Biochimica et Biophysica Acta, 690 (1982) 81–88
Elsevier Biomedical Press

BBA 71288

THE PHYSICAL STATE OF QUICK-FROZEN MEMBRANES AND LIPIDS

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(Received February 2nd, 1982)

Key words: Lipid bilayer; Membrane structure; Rapid freezing; X-ray crystallography; Liquid crystal; Phase transition; Differential scanning calorimetry

Lipid bilayers and biomembranes produce nearly identical calorimeter scans regardless of whether they are slowly cooled under near-equilibrium conditions or rapidly frozen at rates used in freeze-fracture electron microscopy. Except for the melting of ice at 273 K, for both cooling regimens no significant thermal events occur from 100 K to the usual gel to liquid crystal transition. The gel to liquid crystal transition itself is somewhat altered by rapid cooling when bilayers contain mixed lipid species. Combined with X-ray diffraction studies, the results indicate that quickly frozen bilayers are crystalline, but that the crystalline domains are quite small or otherwise disordered. In contrast to the behavior of lipids in bilayers, hexagonal-phase calcium cardiolipid easily forms a glass upon cooling.

Introduction

In freeze-fracture electron microscopy of membranes, samples are quickly frozen at rates fast enough to immobilize fluid bilayers before gross lateral phase separation of lipids and diffusion of proteins can occur [1]. This effect of rapid cooling can easily be seen by examining the electron microscopic images [2,3], and in fact is the main reason why freeze-fracture microscopy is a valuable method for studying membrane dynamics. However, the state of the phospholipid fatty acid chains after quick-freezing is not well understood and cannot be determined directly by microscopy. In analogy with more common fluids, the bilayer could crystallize as it does during slow cooling, or it could merely become a supercooled but very viscous liquid, or it could become a glass possessing a well-defined glass transition. Few investigations to distinguish between these possibilities have been carried out. The most relevant is the X-ray diffraction work of Costello and Gulik-Krzywicki [4], who showed the low-angle diffraction pattern of the hydrocarbon chains in quick-frozen lipid bilayers to be diffuse, as that of fluid bilayers at temperatures above the gel-liquid crystal transition [5], but centered near the position expected for the sharp reflection characteristic of the crystalline phase obtained by slow cooling. The results are consistent with either a supercooled liquid or a glass, or even with a crystalline substance if the crystal domains are very small or otherwise disordered.

Supplementing X-ray diffraction with differential scanning calorimetry or differential thermal analysis has long been a powerful approach for studying the properties and structure of many materials. Thermal data have often been used, for example, to characterize the temperature-dependent properties of polymorphic substances such as polymers [6]. It is especially useful for the problem under consideration here, since the three possibilities suggested can each give rise to unique behavior upon heating. We have carried out a scanning

calorimeter study of a variety of hydrated phospholipids, mostly in the lamellar phase, which have been quick-frozen to liquid nitrogen temperatures at rates comparable to those used in freeze-fracture microscopy. The results allow a definition of the state of the hydrocarbon core of the bilayers. The calcium salt of cardiolipin was also examined, and was found to behave anomalously.

Materials and Methods

Lipids were purchased from Sigma Chemical Company (St. Louis, MO). Their purity was verified by thin-layer chromatography using chloroform/methanol/water (65:25:4, v/v) with detection by iodine or charring. Other chemicals were reagent grade, and the water was twice distilled. Except for cardiolipin, samples were prepared by drying the lipid from chloroform with a stream of nitrogen, putting it under vacuum (100 μmHg) for at least 4 h, then mixing it with approximately two parts solvent by weight. Full hydration and the presence of excess solvent was verified calorimetrically in each case. Calcium cardiolipin was prepared by drying the lipid from ethanol as a thin film in a round-bottom flask, putting it under vacuum as above, suspending it in water by shaking with glass beads, then precipitating by adding this suspension dropwise to a solution of $CaCl_2$ until a final concentration of 0.2 M salt. The precipitate was collected by centrifugation.

Two methods were used to quick-freeze the samples; although perhaps not as fast as the fastest techniques [7,8] both are thought to give greater cooling rates than those used in conventional freeze-fracture electron microscopy [7,9]. For both these methods a large polished brass block partly immersed in a reservoir of liquid nitrogen provided the cold surface. A thin stainless steel sheet with beveled edges placed on top of the block assured that the working surface was a few degrees above liquid nitrogen temperature; this was necessary to prevent condensation of nitrogen gas on the surface that would have slowed the cooling process. The evaporating liquid nitrogen provides a dry atmosphere that prevents condensation of atmospheric water vapor on the block. In one method, samples were splat-cooled on the surface. If temperature control was not essential and the samples had low viscosity, they were simply allowed to fall a distance of several feet onto the surface. A 10–20 μl drop produced a frozen splat a centimeter or more in diameter. If temperature control was important, liquid samples were placed in a thermostatically controlled brass cup mounted on a rod ending in a handle. The device resembles a hammer. The sample was placed in the cup and the hammer was struck against a stop, ejecting the contents onto the cold brass block. In the second method, which is particularly useful for viscous samples or pastes which do not splat easily, the quick-freezing device was a round brass plunger one inch in diameter and one foot long, terminating in a one-inch-long smooth-faced teflon section of the same diameter. The plunger slides vertically through a cylindrical brass guide, and at the end of its stroke squarely contacts the brass block held at about 85 K. A similar device has been used to prepare specimens for freeze-fracture electron microscopy [9]. The pasty sample was loaded onto the teflon face, which was heated if necessary, and the plunger was rapidly pushed down against the brass block. The thin frozen sample was then removed from the face by drawing it across a projection on the surface of the block. After either of the quick-freezing methods the frozen samples were pushed into an aluminum sample pan held in a cavity in the block, sealed, and loaded into the pre-cooled calorimeter head. At no point before scanning did the sample temperature rise above 90 K. In some cases several loadings and strokes of the plunger were needed to obtain enough frozen material for an adequate scan. Samples were routinely scanned up from 100 K through their gel-liquid crystal transition, then cooled in the calorimeter and rescanned up. Unless otherwise indicated, upscan rates were 10 K/min. The calorimeter was either a Perkin-Elmer DSC-2 with the head mount modified to accommodate an 8 l liquid nitrogen reservoir or the Dupont 1090 DSC system.

In all of the scans taken in water, the melting of ice obscured the region in the neighborhood of 273 K. This effect was particularly bothersome for the phospholipids from natural sources, where the gel-liquid crystal transition occurred in the same temperature range. The broad transition in these materials was partially visible, but could not be

completely characterized because of the superimposed ice peak. Samples were therefore examined in both water and 50% (v/v) ethylene glycol, which depresses the freezing point of water by about 40 K.

Results

To illustrate the ability of the calorimeter to distinguish various states, some heating scans of known substances taken in these laboratories are shown in Fig. 1. Stearic acid, Fig. 1A, undergoes the typical first-order melting of a crystalline material; the compound is crystalline at temperatures below the peak and fluid above it. The conventional $L_\beta \rightleftharpoons L_\alpha$ transition of bilayers consisting of pure phospholipid species such as dipalmitoylphosphatidylcholine [5] produces the same type of peak. Cholesterol oleate, Fig. 1B, is a supercooled liquid at the lowest temperature

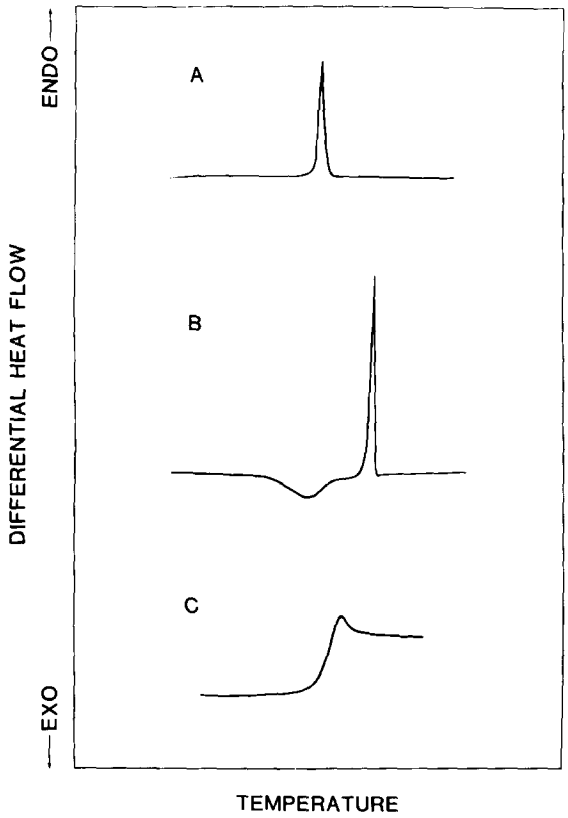

Fig. 1. Calorimeter heating scans of known materials. (A) Stearic acid. (B) Cholesterol oleate. (C) Glycerol.

shown, then crystallization gives rise to an exotherm followed later by endothermic melting of the crystals [10]. Glycerol yields a glass transition, seen as a change in heat capacity without an accompanying enthalpy change. The 'overshoot' is a consequence of differences in the rates of heating relative to the quick-freezing of the sample [6]. Glycerol is glassy below the discontinuity in heat capacity and a supercooled liquid above it. Any of these patterns might be possibilities for quick-frozen phospholipid bilayers.

The lamellar phospholipids examined were dipalmitoyl-, dimyristoyl-, dielaidoyl- and dioleoylphosphatidylcholine; egg phosphatidylcholine both alone and mixed in a 1:1 molar ratio with phosphatidylinositol; and dipalmitoylphosphatidylcholine in a 2:1 molar ratio with cholesterol. *Acholeplasma laidlawii* membranes were also studied. For each material, heating scans beginning at 100 K were compared for samples quick-frozen or slowly cooled from temperatures above the gel-liquid crystal transition, as determined by a separate scan of the material. Dipalmitoylphosphatidylcholine was quick-frozen from 330 K, dimyristoylphosphatidylcholine from 320 K, and *A. laidlawii* membranes from 313 K; the remaining substances were quick-frozen from room temperature. Slow cooling was done directly in the calorimeter; cooling at 320 K/min, the fastest programmed rate available in the DSC-2 calorimeter, gave the same results on subsequent upscans as slower cooling rates. The calorimeter sensitivity was chosen to give full-scale peaks for the gel-liquid crystal transition. As will be shown later, this setting is quite adequate to reveal any anomalous behavior associated with changes in the state of the phospholipids.

Except for minor perturbations of the gel-liquid crystal transition in bilayers which were nonhomogeneous in fatty acid composition or lipid class, in all cases the scans were identical for both slowly and rapidly cooled samples. For both cooling modes, except for thermal phenomena due to water or ethylene glycol-water, baselines were flat and featureless from 100 K to the gel-liquid crystal transition. There were no indications of unexpected thermal events such as those shown in Figs. 1B and 1C. The scan of dimyristoylphosphatidylcholine shown in Fig. 2 is an example. The

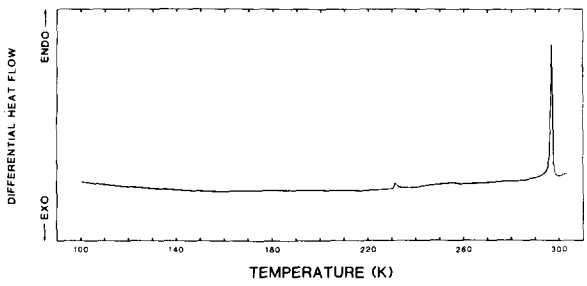

Fig. 2. Heating scans taken at 10 K/min of bilayers of dimyristoylphosphatidylcholine in ethylene glycol quick-frozen to 80 K. The small narrow peak at 231 K and the broad peak at 240–258 K are due to glycol. In water the baseline is featureless until ice melts at 273 K.

small narrow peak at 231 K and the broad peak at 240–258 K are due to ethylene glycol; in water the baseline is featureless until ice melts at 273 K.

As previously pointed out, the results already described and illustrated were obtained at calorimeter sensitivities chosen to give full-scale deflections for the gel-liquid crystal transition. Under such conditions, no thermal events can be seen at lower temperatures. The baseline is flat. However, at much higher sensitivity settings minor effects of quenching are detectable in the region below the ice point. Their magnitude is no more than about one percent of the major transition.

An example of these smaller effects is shown in Fig. 3, a plot of the difference in scans of a rapidly and slowly cooled sample of dimyristoylphosphatidylcholine in water. The sensitivity setting was about 40-times that of Fig. 2 and on this scale the $L_\beta \rightleftharpoons L_\alpha$ transition at 296 K would be many times full scale. The most prominent event is a peak centered at 165 K. This same peak occurs in all lipid samples in water, and also in pure distilled water as well as ^2H_2O which has been quickly frozen by using the teflon plunger or by allowing a 10 μl free-falling water drop to fall on the chilled brass block. It vanishes once the sample has been scanned up then slowly recooled to 100 K. Its origin is unknown, but transitions in this temperature range have been reported in vacuum-deposited glassy water [11]. The other events, which also vanish once the sample has been heated and slowly cooled again, are due to the presence of lipid. Similar peaks at the same temperatures were obtained with dimyristoyl-, dielaidoyl-, dioleoylphosphatidylcholine and egg phosphatidylcholine, the only other lipids examined in this manner. Their origin is also unknown, but it is unlikely that they are associated with gross changes in the state of the bilayers since the same patterns are obtained with phosphatidylcholines having drastically different fatty acid compositions. In addition, major changes such as crystallization or glass transitions of the lipids would be expected to produced calorimetric effects nearly as large as the gel-liquid crystal transition.

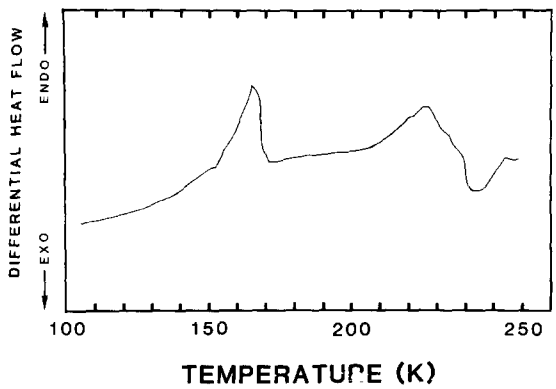

Fig. 3. Heating scans of quick-frozen dipalmitoylphosphatidylcholine bilayers taken at high sensitivity in distilled water. The phenomena seen here are negligible at the sensitivities used for Figs. 1, 2, 4, and 5.

Although of secondary importance to the major conclusion just drawn, rapid cooling can have minor effects upon the shape of the gel-liquid crystal transition itself. Such effects could not be discerned in bilayers of the pure species examined (dipalmitoyl-, dimyristoyl-, dielaidoyl- and dioleoylphosphatidylcholine), where the shape and temperature of the $L_\beta \rightleftharpoons L_\alpha$ peaks appear to be identical whether or not the samples are rapidly cooled. However, quick freezing did affect the shape of transitions in bilayers composed of mixed phospholipids. This phenomenon is not unexpected, since the rate of phase separation is limited by diffusion. Examples of egg phosphatidylcholine and a 1 : 1 molar ratio of phosphatidylinositol and egg phosphatidylcholine quick-frozen and unquenched are shown in Figs. 4A, B and 4C, D, respectively. Repeated scans of slowly cooled samples gave identical thermograms. Similar perturbations of the gel-liquid crystal transitions were found

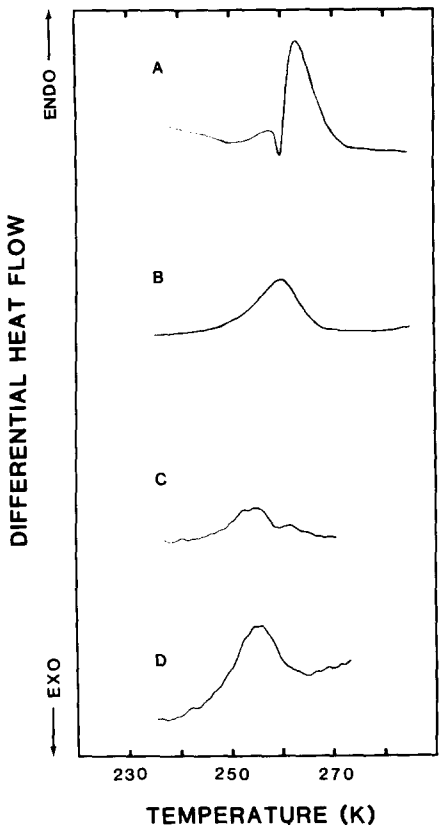

Fig. 4. The order-disorder transition in bilayers of egg phosphatidylcholine (A) quick-frozen and (B) slowly cooled; and of bilayers of a 50/50 molar mixture of egg phosphatidylcholine/soybean phosphatidylinositol (C) quick frozen and (D) slowly cooled. In all cases repeated scans of slowly cooled samples gave identical thermograms. The medium was 50% (v/v) ethylene glycol.

by quick-freezing *Acholeplasma laidlawii* membranes from 313 K, a temperature above the membrane transition.

Comparisons of the effects of slow and rapid cooling were also extended to cholesterol-containing phosphatidylcholine bilayers. Their thermal behavior above 273 K is reasonably well understood. Cholesterol concentrations above about 20 mol% suppress the phosphatidylcholine bilayer transition; a broad transition remains above 20 mol% disappearing at about 50 mol% cholesterol [12,13]. Comparative scans from 100 K were made of rapidly and slowly cooled bilayers of egg phosphatidylcholine/50 mol% cholesterol, dipalmitoylphosphatidylcholine/30 mol% cholesterol and dipalmitoylphosphatidylcholine/50 mol% cholesterol. In no cases were transitions observed; again the scans were flat and featureless from 100 K to 322 K, where the scans were terminated.

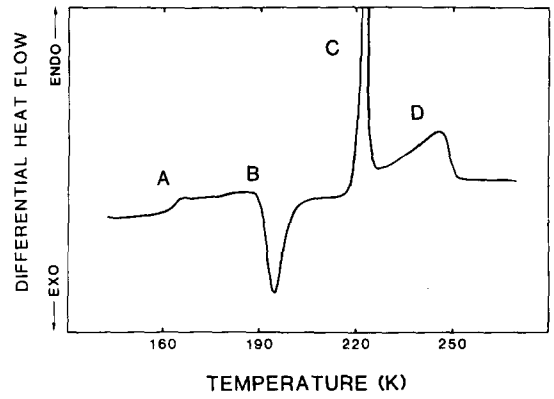

Fig. 5. Heating scans of calcium cardiolipin (bovine heart) in 0.2 M $CaCl_2$. This pattern appears for samples cooled at all rates faster than 5 K/min.

In contrast to lamellar phospholipids, cooled samples of the calcium salt of cardiolipin do in fact clearly show such glass transitions, and therefore, verify the ability of the calorimeter to detect them in lipid systems when they occur. Calcium cardiolipin has been shown by X-ray diffraction to be in the hexagonal phase at temperatures above 273 K [14,15]. An upscan of calcium cardiolipin, cooled from room temperature, is shown in Fig. 5. The calorimeter sensitivity setting was low, the same as that used in Fig. 2, and insufficient to detect minor effects such as those illustrated in Fig. 3. Event A, at about 165 K, is a discontinuity in heat capacity with a slight overshoot, and is typical of a glass transition [16]. This behavior resembles that of glycerol in Fig. 1C. Peak B is an exothermic transition, presumably crystallization of the supercooled material produced by the glass transition at A. As will be discussed shortly, the crystallization of the supercooled material is quite slow. This means that the glass characterized by the transition at A can be produced without quick freezing, and in fact the pattern shown in Fig. 5 is obtained at all cooling rates greater than 5 K/min. The upscans and downscans described here were carried out at 10 K/min. Peak C signifies the melting of the crystalline material produced at B. Peaks B and C, taken together, resemble Fig. 1B.

This interpretation is supported by the fact that the three events are interrelated in the following way. If the sample is scanned from 100 K to 170 K (just above A), then immediately cooled to 100 K and rescanned, the glass transition remains. However, if the sample is incubated at a temperature just below peak B, then cooled and rescanned, both A and B have vanished but C remains. Approx. 6 min are required for complete crystallization at 185 K and 30 min at 180 K. Similarly, if the lipid is scanned down from 270 K to 200 K and immediately scanned up, peak C is absent but D remains. Peak C appears fully if after scanning down from 270 K the sample is incubated for 30 min at 200 K. In this case, A and B are again absent. Peak D, in contrast, is rapidly reversible. It exists independently of C.

Structural assignments and further characterization of the peaks is impossible without X-ray diffraction combined with thermal studies, but it seems possible, in view of the slow kinetics of crystallization, that the form which is produced at B and melted at C is a highly crystalline lipid whose structure is neither hexagonal nor lamellar. Peak D may be either an $L_\beta \rightleftharpoons L_\alpha$ or an $L_\beta \rightleftharpoons H_\alpha$ transition, and the glass transition at A may take place in either an hexagonal or lamellar phase. Although further investigation of the thermal properties of the divalent salts of cardiolipin would no doubt prove to be interesting for its own sake, a detailed understanding of cardiolipin is not needed to strengthen the major conclusion of this investigation: that the calorimeter can identity the glassy state and supercooled condition in lipid systems, and that neither exists in quick-frozen lipid bilayers.

Discussion

No major events beyond the usual gel-liquid crystal transition were seen in rapidly cooled samples scanned from 100 K. For each bilayer-forming lipid, scans of rapidly and slowly cooled samples were essentially identical. Since the hydrocarbon chains of slowly cooled bilayers are known to be in the crystalline L_β phase, and since any additional phase changes would be clearly visible in the calorimeter, the results indicate that the L_β phase, or one thermodynamically identical to it, is retained upon rapid cooling. This behavior is not unexpected, since efforts by other workers to produce glasses of long-chain linear hydrocarbons have been unsuccessful (Moynihan, C.T., personal communication). An even greater tendency to crystallize would be expected for the hydrocarbon chains in bilayers, which in the liquid state possess more order than the simple hydrocarbons.

Two of the systems reported here, egg lecithin alone and the 1 : 1 molar ratio of egg phosphatidylcholine and phosphatidylinositol, have also been studied by X-ray diffraction [4]. Even for slower cooling rates than those used in this work, the hydrocarbon core produced a broad reflection characteristic of fluid, disorganized chains but centered near the spacing characteristic of the L_β phase. One possible explanation of this observation is that the chains are disorganized, as they are in the L_α phase, with the spacing reduced by contraction in the cold. The chilled bilayers would then exist as a supercooled liquid or a glass. Calorimetry eliminates both of these alternatives, for they would be expected to give rise to crystallization of the supercooled liquid as the sample is heated, or even to a glass transition followed by crystallization. However, the production of a diffuse diffraction band can be rationalized with the calorimetric data if the disorder indicated by the diffraction is produced by very small crystal size and/or other distortions of the crystal lattices produced by rapid contraction of the lipid bilayers. Under very rapid cooling, the crystalline bilayer may not be sufficiently plastic to relieve the stresses of contraction.

In the cooling rates used in these studies, quick-freezing versus slow cooling causes changes in the usual order-disorder transition in bilayers containing mixed fatty acid chains. Since the shape of such transitions in slowly cooled bilayers follows an equilibrium phase diagram [17], the changes observed suggest that near-equilibrium conditions are not maintained during rapid cooling. However, as so far as can be estimated, at least partial phase separation of lipids may take place when cooling is rapid, although the size of the separated regions would be expected to be quite small and the distribution of embedded protein molecules seen in the freeze-fracture images of biomembranes is not affected.

The small differences seen between rapidly and slowly cooled lamellar phosphatidylcholine, when scanned below the ice point at very high sensitivity (Fig. 5), cannot be ascribed to crystallization or a glass transition involving the bilayer hydrocarbon core. These possibilities can be rejected, both on thermodynamic grounds and by the results obtained with cardiolipin because the observed enthalpies are too small. Thermodynamically, the enthalpy of a crystallization peak lying T degrees below the usual order-disorder transition would be reduced from its usual magnitude by $C_p \cdot T$, where C_p is the difference in heat capacities between the liquid and solid bilayers. The correction is minor compared to the heat of transition. This conclusion is borne out by calcium cardiolipin, which shows a very large crystallization peak, comparable in size to the melting peak. The magnitudes of heat capacity changes expected during glass transitions, were they to occur in lamellar phospholipids, cannot be as straightforwardly estimated. However, the cardiolipin results indicate that the magnitude would be far beyond the minor effects seen at high sensitivity. In addition, if the high-sensitivity thermal patterns were associated with major changes in the hydrocarbon cores of the bilayers, the scans would be expected to change when fatty acid composition is changed. They do not; for phosphatidylcholine bilayers, the only system studied systematically at high sensitivity, the scans are independent of fatty acid composition. The origin of the events is unknown, and may be quite difficult to determine. Structural alterations in bound water could be a possibility.

Little additional comment can be made on the scans obtained with calcium cardiolipin. They have satisfied the purpose intended here, as a model system to verify the ability of the calorimeter to detect unexpected thermal events in phospholipids. A detailed understanding of the events would require combined calorimetric and X-ray diffraction studies, and possibly freeze-fracture electron microscopy as well. As pointed our earlier, the calorimetry suggests that the exothermic peak at 190 K and the endothermic peak at 220 K may signify the formation and melting of a separate crystalline structure which is neither hexagonal nor the usual ordered bilayers produced by most lamellar phospholipids at temperatures below their order-disorder transition. If this is true, and the lipid is hexagonal above 273 K, one consistent explanation is as follows. When rapidly quenched from room temperature, the hexagonal phase becomes glassy. Upon heating, it first undergoes a glass transition at 165 K to produce a supercooled hexagonal phase which crystallizes at 190 K to the hypothetical crystalline form. At 215 K this form converts to an ordered bilayer, which at 230 K undergoes the usual order-disorder transition and simultaneously changes to the hexagonal phase. Other possibilities can be suggested, but are equally speculative.

Acknowledgments

The authors wish to thank Dr. C.T. Moynihan for helpful discussions of the glassy state. One of us (D.L.M.) is a recipient of an American Diabetes Association Inc. Research and Development Award and would like to acknowledge NSF Grant No. PCM-811577 which allowed the purchase of the DuPont 1090 DSC used in part of these studies. The work was supported in part by National Institutes of Health Biological Research Support grant RR 07085-11.

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