Freeze tolerance: constraining forces, adaptive mechanisms

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For a variety of ectothermic animals, survival of subzero temperatures is aided by a natural capacity to tolerate extracellular freezing. Both low temperatures and freezing place inescapable constraints on the behaviour of molecules and biological structures. For example, low temperature affects metabolic rates, membrane fluidity, and weak bond interactions governing protein structure and function, whereas damage from freezing includes osmotic stress, membrane deformation, dehydration, physical damage by ice, and the consequences of long-term ischaemia. Selected biochemical adaptations permit survival of exposure to freezing by maintaining cell integrity, subcellular structure, energy production, and homeostasis. The key adaptations for freeze tolerance deal with the following: (*i*) control of extracellular ice: ice-nucleating proteins induce ice formation at multiple extracellular sites and at high subzero temperatures, whereas thermal hysteresis proteins inhibit the recrystallization of ice during long-term freezing; (*ii*) regulation of cell volume: the colligative action of high concentrations of polyols limit freeze concentration of the cell beyond a critical cell volume; (*iii*) protection of subcellular organization: trehalose and proline stabilize membrane bilayer structure, polyols stabilize protein structure; and (*iv*) viability in the frozen state: a well-developed tolerance for ischaemia plus mechanisms of facultative metabolic depression support long-term survival. Potential constraints of low temperature on metabolic functions are overcome to produce a metabolism that remains integrated and balanced over a wide temperature range. In addition, temperature change is exploited as a signal for the induction of various freeze tolerance adaptations.

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Pour un bon nombre d'animaux ectothermes, la survie à des températures inférieures à 0 est favorisée par une tolérance naturelle au gel extracellulaire. Des températures froides et le gel imposent des contraintes inévitables au comportement des molécules et des structures biologiques. Par exemple, une température faible affecte les taux de métabolisme, la fluidité des membranes et les interactions entre des liens faibles qui déterminent la structure et le fonctionnement des protéines; d'autre part, le gel entraîne des stress osmotiques, une déformation des membranes, une déshydratation, des dommages physiques causés par la formation de glace, et des ischémies qui ont des conséquences à long terme. Des adaptations biochimiques particulières pemettent la survie au gel en assurant l'homéostasie et le maintien de l'intégrité cellulaire, de la structure infracellulaire et de la production d'énergie. Les principales adaptations reliées à la tolérance au gel sont (*i*) le contrôle de la glace extracellulaire : les protéines de nucléation de la glace déclenchent la formation de glace en plusieurs sites extracellulaires et à des températures sous zéro assez élevées, alors que les protéines de l'hystérèse thermique inhibent la recristallisation de la glace au cours d'un gel prolongé; (*ii*) la régulation du volume cellulaire : l'action regroupante de concentrations élevées de polyols limite la réduction des cellules au cours du gel au-delà d'un volume critique; (*iii*) la protection de l'organisation au niveau infra-cellulaire : le tréhalose et la proline stabilisent la structure double de la membrane, les polyols stabilisent la structure des protéines; (*iv*) la survie en état de gel : une tolérance élevée à l'ischémie et les mécanismes responsables de la diminution facultative du métabolisme assurent la survie à long terme. Les contraintes imposées par la température faible sur les fonctions métaboliques sont compensées par l'existence d'un métabolisme qui demeure intégré et équilibré à un grand éventail de températures. De plus, le changement de température est utilisé comme signal de déclenchement de plusieurs des adaptations qui assurent la tolérance au froid.

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Although the freezing point of water is at about the midpoint of the environmental temperature range on earth, the vast majority of life forms on the planet must elude exposure to subzero temperatures to prevent lethal freezing of body fluids. For some species, however, exposure to subzero environments is unavoidable (notably many terrestrial ectotherms in temperate and polar climates) and they have taken one of two routes for survival: freeze avoidance or freeze tolerance. Animals displaying the freeze-avoidance strategy use an extreme depression of the supercooling point of body fluids to elude lethal freezing and depend upon adaptations that reduce the probability of spontaneous nucleation at subzero temperatures. Freeze-tolerant animals, by contrast, initiate and control ice formation in extracellular fluid spaces while at the same time taking measures to effectively render cytoplasmic water unfreezable. Natural freeze tolerance occurs quite widely among terrestrial insects, most commonly in the Coleoptera, Diptera, Hymenoptera, and Lepidoptera (Ring 1980; Block 1982). In addition, at least four species of terrestrially hibernating frogs tolerate freezing (Storey 1985; Storey and Storey 1986), as do various intertidal marine bivalves, gastropods, and barnacles (Aarset 1982; Murphy 1983). The present article considers the factors involved in cell death from freezing and the adaptive strategies used to confer freeze tolerance in animals.

Living organisms are constrained by two forces: the physicochemical properties of their constituent molecules and the metabolic functions required to perpetuate life. To a large extent, biochemical adaptation (through modulation of quantities, structures, regulatory properties, protective mechanisms, etc.) forms the bridge between the immutable properties of molecules (and their responses to physical conditions such as temperature change) and the functional requirements for life (e.g., at the cellular level the need to maintain energy production, biosynthesis, membrane irritability, cell integrity, and subcellular compartmentation).

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Constraints on life at subzero temperatures

Life at subzero temperatures is challenged by severe physical constraints: (*i*) the effects of low temperature on the properties of molecules and on rate processes, and (*ii*) ice as the only stable physical state of water. Both have inescapable consequences for cell function.

Subzero temperatures, per se, have no uniquely injurious effects on metabolic processes but simply intensify cold sensitivities. Injuries from chilling, hypothermia, or cold shock arise from disruptions of metabolic regulation and imbalances in metabolic rates when organisms are exposed to low temperatures outside the normal thermal experience. The effects of low temperature on molecules have a variety of negative consequences for metabolic processes (Hochachka and Somero 1984; Franks 1985). Temperature-induced changes in the conformation, orientation, and mobility of lipids in membranes result in phase separations and phase changes and affect various metabolic processes including the binding of membrane-associated proteins, transmembrane diffusion or transport, and the functioning of membrane-bound metabolic pathways. Differential effects of low temperature on the various types of weak bond interactions induce conformational changes in proteins and (or) protein denaturation and affect not only the individual functioning of enzymes and other proteins but also the integrated function of enzymes in a pathway, the formation of multienzyme complexes, and enzyme association with structural components of the cell. Differential temperature coefficients for various processes also create imbalances in the rates of cellular functions at low temperature, disrupting metabolic regulation and homeostatic control (e.g., even a minor difference in Q_{10}, 2.0 versus 2.1, will produce a substantial rate difference, 18% between 20 and −20°C, when the temperature range is large).

Injuries from freezing are primarily due to the effects of freeze concentration on cells but also arise from physical damage by ice (Franks 1985). Both of these factors combine to destroy subcellular structure and function when freezing is intracellular with the result that such freezing is lethal in virtually all instances (except under controlled laboratory conditions of ultrafast rates of freezing and thawing, 100−700°C min^{-1}, producing minute ice crystals (Mazur 1984)). In nature, freeze tolerance means survival of extracellular freezing only. However, the consequences of ice formation in extracellular compartments are also damaging without protection. Cells are subject to osmotic shock as extracellular ice formation sets up a rapid redistribution of water and solutes across the cell membrane. The withdrawal of pure water into extracellular ice can also expose cells to dehydration and high solute concentrations (especially ions), both of which can have potentially injurious effects on membrane or protein structure and function. Structural damage from ice can occur when cells are trapped in minute channels of unfrozen extracellular fluid or when ice disrupts cell−cell connections or damages capillaries. Structural damage is intensified by recrystallization, the tendency for small, thermodynamically unstable ice crystals to regroup into larger crystals over time, particularly at the mild subzero temperatures typical of natural exposure to freezing. The properties of molecules are also altered in freeze-concentrated solutions: viscosity increases greatly so that diffusion processes are strongly reduced, pH of buffers can shift dramatically, reactions show extreme deviations from Arrhenius behaviour, and enzyme reaction order can be altered (Franks 1985). In addition, the presence of ice in extracellular fluid spaces stops intertissue transport (e.g., oxygen, fuels, waste products, hormones) in multicellular organisms and requires individual cells to survive using endogenous reserves alone throughout the freezing exposure.

All of the injurious effects of low temperature and freezing are evident when artificial cryopreservation of mammalian organs is attempted. Metabolic regulation and metabolic rates are rapidly disrupted by hypoxia−ischaemia and hypothermia; particularly damaging are imbalances between ATP-driven membrane ion pumps and the opposing ion movement through ion channels (Hochachka 1986). Physical damage to extracellular connections occurs during freezing and is intensified by recrystallization during warming while freeze concentration dehydrates cells and damages membranes by deformation (Mazur 1984; Jacobsen and Pegg 1984). Added cryoprotectants, although alleviating some of the damage brought about by freeze concentration, are toxic to metabolic processes and cause osmotic stress during infusion and removal (Fahy 1986). All of these potential injuries must, to some extent, be eliminated or compensated for by the naturally freeze-tolerant animal.

Adaptive requirements for freeze tolerance

Given the constraints of low temperature on molecular properties and the potentially lethal effects of freezing on cells, what biochemical adaptations are necessary to confer freeze tolerance on a multicellular organism? Certainly, prevention of intracellular ice formation must be primary. Regulation of cell volume, by limiting freeze concentration and maintaining a critical cell volume and (or) unfrozen fraction, is also key (Meryman 1974; Mazur 1984). A lethal limit of about 65% of total water as ice has often been reported, for both naturally freeze-tolerant animals (Williams 1970; Zachariassen et al. 1979*a*; Lee and Lewis 1985) and cryopreserved mammalian cells (Meryman 1974). This limit in freeze-tolerant animals does not appear to be due to injurious effects from either high ion or solute concentrations or dehydration on cellular components, but is probably related to the structural stress of freeze concentration on membranes and membrane functions. Supporting this hypothesis are two observations: (*i*) the lethal limit was 64% tissue water as ice for *Mytilus edulis* adapted to both 50 and 150% seawater, despite the large differences in cellular ion concentrations in the two media (Williams 1970), and (*ii*) substantial free water remained in cells at the lethal limit, unfreezable water (also called osmotically inactive or bound water) accounting for only 20−25% of total water (Williams 1970; Zachariassen et al. 1979*b*). Adaptations for freeze tolerance also must address the realities of freezing in a natural winter environment. These include slow rates of freezing (at most a few degrees per hour), long episodes of freezing (days or weeks), and freezing at relatively moderate subzero temperatures (generally between −5 and −50°C). Natural freezing occurs within a temperature zone (above −60°C) viewed as highly dangerous by cryobiologists compared with the ametabolic state afforded by freezing at ultralow temperatures (e.g., −196°C). Substantial metabolic activity continues within cells at these milder temperatures and recrystallization of ice is rampant (Mazur 1984). Thus, naturally freeze-tolerant organisms must deal with long-term anoxia−ischemia, maintenance of metabolic regulation and homeostasis, and the control of recrystallization.

Control of extracellular ice

Extracellular ice in freeze-tolerant animals is regulated by the actions of two proteinaceous compounds: ice-nucleating proteins (INPs) and thermal hysteresis proteins (THPs). Both have been found in the hemolymph of freeze-tolerant insects and intertidal molluscs (Theede et al. 1976; Duman 1982; Aunaas 1982; Zachariassen 1985; Hayes and Loomis 1985).

INPs induce and control extracellular ice formation. They are *the one molecular adaptation unique to freeze tolerance*; other adaptations such as the accumulation of polyols for cell volume regulation or membrane stabilization by trehalose and proline are also responses to low water (anhydrobiosis) or salt (osmoregulation) stresses. By the action of INPs, extracellular freezing begins close to the hemolymph freezing point (undercooling is less than 2°C), rates of ice formation are kept low, osmotic stress during freezing is minimized, the potential for intracellular nucleation is eliminated, and freeze concentration, membrane deformation, and ischaemia are applied slowly. Nucleation also occurs at multiple extracellular sites, resulting in many small ice crystals.

INPs are proteins or lipoproteins (Aunaas 1982; Duman 1982; Duman et al. 1985; Hayes and Loomis 1985; Zachariassen 1985). The protein from *Vespula maculata* has been purified and shows a molecular weight of 74 000 and an amino acid composition that is highly hydrophilic (20% glutamate–glutamine, 12% serine, 11% threonine) (Duman et al. 1984). The structure of the protein may be such as to provide site(s) that order water into embryo crystal(s), thereby reducing the energy barrier to nucleation (Duman et al. 1984).

Thermal hysteresis proteins are well known as the antifreeze agents in insects that avoid freezing by deep supercooling (Duman 1982; Zachariassen 1985). Thus, their discovery in freeze-tolerant forms was unexpected. However, Knight and Duman (1986) have shown that THPs are extremely effective at inhibiting ice recrystallization. Their probable function, therefore, is to counter the thermodynamic forces that drive the conversion of smaller to larger (physically damaging) ice crystals over time in the frozen state.

Cell volume regulation, unfreezable water, and cryoprotectants

The reduction in cell volume caused by the osmotic outflow of water from cells during extracellular ice formation becomes irreversibly damaging when a critical limit is exceeded. The primary result is structural damage to cell and organelle membranes (also possibly to the microtrabecular lattice) due to folding or collapse as cell volume decreases. Physical deformation of cells by external ice may also be structurally damaging to the cell membrane. Freeze-tolerant animals attack the problem of cell volume regulation by limiting freeze concentration though adaptations that increase the amount of unfreezable water in cells.

The chief mechanism for controlling freeze concentration is the accumulation of low molecular weight cryoprotectants for colligative action in limiting cell volume reduction. Polyhydric alcohols (e.g., glycerol, sorbitol, mannitol, threitol) are the common cryoprotectants of freeze-tolerant insects and are synthesized in concentrations up to several molar during autumn cold harderning (Storey and Storey 1988). Freeze-tolerant frogs accumulate glucose (three species) or glycerol (one species) to levels up to 0.5 M (Storey and Storey 1986). Intertidal marine invertebrates cannot maintain a cryoprotectorant pool because of their osmoconforming habit but the existing pools of intracellular osmolytes (ions, amino acids) undoubtedly play cryoprotective roles during freezing. For example, lower lethal temperature decreases when *M. edulis* is adapted to higher salinities (Williams 1970). It would be interesting to determine, however, whether freezing episodes stimulate short-term increases in the free amino acid pool, as occurs during hyperosmotic stress in these animals.

Polyols and sugars are particularly suited to a cryoprotective function. Few will crystallize spontaneously from aqueous solution, so the content of unfreezable water is stabilized (Franks 1985). Polyols are largely nontoxic to cells, and they are inert both chemically (no nonenzymatic reactions) and biochemically (they are dead-end products). Glycerol is freely penetrating so osmotic equilibrium can be readily maintained. Additional actions of polyols in stabilizing protein–enzyme structure, multienzyme complexes, or enzyme associations with subcellular particles against the effects of dehydration or low temperature may also be important in preserving metabolic regulation (homeostasis) in the frozen state. Such stabilizing effects of polyols are well known for isolated enzymes in dilute solution exposed to low temperature or dehydration (Gekko 1983; Franks 1985; Fink 1986).

Other mechanisms for increasing the content of unfreezable water in cells may also be relevant to freeze tolerance. A significant portion of water in cells is unfreezable because of its intimate association with subcellular components (e.g., water inolved in the tertiary structure of proteins, hydration shells around solutes, water lining membrane pores). Thus, in addition to the proliferation of low molecular weight cryoprotectants, changes to the amounts or structures of other cellular components could improve the content of unfreezable or bound water in cells. In *Eurosta solidaginis*, for example, the amount of water bound by high molecular weight soluble components (protein, glycogen) increased about 5-fold during low-temperature acclimation of the larvae (Storey et al. 1981).

Membrane stabiliztion

Adaptations for the preservation of membrane structure and function in freeze-tolerant animals must address the physical consequences of both low temperature and freeze concentration.

As discussed above, temperature has a variety of effects on the properties of lipids and these effects alter the fluidity of membranes and affect permeability properties, transport processes, and the functioning of membrane-associated proteins. Among ectotherms, acclimation to temperature change elicits membrane restructuring, termed homeoviscous adaptation. This produces membranes with physical properties that are largely independent of acclimation temperature and with physiological processes (e.g., permeability, enzyme and transport activities, receptor and neural functions) that are temperature compensated (Hazel 1984). Such restructuring of membranes is well documented for animal acclimation to a constant low temperature in instances where animals must compensate to retain normal physiological and biochemical functions at low temperature. Alterations to membrane structure are also a facet of cold hardening in plants (Heber et al. 1981). The extent of membrane restructuring during autumn cold hardening of freeze-tolerant animals is not yet known. The only detailed study of seasonal changes in membrane lipid composition of a freeze-tolerant animal (the barnacle *Balanus*

balanoides) found very few seasonal alterations in the composition of various lipid classes and little evidence for homeoviscous adaptation (Tooke and Holland 1985). Thus, the presumption that major restructuring is required during cold hardening may be false. For ectotherms that experience large temperature fluctuations, both summer and winter, only minor changes in lipid composition may be needed to reset optimal membrane function to an overall lower temperature range during winter. In addition, a low temperature induced restriction of various membrane-associated metabolic processes may be noninjurious in the dormant or diapause state that commonly occurs during overwintering or is imposed by extracellular freezing.

However, damage to membranes is a prime cause of freezing injury. The physical stresses on bilayer structure when cells shrink and deform is the primary cause but injury may also arise from dehydration and high salt effects or direct damage from ice crystals (Rudolph and Crowe 1985; Franks 1985). Seasonal changes in membrane lipid composition are related to the maintenance of membrane function (fluidity) but do not influence freeze tolerance (Franks 1985). The relevant adaptations for membrane preservation during freezing are suggested from studies of frost hardening in plants and anhydrobiosis in animals. Three are recognized to date. Firstly, frost hardening in plants appears to include changes to the plasma membrane or associated protein components (a potential function for thermal hysteresis proteins?) that inhibit the propagation of ice crystals across the plasma membrane (Steponkus et al. 1983). Secondly, cell volume reduction during freezing in plants appears to include the removal of membrane material with the mechanisms of removal (exocytosis in plant protoplasts) and storage (a stabilized bilayer) of deleted material being those that allow a ready reincorporation of membrane material during thawing (Williams et al. 1981; Steponkus et al. 1983). Thirdly, membrane structure is stabilized against freezing or dehydration-induced alterations (e.g., phase transitions) by the actions of specific low molecular weight protectants, trehalose and proline (Crowe et al. 1983; Rudolph and Crowe 1985). The relevance of the first two mechanisms to freeze-tolerant animals is not yet known but both trehalose and proline are accumulated during winter hardening in freeze-tolerant insects (Storey and Storey 1988), suggesting the key importance of membrane stabilization by low molecular weight protectants in freezing protection.

Metabolism and metabolic regulation in freeze-tolerant animals

The effects of low temperature on metabolic rates are inescapable for freeze-tolerant animals, as they are for all ectotherms. However, metabolic regulation is not necessarily compromised at low temperatures or in the frozen state. The disastrous effects of hypothermia and (or) freezing-induced ischaemia, which characterize attempts at the cryopreservation of mammalian organs, do not appear in animals with a natural tolerance for freezing. Indeed, far from experiencing disruption of metabolic regulation, cold-hardy animals often exploit low temperature to reorganize metabolism for low-temperature function and to initiate a variety of cold-hardiness adaptations. In general, it is clear that cold-hardy animals (both freeze tolerant and freeze avoiding) can maintain an integrated metabolism over a wide range of body temperatures; for example, alphastat regulation of intracellular pH in *E. solidaginis* is maintained from at least +15 to −20°C (Storey et al. 1984). This is not uncommon for ectotherms but for the freeze-tolerant animal may involve some specific alterations to key rate-limiting functions (compared with more stenothermic ectotherms) to create a system whose components (energy producing, energy utilizing, and passive processes) remain balanced over an extremely wide environmental temperature range. This would allow homeostatic control during both daily and seasonal temperature fluctuations.

Exploitation of low temperature and freezing

Low-temperature effects on certain metabolic functions have been exaggerated and exploited by cold-hardy animals as a means of inducing selected cryoprotective adaptations. Synthesis of carbohydrate cryoprotectants, INPs, and THPs in cold-hardy insects are all triggered by low temperatures (Storey and Storey 1988). For example, polyol synthesis is triggered by a low-temperature activation of glycogen phosphorylase that is, itself, the consequence of a low-temperature inactivation of phosphorylase phosphatase (Hayakawa 1985). The differential temperature-dependent synthesis of glycerol (at 10−15°C) versus sorbitol (below 5°C) during cold hardening of *E. solidaginis* larvae results from a specific low-temperature inactivation of phosphofructokinase (Storey 1982). Thus, modifications of the temperature sensitivities of the kinetic and regulatory properties of only a few selected enzymes can bring about the major metabolic changes underlying cold hardiness.

Freeze-tolerant frogs have chosen freezing itself, not low temperature, as the signal for the stimulation of cryoprotectant synthesis. Synthesis of glucose in liver begins only after ice formation is initiated in the body extremities (Storey and Storey 1985*a*). The mechanism used is likely an adaptation of the catecholamine-mediated fight-or-flight response which stimulates liver glycogenolysis and glucose output into blood in all vertebrates. However, the initiating stimulus, ice nucleation, has been changed and normal feedback mechanisms regulating glucose levels have been removed or inhibited to allow blood and tissue glucose levels to rise to several hundred millimolar.

Metabolic depression and ischaemia tolerance

Extracellular freezing physically isolates each individual cell. Access to blood-borne oxygen and fuels is cut off as are waste disposal routes; cells must survive individually and maintain homeostasis during exposure to freezing. Despite low temperatures, the effects of freezing-induced ischaemia could readily become lethal when episodes of natural freezing stretch out to days or weeks. Survival, then, is dependent upon a well-developed tolerance of anoxia−ischaemia in all tissues and organs and upon the ability to reduce metabolic energy demands in the frozen state.

Freeze-tolerant insects and frogs show no novel mechanisms of anaerobic metabolism. Energy production derives from anaerobic glycogenolysis (with lactate and alanine produced), as well as from the depletion of phosphagen and adenylate reserves. Anoxia tolerance may be enhanced, however, compared with nontolerant species: *E. solidaginis* larvae readily survive 12 weeks of freezing, wood frogs at least 2 weeks (Storey 1985; Storey and Storey 1985*b*). The use of glucose as the cryoprotectant in frogs may have the additional benefit of providing oxygen-sensitive organs (e.g., brain) with an ample endogenous fuel supply in the frozen state.

Natural freeze tolerance is also apparently aided by mechanisms that depress metabolic rate and, therefore, energy demands in the frozen state. Entry into a hypometabolic state is an adaptive response to a variety of environmental stresses and characterizes anaerobiosis, anhydrobiosis, hibernation, estivation, torpor, and diapause (Hochachka and Somero 1984; Storey 1988). Winter dormancy or diapause is common among many cold-tolerant animals (both freeze-tolerant and freeze-avoiding). For many insect, amphibian, and reptile species, such a resting state is, in fact, compulsory before development can continue. Winter dormancy also occurs among marine molluscs, induced by low temperature (e.g, Q_{10} for O_2 consumption was 22 between 4 and 0°C in *Modiolus demissus*), and freeze tolerance is substantially higher for individuals in such a hypometabolic state (induced by either low temperature or anoxia) (Murphy 1983). Cellular energy requirements are substantially reduced in a hypometabolic state (often to $< 10\%$ of normal resting requirements) because of the coordinated reduction or inactivation of numerous cellular processes. Long-term survival of the anoxic–ischaemic conditions accompanying freezing is, therefore, enhanced by a pre-existing hypometabolic state.

Concluding remarks

Broken down into component parts, freeze tolerance requires (*i*) control over ice formation, (*ii*) regulation of cell volume, (*iii*) stabilization of subcellular structure against the effects of low temperature and freeze concentration, (*iv*) maintenance of metabolic regulation over a wide temperature range, and, (*v*) tolerance of anoxia–ischaemia. Of these, only the biochemical adaptations needed to control ice formation are unique to freeze tolerance as an animal response to environmental stress. The others are well-developed animal responses to a variety of environmental stresses and appear widely throughout the animal kingdom as components of anhydrobiosis, osmoregulation, hibernation, estivation, and anaerobiosis, among others. By drawing on our knowledge of the molecular adaptations supporting these animal responses and applying our developing understanding of the molecular actions of INPs and THPs, the biochemistry of natural freeze tolerance is rapidly unfolding. The key question for the future is how much of the knowledge developed from studies of natural freeze tolerance can be applied to the medical use of organ cryopreservation. Certainly, the wide use of glycerol for cell and tissue preservation is validated by the occurrence of glycerol as the major cryoprotectant among freeze-tolerant animals. The key importance of trehalose and proline in membrane stabilization during freezing should also be assimilated by cryobiologists. Freeze-tolerant frogs offer a unique system in which to study, on an organ-specific basis, the requirements for freezing preservation and to develop protocols for the successful cryopreservation of mammalian organs. To date, we can note key features of frog freezing that are applicable to organ cryopreservation. These include the withdrawal of blood from internal organs during freezing (a mechanism that reduces the potential for physical damage by extracellular ice) and the preferred use of glucose as a cryoprotectant in the freeze-tolerant vertebrate (glucose offers both physical protection and metabolic benefits during freezing) (Storey and Storey 1988).

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