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The interaction of vitamin K_1 with phospholipid vesicles

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Vitamin K_1 is a component of the electron transport chain in chloroplasts and also an activator of carboxylases present in microsomes of different tissues. In order to understand its mechanism of action it is necessary to know its interactions, localization and organization in the phospholipid bilayer. We have studied this question using reconstituted systems of vitamin K_1 incorporated in DPPC multibilayer vesicles, using DSC, DPH anisotropy and FT-IR spectroscopy. DSC shows that the pretransition is modified and dissapears as the concentration of vitamin K_1 in the bilayer increases. The main transition is also affected, with a decrease of T_m and ΔH with increasing concentrations of vitamin K_1. Furthermore, a second peak appears at high concentrations of vitamin K_1, which is indicative of a lateral phase separation of a phase rich in vitamin K_1. Fluorescence measurements using DPH show, in agreement with the calorimetric measurements, that the phase transition is shifted to lower temperatures and the anisotropy is increased above T_m but, interestingly, not below T_m. FT-IR spectroscopic measurements are also in good agreement with the calorimetric and fluorescence results, indicating that vitamin K_1 induced a broadening and a shift to lower temperatures in the phase transition. It is deduced from the variation of the frequency parameter of the CH_2 stretching vibration band with temperature that vitamin K_1 perturbs the average number of *gauche* and all-*trans* conformers of DPPC, only during the phase transition interval but neither at temperatures above nor below the phase transition. However, the bandwidth parameter of this vibration indicated a perturbation above, but not below, the phase transition. The possible relationship between these observations and those coming from fluorescence depolarization of DPH are discussed. Finally, it is concluded from the observation of the C=O stretching mode that vitamin K_1 does not produce a very strong perturbation of the interfacial region of the membrane of the type given by, for example, cholesterol.

Introduction

There are several compounds with vitamin K activity. All of them are 2-methyl-1,4-naphthoquinones substituted at the 3-position with a polyisoprenoid chain. One of these compounds is 2-methyl-3-phytyl-1,4-naphthoquinone, known as vitamin K_1. Vitamin K_1 has been found in plants as a component of electron transport chains of chloroplasts participating in the photoreduction steps of the photosynthetic process [1].

In addition, vitamin K_1 is known to have 'vitamin K activity' when fed to vitamin K-deficient animals [2]. 'Vitamin K activity' in animals is thought to be mainly the activation of a carboxylase present in microsomes and found in different tissues which gives post-translational

Abbreviations: DPH, 1,6-diphenylhexatriene; FT-IR, Fourier transform-infrared spectroscopy; DSC, differential scanning calorimetry; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphoshatidylcholine; K_1, vitamin K_1; T_m, midpoint of the transition temperature; MK-*n*, menaquinone-*n*.

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modification of several proteins, including prothrombin, blood clotting factors, other plasma proteins and proteins found in other tissues [3–5]. Vitamin K_1 has not been found itself in animal tissues, however, other naphthoquinones like MK-4 and others are found in animals instead (see Ref. 3 for a review). Hence it seems probable that the isoprenyl chain in position three can be made by the animal [3]. Nevertheless vitamin K_1 has been found to be able to induce in vitro carboxylation by the solubilized vitamin K-dependent enzyme [6]. In fact, vitamin K_1 showed similar activity to MK-1 through MK-10 and other derivatives [6]. Since vitamin K_1 seems to be very representative of the family of naphtoquinones with vitamin K activity, we have used it as a model of these compounds.

Although it is clear that this vitamin is localized in membranes of chloroplasts and microsomes, very little is known about its interaction with phospholipids and its localization and organization in the bilayer. Since it may be important to know that in order to understand its molecular mechanism of action, we have undertaken the study of multilameller liposomes made of DPPC which included different proportions of vitamin K_1. We have studied, by using a number of techniques like DSC, FT-IR and fluorescence depolarization of the probe DPH, the effect of this naphtoquinone on the physical properties of the membrane.

Materials and Methods

Dipalmitoylphosphatidylcholine was obtained from Fluka (Buchs, Switzerland) and it was used without further purification. Vitamin K_1 and ^2H_2O (99.8%) were from Sigma (Poole, Dorset, U.K.). All other chemicals used in this work were of analytical grade.

The lipid mixtures for the microcalorimetry measurements were prepared by combination in a small tube of chloroform solutions containing 2 mg of DPPC and the appropriate amounts of vitamin K_1, giving a final volume of 50 μl. The solvent was evaporated under an O_2-free N_2 stream. The last traces of solvent were eliminated by dessication under vacuum during a period of time of about 3 h. After the addition of 50 μl of twice distilled and deionized water, multilamellar liposomes were formed by carefully mixing using a bench vibrator and keeping the samples at 50–55°C, i.e. above the phase transition of pure DPPC. Mixing was continued until a homogeneous and uniform suspension was obtained. 15 μl of these suspensions containing 0.6 mg of phospholipid were then sealed in small aluminium pans and scanned in a Perkin-Elmer DSC-4 instrument, using a reference pan containing water. The heating rate was 4 K/min. The range of temperature studied was from 25 to 55°C. Peak areas were measured by weighing paper cut-outs of the peaks. The instrument was calibrated using indium as standard.

Samples for fluorescence depolarization measurements were prepared by mixing 2 mg of DPPC, variable amounts of vitamin K_1 and DPH to give a 200 : 1 DPPC/DPH molar ratio, all of them in chloroform solution. After evaporation as in the case for the microcalorimetry samples, 1 ml of twice distilled and deionized water was added and multilamellar vesicles formed by shaking. The sample was diluted in water in the fluorescence cuvette until a maximum and constant reading of anisotropy was obtained, indicating that light scattering was not affecting the measurements. A Shimadzu RF-540 spectrofluorimeter instrument equipped with Polaroid polarizers was used. Fluorescence was measured by excitation of DPH at 360 nm, while emission was monitored at 430 nm, parallel and perpendicular to the plane of excitation. Steady-state fluorescence anisotropy was calculated as described in Ref. 7.

The lipid mixtures for the infrared spectroscopy measurements were prepared as indicated for the calorimetric experiments, but the samples contained 4 mg of DPPC and the appropriate amounts of vitamin K_1 and were resuspended in 40 μl of ^2H_2O. Infrared spectra were obtained using a Nicolet MX-1 FT-IR spectrometer, assisted by a Nicolet 1200-S computer. Samples were examined in a thermostatted Beckman FH-01 CFT cell, equipped with CaF_2 windows and using 25 μm-teflon spacers. 40 μl of the sample suspensions were injected into the cell and 107 interferograms were collected for each spectrum. Underlying ^2H_2O bands were subtracted by computation prior to the measurements of frequencies

and bandwidths. Temperature was controlled by means of a thermocouple inserted onto the cell and calibrated with samples of pure DMPC and DPPC. Measurements at different temperatures were always done by heating, and the samples were previously equilibrated in the cell at 15°C for at least 15 min.

Lipid dispersions were analyzed for organic phosphorus [8] and by absorption spectroscopy for vitamin K_1 in *n*-pentane, using a molar absorbance coefficient of 3825 $M^{-1} \cdot cm^{-1}$ which was determined separately.

The incorporation of vitamin K_1 into liposomes was assayed by the *n*-pentane extraction as described before for ubiquinone [9,10] to remove any vitamin K_1 remaining in the aqueous phase.

Results

Incorporation of vitamin K_1 into liposomes

Homogeneous dispersions were observed to be formed with concentrations of vitamin K_1 lower than 20%. At higher concentrations yellowish droplets were observed apart from liposomes using a light microscope equipped with a Nomarski interference-contrast accesory. These droplets, which were easily distinguishable from liposomes, presumably corresponded to non-incorporated vitamin K_1.

These observations were confirmed by an extraction procedure using *n*-pentane. This procedure was proposed to extract ubiquinone non-incorporated into liposomes [9,10]. Since ubiquinone is similar in structure to vitamin K_1, we used this extraction as an additional test of the incorporation of vitamin K_1 and showed that at concentrations of vitamin K_1 of 20 mol% or lower, only around 5% could be extracted, i.e. incorporation would be around 95%. However at molar concentrations of 33% and 50%, 49.5% and 77%, respectively, of the total vitamin K_1 was extracted by *n*-pentane.

As a consequence of all these measurements, the samples studied in this work had a maximum molar concentration of vitamin K_1 of 20%, i.e. in all the cases vitamin K_1 incorporated is about 95% of the total vitamin used to prepare the samples.

DSC measurements

Fig. 1 shows that the pretransition of DPPC is already considerably broadened at a concentration of vitamin K_1 of 2 mol%, and has totally disappeared at 5 mol%.

The main transition is also affected, so that T_m decreases as more K_1 is incorporated, reaching 38.6°C at 20 mol% (see Table I). This alteration is also reflected in ΔH of this main peak (Table I) which decreases from 8.65 kcal/mol in pure DPPC to 6.88 kcal/mol at 20 mol% of vitamin K_1. Furthermore, the half-height width increases as a function of vitamin K_1 contents, indicating that vitamin K_1 disrupts the cooperative behavior of the lipid bilayer matrix. At concentration of 10% and higher, a second peak is observed at low temperatures, indicating that a phase separation has taken place. This peak does not come from vitamin K_1, since separate scans of pure hydrated K_1 or K_1 in egg yolk phosphatidylcholine do not produce any transition in this range of temperature (data not shown). On the other hand when enough K_1 to give 40 mol% was used, the scan produced was apreciably identical to that of 20 mol% (not shown), probably as a consequence of the lack of incorporation of K_1 above 20 mol% into liposomes, as explained before.

Similar results were obtained using DSPC/K_1 mixtures (not shown).

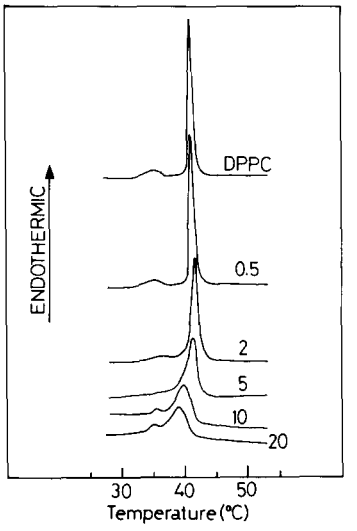

Fig. 1. DSC calorimetric curves for systems containing pure DPPC and DPPC/vitamin K_1. Molar percentages of vitamin K_1 in DPPC are indicated on the curves.

TABLE I

Changes in midpoint of the transition temperature, T_m; enthalpy change of the gel to liquid-crystalline transition, ΔH, and bandwidth at half-height, $\Delta T_{1/2}$, of systems containing differents molar percentages of vitamin K_1 obtained by DSC.

Vitamin K_1 (% mol)	T_m (°C)	ΔH (kcal/mol)	$\Delta T_{1/2}$ (mm)
0	41.5	8.5	2.0
2	41.4	8.4	2.5
5	41.0	8.1	4.2
10	40.0	7.5	6.5
20	39.0	6.8	8.0

Fluorescence anisotropy

In order to investigate the effect of vitamin K_1 incorporation on the fluidity of the DPPC bilayer, the steady-state emission anisotropy of DPPC/K_1 vesicles containing DPH was measured as a function of both temperature and vitamin contents. The results of these experiments are shown in Fig. 2. In agreement with the microcalorimetric results, it was found a shift toward lower temperatures of the phase transition, although this technique did not detect a second peak at high concentrations of K_1. In addition it is apparent from Fig. 3 that the association of increasing amounts of K_1 with the bilayer progresively increased the DPH anisotropy above the phase transition, without significantly modifying it below T_m; such a result suggests that K_1 increases the apparent order of the phospholipid acyl chains in the fluid state but it does not modify this order in the gel state.

FT-IR studies

Infrared spectra of DPPC multibilayers, both

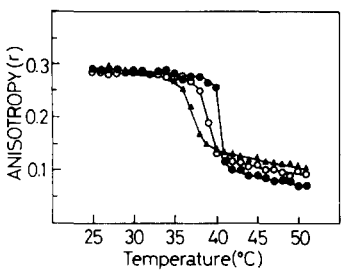

Fig. 2. Dependence of DPH steady-state anisotropy with temperature for multibilayer vesicles composed of pure DPPC (●), and DPPC containing 5 (○) and 20 (▲) mol% of vitamin K_1.

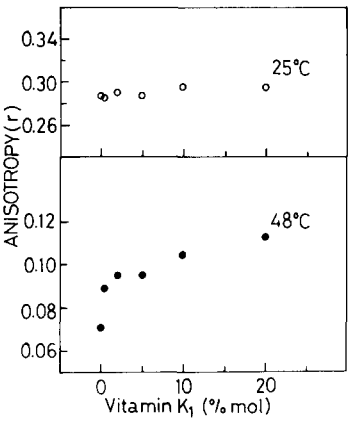

Fig. 3. Dependence of DPH steady-state anisotropy with vitamin K_1 contents incorporated in DPPC multibilayer vesicles below (25°C) and above (48°C) the main transition temperature of pure DPPC.

pure and containing different amounts of vitamin K_1, were collected. The results presented here correspond to the DPPC spectra since the K_1 spectrum is much weaker. Two main vibration bands will be considered, the CH_2 antisymmetric stretching and the C=O stretching mode.

CH_2 antisymmetric stretching

The band which appears at 2920 cm^{-1} corresponds to the antisymmetric stretching mode of the acyl chains of the phospholipid [11,12]. The

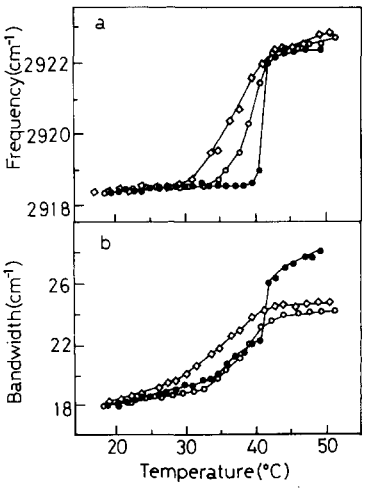

Fig. 4. Temperature dependence of the CH_2 antisymmetric stretching (a) frequency and (b) half-bandwidth. (●), pure DPPC, (○), DPPC containing 5 mol% of vitamin K_1 and (◇), DPPC containing 20 mol% of vitamin K_1.

frequency of this band plotted versus temperature for DPPC multibilayers pure and containing 5 and 20 mol% of vitamin K_1 is shown in Fig. 4a. The shift in frequency of the maximum of the CH_2 stetching modes which takes place during the main endothermic phase transition of DPPC has been associated with the change from all-*trans* to *gauche* conformers [11–13] and hence the frequency of this band is related to the average number of *gauche* conformers. As shown in Fig. 4a the phase transition induces a shift in frequency from 2918.3 cm^{-1} (all-*trans*) to 2922.4 cm^{-1} (*gauche*) in pure DPPC, the onset of the transition being located at 41.3°C. The phase transition is shifted to lower temperatures by the presence of K_1, and the shifts closely agree with those shown by DSC and fluorescence anisotropy measurements of DPH. However, both above and below the phase transition, the presence of K_1 does not change the average number of *gauche* and all-*trans* conformers, respectively.

Something different is observed by plotting the half-height bandwidth versus temperature (Fig. 4b). This parameter has been associated to acyl chain effects which do not involve a variation in *gauche* conformers but rather increases in librational and torsional motion [14,15]. The introduction of K_1 into the bilayer decreases such motions above the phase transition but does not affect them below the phase transition.

C=O stretching mode

Fig. 5 illustrates that a broad asymmetric and intense band appears at 1735 cm^{-1}, being assigned to the C=O stretching mode of the palmitoyl ester groups of DPPC. This asymmetric band has in fact two main components assigned to the carbonyl stretching modes of *sn*-1 (1743 cm^{-1}) and *sn*-2 (1729 cm^{-1}) acyl chains of DPPC [16]. These two components can be distinguished by either Fourier self-deconvolution of the spectra [17] or second derivative spectra as seen in Fig. 5 (see also Ref. 18).

From difference spectra, the total change in the spectral characteristics of this band can be obtained, reflected in the parameter $\Delta A/\Delta t$, obtained as described in Ref. 13. This is shown in Fig. 6a, where it can be observed that the pretransition present in pure DPPC is totally removed by K_1, whereas the main transition is broadened and

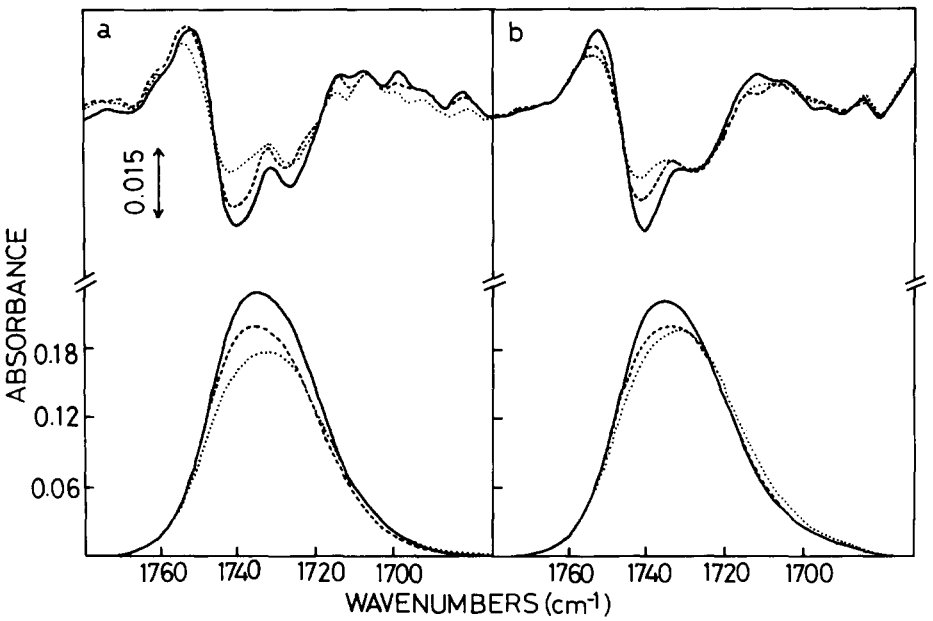

Fig. 5. Infrared spectra of the C=O stretching mode of (a) pure DPPC and (b) DPPC containing 20 mol% of vitamin K_1 at three different temperatures: ————, 25°C; - - - - - -, 37°C and ·····, 44°C. The upper part of the figure shows the second derivative spectra.

shifted to lower temperatures, this pattern being similar to that described before for the CH_2 stretching and it agrees also with the calorimetric and fluorescence experiments.

$\Delta A/\Delta t$ is, however, a complex parameter where changes in frequencies, intensity and bandshape are mixed. Therefore frequency and width were analyzed separately. The frequencies of the maximum of the *sn*-1 and *sn*-2 components as resolved by second derivative spectra, are plotted versus temperature in Fig. 6b, where the frequency of the maximum of the unresolved spectrum is also shown. It can be seen that the frequency of the

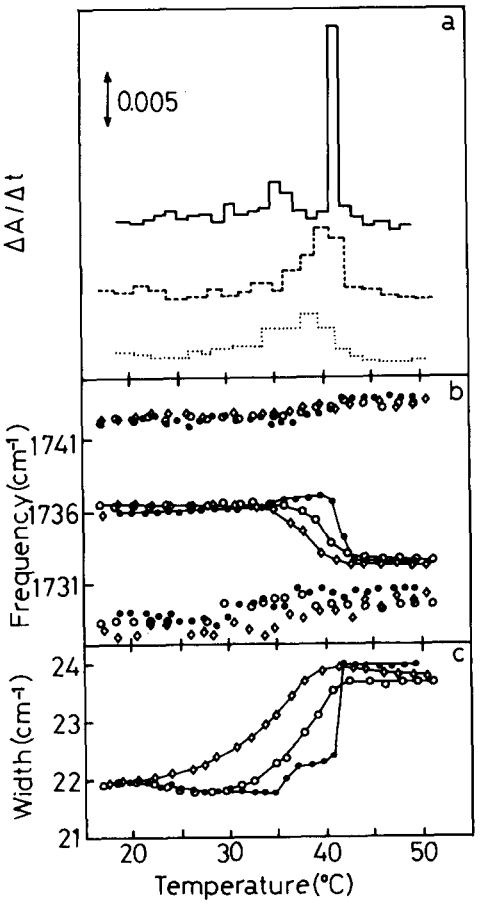

Fig. 6. Temperature dependence of the (a) $\Delta A/\Delta t$, (b) frequency and (c) 0.75 peak height bandwidth of the C=O stretching mode of systems containing pure DPPC and DPPC with vitamin K_1. (a) ————, pure DPPC; - - - - - -, DPPC containing 5 mol% of K_1; · · · · · ·, DPPC containing 20 mol% of K_1. (b) and (c): ●, pure DPPC; ○, DPPC containing 5 mol% K_1; ◇, DPPC containing 20 mol% K_1.

sn-1 component does not change appreciably with temperature neither in pure DPPC nor in K_1 containing samples and that the presence of K_1 does not modify these frequencies. However, the *sn*-2 component, in pure DPPC, is something more sensible to temperature, mainly in the pretransition, with K_1 only slightly decreasing these frequencies. Much bigger changes with either temperature or the presence of K_1, are appreciated in the maximum of the unresolved band, although comparing with the effects of cholesterol [19] or α-tocopherol [20] on DPPC, the effect is relatively modest. It is also clear that the changes which are observed in this band induced by the presence of K_1 are restricted to the phase transition interval of temperatures. This change clearly observed in the plot of $\Delta A/\Delta t$ and in the frequency of the maximum of the unresolved band seems to be due mainly to changes in intensity (see Fig. 5) and in bandwidth (Fig. 6c).

Other infrared absorption bands, corresponding to the polar head group of DPPC, like the phosphate symmetric stretching band at about 1080 cm^{-1} or the $C\text{-}N^+$ stretching vibration of the choline group at about 970 cm^{-1}, were also examined. Since no significant variations were observed with temperature or K_1 concentration, the results are not shown.

Discussion

As a general conclusion from the studies shown above which were obtained using three different physical techniques, vitamin K_1 modifies the physical properties of DPPC and this effect is already appreciable at a 2% molar ratio although it is more evident at higher concentrations.

It is evident from these three techniques that the inclusion of K_1 in multilamellar vesicles of DPPC produces a shift of the phase transition to lower temperatures. The thermograms show in addition that the transition is broadened and the enthalpy decreased. This behaviour is similar to what has been found for other compounds containing a phytanoyl chain of similar lenght like coenzyme Q-3 [21] and α-tocopherol [20,22].

It is, however, distinctive of K_1 that at high concentrations a second peak appeared at temperatures below the main transition. This is attri-

buted to a lateral phase seggregation of a K_1-rich phase. Nevertheless enough K_1 seems to remain in the bulk phase so that there is a shift in the transition temperature, a broadening of the transition and a decrease in the enthalpy, which corresponds to the expected behaviour of a substance which aligns itself principally with the prevailing direction of the phospholipid acyl chains. The shift indicates the formation of an eutectic mixture, the broadening a decrease in cooperativity and the decrease in enthalpy that progressively less phospholipid molecules are participating in the transition, as more K_1 is incorporated in the membrane.

A more detailed study of the formation of eutectic mixtures between vitamin K_1 and DPPC would require the preparation of samples with a K_1 content higher than 20 mol%, so that a complete phase diagram could be elaborated. Unfortunately, and as stated before, the preparation of these samples is hampered by the fact that vitamin K_1 does not incorporate to the DPPC liposomes at concentrations higher than 20 mol%.

The smaller peak at lower temperature (about 35°C) observed by DSC in the presence of 10 and 20 mol% of K_1 was not observed neither in the fluorescence nor in the FT-IR experiments, and this may be due to the higher sensitivity of DSC comparing to the optical techniques, to detect small transitions.

Since the methyl substituents of the phytanoyl chain of K_1 will prevent its accomodation to the all-*trans* configuration adopted by the palmitoyl chains of DPPC, the incorporation of K_1 into the gel phase will be thermodynamically unfavorable. This may explain why K_1 modify the fluorescence anisotropy of DPH above the phase transition but not below (Fig. 3), so that the apparent order of the phospholipid acyl chains will be decreased by the presence of K_1, at temperatures above T_c. A similar observation has been made for coenzyme Q-3 (Aranda, F.J. and Gómez-Fernández, J.C., unpublished data).

It is also observed that K_1 affects the width parameter of the CH_2 stretching band, in the FT-IR spectra, at temperatures above the onset of the transition but not below (Fig. 4). However, the frequency parameter of the same band is not affected by K_1 neither above nor below the phase transition but just only during the phase transition interval of temperatures. How can fluorescence and FT-IR results be correlated?.

The anisotropy parameter varies directly with the rotational relaxation time of the fluorophore and inversely with the motional freedom or 'fluidity' of the membrane lipids. Although it is not straightforward to extract detailed information about fluidity from measurements of steady-state fluorescence anisotropy of DPH in membranes, it has been suggested by a number of authors that an order parameter might be deduced from it [23–25] which is very sensitive to the orientational distribution of the fluorophore. If we accept that both variations in proportion of *gauche*/all-*trans* isomers and librational and torsional motions of the acyl chains are included in this order parameter, it is possible to conclude that K_1 affect the anisotropy of DPH above T_m, influencing the motional freedom of the chains, not including *gauche* effects.

The results obtained by FT-IR for the C=O stretching band seems to indicate that the effect of K_1 on the interfacial region of the membrane is less important than those reported for cholesterol [19] and for α-tocopherol [20]. The changes observed here might solely reveal the changes in acyl chain packing and conformational order to which give rise the presence of impurities in the DPPC multibilayers, hence reflecting the noncooperative nature of the melting of the mixture.

In conclusion we suggest that vitamin K_1 has a limited miscibility with DPPC, being excluded from the gel phase of the phospholipid, this exclusion becoming apparent in DSC at high K_1 concentration where a new phase rich in K_1 is detected. On the other hand, K_1 seems to affect the phospholipid packing above T_c by perturbing the motional freedom of the acyl chains.

Although these results do not clarify by themselves the molecular mechanism of action of vitamin K_1 they could be useful as the base of future studies on the interaction with membranes of different naphthoquinones with vitamin K activity.

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