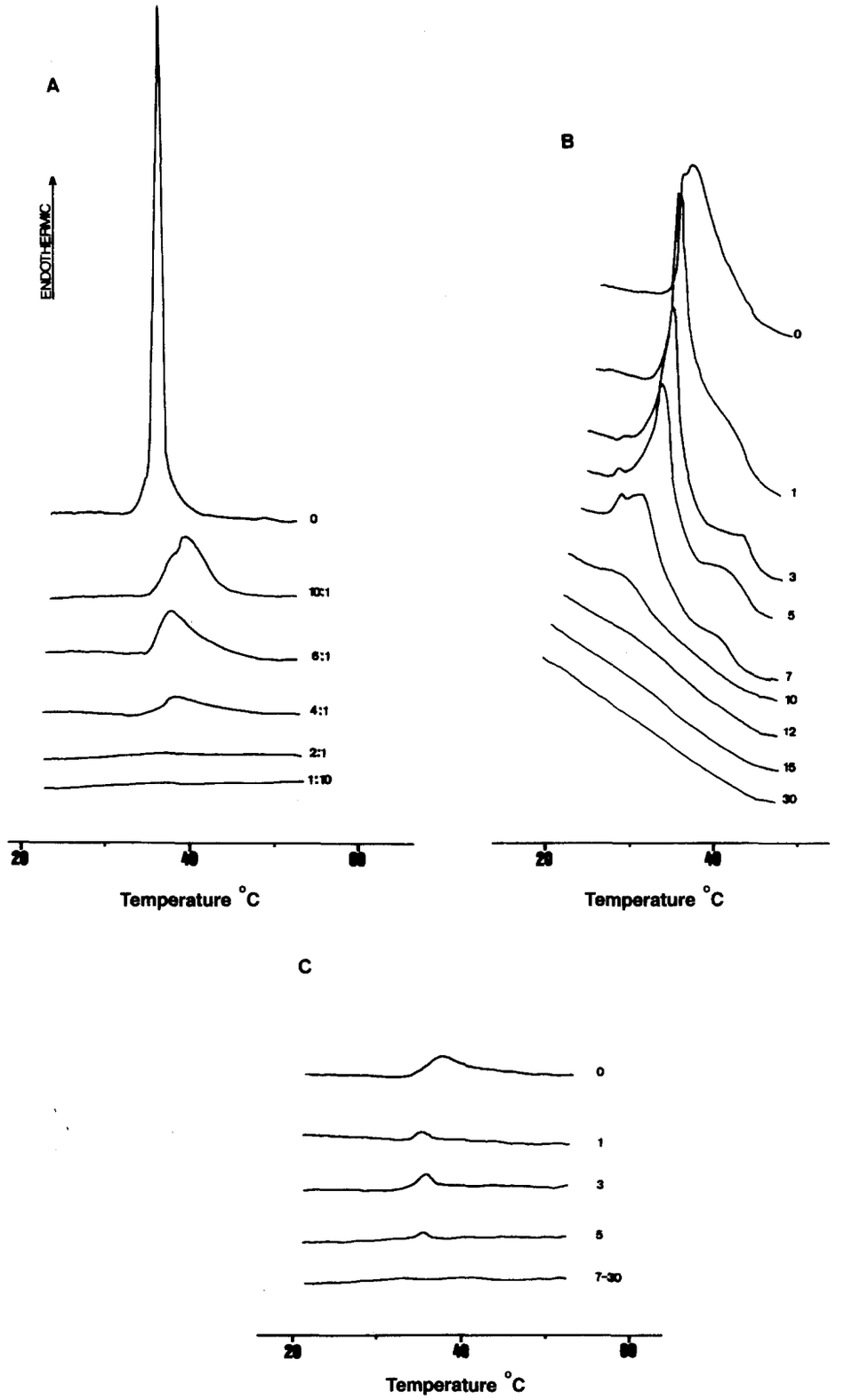

Fig. 5. DSC heating thermograms of (A) pure DMPS in the presence of Ca^{2+} (molar ratios DMPS/Ca^{2+} are indicated on the curves), and mixtures of DMPS/α-tocopherol (molar percentages of α-tocopherol are indicated on the curves) at a DMPS/Ca^{2+} molar ratio of (B) 10:1 and (C) 4:1.

such as 1 mol%, since the peak of the transition was shifted towards lower temperatures and a shoulder was clearly present. The peak was further shifted by higher concentrations of α-tocopherol with a second peak centered at about 42°C, clearly resolved at 3 mol% and higher concentrations up to 10 mol% of α-tocopherol, since at this last concentrations only the main peak remained although considerably broadened. Another small peak, appearing as a shoulder was also observed at the lower temperature side of the main peak between 3 and 7 mol% of α-tocopherol. At concentrations of 12 mol% of α-tocopherol and higher, no transition could be observed in the range of temperatures shown in Fig. 4b. Other results can be observed in Fig. 5c, for DMPS/Ca^{2+} at a 4:1 molar ratio, i.e., still a non saturating concentration. It can be seen that in the last case the phase transition totally disappeared at 7 mol% and higher concentrations of α-tocopherol (up to 30 mol% was tested).

The presence of α-tocopherol into DMPS vesicles also affected to the formation and properties of the DMPS/Ca^{2+} crystalline complex. It is known that above a certain DMPS/Ca^{2+} ratio, a crystalline and dehydrated complex is formed, where the acyl chains and head group of the phospholipid are rigid and immobilized consistent with the elevated transition temperatures observed by DSC [22]. It can be appreciated in Fig. 6a that the phase transition corresponding to the crystalline phase formed in the presence of saturating or nearly saturating Ca^{2+} (DMPS/Ca^{2+} molar ratio of 1:10) was shifted to lower temperatures by the presence of low concentrations of α-tocopherol from 91.6°C in the absence of α-tocopherol to 80°C in the presence of 1 mol%. At 10 mol% the transition totally disappeared. On the other hand, at higher DMPS/Ca^{2+} ratio, like 4:1, i.e., subsaturating Ca^{2+} concentrations, the transition was also shifted to lower temperatures by the presence of very small concentrations of α-tocopherol like 1 mol% and it totally disappeared at 7 mol% and higher concentrations (Fig. 6b)

3.3. $^{45}Ca^{2+}$ binding experiments

In order to explore the possible changes in the binding of Ca^{2+} by DMPS which could take place in the presence of α-tocopherol, measurements were carried out by using $^{45}CaCl_2$. The experimental points of the binding experiments, shown in Fig. 7, were fitted to the following Michaelis-Menten type equation:

$$\frac{[Ca^{2+}]_{bound}}{[DMPS]} = \frac{n[Ca^{2+}]_{free}}{m + [Ca^{2+}]_{free}}$$

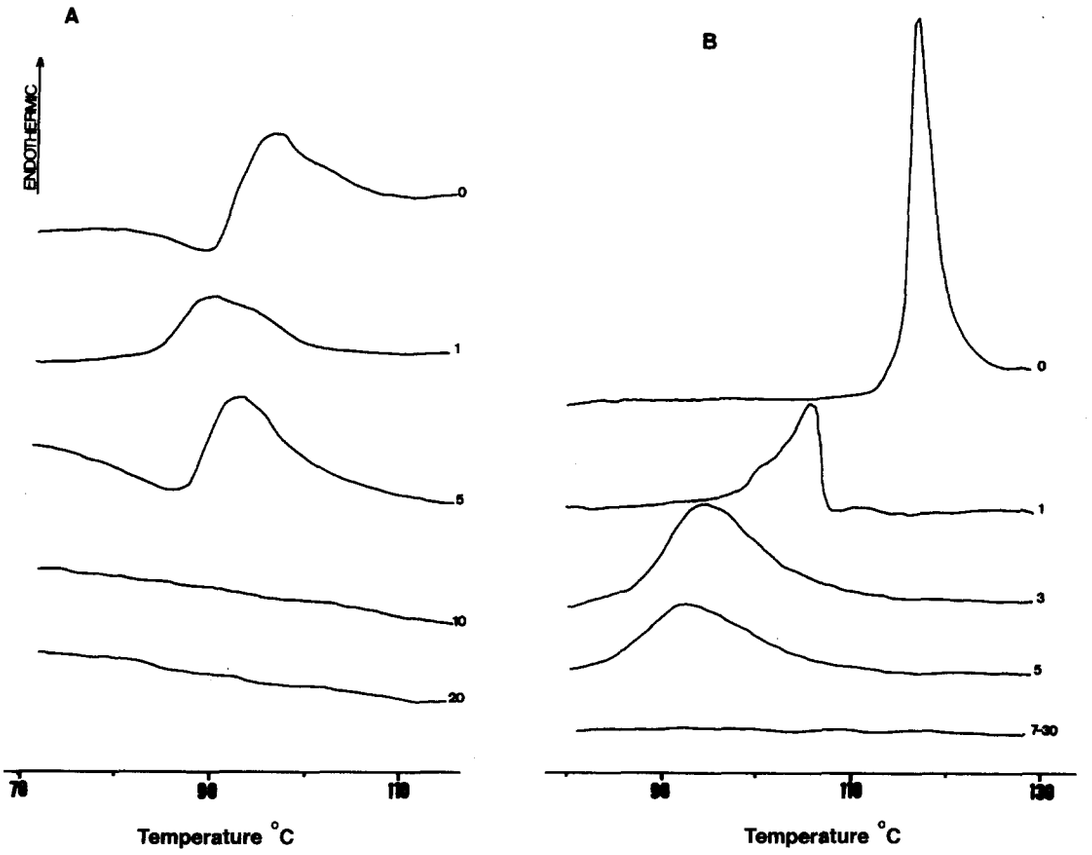

Fig. 6. DSC heating thermograms of the Ca^{2+} complex of DMPS containing different amounts of α-tocopherol (molar percentages of α-tocopherol are indicated on the curves) at a DMPS/Ca^{2+} molar ratio of (A) 1:10 and (B) 4:1.

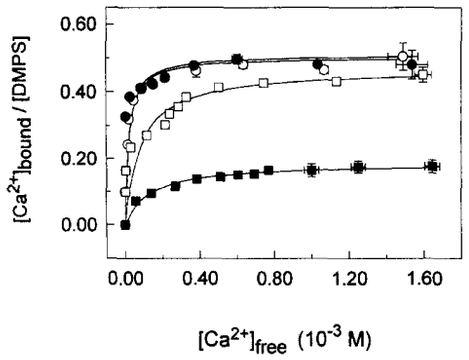

Fig. 7. Ca^{2+}-binding to DMPS for (●) pure DMPS, and DMPS containing 3 mol% (○), 10 mol% (□) and 30 mol% (■) of α-tocopherol, at different DMPS/Ca^{2+} molar ratios. Points represent mean ± standard deviation for three different experiments.

As observed in the figure, the binding of Ca^{2+} by DMPS was saturable. For pure dipalmitoylphosphatidylserine and at high Ca^{2+} concentrations, we previously found a value of 0.51 for n [15], which is the maximum value of Ca^{2+} bound per molecule of phospholipid. This result agreed with the generally observed stoichiometry of about 2 molecules of phosphatidylserine bound to each Ca^{2+} ion [20,21,23]. A value of 0.51 ± 0.02 for n was also deduced for DMPS from the data presented in Fig. 7. This was not sensibly changed by 3 mol% of α-tocopherol, because a value of $0.51 \pm 0.050.50$ was found for n. The number of binding sites slightly decreases in the presence of 10 mol% of α-tocopherol ($n = 0.47 \pm 0.05$). However, in the presence of 30 mol% of α-tocopherol a $n = 0.19 \pm 0.01$ was calculated, indicating that saturation was reached with 1 Ca^{2+} per 5 molecules of DMPS. Therefore, the presence of α-tocopherol prevents that all DMPS molecules will be forming the 2:1 DMPS/ Ca^{2+} complex. Also differences can be deduced from the application of the above equation to the calculation of the apparent dissociation constant (m), which was found to be $2 \pm 1 \cdot 10^{-5}$ M for pure DMPS and DMPS containing 3 mol% of α-tocopherol, but only $8 \pm 4 \cdot 10^{-5}$ M and $13 \pm 2 \cdot 10^{-5}$ M for the mixture containing 10 and 30 mol% of α-tocopherol, respectively. This clearly indicates a reduction in the apparent affinity for Ca^{2+} by phosphatidylserine. Note that this apparent dissociation constant is being estimated through a procedure which calculates membrane-bound Ca^{2+} as all the Ca^{2+} ions accumulated at the membrane/solution interface, and hence it contains both the ions adsorbed at the level of the lipid headgroup and the solvated ions located in the direct proximity of the membrane surface, which act simply as counterions of the negative lipid membranes.

4. Discussion

The results shown in this work indicate that α-tocopherol perturbs the phase behavior of DMPS in the absence, and even more in the presence of Ca^{2+}. Very little was known until now about the interaction of α-tocopherol with phosphatidylserines, and only a few experiments have been reported like that of Ref. [6], where it was said that the effect of α-tocopherol in the phase transition of DMPS was similar to that seen on dimiristoylphosphatidylcholine, although they studied only one concentration, 15.7 mol%, in the absence of Ca^{2+}. Our results show that in the absence of Ca^{2+}, the phase transition of DMPS is decreased by the presence of α-tocopherol and totally suppressed by 30 mol%. These features are certainly similar to those previously reported by our group for dimiristoylphosphatidylcholine [10]. It is however different from the effect given by α-tocopherol on DMPS, that two component are in fact clearly discerned on the thermogram corresponding to the sample containing 10 mol%. Of these components one of them has the same transition temperature than pure DMPS, and the other a lower temperature. A likely explanation for that is that a phase very rich in DMPS, and another containing most of the α-tocopherol are present at low concentration of α-tocopherol (less than 10 mol%). At higher concentration only one phase seems to be present. The FT-IR results support the idea that α-tocopherol greatly perturbs the structure of DMPS in the gel state, as we found that α-tocopherol increased the proportion of *gauche* conformers below the transition, and that α-tocopherol in the liquid crystalline state does not produce any significant effect on fluidity above the transition.

Since the effect of α-tocopherol on T_c of DMPS and on ΔH of the L_β to L_α transition of this phospholipid are similar to those previously reported for phosphatidylcholines [10] it seems plausible to conclude that the localization of α-tocopherol in DMPS membranes must be also similar to the disposition agreed for this vitamin in phosphatidylcholines, i.e., with the chromanol moiety relatively close to the lipid/water interface and the phytyl chain parallel to the fatty acyl chain of the phospholipids [8,24–26]. More drastic was however, the effect of α-tocopherol on DMPS transition in the presence of Ca^{2+}. At relative low Ca^{2+} like a 10:1 DMPS/Ca^{2+} molar ratio, the L_β to L_α phase transition of DMPS was totally abolished by 15 mol%, and at 4:1, i.e., still non saturating, by 7 mol%. α-Tocopherol also affected very considerably to the high-temperature transition of the DMPS/Ca^{2+} complex, so that it was totally abolished by concentrations as low as 7 mol% at a DMPS/Ca^{2+} molar ratio of 10:1 or 4:1, and at 10 mol% at the high concentration of 1:10 which must be practically saturating. This means that α-tocopherol will get incorporated into the cochleate phase formed by DMPS in the presence of Ca^{2+}.

Very interesting are also the results on the binding of Ca^{2+} by DMPS, in the presence of α-tocopherol. The results indicate a decrease in affinity for 10 and 30 mol% of α-tocopherol. The effect of decreasing Ca^{2+}-binding is similar to that observed when phosphatidylcholine [27],

triolein [28] or diacylglycerol [15] are incorporated into phosphatidylserine vesicles. The decrease in the number of binding sites and the affinity of Ca^{2+} for DMPS in the presence of 30 mol% α-tocopherol is certainly remarkable. This may be due to a change in charge density which has been described to lead to a decrease in the affinity for Ca^{2+}, in monolayers studies, particularly in the case of neutral lipids [29,30], Nevertheless, it should be taken into account that Ca^{2+} binds between phosphatidylserine lamellae [21] and it has been reported that neutral lipids, as it the case of α-tocopherol, modify Ca^{2+}-induced bilayer interactions, leading to progressively weaker interactions and larger bilayer separations [31]. This effect could be important since phosphatidylserine and Ca^{2+} are implicated together in many biological events taking place at the lipid/water interface of membrane such as membrane fusion or the activation of enzymes like protein kinase C or phospholipases.

In conclusion, our results show how α-tocopherol affects the thermotropic phase transition of phosphatidylserine in a qualitatively similar fashion to phosphatidylcholine, in the absence of Ca^{2+}, whereas in the presence of Ca^{2+} it has a more drastic effect. α-Tocopherol increases the fluidity of the membrane in the gel state. In addition, α-tocopherol decreases the affinity of DMPS membranes for Ca^{2+}.

Acknowledgements

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