Mechanisms of plant desiccation tolerance

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Anhydrobiosis (‘life without water’) is the remarkable ability of certain organisms to survive almost total dehydration. It requires a coordinated series of events during dehydration that are associated with preventing oxidative damage and maintaining the native structure of macromolecules and membranes. The preferential hydration of macromolecules is essential when there is still bulk water present, but replacement by sugars becomes important upon further drying. Recent advances in our understanding of the mechanism of anhydrobiosis include the downregulation of metabolism, dehydration-induced partitioning of amphiphilic compounds into membranes and immobilization of the cytoplasm in a stable multicomponent glassy matrix.

‘Drought tolerance’ can be considered as the tolerance of moderate dehydration, down to a moisture content below which there is no bulk cytoplasmic water present [~23% water on a fresh weight basis, or ~0.3 (g H₂O) (g dry weight)⁻¹]. ‘Desiccation tolerance’ generally refers to the tolerance of further dehydration, when the hydration shell of molecules is gradually lost. Desiccation tolerance includes also the ability of cells to rehydrate successfully. In nature, anhydrobiosis often bridges periods of adverse conditions.

Living matter has been characterized as depending on two processes: (1) the biosynthesis of...
the appropriate molecules; and (2) their assembly into organized structures 1. For cellular organization, the hydrophobic effect is crucial. Hence, water is the driving force for the assembly of phospholipids into biological membranes and, in part, for the conformation of many proteins. If water completely dissipates from living matter, the driving force for cellular organization is lost. Membranes then undergo structural changes and proteins denature. Some organisms nevertheless manage to survive periods of severe desiccation, indicating that mechanisms have evolved in nature that allow the native cellular structures to be maintained in the absence of water.

On the basis of the presence or absence of bulk water, the mechanisms of protection can be expected to be different. Whereas the mechanisms conferring drought tolerance are mainly based on structural stabilization by preferential hydration, desiccation tolerance mechanisms are based on the replacement of water by molecules that form hydrogen bonds. Of course, during dehydration, anhydrobiotes pass through hydration ranges that also necessitate protection against drought.

Desiccation tolerance is widespread in the plant kingdom, including ferns, mosses and their spores; pollen and seeds of higher plants; and, rarely, even whole angiosperm, but not gymnosperm, plants 2. The phenomenon also occurs in prokaryotes, protists and fungi and animals such as tardigrades, nematodes and crustaceans. Drought and desiccation tolerance are correlated with the presence of considerable quantities of non-reducing di- and oligosaccharides, compatible solutes and specific proteins, such as the late embryogenesis abundant proteins (LEAs) and heat shock proteins (HSPs). In this article, we focus on the mechanisms of structural stabilization during the different stages of water loss.

When is desiccation tolerance acquired?

The desiccation tolerance program can be switched on by dehydration and the plant hormone abscisic acid 3,4. In the green vegetative tissues of mosses, ferns and angiosperm resurrection plants, it is often a slight dehydration that triggers gene expression associated with desiccation tolerance. In anhydrobiotic (orthodox) seeds, this gene expression occurs during development as a part of the maturation program. As a result, seed embryos become desiccation tolerant considerably before maturation drying. Although the water content gradually decreases during the process of seed maturation, this cannot be considered to be dehydration, because the cellular water potential remains constant up to maturation drying 5. This decrease in water content is caused by a gradual accumulation of dry matter 6,7. Signaling to switch on the desiccation tolerance program in developing seeds occurs via abscisic acid, which also inhibits premature germination. Desiccation tolerance in seeds can also be induced by premature, slow drying, which leads to the development of dwarf, anhydrobiotic embryos. However, even if embryos do not develop to the germination-competent stage, the cells in the proembryos nevertheless acquire desiccation tolerance 8.

**Moderate dehydration: removal of bulk cytoplasmic water**

Upon water loss, the decrease in cellular volume causes crowding of cytoplasmic components and the cell contents become increasingly viscous, increasing the chance for molecular interactions that can cause protein denaturation and membrane fusion. For model membrane and protein systems, a broad range of compounds have been identified that can prevent such adverse molecular interactions, among them proline, glutamate, glycine betaine, carnitine, mannitol, sorbitol, fructans, polyols, trehalose, sucrose and oligosaccharides. Although they are chemically dissimilar, these compounds are all preferentially excluded from the surface of proteins, thus keeping the proteins preferentially hydrated 9. Because preferential exclusion is thermodynamically unfavorable, the surface area of a protein will be minimal and the folded conformation will be the most frequent; in the presence of preferentially bound co-solvents, the denatured state will be the most frequent. In the simultaneous presence of both types of solutes, the net result will be the sum of the effects.

In response to cellular dehydration, many plants and microorganisms accumulate compatible solutes, irrespective of whether the dehydration is brought about by drought, freezing or osmotic shock. These solutes are called compatible because they do not interfere with cellular structure and function. They represent the same molecules as the above-mentioned compounds that stabilize proteins by preferential exclusion. Enrichment via external addition or via molecular genetic methods has provided evidence for a causal relationship between elevated concentrations of these compatible solutes and improved stress tolerance 10. Their absolute concentrations are often insufficient to increase the water-holding capacity of cells 11. Therefore, preferential exclusion is likely to be the main mechanism of protecting macromolecules in organisms against moderate water loss (Figs 1, 2). When the concentration of destabilizing molecules (among them some ions) in cells increases during water loss, counteraction by preferential exclusion is necessary to prevent protein denaturation and membrane fusion. Below 0.3 (g H₂O) (g dry weight)⁻¹, the lack of bulk cytoplasmic water implies that the mechanism of preferential hydration would fail to work. Indeed, most of the compatible solutes are unable to protect proteins and membranes against further drying in air or freeze drying 12. Only
sugars can structurally and functionally preserve proteins and membranes below 0.3 \((\text{g H}_2\text{O})/\text{(g dry weight)}\) by water replacement, as we discuss below. Most other compatible solutes can therefore be effective only during the drought stage of water loss.

Some LEAs might act during the drought stage of water loss. The heat-soluble, hydrophilic LEAs are primarily located in the cytoplasm and nuclei of cells\(^{13}\). Their accumulation to high concentrations coincides with the acquisition of desiccation tolerance and so they are thought to play a primary role in desiccation tolerance\(^{14}\). Details about the different classes of LEAs and their assumed functions – among them ion sequestration and replacement of the hydrogen bonding function of water – are available in a few specialized reviews\(^{14–16}\). On the basis of the remarkably high number of polar residues within the structure, some LEAs are thought to coat intracellular macromolecules with a cohesive water layer. This mechanism can be interpreted as a sort of preferential hydration. On further dehydration, LEAs would provide a layer of their own hydroxylated residues to interact with the surface groups of other proteins, acting as ‘replacement water’\(^{14}\). Because LEA transcripts have also been detected in recalcitrant (desiccation-sensitive) seeds and in drought-tolerant tissues submitted to water and/or temperature stress\(^{17}\), LEA proteins might have an essential role during the drought stage of water loss.

Small HSPs are another type of protein that has recently been associated with plant desiccation tolerance\(^{18,19}\). These proteins are induced by the same stresses as LEAs and their synthesis coincides with the acquisition of desiccation tolerance. In desiccation-sensitive mutant seeds of Arabidopsis, their expression is much reduced\(^{20}\). There is little tissue specificity for their expression in mature seeds\(^{20}\), which suggests an overall protective effect of small HSPs during drying. Small HSPs might act as molecular chaperones during seed dehydration and the first few days of rehydration. Generally, HSPs are able to maintain partner proteins in a folding-competent, folded or unfolded state, to minimize the aggregation of non-native proteins, or to target non-native or aggregated proteins for degradation and removal from the cell\(^{21}\). Recently, the LEA-like protein HSP 12 from yeast was observed to be associated with membranes in desiccated yeast cells\(^{22}\). When liposomes are dried in the presence of this protein, it prevents the leakage of entrapped dye from the liposomes. The interaction of the protein with the liposomes is thought to be electrostatic in nature. Therefore, it is likely that the HSPs accumulate at the membrane surface when cells still contain bulk cytoplasmic water.

Amphiphilic metabolites partition from the cytoplasm into the lipid phase when their concentration in the cytoplasm increases upon water loss (Fig. 2).

![Diagram](http://plants.trends.com)
of solutes from membranes keeps membranes preferentially hydrated and hence undisturbed.

Using amphiphilic spin probes inserted into the cytoplasm as a model for small amphiphilic molecules, it has been shown that, during early loss of water from pollen and seeds, the spin probes show up in the membranes during early loss of water from pollen and seeds, the spin probes show up in the membranes during the dehydration of organisms, their membranes become fluidized and perturbed, from which it can be concluded that endogenous amphiphiles also partition into membranes in vivo.

Partitioning of cytoplasmic amphiphiles into membranes during dehydration also has positive aspects because it might assist the automatic insertion of antioxidants or phospholipase inhibitors with amphiphilic properties and thus slow the ageing processes. Examples include glycosylated flavonols and hydroquinones such as rutin and arbutin, both of which occur in some anhydrobiotic plants. These compounds have been shown to increase membrane fluidity and to depress the phase transition temperature of membranes, which was hitherto thought to be the privilege of sugars. Although partitioning at first sight seems to be dangerous for cells, it might nevertheless be beneficial if the membrane-perturbing effects can be effectively neutralized. Indeed, there appears to be a mechanism in anhydrobiotes that immobilizes the membrane surface to a certain extent in a later stage of dehydration, thus negating the perturbation. The nature of this mechanism is not known but it might be linked with the interactions between certain stress-induced proteins and the membrane surface.

Apart from the automatic antioxidant insertion mechanism, the amphiphile-induced membrane perturbation at the onset of drying might have a signaling function. This mechanism would resemble that of the heat shock response, in which a temperature-induced or otherwise evoked perturbation of membranes leads to the expression of HSPs (Ref. 29). In the case of dehydration, this might involve the expression of LEAs and small HSPs.

Partitioning-induced membrane perturbation might be the cause of impairment of the electron transport chains, which is thought to lead to increased formation of reactive O2 species (ROS). These ROS cause an extensive peroxidation and de-esterification of membrane lipids, from which organisms often suffer at the intermediate ranges of water loss. The respiratory upsurge observed during...
drying of recalcitrant Castanea cotyledons can thus be attributed to the collapse of proton gradients in the mitochondrial membrane, which might be linked with amphiphile partitioning into these membranes. There is a growing body of evidence that reduction of metabolism, noticeable as a reduction of respiration rate, coincides with survival of desiccation. Slight osmotic dehydration, which reduces desiccation tolerance in germinated cucumber seeds also reduces respiration rates. However, the nature of this presumed downregulation of metabolism is unknown. A coordinated control of energy metabolism at the onset of dehydration or during the acquisition of desiccation tolerance appears to be essential in avoiding oxidative stress conditions and/or accumulation of byproducts of the metabolism to toxic concentrations.

As ROS increase with drying, free-radical scavenging mechanisms are probably activated. In vegetative tissues, genes encoding enzymatic antioxidants such as ascorbate peroxidase, glutathione reductase and superoxide dismutase are upregulated during drying or rehydration. Furthermore, the accumulation during dehydration of a lipoxygenase inhibitor and anthocyanins with antioxidant capability has been observed in Craterostigma leaves (Fig. 3). It should be realized that the enzymatic antioxidant systems can be active only under conditions of sufficient water and that, in the dried state, only molecular antioxidants (e.g. glutathione, ascorbate, polyols, carbohydrates, proteins such as peroxiredoxin, and amphiphilic molecules such as tocopherol, quinones, flavonoids and phenolics) can alleviate oxidative stress.

Severe dehydration: removal of water shell
When water dissipates from the water shell of macromolecules at a moisture content of <0.3 (g H2O) (g dry weight)−1, the hydrophobic effect responsible for structure and function is lost. It is envisaged that sugars, especially the non-reducing disaccharides but also tri- and tetrasaccharides and fructans that accumulate in anhydrobiotes, can replace the dissipating water – a theory known as the water replacement hypothesis (Figs 1, 2).

With the removal of the last water molecules from the polar head groups, the gel-to-liquid crystalline transition temperature ($T_m$) of model membranes increases by as much as 70°C. This is caused by the reduction in spacing between the head groups and the ensuing increase in packing density of the acyl chains. This increase in $T_m$ is prevented by hydrogen bonding interactions of sugars with the polar head groups, which maintain the lateral spacing and packing density of the acyl chains. An alternative explanation has been suggested, which is based on the alleviation by the solute of the dehydration-induced mechanical stresses in membranes. As a result of the sugar interaction, a phase transition during drying is largely prevented, and this is thought to be pivotal for desiccation tolerance because it avoids possible lateral phase separations of membrane components and excessive leakage during rehydration.

In dry anhydrobiotic pollen and seeds, the $T_m$ of membranes does not greatly exceed that in the hydrated state, which has been attributed to the effect of the abundant sugars. An alternative strategy for the control of $T_m$ could be a high degree of unsaturation of the acyl chains, as found in some pollens. However, the increased lipid peroxidation renders such pollen short-lived. Apparently, the common strategy in dried anhydrobiotes is an external control of membrane dynamics by interaction with cytoplasmic substances such as sugars at the membrane surface. The importance of sugars in the maintenance of cellular structural integrity is exemplified in non-anhydrobiotic human primary fibroblasts engineered to accumulate the disaccharide trehalose. The presence of trehalose alone provided a level of desiccation tolerance. However, the life span of these fibroblasts was limited to a few days, which indicates that other aspects are important for conferring long-term stability.

For proteins, roughly the same story applies in relation to stabilization in the dried state. When compatible solutes cease preferentially to hydrate model proteins during drying because of the lack of free water, sugars can act as a water substitute by satisfying the hydrogen-bonding requirement of polar groups on the surface of the dried protein. Thus, the native folding and activity of proteins are maintained.

http://plants.trends.com
Fig. 4. Relationship between life span (longevity) and intracellular molecular mobility (rotational correlation time, $\tau_R$) in dry anhydrobiotes. There is a linear increase of the life span with decreasing molecular mobility in the cells. Molecular mobility in the cells can be estimated by measuring the rotational correlation time (which is the time it takes for the guest molecule to rotate one radian about its axis) of a polar spin probe that is inserted in the cytoplasm, using saturation transfer electron spin resonance spectroscopy. Using the extrapolation of this linear relationship, it is possible to predict life span after estimating the molecular mobility at a given temperature (red arrows). Figure modified from Ref. 48.

and denaturation and aggregation are prevented (Fig. 1). The results from model protein work also applies to proteins in organisms. In desiccation-sensitive carrot somatic embryos and maturation-defective Arabidopsis seeds, signs of protein denaturation and aggregation are evident after desiccation$^{41,42}$. By contrast, proteins in dried desiccation-tolerant specimens retain their native secondary structure even during heating to 150°C. This emphasizes the stability of the proteins in their dry cytoplasmic environment. It is therefore no surprise that proteins still retain their native secondary structure after decades in dried anhydrobiotic seeds, long after the seeds have died$^{43}$.

As the cytoplasm dries to below 0.3 (g H$_2$O) (g dry weight)$^{-1}$, the molecular mobility in the cytoplasm decreases by more than five orders of magnitude$^{44}$. At $-0.1$ (g H$_2$O) (g dry weight)$^{-1}$ (at room temperature), the cytoplasm vitrifies and exists in a so-called glassy state – an amorphous metastable state that resembles a solid, brittle material, but with retention of the disorder and physical properties of a liquid. In a glass state, the rates of molecular diffusion and chemical reactions are greatly reduced. A glass state is characterized by the glass-to-liquid transition temperature, $T_g$, which depends on water content, temperature and its chemical composition. Carbohydrates in particular have the propensity to form a glass by hydrogen bonding interactions at an appropriate temperature and water content. The higher the molecular weight of the carbohydrate, the higher is $T_g$ over the entire range of water contents.

Intracellular glasses have been detected in dry seeds and pollen. Because oligosaccharides have been found to give more stable glasses of higher $T_g$ in model systems than mono- and disaccharides, and to correlate with the acquisition of desiccation tolerance and seed longevity, the research interest has focused mainly on their role in cytoplasmic glasses. However, the need for oligosaccharides for desiccation tolerance is questionable. For example, desiccation-tolerant anhydrobiotes do not behave in the same way as the same sugars in model glassy systems. Whereas the molecular mobility abruptly increases when the sugar system comes out of the glassy state, that of the cytoplasmic system increases only slightly on melting$^{51}$ (Fig. 5). It is not until the collapse temperature ($T_c$) is reached that the viscosity abruptly drops and a flow on a practical time scale is observed. The $T_c$ in anhydrobiotes occurs at temperatures as high as 55°C above $T_g$ which has important implications for the survival of germ plasm in its natural environment. Any environmental fluctuation in relative humidity or temperature that brings the tissue above its $T_c$ has only a limited effect on its stability. If the intracellular glass were composed of sucrose alone, a small increase in relative humidity or temperature would bring the glass above its $T_c$, resulting in crystallization and loss of macromolecule function and integrity.

On the basis of an elevated $T_c$ of complex glassy model systems, it is likely that the high $T_c$ of cytoplasmic glasses is caused by mixtures of sugars, with the higher molecular weight oligosaccharides and polymers such as proteins. Although more research is needed to determine which types of pollens contain sucrose but lack oligosaccharides, and seeds in which the oligosaccharides have been converted into sucrose by priming are still desiccation tolerant and show no differences in $T_g$ (Ref. 45). In addition, most desiccation-sensitive seeds lose viability at water contents far above those at which glasses are formed, but nevertheless form glasses when air dried$^{46,47}$.

The crucial function of intracellular glasses might be the provision of stability to macromolecular and structural components during dry storage. Because the viscosity of glasses is extremely high (equivalent to a flow rate of $-0.3 \mu$m yr$^{-1}$), glasses prevent the crystallization of embedded chemical compounds, fusion between membrane systems and conformational changes in proteins. They considerably reduce the rates of chemical (ageing) reactions. The storage longevity of anhydrobiotic plants is inversely correlated with the molecular mobility in the cytoplasm$^{48}$. This correlation holds even at low water contents [-0.3 (g H$_2$O) (g dry weight)$^{-1}$], under conditions of which the trend of increasing longevity with further dehydration is reversed$^{49,50}$. Thus, the lower the molecular mobility, the greater the life span (Fig. 4). Elevated water contents and temperatures increase molecular mobility and consequently decrease life span. For long-term survival, it is therefore important that the cytoplasm of the organism is in the glassy state. From the linear relationship between the logarithms of molecular mobility and longevity, it is possible to predict the longevities of anhydrobiotes even at low temperatures, for which experimental determination is practically impossible$^{48}$.

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Fig. 5. The effect of melting the glassy state on the molecular mobility of a guest molecule (carboxy-proxyl, a spin probe) incorporated in different dry glasses. The glasses are composed of sucrose, the polypeptide poly- l-lysine or the intracellular glass of a dried anhydrobiotic plant. Molecular mobility was measured by saturation transfer electron spin resonance spectroscopy according to Ref. 51. The value T - Tg represents the difference between the experimental and glass transition temperatures, respectively; Tg can be different for the different glasses. At a certain temperature above Tg an abrupt increase in mobility is apparent, which represents the critical temperature Tc, at which the dynamics of the system change from solid-like to liquid-like. The interval between Tg and Tc is small for sucrose glass but considerably greater for poly-l-lysine glass and cytoplasmic glass. This indicates that cytoplasmic glasses consist of complex mixtures of (macro)molecules. The glass transition temperature is highlighted by the broken line.

Proteins play roles in the glass formation. LEAs are possible candidates. Improved glass stability has been found to coincide with the synthesis of LEA proteins in slowly dried carrot somatic embryos. Furthermore, purified LEAs from pollen can change the hydrogen bonding properties of model sugar glasses in a comparable way to those of intracellular glasses. However, this is not a unique property of LEAs, because poly-l-lysine can also be effective.

Rehydration
On rehydration, water replaces the sugar at the membrane surface, which is followed by repartitioning of amphiphiles from the membranes into the cytoplasm. The ensuing transient leakage of solutes from the rehydrating cells is associated with the transient residence of endogenous amphiphiles in plasma membranes (Fig. 2). This transient leakage has to be distinguished from the prolonged leakage associated with the often-lethal imbibitional injury. Such injury is particularly severe when imbibition occurs at low temperature and/or the specimens are extremely dry, conditions that lead to rigidification of membranes. Imbibitional injury occurs within seconds of contact with liquid water. Scanning electron micrographs show holes in the plasma membranes of pollen within 10 sec of stressful imbibition. Imbibitional injury has been reported in pollen, fungal conidia, yeast, mosses and seeds, but also in anhydrobiotic animals such as nematodes and Artemia cysts.

The damage can be prevented by warm imbibition or by prehydration of the dried specimens from the vapor phase. These treatments all melt possible gel-phase phospholipids and increase the molecular mobility of the membrane components before the uptake of liquid water. Thus, a possible membrane phase transition during imbibition is avoided and the plasma membrane has become sufficiently flexible to accommodate the expanding protoplast. Insensitivity to imbibitional stress has been reported for dried pollen that has an extremely high proportion (65%) of polyunsaturated linolenic acid in its phospholipids and a low Tm, which suggests that membrane fluidity is an important factor in injury. In seeds, the coat or testa has a moderating effect on the extent of imbibitional damage because it often restricts the penetration of water. Thus, a sort of prehydration from the vapor phase is created for the cells inside the seed covers. Seeds with damaged seed coats take up water more rapidly and are more sensitive to imbibitional stress. In such cases, artificial coatings that are applied before the seeds are hydrated can prevent imbibitional damage. Recently, a class of rehydration proteins was identified that are active in the rehydration phase and might be involved in antioxidant production during rehydration.

Conclusions
During drying, different mechanisms of protection appear to act at different stages of water loss. The survival strategy during early dehydration is to avoid protein unfolding and to restrict membrane disturbance by preferential hydration. Upon further removal of water from the hydration shell, sugar molecules have to replace water at hydrogen bonding sites to preserve the native protein structure and spacing between phospholipids. Meanwhile, curtailing the production of ROS (e.g. by downregulation of the metabolism and free-radical scavenging) is an important mechanism of survival. In the study of plant desiccation tolerance, it has often been found that one specific mechanism does not confer tolerance on its own, but that the interplay of several mechanisms simultaneously is essential. Also, one sensitive component can be protected in different ways so as to guarantee optimal survival.

Most biophysical investigations concerning anhydrobiosis in plants have been focused on phenomena in the dried state. Considering that desiccation-sensitive organisms usually die when the water content is still relatively high (e.g. 0.5–2.0 (g H2O)/(g dry weight)−1), future research should be aimed at mechanisms of protection that operate in this particular range of water contents. Pressing goals for future research are the understanding of the mechanism of protection by LEAs and HSPs in vivo, and how cells cope with membrane destabilization as a result of partitioned amphiphiles.