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Modulation of polymorphic properties of dielaidoylphosphatidylethanolamine by the antineoplastic ether lipid 1-*O*-octadecyl-2-*O*-methyl-glycero-3-phosphocholine

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Abstract

The capacity of the antineoplastic ether lipid 1-*O*-octadecyl-2-*O*-methyl-glycero-3-phosphocholine (ET-18-OCH_3) to modulate the polymorphic properties of dielaidoylphosphatidylethanolamine has been studied using biophysical techniques. Differential scanning calorimetry showed that ET-18-OCH_3 depresses the onset of the L_β to L_α phase transition, decreasing also ΔH of the transition. At the same time, the onset of the transition from L_α to inverted hexagonal H_{II} phase was gradually increased as the ether lipid concentration was increased, totally disappearing at concentrations higher than 5 mol%. Small-angle X-ray diffraction and ^{31}P-NMR confirmed that ET-18-OCH_3 induced that the appearance of the inverted hexagonal H_{II} phase was shifted towards higher temperatures completely disappearing at concentrations higher than 5 mol%. These results were used to elaborate a partial phase diagram and they were discussed as a function of the molecular action of ET-18-OCH_3.

Keywords: Lipid polymorphism; ET-18-OCH_3; DSC; X-Ray diffraction; ^{31}P-NMR

1. Introduction

1-*O*-Octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (ET-18-OCH_3) is the most widely studied member of a family of di-ether lipids, possessing a very well known antineoplastic activity [1,2]. Recent work has established that the cytotoxic effect of ET-18-OCH_3 is due to the ability of this phospholipid to induce apoptosis in sensitive cells [3–6]. Among the many processes which have been claimed to be the targets where ET-18-OCH_3 will cause physiological imbalances it is important to mention the inhibition of phosphatidylinositol phospholipase C [7–9], protein kinase C-regulated functions [10–14], lysophospholipid metabolism [15,16], and phosphatidylcholine synthesis [17–22]. Due to its hydrophobic nature, this molecule is expected to accumu-

Abbreviations: DEPE, 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine; DSC, differential scanning calorimetry; EDTA, ethylenediaminetetraacetic acid; ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-glycero-3-phosphocholine; FT-IR, Fourier transform infrared spectroscopy; ^{31}P-NMR, phosphorus nuclear magnetic resonance; $\Delta\sigma$, chemical shift anisotropy

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late at the membrane level, as has been described [23], and it has also been shown to be a membrane active anti-HIV agent [24]. Furthermore, all of the physiological functions mentioned above are related to cell membranes.

Therefore, although the molecular mechanism through which this ether lipid may develop these biological activities is poorly understood, its membrane localisation suggests that its action should be exerted at this level by changing membrane properties or acting as a membrane-enzyme ligand. Apart from acting on membrane-bound enzymes [7], and interfering with phospholipid synthesis [15], it has been described that it may modulate the activity of an enzyme which is activated by its binding to the membrane as protein kinase C [10,25]. Detailed knowledge of the organisation of these ether lipids in membranes and their capacity to influence the polymorphic phase behaviour of phospholipids may then be relevant to their biological activities, and necessary to understand its mechanism of action. With this purpose several studies have been carried out in the past. It was shown using DSC that ET-18-OCH_3 affects the gel to liquid-crystalline phase transition of a number of phospholipids and increased the membrane fluidity of HL60 leukaemic cells [26]. In another study, DSC experiments revealed that ET-18-OCH_3 affected only slightly the gel to liquid-crystalline phase transition of model membranes and X-ray diffraction was used to show that this ether lipid inserted itself in the bilayer with its long axis parallel to the membrane bilayer chains [27].

1,2-Dielaidoyl-*sn*-glycero-3-phosphoethanolamine (DEPE) is a phospholipid which has been widely used to test the capacity of membrane molecules to affect lipid polymorphism. In particular, aqueous dispersions of DEPE undergo a L_β to L_α phase transition at 36°C and a L_α to inverted hexagonal H_{II} transition at about 63°C [28], and therefore it serves to test the capacity of membrane intrinsic molecules to insert themselves in the bilayer affecting the 36°C transition (T_c), and the 63°C one (T_H). It was known since long ago, that a molecule which inserts itself in the membrane may affect the biological properties of a membrane bilayer and also the tendency to form non-bilayer membranes. In this way, it has been established that the effect of additives on T_H are at least tenfold greater than their effect on T_c [29].

In this paper we have used DSC, small angle X-ray diffraction and ^{31}P-NMR to study the capacity of ET-18-OCH_3 to modulate the polymorphic properties of DEPE, showing that it affected the gel to liquid phase transition and caused the suppression of the lamellar to inverted hexagonal H_{II}, at concentrations higher than 5 mol%.

2. Materials and methods

2.1. Materials

DEPE and ET-18-OCH_3, were from Avanti Polar Lipids (Alabaster, AL). They were judged pure after giving a single spot in thin layer chromatography, and used without further purification. Water was purified by passing it first through a Milli-RX apparatus and afterwards through a Milli-Q apparatus, both from Millipore.

2.2. Differential scanning calorimetry

Samples of 2 mg of DEPE and the appropriate amounts of ET-18-OCH_3 were mixed in chloroform and dried under a stream of O_2-free N_2, and the last traces of solvent were removed by high vacuum for more than 3 h. The samples were kept and dispersed for 30 min at 50°C, a temperature above the gel to liquid-crystalline phase transition of the mixture, in 1 ml of 0.1 mM EDTA, 10 mM MOPS, 100 mM NaCl buffer (pH 7.4), with occasional mixing in a vortex mixer obtaining a homogeneous and uniform suspension. The samples were then scanned in a Perkin Elmer DSC-4 differential scanning calorimeter at a heating rate of 4°C/min, with a sensitivity of 1 mcal/s. The instrument was calibrated with indium as standard, and a pan containing the same buffer was used as reference. Data were obtained from the thermograms as previously described [30]. Peak areas were measured by weighing paper cutouts of the peaks. After the thermal measurements, the phospholipid content of the pans was determined by phosphorus assay of perchloric acid digests [31].

2.3. Small-angle X-ray diffraction

Samples for X-ray diffraction analysis were pre-

pared similarly to those described above for DSC. 10–15 mg of lipid were dried and resuspended in 1 ml of buffer, and spun down in a bench microfuge. The pellet was deposited onto the diffractometer sample holder.

Nickel-filtered Cu K_α ($\lambda = 1.54$ Å) X-ray was obtained from a Philips generator, model PW1830. X-Rays were focussed using a flat gold-plated mirror and recorded using a linear position sensitive detector model 210 (Bio-Logic, France). Unoriented lipid dispersions were measured in aluminium holders using Mylar windows. The sample temperature was kept within ± 0.5°C, using a circulating water bath. The system was allowed to equilibrate for about 5 min at each temperature before measuring. The X-ray exposure times were 10–15 min for each sample. The system was calibrated for the spacings using crystalline cholesterol (33.6 Å spacing).

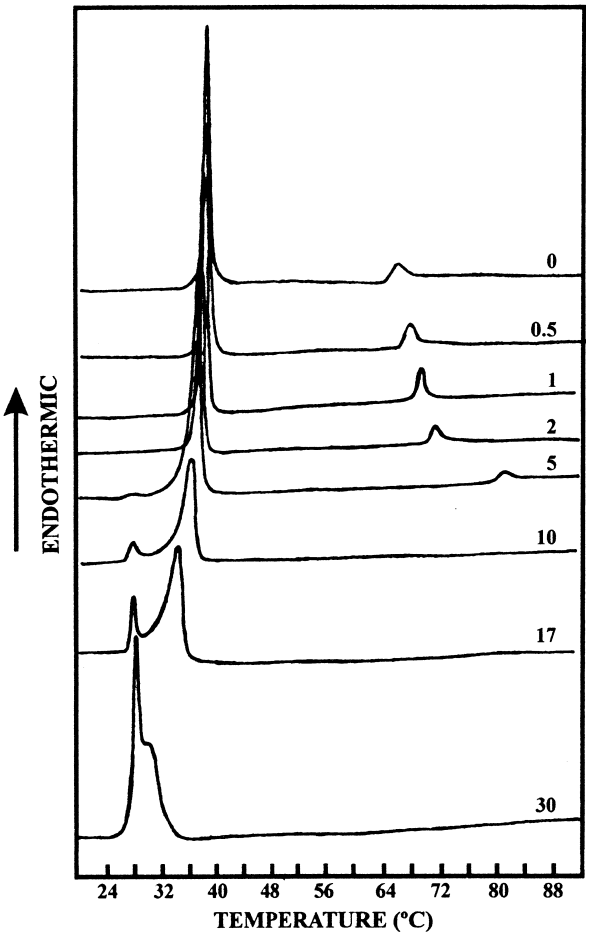

Fig. 1. DSC heating thermograms of aqueous dispersions of ET-18-OCH_3/DEPE mixtures. The mol% of ET-18-OCH_3 in the mixture with respect to total phospholipid is indicated on each thermogram.

2.4. ^{31}P-NMR spectroscopy

The samples for ^{31}P-NMR were prepared by combination of organic solutions containing 20 mg of phospholipid and the appropriate amount of ET-18-OCH_3, evaporation of the solvents and formation of multilamellar vesicles, by adding 1 ml of buffer, and vortexing the samples at temperatures above the phase transition. The suspensions were centrifuged at 10 000 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 spectrometer. All chemical shift 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 proton decoupling (5 W input power during acquisition time) and accumulated free inductive decays were obtained from up to 5000 scans. A spectral width of 25 000 Hz, a memory of 32K data points, a 2 s interpulse time and a 80° radio frequency pulse (11 μs) were used. Prior to Fourier transformation, an exponential multiplication was applied resulting in a 60 Hz line broadening.

3. Results

3.1. Differential scanning calorimetry

Pure DEPE presents a transition from L_β lamellar gel to L_α lamellar liquid crystalline phase at 36°C, and a transition from lamellar liquid crystalline (L_α) to inverted hexagonal H_{II} at 63°C (Fig. 1). When increasing concentrations of ET-18-OCH_3 were added to DEPE, but keeping them at low concentrations such as 0.5–5 mol%, the onset of the transition from L_β to L_α was gradually shifted towards lower temperatures and the transition peak was slightly decreased in size. An even clearer effect was, however, seen on the transition from L_α to H_{II}, so that the onset of the transition was shifted towards a higher temperature, occurring at 68°C at 2 mol% of

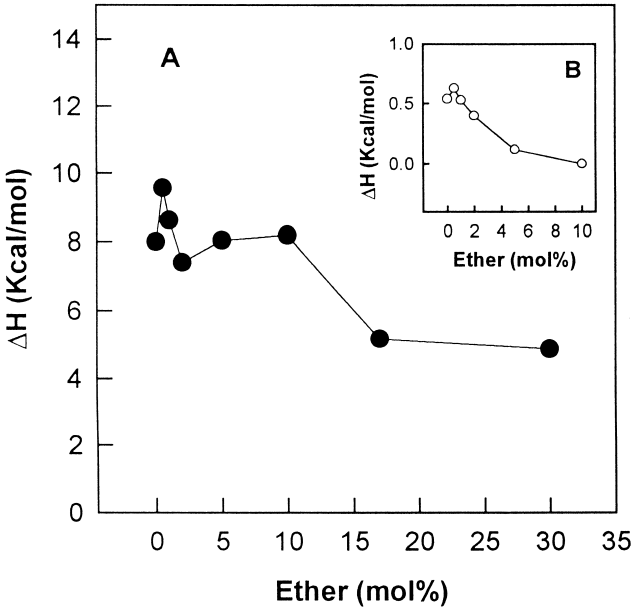

Fig. 2. ΔH of the L_β to L_α phase transition of mixtures with different mol% of ET-18-OCH_3. The same data with respect to the L_α to H_{II} phase transition are plotted in the insert.

ET-18-OCH_3 and at 76°C at 5 mol%. In addition to that, at 5 mol% of ET-18-OCH_3 the L_β to L_α was clearly widened and the onset temperature decreased, with a small peak appearing at 26°C. At 10 mol% of ET-18-OCH_3 the L_β to L_α transition was decreased in temperature and in size, and appeared overlapped to another peak with its onset at 26°C which was already seen in the 5 mol% sample, and the L_α to H_{II} completely disappeared. A pattern very similar to that observed at 10 mol% was observed at 17 mol%, with a further increase of the 26°C transition at the expense of the L_β to L_α transition whose area was decreased. At 30 mol% the same trend was observed, with the peak which has its onset at 26°C increasing in size at the expense of the other one, so that as a consequence of being shifted to a lower temperature both peaks appeared overlapped.

In Fig. 2, ΔH of the transitions are plotted versus ET-18-OCH_3 mol% contents. It can be seen that there was a certain decrease of ΔH of the L_β to L_α transition as the ether concentration increased, going from 8 kcal/mol in pure DEPE to 4.8 in the sample containing 30 mol%. On the other hand, ΔH of the L_α to H_{II} phase transition went down to nil at 10 mol% (see insert of Fig. 2). It should be pointed out that the L_α to H_{II} phase transition which was not detected at all at 10 mol% might be shifted to higher temperatures, beyond the limit of our scan. This shift would follow the trend seen at lower temperatures, where the transition was shifted 8°C when the concentration of the ether was increased from 2 mol% to 5 mol% (see Fig. 1).

3.2. Small angle X-ray diffraction

In order to get information about the structural organisation of the DEPE/ET-18-OCH_3 systems small angle X-ray diffraction was used. The diffractograms obtained were symmetrical as expected for the non-oriented samples used, nevertheless only one side of the diffractograms was considered in Fig. 3. The data are presented in a logarithmic scale for better clarity. Pure DEPE (Fig. 3A) presented a d-spacing of 66.8 Å at 30°C, corresponding to L_β. DEPE presented only the first order reflection and this is in agreement with previous observations [32]. The d-spacing not only informs about the phase organisation of the lipids but also about the interbilayer repeat distance which is indicated by the first order repeat distance. This distance is, however, the addition of the bilayer thickness plus the thickness of the water layer between the lipid bilayers. At 50°C the d-spacing decreased to 51.5 Å, corresponding to the L_α phase. Therefore the L_β to L_α phase transition is accompanied by a 15.3 Å decrease in the first order repeat distance and hence in the interbilayer repeat distance. This is to be expected knowing that the gel to liquid crystalline phase transition gives place to a bigger fluctuation of acyl chains and hence to a decrease in their effective chain length. At 70°C the pattern changed considerably with a first order at 65.2 Å, a second one at 37.4 Å and a third one at 33.0 Å, this series being related with the ratios 1:1/$\sqrt{3}$:1/2, corresponding to an inverted hexagonal H_{II} phase [33]. At 80°C the d-spacings were also typical of a H_{II} phase with 62.3, 35.9, 31.8 Å. In lipidic systems organised in an inverted hexagonal H_{II} system the first order reflection obtained from its small angle X-ray diffractogram is related to the diameter of the lipidic cylinders (denoted by c) by the expression $d = (\sqrt{3}/2) \cdot c$. According to the spacings given above, pure DEPE at 70°C has a c of 75.3, which decreased to 71.9 Å at 80°C.

The presence of 2 mol% of ET-18-OCH_3 (Fig. 3B)

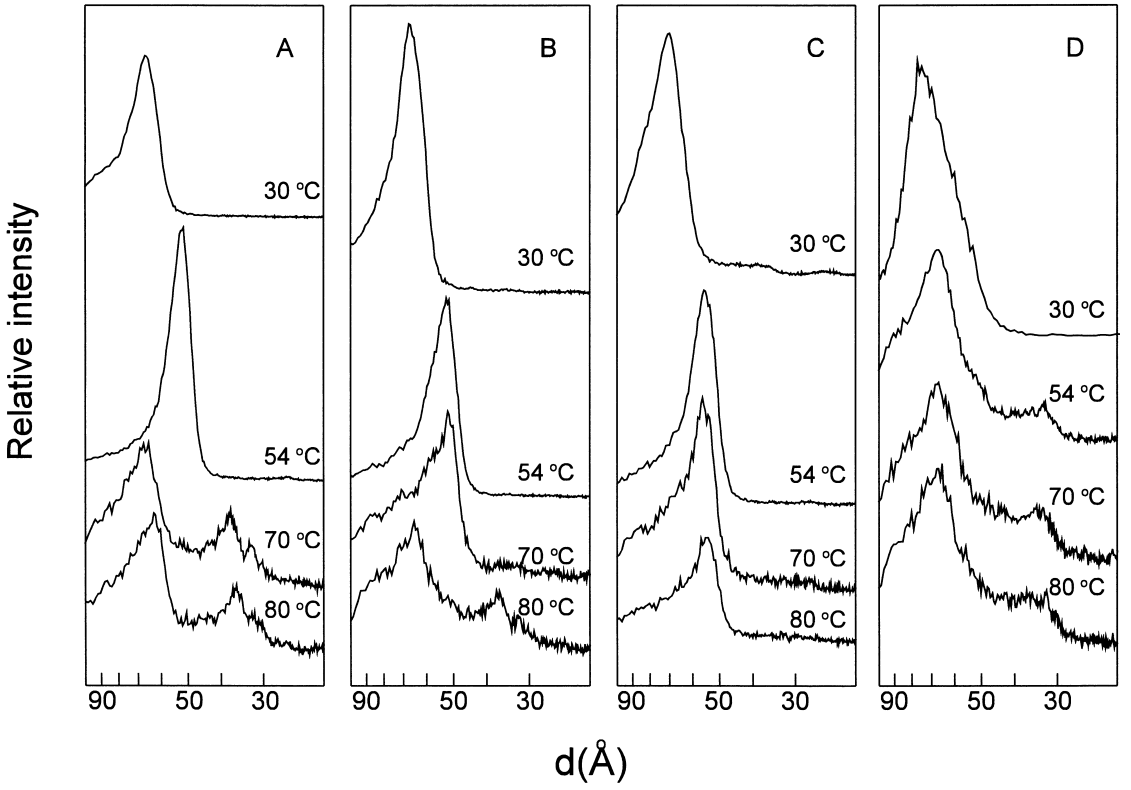

Fig. 3. Small angle X-ray diffractograms obtained from ET-18-OCH_3/DEPE mixtures, at the temperatures shown and at different mol% of ET-18-OCH_3: (A) 0; (B) 2; (C) 5; (D) 17. The data of d-spacings in Å are plotted in a logarithmic scale.

did not appreciably change the pattern of pure DEPE with a d-spacing of 67.6 Å. At 50°C the pattern was also similar to that of pure DEPE at the same temperature with a d-spacing of 52.4 Å. At 70°C this sample presented a predominantly lamellar pattern of 51.9 Å, but given the noise of the diffractogram and the decrease in intensity of the first order reflection, it cannot be excluded that at least a small percentage of the lipids could be organised in an inverted hexagonal H_{II} structure. Such a H_{II} structure seems to be very clearly indicated by the pattern obtained at 80°C with d-spacings at 65.2, 36.7 and 32.6 Å which reveals an increase of *c* (diameter of the cylinders) to 75.2 Å in comparison with pure DEPE which shows 71.9 Å at the same temperature. It can be suggested that this is the result of introducing a positive curvature agent into the membrane that increases the intrinsic radius of curvature.

At 5 mol% of ET-18-OCH_3 (Fig. 3C), an increase in the d-spacing was observed at 30°C, i.e. below the phase transition from L_β to L_α with a first order d-spacing of 70.1 Å, i.e. higher than that of pure DEPE, a second order d-spacing at 35.9 Å and a third one at 24.2 Å. The d-spacing at 50°C was with 55.5 Å also higher than that of pure DEPE at the same temperature. At 70°C and 80°C this sample presented diffractograms with the first spacing at 56.1 and 54.5 Å, respectively, which may correspond to lamellar structures.

Finally, the sample containing 17 mol% of ET-18-OCH_3 presented a peculiar behaviour (Fig. 3D). At 30°C the d-spacing was rather large with 72.8 Å. The same can be said of the 50°C diffractogram with a first order at 66.8 Å and a second one at 3.28 Å. Exactly the same d-spacings were observed at 50°C and at 80°C; the first order was at 65.2 and the second one at 32.2 Å. All these d-spacings can be originated from lamellar structures but for some reasons the spacings are clearly bigger than those of pure DEPE, and they showed second order spacings, all of that indicating that these lamellar structures were different from those of pure DEPE when organised in either L_b or L_α phase.

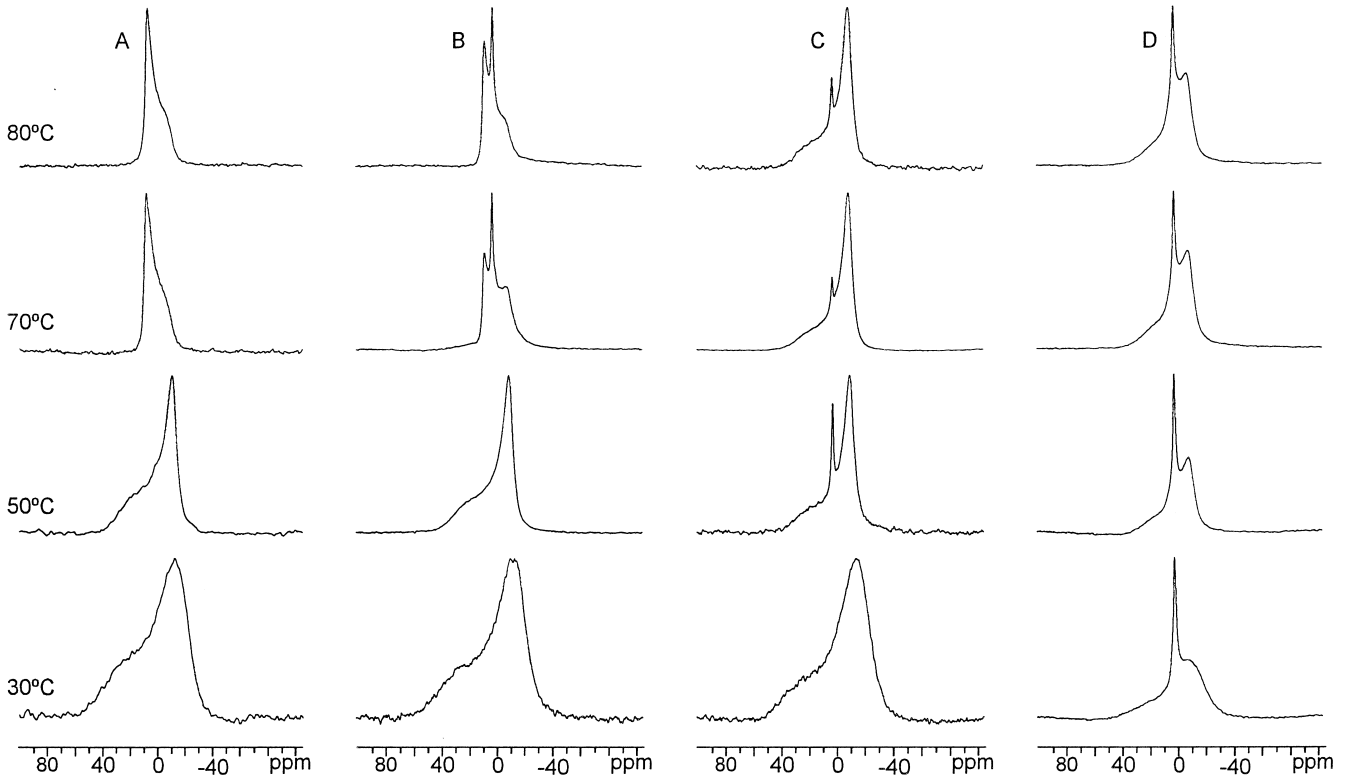

Fig. 4. ^{31}P-NMR spectra of aqueous dispersions of mixtures of ET-18-OCH_3 with DEPE at the temperatures shown. The mol% contents of ET-18-OCH_3 in each sample were as follows: (A) 0; (B) 2; (C) 5; (D) 17.

3.3. *^{31}P-NMR spectroscopy*

The effect of ET-18-OCH_3 on the phase behaviour of DEPE was investigated by means of ^{31}P-NMR. Phospholipids produce a characteristic asymmetrical ^{31}P-NMR line-shape with a high-field peak and a low-field shoulder when they are organised in bilayer structures with a residual chemical shift anisotropy ($\Delta\sigma$) of approx. 64 ppm in the gel state and approx. 42 ppm in the liquid-crystalline state [34,35]. This is the case for pure DEPE (Fig. 4A), showing a $\Delta\sigma$ of 65 ppm at 30°C (gel state), and 45 ppm at 55°C (liquid-crystalline). However, in the hexagonal H_{II} phase, due to rapid lateral diffusion of the phosholipids around the tubes of which this phase is composed, the chemical shift anisotropy is averaged resulting in a shape with reverse asymmetry when compared to the bilayer line-shape, so that now there is a high-field shoulder and a low-field peak with an approx. 2-fold reduction in the absolute value of $\Delta\sigma$ to about 20 ppm [28,36]. This pattern can be seen for

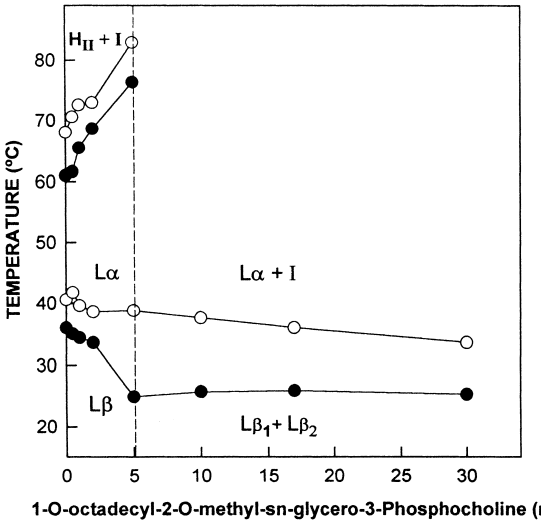

Fig. 5. Phase diagrams for aqueous dispersions of ET-18-OCH_3/DEPE mixtures, based on data derived from DSC, X-ray diffraction and ^{31}P-NMR. The closed and open circles were obtained from T_c of the onset and completion temperatures of the heating scans respectively. L_α and L_β have their usual meaning and I stands for isotropic as judged from ^{31}P-NMR.

pure DEPE (Fig. 4A) at 70°C and at 80°C, with $\Delta\sigma$ of 20 ppm in both cases.

The presence of 2 mol% of ET-18-OCH_3 (Fig. 4B) did not produce a significant perturbation either in the gel state at 30°C or in the fluid liquid-crystalline state at 50°C with spectra showing lamellar patterns. At 70 and 80°C isotropic components appeared superimposed on asymmetric spectra characteristic of inverted hexagonal H_{II} phases. The addition of 5 mol% of ET-18-OCH_3 (Fig. 4C) did not alter the spectrum of pure DEPE at 30°C, i.e. in the lamellar gel state, but a variation appeared in the liquid-crystalline state, at 50°C, because an isotropic component appeared superimposed to a lamellar pattern. At 70°C and 80°C the spectra were similar to that of 50°C, although the isotropic component had different intensities. At 17 mol% of ET-18-OCH_3 (Fig. 4D) the spectra were very similar at all the temperatures studied, 30, 50, 70 and 80°C, with an isotropic peak superimposed on a lamellar component.

4. Discussion

We have studied the influence of the antineoplastic molecule ET-18-OCH_3 on the thermotropic properties of DEPE membranes, with the aim of advancing our knowledge of the way in which this ether lipid may alter the physical properties of membranes. It has been possible to show that ET-18-OCH_3 affected both the L_β to L_α and the L_α to H_{II} phase transitions presented by pure DEPE.

The results obtained through DSC were used as the base to construct a partial phase diagram, and the onset and completion temperatures of both the L_β to L_α and the L_α to H_{II} phase transitions were used to define the line boundaries of both transitions. The data from X-ray diffraction and ^{31}P-NMR were also used to specify the type of phase present in each zone. It can be seen in Fig. 5 that an immiscibility appeared in the gel phase with a horizontal solidus line at concentrations higher than 5 mol% which corresponded to the appearance of a small peak at 5 mol% in the DSC thermograms. The nature of this small peak is not clear. On the other hand, the fluidus line indicated a good mixing in the fluid condition.

Isotropic phases were found trough ^{31}P-NMR in a number of cases. The origin of these isotropic peaks is uncertain, and it could arise from small particles, or from certain areas of the membrane with a very high curvature [37,38]. In the 2% sample where isotropic peaks superimposed to anisotropic spectra characteristic of inverted hexagonal H_{II} phases were observed at 70 and 80°C, it should be concluded that the isotropic signal must come from lipids physically separated from the tubules, forming mixed micelles containing the ether lipid. The formation of mixed micelles would be compatible with the observed decrease in ΔH (Fig. 2), since phospholipids incorporated in these micelles would not undergo the gel to liquid crystalline transition.

With respect to the L_β to L_α transition, DSC experiments clearly showed that the ether lipid depressed both the transition temperature and ΔH. DSC also showed that very low concentrations of ET-18-OCH_3 produced an increase in the transition temperature of the L_α to H_{II} phase transition, so that this transition was no longer seen at concentrations of 5 mol% and higher. Small-angle X-ray diffraction confirmed that at concentrations of ET-18-CH_3 of 5 mol% and higher the inverted hexagonal H_{II} phase was not formed at 80°C. In its place a bilayer phase was observed which presented a d-spacing different to that of pure DEPE in the L_α phase (50°C). In fact, samples containing ET-18-OCH_3, in the L_β phase also had a higher d-spacing than pure DEPE (Fig. 3). It is not possible to distinguish whether these increases in d-spacing were due to either an increase in the bilayer thickness or in the water layer between two different bilayers. Nevertheless, it seems much more likely that the ET-18-OCH_3 increases hydration. It lowers T_c and therefore likely increases acyl chain gauche rotamers, decreasing bilayer thickness. This ether lipid has a phosphatidylcholine-type headgroup which binds much more water than does PE. Therefore it almost certainly increases the d-spacing by increasing the water layer.

Non-bilayer lipid phases such as the inverted hexagonal H_{II} phase (see [39] for a review on the structure of the H_{II} phase) have been related to a great variety of biological processes [40]. Compounds that have a large hydrophobic volume and a small polar headgroup will tend to destabilise membrane bilayers, eventually leading to the formation of a non-bilayer inverted hexagonal (H_{II}) phase, and on

the contrary, compounds with a polar part larger than the hydrophobic one will tend to stabilise the bilayer structure [41,42]. It is interesting to remark in this context that ET-18-OCH_3 is an asymmetrical molecule with a relatively large polar group and a relatively small hydrophobic part, since it has only one chain with a length comparable to the most common membrane phospholipids, and the other chain possesses only one methyl group. This is an inverted cone-shaped molecule which is hence expected to stabilise lamellar phases [41].

According to a large number of studies the tendency to form non-bilayer lipid structures can be associated with increasing activities of enzymes depending on membranes such as phospholipase A_2 [43] and protein kinase C [44]. Note that it is the *tendency* rather than the *presence* of these non-bilayer structures that is blamed to facilitate the activity of these enzymes. This *tendency* has been actually measured through the decrease in the L_α to H_{II} transition (T_H) in phospholipids such as POPE and DEPE by Epand and co-workers [45–47]. This way was used to predict the activating or inhibiting effect of a number of compounds on PKC: those decreasing T_H will be expected to be activators (such as 5β-cholan-24-ol or eicosane) and those increasing T_H will be expected to behave as inhibitors (such as Z-Gly-Leu-NH_2, or cyclosporin A). Using this logic, ET-18-OCH_3 should be expected to behave as an inhibitor of PKC. Contradictory results have been obtained, however, in this respect. Whereas some authors have claimed that ET-18-OCH_3 will indeed inhibit PKC, at least in vitro [10,25,48], others have found inhibition or activation of PKC in vivo, depending on the way of preparing the lipid dispersions used in the PKC assays [49]. A possible way of reconciling these contradictory results could be to consider that ET-18-OCH_3 belongs to a class of compounds which may act as solubilisers at high enough concentrations, being similar to detergents. Since acting like that they will destabilise the bilayer, they could act then as activators of enzymes such as phospholipases and PKC. It has been observed in fact that similar molecules as lysophospholipids can both activate and inhibit PKC depending on the concentration [50], and this biphasic effect may be due to their diverse effects on membranes. A lot of caution should be taken when trying to compare experiments on activation/inhibition of enzymes like PKC, carried out at different concentrations of molecules like ET-18-OCH_3.

We want to stress that the stabilisation of membrane bilayers and the formation of non-bilayer structures are not necessarily the only ways in which ET-18-OCH_3 may affect cell membranes. It has been previously suggested, for example, that membrane permeabilisation [51] and alteration of membrane fluidity [23,26] could be implicated, and other additional perturbations of the membranes may be associated to these effects.

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