

# What Contributes to Freeze Tolerance?

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Unique among vertebrates, some frogs withstand freezing of the whole body during overwintering. How can they tolerate complete stoppage of breathing, heartbeat, and blood flow when the animal freezes, to start again on thawing? They provide a fascinating view of life in a frozen state and offer a model system for possible medical use in organ cryopreservation.

Although alien to human physiology, a natural freeze tolerance is the key to winter survival for many invertebrates (e.g., terrestrial insects and intertidal marine molluscs and barnacles) and plants (2, 10). However, freeze tolerance was unknown among vertebrates until 1982 when Schmid (5) reported freezing survival of terrestrially hibernating frogs. Since then, we have confirmed and extended this work to include four common North American frogs: the wood frog, grey tree frog, spring peeper, and chorus frog (7, 8, 10).

The present article discusses our studies of the wood frog, *Rana sylvatica*, and offers views of the physiological and biochemical factors required for freezing survival of vertebrate cells and organs.

## What contributes to freeze tolerance?

Freeze tolerance in nature denotes a tolerance of ice formation in extracellular fluid spaces (2, 10). Intracellular ice formation, which punctures membranes, destroys subcellular structures, and disrupts metabolic compartmentation, is always lethal (except under laboratory conditions of ultrafast rates, 100–700°C/min, of freeze/thaw). Natural freeze tolerance, therefore, involves mecha-

nisms that effectively render cytoplasmic water unfreezable (2).

Requirements for freezing survival include mechanisms to induce and control extracellular ice formation, regulate and limit cell volume reduction, stabilize subcellular components, and tolerate prolonged ischaemia in the frozen state.

## Adaptations

Adaptations known to serve natural freeze tolerance address these concerns.

*Control of extracellular ice.* Specific ice-nucleating proteins in extracellular compartments induce a slow and regulated freezing at high subzero temperatures ( $>-10^{\circ}\text{C}$ ). As a consequence of their action, transmembrane osmotic stress is applied gradually, cells experience little or no undercooling, and the risk of intracellular nucleation is eliminated. Thermal hysteresis proteins in extracellular compartments inhibit recrystallization to limit physical damage by ice crystals when freezing is long term.

*Cell volume regulation.* The withdrawal of pure water into extracellular ice sets up an osmotic imbalance that causes cell dehydration and shrinkage and elevated ion/solute levels in the remaining intra- and extracellular liquid compartments (termed freeze concentration). Freeze concentration beyond a critical cell volume is prevented by

the colligative actions of high concentrations of low-molecular-weight cryoprotectants (polyols, sugars).

*Stabilization of subcellular organization.* Protectants (e.g., trehalose, proline) stabilize bilayer structure to prevent membrane damage as cell volume is reduced. Polyols stabilize protein/enzyme structure and function against low temperature and dehydration stresses. The content of unfreezable water in cells is raised due to increased water binding by both macromolecules and low-molecular-weight protectants.

*Ischaemia tolerance.* Long-term survival in the ischaemic state imposed by extracellular freezing is served by well-developed anoxia tolerance (including fermentable fuel reserves in all tissues) and mechanisms for reestablishing homeostatic control in the frozen state (2, 10).

## Freeze tolerance in a vertebrate

As the only vertebrate animals known to survive whole body freezing, terrestrially hibernating frogs present intriguing questions to the physiologist and biochemist. Do the vertebrate solutions to the stresses of freezing differ, in principle or in detail, from those of insects and plants? How are breathing, heartbeat, and blood flow regulated to stop in the frozen state and restart on thawing? What are the metabolic responses of individual organs to freezing, and are cryoprotective measures specialized for the needs of each organ?

The answers to these questions are not only the key to understanding natural freeze tolerance in animals but are uniquely applicable to the development of technology for the medical use of cryopreservation. To date all progress in the cryopreservation of mammalian cells, tissues, and organs has come from empirical experimentation (3); the field has never had a useful freeze-tolerant system to use as a model. Freeze-tolerant frogs, however, with organ

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systems equivalent to those of mammals, can finally provide the relevant model with which to study, in an organ-specific manner, the physiological and biochemical requirements for freeze tolerance and to test techniques for artificial cryopreservation. Our studies of the molecular basis of natural freeze tolerance have concentrated on cryoprotectant metabolism and/or function and on life in the frozen state and have revealed a number of novel features supporting freeze tolerance.

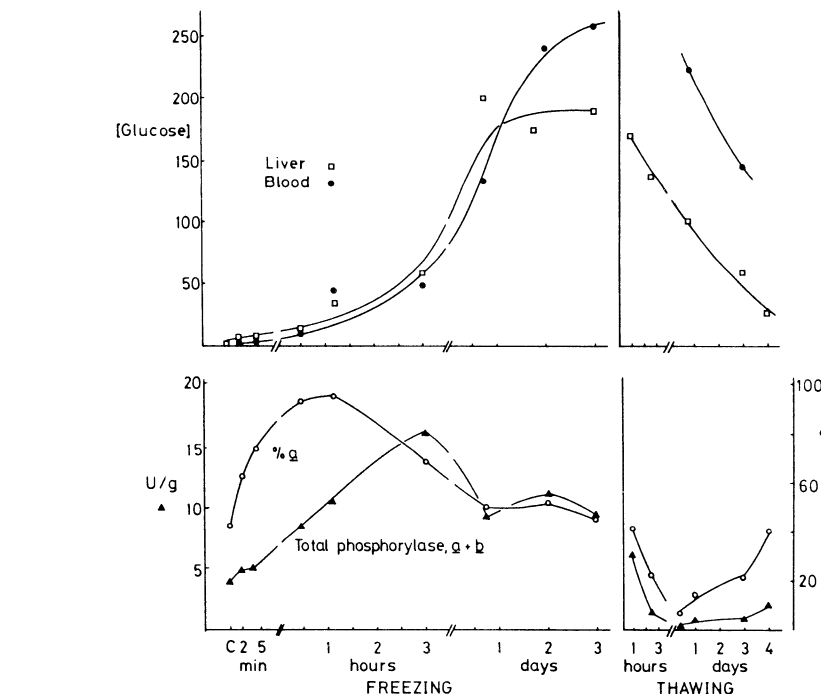
### Characteristics of freezing in frogs

Freeze tolerance appears to be the optimal strategy for cold hardiness in terrestrially hibernating frogs, for it acknowledges the realities of both physiology and environment. Frogs have a very limited capacity for supercooling and, with a water-permeable skin, are highly susceptible to inoculative freezing below the freezing point of body fluids ( $\sim -0.5^{\circ}\text{C}$ ).

Hibernation sites of these frogs are at the soil surface under a cover of damp leaf litter and, as such, give the animals little chance of avoiding subzero exposures and contact with ice. Long-term survival in the frozen state is, therefore, well developed; adult wood frogs show no detrimental effects after two weeks frozen at  $-2.5^{\circ}\text{C}$  (7). Low-temperature limits on freezing survival are not extensive, however. Lethal limits are only about  $-6$  to  $-8^{\circ}\text{C}$  but are well matched to winter microenvironment temperatures, which rarely exceed  $-4$  to  $-7^{\circ}\text{C}$  under the snow, even in the Arctic (10).

The process of freezing begins with nucleation in the body extremities. When freezing is complete, large ice crystals are found under the skin and around skeletal muscles, and they fill the abdominal cavity, surrounding all internal organs. During freezing, breathing, heartbeat, and blood flow gradually slow and then stop. A large mass of frozen blood is found pooled in distended sinuses above the heart, suggesting that blood is drained from organs during freezing (heart and liver appear pallid).

When thawed, a resumption of heartbeat is the first vital sign to reappear, followed by breathing and gulping and finally by motor activity. This process can take 1–2 h at



**FIGURE 1.** Activation of liver glycogen phosphorylase and production of glucose during freezing in wood frogs. An immediate increase in percentage of phosphorylase in the active  $\alpha$  form followed by a more gradual rise in total phosphorylase ( $\alpha + \beta$ ) content in liver underlies rapid biosynthesis of cryoprotectant in response to freezing. Freezing was at  $-3^{\circ}\text{C}$ , timed from appearance of the freezing exotherm (jump in body temperature due to latent heat release on freezing, measured via a thermocouple in contact with ventral abdomen). Thawing was at  $3^{\circ}\text{C}$ . Phosphorylase activities are U/g wet wt of liver; glucose levels are  $\mu\text{mol/g}$  wet wt in liver and  $\mu\text{mol/ml}$  in blood. C, values for control, unfrozen frogs.

room temperature and 12–24 h at  $3^{\circ}\text{C}$ . Likewise, freezing itself is also relatively slow. Maximal ice formation in 14-g wood frogs required  $\sim 24$  h at  $-3^{\circ}\text{C}$ , with a half time of 6.5 h and a linear rate of  $2.9\%/h$  (4). Such slow rates of freezing provide ample time for cell volume regulation, synthesis and distribution of cryoprotectant, a redistribution of blood flow, and a regulated transition to an ischaemic state. Estimates of maximal ice content in frozen frogs range from 35% of total body water for the grey tree frog (*Hyla versicolor*) to 65% for the wood frog (4, 5, 7).

Novel to three species of frog is the use of glucose as a cryoprotectant, with levels reaching  $550 \text{ mmol/l}$  ( $9.9 \text{ g/100 g}$ ) in blood of freezing-exposed wood frogs, compared with  $1\text{--}5 \text{ mmol/l}$  in unfrozen controls (7, 8). The grey tree frog, by contrast, utilizes glycerol, which is the common choice of most cold-hardy invertebrates.

A unique feature of frog freeze tolerance is the mode of triggering involved in the synthesis of cryoprotectant. Production begins only in response to ice nucleation in the

body (Fig. 1; Ref. 6). No anticipatory accumulation occurs, such as is found during cold hardening in insects (10).

The choice of glucose for cryoprotection is significant because mechanisms preexist for the rapid biosynthesis of this sugar. Glucose is synthesized by the liver from massive glycogen reserves (up to  $180 \text{ mg/g}$  wet wt) accumulated for this purpose and is rapidly distributed via the blood to all other organs.

Organ-specific differences in cryoprotectant content occur in the fully frozen animal (highest in core organs such as brain, liver, and heart and progressively lower in peripheral tissues; Fig. 2; Ref. 9). The probable cause is the progressive restriction of blood flow as freezing becomes more complete, but the uneven distribution may also serve the specific needs of individual organs for variable levels of colligative cryoprotection and/or other functions of glucose in the frozen state (e.g., as a fermentable fuel or a metabolic depressant). On thawing, glucose from all tissues is returned to the liver to be restored as glycogen. Mul-

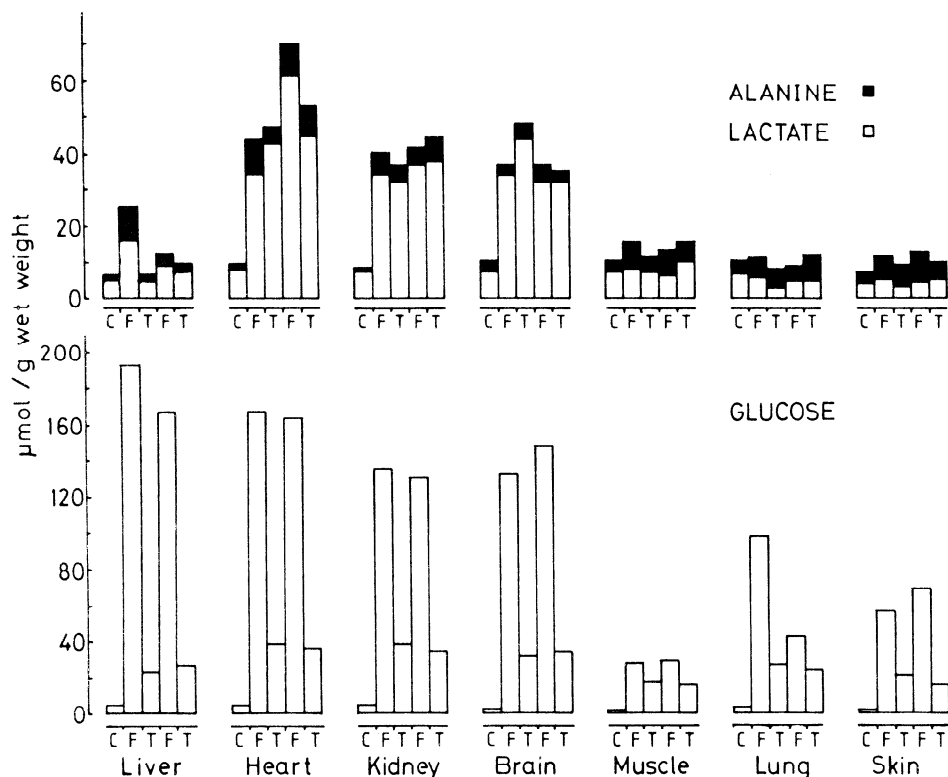


FIGURE 2. Effect of alternating freezing and thawing on organ metabolism in wood frogs. Glucose, lactate, and alanine levels were measured over 2 consecutive cycles of freezing (2 days at  $-2.5^{\circ}\text{C}$ ) and thawing (2 days at  $3^{\circ}\text{C}$ ). C, control animals sampled from  $3^{\circ}\text{C}$ ; F, frozen; T, thawed. Muscle is leg skeletal muscle, skin is from ventral abdomen.

multiple cycles of freeze/thaw repeat the same responses in synthesis, distribution, and subsequent clearance of glucose (Fig. 2).

### Regulation of cryoprotectant synthesis

There are several interesting aspects of cryoprotectant metabolism in the wood frog. Perhaps most striking is that glucose output from liver begins within 5 min of the initiation of ice formation (6). The molecular mechanisms involved in glucose output appear to resemble the vertebrate "fight or flight" syndrome, but the responses differ in that 1) the trigger is peripheral ice formation (we have found no changes in blood catecholamines; K. Storey and S. Perry, unpublished observations), and 2) the normal feedback mechanisms that place an upper limit on glucose levels are overridden.

The key to cryoprotectant synthesis is control of liver glycogen phosphorylase activity. Unique aspects to the regulation of this enzyme are seen in the wood frog. Phosphorylase activation has two components (10). The initial response to ice nucleation

is a rapid conversion of the inactive *b* form of the enzyme to the active *a* form (mediated by enzyme phosphorylation) occurring within minutes (6); this is the typical mode of phosphorylase activation in vertebrates.

Over a longer time course, however, a second component to phosphorylase activation appears: the total activity (*a* + *b*) of phosphorylase in liver rises from a control level of  $\sim 4$  U/g wet wt to a peak level of 16 U/g; this is first apparent after  $\sim 30$  min of freezing exposure and is maximal after 3 h (Fig. 1).

These two mechanisms combine to provide extremely sensitive control over phosphorylase in liver, allowing a 7- to 13-fold increase in activity of the *a* form in response to freezing and facilitating glycogenolysis at rates exceeding  $20 \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$  at  $-2.5^{\circ}\text{C}$  (9). In contrast, a rapid fall in both percent *a* and total phosphorylase combine to produce a 100-fold decrease in liver phosphorylase activity during glucose reconversion to glycogen on thawing (Fig. 1). Contributing to glucose output from liver is an inhibitory block of liver glycolysis at the phosphofructokinase locus (10).

### Cryoprotection by glucose

In common with other cryoprotectants, colligative action by glucose in limiting cell volume reduction is an important function. Although glucose does not reach the molar concentrations seen in freeze-tolerant insects, amounts in the range of  $200 \mu\text{mol/g}$  wet wt appear to be sufficient to hold the freeze concentration of cells within survivable limits at the mild subzero temperatures experienced. Indeed, water loss from liver of frozen wood frogs appeared to be only  $\sim 15\%$  in animals frozen at  $-4^{\circ}\text{C}$  (calculated from freezing-induced increases in liver total protein and enzyme activities, measured per gram wet wt), whereas skeletal muscle showed no indication of dehydration during freezing (7).

Additional protective actions by glucose are suggested by recent *in vitro* studies. Isolated ventricle strips from wood frogs readily survived freezing exposures ( $-5^{\circ}\text{C}$  for 1 h) and regained contractility after thawing when frozen in the presence of 250 mmol/l glucose added to the incubation bath (1). However, in the absence of glucose or when 250 mmol/l glycerol was substituted, physical function did not return after thawing. Since both glucose and glycerol are penetrating cryoprotectants that should offer similar colligative protection during freezing, the basis of the specific glucose effect must rest elsewhere, such as actions in stabilizing subcellular structure or metabolic function in the frozen state.

A metabolic action of glucose is suggested from new studies on isolated hepatocytes from wood frogs. Added glucose preserves the structural integrity of hepatocytes (assayed by trypan blue staining and lactate dehydrogenase leakage) for freezing survival down to  $-10^{\circ}\text{C}$ ; however, metabolic function (urea biosynthesis) of hepatocytes returns only after glucose is washed out of thawed cells (K. Storey and T. Mommsen, unpublished data).

Thus glucose may be acting as a metabolic depressant to limit or inhibit nonessential metabolic functions in the frozen state. Such an action would prolong survival time by limiting energy expenditures and

would dovetail with another potential role for glucose during freezing, that of a fermentable fuel reserve to support energy requirements of the ischaemic state. It is interesting to speculate that multiple actions of glucose (colligative, structural, and metabolic) could make this sugar valuable for artificial cryopreservation of mammalian tissues and organs.

### Life in the frozen state

Extracellular freezing imposes an ischaemic and anoxic state on all cells of the body. Circulatory changes during the early stages of freezing may allow remaining oxygen supplies to be used by the most sensitive organs, but when freezing is complete there are no breathing, no heartbeat, and no blood flow.

Energy metabolism in the frozen state is based on fermentation of endogenous fuel reserves in each individual organ. Organs show a decrease in glycogen content with long-term freezing and an accumulation of lactate and/or alanine as end products of anaerobic glycolysis (9). Amino acid fermentation also appears to occur in some organs (notably skeletal muscle; 9). Some small percentage of cryoprotectant glucose may also be fermented, although end-product accumulation is better correlated with glycogen loss (9).

Organ-specific responses to the frozen state are seen. Accumulation of metabolic end products differed in both amount [net lactate plus alanine accumulation was 10-fold higher in heart than in skeletal muscle (Fig. 2)] and pattern [e.g., predominantly lactate in heart, 2.5:1 lactate:alanine in kidney, 1:4.5 lactate:alanine in skeletal muscle (9)]. Differences in total end-product accumulation suggest substantial differences in organ-specific metabolic rates in the frozen state.

Freezing also appears to place varying levels of metabolic stress on individual organs, as judged from the effects of the frozen state on energy status. Skeletal muscle energy reserves (adenylates, creatine phosphate) are minimally affected by freezing, even after 3 days frozen (9). Liver energy status, however, is much more strongly affected by freezing (ATP levels drop by 50% within 18 h), although recovery is

complete after several days of thawing (9).

Much remains to be explored to determine the molecular mechanisms involved in freeze tolerance and the specific actions of protectants such as glucose. Freeze-tolerant frogs provide cryobiologists with the first good opportunity to study natural freezing survival on an organ-specific basis. For medical applications these animals provide an excellent model system for the development of organ cryopreservation technology.

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## Formate: A Critical Intermediate for Chloride Transport in the Proximal Tubule

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Recent experiments unexpectedly suggest that formate plays a critical role in chloride transport across cell membranes. In particular, active uptake of chloride in the renal proximal tubule cell occurs by chloride-formate exchange. Formate recycles from lumen to cell via nonionic diffusion of uncharged formic acid. In this manner, small amounts of formate can facilitate resorption of large quantities of chloride

The proximal tubule of the mammalian kidney is presented with the

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enormous task of resorbing approximately 60% of the load of NaCl presented to it by glomerular filtration. In humans this amounts to ~10,000 mmol NaCl (~580 g) resorbed by the proximal tubules each day. New evidence surprisingly indicates that formic acid, a short-chain fatty acid present physiologically in low con-