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Mechanism and kinetics of the subtransition in hydrated L-dipalmitoylphosphatidylcholine

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The mechanism of the subtransitions (L_c to $L_{\beta'}$) in L-dipalmitoylphosphatidylcholine bilayers in excess water has been investigated by time-resolved X-ray diffraction using synchrotron radiation. The temperature dependence of the diffraction patterns closely correlate with the asymmetric excess specific heat variation recorded by differential scanning calorimetry. During the subtransition two prominent wide-angle reflections, characteristic of the low-temperature crystalline phase, L_c, gradually change such that a sharp peak at a spacing of 0.430 nm decreases in intensity and ultimately disappears while a broader peak initially located at 0.375 nm progressively shifts to an eventual spacing of 0.410 nm. This behaviour is interpreted as a lateral deformation of the acyl chain packing subcell as the chains begin to rotate until a state is reached where the chains pack on a regular hexagonal array chracteristic of the L_β phase. An increase in lamellar repeat distance from 6.0 to 6.4 nm takes place simultaneously with the acyl chain rearrangement at relatively low (5 K/min) as well as high (6 K/s) heating rates. As judged from the shape of the wide-angle peak, transformation to $L_{\beta'}$ phase occurs some minutes after transition to the L_β phase. The X-ray data characterise the subtransition as a continuous (second order) phase transition in which a presumably orthorhombic subcell is transformed into a hexagonal subcell in a gradual process. In temperature jump experiments at 6 K/s between 0°C and 80°C the relaxation time of the subtrasition was found to be about 5 s while the relaxation time of the main gel to liquid-crystalline transition was about 2 s.

Introduction

Phase transitions and structure of 1,2-dipalmitoylphosphatidylcholine-water systems have been the subject of many studies. Two transitions, namely, lamellar gel to gel ($L_{\beta'} \rightarrow P_{\beta'}$) and gel to liquid-crystalline ($P_{\beta'} \rightarrow L_\alpha$), for example have been fully characterised by calorimetry and diffraction methods [1–7]. They are observed in heating experiments at approximately 34°C and 41°C. Apart from these transitions, a subtransition at about 18°C has been identified by scanning calorimetry in L-dipalmitoylphosphatidylcholine [7] but not in the racemic modification [8]. The crystalline phase is formed when the $L_{\beta'}$ phase is stored for 3.5 days at 0°C. The formation and structure of the low-temperture equilibrium phase (L_c) has been examined by X-ray diffraction methods [9–11] and is characterised by a rotationally more ordered hydrocarbon chain arrangement than the $L_{\beta'}$ form. It was also reported that the subtransition corresponds to a structural

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transformation from the ordered low-temperature crystalline form directly to the $L_{\beta'}$ phase. The kinetics of formation of the L_c phase have been investigated by relatively fast-recording X-ray diffraction methods using a rotating anode and a linear position-sensitive detector to determine parameters associated with the $L_{\beta'}$ to L_c transition as the dispersion undergoes an isothermal phase transition upon standing at $-2°C$ [11]. Two successive stages in the transition were observed over a period of about 15 h. The first involved a rapid shift over the first hour of storage of the two characteristic $L_{\beta'}$ chain spacings and a slower change in these spacings to their position in the L_c phase accompanied by a progressive decrease in the lamellar repeat spacing. The latter change was found to be the result of an expulsion of water from the interlamellar space.

The equipment used in the above kinetic studies was sufficient to resolve isothermal structural changes that take place over periods of hours but different methods are necessary to examine the subtransition in the heating direction. The present report is addressed to structural changes associated with the subtransition in L-dipalmitoylphosphatidylcholine dispersed as multibilayer liposomes in excess water using synchrotron X-ray radiation. Studies have already been reported on the kinetics of lamellar phase transitions in saturated phosphatidylcholines using synchrotron radiation [12–14] where the transition time of $L_{\beta'} \rightarrow L_\alpha$ phase transitions are found to be in the order of a few seconds. The relatively high intensity of synchrotron X-rays enables an improvement in time resolution of some 4 or more orders of magnitude over conventional X-ray sources. Thus, this method is able to provide information concerning the precise mechanism of phase transitions that take place on a time-scale of seconds. In the present study structural changes have been related to thermotropic behaviour, as studied by differential scanning calorimetry, and kinetic measurements on a time-scale of 100 ms have been made during temperature jumps through the subtransition to the L_α phase.

Materials and Methods

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine was purchased from Fluka AG. The lipid was checked by thin-layer and gas chromatography and found to be at least 99% pure. The hydrated samples were prepared by equilibration of appropriate amounts of phospholipid dispersed in 50 mM Hepes (pH 7.2) at 60°C for 6 h. Prior to thermal and X-ray analysis the suspensions were stored for 4 days at 0°C. The lipid concentration was 25% (w/v) for the X-ray study and Microcalorimetry using a DuPont 1090 Thermal Analizer and 0.0003% (w/v) for microcalorimetry using a Privalov differential adiabatic scanning calorimeter (DASM-1M).

Thermograms of concentrated samples (25%, w/v phospholipids) were recorded at 5 K/min using a DuPont 1090 differential scanning calorimeter and compared with thermograms of diluted samples (0.0003%, w/v phospholipid) recorded at 0.5 K/min using a high sensitivity DASM-1M calorimeter. Although the main transition recorded by high-sensitivity differential scanning calorimetry is characterized by several-fold greater cooperativity, the parameters of the subtransition recorded by the two methods were found to be virtually identical. For comparison with X-ray data thermograms recorded by high-sensitivity differential scanning calorimetry have been used.

X-ray experiments were carried out using a monochromatic (0.150 nm) focussed X-ray beam at station 7.25 of the Daresbury Synchrotron Laboratory as previously described [15]. A cylindrically bent single crystal of Ge [16] and a long float glass mirror were used for monochromatization and horizontal focussing, providing $2 \cdot 10^9$ photons/s down a 0.2 mm colimator at 2.0 GeV and 100 to 200 mA of electron beam current. A Keele flat plate camera was used with a linear detector constructed at Daresbury. The dead-time between data acquisition frames was 50 μs, with the temporal resolution of each frame varying from 100 ms for the temperature jump experiment to 1.25 s for temperature scans. X-ray scattering has been plotted as a function of reciprocal spacing, $s = 2 \sin \theta/\lambda$, using teflon (0.48 nm) as a calibration standard [17]. No corrections were applied to path distances from the sample to the linear detector consequently wide-angle spacings will be slightly longer than measured directly by the detector. All mesophase and subcell spacings

were calculated using Bragg's Law [18].

Temperature jumps were produced by water baths connected internally to the sample mount of the X-ray camera; the rate of change of temperature was about 6 K/s. The temperature of the sample was monitored internally using a thermocouple placed adjacent to the sample in the X-ray sample holder. We believe that the thermal diffusion through our samples was approximately the same as that observed in experiments reported by Caffrey [19] since our sample thickness was also 1 mm.

The use of high intensity synchrotron X-ray sources can result in radiation damage of the lipids and structural perturbation of the sample. The first investigation on this problem was carried out by Caffrey [20] who described a number of marked changes in the X-ray diffraction patterns following the damage of dipalmitoylphosphatidylcholine at sufficiently long exposures (up to 1 h). In our experiments the X-ray beam was several times less intense and typical exposure times of 5 min for the scans and less than 30 s during the temperature-jumps would minimize damage. We did not observe any changes in X-ray behaviour or visual appearance of the samples which would indicate noticeable radiation damage and conclude on this basis that the thermotropic behaviour of the samples was not affected by any degradation products that may have been produced. A study on the effect of longer exposures on possible radiation damage is currently in progress.

Results

Structure of the L_c phase

The static wide-angle X-ray pattern of L-dipalmitoylphosphatidylcholine at temperatures below the subtransition temperature show four orders of a lamellar repeat of 6.0 nm and a sharp and broad peak in the wide-angle region at spacings $d_1 = 0.430$ nm and $d_2 = 0.375$ nm, respectively (Fig. 1). The diffraction profile is in close agreement with earlier studies in which values of $d_1 = 0.440$ nm ad $d_2 = 0.387$ nm were reported [10,11]. These bands have been interpreted as representing a hydrocarbon chain arrangement that is rotationally more ordered than that of the $L_{\beta'}$ phase;

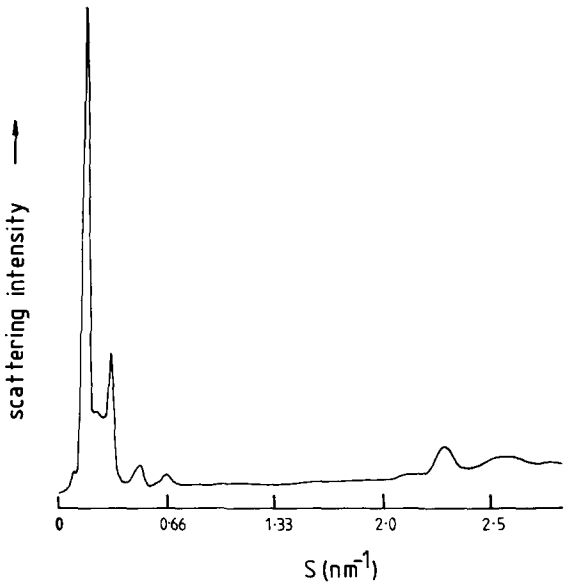

Fig. 1. X-ray diffraction pattern of L-dipalmitoylphosphatidylcholine equilibrated 4 days at 0°C and recorded in 1.2 s at 12°C.

it is assumed to be in an orthorhombic hybrid subcell [11]. A cross-section of this cell, designated HS1 by Abrahamsson et al. [21], parallel to the

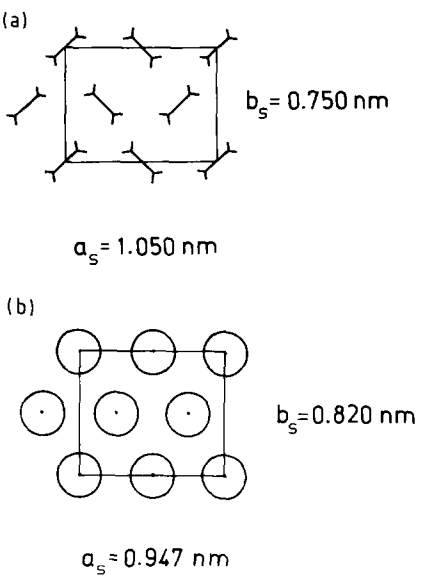

Fig. 2. Proposed cross-sections parallel to the bilayer plane of the acyl chain packing in the L_c phase (a) showing the orthorhombic hybrid subcell (HS1) and (b) immediately after heating above the subtransition temperature where the chains pack on an (ortho)hexagonal subcell (see text for details).

bilayer plane is presented in Fig. 2. Its dimensions, a_s and b_s, were determined by Ruocco and Shipley [11] from the relations

$$a_s = 4d_1 \cdot \tan[\sin^{-1}(d_2/2d_1)]; \quad b_s = 2d_1 \tag{1}$$

which follow an assignment of the two reflections d_1 and d_2 to diffraction planes (020) and (210) of HS1, respectively. The values $a_s = 0.862$ nm and $b_s = 0.880$ nm were obtained in this way (Fig. 11 in Ref. 11). However, the assignments of d_1 and d_2 are ambiguous and can be transposed without any limitations thus producing the version shown in Eqn. 2

$$a_s = 4d_2 \cdot \tan[\sin^{-1}(d_1/2d_2)]; \quad b_s = 2d_2 \tag{2}$$

which gives substantially different values of a_s and b_s ($a_s = 1.070$ nm and $b_s = 0.774$ nm with the data in Ref. 11, and $a_s = 1.050$ nm and $b_s = 0.750$ nm with our data about d_1 and d_2). While there are no apparent physical arguments resolving this ambiguity a comparison can be made with a_s and b_s values for other lipids packed in HS1 including dilauroyl- and dipalmitoylphosphatidylethanolamines and cholesteryl 17-bromoheptadecanoate [21]. The three examples of HS1 arrangement are characterized by $a_s \approx 1$ nm and $b_s \approx 0.75$ nm. These values are comparable with the corresponding values for L-dipalmitoylphosphatidylcholine in the L_c phase derived from Eqn. 2, and quite different from those computed from Eqn. 1. On these grounds, we can conclude that Eqn. 2 provides a more plausible basis for determination of the structural parameters of the L_c phase. The same argument applies even if another type of chain packing is contemplated. A large number of simple orthorhombic subcells which are one half of HS1, for example, are characterized by $a_s \approx 0.5$ nm and $b_s \approx 0.75$ nm (Table I in Ref. 21).

Mechanism and order of the subtransition

The thermal characteristics of the subtransition in L-dipalmitoylphosphatidylcholine are shown in Fig. 3 which presents a differential scanning calorimetric heating curve recorded at a scan rate of 0.5 K/min. The temperature and transition enthalpy calculated from the scan are in agreement with earlier reports [8]. An overview of the

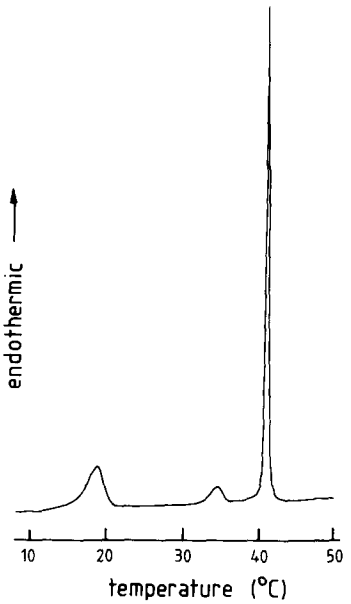

Fig. 3. Differential scanning calorimetric heating thermogram of an aqueous multilamellar dispersion of L-dipalmitoylphosphatidylcholine stored for 4 days at 0°C. The sample preparation for the DASM-1M calorimeter and scan conditions are described in Methods.

corresponding structural changes that take place upon heating the L_c phase through the subtransition is presented in Fig. 4 which is a three-dimensional plot of X-ray scattering intensity versus reciprocal spacing recorded at intervals during a temperature scan at the rate of 5 K/min. The first frame was recorded at 12°C and successive frames corresponding to 0.5 K change in temperature are plotted. Low-angle lamellar repeat spacings with at least four orders of diffraction can be seen on the right of the data set and prominent wide-angle diffractions arising from the acyl chain packing on the left. The subtransition is clearly distinguished in the wide-angle region where the peaks characteristic of the L_c phase merge into one peak typifying the L_β phase over the range of temperature 15°C to 19°C.

A more detailed picture of the changes in the wide-angle region of the diffraction pattern is shown in Fig. 5 in which particular frames of the data set corresponding to different temperatures through the subtransition are plotted. The sharp diffraction peak at 0.430 nm undergoes a progressive decrease in intensity on passing through the

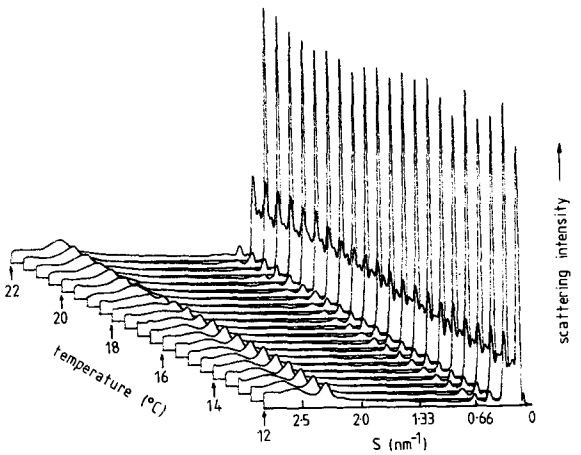

Fig 4. Three-dimensional plot of X-ray scattering intensity versus reciprocal spacing as a function of temperature of a sample of L-dipalmitoylphosphatidylcholine equilibrated for 4 days at 0°C. The sample was heated from 0°C at a rate of 5 K/min and the diffraction data recorded over the temperature range 12°C to 22°C is plotted. Dispersions were accumulated over 1.2 s and frames corresponding to intervals of 0.5 K are shown.

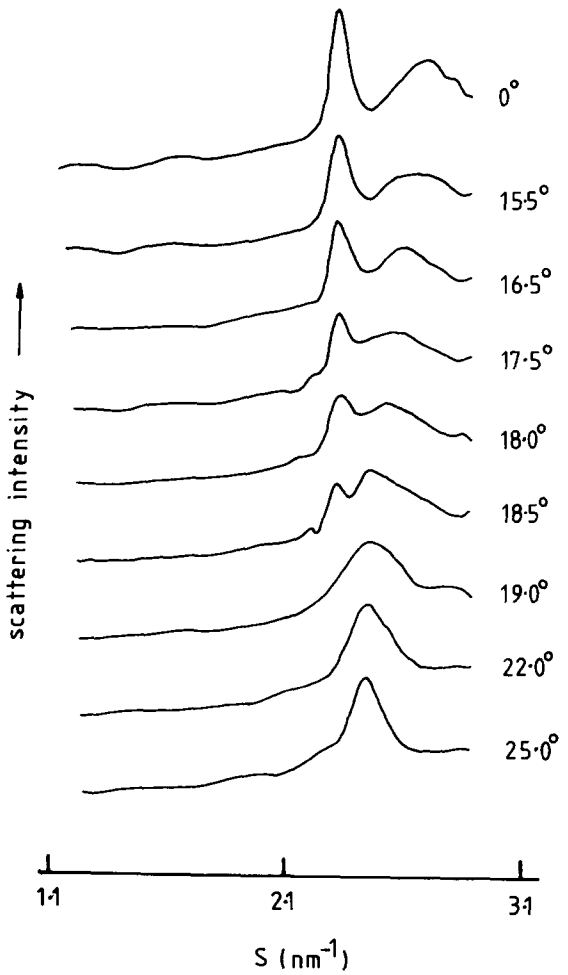

Fig. 5. Plot of representative X-ray scattering profiles of the wide-angle reflections from L-dipalmitoylphosphatidylcholine during heating through the sub-transition at 5 K/min.

subtransition while the broader peak initially at a spacing of 0.375 nm shifts gradually to an ultimate spacing of 0.410 nm. The structural parameters associated with lamellar and chain spacings are plotted together with the corresponding thermal data in Fig. 6. It shows clearly that the increase of the lamellar repeat distance from 6.0 to 6.4 nm during the subtransition occurs simultaneously with the structural rearrangement of the acyl chains reflected in the wide-angle pattern. The relationship of the diffraction changes with the endothermic phase transition recorded by high-sensitivity differential scanning calorimetry is also illustrated in Fig. 6 in the form of the integral of the excess specific heat associated with the subtransition obtained from the thermogram shown in Fig. 3. Although a complete coincidence between the thermal and structural parameters may not be expected because of a less than precise temperature control in the X-ray measurements, it can be seen that the structural changes are closely correlated with the endothermic event. A convincing and interesting feature of this correlation is that the clearly observed asymmetry of the subtransition in the thermogram is also manifest in the temperature dependence of the structural parameters.

Examination of the wide-angle region of the diffraction pattern immediately after completion of the subtransition did not show evidence of a shoulder on the peak centred at 0.410 nm which, according to the interpretation of previous X-ray studies [3], would indicate that the hydrocarbon chains are inclined with respect to the bilayer normal. A shoulder, however, was clearly distinguished when diffraction patterns were recorded 12 min after completion of the subtransition (Fig. 7). This suggests that the L_c phase is transformed firstly into an L_β phase and subse-

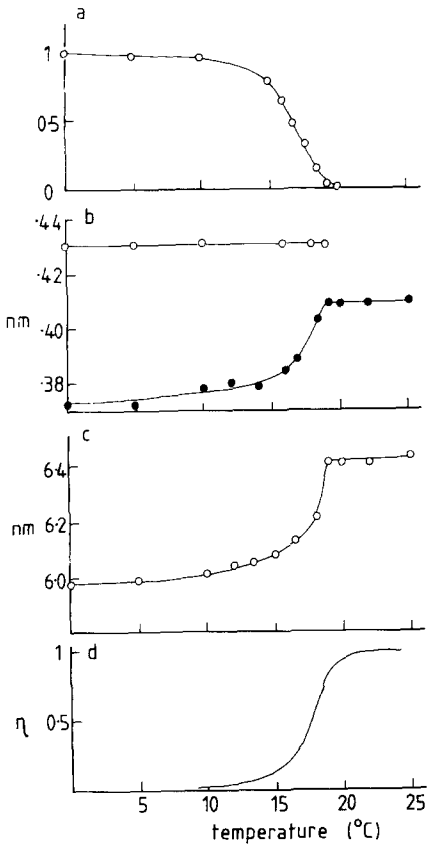

Fig. 6. Changes in parameters associated with the acyl chain packing and lamellar repeat spacing in L-dipalmitoylphosphatidylcholine during heating through the subtransition at 5 K/min. (a) Relative intensity of the sharp wide-angle diffraction peak; (b) diffraction spacing of the sharp (○) and broad (●) wide-angle diffraction peaks; (c) *d*-spacing of the lamellar repeat; (d) integral of the excess specific heat,

$$\eta = \int_{T_0}^{T} C_p \, dT/\Delta H_{cal}, \quad T_0 = 8°C$$

obtained from the thermogram shown in Fig. 1.

quently into an $L_{\beta'}$ phase rather than proceeding directly from L_c to $L_{\beta'}$. It is noteworthy that the lamellar *d*-spacing remains at a value of 6.4 nm during the shape change in the wide-angle pattern shown in Fig. 7.

Kinetics of the subtransition

The relaxation time of the subtransition was investigated by temperature jumps from 0°C to 80°C at an average rate of temperature change of 6 K/s. Diffraction data was recorded with an

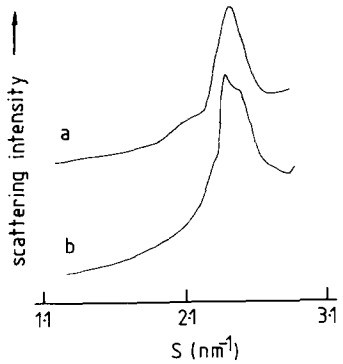

Fig. 7. Wide-angle diffraction patterns (a) recorded at 22°C immediately after a scan at 5 K/min through the subtransition (b) recorded from the same sample 12 min later.

acquisition time of 100 ms for each of 255 successive data frames. Individual 100 ms data frames show considerable fluctuation both in position and shape of the diffraction peaks (Fig. 8a). The significantly greater fluctuations of the broad wide-angle peak suggests that molecular motion along the b_s axis of the subcell is less constrained

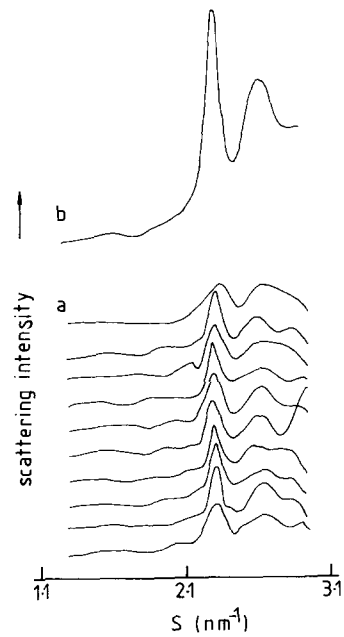

Fig. 8. (a) Wide-angle diffraction intensity recorded in 10 successive time frames each of 100 ms recorded during a temperature jump through the subtransition; temperature of lowest frame, 22°C and highest frame 28°C (b) sum of individual frames shown in (a).

than along the a_s axis. However, summation over 10 successive frames, which is equivalent to accumulation time of 1 s, is sufficient to average these fluctuations (Fig. 8b). Further increase of the data acquisition time up to 100 s does not change the features of the diffraction pattern recorded in 1 s.

A three-dimensional plot of a representative sequence of frames recorded during a jump is presented in Fig. 9. An inspection of the relevant 210 frames of the complete data set shows that the wide-angle diffraction pattern typical of the L_c phase persists for about 3.5 s after the temperature has increased above 20°C. During this time the lamellar repeat distance retains its characteristic L_c value of about 6.0 nm. The first L_β frames appear among the L_c frames at about 7 s (42°C). A mixed sequence of L_β and L_c frames then follows for 1.5 s until the last remnants of the L_c phase eventually disappear from the diffraction pattern. In these 1.5 s the lamellar repeat distance increases from its L_c to its L_β value. Thus, the overall relaxation time is about 5 s in these conditions although a mixed sequence of L_β and L_c frames and an increase of the lamellar repeat distance are observed only during the last 1.5 s.

The behaviour of the two wide-angle L_c peaks is similar to that observed during the temperature scans at 5 K/min. Here again the sharp peak

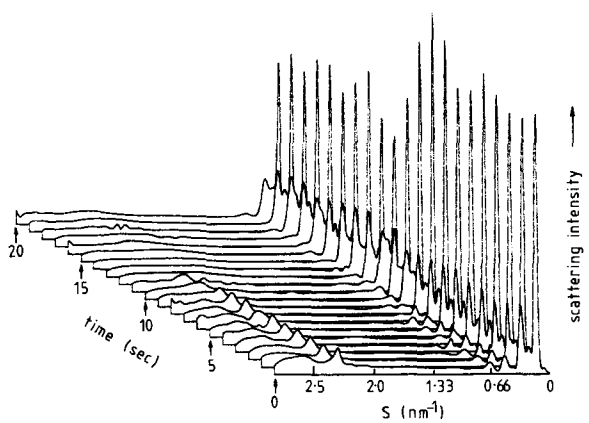

Fig. 9. Three-dimensional plot of X-ray scattering intensity versus reciprocal spacing as a function of time of an aqueous dispersion of L-dipalmitoylphosphatidylcholine equilibrated at 0°C for 4 days and subjected to a temperature jump at 6 K/s above the L_α phase transition temperature. The data set consisted of 255 consecutive frames each of 100 ms duration; every 10th frame from frames 1 to 210 are shown in the plot.

decreases and finally disappears while the broad peak shifts to longer spacings. A specific feature which is absent from the scans is that the wide-angle peak in the first several frames in which the L_β structure can be recognised is rather broad although the position of the peak is not changed. After complete disappearance of the L_c phase the L_β peak sharpens rapidly to its typical width and persists for about 2 s until eventually giving way to the broad diffraction band characterizing conversion into the L_α phase. Thus the relaxation time of the gel to liquid-crystalline phase transition in dipalmitoylphosphatidylcholine is about 2 s which is in close agreement with results published by Caffrey and Bilderback [12].

Discussion

The present experiments clearly demonstrate the advantages of high intensity synchrotron radiation in diffraction analysis of phase changes in lipid-water systems. An analysis of the behaviour of the diffraction peaks arising from the acyl chain packing for example, provides a valuable insight into the mechanism and order of the subtransition in dipalmitoylphosphatidylcholine. The static pattern obtained for the wide-angle spacings of phospholipid in the L_c phase shows a sharp peak (0.43 nm) and a broad peak (0.375 nm). The difference in peak shape suggests that the order of the chains in the two dimensional unit cell are different for the respective coordinates i.e. motion in one direction indexed by the sharp Bragg reflection is relatively constrained while that in the other direction is of greater amplitude. This effect of motional freedom within the lattice is apparent when the system is heated through the subtransition.

The time-resolved X-ray data indicates that the subtransition is not a two-state but rather a continuous process. This is especially apparent in the wide-angle diffraction patterns which show a gradual conversion in the crystalline L_c phase into L_β phase through intermediate states characterised by intermediate positions of the broad wide-angle band (Figs. 5 and 6). This conclusion is supported by the low-angle data which show a monotonous increase of the lamellar repeat distance from 6.0 nm in the L_c phase to 6.4 nm in the L_β phase and provides no evidence for co-ex-

istence of these two structures.

The continuous nature of the structural rearrangement observed during the subtransition is a specific and distinctive mark of second-order phase transitions [22]. A second-order transition separates crystalline phases of different symmetry such that the high-temperature lattice, characterized usually by a higher symmetry, can be obtained by a continuous deformation of the low-temperature lattice. For example, a two-dimensional rhombic lattice can be converted into a square lattice by a suitable deformation. A similar geometric interpretation can be proposed for the molecular rearrangement during the subtransition. Before the subtransition (in the L_c phase) the acyl chains are packed in orthorhombic subcells where chain rotation on the long axis is frozen out. An increase of the rotational disorder during the subtransition will result in a tendency of the chains to assume a cylindrical effective shape. This will cause a lateral deformation of the orthorhombic subcell such that there is an elongation along b_s and a respective contraction along a_s until a final state is reached where the acyl chains packed on a regular hexagonal array. This picture accounts in broad outlines for the observed temperature dependence of the X-ray patterns and is consistent also with the data concerning the thermal response of orthorhombic subcells. With an increase of temperature, the b_s value of these cells increases while a_s remains constant or slightly decreases [21].

Of particular interest is the fact that the subtransition involves conversion of the L_c phase to the L_β phase and not directly to the $L_{\beta'}$. This provides further support for the idea that initially disorder in the chain packing involves increased rotation of the chains about an axis perpendicular to the bilayer plane until the chains are able to pack onto a regular hexagonal subcell whereupon tilting of the molecule can subsequently occur in formation of the $L_{\beta'}$ phase.

Despite the general correspondence, the evolution of the diffraction patterns upon heating through the subtransition is not precisely a reversal of the picture observed by Ruocco and Shipley [11] during a slow isothermal low-temperature conversion $L_{\beta'}$ to L_c. The differences obviously reflect the somewhat different pathways of the L_c to $L_{\beta'}$ and $L_{\beta'}$ to L_c transitions which actually take place in quite different time-scales and temperature conditions.

The reduction of the lamellar repeat distance during isothermal conversion of the gel phase at $-2°C$ into the crystalline phase has been shown to be associated with a reduction of interlamellar water from 19 to 11 molecules per phospholipid [11]. It is relevant to note, however, that the increase of this *d*-spacing during heating through the subtransition is fast enough and occurs synchronously with the acyl chain rearrangement even at high (6 K/s) heating rates. This observation indicates that either penetration of water between the bilayers occurs very rapidly or additional hydration is not a primary cause of the increase of the lamellar spacing.

The kinetic study revealed that the relaxation time of the subtransition is longer than the main gel to liquid-crystalline phase transition in dipalmitoylphosphatidylcholine and is about 5 s compared with 2 s for the main transition. With a heating rate of 6 K/s, the L_c phase was stable for 3.5 s after the temperature had increased above 20°C and after that was converted to the gel phase. This phase remained for about 1.5 s before it was in turn converted to the L_α phase. Careful examination of the data sets indicated that there was considerable fluctuation within the system and appearance, disappearance and reappearance of particular phases during temperature jumps. Clearly further study will be required to fully resolve the processes associated with these kinetic measurements.

Acknowledgements

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