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Influence of retinoids on phosphatidylethanolamine lipid polymorphism

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The interaction of all-*trans*-retinoic acid and all-*trans*-retinol with dielaidoylphosphatidylethanolamine has been studied by differential scanning calorimetry and ^{31}P-NMR spectroscopy. Increasing concentrations of all-*trans*-retinoic acid up to a mol fraction of 0.09 were found to induce shifts to lower temperatures of both the L_β to L_α and L_α to hexagonal-H_{II} phase transitions, with a slight decrease in the enthalpy change of the transitions. At higher concentrations no further effects on the transitions were observed, and this is interpreted as indicative of a limited miscibility of retinoic acid with the phospholipid. ^{31}P-NMR spectroscopy confirmed that the L_α to hexagonal-H_{II} phase transition was shifted to lower temperatures in the presence of retinoic acid. On the other hand increasing concentrations of all-*trans*-retinol up to a mol fraction of 0.166, induced a progressive shift of the L_β to L_α and the L_α to hexagonal-H_{II} phase transitions to lower temperatures. At higher concentrations the main gel to liquid-crystalline phase transition was further displaced to lower temperatures and the lamellar to hexagonal-H_{II} phase transition was not observed in the thermograms. ^{31}P-NMR spectroscopy indicated that retinol was able of inducing the phospholipid to adopt the hexagonal-H_{II} phase at temperatures even below the main gel to liquid-crystalline phase transition temperature of the pure phospholipid.

Introduction

Retinoids (vitamin A and its derivatives) are essential compounds for the maintainance of health. The term retinoids comprises a number of naturally occurring compounds such as retinol, retinal and retinoic acid, as well as a large list of synthetic analogs with or without biological activity. Their structure is very similar and it only differs at the polar end (Fig. 1), however, their physiological functions are markedly different. All-*trans*-retinol, apart from being a metabolic precursor of 11-*cis*-retinal, involved in the mechanism of vision in animals, is essential for reproduction while, retinoic acid is active in the promotion of growth [1,2]. All of them are highly hydrophobic compounds which preferentially partition into non-polar phases. Because of its amphiphylic nature (Fig. 1) they may display a detergent-like effect. Retinol and retinoic acid have been shown to disrupt the membranes of erythrocytes and lysosomes [3]. This fact, together with its marked hydrophobicity points to biological membranes as one of their physiological sites of action.

Protein kinase C activity is also modulated by retinoids [4,5]. Since retinoids do not compete for the phorbol ester-binding site [4], it is possible that they associate with the plasma membrane lipids affecting the activity of protein kinase C associated to the membrane.

Several studies have correlated the membrane effects of retinoids and their biological activity. Furthermore, the ability of retinoids to fluidize membranes has been correlated with their toxic effects [6,7].

Some biophysical studies have dealed with the interaction of retinoids and model membranes. The effect on the phase transition temperature of DMPC and DPPC has been studied by following changes in water permeability and liposome size [8]. EPR studies on the interaction of retinoids and DPPC bilayers have re-

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Abbreviations: DEPE, 1,2-dielaidoylphosphatidylethanolamine; DPPC, 1,2-dipalmitoylphosphatidylcholine; DPPE, 1,2-dipalmitoylphosphatidylethanolamine; DSC, differential scanning calorimetry; ΔH_c, enthalpy change of the gel to liquid-crystalline phase transition; ΔH_h, enthalpy change of the bilayer to hexagonal-H_{II} phase transition; PC, phosphatidylcholine; ^{31}P-NMR, ^{31}P nuclear magnetic resonance spectroscopy; PE, phosphatidylethanolamine; retinoic acid, all-*trans*-retinoic acid; retinol, all-*trans*-retinol; T_c, onset temperature of the gel to liquid-crystalline phase transition; T_h, onset temperature of the bilayer to hexagonal-H_{II} phase transition; $\Delta\sigma$, chemical shift anisotropy.

ported a retinoid-induced restriction of the lipid side-chain motions in the lower portion of the chain [9]. NMR data indicated a large perturbation of the lipid bilayer structure induced by retinol and retinoic acid, as well as the presence of lateral phase separations [10]. The interaction of retinol and retinoic acid with DPPC and DPPE membranes has been also investigated by means of DSC [11].

It is widely known that dispersions of individual or mixtures of phospholipids of biological origin or synthetic ones can adopt several structures, including the micellar phase, the familiar bilayer phase, the hexagonal-H_{II} phase and lipidic particles [12]. The ability of lipids to adopt these different liquid-crystalline structures is known as 'lipid polymorphism'. These non-bilayer structures can greatly affect the functional behaviour of the membrane [13]. They might be intermediates in vesicle fusion, they appear to be involved in lipid flip-flop, and they might act as carriers for polar compounds. Since lipid polymorphism has such a potential biological importance, it is interesting to check whether retinoids, which are very important membrane components, are able to modulate the lipid polymorphism of phosphatidylethanolamine.

In this paper we present a detailed study on the interaction of all-*trans*-retinol and all-*trans*-retinoic acid with DEPE bilayers by means of differential scanning calorimetry and ^{31}P-NMR. Calorimetric data are analyzed by constructing partial phase diagrams. We find that both retinol and retinoic acid promote hexagonal-H_{II} phase formation in DEPE systems. The modulation of DEPE lipid polymorphic behaviour by these compounds is discussed.

Materials and Methods

DEPE was from Avanti Polar Lipids, Birmingham, AL. All-*trans*-retinol was obtained from Fluka and all-*trans*-retinoic acid was from Eastman-Kodak. All the other reagents were of the highest purity available. Water was twice-distilled in an all-glass apparatus and deionized in a Milli-Q system from Millipore. Retinoids were always handled in the dark and under inert atmosphere to prevent light-induced decomposition and peroxidation. Occasionally samples were analyzed by thin-layer chromatography to monitor stability and it was found that there was no appreciable alteration as a consequence of the experiment.

The lipid mixtures for calorimetry measurements were prepared by combination of chloroform solutions containing 5 μmol of the phospholipid and the appropriate amount of retinoid when indicated. The organic solvent was evaporated under a stream of dry N_2, free of O_2, at a temperature above the gel to liquid-crystalline transition temperature of the phospholipid and the last traces of solvent were removed by a further 1–2 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 liposomes were formed by mixing, using a bench-vibrator, always keeping the samples at a temperature above the highest gel to liquid-crystalline phase transition temperature of the mixture. Mixing was continued until a homogeneous and uniform suspension was obtained. The suspensions were centrifuged at 10000 rpm in a bench microfuge and the pellets were collected and placed into small aluminium pans. Pans were sealed and scanned in a Perkin-Elmer DSC-4 calorimeter, using a reference pan containing buffer. The heating and cooling rates were 4 C°/min in all the experiments. The DSC instrument was set at a sensitivity of 1 mcal/s. Peak areas were measured by weighing paper cut-outs of the peaks. For the determination of the total phospholipid contained in a pan, this was carefully opened, the lipid was dissolved with chloroform/methanol (1:1, v/v) and the phosphorus contents were determined using the method of Böttcher [14]. The instrument was calibrated using indium as standard.

Phase diagrams were constructed as previously described [15]. The main transition region was defined by the onset temperatures on heating and cooling experiments.

The samples for ^{31}P-NMR were prepared by combination of chloroform solutions containing 50 mg of DEPE and the appropriate amount of retinoid, evaporation of the solvent and formation of multilamellar vesicles by shaking in the same buffer described above for the calorimetry samples. The samples were left for 30 min at 50°C with occasional vortexing to completely hydrate the phospholipid. The suspensions were centrifuged at 10000 rpm in a bench microfuge and pellets were placed into conventional 5 mm NMR tubes and ^{31}P-NMR spectra were obtained in the Fourier Transform mode in a Varian Unity 300 spectromate. Temperature was controlled to ± 1 C° with a Standard Varian variable temperature control unit. All chemical shifts values are quoted in parts per million (ppm) with reference to pure lysophosphatidylcholine micelles (0 ppm), positive values referring to low-field shifts. All spectra were obtained in the presence of a gated-broad band decoupling (4.5 W input power during acquisition time) and accumulated free inductives decays were obtained from up to 600 scans. A spectral width of 25000 Hz, a memory of 8K data points, a 1.3 s interpulse time and a 80° radio frequency pulse were used. Prior to Fourier transformation, an exponential multiplication was applied resulting in a 100 Hz line broadening. The relative amount of each phase component was determined by directly measuring the part of the total integral corresponding to the area of each signal.

In order to check the incorporation of retinoids into the membranes, vesicles were pelleted down and the

amount of retinoid present in the supernatant was measured. Retinoids were quantitatively extracted using n-pentane and their concentrations were determined by employing $\epsilon_{325} = 52480\ M^{-1}cm^{-1}$ for retinol and $\epsilon_{350} = 45400\ M^{-1}cm^{-1}$ for retinoic acid. It was found that more than 99.5% of the added retinoid was incorporated into the membrane for all the samples.

Results

The effect of two retinoids of great biological importance, namely retinoic acid and retinol (Fig. 1), on the phase behaviour of DEPE was investigated by means of DSC and ^{31}P-NMR. We first studied the effect of these retinoids on the thermotropic phase transitions of DEPE. Fig. 2 shows the calorimetric profiles obtained for pure DEPE and mixtures of DEPE with retinoic acid. Aqueous dispersions of DEPE can undergo a gel to liquid-crystalline phase transition in the lamellar phase and, in addition, a lamellar liquid-crystalline to hexagonal-H_{II} transition [16]. The gel to liquid-crystalline phase transition occurs around 37 ± 0.5°C and the bilayer to hexagonal transition occurs around 65 ± 1°C, in agreement with previous data [16]; the latter transition has a much smaller transition enthalpy due to the fluid character of both the lamellar and the hexagonal-H_{II} phases [17]. Increasing concentrations of retinoic acid in DEPE produce a shift of the main gel to liquid-crystalline phase transition temperature, T_c, to lower values, reaching a value of 34°C at a retinoic acid mol fraction of 0.09. When the concentration of retinoic acid is increased above this value (up to a retinoic acid mol fraction of 0.5), the T_c is not further affected, remaining unchanged and close to 34°C. Highly cooperative thermograms are obtained even for the highest retinoic acid concentration (mol fraction 0.5) and no phase separation is observed. The effect of retinoic acid on the bilayer to hexagonal-H_{II} phase transition temperature, T_h, is similar. As the concentration of retinoic acid is increased a shift of T_h to lower values is observed, reaching a temperature of 49.2°C at a mol fraction of 0.09. Similarly to the effect on T_c, increasing the concentration of retinoic acid up to a mol fraction of 0.5 does not further change T_h.

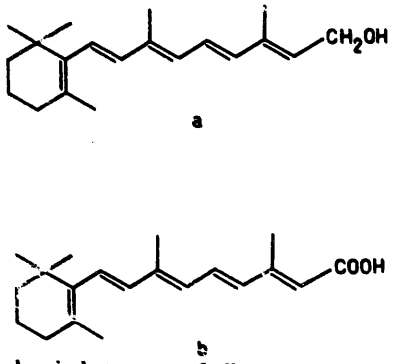

Fig. 1. The chemical structure of all-*trans*-retinol (a) and all-*trans*-retinoic acid (b).

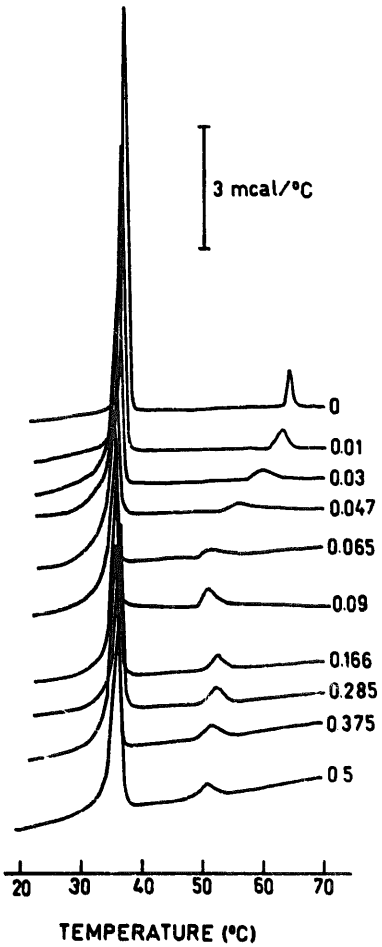

Fig. 2. DSC thermograms for mixtures DEPE/retinoic acid. The concentration of retinoic acid in the membrane (mol fraction) is expressed on the curves. The profiles correspond to heating scans.

The effect of the incorporation of retinoic acid on the enthalpy change of the thermotropic phase transitions of DEPE is shown in Fig. 4b. For pure DEPE the enthalpies for both transitions were estimated to be 8.3 ± 0.6 kcal/mol for the gel to liquid crystalline phase transition and 0.7 ± 0.1 kcal/mol for the bilayer to hexagonal-H_{II} phase transition, in agreement with previous data [16,18]. Increasing the retinoic acid concentration up to a mol fraction of 0.09 produces a progressive decrease of ΔH_c down to a value of 6.7 kcal/mol, which remains unchanged upon a further increase in retinoic acid concentration. There is no significant effect of retinoic acid on ΔH_h of DEPE (Fig. 4b).

Fig. 3 shows DSC thermograms obtained for pure DEPE and mixtures with retinol. The presence of retinol in DEPE gives rise to a progressive shift of T_c to lower temperatures reaching a value of 18.8°C at a retinol mol fraction of 0.5, which is accompanied by a

concomitant broadening of the peaks. Again no phase separation is observed. As shown in Fig. 4a, ΔH_c is progressively decreased as the concentration of retinol is increased, reaching a value of 6.6 kcal/mol for a retinol mol fraction of 0.5.

The effect of retinol incorporation on the bilayer to hexagonal-H_{II} transition is more pronounced. Incorporation of increasing amounts of retinol results in a decrease of T_h, concomitant with a broadening of the peaks (Fig. 3). The effect of retinol on ΔH_h (Fig. 4a) is such that at a retinol mol fraction of 0.285 the bilayer to hexagonal-H_{II} phase transition is not observed anymore. It is interpreted that upon incorporation of retinol, part of the DEPE molecules give rise to a broad bilayer to hexagonal-H_{II} phase transition which is shifted to lower temperatures and which, due to its width and low energy content, cannot be detected in the thermograms, the remainder of the DEPE molecules showing a less perturbed transition. Upon increasing the retinol content the fraction of these molecules decreases and so decreases the enthalpy of the detectable transition, such that eventually only a very broad bilayer to hexagonal-H_{II} phase transition is present which already starts below the gel to liquid-crystalline phase transition, as it is shown below by ^{31}P-NMR.

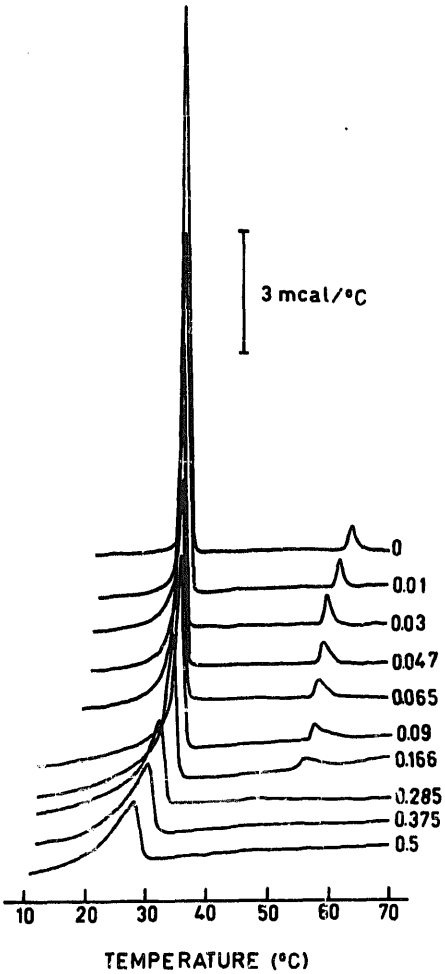

Fig. 3. DSC thermograms for mixtures of DEPE/retinol. The concentration of retinol in the membrane (mol fraction) is expressed on the curves. The profiles correspond to heating scans.

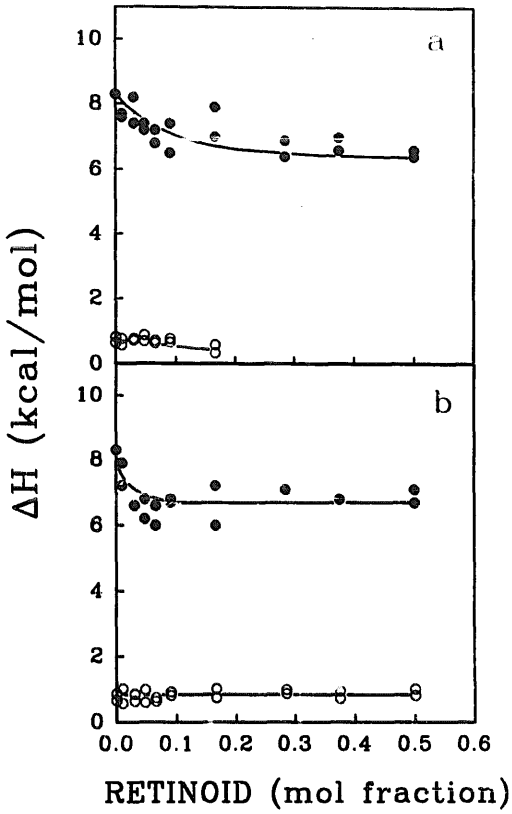

Fig. 4. The dependence of the enthalpy change of the gel to liquid-crystalline phase transition (●) and the bilayer to hexagonal-H_{II} phase transition (○) on the concentration of retinol (panel a) or retinoic acid (panel b). Experimental points corresponding to two independent experiments are shown.

The effect of retinoic acid and retinol on the thermotropic phase transitions of DEPE was further investigated by means of ^{31}P-NMR. DEPE when organized in bilayer structures gives rise to an asymmetrical ^{31}P-NMR line-shape, with a high-field peak and a low-field shoulder [19] (measured as 3-times the chemical shift difference between the high-field peak and the position of isotropically-moving lipid molecules) of approx. 40 ppm, in agreement with previous data [19,20], characteristic of an axially symmetrical shift tensor (Fig. 5A). As shown in Fig. 5A in the gel state (at 20°C), the line-shape is broadened, possibly due to increased (^{1}H–^{31}P) dipolar interactions [21]. In the hexagonal-H_{II} phase (Fig. 5A, 70°C), due to rapid lateral diffusion of the phospholipid around the tubes of which this phase is composed, the chemical shift anisotropy is further averaged, resulting in a line-shape with a reversed asymmetry, i.e., a high-field shoulder and a low-field peak, with a two-fold reduction in absolute value of $\Delta\sigma$ [12,22]. Incorporation of retinoic acid into DEPE results in the appearance of the characteristic spectrum

corresponding to the hexagonal-H_{II} phase at temperatures lower than those obtained for the pure phospholipid (Figs. 5B and C). It is clearly shown that at 60°C for a retinoic acid mol fraction of 0.047 (Fig. 5B) and at 50°C for a mol fraction of 0.285 (Fig. 5C), a hexagonal-H_{II} phase is present, whereas the pure phospholipid is still organized in extended bilayer structures (Fig. 5A).

Low concentrations of retinol (mol fraction 0.047, Fig. 5D) give rise to the appearance of a spectral component with resonance position at 0 ppm, at temperatures of 30°C and above, indicating that the spectrum originated from DEPE is partially replaced by a spectrum characteristic of phospholipid molecules undergoing a rapid motion that leads to a nearly complete averaging of the chemical shift anisotropy. Incorporation of retinol at a mol fraction of 0.285 (Fig. 5E) results in the appearance of the isotropic signal already at 20°C. Interestingly, the spectrum corresponding to the hexagonal-H_{II} phase is also present at 20°C, i.e., at this temperature the spectrum has three components: lamellar gel, hexagonal-H_{II} and isotropic. Increasing the temperature produces a progressive enhancement of the hexagonal component at the expense of the lamellar component and then from the isotropic one. The presence of the characteristic line-shape of a hexagonal-H_{II} phase in the spectra at temperatures of 20°C and 30°C supports the suggestion made above that in the presence of retinol, formation of the hexagonal-H_{II} phase already starts at temperatures even below that of the gel to liquid crystalline phase transition of DEPE. The ^{31}P-NMR spectra shown in this paper were highly reproducible between different samples, which makes improbable that the isotropic signal which appears in the presence of retinol (Figs. 5D and 5E) is a function of the method of sample preparation. In addition it can be observed in Fig. 5D that the isotropic signal, which is not present at 20°C, appears at 30°C and is present at all temperatures above that. An stimate of the relative amount of different phases was made from the spectra shown in Fig. 5. Particularly

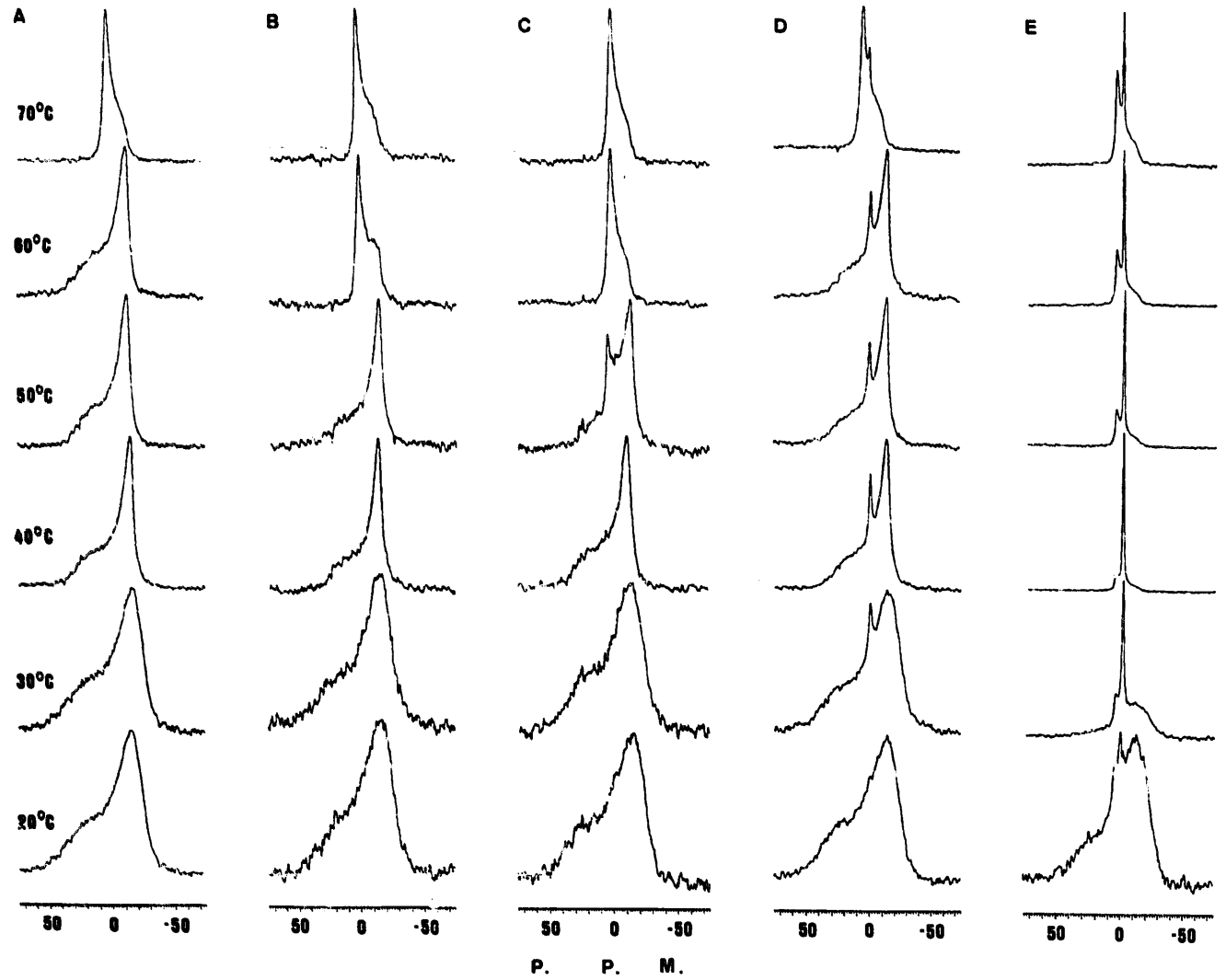

Fig. 5. ^{31}P-NMR spectra corresponding to pure DEPE (A); DEPE/retinoic acid, 0.047 mol fraction (B); DEPE/retinoic acid, 0.285 mol fraction (C); DEPE/retinol, 0.047 mol fraction (D) and DEPE/retinol, 0.285 mol fraction (E).

in the spectra of Figs. 5D and E, the contribution of isotropic signal to the spectra was 3–6% and 10–40%, respectively.

Using our DSC data from heating and cooling experiments partial phase diagrams have been constructed. The phase diagrams extend up to a retinoid mol fraction of 0.5. The information about the structural organization of the phospholipid obtained from ^{31}P-NMR experiments has been incorporated into the phase diagrams as well, in order to get more insight into the polymorphic behaviour of DEPE/retinoids systems.

Fig. 6 shows the partial phase diagram for mixtures of DEPE and retinoic acid. For a retinoic acid concentration range from 0 to 0.1 mol fraction, the system displays a near-ideal behaviour, the temperature for both the gel to liquid-crystalline and the bilayer to hexagonal-H_{II} phase transitions decreasing as the concentration of retinoic acid increases. For a given retinoic acid mol fraction within this range, increasing the temperature gives rise to the consecutive transition from L_β to L_α and then from L_α to H_{II} phase, the regions of co-existence of $L_\alpha + L_\beta$ and $L_\beta + H_{II}$ phases being very narrow which indicates the high degree of cooperativity of these transitions. At retinoic acid concentrations above a mol fraction of 0.1 there is phase immiscibility in both the L_α/L_β and L_α/H_{II} phase boundaries, since the phase lines remain horizontal.

The corresponding phase diagram for the system DEPE/retinol is shown in Fig. 7. From DSC data a near-ideal behaviour for the gel to liquid-crystalline and the bilayer to hexagonal-H_{II} phase transitions is

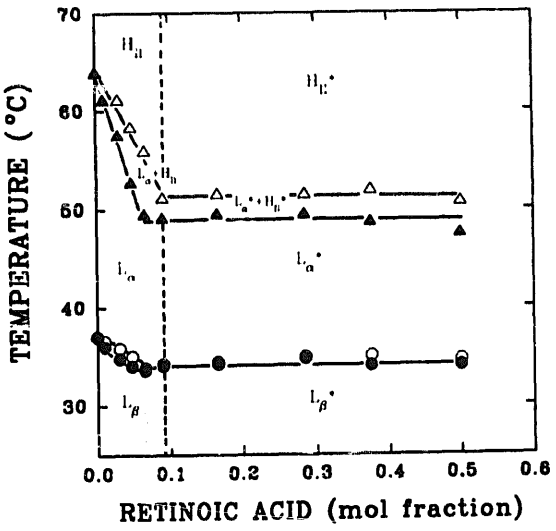

Fig. 6. Partial phase diagram for the system DEPE/retinoic acid. Black symbols are obtained from DSC heating scans and open symbols from DSC cooling scans. Circles correspond to the gel to liquid-crystalline phase transition and triangles to the bilayer to hexagonal-H_{II} phase transition. See text for the explanation of the diagram.

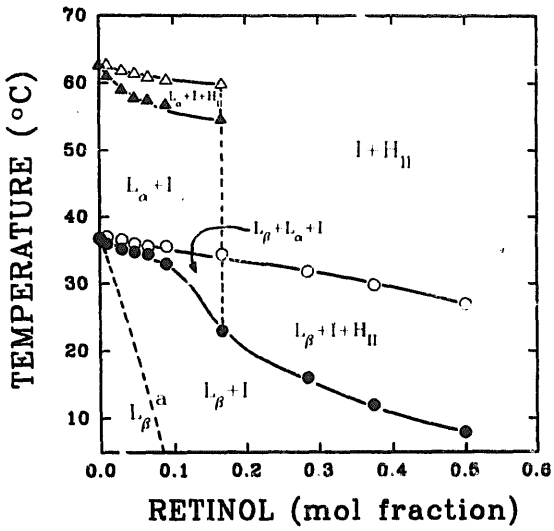

Fig. 7. Partial phase diagram for the system DEPE/retinol. Black symbols are obtained from DSC heating scans and open symbols from DSC cooling scans. Circles correspond to the gel to liquid-crystalline phase transition and triangles to the bilayer to hexagonal-H_{II} phase transition. See text for the explanation of the diagram.

observed. At retinol concentrations higher than a mol fraction of 0.166 the bilayer to hexagonal-H_{II} phase transition cannot be further observed. Inclusion of the ^{31}P-NMR data make the phase diagram considerably more complex. For a retinol mol fraction of 0.047, when the temperature is raised from 20°C to 30°C an isotropic signal in the spectrum appears (Fig. 5D), indicating that a $L_\beta/L_\beta + I$ boundary should exist, and this is hypothetically depicted by line 'a' in Fig. 7. For a retinol concentration range between 0 and 0.166 mol fraction when the temperature is raised the phospholipid consecutively undergoes a gel to liquid-crystalline and a bilayer to hexagonal-H_{II} phase transition, all these phases co-existing with a fraction of the phospholipid organized in a structure responsible for the isotropic signal observed in the ^{31}P-NMR spectra (Figs. 5D and E) and here called 'I'. For a retinol mol fraction higher than 0.166, as the temperature is raised the system evolves from $L_\beta + I$ to $H_{II} + I$ through a region of co-existence of $L_\beta + H_{II} + I$, apparently without an intervening L_α phase.

Discussion

In this study, the influence of two retinoids of major biological importance like retinoic acid and retinol on the lipid polymorphic behaviour of DEPE has been examined using physical techniques such as DSC and ^{31}P-NMR.

The molecule of a retinoid consists of three main sections, a lipophilic part at one end, connected via a polyunsaturated chain as a spacer to a hydrophilic group at the other end of the molecule (Fig. 1). This

amphiphylic nature makes biological membranes one of the most likely sites of retinoid action.

Temperature is an important experimental parameter which determines the macroscopic structure of hydrated membrane lipids. Temperature dependent bilayer to hexagonal-H_{II} phase transitions have been observed in a large variety of both synthetic and natural PE's [13]. We have used in this study an unsaturated PE such as DEPE, whose bilayer to hexagonal-H_{II} transition has been thoroughly characterized [23,24].

The effect of retinoic acid on T_c and ΔH_c may be due to the perturbation of the intermolecular hydrogen bonds present in phospholipid vesicles made of PE [25]. Also, given the structure of retinoic acid (Fig. 1), it should be expected to disrupt the packing of the hydrophobic chains of DEPE in the gel phase, therefore giving place to a decrease in the T_c of the gel to liquid-crystalline phase transition. A similar behaviour is found in relation to the bilayer to hexagonal-H_{II} phase transition. These effects are restricted to retinoic acid concentrations up to a mol fraction of approx. 0.1. Higher concentrations of retinoic acid do not further affect the phase transitions parameters, indicating that only a limited amount of retinoic acid is allowed to interact with DEPE. When the concentration of retinoic acid exceeds a mol fraction of 0.1, this compound will possibly form phase separated retinoic acid-enriched domains within the membrane.

The effect of retinoic acid on T_h indicates that this molecule is able to promote hexagonal-H_{II} phase formation in DEPE membranes, as it is also confirmed by ^{31}P-NMR. As shown in Figs. 5B and C, the presence of increasing concentrations of retinoic acid results in the formation of hexagonal-H_{II} structures at temperatures below those observed for pure DEPE.

The partial phase diagram for the system DEPE/retinoic acid (Fig. 6) clearly presents two parts: below and above a retinoic acid mol fraction of approx. 0.1. Below this value the system behaves near-ideally for both the L_β/L_α and L_α/H_{II} phase transitions, the regions of co-existence of $L_\beta + L_\alpha$ and $L_\alpha + H_{II}$ phases being very narrow, which indicates that retinoic acid does not affect very much the cooperativity of these phase transitions. Above a mol fraction of 0.1, the system presents gel and fluid-phase immiscibility in the bilayer structure as well as bilayer/hexagonal-H_{II} immiscibility. The regions marked * in Fig. 6 will correspond to phases composed of DEPE/retinoic acid with stoichometry 10:1 (retinoic acid mol fraction 0.1) in equilibrium with retinoic acid molecules forming enriched domains.

At difference with retinoic acid, the perturbation of the transitions parameters induced by retinol increases with retinol concentration along the whole range under study. Furthermore, retinol is able to considerably broaden the transition peaks specially at concentrations above 0.1 mol fraction, which indicates a strong perturbation of the cooperativity of the transition. It should be emphasized that although the peak of the lamellar to hexagonal-H_{II} phase transition is not visualized in DSC experiments at concentrations of retinol higher than a molar fraction of 0.166, this transition is not abolished in these cases. As shown by ^{31}P-NMR the onset of the lamellar to hexagonal-H_{II} phase transition is shifted to low temperatures at these concentrations of retinol. This effect may be due to the formation of domains enriched in retinol where the hexagonal-H_{II} phase is stabilized at temperatures lower than that at which the transition takes place in the pure phospholipid. Retinol has been previously found to lower the bilayer to hexagonal-H_{II} transition temperature of DEPE [5]. The larger perturbation of DEPE bilayers produced by retinol vs. retinoic acid is consistent with the greater solubility of retinol that allows a greater perturbation.

Characterization of DEPE/retinol systems by ^{31}P-NMR shows (Figs. 5D and E) that retinol is a better promoter of hexagonal-H_{II} phase formation than retinoic acid. Indeed, hexagonal-H_{II} phases are already observed at temperatures even below the main gel to liquid-crystalline phase transition temperature of pure DEPE. An interesting finding is the presence of an isotropic signal in the spectra. This signal already appears at low retinol concentrations and at temperatures below the gel to liquid-crystalline phase transition. It has been previously described that *N*-methyldioleoylphosphatidylethanolamine can form structures giving rise to isotropic ^{31}P-NMR signals at temperatures well below those at which a hexagonal-H_{II} phase is first observed [26,27] and something similar has been also reported for mixtures of unsaturated PE's and PC's [23,28–30].

As discussed by Ellens et al. [31], the appearance of isotropic components in the ^{31}P-NMR spectra of multilamellar lipid preparations can also be due to the presence of very small liposomes, although this is unlikely to be the case here since they will be fusing and would quickly give place to larger non-isotropic structures and also because the preparation of the samples studied in this paper included a sedimentation step by centrifugation at low speed, which will fail in pelleting liposomes of very small diameter. On the other hand, the kinetic model of the L_α to hexagonal-H_{II} phase transition mechanism suggested by Siegel and coworkers [31–34] includes the formation of inverted micellar intermediates and interlamellar attachment structures which will produce isotropic spectral resonances in ^{31}P-NMR. The appearance of isotropic resonance signals in DEPE has been previously reported for mixtures of this phospholipid with tocopherols [35]. At high retinol concentrations (Fig. 5E) it is clearly observed that upon increasing the temperature the

hexagonal-H_{II} phase is formed at the expense of the isotropic signal. The ^{31}P-NMR isotropic signal may also arise from cubic arrays [23] but in our case it can be speculated that it could be due to disordered bilayer intermediates which upon increasing the temperature will adopt the hexagonal-H_{II} organization.

The effect of retinol on DEPE lipid polymorphic behaviour is considerably more complex than that of retinoic acid, as observed in the corresponding phase diagram (Fig. 7). The phase lines obtained from DSC experiments display a near-ideal behaviour, progressively decreasing with increasing concentration of retinol. However, upon incorporation of the ^{31}P-NMR results into the phase diagram this gets more complicated. From the observation of spectra at 20°C and 30°C in Fig. 5D it can be deduced that a boundary separating regions of L_β from regions of L_β + I phases should exist. We have tentatively depicted this boundary by line 'a' in the diagram. Below line 'a' DEPE will adopt a gel lamellar phase, L_β. Upon increasing either temperature or retinol concentration part of the DEPE molecules interacting with retinol will adopt a structure giving rise to the isotropic signal in ^{31}P-NMR spectra, so that a region of co-existence of L_β + I exists. At a retinol mol fraction of approx. 0.17 the bilayer to hexagonal-H_{II} phase transition is not observed anymore in the DSC thermograms and the peaks corresponding to the gel to liquid-crystalline phase transition are considerably broadened (Fig. 3). ^{31}P-NMR data (Fig. 5) indicate that the bilayer to hexagonal-H_{II} transition is already taking place at temperatures even below the gel to liquid cristalline phase transition of pure DEPE. In order for the system to fulfill the phase rule it is a requisite that the lipid molecules responsible for the isotropic signal do not represent a different separated physical phase from L or H_{II} phases. In this respect it has been suggested for other PE-containing systems [36] that the observed ^{31}P-NMR isotropic signals do not necessarily represent highly ordered cubic phases, but may be disordered bilayer intermediates. It can be speculated that these intermediates are in a dynamic equilibrium with either L or H_{II} phases, which will displace in one or another direction depending on the parameters of the system (temperature and composition), however the rate of exchange of phospholipid molecules between isotropic and lamellar and/or hexagonal phases must be slow or intermediate on the NMR time-scale.

At retinol concentrations with a mol fraction above approx. 0.17 the system seems to directly evolve from an ordered lamellar phase, L_β, to a fluid hexagonal-H_{II} phase, H_{II}, without an intervening fluid lamellar phase, L_α. This observation has been also reported for other systems like dipalmitoylphosphatidylcholine/palmitic acid mixtures at low pH and in dihexadecylphosphatidylethanolamine and distearoylphosphatidylethanolamine in saturated NaCl [37] as well as in dihexadecylphosphatidylethanolamine alone [38].

The capacity of hydrated liquid-crystalline lipid to adopt different structures according to acyl chain or headgroup composition has been thoroughly studied. These studies have given support to the hypothesis that a generalized 'shape' property of lipids determined the phase structure adopted [39] and a shape or packing parameter, S, has been characterized [40] being $S = v/(a_o l_o)$, where v is the hydrophobic volume per molecule, a_o is the 'optimum' area per headgroup at the lipid/water interface and l_o is the length of the hydrocarbon chains. PE's have a smaller, less hydrated headgroup than PC's and this, together with the possibility of forming intermolecular hydrogen bonding, would reduce the area per molecule in the headgroup region, this leading to a cone-shaped molecule, where $S > 1$, having shape properties compatible with inverted structures such as the hexagonal-H_{II} phase. The inclusion of lipidic molecules such as retinoids, having a bulky apolar cyclohexane group and a smaller polar alcohol or carboxyl group and thus constituting a cone-shape, in the PE system will perturb the lipid matrix increasing the acyl chain motion which may be considered to increase the hydrophobic volume, v, and thus giving an average greater value for the shape parameter, S, and in this way facilitating the formation of hexagonal-H_{II} structures by PE.

Although retinoic acid has been described to act by modulating the genetic expression of the cell [41], the incorporation of retinoids into membranes can be of biological importance. It has been described for example, that retinol [5] and retinoic acid [4] may activate protein kinase C and it has been suggested that there may be a relationship between the effect of lipophylic molecules on bilayer stability and on protein kinase C activity [5]. It can be then speculated that the ability of retinoids to promote hexagonal-H_{II} phase described in this paper may be of relevance for the mechanism of action by which retinoids exert this biological activity.

On the other hand retinoids have been claimed to be active in the treatment of a number of cancers [42], but the toxicity of high doses of retinoids is a problem for their use. Certainly, membrane disruption may be a reason for their toxicity and this may be caused by their ability to stabilize non-lamellar structures, as shown here. This ability may be also behind the enhancement in membrane permeability to non-electrolytes and ions which is observed on incorporation of retinoids into membranes [8,43,44].

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