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The Role of Trehalose and Other Carbohydrates in Biopreservation

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General introduction

Trehalose is a non-reducing disaccharide of D-glucose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) which is found in fungi, insects and some lower plants, such as the resurrection plant *Selaginella lepidophylla*. The biological role of trehalose in these organisms is still discussed. In some situations it can function as a reserve carbohydrate, in others as a protectant. For example, it has been noted that trehalose accumulates under conditions of environmental stress, such as desiccation. As the trehalose is rapidly metabolized on rehydration, a reserve function could be inferred. Trehalose can also accumulate in response to other stresses, such as heat or osmotic shock. This response to environmental stress has led to the suggestion that the accumulation of trehalose protects biological structures. Currently, the exact mechanisms of protection are debated. In an effort to identify protective mechanisms, a range of *in vitro* studies have been carried out, examining the effectiveness of trehalose in the preservation of the structure and biological activity of proteins and membrane structures. At the same time, processes for the utilization of trehalose for effective biopreservation have been developed. In part, this has developed as a result of the current need to preserve proteins and other biological structures in the dry state, and a potential increasing requirement to preserve genetically engineered proteins.

Although trehalose is a major disaccharide found in fungi, in other organisms, other carbohydrates and polyols can accumulate when exposed to environmental stress. In the examination of the role of trehalose in biopreservation, it is useful to compare the behaviour of trehalose with these other carbohydrates. Using this approach, it should be possible to identify the specific molecular mechanisms and any special properties or effectiveness of trehalose.

Abbreviations: AMP, adenosine 5′ phosphate; FTIR, Fourier transform infra-red; NMR, nuclear magnetic resonance.

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In this chapter, we will examine the biosynthesis and occurrence of trehalose, and its ability to protect membranes and proteins *in vitro*.

Biosynthesis and occurrence of trehalose

FUNGI AND BACTERIA

A first step in the biosynthesis of trehalose is the formation of trehalose-6-P from UDP-glucose and glucose-6-P with the enzyme UDP-glucose: D-glucose-6-P-1-glucosyl transferase (EC 2.4.1.15). The phosphate group is then removed with a specific phosphatase, trehalose-6-P phosphohydrolase (EC 3.2.1.28), to form trehalose. In its catabolism, trehalose is hydrolysed by trehalase. In yeast there are two main forms of the enzyme, one of which is regulated by a cyclic AMP-dependent phosphorylation.

The levels of trehalose obtained are dependent on the balance between synthesis and catabolism. This balance changes with life-cycle, nutrient availability and environmental stress (Thevelein, 1984). Both gluconeogenesis and glycogenolysis can provide the glucosyl units for synthesis (François, Neves and Hers, 1991). In yeast growing on a glucose medium, the levels of trehalose are generally low during logarithmic growth of the cells, typical values being a few milligrams per gram of cells. As the carbon source becomes limiting and growth slows, there is a rapid accumulation of trehalose, to a level in the region of 80 mg of trehalose per g of cells. At the same time, there is an accumulation of glycogen. In this instance, glycogenolysis is not the source of the glycosyl units of trehalose. This general pattern of accumulation shows some genetic dependency with a few strains not showing this accumulation (Panek *et al.*, 1980).

Mono- and disaccharides are utilized to different extents by different species of yeast (Barnett, 1981), and changing the carbohydrate in the growth medium – for example, substituting maltose for glucose – can change the pattern of accumulation of trehalose (Panek and Mattoon, 1977). In addition to the dependence of trehalose levels on the availability and type of carbon source, the levels of trehalose found are also dependent on the presence of other nutrients (Thevelein, 1984). When growing yeast cells were transferred to media deprived of nitrogen, sulphur or phosphorus, an accumulation of both trehalose and glycogen was observed (Lillie and Pringle, 1980), with levels of trehalose increasing from 0.3% to 16.0% of dry weight. The comparable change in glycogen level was from 2.5 to 14.0 dry weight. The level of these carbohydrates is also affected by oxygen availability, with anaerobic conditions favouring accumulation (Chester, 1963).

While nutrient limitation is a common cause of trehalose accumulation in yeast and other fungi (Thevelein, 1984), resumption of growth, either through the further provision of a carbon source or by adding a deficient nutrient, brings rapid breakdown of the accumulated trehalose. In an NMR study on the mobilization of trehalose during the germination of yeast spores, it was concluded that its usage only accounted for 9% of the energy consumed during germination and that the primary source was exogenous glucose

(Barton *et al.*, 1982). While trehalose may not be an important source of metabolic energy during germination, it was suggested that it could function as a reserve during dormancy (Barton *et al.*, 1982). There have been a number of studies of the regulation of trehalose metabolism in fungi and the reader is referred to Thevelein (1984) for a review. Trehalose is also found in bacteria such as *Streptomyces* (McBride and Ensign, 1987b), *Rhizobium* (Streeter, 1985) and *Micrococcus* (Ahmad, Alden and Montague, 1980). In some bacterial spores (e.g. *Streptomyces griseus*), accumulation of trehalose can be substantial and up to 25% of the dry weight of the spore. Transfer of the spores to conditions which favour germination leads to the metabolism of the accumulated trehalose (McBride and Ensign, 1987b). While the trehalose present could function as a reserve carbohydrate, its presence is also associated with preservation, in that the resistance of the spores to damage by heat or desiccation increases with increasing content of trehalose (McBride and Ensign, 1987a). For example, the spores showed a five-fold improvement in resistance to exposure to 60°C for 10 min, as the trehalose level increased from 0.2 to 0.8 mg/mg protein (McBride and Ensign, 1987a). These spores contain both trehalose and the enzyme responsible for its breakdown, trehalase. For fungal spores, it has been suggested that both the different compartmentation of trehalose and trehalase, and the regulation of trehalase activity through a cyclic AMP-dependent phosphorylation of the enzyme, are responsible for the simultaneous occurrence of trehalose and trehalase (Thevelein, 1984). A study on yeast suggested that the trehalase was located in the vacuole and the trehalose in the cytosol (Keller, Schellenberg and Wiemken, 1982). In a study on *Streptomyces griseus* spores, it was also suggested (McBride and Ensign, 1990) that the level of hydration of the spores was important and that trehalase activity was sensitive to the extent of hydration, increasing as the water content of the spore increased.

PLANTS

Selaginella lepidophylla is a desert xerophyte of North America. It is well known for its ability to survive desiccation. On rehydration, the brown curled-up mass of the plant rapidly opens and becomes green again, and has the general appearance of a healthy plant, hence the popular name for the species is the 'resurrection plant'. There have been a few studies on the carbohydrate metabolism of this species (Adams, Kendall and Kartha, 1990). In the growing plant, two main disaccharides were present – sucrose and trehalose – representing approximately 14% of the dry weight, with trehalose being the major component (90%). On desiccation, there was a small decrease in the total amount of carbohydrate. Sucrose levels increased to 23% of the carbohydrate present, while the amount of trehalose fell slightly (75%). Although the levels of the disaccharides actually fell slightly during desiccation, it was argued that this desert plant must at all times be prepared for drought, hence the high levels of the disaccharides found.

Trehalose is also found in insects (Wyatt and Kalf, 1957); for example, it is the major carbohydrate found in the blood of the blowfly *Phormia terraenovae* (Wilps and Gade, 1990), where it is a metabolic source of energy for flight. The levels of trehalose found in the blood of insects are influenced by diet (Friedman *et al.*, 1991) and environmental stress, particularly as a response to cold temperatures during wintering (Zachariassen, 1985). Trehalose is also found in the cysts of the brine shrimp *Artemia salina*; these cysts are considered to be dormant embryos. Trehalose accumulation only occurs in embryos entering dormancy, probably as a result of the conversion of glycogen. The amount of trehalose accumulated is approximately 15% (w/w) of the dry weight; glycogen (1.5% w/w) and glycerol (3.3% w/w) are also present (Clegg, 1964). At the end of dormancy, there is a dramatic fall in the level of trehalose and an increase in the levels of glycogen and glycerol. Although some of the trehalose is catabolized and could be considered to be a reserve carbohydrate, the interconversion of glycogen and trehalose at different stages in the life-cycle has prompted the suggestion that trehalose has another function, associated with the successful dormancy of the cysts (Clegg, 1965). As it is not metabolized during the dormant period, it is thought that the trehalose functions as a 'preservative'.

Trehalose is also found in nematodes, and accumulates at the onset of desiccation. It was observed that species which showed good survival after desiccation accumulated higher levels of trehalose than the poor survivors (Womersley and Smith, 1981).

CHANGES IN LEVELS OF TREHALOSE IN RESPONSE TO ENVIRONMENT STRESS

Heat shock

The accumulation of trehalose in *Saccharomyces cerevisiae* is dependent upon the temperature of growth. At ambient temperatures, only traces of the disaccharide are found in yeast cells growing exponentially, with glucose as the carbon source and with no deficiency of other nutrients. If these cells are then transferred to a higher, but non-lethal temperature, there is a rapid accumulation of trehalose, with levels approaching 1 g/g protein (Hottiger, Schmutz and Wiemken, 1987). Following the initial rapid synthesis, there is a gradual reduction in trehalose levels to a new 'steady-state' value, which, in the temperature range 27–40°C, increases with increasing temperature. The activities of both trehalose-6-P synthase and trehalase increase with increasing temperature in this range, the dominant effect being on the synthetic enzyme leading to an accumulation of trehalose (Hottiger, Schmutz and Wiemken, 1987). It is also suggested that the primary cause of increased trehalose levels is the increase in the levels of substrate for trehalose synthase. The heat shock response on trehalose levels is rapidly reversed if the cells are transferred back to a lower temperature (Attfield, 1987).

Changes in trehalose levels are associated with improved tolerance to desiccation and heat. Cells containing approximately 1 g trehalose per g protein showed a six-fold improvement in desiccation tolerance, and a similar correlation was noted between trehalose content and the ability to survive heating to 40°C (Hottiger, Boller and Wiemken, 1987). Transfer of the cells back to the temperature of normal growth, with the consequent fall in trehalose levels, resulted in the disappearance of desiccation and thermotolerance. These observations led to the suggestion that trehalose functions as a thermoprotectant, (Hottiger, Boller and Wiemken, 1989) rather than a reserve carbohydrate (Wiemken, 1990). Similar observations were reported on the heat resistance of spores of *Dictyostelium discoideum* with an association between the level of trehalose and resistance (Emianitoff and Wright, 1979).

In addition to the increase in trehalose levels as a response to heat shock, heat shock proteins are also synthesized (Sanchez and Lindquist, 1990). These proteins may be classified as chaperonins, necessary for the successful folding and assembly of proteins (Cheng, 1989). The observation by Winkler *et al.* (1991) that yeast mutants lacking the heat shock-induced protein HSP104 were killed by exposure to 50°C despite accumulating trehalose, has led to debate on the possible protection conferred by trehalose. It has been suggested that the molecular chaperone HSP104 is involved in the repair of damaged structures, while the trehalose confers protection over a limited temperature range (De Virgino *et al.*, 1991). The heat shock protein is required after severe heat stress, which has caused damage to biological assemblies, and is involved in the refolding of the damaged protein.

Osmotic stress

When microbial cells are subjected to osmotic stress, achieved for example by the addition of salt to the growing medium, a common response is the accumulation of low molecular weight solutes, which protect against loss of water from the cell through an osmotic effect. Often these are relatively simple carbohydrates or polyols such as sucrose and glycerol. It has been suggested that the structurally more complex cyclic glucans of *Agrobacterium* and *Rhizobium* could function in this way (Miller, Kennedy and Reinhold, 1986; Breedveld, Zevenhuizen and Zehnder, 1990). It has also been suggested that trehalose could function as an osmoprotectant in rhizobia and bradyrhizobia species (Elsheikh and Wood, 1990). In a survey of the osmoregulatory solutes of cyanobacteria, a number of solutes were identified, including sucrose, trehalose, O-α-D-glucopyranosyl-(1-2)-glycerol, glycine betaine and L-glutamate betaine (Mackay, Norton and Borowitzka, 1984). Trehalose accumulation in response to osmotic stress has also been noted in *Escherichia coli* (Welsh, Reed and Herbert, 1991). In this case, the accumulation occurs when the organism is grown on a glucose/salt medium without added betaine (Strom, Falkenberg and Landfald, 1986; Larsen *et al.*, 1987). In the case of a halophilic bacterium *Ectothiorhodospira halochloris*, betaine was the primary osmoprotectant, although trehalose was synthesized under

nitrogen-limiting conditions and could have a subsidiary role as an osmoprotectant (Galinski and Herzog, 1990).

The main conclusion to be drawn from these studies is that although trehalose is accumulated in response to osmotic stress in some organisms, a variety of other low molecular weight solutes apparently fulfil the same role in other organisms.

Low temperature stress

The association between the accumulation of trehalose and survival at low temperatures has mainly been studied in insects. Generally, this biochemical adaptation is of two forms. In freeze-tolerant species, ice formation in the extracellular fluid is tolerated. In some species, the extent of ice formation can approach 65% of the available water before injury occurs (Zachariassen, 1985). In freeze-avoiding species, a supercooled liquid state is maintained, partly through the avoidance of ice nucleation (Storey, 1990). It has been found that some freeze-tolerant species can survive cooling to –85°C, while the lowest temperature recorded for survival of freeze-avoiding species is in the region of –50°C (Zachariassen, 1985). In both cases, the biosynthesis of cryoprotectants is common. One property of the cryoprotectant – indeed, of all solutes – is to depress the equilibrium melting point of ice through a colligative action. A further property of a cryoprotectant is to inhibit the crystallization, on practical time-scales, of a fraction of the water present (see below). Common cryoprotectants found in insects are carbohydrates and other polyols, including glycerol, sorbitol, mannitol, xylitol, inositol, rhamnitol, fucitol, ribitol, threitol, erythritol and ethylene glycol (Zachariassen, 1985; Storey, 1990). In the freeze-tolerant gall fly, *Eurosta solidaginis*, typical levels of glycerol and sorbitol accumulated are 25 and 18 mg g wet weight^{-1}, respectively. *Eurosta scudderiana* can accumulate up to 19% of its wet weight of glycerol (Storey, 1990).

The disaccharides trehalose and sucrose are also found. In the poplar sawfly *Trichiopcampus populi* – a freeze-tolerant insect – trehalose increases from 10 to 50 mg g wet weight^{-1} during winter. Comparable levels (40 mg g^{-1}) have been found in the overwintering larvae of the red sunflower seed weevil, *Smicronyx fulvus*, which is freeze-avoiding (Rojas, Charlet and Leopold, 1991). It is also suggested that free amino acids and inorganic ions could also play a role. Survival at these low temperatures implies a very substantial accumulation of the cryoprotectant.

Desiccation tolerance

Desiccation of biological cells might involve different environmental stresses. During drying under ambient conditions, the cells might experience a form of osmotic stress. Drying at higher temperatures could involve both heat and osmotic stresses. Freezing of water external to the cell could also lead to desiccation through two effects; first, from freeze-concentration of external solutes with consequent osmotic stress and, second, from the freezing of

water vapour escaping from the supercooled cell. Biochemical and physiological aspects of anhydrobiosis have been reviewed (Womersley, 1981).

In yeast cells and the dormant embryos of the brine shrimp, the accumulation of trehalose is associated with improved desiccation tolerance (see above). Trehalose is found in many fungal spores; for example, the spores of *Phycomyces blakesleeanus* contain up to 35% of their dry weight as trehalose. The spores can support 'complete desiccation' without loss of viability. Germination occurs on rehydration and is associated with a depletion in trehalose levels. Since the breakdown exceeds the respiratory requirement, this strengthens the argument in favour of the proposed role of trehalose as a protective factor against desiccation (Barton *et al.*, 1982; Laere *et al.*, 1987).

In some micro-organisms and invertebrates, trehalose is the major disaccharide present, which increases in response to some environmental stresses, largely as a result of the conversion of glycogen. In higher plants, there is a starch-to-sucrose interconversion, and it has been observed that sucrose accumulates when plants are exposed to cold and desiccation stress. It is thought that sucrose, alone and together with non-reducing oligosaccharides such as raffinose, could play a part in the desiccation tolerance of seeds (Koster and Leopold, 1988) and pollen (Hoekstra and van Roekel, 1988).

Concluding remarks

In vivo studies have shown that trehalose, and other low molecular weight carbohydrates and polyols, might have a number of biological roles. There is a strong association between the accumulation of these substrates on environmental stress, and a subsequent tolerance of that stress. This has led to the suggestion that these molecules have a 'preservative' action *in vivo*, and could be used *in vitro* to preserve delicate biological structures. The preservative action *in vitro* could be improved with a better understanding of the mechanisms involved. *In vivo* studies have largely provided evidence for an association between the accumulation of solutes and preservation action. These have been followed by *in vitro* studies examining the preservation of isolated biological structures such as proteins and membranes. In this chapter, we would like to compare *in vitro* and *in vivo* behaviour.

To aid comparison we have compiled a table of the levels of trehalose, and other carbohydrates and polyols, in various micro-organisms and invertebrates, and the changes in these levels that occur under certain conditions of environmental stress (*Table 1*). We will review current understanding on the role of trehalose and other low molecular weight carbohydrates in conferring protection to biological structures on exposure to sub-ambient temperatures, freezing, elevated temperatures and desiccation.

Solid and solution conformations of trehalose

Trehalose is the name commonly given to α, α-trehalose, although two other possible isomers, α,β-trehalose (neotrehalose) and β,β-trehalose (isotrehalose), are known. Trehalose consists of two D-glucopyranosyl residues which

Table 1. Levels of trehalose found in different organisms and their change with environmental stress

Organism	Condition	% Trehalose (w/w)	Reference
Nematodes			
Anguina tritici	Desiccated	9.1	Womersley and Smith (1981)
Ditylenchus dipsaci	Desiccated	4.6	Womersley and Smith (1981)
Brine shrimp			
Artemia salina	Dormant embryo	15.0	Clegg (1965)
Artemia francisana	Dormant embryo	16.9	Clegg and Jackson (1992)
Seed weevil			
Smicronyx fulvus		7.3	Rojas, Charlet and Leopold (1991)
Blowfly			
Phormia terraenovae	Blood	30 mg ml^{-1}	Wilps and Gade (1990)
Yeast			
Saccharomyces cerevisiae	Heat shock	1.12 g g protein^{-1}	Hottiger, Boller and Wiemken (1987a)
	Nutrient limit	16	Lillie and Pringle (1980)
Bacterial spore			
Streptomyces griseus		25	McBride and Ensign (1987a)

are linked by a glycosidic oxygen bridge between carbons 1 and 1′.

Trehalose can exist in several solid forms. The common form is that of the crystalline dihydrate which crystallizes from 80% ethanol (Birch, 1963) and has a melting point of 97°C. If the dihydrate is heated to 130°C, the trehalose loses water of crystallization and resolidifies to form anhydrous crystals which melt at 214–216°C (Shafizadeh and Susott, 1973). A new form of anhydrous trehalose which has different vibrational and NMR characteristics has recently been reported (Belton and Gil, in press). Additionally, an amorphous glassy solid may be obtained by supercooling the molten crystalline dihydrate (Green and Angell, 1989).

The crystal and molecular structures of the dihydrate have been confirmed by several independent X-ray studies (Brown *et al.*, 1972; Taga, Senma and Osaki, 1972), which show four $C_{12}H_{22}O_{11}.2H_2O$ units arranged in an orthorhombic cell. The two glucopyranose residues exist in the chair $^4C_1$ form, and the trehalose molecule has an approximate two-fold symmetry. Departures from symmetry are found in the torsion angles about the glycosidic linkages. In most of the common disaccharides (e.g. sucrose, lactose and maltose) the conformation is influenced by intramolecular hydrogen bonding between monosaccharide residues. For trehalose dihydrate, indirect inter-residue hydrogen bonds through water affect the conformation. The average length of these hydrogen bonds is relatively short (1.825 Å) and therefore unusually strong (Jeffrey and Saenger, 1991). For this reason, the crystalline dihydrate readily crystallizes from concentrated aqueous solutions.

The crystal structure of the anhydrous form of trehalose has also been

studied (Jeffrey and Nanni, 1985). This was carried out using X-ray analysis at low temperature (–150°C). The molecular structure was similar to that found for the dihydrate crystals.

From ^{1}H NMR studies and optical rotation data (Duda and Stevens, 1990) it has been shown that in aqueous solution the D-glucopyranosyl residues of the trehalose are physically equivalent and the expected $^4C_1$ conformation is adopted. There have been a few studies on the hydration of trehalose in aqueous solution, using different experimental approaches, including measurement of the enthalpy of solution (Gaffney *et al.*, 1988), partial molar volumes and isoentropic compressibilities (Galema and Hoiland, 1991) and, finally, determination of the unfrozen water content associated with trehalose in frozen aqueous solutions (Kawai *et al.*, 1992). These data were used to estimate or evaluate a hydration number for trehalose which was then compared to that of other disaccharides. The values obtained for trehalose were at the upper end of the range of values found for the disaccharides. The significance of this observation for the preservative action of trehalose is difficult to assess at the current time.

The effect of carbohydrates on the thermal transitions of proteins and lipids

PROTEINS

The stability of the native conformation of proteins is a balance of stabilizing and destabilizing forces and effects. Intermolecular forces stabilizing the structure include van der Waals interactions and hydrogen bonding. As the temperature is raised, these interactions weaken, thereby contributing to the change in the protein conformation from the native to the denatured state. A further interaction to be considered is the interaction of water with the native and denatured forms of the protein and should include consideration of the intermolecular forces between water and both polar and non-polar groups on the protein, and the effect of these forces on the configurational entropy of the system. For example, a major stabilizing contribution is the unfavourable entropic interaction between water and non-polar residues which would occur if these residues are exposed at the protein surface, as in the denatured form. Often the interaction between water and the non-polar residues of the protein is separately identified and described under a general hydrophobic interaction or hydrophobic effect (Tanford, 1980; Privalov and Gill, 1988).

As the stabilizing entropic contribution of the hydrophobic interaction decreases with decreasing temperature, the stability of the protein can also decrease (Jaenicke, 1990), and a phenomenon known as cold denaturation has been predicted and observed to occur (Brandts, Fu and Nordin, 1970; Franks, Mathias and Hatley, 1990). A further influence arises as a result of the temperature dependence of the pK of charged low molecular weight solutes, water and charged groups on macromolecules. This dependence affects the solvent qualities of the aqueous medium, solvent–macromolecule interactions, and the interaction between charged groups on the macromolecule. The net result is temperature-dependent changes in conformation and

biological activity of the macromolecule (Franks, Mathias and Hatley, 1990). A further observed effect on multi-subunit proteins, is the phenomenon of cold dissociation of subunits (Jaenicke, 1990).

The denaturation of proteins is conveniently studied by differential scanning calorimetry (Privalov, 1979, 1982), where the denaturation process on heating is observed as an endothermic transition, from which both the denaturation temperature T_d and the enthalpy change on denaturation $\triangle H_d$ can be determined. In some cases, the denaturation process can be reversed on cooling and, as a result, denaturation has been likened to, and analysed as, a melting process. For the ordered crystalline structures of synthetic polymers, the observed melting temperature T_m increases with decreasing volume fraction of solvent. Similar behaviour has been observed for the denaturation of proteins (Rupley and Careri, 1991). For example, in a study on the denaturation behaviour of β-lactoglobulin, T_d increased from 80°C to 99°C as the water content decreased from 4.6 to 0.18 g g protein $^{-1}$. Similarly, in a study of the denaturation of ovalbumin, the T_d of ovalbumin increased sharply from 77°C as the water content decreased below 0.4 g g^{-1} and approached 115°C at a water content of 0.1 g g^{-1}. The drying of a protein therefore increases its thermal stability, although whether after drying the protein can be rehydrated and regain its biological activity is another matter.

As it has been observed *in vivo* that carbohydrates and polyols are biosynthesized in response to heat shock or cold, it is useful to examine *in vitro* behaviour, and to see if the magnitude of the stabilizing effect observed is consistent with the proposed protective role *in vivo*. To do this, we need to examine the way in which carbohydrates interact with, and affect, the denaturation process.

The interaction of carbohydrates with proteins in aqueous solution has been conveniently studied through the measurement of solution densities (Arakawa and Timasheff, 1982). In the usual form of this experiment (Lee and Timasheff, 1981), the density of an aqueous carbohydrate solution of known molality is determined as a function of added protein. The apparent partial specific volume of the protein, ϕ_2^0, in a carbohydrate solution of constant molality, may then be obtained by extrapolation of the data to zero concentration. For the second measurement, the aqueous carbohydrate solution containing protein is dialysed against the carbohydrate solution alone. During the course of the dialysis, solvent components redistribute across the dialysis membrane. The redistribution depends on the relative affinity of the solvent components for the macromolecule. From measurement of the density of the protein solution, it is possible to determine the apparent partial specific volume of the protein at constant chemical potential of the carbohydrate, ϕ_2^0. This term contains information on the redistribution of solvent components. For example, if the carbohydrate had a preferred interaction with the protein, the apparent partial specific volume of the protein at constant chemical potential would be smaller than that at constant molality, reflecting an increase in density in the domain of the protein arising from its interaction with the carbohydrate. A more formal way of expressing this interaction is through a preferential interaction parameter of the carbohy-

drate (component 3) with the protein which is calculated (Casassa and Eisenberg, 1964; Lee, Gekko and Timasheff, 1979):

$$\xi_3 = \rho_0 (\phi_2^0 - \phi'_2{}^0) / (1-\rho_0 \nu_3) \quad (1)$$

where ρ_0 is the density of the solvent medium and ν_3 is the partial specific volume of the carbohydrate.

The preferential interaction parameter obtained for carbohydrates and polyols mixed with proteins in aqueous solution is negative (Gekko and Morikawa, 1981; Arakawa and Timasheff, 1982), indicating that the protein has a preferred interaction with water, with the carbohydrate or polyol being excluded from the domain of the protein. The interaction parameter increases with increasing concentration of carbohydrate (Arakawa and Timasheff, 1982). This means that the aqueous solution of the carbohydrate is a less effective solvent than water alone. A consequence of this is that the ordered or native conformation of the protein is stabilized and the denaturation is shifted to higher temperatures. In contrast, reagents which promote the denaturation of proteins such as guanidine hydrochloride (Lee and Timasheff, 1974) have a preferred interaction with the protein.

The preferential interaction parameter shows a small dependence on carbohydrate/polyol structure. On a molar basis, it increases with molecular weight on going from a low molecular weight polyol (glycerol) to a monosaccharide and disaccharide (Lee and Timasheff, 1981; Gekko and Timasheff, 1981; Arakawa and Timasheff, 1982). The thermal stability of α-chymotrypsin in aqueous solution increased by approximately 8°C on the addition of sucrose to produce a 1 M solution (Lee and Timasheff, 1981). In comparisons (Back, Oakenfall and Smith, 1979) of the stabilizing effect of different carbohydrates and proteins on the denaturation of ovalbumin (Back, Oakenfall and Smith, 1979) and lysozyme (Gekko, 1982), it was found that polyols and carbohydrates generally increased the denaturation temperature (an exception was ethylene glycol). Typical values of the increase were in the region of 4–6°C for a 1.5 M solution of the solute (Back, Oakenfall and Smith, 1979).

In a recent study on the interaction of trehalose with bovine serum albumin in aqueous solution, it was found that, as for the other carbohydrates, the protein was preferentially hydrated and the magnitude of the preferential interaction parameter was comparable to that of other disaccharides. The stabilizing effect of trehalose in aqueous solution on protein denaturation was similar to that of the other carbohydrates. Trehalose at a concentration of 500 g l^{-1} (~1.5 M) would generally be expected to raise the denaturation temperature by about 5°C. In the heat shock response in yeast, the maximum concentration of trehalose obtained is in the region of 1.1 g trehalose per g protein. For a yeast cell containing 80% (w/w) water, this level of trehalose would represent an average concentration of approximately 0.25 M. At this concentration, the protective effect on protein denaturation would be relatively small, perhaps less than a degree. The idea that trehalose is protective against heat shock through its effect on protein denaturation is not, at present, convincing. *In vitro* experiments can give indications of the *in vivo*

effect of the accumulation of these solutes, although the *in vivo* situation differs in that the protein content is appreciably greater. This in itself will have an effect both on the denaturation temperature and the aggregation of partially unfolded proteins. Additionally, the distribution of trehalose within the cell might lead to high local concentrations, which could be effective in conferring some protection. As far as we are aware, the effect of carbohydrates and polyols on cold denaturation have not been studied to date, although increased stabilization would be expected.

When considering the effect of carbohydrate on the drying of protein solutions, it might be predicted – by analogy with the behaviour of synthetic polymers in mixed solvent systems (Flory, 1953) – that as drying proceeds and the concentration of the macromolecule increases, a phase separation would occur in which a macromolecule/water phase separates from a carbohydrate/water phase. In this case, the function of the carbohydrate might be to maintain some hydration of the protein in the 'dry' state.

LIPIDS AND MEMBRANES

Carbohydrates and polyols are also thought to play a role in the stabilization of biological membranes. The stabilizing action is thought to arise as a result of the ability of the carbohydrate species to maintain the lipid in a particular phase (Crowe *et al.*, 1988). The structure of biological membranes is very complex, containing proteins, a variety of phospholipids and glycolipids and other lipid species. Although the phase behaviour of individual classes of lipids may be well defined (Small, 1986), the phase behaviour of the mixture of compounds found in the membrane is less easy to measure. As a consequence of the structural complexity, it is extremely difficult to predict the temperature dependence of the phase behaviour of the membrane, the effect of changing the level of hydration and, finally, the effect of solutes such as polyols and carbohydrates on the observed behaviour.

One approach is to study the interaction of lipids of defined structure with carbohydrates. In one experimental study, phospholipids such as 1,2-dipalmitoyl-L-phosphatidylcholine were spread as a monolayer onto water, and the surface tension measured as a function of the extent of compression of the film (Johnston *et al.*, 1984). From the surface tension, it is possible to calculate the surface pressure. It was found that if carbohydrates were present in the aqueous phase, then at a given surface pressure the film was more expanded than a similar film spread over pure water. The extent of expansion was dependent on the type of carbohydrate present. An interpretation of the observed behaviour was that the carbohydrate had a preferred interaction with the lipid head group, the relative increase in concentration of the carbohydrate at the film surface leading to the observed expansion of the monolayer (Johnston *et al.*, 1984). A similar approach was used in an earlier study which compared the effect of glycerol and oligosaccharides such as trehalose and sucrose (J.H. Crowe *et al.*, 1984). The effectiveness of the carbohydrates in preserving membrane structures was correlated with their ability to expand the monolayer. Since that time, a further study has

demonstrated that the observed effects could be due to very potent surface active impurities, which were present in the 'pure' preparations of the carbohydrate (Arnett *et al.*, 1986).

In other experimental studies, the effect of carbohydrates on the 'phase behaviour' of lipids has been determined using techniques such as differential scanning calorimetry. Amphiphilic lysophosphatidylcholines swell when placed in water, the extent of water uptake depending on the acyl chain, ranging from 20 to 40 water molecules per lipid molecule (Small, 1986). The swollen lipid forms a bilayer structure with the head group on the external surface. There are several polymorphic forms of this structure in which the arrangement and packing of the acyl chains differ. In the Lα form of the lamellar structure, the acyl chains retain fluidity. On cooling the fluidity of the chains is reduced, and at defined transition temperatures the acyl chains can adopt more ordered conformations, such as a lamellar Lβ form. The extent of polymorphism of the lamellar form is dependent on the structure of the phospholipid. At higher temperatures, the lamellae may be disrupted to form a structure based on the hexagonal packing of cylinders, hexagonal II, in which the head group and associated water occupy the centre.

The transition from a liquid crystalline lamellar structure to a more ordered lamellar structure such as Lβ, is dependent on water content. For example, for a 1,3-dipalmitoylphospatidyl choline, this transition decreases from 70°C to 40°C as the water content is increased from 2 to 20% (w/w) (Small, 1986). In this temperature range, the addition of water encourages the stabilization of a more fluid lamellar structure. A decrease in temperature, or drying, promotes the occurrence of the lamellar gel structure Lβ. This transition to the gel form is associated with a reduction in integrity of lamellar structures containing a single lipid, and also membranes containing a range of lipids and proteins. In the former case, it can be envisaged that this occurs as a result of the reduction in flexibility of the lamella as the acyl chains adopt an ordered conformation. For the membrane, the phase behaviour of the lipid mixture, the potential phase separation of different types of lipids and the aggregation of the proteins present must also be considered. The protein aggregates may lead to defects in the membrane. Damage to the membrane may also occur both during the initial cooling, inducing a particular species of lipid to undergo a transition from liquid crystalline to gel, and also during heating through the same transition (Quinn, 1985).

The phase behaviour of mixtures of 1,2-dipalmitoyl phosphatidylcholine mixed with sucrose (Hentschel, Miethe and Meyer, 1989) and trehalose (Crowe, Crowe and Chapman, 1984) was investigated by differential scanning calorimetry. The events observed in the calorimeter were associated with transitions from a fluid lamellar form to a more ordered form. It was found that the addition of sucrose and other carbohydrates to the 'dry' lipid depressed the transition. For example, the addition of sucrose to a mole fraction of 0.7 (2 g sucrose per g lipid: Hentsche, Miethe and Meyer, 1989) depressed the transition at 70°C by 10°C. The same depression in transition temperature can be achieved through the addition of 10% (w/w) water. In a study on the effect of trehalose on this transition, it was found that a maximal

depression of the transition temperature to 24°C was observed at concentrations of trehalose in excess of 1.25 g g lipid^{-1}. The extent of depression observed was dependent on the method of preparation of the mixed system (Crowe and Crowe, 1988).

As a result of these and similar studies on dry phospholipids, it is argued that carbohydrates could stabilize membranes in the dry state through their effect on depressing the transition from lamellar liquid crystalline phase to a lamellar gel phase, thus ensuring the retention of fluidity in the dry state (Crowe *et al.*, 1987). There have been a number of structural studies on the interaction of trehalose with phosphatidylcholines. NMR experiments (Lee, Waugh and Griffin, 1986; Lee *et al.*, 1989) have established that in mixtures of trehalose with dry phospholipid, the trehalose hinders the motion of the head group, suggesting a direct interaction. X-ray diffraction studies have confirmed that the lipid was in a lamellar structure. From experiments on bilayer periodicity (Nakagaki, Nabase and Ueda, 1992), it was deduced that the trehalose was intercalated between the lamellae either as a monolayer, or at higher trehalose concentrations as a bilayer. This intercalation affected the extractability of the trehalose on subsequent rehydration (Viera *et al.*, 1993).

The effect of carbohydrates on the polymorphism of hydrated phospholipids has also been examined. It was found that the addition of trehalose and sucrose lead to a small increase in the temperature of the transition from lamellar gel to lamellar liquid crystalline phase (Ram, Mazzola and Prestegard, 1989). Additionally, it was shown that carbohydrates promote the formation of a hexagonal 11 phase at the expense of the lamellar liquid crystalline phase (Bryszewska and Epand, 1988; Aurell Wistrom *et al.*, 1989; Koyonova, Tenchov and Quinn, 1989). This effect would cast some doubt on the function of trehalose in the preservation of membrane structures during heat shock.

Biological membranes are considerably more complex, since they consist of mixtures of lipids and proteins. In one example (Williams, 1990), the lipid extract of a chloroplast membrane when dispersed in aqueous media did not reform a bilayer structure, and it was suggested that the proteins in the membrane help stabilize the bilayer. An attempt has been made to define the general phase behaviour of membranes (Williams, 1990), the stability of the membrane being associated with the preservation of a fluid bilayer structure. Environmental changes which potentially destabilize the membrane, include heating, cooling and drying.

A biological response to a change in the ambient temperature could be a change in the chemical composition and hence phase behaviour of the membrane. Alternatively, it is suggested that protectants such as polyols could function by helping the retention of a suitable membrane structure under adverse conditions.

Drying of biological molecules

If an aqueous glucose solution is dried at room temperature, the viscosity of the solution will increase progressively as it becomes more concentrated. At

sufficiently high concentrations, the liquid glucose–water mixture will vitrify if crystallization of the carbohydrate does not intervene.

Relaxation is the time-dependent response in a property following a perturbation. The shear stress relaxation time, τ, is related to the shear viscosity, η, by the relationship:

$$\tau = \eta / G_\infty$$

where G_∞ is the high-frequency limit of the shear modulus which is 10^{10} N m^{-2} for many materials. A commonly accepted value for the shear viscosity of a glass is 10^{12} Pa s, giving a shear stress relaxation time of 100 s. On the time-scale of a few seconds the material behaves as a solid, whereas if observations are conducted over longer periods of time fluid-like behaviour is observed. Once vitrified, the carbohydrate glass is relatively stable. A consequence of the enormous slowing of translational diffusion in the very viscous liquid is that even if nuclei of the crystalline solid are present, the rate of growth at these very high viscosities is slow. In fact, carbohydrate glasses have stabilities to crystallization of several years, crystallization of the carbohydrate generally commencing at a defect in the glassy structure (Parks, Huffman and Cattoir, 1928). A further effect of the very high viscosity of the glass and the predicted retardation in diffusion, is that the rate of drying of these materials is very slow. One consequence of the vitrification of biological structures may be the entrapment of sufficient water so that the material never really drys to completion, and subsequent rehydration is facilitated.

The glass transition temperature, T_g, of carbohydrates and polyols shows a dependence on molecular weight, and a weak dependence on molecular structure (Slade and Levine, 1988). Some data (Green and Angell, 1989; Finegold, Franks and Hatley, 1989; Roos, 1993; Orford, Parker and Ring, 1990) on carbohydrates and polyols are shown in *Table 2*. The T_g of the alditol is less than that of the corresponding aldose; for example, D-glucitol has a T_g of 0°C, whereas that of D-glucose is 38°C. For hexoses, pentoses and their alditols, there are relatively small differences in observed T_g within any group. The T_g of a series of oligomers increases with increasing degree of polymerization. For a series of dry malto-oligomers, the T_g increases from 38°C to 95°C to 175°C on going from monomer to dimer to hexamer.

For carbohydrates and polyols, water is a very effective plasticizing agent (Orford *et al.*, 1989; Roos, 1993), and the addition of even small amounts of

Table 2. Glass transition temperatures, T_g, of low molecular weight carbohydrates[a]

Carbohydrate	T_g (°C)
Fructose	5–13
Glucose	31–39
Glucitol	–9 to 0
Trehalose	78
Maltose	87–95
Sucrose	57–70

[a] From Finegold, Franks and Hatley (1989), Green and Angell (1989), Orford, Parker and Ring (1990) and Roos (1993).

water to the dry carbohydrate leads to a marked depression in T_g. The glass transition behaviour of trehalose and its plasticization by water is comparable to that of other disaccharides (Green and Angell, 1989). It is also useful to examine the molecular weight dependence. For the malto-oligomer series, the water content of the malto-oligomer/water mixture with a T_g of 25°C is 7.5%, 10% and 15% for the dimer, trimer and hexamer, respectively. For the high molecular weight polymer, the corresponding value is 18%. If it is accepted that glassy matrices might have a protective role through the trapping and encapsulation of water, then we predict that the high molecular weight polymer should be the most effective.

Structural proteins and enzymes can also show glassy behaviour. The glass transition behaviour of several structural proteins has been described, including elastin (Kakivaya and Hoeve, 1975) and cereal proteins (Hoseney, Zeleznak and Lai, 1986; Kalichevsky and Blanshard, 1992; Kalichevsky, Jaroszkiewicz and Blanshard, 1992). The glass transition behaviour of these proteins are broadly similar. Water is an effective plasticizing agent and its addition depresses the T_g of a protein/water mixture. For an elastin/water mixture, the water content of the mixture with a glass transition at ambient temperature is in the region of 24% (w/w). Although it is likely that amorphous biopolymers, including proteins and polysaccharides, show a glass transition, it is less likely for proteins which have an ordered conformation in aqueous solution. Even proteins with a defined three-dimensional structure (Iben *et al.*, 1989) can show structural fluctuations, ranging from the local dynamics of a few amino acid residues, to global structural changes as in the hinge bending motion of lysozyme and L-arabinose-binding protein (McCammon and Harvey, 1987). The ability of the structure to fluctuate on a practical time-scale is temperature-dependent (Frauenfelder, Sligar and Wolynes, 1991), and in calorimetric experiments sharp changes in heat capacity are observed, indicative of a change from a rigid to a mobile structure. These transitions are analogous to the glass transitions of amorphous polymers and low molecular weight solutes. A further indication of these dynamic transitions is the disappearance of enzyme activity or the ability to bind ligands over a narrow temperature range (Steinbach *et al.*, 1991; Rasmussen, Stock and Petsko, 1992). For example, in a study of the heat capacity of lysozyme/water mixtures (Yang and Rupley, 1979), a relatively sharp increase in heat capacity, indicating a change from a rigid to a mobile structure, was observed on increasing the water content from 0.1 to 0.27 g water per g protein. The onset of detectable enzymic activity coincided with the transition to a mobile structure (Careri *et al.*, 1980).

The concept that the vitrification of biological structures is a useful preservative mechanism is attractive. For example, a 'glassy' enzyme would not show enzymic activity on practical time-scales. If the translational diffusion of components in the glassy matrix was determined by the macroscopic shear viscosity, then the rate of diffusional encounter of an enzyme and its substrate would be greatly retarded. In a matrix with the shear viscosity of a glass of 10^{12} Pa s, a water molecule would take several hours to diffuse its molecular diameter. The enzyme and substrate could be mixed and there

would be no significant reaction on practical time-scales until the mixture was plasticized, for example by the addition of water, leading to a sharp reduction in the observed viscosity.

Drying as a means of preservation could have several practical advantages. It is probable that the drying of biological structures results in the formation of a glassy matrix containing biopolymers and low molecular weight solutes. Bacterial and fungal spores, and plant seeds (Koster and Leopold, 1988), might be examples of this phenomenon. The high viscosity of the glass would be protective through its effect on diffusive processes. Enzymic action is effectively arrested, and the glassy matrix would prevent further loss of water. It is difficult from the current literature to estimate the magnitude of the latter effect. In many experiments on the drying of an organism or a protein preparation, the material is dried to a constant weight under a defined set of conditions for a specified length of time, often overnight. The achievement of an apparent constant weight could simply mean that the rate of water loss from the sample is very low. Drying a protein could have further advantages in improving its thermal stability. Additionally, biopolymer glasses could potentially trap more water at ambient temperatures than glasses prepared from low molecular weight solutes and this may benefit subsequent rehydration. A remaining question concerns the usefulness and effectiveness of polyols and carbohydrates for the protection of biological structures on drying. A disadvantage of drying proteins or lipid structures alone is that the drying encourages protein aggregation and fusion of the lipid structures. The carbohydrate could therefore function as a diluent, preventing contact and aggregation of the active species. As the carbohydrate is a polyhydroxy compound, it is sufficiently like water in its solution properties, that the aqueous solution conformation of a protein, or the organization of a lipid structure in aqueous dispersion, is retained. This has led to the proposal that the carbohydrate or polyol replaces water in these systems (Clegg *et al.*, 1982; Clegg, 1986).

Freezing of biological materials

The biophysics and biochemistry of biological materials at low temperatures were considered in a recent textbook (Franks, 1985). Pure water may be supercooled below the equilibrium melting temperature of the crystalline solid. Bulk samples of the order of a gram in weight can, with care, usually be supercooled to –12°C. If smaller samples are used, contained in capillaries of 0.5 mm diameter, then it is possible to supercool to –34°C. The extent of supercooling that can be achieved depends on the initial elimination of nucleating impurities. One approach is to use very small sample volumes (e.g. μm, such as in emulsion droplets). The lower limit of supercooling obtained using this approach is –42°C (Angell, 1983).

It has been suggested that insects and other organisms utilize two approaches to prevent ice crystallization. The first is the 'removal' of nucleating impurities. Second, polyols are synthesized, which both depresses the equilibrium melting point of water through a colligative action, and more

importantly extends the limit of undercooling (Franks, Mathias and Hatley, 1990; Storey, 1990).

On cooling below the limit of supercooling, crystallization of the water is inevitable. Through the condensation of water vapour on a liquid helium cooled cryoplate, an amorphous solid form of water can be prepared. On heating, the material undergoes a glass transition and then rapidly crystallizes. Recently, through the employment of extremely fast cooling rates in the region of 10^7 °C per second, it has been possible to vitrify liquid forms of water. Calorimetric experiments have permitted the determination of a glass transition in the region of 136 K (Hallbrucker and Mayer, 1987; Hallbrucker, Mayer and Johari, 1989).

Compared to water, supercooled liquid carbohydrates are much more readily vitrified, and crystallization is avoided even using comparatively slow cooling rates of the order of a few degrees per minute. The behaviour of aqueous solutions of polyols and carbohydrates has also been examined (Slade and Levine, 1988; Levine and Slade, 1988; Franks, 1990). If an aqueous solution of the carbohydrate is cooled below the melting temperature of ice, and ice growth is initiated through nucleation, ice growth will proceed and the remaining aqueous solution of the carbohydrate will become progressively more concentrated. Providing the crystallization of the carbohydrate does not intervene, the viscosity of the solution will also increase progressively until ice crystal growth is not observed on experimental time-scales, and a vitreous carbohydrate/water mixture is obtained. This behaviour is usually described using a solid/liquid state diagram (Levine and Slade, 1986, 1988), which incorporates information on the effect of the solute on the depression of the freezing point, and the dependence of the glass transition temperature of the amorphous carbohydrate/water mixture on composition.

The freezing behaviour of biological materials is relevant to the behaviour of these materials during the process of freeze-drying.

Preservation of proteins by carbohydrates

Investigations have shown that some protection of proteins during preparation and storage, both in aqueous solution and the dry state, can be attained by the addition of stabilizing solutes. Amino acids, salts, polyols and sugars have been found to stabilize proteins in aqueous solution. Carbohydrates and polyols might protect proteins in a number of ways. We have already pointed out that when sugar and protein are mixed in aqueous solution, there is a deficiency in the stabilizing solute in the immediate vicinity of the protein, and the protein is preferentially hydrated. If this effect persists during drying, the added carbohydrate may help the protein retain sufficient water to maintain its conformation. In the dry state, the carbohydrate may also be sufficiently like water in its solvent properties to stabilize protein conformations, similar to those found in aqueous solution. The carbohydrate may also prevent protein aggregation through its effect as a diluent. Another suggestion is that a specific interaction occurs between the sugar and the protein.

Studies of Fourier transform infra-red (FTIR) data (Carpenter and Crowe, 1989) have led to the proposal that intermolecular hydrogen bonds are formed between trehalose and a protein when dried.

There have been a number of studies on the preservation of phosphofructokinase, both during and after freeze-drying (Crowe *et al.*, 1987; Carpenter, Crowe and Crowe, 1987; Carpenter *et al.*, 1988; Carpenter and Crowe, 1988). It was found that the activity of this enzyme was easily lost on freeze-drying, and therefore that it was a good protein on which to test the preservative action of different solutes. The preservative action immediately after drying a number of mono- and disaccharides was tested but the two monosaccharides examined, glucose and galactose, showed no significant preservative effect (Crowe *et al.*, 1987). The disaccharides were effective, their effectiveness increasing as the weight ratio of protein to carbohydrate increased from 400 : 1 to 3500 : 1. The protein activity was monitored soon after freeze-drying, and it was found that at high relative concentrations of the carbohydrate, it was possible to recover between 60 and 80% of the original enzyme activity. In this study, trehalose and maltose were marginally more effective than sucrose. The origin of the increased effectiveness of the disaccharides relative to the monosaccharides is unclear. It cannot simply be that the carbohydrate is functioning as a diluent, since the monosaccharides were found to be ineffective (Crowe *et al.*, 1987; Carpenter, Crowe and Crowe, 1987). In a subsequent study, it was demonstrated that glucose, a monosaccharide, was partially effective (Carpenter *et al.*, 1988). A protective mechanism based solely on a specific molecular interaction is also ruled out, since the disaccharides tested showed comparable protective ability.

At this stage, it is useful to examine some of the potential interactions and reactions that could occur during the freeze-drying process. The protein could denature, or multi-subunit proteins dissociate as a result of cooling prior to freezing. On freezing, the crystallization of water freeze-concentrates the remaining solutes. Depending on the freezing rate, and the rate of freeze concentration, the remaining solutes could either crystallize, phase-separate or form an amorphous glass. A consequence of phase separation or crystallization is the separation of a protein-rich phase, in which the aggregation of protein molecules would be encouraged. The observation by Izutsu, Yoshioka and Takeda (1991) that carbohydrates which crystallize either during the freeze-drying process or on storage are less effective preservatives, would support this suggestion and the general conclusion that for successful freeze-drying, the crystallization of additives should be avoided (Franks, 1990).

Although trehalose readily crystallizes from aqueous solution, it has been shown that during its drying with proteins an amorphous form is retained. FTIR spectra of freeze-dried preparations of trehalose (Carpenter and Crowe, 1989) clearly show the presence of the crystalline dihydrate, whereas on freeze-drying with protein the trehalose gives a different spectrum. Although this spectral change has been interpreted as showing a direct interaction between trehalose and the protein, the spectrum obtained is that of an amorphous form of trehalose (Newman *et al.*, submitted). The presence

of the protein has therefore interfered with the crystallization of the trehalose.

If it is assumed that an amorphous matrix has been obtained, then it is worthwhile to examine other factors which might influence the ability of carbohydrates to preserve proteins. A potentially important factor is the reactions which could occur between reducing sugars and proteins. It is well known that on heating a protein with a reducing sugar, a brown product is obtained. If these reactions can occur in the dry state at ambient temperature, then non-reducing sugars would be predicted to be better preservatives, and would therefore be preferred. Nevertheless, it is still useful to examine this reaction for a number of reasons. First, reducing sugars are invariably present with non-reducing sugars *in vivo*, and it is useful to consider whether the reducing sugars have the potential to react with the protein and alter its activity. Second, since non-reducing sugar preparations often contain reducing sugar impurities, it is therefore useful to be able to define the required degree of purity. Finally, one must also consider the potential cleavage of the glycosidic bond of non-reducing disaccharides to produce reactive products. Although the glycosidic linkage of trehalose is relatively resistant to acid hydrolysis, that of sucrose is much more labile, and for this reason trehalose may be more suitable.

THE MAILLARD REACTION

The Maillard reaction (for a review, see Ellis, 1959) is defined as the reaction that occurs initially between the amino groups of amino acids, peptides or proteins with the glycosidic hydroxyl group of sugars. More complex reactions then occur after this initial reaction, which lead to the formation of brown pigments and polymers. The reaction has also been termed the 'browning reaction' and 'non-enzymic browning'. The formation of Maillard polymers is the result of a number of chemical reactions which are dependent on reaction parameters such as pH, reactant concentration, temperature and type of reactant. A general reaction scheme for the initial stages of this reaction is shown in *Figure 1*. The initial reaction occurs between the sugar and the amino group. This leads to the formation of a Schiff base, which then undergoes cyclization to the corresponding *N*-substituted glycosylamine. The glycosylamine is then converted into the 1-amino-2-ketose via the Amadori rearrangement.

The Maillard reaction has been studied because of its relevance to the colour, flavour and nutritional quality of foods (Mauron, 1981). These studies generally examine the reaction products formed on incubation of a protein and a reducing sugar at relatively high temperatures (*c.* 100°C or higher). At these temperatures, degradation products of the Amadori rearrangement are formed. It is also recognized that the initial Maillard reactions are clinically important (Ahmed, Baynes and Thorpe, 1986; Ahmed *et al.*, 1988; Negia *et al.*, 1985; Baynes, 1991; Baynes *et al.*, 1989, 1991; Bunn, 1979; Higgins and Bunn, 1981; Shapiro *et al.*, 1980) and contribute to the cross-linking and thus ageing of tissue proteins (Baynes *et al.*, 1991). Furthermore, acceleration of

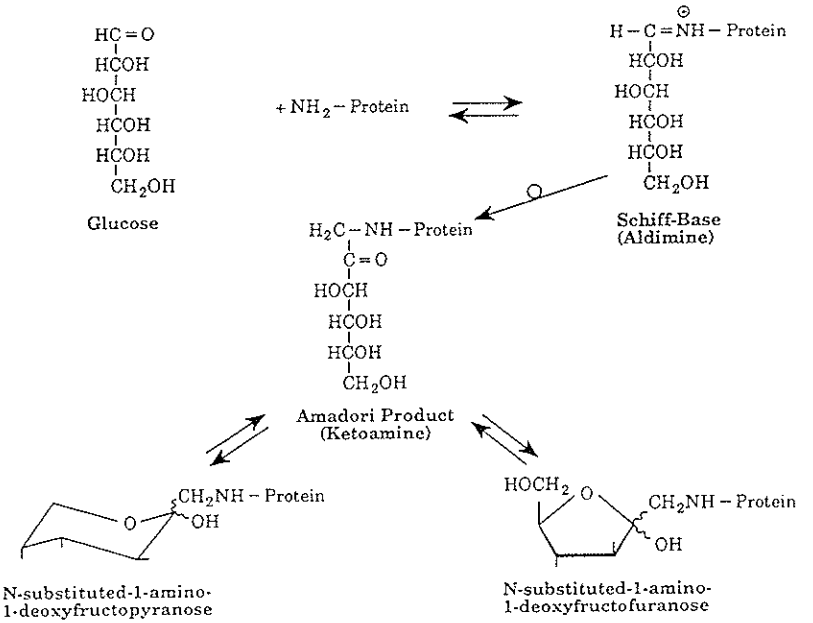

Figure 1. General scheme for the reaction of reducing sugars with proteins.

these reactions through elevated blood glucose levels, is relevant to the pathogenic effects associated with diabetes. The non-enzymatic glycosylation of proteins can alter their conformation and function (Bunn, Garlick and Shaklai, 1984), and lead to progressive enzyme inactivation (Watkins, Thorpe and Baynes, 1985). Further reaction can lead to the cross-linking of proteins (Grandhee and Monnier, 1991) and further affect their biological activity. Recent studies have concentrated on the initial stages of the Maillard reaction, and have shown that even at 37°C the extent of reaction obtained can significantly affect biological function and activity. The reaction is therefore relevant to the use of carbohydrates in the preservation of proteins in the dry state.

In considering potential reaction rates between proteins and reducing sugars in the dry state, it is useful to examine the dependence of the viscosity of the supercooled carbohydrate on both the type of carbohydrate and water content. The viscosity of the liquid influences the predicted rate of diffusional encounter of reacting species, and hence the rate of reaction. *Figure 2* shows the variation in viscosity with temperature for a hexitol, a hexose and a disaccharide. The temperature dependence of viscosity is of the same general form. As the liquid is cooled, its viscosity increases progressively, until a vitreous solid is obtained with a viscosity in excess of 10^{12} Pa s. As this viscosity is approached, very marked changes in viscosity are observed with relatively small changes in temperature. At ambient temperature, the viscosity of the hexitol is in the region of 10^{4} Pa s, whereas that of the disaccharide is effectively infinite. At this temperature, the disaccharide matrix is expected to be much more effective in retarding the rate of reaction. In addition, the stability of the matrix with respect to crystallization is much reduced as the

viscosity is decreased. While glassy matrices of a viscosity of 10^{12} Pa s may be stable for several years, those with a lower viscosity may only be stable for hours or days.

In practical situations, it is very difficult to maintain the amorphous carbohydrate in a completely dry state. The addition of water to the carbohydrate will dramatically affect its viscosity. Water is a very potent plasticizing agent for carbohydrates, and the addition of 10% (w/w) water can depress the glass transition temperature of a disaccharide by 60°C. For storage at ambient temperature, the disaccharide can tolerate the addition of 7% (w/w) water and still be in a glassy state. The addition of the same amount of water to the hexitol leads to a further, dramatic reduction in its viscosity and hence effectiveness as an encapsulating matrix.

Preservation of lipid structures by carbohydrates

The phase behaviour of phospholipids and other triacylglycerols has been much studied in an attempt to gain insight into the behaviour of membranes such as liposomes, which have potential utility for the encapsulation and delivery of active agents such as pharmaceutical products. It is necessary to preserve and stabilize these products prior to use. One strategy is to use carbohydrates as protecting and stabilizing matrices. The usual approach is to freeze-dry a liposome preparation which is dispersed in an aqueous solution of the carbohydrate. The freeze-drying process usually results in the formation of an amorphous matrix of the carbohydrate. The effectiveness of the carbohydrate in preserving the liposome is judged from its ability to encapsulate and retain an active agent.

The mechanism of action of the carbohydrate in preserving the liposome structure is not entirely clear, and one effect of the carbohydrate might be the preservation of the appropriate polymorph of the lipid (e.g. a particular liquid crystalline lamellar structure) during both drying and cooling to freeze-drying

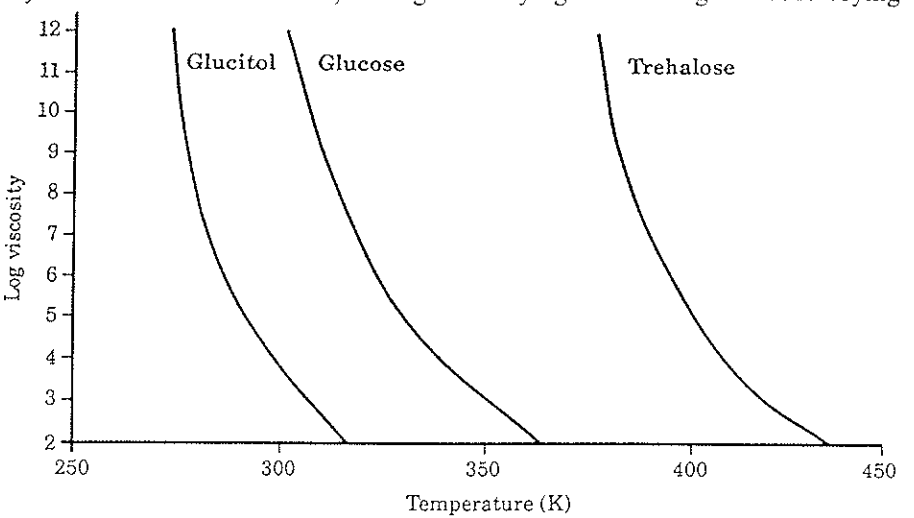

Figure 2. Plot of log viscosity *vs* temperature for a hexitol, hexose and disaccharide.

temperatures. In the freeze-drying operation, the sample may be cooled in liquid nitrogen prior to freeze-drying at, for example, –40°C. From model studies on the effect of carbohydrates on the phase behaviour of phospholipids, it is difficult to imagine how the carbohydrate could prevent the appearance of different polymorphs of the lipid, such as a lamellar gel or lamellar crystalline phase, when the sample is quenched to such an extent. It it more likely that the carbohydrate is more important in preserving the desired structure in the dry state at ambient temperature and on initial rehydration.

The carbohydrate may also prevent fusion of the lipid vesicles, which would result in loss of the encapsulated contents, and it may achieve this in several ways. First, it has been suggested that carbohydrate has a preferred interaction with the head group of the phospholipid. This 'layer' of carbohydrate on the liposome surface could inhibit contact between droplets and subsequent fusion. The greater the matrix viscosity of the undercooled liquid carbohydrate, the slower the rate of diffusional encounter of vesicles, and the greater the stability. From this consideration it would be predicted that the effectiveness of preservation would be in the order disaccharide>monosaccharide>hexitol>glycerol. Second, carbohydrate could reduce the rate of encounter of vesicles simply through its action as a diluent.

The effectiveness of different carbohydrates in preventing fusion and leakage in freeze-dried liposomes has been explored in a number of studies (Crowe *et al.*, 1984, 1986; Strauss and Hauser, 1986; Strauss, Schurtenberger and Hauser, 1986; Madden *et al.*, 1985). The extent of preservation increases with increasing carbohydrate content and approaches a plateau at around 0.5 g carbohydrate per g lipid. Trehalose is generally found to be among the most effective of the disaccharides, which are more effective than monosaccharides and low molecular weight polyols.

The possible mechanisms through which trehalose and other carbohydrates could exert a preservative effect on liposomes were evaluated in a recent review by Baltes and Bochman (1986). A continuing theme of many studies is that the interaction of the phospholipid head group with the carbohydrate, particularly trehalose, is an important factor. In studies of this nature, it is extremely difficult to identify the dominant protective mechanism, and the preservation of these structures probably depends on a range of equilibrium and non-equilibrium factors. In accounts of the biopreservation of liposomes, there has been a tendency to emphasize specific molecular interactions between the carbohydrate and the lipid, but it is also necessary to consider other factors such as the role of the matrix viscosity in influencing aggregation and fusion in these systems.

Current biotechnological use

Air-drying and freeze-drying methods have been used by the food industry for many years to preserve food. These methods of preservation increase the shelf-life of product and there is the advantage of reducing the weight and

volume of the product. There are disadvantages though. By using these methods, the appearance of food may be altered, and the taste and aroma changed. Roser (1991) has claimed that foods dried in the presence of trehalose retain their original texture, colour, taste and cooking properties. Pureed fresh fruit has been dried in the presence of trehalose at 25–50°C and after prolonged storage showed no detectable changes in colour, but it had no aroma. On rehydration, the colour, viscosity and texture of the fresh purees were restored, and after a few minutes the aroma of the fresh fruit was noticeable. Roser (1989) has patented the use of trehalose as a method of protection against denaturation in proteinaceous foodstuffs during drying at temperatures above ambient. Examples of foods that have been treated with trehalose include milk, coffee, orange juice and egg. Trehalose was claimed to be more efficient than both sucrose and glucose in retaining the freshness and structure of the original food when the food was dried at elevated temperatures and then reconstituted.

Restriction enzymes are important in genetic engineering, cutting the DNA into precise and reproducible fragments. Some of the members of this class of enzyme are stable at elevated temperatures, but others are extremely unstable. As a result, these reagents usually have to be stored in solid CO_2, making them expensive to store, difficult to handle and the shipping costs are high. A technique has been developed (Colaco *et al.*, 1992) which enables even the most unstable restriction enzymes to be stored at up to 55°C for prolonged periods with no detectable loss of activity. This patent describes how trehalose and other polyhydroxy compounds have been used to preserve delicate biological substances such as enzymes, antibodies and vaccines, in the dry state and/or at elevated temperatures and/or under irradiated conditions.

Trehalose is an important storage compound for yeasts and fungi. It has also been found that high levels of trehalose in fungal spores enhances tolerance to stress conditions such as increased osmotic pressure, elevated temperature and desiccation. Yeast strains have been developed that provide a yeast with a higher content of trehalose, and as a result the strain has improved stress resistance (Driessen, Osinga and Herweifer, 1991).

Trehalose and other polyhydroxy compounds have been considered for use in medicine as cryoprotectants (Borelli, Semino and Hernandez, 1987; Fahy *et al.*, 1984; Blakeley *et al.*, 1990). It is common practice for organs, etc, to be frozen and then thawed, but there are problems involved with this method. It has been reported (Borelli, Semino and Hernandez, 1987) that isolated islets of Langerhans (used in the treatment of clinical and experimental diabetes) can be cryopreserved using trehalose.

Conclusions

At the beginning of this chapter, we examined the occurrence of trehalose and the association between its accumulation and protection against environmental stress. From *in vitro* studies, it appears that trehalose is best suited to the protection of biological materials in the dry state. Other molecular species

are better osmoprotectants and protectants against chill damage. As for the mechanisms of protection, these have been clarified to a certain extent. There is good evidence that trehalose interacts with the head group of charged phospholipids and can intercalate between bilayers and prevent their fusion. Evidence of a specific molecular interaction with proteins has not yet been obtained. In the preservation of both proteins and lipids *in vitro*, the matrix properties of the carbohydrate, such as its reactivity, stability, concentration and viscosity, are important and need to be recognized so that experiments may be designed to test for specific interactions. The current use of trehalose as a preservative is increasing, and should expand further. The elucidation of the mechanisms of action should improve technological use.

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