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Partial dehydration of phosphatidylethanolamine phosphate groups during hexagonal phase formation, as seen by i.r. spectroscopy

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The gel-to-fluid and lamellar-to-H_{II}-hexagonal thermotropic phase transitions of egg-yolk phosphatidylethanolamine have been examined by Fourier-transform infrared spectroscopy under a variety of conditions, namely excess water at pH 5.0, excess water at pH 9.5 and low hydration. The various lamellar and hexagonal phases have been characterized by X-ray diffraction. At pH 5.0, gel–fluid and lamellar–hexagonal transitions were detected at 10 and 32 °C respectively, in accordance with previous data. At pH 9.5, only the first of these two transitions was detected. In the partially hydrated sample a single phenomenon was observed, probably encompassing both transitions, so that, in practice, a gel–H_{II}-hexagonal transition appears to occur. The region of the i.r. spectrum corresponding to the phospholipid phosphate group reveals that the lamellar–hexagonal, but not the gel–fluid, transition is accompanied by a weakening in the shell of hydrogen-bonded water, thus providing direct evidence that, in a pure lipid/water system, hexagonal phase formation requires partial dehydration of the phospholipid phosphate group. X-ray diffraction data support this conclusion, since, at least in the low-hydration system, the average surface area per lipid polar group decreases with the thermotropic lamellar–hexagonal transition.

INTRODUCTION

In the last few years, considerable effort has been directed towards the characterization of lamellar to non-lamellar phase transitions in lipid/water systems, mainly in view of the putative biological implications of non-lamellar structures (Cullis *et al.*, 1986; Siegel, 1986). With this aim, various physical techniques have been applied to such systems (Mantsch *et al.*, 1981; Hardman, 1982; Seddon *et al.*, 1983; Gruner *et al.*, 1985; Goñi & Arrondo, 1986), and the corresponding information has been used to develop molecular models that reflect the physical and chemical properties governing the process (Seddon, 1990). In particular, factors influencing the effective molecular shape of the lipid and its packing constraints, such as temperature, water content, head-group size, ionization or solvation, and hydrocarbon-chain properties, have been extensively investigated (Seddon *et al.*, 1984; Gruner *et al.*, 1985; Tate & Gruner, 1987, 1989; Lewis *et al.*, 1989; Rand *et al.*, 1990).

Aqueous dispersions of egg-yolk phosphatidylethanolamine (PtdEtn) exhibit at about neutral pH two main thermotropic phase transitions, a gel (L_β) to fluid-lamellar (L_α) transition and a lamellar to H_{II}-hexagonal transition, at 10–15 °C and 25–30 °C respectively (Reiss-Husson, 1967; Cullis & de Kruijff, 1978; Mantsch *et al.*, 1981). We have studied these transitions with PtdEtn in excess water (at pH 5.0 and pH 9.5) and under limited hydration conditions. The resulting lamellar and hexagonal phases have been characterized by X-ray diffraction (Luzzati, 1968; Gruner *et al.*, 1988), and the phase transitions have been examined by Fourier-transform infrared (FT-i.r.) spectroscopy. The latter is a powerful technique for monitoring the physical state and thermal phase behaviour of lipids, since it can simultaneously yield information on the different parts of the molecule (Fringeli & Günthard, 1981; Casal *et al.*, 1984; Mendelsohn & Mantsch, 1986; Lee & Chapman, 1986). Mantsch *et al.* (1981), studying unbuffered aqueous PtdEtn dispersions by this technique, found that the bands corresponding to C–H (symmetric) and C=O stretching vibrations were most useful in the detection of phase transitions. However, since the phosphate group has been proposed to undergo an important reorganization during the lamellar to non-lamellar phase transition (Cullis & de Kruijff, 1978; Boggs, 1987; Seddon, 1990), we have carefully investigated the vibrational bands arising from this lipid moiety. A distinct spectral change was observed, concomitant with the lamellar–hexagonal phase transition, which could be explained as a change in the degree of water hydrogen bonding to the phosphate group. X-ray measurements of lipid phase dimensions are also compatible with this hypothesis.

MATERIALS AND METHODS

Egg-yolk PtdEtn was purchased from Lipid Products (South Nutfield, Surrey, U.K.). Its fatty acid composition was very similar to that published by Mantsch *et al.* (1981) for the same material and source. Samples in excess water were prepared by swelling freeze-dried lipid in borate buffer (5 mM-borate, 150 mM-NaCl and 0.1 mM-EDTA, pH 9.5) at room temperature. When required, the pH was changed by dialysing the above preparation against citrate buffer (10 mM-citrate, 150 mM-NaCl and 0.1 mM-EDTA, pH 5.0), also at room temperature. Low-hydration samples were prepared by adding known volumes of citrate buffer to pre-weighed amounts of freeze-dried lipid in the bottom of a constriction tube; the tube was then flame-sealed and the lipid/water mixture was centrifuged (1000 *g*) twenty times backwards and forwards, through the constriction, in a bench-top centrifuge at room temperature. The resulting mixture was very homogeneous in appearance; the added water content was ~ 8 mol of water/mol of PtdEtn.

I.r. spectra were collected in a Nicolet 620 spectrometer equipped with a DTGS detector. Samples were placed in a thermostatted cell with BaF_2 windows. A total of 100 scans for the sample and 100 scans for the background was taken for each spectrum using a shuttle device; these were averaged, apodized

Abbreviations used: PtdEtn, phosphatidylethanolamine; FT-i.r., Fourier-transform infrared.

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with a Happ–Gentzel function and Fourier-transformed to give a nominal resolution of 2 cm^{-1}. Buffer contributions were subtracted and band positions were obtained using a centre-of-gravity algorithm (Moffatt *et al.*, 1986). Measurements were taken at 2 °C intervals; at each temperature, samples were allowed to equilibrate for 8 min. Data from the first heating/cooling cycle were neglected; otherwise subsequent runs gave similar results.

For X-ray diffraction, an X-ray beam from a fine focus sealed-off X-ray tube was focused via a germanium monochromator in a Stoe focusing monochromatic beam transmission diffractometer. The diffraction pattern was recorded using a position-sensitive detector by a step-scanning technique (180 s/step). The overall recording time was approx. 45 min. Only the low-angle regions (Bragg angles $2\theta < 10.0$) were explored. Unoriented lipid dispersions in Lindemann glass capillaries were used in all diffraction studies.

RESULTS

Thermotropic transitions of egg-yolk PtdEtn, as seen by FT-i.r. spectroscopy, are shown in Figs. 1–3 for various lipid dispersions, namely excess water at pH 5.0 (Fig. 1), excess water at pH 9.5 (Fig. 2) and low hydration (~ 8 mol of water/mol of lipid) (Fig. 3). In all cases the position of the C–H (symmetric methylene), PO_2^- (asymmetric) and C=O stretching vibration bands is plotted as a function of temperature. The preparation in Fig. 1 is comparable with the one described by Mantsch *et al.* (1981); X-ray diffraction data of this sample confirmed the existence, at 21 °C, of a lamellar phase [repeat distance $d = 5.34$ nm (53.4 Å)] which, upon heating to 60 °C, becomes a H_{II}-hexagonal phase [$d = 6.11$ nm (61.1 Å)]. By FT-i.r. spectroscopy we could detect the gel–fluid and lamellar–hexagonal phase transitions, which were respectively centred at about 10 and 32 °C, through changes in the C–H and C=O vibrations, in agreement with previous observations (Gruner *et al.*, 1988). In addition, the lamellar–H_{II}-hexagonal transition was also marked by an increase in the wave number of the asymmetric PO_2^- stretching vibration band. At an alkaline pH (Fig. 2), the methylene vibration band detected only the gel–fluid transition, which occurred at a lower temperature than at pH 5.0; this transition was also accompanied by a decrease in the C=O band, but no changes in the PO_2^- signal were observed. Both the lack of hexagonal phases (in this temperature range) and the shift to lower temperatures of the $L_\beta \rightarrow L_\alpha$ transition at alkaline pH are well documented for unsaturated PtdEtn (Eibl & Woolley, 1979; Cevc *et al.*, 1981; Seddon *et al.*, 1983; Seddon, 1990). The lack of a lamellar–hexagonal transition at pH 9.5 has also been confirmed for our sample by X-ray measurements.

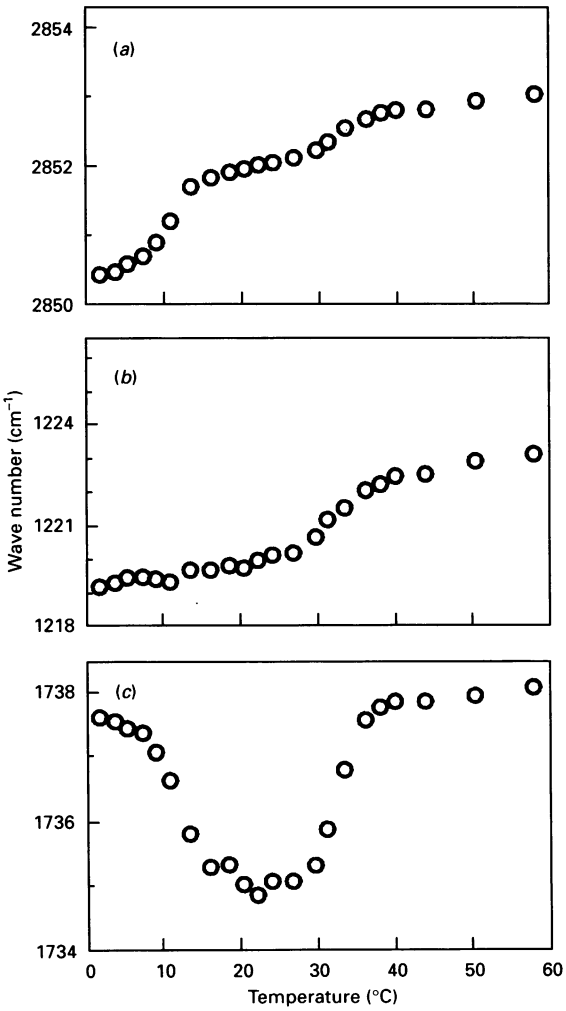

Fig. 1. Temperature-dependence of the positions of three characteristic vibrational modes of egg-yolk PtdEtn at pH 5 with excess water

(*a*) CH_2, (*b*) PO_2^-, (*c*) C=O.

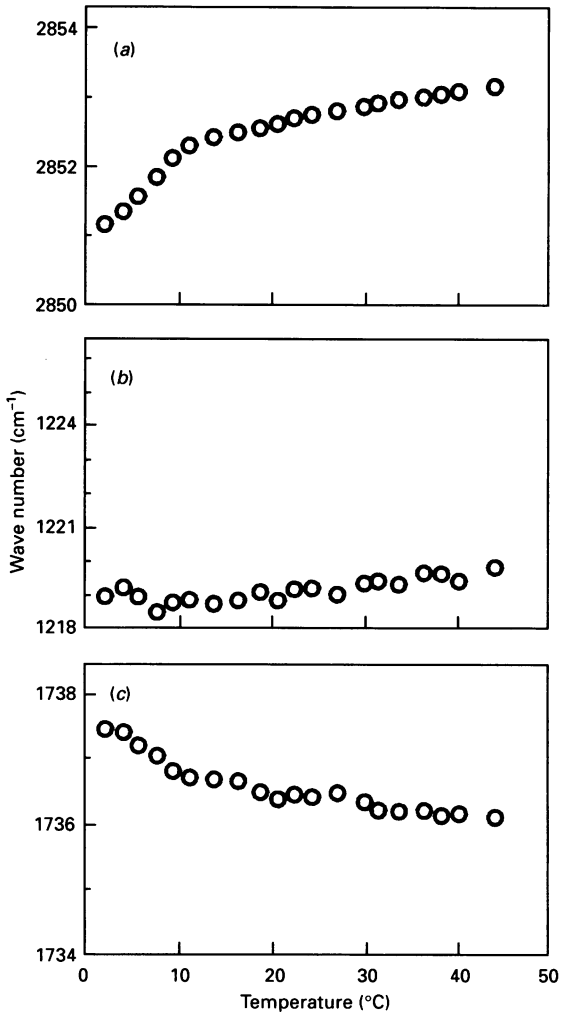

Fig. 2. Temperature-dependence of the positions of three characteristic vibrational modes of egg-yolk PtdEtn at pH 9.5 with excess water

(*a*) CH_2, (*b*) PO_2^-, (*c*) C=O.

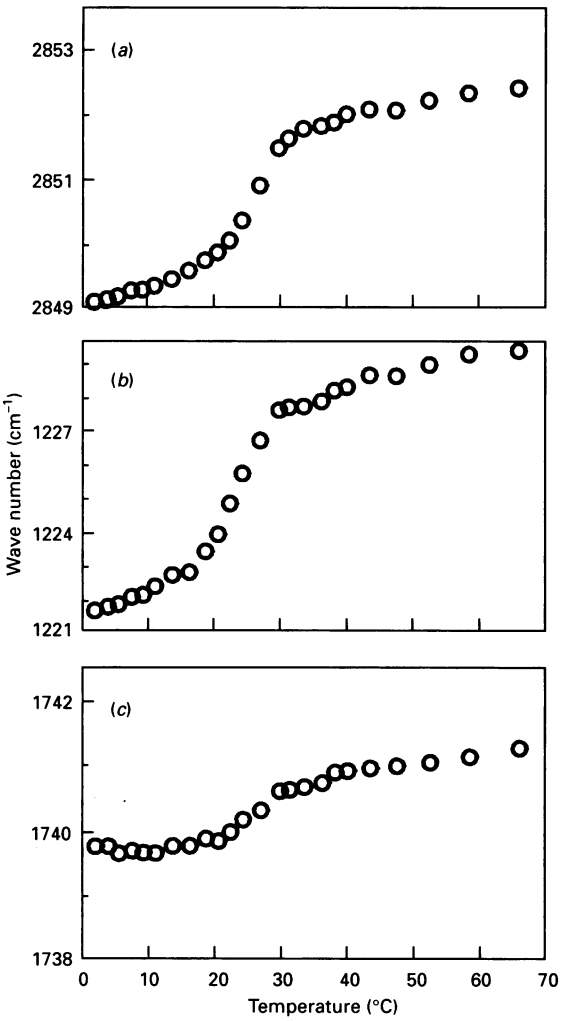

Fig. 3. Temperature-dependence of the positions of three characteristic vibrational modes of egg-yolk PtdEtn under low-hydration conditions

(*a*) CH_2, (*b*) PO_2^-, (*c*) C=O.

The low-hydration sample (Fig. 3) undergoes a single transition in the 0–70 °C interval, centred at about 25 °C. The distinct increase in the centre of gravity of the PO_2^- vibration band suggests that a lamellar–hexagonal phase transition is taking place. In addition, the extent and interval of the concomitant change in the C–H band (starting at 2849 cm^{-1} and extending over more than three wave numbers) may indicate that both transitions (L_β to L_α to H_{II}) occur simultaneously or one immediately after another, or else that a single L_β to H_{II} transition is taking place. The change in the C=O band position, also showing a single transition, is compatible with these possibilities. X-ray diffraction showed a pure lamellar phase with a repeat distance of 5.18 nm (51.8 Å) at 21 °C, while at 42 °C the system exhibited a pure H_{II} hexagonal phase with a repeat distance of 4.89 nm (48.9 Å) between the cylinders.

Since the latter sample has a defined water content, the phase dimensions may be calculated as described by Luzzati (1968) from the X-ray repeat spacing in combination with the lipid concentration and densities. In order to determine the structural parameters, the implicit hypotheses are that the paraffin chains are clustered into regions from which water is excluded, and that the interface between water and paraffin is covered by the hydrophilic groups of the lipid molecules (Luzzati, 1968). X-ray diffraction results do not provide data on the degree of penetration of water molecules amongst the lipid head-groups; thus our study cannot directly address this question.

The lipid volume fraction, ϕ_L, is:

$$\phi_L = [1 + (\bar{v}_w/\bar{v}_L)(1-c)/c]^{-1}$$

where $\bar{v}_w$ and $\bar{v}_L$ are the partial specific volumes of water and lipid respectively, and c is the lipid concentration (weight fraction), calculated on the basis of 8 water molecules per lipid molecule.

According to Seddon *et al.* (1984), it is assumed that the bound water has the same partial specific volume as free water, i.e. $\bar{v}_w = 1$ ml/g. The partial specific volume of the lipids is a temperature-dependent parameter: values of $\bar{v}_L = 0.91$ ml/g in the lamellar phase (21 °C) and $\bar{v}_L = 0.93$ ml/g in the hexagonal phase (42 °C) were used, according to measurements by Seddon *et al.* (1984) for dipalmitoyl-PtdEtn. In any case, variations in $\bar{v}_L$ of 0.02 ml/g result in negligible changes in the parameter dimensions.

For the lamellar phase (21 °C), $\phi_L = 0.818$. Bilayer thickness (D_L) can be calculated as $D_L = \phi_L d$, where d is the X-ray repeat distance; thus $D_L = 0.818 \times 5.18 = 4.24$ nm (42.4 Å). The area per polar group (S, in nm^2) is given by:

$$S = (2M\bar{v}_L \times 10^{24})/(D_L N)$$

where M is the lipid molecular mass and N is Avogadro's number. For the lamellar phase, $S = 0.51$ nm^2 (51.0 $Å^2$).

The diameter of the water cylinders in the H_{II} hexagonal phase (d_w) is computed as follows:

$$d_w = [(2\sqrt{3}/\pi)(1-\phi_L)a^2]^{1/2}$$

where $a = (2/\sqrt{3})d$ is the lattice spacing of the H_{II} phase, d being the X-ray repeat distance, 4.89 nm (48.9 Å). The lipid volume fraction for this phase (at 42 °C) is $\phi_L = 0.821$. According to the above, $d_w = 2.51$ nm (25.1 Å). The area per polar group in the H_{II}-hexagonal phase, S, is the value at the lipid/water interface:

$$S = 2\pi d_w M\bar{v}_L \times 10^{24}/(\sqrt{3} \times d^2 \phi_L N)$$

The computed value of S is 0.389 nm^2 (38.9 $Å^2$).

DISCUSSION

One interesting aspect of our study is that a change is detected in the position of the asymmetric PO_2^- stretching vibration band that can be associated with a lamellar–hexagonal phase transition of a phospholipid. Mantsch *et al.* (1981) and Goñi & Arrondo (1986) were unable to detect any such changes. The difference between their results and ours is probably due to improvements in present-day instrumentation and/or the use of buffer solutions for pH control in the present study. In our case, the change consisted of a shift towards higher wave numbers. There is ample evidence in the literature demonstrating that such shifts occur when the phosphate–water hydrogen bonds are weakened and the number of water molecules hydrogen-bonded to the phosphate group is decreased. This has been shown for phosphatidylcholine (Arrondo *et al.*, 1984; Mellier & Diaf, 1988), PtdEtn (Sen *et al.*, 1988) and phosphatidylserine (Dluhy *et al.*, 1983; Casal *et al.*, 1984, 1989). No shift of the asymmetric PO_2^- band occurred during the gel–fluid transition, at either pH 9.5 or pH 5.0, but a shift was clearly observed associated to the lamellar–hexagonal phase transition. Since such a shift is indicative of some degree of dehydration of the phosphate group, we conclude that the dehydration step is specifically associated with the lamellar–hexagonal transition. In fact, the gel–fluid transition may be considered as an internal control, being a well-defined phase transition with no changes in the P–O band position. The X-ray data demonstrate, at least for the low-hydration sample, a decrease in the average surface occupied per lipid polar head-group accompanying the lamellar–hexagonal transition. This

calculation does not take into account the relative hydration level of the polar groups in both phases, but these results are also in agreement with the concept of phosphate group dehydration concomitant with the thermotropic transition.

The view that the phosphate group has a weaker shell of hydrogen-bonded water in the hexagonal phase than in the lamellar phase is in agreement with the proposals by previous authors (Boggs, 1987; Rand *et al.*, 1990). However, it should be noted that, when compared with the large shifts produced by extensive dehydration (e.g. see Sen *et al.*, 1988), our observations can only account for a small decrease in the proportion of bound water. Moreover, one should distinguish between local hydration of the lipid head-group and the volume requirements of a given phase for water. In fact, the limiting hydrations for the H_{II} phases for PtdEtn and certain lipid mixtures are often greater than those for the corresponding lamellar phases (Seddon *et al.*, 1984; Rand *et al.*, 1990). In spite of this, it is more difficult, at low water activities, to remove water from the same polar groups when they are in the lamellar phase than when they are in the hexagonal phase (Rand *et al.*, 1990).

Also significant in this respect is our observation that the position of the asymmetric PO_2^- stretching vibration band, and thus the level of local hydration, is similar for the L_β phases at both pH 5.0 and pH 9.5, although Seddon *et al.* (1983) had proposed a decreased hydration of the phosphate group upon protonation of the amino group (i.e. below pH $\sim$ 8). However, it is still possible that amino group protonation, though not inducing direct phosphate dehydration by itself, renders the phosphate group more prone to losing water molecules upon heating.

The low-hydration sample (Fig. 3) displayed unusual behaviour in that a single transition was observed. From the available data it cannot be concluded that a direct $L_\beta \rightarrow H_{II}$ transition is taking place; however, the combined C–H and PO_2^- spectral changes are suggestive of this possibility. Such direct transitions have already been observed in related systems (Marsh & Seddon, 1982; Seddon *et al.*, 1984; Wistrom *et al.*, 1989).

Mantsch *et al.* (1981) proposed that the driving force for the bilayer to non-bilayer phase transition in egg-yolk PtdEtn was the introduction of additional conformational disorder into the already disordered liquid-crystalline bilayer phase (see our Fig. 1*a*). Although establishing cause–effect relationships may be difficult, our results with excess water suggest that the increase in gauche rotamers must be accompanied by a certain degree of head-group dehydration for the phase transition to occur, while the data under low-hydration conditions indicate that a single step of simultaneous phosphate dehydration and alkyl chain disordering may be sufficient to bring about the transition from gel phase to inverted hexagonal phase.

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