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REVIEW ARTICLE

Stabilization of dry phospholipid bilayers and proteins by sugars

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Introduction

Water is usually indispensible for maintenance of the integrity of biological membranes, phospholipid bilayers and many soluble proteins (Tanford, 1980), but it has now become possible to remove essentially all the water from many such labile molecular assemblages by drying them in the presence of certain sugars. In this Review we will review the current state of knowledge concerning which sugars are effective in this regard and also the mechanism by which the sugars exert their effects.

This work has its origins in investigations on certain organisms that possess the ability to survive complete dehydration. These organisms, said to be in a state of 'anhydrobiosis', may persist in the dry state for decades (Crowe & Clegg, 1978). When they become wet again they rapidly swell and resume active metabolism, often within minutes. We believe that elucidating how such organisms escape irreversible damage from dehydration would contribute to our understanding of the role of water in maintaining structural and functional integrity in fully hydrated membranes and cells. Many of these organisms (reviewed in Crowe & Clegg, 1978; Crowe, J. H., *et al.*, 1984*b*; Womersley, 1981), including spores of certain fungi, macrocysts of the slime mould *Dictyostelium*, dry active baker's yeast, brine shrimp cysts (dry gastrulae of the brine shrimp *Artemia salina*) and the dry larvae and adults of several species of soil-dwelling nematodes, contain large amounts of sugars, particularly trehalose, and their survival in the dry state is correlated with the presence of this molecule (Madin & Crowe, 1975). In dry cysts of the brine shrimp, *Artemia*, for example, trehalose often constitutes more than 20% of the dry weight. We will show in this Review that this molecule is particularly effective at stabilizing dry membranes, phospholipid bilayers, and proteins.

Preservation of soluble proteins during drying

It is well established that many sugars stabilize proteins in solution (Back *et al.*, 1979; Lee & Timasheff, 1981; Arakawa & Timasheff, 1982) and afford cryoprotection to isolated enzymes (Shikama & Yamazaki, 1961; Chilson *et al.*, 1965; Brandts *et al.*, 1970; Whittam & Rosano, 1973; Carpenter *et al.*, 1986*a*). However, freeze-drying is generally thought to be more disruptive to enzyme function than freeze–thawing or thermally-induced perturbations. For example, Hanafusa (1969) found that freeze–thawing of catalase or myosin ATPase resulted in at most a 50% decrease in enzyme activity, while after freeze-drying and dissolution only 13% and 30%, respectively, of the original activities remained. An even more extreme situation exists with L-asparaginase, which is fully active after freeze–thawing, but which can be inactivated by over 80% during freeze-drying (Marlborough *et al.*, 1975; Hellman *et al.*, 1983). Despite the stresses to which proteins are subjected during dehydration, it has been found that a great degree of protection is provided by sugars. In the following paragraphs, we summarize briefly results on the stabilization of proteins by sugars during freeze-drying, as well as preliminary data on air-drying.

In Hanafusa's pioneering studies on freeze-dried enzymes, he found that, in the presence of 100 mM-sucrose, myosin ATPase retains 92% of its initial activity after freeze-drying and rehydration, and that the addition of 30 mM-sucrose to catalase preparations prior to freeze-drying results in a recovery of 85% of the original activity (Hanafusa, 1969). Similarly, L-asparaginase is fully active after freeze-drying in the presence of 2% (w/v) glucose, mannose, sucrose or ribose (Hellman *et al.*, 1983). Sugars have also been used to protect the functional integrity of respiratory proteins during dehydration. For example, glucose, galactose, laevulose or mannose almost completely block the formation of methaemoglobin (up to 60% without added protectants) noted when haemoglobin is freeze-dried (Labrude *et al.*, 1976). In addition, the Hill constant, which is 1.87 for the unprotected protein, is 2.58 for haemoglobin freeze-dried in the presence of glucose, a value similar to that noted (2.74) for the untreated control (Thirion *et al.*, 1983).

The most dehydration-sensitive enzyme tested to date is phosphofructokinase purified from rabbit skeletal muscle. We have found that this enzyme is completely and irreversibly inactivated during freeze-drying, a characteristic that makes it especially suited for use in testing stabilizing compounds (Carpenter *et al.*, 1986*b*). Of the organic solutes we have studied in this role, trehalose and maltose are the most effective, and sucrose provides somewhat less stability (Fig. 1). In contrast, the protection afforded by glucose is much less than that seen for the disaccharides of glucose, trehalose and maltose, even if the data are considered on the basis of equimolar amounts of glucose (Fig. 2). These results indicate that it is not just the type of sugar moiety present that is important for enzyme stabilization, but that subunit orientation is also critical.

However, we have found that monosaccharides, which alone afford virtually no protection to phosphofructokinase, when combined with Zn^{2+} (0.9 mM) greatly stabilize the enzyme (Fig. 2). Similar results are also seen with the disaccharides (Fig. 1). The enhanced preservation noted with Zn^{2+} is clearly not due simply to the summation of the individual effects of the sugars and Zn^{2+}; Zn^{2+} alone (0.9 mM) affords no protection to phosphofructokinase during freeze-drying, but when this cation is added to a sugar solution, at a concentration that is also ineffective at preserving phosphofructokinase, a great degree of stabilization is seen (Figs. 1 and 2). The addition to trehalose/phosphofructokinase solutions of ionic copper, cadmium, nickel, cobalt, calcium and manganese, which alone provide no protection, also results in increased recovery of enzyme activity after

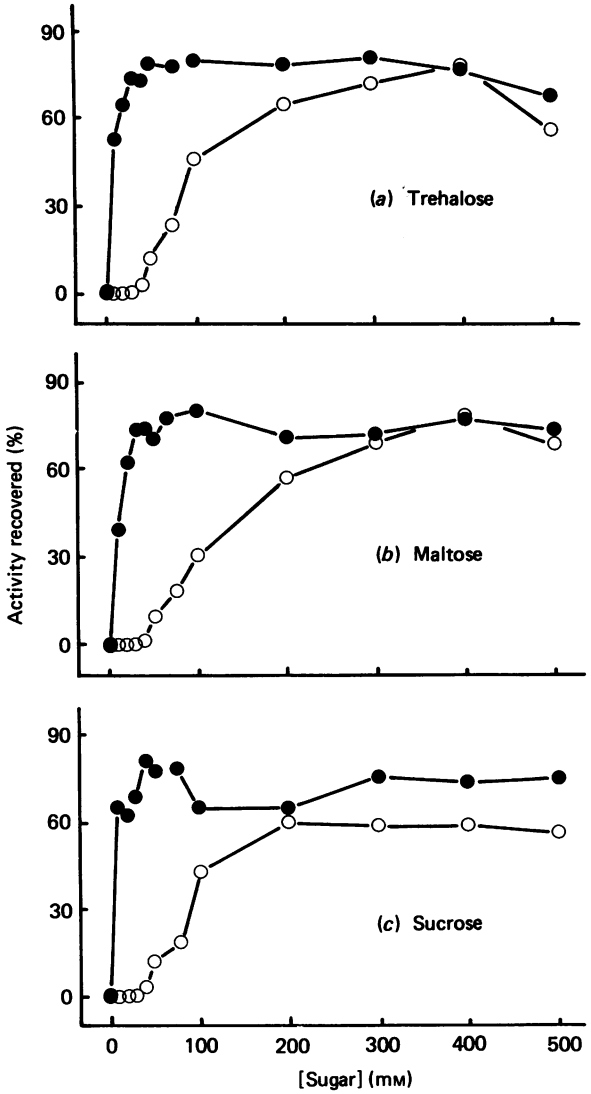

Fig. 1. Comparison of percentage of phosphofructokinase activity recovered after freeze-drying and dissolution in the presence of (*a*) trehalose, (*b*) maltose, and (*c*) sucrose alone (○) and with the addition of 0.9 mM-Zn^{2+} (●)

Data from Carpenter *et al.* (1986*b*).

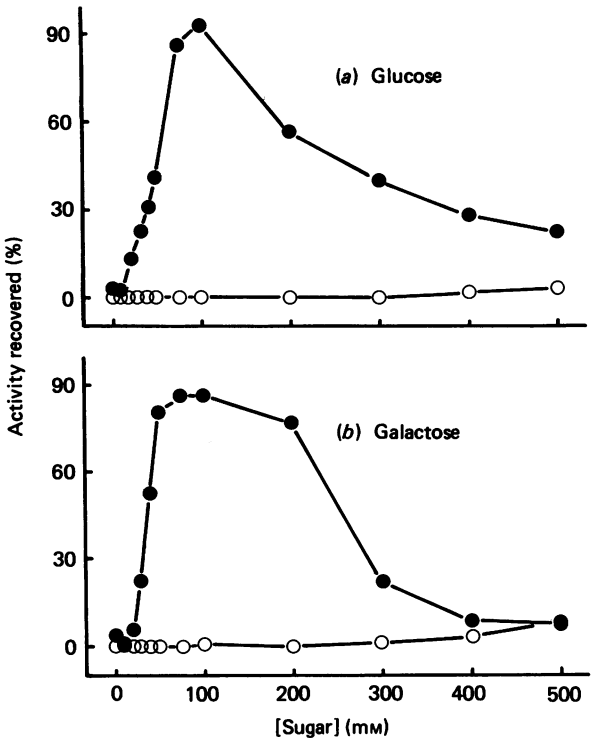

Fig. 2. Comparison of percentage of phosphofructokinase activity recovered after freeze-drying and dissolution in the presence of (*a*) glucose and (*b*) galactose alone (○) and with the addition of 0.9 mM-Zn^{2+} (●)

Data from Carpenter *et al.* (1986*b*).

dissolution, while magnesium is ineffective in this respect (Carpenter *et al.*, 1986*b*). Finally, it is interesting that several other organic solutes (glycerol, inositol, proline, 4-hydroxyproline, glycine and trimethylamine *N*-oxide), either alone or in concert with Zn^{2+}, that afford cryoprotection to phosphofructokinase (Carpenter *et al.*, 1986*a*) do not stabilize the enzyme during freeze-drying (Carpenter *et al.*, 1986*b*). These results provide further support for the contention that dehydration is much more deleterious to enzyme function than freeze–thawing.

Although freeze-drying provides a convenient means to test the capacity of sugars to protect proteins under dehydration-induced stress, this type of perturbation may not most accurately simulate the type of dehydration to which enzymes are subjected in anhydrobiotic organisms during desiccation. Darbyshire has developed a technique to dehydrate protein samples without prior freezing. The water potential in a protein sample is reduced by application of pressure to the aqueous preparation backed by a semipermeable membrane, thereby forcing water and low-M_r solutes through the membrane. While this method is not amenable to testing enzyme stabilization by free sugars, Darbyshire (1974) has found that certain glycosylated fungal enzymes, which are not inactivated by reduction of water potential to −10 bar (1.01 MPa), are unable to maintain fully catalytic capacity during this treatment if more than 10% of the associated carbohydrate has been removed from the protein. More direct evidence that sugars protect enzymes during drying (without prior freezing) is provided by our experiments in which phosphofructokinase is dried under a stream of N_2 at room temperature, and then the dehydration process is completed under vacuum. The unprotected enzyme is completely inactivated during this treatment, but the addition of trehalose stabilizes the enzyme (e.g. 50% recovery of activity in the presence of 200 mM-trehalose), and Zn^{2+} enhances the protection afforded by the sugar (e.g. > 95% recovery with 0.9 mM-Zn^{2+} in 200 mM-trehalose; J. F. Carpenter, J. H. Crowe & L. M. Crowe, unpublished work).

Although the phenomenological data presented above clearly demonstrate that sugars, especially disaccharides, protect proteins under dehydration-induced stress, such results offer little insight into the possible mechanism by which the sugars exert their influence. Timasheff and colleagues have suggested that stabilization of proteins in solution is due to the preferential exclusion of the sugar from contact with the protein surface. Thus, as these authors explain, the addition of sugars to an aqueous

protein solution is thermodynamically unfavourable since it would increase the chemical potential of both the protein and the sugar. Protein structure is stabilized because denaturation of the protein molecule would lead to a greater contact surface between the protein and the solvent and, therefore, augment this unfavourable effect (Timasheff *et al.*, 1976; Lee & Timasheff, 1981; Arakawa & Timasheff, 1982; Timasheff, 1982). The finding that sugars prevent dehydration-induced dissociation of catalase and L-asparaginase into inactive subunits (Hanafusa, 1969; Hellman *et al.*, 1983), which would serve to increase the contact surface between the protein and the solvent, could imply that the stabilization imparted by sugars in solution is translated by the same mechanism into stability during freeze-drying. However, it seems unlikely that the thermodynamic arguments presented above would be applicable to freeze-dried proteins since water is removed from the system.

Even less is known about how divalent cations contribute to the stabilization of dried proteins, but there is some evidence that Zn^{2+} can have a dramatic influence on the conformation of proteins and polypeptides in solution. For example, transition metals such as zinc, copper and nickel, which enhance preservation of dried phosphofructokinase, induce the formation of secondary structure in polypeptides, whereas Mg^{2+} does not elicit either of these phenomena (Bere & Helene, 1979; Maeda *et al.*, 1982; Oka *et al.*, 1983). It has also been found that the binding of Zn^{2+} to calregulin and S100B protein results in an increase in the hydrophobicity of these molecules, and that at Zn^{2+} concentrations above 1 mM calregulin precipitates from solution (Baudier *et al.*, 1983; Khanna *et al.*, 1986). Precipitation of human fibrinogen also occurs when Zn^{2+} is added beyond a critical concentration (Maeda *et al.*, 1983). This decrease in solubility is reminiscent of the increase in the activity coefficients, referred to as 'sugaring out', of model compounds (representing portions of protein molecules) noted in the presence of sugars (Lakshmi & Nandi, 1976). The contributions of the zinc-induced alterations in conformation and solubility to protein stability are not known; however, it has been shown that the quaternary structure of some proteins is stabilized in the presence of zinc. For example, zinc fosters the polymerization of tubulin into microtubules and/or sheets (Nicolson & Veldstra, 1972; Larsson *et al.*, 1976; Gaskin & Kress, 1977; Amos & Baker, 1979). Zinc also has been found to contribute to the maintenance of the native structure of alkaline phosphatase; the apoenzyme is dissociated into subunits in the presence of 8 M-urea or 40% dioxan whereas the metalloenzyme is not (Simpson & Vallee, 1969).

Although it is not known if zinc-induced modifications of protein structure are responsible for the enhanced preservation noted when the metal is present in dried protein/sugar preparations, it seems that the induction of an extremely compact, stable protein conformation in solution (e.g. by exposure of domains with hydrophobic character such as tryptophan; cf. Khanna *et al.*, 1986) could be a prerequisite for the maintenance of catalytic activity when water is removed. Clearly, future research on preservation of dried proteins should be centred not only on attempting to determine how sugars influence protein stability during dehydration, but also on trying to discern the mechanism by which divalent cations are involved in enhancing this process.

Preservation of phospholipid bilayers during drying

While our understanding of how sugars and divalent cations stabilize dry proteins is still in a relatively primitive state, there is a growing body of evidence concerning the mechanism by which sugars stabilize dry phospholipid bilayers. Since understanding the mechanism requires some knowledge of the effects of water on physical properties of phospholipids, we will briefly review some of the literature in this field.

Water and phospholipids

Bound water. When dry phospholipids are hydrated they swell and absorb various amounts of water, depending on the head group, the hydrocarbon chains, the composition of the lipid mixtures and the composition of the surrounding medium (reviewed in Crowe & Crowe, 1985; Hauser *et al.*, 1981). The best known lipid class in this regard are phosphatidylcholines, which have been shown by a number of physical techniques to bind about 10–12 mol of water/mol of lipid. This amount of water constitutes the main hydration shell, above and below which major transitions occur in phospholipid bilayers. On the basis of studies of hydrated bilayers by dielectric measurements (Ebihara *et al.*, 1979; White, 1977), neutron diffraction (Buldt *et al.*, 1978; Simon *et al.*, 1982; Zaccai *et al.*, 1979), i.r. spectroscopy (Bertoluzza *et al.*, 1984), and X-ray diffraction (Hauser *et al.*, 1981), the best available evidence suggests that, not surprisingly, the site of hydration of phospholipids is centred around the phosphate.

Effects of hydration on physical properties of bilayers. Janiak *et al.* (1979) have shown that when the water content of dry phosphatidylcholine multilayers exceeds about 20% major transitions are seen in the X-ray spacings, including: (1) an increase in area/phospholipid molecule, probably due to infiltration between the head groups; (2) increased disorder among the hydrocarbon chains; (3) an increase in lamellar repeat distances, with an appearance of free water between the lamellae.

Since the area/phospholipid molecule declines with hydration below about 20% water content, one would expect decreased hydration to lead to increased opportunities for van der Waals' interactions among the hydrocarbon chains. Consequently, the lipid would be more likely to undergo transition from liquid crystalline phase to gel phase. That this is the case was first demonstrated by using differential scanning calorimetry by Chapman *et al.* (1967), who showed that the transition temperature (t_m) for dipalmitoylphosphatidylcholine rises from about 41 °C in the hydrated lipid to about 90 °C in the 'dry' lipid. When the dry lipid was rehydrated, t_m fell with increasing hydration, reaching a limiting value when hydration reached 10–12 mol of water/mol of lipid. Subsequent study has shown that t_m for dipalmitoylphosphatidylcholine can be even higher; when small unilamellar vesicles of the phospholipid were freeze-dried in the presence of ^3H_2O as a marker for residual water, the dry vesicles were seen to contain less than 0.1 mol of water/mol of phospholipid (J. H. Crowe, L. M. Crowe, J. F. Carpenter & C. Aurell Wistrom, unpublished work), and t_m was observed to exceed 100 °C. Even a brief exposure to air drove t_m down by as much as 40 °C.

More subtle effects of water on phase properties of phospholipids have been described by Kodama *et al.* (1985*a*,*b*, 1986). They have provided good calorimetric evidence that variations in the water content can lead to changes in the pretransition and subtransitions in dipalmitoylphosphatidylcholine. In fact, the existence of the pretransition appears to be dependent on the presence of water.

The important conclusion that can be made from these studies is that the dry lipids are in gel phase at temperatures at which they would be in liquid crystalline phase if they were hydrated, a conclusion that may be the key to understanding the damage to phospholipid vesicles that accompanies dehydration.

Hydration state and stability of bilayers

Dehydration of phospholipid bilayers can lead to several sorts of damage, as follows.

Fusion. The hydration shell provides a force that must be overcome for fusion to occur between bilayers. The magnitude of this force has been measured through the elegent studies of Parsegian, Rand, and their colleagues (Parsegian & Rand, 1979; Lis *et al.*, 1982). A corollary of their work is that dehydration of phospholipid bilayers should lead to fusion, and there is good evidence that this is the case. Freeze–fracture shows that when vesicles or liposomes are dehydrated, massive fusion results (Crowe *et al.*, 1983; Crowe *et al.*, 1985*b*). Similar studies using resonance energy transfer (Crowe, L. M. & Crowe, 1986; Crowe, L. M., *et al.*, 1986; MacDonald & MacDonald, 1981; Womersley *et al.*, 1986), freeze–fracture (Madden *et al.*, 1985; Pick, 1981; Rudolph & Crowe, 1986), and n.m.r. (Strauss & Hauser, 1986) to monitor fusion induced by freezing or dehydration also show without question that the result is fusion between neighbouring bilayers.

Leakage. When phospholipid vesicles fuse they leak their contents to the medium (Hammoudah *et al.*, 1981; Blok *et al.*, 1975). Thus, dehydration that leads to fusion would be expected to result in leakage of vesicle contents, but fusion is not the only mechanism that can lead to leakage. When phospholipid vesicles are heated through t_m they tend to leak (Blok *et al.*, 1975; Papahadjopoulos *et al.*, 1973). The usual explanation for this phenomenon is that, as the phospholipids pass through t_m, temporary defects develop in the bilayer at the boundaries between gel phase and liquid crystalline phase lipids (Lee, 1977*a*,*b*). Such defects could develop either because of inhomogeneities in the heating of vesicles composed of a single phospholipid or because the constituents of more complex mixtures pass through t_m at different temperatures. It is likely that when the vesicle is dehydrated it would undergo a phase transition from liquid crystalline to gel phase. Upon rehydration, the gel phase lipids would undergo another transition from gel to liquid crystalline phase. The bilayer would be expected to become leaky during both phase transitions.

Lateral phase separations. Quinn (1985) has proposed a mechanism for temperature-induced lateral phase separations of phospholipids that can be directly modified to explain dehydration-induced lateral phase separations, and we have done so elsewhere (Crowe, J. H. & Crowe, 1986). Briefly, this suggestion is based on evidence in the literature (reviewed in Matubaysi *et al.*, 1986) that several binary pairs of phospholipids that differ in head group or length of the acyl chains have low miscibility in gel phase. Examination of the phase diagrams for mixtures of the few phospholipids for which such data are available shows that the lipids pass through the liquid crystalline to gel phase transition at different combinations of temperature and hydration state. If the mixture is dehydrated at constant temperature, one would expect such lipids to enter gel phase at different times during the dehydration and to undergo phase separation. Such phase separations may be an important source of damage from dehydration since they may lead to displacement of integral membrane proteins (Crowe *et al.*, 1983) and formation of non-bilayer phases (Crowe & Crowe, 1982) in intact membranes.

Lateral phase separations and non-bilayer phases. Formation of non-bilayer phases resulting from lateral phase separations might be expected to be a damaging event. Phosphatidylethanolamine, for example, prefers in the absence of other lipids not to exist in bilayers in the liquid crystalline phase, but instead forms the hexagonal II phase (e.g. Cullis *et al.*, 1985). Such a phase would clearly be inconsistent with a bilayer, and vesicles that contain such domains would be expected to leak. In fact, it has been suggested that formation of hexagonal II phase is a major cause of leakage of solutes from seeds during rehydration (Simon, 1974), a suggestion that stimulated a great deal of research, but which has subsequently become controversial (reviewed in Crowe, L. M. & Crowe, 1986).

Effects of sugars on stability of dry vesicles

Effects of trehalose on membrane stabilization. When unilamellar vesicles were freeze-dried, upon rehydration they leaked all their contents to the medium. With similar vesicles freeze-dried in the presence of trehalose, however, the results seen in Fig. 3 were obtained, showing that the vesicles can be sufficiently stabilized that 100% of the trapped solute is retained. Similar results have been obtained by Madden *et al.* (1985), using different techniques. When Ca^{2+}-transporting vesicles isolated from striated muscle were freeze-dried, the ability to transport Ca^{2+} was reduced to insignificant levels if the membranes were dried without trehalose, but was stabilized to at least 80% of fresh vesicles as the trehalose content of the dry preparations is increased (Fig. 3).

Comparative effects of sugars. There is considerable variability among the sugars in their ability to stabilize the dry vesicles and membranes, trehalose being the most effective and inositol the least (Fig. 3). That is not to say that sucrose, for example, will not stabilize to a high degree the membranes and unilamellar vesicles with which it has been tested; indeed, preservation of membranes freeze-dried in sucrose is as good as in those freeze-dried with trehalose. However, much more sucrose is required to achieve the same level of stabilization (Fig. 3), suggesting that sucrose is less efficient than trehalose. More recently, Strauss *et al.* (1986) and Anchordoguy *et al.* (1986) have reported that sucrose and trehalose were equally efficient at preventing fusion between vesicles of egg phosphatidylcholine and dimyristoylphosphatidylcholine, respectively, during

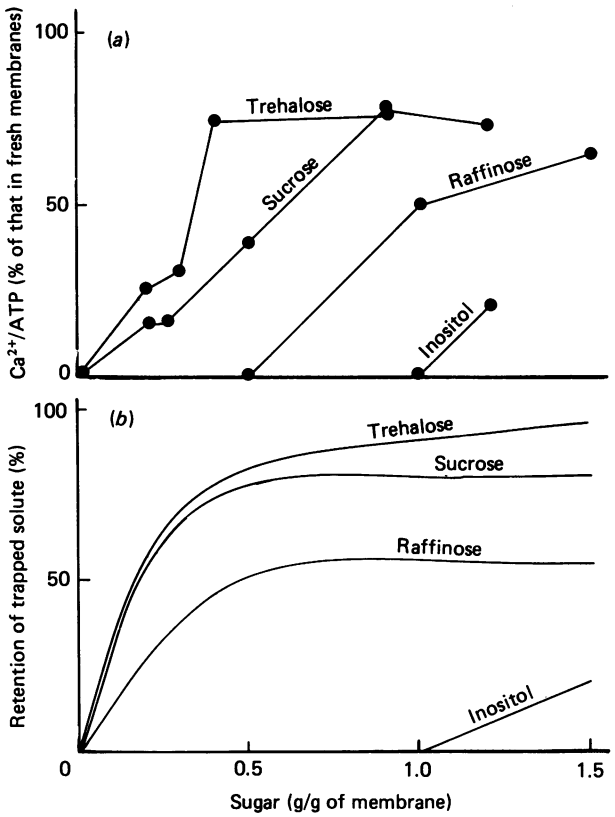

Fig. 3. (*a*) Coupling between Ca^{2+} uptake and ATP utilization in Ca^{2+}-transporting vesicles following freeze-drying and rehydration in the presence of the indicated amounts of sugars, and (*b*) retention of trapped solute (isocitric acid) by vesicles of palmitoylolesoylphosphatidylcholine/phosphatidylserine previously freeze-dried in the presence of various amounts of the indicated sugars

(*a*) Data from Crowe, L. M., *et al.* (1984). (*b*) Data points are omitted for clarity. The curves shown were fitted by least squares, with a regression coefficient for each $\geqslant 0.98$. Data from Crowe *et al.* (1985*b*).

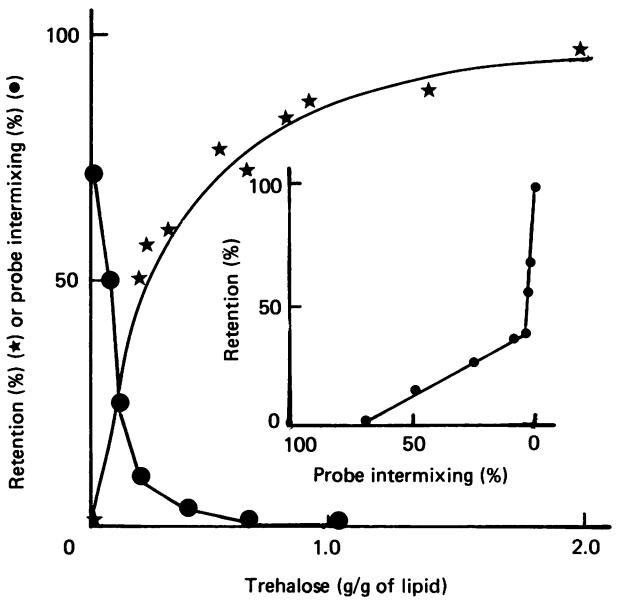

Fig. 4. Probe intermixing assessed by resonance energy transfer in vesicles of palmitoyloleoylphosphatidylcholine/phosphatidylserine previously freeze-dried in the presence of trehalose (●)

Also shown are data for retention of trapped solute (from Fig. 3; ★). Inset: plot of retention of trapped solute against probe intermixing, showing that after probe intermixing reaches minimal values retention of trapped solute continues to increase. Probe intermixing data from Crowe *et al.* (1985*b*).

freezing, so the findings described here cannot necessarily be generalized to all lipids. At this point all we can say is that disaccharides are in general superior to other sugars, but there is also considerable variability among the disaccharides; in our hands trehalose and maltose are significantly more effective at stabilizing both unilamellar vesicles (Crowe, L. M., *et al.*, 1986) and biological membranes (Crowe *et al.*, 1983; Crowe *et al.*, 1985*a*) than the other disaccharides tested. However, since maltose is a reducing sugar, it would be expected to undergo browning reactions with membrane proteins, destabilizing function during storage (Mouradian *et al.*, 1985). Why the sugars differ in their ability to stabilize vesicles and membranes is a subject of some interest that is under study in several laboratories other than our own (e.g. Madden *et al.*, 1985; Gaber *et al.*, 1986; Lee *et al.*, 1986; Strauss & Hauser, 1986).

Mechanism of stabilization of liposomes by sugars

Effects on fusion. Effects of sugars on inhibition of fusion during dehydration have been studied, using freeze–fracture (Crowe, L. M., *et al.*, 1986) and resonance energy transfer (Uster & Deamer, 1981; Crowe, L. M., *et al.*, 1986; Rudolph & Crowe, 1986; Womersley *et al.*, 1986). Typical results with resonance energy transfer (Fig. 4) show that in the absence of sugar, about 70% probe intermixing occurs following freeze-drying and rehydration, suggesting that the vesicles undergo several rounds of fusion. When they are dried in the presence of sugars, probe intermixing can be stopped at moderate concentrations of most of the sugars. The sugars tested show about the same relative abilities to inhibit fusion as they do to prevent leakage; trehalose is the most effective at inhibiting fusion and inositol is the least, with other sugars showing intermediate abilities (Crowe, L. M., *et al.*, 1986). This observation suggests that inhibition of fusion is a key event in stabilizing the dry vesicles, but only about 0.3 g of trehalose/g of lipid is sufficient to inhibit fusion maximally, whereas about three times as much trehalose is required to achieve maximal retention of trapped solute (Fig. 4). It follows, therefore, that while inhibition of fusion is required for the stabilization, the sugars must alter other properties of the dry vesicles that affect their stability.

Effects on phase transitions. Thermotropic phase transitions in dry unilamellar vesicles are complex, and effects of sugars on such transitions may probably be best understood by examination of homogeneous preparations of lipid and sugar. When t_m values for dry homogeneous mixtures (prepared by dissolving the lipids and sugars in organic solvents; see Crowe, J. H., *et al.*, 1984*b*; Crowe, L. M., *et al.*, 1986, for details) of trehalose and palmitoyloleoylphosphatidylcholine and phosphatidylserine in a molar ratio of 9:1 were recorded, for example, the results shown in Fig. 5 were obtained. In the

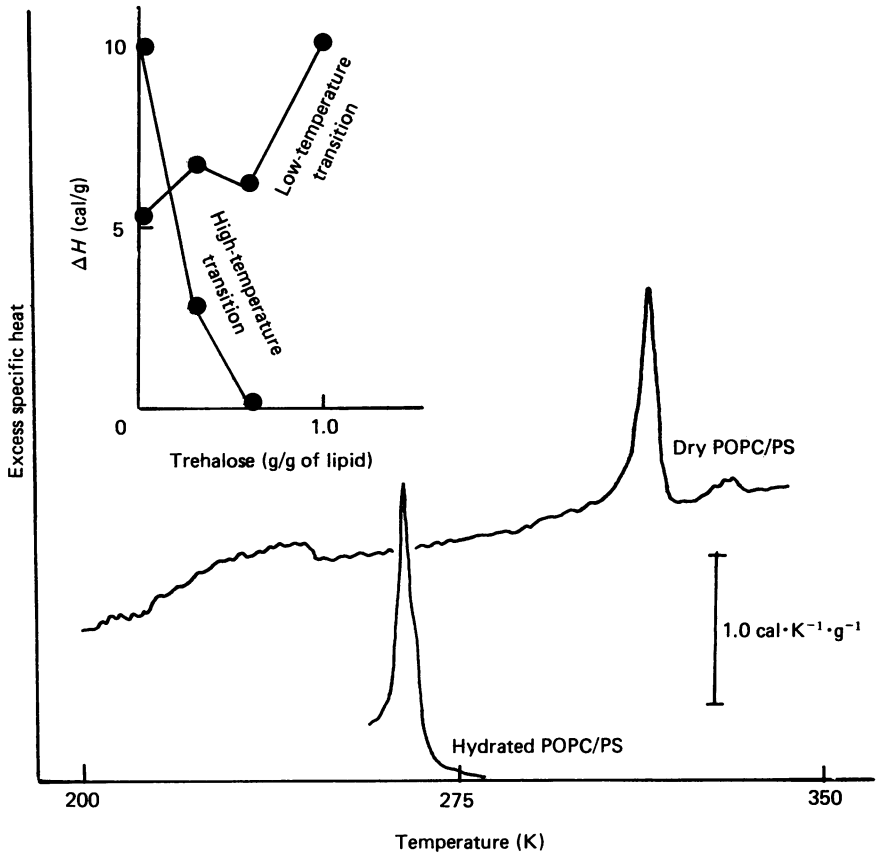

Fig. 5. Melting endotherms for dry and hydrated multilamellar vesicles of palmitoyloleoylphosphatidylcholine/phosphatidylserine (POPC/PS)

Inset: changes in enthalpy of the high-temperature and low-temperature melting endotherms for dry, homogeneous preparations of palmitoyloleoylphosphatidylcholine/phosphatidylserine and trehalose, prepared from organic solvents. Data for dry lipids from Crowe *et al.* (1985*b*) and those for hydrated vesicles from Rudolph *et al.* (1986)

hydrated lipid a single, low-temperature transition is seen in the region of 240 K. But in the dry lipid a high-temperature transition appears that corresponds to the transition for dry palmitoyloleoylphosphatidylcholine alone, and the low-temperature transition persists, with decreased enthalpy (Fig. 5). The low-temperature transition corrresponds to the transition for dry phosphatidylserine. We interpret this result to mean that during the drying there is a lateral phase separation of the palmitoyoleoylphosphatidylcholine and phosphatidylserine. With increasing amounts of trehalose in the dry mixture, t_m for the high-temperature transition falls dramatically to 250 K, a decrease of 80 K. A decrease in the calorimetric enthalpy accompanies the decrease in t_m of the high-temperature transition, whereas there is a corresponding increase in enthalpy of the low-temperature transition (Fig. 5, inset).

Based on these observations and similar ones on other phospholipids (Crowe & Crowe, 1985; Crowe, L. M., *et al.*, 1984, 1986; Rudolph *et al.*, 1986; Strauss & Hauser, 1986), we have suggested that this ability of sugars to depress t_m is critical to stabilization of membranes and phospholipid vesicles in the absence of water; the dry phospholipids are maintained in a liquid crystalline state even though they are dry. Indeed, in the case of palmitoyloleoylphosphatidylcholine, t_m for the dry lipids in the presence of 1 g of trehalose/g of lipid is actually considerably below that for the hydrated lipid. More recently, Lee *et al.* (1986) have shown with high resolution proton n.m.r. that the hydrocarbon chains of phospholipids are in a gel phase when dried without trehalose, but exist in a fluid phase when dried with trehalose. Lee *et al.* (1986) suggest that this is not a genuine liquid crystalline phase, but that it may be a new fluid phase instead.

Unilamellar vesicles are far more complex than homogeneous preparations and yield calorimetric data that are more difficult to interpret (Surkuusk *et al.*, 1976; Bramhall, 1986; van Dijck *et al.*, 1978; Schuh *et al.*, 1982). Nevertheless, the effects of sugars on t_m of such vesicles appear similar to effects on homogeneous mixtures. Dry vesicles composed of palmitoyloleoylphosphatidylcholine and phosphatidylserine of the same composition used to obtain the data shown in Fig. 5 show two transitions, a high-temperature one and a low-temperature one, essentially similar to the homogeneous preparations. That finding is not particularly surprising since the vesicles dried with low concentrations of trehalose fuse to form multilamellar structures that are structurally and thermodynamically similar to the lipids prepared in homogeneous mixtures. When the vesicles are dried in the presence of increasing concentrations of trehalose the enthalpy of the low-temperature transition increases (Fig. 6). This increase in enthalpy coincides with

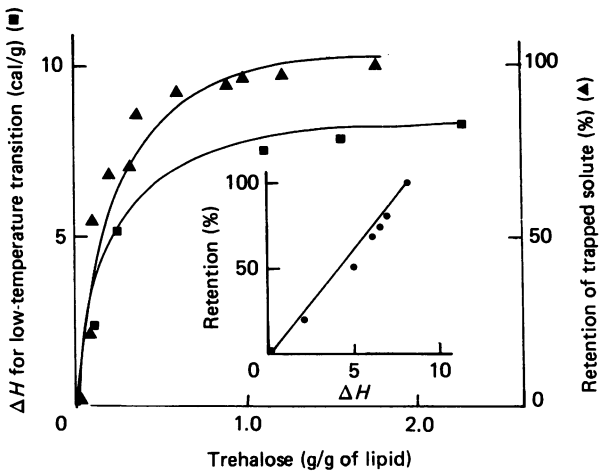

Fig. 6. Changes in enthalpy (■) for the low-temperature transition in dry vesicles of palmitoyloleoylphosphatidylcholine/phosphatidylserine

Also shown for comparison are data for retention of trapped solute by vesicles previously dried in the presence of trehalose, from Fig. 3 (▲). Inset: plot of retention of trapped solute against enthalpy of the low-temperature transition, illustrating that these parameters clearly co-vary. Calorimetric data are from Crowe *et al.* (1985*b*).

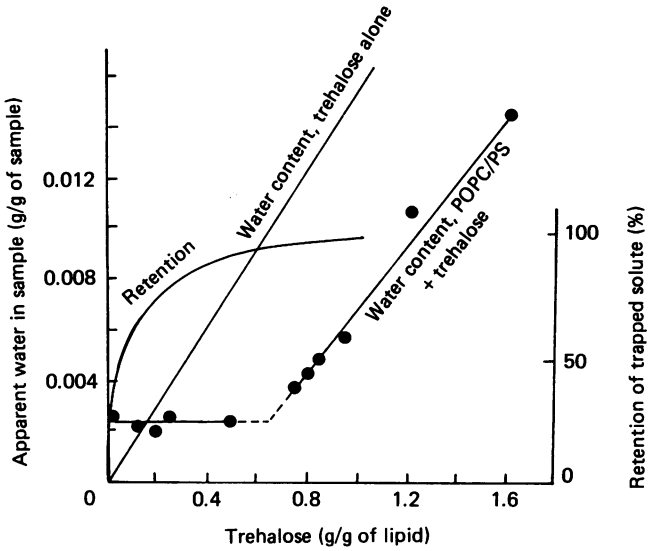

Fig. 7. Residual water content of palmitoyloleoylphosphatidylcholine/phosphatidylserine (POPC/PS) liposomes freeze-dried in the presence of trehalose

Also shown are data for retention of trapped solute by similar liposomes, from Fig. 3. Residual water data are from Crowe, J. H., *et al.* (1986).

an elevation in retention of trapped solute (Fig. 6), suggesting that the change in thermotropic t_m induced by the presence of the trehalose is important in stabilizing the dry vesicles. One problem with this interpretation is that, contrary to the situation for homogeneous preparations (cf. Fig. 5), the high-temperature transition does not disappear with increasing amounts of trehalose. This is probably an artifact of the method of preparation; the vesicles are prepared with a small amount of trehalose inside and then excess trehalose is added to the outside. Thus, the two halves of the bilayer are exposed to vastly different concentrations of trehalose, and may therefore undergo phase transitions independently. This seems an unorthodox idea, but there is, in fact, evidence for independent phase behaviour of the two halves of a bilayer (van Dijck *et al.*, 1978; Schuh *et al.*, 1982).

In summary to this point, we believe that the bulk of the evidence strongly suggests that the sugars that preserve dry phospholipid vesicles do so by maintaining the lipids in a fluid phase in the absence of water. Thus, the dehydration-induced phase separations that accompany liquid crystalline to gel phase transitions are obviated. Further, when the lipids are rehydrated they do not undergo a transition from gel to liquid crystalline phase, and they do not leak.

Mechanism of depression of t_m by sugars

Retention of water by 'dry' vesicles. A potential mechanism by which t_m is depressed by sugars is that the sugars retain water in the 'dry' mixtures. The water and not the sugar would, according to this suggestion, be responsible for driving down t_m. This hypothesis has been tested by drying vesicles in ^3H_2O and various amounts of trehalose (Crowe, J. H., *et al.*, 1986). Residual water in the 'dry' samples is determined by liquid-scintillation counting. Since some proton exchange between the ^3H_2O and exchangeable sites on the trehalose and phospholipid might be expected, this procedure actually yields an overestimate of the residual water content. The results (Fig. 7) show that the water content of the vesicles dried without trehalose is low, of the order of 0.1 mol of water/mol of lipid. As the amount of trehalose in the dry preparations is increased, the residual water content does not rise until the amount of trehalose exceeds 0.65 g of trehalose/g of lipid, after which the apparent residual water content rises linearly and with the same slope as trehalose alone (Fig. 7). We interpret these data as follows: at mass ratios below 0.65 g/g, all the trehalose associates with the lipid and is unavailable for binding water. Above 0.65 g/g free trehalose appears. The data for retention of trapped solute by the same type of vesicles following drying and rehydration, also shown in Fig. 7, indicate that retention increases nearly to the maximal value in the range of trehalose concentrations where the water content of the samples has not yet started to increase. Similarly, the calorimetric data show maximal effects of trehalose on the lipid below the point where water content begins to increase (cf. Fig. 6). We conclude that retention of water by the dry trehalose–lipid preparations cannot account for their stability.

Direct interaction between sugar and the polar head group. The best evidence for this possibility comes from studies on effects of heavy metals on the ability of sugars to stabilize bilayers during freezing. There is good evidence that europium forms a strong ionic linkage with the phosphate of membrane phospholipids (reviewed in Strauss & Hauser, 1986). Strauss & Hauser (1986) reasoned that europium might therefore alter the ability of sugars to interact with phospholipids if an interaction occurs between the sugar and phosphate, and subsequently they showed that addition of europium ions to unilamellar phospholipid vesicles made in sucrose solutions abolishes the ability of the sucrose to inhibit

fusion between the vesicles during freezing. Based on this evidence, Strauss & Hauser (1986) suggested that stabilization of the bilayer by sucrose probably involves a direct interaction between sucrose and phospholipids.

Additional supporting evidence has been obtained from i.r. spectroscopy. In the 'polar head group' region of the i.r. spectrum, dramatic changes are seen when lipids (Crowe, J. H., *et al.*, 1984*b*; Crowe *et al.*, 1985*a*) and biological membranes (Crowe, J. H., *et al.*, 1984*a*) are dried in the presence of sugars. When dry homogeneous palmitoyloleoylphosphatidylcholine/phosphatidylserine and trehalose were prepared, with increasing concentrations of trehalose, the band assigned to the P=O asymmetric stretch shifts to a lower wave number. A similar shift in vibrational frequency is seen when the dry lipid is hydrated. Spectra for trehalose in the presence and absence of the phospholipids show suggestive changes; bands assigned to deformations of –OH groups are broadened to the point where it is difficult to detect them in the presence of the lipid, suggesting an interaction between –OH groups on the sugar and the phosphate of the lipid. In closely related studies, Lee *et al.* (1986) have shown with ^{31}P n.m.r. that the phosphate head groups in dipalmitoylphosphatidylcholine dried with trehalose appear to have low mobility, even though the hydrocarbon chains are maintained in a fluid phase. We conclude from such studies that the phosphate group of the phospholipid interacts with –OH groups on the trehalose, probably by hydrogen bonding. Further support for this viewpoint can be had from the recent work of Gaber *et al.* (1986) who have done molecular modelling studies on the interaction between trehalose and dipalmitoylphosphatidylcholine. Their calculations, which include energy minimization and some *ab initio* calculations, suggest that trehalose forms a hydrogen-bonded complex with the polar head group of the phospholipid. Their models also suggest that the stable structure requires lateral expansion of the bilayer, a result that is clearly consistent with the finding that the sugar depresses t_m.

Effects of trehalose on lamellar to hexagonal phase transitions. It is possible to study the lamellar to hexagonal phase transition with X-ray diffraction and n.m.r. (Cullis *et al.*, 1985), i.r. spectroscopy (Mantsch *et al.*, 1981), and calorimetry (Epand, 1985). We have used Fourier transform i.r. spectroscopy and calorimetry to record the transitions of phosphatidylethanolamine in the presence and absence of trehalose, with results for phosphatidylethanolamine alone similar to those reported by Mantsch *et al.* (1981) (Fig. 8). In the presence of trehalose, however, although t_m is unchanged, the lamellar to hexagonal transition is missing altogether, or it is elevated by at least 30 °C. Calorimetry yields essentially similar results; endotherms assigned to the gel to liquid crystalline and lamellar to hexagonal phase transitions are clearly seen in the thermograms for phosphatidylethanolamine in the absence of trehalose, but the lamellar to hexagonal endotherm is missing for at least 60 °C above its normal position when trehalose is present (Aurell Wistrom *et al.*, 1986). In related studies, Boni *et al.* (1984), in an effort towards understanding the fusogenic properties of poly(ethylene glycol) (Ahkong *et al.*, 1975), have shown that this compound also elevates both t_m and t_h. Since removal of water from phosphatidylethanolamine has similar

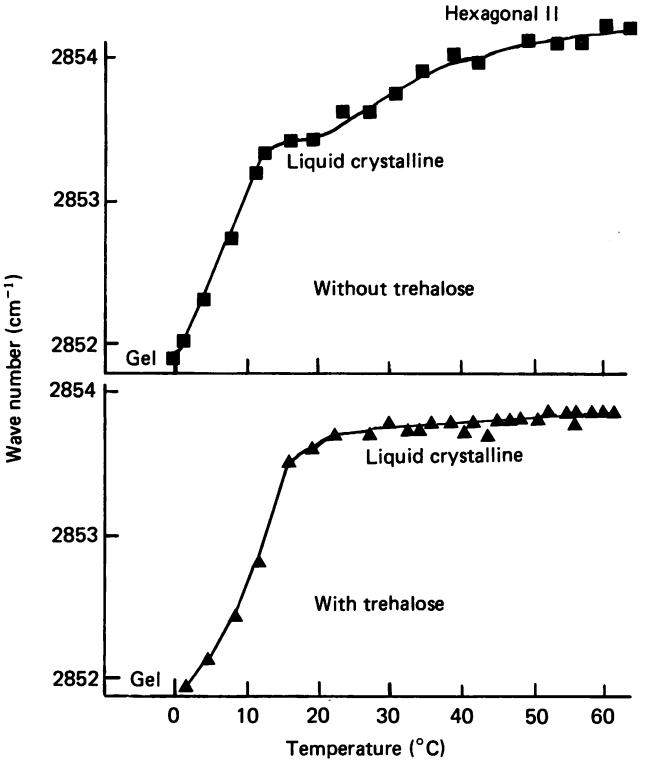

Fig. 8. Vibrational frequency for the CH_2 asymmetric stretch in hydrated egg phosphatidylethanolamine as a function of temperature

The gel to liquid crystalline and lamellar to hexagonal phase transitions can be clearly distinguished in the absence of trehalose, but in the presence of trehalose (4 mol/mol of phosphatidylethanolamine) the lamellar to hexagonal transition is absent, even as high as 30 °C above the normal transition. Data from Aurell Wistrom *et al.* (1986).

effects (Shipley, 1973), they deduced that ethylene glycol probably dehydrates the polar head groups of the phospholipid. This is clearly not the case with trehalose; although t_h is elevated by addition of trehalose, t_m is affected little, if at all (Fig. 8).

We conclude that trehalose is capable of maintaining hydrated phosphatidylethanolamine in bilayers at temperatures at which it would normally be in hexagonal phase. We have not yet done such measurements for dry phosphatidylethanolamine, nor have we tested the ability of other sugars to maintain the phospholipid in bilayers. Nevertheless, it does not seem premature to suggest a potential mechanism by which trehalose and perhaps other sugars may maintain phosphatidylethanolamine in lamellar phase. We suspect that it is a result of direct interaction between trehalose and the phospholipid polar head group, which could have the following effects. The lamellar to hexagonal phase transition is thought to be a result of the fact that the phosphatidylethanolamine head group occupies a small volume and that the head groups can hydrogen bond directly together. The result is that above t_m a curvature is induced in the planar bilayer (see Gruner, 1985, for further discussion). Interaction of trehalose with the head group would increase its volume and would thus be expected to decrease the curvature in the bilayer. We are conducting experiments at present to test this hypothesis.

Conclusions

Removal of water profoundly alters the physical properties of membrane phospholipids, leading to destructive events such as fusion, liquid crystalline to gel phase transitions, and elevation of permeability. In heterogeneous mixtures such as those found in biological membranes, the phase transitions lead to lateral phase separations of membrane constituents. Certain sugars are capable of preventing damage from dehydration not only by inhibiting fusion between adjacent vesicles during drying, but also by maintaining the lipids in a fluid phase in the absence of water. As a result, the changes in permeability and lateral phase separations that would usually accompany dehydration are absent. Although we understand far less concerning the mechanism by which they do so, some of the same sugars are also effective at preserving structure and function of labile proteins in the absence of water, an effect that is remarkably enhanced by the addition of small amounts of transition metals. We believe these effects of sugars on stability of bilayers and proteins may be important in the survival of intact cells and organisms such as seeds in the absence of water. In addition, in view of the practical importance of preserving biological structures such as membrane vesicles, proteins, and intact cells, we suspect that the results reviewed here will ultimately have important applications in biology and medicine.

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