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Lamellar gel–lamellar liquid crystal phase transition of dipalmitoylphosphatidylcholine multilayers freeze-dried from aqueous trehalose solutions. A real-time X-ray diffraction study

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The mechanism of the phase transition of dipalmitoylphosphatidylcholine multilayers freeze-dried from fully hydrated gel phase ($L_{\beta'}$) in the presence of trehalose has been investigated by real-time X-ray diffraction methods. Sequential diffraction patterns were recorded with an accumulation time of 3 s during heating and 1.2 s during cooling between about 20 and 80°C. A transition is observed in the range 47–53°C that involves structural events typical of a lamellar gel–lamellar liquid-crystal (L_{β}–L_{α}) transformation. This transition is completely reversible with a temperature hysteresis of 2–3°C and thereby resembles the main phase transition of fully hydrated dipalmitoylphosphatidylcholine multilayers. The mechanism of the transition from L_{β} to L_{α} as seen in the wide-angle scattering profiles show that the sharp peak at about 0.41 nm, characteristic of the gel phase, broadens and shifts progressively to about 0.44 nm towards the end of the transition. A temperature jump of 6C°/s through the phase transition region of a freeze-dried dipalmitoylphosphatidylcholine: trehalose mixture (molar ratio 1:1) showed that the phase transition had a relaxation time of about 2 s which is similar to that of the main transition in the fully hydrated lipid. X-ray diffraction studies of the melting of dipalmitoylphosphatidylcholine freeze-dried from the lamellar-gel phase in the absence of trehalose showed a transition at above 70°C. The low-angle diffraction data of phospholipid/trehalose mixtures are consistent with an arrangement of trehalose molecules in a loosely packed 'monolayer' separating bilayers of phospholipid. Trehalose appears to reduce the direct interbilayer hydrogen bond coupling thereby modifying the thermal stability and the phase transition mechanism of the bilayers.

Introduction

The study of phase transitions and the phase behaviour of phospholipids in non-aqueous media provides valuable information relating to the fundamental role of the solvent in the stability of model and biological membranes. The structure and phase behaviour of phosphatidylcholines have been examined in organic [1–3] and inorganic [4] solvents and the properties compared to that observed in water [5,6]. Recent interest has centered on a characterisation of phospholipid dispersions hydrated in the presence of high concentrations of trehalose, a non-reducing disaccharide of glucose [7–12]. These studies have been motivated largely

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by the fact that the ability of some species to survive complete dehydration correlates with the metabolism of trehalose [13–16]. The sugar accumulates in these organisms before dehydration, and during subsequent rehydration, it is hydrolysed to liberate two molecules of glucose [16]. It is widely believed that the action of trehalose is to modify the physico-chemical properties of cell membranes such that they are stabilised when dried. This is consistent with reports that trehalose modulates the thermal behaviour of anhydrous phosphatidylcholine (7,10–12). Increasing concentrations of trehalose progressively lowers the phase transition temperature of lyophilised dipalmitoylphosphatidylcholine from above 70 °C to close to that observed in fully hydrated dispersions of the phospholipid. The same effects of trehalose have been reported in mixtures freeze-dried from organic solvents [7,10] or from aqueous trehalose solutions [11,12].

The protective action of trehalose on organisms subjected to dehydration has been rationalised on the basis of a, so-called, water replacement hypothesis [13,17–19]. According to this theory trehalose substitutes for molecules of water bound to the membrame surface thereby preventing membrane damage during dehydration. Useful information about the organisation of phospholipid/trehalose mixtures may be obtained by X-ray diffraction methods. Although detailed structural parameters of such mixtures can be derived under static conditions using conventional X-ray methods, they are not able to provide dynamic information relevant to the transition between phases.

We have undertaken the present study using the high-intensity X-rays from a synchrotron source to follow, in real-time, the structural events during heating and cooling of multilayers of dipalmitoylphosphatidylcholine freeze-dried in the presence of trehalose from an initially hydrated gel (L_β) state. We confirm, by direct structural parameters, that the presence of trehalose in dipalmitoylphosphatidylcholine freeze-dried from aqueous solutions preserves an ordered bilayer arrangement of the phospholipid and reduces the temperature of the gel to liquid-crystal phase transition. The mechanism of this phase transition is shown to be similar to that in hydrated lipids. Finally, temperature jump experiments have been performed to measure the relaxation time of the freeze-dried trehalose/dipalmitoylphosphatidylcholine mixtures.

Materials and Methods

Sample preparation. Synthetic 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine was purchased from Fluka AG and trehalose from Sigma (London). The phospholipid was found to be more than 99% pure by thin-layer and gas chromatography. Multilamellar dispersions were prepared by hydrating the dried phospholipid with excess deionised distilled water or 1 M trehalose at 20 °C with phospholipid concentrations to give molar ratios of 1 : 1 and 1 : 4, phospholipid/trehalose. The dispersions were equilibrated for 1 h at 60 °C after vortexing at this temperature for 3–4 min. The dispersions were finally equilibrated at 20 °C for 1 h and then rapidly frozen by immersion in liquid nitrogen and lyophilised. Gravimetric moisture evaluations showed that the amount of residual water in samples freeze-dried by this procedure is in the range 5–6 wt.% [11,12].

X-ray measurements. Real-time X-ray measurements were performed using a monochromatic (0.150 nm) focussed X-ray beam at station 7.25 of the Daresbury Synchrotron Laboratory as previously described [20]. A cylindrically bent single crystal of Ge [21] and a long float glass mirror were used for monochromatisation and horizontal focussing, providing $2 \cdot 10^9$ photons per s down a 0.2 mm collimator at 2.00 GeV and 200–400 mA of electron beam current. A Keele flat plate camera was used with an area detector constructed at Daresbury modified to act as a linear detector. X-ray scattering intensity was recorded in data sets of 255 consecutive patterns with a 50 μs dead time between aquisition of scattering profiles. A calibration of the spacings was obtained using teflon as a standard [22].

Temperature jumps and scans were produced by water baths connected internally to the sample mount of the X-ray camera. The temperature of the sample was monitored internally using a thermocouple placed adjacent to the sample in the X-ray sample-holder. Heating and cooling rates of 5 and 10 C°/min were used in the temperature scans. The rate of change of temperature during

the jumps was about 6 C°/s. Data acquisition times of the individual frames were 3 and 1.2 s in heating and cooling scans, respectively, and 100 ms in the temperature jumps. Static patterns with 100 s accumulation time were also recorded.

Small- and wide-angle X-ray measurements of dipalmitoylphosphatidylcholine freeze-dried in the absence of trehalose were performed using a Ni-filtered Cu K_α-radiation source in a Kratky compact camera and pinhole camera, respectively (both from A. Paar, Graz, Austria), equipped with position sensitive detectors (M. Braun, Garching, F.R.G. and LETTI from Inel, Buc, France) and Peltier controlled variable temperature cuvettes. The maximum temperatures available were 75 °C and 80 °C for the wide- and low-angle measurements, respectively. The sample-to-detector distances were 26.5 cm for the small-angle camera and 15 cm for the wide-angle measurements, respectively. Prior to each measurement the samples were equilibrated at the designated temperature for at least 20 min. The data acquisition time was 1000 s for the small-angle patterns and between 1000 and 2000 s for the wide-angle patterns.

Results

When an aqueous mixture of dipalmitoylphosphatidylcholine/trehalose in a molar ratio of 1 : 4 is freeze-dried according to the procedure described in Materials and Methods well-ordered multilamellar arrays are formed. This can be seen from the static X-ray diffraction pattern recorded at 21 °C (Fig. 1a). The periodicity of the lamellar repeat is 6.45 nm at the lower temperature and is indexed by at least five orders of reflection in the low-angle region of the scattering pattern. An additional reflection corresponding to a spacing of 4.27 nm was also observed in this mixture but it was less obvious in mixtures containing phospholipid/trehalose molar ratio of 1 : 1. The origin of this reflection could not be determined. In the wide-angle region a prominent scattering band indicated an orthohexagonal packing of the hydrocarbon chains typical of lamellar gel (L_β) phase. The lamellar structure is retained when the sample was heated to 71 °C but the periodicity decreased to about 5.25 nm (Fig. 3). The wide-angle region of the diffraction pattern shows a broad

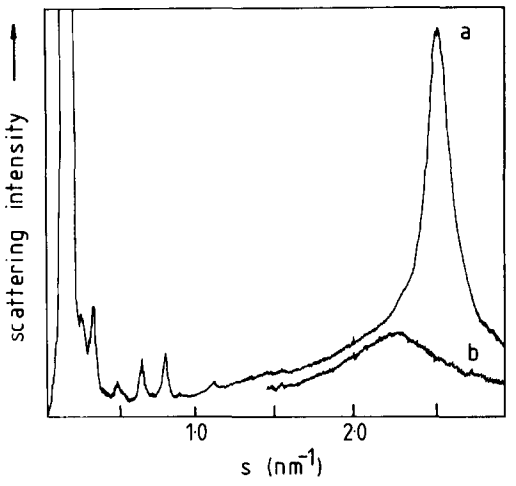

Fig. 1. X-ray diffraction patterns of dipalmitoylphosphatidylcholine: trehalose mixtures (mole ratio 1 : 4) freeze-dried from an initially fully hydrated $L_{\beta'}$ state recorded at (a) 21 °C; (b) 71 °C. Accumulation time 100 s.

scattering band centred at a spacing corresponding to about 0.44 nm and indicates a disordered arrangement of the hydrocarbon chains (Fig. 1b).

The transition between these two phases has already been characterised by calorimetry [11,12]. It was previously shown that with trehalose contents greater than one disaccharide per two phospholipid molecules an endothermic phase transition reaches a lower limiting value of about 48 °C. To examine the structural events associated with this phase transition X-ray diffraction patterns were recorded continuously during heating and subsequent cooling of freeze-dried samples prepared with dipalmitoylphospatidylcholine/trehalose molar ratios of 1 : 1 and 1 : 4. Fig. 2 shows an overview of the X-ray scattering profiles of the two data sets obtained from the 1 : 4 mixture. The phase transition is clearly distinguished from concomitant changes in both low- and wide-angle regions of the diffraction patterns in both the heating and cooling modes.

Spacings obtained from the diffraction maxima of X-ray scattering profiles recorded during heating and cooling have been plotted as a function of temperature in Fig. 3. The lamellar period repeat spacing decreases abruptly upon heating from a constant value of 6.45 nm to 5.60 nm at 55 °C and decreases progressively to about 5.1 nm with fur-

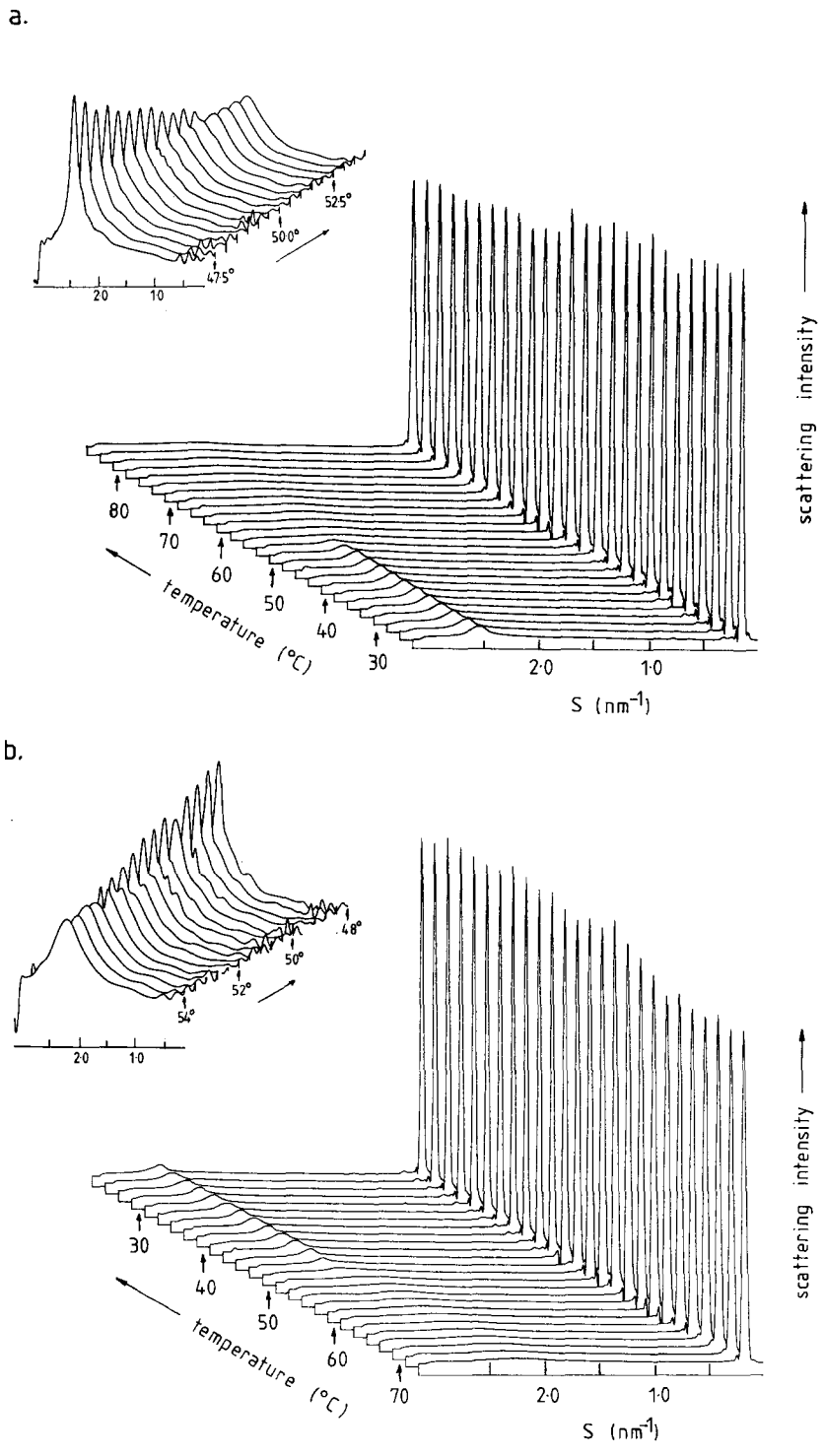

Fig. 2. Three-dimensional plots of X-ray scattering intensity vs. reciprocal spacing of dipalmitoylphosphatidylcholine/trehalose mixture (molar ratio 1 : 4) freeze-dried from initially fully hydrated $L_{\beta'}$ state during (a) heating at 5 C°/min from 21° C to 86° C and (b) subsequent cooling at 10 C°/min from 73° C to 23° C. Every 10th frame of the complete data set is shown. The data acquisition time for each frame was 3 s and 1.2 s in heating and cooling modes, respectively.

ther heating up to a temperature of 85 °C (Fig. 3a). The lamellar period of the high temperature mesophase is characterised by three relatively broad diffraction orders. The sequence of events observed during heating is completely reversible on cooling with a temperature hysteresis of about 2–3C°. The reflection observed at 4.27 nm disappeared from the diffraction pattern at temperatures above the phase transition temperature but reappeared upon cooling to the gel phase. The change in lamellar repeat contrasts with low-angle X-ray measurements of dipalmitoylphosphatidylcholine freeze-dried from water in the absence of trehalose (also shown in Fig. 3a) which shows an abrupt decrease of the lamellar repeat period from 5.82 nm to 5.15 nm in the range between 70 and 80 °C. In the wide-angle region (Fib. 3b) there is a slight, monotonic increase in interchain spacing with increasing temperature up to the transition region (47–57 °C) whereup a sharp increase in the spacing of the diffraction maxima to

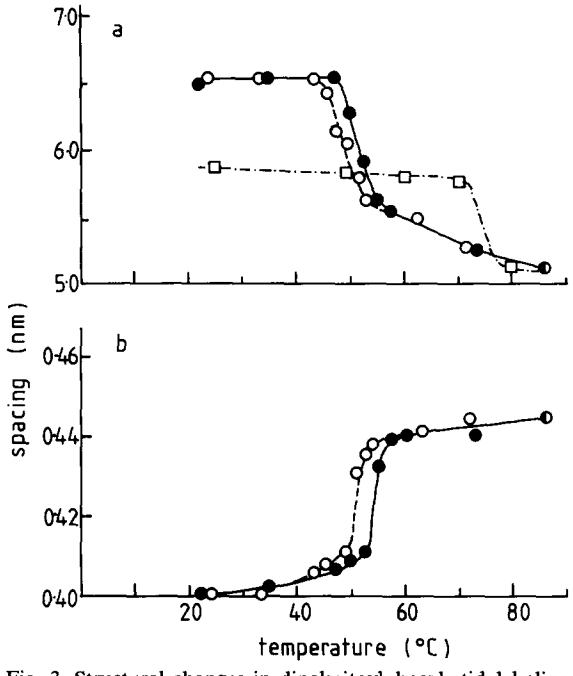

Fig. 3. Structural changes in dipalmitoylphosphatidylcholine/trehalose mixtures (mole ratio 1:4) freeze-dried from an initialy fully hydrated $L_{\beta'}$ state: (a) lamellar period; (b) spacings derived from wide-angle scattering; ●, heating, ○, cooling; □, lamellar period of dipalmitoylphosphatidylcholine freeze-dried in absence of trehalose.

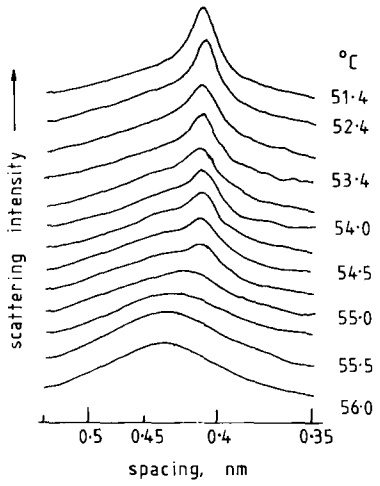

Fig. 4. Consecutive X-ray scattering intensities recorded during the L_β to L_α transition of dipalmitoylphosphatidylcholine/trehalose mixture (molar ratio 1:4) freeze-dried from an initially fully hydrated $L_{\beta'}$ state. The heating rate was 5 C°/min and each pattern was acquired in 3 s. This sequence is reversible on cooling with a hysteresis of 2–3 C°.

values typical of the disordered liquid-crystalline space-chain conformation is observed. Changes in both wide and low-angle X-ray reflections indicate that the mesophase dimension and the hydrocarbon chain inter-chain spacings change simultaneously during the phase transition. In addition, these changes coincide with the increase in the enthalpy of the transition (see also Figs. 3a and 3b). Thus the thermodynamic parameters of the transition reflects both structural changes within the lipid multi-lamellar array.

The mechanism of the transition between L_β and L_α phases can be seen in the sequence of scattering patterns recorded in the wide-angle region on heating through the transition (Fig. 4). This shows that the relatively sharp diffration band characteristic of the L_β phase begins to broaden during the transition with the appearance of an even broader diffraction peak characteristic of the chain packing in the L_α phase. The co-existence of the wide-angle scattering of both phases is similar to the interpretation of the diffraction patterns observed in real-time X-ray measurements of dipalmitoylphosphatidylcholine freeze-dried in the absence of trehalose (Fig. 5a). In this case the chain arrangement at low temperature (20 °C) corresponds to a typical L_β gel phase with

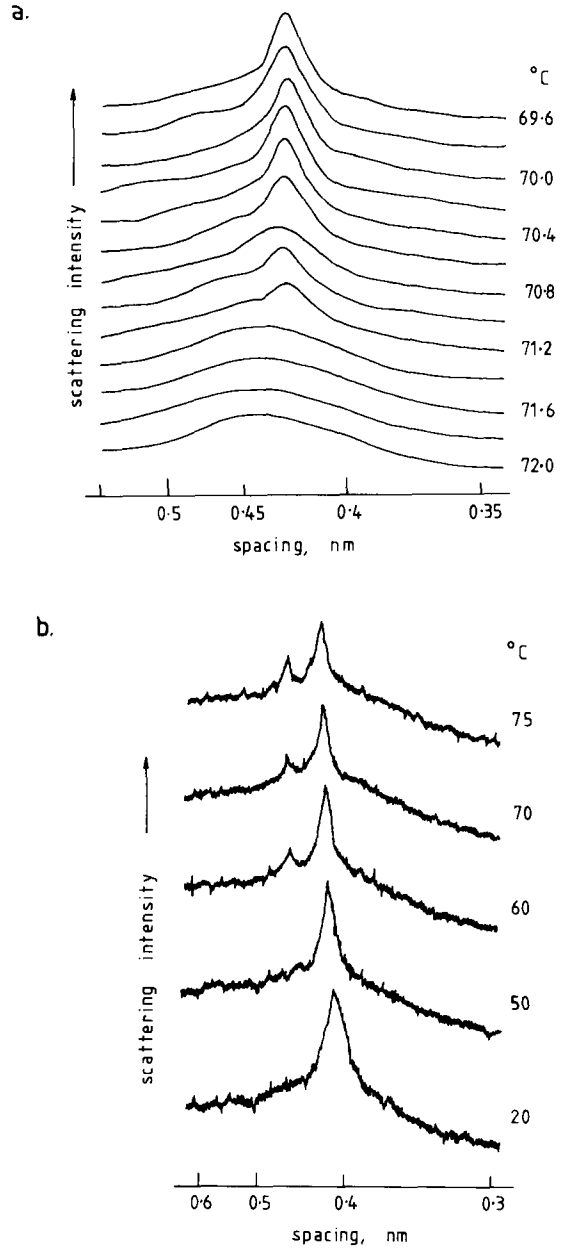

Fig. 5. Wide-angle diffraction patterns of dipalmitoylphosphatidylcholine freeze-dried in the absence of trehalose from an initially fully hydrated $L_{\beta'}$ state at different temperatures. (a) Time-resolved patterns with an accumulation time of 1.2 s recorded during heating at a rate of 10 C°/min. This sequence is reversible on cooling. (b) Static patterns (see Materials and Methods).

a single, symmetric peak at 0.409 nm. With increasing temperature this peak shifts to longer spacings (0.424 nm at 75°C) while a second, weaker scattering band appears at 0.46 nm when the temperature reaches 50–60°C and thereafter increases in intensity. Recent reports by Laggner and co-workers [23], however, have indicated that the equilibrium acyl chain structure, as determined from static X-ray patterns of dihexadecylphosphatidylcholine at low hydration, shows an intermediate state between the L_β and L_α phases. We have obtained static X-ray diffraction patterns of lyophylised dipalmitoylphosphatidylcholine as a function of temperature which are presented in Fig. 5b. In this experiment, the gel state acyl chain packing transforms into an orthorhombic subcellular packing before ultimately reaching a disordered configuration. Thus, in lyophilised dipalmitoylphosphatidylcholine, the high temperature orthorhombic acyl chain phase requires a longer time to stabilize than allowed in our real-time experiments. At this point, we are unable to clearly define all conditions for the formation of unusual high-temperature acyl chain packing [23], however we have noticed its presence in dynamic studies of other lipid systems.

The relaxation time of the L_β to L_α phase transition of dipalmitoylphosphatidylcholine/trehalose mixtures in a molar ratio of 1:1 was determined in temperature jump experiments. The temperature was increased through the transition at 6 C°/s and successive diffraction patterns recorded on a time scale of 100 ms. The pattern of structural changes observed were essentially the same as those recorded during temperature scans of 5 C°/min. The overall relaxation time of the transition was found to be 2 s. In all the experiments the results obtained with dipalmitoylphosphatidylcholine/trehalose (1:4) mixtures were similar to those found for 1:1 molar ratio mixtures.

Discussion

The method of preparation of anhydrous mixtures of dipalmitoylphosphatidylcholine and trehalose appears to determine the structure and phase behaviour of the resulting complex. Preliminary X-ray diffraction studies referred to by other workers [10] have noted the presence of only diffuse scattering bands in the diffraction pattern. This may indicate that there is no highly ordered

multi-lamellar packing of phospholipid and trehalose. By contrast, we observed at least five well-defined orders of diffraction of a lamellar repeat spacing in samples freeze-dried from dispersions of dipalmitoylphosphatidylcholine in aqueous trehalose solutions. This suggests that trehalose enters the interlamellar aqueous spaces during the sample preparation. In the dried state there is a homogeneous expansion of the lamellar spacing from 5.82 to 6.45 nm (Fig. 3a). The magnitude of this expansion was found to be the same irrespective of whether the complexes of dipalmitoylphosphatidylcholine and trehalose were varied from mole ratios of 1 : 1 to 1 : 4. It may be concluded from this observation, therefore, that the actual ratio of phospholipid to interlamellar trehalose does not depend on the amount of the excess trehalose solution.

The structures formed by dipalmitoylphosphatidylcholine in trehalose solutions (1 M) have been shown by X-ray diffraction measurements to cause an expansion of the lamellar period of the four characteristic phases formed by the fully hydrated multilayers [11]. In the gel ($L_{\beta'}$) phase the lamellar repeat period was found to be increased by 0.36 nm (from 6.40 to 6.76 nm) at a dipalmitoylphosphatidylcholine/interlamellar trehalose ratio of approximately 2–3 to 1. A plausible model of the structure of the freeze-dried preparations examined in this study is that trehalose forms a single, loosely packed 'monolayer' separating the adjacent bilayers of phospholipid. The presence of more than one layer of trehalose molecules between the bilayers of phospholipid would probably require an expansion in the lamellar period greater than the observed value of 0.63 nm (Fig. 3a). The freeze-dried samples, however, are not completely devoid of water. When dipalmitoylphosphatidylcholine is freeze-dried from water according to the method described in Materials and Methods the lipid is characterised by a relatively broad endothermic phase transition at about 75 °C [11,12]. Studies of the effect of water activities on the phase behaviour of dipalmitoylphosphatidylcholine [24], suggests that a phase transition at this temperature corresponds to a water content of about two molecules of water per molecule of phospholipid. The lyophilisation procedure described above, therefore, reduces the amount of water in the samples to the level of the dihydrate. This has been confirmed directly by gravimetric moisture evaluations which showed that residual water in the samples freeze-dried in either the presence or absence of trehalose does not exceed 5–6 wt% [11,12].

Characteristics of the phase behavior of phospholipid/trehalose mixtures also distinguish complexes dried from organic solvent from those lyophilised from aqueous solutions. Differential scanning calorimetric studies of dipalmitoylphosphatidylcholine/trehalose mixtures prepared from organic solvent evaporation [7,10] indicate non-reversible transitions which are highly non-cooperative. This suggests the formation of heterogeneous aggregates of dipalmitoylphosphatidylcholine and trehalose which are small in size and consist probably of poorly correlated bilayers. Such an arrangemant is also consistent with a broadening and merging of the X-ray diffraction lines. This was verified in a real-time X-ray diffraction study of a dipalmitoylphosphatidylcholine/trehalose mixture freeze-dried from a chloroform/methanol solution. The data from a heating scan made with this mixture (not shown) indicated that the X-ray reflections were indeed weak and diffuse. In addition, the transition temperature and mechanism of transition is different from that observed for a similar mixture freeze-dried from an aqueous dispersion. When the mixtures are dried from aqueous solutions the phase transition is sharp and completely reversible without significant hysteresis [11].

The dynamic X-ray diffraction results agree with differential scanning calorimetric studies [7,11,12,24] and indicate a significant lowering of the transition temperature from that of the dihydrate of dipalmitoylphosphatidylcholine (Fig. 3). Overall, the low- and wide-angle data unambiguously characterise the phase transition in freeze-dried dipalmitoylphosphatidylcholine/trehalose mixtures as a typical lamellar gel–lamellar liquid-crystal (L_{β}–L_{α}) phase transformation. The consecutive X-ray patterns, particularly in the wide-angle region, show that the transition proceeds by a mechanism remarkably similar to that of the L_{β}-L_{α} transformation of dipalmitoylphosphatidylethanolamine dispersed in excess water (unpublished observations). From these observations,

we find no structural evidence to support the new phase assignments of phospholipid : trehalose mixtures assigned by Lee et al. [10] on the basis of studies of phospholipid/trehalose mixtures prepared by evaporation from organic solvent mixtures. Other calorimetric studies [25] of mixtures prepared from organic solvent have shown that the 'main' endothermic phase transition at 46 °C observed in 1 : 2 mole ratio mixtures of dipalmitoylphosphatidylcholine and trehalose was preceded by a 'pretransition' at 43.4 °C and a 'subtransition' centred at 34 °C. By analogy with the fully hydrated phospholipid, it may be expected that a rippled phase ($P_{\beta'}$) would form in the temperature range 43–46 °C. In the present study, however, we did not find any structural evidence to support the formation of a different phase structure in the temperature range where a $P_{\beta'}$ phase would be expected. In fact, the lamellar period and diffraction line-widths were remarkably constant at temperatures below the phase transformation into the liquid-crystalline phase.

The relaxation time of the phase transition in dried mixtures of dipalmitoylphosphatidylcholine and trehalose measured in the temperature jump experiments was very similar to that observed in fully hydrated dispersions [26,27]. It is also somewhat faster than relaxation times reported for the subtransition in dipalmitoylphosphatidylcholine [27].

It is known from X-ray studies on monocrystals of phosphatidylcholine dihydrate [28] that one water molecule forms intrabilayer hydrogen bonds between the phosphate groups of two neighbouring lipids while the other water molecule takes part in the formation of interbilayer hydrogen bonds. Thus, the phosphatidylcholine dihydrate bilayers are actually coupled into three-dimensional structures. This coupling contributes to an increased stability and increased melting temperature of the lipid. A primary effect of increasing water activity will be to reduce the interbilayer coupling which, in turn, will modify the thermal properties of the bilayers until the fully hydrated state is reached. Since our data suggest that tehalose forms a 'monolayer' between adjacent lipid bilayers, it may be expected that, similarly to additional water, interlamellar trehalose interferes with the formation of direct interbilayer hydrogen bonds and thus reduces the coupling of adjacent bilayers into thermally more stable structures. Calorimetric results [12] show that a 'saturation' of the trehalose effect occurs at roughly one trehalose per two dipalmitoylphosphatidylcholine molecules. This might be an indication that one molecule of trehalose would be sufficient to mediate the interbilayer hydrogen bonding of two dipalmitoylphosphatidylcholine molecules.

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