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GEL-TO-INVERTED HEXAGONAL (L_β-H_{II}) PHASE TRANSITIONS IN PHOSPHATIDYLETHANOLAMINES AND FATTY ACID-PHOSPHATIDYLCHOLINE MIXTURES, DEMONSTRATED BY ^{31}P-NMR SPECTROSCOPY AND X-RAY DIFFRACTION

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The phase behaviour of distearoyl- and dihexadecylphosphatidylethanolamine at high salt concentration and of dipalmitoylphosphatidylcholine + 66 mol% palmitic acid at pH 4.0 have been studied by high-field ^{31}P-NMR spectroscopy and X-ray diffraction. In saturated NaCl, dihexadecylphosphatidylethanolamine undergoes a reversible transition at 74°C directly from the lamellar gel phase L_β to the inverted hexagonal H_{II} phase. A similar transition is observed at 78.4°C for distearoylphosphatidylethanolamine in saturated NaCl (cooling scan). The single, sharp calorimetric transition at 61°C observed in dipalmitoylphosphatidylcholine + 66 mol% palmitic acid at pH 4.0 also corresponds to a transformation directly from the lamellar gel to the inverted hexagonal phase, without an intervening fluid lamellar phase.

Introduction

There is currently much interest in lamellar-hexagonal (H_{II}) phase transitions of aqueous phospholipid dispersions, as a model for non-lamellar lipid transformations involved in activated processes, such as membrane fusion, in biomembranes (see, for example, Refs. 1–3). It is frequently assumed that the transformation to the hexagonal phase takes place only from the fluid lamellar phase (L_α), above the normal gel-to-fluid lamellar (L_β-L_α) phase transition [4]. However, since the lamellar-inverted hexagonal transformation depends on the structural and energetic imbalance between the polar headgroup and the hydrocarbon chain regions of the phospholipid molecules, rather than on the configurational entropy of the chains per se [5], it is possible that the transition may take place directly from the lamellar gel (L_β) phase (provided that the activation barrier is not too great), without the existence of an intervening fluid (L_α) phase. The enthalpy and entropy difference between the L_α and H_{II} phases is small [6], therefore in the absence of kinetic barriers whichever phase is the more stable depends on how great is the 'inverted cone' nature of the phospholipid molecule [5].

In the present work we have investigated three different phospholipid-water systems which are capable of undergoing a direct transformation from the L_β-phase to the H_{II}-phase. The phase transitions were studied using high-field ^{31}P-NMR spectroscopy, which is a diagnostic method for detecting hexagonal phases in phospholipid dispersions (since it is known that the headgroup phosphate conformations of different phospholipids are rather similar and do not change appreciably with temperature, see Refs. 7, 8). Further identification of the phases was also supplied using X-ray diffraction. Two of the lipids involved are disaturated

Abbreviations: DSPE, distearoylphosphatidylethanolamine; DHPE, dihexadecylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine.

phosphatidylethanolamines at high salt concentration, and the third system is a mixture of dipalmitoylphosphatidylcholine with 66 mol% palmitic acid. The phase transition in the latter system, which was previously studied by calorimetry, was formerly assumed to be a purely lamellar gel-to-fluid change [9,10].

Materials and Methods

Distearoyl- and dihexadecylphosphatidylethanolamine (DSPE and DHPE) and dipalmitoylphosphatidylcholine (DPPC) were from Fluka, Buchs, Switzerland, as also was palmitic acid. Phospholipid purity was checked by thin-layer chromatography. No detectable chemical degradation was observed after high temperature cycling of the lipid samples. Typically for ^{31}P-NMR measurements 100 mg of phospholipid was hydrated with 200 μl of water or salt solution by mixing and heating above the phase transition in the NMR tube. This is sufficient to ensure complete hydration (20% for phosphatidylethanolamines) even in the case of substantial evaporation. For the palmitic acid-containing mixtures, the DPPC and palmitic acid were dissolved together in $CHCl_3/CH_3OH$ (2:1, v/v) to ensure good mixing. The solvent was then evaporated off under nitrogen, and the final traces of solvent removed by placing the sample under vacuum for >3 h. The dry lipid was hydrated in a large excess of water in order to adjust the pH, and then the lipid was spun down in the NMR tube using a bench centrifuge. Thin-layer chromatography of the supernatant failed to reveal any unincorporated fatty acid, using conditions under which less than 5% of the total palmitic acid should be detectable.

Proton-dipolar decoupled 109 MHz ^{31}P-NMR spectra were collected with a Bruker WH-270 spectrometer operating in the Fourier transform mode, with a radio frequency pulse length of 10 μs. The decoupling power was 5–10 W, and the duty cycle of the gated decoupling was 0.2%. The error in temperature measurement for the NMR samples was estimated to be less than ± 2 K, as judged from measurements of the gel-fluid phase transition temperature of dimyristoylphosphatidylcholine.

X-ray diffraction measurements were performed using a Guinier camera with a quartz crystal monochromator to isolate the $CuK\alpha_1$ ($\lambda = 0.15405$ nm) radiation. Exposure times for the CEA REFLEX 15 X-ray film (Ceaverhen AB, Strägnäs, Sweden) were between 15 min and 2 h. The hydrated lipid samples were sealed between thin mica plates in specially built, thermostatically controlled, metal holders. The accuracy of temperature calibration in continuous temperature scanning X-ray diffraction measurements was ± 2 K.

Calorimetric measurements were made with a Perkin-Elmer DSC 2 differential scanning calorimeter equipped with Intracooler 1. Centrifuged lipid pellets (approx. 10 mg) were sealed with approx. 40 μl additional buffer in large-volume stainless steel pans. The heating rate was 1.25 K/min.

Calorimetric samples were scanned repeatedly to ensure reversibility. X-ray samples were first scanned in the calorimeter before diffraction measurements. NMR samples were heated above the phase transition for 10–15 min before measurement. A repeated temperature scan of the DHPE sample in saturated NaCl verified the subsequent reproduceability of the NMR spectra.

Results and Discussion

The proton-decoupled ^{31}P-NMR powder spectra of DSPE dispersed in excess saturated NaCl solution are given as a function of temperature in Fig. 1. The low-temperature spectra have strongly broadened lines and a large effective chemical shift anisotropy, $\Delta\sigma \sim -62$ to -55 ppm, characteristic of diacyl phospholipids in the gel phase (see, for example, Ref. 11). The low temperature spectra all correspond to an axially symmetric motion with a negative chemical shift anisotropy, characteristic of phospholipids in a bilayer arrangement (see, for example, Refs. 1 and 2). There is only a slight narrowing of the spectra with increasing temperature, the effective chemical shift anisotropy remaining at $|\Delta\sigma| \gtrsim 55$ ppm, corresponding to phospholipids in the gel phase rather than in the fluid phase (cf. Ref. 11). At about 80°C there is an abrupt decrease in the total spectral width by a little more than a factor of two, and the chemical shift anisotropy, although still axial, changes sign, the peak now being to low field of the shoulder rather than to high field. Such

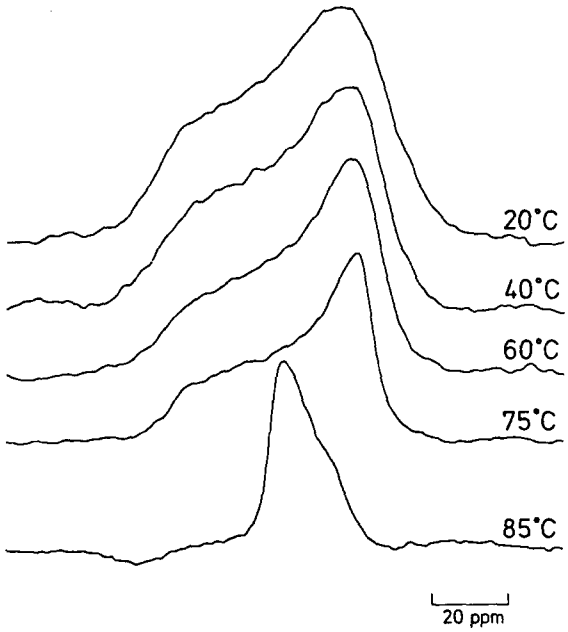

Fig. 1. Proton-dipolar decoupled 109 MHz ^{31}P-NMR spectra of distearoylphosphatidylethanolamine dispersed in saturated NaCl solution. Spectra were recorded at the temperatures indicated.

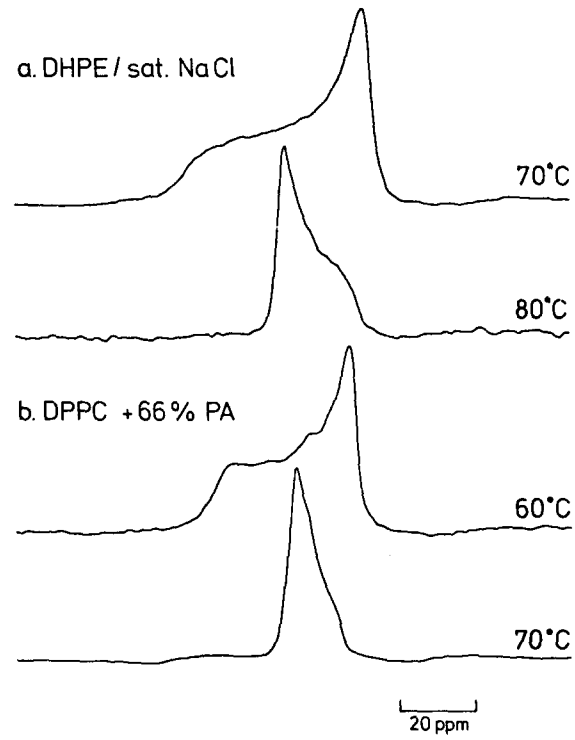

Fig. 2. Proton-doipolar decoupled 109 MHz ^{31}P-NMR spectra of (a) dihexadecylphosphatidylethanolamine in saturated NaCl solution, and (b) dipalmitoylphosphatidylcholine + 66 mol% palmitic acid dispersed in 50 mM triethanolamine, pH 4.0. Spectra were recorded at the temperatures indicated.

spectra are indicative of lipids in an inverted cylindrical arrangement such as is found in the H_{II} phase. The large reduction in spectral anisotropy is due primarily to the additional motional averaging by rotation of the lipid molecules around the cylinder axis as a result of rapid lateral diffusion (cf. Refs. 1 and 2).

Somewhat similar changes are observed with the other two systems studied. Representative spectra of DHPE in saturated NaCl and of DPPC + 66 mol% palmitic acid in 50 mM triethanolamine pH 4.0, both in the L_β and the hexagonal (H_{II}) phase, are given in Fig. 2. The effective chemical shift anisotropies and linewidths of both systems in the low-temperature phase ($\Delta\sigma \sim -55$ ppm for DHPE at 70°C and approx. -38 ppm for DPPC + 66 mol% palmitic acid at 60°C) are less than for DSPE and other diacyl phospholipids in the L_β phase, but nevertheless the temperature dependence of the spectra (see below) indicates no abrupt transition to a more fluid state before transformation to the hexagonal phase.

The identifications of the phases by ^{31}P-NMR have been confirmed by X-ray diffraction. In several aspects the two methods are complementary, whereas the ^{31}P-NMR gives spectra diagnostic of a fluid, cylindrical lipid organization, the X-ray diffraction pattern gives rise to long spacings in the low-angle region which are in the ratio $1:1/\sqrt{3}:1/2$ characteristic of the hexagonal packing of the lipid cylinders (see, for example, Refs. 2 and 3). For DSPE in saturated NaCl at 90°C the long spacing of the hexagonal phase is 6.42 nm and for DHPE in saturated NaCl at 80°C it is 6.14 nm. In the L_β phase the ^{31}P-NMR spectra have linebroadenings and chemical shift anisotropies which are indicative of a more restricted motion of the lipid phosphate group as a result of the closer molecular packing. The X-ray diffraction long spacings in this case are found to be in the ratio $1:1/2:1/3\ldots$, characteristic of a lamellar packing. For DSPE in saturated NaCl the lamellar repeat of the L_β phase is found to be 7.14 nm and 6.92 nm at 24°C and 78°C, respectively, and for

DHPE in saturated NaCl to be 6.36 nm and 6.34 nm at 25°C and 64°C, respectively. In addition, a sharp reflection is observed in the high-angle region, indicating a parallel, pseudo-crystalline packing of the lipid chains. For DSPE in saturated NaCl the high-angle spacing is 0.408 nm and 0.427 nm at 24°C and 78°C, respectively, and for DHPE in saturated NaCl it is found to be 0.410 nm and 0.421 nm at 25°C and 64°C, respectively.

In the above, the lamellar gel phase has been referred to as L_β, i.e. a lamellar structure in which the lipid chains are not tilted relative to the bilayer normal. This assignment is reasonably well established for phosphatidylethanolamines in the gel phase (Ref. 13 and Seddon, J.M., Cevc, G., Kaye, R.D. and Marsh, D., unpublished data), and is confirmed by the sharp, symmetrical X-ray reflection observed in the high-angle region in the present work. For DPPC + 66 mol% palmitic acid at pH 4.0, the L_β structure is again suggested by the sharp, symmetrical high-angle reflection, and also by the absence of the characteristic DPPC pretransition, which is normally associated with tilted chains. In addition the lamellar repeat distance of 6.9 nm for DPPC + 66 mol% palmitic acid at pH 4.0 is considerably greater than the value of approx. 6.3 nm found for DPPC alone in the tilted L'_β phase. This again would be consistent with untilted chains in the palmitic acid-containing mixture. The X-ray diffraction results demonstrate unambiguously that the transitions in the ^{31}P-NMR spectra are due to hexagonal phase formation and not due to a transition in the headgroup phosphate conformation [12]. Although the possibility cannot be excluded, there is currently no evidence for the existence of phospholipid headgroup conformations which would give rise to an erroneous identification of the phases by ^{31}P-NMR [7,8].

The temperature dependence of the effective chemical shift anisotropy, in all three different systems studied, is given in Fig. 3. This gives a more detailed view of the lipid phase behaviour. The effective chemical shift anisotropy measured here is defined as the separation between the points of maximum slope in the outer extremes of the spectrum (cf. Ref. 7). Both for DSPE (Fig. 3a) and DHPE (Fig. 3b) in saturated NaCl, the chemical shift anisotropy varies relatively little with temperature in the L_β phase. For DSPE in saturated

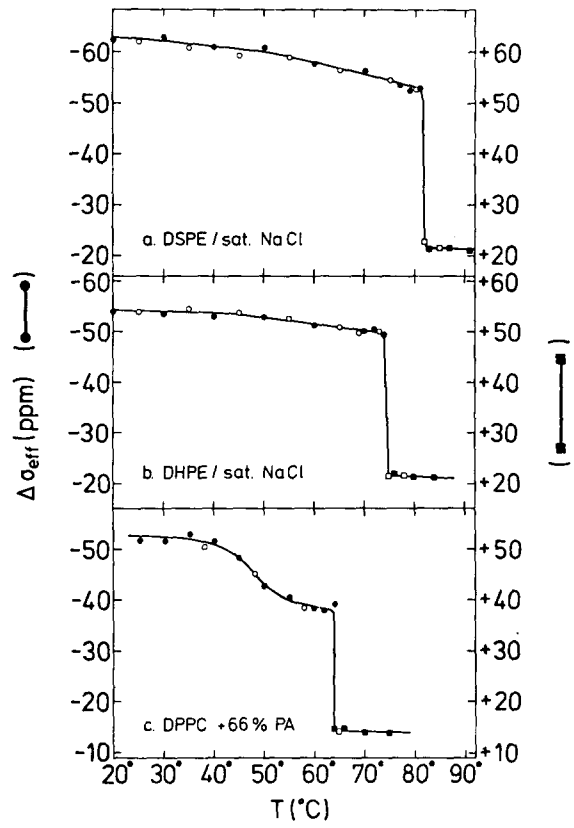

Fig. 3. Temperature variation of the effective chemical shift anisotropy, $\Delta\sigma$, of (a) DSPE in saturated NaCl, (b) DHPE in saturated NaCl, and (c) DPPC+66 mol% palmitic acid in 50 mM triethanolamine, pH 4.0. Filled symbols refer to increasing temperature and open symbols to decreasing temperature. Circles and left-hand scale refer to the lamellar phase; squares and right-hand scale refer to the hexagonal phase.

NaCl there is an abrupt transition between the L_β and the H_{II} phase taking place within 1–2 K. The chemical shift anisotropy in the hexagonal phase is +22 ppm. The corresponding chemical shift in a lamellar phase of equivalent phosphate group mobility would be twice this [1,2], i.e. −44 ppm. This value is characteristic of phospholipids in the L_α phase [7] and is considerably smaller in magnitude than the −55 ppm found in the lamellar phase immediately before the transition, suggesting again that the latter corresponds to the L_β phase. Differential scanning calorimetry provides a more sensitive and continuous method of studying the temperature dependence. DSC traces of DSPE in saturated NaCl are given in Fig. 4a. A single,

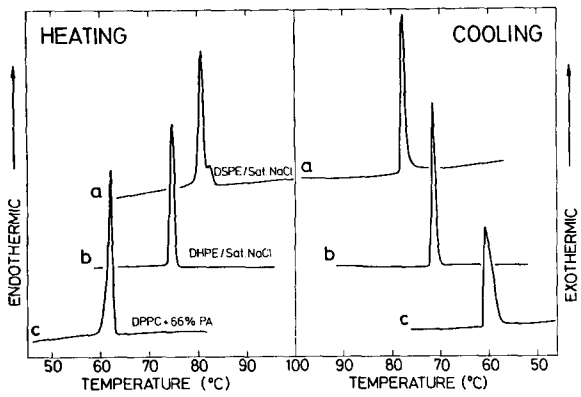

Fig. 4. Differential scanning calorimeter traces of (a) DSPE in saturated NaCl, (b) DHPE in saturated NaCl, and (c) DPPC+ 66 mol% palmitic acid in 50 mM triethanolamine, pH 4.0. Both heating and cooling scans are shown. Scanning rate 1.25 K/min.

high-enthalpy transition is observed in the cooling scan at 78.4°C which agrees reasonably well with the transition observed by ^{31}P-NMR at 81°C (Fig. 3a). In the heating scan, however, a smaller peak is partially resolved on the high-temperature side of the main peak. It appears that the L_α phase exists over a very brief temperature range in the upward scan, but because of hysteresis in the hexagonal transition there is a direct conversion from the H_{II} phase to the L_β phase in the cooling scan.

For DHPE in saturated NaCl there is an abrupt, reversible transition in the ^{31}P-NMR spectra, corresponding to a conversion between the L_β and H_{II} phases taking place at 75°C (Fig. 3b). In the H_{II} phase the chemical shift anisotropy is +21 ppm, appreciably less than half the value of −50 ppm found in the L_β phase immediately below the transition. In this case there appears to be no intervening L_α phase. DSC traces for DHPE in saturated NaCl given in Fig. 4b confirm this fact. A single, sharp, high-enthalpy transition is observed at 73.9°C and 72.1°C in heating and cooling scans, respectively. The transition thus takes place directly between the L_β and the H_{II} phase in both directions of temperature scanning.

For DPPC + 66 mol% palmitic acid at pH 4.0 (Fig. 3c) the low temperature situation appears slightly more complicated. The effective chemical shift anisotropy in the low temperature phase is smaller ($|\Delta\sigma| \sim 52$ ppm) and undergoes a gradual decrease to a value of $|\Delta\sigma| \sim 38$ ppm over the temperature range 40–60°C. At 64°C there is then an abrupt transition to a hexagonal phase with a chemical shift anisotropy of $|\Delta\sigma| \sim 14$ ppm, which is considerably less than half that in the lamellar phase immediately below the transition. This latter strongly suggests that chain melting occurs coincident with the hexagonal transition. In addition DSC measurements on these samples (Fig. 4c) reveal no lower temperature transition, only a single, sharp, reversible, high-enthalpy transition at 61.1°C, in agreement with the results of previous workers [9,10].

To confirm these latter phase assignments a continuous temperature scanning X-ray diffraction experiment was performed on a DPPC + 66 mol% palmitic acid sample at pH 4.0. The results are given in Fig. 5. The presence of the sharp 0.42 nm reflection in the high-angle region, indicating hexagonal closepacking of the hydrocarbon chains, shows that the lipid dispersion remains in the L_β-phase up to 62°C, with a sharp transition to a fluid state at this temperature. In the low-angle region the lamellar spacing remains constant with a repeat distance of 6.9 nm up to 62°C. At this point the lamellar reflections are replaced by reflections with long spacings in the ratio $1:1/\sqrt{3}:1/2$ characteristic of the hexagonal phase ($d = 6.0$ nm). This indicates a direct transition from the L_β to the H_{II} phase. The decrease in effective chemical shift anisotropy, observed in the L_β phase in Fig. 3c, presumably corresponds to lateral expansion of the bilayer. The chain-chain repeat spacing deduced from the high-angle reflections in Fig. 5 increases from 0.410 nm at 25°C to 0.425 nm at 60°C, and the increased curvature in the temperature dependence at 45°C corresponds well with the decrease in $\Delta\sigma_{eff}$ in Fig. 3c.

The results thus demonstrate rather clearly the possibility of transitions directly from an ordered lamellar phase to a fluid inverted hexagonal phase, without an intervening fluid lamellar phase. The origin is clear: the energetic and steric balance between the lipid headgroups and chains favours the hexagonal rather than the lamellar phase immediately on chain fluidization. It is known that increasing salt concentration increases the (L_β-L_α) transition temperature and decreases the (L_α-H_{II}) transition temperature in phosphatidylethanolamines [6,14]. Thus in high salt the hexago-

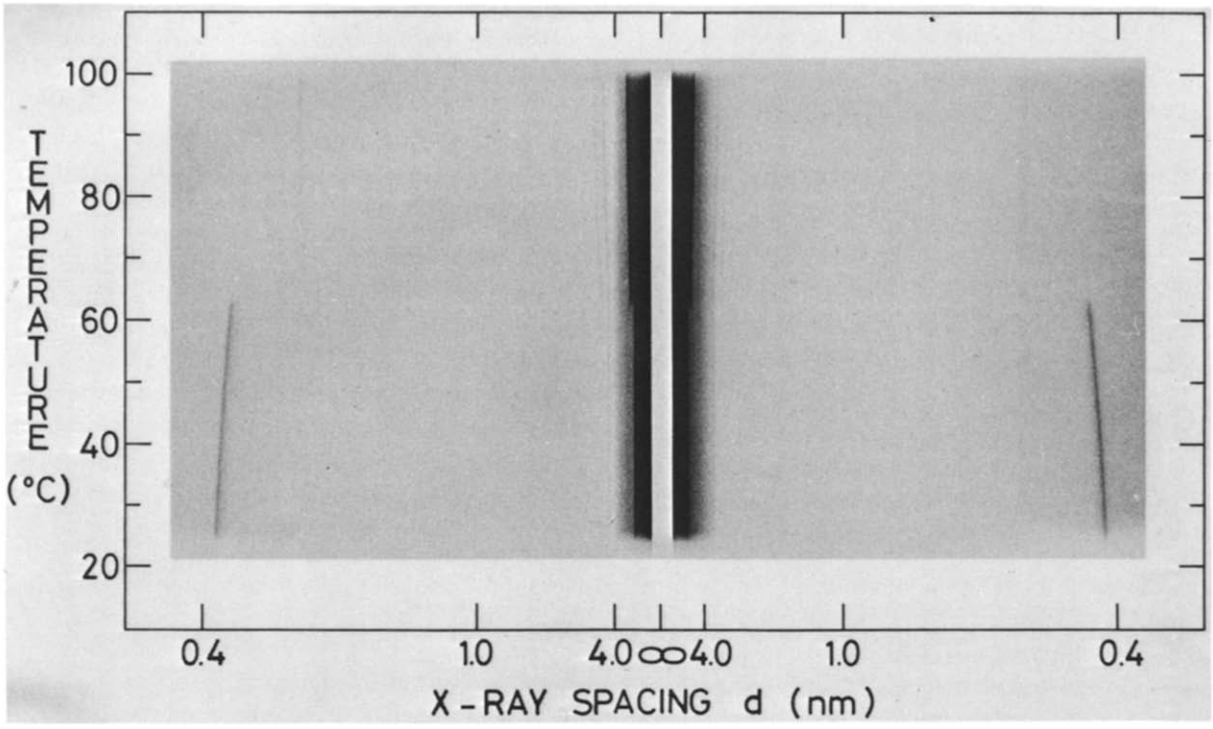

Fig. 5. Continuous temperature scan of the X-ray powder diffraction pattern of DPPC + 60 mol% palmitic acid in 50 mM triethanolamine, pH 4.0. The X-ray film was scanned down behind a masking slit whilst continuously heating the sample. Heating rate 0.1 K/min.

nal phase becomes more stable than the fluid lamellar phase and the two transitions coalesce. (It has previously been reported that the hexagonal transition is only partially resolved from the gel-to-fluid transition in dihexadecyl phosphatidic acid [6]). In the case of DPPC + 66 mol% palmitic acid, it would appear that the steric bulk in the hydrocarbon chain region causes the hexagonal phase to be favoured over the lamellar.

The DPPC + 66 mol% palmitic acid system is particularly interesting since it gives rise to a single, sharp transition in calorimetry which was previously assumed to be a simple lamellar gel-to-fluid transition [9,10]. Parallels were drawn with the gel-to-fluid transition in dipalmitoylphosphatidylethanolamine bilayers at 63°C [9]. Since this latter is known to be a purely lamellar transition [6,14], such comparisons are presumably not valid. In addition, considerable doubt must be cast on the interpretation of 'phase diagrams' for the DPPC-palmitic acid system which assumed that the system remained solely lamellar [10]. In our experiments the DPPC + palmitic acid dispersion was adjusted to pH 4.0, which is well below the surface pK of the palmitic acid [15]. ^{31}P-NMR experiments made in 50 mM triethanolamine at pH 7.0 and above have revealed a complex behaviour at the transition, reversibly yielding a state with an isotropic spectrum at higher temperature. X-ray diffraction studies on the same system at pH 7.0 also indicate the formation of a non-lamellar phase, most probably hexagonal.

It thus seems clear that transitions from an ordered lamellar gel state to a fluid hexagonal state can take place directly. From the biological point of view, these results imply that a lamellar to non-lamellar phase transformation of immobilized lipids in biomembranes can be triggered directly without prior fluidization of the immobilized lipid chains.

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