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The effect of ice on membrane lipid phase behaviour

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The effects of ice on the lipid phase behaviour of di-18:1 PE and di-18:2 PE were studied by comparing the behaviour of these lipids in supercooled and frozen dispersions. The presence of ice raised the onset temperature of the $L_\alpha \rightarrow L_\beta$ phase transition of di-18:1 PE from −10°C to −6.5°C and increased its molar enthalpy from 6.1 to 8.5 kcal/mol but had little effect on the co-operativity of the transition. Real-time X-ray diffraction measurements of the $H_{II} \rightarrow L_\alpha$ phase transition of di-18:2 PE suggested that this transition could take place in the presence of ice but that the corresponding $L_\alpha \rightarrow H_{II}$ phase transition could not take place until the ice melted. Measurements of the temperature dependence of the *d*-spacing of di-18:1 PE and di-18:2 PC in frozen dispersions indicated that the amounts of unfrozen water in such dispersions changes significantly with temperature. It was concluded that the increases in onset temperature and molar enthalpy seen for the $L_\alpha \rightarrow L_\beta$ transition of di-18:1 PE probably reflected the effects of osmotic dehydration. The main effect of ice in the case of the $H_{II} \rightarrow L_\alpha$ phase transition, however, appeared to be to limit the ability of the lipid to undergo structural reorganisation.

Introduction

The response of cells and tissue to cryogenic temperatures is widely believed to be associated with the stability of biological membranes at low temperatures [1–3]. This has led to an increasing interest in the low-temperature phase behaviour of membrane lipids in general and the effects of ice on such behaviour in particular.

Ice formation influences lipid phase behaviour in a number of ways. The freezing process has a severe dehydrating effect. Not all of the water in lipid samples is frozen however. Chapman et al. [4] showed that an amount of water equivalent to about 25% (w/w) of the lipid present in phosphatidylcholine dispersions does not freeze/melt at 0°C. Lowering the temperature of lipid dispersions leads to further freezing. Recent measurements of Bronshtevn and Steponkus [5] indicate that liquid water can persist in such dispersions down to at least as low as −80°C. These latter changes would be expected to have a direct effect on the degree of hydration of lipid headgroups at low temperatures. The removal of liquid water from between adjacent bilayers, and the consequent decrease in their mutual separation, is another potentially important factor.

In order to investigate some of these effects, we took advantage of the fact that concentrated lipid samples commonly show extensive supercooling. The effects of ice on the $L_\alpha \rightarrow L_\beta$ phase transition of di-18:1 PE and the $L_\alpha \rightarrow H_{II}$ transition of di-18:2 PE were investigated by comparing the phase behaviour of lipid samples in the supercooled and frozen states using differential scanning calorimetry and real-time X-ray diffraction techniques.

Materials and Methods

Di-18:1 PE, di-18:2 PE and di-18:2 PC were obtained from Avanti Lipids (Alabaster, AL, USA) and used without further purification.

Differential scanning calorimetry. The lipid samples were hydrated with distilled water in a ratio of 1:1 lipid/water by weight so that water was present in excess. Heating and cooling scans were performed with a Perkin-Elmer DSC-2 fitted with the sub-ambient

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Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; L_α, liquid-crystal lamellar phase; L_β, gel lamellar phase; L_c, lamellar crystalline sub-gel phase; H_{II}, inverted hexagonal phase.

accessory. Samples were scanned at a rate of 5 C°/min. Transition temperatures were taken to be the intercept of the tangent of the rising peak slope with the baseline. Control TLC measurements showed no appreciable degradation of the lipids during the course of the measurements.

X-ray diffraction. Real-time X-ray diffraction was conducted at station 8.2 of the Daresbury Synchrotron Radiation Source as described elsewhere [6]. The samples were subjected to controlled heating and cooling scans at rates of 5 C°/min between defined temperature limits. The data collection system allowed 255 diffraction patterns to be collected consecutively with a 10 μs wait-time between patterns. The exposure time for each pattern was 4 s.

Results

Differential scanning calorimetry

The low-temperature phase behaviour of di-18:1 PE, di-18:2 PE and di-18:2 PC was first investigated using DSC measurements. The lipid samples used in this study all showed marked supercooling; ice formation normally occurred at between about −10°C and −20°C.

Typical thermograms showing the transitions occurring in di-18:1 PE samples are presented in Fig. 1. In the case of this lipid, the $L_\alpha \rightarrow H_{II}$ and $L_\beta \rightarrow L_\alpha$ transitions could be seen both in supercooled and frozen samples. We were unable to detect any thermal changes associated with the $L_\alpha \rightarrow H_{II}$ transition of di-18:2 PE. The X-ray diffraction data detailed below, however, indicates that a $H_{II} \rightarrow L_\alpha$ transition of di-18:2 PE occurs between about −10°C and −16°C in supercooled samples. The $L_\alpha \rightarrow L_\beta$ transitions of di-18:2 PE and di-18:2 PC (data not shown) were both characterised by broad low-enthalpy transitions of the type previously reported by Keough and his co-workers for di-polyenoic PC species [7,8] and by Sanderson and Williams for di-polyenoic monogalacosyldiacylglycerol [6]. These latter transitions, which were centred at about −50°C, were only seen in frozen samples and are discussed in the following paper.

The first two traces shown in Fig. 1 correspond to cooling and heating scans for samples of di-18:1 PE measured under supercooled conditions. On cooling, the low enthalpy exotherm associated with the $H_{II} \rightarrow L_\alpha$ transition occurred at −4°C and the higher entropy transition associated with $L_\alpha \rightarrow L_\beta$ occurred at −10°C. On reheating the supercooled sample, the $L_\alpha \rightarrow L_\beta$ transition was found to be completely reversible and showed no detectable hysteresis. The endotherm corresponding to the $L_\alpha \rightarrow H_{II}$ transition, in contrast, was displaced to about 16°C. The marked hysteresis seen for the $L_\alpha \rightarrow H_{II}$ transition of di-18:1 PE has been noted and commented on by other authors [9].

The third and fourth traces were both measured using frozen samples prepared by first lowering the temperature of supercooled samples to about −20°C to induce freezing and then reheating the samples to −2°C. On cooling, the frozen sample underwent an $L_\beta \rightarrow L_\alpha$ transition with an onset temperature of −6.5°C as opposed to −10°C in the supercooled state. At the same time, the enthalpy of the transition increased from 6.1 kcal/mol to 8.5 kcal/mol. Similar values were obtained on reheating. These increases appear to be direct consequences of the presence of ice, presumably reflecting the osmotic dehydration of the samples occurring on freezing. Apart from these changes in transition temperature and enthalpy, there appears to be little difference in the thermal characteristics of the sample. In particular, the half-widths of

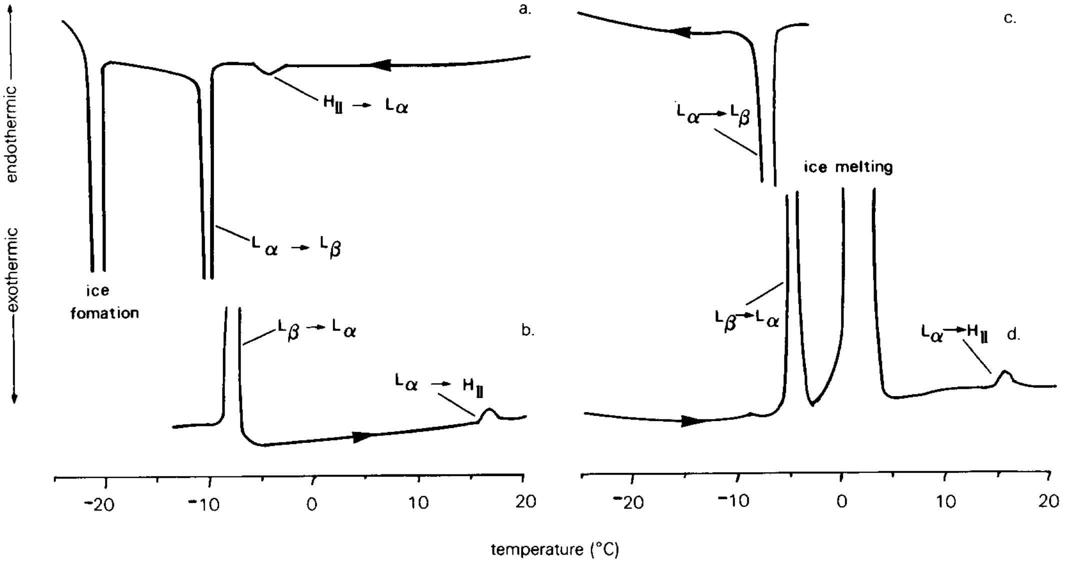

Fig. 1. DSC traces for aqueous dispersions of di-18:1 PE measured in (a) and (b) supercooled and (c) and (d) frozen samples. See text for details.

the endotherms are effectively identical suggesting that there is no significant difference in the co-operativity of the $L_\alpha \rightarrow L_\beta$ transition in the presence of ice.

X-ray diffraction measurements

The effects of ice on lipid phase transitions were also studied using real-time X-ray diffraction measurements. These measurements are conveniently divided into three series of experiments.

The first series of experiments was devoted to the study of the $L_\alpha \rightarrow L_\beta$ and $L_\beta \rightarrow L_\alpha$ transitions of di-18:1 PE. Typical wide-angle diffraction patterns for lipid samples undergoing $L_\beta \rightarrow L_\alpha$ transitions in supercooled and frozen samples are shown in Figs. 2a and 2b, respectively. The lipid transition is reflected in the replacement of the broad diffraction maximum centred at about 0.46 nm, associated with the disorganised acyl chains of the liquid-crystal phase, by a sharper maximum at 0.43 nm, associated with the more organised chains of the gel phase. The presence of ice is indicated by the appearance of new diffraction maxima at spacings of 0.39 and 0.37 nm corresponding to the two most prominent of the three diffraction peaks of hexagonal ice [10]. The 0.43 nm maximum associated with the L_β phase first appears at −12°C in the supercooled sample and at −9°C in the frozen sample. The temperatures for the $L_\alpha \rightarrow L_\beta$ transitions as revealed by X-ray diffraction measurements are about 2 C° lower than those measured using DSC, reflecting the greater difficulty in determining precise onset temperatures in the diffraction measurements, but a similar increase in transition temperature in the presence, as compared to in the absence, of ice is observed.

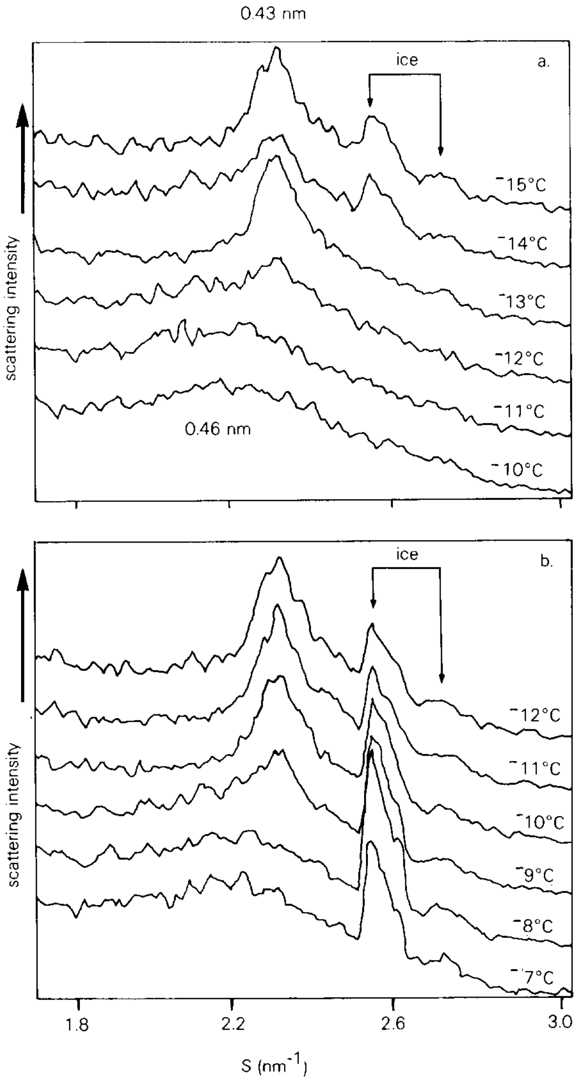

Fig. 2. Typical sets of wide-angle X-ray diffraction patterns for di-18:1 PE showing the $L_\alpha \rightarrow L_\beta$ transition in (a) a supercooled and (b) a pre-frozen sample. The arrows indicate the positions of the 0.39 and 0.37 nm maxima arising from ice crystals.

Small-angle diffraction measurements indicated that the transition between the L_α and L_β states was a standard two-state transition. A limited co-existence of the two phases existed at the transition temperature in the sample but no intermediate states were observed in the presence or absence of ice. Typical small-angle diffraction patterns for the L_β and L_α phases measured in the presence and absence of ice are presented in Fig. 3. The maxima corresponding to the first four orders of diffraction are readily identifiable but the relative heights of the individual maxima vary appreciably. This simply reflects changes in the amplitudes of the Bragg reflections associated with changes in the lamellar repeat distance linked to dehydration occurring on freezing.

A clearer picture of the changes occurring in the sample is seen in the plots of the temperature dependence of the *d*-spacing presented in Fig. 4. The first trace shows the effect of heating a pre-frozen sample. A slow increase in *d*-spacing from 5.3 nm to 5.45 nm occurs as the sample is heated from −18°C to about −6°C suggesting that limited ice melting is allowing a partial rehydration of the L_β phase. This is followed first by a sharp decrease in *d*-spacing to 4.8 nm as the sample undergoes an $L_\beta \rightarrow L_\alpha$ transition and then an increase as the L_α phase begins to hydrate slowly at first and then more rapidly as the sample approaches 0°C.

The second trace shows the effect of recooling the sample under supercooling conditions. The *d*-spacing of the hydrated L_α phase remains effectively constant at about 5.1 nm until a sharp increase to 5.6 nm occurs at about −12°C as the sample reverts to the L_β phase without freezing. The third trace shows the effect of re-cooling a frozen sample from −5°C before the bulk of the ice has melted. In this case, the sample showed a sharp increase in *d*-spacing from about 4.8 nm to 5.5 nm centred at about −9°C associated with the $L_\alpha \rightarrow L_\beta$ followed by a slow reduction in *d*-spacing as the sample was cooled to lower temperatures. As in the wide-

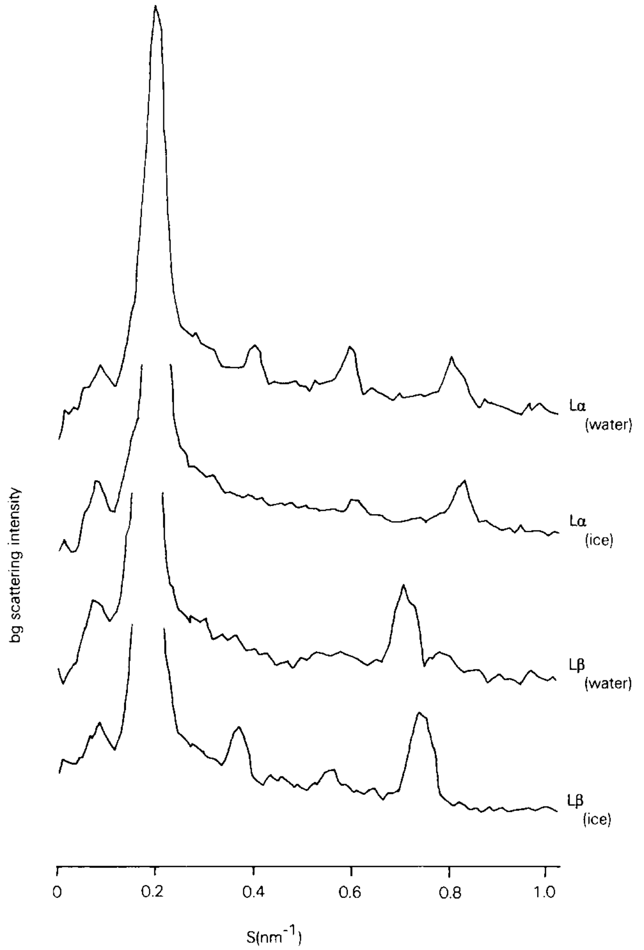

Fig. 3. Typical small-angle X-ray diffraction patterns for di-18:1 PE showing the L_α phase in (a) supercooled water (b) in ice, and the L_β phase in (c) supercooled water and (d) ice.

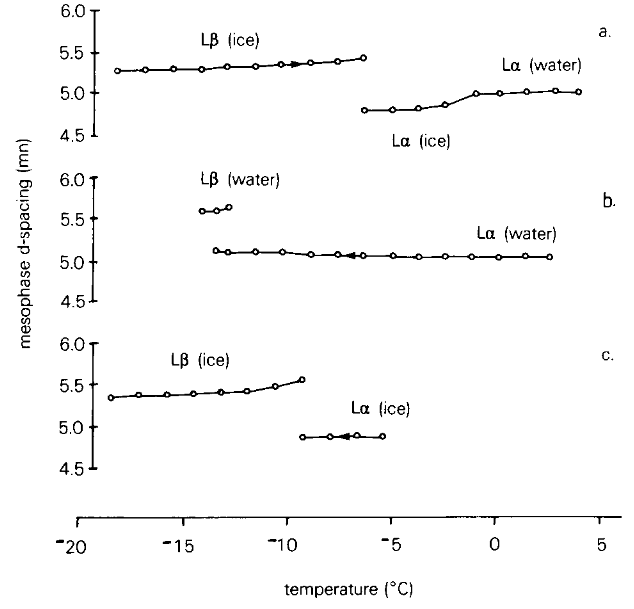

Fig. 4. Plot showing the changes in *d*-spacing of a sample of di-18:1 PE during a heating and cooling cycle. See text for explanation.

angle studies, the transition between the L_β and L_α states can be seen to be shifted to higher temperatures in the frozen as opposed to the unfrozen state. The small decrease in the apparent transition temperature between the heating and cooling runs made under frozen conditions reflects difficulties associated with the accurate determination of onset temperatures in the diffraction measurements.

The second series of experiments was devoted to the study of the effects of ice on the $H_{II} \rightarrow L_\alpha$ transition occurring in di-18:2 PE. A typical set of low angle diffraction patterns, collected during the course of a cooling scan from 0°C to −30°C, are presented in Fig. 5a. Individual diffraction patterns of the lipid collected at 10°C and −25°C, respectively, are presented in Figs. 5b and 5c. Six diffraction maxima, indexing in a ratio of d: $d/\sqrt{3}$: $d/\sqrt{4}$: $d/\sqrt{7}$: $d/\sqrt{9}$: $d/\sqrt{12}$ can be identified for the H_{II} sample. The lamellar phase is less clearly defined but the first and fourth order maxima are readily identifiable.

The sample which was in the supercooled state underwent a clear $H_{II} \rightarrow L_\alpha$ transition between −11°C and −16°C. Again the transition was a conventional two-state transition with no evidence for the formation of any intermediate phases. The formation of ice, signalled by the appearance of a wide-angle diffraction maximum at a spacing of 0.39 nm corresponding to the most intense of the three peaks characteristic of hexagonal ice (not shown) occurred at about −16°C after the completion of the main transition. A second set of data for di-18:2 PE, for a sample in which freezing occurred at −14°C, shortly after the onset of the transition, is shown in Fig. 6. In this case, the transition continues over a much wider temperature range spanning −11°C to about −35°C. The presence of ice appears to impede but not to prevent the completion of the bilayer to non-bilayer transition.

X-ray diffraction measurements carried out on reheating the di-18:2 PE samples (not shown) reversed the changes seen on cooling. The onset of the $L_\alpha \rightarrow H_{II}$ transition was, however, delayed until −2°C coinciding closely with the disappearance of the diffraction peaks associated with the presence of ice. The third and final series of measurements were performed on di-18:2 PC. These were aimed at studying the effect of freezing and melting of ice on the *d*-spacing of a lipid that is not undergoing a low-temperature phase transition. Di-18:2 PC, as detailed in the accompanying paper [11], remains in the L_α phase at temperatures down to at least −30°C. A plot of the temperature dependence of the *d*-spacing of di-18:2 PC as it is first cooled from 10°C to −45°C and then reheated back to 5°C is presented is in Fig. 7. An abrupt decrease in *d*-spacing from 5.62 nm to 4.32 nm associated with osmotic dehydration as the ice-front propogates through sample is observed when the supercooled sample first

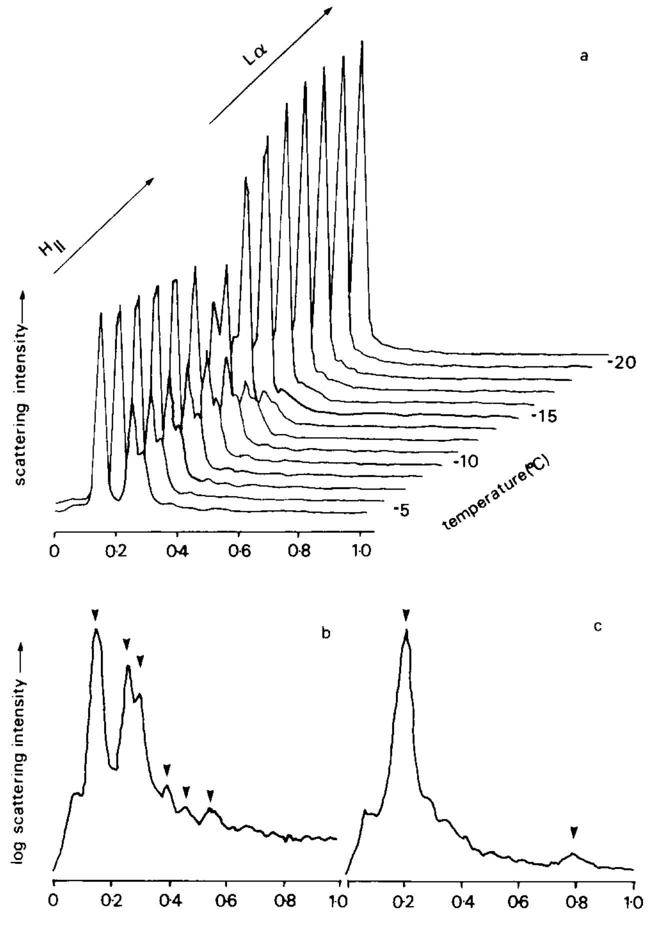

Fig. 5. (a) Set of small-angle patterns X-ray diffraction patterns for di-18:2 PE showing the $H_{II} \rightarrow L_\alpha$ transition occurring in supercooled water together with individual diffraction patterns measured at (b) 10°C and (c) −25°C, respectively. The characteristic diffraction maxima of the H_{II} and L_α phases are indicated by arrows.

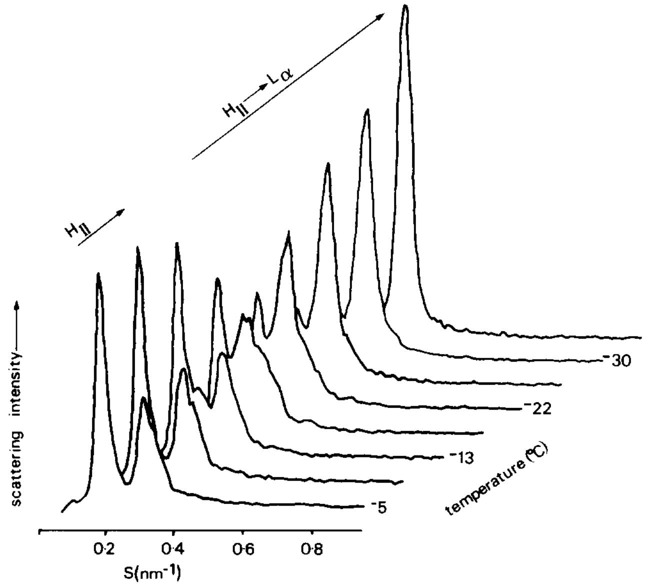

Fig. 6. Set of small-angle patterns for di-18:2 PE showing the $H_{II} \rightarrow L_\alpha$ transition occurring in a sample freezing at −14°C.

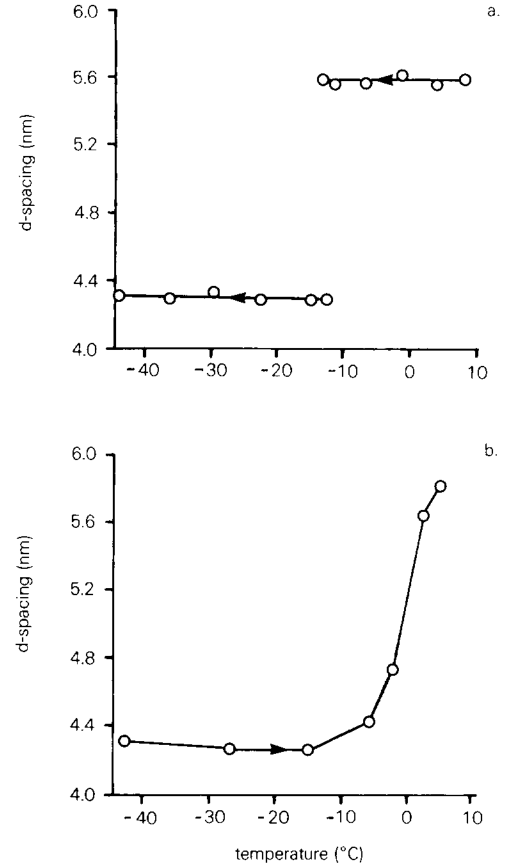

Fig. 7. Plot showing the changes in *d*-spacing of a sample of di-18:2 PC during a heating and cooling cycle.

freezes at about −10°C. Little or no change is seen on further cooling to below −40°C. On re-heating the *d*-spacing remains essentially constant until about −15°C. Above this temperature there is an increase in spacing that rapidly accelerates above about −5°C until the sample regains the spacing typical of the fully hydrated sample at about 5°C. The relatively large changes in *d*-spacing occurring in the frozen sample at sub-zero temperatures further underline the fact that significant changes in the amount of liquid water present in frozen samples occurs at temperatures well below 0°C.

Discussion

Little systematic work has been carried out on the effects of the formation, and presence, of ice on lipid phase behaviour. The effects of ice formation have generally either been ignored or attempts have been made to circumvent the problem by the inclusion of cryoprotectants in the dispersing medium. Recent studies indicating that the presence of high concentrations of soluble co-solutes such as glycerol and sugars influence the phase properties of lipids [12–15] point to the dangers of such an approach.

By comparing results obtained in supercooled and frozen systems, we have been able to obtain a much more direct insight into the effects of ice on lipid phase behaviour. The DSC and X-ray diffraction measurements performed on di-18:1 PE indicate that the presence of ice has relatively little effect on the $L_\alpha \rightarrow L_\beta$ and $L_\beta \rightarrow L_\alpha$ transitions of this lipid. The transition temperature is raised from −10°C to −6.5°C and the molar enthalpy from 6.1 to 8.5 kcal/mol. There is no indication of any significant changes in the co-operativity of the transition.

There are a number of ways in which the presence of ice might affect lipid phase transitions. The most obvious of these is that the presence of ice might impose steric constraints preventing the reorganisation of the lipid. This does not appear to be a significant factor in the case of $L_\alpha \rightarrow L_\beta$ transitions at least. It may, however, play a significant role in influencing transitions between bilayer and non-bilayer phases. Our data suggests that bilayer to non-bilayer phase transitions cannot take place in the presence of ice. In the case of di-18:2 PE, the onset temperature of the $L_\alpha \rightarrow H_{II}$ transition for di-18:2 PE is delayed to −2°C (coinciding with the onset of the bulk melting of ice) as compared to a temperature of about −11°C for the corresponding $H_{II} \rightarrow L_\alpha$ transition. It is not clear whether this reflects a simple hysteretic effect of the type seen for di-18:1 PE or an inherent ability of the system to undergo an $L_\alpha \rightarrow H_{II}$ transition in the presence of ice. The co-incidence of the transition with the bulk melting of the ice does, however, tend to support the latter view.

The data for di-18:2 PE presented in Fig. 6 suggest that non-bilayer to bilayer transitions, can at least proceed in the presence of ice although it is still not clear whether the transition can be initiated in a fully frozen sample. Unfortunately, in our hands freezing always occurred in this system at temperatures below the onset of the $H_{II} \rightarrow L_\alpha$ transition after the initial nucleation of the transition had taken place. Sanderson and Williams [6], however, have reported that the corresponding transition from the H_{II} to the L_β phase does appear to occur in ice, albeit with some difficulty, in monogalactosyldiacylglycerol dispersions.

Another way that the presence of ice might influence lipid phase transitions is by altering the degree of hydration of the sample. The small-angle X-ray diffraction measurements presented in Figs. 3 and 4 indicate that the *d*-spacing of both the L_β and the L_α phases of di-18:1 PE increases with increasing temperature suggesting that significant changes in the amount of liquid water occur over the temperature range of interest. This is emphasised in the measurements of the temperature dependence of the *d*-spacing of di-18:2 PC presented in Fig. 7 where the lamellar repeat distance increases at a steadily increasing rate above about −15°C. Brontshevn and Steponkus [5] have reported independent evidence based on DSC measurements for the melting of ice in hydrated lipid samples over this temperature range.

Lipid dehydration tends to raise the $L_\alpha \rightarrow L_\beta$ transition temperature of phosphatidylethanolamines [16]. It can also raise the molar enthalpy of the gel to liquid-crystal transition by favouring the formation of a crystalline sub-gel phase (L_c) over that of the conventional L_β phase. It must be emphasised, however, that the X-ray diffraction data presented here showed no sign of the formation of such a phase. In the case of transitions between bilayer and non-bilayer phases, reductions in hydration tend to stabilise the non-bilayer phase at the expense of the bilayer phase [15,16]. The fact that the presence of ice tends to prevent, or restrict, the formation of non-bilayer phases together with the fact that a much greater degree of structural rearrangement is associated with such transitions, suggest that steric considerations play the more important role in determining the effect of ice in these systems.

A major outstanding question is whether or not the formation of ice, or changes in the phase of pre-existing ice-crystals can drive lipid phase transitions. Clearly, if the $L_\alpha \rightarrow L_\beta$ transition temperature of a lipid in ice is higher than that in supercooled water, as in the case of di-18:1 PE, samples supercooled to temperatures below this temperature will inevitably undergo a $L_\alpha \rightarrow L_\beta$ transition on freezing. Similarly, freeze-induced concentration of dissolved solutes may raise the lipid phase transition temperature above that of the supercooled system [17]. In this sense ice formation can trigger lipid phase changes. There is, however, as yet no evidence to suggest that phase changes in pre-existing ice crystals can drive such changes.

Another important question is the location of the ice in these systems. Is the ice located between the individual bilayers or is it external to the system dehydrating the bilayers by a mechanism similar to that occurring in the plasmolysis of cells following extracellular ice formation? Preliminary experiments (not shown) performed with partially hydrated lipid samples indicate that the ice diffraction peaks observed in this study are coming from excess water but it is still not clear precisely where this water is located.

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References

1 Morris, G.J. and Clarke, A. (1981) Effects of Low Temperatures on Biological Membranes, Academic Press, London and New York.

2 Gordon-Kamm, W.J. and Steponkus, P.L. (1984) Proc. Natl. Acad. Sci. USA 81, 6373–6377.

3 Morris, G.J. (1987) in The Effects of Low Temperatures on Biological Systems (Grout, B.W.W. and Morris, G.J., eds.), pp. 120–146, Edward Arnold, London.

4 Chapman, D., Williams, R.M. and Ladbooke, B.D. (1967) Chem. Phys. Lipids 1, 445–475.

5 Bronshtevn, V.L. and Steponkus, P.L. (1991) Cryobiology 28, A56.

6 Sanderson, P.W. and Williams, W.P. (1992) Biochim. Biophys. Acta 1107, 77–85.

7 Coolbear, K.P., Berde, C.B. and Keough, K.M.W. (1983) Biochemistry 22, 1466–1473.

8 Keough, K.M.W., Giffin, B. and Kariel, N. (1987) Biochim. Biophys. Acta 902, 1–10.

9 Epand, R.M. and Epand, R.F. (1988) Chem. Phys. Lipids 49, 101–105.

10 Dowell, L.G., Moline, S.W. and Rinfret, A.P. (1962) Biochim. Biophys. Acta 59, 229–242.

11 Williams, W.P., Sanderson, P.W., Cunningham, B.A., Wolfe, D.H. and Lis, L.J. (1993) Biochim. Biophys. Acta 1148, 285–290.

12 Bryszewska, M. and Epand, R.E. (1988) Biochim. Biophys. Acta 943, 485–492.

13 Koynova, R.D., Tenchov, B.G. and Quinn, P.J. (1989) Biochim. Biophys. Acta 980, 377–380.

14 Williams, W.P., Quinn, P.J., Tsonev, L. and Koynova, R.D. (1991) Biochim. Biophys. Acta 1062, 123–132.

15 Sanderson, P.W., Williams, W.P., Quinn, P.J. and Lis, L.J. (1991) Biochim. Biophys. Acta 1067, 43–50.

16 Seddon, J.M. Cevc, G., Kaye, R.D. and Marsh, D. (1984) Biochemistry 23, 2634–2644.

17 Caffrey, M. (1987) Biochim. Biophys. Acta 896, 123–127.