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Preservation of dry liposomes does not require retention of residual water

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ABSTRACT **Certain sugars, particularly trehalose, dramatically alter physical properties of dry phospholipids in ways that mimic the presence of water. As a result, these sugars are capable of preserving the integrity of dry liposomes and membranes. Since these effects could conceivably be due to the presence of small amounts of water in the dry preparations of sugar and lipid, we have done careful measurements of the residual water contents in the dry samples and report the results here. Lyophilized liposomes composed of palmitoyloleoylphosphatidylcholine and phosphatidylserine (9:1) contain at most 0.2 mol of H_2O per mol of lipid. When the trehalose concentration in the dry mixtures is increased, there is no increase in the apparent water content of the samples over a wide range of sugar concentrations. Over the same range of trehalose contents the maximal effect of trehalose on physical properties of the lipids and on stabilization of liposomes is achieved. We conclude that the stabilization does not require retention of residual amounts of water in the dry trehalose–phospholipid preparations. Similar studies with other sugars show a relationship between the amount of sugar interacting with the lipid and the ability of the same sugar to stabilize dry liposomes.**

In view of their practical potential as vehicles for delivery of water-soluble drugs to cells in whole organisms (e.g., ref. 1), stabilization of liposomes for long-term storage is an important, practical problem. We (2, 3) and others (4–6) have addressed this problem and have found that liposomes can be preserved quite effectively by freezing (5–8) or freeze-drying (2–5) in the presence of certain sugars. For example, when liposomes were freeze-dried in the presence of adequate amounts of trehalose, they were found upon rehydration to have retained as much as 100% of their original contents (2–4). By contrast, when they were freeze-dried without sugar, upon rehydration they leaked essentially all of their contents. Some of the same sugars that are effective at preserving dry liposomes are also remarkably effective at stabilizing structure and function in dry biological membranes (9).

In an effort toward understanding the mechanism by which sugars preserve dry liposomes and membranes, we have studied over the past few years the effects of sugars on physical properties of phospholipids. Those studies have shown that the sugars that are the best preservation agents also most strongly inhibit fusion during freezing (5, 6, 8, 10) or freeze-drying (3). However, the amount of sugar required to inhibit fusion is much less than that required to prevent leakage, so inhibition of fusion alone is clearly not the sole mechanism of preservation (3). The same sugars are very effective at reducing the transition temperature (T_m) for the dry lipids, thus potentially maintaining them in a fluid phase in the absence of water (3, 6, 11). We previously suggested that the dry lipids were in liquid crystalline phase above T_m, but more recent evidence (12) suggests that it is a fluid phase, but probably not authentic liquid crystalline phase.

Since maintaining the lipid components of liposomes in fluid phase is of such importance in their stabilization, we are trying to understand the mechanisms by which sugars confer their effects on dry lipids. The best available experimental evidence (refs. 5, 11; reviewed in ref. 13) suggests that the sugars interact directly with the polar head groups of phospholipids, probably by hydrogen bonding to the phosphate and thus changing the lateral spacing of phospholipids in the bilayer. Recent computer-generated models of trehalose–phospholipid associations are consistent with this view (14). An alternative mechanism involves binding of water by the sugars. According to this idea, —OH groups on the sugars would bind water in the "dry" sample, and that water would in turn affect the phase properties of the phospholipids. This notion seems *a priori* unlikely since water bound by the sugar would presumably be unavailable for interaction with the lipid. In addition, at the highest mol ratios of trehalose to lipid studied, T_m for at least two lipids is considerably below that for the fully hydrated lipid (3, 11). Thus, it does not seem plausible that residual water is responsible for depression of T_m in the dry samples. Nevertheless, we have now studied directly the possibility that there is an appreciable amount of water in the dry samples and present results of those studies in the present paper.

MATERIALS AND METHODS

Liposomes. Palmitoyloleoylphosphatidylcholine (PamOle-PtdCho) and crude bovine brain phosphatidylserine (PtdSer) were purchased from Avanti Biochemicals and used without further purification. We have used this mixture of lipids in the present publication because it is the same mixture previously used for studies on stability of dry liposomes (2). However, as we will report elsewhere, similar results can be obtained with other lipids as well. Trehalose dihydrate, raffinose pentahydrate, maltose monohydrate, and *myo*-inositol were obtained from Pfanstiehl Laboratories (Waukegan, IL); sucrose and D-glucose were from Mallinckrodt. PamOle-PtdCho and PtdSer were mixed together in chloroform, dried under nitrogen, and placed under high vacuum (10–20 millitorr; 1 torr = 133 Pa) on a VirTis lyophilizer for at least 12 hr. The lipids were rehydrated to a concentration of 20 mg/ml with double-distilled, deionized water containing the sugar to be tested and sonicated to clarity in a bath sonicator (Laboratory Supplies, Hicksville, NY). Sufficient sugar was present in the rehydration solutions to give mass ratios of sugar to lipid ranging from 0 to 1.5 g of sugar per g of lipid. In previous studies (2, 3), we have shown that liposomes of this type are maximally stabilized by about 1 g of sugar per g of lipid, at least for those sugars that are effective at preserving dry liposomes.

Measurement of Residual Water. To 1.0 ml of each of the solutions containing liposomes and sugar, 1 μCi of ^3H_2O (specific activity = 5 mCi/ml; 1 Ci = 37 GBq) was added, and

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Abbreviations: T_m, transition temperature; PamOle-PtdCho, palmitoyloleoylphosphatidylcholine; PtdSer, phosphatidylserine.

the solutions were thoroughly mixed. Preliminary experiments showed that equilibration of the ^3H_2O with exchangeable protons on the lipids and sugars was achieved within the first 5 min of incubation. Triplicate aliquots of 1 μl and 300 μl were removed from each sample. The 1-μl aliquots were transferred rapidly to scintillation vials; 1.0 ml of H_2O was immediately added, followed immediately by addition of 15 ml of scintillation cocktail (PCS, Amersham). The 300-μl aliquots were placed in scintillation vials, frozen in liquid nitrogen, and then lyophilized for at least 12 hr on a VirTis lyophilizer. During the first 2 hr of lyophilization, a liquid nitrogen-cooled cold trap, in which ^3H_2O and H_2O sublimed from the samples was collected, separated the samples from the lyophilizer. Most of the water was removed from the samples during this period, after which the flasks containing the scintillation vials were connected directly to the lyophilizer for at least an additional 10 hr. The samples were then removed from the lyophilizer and dissolved in 1.0 ml of water, followed by addition of 15 ml of scintillation cocktail. Radioactivity in the samples was measured by liquid scintillation counting with a Beckman LS100c liquid scintillation counter. Using this technique, every sample contained cpm at least 10× background. Water contents of the dry samples were calculated according to the following equation: $H_d = (H_w/\text{cpm}_w)(\text{cpm}_d)$, where H_d = mg of H_2O in the dry sample; H_w = mg of H_2O in the 1-μl aliquots taken before lyophilization—i.e., 1 mg; cpm_w = cpm in the 1-μl aliquots; and cpm_d = cpm in the dry samples. Since the weights of lipid and sugar in each sample are known, mg of H_2O per mg of sample can then be calculated.

Infrared Spectroscopy. To keep the samples as dry as possible during preparation for infrared spectroscopy, the dry lipid–sugar mixtures were removed from the lyophilizer under vacuum by closing the vent valve for the desiccator in which they were lyophilized. The desiccator was then vented in a glove box flushed with dry nitrogen. The samples were crushed lightly between two BaF windows and the windows were mounted in their metal carrier and removed from the glove box. Spectra were immediately recorded in a Perkin–Elmer 1700 Fourier transform infrared spectrometer, assisted by a Perkin–Elmer 7500 laboratory computer. Integrated intensities of selected bands were determined with the "area" routine in the Perkin–Elmer CDS-3 software.

RESULTS AND DISCUSSION

Water Content of Dry Samples Containing Trehalose. When samples of trehalose were lyophilized without the lipid, the water content rose linearly with increasing trehalose, with a slope of 0.16 (Fig. 1). This represents a water content of 3 mol of H_2O per mol of trehalose. Since crystalline trehalose contains 2 mol of H_2O per mol of trehalose (15), we suggest that the apparent extra mol of water in the samples prepared from ^3H_2O is not water but is instead a tritium atom that has exchanged with the most readily exchangeable proton on trehalose, probably on one of the hydroxyl groups. In any case, under the conditions in which these samples were prepared this is a repeatable apparent water content for the trehalose samples in the absence of lipid. By contrast with the more hydrophilic sugar, the lyophilized samples of liposomes in the absence of any trehalose have an apparent water content of at most 0.005 g of H_2O per g of lipid, or about 0.2 mol of H_2O per mol of lipid (Fig. 1). As the trehalose content of the samples was increased, the water content did not change significantly over a broad range of trehalose contents (region I, Fig. 1) but increased sharply when a critical trehalose content was reached (region II, Fig. 1). Above this critical trehalose content, the apparent water content of the samples increased linearly, with a slope of 0.15 (Fig. 1). We interpret these results in the following way: in region I, all of the trehalose present in the samples interacts strongly with

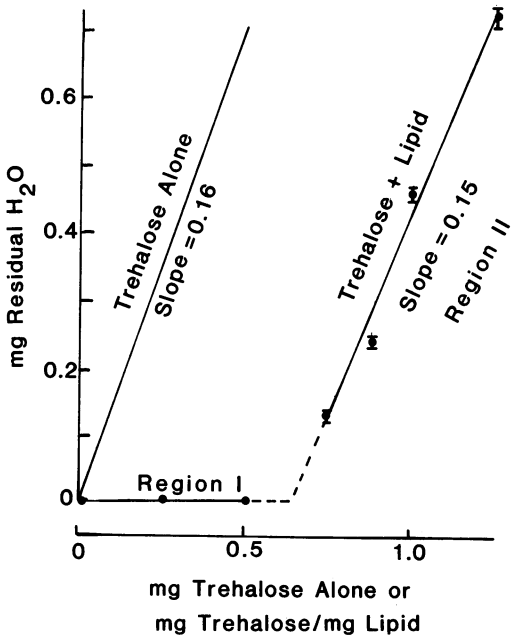

FIG. 1. Apparent water content of PamOle-PtdCho/PtdSer liposomes lyophilized in the presence of various amounts of trehalose and water contents of trehalose lyophilized in the absence of the lipid. Note that in region I, there is no change in the water content but that in region II the slope of the line for the PamOle-PtdCho/PtdSer/trehalose mixtures is the same as that for trehalose alone. Vertical bars represent standard deviations.

the lipid and is unavailable for binding water; in region II, free trehalose appears and binds water at a rate similar to that seen in trehalose lyophilized without lipid. Thus, evaluation of the amount of trehalose interacting with the lipid can be made from a simple calculation of the point of intersection of the two line segments seen in the data for trehalose–lipid preparations in Fig. 1. When this calculation was done, the bound trehalose fraction was found to be 0.67 g of trehalose per g of lipid, or 1.49 mol of trehalose per mol of lipid. This is a reproducible stoichiometry; we have obtained the same figure for bound trehalose in these lipids in four completely independent measurements.

Water Content, Physical Properties of Dry Lipids, and Preservation of Liposomes. The relationship between water content of the dry samples, their stability in the dry state, and the potential influence of any residual water on the physical state of the dry lipids can now be evaluated. We have shown that infrared spectra of dry dipalmitoylphosphatidylcholine (Pam_2-PtdCho) in many ways resemble spectra for hydrated Pam_2-PtdCho (11). We now extend those observations to the PamOle-PtdCho/PtdSer liposomes used in the present study, with a view toward examining the relationship between effects of the sugar on the infrared spectra and water content of the dry preparations.

Segments of typical spectra for hydrated PamOle-PtdCho/PtdSer liposomes and dry PamOle-PtdCho/PtdSer liposomes prepared without trehalose and in the presence of trehalose are shown in Fig. 2. The most important changes seen in these spectra are that when the liposomes were dehydrated the vibrational frequency for the P═O asymmetric stretch shifted from about 1230 cm^{-1} to about 1243 cm^{-1}. In the presence of increasing amounts of trehalose, however, this band in the dry liposomes shifted back to lower frequencies, and at the highest trehalose contents, it is at about the same frequency as that seen in the fully hydrated liposomes. In addition, there is a clear diminution in the intensity of this band in the presence of trehalose. From similar spectra for PamOle-PtdCho/PtdSer liposomes prepared with various trehalose

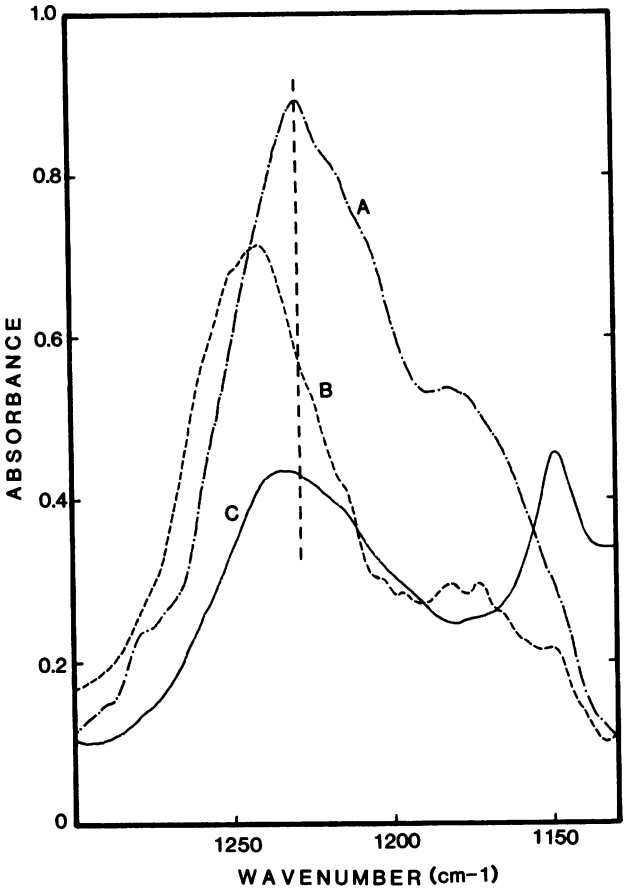

FIG. 2. Infrared spectra in the polar head group region for hydrated PamOle-PtdCho/PtdSer liposomes (trace A), dry liposomes (trace B), and liposomes containing 1 g of trehalose per g of lipid (trace C).

contents, the effects of trehalose on vibrational frequency for the P=O asymmetric stretch were observed (Fig. 3). With increasing amounts of trehalose it declines steadily, reaching a minimal value between 0.5 and 0.62 g of trehalose per g of lipid. Similarly, the integrated intensity of this band declines with increasing trehalose content, reaching a minimal value between 0.62 and 0.75 g of trehalose per g of lipid (Fig. 3). Reproduced in Fig. 3 are the data from Fig. 1 for water contents of the dry mixtures, clearly showing that the maximal effect of trehalose on the infrared spectra is obtained in region I, where water content remains low.

Similar comparisons between water contents of the dry preparations and effects of increasing trehalose contents are equally instructive about the role of water in stabilizing dry liposomes. For example, we previously showed that when PamOle-PtdCho/PtdSer liposomes are lyophilized, two calorimetric transitions (a low temperature one and a high one) are seen, which we tentatively assigned to the inner and outer monolayers of the bilayer (3). With increasing trehalose contents, the enthalpy of the low temperature transition rises, an increase that is strongly correlated with retention of trapped solute by the dry liposomes (3). A comparison is made in Fig. 4 between water contents, enthalpy of the low temperature calorimetric phase transition, and retention of trapped solute by PamOle-PtdCho/PtdSer liposomes dried in the presence of various amounts of trehalose. The data for retention of trapped solute and calorimetric data are reproduced from Crowe *et al.* (2) and Crowe *et al.* (3), respectively. As with the infrared data presented in Fig. 3, here again, maximal effect of trehalose on the calorimetric transition and

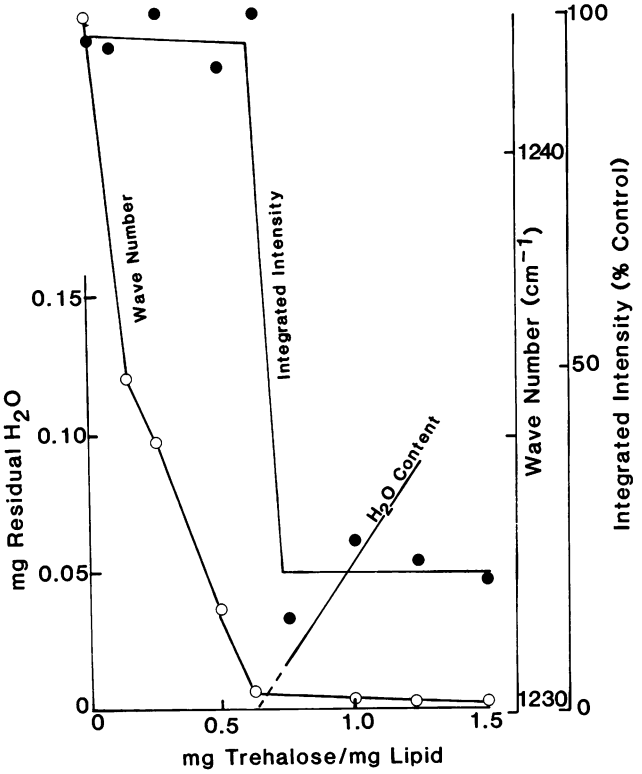

FIG. 3. Vibrational frequency and integrated intensity of the P=O asymmetric stretching vibration of PamOle-PtdCho/PtdSer liposomes dried in the presence of the indicated amounts of trehalose. Also shown are the apparent water contents of the same samples, with data points and standard deviations omitted for clarity.

on retention of trapped solute is obtained in region I, before water content of the dry samples begins to rise.

We conclude from these data that residual water is not involved in stabilizing the dry liposomes. Indeed, the data are entirely consistent with previous suggestions that sugars show a direct interaction with phospholipids (5, 11, 13).

Evaluation of Binding of Other Sugars to Dry Lipids. We have conducted measurements similar to those described above for trehalose for a number of other sugars, with

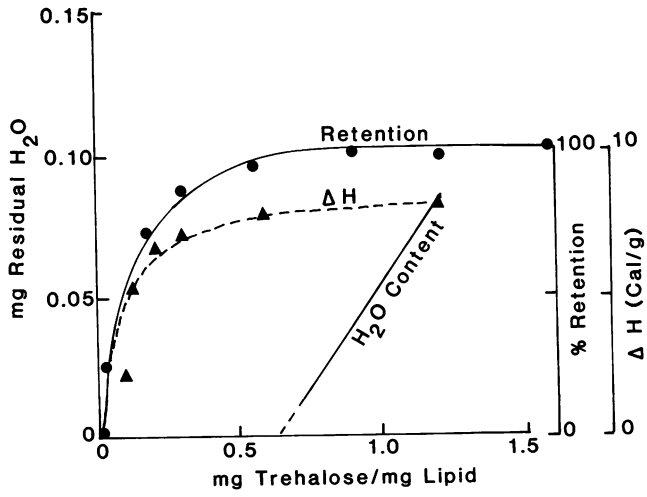

FIG. 4. Retention of trapped solute (data from ref. 2) and enthalpy of the low temperature transition (data from ref. 3) for PamOle-PtdCho/PtdSer liposomes dried in the presence of the indicated amounts of trehalose. (1 cal = 4.18 J.) Also shown are the apparent water contents for similar samples used in the present study, with data points and standard deviations omitted for clarity.

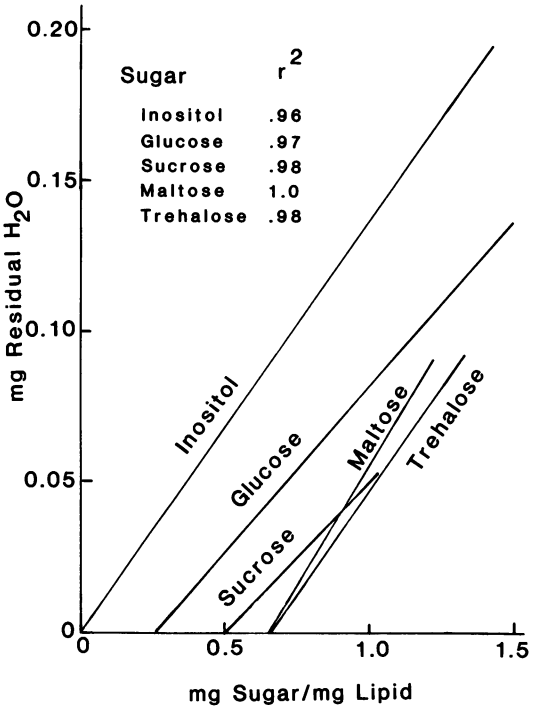

FIG. 5. Apparent water contents of PamOle-PtdCho/PtdSer liposomes dried in the presence of the indicated amounts of various sugars. For clarity, the actual data points and standard deviations are omitted, but correlation coefficients are shown for each line.

representative results shown in Fig. 5. As with trehalose, binding of the sugars to the dry lipids can be evaluated from the intersection of the two line segments seen in the data for each sugar. Considerable variation between sugars is evident; for example, inositol appears to show little or no interaction with the dry lipid, whereas maltose and trehalose show the strongest interaction (Fig. 5). The ability of these sugars to preserve dry liposomes appears to be strongly related to this interaction with the dry lipids; Fig. 6 shows the relationship between the amount of sugar bound to the dry liposomes (evaluated from the data shown in Fig. 5) and the ability of the same sugars to preserve dry liposomes of the same composition. Inositol is the least effective at preserving the dry liposomes and shows the weakest interaction with the dry lipids. By contrast, trehalose and maltose are the most effective at preserving the dry liposomes and at interacting with the dry lipids (Fig. 6). An intermediate series of sugars falls between these two extremes. A similar relationship exists between the ability of some of these same sugars to preserve dry intact membranes (16) and to alter physical properties of dry lipids (17).

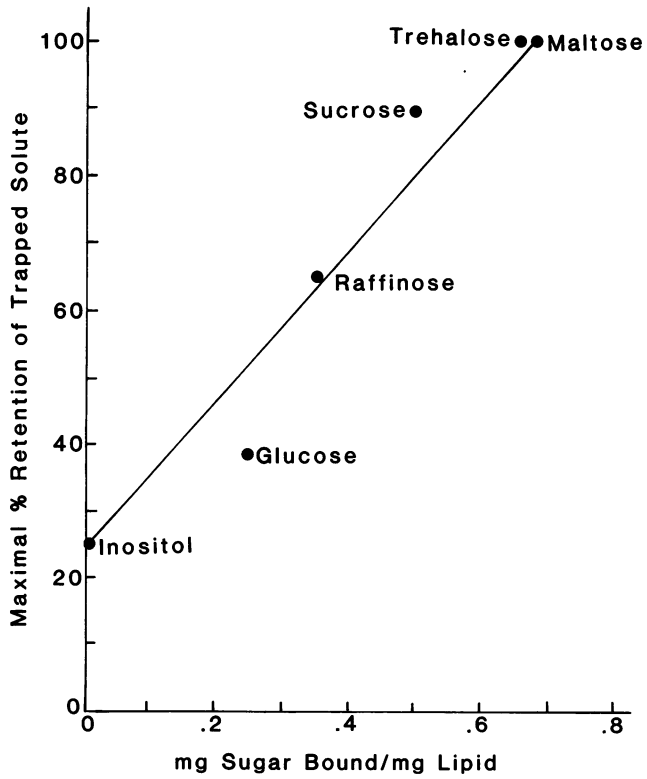

FIG. 6. Relationship between strength of interaction between various sugars and PamOle-PtdCho/PtdSer liposomes (assessed from the intercept with the abscissa for the sugars shown in Fig. 5 and for similar plots for other sugars) and the ability of the same sugars to preserve dry liposomes (data from ref. 3).

We conclude that these data strongly suggest that the ability of sugars to stabilize dry liposomes is related to their strength of direct interaction with phospholipid bilayers, a conclusion that appears to be consistent with our previous suggestions in this regard (17). An unsolved problem resides in the fact that sugars differ so much in their ability to interact with and affect physical properties of phospholipids. It does not appear that the primary structure of the sugars (i.e., number and position of —OH groups) is responsible for the differences seen among the sugars tested (16). Instead, we suspect that the answer lies in the specificity conferred by the stereochemistry of the sugars.

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