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Trehalose and dry dipalmitoylphosphatidylcholine revisited

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Dry mixtures of sonicated vesicles of DPPC and trehalose which contained a maximum of 0.2 mol water/mol lipid were examined by differential scanning calorimetry, Fourier transform infrared spectroscopy and freeze-fracture electron microscopy. Samples of dry DPPC and trehalose prepared from aqueous solution had a minimum T_m of 24°C for the gel to liquid-crystalline transition provided that the vesicles were dried with trehalose while the lipid was in liquid-crystalline phase. This low transition is compared to a transition of 105–112°C for dry pure DPPC and of 42°C for hydrated pure DPPC. The present work is an extension of earlier work from this laboratory using both other lipids and other methods of preparation.

Introduction

A large body of evidence has accumulated over the past several years that trehalose (a disaccharide of glucose) along with several other mono-, di- and trisaccharides have the ability to stabilize biological membranes [1] and unilamellar liposomes [2–9] against fusion and leakage during both freeze-thaw cycles and freeze-drying. Evidence from infrared spectroscopy [10], ^{1}H-NMR spectroscopy, ESR spin labeling [8,9], and resonance energy transfer studies of fusion [7] suggest that trehalose and sucrose, at least, may exert their effects by a direct hydrogen-bonding interaction with the phosphate of phospholipid headgroups.

One might expect that hydrogen-bonded trehalose could have effects similar to hydrogen-bonded water on dry lipids [11], i.e. a lowering of the gel to liquid-crystalline phase transition temperature due to an increase in headgroup spacing and a decrease in van der Waals interactions between acyl chains. That, in fact, has been shown to be the case – trehalose, and to a lesser extent other carbohydrates, do lower the phase transition temperature of dry preparations of lipid and carbohydrates as much as 80°C, to below that of the fully hydrated lipid [6,12,13]. Other studies which used multilamellar preparations in the presence of both trehalose [14] and sucrose [9] report a similar effect although the lowering of the T_m is not as great as has been found in this laboratory.

Earlier work from this laboratory focused on the interactions of carbohydrates with dipalmitoylphosphatidylcholine, probably the most widely studied amphipathic lipid. These early studies were experimentally unsatisfactory in several respects, in that much of the equipment and procedures we now use were not available to us. Thus, it was necessary to make homogeneous multilamellar preparations of lipid and carbohydrate from organic solution, and to handle the preparations in ambient air following freeze-drying. These pro-

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cedures resulted in lipid/sugar mixtures that contained some small and unmeasured amount of water, probably about 2 mol H_2O per mol dipalmitoylphosphatidylcholine [13]. This led to the possibility that the water in the samples, although low, was in some way able to interact with the carbohydrates and phospholipid and produce the effect reported. Later work using other lipids for leakage [2,6] and residual water experiments [15] demonstrated that water in these preparations was extremely low and not related to liposome preservation during drying. The experiments reported in the present paper were done to assess, as carefully as possible, the effects of trehalose on the thermotropic properties of dry dipalmitoylphosphatidylcholine. These data demonstrate that in very dry dipalmitoylphosphatidylcholine the effects of interaction with trehalose are even more striking than when a small amount of water is present [14]. They also suggest that an expanded headgroup spacing is necessary for complete interaction of the trehalose with the phospholipid as suggested by Gaber and his colleagues [16,17]. If stored at or below the gel to liquid crystalline transition temperature the mixture reverts to a more gel-like state.

Materials and Methods

Materials. Dipalmitoylphosphatidylcholine in chloroform was purchased from Avanti Polar Lipid (Birmingham, AL) and used without further purification. Trehalose was purchased from Pfanstiehl, (Waukegan, IL) and was stored in glass following evacuation overnight. All solutions were prepared in distilled, deionized water.

Liposome preparation. DPPC in chloroform was dried under nitrogen in tared glass tubes and then dried under vacuum (10–15 mTorr) for several hours. The tubes were then weighed and rehydrated either with water or with a solution of trehalose which contained enough sugar to give specified trehalose/lipid mol ratios. The lipid concentration in the hydrated samples was 55 mM except where indicated in the figure legends. The highest trehalose/lipid mol ratio studied was 2.15 : 1. Rehydration was carried out at 55–60 °C, and the lipid was suspended by repeated vortexing. Multilamellar vesicles resulted from this treatment.

Small unilamellar vesicles (SUV) were formed by bath sonication (Lab Industries, Hicksville, NY) of multilamellar preparations at 55–60 °C. This method of vesicle preparation was used to ensure that the trehalose was interacting with all of the DPPC in the sample. At the end of sonication, the lipid preparation was faintly opalescent. Thin-layer chromatography in chloroform/methanol/water (65 : 35 : 4, v/v) showed a single spot for both DPPC directly from Avanti and DPPC in sonicated vesicles.

Liposome preparations were dried in two different ways. Each preparation was divided in half, and one half was quickly frozen in liquid nitrogen and freeze-dried at 10–20 mTorr for at least 12 h. The other half of the preparation was placed in a plastic weigh dish or small aluminum cup in a desiccator over anhydrous $CaSO_4$. The desiccator was placed in a 60 °C oven for two or more days until the preparation appeared dry. Then it was placed under vacuum (10–20 mTorr) at room temperature for at least 12 h to remove residual water.

Differential scanning calorimetry. Samples were transferred under vacuum from the lyophilizer to a glove box flushed with dry nitrogen and flushing was continued for several more minutes. Samples were then loaded into airtight ampoules while still in the dry box. Calorimetric scans were run on a Hart 7707 Series differential scanning calorimeter (Hart Scientific, Provo, UT). Data were stored and manipulated with an IBM PC using Hart Scientific software. The samples were scanned from −10 to 110 °C at a rate of 20 C°/h. A few preparations of pure DPPC were scanned to 115 °C.

A few experiments on the effect of storage of the samples below their gel to liquid-crystalline transition temperature were done using a Perkin-Elmer DSC 2C differential scanning calorimeter as well as the Hart calorimeter. The scanning rate of the Perkin-Elmer calorimeter was 5 C°/min. Data were collected and manipulated on the Perkin-Elmer 3600 data station, using Perkin-Elmer software.

Residual water content. The residual water content of both MLV and SUV were determined from the retention of $[^3H]H_2O$ as described in Crowe et al. [15]. This procedure results in an estimate of

the maximal retention since it ignores any ^3H exchange between water and lipid.

Freeze-fracture electron microscopy. Dry samples were fractured as described previously [18], and cleaned in chloroform-methanol solutions followed by chloroform.

Fourier transform infrared spectroscopy (FTIR). Spectra were obtained with a Perkin-Elmer 1750 Fourier transform infrared spectrometer assisted by a Perkin-Elmer 7500 laboratory computer. The spectral data were changed to absorbance and expanded in the phosphate asymmetric stretch region.

Results

DPPC in the absence of trehalose

The residual water content of pure dry DPPC MLV or SUV were 0.5 mol/mol lipid or 0.2 mol/mol lipid, respectively. All samples of dry DPPC examined had a thermotropic gel to liquid-crystalline transition peak (T_m) at 105°C or above; the highest T_m recorded for samples of pure, dry DPPC in this study was 112°C. A representative scan of this type of preparation is shown in Fig. 1. The transition of any preparation of pure dry DPPC was stable on all subsequent scans, and there were no consistent differences between MLV or SUV whether lyophilized or dried at 60°C followed by vacuum.

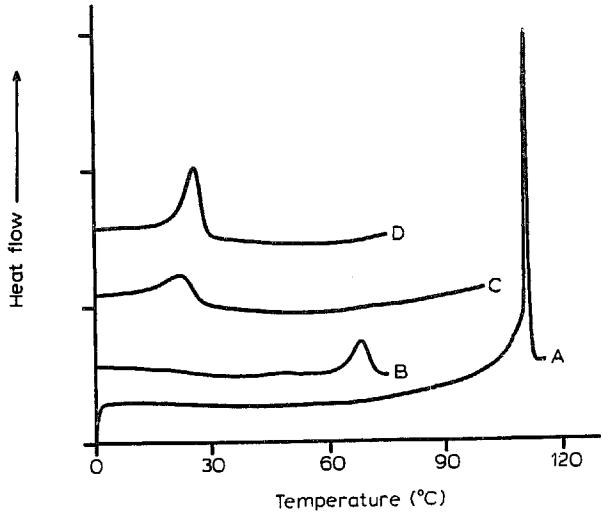

Fig. 1. Transitions of dry DPPC. A, pure DPPC dried at 60°C; T_m is 112°C. B, C, D, a sample of sonicated, lyophilized trehalose/DPPC (2.15 mol/mol). B, first scan, to 75°C; a broad transition is seen with a peak at 60.9°C. C, second scan, up to 110°C; the single peak has a T_m 24°C. D, third scan, to 75°C; the single peak has a T_m of 26°C.

Lyophilized DPPC at high trehalose concentrations

SUV that were lyophilized at trehalose/lipid mol ratios of 1.5:1 and above behaved similarly on the first scan, showing a small peak or shoulder at about 40°C and a large peak with a T_m between 60 and 70°C (Fig. 1). The exact location of the peaks showed some variability, which probably reflects small errors in the actual amount of trehalose added, in handling of the samples and possibly inhomogeneities during the drying process. These same samples, once they had been taken through a complete transition, had a single transition on the second scan with a T_m of 24–25°C (Fig. 1). Scanning these samples past the trehalose melting transition (around 100°C) caused a sharpening of the DPPC transition with a small upward shift in temperature of about 1 C°. Although pure trehalose dihydrate has a sharp melting transition around 100°C, this was never seen in the DPPC/trehalose preparations: however, scanning past 100°C produced the changes noted in the DPPC transition.

SUV lyophilized at a lower mass ratio of 0.65 mol trehalose/mol DPPC had two transitions with T_m values of 58°C and 67°C. At a trehalose/DPPC mol ratio of 0.42 the SUV had two to three transitions with T_m values between 61–83°C (not shown), i.e. the less trehalose present, the higher the temperature range of the multiple transitions. As with the SUV at higher mass ratios of trehalose, these samples with less trehalose showed a single stable transition upon repeated scanning.

To summarize the effects of trehalose on the gel to liquid crystalline transition of lyophilized SUV: at every mass ratio of trehalose/DPPC studied, trehalose lowered the thermotropic gel to liquid-crystalline phase transition temperature. On the first scan, at all trehalose/DPPC mass ratios there were at least two transitions. The range of temperature over which these transitions occurred was progressively lowered as more trehalose was present in the preparation. Once a preparation of trehalose/DPPC SUV had been scanned above 100°C, subsequent scans showed a stable and repeatable gel to liquid-crystalline transition with

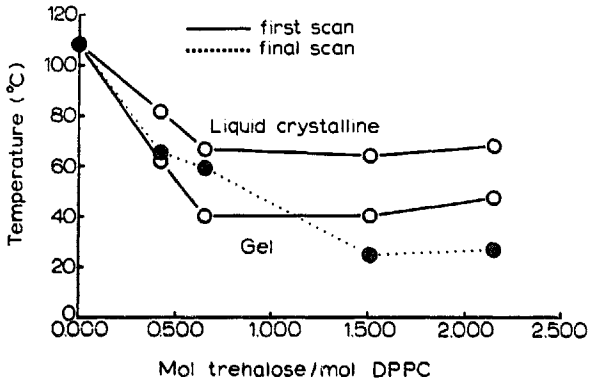

Fig. 2. Phase diagram of sonicated, lyophilized vesicles of DPPC at various mol ratios of trehalose, based on heating scans. Initial and final stable T_m values are shown.

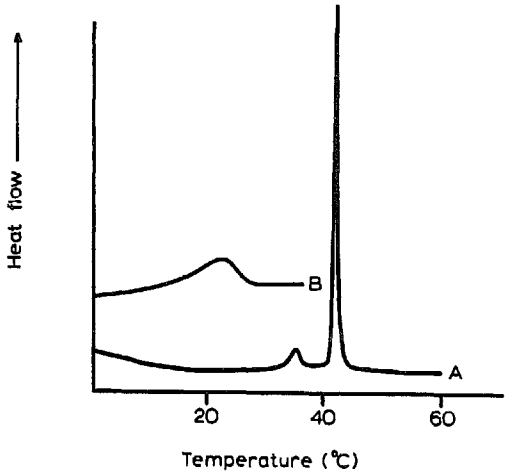

Fig. 3. Thermotropic transition of hydrated DPPC multilamellar vesicles (A) and the initial transition of sonicated vesicles dried at 60 °C as described in Methods in the presence of 2.15 mol trehalose/mol lipid (B). Scan B was taken to 75 °C, and a second scan (not shown) was identical to the first.

a T_m which was progressively lowered as more trehalose was present until it reached a stable value of 24–25 °C at 1.5 mol trehalose/mol DPPC. These results are summarized in the phase diagram shown in Fig. 2. It should be emphasized that this phase diagram is based solely on calorimetric data.

DPPC/trehalose SUV dried at 60 °C

The fact that the above preparations showed a single, stable transition once the DPPC had been melted in the presence of trehalose suggested that if the DPPC were dried while in liquid crystalline phase the trehalose present might be more effective in lowering the T_m of the transition on the initial scan. To investigate this possibility, we dried a series of samples of trehalose/DPPC SUV at 60 °C as described in Methods.

SUV dried at 60 °C and a trehalose/lipid mol ratio of 1.5 : 1 had a single transition on the first scan with a T_m of 24 °C which was stable on subsequent scans until the sample was scanned above the 100 °C trehalose transition at which time the DPPC transition was sharpened and the T_m was shifted slightly upwards (Fig. 3).

Preparations with 1.3 mol trehalose/mol lipid and below showed multiple transitions over the temperature range of approximately 40–70 °C on the first scan. On the second and subsequent scans, the low temperature transition shifted to 24 °C, with the enthalpy of that low transition increasing with increasing amounts of trehalose in the sample, i.e. with more trehalose present, more of the DPPC was able to fully interact with the sugar. These results are summarized in the phase diagram shown in Fig. 4. As in Fig. 2, this phase diagram is based on calorimetric data only.

Multilamellar vesicles dried at 60 °C

In contrast to the small unilamellar vesicles either lyophilized or dried at 60 °C, dry multilamellar vesicles always showed multiple transitions even after repeated scanning at high mol ratios of trehalose (Fig. 5). Repeated scanning increased the enthalpy of the lowest (24 °C) tran-

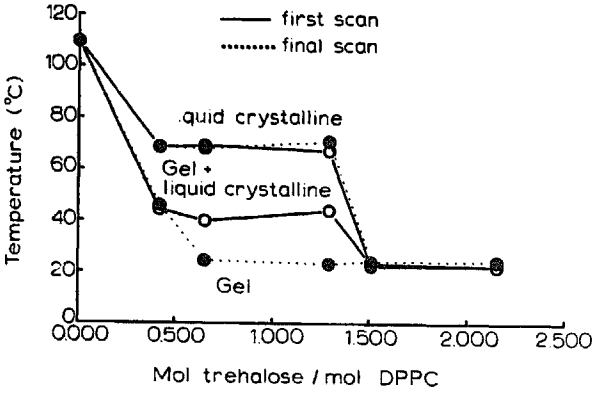

Fig. 4. Phase diagram of sonicated vesicles of DPPC at various mol ratios of trehalose which were dried at 60 °C as described in Methods. T_m values for initial and final scans are shown. A minimal value for T_m of 24 °C was reached at 1.5 mol trehalose/mol lipid.

sition, but higher transitions persisted. These results indicated inhomogeneities in the distribution of trehalose during the preparation of the multilamellar vesicles which is not present in sonicated preparations.

Fourier transform infrared spectroscopy

The frequency of the $-PO_4$ asymmetric stretch is affected by the presence of trehalose. Very dry samples have a wavenumber of 1254 cm^{-1}, while the $-PO_4$ asymmetric stretch is shifted to about 1230 cm^{-1} in hydrated samples due to hydrogen-bonding of water [19]. Sonicated vesicles, either lyophilized or 60 °C dried with high trehalose, have a similar wavenumber shift although there are small differences in the hydrated and dry lipid/trehalose spectra (Fig. 6).

Freeze-fracture electron microscopy

Preparations of sonicated DPPC which are lyophilized at low trehalose concentrations (Fig. 7A) show high fusion. At high trehalose (2.15 mol/mol DPPC), the individual liposomes persist in the lyophilized samples, embedded in a matrix of trehalose (Fig. 7B). However, when sonicated vesicles at any trehalose/DPPC mol ratio are dried at 60 °C, fusion takes place and a multilamellar preparation results (Fig. 7C, D).

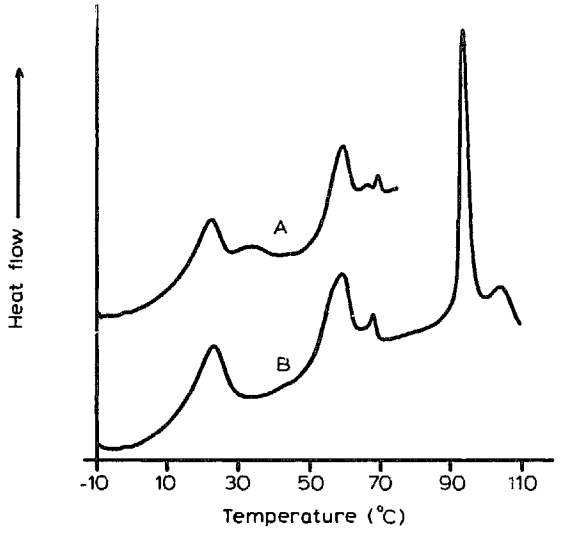

Fig. 5. Thermotropic transitions of a multilamellar preparation of trehalose/DPPC (2.15 mol/mol). In this case, 45 mg of DPPC was rehydrated at 60 °C with 132 µl of 1 M trehalose and repeatedly vortexed. A, the first scan had peaks at 23, 59, and 69 °C. B, the second scan, had peaks at 23, 59, 68, 94, and 105 °C.

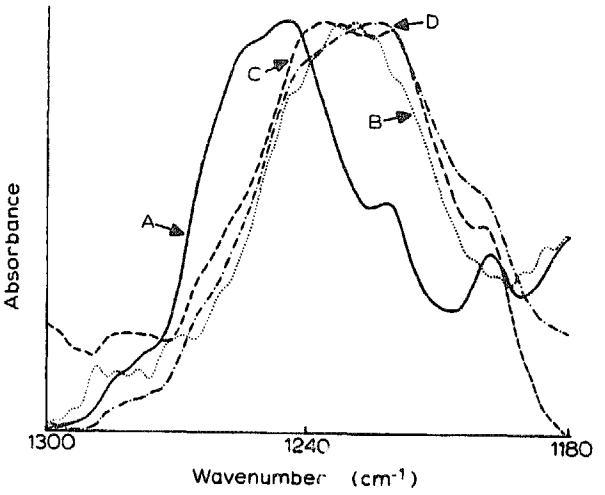

Fig. 6. Infrared absorbance of the asymmetric PO_4 stretch region for DPPC and a 2.15 mol trehalose/mol DPPC sonicated dry vesicles. A, sonicated, lyophilized vesicles of dry pure DPPC have a wavenumber of approx. 1254 cm^{-1}, typical of dehydrated phospholipids. B, hydrated DPPC multilamellar vesicles with a wavenumber shift to approx. 1230 cm^{-1} due to hydrogen bonding of the headgroup to water. C, dry, sonicated vesicles of DPPC/trehalose and D, sonicated vesicles of DPPC and trehalose that have been dried at 60 °C have a similar wavenumber shift as the hydrated sample, although there are slight differences in the spectra. All spectra were taken at room temperature.

Metastability of the dry samples

Dry samples which were stored at or below 24 °C were metastable and a reversion to higher transitions occurred. No systematic study of this phenomenon was undertaken, but it was noted at both low and high mol ratios of trehalose/DPPC. A representative example is shown in Fig. 8. We do not know at present if DPPC/trehalose preparations will undergo these changes when stored at higher temperatures, but we suspect not, since the DPPC should be in liquid crystalline phase in the presence of trehalose and the sugar–lipid interaction should be maximized.

Discussion

When hydrated DPPC vesicles are carefully dried and kept dry, preparations with a gel to liquid-crystalline phase transition temperature of 105–112 °C result. Although these T_m values seen

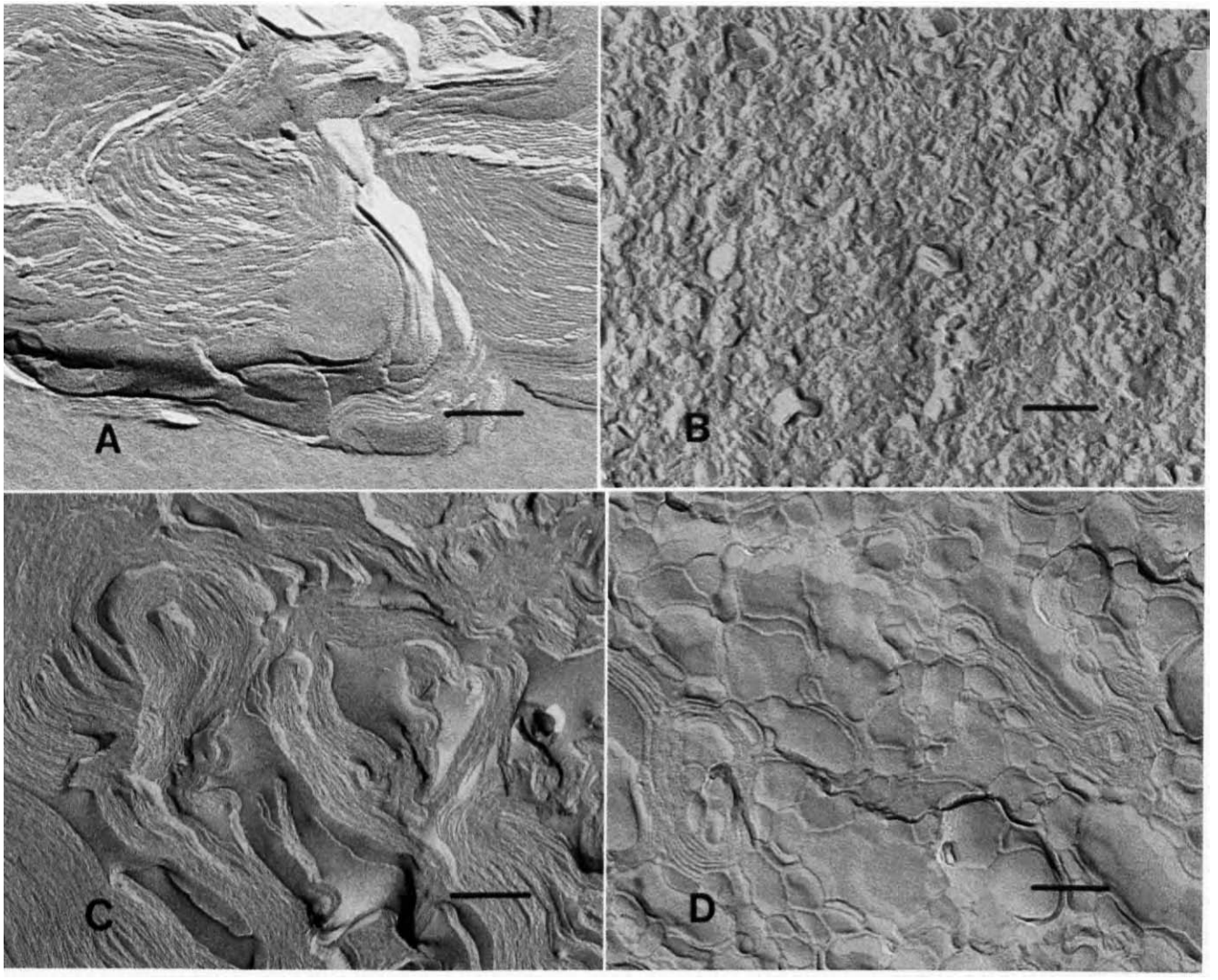

Fig. 7. Micrographs of freeze-fractured dry vesicle preparations. (A) Sonicated lyophilized vesicles with 0.3 mol trehalose/mol DPPC show complete fusion to multilamellar structures. (B) Sonicated vesicles lyophilized at 2.15 mol trehalose/mol DPPC persist as close-packed individual vesicles embedded in a matrix of trehalose. Vesicles which are dried at 60°C show extensive fusion at any trehalose/DPPC mol ratio, although there is clearly more fusion at 0.4 mol trehalose/mol DPPC (C) than there is at 2.15 mol trehalose/mol DPPC (D). Magnification bars on all micrographs represent 100 nm.

here are very high, even higher T_m values have been reported in the literature [20] for those samples had been dried under ultrahigh vacuum. In keeping with the elevation in T_m, we found that the water content of the present samples is low, on the order of 0.2 mol/mol DPPC. We have previously measured the water content of similar preparations of lipid in the presence of trehalose and found that it was also 0.2 mol/mol lipid [15].

The addition of increasing amounts of trehalose to sonicated, dried vesicles results in a progressive lowering of the gel to liquid crystalline transition temperature of DPPC. At trehalose/DPPC mol ratios of 1.5:1 or above, the T_m of the gel to liquid-crystalline transition is lowered maximally, to 24°C. This temperature is over 80°C below the transition of dry pure DPPC. We have previously shown that trehalose can lower the gel to liquid crystalline T_m of palmitoyloleoylphosphatidylcholine (POPC) 80°C, from 57°C for dry pure POPC to −23°C for POPC with 2.15 mol trehalose/mol lipid [6]. These POPC/trehalose samples were prepared from benzene/methanol solutions, while the DPPC/trehalose samples were prepared from

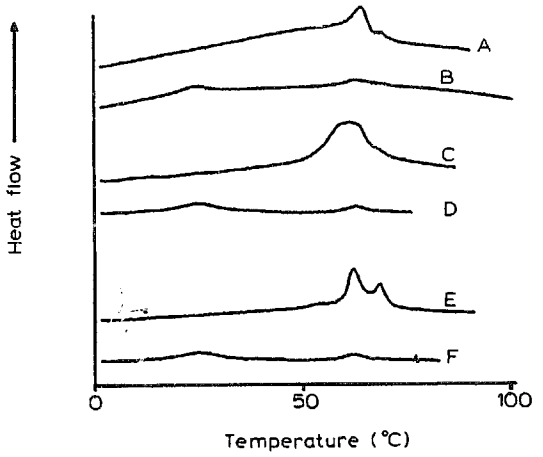

Fig. 8. A series of thermograms showing the metastability of a dry sample that had 0.4 mol trehalose/mol DPPC. The first scan of the fresh sample (A) had a high transition at 64°C, while the second scan (B) had two transitions at 24 and 63°C. After storage at 6°C for four weeks, there was a single transition 62°C (C), but upon re-scanning, the 24°C transition reappeared (D). The sample was then stored on the lyophilizer for four weeks at room temperature. The initial scan following this storage showed transitions at 63 and 70°C (E), while a re-scan again showed the appearance of the low temperature transition (F).

aqueous solution. Thus, neither the method of preparation from organic solution or the presence of water in the sample can account for the large effect seen upon interaction of trehalose with phosphatidylcholines.

For the maximal effect of the trehalose on the DPPC, it is necessary for the trehalose to interact with DPPC which is in liquid-crystalline phase. This result supports the molecular modeling studies of Gaber et al. [16,17], which suggest that phosphatidylcholine headgroup spacing must be expanded in order to accommodate the trehalose molecule into the crystal structure. Very recently, the importance of drying DPPC/trehalose mixtures above the gel to liquid-crystalline phase transition has also been demonstrated for DPPC dihydrate multilamellar vesicles [14]. In our experiments, this was accomplished either by taking a lyophilized sample through the gel to liquid-crystalline transition in the presence of trehalose or by drying the DPPC/trehalose sonicated vesicles at 60°C. In the latter case, the DPPC will not go into gel phase at 60°C until virtually all of the water has been removed [11]. A previous study from this laboratory [6] using sonicated lyophilized vesicles of palmitoyloleoylphosphatidylcholine and phosphatidylserine (9:1 mol ratio) also showed a maximal lowering of T_m of approx. 80°C in the presence of high trehalose. In this case, the vesicles were in liquid-crystalline phase at room temperature before freezing, thus allowing maximal interaction of the lipid and trehalose.

The large frequency shift of the PO_4 asymmetric stretch in the presence of trehalose suggests that the sugar–lipid interaction occurs via hydrogen bonding of -OH of the sugar with the -PO_4 of the lipid. We have seen this effect previously [10,12,13] using various sugars and both dry lipids and dry sarcoplasmic reticulum membranes.

The persistence of multiple melting transitions in the 60°C dried SUV at trehalose/DPPC mol ratios of 1.3:1 or less is most likely due to inhomogeneities during drying. Although the preparations are homogeneous when made, there is ample opportunity for components to move around during the drying at elevated temperatures and for local concentrations of trehalose to vary, while in lyophilized preparations the components are fixed and held in place by the freezing which is maintained during the drying process. Thus, in the lyophilized preparations at lower trehalose contents, a single, intermediate transition results upon repeated scanning while multiple stable transitions persist in the 60°C dried preparations at lower trehalose contents.

In sonicated vesicle preparations dried by either protocol, sufficient trehalose was present to lower the DPPC phase transition to a minimal value of about 24°C when the trehalose/lipid ratio was 1.5:1. We have obtained an identical stoichiometry for the interaction of trehalose and POPC previously by a totally independent method [15]. This previous work, which measured the maximum residual water content of lipid preparations dried with trehalose (and other carbohydrates), suggested that above 1.5 mol trehalose/mol POPC, free trehalose dihydrate appeared in the preparation. However, no transitions attributable to trehalose dihydrate at about 97–100°C were ever detected in either the lyophilized or 60°C dried SUV preparations (see Fig. 1, for example). There are two possible explanations for the absence of the trehalose dihydrate transition. First, although trehalose dihydrate is present, it dries so

amorphously (perhaps under the influence of the DPPC) that a crystalline transition is not seen. Secondly, the ^3H seen in the trehalose samples dried from tritiated water for the residual water experiments [15] may all result from proton exchange between the water and the trehalose, and the dihydrate may not be present at all. At present there are no data available to enable us to distinguish between these two possibilities.

The results of drying DPPC multilamellar vesicles at high trehalose concentration suggest that trehalose is not homogeneously distributed in the vesicles during their preparation. In contrast to sonicated vesicles that are either lyophilized or dried at 60 °C, multilamellar vesicles maintain multiple transitions upon repeated scanning, even at 2.15 mol trehalose/mol lipid.

These results differ from those of Tsvetkova et al. [14], for which there are at least two possible explanations. Their method of preparation of multilammellar vesicles may well result in a more homogeneous sample, but, more importantly, the presence of two moles of water/mole DPPC may allow a more constant and stable structure to form in the presence of the trehalose. The presence of the small amount of water may also explain the stability of the sample upon repeated scanning, although there are no data presented about metastability during storage. Both this study and that of Lee et al. [21] show a slow reversibility of the low-temperature transition. A similar metastability has been mentioned by Finegold and Singer [22] although no data were shown.

We interpret the calorimetric transitions that we have reported in this study as transitions from a gel to liquid-crystalline phase. The exact structure of each phase cannot be determined by calorimetry, but is best examined by X-ray diffraction methods, a line of inquiry we are now pursuing. The structure of a DPPC/trehalose mixture which has a transition some 18 C° below the hydrated transition should be particularly interesting.

Cell membranes of anhydrobiotic organisms are not likely to be composed of pure DPPC. Most membranes for which a transition has been determined are formed from mixtures of lipids with a transition below ambient temperature [23]. Thus, the membranes of anhydrobiotic organisms are likely to be in the liquid-crystalline phase as the organism is synthesizing trehalose and undergoing dehydration, and maximal interaction of the trehalose and membrane can occur. Additionally, anhydrobiotic organisms in the dry state occur in environments where there is some small but variable water content. Small amounts of water can strongly affect of T_m of dry *Typha latifolia* pollen, which used sucrose instead of trehalose as its protective disaccharide [24]. It would be interesting to know how small amounts of water affect the T_m and structure of a model system such as has been presented here.

Acknowledgements

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