Is Trehalose Special for Preserving Dry Biomaterials?

Lois M. Crowe,* David S. Reid,# and John H. Crowe*

*Section of Molecular and Cellular Biology, and #Department of Food Science and Technology, University of California, Davis, California 95616 USA

ABSTRACT   Simple sugars, especially disaccharides, stabilize biomaterials of various composition during air-drying or freeze-drying. We and others have provided evidence that direct interaction, an interaction that we believe is essential for the stabilization, between the sugar and polar groups in, for example, proteins and phospholipids occurs in the dry state. Some researchers, however, have suggested that the ability of the sugar to form a glass is the only requirement for stabilization. More recently, we have shown that both glass formation and direct interaction of the sugar and headgroup are often required for stabilization. In the present study, we present a state diagram for trehalose glass and suggest that the efficacy of this sugar for stabilization may be related to its higher glass transition temperatures at all water contents. We also show that trehalose and trehalose:liposome preparations form trehalose dihydrate as well as trehalose glass when rehydrated with water vapor. Formation of the dihydrate sequesters water, which might otherwise participate in lowering the glass transition temperature to below ambient. Because samples remain in the glassy state at ambient temperatures, viscosity is high and fusion between liposomes is prevented.

INTRODUCTION

Disaccharides such as sucrose, lactose, and more recently trehalose have been used widely as excipients during freeze-drying of a variety of materials, including products in the pharmaceutical industry. Stabilization during drying is also widespread in nature; many plants and animals from several phyla have the ability to survive complete dehydration, a condition known as anhydrobiosis. A common theme in these organisms is the accumulation of large amounts of sugars, especially the disaccharides sucrose and trehalose (Bianchi et al., 1991; Hoekstra et al., 1989; Koster and Leopold, 1988; Suau et al., 1991; Madin and Crowe, 1975; Clegg, 1965).

These sugars (along with many others, including polysaccharides such as hydroxyethyl starch and dextran) have the ability to form glasses, which have very high viscosity and low mobility leading to the increased stability of the material being preserved. Upon heating, glasses can undergo a second-order transition to a rubbery state, which has lower viscosity and higher mobility than the glass.

We and others have provided evidence for direct interaction between sugars and both membrane components and proteins using isolated biological membranes, liposomes, and purified proteins. Methods of study have included differential scanning calorimetry, ^{1}H and ^{31}P NMR, Fourier transform infrared spectroscopy, and estimates of residual water in dry products (Carpenter and Crowe, 1989; J.H. Crowe et al., 1984a,b; J.H. Crowe et al., 1989; Crowe and Crowe, 1988; L.M. Crowe et al., 1985; Hoekstra et al., 1991; Lee et al., 1986, 1989; Tsvetkov et al., 1989).

Despite the published evidence for direct interactions, some researchers have suggested that the only requirement for preservation of structure and function in membranes, liposomes, and proteins is the ability of the additive (e.g., sugar or polysaccharide) to form a glass at all water contents and temperatures during freeze-drying (Levine and Slade, 1992; Aldous et al., 1995). Others have claimed special properties for trehalose (Roser, 1991) over other excipients. We have attempted to clarify the respective contributions of glass formation and direct interaction in the preservation of liposomes (J.H. Crowe et al., 1994; J.H. Crowe et al., 1996). Briefly, we have found that the polymers dextran and hydroxyethyl starch, both good glass formers, are not effective in preventing leakage from egg phosphatidylcholine liposomes during drying. These polymers show no evidence of direct interaction with the headgroup phosphates and do not lower T_m of the lipid. They do, however, inhibit fusion between liposomes during drying, a property that is probably due to glass formation (J.H. Crowe et al., 1994; J.H. Crowe et al., 1996). Trehalose, which is a good glass former and also shows direct interaction with bilayers, stabilizes liposomes during freeze-drying. Glucose, which lowers the T_m of dry egg PC and shows hydrogen bonding with headgroup phosphates, does not remain in a glass under our conditions of freeze-drying and does not stabilize liposomes (Crowe and Crowe, unpublished data). All of these results indicate that as long as both conditions (direct interaction and glass formation) are satisfied, a wide variety of sugars and higher polymers can stabilize liposomes and proteins.

Our results generally have not supported a special role for trehalose; sucrose, for example, works equally well with liposomes and appears to be the preferred sugar accumulated in higher plants. However, some recent reports suggest that trehalose may be more effective than other disaccharides, especially during storage (Leslie et al., 1995; Uritani et al., 1995). A report on the state diagram for trehalose

glasses suggests that the trehalose curve lies somewhat above that for glucose, a result that has been called the "trehalose anomaly" (Green and Angell, 1989). The T_g of anhydrous trehalose reported by Green and Angell appeared low to us, based not only on unpublished work from our laboratory but also a published report showing a T_g of 100°C for anhydrous trehalose (Roos, 1993). This latter study provided a single value for T_g at a single water content. Accordingly, in the current study we have constructed a state diagram for trehalose/water. The results of our experiments show that T_g for dry trehalose is much higher than that of sucrose and remains higher even when the two sugars are partially rehydrated by exposing them to water vapor. The ability of trehalose glass to form the dihydrate as it absorbs water appears to be responsible for this remarkable effect, the result of which is that biomaterials may be more stable when stored with trehalose under high humidities and temperatures.

MATERIALS AND METHODS

Trehalose dihydrate was prepared by and a kind gift from Ajinomoto Inc. (Tokyo, Japan).

Differential scanning calorimetry (DSC) was carried out mainly on a Perkin-Elmer DSC 7 with liquid nitrogen cooling and a helium gas purge. Some samples were scanned in a Perkin-Elmer DSC 2C using the Perkin-Elmer Infracooler II and a nitrogen gas purge, or in a high sensitivity DSC from Calorimetry Sciences Corporation (Provo, UT). Scanning rates were 10 or 20 K/min with the Perkin-Elmer DSC and 1 K/min with the Calorimetry Sciences DSC. Sample pan sizes were 20 μl for the Perkin-Elmer DSC and 1 ml for the Calorimetry Sciences DSC.

Samples were prepared in several ways. 1) Trehalose dihydrate was weighed in tared calorimetry pans and held at 125–130°C for 3–4 h, then placed while hot in a dry box attached to a Balston dry air generator (Balston, Inc., Haverhill, MA). Humidity in the dry box was monitored with an Omega RH201 meter (Omega Engineering, Stamford, CT). Some of the samples were sealed directly in the dry box to run as anhydrous trehalose; others had small amounts of water directly added to them followed by sealing and annealing at 125–130°C for ~2 h. Some samples of anhydrous trehalose, prepared as above, were placed at various relative humidities (RH) for varying lengths of time to produce a range of water contents, then sealed. 2) 20-μl aliquots of a 1.5 M solution of trehalose were placed in calorimeter pans and dried for 6 h at 0% RH followed by one week over anhydrous $CaSO_4$. The samples were white at this point, indicating the formation of trehalose dihydrate. They were then placed in a 130°C oven for 1.5 h, at which point the samples were clear. One sample was placed in the dry box while hot, sealed, and run. Others were held at 58 or 65% RH for various times to produce various water contents. 3) 20-μl aliquots of an 0.5 M solution of trehalose were placed in calorimeter pans, air-dried at 0% RH for 6 h, then placed over anhydrous $CaSO_4$ for 10 days. One sample was sealed directly from the desiccator in the dry box, whereas others were held at 65% RH for one to 2 h. 4) A 0.25 M solution of trehalose was lyophilized in a 0.5-ml volume at 10 milliTorr for 52 h. After lyophilization, one sample was sealed immediately in the dry box, and one was placed at ambient relative humidity (22–25%) for 66 h, then sealed and run. 5) Trehalose dihydrate was placed under high vacuum (10 milliTorr) for various amounts of time, removed from the lyophilizer under vacuum and sealed in the dry box.

Samples were weighed on a Cahn C-33 microbalance (Analytical Technology, Inc., Boston, MA). If it was suspected that water loss occurred during a scan, the sample was reweighed immediately. All samples were weighed after scanning was completed to check for water loss. After scanning, the sample pans were punctured: high water content samples were placed over anhydrous $CaSO_4$ for a couple of days and then placed in

the 125–130°C oven for 2–3 h. Low water content samples were placed directly in the oven for 2–3 h. Following the oven, all samples were placed under high vacuum (10 milliTorr) for at least 24 h. Samples were removed from the lyophilizer under vacuum and then placed in individual sealed vials in the dry box until weighed. Weighing was done by quickly opening the vial, placing the pan on the balance, and recording the first stable weight. Samples gained weight as they remained on the balance. After the final dry weight was taken, pans were opened to check that there was no decomposition of the sugar.

Calorimetry Sciences software was used to calculate a standard calorimetric enthalpy of the crystalline melt of trehalose dihydrate by high sensitivity DSC, and this value was used to calculate the amount of dihydrate in some of the samples run on the Perkin-Elmer DSC 7. The measured enthalpy from the dihydrate transition was compared to the expected enthalpy for a sample of that weight. This then was the fraction of the trehalose in the dihydrate form. Perkin-Elmer software was used to calculate enthalpies and onsets of crystalline melts of trehalose dihydrates, and onsets and ΔC_p values for glass transitions. Glass transition values were determined from samples that had been scanned at least twice.

Liposomes were prepared by drying aliquots of egg phosphatidylcholine (egg PC) in chloroform under a stream of dry nitrogen in a tared tube, followed by at least 2 h on the lyophilizer to remove residual solvent. 100 mM carboxyfluorescein (CF) was added to the egg PC to give 20 mg lipid/ml, and the tube was vortexed until all the lipid was suspended as multilamellar vesicles. Unilamellar vesicles were formed by extruding the multilamellar vesicles through 100-nm polycarbonate filters (Poretics, Inc., Livermore, CA) in a hand held extruder (Liposofast, Avestin Inc, Ontario, Canada). Excess CF (outside the liposomes) was removed by passage over a G50 Sephadex (Sigma Chemical Co., St. Louis, MO) column, which resulted in a lipid concentration of ~10 mg/ml. The resulting liposomes were diluted with either sucrose or trehalose solution to give a final concentration of 5 mg/ml of both sugar and lipid. Ten-microliter aliquots were lyophilized overnight. Following lyophilization, the aliquots were placed in 58% RH chambers at either 22 or 40°C. Aliquots were removed at various times, rehydrated with 10 μl of water and the percentage retention of CF was determined. CF is self-quenching at high concentrations but highly fluorescent when the trapped CF is leaked into the much larger volume of the cuvette. The initial fluorescence of the sample was determined, Triton X-100 (Sigma Chemical Co., St. Louis, MO) was added to lyse the liposomes, and a final fluorescence (which represented total leakage) was read. Percentage trapped inside the liposomes is calculated by:

$$\% \text{ trapped} = \frac{\text{final fluorescence} - \text{initial fluorescence}}{\text{final fluorescence}} \cdot 100$$

Percentage retention of the dried samples was calculated by comparing the percentage trapped after drying to the percentage trapped by freshly prepared liposomes.

The amount of trehalose in the dihydrate form in liposome:trehalose samples was determined from the area under the crystalline melt (mJ) divided by the known weight of sugar in the sample to give a calorimetric enthalpy in J/g. This value was then divided by the expected enthalpy of the crystalline melt to give the percentage trehalose present as dihydrate.

RESULTS

Formation of trehalose glasses

Vitrification of trehalose strongly depends on sample preparation. Thus, we wish to comment first on the conditions under which this state is achieved.

The trehalose dihydrate produced by Ajinomoto appeared to be exceptionally pure. It was white, crystalline, and when melted and dried at 130°C it lost only as much water as could be accounted for by the dihydrate water. The onset, T_f, of the crystalline melt, 97.4°C determined at a scan rate

of 10 K/min, agrees with literature values (Green and An-gell, 1989; Roos, 1993). The calorimetric enthalpy of 146 J/g, determined in the DSC 7, agrees with the value determined in this study by high sensitivity differential scanning calorimetry (144 J/g), but is somewhat higher than the value reported by Roos (1993), 127 J/g. However, the melting behavior of trehalose dihydrate was found to be very sensitive to its transition-metal content (Green and Angell, 1989), and we have found that some less pure samples supplied by other manufacturers can contain more than the dihydrate water (L. M. Crowe, unpublished). Either of these factors might account for the differences in the enthalpy of the crystalline melt.

Freeze-drying 0.25 M trehalose does not remove all the water. After 48 h below 10 milliTorr, the sample retained ~0.02 g water/g trehalose and had a glass transition at ~92°C. When this sample was stored at room temperature and ambient relative humidity (~25%) for 66 h, it adsorbed water and 70% of the sample reverted to trehalose dihydrate.

High vacuum can remove almost all the water from crystalline trehalose dihydrate. A sample was held at <10 milliTorr for 48 h, removed under vacuum, and loaded into calorimeter pans at 0% RH. Residual water was 0.003 g/g sugar and T_g was 111.3°C. This value for T_g falls within the 95% confidence limits for an anhydrous sample prepared by melting and drying trehalose dihydrate.

Air-drying samples at 0% RH resulted in a residual water content of ~0.01 to 0.02 g water/g sugar, even if the samples subsequently were held over anhydrous $CaSO_4$ for up to 10 days. Whether the air-dried sample formed large amounts of dihydrate or a glass when rehydrated with water vapor depended on the sample preparation conditions. Samples that were dried in 20-μl aliquots of a 1.5 M solution or in 0.25-ml aliquots of a 0.25 M solution of trehalose-formed dihydrate on drying (data not shown). However, 20-μl aliquots of 0.5 M trehalose that were dried in calorimeter pans formed glasses (Fig. 1), which remained as glasses when held at 65% RH. Thus, there is an interaction between the size and the concentration of the aliquot that leads to formation of the dihydrate (high concentration or large volume) or glass (lower concentration and volume). These differences may be due to the differing ability of the samples to nucleate and crystallize into the dihydrate.

Construction of the state diagram

Samples prepared by methods 1–5 (see Materials and Methods) gave results that were consistent with each other and were used to construct the curve indicated by the solid line in Fig. 2. Curve-fitting gave a large number of similar curves with r^2 values of 0.99. We chose one simple equation to aid in predicting T_g as a function of water content:

$$y = a + bx + cx^{0.5}$$

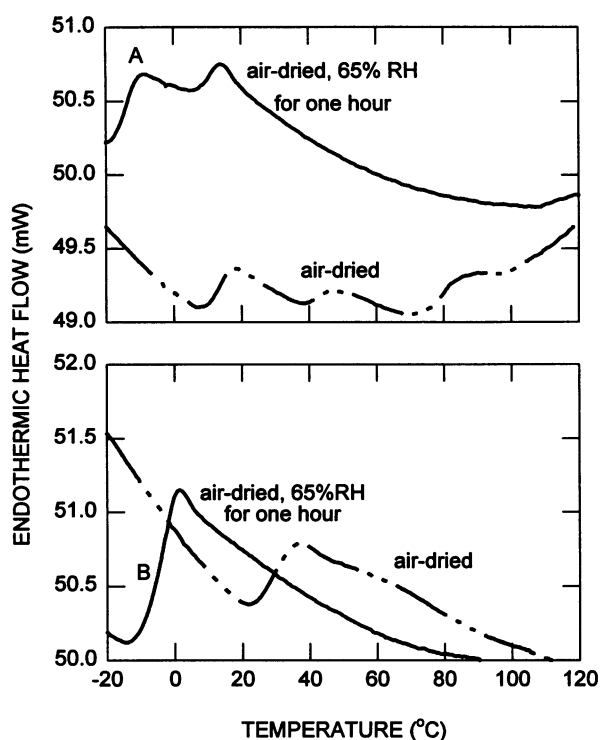where $a = 115.5$, $b = 202.5$, and $c = -405.6$.

FIGURE 1   (A) First scans of a sample air-dried from low volume and concentration as described in the Methods, and a similar sample that was rehydrated at elevated RH. Samples prepared in this manner did not form trehalose dihydrate upon rehydration. There is some heterogeneity in these samples, which disappears when the samples are scanned again (B).

T_g values for trehalose:water glasses lie above those for sucrose, especially at the lowest water contents. The T_g for anhydrous trehalose glass is 113.9 ± 1.4 (SE)°C, ~50°C above that for anhydrous sucrose (~65°C). The sucrose data in Fig. 2 were obtained by Sun et al. (1996). The ΔCp of the trehalose glass transitions was 0.65 ± 0.03 (SE) J/gK.

Is trehalose anomalous?

The T_g of dry trehalose determined in this study, the T_g of water (−135°C), and T_g of the trehalose at various weight fractions of water were used to determine empirically the constant k of the Gordon-Taylor equation (Gordon and Taylor, 1952). This equation was originally developed for the calculation of T_g values of copolymers from known weight fractions of the polymers and the T_g values of the pure polymers. It is frequently used for the same purpose in the determination of T_g in sugar/water glasses in the form (Roos, 1993; Levine and Slade, 1988):

$$T_g = \frac{w_1 T_{g1} + kw_2 T_{g2-}}{w_1 + kw_2},$$

where w_1 and w_2 are the weight fractions of the solute and water, respectively, T_{g1} is the T_g of the anhydrous solute, T_{g2} is the T_g of water (−135°C), and k is a constant. If k is known, the value of T_g of the glass for any water content can
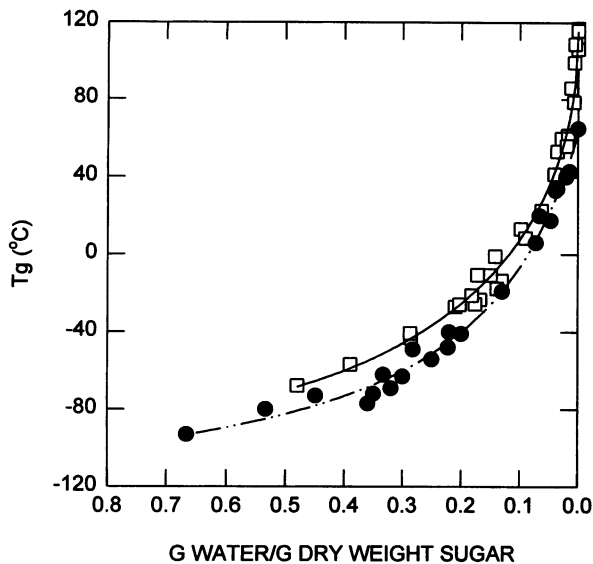
FIGURE 2   State diagrams for sucrose (data obtained by Sun et al. (1995)) and trehalose (this study). The data points are fitted using Table-curve software from Jandel Scientific (San Rafael, CA). The equation for the curve (see Results) can be used to predict T_g of trehalose glasses at various water contents. The equation for the sucrose line can be found in Sun et al. (1995).

be predicted. However, k must be determined experimentally for several water contents, as well as the dry T_g of the sugar glass. Our empirical value for k, 7.5 ± 0.4 (SE) was compared to other values of k (extrapolated from Fig. 1 of Roos (1993)) and a plot was made from the combined results (Fig. 3). A value for k of 7.3 was estimated for trehalose from the refitted line, very close to the empirical value. Trehalose is thus similar to other sugars in the relationship between T_g and water content. It appears to lie at the end of a continuum in this regard, but is not anomalous (cf. Fig. 3).

Effects of T_g on stability of freeze-dried liposomes

Some consequences of the elevated T_g observed in trehalose were studied using leakage of the trapped fluorescent dye CF during storage of freeze-dried liposomes as a measure of stability. Either sucrose or trehalose was added to the liposomes at a lipid:sugar weight ratio of 1:1 before freeze-drying. The sugars prevent fusion during the drying process. When the liposomes were removed from the freeze-drier and rehydrated immediately, retention of CF was high, almost 90%; values of 100% can be obtained if sugar is also inside the liposomes (L.M. Crowe et al., 1986). Other samples were stored at 58% RH at either 22 or 40°C. Samples were removed from the relative humidity chambers at various times, rehydrated, and leakage was measured. As can be seen from Fig. 4, liposomes stored in sucrose at either temperature underwent leakage much more readily than those stored in trehalose. The most likely explanation of
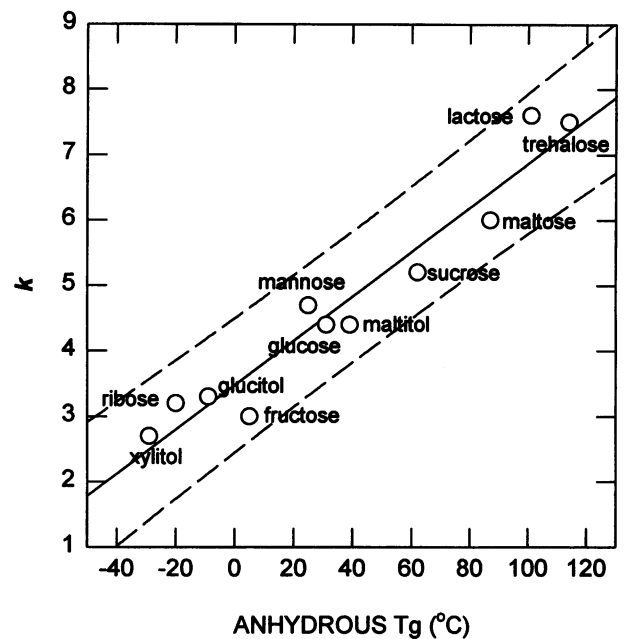

FIGURE 3   Results of the calculation of constant, k, of the Gordon-Taylor equation. k was calculated from our data for trehalose, and extrapolated from Roos (1993) for the other sugars. The regression line of Roos (1993) was recalculated including trehalose. The calculated k from the new regression line, 7.35, was not significantly different from the empirical value of 7.5 ± 0.4 (SE). Solid line, regression (r^2) = 0.94; dashed lines, 95% confidence intervals.

these data is that the sucrose:liposome samples reach a lower glass transition temperature more readily than the trehalose:liposome samples when treated the same way, an explanation that we tested next.
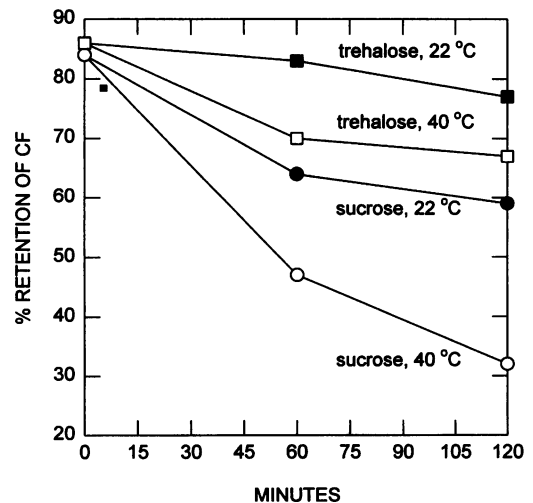

FIGURE 4   Leakage of carboxyfluorescein from lyophilized egg PC:trehalose and egg PC:sucrose liposomes held at 58% RH at either 22 or 40°C for up to 2 h. Leakage is greater for sucrose:egg PC liposomes than for trehalose:egg PC liposomes at both temperatures.

Effects of hydration on trehalose and sucrose glasses

Egg phosphatidylcholine liposomes were dried with either sucrose or trehalose in the same way as the liposomes for the leakage experiments, with the omission of CF. They were freeze-dried as 150-μl aliquots at 10 milliTorr for 42 h, removed under vacuum, and placed in a dry box at 0% RH. Calorimeter pans were loaded and either sealed directly in the dry box or held at 40°C and 58% RH for 15, 30, or 120 min before sealing. After scanning, the water content of the samples was determined by drying, as described for the pure sugar samples. Melting of the lipid was seen as an endotherm, whereas a glass transition was seen as a shift in the baseline. For some very small thermal events it was not possible to determine the type of transition.

T_g for the lyophilized trehalose:liposome sample was 79.5°C, whereas the freeze-dried sucrose:liposome sample, which had slightly more residual water, had a T_g ~56°C and another possible glass transition at ~37°C (Fig. 5). These freeze-dried samples were loaded at ambient temperature, so both were in the glassy state. Placing other samples at 40°C and 58% RH caused a rapid absorption of water to ~0.08 g water/g sample for both the sucrose and trehalose samples, which remained constant for 2 h for the trehalose sample. The trehalose:lipid sample also had a constant glass transition temperature, which never fell below 65°C. T_g for the sucrose:lipid sample fell to -14.5°C within 15 min of treatment and remained low. At 2 h, the sucrose:lipid sample had no apparent glass transition, although it may have been hidden under the broad lipid transition (Fig. 6). At 15

FIGURE 6 Over time, T_m for egg PC rose from ~ -35°C (typical of a fluid lipid dried with disaccharide) to close to the hydrated T_m. This is consistent with uptake of water by the phospholipid and displacement of disaccharide from its interaction with the phospholipid. As the trehalose: egg PC samples hydrated, increasing amounts of dihydrate were formed. The percentage of the trehalose in the dihydrate form was calculated from the calorimetric scans, using the dry weight of the sugar, the area under the curve, and the expected enthalpy of the transition as described in the Methods. □, percent dihydrate; ●, Tm egg PC/sucrose; ■, Tm egg PC/trehalose.

and 30 min, the sucrose:liposome sample looked considerably wetter than the trehalose:liposome sample, but at 2 h the sucrose:liposome sample appeared to have dried out and looked somewhat crystalline. Measurement of the water content of this sample showed that it had lost about half the water it had originally absorbed (Fig. 5).

In the driest samples the melting peak of the egg PC transition in the presence of either trehalose or sucrose was -35°C or below, consistent with other reports in the literature for several phosphatidylcholines (Tsvetkov et al., 1989; L.M. Crowe et al., 1985, 1986, 1988; J.H. Crowe et al., 1996; Nakagaki et al., 1992). In a careful study of the rehydration of dipalmitoylphosphatidylcholine, Nakagaki et al. (1992) found that trehalose does indeed lower the T_m of DPPC, but with small amounts of rehydration the T_m rises as water displaces trehalose from its interaction with the lipid headgroup. This effect was also seen during the rehydration of the liposome:sugar samples in this study (Fig. 6) where the T_m of egg PC increases as water is absorbed by the sample over time. Because the water content of the samples was reasonably constant and similar between the liposomes in trehalose or sucrose, this suggests that over time, more of the water is associating with the lipid.

Perhaps the most surprising result from the rehydration studies employing water vapor absorption was that increasing amounts of trehalose dihydrate were formed in the trehalose:liposome preparation with time of storage at elevated temperature and relative humidity. This result also was seen with anhydrous bulk trehalose and with anhydrous trehalose prepared from a concentrated solution. The results
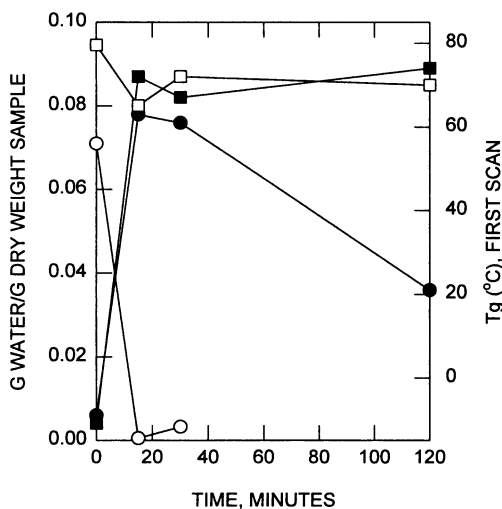

FIGURE 5 Liposomes of the same composition as those in Fig. 5, but prepared without carboxyfluorescein and stored at 40°C and 58% RH. Both sucrose and trehalose protected liposomes absorbed the same amount of water by 15 min, but the glass transition of the trehalose:egg PC liposomes was at 65°C, whereas the sucrose:egg PC liposomes had fallen to -14.5°C. With time the water content of the sucrose:egg PC preparation falls, whereas the trehalose:egg PC preparation remains constant. (●, water content of sucrose:egg PC; ○, T_g sucrose; ■, water content of trehalose:egg PC; □, T_g trehalose).
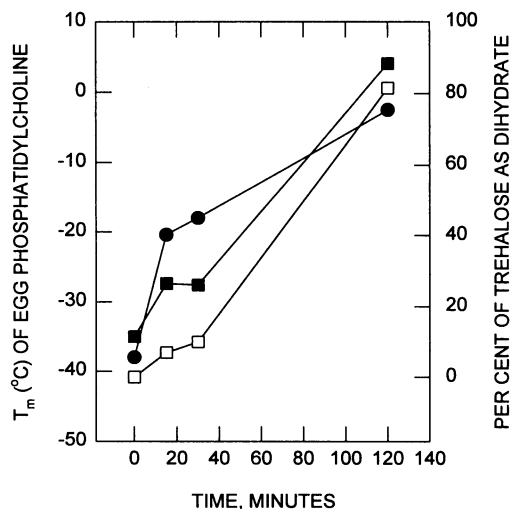
from trehalose:liposome preparations are shown in Fig. 7. In each case, the crystalline melt(s) was preceded by what appears to be a glass transition. These samples differ from the pure trehalose samples air-dried from solution at low volume and low concentration, which remained as glasses when slightly rehydrated (Fig. 1). However, trehalose dihydrate was formed from trehalose samples air-dried from high concentration or volume or samples of bulk trehalose anhydrous glass when held at high humidity (see above).

DISCUSSION

Values of T_g determined in this study for trehalose at all water contents are higher than literature values (Green and Angell, 1989; Roos, 1993), especially at the lowest water contents. Because the curve rises so steeply at the lowest water contents (Fig. 2), small differences in water content
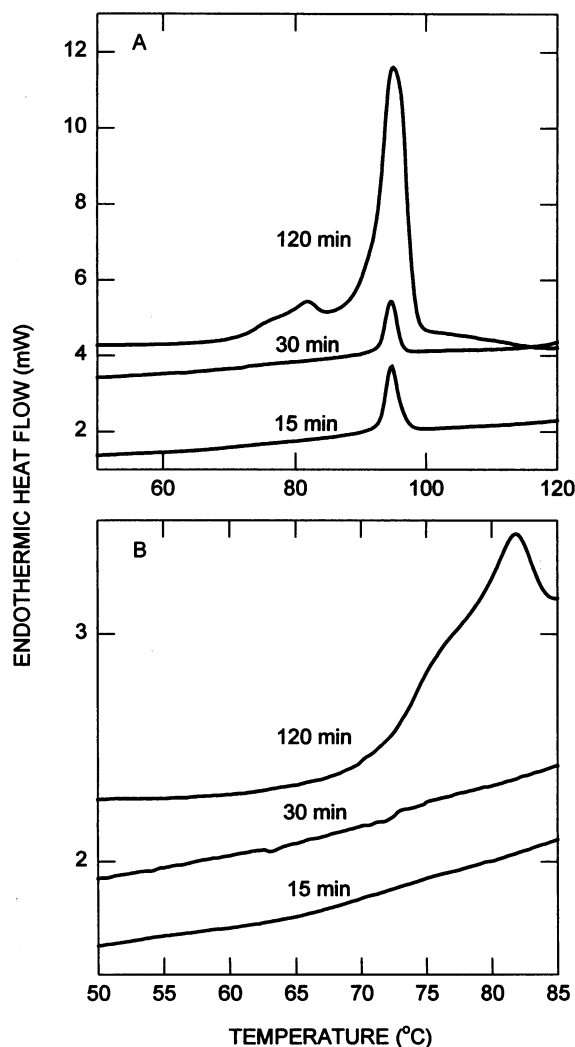

FIGURE 7 (A) Scans of the dihydrate transition with increasing time of hydration of the trehalose:egg PC samples. An apparent glass transition occurred before the main crystalline melt in these samples as seen in the enlargement of this region in (B).

may lead to very different behavior between dry trehalose and sucrose samples when they are stored under the same conditions of temperature and relative humidity.

When the rehydration behaviors of liposomes prepared with trehalose or sucrose and held at 40°C and 58% RH are compared, it can be seen that initially they take up similar amounts of water, but more importantly, much of the absorbed water produces the dihydrate in the trehalose:liposome sample; whereas in the sucrose:liposome sample the absorbed water results in a decreased temperature of the glass transition. The glass transition of the trehalose never fell below 65°C, so the sugar remained in the glassy state, despite the addition of water. Thus, the suggestion of Aldous et al. (1995), that carbohydrates that can form hydrated crystals may do so at the expense of water that might otherwise be in glasses is supported by the current study. Aldous et al. arrived at their results by studying the desorption of water, whereas we have formed dihydrate by the absorption of water, so the two processes may not be equivalent. Nevertheless, we agree that the formation of the dihydrate may be a critical event in maintaining an elevated T_g in the rest of the sample. In this regard, trehalose is clearly unusual, although perhaps not unique.

Uptake and subsequent loss of water from amorphous sucrose glass at elevated temperature and humidity has been reported previously (Islesias and Chirife, 1978), and although the time scale used was different from ours it is clear that pure sucrose took up and then lost water rapidly. This has been ascribed to the crystallization of sucrose from the glass (Islesias and Chirife, 1978; Roos and Karel, 1991). We did not check for sucrose crystallization because heating to such high temperatures would have caused decomposition of the egg PC in our samples. Nevertheless, the initial rapid uptake and lowering of the glass transition within the first 15–30 min of rehydration of the sucrose:liposome sample can account for the leakage seen from the liposomes, as the glass in which they are encased becomes more mobile and opportunities for fusion occur. We have previously shown that the leakage due to storage above T_g is accompanied by fusion (J.H. Crowe et al., 1994). Although leakage from a liposome can occur if the lipid undergoes a phase transition during rehydration (see Crowe and Crowe (1995) for review), the T_m of the egg PC in our liposome samples was below the hydrated transition (except for our 2 h trehalose:liposome sample, which was only slightly above the hydrated T_m), so the egg PC was always in liquid crystalline phase.

Trehalose:liposome samples, although they initially took up as much water as the sucrose:liposome samples, behaved quite differently. Even at 15 min, trehalose dihydrate began to be formed, and T_g remained high. There were some small, uninterpretable thermal events at 9°C in the 15 min sample and at 19°C in the 30 min sample, which might possibly have been glass transitions. If so, this could account for the slight leakage of carboxyfluorescein seen in the trehalose:liposome samples at 1 and 2 h.

Thus, it appears that in a model liposome system, trehalose is superior to sucrose as a stabilizer, even though they appear to hydrate equally, at least initially. The glass transition of the trehalose samples remains high, possibly because much of the absorbed water goes to form trehalose dihydrate. Temperatures of 40°C and humidity of at least 60% can frequently be found in many parts of the world, under which conditions we infer that trehalose might well be superior to other sugars for preserving biomaterials.

REFERENCES

Aldous, B. J., A. D. Auffret, and F. Franks. 1995. The crystallization of hydrates from amorphous carbohydrates. *Cryo-Lett.* 16:181–186.

Bianchi, G., A. Gamba, C. Murellie, F. Salamini, and D. Bartels. 1991. Novel carbohydrate metabolism in the resurrection plant *Craterostigma plantagineum. Plant J.* 1:355–359.

Carpenter, J. F., and J. H. Crowe. 1989. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. *Biochemistry.* 28:3916–3922.

Clegg, J. S. 1965. The origin of trehalose and its significance during the formation of encysted dormant embryos of *Artemia salina. Comp. Biochem. Physiol.* 14:135–143.

Crowe, J. H., L. M. Crowe, and D. Chapman. 1984a. Preservation of membranes in anhydrobiotic organisms: the role of trehalose. *Science.* 223:701–703.

Crowe, J. H., L. M. Crowe, and D. Chapman. 1984b. Infrared spectroscopic studies on interactions of water and carbohydrates with a biological membrane. *Arch. Biochem. Biophys.* 232:400–407.

Crowe, J. H., F. A. Hoekstra, K. H. N. Nguyen, and L. M. Crowe. 1996. Is vitrification involved in depression of the phase transition temperature in dry phospholipids? *Biochim. Biophys. Acta.* 1280:187–196.

Crowe, J. H., S. B. Leslie, and L. M. Crowe. 1994. Is vitrification sufficient to preserve liposomes during freeze-drying? *Cryobiology.* 31:355–366.

Crowe, J. H., B. J. Spargo, and L. M. Crowe. 1989. Preservation of dry liposomes does not require retention of residual water. *Proc. Natl. Acad. Sci. USA.* 84:1537–1540.

Crowe, L. M., and J. H. Crowe. 1988. Trehalose and dry dipalmitoylphosphatidylcholine revisited. *Biochim. Biophys. Acta.* 946:193–201.

Crowe, L. M., and J. H. Crowe. 1995. Freeze-dried liposomes. *In* Liposomes, New Systems and New Trends in Their Applications. G. Puisieux, P. Coubreur, J. Delattre, and J.-P. Devissaguet, editors. Editions de Santé, Paris, France. 237–272.

Crowe, L. M., J. H. Crowe, and D. Chapman. 1985. Interaction of carbohydrates with dry dipalmitoylphosphatidylcholine. *Arch. Biochem. Biophys.* 236:289–296.

Crowe, L. M., C. Womersley, J. H. Crowe, D. Reid, L. Appel, and A. Rudolph. 1986. Prevention of fusion and leakage in freeze-dried liposomes by carbohydrates. *Biochim. Biophys. Acta.* 861:131–140.

Gordon, M., and J. S. Taylor. 1952. Ideal copolymers and the second-order transitions of synthetic rubbers. I. Non-crystalline copolymers. *J. Appl. Chem.* 2:493–500.

Green, J. L., and C. A. Angell. 1989. Phase relations and vitrification in saccharide-water solutions and the trehalose anomaly. *J. Phys. Chem.* 93:2880–2882.

Hoekstra, F. A., L. M. Crowe, and J. H. Crowe. 1989. Differential desiccation sensitivity of corn and *Pennisetum* pollen linked to their sucrose contents. *Plant Cell Environ.* 12:83–91.

Hoekstra, F. A., J. H. Crowe, and L. M. Crowe. 1991. Effect of sucrose on phase behavior of membranes in intact pollen of *Typha latifolia* L., as measured with Fourier transform infrared spectroscopy. *Plant. Physiol.* 97:1073–1079.

Islesias, H. A., and J. Chirife. 1978. Delayed crystallization of amorphous sucrose in humidified freeze dried model systems. *J. Food Technol.* 13:137–144.

Koster, K. L., and A. C. Leopold. 1988. Sugars and desiccation tolerance in seeds. *Plant Physiol.* 88:829–832.

Lee, C. W. B., S. K. Das Gupta, J. Mattai, G. G. Shipley, O. H. Abdel-Mageed, A. Makriyannis, and R. G. Griffin. 1989. Characterization of the L$_\lambda$ phase in trehalose-stabilized dry membranes by solid-state NMR and x-ray diffraction. *Biochemistry.* 28:5000–5009.

Lee, C. W. B., J. S. Waugh, and R. G. Griffin. 1986. Solid-state NMR study of trehalose/1,2 dipalmitoyl-*sn*-phosphatidylcholine interactions. *Biochemistry.* 25:3737–3742.

Leslie, S. B., E. Israeli, B. Lighthart, J. H. Crowe, and L. M. Crowe. 1995. Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Appl. Environ. Microbiol.* 61:3592–3597.

Levine, H., and L. Slade. 1988. Water as a plasticizer: physico-chemical aspects of low-moisture polymeric systems. *In* Water Science Reviews, vol 3. F. Franks, editor. Cambridge University Press, Cambridge, England. 79–185.

Levine, H., and L. Slade. 1992. Another view of trehalose for drying and stabilizing biological materials. *BioPharm.* 5:36–40.

Madin, K. A. C., and J. H. Crowe. 1975. Anhydrobiosis in nematodes: carbohydrate and lipid metabolism during dehydration. *J. Exp. Zool.* 193:335–342.

Nakagaki, M., H. Nagase, and H. Ueda. 1992. Stabilization of the lamellar structure of phosphatidylcholine by complex formation with trehalose. *J. Membr. Sci.* 73:173–180.

Roos, Y. 1993. Melting and glass transitions of low molecular weight carbohydrates. *Carbohydr. Res.* 238:39–48.

Roos, Y., and M. Karel. 1991. Plasticizing effect of water on thermal behavior and crystallization of amorphous food models. *J. Food Sci.* 56:38–43.

Roser, B. 1991. Trehalose drying: a novel replacement for freeze-drying. *BioPharm.* 4:47–53.

Suau, R., A. Cuevas, V. Valpuesta, and M. S. Reid. 1991. Arbutin and sucrose in the leaves of the resurrection plant *Myrothamnus flabellifolia. Phytochemistry.* 30:2555–2556.

Sun, W. Q., A. C. Leopold, L. M. Crowe, and J. H. Crowe. 1996. Stability of dry liposomes in sugar glasses. *Biophys. J.* 70:1769–1776.

Tsvetkov, T. D., L. I. Tsonev, N. M. Tsvetkova, R. S. Koynova, and B. G. Tenchov. 1989. Effect of trehalose on the phase properties of hydrated and lyophilized dipalmitoylphosphatidylcholine. *Cryobiology.* 26:162–169.

Uritani, M., M. Takai, and K. Yoshinaga. 1995. Protective effect of disaccharides on restriction endonucleases during drying under vacuum. *J. Biochem.* 117:774–779.