Journal of Thermal Analysis, Vol. 47 (1996) 1581-1596

THE EFFECT OF STORAGE TEMPERATURE ON INTERACTIONS BETWEEN DEHYDRATED SUGARS AND PHOSPHATIDYLCHOLINE

Karen L. Koster, Catherine L. Sommervold and Yao Ping Lei*

Department of Biology, University of South Dakota, Vermillion, SD 57069, USA

Abstract

We studied the effects of storage temperature on the stability of dehydrated POPC (1-palmitoyl-2-oleoyl-phosphatidylcholine) mixed with sucrose, trehalose, or a sucrose/raffinose mixture. We used DSC to measure the gel-to-fluid phase transition temperature (T_m) of POPC after incubation either below or near the glass transition temperature (T_g) of the sugars in the mixture. Glass formation by the sugars around fluid-phase POPC led to the lowering of T_m below that of the fully hydrated lipid. Phospholipid phase behavior did not change during storage below T_g. In some samples stored above T_g, trehalose crystallized completely; in these samples, the T_m of POPC increased to that of the partially dehydrated phospholipid. Melting the crystalline sugar re-established its ability to lower POPC's T_m. We conclude that prevention of complete sugar crystallization was important for stability in the dry state, and that storage below T_g conferred long-term stability to the dehydrated sugar-lipid mixtures.

Keywords: crystallization, dehydration, phase transition, phosphatidylcholine, sugars, vitrification

Introduction

Stability in the dry state is important to many systems, including both dehydrated foods and desiccated organisms. Many biological organisms have the ability to survive extended periods of desiccation (water contents generally less than 0.2 gH_2O/gDW during some stage of their life cycle [1, 2]. This phenomenon, often called anhydrobiosis, confers some benefits to organisms. Because bulk water is removed from their tissues, anhydrobiotic organisms may avoid damage caused by freezing or extreme heat. Thus, by entering a quiescent desiccated state, these organisms may survive periods during which the environment is not favorable for active metabolism.

Examples of anhydrobiotic organisms are many and include some nematodes, brine shrimp cysts, yeast, some mosses and ferns, and, perhaps most

* Author to whom all correspondence should be addressed.

0368–4466/96/ $ 5.00

John Wiley & Sons, Limited
Chichester

familiar, the seeds of many flowering plants [1, 2]. How these diverse organisms are able to survive desiccation is a fascinating question of basic biology; furthermore, understanding the mechanisms by which some seeds tolerate desiccation is of agronomic importance. Long-term storage of seeds is essential for continued diversity of germplasm reserves. Desiccation tolerance and stability in the dry state are important facets of successful long-term storage of seeds [3].

One aspect of desiccation tolerance and stability in the dry state is the maintenance of cellular membranes as intact bilayers. It has been shown that dehydration of phospholipid bilayers can significantly alter the phase behavior of the phospholipids, in some cases leading to demixing of membrane components, transitions to the gel phase, and the possible formation of non-bilayer phases [4, 5]. The formation of the non-bilayer hexagonal II phase has been correlated with the loss of viability in dehydrated cells and is usually considered to be a lethal event [6, 7].

In a recent paper [8], we have described how the presence of soluble sugars around phospholipid bilayers during dehydration can prevent increases in the gel-to-fluid phase transition temperature, T_m, of the phospholipids during dehydration.This phenomenon is well-documented, and a variety of mechanisms have been proposed to explain it [4, 5, 8–10]. We also demostrated that vitrification of sugars around fluid-phase phospholipids during desiccation caused an unforeseen lowering of the phospholipid's T_m, in effect, deferring the formation of the gel phase to lower temperatures. This effect was only observed when sugars vitrified around fluid-phase phospholipids (i.e., when the glass transition temperature, T_g, was higher than the T_m of the fully hydrated phospholipid). A satisfactory theoretical explanation for this effect has not yet been established.

In our previous study [8], we used mixtures of sugars, modeled upon the sugars found in desiccation-tolerant (DT) and non-desiccation-tolerant (NT) seed embryos, in combination with a representative membrane phosphatidylcholine, POPC (1-palmitoyl-2-oleoyl-phosphatidylcholine). The DT sugar mixture consisted of sucrose and raffinose, while the NT sugar mix contained predominantly glucose and some sucrose. In addition to the mixed sugar samples, we prepared some samples containing only sucrose with the phosphatidylcholine. A comparison of these three samples at low hydration revealed that the DT sugar mix consistently vitrified at ambient temperatures during desiccation, which led to the lowering of the phospholipid T_m below its hydrated value. As determined using differential scanning calorimetry (DSC), the NT- and sucrose-containing samples also vitrified as they dried, leading to a lowering of the phospholipid T_m. However, subsequent examination of the samples by X-ray diffraction revealed an abundance of reflections, presumed to arise from crystalline sugars, in the NT and sucrose samples. X-ray diffraction data were interpreted to indicate that the phospholipid T_m was no longer lowered below its

hydrated value. We concluded from these observations that pure sucrose and the NT sugar mixture were more prone to crystallization during storage than was the DT sugar mixture. This was not unexpected in light of previous studies showing that the crystallization of sucrose was slowed by the presence of raffinose [11, 12]. We also inferred that crystallization of sugars in the sample abolished the effect of vitrification, whereby the phospholipid T_m was lowered.

In the work described below, we have taken a more systematic approach to study the stability of dehydrated sugar-lipid mixes during prolonged storage. We have used three sugar solutions (sucrose, the DT mix, and trehalose), alone and in combination with the phospholipid POPC, to examine the effect of storage temperature on the crystallization of the sugars and the effect of crystallization on the phase behavior of the phospholipid.

Abbreviations

DSC, differential scanning calorimetry; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; T_m, gel-to-fluid phase transition temperature; T_g, glass transition temperature; gDW, dry weight (g) of samples.

Materials and methods

Materials

Samples used in this study contained one of three sugar solutions: pure sucrose, pure trehalose, and the DT mix. The DT sugar mix was modeled on sugars found in desiccation-tolerant maize embryos and contained sucrose and raffinose in an 85:15 weight ratio [13]. The sugars were all purchased from Sigma Chemical Company and were used with no further purification. Sucrose was ACS reagent grade, raffinose pentahydrate had a minimum purity of 99%, and trehalose dihydrate with a reduced metal ion content was used.

The phospholipid used in this work was POPC (1-palmitoyl-2-oleoyl-phosphatidylcholine), a mixed chain, monounsaturated phosphatidylcholine that is representative of many membrane phospholipids. POPC was purchased from Avanti Polar Lipids as a chloroform solution. Thin-layer chromatography of the solution yielded a single spot, so the lipid was used with no further purification.

Sample preparation

Samples for calorimetry were prepared as described previously [8] with some minor changes. Sugars were dissolved in purified Type I water (Nanopure, Barnstead, Inc.) during heating in a water bath at 333 K to ensure complete dissolution. For samples containing only sugars, aliquots of these sugar solutions were transferred into preweighed aluminum volatile-sample

pans. For samples containing both sugars and phospholipid, and equal volume of methanol was added to the cooled sugar solution to facilitate blending with the phospholipid. POPC in chloroform was first dried under a stream of N_2 at 323 K, then aliquots of the sugar solutions were added to the dry phospholipid film. Sugars were added to obtain a weight ratio of 2:1 (sugar:lipid). In a separate experiment, some samples were prepared with sugar and phospholipid at equal weights. The resulting suspensions were thoroughly mixed using a vortex mixer, then the suspensions were dried under a stream of N_2 at 323 K to remove the methanol. The dry mixtures of sugar and phospholipid were then incubated overnight in a vacuum oven with phosphorus pentoxide at 323 K. Sample mixtures were resuspended with purified water and were vortex-mixed and sonicated to obtain suspensions that appeared to be homogeneous. Aliquots of these suspensions were transferred to preweighed aluminum volatile-sample pans.

Samples were incubated at one of three temperatures, 253 K, 283 K, or 300 K, which represent temperatures below (253 K) or near (283 K and 300 K) the glass transitions of the sugars at the water contents attained during incubation. Samples were incubated over saturated solutions of LiCl to dry the samples and maintain constant low relative vapor pressures. Samples incubated at 253 K were first incubated for 24 h at 300 K over a saturated LiCl solution to lower the sample hydration to prevent ice formation within the sample at 253 K. Duplicate samples were removed weekly during the first eight weeks of the experiment, and less frequently thereafter. Pans were sealed with preweighed lids, and samples were examined using DSC. After calorimetry, dry weights were obtained by puncturing the sample pans and drying them in a vacuum oven over fresh phosphorus pentoxide at 323 K for at least 16 h. Water contents were calculated based on the sample dry weight.

Calorimetry

DSC was performed using a Perkin-Elmer DSC-7 with liquid N_2 cooling. Samples containing only sugars were loaded into the calorimeter at 298 K, then scanned at a rate of –20 K min^{-1} to 223 K. Samples were then scanned while heating at a rate of 20 K min^{-1} to 473 K. After seven weeks of sample incubation, this procedure was modified such that samples were scanned during cooling to 223 K, heating to 393 K, then a second cooling and heating cycle to 223 K then 473 K. Samples containing both sugar and POPC were loaded into the calorimeter at their incubation temperature, scanned at –20 K min^{-1} to 173 K, then at 20 K min^{-1} to 393 K, followed by a second cooling and heating cycle from 393 K to 173 K to 373 K. Some of the sucrose-containing samples were scanned to 473 K during the second heating scan to check for a melting peak of anhydrous crystalline sucrose.

Data were analyzed using software supplied by Perkin-Elmer for the Model 1020 controller of the DSC-7. All data that appear in the graphs were taken from the heating scans. T_g represents the glass transition temperature, determined as the midpoint of the temperature range over which the change in specific heat occurred. Because many scans had multiple peaks, T_m is used to represent the temperature of the peak maximum for the low temperature gel-to-fluid phase transition of POPC, while T_c is used to represent the peak maximum for high temperature peaks, possibly caused by crystalline melts of the sugars.

Peak enthalpies of lipid and sugar melting endotherms were calculated as the area under the peak divided by the dry weight of the sample component presumed to be responsible for that peak. For example, measured enthalpies of low temperature (239 to 258 K) peaks were divided by the dry weight of the POPC in the sample because these peaks were believed to represent the gel-to-fluid phase transition of the phospholipid [8]. Enthalpies of high temperature peaks (310 to 371 K) were divided by the dry weight of the sugars because it was believed that these peaks were produced by sugars.

Microscopy

To visually determine whether crystallization of sugars had taken place in some samples incubated at 300 K, randomly selected samples were examined using polarized light microscopy instead of DSC. Chosen samples were removed from the volatile-sample pan in which they had been incubated and were spread onto a microscope slide. Samples were flattened carefully using a cover slip and were then examined on an Olympus BH-2 microscope using polarized light. Examination of the samples was done at room temperature, then some slides were heated to view the effect on the sample. Slides with the sample mixture spread upon them were warmed on a heating block next to the microscope, while the surface temperature of the slides was monitored using a thermocouple. The heated slides were then quickly returned to the microscope for observation. Light transmitted by the sample when it was viewed through crossed polars was taken as evidence of the presence of crystals within the sample [14].

Results and discussion

We used dehydrated samples containing sugars or sugars mixed with POPC to examine the effect of storage temperature on sugar crystallization and the effect of sugar crystallization on the phase behavior of POPC. During incubation over saturated solutions of LiCl, the samples dried to water contents between 0.01 and 0.10 gH_2O/gDW (Table 1). Samples incubated at 300 K dried to lower water contents than did similar samples incubated at lower temperatures, although there was some overlap in the measured values. For samples containing

either sucrose or the DT mix, there was also a tendency for samples mixed with POPC to reach lower water contents than did samples containing only the sugars. This difference was more pronounced at the higher incubation temperatures and can presumably be ascribed to the hydrophobic nature of the phospholipids. For trehalose-containing samples, no differences in hydration between samples with and without the phospholipid were observed, with the exception of samples stored at 253 K, in which the pure sugar samples had a slightly higher water content than those also containing POPC ($p<0.05$). Water contents remained relatively constant during the time course of incubation.

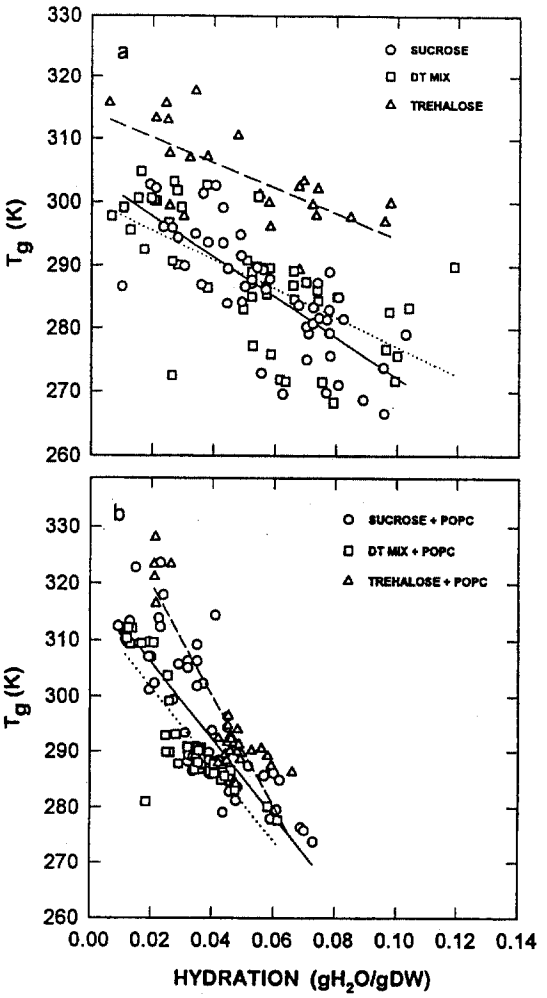

Fig. 1 (a) T_g's of pure sugar samples after incubation over saturated LiCl. (b) T_g's of sugars in samples containing POPC after incubation over saturated LiCl. In both graphs, lines represent first-order regressions of the data. Solid lines = sucrose; dotted lines = DT mix; dashed lines = trehalose

Table 1 Hydrations and peak midpoint temperatures of sugar-containing samples incubated at different temperatures

Sample	$T_{incubation}$/ K	Hydration (mean±/std. dev.)/ (gH_2O/gDW)	POPC T_m (mean±std. dev.)/ K	T_c (mean±std. dev.)/ K
Sucrose	300	0.036±0.012		331.6±1.5
Sucrose+POPC	300	0.014±0.004	249.6±1.3	331.3±2.2
Sucrose	283	0.078±0.010		no peaks
Sucrose+POPC	283	0.035±0.002	250.9±4.2	316.9±3.0
Sucrose	253	0.057±0.011		no peaks
Sucrose+POPC	253	0.041±0.005	254.5±2.5	no peaks
DT mix	300	0.022±0.008		333.6±1.3
DT mix+POPC	300	0.013±0.002	249.9±0.2	335.2±2.1
DT mix	283	0.068±0.018		no peaks
DT mix+POPC	283	0.033±0.006	246.6±1.8	315.5±2.9
DT mix	253	0.065±0.021		no peaks
DT mix+POPC	253	0.046±0.006	255.9±1.8	no peaks
Trehalose	300	0.028±0.010		339.9±2.0
Trehalose+POPC	300	0.028±0.012	247.4±2.5	345.1±2.3
Trehalose	283	0.046±0.014		326.2±2.4
Trehalose+POPC	283	0.048±0.008	241.0±0.8	320.1±1.8
Trehalose	253	0.071±0.020		326.7±3.8
Trehalose+POPC	253	0.059±0.013	250.5±4.5	330.7±1.1

Glass transition temperatures varied inversely with sample hydration, and they ranged from approximately 268 K to 318 K over the hydration range of the samples (Fig. 1). The inverse relationship between hydration and T_g is due to the well-known plasticizing effect of water on the amorphous sugars [15–17]. For samples containing only sugars, T_g values measured in samples containing trehalose were generally higher than values measured in samples containing either pure sucrose or the DT mixture (Fig. 1a). Over the narrow hydration range achieved in these samples, linear regressions of the T_g data provided good fits. An analysis of covariance of the lines thus obtained indicated that the lines for sucrose and the DT mix were not different, but the line for trehalose was significantly different from the others ($p < 0.01$). A similar pattern of higher T_g values for trehalose than for the other sugars was noted in samples containing POPC (Fig. 1b). These data are consistent with a report by Green and Angell [18] of significantly higher values of T_g for trehalose than for some other disaccharides, including sucrose. These high T_g values for trehalose have been further discussed by Levine and Slade [16,19] and may contribute to trehalose's well-known role as a dehydro-protectant [20].

For those samples that had water contents less than approximately 0.03 gH_2O/gDW, T_g values in samples containing the phospholipid POPC were higher than T_g values for pure sugar samples (Fig. 1). Two possible explanations for this disparity can be hypothesized. First, it may reflect unequal distribution of the water within the samples containing POPC, so that the sugar component forming the glass was actually drier than the average water content within the sample. One may infer that other portions of the sample were, therefore, more hydrated than the glassy portion. This point will be addressed further below in the context of the possible formation of sugar hydrate crystals. An additional explanation for the difference may exist if anhydrous sugar crystals formed in the pure sugar samples. In this event, water excluded from the anhydrous crystalline matrix may have plasticized the remaining sugar in the sample and, thus, lowered its T_g. Practically speaking, for this experiment, the disparity in T_g values means that sucrose and DT-mix samples with no POPC were stored above their T_g values at both 283 K and 300 K, while samples containing the phospholipid were stored near or below the onset temperature of the glass transition. Trehalose had higher T_g values than the other sugars; thus, all trehalose-containing samples were stored below T_g.

The gel-to-fluid phase transition temperature, T_m, of POPC ranged from 241 K to 256 K in samples that had been incubated over LiCl (Table 1). These values are lower than the T_m at 270 K reported for the fully hydrated phospholipid [8]. The lowered values observed in the current study correspond to those previously reported for POPC bilayers surrounded by vitrified sugars [8]. The POPC T_m values measured in this work vary among the samples and depend upon the sugar used and the incubation temperature (Table 1). These vari-

ations in the extent to which T_m was lowered by the vitrified sugars may reflect slight differences among the glassy matrices formed under each experimental condition of sugar composition and incubation temperature. Peak enthalpies of the gel-to-fluid phase transition did not vary significantly between the first and subsequent heating scans of the samples. One can infer from this that the phase behavior of the phospholipid was stable in these samples and was not affected by other thermotropic events within the sample.

During the incubation period, additional peaks began to appear in the DSC scans of both the sugar and the sugar-plus-phospholipid samples. These peaks had midpoint temperatures (designated as T_c to distinguish them from the low temperature phospholipid T_m) ranging from 310 K to 345 K, depending upon the sample composition and incubation temperature (Table 1). These high temperature peaks were present only in the first heating scan of any sample in which they appeared (Fig. 2, lines 1a and 2a). In the second and any subsequent heating scans, only glass transitions were observed in this temperature range (Fig. 2, lines 1b and 2b). In many DSC curves, the peaks were clearly distinct from the glass transition (Fig. 2, line 1a); however, when T_g was higher and the

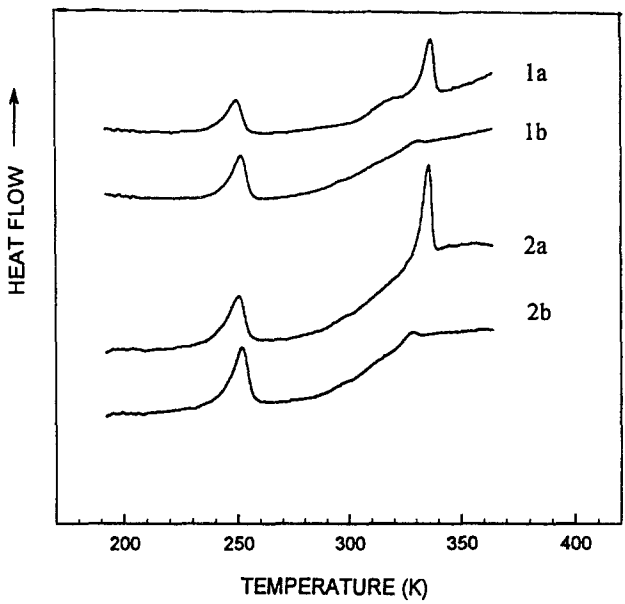

Fig. 2 DSC heating curves of samples containing sugars mixed with POPC stored over a saturated LiCl solution at 300 K. Lines 1a and 1b are from a sample of DT sugars with POPC stored for 7 weeks. Line 1a, from the first heating scan, shows a peak at 250 K, the T_m of POPC, a glass melt from 300 to 320 K, and a high temperature peak at 335 K. Line 1b is a subsequent heating scan of the same sample. Lines 2a and 2b are from a sample of sucrose with POPC stored for 8 weeks. Line 2a, from the first heating scan, shows the POPC transition at 250 K, but the glass transition and high temperature peak have merged, so that the peak now resembles an overshoot of the glass transition. Line 2b is the second heating scan of the sample

peaks were larger, the peak onsets merged with the glass transition (Fig. 2, line 2a). In those cases where the peak overlapped the glass transition, it sometimes appeared as though the peaks were large heat-flow overshoots of the glass transition. The thermal behavior exhibited in some of our samples stored at 283 K and 300 K was not inconsistent with previously described behavior of heat-flow overshoots [15, 21]; however, peaks that were clearly separate from the glass transition were seen in many samples, and these peaks did not have the characteristic appearance of heat-flow overshoots (Fig. 2). We chose to consider the clearly separate peaks as thermal events distinct from the glass transition and to consider the possibility that they represented the melting of crystalline sugars.

In DSC scans of samples containing just sucrose or the DT mixture with no POPC, these peaks appeared only for some samples incubated at 300 K, and they began to appear during the fourth week of incubation (Fig. 3). Enthalpies of these peaks ranged from 0.1 J g^{-1} to 1.8 J g^{-1}, and they increased significantly during incubation ($p<0.01$). Values of T_c measured in samples containing sucrose and the DT mix were not significantly different from one another ($p<0.01$). No distinct peaks were observed at 458 K, the melting point of anhydrous sucrose [22]; however, gradual rising in the DSC baseline between 400 K and 450 K sometimes occurred, with small peaks around 440 K. Microscopic examination of a pure sucrose sample stored five weeks at 300 K revealed extensive regions that transmitted light through crossed polars and appeared to be crystalline. These crystalline regions did not melt when the slide was heated to 373 K and re-examined, but they did melt when the slide was heated to 450 K. It is possible that anhydrous sucrose crystallized in some of these samples during incubation, although it is puzzling that no DSC scans exhibited clear melting peaks for anhydrous sucrose. The T_c values measured near 332 K (Table 1) in some of these samples are consistent with melting points measured by Young and Jones [23] for sucrose hydrate crystals at low water contents.

In samples containing only trehalose, peaks appeared in DSC curves of some samples from all incubation temperatures. These peaks appeared after two weeks of incubation in samples incubated at 300 K and 253 K, but they appeared only after five weeks in samples incubated at 283 K. For trehalose samples incubated at 300 K, peak enthalpies ranged up to 14.6 J g^{-1}, and they increased significantly during incubation ($p<0.01$) (Fig. 3). For trehalose samples incubated at 253 K and 283 K, peak enthalpies were between 0.7 J g^{-1} and 5.9 J g^{-1}, and they did not vary as a function of the incubation time. No peaks were observed at 371 K, the melting point of trehalose dihydrate [22].

In samples containing both sugar and phospholipid, these high temperature peaks only appeared in samples that had a 2:1 weight ratio of sugar to phospholipid, they appeared more frequently than they did in samples that had only sugars, and they appeared early during incubation, sometimes during the first week. The fact that these peaks appeared in DSC scans of samples that had a 2:1 weight ratio of sugar to phospholipid and did not appear in scans of samples

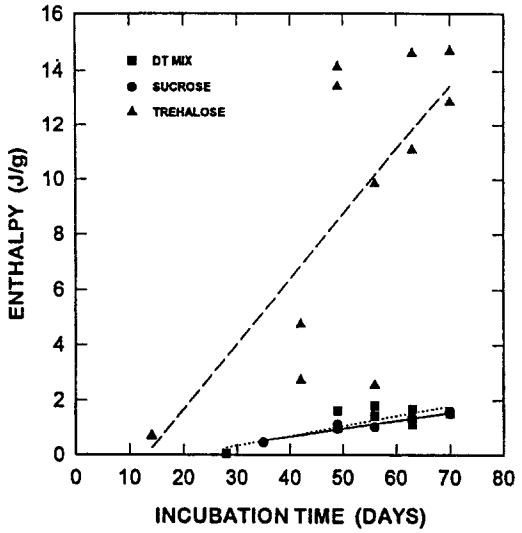

Fig. 3 The increase in enthalpy with time of the high temperature peaks observed in thermograms of sugars incubated at 300 K. Lines represent first-order regressions of the data. Solid line=sucrose; dotted line=DT mix; dashed line=trehalose

with 1:1 weight ratios may indicate that the presumed crystallization event only occurred in a bulk sugar phase that was not interacting with the phospholipids in the sample or was not intercalated between adjacent bilayers.

For samples of sucrose mixed with POPC and incubated at 283 K and 300 K, the high temperature peaks appeared in DSC scans after one week of incubation, while for samples incubated at 253 K, no peaks were observed. The enthalpy of these peaks increased significantly during the course of incubation ($p<0.01$) (Fig. 4). The rate of increase of the peak enthalpy, calculated as the slope of a regression line fit to the data, was greater in samples incubated at 300 K than in samples incubated at 283 K. If these peaks are considered to represent the melting of a crystalline sugar, the growth of the peaks during incubation reflects the kinetics of crystallization in these samples. Roos and Karel [24] measured crystallization rates in amorphous lactose held at constant relative humidity and found that the rates depended upon the difference between the incubation temperature and the onset temperature of the glass transition. Amorphous sugars stored near their onset temperatures had slower rates of crystallization than did sugars held at temperatures far above the glass transition [24]. Our samples were stored near the onset temperatures of their glass transitions and, thus, would be expected to have relatively slow rates of crystallization.

Conversely, if the measured peak enthalpies in these samples include heat-flow overshoots of the glass transition, one would also expect the enthalpy to increase with prolonged storage. Chang and Baust [21] found that, in samples

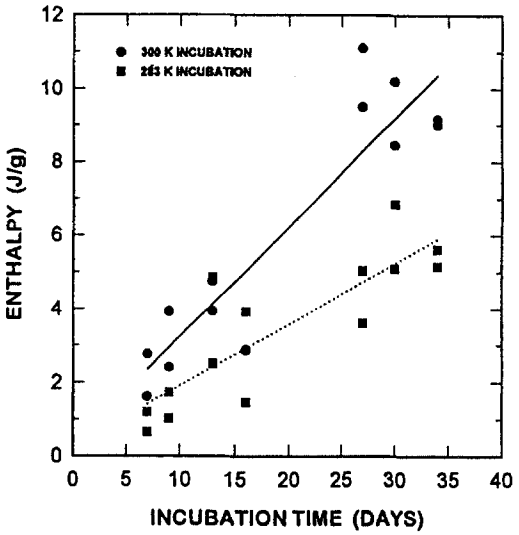

Fig. 4 The increase in enthalpy with time of the high temperature peaks observed in thermograms of sucrose mixed with POPC and incubated at 283 K or 300 K. Lines represent first-order regressions of the data. Solid line=300 K; dotted line=283 K

of vitrified glycerol annealed at a temperature just below T_g, the magnitude of the glass transition overshoot increased with increased annealing time. Thus, both crystal growth and larger heat-flow overshoots may have contributed to the increases in peak enthalpy observed in our samples.

We emphasize that, regardless of the nature of the peaks observed between 310 K and 345 K, no effects on the phospholipid phase behavior were observed in these samples. The T_m of the phospholipid was lowered below its fully hydrated value in all samples containing vitrified sugars. The peaks presumably do not reflect transitions of POPC because they were found in samples both with and without the phospholipid (Table 1). Also, the enthalpy of the POPC gel-to-fluid phase transition did not change between the first and subsequent heating scans, suggesting that the phase transition was unaffected by the presence of the high temperature melting peak.

High temperature peaks also appeared in thermograms of samples containing the DT mix with POPC and trehalose with POPC. The melting temperatures of the peaks observed in the DT mix samples were similar to those of the peaks in the sucrose-containing samples; therefore, we infer that the same substance, presumably sucrose, was responsible for the peaks in both sample sets. The T_c values correspond to melting temperatures reported for sucrose hydrate crystals [23]. The peaks observed in DSC scans of samples containing trehalose and POPC had significantly higher melting temperatures than those in the sucrose and DT mix samples ($p<0.001$), and we infer from this that a different substance was melting in these samples. We earlier hypothesized that micro-

domains having different hydrations existed within these samples, as evidenced by the differing T_g values measured in the sugar-plus-phospholipid samples when compared to T_g values in the pure sugar samples at the lowest water contents. If this is true, the wetter domains may be those containing crystalline hydrates of the sugars.

Microscopic examination of randomly chosen samples containing both sugar and phospholipid incubated for many weeks at 300 K revealed that these samples contained large regions that transmitted light when viewed through crossed polars, which can be taken as evidence that crystals were present [14]. However, pure POPC was also found to transmit polarized light diffusely. Therefore, in samples containing both sugar and phospholipid, only regions within the sample that had sharp transmission of polarized light and distinct crystalline structure were counted as evidence that crystals were present within the sample.

In many of the samples containing both sugar and phospholipid that had been incubated at 300 K, we observed very small, distinct structures that appeared to be crystalline, although we saw no single crystals clearly enough to determine their shape. Within the same sample, other regions sometimes appeared that had no apparent structure and only transmitted polarized light weakly. These amorphous regions also appeared very viscous in consistency. When the microscope slides were heated to approximately 373 K, then re-examined, the crystalline structures disappeared, and the samples transmitted polarized light more diffusely than they did before heating. These observations provide further evidence of the existence of very small crystals within many of the samples that had been incubated at 300 K. These crystals melted below 373 K, which is consistent with the behavior of the high temperature peaks in the DSC curves.

In two of the samples containing trehalose and POPC that had been incubated at 283 K and in two others incubated at 300 K, sharp melting peaks appeared at 371 K (Fig. 5, line a), the melting point of trehalose dihydrate crystals [22], instead of the more frequently observed peaks at 320 K and 345 K. These sharp peaks were very large, with enthalpies ranging from 196 to 221 J g^{-1}. In these few samples, no glass transitions were observed, and the apparent phospholipid T_m was at 306 K. No other peaks were observed in these samples during the first heating scan. During the second heating scan, the phospholipid T_m was lowered to 251 K, a glass transition appeared at approximately 296 K, and in three of the samples, very small peaks appeared at 281 K. The large peaks at 371 K were not present in the second heating scans (Fig. 5, line b). Water contents of these four samples were 0.058 to 0.072 gH_2O/gDW, values which were slightly higher than those of the other samples of trehalose and POPC incubated at these temperatures (Table 1). Thus, it is likely that these samples of trehalose were being stored above the T_g's for their water contents, unlike most of the other trehalose-containing samples. Incubation above T_g would facilitate crystallization, and the rate of crystallization would be higher in those samples stored well above T_g than in samples stored near or below T_g [16, 17, 24].

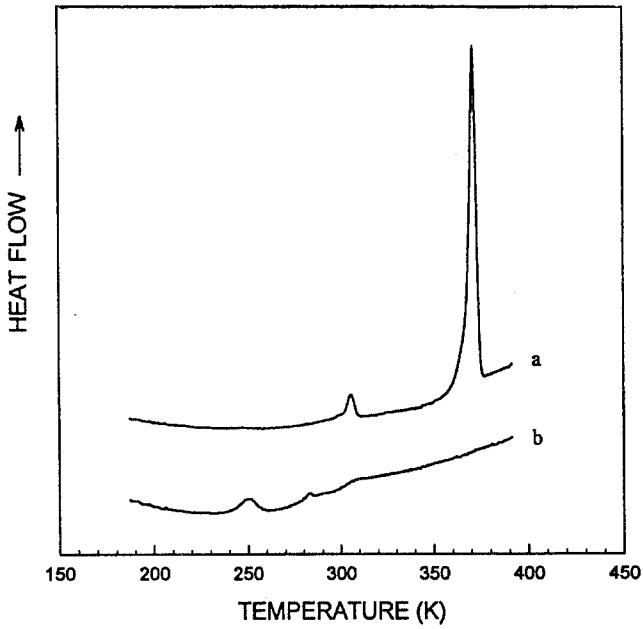

Fig. 5 DSC curves of trehalose mixed with POPC, then incubated at 300 K over saturated LiCl solution for several weeks. Line a, the first heating scan, shows a peak at 306 K, the T_m of partially dehydrated POPC, and a peak at 371 K, the melting point of trehalose dihydrate crystals. Line b, the second heating scan of the sample, shows the T_m of POPC at 251 K, a small peak at 281 K, and a glass transition around 296 K

An important aspect of these few samples in which trehalose crystallized during storage is that the interaction between the sugar and the phospholipid was abolished when trehalose was completely crystalline (Fig. 5, line a). The T_m of POPC was at 306 K, which corresponds to the T_m of POPC at approximately 0.06 gH_2O/gDW [8]. When the crystalline trehalose was melted and the sample cooled, the effect of the trehalose glass was re-established, and POPC's T_m was lowered to 251 K (Fig. 5, line b). The complete crystallization of the sugars was not observed in any other samples in this study. In samples that had peaks at 310 K to 345 K, glass transitions were still evident, and POPC's T_m was lowered below its hydrated value.

In a previous study [8], we reported that X-ray diffraction data of sucrose samples revealed extensive reflections presumed to be from crystalline sugars. In those samples, POPC was in a gel phase at 263 K and in a fluid phase at 303 K. Based on the X-ray diffraction data, we concluded that sugar crystallization abolished the ability of the sugar glass to lower the phospholipid T_m below its hydrated value (270 K) [8]. In the present study, we have confirmed that complete crystallization of sugars in a few samples prevented the lowering of

POPC's T_m. Using DSC, we observed that, in the presence of anhydrous trehalose crystals, the T_m of POPC was elevated to the temperature corresponding to that of the partially dehydrated phospholipid. Thus, complete crystallization of the sugars precluded the interactions between sugars and phospholipid that were observed in the majority of the samples. Melting of the sugar crystals restored the sugars' ability to intercalate between adjacent bilayers and thereby prevent increases in the T_m of POPC.

Conclusions

In this study, we observed that incubation temperature affected the long-term stability of dehydrated sugar-lipid mixes. Both with and without POPC, samples containing sucrose or the DT mix that were dried, then incubated at 253 K, did not change noticeably during the 8 weeks of the study. When stored at 253 K, all samples were well below their T_g and, thus, were vitrified. The glassy state is known to confer stability to systems by hindering diffusive motions within the system [17, 18, 25]; thus, the water contents remained constant, and no new peaks appeared in DSC scans of these samples.

In samples that were stored at temperatures near or above T_g, we detected evidence of sample demixing. The appearance of high temperature melting peaks in the DSC curves and the growth of these peaks during the incubation period may reflect the formation of sugar hydrate crystals within domains of the samples. The scattered and disparate T_g values may indicate that regions within the samples had different hydrations, possibly because of crystallization within other microdomains of the sample. Incubation of samples at temperatures near or above T_g may permit such changes to occur because of the diffusive motions possible in the rubbery state [17, 18, 25].

The high temperature melting peaks appeared more frequently in samples containing POPC, and they usually appeared earlier during the incubation period in samples with the phospholipid. It may be that the heterogeneity of the samples containing POPC increased the likelihood that a microdomain might exist that had conditions, such as water content, favorable to crystallization. It is clear that the partial crystallization observed in the sucrose and DT mix samples did not affect the phospholipid phase behavior. Neither the T_m of the phospholipid nor the enthalpy of the phospholipid transition were altered by the presence of the high temperature peaks. Presumably, crystallization within these samples was incomplete, and sufficient sugar was still intercalated between adjacent phospholipid bilayers to prevent their close approach and the resultant effects on the phospholipid T_m.

Complete crystallization of sugar was only observed in a few samples made with trehalose that were incubated above T_g. Calorimetry of these samples revealed large peaks at temperatures corresponding to the melting temperature of the crystalline dihydrate of trehalose (Fig. 5). Complete crystallization abolished the effect of sugars on the phospholipid bilayers; the T_m of POPC in these

samples was the same as for a partially dehydrated sample of pure POPC. Melting of the crystalline sugar re-established the sugar's effect on the phospholipid T_m. Thus, prevention of complete sugar crystallization is important for stability in the dry state. Storage below T_g impeded crystallization and conferred long-term stability to the dehydrated mixtures of sugar and phospholipid.

* * *

We thank Karen Sicard for her technical assistance, Dr. Karen Olmstead for her statistical expertise, and Dr. Royce Engstrom for his generous help with polarized light microscopy. We also thank two anonymous reviewers for their helpful comments about the manuscript. Funding for this work was provided by the Cooperative State Research Service, NRI Competitive Grants Program/U.S. Department of Agriculture, under Agreement No. 93-37100-8993 to KLK and by NSF-EPSCoR Grant #OSR-9108773 and the South Dakota Future Fund. CLS was supported by a Grant-in-Aid from Sigma Xi.

References

1 J. H. Crowe and J. S. Clegg, eds., Dry Biological Systems, Academic Press, NY 1978.
2 A. C. Leopold, ed., Membranes, Metabolism, and Dry Organisms, Comstock, Ithaca, NY 1986.
3 C. W. Vertucci and E. E. Roos, Plant Physiol., 94 (1990) 1019.
4 G. Bryant and J. Wolfe, Cryo-Lett., 13 (1992) 23.
5 J. Wolfe and G. Bryant, Physical Principles of Membrane Damage Due to Dehydration and Freezing, in Mechanics of Swelling: From Clays to Living Cells and Tissues, NATO ASI series H., 64 (1991) 205.
6 L. M. Crowe and J. H. Crowe, Arch. Biochem. Biophys., 217 (1982) 582.
7 W. J. Gordon-Kamm and P. L. Steponkus, Proc. Natl. Acad. Sci. USA, 81 (1984) 6373.
8 K. L. Koster, M. S. Webb, G. Bryant and D. V. Lynch, Biochim. Biophys. Acta, 1193 (1994) 143.
9 L. M. Crowe and J. H. Crowe, Biochim. Biophys. Acta, 946 (1988) 193.
10 J. H. Crowe, L. M. Crowe, J. F. Carpenter, A. S. Rudolph, C. A. Wistrom, B. J. Spargo and T. J. Anchordoguy, Biochim. Biophys. Acta, 947 (1988) 367.
11 B. M. Smythe, Aust. J. Chem., 20 (1967) 1097.
12 M. Caffrey, V. Fonseca and A. C. Leopold, 86 (1988) 754.
13 K. L. Koster and A. C. Leopold, Plant Physiol., 88 (1988) 829.
14 W. C. McCrone, L. B. McCrone and J. G. Delly, Polarized Light Microscopy, McCrone Research Institute, Chicago, IL 1987.
15 L. Finegold, F. Franks and R. H. M. Hatley, J. Chem. Soc., Faraday Trans. I, 85 (1989) 2945.
16 H. Levine and L. Slade, Crit. Rev. Food Sci. Nutr., 30 (1991) 115.
17 L. Slade and H. Levine, Adv. Food Nutr. Res., 38 (1995) 103.
18 J. L. Green and C. A. Angell, J. Phys. Chem., 93 (1989) 2880.
19 H. Levine and L. Slade, BioPharm, 5 (1992) 36.
20 J. H. Crowe, L. M. Crowe and D. Chapman, Science, 223 (1984) 701.
21 Z. H. Chang and J. G. Baust, Cryobiol., 28 (1991) 87.
22 CRC Handbook of Chemistry and Physics, 61st ed. , CRC Press, Inc., Boca Raton, FL 1981.
23 F. E. Young and F. T. Jones, J. Phys. Colloid Chem., 53 (1949) 1334.
24 Y. Roos and M. Karel, J. Food Sci., 57 (1992) 775.
25 F. Franks, Biophysics and Biochemistry at Low Temperatures, Cambridge University Press, Cambridge 1985.