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The effect of the cryoprotective sugar, trehalose on the phase behaviour of mixed dispersions of dioleoyl derivatives of phosphatidylethanolamine and phosphatidylcholine

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The effect of the cryoprotective sugar, trehalose on the phase behaviour of mixed dispersions of dioleoyl derivatives of phosphatidylethanolamine and phosphatidylcholine

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The phase behaviour of a mixed dispersion of dioleoyl derivatives of phosphatidylethanolamine and phosphatidylcholine (3:1, by weight) in an excess of water was compared with that in 1·8 M trehalose using dynamic X-ray diffraction, differential scanning calorimetry and freeze–fracture electron microscopy. The phase transitions of the fully-hydrated dispersion observed upon heating, proceeded with lamellar-gel → lamellar liquid crystalline → inverse cubic → inverse hexagonal phase at temperatures of −10°C, 49°C, and 67°C, respectively. Our results confirm the existence of an inverse cubic phase of this system and support the model mechanism for the lamellar/non-lamellar phase transition previously suggested by Siegel (1986, *Biophys. J.* **49,** 1155, 1171). Dispersion of the binary lipid mixture in a 1·8 M solution of the naturally occurring cryoprotective disaccharide, trehalose, inhibited the formation of both lamellar liquid crystalline and inverse cubic phases and resulted in a direct transition from lamellar-gel to inverse hexagonal phase at about −6°C. The effect of trehalose is discussed in terms of a 'water replacement' model and Hofmeister effects on water structure. Trehalose is regarded as a kosmotropic agent that may also interact directly with the lipid polar groups. Comparison of the relationship between temperature and the dimensions of the inverse hexagonal phase formed in the presence and absence of trehalose suggests that the osmotic effect of the impermeant trehalose prevents water molecules from being taken up by the hexagonal mesophase which is normally more hydrated that the lamellar phase.

1. Introduction

During the past decade, there has been considerable interest in the way sugars interact with polar lipids. One reason for this is that such interactions are believed to underlie the mechanisms whereby sugars prevent injury of cell membranes associated with freeze–thawing, freeze-drying, and dehydration–rehydration [1–10]. A general approach to the question has been to examine the effects of sugars and related cryoprotective agents on the phase behaviour of pure lipid dispersions and

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lipid mixtures. In particular, studies of the kinetics and the mechanisms of lipid phase transitions including lamellar-subgel → lamellar-gel ($L_c \rightarrow L_\beta$), lamellar-gel → lamellar liquid crystal ($L_\beta \rightarrow L_\alpha$), and lamellar liquid crystal → inverse hexagonal ($L_\alpha \rightarrow H_{II}$) have been reported and their possible biological relevance has been discussed [11–29]. Identification of different phase structures and the mechanisms of transitions among them have been summarized in a review [30].

A conspicuous effect of sugar alcohols and disaccharides is their ability to lower the temperature of the $L_\alpha \rightarrow H_{II}$ phase transition of dielaidoylphosphatidylethanolamine [5] and the dihexadecyl- and distearoyl-derivatives of phosphatidylethanolamine (DSPE and DHPE) [10]. Direct $L_\beta H_{II}$ transitions were observed in DSPE and DHPE dispersions when the concentration of sucrose was sufficiently high [10]. Similar effects were subsequently reported for dispersions of unsaturated dioleoylphosphatidylethanolamine (DOPE) [9]. The effect of sugars on these phase transitions appears to be analogous to dehydration, as studies of lipids at low hydration have implied [8, 16, 31–33].

Another point of interest is the mechanism of the $L_\alpha \rightarrow H_{II}$ phase transition. A kinetic model, which involves the formation of an intermediate inverse cubic phase (I_{II}), has been proposed by Siegel [17, 18]. And a metastable I_{II} phase has been observed in *N*-monomethylated DOPE by both X-ray diffraction [21] and ^{31}P NMR spectroscopy [24] and in a mixed dispersion of DOPE and dioleoylphosphatidylcholine (DOPC) (3 : 1,wt/wt) by ^{31}P NMR [24].

In order to extend these observations, the present study was undertaken to examine the phase behaviour of mixed dispersions of DOPE/DOPC (3 : 1 by weight) in an excess of water and in a concentrated (1·8 M) trehalose solution using dynamic X-ray diffraction, differential scanning calorimetry and freeze–fracture electron microscopy. Trehalose was chosen for this study because it is a naturally-occurring cryoprotectant found abundantly in some animals and plant seeds. Emphasis is placed on characteristics of the intermediate cubic phase and the effect of trehalose on the lamellar to non-lamellar phase transition.

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2. Materials and methods

2.1. *Source materials*

Synthetic dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids Inc. (Birmingham, Alabama) and used without further purification. Trehalose was obtained from Fluka AG.

2.2. *Sample preparation*

Multilamellar liposomes consisting of DOPE/DOPC (3:1, wt/wt, which is equivalent to a molar ratio of 3·17 : 1) were prepared according to methods described by Tate and Gruner [19]. To ensure full and uniform hydration, 60 wt % of either distilled, deionised water or 1·8 M trehalose solution was added to freeze-dried mixtures of the two lipids. The samples were extensively vortex mixed and subjected to several freeze–thaw cycles between −196° and 70°C. Finally, the frozen lipid was slowly thawed and centrifuged at 4000 rpm at 2°C. The pellet was used for conventional differential scanning calorimetry, freeze–fracture electron microscopy and synchrotron X-ray diffraction measurements. The samples for high-sensitivity differential scanning

calorimetry were prepared separately using the same procedure as described above, except that water or sugar solution was added to the freeze-dried lipid mixture to yield a final concentration of the lipid of 0·003 wt %.

2.3. *Differential scanning calorimetry*

Conventional differential scanning calorimetry was performed using a Dupont 1090 Thermal Analyser fitted with a sub-ambient liquid nitrogen accessory. This was interfaced with a Dupont 1091 Disc Memory which was used to record, plot and calculate the thermodynamic parameters from the thermograms. The well-sealed sample pans, each containing about 5 mg of lipid were scanned from $-100°$ to 85°C at 5°, 10° and 20° min^{-1}. High sensitivity differential scanning calorimetry was used to obtain thermograms from 0° to 70°C at heating rates of 0·5° min^{-1} using a Privalov differential scanning calorimeter, DASM-4M. It was possible to duplicate, to some extent, conventional differential scanning calorimetry by heating the diluted lipid suspensions (2 ml) outside the calorimeter to 47°C and then plunging them into liquid nitrogen. The calorimeter cell was loaded with the sample thawed to 0°C.

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2.4. *X-ray diffraction*

Real-time X-ray diffraction results were obtained at Station 8.2 of the Synchrotron Radiation Source at the SERC Daresbury Laboratory U. K. as previously described [30, 34]. A curved single crystal of Ge and an optically flat mirror were used for monochromatization (0·15 nm) and horizontal focusing to provide approximately 10^{10} photons s^{-1} mm^{-2} at 2·0 GeV and 100–200 mA of electron beam current. A camera and linear delay line detector constructed at the Daresbury Laboratory were used to record scattered X-ray intensity. Powdered teflon codispersed with fully hydrated dipalmitoylphosphatidylcholine served as a reference standard to define the small-angle spacing and the centre of the incident X-ray beam. Lipid mixtures were loaded into a sample holder (16 µl capacity) and sealed between two thin mica windows 1 mm apart and mounted on a modified cryomicroscope stage (Linkam, Tadworth, U.K.). Temperature controlled scans were performed at heating or cooling rates of 5° min^{-1} from $-30°$ to 70°C. Data were acquired in files consisting of 255 consecutive diffraction patterns, each of 3 or 5 s duration and separated by 50 µs dead time, and stored on a VAX-11/750 computer. Selected frames were averaged to improve signal/noise ratio.

2.5. *Freeze–fracture electron microscopy*

Prior to the thermal quenching, all samples were heated to give the H_{II} phase. The dispersions were sandwiched between two copper sample mounts (Balzers Union 120557/1205 JT) and subsequently cooled at a rate of approximately 5° min^{-1} to the desired temperature and equilibrated at that temperature using a thermally stable gas flow for 3 min prior to thermal quenching. Samples were thermally quenched in a liquid nitrogen jet freezer. Fracturing and replication were performed at $-150°$C using a Polaron E7500 freeze–fracture device. Replicas were cleaned in a solvent consisting of chloroform : methanol (2 : 1 v/v) before examination in a Joel JEM100CX electron microscope.

3. Results

3.1. *Lipid mixtures without trehalose*

The structural changes of the mixed dispersion of DOPE/DOPC (3:1, by weight) in water during a heating scan from −30°C to 70°C at 5° min^{-1} are evident in the X-ray data shown in figure 1 (*a*). The characteristic features indicate the sequence $L_\beta \rightarrow L_\alpha \rightarrow I_{II} \rightarrow H_{II}$. The repeat spacings of the different phases derived from the X-ray data are shown in figure 2. In the low temperature region, −30°C to −20°C, and L_β phase predominates which has a repeat spacing of 5·6 nm and a wide-angle reflection centred at about 0·43 nm. Further heating induces an $L_\beta \rightarrow L_\alpha$ transition at about −10°C; this is consistent with the thermal data obtained from the mixed dispersion heated under identical conditions and shown in figure 3 (thermogram 2). The dramatic decrease in lamellar repeat spacing to about 5 nm at the phase transition is due to a

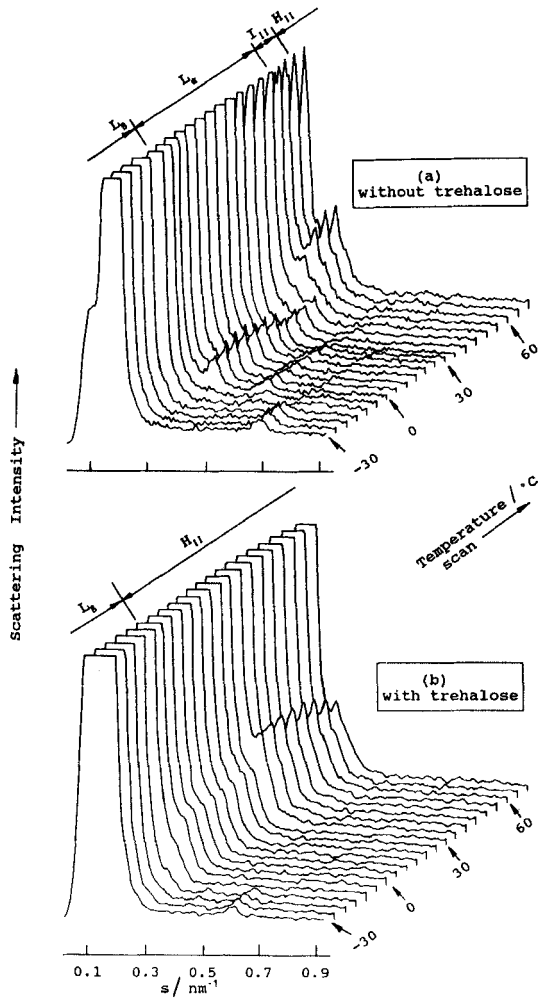

Figure 1. Three-dimensional plot of X-ray scattering intensity versus reciprocal spacing during heating from −30°C to 70°C of dispersions of DOPE/DOPC (3:1, by wt) fully hydrated in (*a*) water; (*b*) 1·8 M trehalose. Temperature was scanned at 5° min^{-1} and individual diffraction patterns were accumulated over 3 s.

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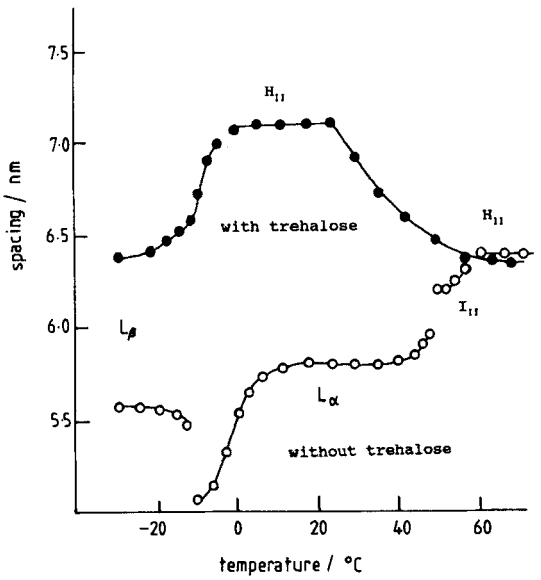

Figure 2. Relationships between temperature and repeat spacings of lamellar-gel (L_β), lamellar liquid crystalline (L_α), inverse cubic (I_{II}), and inverse hexagonal phase (H_{II}) derived from the dynamic X-ray diffraction data for a DOPE/DOPC (3:1, by wt) mixture dispersed in water (○) and 1·8 M trehalose (●).

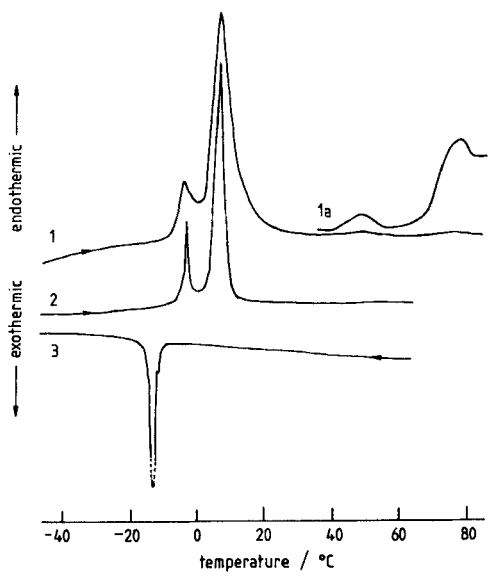

Figure 3. Heating and cooling thermograms obtained by differential scanning calorimetry of a dispersion of DOPE/DOPC (3:1, by wt) in water. Scan 1 was recorded during heating at 20° min^{-1}; the scale of the high temperature region has been expanded 10-fold in 1 *a*. Scans 2 and 3 were recorded during heating and cooling, respectively, at 5° min^{-1}.

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decrease in bilayer thickness associated with the melting of the hydrocarbon chains. The subsequent increase in lamellar *d*-spacing at about 0°C results from the penetration of water into the bilayer as it is made available from the thawing of ice. The repeat distance 5·8 nm of L_α phase is in good agreement with data obtained by others for this lipid [21].

At a temperature of *c.* 50° to 60°C the L_α phase transforms into another phase characterized by diffraction maxima with reciprocal spacings in a ratio of $1:\sqrt{2}:\sqrt{3}:\sqrt{4}$, characteristic of cubic phases, with the 2nd of these peaks being relatively weak. At temperatures above 60°C, a different pattern with the reciprocal spacing ratio $1:\sqrt{3}:\sqrt{4}:\sqrt{7}:\sqrt{9}$, characteristic of hexagonal phases, was observed. A clear shift of the first order diffraction towards smaller angle was observed above 50°C. We assign the two structures as inverse cubic phase I_{II} and inverse hexagonal phase H_{II}. It is still problematic, however, as to whether the cubic phase is the only phase formed between 50° and 60°C or whether it coexists with a hexagonal phase. The repeat spacing of the H_{II} phase is 6·4 nm giving a centre-to-centre distance of the cylinders of 7·4 nm at 70°C. This is in good agreement with values of 7·3 and 7·1 nm reported by Gruner *et al.* [21], and Ellens *et al.* [24], respectively, for this phospholipid mixture. This spacing is somewhat greater than that for pure DOPE (6·5 nm) at 70°C and indicates that DOPC probably acts to prevent close packing of the head groups required for the formation of a lipid–water interface with a low radius of curvature.

The transitions observed in cooling scans at 5° min^{-1} were different from those recorded upon heating. A pure H_{II} phase was present from 70° down to 40°C. Cooling below 40°C produced a mixture of L_α and H_{II} phases which persisted to − 10°C, where there was a sharp transition to a pure L_β phase. Freeze–fracture studies of dispersions quenched from 22°C (see figure 4 (*a*)) show a variety of hexagonal forms. Areas of 'conventional' H_{II} with straight bundles of cylinders with a periodicity of approximately 4·5 nm (right-hand area, see figure 4 (*a*)) were interspersed with areas containing bent and convoluted cylinders of periodicities varying from approximately 5·4 to 6·4 nm

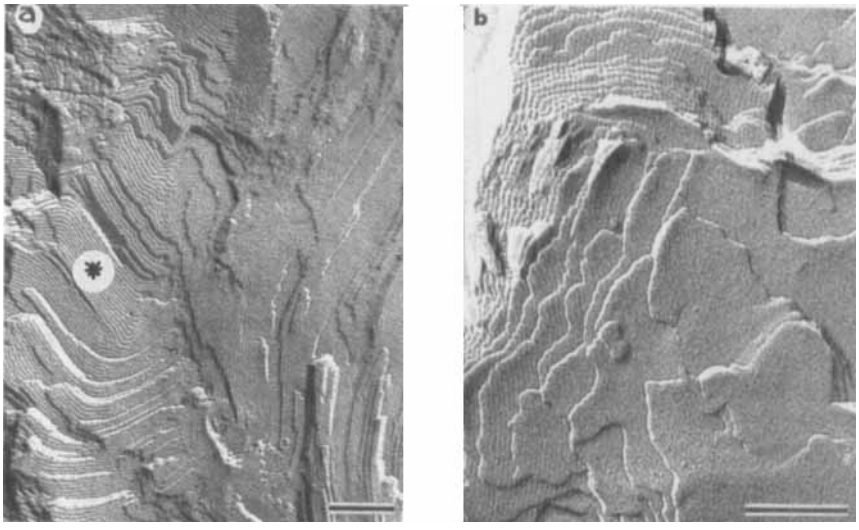

Figure 4. Freeze–fracture electron micrographs of DOPE/DOPC (3:1, by wt) dispersed in water and thermally quenched from (*a*) 22°C showing a variety of H_{II} forms including bent and convoluted cylinders (*); and (*b*) 0°C showing the transition from H_{II} to lamellar phase. (Bar, 100 nm.)

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(left-hand area, *, figure 4(*a*)). The same sample, thermally quenched from 0°C (see figure 4(*b*)), showed a predominantly bilayer appearance with small stacks of bilayers arranged at random angles throughout the sample. In some areas of the replica, the transition from inverse hexagonal to bilayer was observed. The coexistence of H_{II} and L_α was typical of this intermediate temperature.

The enthalpy changes in the same system associated with the phase transitions observed by dynamic X-ray diffraction were determined by differential scanning calorimetry over a temperature range − 45° to 85°C (see figure 3). In addition to the ice melting peak, a single endotherm at about − 10°C is observed upon heating which is due to the order–disorder transition of the hydrocarbon chains at the $L_\beta \rightarrow L_\alpha$ transition. There is no evidence of any phase separation during the cooling scan and a discrete exotherm was often observed prior to freezing of the supercooled aqueous medium. Reheating the dispersion to − 2°C and subsequent cooling, a procedure that avoids an ice melting endotherm, revealed the presence of a cooperative transition with no hysteresis and an identical enthalpy value. No enthalpy changes could be detected corresponding to the lamellar to non-lamellar phase transitions at a scan rate of 5° min^{-1}. Increasing the heating rate to 20° min^{-1} showed clear endotherms with a T_{max} of 49°C associated with the $L_\alpha \rightarrow I_{II}$ transition, followed by a larger endotherm with an onset temperature of 67°C and a T_{max} of 78°C corresponding to the $I_{II} \rightarrow H_{II}$ transition.

3.2. *Lipid mixtures with trehalose*

The effect of trehalose on the phase transition behaviour was studied with the binary lipid mixture dispersion in 1·8 M aqueous trehalose. When the dispersion is cooled to − 30°C, a typical L_β phase, with a repeat distance of about 6·4 nm, is observed. The strong first-order diffraction peak and prominent fourth-order peak can be seen in figure 1 (*b*) in the small-angle X-ray diffraction region and there is a relatively sharp diffraction peak centred at 0·43 nm in the wide-angle region. Upon heating, there is a slight increase in the intensities of the second- and third-order reflections of the lamellar repeat, together with a displacement of the peaks, indicating some lattice expansion (which may be associated with some disordering of the acyl chain packing). At about

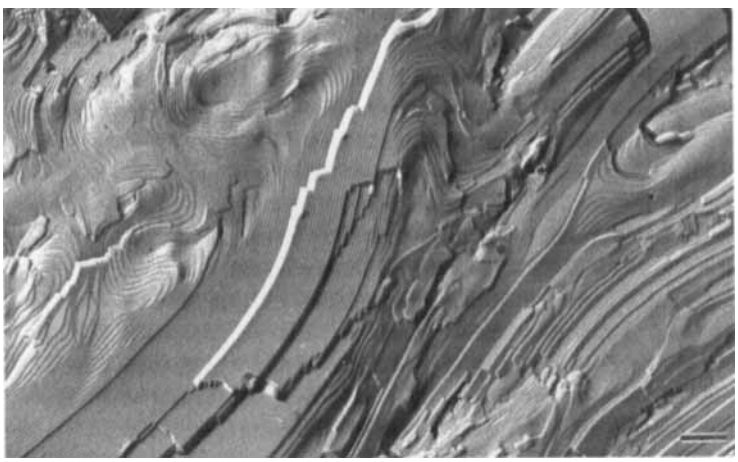

Figure 5. Freeze–fracture electron micrographs of DOPE/DOPC (3:1, by wt) dispersed in 1·8 M trehalose and thermally quenched from 0°C showing the transformation from H_{II} to lamellar phase during cooling. (Bar, 100 nm.)

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− 6°C, a direct transition from a lamellar to a non-lamellar phase is observed. The latter phase was characterized as a hexagonal structure by the first- and second-order X-ray reflections and the hexagonal structure was confirmed by freeze–fracture electron microscopy (see figure 5). The repeat spacing around 10°C for this phase was 7·1 nm, corresponding to a centre-to-centre spacing of 8·2 nm. This is somewhat less than would be predicted from the dimensions of the binary mixtures in an excess of water [35] and may be due to the fact that water is unable to penetrate into the lattice because of osmotic constraints imposed by the impermeant trehalose. Heating to higher temperatures results in a decrease of the periodicity of the H_{II} phase. The value obtained at 70°C was comparable to that of the H_{II} phase calculated for a dispersion in an excess of water at this temperature.

When the dispersion in 1·8 M trehalose was subsequently cooled to 2°C, the sample gave reflections arising from both H_{II} and L_{β} phases. The coexistence of these two phases was observed in a temperature range from about 2°, to − 16°C, and evidence

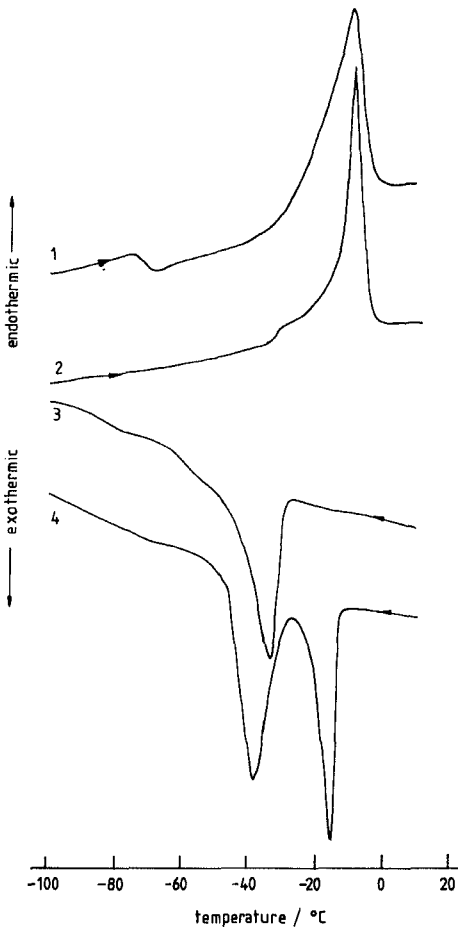

Figure 6. Thermograms obtained by differential scanning calorimetry of 1·8 M trehalose (Scans 1 and 3) and DOPE/DOPC (3:1, by wt) dispersed in 1·8 M trehalose (Scans 2 and 4). The scan rate was 10° min^{-1} and the cooling scans (3 and 4) were recorded immediately after the heating scans (1 and 2).

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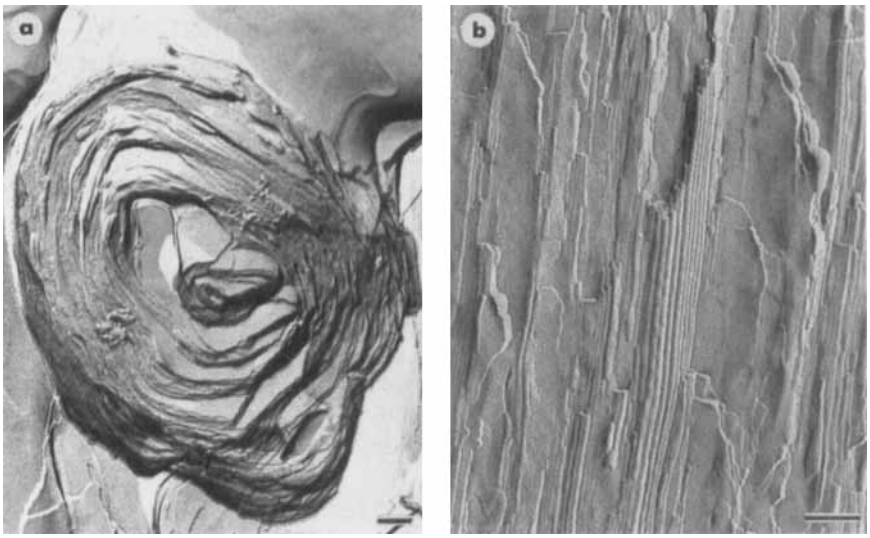

Figure 7. Freeze–fracture electron micrographs of DOPE thermally quenched from 0°C. (*a*) dispersion in water showing characteristic bilayer structures with multi-lamellar vesicles; (*b*) dispersion in 1·8 M trehalose showing the coexistence of lamellar and H_{II} phases. (Bar, 100 nm.)

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for it was also given by the freeze–fracture replica of the dispersion thermally quenched from 0°C (see figure 5). At temperatures lower than − 16°C, the system transforms into a pure lamellar-gel phase.

Thermograms recorded over the temperature range − 100° to 20°C with a scan rate of $10° \text{ min}^{-1}$ for the DOPE/DOPC mixture in 1·8 M trehalose and for 1·8 M trehalose in the absence of lipid are shown in figure 6. It can be seen that the trehalose solution exhibits a marked freezing point depression and supercooling effects at this scan rate. Nevertheless, a clear exotherm due to the lipid is observed in the cooling scan with a T_m at − 15·7°C. This corresponds to a direct $H_{II} \rightarrow L_\beta$ phase transition of the phospholipid mesophase (see figure 6, curve 4).

The effect of trehalose on the phase behaviour of dispersions of pure DOPE thermally quenched from 0°C was also examined by freeze–fracture electron microscopy (see figure 7). The dispersion in water (see figure 7 (*a*)) exhibits a typical lamellar appearance in which bilayer sheets and multilamellar vesicles are seen. The dispersion in 1·8 M trehalose, on the other hand, shows hexagonal-II phase (periodicity of approximately 6·4 nm) coexisting with lamellar phases (see figure 7 (*b*)).

4. Discussion

The results obtained in this study extend our understanding of the structural changes of DOPE/DOPC dispersions as a function of temperature and the nature of the direct $L_\beta \rightarrow H_{II}$ transition in the presence of concentrated trehalose solutions.

Limited by the resolution of the X-ray diffraction, we cannot be sure whether the I_{II} phase existed alone or coexisted with hexagonal phase. But with a heating scan of $20° \text{ min}^{-1}$ in the DSC measurement, two separate transitions were revealed. The low-enthalpy peak centred at 49°C corresponds to the $L_\alpha \rightarrow I_{II}$ transition and the endotherm beginning at about 67°C corresponds to the $I_{II} \rightarrow H_{II}$ transition. Thus we

come to the conclusion that the phase transition sequence of the fully hydrated DOPE/DOPC (3:1 by weight) dispersion observed in this study is $L_\beta \rightarrow L_\alpha \rightarrow I_{II} \rightarrow H_{II}$ with transition temperatures of $-10°$. 49°, and 67°C, respectively. The slightly higher value observed for the temperature of the transition to H_{II} in comparison with published data [21, 24] is probably due to the faster heating rate used to detect the phase sequence.

The thermal and dynamic X-ray diffraction results are consistent with the kinetic model for the $L_\alpha \rightarrow H_{II}$ phase transition mechanism proposed by Siegel [17], which involves an intermediate structure. Two types of bilayer/non-bilayer phase transition have been suggested [18]. The first type, exemplified by DOPE and egg PE, is rapid and comparatively non-hysteretic, whereas the second type, typified by *N*-monomethylated DOPE, shows pronounced hysteresis and could involve the formation of an inverse cubic or isotropic mesophase during the slow process. There has been a report of the I_{II} phase corresponding to the isotropic ^{31}P NMR signal at 45°C in the same mixed phospholipid dispersion [24]. The present results confirm this observation. The lipid mixture DOPE/DOPC (3:1 by weight) also appears to show this second type of bilayer/non-bilayer phase transition.

The introduction of trehalose to the mixed lipid dispersion lowered the onset temperature of the transition to non-lamellar phase and resulted in an apparently direct $L_\beta \rightarrow H_{II}$ transition. Compared with the phase transition sequence of the same lipid mixture in pure water, the temperature of disappearance of the gel phase is raised by about 4° and the onset of the transition to non-lamellar phase is lowered by about 50°.

Koynova *et al.* [10], have discussed such phenomena in some detail. They suggested that indirect (Hofmeister effect) interactions play a major role in the effect of sugars on lipid/water phase behaviour. As a kosmotropic reagent (water structure promoter), trehalose can stabilize the structure of bulk water. This tends to reduce the area of the unfavourable interfaces between aqueous and lipid phases and thus favours the formation of hexagonal and gel phases.

However, direct interactions between sugar and lipids may also play a part in this effect. Like that of anhydrous or less hydrated cases in the 'water replacement' model, which assumes the replacement of the water layer on the surface of the membrane by carbohydrates [36], there may well be some hydrogen-bonding between the lipid head groups and the sugar molecules. This will increase the hydrophilic/hydrophobic interaction ratio of lipid molecules which is believed to be vital for the formation of the inverse hexagonal phase [37]. On the other hand, such a change in the hydrophilic–hydrophobic balance also affects the temperature of the $L_\beta \rightarrow L_\alpha$ transition. It is known that phosphatidylethanolamines have higher temperatures for this transition than do phosphatidylcholines of the same acyl-chain-length, because there is hydrogen-bonding among PEs but not among PCs. Such an explanation is parallel to that proposed for the Hofmeister effect. Unfortunately, because of the lack of convincing spectroscopic or other evidence, the question as to whether there are direct interactions among lipid head groups and sugar molecules or whether the direct or indirect interactions among sugars and lipids play the main part in the 'sugar effect' cannot be answered at present.

Another interesting feature of the effect of trehalose on the transition is that the hexagonal-II phase has a significantly smaller lattice spacing than might be expected from an extrapolation of the *d* versus temperature relationship observed at higher temperatures. Hexagonal-II phases, in general, contain more solvent than the corresponding lamellar phases [32], but the uptake of water in the presence of the impermeant trehalose is prevented by osmotic effects, thereby restricting the diameter

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of the water-filled tubes. If water movement between the lipid and the aqueous phase is prevented over the entire temperature range studied, then any change in the centre-to-centre spacings between the water-filled tubes with temperature must be due to either a change in the dimension of the lipid hydrocarbon domain or a change in the dimension of the lipid hydrocarbon domain or a change in the diameter of the water cylinders. Since the decrease in *d*-spacing is less significant upon heating above 50°C, a reasonable explanation could be an increase of the diameter of the water-filled lipid tubes at the expense of their length, so as to create more space to meet the expansion of solvent they have encapsulated.

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