Stabilization of Dry Mammalian Cells: Lessons from Nature

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SYNOPSIS. The Center for Biostabilization at UC Davis is attempting to stabilize mammalian cells in the dry state. We review here some of the lessons from nature that we have been applying to this enterprise, including the use of trehalose, a disaccharide found at high concentrations in many anhydrobiotic organisms, to stabilize biological structures, both in vitro and in vivo. Trehalose has useful properties for this purpose and in at least one case—human blood platelets—introducing this sugar may be sufficient to achieve useful stabilization. Nucleated cells, however, are stabilized by trehalose only during the initial stages of dehydration. Introduction of a stress protein obtained from an anhydrobiotic organism, Artemia, improves the stability markedly, both during the dehydration event and following rehydration. Thus, it appears that the stabilization will require multiple adaptations, many of which we propose to apply from studies on anhydrobiosis.

Trehalose and Biostability

More than 20 years have passed since we first reported that biomolecules and molecular assemblages such as membranes and proteins can be stabilized in the dry state in the presence of a sugar found at high concentrations in many anhydrobiotic organisms, trehalose (J. Crowe, et al., 1983). We also reported that when comparisons were made with other sugars, trehalose was clearly superior (Crowe, et al., 1984). The superiority of trehalose seemed so clear it quickly led to wide-spread, and often uncritical, use of the sugar for preservation and other purposes. In fact, an astonishing array of applications for trehalose have been reported, ranging from stabilization of vaccines and liposomes to hypothermic storage of human organs (reviewed in Crowe et al., 2001). Other studies suggested that it might even be efficacious in treatment of dry eye syndrome (Matsuo, 2001) or dry skin (Norcia, 2000) in humans. Trehalose is prominently listed as an ingredient in cosmetics (reviewed in Higashiyama, 2002). Apparently the only basis for its use in cosmetics is that trehalose is reputed to inhibit oxidation of certain fatty acids in vitro that might be related to body odor (Higashiyama, 2002). Trehalose has been shown by several groups to suppress free radical damage (e.g., Benaroudj et al., 2001), protect against anoxia (Chen et al., 2002, 2004), inhibit dental caries (Neta et al., 2000), enhance ethanol production during fermentation (Gimeno-Alcainiz et al., 1999; Pataro et al., 2002), stabilize the flavor in foods (e.g., Komes et al., 2003), and to protect plants against abiotic stress (Garg et al., 2002). According to one group, trehalose inhibits bone resorption in ovariectomized mice (Nishizaki et al., 2000), apparently by suppressing osteoclast differentiation (Yoshizane et al., 2000); the suggestion followed that trehalose might be used to treat osteoporosis in humans. More recently, Tanaka et al. (2004) reported that trehalose could be used to inhibit the protein aggregation associated with Huntington’s disease in vivo in a rat model for this disease. That report that has already led to an unorthodox clinical trial in humans (Couzin, 2004).

The point we want to make is that a myth has grown up about trehalose and its properties, as a result of which it is being applied, sometimes rather uncritically, to a myriad of biological and clinical problems. Thus, we are making special efforts in the literature to clarify the properties of trehalose that make it useful for stabilization of biomaterials and to dispel the most misleading aspects of this myth.

Origins of the Trehalose Myth

We recently reviewed the history of this field (Crowe et al., 2001) and provide only a brief summary here. The key observations were: (1) The first model membrane investigated was sarcoplasmic reticulum, isolated from lobster muscle (reviewed in Crowe et al., 1987). When we compared the ability of a variety of sugars to preserve the SR during drying, we found that trehalose was without question superior to all other sugars tested. Some years later, however, we obtained evidence that these SR membranes have a mechanism for translocating trehalose across the bilayer. We suggest that other sugars such as sucrose might preserve the membranes at concentrations similar to those seen with trehalose if they had access to the aqueous interior. (2) Initial studies with liposomes, from the mid-1980s (reviewed in Crowe et al., 1992), were done with a phospholipid with low Tm. When the liposomes were freeze dried with trehalose and rehydrated, the vesicles were seen to be intact, and nearly 100% of the trapped solute was retained. It quickly emerged


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against a role for sugars in anhydrobiosis (Crowe et al., 2001). These effects were reported first for trehalose (reviewed in Crowe et al., 1992). When we compared the effects of other sugars and polymers on the preservation, we found that, with vesicles made from lipids with low Tm, trehalose appeared to be significantly superior to the best of the additives tested. Oligosaccharides larger than trisaccharides did not work at all (Crowe and Crowe, 1988). Other sugars, particularly disaccharides, did provide good stabilization of egg PC vesicles in the dry state, but much higher concentrations than trehalose were required, at least according to initial reports. However, as freeze-drying technology improved, the differences between disaccharides tended to disappear, and the myth eventually got modified to encompass disaccharides in general. Nevertheless, the observation that trehalose was significantly more effective at low concentrations under suboptimal conditions for freeze drying requires explanation, which we provide later. (3) At first it appeared that the ability to preserve liposomes in the dry state is restricted to disaccharides. Subsequently, we found this is not the case. For example, DPPC is a lipid with saturated acyl chains and thus an elevated Tm (41°C). When it is dried without trehalose Tm rises to about 110°C; with trehalose present Tm rises to about 65°C (reviewed in Crowe et al., 1998). Thus, DPPC is in gel phase at all stages of the freeze-drying and rehydration process, and one would expect that inhibition of fusion might be sufficient for the stabilization. In other words, any inert solute that would separate the vesicles in the dry state and thus prevent aggregation and fusion should stabilize the dry vesicles. That appears to be the case; a high molecular weight (450,000) HES (hydroxyethylstarch) has no effect on Tm in dry DPPC, but preserves the vesicles, nevertheless.

**There is More Than One Way to the Same End**

Although the occurrence of trehalose at high concentrations is common in anhydrobiotic animals, some such animals have vanishingly small amounts of trehalose (Womersley, 1990; West and Ramlov, 1991) or none at all (Lapinski and Tunnacliffe, 2003; Tunnacliffe and Lapinski, 2003; Caprioli et al., 2004). It is tempting to construe these findings as evidence against a role for sugars in anhydrobiosis (cf., Tunnacliffe and Lapinski, 2003). We suggest that it is not the sugars per se that are of interest in this regard, but rather the physical principles of the requirements for stabilization, as described above. There are multiple ways to achieve such stabilization: (1) HES (hydroxyethylstarch) alone will not stabilize dry membrane vesicles composed of lipids with low Tm, but a combination of a low molecular weight sugar such as glucose and HES can be effective (Crowe et al., 1997). Here is the apparent mechanism: glucose depresses Tm in the dry lipid, but has little effect on inhibiting fusion, except at extremely high concentrations. On the other hand, the polymer has no effect on the phase transition, but inhibits fusion. Thus, the combination of the two meets both requirements, while neither alone does so (Crowe et al., 1997). It seems likely that such combinations of molecules might be found in anhydrobiotes in nature. (2) In fact, a glycan isolated from the desiccation tolerant alga *Nostoc* apparently works in conjunction with oligosaccharides (Hill et al., 1997). Similarly, certain proteins have been shown to affect the phase state of the sugars and either enhance or are required for stabilization (reviewed in Buitink and Leprince, 2004). (3) Hincha et al. (2000) have shown that fructans from desiccation tolerant higher plants will by themselves both inhibit fusion and reduce Tm in dry phospholipids such as egg PC. The mechanism behind this effect is still unclear. Vereyen et al. (2003) provided evidence that the interaction is similar to that shown by sugars, but that it is also specific to fructans and is not shown by other polymers. In related studies, Hincha et al. (2003) reported that a series of raffinose family oligosaccharides are all capable of stabilizing dry liposomes. (4) Hincha and Hagemann (2004) recently studied effects of other compatible solutes on stabilization of liposomes by sugars. This approach is in its earliest stages, but those authors found that some compatible solutes improve the stabilization in the presence of sugars, suggesting that the solutes might decrease the amount of sugar required *in vivo*. (5) Hoekstra and Golovina (2002) have reported that amphiphiles that are free in the cytoplasm in fully hydrated cells of anhydrobiotes apparently insert into membranes during dehydration. The role of this phenomenon in stabilization is uncertain, but presumably the amphiphiles alter the order of the acyl chains. Popova and Hincha (2004) have shown that this is the case for a model amphiphiles, tryptophan, that the interaction depends on the lipid composition. This latter finding suggests that the protective molecules themselves may vary depending on lipid composition. Further evidence for this proposition appears in Hincha et al. (1999) and Oliver et al. (2001, 2002). (6) Goodrich et al. (1991) reported that disaccharides tethered to the bilayer surface by a flexible linker esterified to cholesterol have an effect on membrane stability similar to that seen in the free sugar. Such molecules could provide stability in anhydrobiotes, although they have not yet been reported.

The point is there are many ways to achieve stabil-
GLASS TRANSITIONS AND STABILITY

Using liposomes as a model, we attempted to find a mechanism for long term stability in the presence of trehalose. As with the bacteria and immunoconjugates, the dry liposomes exposed to increased relative humidity rapidly leaked their contents when they were dried with sucrose, but not when they were dried with trehalose (Crowe et al., 1996; Sun et al., 1996). The liposomes underwent extensive fusion in the moist air when dried with sucrose, but not with trehalose.

Trehalose, along with many other sugars, forms a glass when it is dried. This glass undergoes a transition from a highly viscous fluid to a highly mobile system when it is heated above a characteristic temperature, \( T_g \), which increases sharply as dehydration progresses, resulting in what is known as a state diagram (Fig. 1). The importance of the state diagram is as follows. It has become widely accepted that stability of dry materials in which close approach of surfaces must be prevented requires that the material remain below the curve for the state diagram, i.e., it must be maintained in the glassy state. Above the curve the mobility of the system increases, while below it the materials are held in a relatively rigid matrix (Fig. 1). For instance, heating a sample containing liposomes above \( T_g \) results in increased mobility to the point where fusion occurs in the concentrated solution. (Brief excursions above the curve are not necessarily damaging, since the surface to surface interaction has a kinetic component. Because of this kinetic component, there is a lot of confusion in the literature concerning whether the glassy state is even required for stabilization.)

\( T_g \) for trehalose is much higher than that for sucrose (Fig. 2), a finding first reported by Green and Angell (1989). As a result, one would expect that addition of small amounts of water to sucrose by adsorption in moist air would decrease \( T_g \) to below the storage temperature, while at the same water content \( T_g \) for trehalose would be above the storage temperature. Indeed, at water contents around 5%, \( T_g \) for trehalose is about 40°C, while that for sucrose is about 15°C. \( T_g \) for glucose at a similar water content is about \(-10°C\) (Fig. 2). One would predict that at such water contents
trehalose would be the only one of these three sugars that would stabilize the sample, and this appears to be the case. This would seem to provide an explanation for the superior stability of, for example, the immunonoconjugates stored in sucrose or trehalose described above. We stress, however, that the elevated $T_g$ seen in trehalose is not anomalous. Indeed, trehalose lies at the end of a continuum of sugars that show increasing $T_g$ (Crowe et al., 1997), although the basis for this effect is not understood.

**NON-ENZYMATIC BROWNING AND STABILITY OF THE GLYCOSIDIC BOND**

The Maillard (browning) reaction between reducing sugars and proteins in the dry state has often been invoked as a major source of damage (e.g., Li et al., 1996), and the fact that both sucrose and trehalose are non-reducing sugars may explain at least partly why they are the natural products accumulated by anhydrobiotic organisms. However, the glycosidic bonds linking the monomers in sucrose and trehalose have very different susceptibilities to hydrolysis (reviewed in O’Brien et al., 1996; Schebor et al., 1999). For instance, the activation energy for acid hydrolysis in aqueous solution is nearly twice that for other disaccharides (Fig. 3). When O’Brien (1996) and subsequently Schebor et al. (1999) incubated a freeze dried model system (albumin, with the addition of lysine) with sucrose, trehalose, and glucose at relative humidities in excess of 20%, the rate of browning seen with sucrose approached that of glucose—as much as 2,000 times faster than that with trehalose, although they observed a distinct lag in the onset of browning (Fig. 4). Schebor et al. (1999) found that a peak in the appearance of monosaccharides occurs prior to the onset of browning, after which free monosaccharides decline, coincidentally with the onset of browning (Fig. 4). These observations strongly suggest that the browning seen with sucrose—but not with trehalose—is due to hydrolysis of the glycosidic bond during storage.

The glassy state is undoubtedly related to these effects; if the samples are stored at very low humidities only minimal amounts of hydrolysis and subsequent browning were seen in the sucrose preparations (O’Brien, 1996; Schebor et al., 1999). Nevertheless, since sucrose is the major sugar associated with desiccation tolerance in higher plants, consideration of the mechanisms by which devitrification at moderate water contents and hydrolysis of the glycosidic bond in sucrose glasses are obviated in anhydrobiotic plants is instructive.

**SUGAR GLASSES IN PLANT ANHYDROBIOTES**

Buitink has published an elegant series of studies of the properties of glasses in vivo in anhydrobiotic plants (Buitink et al., 1996, 2000), along with a superb review of the work (Buitink and Leprince, 2004). Briefly, Koster (1991) found that mixtures of sugars similar to those found in desiccation tolerant corn embryonic axes [85% (w/w) sucrose, 15% (w/w) raffinose]
formed glasses at temperatures above 0°C, while sugar mixtures similar to those found in desiccation-sensitive axes [75% (w/w) glucose, 25% (w/w) sucrose] formed glasses only at subzero temperatures. These and similar data suggested that sugar mixtures form glasses in plant anhydrobiotes, but subsequent studies indicated that the intracellular glasses are not composed of sugars alone. The state diagram for intact pollen of cat-tail at first glance seems to agree reasonably well with that for sucrose (the major sugar in these pollen grains). However, subtle differences can be seen that have turned out to be significant: at low water contents Tg in the intact pollen is higher than predicted based on the state diagram for the sugar, while at low water contents it is lower than predicted (Buitink et al., 1996). Furthermore, the temperature at which the glass collapses (Tc), which occurs several degrees above Tg, is elevated by as much as 40°C in intact pollen and other anhydrobiotes (Buitink et al., 2000; reviewed in Buitink and Leprince, 2004). The outcome of these studies is a clear indication that glasses in intact anhydrobiotic plants are not composed simply of mixtures of sugars.

Wolkers et al. (1998a) developed a powerful approach based on infrared spectroscopy that permitted characterization of cytoplasmic glasses. The measurement—vibrational frequency of the O–OH stretch in sugars—permitted an estimate of the length and strength of hydrogen bonds within the glass. Using this technique, Wolkers et al. (1998b, 1999) found that the molecular density of the cytoplasm resembled that of protein glasses more than that of sucrose, a finding that initially suggested that sucrose may be a relatively minor player in formation of the cytoplasmic glass. However, studies on molecular motion in protein glasses have shown that rotational mobility of the proteins is almost twice that seen in the cytoplasmic glass (Buitink et al., 2000). The conclusion is that the cytoplasmic glass is likely to consist of a mixture of sucrose and proteins. The most likely candidates for the protein component are the late embryogenesis abundant (LEA) proteins that are accumulated in seeds and pollen late in development, and there is some evidence suggesting that this is the case. Walters et al. (1997) showed that when extracts are made from wheat embryos sucrose in large amounts was co-isolated with the LEA proteins. Exhaustive dialysis removed only a fraction of the sucrose, indicating that it is tightly bound to the protein. Subsequently, Wolkers et al. (2001) isolated a specific LEA protein that increased Tg of sucrose by about 20°C and altered the molecular packing so that it more closely resembled that seen in cytoplasmic glasses.

The conclusion from these studies is that at least in plants cytoplasmic glasses consist of sugar-protein mixtures. The apparent elevation of Tg and the collapse temperature by addition of the protein to the glass is likely to lead to increased stability of the kind seen in trehalose alone in vitro, owing to its elevated Tg. Thus, devitrification at moderate water contents is obviated. The problem of stability of the glycosidic bond in sucrose during storage in the dry state is somewhat more problematic, but it seems likely that the association with the protein fraction, leading to the elevated Tg, could limit accessibility of water to the bond, thus limiting hydrolysis. In any case, the possibility that proteins may be involved in stabilization of cells in the dry state is a lesson from nature that has not been explored extensively as yet, but one that we will raise again in the section on nucleated cells.

Lessons from Nature Can be Used to Preserve Intact Cells in the Dry State

Clearly, trehalose must be introduced into the cytoplasm of a cell if it is to be effective at stabilizing intracellular proteins and membranes during dehydration. Previous efforts centered around this fundamental problem involving molecular engineering have not been particularly successful (Eroglu et al., 2000; Guo et al., 2000). More recently, Wolkers et al. (2001a) made the surprising discovery that when human blood platelets are placed in the presence of modest amounts of trehalose, they take it up by fluid phase endocytosis, and the intact sugar ends up in the cytoplasm. Wolkers et al. were able to show only indirectly that trehalose is in the cytoplasm, but subsequent studies have shown that this is so. Oliver et al. (2004) have followed the fate of the sugar once it enters the endocytotic pathway in a stem cell line, using fluorescence microscopy, with the results summarized in Figure 5. The fluorescence initially appears in vesicles, but with time it becomes diffuse, suggesting that the sugar is released into the cytoplasm, although the mechanism of release is not entirely clear. The endocytotic vesicles progress through the normal pathway to lysosomes. It is well established that low molecular weight compounds such as glucose readily cross the lysosomal membrane into the cytoplasm (Lloyd, 2000), but there is very little evidence concerning the fate of disaccharides in lysosomes. Lloyd (2000) showed that incubation of cells in sucrose led to persistent vesiculation, suggesting that, apparently unlike trehalose, sucrose is retained in the lysosomes. This seems surprising because even though lysosomes lack invertase (Lloyd, 2000) and thus cannot break the glycosidic bond in sucrose enzymatically, the glycosidic bond in sucrose should be hydrolyzed at the pH known to occur in lysosomes, while that of trehalose should not. Thus, if anything, one would expect that the sucrose, broken down into component glucose and fructose, should cross the lysosomal membrane into the cytosol by means of the glucose carrier, while the still intact trehalose should be retained. This matter is unresolved, but we suspect that the lysosomal pH itself might lead to leakage; when liposomes loaded with a polar fluorescent marker about the same size as trehalose were subjected to lysosomal pH, the marker leaked across the bilayer into the external medium (Hays et al., 2001). We suggest that the pH gradient across the lysosomal membrane might lead to leakage of trehalose and other low
molecular weight molecules into the cytosol. At any rate, we have found that trehalose can be introduced into the cytoplasm of every cell we have tested, so long as the cell has a functional fluid phase endocytotic pathway.

**SUCCESSFUL FREEZE-DRYING OF TREHALOSE-LOADED CELLS**

We have recently reported successful freeze-drying of platelets, with a detailed discussion of the procedure, which results in survival exceeding 90% (Wolkers et al., 2001a). We started this project at the invitation of the Department of Defense, where there is an obvious need for platelets for use in severe trauma cases. At present, platelets are stored in blood banks for a maximum of 3–5 days, by Federal regulation, after which they are discarded. Furthermore, the platelets are stored at room temperature; they cannot even be refrigerated without rendering them useless therapeutically, a phenomenon for which we have provided an explanation (Tablin et al., 1996, 2001; Oliver et al., 1999; Crowe et al., 1999, 2001, 2003; Tsvetkova et al., 2000). There is a chronic shortage of platelets in hospitals, and field hospitals operated by the military rarely have access to platelets at all. Thus, prolonging the shelf life of platelets would be a valuable contribution. The freeze-dried platelets have the following properties: (a) The dry platelets are stable for at least two years when stored at room temperature, under vacuum. During that time we have seen no loss of platelets. (b) The freeze-dried, rehydrated cells respond to normal platelet agonists including thrombin, ADP, collagen, and ristocetin. (c) Studies on the morphology of the trehalose-loaded, freeze-dried, and rehydrated platelets show that they are affected by the drying, but are morphologically similar to fresh platelets (Fig. 6). When they were dried without trehalose, on the other hand, most of the platelets disintegrated during the rehydration event, but of the small number that were left, most had fused with adjacent cells, forming an insoluble clump. (d) We have extended the freeze-drying to mouse and pig platelets as animal models for in vivo testing.

The rehydrated platelets are far from perfect, but they nevertheless show surprisingly good regulation of key elements of cellular physiology such as intracellular pH (Tang et al., in preparation) and calcium (Auh et al., 2004). For instance, when fresh platelets are

![Fig. 5. Proposed mechanism for loading disaccharides into cell, based on data of Oliver et al., 2004 and unpublished data of Auh et al. A. endocytic vesicle. B. secondary lysosome. Trehalose (solid double dots, representing two glucose monomers) and sucrose (open and solid mixed dots, representing glucose and fructose monomers) enter the cell by fluid phase endocytosis (A) and are passed down the lysosomal pathway (B). We suggest that the stability of the glycosidic bond in trehalose will permit trehalose to survive at lysosomal pH, but that sucrose might be hydrolyzed. We further propose that trehalose, sucrose, glucose, and fructose will all leak into the cytoplasm due to the known effects of pH on permeability of phospholipid bilayers (Hays et al., 2001).](image)

![Fig. 6. Fresh dog platelets (left) and dog platelets that had been loaded with trehalose, freeze-dried, and rehydrated (right).](image)
challenged with thrombin they show an increase in 
Ca_i] that is dose dependent. The rehydrated platelets 
show a similar response, although it is strongly atten-
uated. Nevertheless, the increase in [Ca_i] appears to be 
sufficient to trigger morphological and physiological 
changes necessary for coagulation (Auh et al., 2004).

CAN NUCLEATED CELLS BE STABILIZED IN THE 
DRY STATE?
Platelets are admittedly specialized cellular frag-
ments, so it seemed likely at the outset that the single 
perturbation of adding trehalose might not be sufficient 
to stabilize more complex living cells. Indeed, this ap-
pears to be the case. Ma et al. (2005) have recently 
found that when 293 cells were dried without prior 
loading with trehalose by the fluid phase endocytosis 
described previously they all died at fairly high water 
contents (Fig. 7). When they were loaded with treha-
lose survival was extended considerably, but the cells 
nevertheless died when water content was reduced be-
low about 0.3 g H_2O/g dry wgt (Fig. 7). Thus, we have 
begun studies on effects of stress proteins on improv-
ing survival of nucleated cells at lower water contents 
and MacRae, 1999; Day et al., 2003; Ma et al., 2005).
In addition, p26 has been shown to protect synergis-
tically with trehalose in vitro conditions (Viner and 
Clegg, 2001) or when loaded artificially into mam-
malian cells (Collins and Clegg, 2004). Along the 
same lines, Singer and Lindquist (1998a, b) previously 
showed that trehalose acts synergistically with heat 
shock proteins in protein folding.

Sun et al. (2004) isolated and cloned the gene for 
this protein and transfected 293 cells with it. They gen-
erously supplied us with the transfected cells. To our 
surprise, this protein significantly improved the sur-
vival to low water contents (Fig. 7), even though the 
levels of expression have been very low—much less 
than that reported by Clegg for Artemia cysts (e.g., 
Collins and Clegg, 2004). The protein alone does noth-
ing to improve survival; trehalose is required as well, 
and the two appear to act synergistically. The effects 
of expression of this gene become even more pro-
nounced in the time following rehydration; the cells 
expressing p26 showed a ten fold increase in colony 
growth over those without the protein. Metabolism, 
expressed in terms of Alamar blue reduction, improved 
at least five fold compared with cells dried with tre-
halose alone (Fig. 8).

WHAT IS THE ROLE OF P26 IN STABILIZING DRY 
NUCLEATED CELLS?
One hypothesis is that p26 participates in modulat-
ing the structure of the sugar glass, as suggested from 
the findings of Wolkers (2001b) and Buitink and Le-
prince (2004). However, the expression levels are so 
low in this case that we doubt that the protein would 
have much effect on the glass. We favor instead the 
catalytic-like functions ascribed to stress proteins. A 
possible role for heat shock proteins in the protection 
of mammalian cells during dehydration stress has not
been addressed, but there are indications from other organisms implicating HSPs in this regard. For example, a drought-resistant form of maize expresses a 45 Kd HSP that is not found in drought-sensitive lines (Ristic et al., 1996). Further, crossing the drought-resistant and sensitive lines led to F2 plants in which tolerance to soil drying was associated with expression of the 45Kd HSP (Ristic et al., 1998). In addition, the flesh fly Sarcophaga crassipalpis expresses two inducible HSPs (HSP23 and HSP70) during dehydration of non-diapausing pupae (Hayward et al., 2004). In diapausing pupae, these proteins are already highly expressed, and desiccation does not cause a further increase in expression.

The most important mechanism by which heat shock proteins protect cells from various stresses has traditionally been considered the protein chaperone function, assisting nascent and misfolded proteins to gain their proper folded configuration (Hartl and Hay-er-Hartl, 2002; Barral et al., 2004). However, an association of stress proteins with membranes has more recently been described, including work from our laboratory (Trent et al., 2003; Torok et al., 2001, 2003; Tsvetkova et al., 2002). In fact, the “membrane trigger” hypothesis suggests that the membrane may serve as an indicator, sensing the initial stress and leading to the expression of heat shock proteins within the cell (Vigh et al., 1998; Horvath et al., 1998). Finally, in addition to the protein and membrane effects, heat shock proteins have also been implicated in the inhibition of apoptosis (Beere and Green, 2001; Concan-non et al., 2003; Samali and Orrenius, 1998) and ox-idative damage (Collins and Clegg, 2004; Downs et al., 1999; Gill et al., 1998; Park et al., 1998). In fact, we already have some evidence that apoptosis is a major problem in rehydrated nucleated cells (Zhu et al., in preparation), so this seems to be a reasonable hypothesis to pursue first.

SUMMARY AND CONCLUSIONS

Under ideal conditions for drying and storage, trehalose is probably no more effective than other oligosaccharides at preserving biomaterials. However, under suboptimal conditions it can be very effective and is thus still a preferred excipient. There is growing evidence that additional modifications to the cellular milieu will probably be required if we are to achieve a stable, freeze-dried mammalian cell, including expression of stress proteins, as reported here, and administration of antioxidants and inhibitors of enzyme activity, as described elsewhere (Oliver et al., 2002). Thus, we have come full circle over the past decades on the requirements for stabilization of cells in the dry state; 34 years ago, we suggested that survival of living cells in the dry state is a complex phenomenon that is likely to involve multiple adaptations (Crowe, 1971). With the discovery that membranes and proteins (reviewed in Crowe et al., 1987), and human platelets (Wolkers et al., 2001) can be stabilized by the single perturbation of adding trehalose, we suggested that this single lesson from nature might be sufficient (Crowe et al., 1987), at least under ideal storage conditions. However, the studies on nucleated cells summarized here indicate that the original viewpoint on this matter (Crowe, 1971) is most likely the correct one.

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