Chapter 11
Anhydrobiosis: An Unsolved Problem with Applications in Human Welfare

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Abstract Anhydrobiosis (Life Without Water) has been known for millennia, but the underlying mechanisms have not been understood until recent decades, and we have achieved only a partial understanding. One of the chief sites of damage from dehydration is membranes, and we and others have provided evidence that this damage may be obviated by the production of certain sugars, particularly trehalose. The sugar stabilizes membranes by preventing fusion and fluidizing the dry bilayers. The mechanism by which this is accomplished has been controversial, and I review that controversy here. In the past decade evidence is accumulating for a role of stress proteins in addition to or as a substitute for trehalose. Genomic studies on anhydrobiotes are yielding rapid progress. Also in the past decade, numerous uses for trehalose in treating human diseases have been proposed, some of which are in clinical testing. I conclude that the mechanisms underlying anhydrobiosis are more complex than we thought 20 years ago, but progress is being made towards elucidating those mechanisms.

Keywords Anhydrobiosis • Trehalose • Microdomains • Water entrapment

11.1 Introduction

Investigations on the phenomenon of anhydrobiosis ("life without water") have a long history, dating back to the time of Leeuwenhoek (see Keilin 1959, for a fascinating, scholarly review of the old history). Nevertheless, the underlying mechanisms by which a wide array of organisms such as seeds of many plants, yeasts, mosses, cysts of crustaceans, and certain microscopic animals such as nematodes, rotifers, and tardigrades all survive loss of essentially all of their water without being killed were completely unknown until the last few decades. The first concrete suggestion concerning the mechanism came from studies on anhydrobiotic nematodes (reviewed in Crowe and Hoekstra 1992). The worms with which these

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E.A. Disalvo (ed.), *Membrane Hydration*, Subcellular Biochemistry 71,
DOI 10.1007/978-3-319-19060-0_11

studies were done must be dried slowly, during which time they synthesize large quantities of the disaccharide, trehalose. There is a strong correlation between survival in the dry state and the production of trehalose. This sugar had been known to be present at high concentrations in a large number of anhydrobiotes, but it was thought to be a metabolic storage product, and had not been implicated as having a role in anhydrobiosis. Subsequent investigations established a similar correlation between survival of many organisms in anhydrobiosis and the presence of disaccharides, usually trehalose or, in the case of higher plants, sucrose.

Studies with model systems (isolated biological membranes, liposomes, and proteins) showed that trehalose has a remarkable ability to preserve these biomolecular assemblages in the dry state (reviewed in Crowe 2008). Physical studies on the dry materials indicated a mechanism by which it does so; it depresses the gel to liquid crystalline phase transition in dry phospholipids, often by values approaching 100 °C (reviewed in Crowe 2008). In other words, the presence of the sugar fluidizes dry phospholipid bilayers and mimics the presence of water. We first reported this phenomenon more than 30 years ago (Crowe et al. 1984), but the mechanism by which the sugar depresses Tm is still not entirely clear. Based on spectroscopic evidence presented in the original report, we suggested that the sugar hydrogen bonds to polar residues in dry phospholipids (mainly the phosphate of the headgroup), which changes their lateral spacing, much as water does. This mechanism is known as the water replacement hypothesis (reviewed in Crowe 2008).

Thus, because the sugar has these effects both in vitro and in vivo and because its presence was correlated with survival of anhydrobiosis, we suggested some decades ago that a central role for the sugar in anhydrobiosis seemed to be reasonable and remains so to this day, at least for many, but not all, anhydrobiotes. Nevertheless, the role of trehalose has been based on such correlations or on in vitro data, which in themselves are not convincing that it is important in intact cells and organisms. Furthermore, a key part of this phenomenon is the mechanism by which the sugar depresses the transition temperature, which has come under some considerable discussion. I have summarized more recent findings in this regard in sections following.

Is trehalose special in its ability to preserve biomolecules and assemblages? Yes and no. Other sugars can be just as effective, under special conditions. Trehalose works particularly well for the following reasons: (a) the glycosidic bond linking the two glucose monomers is remarkably stable, far more stable than the bond in sucrose, for example (Schebor et al. 1999); (b) trehalose forms a glass in the dry state, which is essential to its ability to stabilize biomaterials. The temperature at which this glass undergoes its transition to a more fluid state is remarkably high for trehalose—much higher than in comparable sugars. The significance of the glass in vivo has been demonstrated a number of times (see Hengherr et al. 2009 for an interesting example). If conditions are such that the glycosidic bond is protected and the temperature is low enough to maintain the glassy state other sugars can be just as effective as trehalose.

Trehalose has been used successfully to preserve mammalian blood platelets (Wolkers et al. 2001), with excellent recovery and functionality in the rehydrated cells (e.g. Auh et al. 2004). Several groups have tried to use trehalose to preserve nucleated cells in the dry state, and it became apparent that adding trehalose alone was not sufficient, as I will describe below. The cells survived drying initially, but they rapidly died. However, when they were transfected with the gene for a stress protein obtained from anhydrobiotic cysts of a crustacean, Artemia, survival was improved, and the ability of the rehydrated cells to reproduce was elevated dramatically (Ma et al. 2005; Zhu et al. 2006). The stress protein alone was insufficient to protect the cells during drying, but the stress protein and trehalose acted synergistically to do so. Clearly, there are requirements in addition to trehalose to stabilize nucleated cells in the dry state. Recent studies on the apparent synergism between trehalose and stress proteins will be discussed in a following section.

In some anhydrobiotic microorganisms only small amounts of disaccharides have been detected (Tunnacliffe et al. 2005; reviewed in Wharton 2014), suggesting that there must be alternative ways to achieve the same end as those seen with the sugars present. It is not clear yet just what those means might be, but several groups around the world are seeking elucidation of what might be the significant adaptations, using genomics and proteomics (e.g. Leprince and Buitink 2010; Terrasson et al. 2013; Dupont et al. 2014). These powerful methods look well beyond the rather primitive approaches we were using decades ago in which we looked for metabolic products that are downstream from the transcripts and translated products that are now being detected. This approach has been remarkably productive, as I will describe below.

11.2 Lipid Phase Transitions in Membranes and Anhydrobiosis

11.2.1 An Example of Effects of Trehalose on Dry Phospholipids

Most of the following was reviewed in detail in Crowe (2008). Liposomes were prepared from a lipid with low *T*m, palmitoyloleoylphosphatidylcholine (POPC), with a fluorescent marker, carboxyfluorescein trapped in the aqueous interior. When the liposomes were freeze-dried with trehalose and rehydrated, the vesicles were seen to be intact, and nearly 100 % of the carboxyfluorescein was retained. It quickly emerged that stabilization of POPC liposomes, and other vesicles prepared from low-melting-point lipids, had two requirements, inhibition of fusion between the dry vesicles and depression of *T*m in the dry state. In the hydrated state, *T*m for POPC is about −1 °C and rises to about +70 °C when it is dried without trehalose. In the presence of trehalose, *T*m is depressed in the dry state to −20 °C. Thus, the lipid is maintained in the liquid-crystalline phase in the dry state, and phase transitions are not seen during rehydration at room temperature.

The significance of this phase transition during rehydration is that when phospholipids pass through such transitions, the bilayer becomes transiently leaky, which resembles the effects resulting from passage through a main phase transition in fully hydrated membranes during changes in temperature, as discussed earlier. The physical basis for this leakiness has been investigated in some detail by Hays et al. (2001). Thus, the leakage that normally accompanies this transition must be avoided if the contents of membrane vesicles and whole cells are to be retained. During drying, leakage is probably not a problem because *T*m is not affected until all the bulk water has been removed. But during rehydration, it is a serious problem; the membranes are placed in water and will undergo the phase transition in the presence of excess bulk water, thus allowing leakage. In addition to the damage that occurs during passage through the phase transition, and perhaps even more importantly in the present context, phase separation of membrane components can occur in the absence of trehalose during drying, as the membranes undergo the transition into the gel phase, an event that is often irreversible.

Low-temperature-melting lipids such as POPC all seem to behave as described previously; *T*m is depressed to a minimal value immediately in the presence of trehalose after drying, independent of the thermal history. Saturated lipids with high *T*m, such as DPPC, behave quite differently, and effects of trehalose on *T*m depend strongly on the thermal history. When *T*m in DPPC dried without trehalose is measured, it is seen to rise from 41 °C in the hydrated lipid to 110 °C when it is dried. In the presence of trehalose, *T*m is about 60 °C until the acyl chains are melted once, after which *T*m is depressed to 24 °C (Crowe 2008). If the lipid is then incubated at temperatures <24 °C, *T*m rapidly reverts to about 60 °C. Thus, the stable *T*m renders DPPC in the gel phase at physiological temperatures, regardless of whether it is hydrated or dry. This effect may have special relevance for biological membranes, because microdomains contain lipids with elevated *T*m.

11.2.2 Trehalose Maintains Microdomains in Dry Membranes

We have carried out modeling studies in an attempt to discover whether trehalose can maintain the structural integrity of rafts in the dry state and, if so, to elucidate the mechanism. Because the phase behavior of the binary mixture DLPC/DSPC is so well characterized in the hydrated state, we started there, rather than with the more complex mixture seen in a native membrane. Using deuterated DPPC and hydrogenated DLPC, it has been possible to monitor phase behavior of the two lipids in a 1:1 mixture, using FTIR, with the following results: (i) the freshly prepared fully hydrated mixture is completely phase-separated in the gel/liquid-crystalline coexistence temperature regime, as expected; (ii) when this mixture is dried without trehalose, mixing occurs; (iii) if the mixture is dried with trehalose, the lipid phase separation is maintained, although a small fraction of the DLPC is mixed with the DSPC; and (iv) in the mixture dried with trehalose, most of the DLPC fraction has a transition below 0 °C in the dry state, while the DSPC transition is seen at about 80 °C (Leidy et al. 2004).

We propose that trehalose maintains phase separation in this mixture of lipids in the dry state by the following mechanism (Ricker et al. 2003). The DLPC fraction, with its low *T*m in the hydrated state, might be expected to behave like unsaturated lipids described earlier, in that *T*m in the dry state is reduced to a minimal and stable value immediately after drying with trehalose, regardless of the thermal history. That appears to be the case. The DSPC fraction, by contrast, would be expected to be in the gel phase in the hydrated state at room temperature, and it remains in the gel phase when it is dried with trehalose. In other words, we are proposing that by maintaining one of the lipids in the liquid-crystalline phase during drying, while the other remains in the gel phase, trehalose maintains the phase separation. We suggest that this is the fundamental mechanism by which trehalose maintains microdomains in native membranes during drying (Ricker et al. 2003). By maintaining the liquid-ordered/liquid-crystalline microdomain structure that we observed in platelets during drying and rehydration, trehalose could preserve the small-scale phase separation that appears to be important for membrane function and thus prevent macroscopic phase separation.

Moiset et al. (2014) recently presented data that would appear to be in conflict with the mechanism proposed above. They found both by experiment and modeling that trehalose and sucrose destabilize phase separated model membranes and lead to mixing of the components. However, these studies were all done in the presence of excess water. Furthermore, the mixing effect was seen at high sugar concentrations, in the molar range. The effects of trehalose on stabilizing domains were seen at low sugar concentrations, and the studies were done in the dry state, which is a completely different thing.

Thus, it is likely that effects of sugars on thermal transitions in the dry state are a central factor in stabilizing membranes in the absence of water, and the mechanism by which this is accomplished is critical to our understanding of this phenomenon.

11.3 How Does Trehalose Depress *T*m in the Dry State?

The mechanism of depression of *T*m has received a great deal of attention since the discovery of this effect. Three main hypotheses have emerged:

11.3.1 The Water Replacement Hypothesis

The water replacement hypothesis suggests that sugars can replace water molecules by forming hydrogen bonds with polar residues, thereby stabilizing the structure in the absence of water (Crowe 2008). Direct interaction, on the other hand, has been demonstrated by a wide variety of physical techniques, including IR spectroscopy (Crowe et al. 1984; Tsvetkova et al. 1998), NMR (Lee et al. 1986; Tsvetkoa et al. 1998; Wolkers et al. 1998), and X-ray (e.g. Nakagaki et al. 1992). Theoretical

analyses have contributed greatly to this field in recent years. Chandrasekhar and Gaber (1988) and Rudolph et al. (1990), in the earliest studies, showed that trehalose can form energetically stable conformations with phospholipids, binding three adjacent phospholipids in the dry state. Sum et al. (2003) showed by molecular simulations that the sugars adapt molecular conformations that permit them to fit onto the surface topology of the bilayer through hydrogen bonds. The sugars interact with up to three adjacent phospholipids. Golovina et al. (2010) confirmed these results with molecular simulations and showed that trehalose increases the area per lipid in the dry state under conditions that seem to be inconsistent with any model that does not require direct interaction between the sugar and polar head group. Modifications of the gel state of hydrated phospholipids by trehalose can only be achieved if a drastic dehydration is performed in the presence of the sugar. The results suggest that trehalose is still intercalated between the phospholipids after restoring water to the dried liposomes either at temperatures below or above the phase transition (Viera et al. 1993).

The water activity in dimyristoylphosphatidylcholine (DMPC) decreases by 60 % when the lipid is dehydrated in the presence of trehalose concentrations higher than 0.02 M. FTIR in these conditions indicated that trehalose binds to the carbonyl groups, replacing 11 of 14 water molecules per lipid molecule. About four are displaced by changes in the water activity of the bulk solution, and seven by specific interactions with the phospholipids. In this last case, at least two of them are linked to the carbonyl group. This appears to be the cause of the decrease in the dipole potential of lipid monolayers spread on an air/water interface from 480 mV in pure water to 425 mV in 0.1 M trehalose (Luzardo et al. 2000).

Molecular dynamics simulations from this same group of investigators showed that trehalose binds to the phospholipid headgroups with its main axis parallel to the membrane normal. It establishes hydrogen bonds with the carbonyl and phosphate groups and replaces water molecules from the lipid headgroup. Notably, the number of hydrogen bonds that the membrane made with its environment was conserved after trehalose binding. The H-bonds between lipid and trehalose have a longer lifetime than those established between lipid and water. The binding of the sugar does not produce changes either in the lipid area or in the lipid order parameter. The effect of trehalose on the dipole potential is in agreement with experimental results. The contribution of the different components to the membrane dipole potential was analyzed. It was observed that the binding of trehalose produces changes in the different components and the sugar itself contributes to the surface potential due to the polarization of its hydroxyl in the interface (Villarreal et al. 2004).

11.3.2 The Water Entrapment Hypothesis

The water entrapment hypothesis suggests that sugars concentrate water near surfaces, thereby preserving its salvation (Belton et al. 1994; Cottone et al. 2002; Lins et al. 2004). While there is some evidence, both experimental (Crowe et al. 1987) and modeling (Golovina et al. 2010) that significant amounts of water are

retained in "dry" membranes in the presence of trehalose, there is little evidence that there is sufficient water to depress Tm to the levels seen experimentally. Indeed, since Tm is often well below that of the fully hydrated lipid it is difficult to see how residual water can be responsible for depression of Tm to such a level. That is not to say that a few water molecules around the headgroup are not important, as well they might be for the details of the interaction.

11.3.3 The Hydration Forces Explanation

The hydration forces explanation invokes the mediation of the forces between bilayers by the sugars as membranes come close together, and the subsequent reduction in the induced lateral compression in the plane of the bilayer responsible for deleterious phase transitions (Bryant et al. 1992, 2001; Koster 2001). Lenne et al. (2009) reported that The presence of sugars has no effect on the average spacing between the phospholipid chains in either the fluid or gel phase. Using this finding, they observed that for low sugar concentrations only a small amount of sugar exclusion occurs and that under these conditions, the effects of sugars on the membrane transition temperatures can be explained quantitatively by the reduction in hydration repulsion between bilayers due to the presence of the sugars. They suggested that specific bonding of sugars to lipid headgroups is not required to explain this effect. More recently, Kent et al. (2014) used neutron diffraction to localize deuterated trehalose between opposing bilayers. According to the water replacement hypothesis the trehalose should be found preferentially in association with the polar headgroups. Instead, Kent et al. (2014) reported that the sugar was distributed predominantly in the aqueous phase between adjacent bilayers. This finding would seem to be in disagreement with the proposal for direct interaction with the headgroups. However, since the measurements were done in excess water it is difficult to extend these results to exclude direct interaction between trehalose and dry bilayers. Furthermore, since all these studies were done with multilamellar vesicles, for technical reasons the results cannot easily be compared with those done with unilamellar vesicles. In the former, the solutes are restricted to confined spaces, as opposed to the single bilayer to bilayer seen in unilamellar vesicles used in the studies described above. Furthermore, solutes can be excluded from multilamellar vesicles, as Koster et al. (2003) reported. But such exclusion may be absent in unilamellar vesicles, which has led to some confusion. For example, when unilamellar vesicles are dried with a large polymer fusion during drying was strongly inhibited (Crowe et al. 1998). It is difficult to suppose that the polymers and the vesicles are in different phases under these conditions since fusion is inhibited. Since Tm in the dry lipids is not affected (Crowe et al. 1998), this finding seems clearly inconsistent the hydration forces explanation. Such a polymer is excluded from the interbilayer spaces in multilamellar vesicles and thus would not be expected to have the same effects.

A consensus is slowly emerging that these three mechanisms might not be mutually exclusive. First, in the dry state, vitrification may occur simultaneously with direct interactions between the sugar and polar residues, that is, the sugar and phospholipids form a glass together, with direct interaction between the lipid and sugar, which seems consistent with all three hypotheses. The situation for hydrated systems is perhaps more complex, but data from Andersen et al. (2011) suggest a solution to this apparent disagreement. They reported data using small angle neutron scattering and thermodynamic measurements that seem to show that sugars may be either bound or expelled, depending on the concentration of sugar. At low concentration, small sugars bind quite strongly to a lipid bilayer, and the accumulation of sugar at the interface makes the membrane thinner and laterally expanded, apparently in agreement with the water replacement hypothesis. Above ~0.2 M the sugars gradually become expelled from the membrane surface, and this repulsive mode of interaction counteracts membrane thinning. The dual nature of sugar–membrane interactions, Andersen et al. suggest, offers a reconciliation of conflicting views in earlier reports on sugar-induced modulations of membrane properties.

I suggest that the hydration forces explanation probably does apply under the conditions described by Andersen et al. (2011). Indeed, we have reported data on effects of sugars on Tm in fully hydrated systems that are consistent with this viewpoint (Crowe and Crowe 1991). However, when the bulk water is removed the solute must come in contact with the bilayer surface; there is no water left into which the sugar can partition. At this point I believe the matter is still unresolved, mainly because of technical differences in the way the studies have been done.

11.4 Is Trehalose Necessary and Sufficient for Cellular Stabilization in Anhydrobiotes?

Nearly 40 years ago we reported that a nematode, *Aphelenchus avenae* can be induced to enter an anhydrobiotic state by slowly drying them over a three day period. During the slow dehydration the animals synthesized trehalose, and the ability to survive more extensive water loss was strictly correlated with this synthesis. Similar studies have been reported by Watanabe et al. for (2002) and Mitsumasu et al. (2010). These animals are of special interest because they are the largest anhydrobiotes known In vitro studies by numerous investigators showed the ability of trehalose to stabilize dry biological structures, which led to the widely accepted paradigm that trehalose is a key factor in anhydrobiosis. Nevertheless, that paradigm was based on correlations without convincing evidence for a role *in vivo*. Several groups have addressed that need in recent years, as follows.

11.4.1 Genetic Models

In a remarkable series of recent papers, a group of investigators at research institutes in Dresden have made rapid progress in this regard in the last 3 years. First, Erkut et al. (2011) have shown that trehalose is absolutely required for survival in a nematode and that mutants lacking the ability to synthesize the sugar did not survive even mild dehydration. They used the popular genetic model organism *Caenorhabitidis* as a model organism and reported that the dauer larva is a true anhydrobiote: under defined conditions it can survive even after losing 98 % of its body water. This ability is correlated with a several fold increase in the amount of trehalose. Mutants unable to synthesize trehalose cannot survive even mild dehydration. Light and electron microscopy indicate that one of the major functions of trehalose is the preservation of membrane organization. Fourier-transform infrared spectroscopy of whole worms suggests that this is achieved by preserving the native packing of lipid acyl chains. In the next paper in this series Erkut et al. (2013) used microarray analysis, proteomics, and bioinformatics to identify genes, proteins, and biochemical pathways that are upregulated during the induction process. These pathways were validated by testing the desiccation tolerances of mutants. The data suggest that the desiccation response is activated by sensing the desiccative environment via neurons, leading to elimination of reactive oxygen species and xenobiotics, expression of heat shock and intrinsically disordered proteins, polyamine utilization, and induction of fatty acid desaturation pathways. Erkut et al. (2013) suggest that this response is specific and involves a small number of functional pathways. They make the remarkable suggestion that these pathways represent the genetic toolkit for anhydrobiosis in both animals and plants. In the most recent paper in the series, Abusharkh et al. (2014) showed that, in addition to trehalose accumulation, the dauer larvae reduce their phosphatidylcholine (PC) content. Using Langmuir – Blodgett monolayers, they found that phospholids from preconditioned larvae with reduced PC content exhibit a higher trehalose affinity, a stronger hydration-induced gain in acyl chain free volume, and a wider spread of structural relaxation rates of their transitions and headgroup H-bond interactions. I suggest that this genetic approach, using this well-defined model, will continue to provide powerful evidence concerning the fundamental mechanisms underlying anhydrobiosis.

Using another well-defined genetic model, the yeast using Saccharomyces cerevisiae, Tapia and Koshland (2014) showed that intracellular trehalose is essential for survival to long-term desiccation, in agreement with previous investigations (e.g. Eleutherio et al. 1993). Maintaining long-term desiccation tolerance consists of a balance of trehalose stockpiled prior to desiccation and trehalose degradation by trehalases in the desiccated cells. The activity of trehalases in desiccated cell reveals a surprising enzymatic activity while desiccated. Interestingly, the protein chaperone Hsp104 compensates for loss of trehalose during short-term but not long-term desiccation. Tapia and Koshland (2014) found that desiccation induces protein misfolding/aggregation of cytoplasmic and membrane proteins and demonstrated

that trehalose but not Hsp104 mitigates the aggregation of both cytoplasmic and membrane prions. They proposed that cells are initially protected against desiccation by both protein and chemical chaperones, like Hsp104 and trehalose, respectively. As desiccation extends, the activities of the protein chaperones are lost because of their complexity and requirement for energy, leaving trehalose as the major protector against the aggregation of cytoplasmic and membrane proteins. Tapia and Koshland (2014) suggest that trehalose is both a more stable and more versatile protectant than protein chaperones, explaining its important role in desiccation tolerance and emphasizing the translational potential of small chemical chaperones as stress effectors.

11.4.2 The Dessicome

Clearly, it is emerging that multiple adaptations, not just trehalose synthesis, are required for survival of anhydrobiosis. A number of investigators around the world are investigating such adaptations, and among the most fruitful approaches are genetic ones, involving microarray analysis to elucidate the chorus of genes and downstream products that are involved. The large suite of genes, proteins, and metabolites involved in protection against dehydration damage and in repair has been called "the desiccome" (Leprince and Buitink 2010; Terrasson et al. 2013). Out of this array of participants, four categories have been recognized, which appear to act synergistically (for reviews see Hand et al. 2011; Hincha and Thalhammer 2012; Hoekstra et al. 2001; Leprince and Buitink 2010; Gaff and Oliver 2013; Terrasson et al. 2013; Tunnacliffe et al. 2010; Forster et al. 2012). Protection is accomplished by: (1) stabilization of proteins by non-reducing sugars, late embryogenesis abundant (LEA) proteins and heat shock protein (HSP); (2) preventing oxidative damage by a range of antioxidant compounds such as tocopherols, glutathione, together with a coordinated response of metabolism during drying; (3) obviating damage from structural stresses imposed by drying such as cell wall modification, reorganization of intracellular membranes and cytoskeleton, vacuolization and chromatin condensation; (4) altering the regulatory mechanisms and signaling pathways controlling the induction of these protective mechanisms.

11.4.3 LEA Proteins

The adaptation that has seen the most intense investigation in recent years is synthesis of the so-called "late embryogenesis abundant" (LEA) proteins, which were discovered in plant embryos, but more recently have been found in a wide variety of other anhydrobiotic organisms (e.g. Tunnacliffe 2007; Hand 2011). Despite the fact that they were discovered many years ago and have now been found to be widespread among anhydrobiotic organisms, the role of LEA proteins

in anhydrobiosis is still not clear, but it appears that they may play a role similar to that ascribed to trehalose. For example, Tolleter et al. (2007) showed that LEAM, a mitochondrial LEA protein expressed in seeds, is a natively unfolded protein, which reversibly folds into a-helices upon desiccation. Structural modeling revealed an analogy with class A amphipathic helices of apolipoproteins that coat low-density lipoprotein particles in mammals. LEAM appears spontaneously modified by deamidation and oxidation of several residues that contribute to its structural features. LEAM interacts with membranes in the dry state and protects liposomes subjected to drying. The overall results suggest that LEAM protects the inner mitochondrial membrane during desiccation. According to sequence analyses of several homologous proteins from various desiccation tolerant organisms, a similar protection mechanism likely acts with other types of cellular membranes. Subsequently, Tolleter et al. (2010) used Fourier transform infrared and fluorescence spectroscopy to gain insight into the molecular details of interactions of LEAM with phospholipid bilayers in the dry state and their effects on liposome stability. LEAM interacted specifically with negatively charged phosphate groups in dry phospholipids, increasing fatty acyl chain mobility. This led to an enhanced stability of liposomes during drying and rehydration, and also upon freezing. Protection depended on phospholipid composition and was strongly enhanced in membranes containing the mitochondrial phospholipid cardiolipin. Collectively, the results provide strong evidence for a function of LEAM as a mitochondrial membrane protectant during desiccation and highlight the role of lipid composition in the interactions between LEA proteins and membranes. Hand (2015) has estimated that the concentration of LEAM required for stabilization of the inner mitochondrial membrane (from Tolleter et al.'s data) closely matches the concentration of LEAM actually observed in mitochondria from another anhydrobiote, *Artemia*, which is reassuring that the suggested role is realistic.

Hand et al. have in the last few years been investigating the role of a number of LEA proteins in *Artemia*, with very instructive results. First, fully hydrated in solution, the CD spectrum of one of the LEAS from *Artemia* exhibited features typical of a disordered, random-coiled protein, but when the protein was dried a shift in α-helix content from 4 % in solution to 46 % was observed. A similar shift in conformation from disorder to order has been seen in a number of LEA proteins from different sources (cf., Tunnacliffe and Wise 2007; Hand et al. 2011b) and certain other stress proteins that have been thought to interact with membranes during chilling or drying (Tomczak et al. 2002; Torok et al. 2003). Such conformational shifts may be key to the ultimate function of these proteins. Hand and Menze (2015) have suggested that the proteins could act as a molecular shield in solution, and the same LEA protein could gain structure as water is removed to further protect the cell in the dry state by interacting with membranes, stabilizing sugar glasses, and forming filamentous networks (Hand et al. 2011; Tunnacliffe and Wise 2007). Hand and Menze (2015) recently studied effects of trehalose and a LEA protein from *Artemia* on stability of a model enzyme, phosphofructokinase, which is very labile; drying with no protection irreversibly reduced enzymatic activity to zero when the enzyme was rehydrated. Addition of trehalose or the LEA protein

improved enzymatic activity by a small amount, but when both trehalose and the LEA protein were added nearly 100 % of the original activity was recovered. Clearly, there is a marked synergism between the trehalose and the LEA protein. However, the effects of the protein appear not to be specific; when BSA was added instead of the LEA protein recovery of enzymatic activity was almost as good. Along the same lines, Li et al. (2012) dried human fibroblasts transfected with genes for two LEA proteins and loaded the cells with trehalose using a trehalose transporter. With nothing added the cells showed loss of al membrane integrity, but with both trehalose and LEA proteins membrane integrity approached 100 %, again suggesting a synergism between the LEA proteins and trehalose. Ma et al. (2005) had previously shown that when fibroblasts were transfected with a stress gene from *Artemia* and loaded with trehalose, survival of drying was very poor, but the cells that did survive showed markedly more robust cell divisions following rehydration if the stress gene was present and being expressed—yet another case of a synergism between trehalose and the stress protein.

In summary, there is a growing body of evidence that proteins associated with stress act synergistically with trehalose or other small molecules in stabilizing biological materials, including intact cells. However, very little is understood about the mechanism by which the proteins and sugar interact to produce these effects.

11.5 Some Surprising Uses for Trehalose in Humans

Since there is a large body of evidence that trehalose is an effective stabilizer of biological materials, some considerable attention is being paid to the possibility that it might be useful in humans, particularly as a therapeutic agent in protein folding and protein aggregation diseases, treatment of corneal dysfunctions, preservation of materials of interest in human welfare, and in cosmetics. I will not to attempt to review the large literature in this rapidly growing field and instead refer the reader to an excellent review by Ohtake and Wang (2011). The following is merely a sampling of what is happening in this area.

11.5.1 Cosmetics

Trehalose is being used in a wide variety of cosmetic products, often with little rationale. However, it is also included in some deodorants as a key ingredient in suppressing human body odor, with good evidence concerning its efficacy. Odor in the elderly is caused by the formation of unsaturated aldehydes such as 2-nonenal and 2-octenal, produced by the degradation of unsaturated fatty acid (palmitoleic acid) in the skin. The body of seniors (>55 years) was sprayed with 2 % trehalose after showering, and 20 h later, the amount of unsaturated aldehydes produced was analyzed from the subjects' shirts. Aldehyde production was seen to be reduced by

70 % in subjects with trehalose. Besides the unsaturated aldehydes, free radicals and HPOs are produced during oxidation of fatty acids and may result in more than foul odor. These products can react with proteins and DNA, resulting in cleaving of DNA chain or its irregular production, potentially leading to deleterious conditions, including cancer, as Ohtake and Wang (2011) suggested. This scenario might be avoided with the use of trehalose, which can suppress the breakdown of fatty acids.

11.5.2 Osteoporosis

Osteoporosis results from an imbalance in bone formation and resorption. There are several causes, but the lack of estrogen production after menopause is considered to be the major contributor. Estrogen replacement therapy is effective in preventing bone loss in animals and humans (Gadducci et al. 1997). However, continuous administration of estrogen is accompanied by adverse side effects (Vessey 1984). The effects of trehalose on bone resorption were studied using ovariectomized mice, with trehalose administered orally five times a week for 4 weeks, and the changes in bone weight and calcium/phosphorous contents were analyzed. Bone weight loss was prevented in a dose-dependent manner (Arai et al. 2001). Furthermore, the increase in osteoclast formation was significantly inhibited by trehalose. Trehalose is unlikely to have an estrogen-like function, and the authors of the study speculated that it suppresses osteoclast differentiation.

11.5.3 Huntington's Disease

The cause of the disease is thought to be the aggregation of mutant Huntingtin protein, characterized by long glutamine repeats. When The efficacy of trehalose in reducing aggregation of a model protein (Tanaka et al. 2004) aggregation was decreased by about 50 %. Trehalose was shown to suppress the formation of aggregates in mammalian cells and to improve their viability. Furthermore, oral administration of 2 % trehalose solution to a transgenic model mice resulted in reduction of aggregate formation in the motor cortex and in improved motor function and survival. In addition, trehalose was reported to be effective in reducing the aggregation of amyloid peptides (Liu et al. 2005) which is a key step in the pathogenesis for Alzheimer's disease. Although *in vitro* studies indicate that trehalose suppresses peptide aggregation, their effects *in vivo* are difficult to interpret, as trehalose administered orally should have been degraded to glucose in the intestine. For most of the studies, glucose was examined as a control and was much less effective than trehalose. how trehalose administration resulted in the reduction of aggregate formation in the brain of rats is a mystery. Nevertheless, the efficacy in inhibiting polyglutamine aggregation may place trehalose as one of the leading candidate as a therapeutic compound for the treatment of HD and other protein

aggregation diseases. In fact, one clinical trial has already commenced (Couzin 2004). There is even a suggestion that trehalose might emerge as a leading therapeutic agent for treating a wide variety of neurogenerative diseases (Emanuele 2014).

11.5.4 Eye Dysfunctions

Matsuo et al. (2004) have shown that trehalose administered directly on the cornea of patients with dry eye syndrome significantly relieved the discomfort. Part of the discomfort appears to be due to loss of smoothness of the cornea due to desiccation. In more recent studies, the same group showed that administration of the trehalose solutions maintains that smoothness (Izawa et al. 2006). Some recent suggestions concerning how this might work include inhibition of fibroblast differentiation by trehalose (thus, inhibiting formation of scar tissue; Takeuchi et al. 2010), direct protection of the corneal epithelium from desiccation damage (Hovakimyan et al. 2012), and inhibition of cytokine mediated inflammation in the cornea (Cejkova et al. 2011). It is unclear how the sugar imparts any of these effects, but it continues to be of interest in ophthalmic applications (e.g. Luyckx and Baudouin 2011).

11.5.5 Trehalose and Autophagy

Administration of trehalose has been reported to stimulate autophagy in several cells types in vitro. Kruger et al. (2012) reported that autophagy can be activated in neurons in vitro expressing the tau protein, which is thought to be involved in formation of the aggregates in Alzheimer's disease. The cells showed depressed levels of tau aggregation and much less cytotoxicity. In a related study, Lan et al. (2012) showed that production of synuclein (a protein related to Parkinson's disease) was suppressed by trehalose induced autophagy. The authors of both these studies suggested that trehalose might be developed as a therapeutic agent for these diseases, but it is, of course, unclear how such a therapy could be developed for clinical application.

11.6 Conclusions

More than 40 years ago, I wrote a review for The American Naturalist (Crowe 1971) with the same title I've given to this commentary. I've recycled the title because in the not too distant past, at the apex of the excitement about trehalose, we thought for a short time that the problem might have been solved. Now it is clear that we were optimistic in this conclusion, and that what we had thought to be a simple solution is not so simple at all. Furthermore, the outgrowth of the field into far flung applications is at once both gratifying and flabbergasting.

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