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Biochimica et Biophysica Acta 1280 (1996) 187–196

BBA Biochimica et Biophysica Acta

Is vitrification involved in depression of the phase transition temperature in dry phospholipids?

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Received 27 June 1995; revised 10 October 1995; accepted 23 November 1995

Abstract

Recent literature has suggested that the depression of the phase transition temperature (T_m) in dry phospholipids by sugars may be ascribed to vitrification of the stabilizing solute, rather than by the direct interaction between sugar and phospholipid we have proposed. Koster et al. ((1994) Biochim. Biophys. Acta 1193, 143–150) claim that the only necessity is that the glass transition (T_g) for the sugar exceed T_m for the lipid. Evidence is presented in the present paper that this is not sufficient. Based on the vitrification hypothesis of Koster et al., the predicted order of effectiveness in depressing T_m in dry dipalmitoylphosphatidylcholine (DPPC) is dextran ≥ hydroxyethyl starch > stachyose > raffinose > trehalose > sucrose > glucose. In fact, the opposite order was seen. The effect of raffinose, sucrose, or trehalose on T_m in dry DPPC depends on the thermal history of the sample, as we have reported previously. When DPPC dried with trehalose is heated for the first time, T_m is about 55°C, but on the second and subsequent heating scans T_m falls to about 25°C. Koster et al. suggest that this effect is due to heating the sample above T_g rather than to melting the hydrocarbon chains. We present evidence here that all that is required is for the chains to be melted. Further, we show that retention of residual water by DPPC dried with trehalose depends on the drying temperature, but is independent of drying temperature with glucose, a finding that is consistent with direct interaction. We conclude that vitrification is not in itself sufficient to depress T_m in dry phospholipids.

Keywords: Phase transition temperature; Vitrification; Phospholipid; Depression; Sugar

1. Introduction

The ability of sugars to depress the gel-to-liquid crystalline phase transition temperature (T_m) of phospholipid bilayers is a well known phenomenon that was first described by us more than a decade ago [1]. There is considerable evidence that it is involved in stabilization of liposomes [2], intact membranes [3], and whole cells [4,5] during drying. We have proposed that the depression of T_m involves direct interaction between the sugar and phospholipid head groups, a proposal called the 'water replacement hypothesis' (reviewed in [6]). According to this hypothesis, the sugars maintain the lateral spacing between polar headgroups in the dry state, thus minimizing van der Waals' interactions among the hydrocarbon chains. The result, we proposed, would be a reduction in T_m. Evidence for similar direct interactions between sugars and dry proteins has also been provided (reviewed in [6]). Incidentally, Mansure et al. [7] have recently shown that trehalose also inhibits the toxic effects of ethanol in yeast cells.

Koster et al. [8] have suggested an alternative mechanism to direct interaction. They proposed, based partly on previous work of Green and Angell [9], that vitrification of the sugar is in itself sufficient to reduce T_m. An excellent review of vitrification and its role in stabilization of macromolecules has recently been published [10]. The analysis of Koster et al. [8], derived from a physical model of Bryant and Wolfe [11], proposes that vitrification would inhibit the increase in T_m during dehydration, perhaps by limiting lateral stresses in bilayers during dehydration. They admit they do not understand how this could lead to depression of T_m below that of the hydrated phospholipid, but nevertheless suggest that while "...specific sugar/lipid interactions may exist,... they probably contribute little to the effect of preventing increases in T_m".

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; FTIR, Fourier transform infrared spectroscopy; POPC, palmitoyloleoylphosphatidylcholine.

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SSDI 0005-2736(95)00287-1

Table 1
Glass transition temperatures (T_g) for several dry solutes used in stabilization of dry biomolecules

Solute	T_g	References
Glucose	30°C	[9,10,25]
Sucrose	65°C	[10,25]
Trehalose	80°C	[9]
	107°C	[16]
	110°C	our measurements
Raffinose	90°C	W.Q. Sun, personal communication
Stachyose	98°C	W.Q. Sun, personal communication
Hydroxyethyl starch	> 110°C	our measurements
Dextran (360 kDa)	> 110°C	our measurements

A key finding reported by Koster et al. [8] is that T_g (the glass transition temperature for the dry sugar) must exceed T_m in order for it to depress T_m in the dry lipid. Koster et al. [8] suggest that the state diagram for the sugar and the phase diagram for the lipid must intersect and cross at some point during dehydration, after which T_g would exceed T_m. We cannot see how this could lead to depression of T_m, even though it could limit further increases in T_m as dehydration progresses. Even so, there is no ambiguity in the proposal of Koster et al. [8] that T_g must exceed T_m of the hydrated phospholipid in order to depress T_m of the dry phospholipid.

We test the validity of this proposal in the present work, in which we have studied the effects of various carbohydrates on the T_m of dry DPPC. This phospholipid has a T_m in the hydrated state of 42°C, which rises to nearly 120°C when the lipid is fully dehydrated [12]. Table 1 shows T_g values for a number of dry carbohydrates, which are suggestive concerning their predicted effectiveness at depressing T_m in dry DPPC, if the Koster et al. [8] hypothesis is correct. Glucose, for example, has a T_g of about 30°C, so T_g never exceeds T_m for the lipid, in either the hydrated or dry states. Dextran, by contrast, has a $T_g > 110$°C and is predicted by the vitrification hypothesis to be particularly effective at reducing T_m in dry DPPC. The predicted order of effectiveness, based on T_g alone, would be dextran ≥ hydroxyethyl starch > stachyose > raffinose > trehalose > sucrose > glucose. Glucose should, in fact, have no effect on T_m in the dry lipid at all. We test that prediction here.

2. Materials and methods

2.1. Chemicals

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Glucose, raffinose, stachyose, sucrose, and trehalose were all from Pfanstiehl Carbohydrates (Waukegan, IL). Dextran T400 was from purchased from Pharmacia. Hydroxyethyl starch was a gift of Dr. Barry Spargo (Naval Research Laboratory, Washington, DC). These polymers were hydrated at a concentration of 100 mg/ml and dialyzed against distilled water (50 × the volume of the dialysate, with three changes) for at least 24 h. The dialysate was then freeze-dried and rehydrated at a concentration of 100 mg/ml before use.

2.2. Preparation of samples

Phospholipids were dried from chloroform in a tared tube, then re-weighed to obtain the weight of lipid used. The dry lipids were then rehydrated at a concentration of 20 mg/ml and extruded through 100 nm polycarbonate filters, using an Avestin (Ottawa, ON, Canada) extruder. The extruded liposomes were maintained at room temperature before use. Carbohydrates were added externally to the liposomes to give a mass ratio of 5:1, after which the liposomes were air dried at 20°C for at least 4 h in a glove box attached to a Balston (Haverhill, MA) dry air generator. The relative humidity during the drying was < 1%. Water contents of the samples after this protocol, determined by drying the samples to constant weight at 110°C, were less than 0.02 g H_2O/g sample. For infrared spectroscopy, 10 μl samples were dried directly on the windows. Before the samples were removed from the glove box, the windows were sealed with vacuum grease to prevent rehydration of the samples as it was transferred to the spectrometer. For calorimetry (DSC), the samples were freeze dried. After drying in the glove box for at least 3 h, the samples were sealed in the DSC pans (1 ml volume) before transfer to the calorimeter (Calorimetry Sciences Corporation, model 4207, Provo, UT).

2.3. Measurement of phase transitions

We used Fourier transform infrared spectroscopy (FTIR) to measure the lipid phase transitions rather than calorimetry, primarily because this method also can be used to study effects of the experimental treatment on functional groups in both the phospholipid and sugar. FTIR studies were done with a Perkin-Elmer 1760 optical bench assisted by a microcomputer equipped with Perkin-Elmer IRDM software and a Peltier temperature controller [13]. Alternatively, the measurements were done with a Perkin-Elmer 1650 FTIR coupled with a Perkin-Elmer FTIR microscope. Temperature control in this instrument is with a liquid nitrogen cooled cell with a resistance heater under computer control. Both instruments are purged of water vapor with the Balston dry air generator.

2.4. Measurement of fusion

Fusion and aggregation of DPPC unilamellar vesicles during drying were assessed with a Brookhaven BI90 quasielastic light scattering device. The vesicles (20 mg

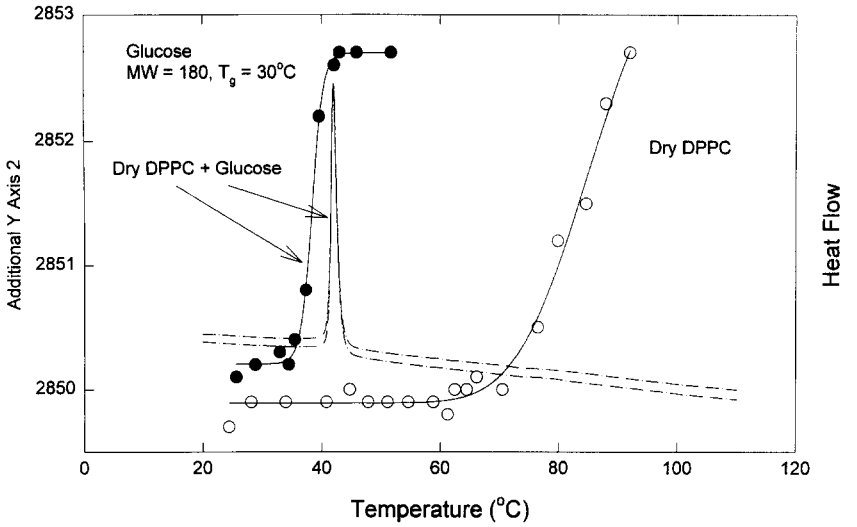

Fig. 1. Effects of glucose on gel to liquid crystalline transition temperatures in dry DPPC. Measurements, done both with FTIR and DSC, show that the transition occurs slightly below (about 40°C) that of the hydrated lipid and that it is a stable transition, occurring at the same temperature on the first and subsequent scans. In separate measurements, the samples were cooled to below T_m for up to a week or heated to temperatures as high as 100°C. These treatments had no effect on T_m (data not shown).

lipid/ml) were dried in 100 μl aliquots, then rehydrated in 2.5 ml of water. The entire sample was transferred to a plastic cuvette and particle size was determined using 2000 scans at 25°C.

3. Results and discussion

3.1. Results with glucose

DPPC with no glucose added showed a transition centered on about 42°C in the presence of excess water (data not shown), and about 90°C in the dry state (Fig. 1). The latter result suggests that these samples are actually DPPC monohydrate [14], in agreement with the measured water content of about 0.02 g H_2O/g dry weight, as described in Section 2. In the presence of glucose, the lipid shows a transition slightly lower (about 40°C) than that seen in the fully hydrated sample, as measured both with FTIR and DSC (Fig. 1). This transition is stable, persisting at this same value after several experimental treatments, including repeated scanning, storage below T_m for up to 1 week, and heating to over 100°C (data not shown). We emphasize that, unlike the results presented below for oligosaccharides, the thermal history of the samples made with glucose has no effect on T_m; regardless of the treatment it is stable at about 40°C.

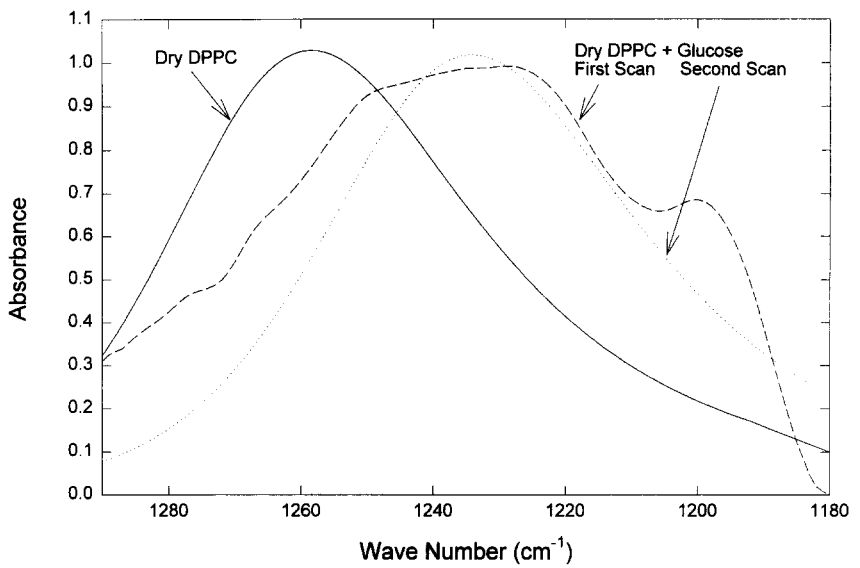

Fig. 2. FTIR spectra of the phosphate asymmetric stretch in DPPC. In dry DPPC this band is centered on 1260 cm^{-1}, and falls to 1230 cm^{-1} when the lipid is hydrated (data not shown). In DPPC dried with glucose, it is seen at 1230 cm^{-1} on the first and subsequent scans.

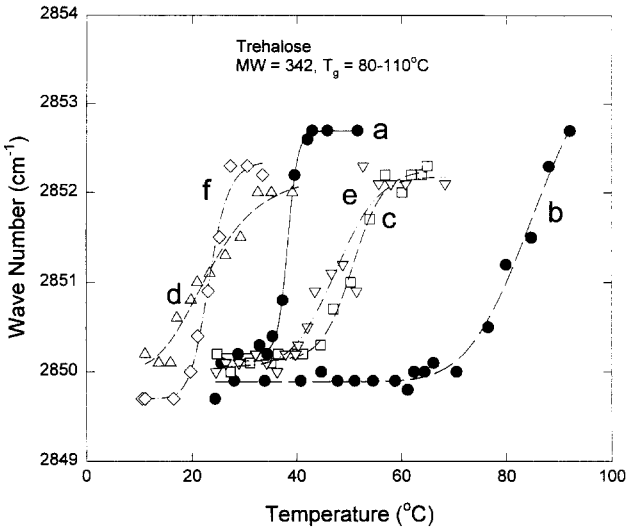

Fig. 3. Effects of trehalose on transition temperatures in dry DPPC. a: Hydrated DPPC; b: dried without trehalose; c: dried with trehalose, first scan; d: dried with trehalose, scan made immediately after the first scan; e: dried with trehalose, scan made after incubating the same sample at 4°C for 12 h; f: dried with trehalose, scan made immediately after scan 'e'.

We have previously reported that when phospholipids are dried with trehalose, the vibrational frequency of the phosphate of the polar headgroup of various phospholipids is depressed relative to the controls (e.g., [1,2]); when the phospholipid is dried without the sugars, the frequency increases, but with the sugar present the phosphate stretch is reduced to that seen in the fully hydrated phospholipid or, in the case of trehalose, to an even lower frequency [15]. We have interpreted this effect to be due to hydrogen bonding between the sugar and phosphate of the polar headgroup [1,15]. A similar effect is seen with glucose on the first scan after drying (Fig. 2). The phosphate asymmetric stretch rises from 1230 cm^{-1} in hydrated DPPC (data not shown) to 1260 cm^{-1} in DPPC dried without glucose (Fig. 2). With glucose present, this band decreases in frequency to about 1230 cm^{-1} (Fig. 2). Although the first scan suggests the presence of a small amount of heterogeneity, this band remains at the same frequency in subsequent scans, regardless of the experimental treatment.

3.2. Results with sucrose and trehalose

These sugars show similar effects, and will be treated together. In the samples dried with trehalose, T_m was about 55°C (Fig. 3), in reasonable agreement with previous results [15]. We previously reported that when DPPC was freeze dried with trehalose, T_m was found at 60°C on the first scan, but fell to 24°C on the subsequent and following scans [15]. We interpreted this effect in the following way. During the drying, the headgroups in DPPC are tightly packed, thus limiting access by the trehalose to the polar headgroups; as a result, on the first scan T_m is depressed partially but not completely. When the hydrocarbon chains are melted, thus decreasing the headgroup packing, the sugar might then be expected to penetrate and, as a result, depress T_m on the subsequent scans.

In the initial studies in which we reported this effect [15], we heated all samples to about 90°C, since this was the temperature at which the controls showed their transition. Koster et al. [8] suggested that the heating might actually have exceeded not only T_m but also T_g for the sugar, and that the important event here is exceeding T_g. In the case of sucrose, it is not possible to heat the samples above T_m without exceeding T_g (cf. Table 1), but with trehalose it is possible to do so. The published value for T_g

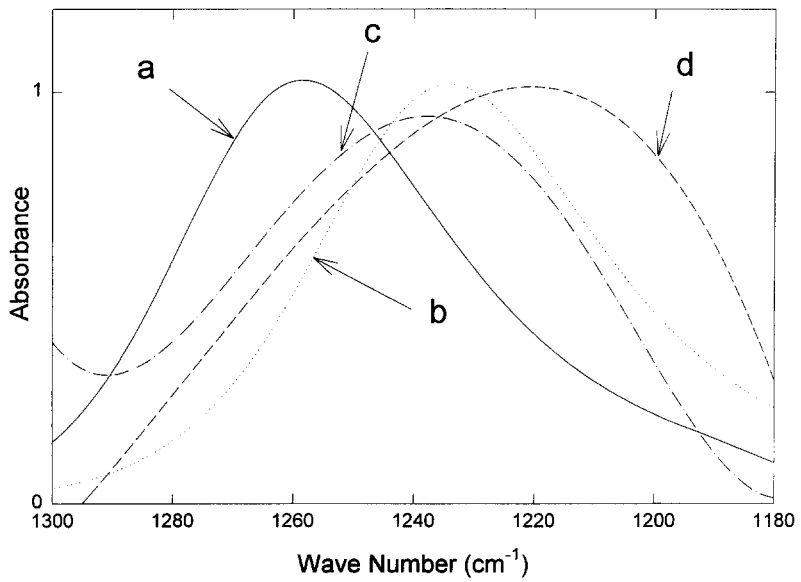

Fig. 4. FTIR spectra of the phosphate asymmetric stretch in DPPC. a: Dried without trehalose; b: hydrated; c: dried with trehalose, spectrum obtained at 25°C, before heating; d: dried with trehalose, spectrum obtained at 25°C immediately after heating to 65°C, and then cooling to 25°C.

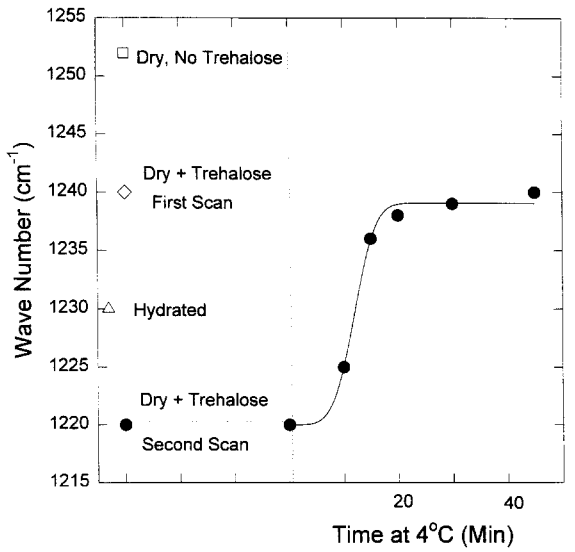

Fig. 5. Vibrational frequency (wave number) of the phosphate asymmetric stretch under the indicated conditions. All spectra taken before time zero were obtained at 25°C. The sample marked 'dry + trehalose, second scan' had been heated to 65°C, then cooled to 25°C before obtaining the spectrum. The same sample was then cooled to 4°C, after which spectra were recorded continuously.

in trehalose is about 80°C (Table 1). Those measurements were done by starting with what was assumed to be 'anhydrous' trehalose, although the method of dehydration was not specified. Crystalline trehalose, which is actually trehalose dihydrate, contains about 0.1 g H_2O/g dry weight. When this water is removed by drying above the crystalline melt, T_g rises to greater than 100°C [16]. We have found that trehalose freeze-dried as 0.4 ml aliquots of a 200 mM solution contains 0.07–0.08 g H_2O/g trehalose after 24 h on the freeze dryer. If such a sample is left under vacuum on the freeze dryer water content continues to fall. By contrast, the air-dried samples used here contained about 0.02 g H_2O/g trehalose. We will report a revised state diagram for trehalose elsewhere taking the residual water seen in freeze dried trehalose into account. We estimate that at the water contents seen in the air dried samples here, T_g is at least 80°C, despite the presence of 0.02 g residual water/g trehalose.

The dry sample was heated to 65°C – just above T_m for the DPPC in the presence of the sugar, but well below T_g – after which it was cooled to 10°C, and then re-scanned. On the second scan, T_m was seen to be at about 24°C ('d', Fig. 3), in agreement with our previous results [15]. In order to achieve this remarkable effect it is necessary only to exceed T_m for the lipid. In the procedure used here most of the trehalose would always be vitrified, and T_g is always well above T_m. In subsequent scans, T_m remained constant at 24°C, but if the sample was chilled to 4°C and held there overnight, the results marked 'e' in Fig. 3 were obtained; T_m clearly returned to its original value of about 55°C. When the same sample was again chilled to 10°C and immediately scanned again, T_m was seen to have returned to 24°C ('f', Fig. 3).

The phosphate stretch seen in FTIR is seen at about 1240 cm^{-1} on the first scan ('c', Fig. 4), falling to about 1225 cm^{-1} on the second and subsequent scans ('d', Fig. 4). When the samples were stored at 4°C overnight, the phosphate stretch returned to its original value of 1240 cm^{-1} (data not shown). We interpret this effect to be due to a forcing of the trehalose out of the bilayer when it is

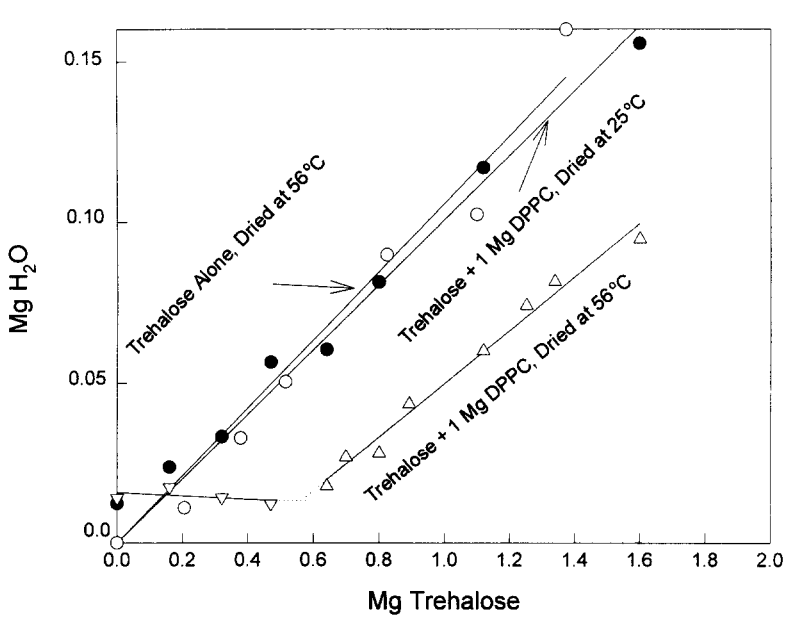

Fig. 6. Residual water contents in trehalose or trehalose + DPPC, air dried under the indicated conditions. Slopes of the lines were calculated by least squares.

stored below T_m, during which time the chain packing would be expected to increase. We measured the time required to see this effect by recording spectra continuously while the samples were kept at 4°C. The results show that the phosphate reverts to its original frequency (1240 cm^{-1}) in as little as 20 min (Fig. 5). When we measured T_m immediately after the 45 min incubation at 4°C, we found a T_m of about 55°C. Thus, T_m can be shifted by thermal manipulation of the sample without ever exceeding T_g of the sugar. We emphasize that the sugar must be present to obtain this result. When DPPC alone was given the same treatment, T_m remained constant at 90°C, regardless of the thermal history.

3.3. Residual water in 'dry' samples

We previously showed that when POPC vesicles were freeze dried with trehalose minimal residual water was retained in the samples until more than about 0.7 g trehalose/g lipid was added [17]. At higher mass ratios of trehalose to lipid residual water content rose linearly, and at a rate similar to that seen in trehalose without the POPC. We interpreted this result to mean that at mass ratios of trehalose/POPC < 0.7, all of the trehalose interacts directly with the lipid and is unavailable for retaining residual water. We did similar experiments here with DPPC, with the results shown in Fig. 6. When trehalose was air dried at 56°C, and water content was determined by further drying at 110°C, as described in Section 2, residual water was seen to rise linearly. When DPPC was added to the trehalose, residual water content rose linearly and at the same rate as in trehalose alone when the sample was dried at 25°C. But if the samples containing DPPC were dried at 56°C, well above T_m for hydrated DPPC, water content was seen to remain constant at about 0.015 g H_2O/g dry weight until the trehalose:DPPC mass ratio exceeded 0.62 – in good agreement with previously reported results for trehalose and POPC [17]. These results seem consistent with the suggestion that at mass ratios < ca. 0.7 all of the trehalose interacts directly with the lipid. This viewpoint is supported by the finding that samples of DPPC containing trehalose must be dried above T_m in order to see this effect.

A strikingly different result was obtained with glucose. When residual water was measured with glucose alone, it was seen to rise linearly, and at about the same rate as with trehalose (Fig. 7). But unlike trehalose, residual water contents in samples with DPPC remained low (about 0.009 g H_2O/g dry weight) in samples dried at 25°C until the mass ratio of glucose:DPPC exceeded about 0.3, after which residual water rose at the same rate as in free glucose. This finding that it is not necessary to heat the sample above T_m for hydrated DPPC during the drying is consistent with the results shown in Fig. 1, in which we showed that glucose depresses T_m of DPPC on the first scan when the samples are dried at 25°C.

Since the molecular weight of glucose is half that of trehalose, the mass ratios of trehalose (0.62) and glucose (0.3) are equivalent mole ratios of sugar to DPPC – about 1.5 mol sugar/mol lipid.

3.4. Results with raffinose

On the first scan, samples dried with this trisaccharide showed a T_m similar to that seen with sucrose or trehalose – about 55°C ('a', Fig. 8). But after the samples were heated to 65°C (as with trehalose, above T_m but below T_g) and then cooled, during the cooling direction scan the

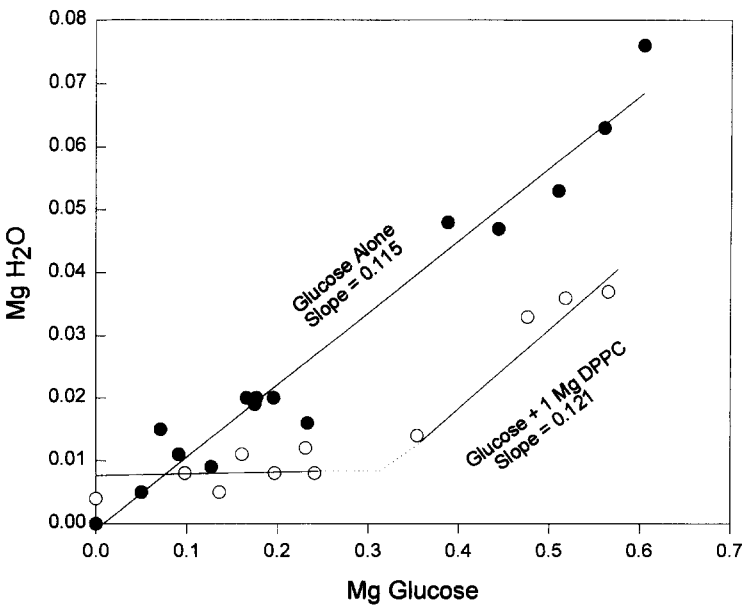

Fig. 7. Residual water contents in glucose or glucose + DPPC, air dried at 20°C. Slopes of the lines were calculated by least squares.

transition was seen to be centered on about 18°C ('b', Fig. 8). This remarkable depression of T_m is not stable when the lipids are stored below the transition temperature, however. When the sample was cooled to 2°C and then immediately scanned in the warming direction, curve 'c' in Fig. 8 was obtained. The melting curve is spread over a broad range, and is biphasic, with one part of the transition extending up to about 55°C ('c', Fig. 8). We suggest that the raffinose may be expelled from the bilayer rapidly below the transition, thus resulting in heterogeneity when the sample is re-warmed. In keeping with this suggestion, we found that when the samples were kept at −2°C for as 1 h and then rewarmed, T_m was seen to have returned to 55°C ('e', Fig. 8). Heating the samples above T_g (to 150°C, in fact) had no effect; the transition seen at 15°C remained unstable, reverting to 55°C rapidly when the samples were cooled below the transition. Results with the phosphate stretch were similar to those seen with trehalose. Before the first scan the phosphate stretch was seen at about 1240 cm^{-1}, falling to 1220 cm^{-1} after the hydrocarbon chains were melted once. With as little as 2 min at 2°C, this band returned to 1240 cm^{-1} (data not shown).

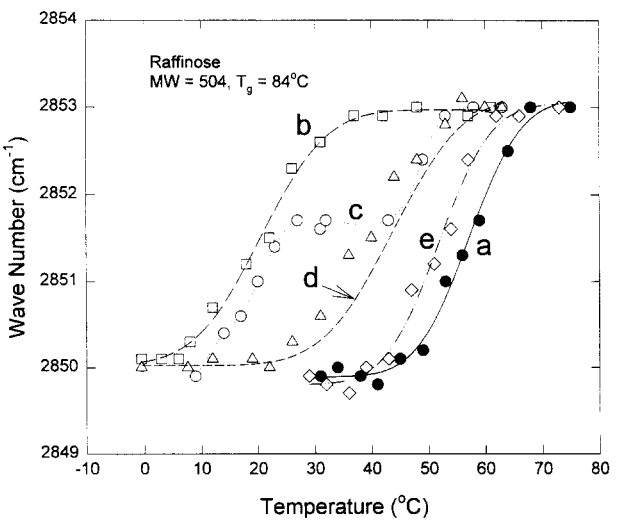

Fig. 8. Effects of raffinose on transition temperatures in dry DPPC. The scans were made in sequence on the same samples, without interruption, except as indicated a: First scan; b: second scan, in the cooling direction; c: third scan, in warming direction; d: fourth scan, warming direction, after 2 min at −0.5°C; e: fifth scan, warming direction, after 1 h at −2°C.

3.5. Results with stachyose

As with the other oligosaccharides, samples dried with stachyose showed a transition centered on about 55°C on the first scan ('a', Fig. 9). However, unlike the other oligosaccharides this transition remained at the same value, regardless of the treatment. During both cooling and warming after the first scan, the transition was seen at 55°C (Fig. 9). When the sample was heated well above T_g, to 150°C, no effect was seen; the transition remained at 55°C.

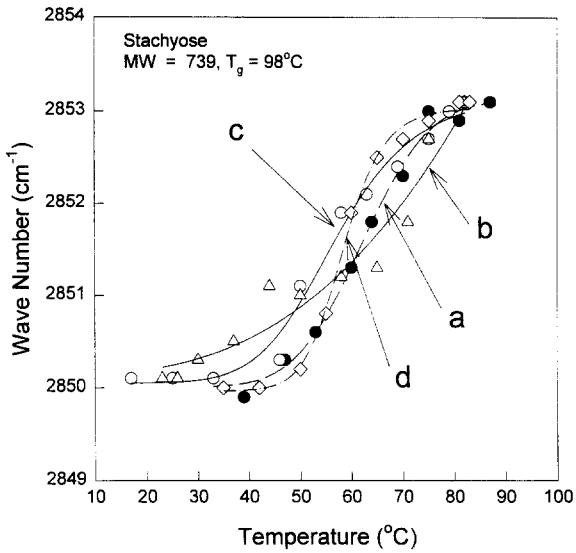

Fig. 9. Effects of stachyose on transition temperatures in dry DPPC. a: First scan; b: second scan, cooling direction; c: third scan, warming direction; d: fourth scan, after incubation at 150°C for 5 min, followed by cooling to 35°C.

Unlike the case with other oligosaccharides, the phosphate band showed two distinct sub-peaks, one at 1260 cm^{-1}, corresponding to the band seen in the control, and a second one at 1240 cm^{-1}, corresponding to that seen with the other oligosaccharides. These bands are stable, regardless of the experimental treatment (data not shown).

3.6. Results with dextran and hydroxyethyl starch

We have previously reported when egg PC was freeze-dried with dextran, T_m was not significantly different from that of the control [18]. Similar results were obtained here with dextran and hydroxyethyl starch (HES) air dried with DPPC. On the first and all subsequent scans a very small effect was seen on T_m with dextran, and no effect at all was seen with HES (Fig. 10). When the samples were heated well above T_g (to 150°C), no change was seen in T_m. Again, the vitrification hypothesis [8] predicts that this treatment would reduce T_m. The phosphate band was unaffected by the presence of the polymers, remaining undistinguishable from the control at 1260 cm^{-1}, regardless of the experimental treatment.

3.7. Why is T_m depressed to 55–60°C on the first scan?

With all the oligosaccharides tested, T_m is depressed from about 90°C to 55–60°C on the first scan. We suggest the following possible explanations for this effect. (1) The sugars retain water in the dry sample, and this water is responsible for depressing T_m. This would seem to be a likely explanation, since depression of T_m by this amount would require about one additional molecule of water/lipid [14]. However, in all the additives residual water was

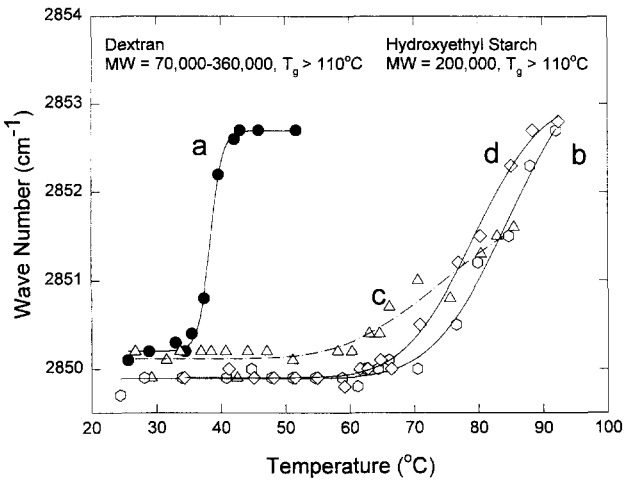

Fig. 10. Effects of dextran and hydroxyethyl starch on transition temperatures in dry DPPC. a: Hydrated DPPC; b: DPPC dried without dextran or hydroxyethyl starch; c: dried with dextran; d: dried with hydroxyethyl starch. The samples dried with additives were subsequently incubated at 4°C for 1 h or at 150°C for 15 min. Neither treatment significantly altered the phase transition.

nearly constant, at about 0.02 g H_2O/g dry weight. But dextran and hydroxyethyl starch hardly affect the transition at all, while glucose reduces it to about 40°C. If residual H_2O were the explanation, it is clear that all the molecules tested should have similar effects. While we cannot rule out this small amount of H_2O as a participant, the data do not support this hypothesis. (2) A small fraction of the oligosaccharides form a stable complex with the lipids and may, depending on thermal history, depress T_m even further, as we have shown. On first consideration, it might seem that exceeding the glass transition would be required for the sugar to have sufficient mobility to change orientation. However, recent studies have shown that several glass forming molecules, including sucrose, have significant molecular mobility at temperatures as low as 50°C below T_g [26], thus permitting the hypothesized change in orientation of the sugar. (3) T_m is limited in its increase by glass formation, as others have suggested [8,11]. We present further evidence having bearing on that hypothesis in the following section.

3.8. Effects of additives on fusion

The mechanism for limiting the rise in T_m during dehydration, according to Bryant and Wolfe [11], involves relaxation of lateral compression in the plane of the bilayer due to inhibition of close approach of adjacent bilayers. It follows that if the additive does not inhibit fusion during dehydration, it should have no effect on limiting the rise of T_m. It is not entirely clear that additives that inhibit fusion would necessarily also limit the rise in T_m, but that prediction is also implicit in the model of Bryant and Wolfe [11].

When we tested these predictions, the results shown in Fig. 11 were obtained. The original unilamellar vesicles were approx. 100 nm in diameter, but after drying the average diameter rose to about 3000 nm in the controls. With a single exception, every molecule tested strongly inhibited this fusion ('c–h', Fig. 11). The exception to this pattern, glucose, reduced fusion relative to the controls, but average diameter nevertheless increased to about 800 nm after drying ('i', Fig. 11). This lack of effectiveness on the part of glucose is immediately understandable; in samples dried at room temperature, this sugar does not achieve the glassy state until very late in the dehydration process (cf. Table 1), while the other molecules tested, with their much higher values for T_g, become vitrified much earlier during drying. Since fusion is a time dependent process, the longer the vesicles remain above T_g the greater the probability that they would undergo fusion. We have previously shown that vitrification is important in limiting fusion between liposomes during freeze drying [18] and will present additional evidence elsewhere concerning its role during air drying liposomes (Hoekstra et al., unpublished

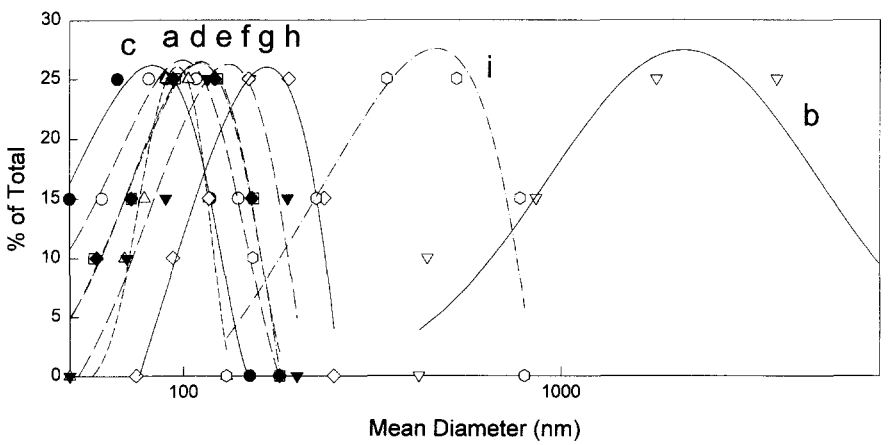

Fig. 11. Size distribution of DPPC vesicles under the indicated conditions. Samples that had been dried were rehydrated in order to make the measurements. a: Hydrated, before drying; b: dried without additives; c: dried with stachyose; d: dried with trehalose; e: dried with sucrose; f: dried with hydroxyethyl starch; g: dried with dextran; h: dried with glucose.

data). For the present, we point out that, contrary to predictions from the Bryant and Wolfe [11] model, glucose does not inhibit fusion (and clearly cannot limit close approach of adjacent bilayers), but it nevertheless limits the rise in T_m during drying. Similarly, dextran and HES inhibit fusion but have no effect on T_m in dry DPPC. We see no way to rectify these results with the Bryant and Wolfe [11] model.

Pincet et al. [19] have recently published findings that they suggest also call the water replacement hypothesis into question. They found that trehalose does not affect interbilayer surface pressures between two fully hydrated bilayers and conclude that they could find no evidence for direct interactions between the sugar and hydrated bilayer. That finding is not surprising; we [20,21] and others [22,23] have provided evidence previously that direct interactions do not occur in the fully hydrated lipids. Indeed, Nakagaki et al. [24] reported that when as little as two moles of water per mole lipid is added to DPPC dried with trehalose T_m rose from 24°C to about 60°C. It then declined towards T_m for the hydrated lipid as additional water was added. They interpreted these findings to mean that addition of small amounts of water to the dry sample displaces the trehalose. The point we wish to make is that results obtained with fully hydrated bilayers cannot be extrapolated to dry ones.

3.9. Summary and conclusions

There is no doubt that vitrification is an important factor in stabilizing biomolecules in the dry state, and we have provided evidence ourselves that this is true for liposomes [18]. However, the evidence presented here shows that the effects predicted from the vitrification hypothesis concerning T_g and its relationship to T_m are the opposite of those actually measured. In fact, when the effects of the molecules used here on T_m were plotted against T_g, the results shown in Fig. 12 were obtained. With the low molecular weight molecules, the predictions of the vitrification hypothesis seem to be fulfilled; as T_g increases, the effect on T_m gets larger, but that relationship breaks down above the trisaccharide level (Fig. 12), when increasing molecular weight and T_g have no effect on T_m. The most logical explanation for these effects is that they are related to molecular weight and have nothing to do with T_g: with increasing molecular weight, the polar headgroups are spread more extensively, leading to a further depression of T_m. But a point is reached at which the bilayer cannot accommodate larger sugars. Above that molecular weight (which seems to be about the size of a trisaccharide), the effects are diminished.

We have also plotted in Fig. 12 the effects of the molecules used here on the frequency of the phosphate stretch against effects on T_m. Clearly, the larger the effect on the phosphate the larger is the effect on T_m. We believe this observation is in good agreement with the water

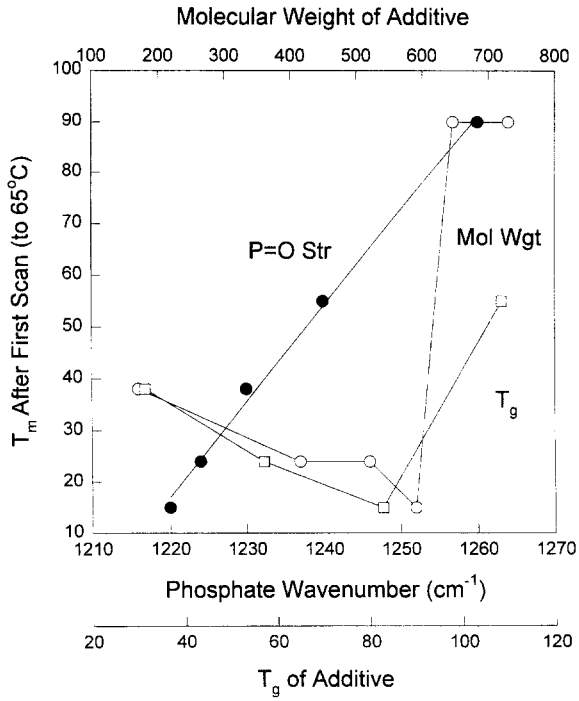

Fig. 12. Relationship between T_m in dried DPPC (after heating to 65°C) and molecular weight of the additive, T_g of the additive, and the phosphate asymmetric stretch.

replacement hypothesis and see no way to explain it based on vitrification.

We suggest that vitrification alone is insufficient to reduce T_m in dry DPPC, and probably in all phospholipids. We stress that we do not believe that the requirement for vitrification and direct interaction are necessarily mutually incompatible; indeed, the bulk of the evidence suggests to us that both are required.

Acknowledgements

Supported by grant IBN 93-08581 from the National Science Foundation and grant N00014-94-0379 from the Office of Naval Research. We also gratefully acknowledge the critical comments of Dr. Ann Oliver and an anonymous reviewer, who detected several errors in the manuscript.

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