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The influence of vitamin K_1 on the structure and phase behaviour of model membrane systems

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Abstract

Vitamin K_1 is a component of the Photosystem I of plants which constitutes the major dietary form of vitamin K. The major function of this vitamin is to be cofactor of the microsomal γ-glutamylcarboxylase. Recently, novel roles for this vitamin in the membrane have been postulated. To get insight into the influence of vitamin K_1 on the phospholipid component of the membrane, we have studied the interaction between vitamin K_1 and model membranes composed of dimyristoylphosphatidylcholine (DMPC) and dielaidoylphosphatidylethanolamine (DEPE). We utilized high-sensitivity differential scanning calorimetry and small-angle X-ray diffraction techniques. Vitamin K_1 affected the thermotropic properties of the phospholipids, broadened and shifted the transitions to lower temperatures, and produced the appearance of several peaks in the thermograms. The presence of the vitamin gave rise to the formation of vitamin-rich domains which were immiscible with the bulk phospholipid in both the gel and the liquid–crystalline phases. Vitamin K_1 was unable to alter the lamellar organization of DMPC, but we found that it produced an increase in the interlamellar repeat spacing of DMPC at 10°C. Interestingly, vitamin K_1 promoted the formation of inverted hexagonal H_{II} structures in the DEPE system. We discuss the possible implications that these vitamin K_1–phospholipid interactions might have with respect to the biological function of the vitamin.

Keywords: Differential scanning calorimetry; X-ray diffraction; Lipid polymorphism

1. Introduction

All naturally occurring vitamins K possess the same 2-methyl-1,4-naphthoquinone ring, but differ in the structure of the side chain at the 3-position. Vitamin K_1 (phylloquinone, 2-methyl-3-phytyl-1,4-naphthoquinone, see Fig. 1) which has the same phytyl side chain as chlorophyll, is the only major form found in plants. Vitamin K_1 is a component of the Photosystem I, a pigment protein complex embedded in the photosynthetic membrane of oxygenic photosynthetic organisms, where it acts as a transient electron acceptor [1].

Vitamin K_1 is the major dietary form of vitamin K [2]. The major role of vitamin K is to be cofactor of the vitamin K-dependent carboxylase, a microsomal enzyme catalysing the post-translational conversion of glutamyl residues in precursor proteins to γ-carboxyglutamyl residues in mature proteins. Several coagulant and anticoagulant proteins needed for

Abbreviations: DEPE, dielaidoylphosphatidylethanolamine; DMPC, dimyristoylphosphatidylcholine; DSC, differential scanning calorimetry; SAXD, small-angle X-ray diffraction

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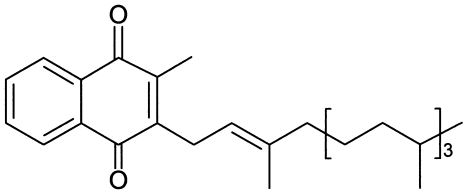

Fig. 1. Structure of vitamin K_1 (phylloquinone, 2-methyl-3-phytyl-1,4-naphthoquinone).

blood clotting were the first known to contain γ-carboxyglutamyl residues. Novel vitamin K-dependent proteins were subsequently discovered in other tissues, most notably bone (in which the dominant form is vitamin K_1), and activity of the enzyme has also been detected in numerous non-skeletal tissues and organs [3,4].

In addition to these γ-glutamylcarboxylase related functions, a number of novel direct roles are emerging for vitamin K in the membrane. In the vitamin K cycle, the active cofactor for γ-glutamylcarboxylase is continuously regenerated. This vitamin K cycle has been shown to act as a potent antioxidant suppressing lipid peroxidation in rat liver microsomes [5]. Kinetic studies of free-radical-scavenging action of biological hydroquinones in solution showed that the rate constant of the hydroquinone form of vitamin K_1 was 31-fold larger than that of α-tocopherol [6]. It has been suggested that mixtures of vitamin E and this hydroquinone may function synergistically as antioxidants in various tissues and mitochondria [7]. Vitamin K has been recently shown to prevent the changes in lipid composition of biomembranes of different tissues produced by chronic phenol poisoning [8], and to exhibit antitumour activity against human prostatic carcinoma cells [9]. In the latter, an effect on the mitochondria and other membranous components of the cell was suggested for vitamin K. Finally, vitamin K_1 has been suggested to increase the immune response in rats after intensive physical load, partly by the direct action on the cell membranes [10].

In the light of the above reports and the extreme lipophilicity of vitamin K_1, a possible role of vitamin K_1–membrane interactions in the mechanism of action of this vitamin is readily suggested. Despite the obvious importance that the knowledge of the interaction between vitamin K_1 and the lipidic component of the membrane would have in the understanding of the function of the vitamin, very little is known about the effects of vitamin K_1 on the structure and dynamics of phospholipid membranes. A former study from our laboratory previously addressed the interaction between vitamin K_1 and dipalmitoylphosphatidylcholine model membranes by using DSC, diphenylhexatriene fluorescence anisotropy and infrared spectroscopy [11]. When the content of vitamin K_1 was increased, DSC experiments showed that the pretransition disappeared and that the midpoint transition temperature and the enthalpy change of the main transition decreased. The appearance of a second peak in the thermograms corresponding to samples containing high concentration of vitamin K_1 suggested the presence of an enriched vitamin K_1 phase laterally separated. Fluorescence measurements using diphenylhexatriene showed that the presence of vitamin K_1 shifted the transition to lower temperatures and produced an increase of the anisotropy above but not below the midpoint transition temperature of the phospholipid. Infrared spectroscopy measurements agreed with the calorimetric and fluorescence results and showed that vitamin K_1 induced a broadening and shifting to lower temperatures of the phase transition. From the study of variation of the frequency parameter of the CH_2 stretching vibration band, it was concluded that vitamin K_1 did not perturb the average number of *gauche* and *trans* conformers neither above nor below the phase transition. It was also concluded from the observation of the $C=O$ stretching mode that vitamin K_1 did not produce a very strong perturbation of the interfacial region of the membrane [11].

The purpose of the present study is to investigate in detail the interaction of vitamin K_1 with membranes using phospholipid vesicles as model systems. Membrane model systems are widely used membrane-mimicking systems by which lipid–lipid interactions, far away from the complexity of a cellular membrane, can be more easily recognized [12–14]. Phospholipids, in addition to the lamellar organization, can adopt several non-lamellar structures. This ability, known as lipid polymorphism, can greatly affect the functional behaviour of the membrane [15], and there is strong evidence that the function of membrane proteins can be affected by the modification of the properties of the lipid matrix in which they are embedded [16]. In this investigation we focus

on the effect of vitamin K_1 on the thermotropic properties of two major phospholipid components of membranes: phosphatidylethanolamine (a major component of eukaryotic membranes which spontaneously adopts hexagonal H_{II} phases at physiological conditions) and phosphatidylcholine (the most important class of membrane phospholipids). Given the potential biological importance of non-lamellar structures, we checked the possibility that vitamin K_1 might modulate lipid polymorphism. Using high-sensitivity differential scanning calorimetry (DSC) and small angle X-ray diffraction (SAXD) techniques, we have characterized the interaction between vitamin K_1 and lipid membranes composed of dimyristoylphosphatidylcholine (DMPC), a species with acyl chains shorter than the previously studied palmitoyl derivative [11] and extended the study to the system composed by dielaidoylphosphatidylethanolamine (DEPE). Evidence is provided suggesting that vitamin K_1-rich domains, with thermotropic properties depending on the concentration of the vitamin, are formed in both phosphatidylcholine and phosphatidylethanolamine systems. Vitamin K_1 was also shown to promote the formation of non-lamellar phases in phosphatidylethanolamine. These findings should help to understand the nature of the interaction between vitamin K and membranes.

2. Materials and methods

DMPC and DEPE were obtained from Avanti Polar Lipids (Birmingham, AL, USA). 2-Methyl-3-phytyl-1,4-naphthoquinone (vitamin K_1, phylloquinone) was obtained from Sigma (Poole, Dorset, UK). All other reagents were of the highest purity available. Phospholipid concentrations were determined using de method of Böttcher et al. [17].

The lipid mixtures for DSC measurements were prepared by combination of chloroform/methanol (1:1) solutions containing the phospholipid and the appropriate amount of vitamin K_1 as 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. Multilamellar liposomes were prepared in 0.1 mM EDTA, 100 mM NaCl, 10 mM Hepes (pH 7.4) buffer by mixing, using a bench mixer, always keeping the samples at a temperature above the lamellar gel to lamellar liquid–crystalline phase transition temperature of the lipid. The suspensions were sonicated for 1 min in a Branson 1200 bath sonicator (Branson Ultrasonic, Danbury, CT, USA) to aid with the homogenization. Experiments were performed using a MicroCal MC2 calorimeter (MicroCal, Northampton, USA). The final phospholipid concentration was 1 mg ml^{-1}. The heating scan rate was 30°C h^{-1}. Samples were incubated for 50 min at the starting temperature before scans were initiated. The construction of partial phase diagrams was based on the heating thermograms for a given mixture of phospholipid and vitamin K_1 at various vitamin concentrations. The onset and completion temperatures for each transition peak were plotted as a function of the mole fraction of vitamin K_1. These onset and completion temperature points formed the bases for defining the boundary lines of the partial temperature–composition phase diagram.

Samples for SAXD were prepared as described previously [18]. Briefly, 15 mg of phospholipid and the appropriate amount of vitamin K_1 in chloroform/methanol (1:1) were mixed and the solvents were removed as described above. Dry lipid films were hydrated with the same weight of buffer during 1 h above the temperature of the gel to liquid–crystalline phase transition of the pure phospholipid and pelleted by centrifugation at 12 000 rpm for 30 min. Nickel-filtered Cu K_α ($\lambda = 1.54$ Å) X-ray was obtained from a Philips PW1830 X-ray Generator. X-Rays were focused using a flat gold-plated mirror and recorded using a linear position sensitive detector model 210 (Bio-Logic, Echirolles, France). Samples were mounted in aluminium holders with cellophane windows. The sample temperature was controlled to ± 0.5°C using a circulating water bath. X-Ray diffraction profiles were obtained from 15-min exposure times after 10 min of temperature equilibration. Calibration of the detector was carried out using crystalline cholesterol ($d = 33.6$ Å).

3. Results

High-sensitivity DSC profiles for pure DMPC and mixtures of DMPC with vitamin K_1 are shown in

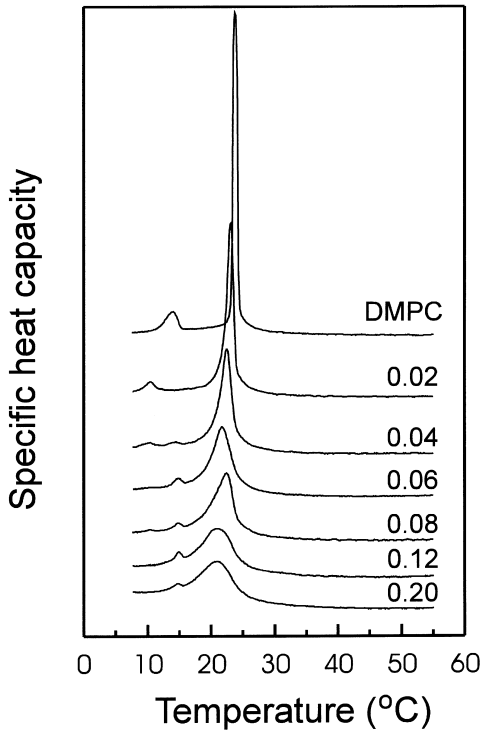

Fig. 2. Representative DSC thermograms for DMPC and mixtures DMPC/vitamin K_1. The concentration of vitamin K_1 in the membrane (mol fraction) is expressed on the curves.

Fig. 2. In the absence of vitamin K_1, DMPC exhibits two endotherms upon heating, a pretransition at 11.8°C and the main gel to liquid crystalline phase transition at 23.5°C, in agreement with previous reports [19]. Fig. 2 shows the overall effect of the incorporation of increasing amounts of vitamin K_1 on the thermotropic phase transitions of DMPC. Both the temperature and the enthalpy of the pretransition decreased with increasing concentration of vitamin K_1 and completely disappeared above a 0.08 vitamin K_1 mol fraction. The temperature of the main gel to liquid crystalline phase transition decreased with increasing concentrations of vitamin K_1, concomitantly with a reduction in the apparent cooperativity of the overall transition (inversely related to the width of the transition). The transition peak became broader and asymmetric but the presence of vitamin K_1 produced only a slight decrease in the enthalpy change of the lamellar gel to lamellar liquid–crystalline phase transition of DMPC (Table 1). Interestingly, a new peak appeared below the main transition peak for 0.04 mol fraction and higher concentrations of vitamin K_1. A more detailed view of the effect of vitamin K_1 on the pretransition and the appearance of the new peak is presented in Fig. 3. It can be seen that the incorporation of already small amounts of vitamin K_1 (0.03 mol fraction) produced a decrease in the pretransition temperature (ca. 3°C). The addition of more vitamin K_1 had no further effect on the temperature of the pretransition. As shown in the insert of Fig. 3, the enthalpy of the pretransition was progressively decreased with increasing vitamin K_1 concentrations, being completely abolished at a vitamin K_1 mol fraction of 0.10. Fig. 3 also shows the presence of the new peak below the main transition. This peak appeared at a vitamin K_1 mol fraction of 0.04, and the increase in vitamin K_1 concentration did not significantly change its transition temperature. The inset of Fig. 3 shows that the enthalpy change associated with this new peak increased from the sample containing 0.04 to the sample containing 0.06 mol fraction of vitamin K_1, and then it became invariant with the increase in vitamin content until it decreased again at the highest content of vitamin K_1 tested (0.20 mol fraction). The different origin of the pretransition and this new peak are verified by the coexistence of both transitions in the thermograms corresponding to concentrations of vitamin K_1 ranging from 0.4 to 0.8 mol fraction.

Further information on structural characteristics of the DMPC/vitamin K_1 systems was obtained

Table 1
The enthalpy changes (ΔH, kcal/mol) for the lamellar gel to lamellar liquid–crystalline phase transition of mixtures of DMPC/vitamin K_1 and DEPE/vitamin K_1 at different vitamin K_1 mol fractions

Vitamin K_1 mol fraction	ΔH (kcal/mol)	
	DMPC	DEPE
0	5.53 ± 0.43	5.63 ± 0.40
0.01	5.49 ± 0.22	5.92 ± 0.30
0.02	5.17 ± 0.09	5.94 ± 0.15
0.03	5.52 ± 0.24	5.30 ± 0.30
0.04	5.92 ± 0.34	4.83 ± 0.04
0.06	5.46 ± 0.47	5.13 ± 0.10
0.08	5.17 ± 0.19	5.05 ± 0.32
0.10	5.11 ± 0.24	5.68 ± 0.10
0.12	4.90 ± 0.28	6.88 ± 0.22
0.14	–	6.25 ± 0.03
0.16	4.76 ± 0.32	–
0.20	4.22 ± 0.02	5.92 ± 0.03

Values are means ± S.D. of three different experiments.

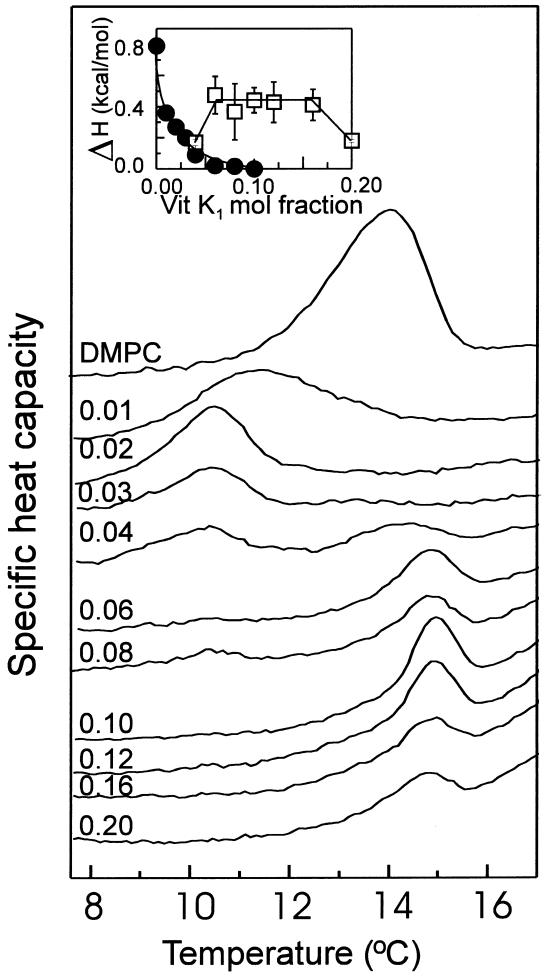

Fig. 3. DSC thermograms, showing the enlarged region of the pretransition, for DMPC and mixtures DMPC/vitamin K_1. The concentration of vitamin K_1 in the membrane (mol fraction) is expressed on the curves. Inset: enthalpy change for the pretransition (●) and the low temperature melting peak (□) as a function of vitamin K_1 concentration. Points represent the average of three experiments (standard error bars are shown when larger than the symbols).

with the use of SAXD. Phospholipids organized in multilamellar structures are expected to give rise to reflections with distances which relate as 1:1/2:1/3... [20]. Fig. 4 shows the diffraction pattern profiles corresponding to pure DMPC and DMPC containing vitamin K_1 at different temperatures. Fig. 4A shows a typical SAXD pattern obtained for pure DMPC showing the presence of sharp Bragg reflections with a good signal to noise ratio. The diffractograms were symmetrical as expected for the non-oriented samples used. The centre of the diffractograms had a deep trough as a normal consequence of the presence of the non-diffracted beam stopper. For the results shown in Fig. 4, only one side of the diffractogram was considered. The detector was calibrated and the channel number units were transformed into distance in angstroms, presenting the data in a logarithmic scale for better clarity. Fig. 4B shows that pure DMPC gave rise to two reflections with distances which relate as 1:1/2 (Table 2), consistent with the expected multilamellar organization. With this technique not only the macroscopic structure itself can be defined, but also the interlamellar repeat distance in the lamellar phase can be obtained. The largest first-order reflection component corresponds with the interlamellar repeat distance, which is comprised of the bilayer thickness and the thickness of the water layer between the bilayers. As seen in Table 2, DMPC gave rise to a first-order reflection with *d*-value of ca. 60 Å in the gel state (10°C and 17°C) and of ca. 50 Å in the liquid–crystalline state. Samples containing 0.02 and 0.10 mol fraction of vitamin K_1 gave rise to two reflections which related as 1:1/2 in the whole range of temperatures under study, indicating that the presence of vitamin K_1 did not alter the structural lamellar organization of DMPC. However, at 10°C, some differences were found in the distances of the reflections. At 10°C (Table 2), i.e., below the pretransition and the transition of the lower melting component of the main transition (see Figs. 2 and 3), the interlamellar repeat distance increased from 60 Å for pure DMPC to 62 Å for the sample containing 0.02 mol fraction of vitamin K_1 and to 70 Å for the sample containing 0.10 mol fraction of vitamin K_1. In this latter case there appears to be a reduction in the total intensity of defined reflections (Fig. 4D, upper part), which may indicate a loss of long-range order in the system. The increase in *d*-spacing was ca. 10 Å, a magnitude similar to the change in *d*-spacing observed for the transition from the gel to the liquid–crystalline phase of pure DMPC. At 17°C (Table 2), i.e., above the pretransition and the transition of the lower melting component but still below the main transition, the presence of vitamin K_1 had no effect on the interlamellar repeat distance being the *d*-value for the first-order reflection ca. 60 Å for all the samples. Finally at 40°C (Table 2), i.e., above the main transition, the lamellar repeat distance was also not

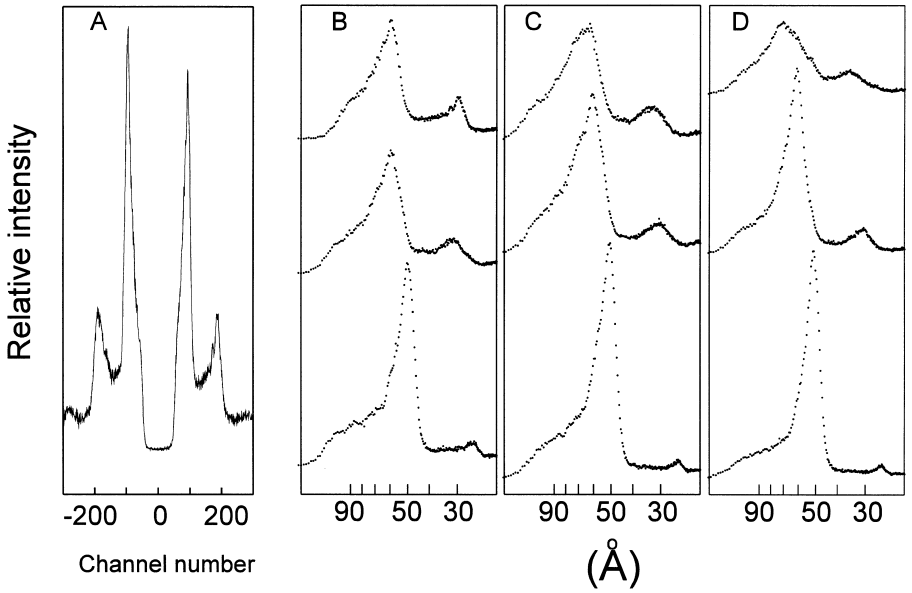

Fig. 4. (A) Raw representative X-ray diffraction pattern obtained for pure DMPC at 10°C. (B–D) X-Ray diffraction profiles from pure DMPC (B), DMPC containing 0.02 vitamin K_1 mol fraction (C), and DMPC containing 0.10 vitamin K_1 mol fraction (D) obtained at 10°C, 17°C, and 40°C (from top to bottom).

affected by the presence of vitamin K_1 being the *d*-value ca. 50 Å.

Using the thermograms shown in Fig. 2, a partial phase diagram for the system DMPC/vitamin K_1 was constructed (Fig. 5). Vitamin K_1 did not undergo any phase transition in the range of temperature under study, and therefore all the observed thermotropic transitions arose from the phospholipid component in the mixture. The onset and completion temperatures of the main transition gave us the points to obtain the solid and fluid lines (boundary lines of the gel to liquid–crystalline phase transition), respectively. Increasing the concentration of vitamin K_1 from 0 to 0.04 mol fraction produced a decrease

Table 2
X-Ray diffraction data of pure DMPC and DMPC/vitamin K_1 systems at different temperatures

Sample	Reflections (Å)	
Pure DMPC		
10°C	59.6	29.1
17°C	59.6	31.1
40°C	50.4	25.2
DMPC/vitamin K_1 0.02 mol fraction		
10°C	62.3	31.3
17°C	60.9	30.5
40°C	50.5	25.0
DMPC/vitamin K_1 0.10 mol fraction		
10°C	70.1	35.5
17°C	60.9	30.5
40°C	51.0	25.2

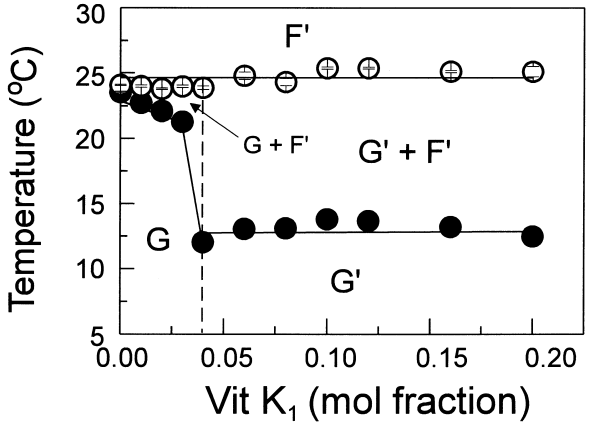

Fig. 5. Partial phase diagram for DMPC in mixtures of DMPC/vitamin K_1. Closed (●) and open circles (○) were obtained from the onset and completion temperatures of the main gel to liquid–crystalline phase transition (see text for details). The phase designations are as follows: G, DMPC gel phase; G′, immiscible DMPC/vitamin K_1 domains in the gel phase; F′, immiscible DMPC/vitamin K_1 domains in the liquid–crystalline phase. Points represent the average of three experiments (standard error bars are shown when larger than the symbols).

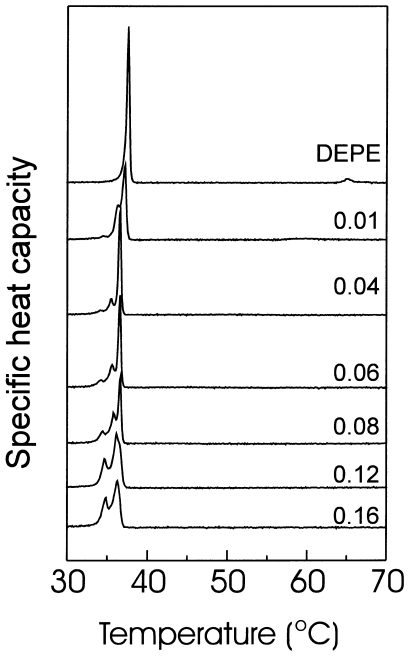

Fig. 6. Representative DSC thermograms for DEPE and mixtures of DEPE/vitamin K_1. The concentration of vitamin K_1 in the membrane (mol fraction) is expressed on the curves.

of the temperature of the solid line. At higher concentrations of vitamin K_1, the solid line stayed horizontal, i.e., at a constant temperature, while the fluid line remained horizontal in the whole range of vitamin K_1 concentrations under study. The observation that vitamin K_1 did not perturb the temperature of the boundary lines indicated the presence of immiscibilities, suggesting the formation of different immiscible DMPC–vitamin K_1 domains both in the gel and the liquid–crystalline states. In Fig. 2 it can be observed that the DSC profiles corresponding to the samples containing 0.12 and 0.20 mol fraction of vitamin K_1 are almost identical and, hence, an alternative interpretation to the immiscibility would be that the membrane becomes saturated with vitamin K_1 at these high concentrations of the vitamin. Below 0.04 mol fraction of vitamin K_1, when the temperature increases the system evolves from a gel phase (G), to a liquid–crystalline phase (F′) (composed of pure DMPC in the fluid state and immiscible DMPC–vitamin K_1 domains in the same phase) through a region of coexistence of both phases. At vitamin K_1 concentrations higher than 0.04 mol fraction, the system evolves from a gel phase (G′) (composed of immiscible DMPC–vitamin K_1 domains and pure DMPC in the gel state), to the liquid–crystalline phase (F′), through a broad region of coexistence of both phases.

The effect of vitamin K_1 on the thermotropic phase transitions of DEPE is shown in Fig. 6. Aqueous dispersions of DEPE can undergo a gel to liquid–crystalline phase transition in the lamellar phase and in addition a lamellar to hexagonal H_{II} structural phase transition [21]. This is shown in the scan corresponding to pure DEPE (Fig. 6, upper part). The lamellar gel to lamellar liquid–crystalline phase transition takes place at 37.2°C and the lamellar to hexagonal H_{II} structural phase transition occurs at 63.8°C, in agreement with previous data [21]. The latter has a much smaller transition enthalpy

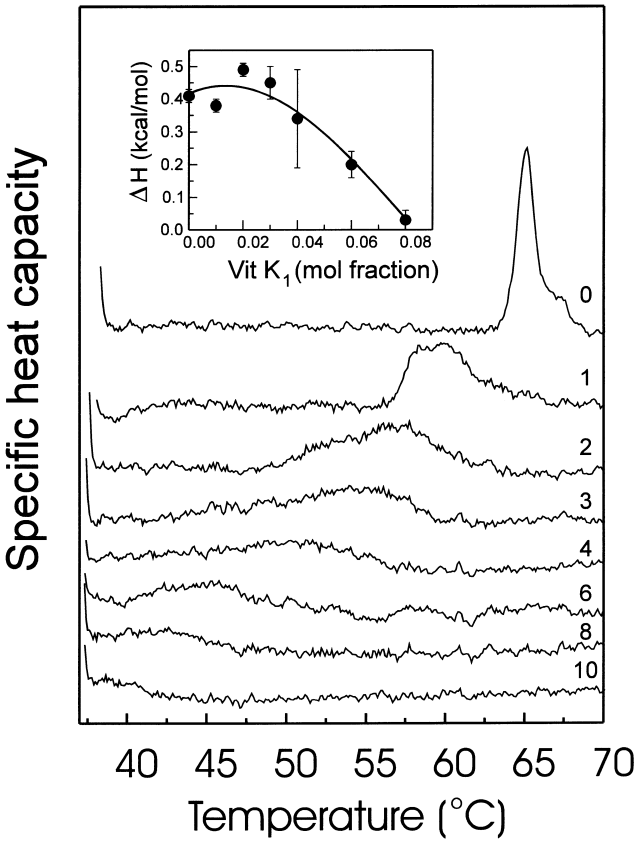

Fig. 7. DSC thermograms, showing the enlarged region of the lamellar to hexagonal H_{II} phase transition, for DEPE and mixtures DEPE/vitamin K_1. The concentration of vitamin K_1 in the membranes (mol fraction) is expressed on the curves. Inset: enthalpy change of the lamellar to hexagonal H_{II} phase transition as a function of vitamin K_1 concentrations. Points represent the average of three experiments (standard error bars are shown when larger than the symbols).

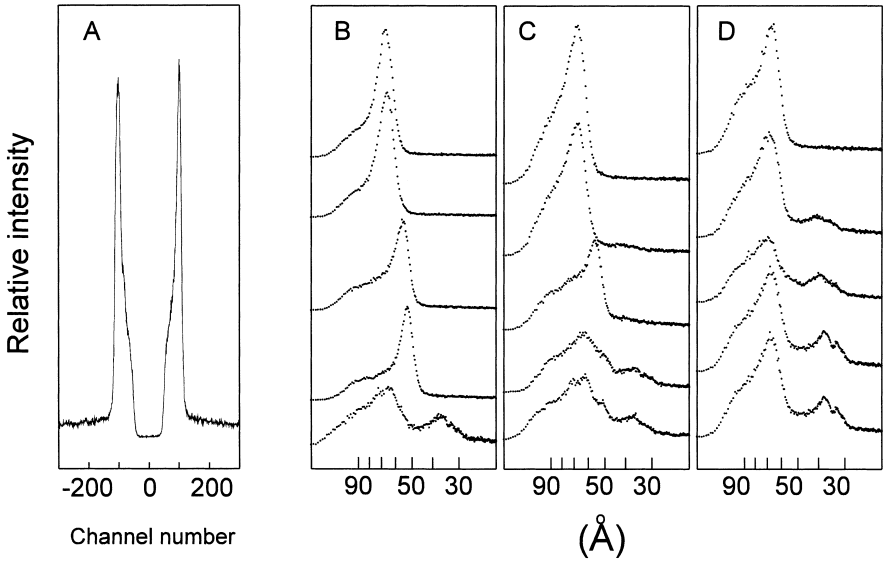

Fig. 8. (A) Raw representative X-ray diffraction pattern obtained for pure DEPE at 45°C. (B–D) X-Ray diffraction profiles from pure DEPE (B), DEPE containing 0.03 vitamin K_1 mol fraction (C), and DEPE containing 0.10 vitamin K_1 mol fraction (D) obtained at 30°C, 35°C, 45°C, 60°C, and 70°C (from top to bottom).

due to the fluid character of both the lamellar and the hexagonal H_{II} phase [22]. The presence of vitamin K_1 did not produce a significant effect on the enthalpy change of the lamellar gel to lamellar liquid–crystalline phase transition of DEPE (Table 1). As shown in Fig. 6, the presence of increasing concentrations of vitamin K_1 produced a broadening in the lamellar gel to lamellar liquid–crystalline phase transition peak and the appearance of several components in the thermogram. The effect of vitamin K_1 on the lamellar to hexagonal H_{II} phase transition was more drastic, resulting in the disappearance of the transition. A detailed picture of the effect of vitamin K_1 on the lamellar to hexagonal H_{II} phase transition is presented in Fig. 7. Vitamin K_1 produced a broadening and shifting of the transition peak to lower temperatures with a concomitant decrease in the enthalpy of the transition (Fig. 7, inset). At 0.10 mol fraction of vitamin K_1 the transition is observed as a mere shoulder on the upper part of the main transition and it could not be detected at higher concentrations of vitamin K_1.

The interaction of vitamin K_1 with DEPE was further studied by means of SAXD. Fig. 8 shows the diffraction patterns corresponding to pure DEPE and DEPE containing vitamin K_1 at different temperatures. A typical X-ray diffraction pattern obtained for DEPE containing samples is presented in Fig. 8A. The channel number units were transformed into distance in angstroms, as described above for DMPC samples, and are presented in Fig. 8. It can be seen that DEPE systems in the lamellar states showed only the first-order reflection. It has been previously observed [23,24] that in DEPE systems in the lamellar phases no higher order reflections are found. For pure DEPE (Fig. 8B) below the gel to liquid–crystalline phase transition (30°C and 35°C) the interlamellar repeat distance in the gel state was ca. 66 Å (Table 3). The transition to a liquid–crystalline phase was accompanied by a decrease of ca. 10 Å in first-order repeat distance, due to the decrease in the effective acyl chain length. Increasing the temperature in the liquid–crystalline state produced a reduction of the interlamellar distance from 55.2 Å at 45°C to 52.6 Å at 60°C (Table 3), due to the resulting increase in chain motion which leads to a reduction of the effective chain length. A similar behaviour has been previously reported [25]. Lipids organized in hexagonal H_{II} structures give rise to reflections at distances which relate as $1:1/\sqrt{3}:1/\sqrt{4}:1/\sqrt{7}...$ [20], the largest spacing of the hexagonal H_{II} (d) relates to the diameter of the lipidic cylinders (a) as $d=(\sqrt{3}/2)\cdot a$. Pure DEPE in the hexagonal H_{II} phase, i.e., at 70°C (Fig. 8B), gave

Table 3
X-Ray diffraction data of pure DEPE and DEPE/vitamin K_1 systems at different temperatures

Sample	Reflections (Å)		
Pure DEPE			
30°C	66.4		
35°C	65.6		
45°C	55.2		
60°C	52.6		
70°C	64.8	37.4	
DEPE/vitamin K_1 0.03 mol fraction			
30°C	67.2		
35°C	67.2		
45°C	55.7		
60°C	63.4	36.4	
70°C	61.9	35.7	
DEPE/vitamin K_1 0.10 mol fraction			
30°C	65.6		
35°C	68.8	40.1	
45°C	69.7	40.1	
60°C	65.6	37.9	32.8
70°C	65.6	37.6	32.4

rise to two reflections with distances which relate as $1:1/\sqrt{3}$ (Table 3). The first-order spacing was 64.8 Å, and this means that the diameter of the tubes of the DEPE hexagonal H_{II} phase was ca. 53 Å. The presence of 0.03 mol fraction of vitamin K_1 (Fig. 8C) had no effect on the interlamellar spacing neither below (30°C) nor above (35°C and 45°C) the gel to liquid–crystalline phase transition (Table 3). However, at 60°C, temperature at which pure DEPE was organized in the lamellar liquid–crystalline state, the presence of 0.03 mol fraction of vitamin K_1 produced the appearance of reflections which distances related as $1:1/\sqrt{3}$, characteristic of lipid organized in hexagonal H_{II} structures (Table 3). At 30°C, the presence of 0.10 mol fraction of vitamin K_1 did not affect the interlamellar repeat distance (Fig. 8D). At 35°C, DEPE samples containing no vitamin K_1 or a 0.03 mol fraction of vitamin K_1 (Fig. 8B,C), were organized in the lamellar gel state. However, in Fig. 8D it can be clearly seen that the sample containing 0.10 mol fraction of vitamin K_1 at the same temperature (35°C) shows a diffraction pattern with reflections which distances relate as $1:1/\sqrt{3}$, characteristic of a hexagonal H_{II} phase. The latter clearly indicates the ability of vitamin K_1 to promote hexagonal H_{II} phase formation in DEPE systems. At 35°C (Table 3) the first-order repeat distance of the hexagonal H_{II} phase for the sample containing 0.10 mol fraction of vitamin K_1 was ca. 69 Å. This value decreased with increasing temperature, reaching a value of ca. 65 Å at 70°C, otherwise similar to those values obtained for pure DEPE and DEPE containing 0.03 mol fraction of vitamin K_1 at the same temperature.

Using the DSC data shown in Figs. 6 and 7 and the information of phospholipid structural organization obtained from SAXD presented in Fig. 8, a partial phase diagram for DEPE in mixtures with vitamin K_1 (Fig. 9) was constructed. In a concentration range of vitamin K_1 from 0 to 0.08 mol fraction, both the main lamellar gel to lamellar liquid–crystalline phase transition and the structural lamellar to hexagonal H_{II} phase transition of DEPE were clearly distinguishable (Figs. 6 and 7). The onset and completion temperatures of the lamellar gel to lamellar liquid–crystalline phase transition were used to define the solid and fluid boundary lines, whereas the onset

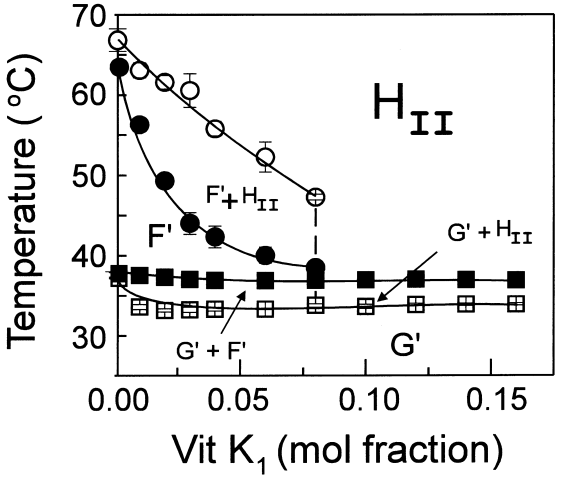

Fig. 9. Partial phase diagram for DEPE in mixtures of DEPE/vitamin K_1. Closed (■) and open squares (□) were obtained from the completion and onset temperatures of the main gel to liquid–crystalline phase transition, and closed (●) and open circles (○) were obtained from the onset and completion temperatures of the lamellar to hexagonal H_{II} phase transition (see text for details). The phase designations are as follows: G′, immiscible DEPE/vitamin K_1 domains in the gel phase; F′, immiscible DEPE/vitamin K_1 domains in the liquid–crystalline phase; H_{II}, hexagonal H_{II} phase. Points represent the average of three experiments (standard error bars are shown when larger than the symbols).

and completion temperatures of the lamellar to hexagonal H_{II} phase transition were used to define the lamellar and hexagonal boundary lines. At concentrations of vitamin K_1 higher than 0.08 mol fraction, the lamellar to hexagonal H_{II} phase transition peak was not observed by DSC (Fig. 7). In these cases the onset and completion temperatures of the only observed transition were used to define the solid and fluid lines. According to SAXD data, the boundaries defined by these solid and fluid lines are different from those defined at lower vitamin K_1 content. The solid and fluid lines kept horizontal, i.e., at a constant temperature, in the whole range of vitamin K_1 concentration under study, suggesting the presence of immiscible DEPE/vitamin K_1 domains in both lamellar states. The temperature of the lamellar and hexagonal lines decreased as the concentration of vitamin K_1 increased. Up to a concentration of vitamin K_1 of 0.08 mol fraction, the system evolved from a lamellar gel phase (G') with different DEPE–vitamin K_1 domains to a lamellar liquid–crystalline phase (F') with different DEPE–vitamin K_1 domains through a very narrow coexistence region ($G'+F'$), and then to the hexagonal H_{II} phase (H_{II}) through a coexistence region ($F'+H_{II}$). At concentrations of vitamin K_1 higher than 0.08 mol fraction, the system evolved from the immiscible lamellar gel phase (G') to the hexagonal H_{II} phase (H_{II}) through a coexistence region ($G'+H_{II}$), apparently without an intervening lamellar liquid–crystalline phase.

4. Discussion

In this study, the nature of the interaction between vitamin K_1 and membranes has been investigated using lipid vesicles formed by two different classes of phospholipids, namely phosphatidylcholine and phosphatidylethanolamine. DSC has been used in order to characterize the influence of vitamin K_1 on the thermotropic properties of the phospholipids. The profile of a DSC thermogram of a phospholipid phase transition is largely determined by the transition temperature and the enthalpy change. Determining the temperatures of the transitions allows the construction of phase diagrams, which provide information regarding the equilibrium between different phases. The effect of vitamin K_1 on the macroscopic organization of the phospholipids was assessed by SAXD.

For the DMPC/vitamin K_1 system we observed a broadening of the peak and a shifting of the transition temperature to lower values, together with the appearance of a new peak located below the main transition peak. The same type of perturbation was previously reported for the effect of vitamin K_1 on the thermotropic transition of dipalmitoylphosphatidylcholine [11]. This similarity suggests that the effect of vitamin K_1 on the phase transition of phosphatidylcholines does not depend on the length of the phospholipid acyl chains. In the case of DMPC, the larger difference between the pretransition and the main transition temperatures of this shorter phosphatidylcholine enabled us to detect both transitions, the pretransition and the new transition peak, in the same thermogram. This coexistence, which confirmed the different origin of both transitions, was not possible to detect when using dipalmitoylphosphatidylcholine [11]. These results suggest that in the presence of vitamin K_1 some of the phospholipid molecules leave the main transition domain to assemble into a vitamin K_1-richer domain with lower melting temperature. Vitamin K_1 was able to shift the gel to liquid–crystalline phase transition of phosphatidylcholines to lower temperatures. This promotion of the liquid–crystalline phase could be the consequence of the intercalation of the vitamin K_1 molecules between the DMPC ones. However, this perturbation, which is in line with the type of behaviour that could be expected for an uncharged very apolar molecule, is not very effective given the high tendency of vitamin K_1 to aggregate into the bilayer, thereby reducing the effective vitamin–phospholipid contact area. The interaction of the vitamin with the phospholipid molecules is demonstrated by its effect on the pretransition, which is more sensitive than the main transition to the presence of other compounds into the bilayer. A similar behaviour has been described for other isoprenoid molecules like coenzyme Q_3 [26] and α-tocopherol [27] in phosphatidylcholine membranes. In those cases a broadening and shifting of the phase transition to lower temperatures was found but no evidence of new peaks was reported, suggesting that the naphthoquinone ring could be important for the enhanced aggregational behaviour of vitamin K_1 in phosphatidylcholine systems.

Vitamin K_1 did not alter the structural lamellar organization of DMPC as evidenced by the lamellar reflections pattern found in all the DMPC/vitamin K_1 mixtures. We found that the presence of vitamin K_1 produced an increase of the interlamellar repeat distance of DMPC, at 10°C, but it did not affect the interlamellar repeat distance of DMPC at higher temperatures. The observed increase in the interlamellar repeat distance can be a consequence of the increase of the water layer between the phospholipid bilayers or it can be due to the effective increase of the bilayer thickness. At present there is no experimental evidence which can unambiguously support either one of the possibilities. From the reported influence of vitamin K_1 on the C=O stretching mode of DPPC [11] it was concluded that the vitamin did not produce a strong perturbation of the interfacial region of the membrane; however, the possibility that vitamin K_1 could produce an increase of the hydration layer of the bilayer cannot be totally excluded. An increase of the effective bilayer thickness would be apparently not compatible with the previous diphenylhexatriene fluorescence and infrared spectroscopy data obtained for a dipalmitoylphosphatidylcholine system [11]. Infrared spectroscopy data showed that vitamin K_1 did not change the content of the *trans–gauche* conformers both above and below the midpoint transition temperature of the phospholipid. It was also shown that vitamin K_1 did not alter diphenylhexatriene steady-state anisotropy below the midpoint transition temperature of the phospholipid and increased it above this temperature. The reported increase in diphenylhexatriene anisotropy above the midpoint transition temperature of the phospholipid was in contradiction to the absence of change in the content of *gauche* conformers observed by infrared spectroscopy at this temperatures. It was suggested [11] that vitamin K_1 would influence the motional freedom of the acyl chains of the phospholipid without including *gauche* effects. In the light of the present results, a simpler alternative explanation can be suggested for the observed increase in diphenylhexatriene anisotropy. Similar to what has been recently proposed for coenzyme Q in phosphatidylethanolamine systems [18], we suggest that vitamin K_1 is able to form enriched domains in the membrane, moreover it is possible that vitamin K_1 forms aggregates which could be located in the middle of the bilayer. The space between the two layers of phospholipids is precisely the place in which diphenylhexatriene is located. In this way, diphenylhexatriene may interact with these aggregates or with vitamin K_1-enriched domains and this would lead to the perturbation of the motional properties of the probe. The increase of diphenylhexatriene anisotropy above the transition temperature could reflect the interaction of the probe with the vitamin and will not be due to a change in *gauche* conformers of the phospholipid. Then, how can this absence of change in *gauche* conformers be reconciled with the possibility that vitamin K_1 would increase the bilayer thickness at 10°C? It has to be noted that the increase in *d*-spacing is observed at 10°C, i.e., below the pretransition and the transition of the small lower melting peak, but it is not observed at 17°C. At 17°C the system is mostly in the gel phase because this temperature, being higher than that of the pretransition and the transition of the small melting peak, is still lower than the temperature of the main transition. It seems that the increase in *d*-spacing occurred only when the phospholipid acyl chains are in the tilted conformation characteristic of the $L\beta'$ phase. A possibility for the presence of these unusual large repeat distances could be the stabilization by vitamin K_1 of rippled phases in the DMPC system. Large *d*-spacing values were found previously to correspond to rippled phases in mixtures of diacylglycerol with phosphatidylcholines [28]. Another possibility is that the vitamin K_1 aggregates located between both monolayers would produce an effective increase of the bilayer thickness if the short and ordered acyl chains of DMPC are not able to accommodate these aggregates in the inner space of the bilayer. Both possibilities would increase the *d*-spacing without requiring a change in the *trans–gauche* conformers. However, DMPC above the transition temperature of the lower melting peak and in the disordered liquid–crystalline state could accommodate vitamin K_1 without affecting the thickness of the bilayer.

When a foreign molecule like vitamin K_1 is added to a phospholipid system, a change in the transition temperature of the phospholipid would be expected if both molecules are miscible. We observed that for very low vitamin K_1 concentrations (up to 0.04 mol fraction) the temperature of the solid line decreased

as more vitamin was present in the system, indicating that DMPC and a small amount of vitamin K_1 are miscible in the lamellar gel phase. The intercalation of the vitamin molecules into the phospholipid palisade would perturb the thermotropic properties of the phospholipid. The same applied to the pretransition at these very low concentrations of vitamin K_1. When the contents of vitamin K_1 was increased, the solid line remained horizontal, whereas the fluid line remained horizontal along the whole range of vitamin K_1 under study. This lack of perturbation in the temperature of the boundary lines confirmed the presence of immiscibilities which were suggested by the calorimetric thermograms, and suggested the formation of DMPC–vitamin K_1-rich domains both in the gel and in the liquid–crystalline phases.

The effect of vitamin K_1 on the lamellar gel to lamellar liquid–crystalline phase transition of DEPE was qualitatively similar to that commented above for DMPC, i.e., it produced the appearance of several peaks in the thermograms suggesting the formation of vitamin K_1-rich domains. It is interesting that α-tocopherol has been shown to give rise to multicomponent thermograms in saturated phosphatidylethanolamines, like dimyristoyl- [29] and dipalmitoylphosphatidylethanolamine [27] but not in unsaturated ones, like DEPE [29]. This emphasized the importance of the naphthoquinone ring for the aggregational behaviour of vitamin K. From SAXD it is concluded that DEPE could accommodate vitamin K_1 aggregates in both the lamellar gel and the lamellar liquid–crystalline state without altering the interlamellar repeat distance. The most interesting finding is that vitamin K_1 was able to promote the formation of hexagonal H_{II} structures in DEPE systems. Incorporation of increasing amounts of vitamin K_1 resulted in a progressive decrease of both transition temperature and enthalpy change of the lamellar to hexagonal H_{II} phase transition, indicating that those DEPE molecules interacting with vitamin K_1 give rise to a broad phase transition which is shifted to lower temperatures. This interpretation was confirmed by our SAXD data which evidenced that vitamin K_1 was a very effective promoter of the hexagonal H_{II} phase, such that the characteristic reflections of the hexagonal H_{II} phase appeared at temperatures at which pure DEPE, in the absence of vitamin K_1, was organized in the lamellar gel state. It has been found [30] that when lipids are organized in hexagonal H_{II} structures, due to an increased motion of the acyl chains, the first-order spacing and therewith the tube to tube distance decreases with temperature. The larger repeat distance found at 35°C in the sample containing 0.10 mol fraction of vitamin K_1 probably reflects the decreased motion of the acyl chains in the gel state. In the sample containing 0.10 mol fraction of vitamin K_1 there are two distinct changes in the diffraction pattern of the hexagonal H_{II} phase (Fig. 8D). First, there appears to be an increase in the total intensity of a defined reflection, and second, the third-order reflection of the hexagonal H_{II} pattern is clearly observed. These observations indicate a gain of long-range order in the hexagonal H_{II} phase.

The observation that both the solid and fluid lines in the DEPE–vitamin K_1 partial phase diagram remain horizontal along the whole range of vitamin under study, evidences the formation of vitamin K_1-rich domains laterally segregated in both the lamellar gel and the lamellar liquid–crystalline state of DEPE. However, these domains enriched in vitamin K_1 greatly affected the formation the hexagonal H_{II} phase, with both the lamellar and the hexagonal H_{II} boundary lines decreasing as more vitamin K_1 is present in the system, suggesting that vitamin K_1 is more miscible with DEPE in the hexagonal H_{II} phase. The ability of lipids to adopt different non-lamellar structures has been thoroughly studied. The cone-shaped molecule of phosphatidylethanolamines makes them compatible with inverted structures such as the hexagonal H_{II} phases [31]. The intercalation of vitamin K_1 between the DEPE ones, although very limited, will perturb the lipid matrix increasing the hydrophobic volume and will give a more effective cone-shaped molecule, and in this way will facilitate the formation of the hexagonal H_{II} structures. A similar mechanism has been previously suggested for the promotion of non-lamellar structures found for α-tocopherol [29]. On the other hand, the presence of vitamin K_1 aggregates in the centre of the bilayer protruding toward the lipid palisade will also perturb the lower part of the lipid matrix and will facilitate the formation of hexagonal H_{II} structures. A promotion of hexagonal H_{II} structures by aggregates of coenzyme Q in the centre of the DEPE bi-

layer has been recently reported by our laboratory [18].

We believe that the two major findings of this study, namely the formation of vitamin K_1-rich domains laterally segregated within the plane of the bilayer, in two major membrane phospholipids systems (phosphatidylcholine and phosphatidylethanolamine), and the modulation of lipid polymorphism by the vitamin (promotion of non-lamellar structures), could be of relevance for the biological role of vitamin K. Although the tissue distribution of vitamin K may vary with the nutritional regimens [32], the median hepatic concentration of vitamin K_1 in adults is ~ 5 ng g^{-1} [33], this value being 40 000-fold lower than the hepatic store of, for example, vitamin A. The tendency of vitamin K_1 to form domains would be thus important for a molecule whose concentration in the membrane is very low, because it would allow vitamin K_1 to be present in certain parts of the membrane at a sufficiently high concentration for its optimal function. From the comparison between the amount of glutamate carboxylation and the daily vitamin K requirements, it has been assumed that an average vitamin K molecule is recycled several hundred times [34]. It is apparent that the presence of vitamin K domains would facilitate the establishment of such a regenerating vitamin K cycle, such domains being very important for the well-known function of vitamin K as a cofactor for γ-glutamylcarboxylases. The segregation of vitamin K would be also significant for the postulated role of the vitamin K cycle as a potent antioxidant against lipid peroxidation [5]; however, further studies are required to establish the possible preferential association of vitamin K with unsaturated phospholipids, as has been postulated for α-tocopherol [27,35]. Vitamin K has been shown to be able to regenerate vitamin E, and it has been suggested that mixtures of both vitamins may function synergistically as antioxidant in various tissues [7]. The analogous aggregational behaviour of vitamin E and vitamin K suggests the possibility, which will require further examination, that both vitamins might be associated, sharing the same domains in the membrane, which would facilitate their antioxidant function.

The efficient non-lamellar promotion ability described in this study for vitamin K_1 also deserves consideration. There is increasing evidence that lipids with tendency to form hexagonal H_{II} phases play an important role in modulating membrane attached proteins. Activation of protein kinase C [36] and phospholipase A_2 [37] by diacylglycerols, which are well-known modulators of lipid polymorphism, constitute paradigmatic examples of such a membrane–protein interaction. α-Tocopherol, a isoprenoid molecule with a similar hexagonal H_{II} promotion ability, has been shown to inhibit protein kinase C activity [38] and modulate phospholipase A_2 activity [39]. The latter evidence together with the finding that a synthetic vitamin K congener inhibits phospholipase D activity [40] suggest a potential role, which has to be evaluated, for vitamin K_1 in the modulation of the function of membrane attached proteins. The fact that the vitamin K requirements of the γ-glutamylcarboxylase appear to be dependent on whether intact microsomes or a detergent-solubilized system is studied [3] opens the interesting possibility that the interaction between vitamin K and the membrane might be also important for the activation of the carboxylase. Finally, the modulation of lipid polymorphism by vitamin K_1 described in this study might be related to the recently described alterations in membrane structure responsible for the cytotoxicity of vitamin K to certain tumour cells [9] or the increase of the immune response during physical load [10]. Further studies, using mixtures of relevant phospholipids, including charged species, will be required to more deeply explain the general features of vitamin K-membrane interactions revealed in this study and to provide a clearer understanding of the influence that these interactions might have on the function of vitamin K in membranes.

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