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Phase transition from a gel to a fluid phase of cubic symmetry in dimyristoylphosphatidylcholine/myristic acid (1 : 2, mol/mol) bilayers

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Aqueous dispersions (pH 4.0) of a 2:1 (mol/mol) mixture of myristic acid with dimyristoylphosphatidylcholine undergo a sharp transition at 45–47 °C from a lamellar gel phase to a fluid phase which is optically isotropic. This fluid phase gives rise to ^{31}P-NMR spectra, and ^2H-NMR spectra of the chain-deuterated components, which are also isotropic. X-ray diffraction studies of the fluid phase at 49 °C, reveal reflections with spacings in the ratio $\sqrt{2}:(\sqrt{3}):\sqrt{4}:\sqrt{6}:\sqrt{8}$, accompanied by a strong diffuse scatter. These reflections index on a cubic lattice of primitive space group Pn3 or Pn3m, or possibly the body-centered group Im3m, with a lattice constant of 21.2 nm. The dimensions of the phase are consistent with a structure composed of two systems of tetrahedrally (octahedrally) oriented inverted lipid cylinders, found for other cubic lipid phases with Pn3m (Im3m) symmetry. At higher temperatures the cubic phase gradually converts, with increasing temperature, to a coexisting inverted hexagonal phase.

Introduction

Mixtures of fatty acids with diacyl phosphatidylcholines of the same chainlength form a 2:1 (mol/mol) stoichiometric compound which is characterized by a sharp chain melting phase transition [1–5]. For the palmitic chain system, it has been shown that the low temperature phase is a lamellar gel, but the high temperature fluid phase is non-lamellar, being of the inverted hexagonal (H_{II}) type [2]. A direct transition occurs from the gel phase to the inverted hexagonal phase in this system.

Of the other non-lamellar phases for lipid/water systems, those with cubic symmetry are known for systems with limited water content (see, for example, Ref. 6). Cubic structures with the space groups Pn3m and Ia3d are amongst those which have been characterized [7,8]. Recently, it has been found that cubic phases exist between the fluid lamellar and inverted hexagonal phases of both didodecyl- and dioleoylphosphatidylethanolamines for samples in excess water (Refs. 9 and 10, respectively). Thus they provide attractive models for localized intermediates in membrane fusion processes where the normal lamellar membrane topology is disrupted.

Here we show that the 2:1 myristic acid/dimyristoyl phosphatidylcholine system undergoes cooperative chain melting from a lamellar gel phase to an isotropic phase of cubic symmetry. The fluid phase has a space group consistent with Pn3m (or possibly Im3m) symmetry, and a large lattice constant of 21.2 nm. Either the repeating units display irregularities, or large parts of the phase do not possess long-range coherence.

Materials and Methods

Specifically deuterated myristic acid 2-d_2-MA was synthesized by exchange with alkaline ^2H_2O at elevated temperature, as described in Ref. 11. Perdeuterated myristic acid, d_{27}-MA, was obtained from Larodan, Malmö, Sweden. Deuterated dimyristoylphosphatidylcholines 2-d_2- and d_{27}-DMPC were synthesized from the corresponding deuterated myristic acids by coupling to 1-myristoyl-2-lysophosphatidylcholine according to the method of Ref. 12, except that a smaller excess of

Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; MA, myristic acid; *n*-d_2-DMPC, 1-myristoyl-2-([*n*-$^2H_2$]myristoyl)-*sn*-glycero-3-phosphocholine; d_{27}-DMPC, 1-myristoyl-2-([$^2H_{27}$]myristoyl)-*sn*-glycero-3-phosphocholine; *n*-d_2-MA, [*n*-$^2H_2$]myristic acid; d_{27}-MA, [$^2H_{27}$]myristic acid; H_{II}, inverted hexagonal phase; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance.

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fatty acid was used. The lysophosphatidylcholine was prepared from DMPC (Sigma, St. Louis, MO) by phospholipase A_2 digestion, under the conditions for the activity assay given by the suppliers (Boehringer, Mannheim, F.R.G.).

Lipid samples were prepared by first dissolving the required amounts of MA and DMPC in chloroform, reducing to dryness by rotary evapouration, and removing the traces of residual solvent under vacuum. Typically the samples contained 100–200 mg of MA. The dry lipid mixture was then warmed briefly to approx. 55 °C to obtain an isotropic melt, in order to ensure good mixing. The lipid was then suspended in 100 mM sodium acetate, 1 mM EDTA (pH 4.0) by vortex mixing, whilst cycling the temperature between room temperature and 65 °C. The dispersion was then transferred to a short, 10 mm diameter tube for NMR studies, or to a 1 mm diameter, fine-wall glass capillary for X-ray diffraction studies. In the latter case, the capillary was placed within a heated metal block, which could be used in a bench centrifuge.

High power ^{2}H-NMR spectra were recorded at a frequency of 46.1 MHz on a Bruker MSL-300 spectrometer, using the quadrupolar echo pulse sequence with full phase cycling. The $\pi/2$ pulse width was 6 μs and the interpulse spacing was 30 μs. Recycle delays were always in excess of $5 \times T_1$. Proton dipolar decoupled ^{31}P-NMR spectra were recorded at a frequency of 109 MHz on a Bruker WH-270 spectrometer operating in the Fourier transform mode. The decoupling power was approx. 20 W and the duty cycle of the gated decoupling was approx. 0.2%.

Polarizing microscopy was performed with an Optiphot Type 104 microscope from Nikon, equipped with a THM 600 thermostatted stage from Linkam Scientific Instruments.

X-ray diffraction measurements were performed using a Kratky small-angle X-ray scattering apparatus with slit geometry, which is described in Ref. 13. Sample capillaries were mounted in a massive copper housing which was thermostatted with a Peltier device. Temperature stability and gradients were better than 0.1 K. Nickel-filtered Cu Kα radiation ($\lambda = 0.154$ nm) was obtained from an AEG fine-focus tube, and diffracted intensity recorded with a linear position-sensitive detector mounted on an extension tube at a distance of 98.35 cm from the sample.

Results and Discussion

The temperature dependence of the ^{2}H-NMR spectra of 2-d_2-DMPC in a MA-DMPC (2:1, mol/mol) aqueous dispersion at pH 4.0 is given in Fig. 1. At low temperatures the ^{2}H-NMR powder patterns are broad and structureless, characteristic of the slow rotational motion of phospholipids in the gel phase (see, for example, Ref. [14]). At temperatures around 40 °C, the spectra narrow whilst maintaining approximately the same overall quadrupole splitting of 43.5 kHz, characteristic of the gel phase (cf. Ref. 15). At 45–47 °C, the sample goes through a sharp thermotropic phase transition from a gel to a fluid phase, as recorded by differential scanning calorimetry (data not shown). The ^{2}H-NMR spectra are dominated by the extremely sharp resonances from the fluid phase and record somewhat lower effective transition temperatures. At 42 °C there is a complex coexistence of two pairs of quadrupole splittings in the ^{2}H-NMR spectra. This pretransitional behaviour may indicate the coexistence of the lamellar gel phase and an intermediate fluid lamellar phase during the transition, the two deuterons being inequivalent in both phases. Above the transition, however, the fluid phase is one which gives rise to an isotropic ^{2}H-NMR spectrum, as seen by the spectrum at 46 °C. Only very small contributions from the lamellar powder pattern (observable at higher vertical gain) are still present in this spectrum. At 68 °C, the spectrum consists almost solely of the isotropic component. As the temperature is increased further to 84 °C, an axial powder pattern appears, with a small quadrupole split-

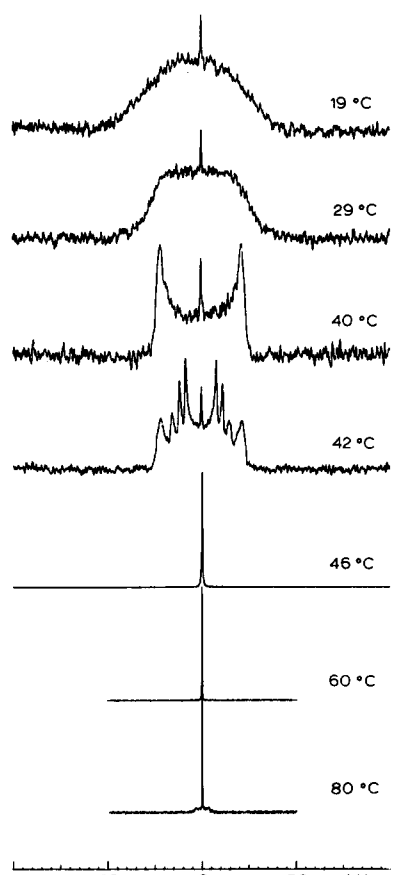

Fig. 1. ^{2}H-NMR spectra of [2-$^2H_2$]dimyristoylphosphatidylcholine in a myristic acid/dimyristoylphosphatidylcholine (2:1, mol/mol) dispersion at pH 4.0, as a function of temperature.

ting of 9 kHz, which is typical for an inverted hexagonal phase. The hexagonal powder pattern is dominated by the much sharper isotropic resonance in Fig. 1, but is clearly evident at higher vertical expansion. In addition, its relative intensity is greater in the NMR spectra of the deuterated MA component as compared with that of the deuterated DMPC.

Qualitatively similar effects were seen in the temperature dependence of the ^{2}H-NMR spectra of the corresponding ^{2}H-labelled myristic acid, as well as for other positions of labelling, and were also seen in the temperature dependence of the broad line ^{31}P-NMR spectra of the DMPC component. In the case of the ^{31}P-NMR spectra, the axial powder pattern observed at high temperature had a positive chemical shift anisotropy, which is indicative of a cylindrical structure such as the H_{II} phase (see also Ref. 20). The salient features of the temperature dependence deduced from all the NMR spectra were: a low temperature lamellar gel phase which undergoes a cooperative chain-melting transition to an isotropic phase, which then gradually converts to an inverted hexagonal phase. For a given temperature, the proportion of the hexagonal component to that of the isotropic component was greater for the myristic acid than for the DMPC. This suggests that the high temperature region may be one of phase separation between fluid components of different composition.

When the hydrated MA/DMPC (2:1, mol/mol) mixture at pH 4.0 is viewed under polarized light, the sample becomes optically isotropic at temperatures just above the main phase transition. This change corresponds to the appearance of the isotropic NMR spectrum. On further heating, a more granular structure gradually appears, which presumably corresponds to the hexagonal component seen in the NMR spectra. The focus here is primarily on the identity of the fluid phase which is optically isotropic when viewed under polarized light and which gives rise to isotropic ^{2}H- and ^{31}P-NMR spectra.

The temperature dependence of the low angle X-ray diffraction patterns (not corrected for the slit effect) of the MA/DMPC (2:1, mol/mol) mixture at pH 4.0 is given in Fig. 2. At 45°C, the first two orders of a lamellar diffraction pattern with repeat spacing of 6.75 nm are clearly seen. Measurements at lower temperature confirm that this corresponds to the lamellar gel phase. At 49°C, the diffraction pattern consists of a series of distinct reflections apparently superimposed on a background of diffuse scatter. This pattern corresponds to the optically isotropic phase which gives rise to isotropic NMR spectra. The reflections at very low angle at 49°C index on a cubic lattice, as indicated by the diffraction spacings which are given in Table I. At 60°C, the intensity of the diffuse scatter is reduced, the reflections at very low angle are extremely weak and have been shifted to shorter spacings, and a strong

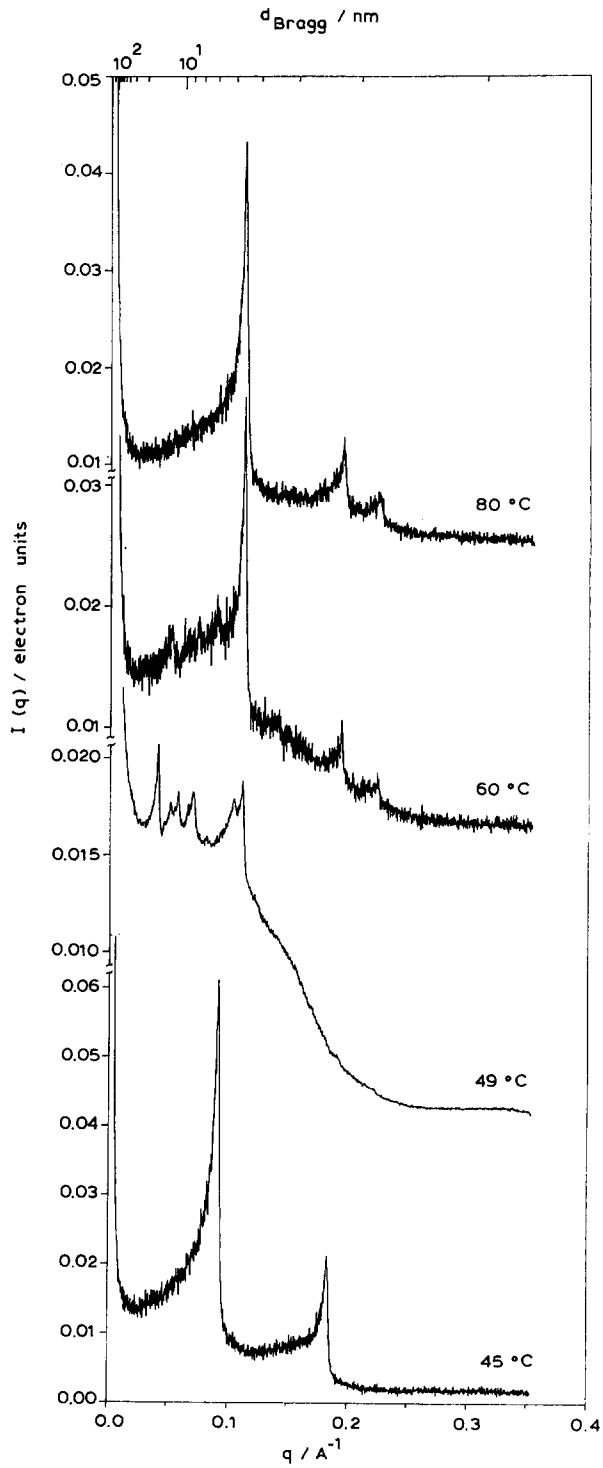

Fig. 2. Low-angle X-ray diffraction patterns of a myristic acid/dimyristoylphosphatidylcholine (2:1, mol/mol) dispersion at pH 4.0, as a function of temperature. From top to bottom, diffraction patterns were recorded at: 80°C, 60°C, 49°C and 45°C, respectively. Scattering intensity, not corrected for the slit geometry, is plotted as a function of the scattering vector, $q = 2\pi/\lambda$, and the repeat spacing, d_{Bragg}.

diffraction pattern with reflections in the ratio $1:\sqrt{3}:2$, corresponding to a hexagonal phase, is seen. At 80°C, the diffuse scatter is absent and only reflections from

TABLE I

X-ray diffraction spacings of the cubic phase of dimyristoylphosphatidylcholine/myristic acid (1:2, mol/mol) mixture, pH 4.0, at 49 °C

h k l	d_{hkl} (nm)	*a* (nm)
1 1 0	15.0	21.3
1 1 1	12.0	20.8
2 0 0	10.6	21.2
2 1 1	8.68	21.3
2 2 0	7.53	21.3

the hexagonal phase are apparent. The first order of this pattern corresponds to a repeat spacing of 5.55 nm, i.e., a hexagonal lattice constant of 6.4 nm.

The indexing of the reflections of the cubic phase at 49 °C is illustrated in the expanded region of the pattern in Fig. 3. Also seen is the first order [10] of the H_{II} phase which grows in at higher temperature. The peak at the inner flank of this reflection has not been identified. It would correspond to a fundamental repeat of 6.0 nm and does not index on the cubic lattice. The presence of the 110, 111 and 200 reflections, together with the absence of the 100 and 210 reflections, suggests that the diffraction pattern from the cubic phase corresponds to the first five orders of the primitive space group Pn3m or possibly Pn3 [16]. In particular, the body-centered cubic space group, Ia3d, which is also observed in some lipid-water systems [6,7] can be excluded. Other cubic structures which have recently been identified in lipid systems [18] are also not compatible with this indexing. A possible exception is the body-centred space group Im3m, which is distinguished from Pn3m only by the absence of the [111] reflection over the range of observed reflections. The reflection assigned to [111] is relatively weak and variable in its appearance. Therefore the Im3m space group cannot be completely excluded.

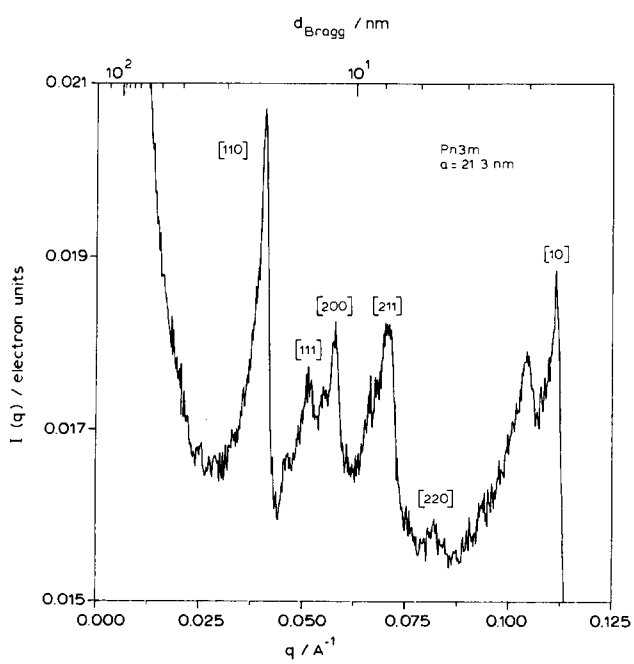

Fig. 3. Expanded version of the low-angle diffraction pattern of a myristic acid/dimyristoylphosphatidylcholine (2:1, mol/mol) dispersion at pH 4.0, recorded at 49 °C. Indexing of the reflections [*hkl*], according to Table I is indicated. The reflection designated [10] is the first order of the hexagonal diffraction pattern. Data are presented as described in the legend to Fig. 2.

From Table I, the cubic lattice constant is seen to be 21.2 nm, which is considerably larger than that observed for several other lipid-water phases with space group Pn3m/Pn3. For instance, the lattice constant of the cubic phase of glycerol mono-oleate is 10.4 nm [8], and those of didodecyl- and dioleoylphosphatidylethanolamines are 9.0 nm [9] and 12.2 nm [10], respectively. The background scatter seen in Fig. 2 may therefore correspond to the loss of long-range coherence in parts of the cubic phase, or to irregularities in the repeating unit. It is interesting to note that a fluid phase characterized by an intense diffuse scatter, extending to large spacings, has been observed in 1:1 (mol/mol) mixtures of palmitic acid and dipalmitoylphosphatidylcholine [17]. In this latter case, however, no sharp reflections were detected.

A model has been established for the structure of the cubic lipid phase of space group Pn3m/Pn3. This consists of two interpenetrating networks of tetrahedrally oriented cylinders with an aqueous core [8,18]. Expressions for the dimensions of this phase have been given in Ref. 19. Taking the mean molecular weight and partial specific volume of the MA/DMPC lipid system, respectively as 577 and 1.028 $cm^3 \cdot g^{-1}$, the dimensions and composition of the phase may be estimated from the lattice spacing, for various values of the mean molecular area, A, per two lipid chains. For $A = 0.6$ nm^2, the radius of the water cylinders is $R = 6.2$ nm, the maximum and minimum lengths of a lipid molecule are $L_{max} = 2.5$ and $L_{min} = 1.3$ nm, and the mass concentration of lipid is $c = 0.31$. The value of L_{max} is less than that for a DMPC molecule with all-*trans* chains and that for L_{min} is less than that for a fully extended MA molecule. Therefore these values lie within the realistic range. The cubic lipid phase with space group Im3m has been characterized by two interpenetrating networks of octahedrally oriented cylinders with an aqueous core [18]. The dimensions of the phase have been given in Ref. 18. For an area per molecule $A = 0.6$ nm^2, the radius of the water cylinders would be correspondingly $R = 4.8$ nm and the mass concentration of lipid $c = 0.38$. The predicted water concentration and the value of R, in either case, suggest a very highly hydrated structure. This may account for the lack of long-range coherence, or alternatively irregularities in the aqueous cylinders, which is most evident in the X-ray scattering profiles at higher angles.

In summary, the fatty acid/phosphatidylcholine (2 : 1, mol/mol) mixture with myristoyl chains differs from that previously investigated with palmitoyl chains [2]. A sharp thermotropic transition takes place from a lamellar gel phase to an inverted phase with cubic rather than hexagonal structure. This demonstrates that a rich range of lipid polymorphism may be displayed by simple fatty acid/phospholipid mixtures. Such results may have implications for the functional role of fatty acids in cases where they are (transiently) present in high local concentrations in biological membranes, e.g., as a result of the action of lipolytic enzymes.

Note added in proof: (Received 25 April 1990)

In a recent neutron diffraction study, Seddon et al. [21] have observed three reflections from a cubic phase, in addition to a hexagonal phase, for a MA/DMPC (2 : 1, mol/mol) mixture. These reflections index as the first three orders of the Im3m space group, with a lattice constant that decreases steeply with increasing temperature from 20.5 nm at 54.5 °C to 15.1 nm at 65.9 °C. A [111] reflection was not detected in this study. We thank Dr. J.M. Seddon for correspondence on this topic.

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