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Water, Temperature and Life [and Discussion]

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Water, temperature and life

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Cold is the fiercest and most widespread enemy of life on earth. Natural cold adaptation and survival are discussed in terms of physicochemical and biochemical water management mechanisms, relying on thermodynamic or kinetic stabilization. Distinctions are drawn between general effects of low temperature (chill) and specific effects of freezing.

Freeze tolerance is a misnomer because tolerance does not extend to the cell fluids. Freezing is confined to the extracellular spaces where it acts as a means of protecting the cytoplasm against freezing injury. Freeze resistance depends on the phenomenon of undercooling, a survival mechanism that relies on the long-term maintenance of a thermodynamically highly unstable state. Correct water management involves many factors, among them the control of membrane composition and transmembrane osmotic equilibrium, the biosynthesis of compounds able to afford protection against injury through freeze desiccation and the availability (or inactivation) of biogenic ice nucleation catalysts.

Introduction

'*Who speaks of victory? Survival is all that counts*' (*Rilke*)

The life processes of all organisms are adapted for optimum functioning in their respective 'normal' physiological environments. Any change in the environmental conditions is experienced as a stress that threatens the normal metabolic balance and produces a response designed to counter the effects of the disturbing influence. Physiological stress conditions can arise from climatic changes (pressure, temperature, water balance) or from changes in the chemical environment of the organism (pH, salinity, starvation). They can also be brought about artificially, e.g. by the substitution of H_2O by D_2O (Franks 1982). Of all the natural stress conditions that threaten life on this planet, cold is by far the most widespread. A substantial proportion of the ecosphere is subject to subzero temperatures either permanently or seasonally.

It is surprising, therefore, that until recently, comparatively little effort had been made to strive for a fundamental understanding of the mechanisms that govern cold tolerance, resistance and acclimation in living organisms. What information does exist is of a mainly qualitative nature (Levitt 1980), although descriptive studies of the impact of freezing on plants have a long and distinguished history (Molisch 1897).

It is the purpose of this contribution to analyse the consequences of exposure to sub-optimal temperatures in terms of individual physicochemical and biochemical factors. As water is the major constituent of all forms of life, it is to be expected that the manner in which its physical properties depend on temperature must figure prominently in any study of cold stress.

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Chill and freezing

A clear distinction must be drawn between the chemical and physiological consequences of low temperatures *per se* and those specifically caused by freezing. The former condition is described as chill and does not involve the separation of water as a pure phase (ice). Thus chill conditions are not accompanied by changes in the concentrations of water-soluble components, except in rare cases where such substances might be present close to saturation at normal temperatures, in which case precipitation under chill conditions becomes a possibility.

Before discussing mechanisms of adjustment to low temperature stress in living organisms, it is necessary to consider the physical and chemical events that accompany chill and freezing.

Undercooled water

Because of the ability of water, like most other molecular liquids, to suffer substantial undercooling, chill conditions in aqueous systems can persist down to −40 °C and even below. Angell (1982) has performed the most comprehensive study of liquid water at subzero temperatures. Table 1 summarizes the changes of some important properties of water over the temperature range +25 to −25 °C, the latter being a common seasonal temperature in the natural environment of many species. The temperature dependences are not linear but become more pronounced at lower temperatures. In fact, many physical properties appear to diverge at −45 °C; the origin of this temperature singularity has for some time been the subject of considerable discussion.

Table 1. Physical properties of liquid water at −25 and +25 °C

(After Angell (1982).)

	−25 °C	+25 °C
density/(g cm^{-3})	0.987	0.996
heat capacity (C_p)/(J $(mol\ K)^{-1}$)	80	75
isothermal compressibility/(10^6 MPa^{-1})	720	440
hypersonic sound velocity/(m s^{-1})	1220	1480
dielectric permittivity	102	79
self-diffusion coefficient/(10^5 cm^2 s^{-1})	0.321	2.23
viscosity/(mPa s)	6.5	0.89
pK_w	17.3	14.0

The most dramatic change that takes place in water, at least from the point of view of biochemistry, is probably in the degree of ionization, which in the temperature interval referred to in table 1, decreases by almost two orders of magnitude (Hepler & Woolley 1973). As H^+ and OH^- ions are involved in most life reactions (condensation, hydrolysis, oxidation, reduction) it is likely that this large decrease in K_w will affect equilibrium and kinetic processes. In an aqueous environment the solvent acts as conjugate acid or base and any change in K_w will produce changes in the respective dissociation constants of acids and bases that define ionization equilibria in solution. Figure 1 illustrates such temperature-induced changes in some pK_a values of acids that commonly occur in pH buffer systems (Franks 1985).

Because of the polyelectrolyte nature of most biopolymers, it is to be expected that even small pK changes will affect their conformational stabilities and biological activities. Any such influences would presumably be compounded by the increased dielectric permittivity of the

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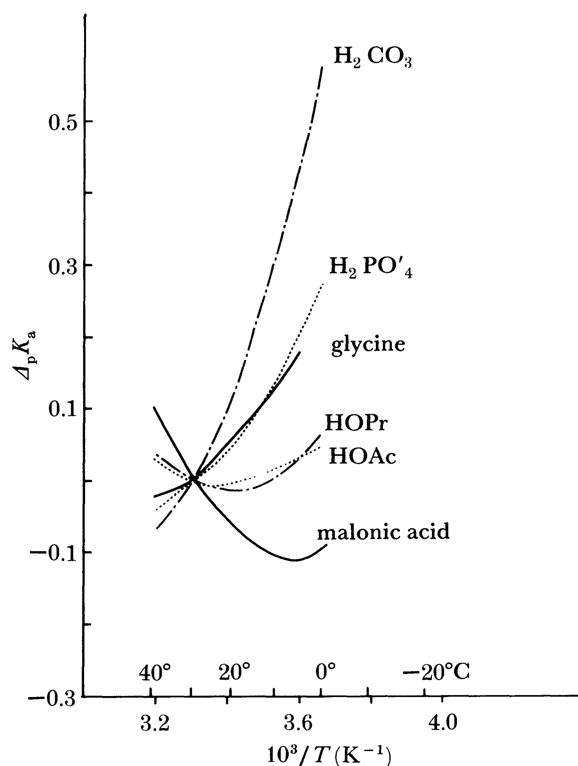

FIGURE 1. The effect of temperature on the pK_a values of some acids used in the preparation of pH buffers, normalized to 30 °C. (Reproduced from Franks (1985).)

solvent, especially in structures with high charge densities, such as nucleotides and sulphated polysaccharides.

Turning now to the dynamic properties of water at low temperatures, the decrease in diffusion rates may also contribute to low temperature stress symptoms. As the temperature is decreased, the transport properties of water in aqueous solutions increasingly deviate from the Arrhenius equation and exhibit a behaviour that becomes increasingly more sensitive to temperature changes than that predicted by a linear log $D(T^{-1})$ plot. Thus at −90 °C, undercooled water has a viscosity comparable to that of glycerol at ordinary temperatures (Lang & Luedemann 1977).

Little is known about the manner in which chill affects the kinetics of coupled biochemical reaction sequences. As evolution has produced efficient coupling mechanisms whereby the product of a given reaction becomes the substrate for a subsequent reaction, it is to be expected that a temperature perturbation must lead to a decrease of the coupling efficiency, because the rates of the various reactions will be affected differently by the temperature change (Franks 1985). ATP production at low temperatures, may also become insufficient to meet the demand (Ellory & Willis 1981). This can lead to different types of chill injury, e.g. the leakage of K^+ from cells.

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Protein stability at low temperatures

Protein integrity is also affected by temperature changes. Whereas the phenomenon of heat inactivation (denaturation) is well documented and has been exhaustively studied (Privalov 1983), the influence of chill conditions on protein stability and function require more attention, if only because, unlike the temperatures above physiological tolerance, such chill conditions are common in the ecosphere.

The possibility of cold unfolding/inactivation of proteins was suggested by Brandts from a consideration of the solution thermodynamics of chymotrypsinogen (Brandts 1964) and ribonuclease (Brandts & Hunt 1967). The phenomenon was first demonstrated experimentally for chymotrypsinogen by Franks & Hatley (1985). Reversible deep chill inactivation has since been reported for lactate dehydrogenase (Hatley & Franks 1986) (see figure 2), and later for metmyoglobin (Privalov *et al.* 1986). Under physiological conditions of pH and medium composition, the natural inactivation temperatures usually lie well below 0 °C. To bring cold denaturation within an experimentally accessible temperature range, most workers have studied the phenomenon after first destabilizing the protein by the addition of urea or the employment of mixed aqueous/organic cryosolvents (Brands 1964; Brandts & Hunt 1967). Neither of these devices is very satisfactory in simulating natural chill conditions. On the other hand, Privalov *et al.* (1986) were unable to follow the cold unfolding to its completion, because of fear of inadvertent freezing. By the use of special techniques to prevent ice nucleation in undercooled solutions, Franks & Hatley (1985) extended the experimentally accessible temperature range to −40 °C and were thus able to study cold unfolding and refolding transitions completely and under physiologically more realistic conditions.

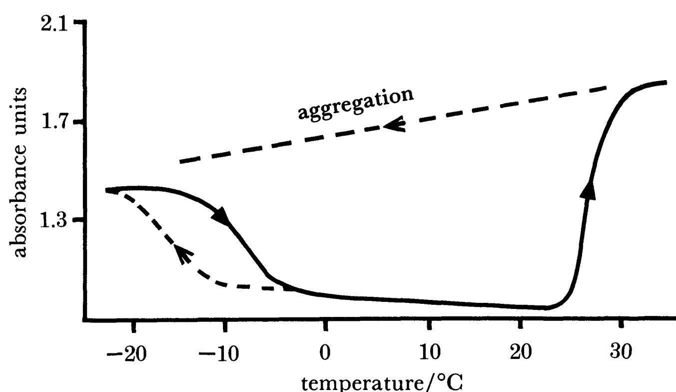

Figure 2. The effect of temperature on the stability of native (active) lactate dehydrogenase (ex rabbit muscle) in 40% (by volume) aqueous methanol, as described by Hatley & Franks (1986). Broken line indicates cooling and solid line indicates heating. The cold inactivation is fully reversible, whereas the heat denaturation is followed by aggregation and precipitation.

None of the earlier thermodynamic models and theories of the heat denaturation of proteins took the possibility of cold inactivation into account (see for example, Privalov (1983)). A recent thermodynamic analysis of the temperature dependences of the heat capacities C of two-state protein equilibria of the type $\mathrm{N} \rightleftharpoons \mathrm{D}$ has demonstrated that, when no restrictions are imposed on the functions $C_N(T)$ and $C_D(T)$, then for the general case where $\mathrm{d}C_D/\mathrm{d}T > \mathrm{d}C_N/\mathrm{d}T$, a cold-induced transition is indicated at some transition temperature T_L, in addition to the

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well-known heat induced denaturation at T_H (Franks *et al.* 1988). The above inequality appears to be physically realistic, even without the need to invoke solvent effects, because the N-state (native) is ordered and compact, whereas the D-state (denatured) possesses a higher degree of chain flexibility and solvent accessibility.

A finite temperature range for stability can be expected whenever the ordered N-state stability depends on two or more types of interactions with temperature dependences of opposite signs. For native proteins, the major stabilizing contribution is the hydrophobic interaction that increases in strength with increasing temperature. Apart from the configurational entropy, the chief destabilizing effects derive from direct hydrogen bonding interactions between water and polar or ionic protein groups; this type of interaction becomes stronger at low temperatures, especially at subzero temperatures (Franks & Wakabayashi 1987).

A balance is achieved at T_L and maintained over the temperature range of the N-state, with a maximum stability at a characteristic temperature in the neighbourhood of $(T_H - T_L)/2$. The thermal denaturation of proteins at T_H, which usually lies above 40 °C, is probably not of any general physiological importance. However, the cold inactivation of many proteins is likely to occur at temperatures commonly suffered by over-wintering or Alpine organisms. Such cold-induced transitions have also been shown to alter the substrate specificities of some enzymes and to change the nature of the particular chemical reactions that certain enzymes catalyse at 'normal' temperatures (Storey *et al.* 1981). This, in turn, may result in the cold-induced biosynthesis of certain substances that can act as natural cryoprotectants, metabolically, by modifying the properties of membranes, or by depressing the freezing point of water.

Phase relations

Solubilities and other phase relations are also affected by temperature, and it is possible that chill can cause changes in the co-existence conditions of heterogeneous equilibria. Such changes are well known in *in vitro* lipid–water systems. They can be of several types: in pure lipid systems, solid–liquid (thermotropic) or solid–solid (polymorphic) transitions are common. In mixed lipid systems, the degree of miscibility can also undergo a discontinuous change at a characteristic transition temperature, as can also the degree of hydration. The phase behaviour of lipid–water systems is subject to great complexity (Pringle & Chapman 1981).

As lipid molecules, like proteins, are amphiphilic, the arguments presented above for the temperature dependence of protein stability–instability relations should also apply to the integrity of ordered lipid phases in aqueous media. Similar effects have been observed for surfactant micelles, with critical micelle concentrations exhibiting minima at temperatures that are specific for given compounds (Kreshek 1975).

Ice nucleation in aqueous systems

Freezing, like other crystallization processes from the melt or from solution, requires the formation of nuclei within the liquid phase (Hobbs 1974). The molecules in a liquid are subject to random density and energy fluctuations as a result of Brownian diffusion. This process can lead to the spontaneous formation of domains in which, for limited periods, a group of molecules adopts a configuration that resembles that of ice. If such a domain has a sufficiently

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long lifetime, then it is capable of initiating the growth of a crystal; otherwise it decays before such growth can occur.

The calculation of nucleation rates requires a knowledge of the physical properties of undercooled water. Such data for deeply undercooled water are now available (Angell 1982) and their substitution into the nucleation equations has led to major changes in the estimated nucleation densities in liquid water, as shown in figure 3 (Franks *et al.* 1984). Nucleation exhibits first-order kinetics, but with an inverse fifth-order temperature dependence. Nucleation thus increases rapidly over a very narrow temperature range and, for water, this occurs in the neighbourhood of -40 °C, which is usually referred to as the homogeneous nucleation temperature T_{hom} of ice.

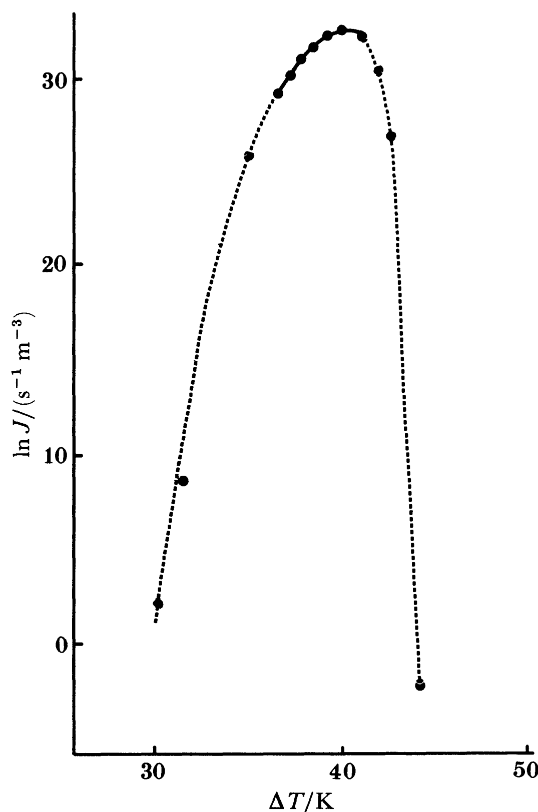

FIGURE 3. The nucleation rate of ice in water as a function of the degree of undercooling ΔT. The solid line corresponds to the experimentally accessible range; calculated values (Franks *et al.* 1984) are shown by the broken line.

In most practical situations, and also in living cells or organisms, ice nucleation is catalysed by particulate matter, such as supramolecular biological structures or crystallites, and therefore the process occurs at temperatures higher than T_{hom}; the process is then referred to as heterogeneous nucleation and the relevant temperature is T_{het}. It must however be emphasized that the actual ice nuclei are identical to those generated in the absence of the catalyst by random molecular fluctuations.

The properties of biogenic catalysts have become the subject of lively interest, since it was discovered that certain microorganisms, particularly *Pseudomonas syringae* and *Erwinia herbicola*,

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contain powerful catalysts for ice nucleation, able to promote ice growth at temperatures as high as −4 °C (Schnell & Vali 1976). The nucleation mechanism has been the subject of intensive study and so-called ice-minus mutants have been grown (Lindow 1983). By contrast, the catalytic sites within human red blood cells that reside on the interior surface of the plasma membrane, only become active at −38 °C (Franks *et al.* 1983). Red blood cells can thus be undercooled to this temperature and recovered without signs of injury. The undercooling abilities of other biological cells lie within these two extremes.

Ice crystallization and freeze concentration

Once nuclei of a critical dimension exist, ice crystallization follows rapidly. As regards biochemical and biophysical processes, the primary and singly most injurious consequence of freezing is the accompanying increase in the concentrations of all water soluble substances. The extracellular spaces (plasma) provide a continuous aqueous phase of large volume, so that heterogeneous nucleation and ice crystallization occur in the extracellular phase before the cytoplasm is nucleated and freezes. The osmotic perturbation thus set up across the plasma membrane results in an efflux of water that is controlled by the excess solute concentration in the extracellular phase and the membrane permeability. Under conditions of natural freezing, i.e. at low cooling rates, the high membrane permeability ensures that osmotic equilibrium is maintained during the gradual freeze concentration of the plasma. Under extremes of temperature, the osmotic cell dehydration may cause mechanical deformation of the membrane and this eventually results in leakage of cytoplasmic solutes or delocalization of membrane-bound proteins, or both.

Concentration and crystallization of other components

Just as ice requires nucleation for growth, so all other components in an aqueous system that are expected to undergo precipitation or other phase changes during cooling, require nuclei for such transitions to occur spontaneously. In a system subjected to freezing, a predicted equilibrium phase change may be partially, or even completely inhibited, resulting in undercooled/supersaturated states. Such systems are thermodynamically unstable but may

Table 2. Eutectic data of phosphate solutions

(From van den Berg & Rose (1959).)

	T_e/°C	$C_e(1)^a$/M	$C_e(2)^b$/M	crystal type	$C_e(1)^a/C_e(2)^b$
NaH_2PO_4	−9.7	3.42	—	$NaH_2PO_4 \cdot 2H_2O$	—
Na_2HPO4	−0.5	—	0.11	$Na_2HPO_4 \cdot 12H_2O$	—
NaH_2PO_4–Na_2HPO4	−9.9	3.42	0.06	$NaH_2PO_4 \cdot 2H_2O$, $Na_2HPO_4 \cdot 12H_2O$	57 (0.72)[c]
KH_2PO_4	−2.7	0.92	—	KH_2PO_4	—
K_2HPO_4	−13.7	—	2.85	$K_2HPO_4 \cdot 6H_2O$	—
KH_2PO_4–K_2HPO_4	−16.7	1.30	2.70	KH_2PO_4, $K_2HPO_4 \cdot 6H_2O$	0.48 (0.72)[c]

[a] (1), monosodium salt (NaH_2PO_4) or monopotassium salt (KH_2PO_4).
[b] (2), disodium salt (Na_2HPO_4) or dipotassium salt (K_2HPO_4).
[c] Corresponds to the pH 7 buffer ratio.

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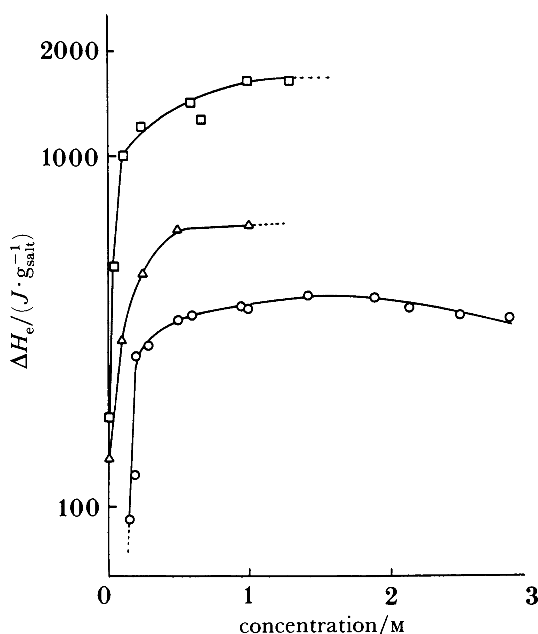

FIGURE 4. Enthalpy of eutectic melting of salts from frozen solutions as a function of the concentration, after Murase & Franks (1990), indicating incomplete precipitation at low concentrations. The plateau value of ΔH is assumed to reflect complete precipitation; (□), KH_2PO_4; (△), $NaCl \cdot 2H_2O$; (○), $K_2HPO_4 \cdot 6H_2O$.

persist for long periods. Buffer solutions provide a good example of such thermodynamic instability. Table 2 summarizes the eutectic details for phosphate buffer solutions. From these data, pH changes during freezing have been predicted (van den Berg & Rose 1959). However, such predictions are based on the assumption that binary and ternary eutectic phase separations actually take place in the freezing system. The data in table 2 suggest that a sodium buffer mixture (pH 7) would become subject to major pH changes during freezing. However, the *actual* proportions of phosphates that crystallize spontaneously during freezing depend on their initial concentrations (Murase & Franks 1990). This is shown in figure 4 and suggests that under physiological conditions, in the range up to 300 mM, precipitation of most salts would be incomplete. Indeed, some salts, e.g. NaH_2PO_4, do not precipitate at all without seeding; others, while spontaneously forming binary eutectics with ice, remain in supersaturated solutions in the presence of other salts. Altogether, the *actual* phase behaviour of ternary aqueous mixtures can be extremely complex and may bear little resemblance to the predicted equilibrium phase diagram.

SUPERSATURATED SOLUTIONS AND GLASS TRANSITIONS

As a direct consequence of supersaturation, the viscosity of a solution will increase, at first as predicted by the Arrhenius equation. Eventually the viscosity increases ever more steeply with decreasing temperature and increasing concentration. As diffusion slows dramatically, so that ice cystallization is inhibited and eventually ceases altogether, at least on practical timescales. The heterogeneous mixture then consists of a pure ice phase dispersed in a freeze-concentrated supersaturated solution that is in the form of a glass, i.e. an amorphous solid with

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a viscosity of the order of 10^{12}–10^{14} Pa s. With increasing temperature, the freeze-concentrate undergoes a glass–rubber transition at a characteristic temperature T_g' (Slade & Levine 1988).

Water increases the plasticity of all naturally occurring materials, in that it depresses T_g, so that the locus of $T_g(w)$, where w is the water content, corresponds to an isoviscosity curve that meets the liquidus curve at T_g' at which temperature the water content per gram solid is w_g'. A typical state diagram for the sucrose–water system is shown in figure 5 (MacKenzie 1977). For the maximally frozen solution $T_g' = -32$ °C, where the freeze concentrated mixture contains 0.56 g water per g sucrose.

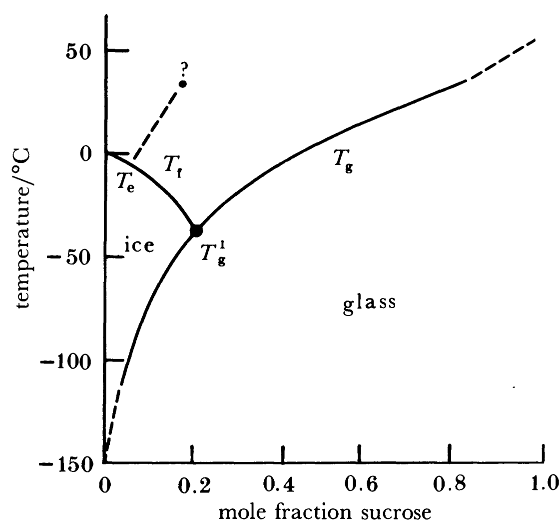

Figure 5. Solid–liquid state diagram for the sucrose–water system, adapted from MacKenzie (1977). T_f, equilibrium freezing point; T_e, hypothetical eutectic temperature; T_g, glass transition (isoviscosity) curve, with T_g', freezing point of the maximally freeze-concentrated mixture.

In the glassy state, translational diffusive motion is almost completely inhibited. Thus, at T_g, diffusion occurs at a rate of approximately 0.3 μm per year, but increases rapidly once the system has gone through the glass transition. The physiological significance of aqueous glassy states is related to the possibility that under extremes of freezing the cytoplasm of certain cells or organisms may become so concentrated in potentially glass-forming substances that at low environmental temperatures T_g' is reached. Similar reasoning can be advanced to account for dormant states of bacterial spores and for the phenomenon of anhydrobiosis. In each case low molecular mass carbohydrates appear to be implicated, but the thermophysical properties of amorphous sugars and sugar alcohol are as yet largely unexplored (Finegold *et al.* 1989).

Kinetics in part-frozen systems

Another consequence of freeze concentration is its effect on chemical kinetics. Reaction rates in part-frozen mixtures exhibit complex temperature dependencies, because the rate-retarding effect of cooling competes with a rate enhancement caused by increasing concentration during freezing. The result is usually a dramatic increase in the rate at the onset of freezing. The phenomenon was discussed in detail by Fennema (1975); more recent investigations in our laboratory have compared the kinetics of frozen and undercooled systems (Hatley *et al.* 1986).

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The effects of chill and freezing on the oxidation rate of ascorbic acid are compared in figure 6.

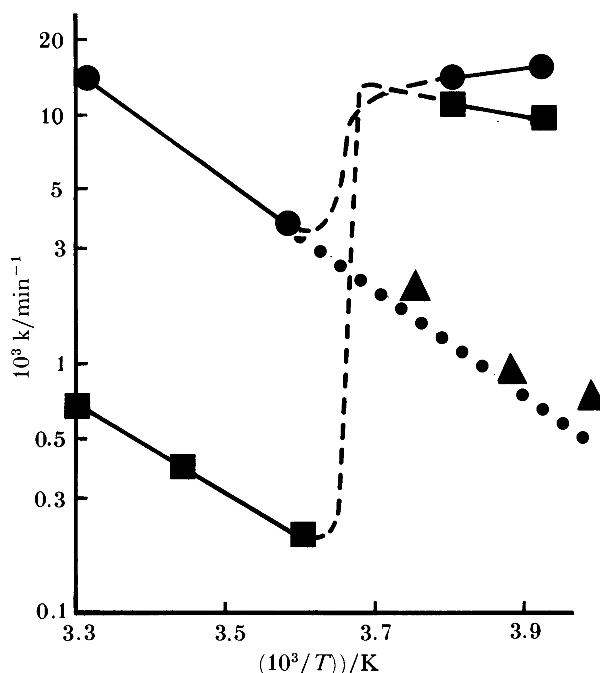

FIGURE 6. Oxidation rates of ascorbic acid in dilute (■) and concentrated (●) solution as a function of temperature, under conditions of freezing and undercooling (▲). The broken lines indicate the effects of freeze concentration. (Redrawn from Hatley *et al.* (1986).)

In summary, for cells or organisms to survive chill and freezing, they must change their chemistry so as to be able to cope with the various deleterious influences described in the preceding sections. We shall now examine a number of possible survival strategies.

Stress tolerance and resistance

A living organism can respond to an environmental stress in one of two ways. It can either yield to the stress conditions and make suitable provisions for survival, or it can attempt to resist the stress. The mechanical analogues correspond to plastic deformation or visco-elastic flow on the one hand, and an elastic response on the other. In the latter case, the removal of the force is followed by complete recovery, but a critical stress exists, the elastic limit, beyond which damage is irreversible.

The term chill tolerance is commonly applied to organisms that can survive temperatures that, although suboptimal, are never low enough for freezing to occur. Where subzero temperatures do occur, but the organism does not freeze, then chill tolerance can be equated to freeze resistance, because even under deep chill (undercooled) conditions, no concentration of solutes occurs and isotonic equilibrium is maintained. Real freeze resistance is rare in fully hydrated higher forms of life, especially in those plants and animals that seasonally experience low subzero temperatures.

In the biological context, the term freeze tolerance is applied to organisms that can survive partial freezing of their tissue water. However, such tolerance does not extend to the freezing

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of cytoplasmic water; the description is thus somewhat misleading. Indeed, the freezing of extracellular water with the concomitant concentration of the cytoplasmic contents is one of the factors that prevents the freezing of the cell.

Chill injury and acclimation

For the purposes of the following discussion, we distinguish between chill and undercooling, although in terms of the physical and chemical consequences such a distinction can hardly be justified. Chill refers to suboptimal temperatures lying above the normal freezing point of water; it can be treated by using equilibrium thermodynamics. The undercooled state, below the normal freezing point of water, is thermodynamically unstable and relies for its kinetic stability on the prevention of ice nucleation.

Chill acclimation does not require the obligatory biosynthesis of cryoprotectants but may, nevertheless, involve major metabolic changes, some of them discussed in detail in other contributions to this volume. The triggers for the hardening (acclimation) process involve a combination of temperature and photoperiod, even in cold hardy animals (Duman 1982). Most attention has in the past been paid to membrane lipid changes that accompany hardening (Heber *et al.* 1981). On the other hand, there is mounting evidence of changes in metabolic processes, such as enzyme cold inactivation and an increased substrate sensitivity of certain enzymes (Martin 1963). Comparative studies involving cold-sensitive and cold-hardy mutants have helped with the cataloguing of metabolic changes and various kinetic models have been proposed which, although providing a fit to the data, do not explain the complex interplay of competing processes, such as biosynthesis, enzyme inhibition, etc.

Strategies for freeze tolerance

Unlike chill stress, which is purely a temperature effect, freezing is described as a water stress, as it causes an osmotic water flux from the cell. In this sense, the stress effects of freezing, drought and salinity resemble each other, except that drought and salinity can be studied under isothermal conditions and in homogeneous systems. Freezing is more complicated, as the observed effects result from a superposition of freeze concentration and low temperature. The dehydration of living cells during freezing is usually countered by the accumulation of so-called compatible solutes and the process is referred to as osmoregulation (Gould & Measures 1977). Little is known about the particular attributes of certain molecules or ions that make for compatibility with the cytoplasmic components. The main two groups of substances that are accumulated in organisms during the hardening process are free amino acids and low molecular mass polyhydroxy compounds (PHCs). Compatible solutes must be soluble and not crystallize from aqueous solution, must not be metabolic intermediates and must protect proteins against salt denaturation.

The ability of sugars and sugar alcohols to stabilize proteins in solution has been studied in detail by Gekko & Morikawa (1981) and Gekko & Timasheff (1981). The observed effects resemble those produced by sulphates and phosphates, which are referred to as salting-out. Such effects appear to involve the hydrophobic domains of proteins. PHCs are quite remarkable in this respect because they can protect biological and technological functions of complex structures against quite extreme dehydration, whether caused by freezing or heat. The

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mechanisms are obscure, but the ability of sugars, especially trehalose, to protect, not only isolated proteins and lipid phases (Crowe & Crowe 1982), but microorganisms and even small animals, against almost complete desiccation is well documented (Crowe & Clegg 1978); the technological implications are not escaping the attention of relevant industries (Roser 1988). The detailed mechanism whereby glycogen degradation and the accumulation of low molecular mass PHCs are triggered, is not yet fully understood, nor is the molecular nature of the actual protection.

Although PHCs are also found in some cold-acclimated plants, free amino acids are more common, in particular glutamate and proline (Pollard & Wyn-Jones 1979). In addition, non-protein amino acids also figure as freeze protectants; glycinebetaine is found in many frost and drought hardy species. Proline and betaine transport genes (*proP proU*) have been identified in microorganisms (Higgins *et al.* 1987). Permeases exist in membranes and also betaine-binding proteins with high binding affinities. The permease only allows betaine transport when the cell is stressed. Thus an osmotic stress turns on gene expression but without being ion specific. For instance, when the NaCl concentration is increased from 0.1 to 0.5 M, the rate of expression increases 500-fold. Of the two genes, *proU* is only activated in the presence of potassium. Expression is then a linear function of potassium ion activity. Genes have also been identified for trehalose synthesis, polyamine transport, glutamate transport/synthesis and membrane-derived oligosaccharides. All genes are activated by potassium transport, either by turgor or by the action of potassium on internal enzymes. It is one of the intriguing puzzles of biology why K^+ should be a compatible solute, whereas Na^+ is not.

Whereas the biochemistry of frost hardening and protecting is probably similar for different species, the acclimation periods vary dramatically. Thus the fully hardy state that takes several months to reach completion in a tree, can be achieved in a few days by insects and in a few minutes by microorganisms. Changes in membrane lipids generally take place more slowly than the synthesis of low molecular mass cytoplasmic protectants, and the same is true for the dehardening process, which commences as soon as the stress is relaxed.

The onset of extracellular freezing produces a water stress on the cell. The rate at which water is drawn from the cell is proportional to the osmotic gradient across the cell membrane. The stress is therefore minimized if the extracellular fluids freeze at their equilibrium freezing point, i.e. without undercooling. Efficient nucleation would help in this process. Reference has already been made to biogenic ice nucleation catalysts in microorganisms, but the phenomenon of catalysed ice nucleations has also been observed in some winter hardy insects in their fully acclimated state (Duman 1982).

Strategies for freeze resistance

As *in vivo* freezing always commences in the extracellular water, the complete inhibition of nucleation at subzero temperatures is a primary requirement for freeze avoidance. Antarctic fish species are the most intensively studied example of antifreeze activity (Feeney & Yeh 1978). The antifreeze factor (AFGP) has been identified as an oligomer of a simple triglycopeptide Ala-Ala-Thr, with disaccharide residues linked to each threonine. The peptide has been shown to inhibit the growth of ice crystals in slightly undercooled water (*ca.* -2 °C), but it loses this ability at lower temperatures. A solution conformation of the antifreeze glycopeptide has recently been suggested (Bush & Feeney 1986). There is, however, as yet no

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convincing mechanism for the ability of the glycopeptide to retard the nucleation rate of ice *in vivo*, although it has been demonstrated that the homogeneous nucleation of ice (at −40 °C) is affected by low AFGP concentrations (Franks *et al.* 1987). Antifreeze proteins have also been detected in several fish species in the Northern hemisphere and in some freeze-intolerant insects. Their peptide sequences are more complex and no connection has yet been established between specific secondary structures and ice nucleation inhibition.

In principle, freezing could also be avoided by a colligative freezing point depression, but the concentrations of solutes required probably make this an unrealistic alternative. In any case, to avoid large osmotic stresses on the cell, similar concentrations of solutes would need to be accumulated in the cytoplasm.

Moisture management is yet another method for preventing freezing. This is the controlled and selective dehydration of those domains of a system that would suffer injury. Although moisture management is practised in the food industry to achieve microbial safety, it is not certain whether the mechanism also exists *in vivo*. It has been suggested that the resistance of bacterial spores to environmental extremes is based on the selective dehydration and compression of parts of the cell (Gould & Measures 1977).

Probably the most extreme example of freezing resistance by deep chill is found in some plant species, e.g. hickory. Cold hardening takes place during the months of September–January and in its fully hardened state the tissue water is able to tolerate undercooling to temperatures of *ca.* −45 °C before freezing occurs suddenly; freezing is then invariably lethal (Burke *et al.* 1976).

Conclusion

The fact that water freezes at the centre of the temperature range that is normally associated with life is coincidental but emphasizes that many organisms have to survive periods at temperatures at which their tissue water becomes vulnerable to freezing. They can achieve such survival either by a controlled cell dehydration in response to an osmotic stress or by deep chill, i.e. by avoiding freezing altogether, a themodynamically highly unstable state.

In any event, ice nucleation is controlled *in vivo* so as to assist the particular survival strategy adopted, by the biosynthesis of antifreezes or nucleation catalysts. Hardening is also accompanied by the accumulation of water soluble protectants (amino acids, PHCs), which reduce the amount of water able to freeze or able to leave the cell during the applied stress. The concentration of such protectants may, in some cases, reach levels that would enable the cytoplasm to undergo a rubber–glass transition and thus become completely resistant to any further applied stress perturbation.

As water is the basic substrate for all forms of life, it is not surprising that effective water management is basic to all cold survival strategies.

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Discussion

J. G. Baust (*Centre for Cryobiological Research, State University of New York, U.S.A.*). The lists of published T_g' are typically obtained from saturated sugar–water solutions and therefore may not be relevant to the 'ecological' state of some organisms. For example, the T_g' of a trehalose–H_2O solution is approximately −30 °C. In anhydrobiotic species such as *Artemia salina* the encapsulated cyst's T_g' is +2 °C, which corresponds to the T_g of trehalose dihydrate. Could Professor Franks comment on this point?

F. Franks. I agree that the published glass transitions of *binary* sugar–water blends have little ecological significance. However, they can serve as guidelines in the estimation of T_g values for 'real' mixtures, because, as a first approximation, T_g is a function of the nominal molecular mass of a mixture. As regards the specific example of trehalose, the dihydrate melt has a water content of 9.5% and at this composition, $T_g = +2$ °C. When a trehalose solution is frozen, the ultimate freeze concentrate has a water content of 17% and at this composition $T_g = T_g' = -30$ °C.

J. G. Baust. Professor Franks stated that life in the undercooled state is tenuous because of the increased likelihood of nucleation below the solution melting point once freezing, crystallization, occurs, there is, in your words, '... no way back...' until the melting point. Is it not important to keep in mind that there is an ice–liquid water continuum between the approximate homogeneous nucleation temperature and the melting point? That is, ice content is temperature dependent and begins at low temperatures, *ca.* −40 °C, upon warming. Therefore, as temperatures fluctuate, the unfrozen fraction surrounding a cell can vary. I would appreciate Professor Franks' clarification of this point.

F. Franks. It is, of course, absolutely correct that an ice–liquid-phase equilibrium exists over the whole of the ecologically important temperature range, say −40 °C up to the melting point of the body fluids. The significance of my statement was that there is no way in which ice (the stable phase), once it has formed, can be converted back to undercooled water (the unstable phase) isothermally.

J. G. Baust. Recent reports have focused on the *in vitro* analysis of antifreeze proteins and their proposed role of inhibiting migratory recrystallization in fish and insects. Would Professor Franks comment on the probable *in vivo* role of AFP in 'frozen' insects (freeze tolerant) and polar fish, the latter of which are said to survive with levels of circulating ice crystals.

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F. Franks. The physiological role of antifreeze proteins is still subject to uncertainty. The ability of AFP to inhibit ice crystal growth is now beyond doubt and this presumably includes the inhibition of migratory recrystallization of existing ice crystals.

The effect of AFP in nucleation of ice is still not clear. In bulk water AFP activity is confined to temperatures above −2 °C, suggesting that heterogeneous nucleation is marginally affected. Droplet emulsion experiments (Franks *et al.* 1987) have indicated that homogeneous nucleation of ice (at −40 °C) is also affected by AFP, but the mechanism is still unclear.

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A. Hvidt (*University of Copenhagen, Denmark*). Professor Franks presented interesting eutectic data on aqueous phosphate solutions. The enthalpy of eutectic melting, ΔH_e, depends on concentration. Could Professor Franks explain in more detail, what is meant by ΔH_e?

F. Franks. The eutectic point (at constant pressure) is an invariant point in terms of the phase rule, i.e. zero degrees of freedom. The latent heat of fusion of a given eutectic mixture therefore has a fixed value. Once the eutectic mixture melts, but only then, depending on the initial composition of the mixture, either ice will melt or salt will dissolve, both of which processes are associated with ΔH values that are concentration dependent. In DSC, the eutectic melt invariably appears as a very narrow endotherm, like all other first-order phase transitions of pure compounds.

D. Chapman (*Department of Protein and Molecular Biology, Royal Free Hospital School of Medicine, London, U.K.*). What is the physical evidence for the protein inactivation process that Professor Franks suggests occurs at low temperature? Have techniques such as FTIR spectroscopy been applied to study changes of secondary structure?

F. Franks. The evidence for low temperature protein inactivation is reviewed in several contributions to this volume. The probability of low temperature destabilization was first suggested on thermodynamic grounds, because the free energy of protein denaturation ΔG (N → D) appears to be a parabolic function of temperature. The high temperature transition T_H has been the subject of intensive study, but the low temperature, T_L, at which $\Delta G = 0$, usually lies below the freezing point of water in the solution, and therefore requires special techniques for its identification (Franks & Hatley 1985). Low temperature unfolding/dissociation has been studied *in vitro* by UV spectroscopy, NMR, CD and microcalorimetry (Franks & Hatley 1985; Hatley & Franks 1986; Privalov *et al.* 1986). Low-temperature inactivation of enzymes *in vivo* is a well-known phenomenon and is discussed by Jaenicke and Storey in this volume.

R. Jaenicke (*Institut für Biophysik und physikalische Biochemie, Universität Regensburg, F.R.G.*). In connection with Professor Franks' statement that lyophilization kills enzymes, there are two questions: (i) how is it that in the study by Careri *et al.* of the dehydration–rehydration of lysozyme, the enzyme regains full activity after 'complete removal of water'? (ii) Would not

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the decreased diffusion coefficient in the glass state take care of the reversibility of dehydration because 'surface denaturation' cannot occur because the enzyme is trapped in its native state?

Reference

Careri *et al.* 1980 *Nature, Lond.* **73**.

F. Franks. In the studies referred to, the pure lysozyme–substrate complex was carefully dried at ambient temperatures to a nominal zero water content. The dehydrated complex was then in a glassy state and upon rehydration to 0.3 g water g^{-1} complex, enzyme activity was found to return, suggesting that at this level of water induced plasticity, the glass transition of lysozyme is close to room temperature.

In many practical freeze drying operations the starting material contains proteins, the stability of which is much more marginal than that of lysozyme. The mixtures also contain salt and upon freezing, the high salt concentrations can, and do, cause irreversible inactivation of the protein.

I believe that the reason for inactivation during freezing is not surface denaturation but salt concentration or pH denaturation, or both; both of these can be reduced by additives that can raise T_g of the freeze concentrate. With very few exceptions, of which lysozyme may be one, proteins are not easily trapped in their native states. It needs very careful processing to achieve complete recovery of activity after freeze drying (Franks 1989).

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