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Prevention of fusion and leakage in freeze-dried liposomes by carbohydrates

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The ability of various carbohydrates to prevent leakage of a small, water-soluble marker from unilamellar liposomes during freeze-drying has been investigated and compared with the ability of trehalose to prevent such leakage. Mechanisms of the stabilizing effect of the carbohydrates have also been studied. While the ability of the sugars to prevent lipid mixing during freeze-drying is related to their ability to prevent leakage more sugar is required for prevention of leakage than for prevention of lipid mixing. The mass ratio of carbohydrate: lipid (rather than the molarity of the solution) is the important parameter for prevention of lipid mixing and leakage. Calorimetric results suggest that it is the ability of trehalose to prevent phase separation during drying and phase transitions during rehydration that accounts for the stabilizing effect of this carbohydrate.

Introduction

Liposomes of defined composition have been widely studied both as models for membrane structure and function, and because of their practical potential as vehicles for specific drug delivery to cells in whole organisms. We have been using liposomes as a model for understanding membrane-carbohydrate interactions. These studies were undertaken because of the finding that various carbohydrates were effective in stabilizing a biological membrane, sarcoplasmic reticulum, by preventing fusion and phase separations of proteins and lipids during freeze-drying [1,2]. These sarcoplasmic reticulum membranes when rehydrated were similar to fresh membranes in size, intramembrane particle distribution, and ability to transport and sequester Ca^{2+}. In these earlier studies we found that the non-reducing disaccharide, trehalose, was by far the most effective carbohydrate for this stabilization. This was a satisfying result since trehalose is synthesized in large quantities during dehydration by organisms that normally survive complete dehydration (anhydrobiotic organisms; for references see Ref. 3). The amounts found in anhydrobiotic plants and animals are similar to the amount of trehalose needed to stabilize biological membranes fully – on the order of 15–20% of the dry weight. Trehalose has also been shown to be effective in preventing fusion and leakage from liposomes during freeze-drying: if trehalose is present inside and outside of unilamellar liposomes they will retain up to 100% of a trapped water soluble marker and be prevented from fusing [4,5]. The absolute amount of trehalose needed for this stabilization is

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about 3–4-times that needed to stabilize sarcoplasmic reticulum membranes [4]. This difference may be related to differences in sample preparation rather than fundamental differences between liposomes and membranes. One practical result of these studies with liposomes is that it may now be possible to store liposomes for drug delivery in the freeze-dried state.

In the present study we examine the relative effectiveness of other carbohydrates at preventing fusion and leakage of liposomes during freeze-drying and rehydration. In addition, we show that while the ability of the carbohydrates tested to preserve dry liposomes is related to the ability of the same carbohydrates to prevent lipid mixing during drying, prevention of lipid mixing is not in itself sufficient to prevent leakage from the dry liposomes. In previous studies on this subject [1,6] we have suggested that the mechanism of stabilization against fusion and leakage involves maintaining the dry lipids in a liquid crystalline state in the presence of trehalose. We now present a refined version of that model, based on calorimetric data.

Materials and Methods

Liposomes. Palmitoyloleoylphosphatidylcholine (POPC) and crude bovine brain phosphatidylserine (PS) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL) and used without further purification. Trehalose dihydrate, raffinose pentahydrate and *myo*-inositol were obtained from Pfanstiehl Laboratories (Waukegan, IL.), galactose, maltose monohydrate and lactose monohydrate from Sigma (St. Louis, MO), and D-glucose from Mallinckrodt (Paris, KY). POPC and PS, at a 9:1 mole ratio, were mixed together in chloroform solution, dried under nitrogen, and placed under high vacuum (15–25 mTorr) on a Virtis lyophilizer (The Virtis Co., Inc., Gardiner, NY) for at least 12 h. The lipids were rehydrated with double-distilled deionized water and sonicated to clarity in a bath sonicator (Laboratory Supplies Co., Inc., Hicksville, NY). Carbohydrate and sodium isocitrate were added to the solution to give a mass ratio of 0.75 carbohydrate: 0.25 isocitrate: 1 lipid, and it was dried under nitrogen on the wall of a round flask. This mixture was rehydrated with a 10 mM Tes, 0.1 mM EDTA, pH 7 buffer to form unilamellar vesicles [7]. Freeze-fracture of freshly prepared vesicles made in the presence of trehalose had previously shown these vesicles to have an average diameter of 50 nm, not much larger than vesicles prepared by sonication [4]. Excess carbohydrate and isocitrate not trapped by the vesicles was removed by passing the vesicles over a Sephadex G-50 fine column (15 × 180 mm). The liposome concentration of the eluate was determined [8] and various amounts of each carbohydrate were added back externally to aliquots of the vesicles to provide a range of mass ratios of carbohydrate: lipid. The aliquots were frozen in liquid nitrogen and lyophilized in 0.5 ml amounts for at least 12 h. The dry samples were rehydrated to their original volume with deionized distilled water. At the end of each experiment the liposome and carbohydrate concentration of the samples were determined again [8,9].

Measurement of leakage. Isocitrate present in the extravesicular and intravesicular volume of fresh and rehydrated unilamellar liposomes was determined by a fluorometric assay for the reduction of NADP accompanying the oxidation of isocitrate in the presence of isocitrate dehydrogenase [10]. The enzyme was added to the outside of the vesicles, and after the extravesicular isocitrate was assayed the vesicles were lysed by addition of a drop of Triton X-100 and intravesicular isocitrate was measured. Retention of isocitrate by the freeze-dried vesicles was calculated as a per cent of the isocitrate trapped by the freshly prepared sample (before freeze-drying). The curves shown were fitted by a BASIC curve fitting program [11].

To determine whether carbohydrates leak from the freshly prepared liposomes, POPC/PS liposomes were prepared in the presence of 1 g/g trehalose with the addition of [^{14}C]trehalose and [^{3}H]glucose. Extravesicular trehalose and glucose were removed on a Sephadex column as described above. At various intervals, 50-μl aliquots of the vesicles were removed, leaked carbohydrate was removed on small Sephadex columns made in disposable glass pipets, and the eluate was analyzed for retained radioactivity and liposome concentration.

Measurement of lipid mixing. Lipid mixing be-

tween vesicles during the whole process of freezing and freeze-drying was estimated by the technique of resonance energy transfer [12,13]. The fluorescent probes cholesteryl anthracene-9-carboxylate (donor) and 7-nitrobenzo-2-oxa-1,3-diazol-4-yl phosphatidylethanolamine (acceptor) were purchased from Molecular Probes, Inc. (Junction City, OR). The probes were incorporated into separate populations of vesicles by addition of 2 mol% of either probe in chloroform solution with the POPC and PS. The vesicles were prepared in the presence of the carbohydrates as described above, using a mass ratio of 1 : 1 and no isocitrate. After removal of extravesicular carbohydrate, aliquots of donor and acceptor vesicles were mixed together and sufficient carbohydrate was added to give a range of mass ratios similar to those used in the retention experiment. The mixtures of vesicles were frozen, lyophilized and rehydrated as above, and probe intermixing was determined fluorometrically as the quenching of the donor emission [12,13]. A sample with maximal donor quenching was made by mixing both probes in chloroform solution with the POPC and PS (mock-fused vesicles), and a sample with no quenching was made by mixing separate populations of unlyophilized donor and acceptor probe-labelled vesicles. The amount of probe intermixing after lyophilization was calculated as a per cent of the difference in the donor emission between freshly mixed and mock-fused vesicles [4].

Calorimetry. Freeze-dried unilamellar liposomes for calorimetry were made essentially as above, but using 1 g trehalose : g lipid during the preparation of the liposomes and rehydrating the rotary dried lipid/trehalose mixture with distilled water instead of buffer. Aliquots of the freeze-dried liposomes used for the calorimetry were assayed for lipid and trehalose as indicated above. Homogeneous dry multilamellar preparations of POPC, PS, and POPC/PS with various amounts of trehalose were prepared as follows. The lipids were dried under nitrogen in tared tubes and placed under vacuum for at least 12 h. The lipids were then resuspended in methanol, and the desired amount of trehalose was added. The trehalose had been dissolved in methanol at a concentration of 10 mg/ml immediately prior to use. To this mixture was added two volumes of benzene: if necessary, sufficient methanol was added prior to the benzene so that the final concentration of trehalose was no more than 3 mg/ml of methanol. Any more would precipitate upon addition of the benzene. The final mixture of lipid, trehalose, methanol, and benzene was placed as 2 ml aliquots into shell vials (17 ml) and frozen in as thin a layer as possible in liquid nitrogen. The vials were lyophilized in a large side-arm flask on a Virtis lyophilizer with a liquid nitrogen trap between the samples and the lyophilizer. The samples were freeze dried for about 6 h or until they appeared dry. They were then pumped directly by the lyophilizer at a vacuum of 15–25 mTorr for at least 12 h.

For calorimetry, samples were removed from the lyophilizer under vacuum, and placed in a dry box purged with dry nitrogen. The dry liposomes were packed into the calorimetry pans (Perkin-Elmer volatile sample pans) in the dry box. The pans were sealed using a volatile sample pan sealer (seal tight to > 2 atmospheres pressure) to avoid hydration of the lipid. As a further precaution, the sample pans were placed in small vials capped with parafilm until just before being placed in the calorimeter. Calorimetry was carried out on a Perkin-Elmer DSC-2C differential scanning calorimeter assisted by a Perkin-Elmer 3600 data station. Since calorimetric scans were run over a range of more than 150°C, each scan included a baseline subtraction of a scan made with empty pans in both the sample and reference chambers. Scans were made at a rate of 10 Cdeg(K)/min. This high scan rate was necessary because of the small size of the samples and the relative insensitivity of the calorimeter.

Results and Discussion

Dehydration and liposome leakage

Disaccharides and a trisaccharide were able to provide a fair measure of stabilization of unilamellar liposomes against leakage during freeze-drying as shown in Fig. 1. Under the conditions of our experiments maltose appeared to stabilize the liposomes as well as trehalose. Unlike trehalose however, maltose is a reducing sugar and may undergo non-enzymatic reactions between its aldehyde group and amino groups of proteins and

some phospholipid head groups in natural membranes and defined lipid systems (Ref. 14, and references therein). Sucrose, another non-reducing sugar, achieves only about 80% protection at the highest concentrations used while trehalose and maltose can provide complete protection. Lactose and raffinose provide a maximum of about 60% and 50%, respectively. Inositol, which was previously shown to be ineffective at preserving biological membranes [1], reducing the gel-liquid crystalline transition temperature of dipalmitoylphosphatidylcholine (DPPC) [15], and forming hydrogen bonds with the phosphate of the DPPC head group [15], has some small effect on the liposomes at very high mass ratios. Glucose and galactose, the only monosaccharides tested, gave maximum protection on the order of 50% retention of isocitrate with a falling off of retention at higher carbohydrate mass ratios (Fig. 1).

In a leakage experiment done with freshly made liposomes, glucose leaked out rapidly with only 20% of the original trapped glucose left after 24 h. Trehalose, by contrast, leaked very little during the same time period (Fig. 2). We also determined with the isocitrate assay that isocitrate did not leak out of the liposomes under the same storage conditions. Although we have no leakage data for galactose, it is likely that it also shows rapid leakage from fresh liposomes. Thus, the liposomes made with glucose and probably galactose have very little sugar left inside when carbohydrate is added externally and they are frozen. We know from previous experiments [4] that it is necessary to have carbohydrate on both sides of the membrane for good stabilization of liposomes during freeze-drying. The low retention of isocitrate in liposomes stabilized with glucose and galactose may reflect the fact that much of the internal carbohydrate leaked out during preparation of the liposomes. To test that hypothesis, liposomes were prepared with glucose as described above (Methods), except that they were stored overnight in the presence of various concentrations of glucose which had been added back externally. Retention of isocitrate by the liposomes was assayed when they were first made and also following storage: no leakage of isocitrate had occurred overnight. Since glucose leaks out of the liposomes, we assumed that externally added glucose could leak into the fresh liposomes during the storage period, thereby increasing internal carbohydrate and improving retention of isocitrate during freeze-dry-

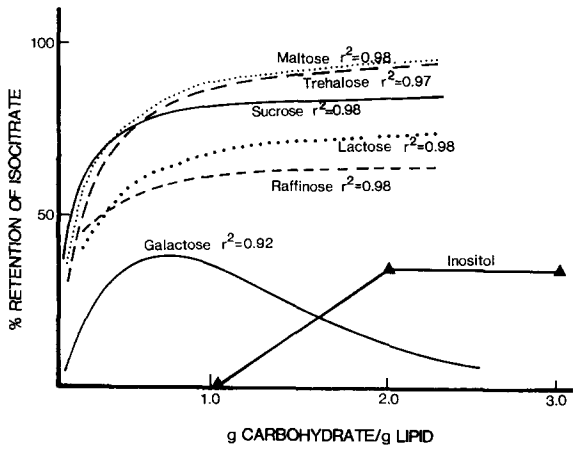

Fig. 1. Retention of trapped marker (isocitrate) by freeze-dried vesicles as a function of carbohydrate/lipid mass ratio. Retention was calculated as a percentage of isocitrate trapped by the freshly made liposomes. All of the curves, except for inositol, were fitted from the original data by a BASIC curvefitting program [11]. Coefficients of determination (r^2) for the curves are given on the figure. The effect of increasing carbohydrate concentration is maximal at about 1 g/g lipid, except for inositol. Glucose (not shown) was very similar to galactose in its effect on retention.

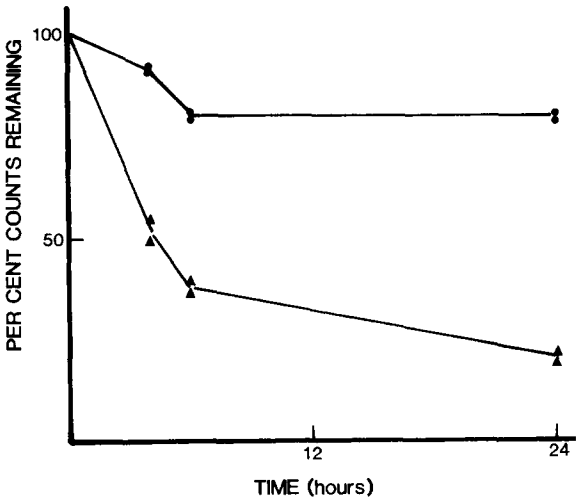

Fig. 2. Leakage of [^{14}C]trehalose (●) and [^{3}H]glucose (▲) over a period of 24 h. Leakage was most rapid in the first 6 h. By 24 h, 80% of the trehalose was still in the vesicles, but only 20% of the glucose was left inside the same vesicles. The liposomes were stored at 4°C, above the phase transition of the hydrated POPC/PS.

ing. The samples were freeze-dried, rehydrated, and isocitrate leakage assayed. The results of this experiment were similar to the original one, with low retention. Two possibilities exist: either the glucose cannot leak into the vesicles or monosaccharides such as glucose and galactose are not able to stabilize liposomes effectively. We feel that the latter explanation is the correct one for the low retention of trapped solute in the presence of glucose and galactose.

A study by Madden et al. [5] gives different results for retention of a trapped water soluble marker by liposomes in the presence of various carbohydrates. In their study, glucose is considerably more effective than we have found. However, these investigators used egg phosphatidylcholine, made their unilamellar liposomes by extrusion, and used much higher concentrations of carbohydrate when preparing the liposomes. Any of these differences in experimental conditions could be expected to affect the results.

Fusion and liposome leakage

Studies with hydrated liposomes show that they leak during fusion [16,17], possibly through defects in the bilayer or non-bilayer structures during the fusion event [18]. The mechanism for leakage in the presence of the various carbohydrates could be simply fusion of the vesicles during the freezing and drying process. This possibility was investigated by the method of resonance energy transfer [4,12,13], and the results are presented in Fig. 3. Strictly speaking, the resonance energy transfer method measures probe intermixing (as described in Methods); this is usually taken as a measure of lipid mixing between different samples of lipids. We have shown previously by freeze-fracture that a sample which would show maximal probe intermixing is also highly fused [4]. As before, lipid mixing in the presence of trehalose declined rapidly and reached a minimum at between 0.2 and 0.4 g trehalose/g lipid (see also Ref. 4). It should be pointed out that under the conditions of these experiments (lipid concentration of 4 mg/ml) the trehalose concentration of a sample with 0.4 g trehalose/g lipid is approx. 5 mM. Glucose and galactose do not minimize lipid mixing until they are present at 0.75 g/g lipid, or about 17 mM. These two

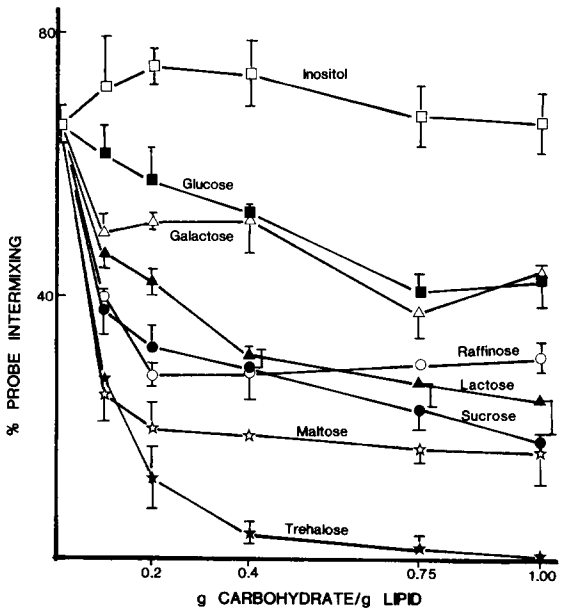

Fig. 3. Lipid mixing in liposomes during freeze-drying as estimated by the technique of resonance energy transfer. Per cent probe intermixing was determined by comparison with the difference between mock-fused vesicles (100% probe intermixing) and freshly made mixed donor and acceptor vesicles (0% probe intermixing). Values shown are means ± S.D. Per cent probe intermixing is minimized at trehalose concentrations between 0.2 and 4 g/g lipid. There is no significant difference in the probe intermixing at 0.4 g trehalose/g lipid and higher. The values for probe intermixing in the presence of inositol are not significantly different from each other or from the control.

carbohydrates are much less effective at preventing lipid mixing than the di- and trisaccharides tested, thus original molarity of the solution is not related to the protective effect. The protective effect of carbohydrates against lipid mixing during freeze-drying is related to their protection of liposomes against leakage, however, it takes about two to four times as much carbohydrate to maximize retention as it does to minimize lipid mixing. Thus, leakage during freeze-drying is not simply due to fusion of the liposomes.

Dehydration and phase transitions in liposomes

Many studies have shown that liposomes composed of one or two phospholipids show significant increases of permeability to water, ions, or carbohydrates at their phase transition temperature [19–22]. Theoretical treatments of some of these results [20,23] correlate permeability char-

acteristics of liposomes with the proportion of interfacial lipid at the boundary between gel and liquid crystalline regions. One might expect such boundaries to be more extensive and occur over greater temperature ranges in mixtures of lipids which could phase separate during cooling or dehydrating treatments.

Although phase separation of different phospholipids has been demonstrated frequently in hydrated lipid systems [19,24–27], so far as we know it has not been directly demonstrated in dry lipid systems. It has been suggested that such phase separations do occur, based on phase diagrams of single phospholipids compared to each other [28,29]. Fig. 4 shows a calorimetric scan of a mixture of POPC/PS which has been lyophilized and then kept as dry as possible as described in the methods. Three separate transitions can be seen; the lowest temperature transition is due to the PS, while the two higher temperature transitions are due to the POPC. Pure dry POPC, lyophilized alone, has a transition temperature of 57°C, thus the transition at 41°C is probably caused by incomplete separation of POPC and PS. The crude bovine brain PS used in this study has a dry T_m of −11°C, so the lowest transition shown probably represents PS incompletely phase separated from POPC.

Homogeneous dry mixtures of trehalose with phospholipids, lyophilized from a solution of methanol and benzene, are multilamellar as determined by X-ray diffraction (Caffrey, M., personal communication). Multilamellar preparations made in this way should have the trehalose uniformly distributed between the bilayers, in contrast to unilamellar vesicles which will not have a uniform distribution of the carbohydrate on both sides of the bilayer when dry. The transition temperature of multilamellar PS is very little altered in the presence of trehalose, while the T_m of POPC is steadily decreased by the addition of trehalose to the mixture (Fig. 5A). At the highest amount of trehalose tested, the T_m of POPC falls to about −23°C, a decrease of 80°C. Mixtures of POPC/PS with trehalose show two transitions at lower trehalose concentrations, but at between 0.3 and 0.6 g trehalose/g lipid the high temperature transition disappears and only a low temperature transition is seen (Fig. 5A). At the same time, the

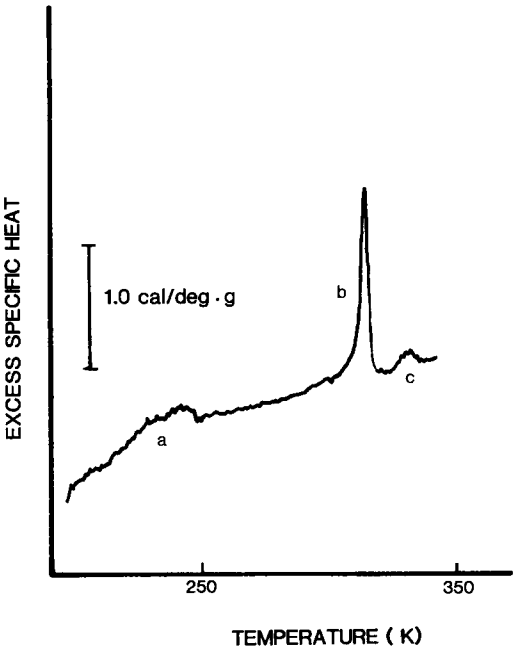

Fig. 4. Calorimetric scan (10 K/min) of dry multilamellar POPC/PS. The transition shown at (a) was due to PS incompletely phase separated from POPC and had a T_m of −32°C. The transition shown at (b) had a T_m of 40°C and was due to POPC incompletely phase separated from PS. The small peak at (c) had a T_m of 57°C, which is the transition temperature of pure dry POPC. Phase separation of POPC and PS is not complete since the transitions at (a) and (b) have peak transition temperature lower than for pure PS and POPC, but the freeze-drying process has effected a substantial separation of these two lipids. The scan shown was the first heating scan on this sample. All samples, with and without trehalose present showed complex behavior which depended on the amount of carbohydrate present, whether the sample was multilamellar or vesicular, and the thermal history of the sample. The first heating scan provides information about the state of the lipids when they are first rehydrated for the leakage studies.

calorimetric enthalpy (ΔH_{cal}) of the high temperature transition decreases steadily while the ΔH_{cal} of the low temperature transition increases (Fig. 5B). We interpret these results to mean that as trehalose is added to the POPC/PS mixture, more of the POPC is interacting with it and participating in the low temperature transition. That this effect does not depend on the presence of PS is shown by the lowering of T_m of binary mixtures of POPC and trehalose.

Lyophilized unilamellar liposomes, in contrast to the multilamellar preparations, had two transi-

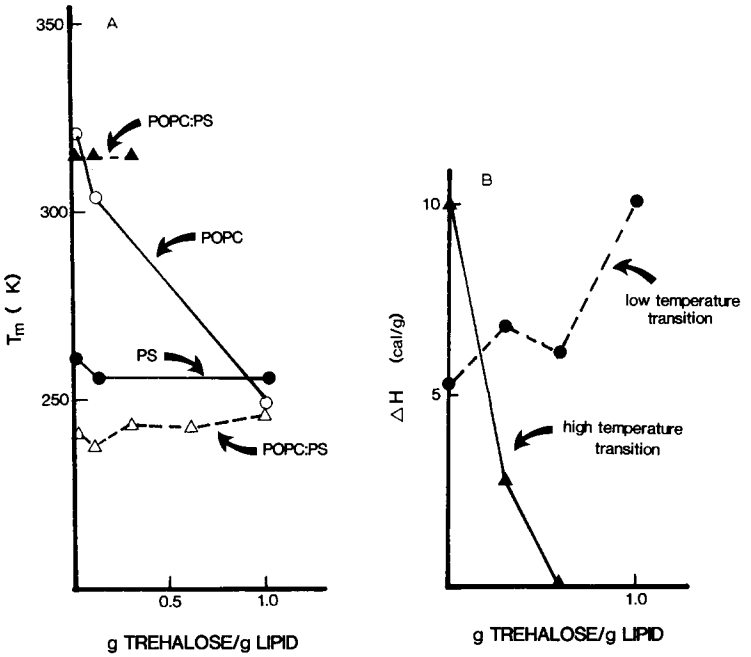

Fig. 5. (A) Peak transition temperatures of the first scans done on homogeneous multilamellar preparations of POPC, PS, POPC/PS, and trehalose lyophilized from benzene-methanol solution. The presence of trehalose had little effect on the transition of PS (●), but lowered the T_m of POPC (○) by 80°C at concentrations of 1 g/g lipid. Mixtures of POPC/PS with trehalose showed both a high (▲) and low (△) temperature transition at low trehalose/lipid mass ratios, but above 0.3 g trehalose/g lipid only a low-temperature transition was seen. (B) Calorimetric enthalpy of the high- (▲) and low- (●) temperature transitions of homogeneous multilamellar preparations of POPC/PS and trehalose. As the mass ratio of trehalose in the preparations is increased, the enthalpy of the high-temperature transition falls. At higher trehalose concentrations, the high-temperature transition disappears. At the same time, the enthalpy of the low-temperature transition increases, suggesting that an increasing amount of the POPC is participating in the low temperature transition.

tions at every trehalose concentration tested as shown in Fig. 6A. The variability of T_m for the low temperature transition may be due to the fact that it was difficult to choose a baseline for this very broad transition (e.g., Fig. 4). Small differences in the limits set for the computer determination of the peak can produce differences in T_m, especially for broad transitions. For the same reason, very accurate determinations of ΔH_{cal} were not possible, however differences in ΔH_{cal} between samples with different trehalose concentrations outside the liposomes were clear. The increase in T_m with increasing trehalose concentration outside the liposomes for the high-temperature transition was reproducible with different preparations.

The persistence of the high-temperature transition in the dry unilamellar liposomes at all trehalose concentrations suggested that there was a population of lipid molecules which was not being affected by trehalose and was maintaining a phase separation from the rest of the lipid. Examination of the ΔH_{cal} of the high transition showed that with the addition of the lowest amount of trehalose, the enthalpy fell to 1/3 to 1/2 of the value without any trehalose present. At the same time, the ΔH_{cal} of the low temperature transition increased. The method of preparation of these liposomes (see Methods) gave a constant small amount of trehalose inside the vesicles and increasing amounts outside the vesicles as trehalose was added back to the preparations prior to lyophilization. Thus, one might expect the inner monolayer of the liposome to exhibit a nearly constant ΔH_{cal} during the gel to liquid crystalline transition while the outside monolayer would show an increased ΔH_{cal} as the mass ratio of trehalose/lipid increases. This latter has been plotted in Fig. 6B,

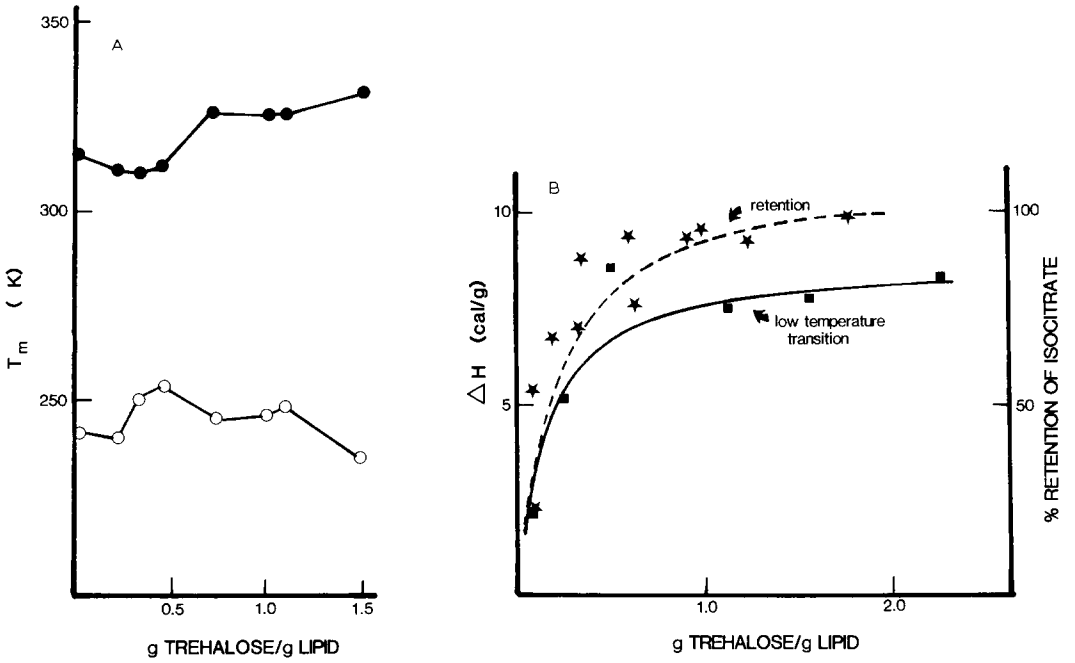

Fig. 6. (A) Peak transition temperatures of transitions of unilamellar vesicles prepared at various mass ratios of trehalose as described in Materials and Methods. All of the vesicles (except for the zero trehalose sample) had a constant small amount of trehalose, about 0.1 g/g lipid, inside them while increasing amounts of trehalose outside gave the increasing mass ratios shown. In contrast to the homogeneous multilamellar preparations (Fig. 5A), the unilamellar vesicles had both a high- (●) and low- (○) temperature transition at all trehalose/lipid mass ratios. The variability of the T_m of the low-temperature transition may be due to the broadness of the transition, the difficulty of choosing endpoints, and the relative insensitivity of the calorimeter. The T_m of the high-temperature transition was reproducible between experiments, and the rise of T_m at higher trehalose concentrations was consistent. Possible reasons for this increase in T_m are given in the text. (B) Calorimetric enthalpy of the low temperature transition (■) of lyophilized unilamellar vesicles and the retention of trapped marker (★) by similar vesicles are compared as a function of trehalose/lipid mass ratio. The hyperbolic curves (enthalpy, ————; retention, — — —) are fitted from the data using a BASIC curvefitting program [11]. The coefficient of determination, r^2, for the retention curve is 0.97 and for the ΔH_{cal}, 0.99.

where the mass ratio of trehalose/lipid is calculated from the amount of trehalose outside the liposomes and the lipid (60% of total) estimated to be in the outer monolayer. The persistence of the high temperature transition in the lyophilized liposomes then would be due to phase separation of the inner and outer monolayers.

This suggestion of phase separation of the inner from the outer monolayer of the lyophilized liposomes has some basis in other experimental data. A number of workers have demonstrated that the packing of phospholipid head groups and chains is different in the two halves of the bilayer of small unilamellar vesicles [30–33]. This difference in packing might be expected to lead to differences in behavior during the phase transition. High resolution ^{1}H-NMR of small unilamellar DPPC liposomes does show differences in behaviour of resonances originating from the two monolayers as the temperature is lowered through T_m [31]. The liposomes used in our calorimetric studies are not much larger than the small sonicated vesicles used in the cited papers.

When the ΔH_{cal} of the low temperature transition of dry unilamellar liposomes is plotted together with the per cent retention of solute at various mass ratios of trehalose, it can be seen that there is a strong correlation between the two (Fig. 6B). Both curves can be fit with a hyperbolic function [11] with a coefficient of determination (r^2) of 0.97 or better. These fitted curves show that values for retention or excess enthalpy reach 95% of full value at about 1.2 g trehalose/g lipid, while the raw data suggests that the effect of

trehalose is maximal by a mass ratio of 1 : 1. Such results are suggestive that the important factor for retention is prevention of phase separation during drying and phase transition during rehydration of the outer monolayer of these unilamellar vesicles.

At present we have no explanation for the increasing T_m of the high temperature transition in the unilamellar liposomes, although two possibilities suggest themselves. The increased T_m could be due to increased packing of the head groups of the inner monolayer. The shape of the dried liposomes is a collapsed cup [1,4] which topologically is a closed convex surface with some concave elements [34]. We have not yet attempted to predict the disposition and spacing of the phospholipid molecules in each monolayer. Another possibility is that there is an asymmetric distribution of the PS in the two monolayers. A number of studies have found that phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol and phosphatidic acid will distribute asymmetrically between the two halves of the bilayer of unilamellar vesicles of phosphatidylcholine [35–37]. The asymmetric distribution is affected by the charge and packing requirements of the phospholipid headgroup and the size of the vesicles [36–38]. The PS used in these studies has a low transition temperature indicating a high degree of unsaturation, and might more easily fit into an outer monolayer expanded by the interaction of trehalose with the head groups. If an increasing proportion of PS is located in the outer monolayer under the influence of increasing amounts of trehalose associated with the outside of the vesicle, the T_m of the inner monolayer should increase and approach that of pure POPC.

Conclusions

Most, but not all of the carbohydrates tested can to some extent prevent lipid mixing of liposomes during freeze-drying. The prevention of lipid mixing is related to the effectiveness of the carbohydrates in preventing leakage of trapped solute from liposomes during lyophilization and rehydration, but considerably more carbohydrate is needed to maximize retention than is needed to minimize fusion. Trehalose, which is found in high concentration in anhydrobiotic organisms, is most effective against preventing fusion, and trehalose and maltose are most effective in preventing leakage during dehydration and rehydration. The greater amounts of carbohydrate needed to maximize retention may be related to their effect on the outer monolayer of liposomes. Trehalose has been found to drive down the T_m of dry POPC and prevent phase separation of POPC and PS in homogeneous dry multilamellar preparations of a mixture of these phospholipids. An interpretation of the calorimetric data from the lyophilized liposomes suggests that the inner and outer monolayers of the liposome undergo the gel to liquid crystalline phase transition independently of each other in the dry state. When sufficient trehalose is present outside the liposomes, the T_m of all of the phospholipid of the outer monolayer is driven to a low value and phase separation of POPC and PS is prevented. The mass ratio of trehalose/lipid needed to achieve this is similar to that needed to maximize retention. Trehalose and some other carbohydrates may serve as protectants against desiccation damage in anhydrobiotic organisms by suppressing fusion, phase separations and phase transitions in dry membranes.

Acknowledgments

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References

1 Crowe, J.H., Crowe, L.M. and Jackson, S.A. (1983) Arch. Biochem. Biophys. 220, 477–484
2 Crowe, L.M., Mouradian, R., Crowe, J.H., Jackson, S.A. and Womersley, C. (1984) Biochim. Biophys. Acta 769, 141–150
3 Crowe, J.H., Crowe, L.M. and Chapman, D. (1984) Science 223, 701–703
4 Crowe, L.M., Crowe, J.H., Rudolph, A., Womersley, C. and Appel, L. (1985) Arch. Biochem. Biophys. 242, 240–247
5 Madden, T.D., Bally, M.B., Hope, M.J., Cullis, P.R., Schieren, H.P. and Janoff, A.S. (1985) Biochim. Biophys. Acta 817, 67–74
6 Crowe, J.H., Whittam, M.A., Chapman, D. and Crowe, L.M. (1983) Biochim. Biophys. Acta 769, 151–159
7 Shew, R. and Deamer, D.W. (1985) Biochim. Biophys. Acta 816, 1–8

8 London, E. and Feigenson, G. (1978) Anal. Biochem. 88, 203–211
9 Dimlerr, R.J., Schaeffer, W.C., Wise, C.S. and Rist, C.E. (1952) Anal. Chem. 24, 1411–1414
10 Plaut, G.W.E. (1962) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 5, pp. 645–651, Academic Press, New York
11 Spain, J.D. (1982) Basic Microcomputer Models in Biology, Addison Wesley, Reading, MA
12 Uster, P.S. and Deamer, D.W. (1981) Arch. Biochem. Biophys. 209, 385–395
13 Rudolph, A.S. and Crowe, J.H. (1985) Cryobiology 22, 367–377
14 Mouradian, R., Womersley, C., Crowe, L.M. and Crowe, J.H. (1985) Cryobiology 22, 119–127
15 Crowe, L.M., Crowe, J.H. and Chapman, D. (1985) Arch. Biochem. Biophys. 236, 289–296
16 Hammoudah, M.M., Nir, S., Bentz, J., Mayhew, E., Stewart, T.P., Hui, S.W. and Kurlan, R.J. (1981) Biochim. Biophys. Acta 645, 102–114
17 Wilschut, J. and Papahadjopoulos, D. (1979) Nature 281, 690–692
18 Hui, S.W., Stewart, T.P., Boni, L.T. and Yeagle, P.L. (1981) Science 212, 921–922
19 Carruthers, A. and Melchior, D.L. (1983) Biochemistry 22, 5797–5807
20 Papahadjopoulos, D., Jacobsen, K., Nir, S. and Isac, T. (1973) Biochim. Biophys. Acta 311, 330–348
21 Blok, M.C., Van der Neut-Kok, E.C.M., Van Deenen, L.L.M. and De Gier, J. (1975) Biochim. Biophys. Acta 406, 187–196
22 Blok, M.C., Van Deenen, L.L.M. and De Gier, J. (1976) Biochim. Biophys. Acta 433, 1–12
23 Marsh, D., Watts, A. and Knowles, P.F. (1976) Biochemistry 15, 3570–3578
24 Phillips, M.C., Hauser, H. and Paltauf, T. (1972) Chem. Phys. Lipids 8, 127–133
25 Cullis, P.R. and De Kruijff, B. (1978) Biochim. Biophys. Acta 507, 207–218
26 Luna, E.J. and McConnell, H.M. (1978) Biochim. Biophys. Acta 509, 462–473
27 Boni, L.T. and Hui, S.W. (1983) Biochim. Biophys. Acta 731, 177–185
28 Crowe, J.H. and Crowe, L.M. (1986) in Membranes, Metabolism, and Dry Organisms (Leopold, A.C., ed.), Cornell University Press, Ithaca, NY, in the press
29 Crowe, L.M. and Crowe, J.H. (1986) in Membranes, Metabolism, and Dry Organisms (Leopold, A.C., ed.), Cornell University Press, Ithaca, NY, in the press
30 Sheetz, M.P. and Chan, S.I. (1972) Biochemistry 11, 4573–4581
31 Schuh, J.R., Banerjee, U., Muller, L. and Chan, S.I. (1982) Biochim. Biophys. Acta 687, 219–255
32 Mason, J.T. and Huang, C. (1978) in Liposomes and Their Uses in Biology and Medicine (Papahadjopoulos, D., ed.), pp. 29–49, New York Acad. Sci., New York
33 Bramhall, J. (1986) Biochemistry 25, 3479–3486
34 DeHoff, R.T. and Rhines, F.N. (1968) Quantitative Microscopy, McGraw-Hill Book Company, New York
35 Litman, B.J. (1974) Biochemistry 13, 2844–2848
36 Berden, J.A., Barker, R.W. and Radda, G.K. (1975) Biochim. Biophys. Acta 375, 186–208
37 Nordlund, J.R., Schmidt, C., Dicken, S.N. and Thompson, T.E. (1981) Biochemistry 20, 3237–3241
38 Litman, B.J. (1975) Biochim. Biophys. Acta 413, 157–162