Frozen and Alive

A variety of animals freeze solid during the winter months and thaw in the spring. This natural ability to survive freezing may yield clues to the cryopreservation of human tissue

by Kenneth B. Storey and Janet M. Storey

When the mercury dips below zero degrees Celsius, we retreat to our warm houses, don parkas if we venture outdoors and perhaps look forward to a vacation somewhere tropical. Few animals remain active during the winter months. Birds have flown south, and many terrestrial animals hibernate in dens or on lake bottoms. But what happens to ectothermic, or cold-blooded, animals—frogs and turtles, beetles and spiders—that cannot find a relatively warm haven? How do they endure when environmental temperatures fall below the freezing point of their body fluids? Some species avoid freezing through biochemical changes in their bodies. But, remarkably, the answer for many other animals is that they freeze solid and survive.

Hundreds of species of terrestrial insects survive long periods of freezing while they overwinter. At the extreme, insects of the high Arctic, such as the woolly bear caterpillars (*Gynaephora groenlandica*), may spend 10 months of the year frozen solid at temperatures that descend to -50 degrees C (-58 degrees Fahrenheit) or even lower. A variety of invertebrate animals that colonize the intertidal zone of northern seashores, such as barnacles, mussels and periwinkles, also freeze when exposed to subzero air temperatures at low tide. But of greatest interest to our laboratory at Carleton University in Ottawa, Ontario, are a group of amphibians and reptiles that survive freezing during their winter hibernation.

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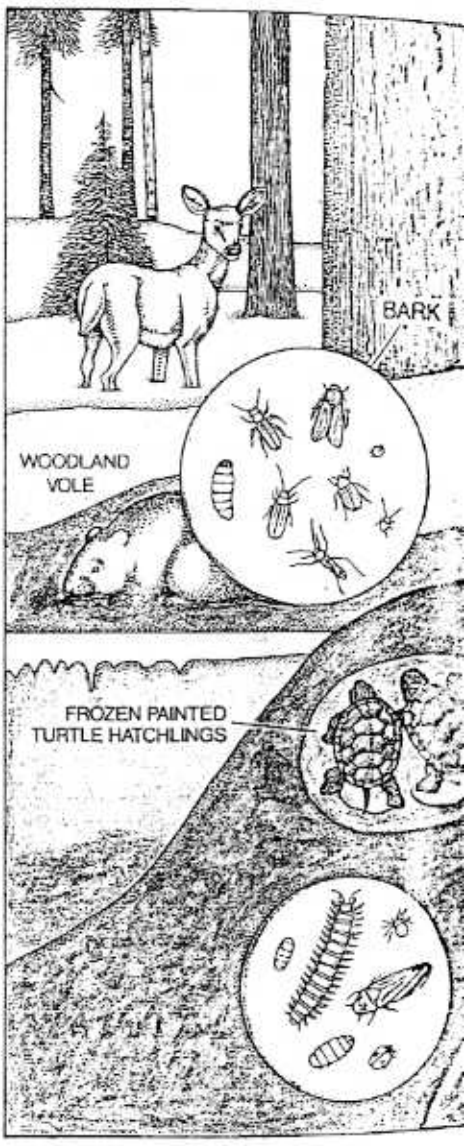
William D. Schmid of the University of Minnesota at Minneapolis stimulated our work on this striking adaptation with a 1982 report on frogs that survive freezing. Following up on Schmid's study, we have shown that four common species of frogs—the wood frog (*Rana sylvatica*), the spring peeper (*Hyla crucifer*), the gray tree frog (*Hyla versicolor*) and the striped chorus frog (*Pseudacris triseriata*)—that hibernate on the forest floor can survive days or weeks of freezing with as much as 65 percent of their total body water as ice. Scientists in the U.S.S.R. have also reported that the Siberian salamander (*Hynobius keyserlingi*) can survive freezing. This species, the only land-hibernating amphibian found on the tundra, may survive exposure to -35 degrees C.

Then, in 1988, we identified a reptile that freezes during the winter. Our colleague Ronald J. Brooks of the University of Guelph told us about the unusual behavior of newly hatched painted turtles (*Chrysemys picta*). Instead of leaving their nests after hatching in late summer, the young turtles stay put, safely hidden from predators, until spring arrives. Their nests, only three to four inches deep and placed on exposed banks of lakes and rivers, offer little insulation.

Brooks recorded nest temperatures of -6 to -8 degrees C during January and February of 1988, but our laboratory tests showed that the turtles froze whenever the temperature fell below -3 degrees C. Therefore, the hatchlings must freeze and thaw repeatedly over the winter before emerging in the spring. Studies by Jon P. Costanzo, Dennis L. Claussen and Richard E. Lee, Jr., of Miami University in Oxford, Ohio, also showed that adult box turtles and garter snakes have some ability to survive freezing.

While frozen, all these animals show no movement, respiration, heart beat or blood circulation, and our latest ex-



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periments show barely detectable neurological activity. Ice accumulates in all extracellular fluid compartments and fills the abdominal cavity and the bladder; crystals run under the skin and in between muscles. These animals have mastered the tricks of organ cryopreservation—the freezing of live tissue for storage and subsequent use—and our studies of frozen frogs and turtles are revealing the molecular mechanisms essential to life in a frozen state.

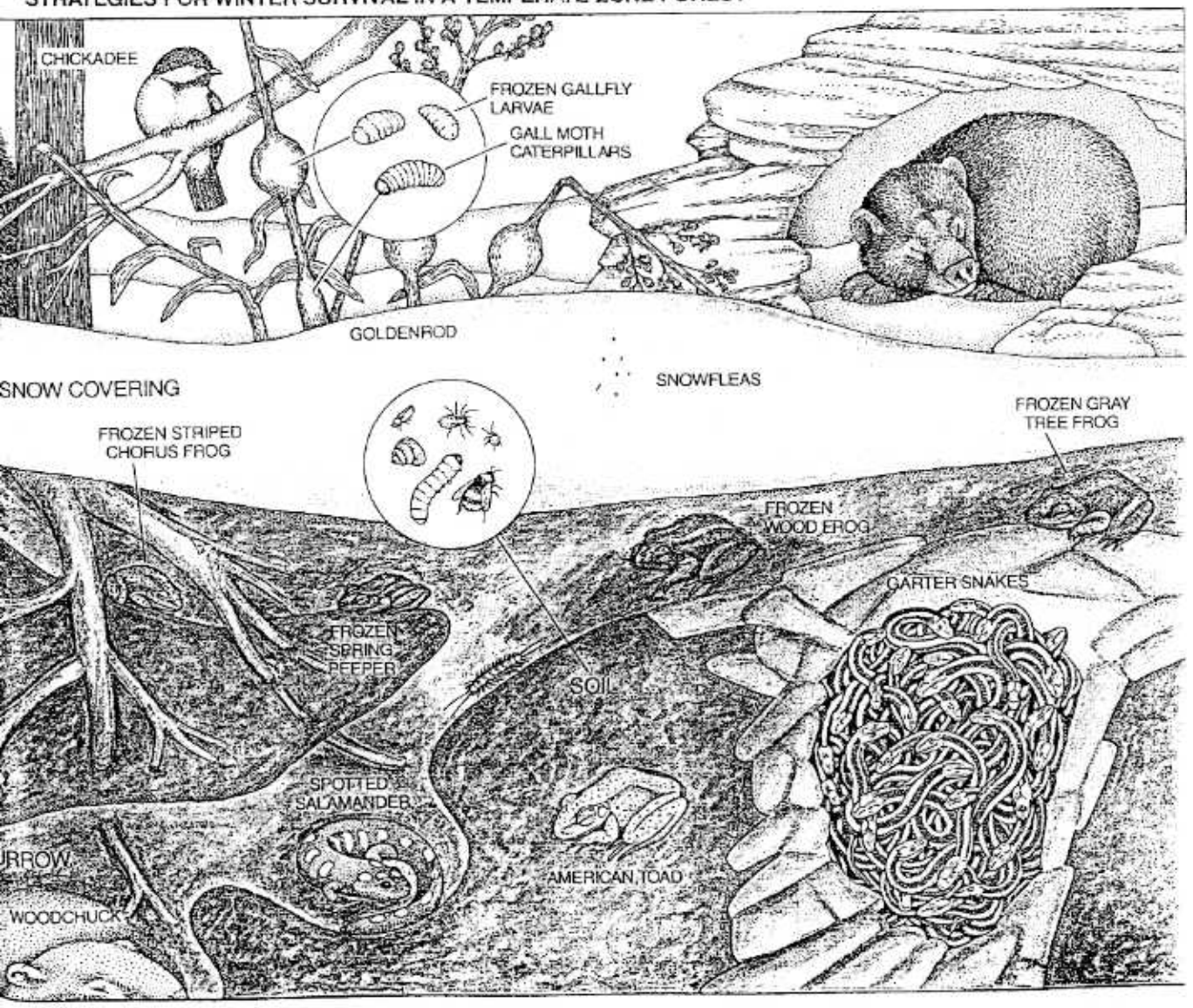
Spending the winter frozen seems to be an incredibly dangerous adaptive strategy—freezing is lethal for most cells. As any gardener knows, the first hard frost will transform a lush autumn flower bed into a pile of brown mush. Ice crystals rip through cell membranes and damage subcellular organelles; cell contents spill out, and the discrete localization of individual metabolic processes within the cell becomes scrambled. And even if ice formation can be controlled, freezing stresses cells in other ways. For example, because freezing halts breathing and blood circulation, all organs are cut off from access to oxygen and blood-borne fuels for the duration of the freeze. Instead of tolerating the frozen state, are there not easier ways for cold-blooded animals to deal with subzero temperatures?

In fact, two alternatives exist. The first—and most familiar—strategy is to avoid exposure to temperatures below the freezing point of body fluids. Animals simply "choose" relatively warm hibernation sites under water or deep underground. Numerous insect species overwinter as aquatic larvae, and many types of frogs and turtles hibernate at the bottom of ponds, where they are safe unless the body of water freezes completely. On land, toads may dig into the earth to remain below the frost line, and snakes may congregate in underground communal dens.

The second alternative to freezing is to use specific adaptations that stabilize the liquid state at subzero temperatures. All water solutions, including body fluids, have an equilibrium freezing point, or the temperature at which an ice crystal placed in the solution will begin to grow. But all water solutions can also be supercooled—that is, they can be chilled well below the equilibrium freezing point before the water crystallizes spontaneously into ice. Human plasma, for example, has a freezing point of –.8 degree C but, if chilled in a controlled manner, can be supercooled to –16 degrees C.

The presence of nucleators, however, limits the extent of supercooling. Nucleators are compounds that seed ice

STRATEGIES FOR WINTER SURVIVAL IN A TEMPERATE ZONE FOREST



growth by providing binding sites that can order water molecules into the ice-lattice structure. Ice itself is the best nucleator, but plasma proteins, foreign bacteria and food particles also act as effective nucleators. To stabilize the liquid state, then, animals must eliminate nucleators or prevent the nucleators from triggering widespread crystallization—in effect, animals must lower the supercooling point of their body fluids.

Arthur L. DeVries and his colleagues at the University of Illinois at Urbana-Champaign discovered that polar marine fish use such a strategy. The fish avoid freezing because they have antifreeze proteins in their body fluids. When embryonic ice crystals form within the fish, the proteins quickly bind to the crystal and effectively impede the further addition of water molecules to the crystal growth plane [see "Antarctic Fishes," by Joseph T. Eastman and Arthur L. DeVries; SCIENTIFIC AMERICAN, November, 1986]. Many terrestrial arthropods, including spiders, ticks, mites and numerous insects, have also developed antifreeze proteins. In many cases, insect antifreeze proteins are so potent that they can prevent ice formation at temperatures as low as -15 degrees C, enabling many insects to remain active under the winter snowpack.

Other insects require greater winter protection and have developed an additional antifreeze besides proteins to depress the supercooling point. These insects load their body fluids with an antifreeze made of low molecular weight polyhydroxy (sugar) alcohols. A solution of 50 percent ethylene glycol, providing protection to -30 degrees C, is the standard level of antifreeze added to the radiators of cars in southern Canada. By comparison, our studies of caterpillars of the gall moth (*Epiblema scudderiana*) found that they have body fluids that are about 40 percent glycerol in midwinter, representing an enormous 19 percent of the total body weight of the animal. This amount allows the insects to supercool to -38 degrees C.

Given that a variety of terrestrial animals can successfully avoid freezing by deep supercooling, it may seem odd that other animals have become tolerant to freezing, taking on the much more difficult job of regulating and surviving the freezing of body fluids. But avoiding freezing has its risks. The supercooled state is metastable, and the probability of spontaneous nucleation below the freezing point increases as the period of cooling lengthens and the temperature continues to drop. Cooling below the supercooling point or contact with nucleators (for example, as a result of injury to the skin) results in instant and lethal flash freezing. Many freeze-tolerant animals may have opted to forgo the probabilistic nature of supercooling in favor of a slow and controlled freezing that, if done properly, is readily survivable.

To some extent the rationale for "choosing" to freeze rather than to supercool is lost in the evolutionary history of each species. For instance, the gall moth caterpillars that we study share their winter home on goldenrod stems with larvae of the gallfly (*Eurosta solidaginis*). The caterpillars supercool and avoid freezing, but the gallfly larvae freeze when temperatures drop below about -8 degrees C. Both experience the same winter weather conditions but use opposite strategies to survive.

We have no good answer to this dichotomy except the following obvious observation: one species has evolved mechanisms to avoid freezing, the other to tolerate it. The gall moth caterpillars succeeded in eliminating internal nucleators from their bodies, spun a waterproof cocoon to prevent seeding by environmental ice and then perfected the supercooling strategy. In contrast, the gallfly larvae cannot or do not block the action of nucleators and have perfected ways to tolerate freezing instead.

How, then, do animals such as the gallfly larvae survive freezing? We noted earlier that ice crystals can cause extensive physical damage to the internal structure of cells as well as to the greater organization of connections between cells or the integrity of capillaries. Indeed, the destruction caused by ice inside cells is so massive that even freeze-tolerant animals do not survive intracellular ice. The same is true, for all practical purposes, for all types of mammalian cells and tissues that have been successfully cryopreserved to date. Freeze tolerance in nature consequently means a tolerance for ice growth in extracellular fluid spaces coupled with mechanisms that keep the cytoplasm liquid.

To survive freezing, animals must use specific biochemical adaptations that satisfy three basic conditions. The first condition is that ice formation must be controlled. Ice growth must be initiated in extracellular fluids (for example, blood plasma, abdominal fluid and urine) in a way that keeps the rate of freezing slow and the size of the crystals small. To accomplish these tasks, freeze-tolerant animals add specific nucleating agents to their extracellular fluids. By providing binding sites that order water molecules into an ice-lattice structure, the nucleating agents stimulate crystallization and enable it to occur more easily.

Biological nucleators in freeze-tolerant animals are most often specific blood proteins (called ice-nucleating proteins) that are synthesized during the autumn months. The regulation of their production probably comes from the same types of photoperiod cues and hormonal stimulation that control the synthesis of antifreeze proteins in insects that avoid freezing. Ice-nucleating proteins seed ice formation, generally initiating crystallization at a temperature less than two degrees C below the freezing point of body fluids.

Such a process minimizes the extent of supercooling, so that freezing becomes a relatively slow and controlled event that allows plenty of time for cells to adjust both physically and metabolically during the transition to the frozen state. Our studies of ice-nucleating proteins in the blood of wood frogs (done in collaboration with Jan P. Wolanczyk and John G. Baust of the State University of New York at Binghamton) have shown that these proteins are quite potent. As little as .5 percent by volume of frog blood added to human plasma effectively raises the nucleation temperature of the plasma by seven degrees C.

The action of ice-nucleating proteins ensures that the initial freezing process results in the dispersal of thousands of small ice crystals throughout the extracellular spaces of the animal. Small ice crystals, however, are thermodynamically unstable, and they tend to re-form over time into larger and larger crystals, much as sizable ice crystals appear in ice cream kept too long after opening. For animals, such recrystallization could do physical damage, especially in delicate spaces such as the lumina of capillaries; therefore, freeze-tolerant animals require a mechanism that controls the size of ice crystals.

John G. Duman and his colleagues at the University of Notre Dame identified such a mechanism. They noted the puzzling presence of both ice-nucleating and antifreeze proteins, which apparently perform opposite functions, in freeze-tolerant insects. Experiments soon showed, however, that the same molecular actions that enabled antifreeze proteins to block the growth of embryo ice crystals were equally effective in blocking the recrystallization of existing crystals. Together, then, the two proteins control ice structure: ice-nucleating proteins seed the formation of extracellular ice, and antifreeze pro-

teins stabilize the ice crystals at a small, harmless size.

The second condition for freezing survival involves the protection of cell structure and function. The semipermeable cell membrane, which separates the extracellular and intracellular compartments, allows the free passage of water and some solutes but restricts movement of other compounds. So when ice forms outside cells, it immediately changes the water and solute balance inside cells.

Ice is a crystal of pure water, and as extracellular ice forms, it excludes from its structure solutes such as salts, sugars and proteins. Thus, the remaining extracellular fluid becomes more and more concentrated. This process places osmotic stress on the cell because the total concentration of solutes on either side of the cell membrane must always balance. In response to such stress, water flows out of the cells, and the solutes move in. The process stops when the concentration of solutes becomes great enough to prevent the further loss of water into ice.

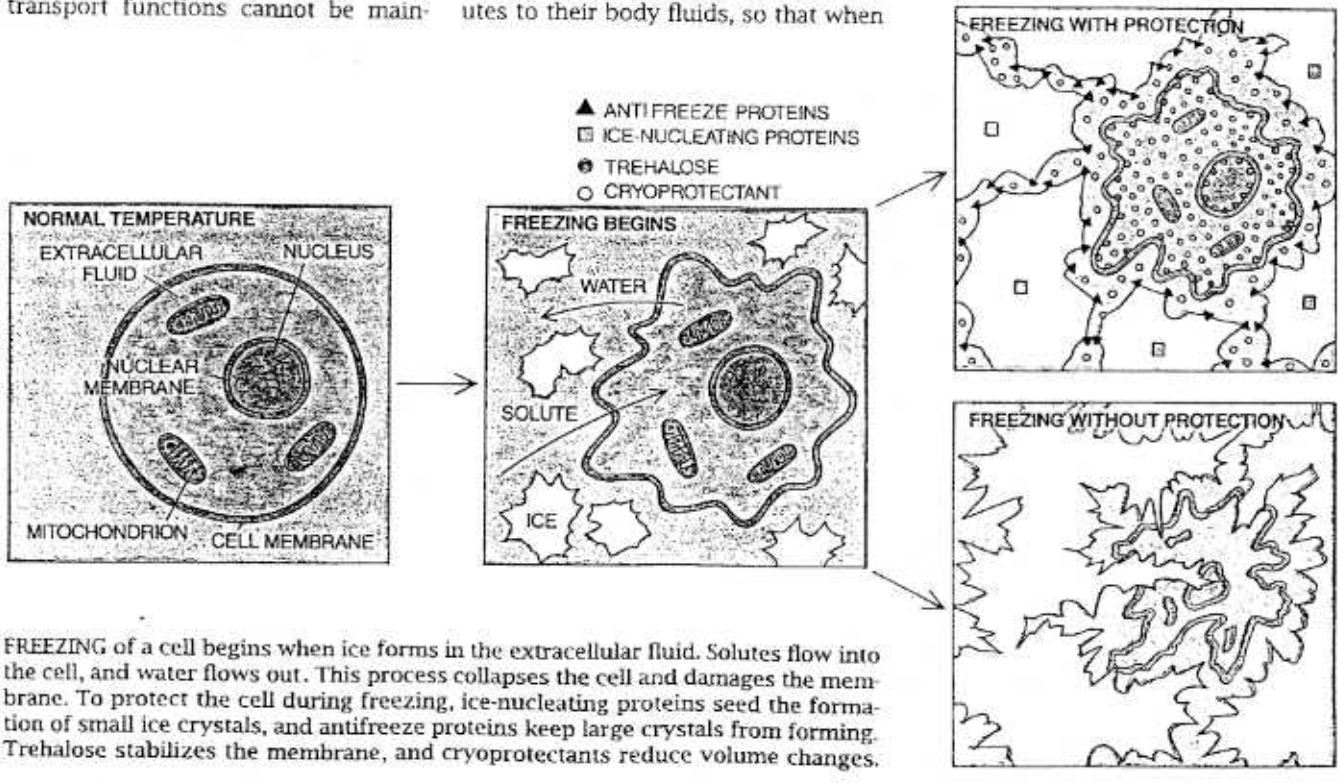
The most serious injury that can potentially occur during freezing is to the cell membrane. The outflow of cell water caused by extracellular ice formation rapidly reduces cell volume, and the cell membrane collapses inward. If the cell volume falls below a critical minimum, then the bilayer of phospholipids in the membrane becomes so greatly compressed that its structure breaks down. Membrane transport functions cannot be maintained, and breaks in the membrane spill cell contents and provide a gate for ice to propagate into the cell. Most freeze-tolerant animals reach the critical minimum cell volume when about 65 percent of total body water is sequestered as ice.

To counter these stresses on cell structure, freeze-tolerant animals use both membrane and colligative cryoprotectants—low molecular weight compounds that in various ways prevent the injuries that would result from massive cell volume changes during freezing. Membrane cryoprotectants interact with the membrane phospholipids to spread the bilayer and stabilize membrane structure as the cell volume collapses. Trehalose, a disaccharide, and proline, an amino acid, are the natural compounds known to perform this function. Not surprisingly, freeze-tolerant animals such as the gallfly larvae accumulate substantial amounts of both compounds during the autumn months prior to their first exposure to freezing temperatures.

Colligative cryoprotectants help to limit by osmotic action both the amount of ice that can form and the degree to which cells lose water and hence the extent to which cell volume decreases during freezing. The higher the concentration of solutes in a fluid, the less ice forms at any given temperature and the lower an animal's temperature can be pushed before the lethal 65 percent ice content is reached. Therefore, freeze-tolerant animals add high concentrations of nontoxic solutes to their body fluids, so that when extracellular freezing occurs the resulting reduction of cell volume can be minimized.

For this purpose freeze-tolerant insects use the same polyhydroxy alcohols that freeze-avoiding species use for antifreeze protection. Gallfly larvae build up a huge reserve of carbohydrate in their fat body (the insect equivalent of a liver) during the final weeks of summer feeding. During the autumn months this stored glycogen, making up about 8 to 12 percent of the total body weight of the larvae, is completely converted into two polyhydroxy alcohols: glycerol and sorbitol.

Key enzymes involved in the synthesis of these compounds respond uniquely to low temperatures. Whereas the activity of most enzymes and other metabolic processes lessens with decreasing temperature, temperatures between zero and five degrees C actually raise the activity of an enzyme called glycogen phosphorylase by stimulating it to convert from its inactive to its active form (the enzyme chops hexose sugar units off glycogen to begin synthesis). In addition, low temperatures inactivate other enzymes, resulting in a redirection of the flow of carbon from the normal routes of carbohydrate catabolism (used to produce cellular energy) to special pathways that lead to cryoprotectant syn-



FREEZING of a cell begins when ice forms in the extracellular fluid. Solutes flow into the cell, and water flows out. This process collapses the cell and damages the membrane. To protect the cell during freezing, ice-nucleating proteins seed the formation of small ice crystals, and antifreeze proteins keep large crystals from forming. Trehalose stabilizes the membrane, and cryoprotectants reduce volume changes.

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thesis. Cryoprotectants persist throughout the winter, and then as spring begins they are converted back into sugars to fuel the continued development of the insects through the pupal and adult stages.

Glycerol, sorbitol and related compounds represent excellent choices of cryoprotectant in biochemical terms. Not only do these compounds provide the osmotic actions needed to regulate cell volume during freezing, but they also remain nontoxic to cells even at very high concentrations. They do not crystallize spontaneously from aqueous solutions at low temperature, and they pass freely across membranes. In addition, these polyhydroxy alcohols stabilize the structure of proteins and enzymes and protect them from the denaturing effects of low or freezing temperatures.

Our studies of freeze-tolerant frogs have revealed a system unlike the one insects employ. Frogs use a different cryoprotectant and an unusual way of triggering its synthesis. Wood frogs, spring peepers and striped chorus frogs all accumulate massive quantities of glucose, the normal blood sugar of vertebrate animals, during freezing episodes (gray tree frogs use glycerol). Whereas human blood has a normal glucose content of about 50 to 100 milligrams per 100 milliliters (in diabetics, glucose levels may be three to four times higher), wood frogs after freezing have blood glucose levels that average 4,500 milligrams per 100 milliliters. All the organs of the frog body also contain glucose in concentrations that seem optimal for the protection of each organ.

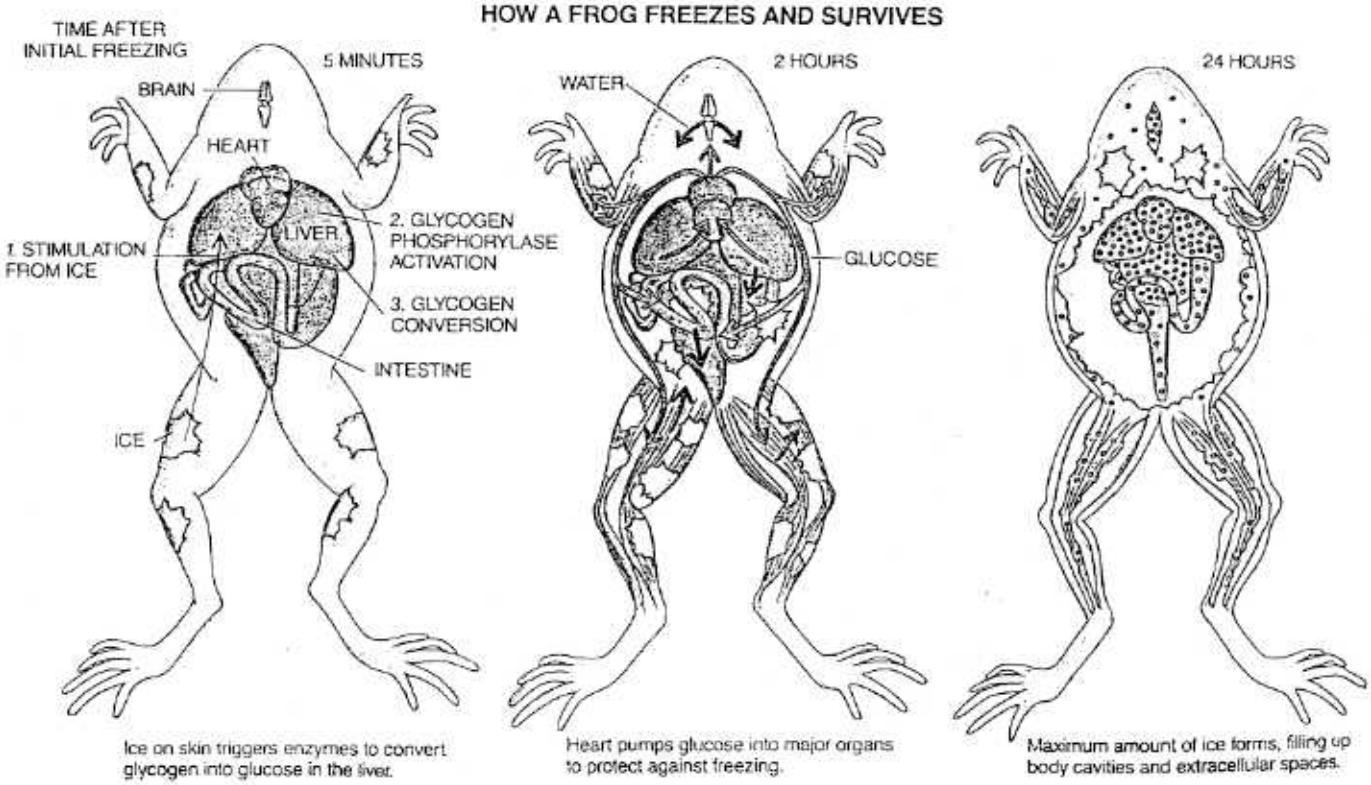
But frogs do not gradually build up cryoprotectant pools over the autumn months as insects do. Instead they wait until actual freezing begins on their skin surface. Ice on the skin triggers a hormonal or nervous response that instantly activates glycogen breakdown in the liver, flooding glucose into the blood. Indeed, we have detected rising blood glucose levels within as little as five minutes after initial ice formation, and organs become well packed with glucose in less than eight hours—well before the approximately 24 hours required to reach maximum survivable levels of ice in the body. The rapid synthesis of cryoprotectant during freezing, and the similarly rapid reconversion to liver glycogen when the frogs thaw, may be the key to circumventing various negative effects of sustained high glucose levels that are associated in humans with, for example, diabetes or the aging process.

But if high levels of glucose can be damaging, why do frogs use the sugar for cryoprotection? One reason is that glucose can be produced quickly from liver glycogen. Cryoprotectant synthesis in frogs appears to be an extreme exaggeration of the adrenaline-mediated "fight or flight" response that occurs in all vertebrate animals and that rapidly increases blood glucose during stress. Indeed, compounds that block the action of adrenaline on the liver, such as propranolol, also effectively block the synthesis of glucose by the frog liver during freezing.

Our studies with heart strips and liver cells from the wood frog, however, suggest a more crucial reason for the choice of glucose: the sugar has specific, beneficial effects for the cryopreservation of vertebrate organs. For example, ventricle strips regained their ability to contract after thawing if they were frozen in the presence of high glucose levels but not if they were frozen in an equivalent concentration of glycerol. Because both glucose and glycerol should provide the same osmotic effects to control the decrease in cell volume during freezing, the superiority of glucose must result from other specific actions that aid cell survival.

One of these actions may be the use of glucose as a fuel to provide adenosine triphosphate (ATP) energy in cells that have no access to blood-borne oxygen while frozen. In addition, we have observed that high levels of glucose (but not glycerol) can depress the biosynthesis of urea in the frog liver. This evidence suggests that high glucose levels may help arrest the metabolism of frozen organs, limiting cellular ener-

HOW A FROG FREEZES AND SURVIVES



Ice on skin triggers enzymes to convert glycogen into glucose in the liver.

Heart pumps glucose into major organs to protect against freezing.

Maximum amount of ice forms, filling up body cavities and extracellular spaces.

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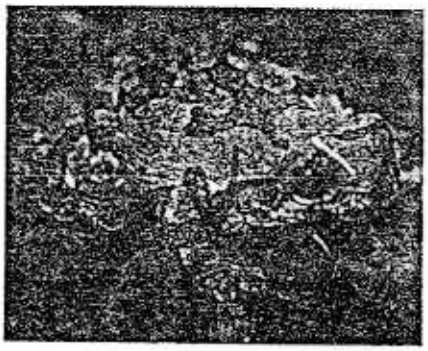
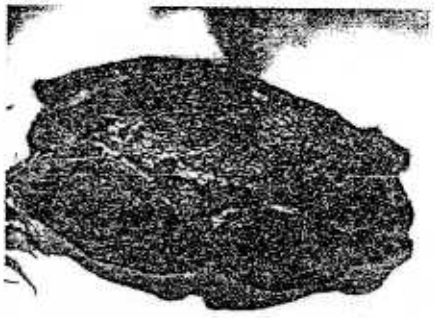
gy needs and thereby enhancing long-term survival.

The third and final condition to surviving freezing is the maintenance of cell viability. Although the low body temperature of frozen animals automatically lowers their metabolic rate, the cells of freeze-tolerant animals must have a well-developed ability to survive without oxygen, without access to blood-borne fuels and without being poisoned by a buildup of the metabolic end products that are normally carried away by the blood. A human brain can survive perhaps three minutes of interrupted blood flow before tissue necrosis begins; a kidney or heart removed for transplantation can be stored for about six to 12 hours if packed in ice. But in our laboratory we routinely revive wood frogs after one to two weeks of constant freezing.

Such animals must continue to generate cellular energy in the absence of oxygen. We found that frozen gallfly larvae showed no disruption at all of their cellular levels of ATP when frozen for one week, and energetics in frog organs remained stable over at least three days of freezing. Even when ATP levels fell during prolonged freezing, both gallfly larvae and frogs could rapidly restore cellular energy levels after thawing. Consequently, freeze-tolerant animals can survive without oxygen for long periods. They have mechanisms for generating sufficient ATP from the fermentation of glycogen or glucose, and all organs tolerate quite well the prolonged periods of low energy levels.

The metabolic arrest that occurs during freezing may also figure highly in subsequent recovery. The ability to reduce metabolic rate greatly, frequently to as low as 1 to 10 percent of the normal resting rate, is a key adaptive strategy used by many animals to survive environmental extremes. A tenfold drop in metabolic rate, for instance, gains for the animals a tenfold extension of the time that a fixed store of body fuels can sustain life. The most familiar example is mammalian hibernation: by entering a dormant state and dropping body temperature to near zero degrees C, small mammals can save up to 88 percent of the energy that would otherwise be expended for winter survival. Numerous insects overwinter in diapause (a state of arrested development), and turtles hibernating at the bottom of ponds drop their metabolic rate to survive the whole winter without breathing. For freeze-tolerant species, then, the ability to lower their metabolic rate while frozen can greatly enhance the prospects for long-term survival.





GRAY TREE FROG (*left*) freezes beneath the winter snowpack, where temperatures fall to –8 degrees Celsius. Its skin pigments turn blue in the frozen state (*right*).

To us, the adaptive strategies that animals use to survive freezing are marvelous in themselves, but we are always asked about the application of our studies to medical cryopreservation, particularly for organs used in transplants. The first successful cryopreservation occurred in 1949, when sperm were revived after having been frozen in a glycerol solution. Since then, techniques have been developed for freezing many single-cell suspensions (sperm, red and white blood cells, platelets) and simple tissues (embryos, skin, cornea, pancreatic islets).

Physical problems remain, however, for complex tissues, and researchers do not yet have adequate technology to restore a functional organ after freezing. Obstacles to organ freezing include the difficulty of evenly chilling or warming a large organ mass to prevent physical damage by ice and the problem of infusing or removing large amounts of cryoprotectants from cells that are not naturally adapted to dealing well with major osmotic stresses. Furthermore, metabolic problems exist. Unnatural cryoprotectants (such as the commonly used dimethyl sulfoxide) offer excellent physical protection but have toxic effects on cell metabolism. In addition, metabolic decay occurs within minutes after an organ is removed from its blood-oxygen supply, and the chilling process further damages mammalian organs that are not designed to function at temperatures far lower than their normal 37 degrees C.

But the injuries caused by freezing and the principles of circumventing them are the same in cryopreservation as in natural freeze tolerance, and some answers are identical. For instance, glycerol and other low molecular weight alcohols and carbohydrates are commonly used for medical cryopreservation because they are relatively nontoxic and can move rapidly across membranes. Workers also lower the temperature in stages to trigger extracellular nucleation at a mild subzero temperature and to avoid spontaneous crystallization in a supercooled cytoplasm. Some of the newest studies in the field are investigating whether membrane stabilizers or metabolic inhibitors (which impose metabolic arrest by inhibiting ATP-using processes) can improve survival during freezing.

Our studies suggest additional approaches. Synthetic compounds that mimic the actions of ice-nucleating or antifreeze proteins could be developed to regulate extracellular ice formation more effectively, and a careful choice of cryoprotectant (such as glucose) could provide both physical and metabolic protection during freezing. Research should also examine other metabolic arrest strategies to preserve the viability of an organ while frozen. We are beginning to test these ideas by comparing freezing survival in wood frog and rat tissues. We already know some secrets of freeze tolerance; we hope to continue to unravel more.

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