17

COLD HARDINESS AND FREEZE TOLERANCE

JANET M. STOREY AND KENNETH B. STOREY

INTRODUCTION

The Earth is a cold place. About 90% of the water in the oceans is colder than 5°C and surface waters in temperate and polar regions drop to −1.9°C, the freezing point of seawater, for many months of the year. Land is even colder. Winter temperatures of −30°C in temperate zones and −70°C in polar regions are not uncommon. Yet life endures in all cold places. Indeed, many organisms can survive only in the cold; for example, psychrophilic bacteria typically cannot grow above 15°C but grow well at subzero temperatures.

For most multicellular organisms, freezing means death, and, therefore, when winter arrives, strategies of adaptation are needed to survive at subzero temperatures. The fundamental options for protection against the cold are: (a) migration to a warmer climate or microclimate, (b) insulation, (c) thermogenesis, and (d) development of cold hardiness. Migration can be long distance such as birds and butterflies that fly south or short-distances such as frogs that move from their summer haunts in wetlands to spend the winter at the bottom of lakes or garter snakes that travel several kilometers to mass together by the thousands in deep underground dens. Short-distance migration is often, in effect, a way of achieving insulation; animals retreat underground to sites well below the frost line or into deep water that will not freeze to the bottom. Many other organisms also burrow underground (e.g., toads, ground squirrels), seek sheltered sites (e.g., under leaf litter) or dens (e.g., bears, bats), or winter under water (e.g., dragonflies and other insects overwinter in aquatic life stages). Recall also from Chapter 15 that underwater hibernation by turtles, although insulating them from the cold, has required that they optimize their ability to survive without oxygen. Individual insulation consisting of thicker fur or down feathers and layers of fat are key for endotherm survival, as is thermogenesis. Indeed, although hibernating mammals may be exposed to subzero ambient temperatures, they never really have to deal with the possibility of their body fluids freezing because they can always activate nonshivering thermogenesis to maintain a core body temperature, T_b, between about 0 and 5°C. For the vast majority of ectotherms, however, such options are impossible (for an exception see thermogenesis by honeybees in Text Box 16.1). When ambient temperature falls below 0°C, most ectotherms have no physiological or biochemical mechanisms to prevent T_b from falling, and, when T_b falls below 0°C, the risk of body fluids freezing becomes high. For most organisms, freezing is lethal and, hence, their only choice for survival is to develop cold hardiness. Cold hardiness is frequently expressed only in the winter (organisms lose the ability in summer) and is often restricted to one simple life stage (e.g., egg, seed, spore, cyst), which is easier to protect than is a multicellular adult or vegetative form that dies off. Cold hardiness covers a range of biochemical strategies that sustain life at subzero body temperatures. These basically involve multiple options for dealing with the reality that water turns to ice at temperatures below 0°C.

The present chapter explores metabolic regulation as it applies to ectotherm survival at cold temperatures, with a primary focus on survival below 0°C. The subjects of cryobiology and the related medical field of cryopreservation are huge ones that fill books of their own. The focus here will be on the mechanisms of metabolic regulation and biochemical adaptation that contribute to cold hardiness and subzero survival with a major emphasis on the biochemistry of vertebrate freeze tolerance. Advances in understanding these phenomena have provided novel insights about the

Functional Metabolism: Regulation and Adaptation, edited by Kenneth B. Storey.
ISBN 0-471-41090-X

plasticity and adaptability of the mechanisms of metabolic regulation that have been discussed in earlier chapters.

The central theme for preserving life below 0°C is protection. Organisms must arrange protective measures that either prevent their body fluids from freezing or, if managed freezing is allowed to occur, that protect their cells and tissues from the associated physical and metabolic insults. Hence, much of the study of metabolic regulation in the field of cryobiology has centered on the regulation of cryoprotectant production and the synthesis of specific proteins that are involved in ice management or macromolecular stabilization. These will also be our primary focus.

INJURY AND SURVIVAL AT SUBZERO TEMPERATURES

Freezing is lethal for most organisms for several reasons. Ice formation inside cells can destroy both subcellular architecture and metabolic microcompartmentation, and, with only one or two documented exceptions, intracellular ice formation is uniformly lethal for all species and cell types. Both in nature and in the laboratory, freezing typically begins in extracellular spaces and ice propagates through extraorgan spaces (e.g., the abdominal cavity, between skin and muscle layers), through the lumen of the vasculature, and throughout the extracellular fluid spaces surrounding cells, tissues, and organs. Ice does not usually penetrate into cells unless membranes are broken due to physical damage from ice or freezing-related stresses. However, ice growth in extracellular spaces still causes multiple injuries (Fig. 17.1). These include:

1. Ice expansion in the vasculature ruptures capillaries so that upon thawing vascular integrity is lost and there is extensive internal bleeding.
2. The withdrawal of pure water into crystals of extracellular ice elevates the osmotic concentration of remaining extracellular fluid and sets up a powerful osmotic gradient that sucks water out of cells. For example, if the freezing temperature is −10°C, the solute concentration in the unfrozen extracellular liquid of nonacclimated organisms will rise to about 5 osmolar (osM), and more than 90% of the osmotically active water will move out of cells. This extreme cell volume reduction can severely damage membranes and elevates cytoplasmic ionic strength to dangerous levels. Furthermore, during thawing, volume increase can be so rapid as to burst cells.
3. Freezing stops blood circulation so that tissues are deprived of oxygen and blood-borne nutrients as well as of the means to dispose of accumulating waste products. This condition is called ischemia and also characterizes multiple pathological conditions (e.g., heart attack, stroke) and limits the survival time of organs that are removed for transplant (see Chapter 18).
4. Freezing halts multiple vital processes including breathing, heart beat, nerve transmission, and muscle movement that may be impossible to reactivate after thawing.

Because of the dangers of freezing, most ectotherms faced with seasonal exposure to subzero environmental temperatures take steps to avoid ice formation in their bodies, but selected species actually embrace freezing. Ectothermic species use four basic options for survival at subzero temperatures:

1. *Anhydrobiosis* Extensive dehydration that removes all free water from an organism so that there is no water left to freeze
2. *Vitrification* Solidification of water into an amorphous glass state in order to circumvent the physical and osmotic injuries caused by water freezing into ice crystals
3. *Freeze Avoidance* Deep supercooling of body fluids achieved with the use of multiple kinds of antifreezes
4. *Freeze Tolerance* The controlled freezing of body water in extracellular fluid spaces while preserving the liquid state of the cytoplasm

Anhydrobiosis and vitrification strategies are primarily used by microfauna or by "simple" life stages (e.g., eggs, embryos, seeds, cysts), and some unique aspects of these strategies are dealt with in Text Box 17.1. Note, however, that an integral part of both of these strategies, as well as of freeze tolerance and freeze avoidance, is the synthesis and accumulation of high quantities of carbohydrate protectants. Our discussion below of the pathways and regulatory mechanisms that control the synthesis of these protectants applies equally to all four mechanisms of cold hardiness.

At first glance, the freeze avoidance and freeze tolerance options appear to be opposite strategies. The freeze avoidance option prevents freezing by deep supercooling of body fluids, whereas freeze-tolerant organisms allow ice to form in extracellular fluid spaces. However, the two options share at least two key biochemical adaptations: (1) the accumulation of low-molecular-weight carbohydrate protectants and (2) the production of antifreeze proteins. The reason for this is that freeze-tolerant organisms are actually something of a paradox. Ice is allowed to form in extracellular and extraorgan fluid spaces, but intracellular water is prevented from freezing. Hence, freeze-tolerant organisms defend the liquid state of the cytoplasm with the same mechanisms that freeze-avoiding animals use to

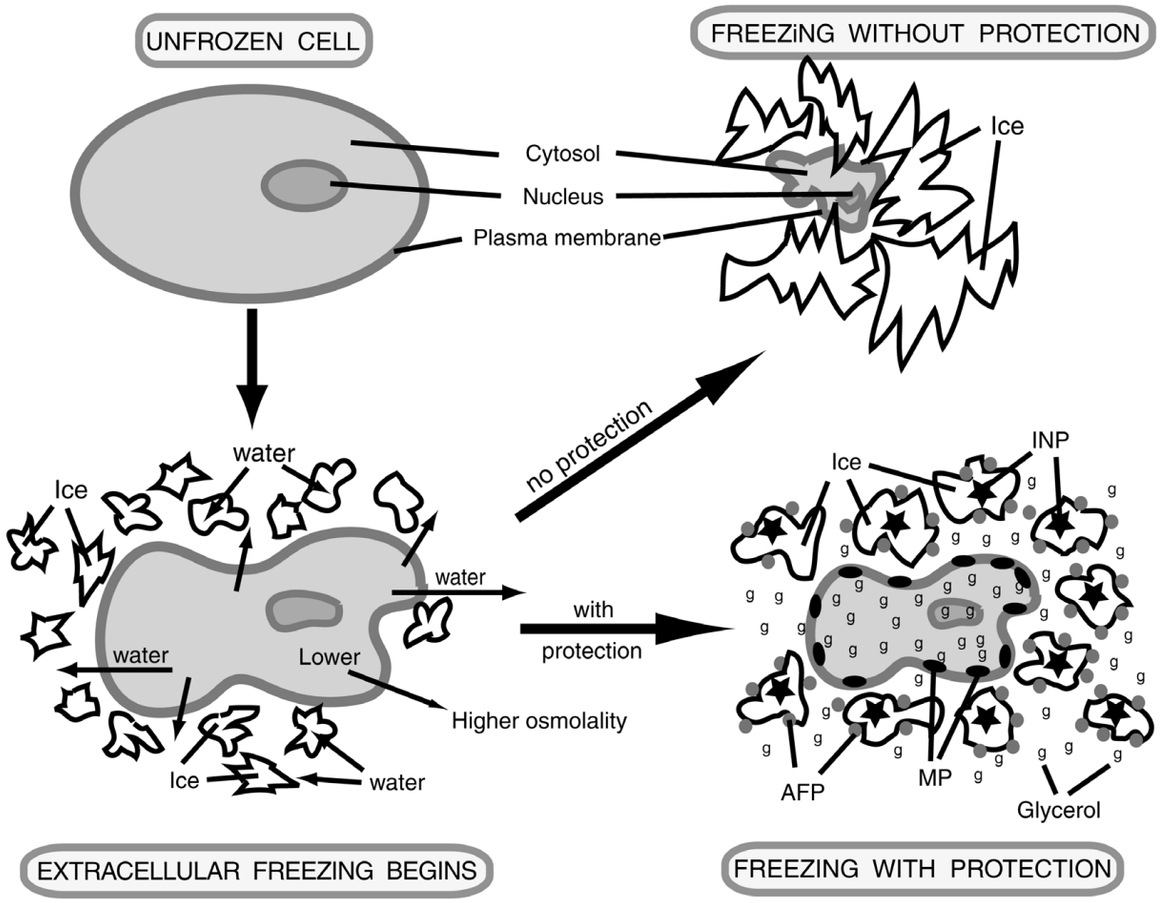

Figure 17.1 Cell responses to extracellular freezing. For an unprotected cell, extracellular ice nucleation results in a rapid growth of ice in large crystals. Solutes are excluded from the crystals and the osmolality of the remaining extracellular fluid rises quickly. Cells respond by loosing water. Cell shrinkage beyond a critical minimum cell volume causes permanent damage to cell membranes so that upon thawing the integrity of the plasma membrane is lost. Cells may also be squeezed and sheared when trapped within growing crystals. Freeze-tolerant organisms use various protective strategies. Freezing is seeded by ice nucleating proteins (INP) at a temperature just under the equilibrium FP of body fluids so that ice growth is slow and controlled. Antifreeze proteins (AFP) regulate the shape of crystal growth and inhibit recrystallization so that crystal size stays small. Low-molecular-weight carbohydrate cryoprotectants such as glycerol (g) or glucose are distributed to all cells and act in a colligative manner to minimize cell volume reduction, whereas others, such as trehalose or proline, act as membrane protectants (MP) to stabilize membrane bilayer structure.

defend the liquid state of the entire organism. Before discussing the biochemical adaptations involved, a further brief explanation of the strategies of freeze avoidance and freeze tolerance are needed.

Freeze Avoidance

Many organisms have mastered the ability to maintain a liquid state at temperatures well below the equilibrium freezing point (FP) of their body fluids. Among animals, the phenomenon has been best studied in two groups: cold-water marine fish and terrestrial insects. Marine fish use this strategy to keep from freezing in surface and inshore waters that chill to -1.9°C in the winter (or year-round in polar regions). This value is well below the -0.5°C FP of the body fluids of teleost fish, and so the potential for inoculative freezing due to contact with environmental ice is a constant hazard. Many insects and

TEXT BOX 17.1 ANHYDROBIOSIS AND VITRIFICATION

Anhydrobiosis

Anhydrobiosis means literally "life without water," and, although this seems a contradiction in terms, it is in fact a critical survival strategy for thousands of species of procaryotes, invertebrate microfauna (e.g., brine shrimp, rotifers, nematodes, tardigrades), some plant seeds, and even the vegetative tissues of a selected group of "resurrection plants." Also called cryptobiosis, the strategy is not just for cold hardiness but allows organisms to endure multiple environmental extremes including very low ($-196^{\circ}C$) or very high ($100^{\circ}C$) temperatures, desiccation, and oxygen lack. Anhydrobiotic organisms can frequently survive for years, decades, or even centuries. Among animals, the phenomenon has been best-studied in the brine shrimp, *Artemia*. Encysted embryos have as little as 0.1 g of water per gram dry mass (an active animal that is ~80% water would have ~4 g/g dry mass), an amount of water that is insufficient to hydrate intracellular proteins and is therefore incompatible with enzymatic activity or cellular metabolism. Indeed, studies with *Artemia* have found only one indicator of continuing life in dry or anoxic embryos. This is a very slow catabolism of the "energy storage" pool of guanosine polyphosphate (P^1,P^4-diguanosine-5′-teraphosphate) that is built up as animals enter dormancy and appears to support a very slow production of GTP or ATP.

The shell of vicinal water can be stripped off macromolecules during dehydration and, hence, the key to survival in this situation is the choice of effective protectants. Polyhydroxy sugars play a prominent role, not in retaining cellular water as we discussed for freeze-tolerant organisms, but in actually replacing the primary water of hydration surrounding macromolecules (this is called the *water replacement hypothesis*). *Artemia* and many other anhydrobiotes accumulate the disaccharide trehalose; ~15% of the dry mass of encysted Artemia is trehalose. Trehalose is an excellent stabilizer of both biological membranes and proteins (Fig. 17.3) and also has the ability to form an amorphous glass at low water contents (as does the sucrose accumulated by desiccation-tolerant plants). An important feature of such glasses is the huge reduction in diffusion coefficients of metabolites and macromolecules within the glass, which can effectively bring metabolism to a stand-still.

Protection is also provided by the synthesis of stress proteins that prevent protein unfolding or aggregation. In *Artemia*, a protein called p26 plays this chaperone role and makes up 10 to 15% of the nonyolk protein in the dry embryos. The protein is a member of the small heat shock/α-crystallin family. It polymerizes into oligomers of ~500 kD and displays a GTPase activity that may aid the extensive translocation of p26 to the nucleus during stress. A second stress protein found in encysted *Artemia* is artemin. It shows derivation from the iron storage protein, ferritin, but artimen multimers lack the central space used for metal storage. Artemin is enriched in cysteines, and its proposed function is in the protection of encysted embryos from oxidative damage.

Vitrification

Vitrification is a process by which water is solidified, not into a crystal but into an amorphous glass. As mentioned above, trehalose or sucrose glasses are an integral part of anhydrobiosis so the "line" between anhydrobiosis and vitrification as a winter cold-hardiness strategy is sometimes vague. The difference is that various organisms can solidify water into sugar glasses without having to undergo the massive dehydration of the anhydrobiotes. Sugar glasses form in many plant seeds and are key to subzero survival in the twigs of various subarctic woody plants such as poplar and birch. In poplar, for example, sugar glasses form below $-20^{\circ}C$, and twigs that are cold-hardened at $-20^{\circ}C$ to optimize sugar production can subsequently endure exposure to liquid nitrogen. The advantage of vitrification as a strategy for cold hardiness is that a sugar glass incorporates all of the dissolved solutes present in the extra- and/or intracellular water of an organism and, hence, vitrified cells are not under osmotic, ionic strength, or volume stresses as frozen ones are.

Vitrification has been widely explored as a strategy for the applied cryopreservation of cells, tissues, and organs, and, although successful in some cases, researchers have often found that the requirements for achieving a glass transition can be daunting. Necessary conditions can include the need to add extremely high concentrations of solutes (about 40% solutions), to achieve rapid cooling to the glass transition temperature (T_g), that is often well below $-30^{\circ}C$, and to use warming rates in the order of 30 to $50^{\circ}C$/min to prevent devitrification, the instantaneous crystallization of ice that can occur during warming at any temperature between the T_g and the melting point (MP) of the solution. How, then, do organisms manage to achieve vitrification in nature?

Natural vitrification occurs under much less rigorous conditions due to at least two factors that cannot always be used in cryomedical situations: (1) vitrifying systems undergo at least partial dehydration, and (2) cells

produce and accumulate high concentrations of sugars that have particularly high glass transition temperatures (e.g., trehalose in animals and sucrose, raffinose or stachyose in plants). High glass transition temperatures are very important so that organisms can make the transition to the vitrified state before there is any risk of spontaneous freezing. Indeed, work with both resurrection plants and *Artemia* shows evidence that vitrification occurs during drying at temperatures well above 0°C.

Vitrification is now used quite widely for the ultralow storage of medically important cells and some tissues, but successful preservation of vitrified organs remains elusive. There are several reasons for this including the low tolerance of mammalian organs for substantial dehydration, the requirement for extremely high amounts of cryoprotective agents (such as dimethylsulfoxide, ethylene glycol, glycerol), which may have cytotoxic effects, the impermeability of mammalian cells to the best protective agents with high T_g values (e.g., trehalose), and the need for very fast and even cooling and warming throughout the entire organ mass to both induce vitrification during cooling and avoid devitrification during warming.

other invertebrates are even more adept at freeze avoidance and can remain unfrozen down to −40°C or even lower (see Text Box 17.2). Deep supercooling also occurs within the primordium tissue of the buds and xylem of many woody plants.

The freeze avoidance strategy exploits two of the physical properties of water solutions. The first is a colligative property: the greater the concentration of dissolved solutes, the lower the FP of a solution. The second is the phenomenon of supercooling: the ability of water solutions to chill to temperatures below their equilibrium FP without freezing. All solutions can supercool to some extent, but prolonged supercooling by more than a few degrees below the FP does not normally occur in nature because crystallization is triggered either by contact with environmental ice or by the presence of heterogeneous nucleators such as proteins, food particles in gut, or bacteria or surfaces that can orient water molecules into the crystal lattice and trigger freezing. Freeze-avoiding organisms exploit both of these properties using an accumulation of high concentrations of solutes to achieve strong colligative suppression of the temperature at which body fluids freeze as well as mechanisms that stabilize the supercooled state. The implementation of both of these principles present us with interesting case studies in metabolic regulation and biochemical adaptation.

Freeze Tolerance

The negative side to freeze avoidance is that the supercooled state is not stable, and, if a supercooled organism is seeded by random contact with ice or another nucleator, it will flash freeze and die. Therefore, some organisms have made the "choice" to let themselves freeze but dictate the conditions of the freeze. Freezing is initiated at a high subzero temperatures (usually well above −10°C) so that the rate of ice formation is slow, crystal size is regulated, and ice growth is limited to extracellular and extraorgan spaces only. The best-studied groups of freeze-tolerant animals are certain insects (Text Box 17.2), some intertidal marine mollusks, and several kinds of frogs that hibernate

TEXT BOX 17.2 ONE HABITAT—TWO INSECTS—TWO STRATEGIES

Many kinds of insects make their homes inside galls—hollow nodules on plant stems, leaves, or roots that provide the insects with shelter and food while doing little or no harm to the plant. Life in a gall is restrictive, and insects that live in stem or twig galls may be imprisoned in them throughout the winter and exposed to deep subzero temperatures. The woody stems of goldenrod are home to two gall insects with contrasting strategies of winter survival. Each of these species has become a major model animal for studies of insect cold hardiness.

Caterpillars of the gall moth *Epiblema scudderiana* live inside elliptical galls. They have perfected the freeze avoidance strategy that allows them to stay liquid down to nearly −40°C (see Fig. TB17.1). Triggered when autumn temperatures fall to ∼5°C, the insects convert their huge stores of glycogen to glycerol, which reaches levels of over 2M or ∼19% of larval body mass by early winter. High glycerol plus the production of antifreeze proteins results in a suppression of the insect's supercooling point (SCP) from −14°C in September to −38°C by December, a value well below the lowest expected environmental temperature. Freeze avoidance is also aided by a decrease in total body water content and by the presence of a waterproof silk cocoon that lines the

interior of the stem cavity to prevent inoculative freezing by contact with environmental ice.

Larvae of the gall fly *Eurosta solidaginis* live inside ball galls, frequently sharing the same stem with a gall of *Epiblema*, but they use the opposite strategy of winter survival—freeze tolerance. Gall fly larvae actually raise their SCP to about −8°C during the winter and, lacking a cocoon, they are susceptible to freezing at even higher temperatures due to contact with frozen detritus or ice in the gall interior. Freeze-tolerant animals encourage ice formation at high subzero temperatures because this allows a slow rate of ice formation that gives cells the greatest amount of time to adjust to the stresses imposed by ice growth through their tissues. Gall fly larvae use a dual system of colligative cryoprotectants: glycogen is converted to ~300 mM glycerol and ~150 mM sorbitol each protectant accumulating on different time frames. Glycerol synthesis is triggered when ambient temperatures cool below 15°C and sorbitol by 5°C exposure. The two protectants are also catabolized separately with sorbitol being reconverted to glycogen, whereas glycerol is probably oxidized as an aerobic fuel during pupal/adult development in the spring. Larvae also accumulate 50 to 60 mM proline, which contributes to membrane stabilization during freezing.

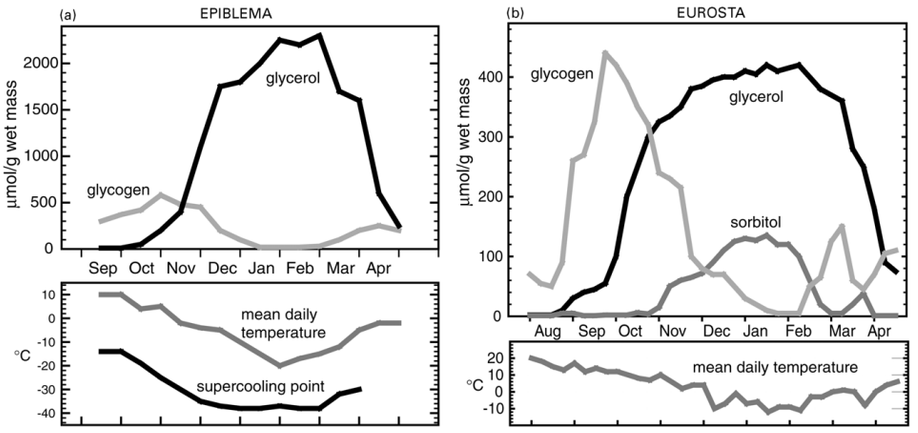

Figure TB17.1 Seasonal synthesis of cryoprotectants by goldenrod gall insects, (a) the freeze-avoiding *Epiblema scudderiana* and (b) the freeze-tolerant *Eurosta solidaginis*. In *Eurosta* note the mid-February loss of sorbitol during a warm spell (with carbon returned to glycogen) but renewed production during colder weather in March. [Data are compiled from J. Rickards, M. J. Kelleher, and K. B. Storey (1987). *J Insect Physiol* **33**:443–450 for *Epiblema* and from J. M. Storey and K. B. Storey (1986). *J Insect Physiol* **32**:549–556 for *Eurosta*.]

on land (Text Box 17.3). Freeze tolerance is also widely studied in agricultural science since the mechanisms of inducing or enhancing frost hardiness have great benefit for the engineering of plant cultivars that survive in cold climates. Freeze-tolerant organisms typically regulate the conversion of up to 65% of their total body water into extracellular ice while using high concentrations of colligative protectants within their cells to prevent the cytoplasm from freezing. Multiple biochemical adaptations address issues associated with freezing. These include:

1. *Ice Management* Ice nucleating agents (often specific proteins) are used to trigger and regulate ice crystal formation in extracellular compartments. Antifreeze proteins help to manage crystal size and prevent recrystallization. Enhanced synthesis of blood clotting proteins in vertebrates also helps to repair any internal bleeding injuries caused by ice when animals thaw.

2. *Cell Volume Reduction* The growth of ice (a crystal of pure water that excludes solutes) in extracellular spaces creates a powerful osmotic force that sucks water out of cells and causes a major decrease in cell volume and a large increase in intracellular ionic strength (Fig. 17.1). Volume changes and the structural pressure that they place on cellular membranes must be managed. The action of membrane protectants stabilizes bilayer structure in response to the compression stress imposed by cell volume loss, whereas low-molecular-weight carbohydrate

TEXT BOX 17.3 FROZEN AMPHIBIANS AND REPTILES

There is nothing quite so amazing as removing an ice-coated, stiff and frozen solid frog from a freezer, setting it on the lab bench, and then watching as ice melts away. The frog relaxes into a seemingly lifeless blob, and then after about an hour a flutter of life appears as a heart beat becomes discernible through the chest wall. Minutes later, the frog takes a first gulp of air, then an eyelid blinks, then legs shuffle and push the frog up into a sitting position, and then it leaps away!

Freezing and thawing several times a winter is part of normal life for several species of woodland frogs that hibernate on the forest floor. They are insulated by layers of leaf detritus and snow from the harshest air temperatures above the snowpack, but under the snow, midwinter temperatures may still fall to $-5^{\circ}C$ or lower for days or weeks. Frog skin is no barrier to water or ice, and so, when ice forms on the skin surface, body water is quickly seeded and ice propagates through the frog's body over several hours. Ice nucleation triggers an immediate outpouring of glucose from the liver, and sugar is packed into all cells to provide intracellular cryoprotection at the same time as 50 to 70% of the frog's total body water turns to ice in extracellular spaces. When fully frozen, ice fills all extracellular compartments including the abdominal cavity, the ventricles of the brain, the bladder, and runs in sheets between the skin and skeletal muscle. Breathing and heart beat stop, muscles cannot move, no electrical activity can be detected in the brain, and the frog waits, in an icy suspended state, until temperature rises and the melt begins.

The best-studied of the freeze-tolerant frogs is the wood frog, *Rana sylvatica*, the subject of most of the discussion in this chapter, but others include the spring peeper (*Pseudacris crucifer*), the chorus frog (*P. triseriata*), and the tree frogs (*Hyla versicolor*, *H. chrysoscelis*). Several reptiles also show sufficient freezing survival to indicate that freeze tolerance contributes to their winter survival. These include the European common lizard (*Lacerta vivipara*), the box turtle (*Terrapene carolina*), and hatchlings of the painted turtle (*Chrysemys picta*). Various other reptiles and amphibians have a capacity to endure brief freezing exposures but succumb during long-term exposures where ice content rises above 50% and vital processes such as heart beat are interrupted.

protectants provide colligative action to increase cellular osmolality and keep cell volume from falling below a critical minimum.

3. *Ischemia* The freezing of blood plasma halts circulation so that while frozen organs must survive without supplies of oxygen and blood-borne fuels. Survival requires good anoxia tolerance as well as antioxidant defenses that prevent damage due to the formation of reactive oxygen species when oxygen is reintroduced upon thawing. Mechanisms of metabolic rate depression may also be employed to minimize cellular energy requirements and, hence, maximize survival time in the frozen state.

4. *Vital Signs* Freezing halts all vital signs including heart beat, breathing, muscle movement, and nerve transmission. All are sequentially reactivated during thawing, but the molecular mechanisms underlying these processes are still largely unknown.

BIOCHEMISTRY OF CARBOHYDRATE PROTECTANTS

Common to most cold-hardy organisms, both animal and plant, is the accumulation of high concentrations of low-molecular-weight sugars and polyhydric alcohols as protectants. For anhydrobiotic organisms these stabilize macromolecular structure and substitute for the hydration shells that normally contribute significantly to stabilizing protein conformation. For vitrification, high concentrations of protectants allow the formation of sugar glasses in which water is solidified but not crystallized. For freeze-avoiding organisms, high levels of carbohydrate protectants provide the colligative suppression of FP and supercooling point (SCP) of biological fluids that is integral to maintaining body water in a liquid state at subzero temperatures. This use of biological protectants is the functional equivalent of adding ethylene glycol to the water in the radiator of a car (indeed, some insects actually accumulate ethylene glycol). Concentrations of 2 M or more are often achieved in the body fluids of freeze-avoiding insects (see Fig. TB17.1). For freeze-tolerant organisms, carbohydrate protectants preserve the liquid state of the cytoplasm and defend a minimum cell volume by limiting water loss into extracellular ice. Although total cryoprotectant levels, measured per gram wet mass (gwm), are lower in freeze-tolerant animals (typically 300 to 500 μmol/gwm) (see Text Box 17.2 for freeze-tolerant insects), once 65% of body water is frozen as ice, protectant concentrations in remaining intracellular water will rise to 1 M or more.

Glycerol is by far the most common protectant used by insects and other terrestrial arthropods, but other carbohydrates include sorbitol, mannitol, ribitol, xylitol, erythritol, ethylene glycol, glucose, trehalose, and sucrose

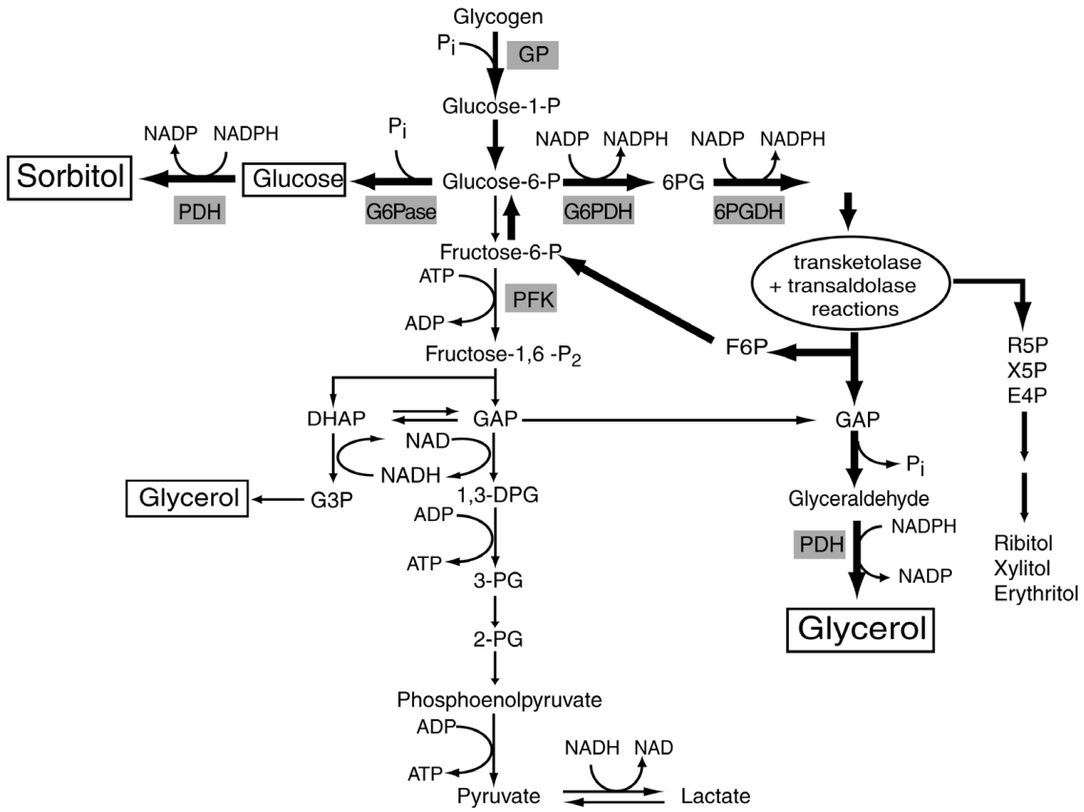

Figure 17.2 Biosynthetic pathways for glycerol, sorbitol, and glucose cryoprotectants. Glycogen is the carbon source and enzymes of glycolysis and the pentose phosphate cycle (PPP) are used. Glycerol in insects is primarily made from the glyceraldehyde-3-phosphate (GAP) output of the PPP using NADPH reducing power that is also generated by the PPP. An alternative output of glycerol from glycerol-3-phosphate (G3P) is shown and is the route used in vertebrate liver. Outputs of four and five carbon sugar phosphates from the PPP can also be converted to their corresponding polyols after phosphate removal and then conversion of sugar to polyol by the enzyme polyol dehydrogenase (PDH). Other enzymes in shaded boxes are: GP, glycogen phosphorylase; G6Pase, glucose-6-phosphatase; G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphgluconate dehydrogenase; PFK, 6-phosphofructo-6-kinase.

(Fig. 17.2). A few marine fish of the smelt family also produce large amounts of glycerol in the winter to raise their body fluid osmolality to match that of seawater. This is a expensive proposition for these fish because glycerol passes easily across biological membranes, and about 10% of the glycerol pool is lost each day to the surrounding seawater, necessitating continuous repletion. Glucose is used as the protectant by three kinds of freeze-tolerant frogs, but one species (*Hyla versicolor*) accumulates glycerol. Freeze-tolerant marine mollusks and barnacles do not make carbohydrate protectants, but they do maintain constant high levels of free amino acids in their cells to balance their internal osmolality with that of seawater. During freezing, these amino acids provide the same colligative resistance to cell water loss as is provided by carbohydrates for most other species. Sucrose is the most common carbohydrate protectant found in cold-hardy plants but raffinose, sorbitol, and others have been reported.

Most of the protectants mentioned above function as colligative protectants, their actions being primarily a function of their high concentration in body fluids, but some have main actions as membrane protectants. These include the disaccharide, trehalose, and the amino acid, proline. Multiple forms of damage to membranes can occur during freeze/thaw; for example, in plants these include expansion-induced lysis, lamellar to hexagonal-II phase transitions, and freeze fracture jump lesions. Protective metabolites act to stabilize the bilayer structure of membranes. For example, trehalose appears to inhibit the phase transition to the gel phase that is induced by

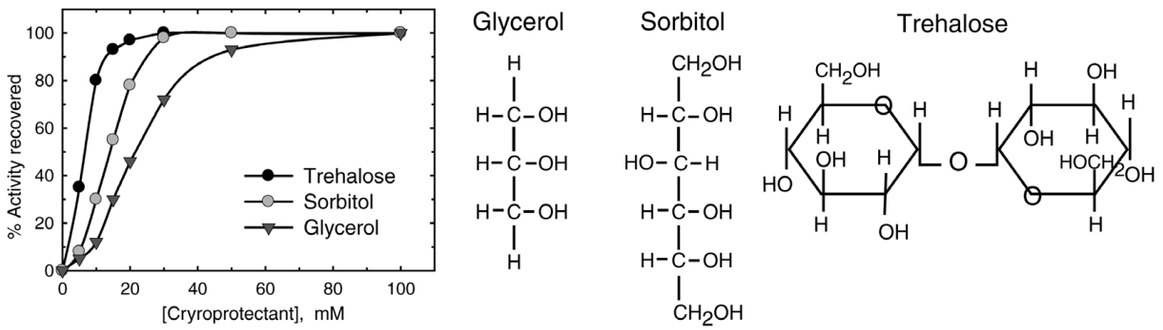

Figure 17.3 Chemical structures of the three most common carbohydrate cryoprotectants and their effects in protecting an enzyme from freeze denaturation. Graph shows the effect of increasing cryoprotectant concentration on the activity of *Eurosta solidaginis* glucose-6-phosphate dehydrogenase recovered after 1 h of freezing at −77°C. Half-maximal protection occurs with 7 mM trehalose, 14 mM sorbitol, or 20 mM glycerol. [Data are from K. B. Storey, D. Keefe, L. Kourtz, and J. M. Storey (1991). *Insect Biochem* **21**:157–164. Used with permission.]

low water stress (freezing or dehydration) (also see Text Box 17.1) apparently by hydrogen bonding with polar head groups on phospholipids to spread the phospholipid monolayers. The importance of these membrane protectants has been emphasized in studies with the freeze-tolerant plant *Arabidopsis thaliana*. Multiple genes associated with proline metabolism are among the suite of cold-responsive genes in this species and transgenic studies traced the constitutive freeze tolerance in a mutant, *eskimo1* (*esk1*), to the accumulation of proline even at warm temperatures; levels of proline were 30-fold higher in *esk1* mutants than in wild-type plants. In recent years a number of small hydrophilic proteins have also been found in plants that are cold/freeze-responsive and appear to act as membrane stabilizers (more about these in the section on gene expression later). Analysis of the damage caused to membranes by freezing (and/or dehydration) and the molecular mechanisms of membrane stabilization by protectants (both trehalose/proline and small proteins) is a huge field of its own that we cannot do justice to in this chapter, but the interested reader is directed to reviews cited in the Suggested Reading list at the end of this chapter.

Why Choose Carbohydrates for Colligative Cryoprotection?

Polyhydric alcohols such as glycerol are a common choice for a protectant role for several reasons: (a) They are highly soluble and therefore can be accumulated in very high concentrations; for example, glycerol levels reach 2 M or more in cold-hardy insects to provide strong colligative suppression of FP and SCP (see Text Box 17.2); (b) they are easily synthesized from glycogen (or from starch in plants) with near-perfect stoichiometry and with little or no adenosine 5′-triphosphate (ATP) input needed; (c) they are synthesized as offshoots from central pathways that are typically present in all cells and organisms; (d) they are easy to remove when no longer needed either by reconversion to glycogen (or starch) or by oxidation as an aerobic fuel; (e) they are good stabilizers of protein conformation in the face of multiple stresses including dehydration, dilution, heat/cold, and freezing; and (f) they are "compatible solutes," which means that their presence in high concentrations has little or no effect on enzyme kinetic parameters. The use of glucose as a cryoprotectant by frogs also fits most of these criteria, particularly its ease of synthesis in three ATP-independent steps from glycogen, although the potential injurious effects of extremely high glucose levels (as are seen in the disease diabetics mellitus) must be addressed.

The stabilizing effects of sugars and polyols in the preservation of enzyme activity are illustrated in Figure 17.3. The figure shows that glycerol, sorbitol, and trehalose protect the enzyme glucose-6-phosphate dehydrogenase (G6PDH) from freeze denaturation. As little as 40 to 50 mM protectant was needed to provide full recovery of enzyme activity after freezing, whereas in the absence of protectant, no enzyme activity could be recovered. Similar results have been documented for many other enzymes and also for isolated cell systems. The actions of polyols as compatible solutes that have little effect on enzyme properties were discussed in Chapter 14. Table 14.6 showed that glycerol and sorbitol themselves have little effect on enzyme properties and also stabilize enzyme kinetic properties against the disruptive influences of low temperature and high salt. For freeze-tolerant animals, this latter effect of minimizing the negative consequences of high ions (e.g., K^+, Na^+, Ca^+) on metabolic reactions is probably very important. By introducing high concentrations of polyhyd-

ric alcohols as solutes to minimize the amount of ice that can form at any given subzero temperature, the increase in cytoplasmic ionic strength during freezing is also minimized.

Biosynthesis of Polyhydric Alcohols

Glycerol can be synthesized in two steps from one of the triose phosphate intermediates of glycolysis, dihydroxyacetone phosphate (DHAP). DHAP is converted to glycerol-3-phosphate (G3P) by the enzyme glycerol-3-P dehydrogenase (G3PDH) (Fig. 17.2); this enzyme is found in all cells because of its role in providing G3P as the "backbone" to which fatty acids are esterified in the formation of mono-, di-, and triglycerides. Phosphatase action on G3P produces glycerol. This route appears to be used by vertebrates (e.g., marine smelt, gray tree frogs) that accumulate glycerol as a protectant. In support of this, these species show much higher activities of G3PDH in liver than do comparable species in the same environment that do not synthesize glycerol. For example, we found that G3PDH activity in liver of cold-acclimated gray tree frogs (*H. versicolor*) was four- to fivefold higher than in wood frogs (*Rana sylvatica*), reflecting the accumulation of glycerol versus glucose, respectively, by these freeze-tolerant frogs. G3PDH activity in smelt liver also peaks during the winter months when glycerol biosynthesis is highest. The carbon source for glycerol synthesis is liver glycogen that is accumulated during summer/autumn feeding or, in the case of smelt, dietary carbohydrate and amino acids are also used, the latter being processed by gluconeogenic reactions in liver.

The synthesis of glycerol (or other polyols) in insects seems to be accomplished by a more specialized coupling of two metabolic pathways: glycolysis and the pentose phosphate pathway (PPP). In the insect fat body (its liver-like organ), glycogen breakdown proceeds to the level of glucose-6-phosphate (G6P), and then carbon flow is diverted into the PPP from which both sugar phosphate precursors (hexose, pentose, tetrose, or triose phosphates) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) emerge (Fig. 17.2). After removal of the phosphate group, sugars are converted to sugar alcohols by the enzyme polyol dehydrogenase, whose activity increases strongly in the autumn during the time of active polyol synthesis:

$$\text{Sugar} + \text{NADPH} + \text{H}^+ \rightarrow \text{polyol} + \text{NAD}^+ + \text{H}_2\text{O}$$

$$\text{Glyceraldehyde} + \text{NADPH} + \text{H}^+ \rightarrow \text{glycerol} + \text{NAD}^+ + \text{H}_2\text{O}$$

$$\text{Glucose} + \text{NADPH} + \text{H}^+ \rightarrow \text{sorbitol} + \text{NAD}^+ + \text{H}_2\text{O}$$

Glycerol and sorbitol are the two most common polyol products in insects, but, as Figure 17.2 shows, other sugar phosphates can be drained from the PPP to synthesize alternative polyols. Because 2 mol of NADPH are produced for every G6P moiety that enters the PPP, the synthesis of sorbitol is balanced when about half of the G6P first cycles through the PPP, whereas the other half is converted directly to glucose and then to sorbitol. For the synthesis of glycerol, however, almost all the carbon must cycle through the PPP to achieve a near equal output of 2 glyceraldehyde-3-P (GAP) and 2 NADPH per G6P that enters the PPP. We say "near equal" because each cycle of the PPP leads to the loss of 1 CO_2 at the 6-phosphogluconate dehydrogenase reaction so that the input of 6 G6P results in the net output of 10 GAP and 12 NADPH. Hence, to use up all the NADPH and maintain redox balance, one-sixth of the G6P must be converted to GAP (or G3P) via the nonoxidative reactions of glycolysis that require ATP input at the 6-phosphofructo-1-kinase (PFK-1) reaction (Fig. 17.2). Furthermore, the ATP requirement of this route has to be satisfied by utilizing a small portion of the G6P to generate ATP via oxidative phosphorylation. When all demands are accounted for, the theoretical efficiency of carbon conversion from glycogen to glycerol can be calculated as 84 versus 95% if sorbitol is the product.

Documenting the Role of the PPP

The obligatory coupling of the PPP and glycolysis during polyol synthesis in insects is another excellent example of the reorganization or adaptation of central metabolic pathways to achieve a very specific goal (recall the multiple options for modifying glycolytic outputs during anaerobiosis discussed in Chapter 15). This coupling was confirmed in a novel way in studies that we undertook with freeze-tolerant gall fly larvae (*Eurosta solidaginis*), and these illustrate how the use of a simple stress situation (e.g., anoxia) can be employed to gain information about the integration of metabolic pathways. As discussed in Text Box 17.2, gall fly larvae produce both glycerol and sorbitol as their cryoprotectants. Glycerol synthesis is triggered during early autumn cooling when ambient temperatures drop below $\sim 15^\circ$C, whereas sorbitol synthesis is triggered by colder temperatures below 5°C (see Fig. TB17.1). Since glycerol synthesis has an ATP requirement because a portion of the carbon flow must be routed via PFK-1 to reach GAP (or G3P) (Fig. 17.2), we wondered if glycerol synthesis could go forward under anoxic conditions.

It is obvious from looking at Figure 17.2 that if glycerol was produced using *only* glycolysis and the G3PDH reaction (with no PPP involvement), then when insects were placed under anoxia (nitrogen gas atmosphere) glycerol synthesis could only continue if G3PDH used the NADH that was produced by the glyceraldehyde-3-P dehydrogenase (GAPDH) reaction of glycolysis. This would necessitate a 1 : 1 split of triose phosphates into the G3PDH versus GAPDH reactions and, furthermore, the 50% of carbon

that continued through glycolysis to reach pyruvate could not be converted to lactate because the NADH needed by the lactate dehydrogenase reaction would have already been used by G3PDH. Pyruvate would have to be converted to a product that does not require reducing equivalents, such as transamination to form alanine. Hence, without PPP participation, glycerol synthesis under anoxia would result in a 1 : 1 accumulation of glycerol and alanine; notably ATP production from glycolysis ending in alanine would provide the necessary ATP for PFK-1. However, when we actually evaluated the metabolic responses of *Eurosta* to anoxic conditions at 13°C, a very different result was seen (Table 17.1). Under anoxic conditions, glycerol accumulation by the larvae at 13°C was still substantial (about half as much as under aerobic conditions), both lactate and alanine accumulated as glycolytic end products, glycerol accumulation was about four times greater than alanine, and a considerable amount of sorbitol was also made. What does this mean? First of all it means that the PPP must be at work because the total reducing equivalents needed to produce the observed glycerol + sorbitol output was 85 μmol NAD(P)H per gram wet mass, a value over 5 times greater than the amount of NADH that would have been available from anaerobic glycolysis alone, based on alanine accumulation. At 3°C the imbalance was even greater, an 8-fold higher output of sorbitol, compared with alanine.

However, when viewed in another way, what is interesting about this partnership between glycolysis and the PPP in cold-hardy insects is that having created a system where G6P must cycle through the PPP to optimize polyol synthesis, insects appear to be "stuck" with this system for all their metabolic needs during the period of autumn cold hardening. Thus, under anoxia, organisms typically suppress biosynthesis and focus their carbohydrate use into glycolytic ATP production (Chapter 15). There is no energetic benefit to be gained from sorbitol or glycerol synthesis in anoxia. So, why does sorbitol biosynthesis go forward at high rates in anoxia? Note that sorbitol is not normally produced at 13°C in *Eurosta* and that under anoxia at 3°C sorbitol was accumulated at 60% higher levels than under aerobic conditions. The answer is that if the route of carbohydrate degradation in autumn larvae is obligately linked to channeling a very high percentage of carbon through the pentose phosphate cycle, then an anoxia-induced activation of carbohydrate catabolism for the purpose of anaerobic ATP synthesis would elicit an enormous overproduction of NADPH. This is because hexose phosphates have to be channeled through the NADPH-generating reactions of the PPP in order to deliver GAP to the ATP-generating reactions of the lower half of glycolysis. For example, look at the situation during anoxia at 3°C. If carbon flowed directly through glycolysis, then an accumulation of 10 mol alanine as the glycolytic end product would have come from 5 mol of G6P (derived from glycogen) and would have produced 10 mol of NADH at the GAPDH reaction and an output of 15 mol of ATP. However, if carbon must go through the PPP instead, we calculated that 10 mol of alanine would have come from the mobilization of 6 mol of G6P (one-sixth of carbon is lost as CO_2) and would be accompanied by the production of 12 mol of NADPH in the PPP plus 10 mol of NADH at the GAPDH reaction and produce 20 mol of ATP (since the PFK-1 reaction is circumvented). Although the ATP yield of this routing is seemingly marginally better than for glycolysis alone, the output of reducing equivalents is more than doubled, and redox balance can only be maintained by the production of large amounts of sorbitol. Hence, this example shows how the "choice" of metabolic pathways needs to consider various pros and cons. Highly efficient polyol synthesis can be accomplished using the PPP to output both NADPH and sugar phosphates. Under anoxic conditions, however, carbon routing through the PPP is highly inefficient because a high percentage of the carbohydrate substrate ends up in a "dead end" product, sorbitol, that cannot be mobilized to contribute to the anaerobic generation of ATP.

TABLE 17.1 Effect of Anoxia Exposure on Balance of Carbon Compounds, Hydroxyl Equivalents, and ATP Produced by Freeze-Tolerant Insect *Eurosta solidaginis* at 13 vs. 3°C [a]

	13°C Exposure		3°C Exposure	
	Aerobic	Anoxic	Aerobic	Anoxic
Net synthesis of carbon compounds				
C6				
Sorbitol	0.2	21.3	49.7	79.3
Glucose	0.2	4.0	2.2	27.0
C3				
Glycerol	112	64	15	0
Glycerol-3-phosphate	14.4	23.7	6.8	0
Lactate	1.4	15.0	0.5	0
Alanine	0.5	15.8	1.9	10.4
Total products in C6 equivalents	64.6	84.5	64.0	125

[a]All data are μmol/g wet weight.

Source: Compiled from K. B. Storey and J. M. Storey (1992). *Adv Low Temp Biol* **1**:101–140.

Enzymatic Regulation in Polyol Biosynthesis

The production of polyols in insects also involves some interesting variations in metabolic regulation. With polyols representing as much as 20 to 25% of the insect's body mass in midwinter, it is obvious that major preparations are needed. These include the accumulation of huge amounts

of glycogen in the fat body during summer/autumn feeding and the synthesis of increased amounts of key enzymes. For example, in caterpillars of the glycerol-producing *Epiblema scudderiana* (see Text Box 17.2), we found that the amount of active glycogen phosphorylase (GP*a*) rose from about 0.4 units/g wet mass in early autumn (total GP $a + b = 4$ U/g with 10% *a*) to 7.2 U/g during peak glycerol synthesis in November (total GP = 12 U/g with 60% *a*). At the same time, polyol dehydrogenase activity rose over sevenfold and G3PDH increased threefold.

Once preparatory measures are made, the actual initiation of polyol synthesis relies on low-temperature triggers that are positioned such that polyol accumulation can be complete before the insect experiences the subzero temperatures that require cryoprotection. For species that produce only glycerol, synthesis is initiated by exposure to ~5°C (in nature three to four consecutive nights with lows in this range are needed), and glycerol accumulates at the highest rate at temperatures between 0 and −5°C (insects are not frozen in this range because SCPs of cold-hardy species are typically −8°C or lower). Species that accumulate two protectants, such as glycerol and sorbitol, do so with two independent synthesis events; for example, in *Eurosta* the triggers are 15°C for glycerol and 5°C for sorbitol (Text Box 17.2 and Table 17.1). The primary metabolic focus of these temperature triggers is GP. The percentage of GP in the active *a* form rises from low to high values within 1 to 2 h when insects are transferred from warm to cold temperatures. The basis of this effect has been traced to differential temperature effects on the activities of the two enzymes that regulate GP, phosphorylase kinase and phosphorylase phosphatase. Over the range between 30 and 0°C the kinase undergoes a normal reduction in activity (Q_{10} ~2) and the K_m value for phosphorylase *b* is conserved. The phosphatase, however, is rapidly inactivated at temperatures below ~8°C with a functional Q_{10} of about 23 at low temperatures. Thus, when silkmoth fat body GP was incubated *in vitro* with partially purified preparations of its phosphorylase kinase (+ATP) and phosphorylase phosphatase, the phosphatase was unable to prevent GP*a* content from soaring to maximum values when incubations were at 0°C, whereas at 25°C competition between the two enzymes maintained GP*a* at about 25% of its maximum value. Although other factors may also be involved *in vivo*, this elegant experiment showed that simple temperature effects on a single enzyme can have a huge effect on a major metabolic function and provides a very simple way of modulating the rate of polyol synthesis in direct response to environmental temperature change.

Cold activation of GP*a* is further potentiated by the effects of low temperature on the kinetic properties of the enzyme and by oppositely directed controls of glycogen synthase (GS), which ensure that no recycling of polyols to glycogen places in the cold. As Table 17.2 shows the affinity of *Epiblema* GP*a* for both glycogen and inorganic phosphate (P_i) increased strongly at low temperature (K_m values decreased to just 20% of the values at 22°C), as did sensitivity to adenosine 5′-monophosphate (AMP) as an activator (K_a dropped just 30% of the value at 22°C). Notably, the addition of 500 mM glycerol, at about 25% of the maximum level accumulated in *Epiblema*, further potentiated GP*a* affinity for its substrates. However, low temperature had just the opposite effect on GS. Like GP, GS is interconvertible between an active, phosphorylated, G6P-independent (I) form and an inactive, dephosphorylated form (D) that is dependent on G6P to show activity *in vitro*. Only the inactive D form could be found in autumn-collected *Epiblema*, which fits with the idea that the larvae are poised for unidirectional glycogenolysis during the season of cold-hardening. Low-temperature effects on GS kinetic properties further show an enzyme that is much less active in the cold. At 5°C our studies revealed that the enzyme showed much lower affinities for both substrates; K_m values for glycogen and uridine diphosphoglucose were 4.4- and 1.8-fold higher at 4°C compared with 22°C. The enzyme was also much less sensitive to activation by G6P at 4°C.

Regulation of GP activity controls the input of carbon into polyol synthesis but cannot determine which polyol product is made. How is that decision made? This question is particularly interesting for a species such as *Eurosta* that produces glycerol as the result of glycogenolysis at cool temperatures (below ~15°C) but accumulates sorbitol when a second round of glycogenolysis is activated by

TABLE 17.2 Effect of Assay Temperature on Kinetic Properties of Glycogen Phosphorylase and Glycogen Synthase from *Epiblema scudderiana*[a]

	22°C	5°C
Phosphorylase a		
K_m glycogen, μg/ml		
No additions	120 ± 4	24 ± 1[b]
+ 0.5 M glycerol	73 ± 2[b]	15 ± 1[b]
K_m inorganic phosphate, mM	6.5 ± 0.1	3.7 ± 0.1[b]
K_a AMP, nM	176 ± 4	53 ± 1[b]
Synthase D		
K_m glycogen, μg/mL	1170 ± 350	5160 ± 160[b]
K_m UDPG, μM	48 ± 4	88 ± 3[b]
K_a G6P, mM	2.1 ± 0.2	6.2 ± 0.2[b]

[a]Note that addition of glycerol further increases the affinity of phosphorylase for glycogen. UDPG is uridine diphosphoglucose.
[b]Significantly different from the corresponding value at 22°C with no glycerol added.

Source: Compiled from C. P. Holden and K. B. Storey (1993). *J Comp Physiol B* **163**:499–507, and A. M. Muise and K. B. Storey (1999). *Cryo-Lett* **20**:223–228.

5°C exposure. Sorbitol synthesis is a less efficient use of the carbon pool for the production of colligatively active molecules (1 G6P makes 1 sorbitol versus 2 glycerol) so we reasoned that there must be a metabolic block on the production of triose phosphates by the larvae at low ambient temperatures. To determine where this block might be, we used a technique called crossover analysis (the theory is explained in Chapter 1) that looks at the changes in levels of metabolites in a pathway as the result of a metabolic stress and evaluates the relative change, compared with control values, of the substrate versus product concentrations of the each enzyme. A crossover occurs when a stress has a differential effect on substrate versus product concentrations of an enzyme; that is, the ratio of experimental/control concentrations for substrate rises to >1.0, whereas the ratio for product concentrations decreases to <1.0, or vice versa. Relative changes in substrate versus product levels "cross over" the line where experimental/control concentrations $= 1$. This analysis was applied to assessing the changes in glycolytic intermediates in *Eurosta* when the larvae were acutely transferred from 13 to 3°C, a temperature shift that rapidly initiates sorbitol synthesis (Table 17.1). Within 1 h of the shift to the cold temperature, an activation of glycogenolysis was obvious from the significant rise in G6P levels, and by 2 h G6P had tripled, glucose had risen by 15-fold, and sorbitol accumulation had started (Table 17.3; see also Fig. 17.2 for the reactions involved). When G6P rose, so did fructose-6-phosphate (F6P), the substrate of PFK-1; this is because phosphoglucoisomerase is an equilibrium enzyme. The ratio experimental/control [F6P] rose to 38 within the first 2 h of cold exposure, but, despite this massive proportional increase in the substrate of PFK-1, the experimental/control concentrations of the product of PFK-1, fructose-1,6-bisphosphate ($F1,6P_2$), behaved oppositely and fell to 0.33 within 2 h and to 0.50 by 4 h of cold exposure. This indicates a very strong inhibition of PFK-1 because, despite the huge increase in F6P substrate concentrations, $F1,6P_2$ product was depleted because it was consumed faster by the rest of glycolysis than it was produced by PFK-1. By contrast, when the same analysis was applied to pyruvate kinase, a regulatory enzyme in the lower part of glycolysis, no such changes in its substrate and product concentrations were seen; levels of both the substrate (phosphoenolpyruvate) and product (pyruvate) remained constant (Table 17.3). The crossover theorem can be applied to many different pathways and effectively pinpoints rate-limiting steps in pathway response to different stresses. In this case, it indicated strong negative control of glycolytic flux at the PFK-1 reaction as a result of the decrease in temperature. Such a block on glycolysis would promote the diversion of carbon into the reactions of sorbitol synthesis. The mechanism of PFK-1 inhibition by cold was subsequently explored, and we found that it was due to multiple factors: (1) a large negative effect of low temperature on enzyme activity ($Q_{10} = 3.6$), (2) a decrease in enzyme affinity for F6P (K_m increased) and a decrease in enzyme sensitivity to activators, AMP, and fructose-2,6-bisphosphate ($F2,6P_2$) when assayed at low temperature, and (3) elevated levels of PFK-1 inhibitors (G3P, sorbitol) and decreased levels of PFK-1 activators ($F2,6P_2$) *in vivo* at low temperature.

TABLE 17.3 Changes in Concentrations of Intermediates of Sorbitol Synthesis When Production is Stimulated by Acute Temperature Decrease from 13 to 3°C[a]

	Control (time zero)	2 hours cold	Ratio Cold : Control
Glycogen	304	299	
Glucose	0.1	1.5	15
Sorbitol	2	4	2
G6P	0.15	0.45	3
F6P	0.002	0.076	38
$F1,6P_2$	0.105	0.07	0.67
PEP	0.07	0.08	1.1
Pyruvate	0.055	0.055	1.0

[a]All data are μmol/g wet weight.
Source: Data are excerpted from J. M. Storey and K. B. Storey (1983). *J Comp Physiol* **149**:495–502.

Biosynthesis of Glucose as the Cryoprotectant in Frogs

The glucose used for cryoprotection by woodland frogs is produced in liver using the same three-step pathway present in the liver of all vertebrates (Fig. 17.2):

$$\text{Glycogen} \rightarrow \text{glucose-1-phosphate} \rightarrow \text{glucose-6-phosphate} \rightarrow \text{glucose}$$

The enzymes involved are GP, phosphoglucomutase, and glucose-6-phosphatase. Preparatory measures for winter cryoprotectant synthesis include the accumulation of large amounts of glycogen in liver during summer/autumn feeding, so that about 20% of liver mass is glycogen when hibernation begins, and the presence of high activities of GP (e.g., activity in wood frog liver is 12-fold higher than in liver of freeze-intolerant leopard frogs). Unlike the situation in insects, however, frogs do not show anticipatory accumulation of their cryoprotectant during an autumn cold-hardening period but only trigger glucose synthesis when the frog's body actually begins to freeze. The reason for this may be to minimize the time that tissues are exposed to extreme hyperglycemia because, unlike glycerol or other polyols, glucose is not a chemically or metabolically inert carbohydrate.

Indeed, diabetes research has shown that many of the pathophysiological features of the disease are directly related to chronic hyperglycemia. For example, the nonenzymatic attachment of glucose to long-lived proteins underlies diabetic microvascular disease (due to glycation of collagen and other basement membrane proteins) and diabetic cataract (glycation of lens crystallins). Glucose and glycated proteins are also effective pro-oxidants. Free glucose is prone to auto-oxidation in the presence of transition metals (iron, copper) to form protein-reactive dicarbonyl compounds and hydrogen peroxide (see Chapter 12). The potential for hyperglycemic damage to frog tissues can be appreciated from the glucose concentrations involved. Glucose is normally regulated at 4 to 5 mM in vertebrate blood but may rise to 7 to 8 mM after a meal with insulin secretion typically acting to prevent higher values (see Chapter 9). Multiple metabolic injuries arise in uncontrolled diabetes when glucose ranges between 10 to 50 mM. However, freeze-tolerant frogs elevate glucose to 150 to 300 mM, and, when as much as two-thirds of total body water freezes out as extracellular ice, the resulting concentration of glucose in the cytoplasm can be nearly 1 M!

The production of glucose for cryoprotection is triggered within seconds when the frog's body begins to freeze. The initial freezing event for frogs is typically ice nucleation on the skin surface, either seeded by contact with environmental ice crystals (at any temperature below of -0.5°C FP of frog body fluids) or as a result of the ice nucleating activity of skin bacteria that stimulate freezing at -2.5 to -3°C (see section below on ice nucleators). Within 2 to 4 min after nucleation, liver glucose levels have doubled, and significant export into the blood can be detected within 4 to 5 min. Glucose output continues at high rates so that levels in blood and other organs rise from <5 to over 100 mM within just a few hours (Fig. 17.4), quite a feat when you remember that the frog's body temperature is below 0°C!

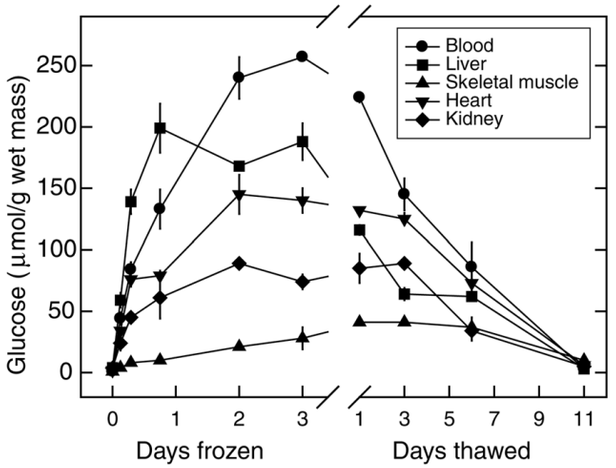

Figure 17.4 Freezing triggers high rates of glycogenolysis in frog liver, and glucose builds up quickly and is exported via the blood and taken up by all other organs. Shown is the time course of changes in glucose levels during freezing at -2.5°C and thawing at 5°C; data are means + SEM, $n = 3$.

Signal Transduction and Enzymatic Regulation

The regulation of freeze-induced glucose production has been extensively studied in wood frogs, *R. sylvatica*. Much is known but some intriguing questions remain. One is the nature of the signal that links ice formation at a peripheral site on the skin with the immediate activation of glycogenolysis in the liver. The signal may be nervous or hormonal, but its action is a stimulation of β_2-adrenergic receptors on the plasma membrane of liver cells. β-Adrenergic reception was identified when we showed that injections of β-adrenergic blockers (propranolol) impaired the hyperglycemic response to freezing, whereas α-adrenergic blockers (phenolamine) did not. β_2-Receptor activation triggers the production of cyclic 3′,5′-adenosine monophosphate (cAMP) in liver, whose levels rise twofold within 2 min postnucleation, and this, in turn, activates cAMP-dependent protein kinase (PKA). Binding of cAMP to regulatory subunits on PKA triggers the dissociation of the inactive PKA tetramer to release the two catalytic subunits and the percentage of PKA present as the active subunit in frog liver rises from ~7% in controls to over 60% within 5 min after freezing begins. PKA phosphorylates glycogen phosphorylase kinase, which in turn phosphorylates the inactive *b* form of GP to convert it to the active *a* form. The percentage of GP in the *a* form rises quickly to nearly 100% and the production of glucose cryoprotectant is underway. This cascade of events is well-known as the standard way of activating glycogenolysis in vertebrate liver in response to glucagon, epinephrine, or other hyperglycemic stimuli (see Chapters 9 and 10).

However, as we learned in Chapters 9 and 10, blood glucose levels are normally under tight homeostatic controls in vertebrates due mainly to the actions of insulin versus glucagon and their oppositely directed effects on the activities of GP versus GS. Indeed, this system of homeostatic regulation of glucose is fully functional in unfrozen frogs, but, obviously, one or more novel regulatory mechanisms must come into play to allow the massive freeze-induced hyperglycemia. The full picture is not yet known but significant elements are.

Once again, similar to the situation in cold-hardy insects, one key factor in GP control is the behavior of protein phosphatase-1 (PP-1) that acts to dephosphorylate active GP*a* and return it to its inactive *b*. In normal vertebrate liver, PP-1 activity rises as glucose levels increase and the enzyme dephosphorylates GP*a* to halt further glycogenolysis. PP-1 activity also rises in wood frog liver

during cryoprotectant production (a 70% increase over the first 20 min) but GP*a* activity remains high and unaffected. Why is this? The answer seems to be that during freezing PP-1 is kept out of physical contact with liver glycogen granules. In normal vertebrate liver, a high proportion of PP-1 catalytic subunits occur as dimers, bound 1 : 1 with a G protein that confers glycogen-binding ability (see Fig. 4.14). The α and δ isoforms of PP-1 are the main ones that associate with glycogen. Subcellular fractionation and immunoblotting were used to follow PP-1 isoform distribution in wood frog liver. The results showed that in control frogs PP-1 α and δ were overwhelmingly associated with glycogen particles with little enzyme free in the cytosol (Fig. 17.5). However, when frogs were frozen for 12 h at -2.5°C, the opposite response was seen. PP-1 was translocated to the cytosolic fraction with virtually no enzyme left bound to glycogen particles. Hence, PP-1 cannot halt glycogenolysis in liver during freezing and glucose output continues unabated. The mechanism of PP-1 translocation may be linked with PKA-mediated phosphorylation of the G subunit, which in mammals increases the rate of PP-1 inactivation by inhibitor-1 and promotes PP-1 release from glycogen. But, whereas PKA activation is transient under normal circumstances in vertebrate liver, the percentage of PKA present as the active catalytic subunit was sustained at high levels in frog liver for at least the first 8 h during freezing.

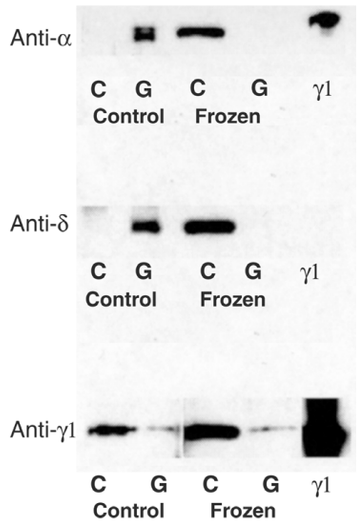

Figure 17.5 The distribution of protein phosphatase-1 (PP-1) isozymes between the cytosolic (C) and glycogen particle (G) fractions of wood frog liver was investigated by using Western blotting with antibodies to rat liver α, δ, and γ1 PP-1. The blots compare isozyme distribution between control and 12-h frozen wood frogs, clearly showing a shift by the α, δ isozymes from the glycogen fraction in control frogs to the cytosolic fraction when frogs were frozen. Recombinant PP-1-γ1 is present in the fifth lane as a positive control. [From J. A. MacDonald and K. B. Storey (1999). *Cryo-Lett* **20**:297–306. Used with permission.]

A rise in blood glucose levels beyond ~7 mM in vertebrate blood is typically aggressively reversed by the secretion of insulin to promote uptake and storage of the sugar by insulin-sensitive organs. How then does blood glucose continue to rise to over 200 mM in frogs during freezing? Freezing must interfere with the normal homeostatic control of glucose perhaps by interfering with insulin action. Several possibilities exist but the answer is still not known. Insulin secretion by the pancreas could be blocked, although, in fact, we found that plasma insulin levels do double during freezing. Alternatively, insulin interaction with its receptors on hepatocyte membranes could be blocked. However, one of the main intracellular responses that is activated by insulin binding to its receptor is the activation of Akt (protein kinase B) (see Chapters 5 and 9), and Akt activity rises by threefold in the liver of freezing frogs. This suggests that the initial steps of insulin signal transduction may continue during freezing. However, subsequent actions of Akt are clearly disrupted during cryoprotectant synthesis. A main action of Akt signaling in liver cells is to activate GS and thereby stimulate glycogen synthesis when glucose is high. This clearly does not occur during freezing; we have shown that GS activity remains very low throughout freezing (exactly the opposite of GP) and only rises when frogs thaw. Akt phosphorylates glycogen synthase kinase 3 (GSK3) to inactivate it and prevent it from phosphorylating and inactivating glycogen synthase. Hence, the disruption of insulin action may occur in the link between Akt, GSK3, and GS.

A structural modification of wood frog insulin could also reduce its effectiveness in regulating glucose levels in freezing frogs and, indeed, sequence analysis of the wood frog hormone showed some anomolous features that could limit its effectiveness (Fig. 17.6). One unusual feature was the presence of a two-amino-acid extension (lysine–proline) on the N terminus of the A chain. Although shared by other ranid frogs, this extension does not occur in any other vertebrates and its role remains unknown. The rest of the A chain, as well as the B chain, are highly conserved in other frogs as compared with the mammalian hormone, but wood frog insulin showed two anomolies. The serine residue at position A23 in wood frog insulin (A21 of human) is an asparagine in all other species, and the aspartic acid at B13 in wood frog insulin is glutamic acid in nearly all tetrapods. Both of these residues have been shown to play important roles in insulin action. A21 is key to the maintenance of the biologically active conformation due to its bonding to B22/23, whereas B13 aids binding to the insulin receptor. Both of the amino acids substitutions in wood frog insulin, as minor as they may seem, could significantly impair its function. Indeed, the only other known instance of a Glu to Asp substitution at B13 results in a

Insulin A-chain

Rana sylvatica	KP GIVEQ CCHNM CSLYD LENYC S…
Rana catesbeiana	-- ----- ----T ----- ----- N…
Rana ridibunda	-- ----- ----T ----- ----- N…
Xenopus laevis	----- ---ST --F-- --S-- N…
Homo sapiens	----- --TSI ----Q ----- N…

Insulin B-Chain

Rana sylvatica	FPNQH LCGSH LVDAL YMVCG DRGFF YSPRS…
Rana catesbeiana	----Y ----- --E-- ----- ----- -----…
Rana ridibunda	----Y ----- --E-- ----- E---- -----…
Xenopus laevis	LV--- ----- --E-- -L--- ----- -Y-KV…
Homo sapiens	-V--- ----- --E-- -L--- E---- -T-KT…

Figure 17.6 N-terminal sequences of insulin A and B chains in wood frog, bullfrog, green frog, African clawed frog and humans. [From J. M. Conlon, K. Yano, N. Chartrel, H. Vaudry, and K. B. Storey (1998). *J Mol Endocrinol* **21**:153–159.]

low potency insulin. Interestingly, although wood frog insulin displays novel features, its glucagon is identical with the hormone from the bullfrog and has only one amino acid substitution as compared with human glucagon.

Control over GP regulates the production of glucose-1-P (which is interconverted with G6P) but, as mentioned previously, cannot determine the ultimate product of glycogenolysis. Multiple metabolic fates are open to G6P including (a) hydrolysis to glucose by glucose-6-phosphatase (G6Pase), (b) entry into the PPP via the G6PDH reaction, and (c) catabolism via glycolysis (Fig. 17.2). In the liver of cold-acclimated wood frogs, competition between G6Pase and G6PDH seems to be ruled out by G6Pase activities that are >200-fold higher than G6PDH. In addition, freezing stimulates a further 70% increase in G6Pase activity while reducing G6PDH activity to 20% of its former value. G6P use by glycolysis is also effectively shut down during freezing. Once again we demonstrated that inhibition is centered on PFK-1. The maximal activity of PFK-1 in wood frog liver is similar to that of G6Pase, and PFK-1 activity does not change during freezing, so a different approach to PFK-1 control is needed. When levels of glycolytic metabolites were measured over the initial minutes of freezing, all hexose phosphates (G1P, G6P, F6P) were strongly elevated within 5 min postnucleation, whereas $F1,6P_2$ levels were unaffected; when crossover analysis was applied, strong negative control of glycolytic flux was again indicated at the PFK locus. The mechanism of PFK-1 inhibition was traced to significant changes in the kinetic and regulatory properties of the frog liver enzyme occurring over the first 70 min postnucleation, changes that are characteristic of protein phosphorylation of the vertebrate liver enzyme. Furthermore, levels of the PFK-1 activator, $F2,6P_2$, dropped sharply during freezing as a result of freeze-induced changes in the activity and properties of 6-phosphofructose-2-kinase (PFK-2), which produces $F2,6P_2$ (see Chapter 14 for a discussion of PFK-1 and PFK-2 relationships). Kinetic changes to PFK-2 (e.g., at 10-fold increase in K_m for F6P) are also consistent with freeze-stimulated phosphorylation of this enzyme to produce a less active form. Indeed, the activation of GP and the inhibition of PFK-2 are linked because they are both regulated by PKA; the same activation of PKA that stimulates glycogen phosphorylase kinase inhibits PFK-2, causing the fall in $F2,6P_2$ levels that inhibits PFK-1 during freezing.

Glucose Distribution

Once synthesized, glucose needs to be quickly exported to other organs of the frog to provide them with cryoprotection. This must be accomplished within just a few hours because, as ice grows inward from the peripheral regions of the frog's body and into the core, blood flow is progressively cut off and, with it, the ability to distribute glucose. Hence, an organ-specific pattern of glucose distribution results with lowest levels in skeletal muscle and skin, intermediate levels in organs such as brain and kidney, and highest amounts in liver and heart (Fig. 17.4). Significantly, the very high levels of glucose in liver and heart prove to be a bonus to the frog during thawing because these organs have the highest concentrations of colligative solutes and, therefore, the lowest melting points. Hence, the heart and liver melt first, and this allows for the resumption of heart beat as the first vital sign detectable in the thawed animal and means that circulation of oxygenated and nutrient-laden blood can resume to each other organ as soon as it thaws.

The uptake of glucose into vertebrate cells depends on the carrier-mediated transport of glucose across the plasma membrane by glucose transporters (GLUT), and

this transport system is also the focus of adaptive change in freeze-tolerant frogs. Our studies suggest that the mechanism is to be purely quantitative with no evidence of altered kinetic properties, at least for the liver isoform of the transporter (GLUT2). Thus, we found that the number of glucose transporters in liver plasma membrane of freeze-tolerant wood frogs is 5 times greater than in freeze-intolerant leopard frogs (*Rana pipiens*) and, between summer and autumn, the number of GLUT2 transporters in wood frog liver plasma membranes increases by 8.5-fold. Glucose transporter numbers in the receiving organs are also high in freeze-tolerant frogs; for example, the maximal rate of glucose transport by skeletal muscle of wood frogs was 8-fold higher than in leopard frogs. New data from complementary deoxyribonucleic acid (cDNA) array screening of wood frog heart has also identified the GLUT4 transporter, the insulin-responsive form, as one of several prominently up-regulated genes in 24-h frozen frogs; transcript levels are 2- to 3-fold higher in heart of frozen versus control frogs. This indicates that an acute (as well as seasonal) up-regulation of glucose transport capacity helps frog organs to rapidly accumulate cryoprotectant.

Once glucose is transported into the cells of freezing frogs, the next question is: Why is it not catabolized? The normal response of most organs after glucose uptake is immediate phosphorylation via the ATP-dependent hexokinase reaction followed by the use of G6P by one of several pathways. Indeed, glucose would be a valuable substrate for anaerobic glycolysis under the ischemic state imposed by freezing. Although the mechanism is not yet known, it is clear that glucose catabolism by wood frog organs is specifically inhibited at low temperatures. This was shown in our studies with wood frog erythrocytes. Red blood cells incubated with [U-^{14}C]-glucose took up glucose just as effectively at 4°C as they did at 12, 17, or 23°C. However, when ^{13}C nuclear magnetic resonance (NMR) was used to track the change in intracellular glucose levels over time, red blood cells at 17 or 12°C showed constant temperature-dependent rates of glucose depletion, whereas cells incubated at 4°C showed no glucose catabolism. The mechanism of this inhibition may be control over the enzyme, hexokinase. Although total hexokinase activity is not altered in most organs during freezing (although a 40% decrease occurred in kidney), the physical association of hexokinase with subcellular binding sites is key to its function (see Chapter 14), and these physical interactions may be disrupted in the frozen state to prevent glucose catabolism.

Development of the Cryoprotectant Response in Freeze-Tolerant Frogs

How did the use of glucose as a cryoprotectant get started in frogs? As we have seen in other instances in these chapters on biochemical adaptation, novel adaptive strategies typically develop from some underlying metabolic capacity. In this case, the origin of glucose use as a cryoprotectant probably grew out of an underlying hyperglycemic response to dehydration that is shared by both freeze-tolerant and freeze-intolerant frogs. Amphibians as a group have highly water-permeable skins. This tends to restrict them to living in moist habitats and has required them to develop the greatest tolerance of all vertebrates for variations in body water content and body fluid ionic strength. For example, mammals manifest nervous system dysfunction when plasma sodium rises by 30 to 60 mM, whereas amphibians can tolerate sodium at 90 to 200 mM above normal. Many amphibians can readily endure the loss of 50 to 60% of their total body water, and desert, arboreal, and freeze-tolerant frogs and toads are all at the top of the list. From the point of view of a cell, intracellular dehydration due to evaporative water loss is really no different from intracellular dehydration due to water loss into extracellular ice masses.

To experimentally test the idea that freeze tolerance and desiccation tolerance were related, we analyzed the metabolic responses of wood frogs to dehydration. Frogs were placed in dry containers where they lost body water at a rate of ~1% of total per hour to a final total of 50% lost (both the rate and the final maximum are fully survivable by wood frogs). Dehydration stimulated hyperglycemia in wood frogs just as effectively as freezing did, and glucose levels rose to values just as high as in frozen frogs (e.g., a 300-fold increase in liver) and with the same β_2-adrenergic signal transduction pathway activated in liver. Amazingly, however, the hyperglycemic response to dehydration also occurred in a freeze-intolerant frog, the leopard frog *R. pipiens*, although the response was more muted (a 24-fold increase in liver glucose). These results strongly indicated that the cryoprotectant biosynthesis response to freezing grew out of a preexisting hyperglycemic response by frogs to water stress. Notably, however, wood frogs given anoxia exposure at 5°C (to mimic the oxygen deprivation that is another component of freezing) did not show elevated glucose. As will be discussed in the later section on gene expression, many of the genes that are up-regulated in response to freezing also respond to either dehydration or anoxia stresses, and this information has provided valuable clues, particularly for novel genes of unknown function, as to the possible functional roles of gene products and to the signal transduction pathways that may be involved in regulating expression.

ICE MANAGEMENT BY ANTIFREEZE PROTEINS AND ICE NUCLEATING PROTEINS

Antifreeze proteins (AFPs) and ice nucleating proteins (INPs) are superb examples of novel proteins that have

been developed in selected organisms for very specific purposes. AFPs are widely distributed in coldwater marine fishes, terrestrial arthropods, and many plants where they act to inhibit the growth of microscopic ice crystals and thereby prevent the freezing of body fluids among organisms using the freeze-avoiding strategy of cold hardiness. INPs have the opposite action—they act to order water molecules into the ice lattice structure and thereby trigger crystallization in freeze-tolerant species. Interestingly, some freeze-tolerant insects have both INPs and AFPs, and the postulated role of an AFP in a freeze-tolerant organism is to inhibit recrystallization, the process whereby small ice crystals regroup over time into larger and larger crystals that could be physically damaging to tissues (Fig. 17.1). Hence, AFPs are employed to provide long-term stability of crystal size and shape in organisms that could be frozen for weeks or months. Many reviews cover the protein chemistry of AFPs and INPs and their mechanisms of physical interaction with ice (see Suggested Reading at the end of this chapter), and in Chapter 14 fish AFPs were presented as an excellent example of gene/protein evolution. Our remaining discussion here will focus mainly on physiological function and the metabolic regulation of AFP and INP synthesis.

Antifreeze Proteins

Antifreeze proteins come in many types. Five different groups have been described in coldwater fish and many other kinds occur in insects and plants. Although each type of AFP is structurally distinct, the mechanism by which they inhibit the growth of ice is much the same in all cases. AFPs adsorb onto the surface of seed crystals via hydrogen bonding to the surface water molecules and thereby prevent crystals from growing beyond a microscopic size. Bonds form with the polar side chains of amino acids on most AFP types or with the hydrophilic groups on the sugar side chains of antifreeze glycoproteins. This forces crystal growth into highly curved (high surface free energy) fronts rather than the preferred low curvature fronts (low surface free energy) and growth stops (although growth will resume with the impetus of a lower temperature). The presence of AFPs causes a reduction in the apparent FP of body fluids without changing the melting point; this thermal hysteresis is exploited for AFP detection and assay. The thermal hysteresis provided by AFPs to the blood of marine fish is only about 0.7 to 1.5°C, but this is enough to lower plasma FP (about −0.5°C for most teleost fish but higher in glycerol-accumulating smelts) below the FP of seawater (−1.9°C). Insect and plant AFPs are much more powerful because they must act in much colder terrestrial environments. Thermal hysteresis values for the AFP-containing hemolymph (the blood equivalent) of terrestrial arthropods in midwinter are generally 3 to 6°C, and together with high levels of carbohydrate protectants and a nucleator-free environment this allows many insects to chill to −20 to −40°C without freezing.

Whereas polar fish retain AFPs year-round, temperate-zone fish show a seasonal cycle of blood AFP levels that peaks in midwinter. Multiple influences on AFP production have been demonstrated using species that inhabit the waters off the Northeast coast of North America. These include (a) geographic variation—AFP gene dosage and AFP levels in plasma are higher in more northerly populations of individual species (e.g., the technique of Southern blotting showed that ocean pout from Newfoundland have 150 AFP gene copies, whereas New Brunswick pout have only 30 to 40 copies; correspondingly, plasma AFP levels that are 5- to 10-fold higher in the more northerly populations), and (b) environmental niche—inshore species that could frequently encounter surface ice show an anticipatory increase that elevates blood AFPs before water temperature drops to subzero values, whereas deep-water fish synthesize AFPs only if exposed to subzero temperatures. Deep-water species also show lower gene dosage and lower maximal AFP levels than do closely related inshore species.

Recent studies with the AFPs of winter flounder have contributed information about the metabolic regulation of AFPs. Liver is the production site for the plasma AFPs. The type I AFP of the flounder is encoded as a preproprotein; the presequence is removed cotranslationally and the pro-AFP is secreted into the blood where the prosequence is removed within 24 h to yield a 37-amino-acid mature AFP. AFP messenger ribonucleic acid (mRNA) appears in the liver in October, about one month prior to AFP appearance in the blood, which occurs when water temperature drops below 8°C. Low temperature appears to be needed to allow AFP mRNA to accumulate (the mRNA is destabilized at warmer temperatures) but does not regulate AFP gene expression. Photoperiod is the main trigger of gene expression; long day lengths (>14 h) strongly delay AFP production, whereas short days (4 to 8 h) are without effect. Insect AFPs also respond to photoperiod as the primary trigger for AFP synthesis (critical daylength is between 10 and 11 h), but other cues include temperature, thermoperiod (lengths of warm versus cold phases each day), and low relative humidity. In insects, information from environmental cues is integrated in the central nervous system and results in the release of juvenile hormone that stimulates the production of AFPs in the insect fat body.

In fish, photoperiod effects also act via the central nervous system to control the release of growth hormone (GH) from the pituitary. High GH levels repress AFP gene transcription, apparently via the action of insulin-like growth factor (IGF-1) (see Chapter 10) as the intermediary. The annual life cycle of winter flounder includes the cessation of feeding and growth from about October through

March, which leads to reduced GH and IGF-1 levels over the winter months. This releases the repression of AFP genes and allows the synthesis and accumulation of AFPs. At the molecular level, IGF-1 can inhibit liver AFP gene enhancer activity. Analysis of the liver AFP gene promoter showed DNA-binding motifs for the C/EBPα transcription factor and a novel AP-1-binding complex that has been termed the antifreeze enhancer-binding protein (AEP). In other systems, IGF-1 causes a dephosphorylation and deactivation of the cCAAT enhancer binding protein (c/EBPα), and it is proposed that this effect and/or an effect on AEP expression causes transcriptional inhibition of the liver AFP genes. With the loss of long day length in the fall, GH production is inhibited, IGF-1 levels fall, and C/EBPα and AEP are activated to enable AFP gene expression.

Whereas liver produces the secreted form of fish AFP, other studies have recently shown that AFP production is not limited to the liver in winter flounder. When a liver cDNA probe was used to screen a skin cDNA library, other AFP clones were found. Distinct but homologous alanine-rich AFPs are produced in skin, their main differences from liver AFP being a lack of the signal and prosequences that allow export of liver AFPs. Hence, skin AFPs remain *in situ* to protect the integument from intracellular freezing. Northern blot analysis showed that the skin type AFP was also strongly expressed in other exterior tissues such as scales, fin, and gills. Hence, two multigene AFP families occur in winter flounder, both with the same functional amino acid repeats. One produces the secretory liver type of AFP and the other nonsecretory AFP that provides antifreeze protection to surface tissues of the fish.

Ice Nucleating Agents

Ice nucleating agents are found in most freeze-tolerant organisms where they are employed to trigger ice formation in the extracellular body fluids. By their action, nucleation is triggered at mild subzero temperatures (usually well above -10°C), extensive supercooling is avoided, and slow ice growth through extracellular spaces is managed so as to minimize injury to the organism. Ice nucleating agents are sometimes novel INPs that apparently evolved specifically for this purpose, but other agents can have ice nucleating activity including heterogeneous material in the gut or in the external environment around the organism, bacteria, and in the case of insects, various mineral crystals (e.g., calcium phosphate) that are stored in fat body or Malpighian tubules. The presence of such material is the reason that freeze-avoiding species typically clear their gut and/or spin a protective cocoon to minimize the risk of nonspecific nucleation over the winter months, but freeze-tolerant species can exploit these actions.

Wood frogs, *R. sylvatica*, appear to have three modes of ice nucleation. When cooled on a wet substrate (typical of their forest floor hibernaculum), freezing can begin at any temperature below the -0.5°C FP of frog blood due to seeding across the skin by contact with environmental ice crystals. If cooled on a dry substrate, however, wood frogs chill to a characteristic SCP of -2.5 to -3°C before freezing is stimulated by the nucleating activity of *Pseudomonas* and *Enterobacter* bacteria on the surface of the frog's skin or in the gut. The third option is the presence of a plasma INP. We have found that the INP appears seasonally, triggers freezing *in vitro* at -6 to -8°C (as assessed by differential scanning calorimetry), and is susceptible to denaturation by heat and various chemical treatments that confirm that it is a protein. However, it is difficult to envision the role of the plasma INP if it acts only at these low temperatures *in vivo* for such temperatures are near the survival limits of wood frogs, but it is possible that within the body the protein is effective at higher temperatures. Furthermore, this plasma INP may not actually trigger ice formation but, instead, may aid the propagation of ice through the frog's body, perhaps by modulating or regulating crystal growth through the vasculature.

Many bacteria show powerful ice nucleating activity that is a major cause of frost damage to crop plants. From the bacterial point of view, their INP activity is beneficial and has functions that could be nutritive (frost injury to plant cells causes them to leak nutrients that can be used by the bacteria) or protective (INPs can provide resistance to desiccation for the bacteria by sequestering a reserve of water close to the cell surface as well as dispersing ice crystals away from the cell to limit the chance of intracellular inoculation). The structure and function of bacterial INPs has been extensively studied for two reasons: (1) to find a way of impeding their action to reduce frost damage to crops, and (2) to optimize their function as nucleators in commercial snow-making operations. The INP monomers of *P. syringae* have a molecular mass of 120 to 180 kD, but the relationship between nucleation temperature and molecular mass predicts that a nucleator must be $\sim$20,000 kD to trigger crystallization at -2°C. Not surprisingly, then, huge aggregates of INP monomers are found on the bacterial cell membrane, and a phosphatidylinositol (PI) component of the protein appears to anchor them in groups.

Insect INPs Hemolymph INPs have been found in a number of freeze-tolerant insects where they act to raise the SCP during the winter months so that the insects freeze at relatively high subzero temperatures. Only the INP of the cranefly, *Tipula trivittata*, has been extensively characterized. It is an 800-kD globular lipoprotein (45% protein, 4% carbohydrate, 51% lipid) with two protein subunits (265 and 80 kD) that is present year-round and also functions as a normal lipophorin in the transport of lipids through the hemolymph. A distinguishing feature of the

INP compared with other lipophorins is the presence of PI (not previously found in insect lipophorins), which accounts for 11% of the bound phospholipids. Surface phospholipids on insect lipophorins can interact with water, and the PI content of the protein appears to be key to the ice nucleating action. Thus, treatments that affect the PI head groups inactivate INP function, and the data suggest that the hydroxyl groups on the inositol rings form the active water-organizing site. As with bacterial INPs, function of the *T. trivittata* INP also requires protein aggregation, in this case into long chains with two to three chains closely associated.

ENERGETICS, ISCHEMIA RESISTANCE, AND METABOLIC RATE DEPRESSION

Freezing is an ischemic event. When blood plasma freezes, each cell in each organ is isolated and cut off from the delivery of oxygen and nutrients, the removal of wastes, and interorgan communication via hormones and other signals for the duration of the freeze. Although metabolic rate is very low in a frozen organism, freezing may extend for many weeks, and hence, each cell of a freeze-tolerant organism must have fermentative fuel reserves and maintain a viable basal metabolism throughout the freeze. Indeed, the injurious effects of ischemia on human organ explants are one of the biggest challenges faced by researchers working in the field of organ transplantation (Chapter 19) and the main driving force behind continuing research that aims to extend cryopreservation technology for ultralow temperature storage ($-196^{\circ}C$) to more and more types of tissues and organs.

Freeze-tolerant animals show well-developed anoxia tolerance; in laboratory trials we have shown that wood frogs readily endure at least 2 days under a nitrogen gas atmosphere at 5°C, whereas *Eurosta* survived an 18-day exposure. Known freezing survival times extend to at least 4 weeks for frogs at $-3^{\circ}C$ and 16 weeks for *Eurosta* at $-15^{\circ}C$. Over the course of a freezing episode, both frogs and insects show typical animal responses to oxygen limitation: a slow progressive depletion of organ glycogen, phosphagen, and ATP over time and an accumulation of lactate and alanine as glycolytic end products. No unusual anaerobic end products are made although, as noted earlier, the peculiarities of carbohydrate pathways in *Eurosta* during the autumn cold-hardening period mean that large amounts of sorbitol accumulate under anoxic conditions. All parameters normalize again within 3 to 11 days postthaw. Although the glucose that frogs accumulate as a cryoprotectant is a fermentable fuel, frog organs appear to deplete only glycogen in support of anaerobic metabolism while frozen. As mentioned earlier, this may be due to strong inhibition of hexokinase in the cells of frozen frogs that prevents cryoprotectant pools from being catabolized.

When we analyzed the effects of freezing on many enzymes of glycolysis, gluconeogenesis, the tricarboxylic acid cycle, amino acid metabolism, fatty acid metabolism, and adenylate metabolism in wood frog organs we found that, in general, there are very few changes in enzyme activities in response to freezing (apart from those involved in cryoprotectant production in liver); equivalent studies assessing responses to anoxia exposure concurred. Enzyme changes that did occur during freezing generally involved a decrease in activities (in 75% of cases), often of biosynthetic enzymes, that was reversed after thawing. Hence, freeze-tolerant animals, like anoxia-tolerant animals, are well-prepared with constitutive activities of enzymes for dealing with aerobic-anoxic transitions during freezing. Interestingly, our recent application of cDNA array screening to identify freeze-responsive genes in wood frog organs identified the hypoxia-inducible factor 1 (HIF-1) as a freeze up-regulated gene but did not find up-regulation of any of the glycolytic enzymes that are usually under HIF-1 control. HIF-1 is also well-known to stimulate the up-regulation of the GLUT1 transporter (the ubiquitous form) so it may be that during freezing in wood frog organs HIF-1 triggers an acute up-regulation of glucose transporter levels to aid cryoprotectant uptake without triggering accompanying changes in glycolytic enzymes. The regulatory mechanisms involved in selectively activating just a subset of genes that are normally under HIF-1 control are not yet known, but we are sure that they will provide a fascinating study in adaptation that is likely intertwined with other modified regulatory mechanisms to accomplish and sustain the extreme hyperglycemia of the frozen state.

Freezing survival seems to be associated with metabolic rate depression (MRD), although there have been few studies that specifically address the issue. Many freeze-tolerant organisms are in preexisting hypometabolic states (dormancy, diapause, anhydrobiosis) when they are exposed to freezing, and this would clearly facilitate long-term freezing survival. For example, many insects undergo a winter diapause with metabolic rates typically $<10\%$ of normal values at the same temperature. The strong suppression of aerobic metabolism during diapause is associated with decreased activities of mitochondrial enzymes. For example, Figure 17.7*c* shows that activities of two enzymes of the tricarboxylic acid cycle, citrate synthase and NAD-dependent isocitrate dehydrogenase, decrease by about 50% in *Eurosta* over the midwinter months and then rebound in the spring; the activity of glutamate dehydrogenase, another enzyme located in the mitochondrial matrix, follows suit. The same thing happens in *Epiblema*. In some species, an actual degradation of mitochondria is seen so that only low numbers of the organelles remain throughout the dormant period.

The probability that freezing stimulates MRD in wood frogs is supported by data showing a freeze-induced

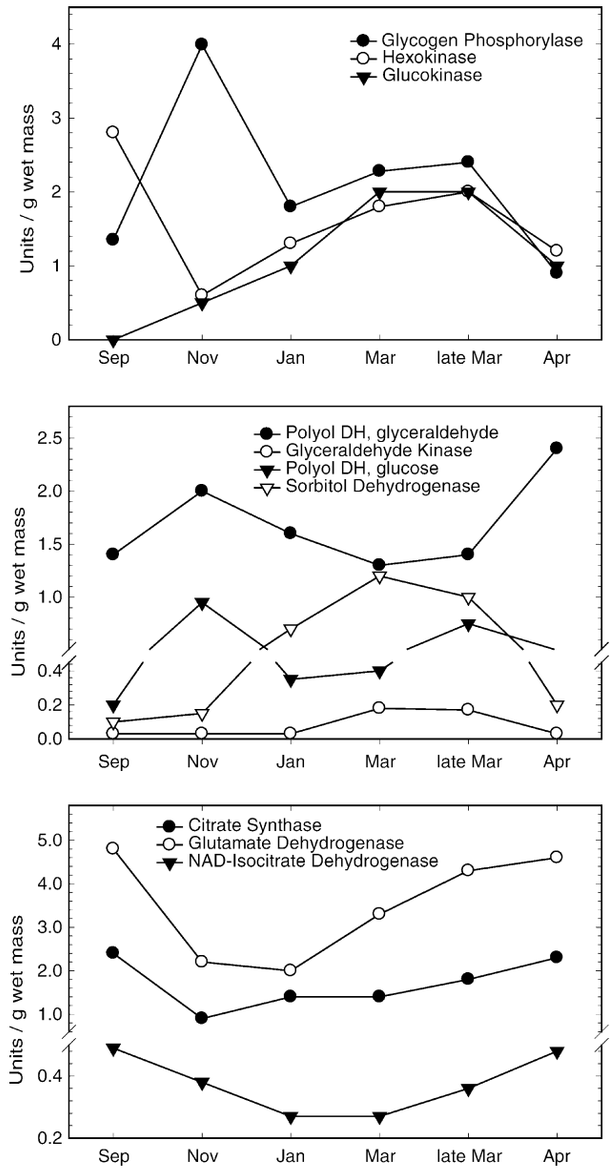

Figure 17.7 Changes in the activities of selected enzymes in goldenrod gall fly larvae, *Eurosta solidaginis* over the winter months. For polyol dehydrogenase, activity was measured with each of two substrates: with glyceraldehyde the product is glycerol, whereas with glucose the product is sorbitol. Animals were sampled within the first week of the month except for a late March sampling point, and the final sample was on April 21, just before larvae pupated. [Data compiled from D. R. Joanisse and K. B. Storey (1994). *J Comp Physiol B* **164**:247–255 and D. R. Joanisse and K. B. Storey (1994). *Insect Biochem Mol Biol* **24**:145–150.]

increase in the content of phosphorylated eIF2α in organs of frozen frogs. Recall that a high phospho-eIF2α is indicative of an inhibition of protein synthesis and commonly accompanies MRD in other systems. We have also found that freeze exposure suppresses the activities of ion-motive ATPases in wood frog skeletal muscle, again indicating MRD. Na^+K^+–ATPase and sarcoplasmic reticulum (SR) Ca^{2+}–ATPase activities were reduced by 30 to 50% during freezing as was calcium binding by SR fractions; furthermore, oxalate-stimulated calcium uptake was reduced by 92% in SR fractions from frozen frogs. All rebounded partially or fully after 24 h thawing. This strongly indicates that active steps are taken to conserve organ energy expenditure on ATP-dependent ion pumping in the frozen state.

Once again, our new data from cDNA array screening provide additional indications that freeze-induced MRD occurs, probably as a mechanism of ischemia resistance. Screening identified adenosine A1 and A2A receptors and the enzyme 5′-nucleotidase (that synthesizes adenosine from AMP) as putatively up-regulated during freezing in frog heart. Recall from Chapter 15 that adenosine accumulates quickly in response to anoxia in organs (particularly brain) of anoxia-tolerant turtles and acts via adenosine A1 receptors to trigger ion channel arrest. Overexpression of adenosine A1 receptors is also known to increase myocardial tolerance of ischemia in transgenic mice. Hence, the putative up-regulation of adenosine receptors and 5′-nucleotidase in frog heart provides evidence that as wood frogs freeze they activate a set of ischemia-protective signals and receptors and implement hypoxia/anoxia-induced suppression of ATP-expensive metabolic processes. Interestingly, in the study with transgenic mice, overexpression of adenosine A1 receptors was also associated with enhanced GLUT4 expression, suggesting yet another mechanism that frog organs adapt and use to acutely enhance cryoprotectant uptake during freezing.

ANTIOXIDANT DEFENSES

All organisms maintain antioxidant defenses, including enzymes, metabolites (e.g., glutathione, ascorbate) and proteins (thioredoxin), that deal with the continual assault to cellular metabolism by reactive oxygen species such as superoxide, hydrogen peroxide, hydroxyl radicals, and peroxynitrite (see Chapter 12). The latter two, in particular, are highly reactive species that can cause serious damage to cellular lipids, proteins, and DNA. Studies in mammalian systems have linked a substantial portion of ischemic damage to organs, not to the period of oxygen deprivation itself, but to the reperfusion phase when oxygen is reintroduced and a burst of oxyradical production can temporarily overwhelm the antioxidant defenses of an organ. Ischemia-linked oxidative damage is also a serious issue in hypothermic organ preservation and is lessened by the addition of antioxidants to the perfusion medium during organ retrieval and storage (see Chapter 19). Freeze–thaw is

an ischemia-reperfusion event that has the potential to result in oxidative damage to organs when heart beat and breathing are restored during thawing.

Furthermore, in the case of freeze-tolerant frogs that use glucose as a cryoprotectant, the potential for glucose-mediated oxidative damage is high since glucose-related oxidative damage is known to play a role in tissue damage in diabetes. Persistent high glucose causes damage to tissues for two main reasons: (1) the nonenzymatic glycation of proteins impairs their function and leads to the accumulation of damage products called advanced glycation end products (AGEs), and (2) the prooxidant actions of glucose, glycated proteins, and AGEs stimulate the production of reactive oxygen species that cause further damage to macromolecules. Indeed, we suspect that the damage that could accrue as a result of sustained high glucose is a major reason why cryoprotectant biosynthesis by frogs is triggered only when the animals actually begin to freeze.

Two strategies contribute to the defense against oxidative stress associated with freeze–thaw in freeze-tolerant animals. The first and most important is elevated constitutive defenses. For example, the activities of six antioxidant enzymes (superoxide dismutase, catalase, glutathione *S*-transferase, glutathione reductase, and total and Se-dependent glutathione peroxidases) were generally at least 2-fold higher in organs of wood frogs compared with the same organs in freeze-intolerant leopard frogs that hibernate underwater. Furthermore, the levels of reduced glutathione (GSH) were up to 10-fold higher in wood frog organs. Indeed, the antioxidant defense capacity of wood frog organs is very close to that of anoxia-tolerant turtles, and both are high for ectothermic vertebrates and closer to the antioxidant capacities of mammalian organs. This indicates that good antioxidant defenses are a necessary aid for freezing survival. Not surprisingly, then, wood frog organs showed no signs of oxidative damage to lipids over a course of freeze–thaw. The freeze-tolerant insect, *Eurosta*, similarly showed no accumulation of oxidative damage products as a result of freeze–thaw, although a 5-fold increase in the amount of oxidized glutathione (GSSG) suggested that some oxidative stress occurred during freezing that consumed GSH.

The second principle of antioxidant defense in freeze-tolerant animals is selective changes to the activities of some enzymes in response to freezing. *Eurosta* showed no change in the activities of antioxidant enzymes in response to either freezing or anoxia exposure but wood frogs did. Both total glutathione peroxidase and selenium-dependent glutathione peroxidase activities increased in frog organs during freezing and returned to near control values after 24 h thawing. This suggests that the need for enhanced defenses against peroxidative damage is greatest during freezing or immediately after thawing, a time when glucose is also very high. These changes may minimize the potential for glucose-mediated oxidative damage to macromolecules, which is a significant problem associated with sustained high glucose levels in diabetes.

COLD HARDINESS AND GENE EXPRESSION

Polar species are typically cold-hardy all year round, but organisms living in temperate zones of Earth acquire their cold hardiness on a seasonal basis. Acquisition of cold hardiness is often linked with photoperiod cues and is accelerated by decreasing environmental temperatures (similar to the situation for AFP induction, discussed earlier). Insects that have only one generation a year (univoltine) often show an obligatory acquisition of cold hardiness with the transition to the overwintering life stage (e.g., in *Eurosta* this is the third instar larva) as well as an obligatory period of diapause (arrested development) before they can resume growth and development. Thus, the goldenrod gall formers, *Eurosta* and *Epiblema*, need a minimum of about 3 months of cold exposure before they can break diapause. They will not resume development if removed from cold storage earlier, and after diapause ends (usually in early February in Canada), they remain in a quiescent state until warming temperatures in the spring facilitate pupation. Insects with two or more generations over the year (bi- or polyvoltine) are typically cold-hardy only in the last generation that overwinters. This generation is usually preprogrammed by photoperiod triggers (day shortening) received by the female in the previous generation that cause the production of diapause hormone that acts on the developing eggs of the next generation to set them on the course for diapause and cold hardiness. For example, diapause-bound eggs laid by silkmoths *Bombyx mori* already contain higher glycogen levels than nondiapause eggs as well as the enzyme complement to immediately begin glycerol and sorbitol synthesis. After these eggs are laid, development arrests at an early embryonic stage, and in eggs stored at 5°C diapause persists for several months before an induction of sorbitol dehydrogenase (to catabolize sorbitol) and other enzymes signals its end.

The seasonal acquisition of cold hardiness involves changes in the expression of multiple genes. Gene expression responses can occur on two time frames: (1) seasonal and/or cold acclimation responses that accrue over days or weeks prior to exposure to potentially damaging subzero temperatures, and (2) rapid responses triggered within minutes to hours when temperature drops (cold shock) or freezing begins. For example, the appearance of AFPs, INPs, and increased activities of enzymes involved in cryoprotectant synthesis all result from seasonal changes in gene expression. Other factors can also come into play such as changes in food availability; goldenrod gall forming

insects also seem to take cues from the senescence of the plant galls in which they live.

Figure 17.7 shows the seasonal changes in activities of selected enzymes of polyol metabolism by freeze-tolerant gall fly larvae; these changes can be compared with the acquisition of cryoprotectants by the larvae as shown in Figure TB17.1. Figure 17.7*a* shows that hexokinase, which is involved in the uptake, phosphorylation, and storage of dietary sugars as glycogen is highest in early autumn, coincident with the time of major glycogen storage. Glycogen phosphorylase *a* activity peaks later in the fall, corresponding with high rates of glycerol and sorbitol synthesis (see Fig. TB17.1) and with peaks of polyol dehydrogenase activity using either glyceraldehyde (making glycerol) or glucose (making sorbitol) as the substrate (Fig. 17.7*b*). However, enzymes that catabolize polyols do not appear until later in the winter. Sorbitol dehydrogenase (SDH), which converts sorbitol to fructose, is induced in January and provides the larvae with flexibility in their sorbitol pools so that these can be catabolized whenever with weather warms up. Notably, SDH induction is linked with the end of diapause in silkmoth eggs, and this may also be so in *Eurosta*, the larvae entering a quiescent state after diapause ends, which endures until warming temperatures trigger massive losses of polyols and the beginning of renewed development. Rising glucokinase and hexokinase activities over the late winter months would phosphorylate the fructose produced by SDH so that it can reenter the hexose phosphate pool for reconversion to glycogen. Glyceraldehyde kinase, which catabolizes the glycerol pool, does not appear till March and this correlates with the beginning of glycerol clearance at this time (see Fig. TB17.1).

Thus, for many insects the acquisition of cold hardiness and/or freeze tolerance is an obligate seasonal event, often intertwined with development and virtually always completed before subzero temperatures are experienced in nature. For this reason, insects are generally poor models for studying acute effects of cold and/or freezing on gene expression. Indeed, when we tried such experiments with *Eurosta* and *Epiblema*, we found very few gene expression changes that are directly triggered by cold or freezing exposures. For example, a search for freeze-induced gene expression in *Eurosta* using differential display polymerase chain reaction (PCR) found only eight transcripts that were putatively up-regulated by freeze exposure, only one of which could be tentatively identified by its high sequence identity with the *Drosophila* ribosomal protein, rpA1. It appears that the program of protein changes needed for cold hardiness in this univoltine species is so "hard-wired" into the developmental and seasonal patterns of the species that few, if any, gene expression and protein synthesis changes are needed when tissue freezing actually begins. With the small body size of insects (gall fly larvae are 50 to 60 mg), this result is not surprising for these insects freeze very fast, probably without sufficient time for significant gene induction and protein synthesis responses.

However, other models offer good opportunities to study cold- and freeze-induced gene expression, and we will consider recent advances with two of these: the model plant *Arabidopsis thaliana* and the wood frog *R. sylvatica*. Recent work with both of these models has employed state-of-the-art gene screening techniques (e.g., cDNA library screening, differential display PCR, cDNA array screening, reverse transcription (RT)-PCR) to provide a wide view of the many different types of cellular proteins and enzymes that respond to cold or freezing stress. Our focus below, then, is on cold-induced gene expression in *Arabidopsis* and freeze-induced gene expression in wood frogs.

Cold-Induced Gene Expression in *Arabidopsis*

Arabidopsis is the "*Drosophila*" of the plant world, a very widely used model whose metabolism and genetics have been extensively characterized and that can be easily manipulated with deletion or overexpression mutants to characterize the phenotypic effects of genes. Until recently, about 40 cold-induced genes had been identified in *Arabidopsis*, representing a wide variety of cell functions (with additional novel unidentified genes indicated from cDNA array screening). The plant is freeze-tolerant so these include a mixture of genes that may have primary functions in dealing with either cold or freezing stress. Many of these genes are responsive to abscisic acid (ABA), and it appears that this plant hormone is the major mediator of the cold or freezing signal in all plants. In *Arabidopsis*, treatment with ABA at normal growth temperatures improved survival in subsequent tests of frost hardiness, whereas ABA-deficient mutants were impaired in developing freezing tolerance during cold acclimation. Among the first genes shown to be cold-inducible were several that encoded enzymes/proteins involved in membrane protection including (a) desaturases that increase the extent of lipid unsaturation to adjust membrane fluidity for low-temperature function, (b) enzymes of proline biosynthesis that produce the low-molecular-weight membrane-protectant, proline, and (c) dehydrins that appear to function as membrane protective small proteins. Alcohol dehydrogenase (ADH) was also up-regulated in the cold to increase the capacity for glycolytic energy production during freezing since plants produce ethanol, not lactate, as their glycolytic end product. Other cold-responsive genes included those encoding sugar transporters, water channel proteins, Ca^{2+} and iron-binding proteins, a wound-inducible protein, and several antioxidant enzymes. Several kinases are also cold-induced, including members of the mitogen-associated protein kinase (MAPK) signaling pathway and the ribosomal S6 kinase,

implicating their roles in regulating the functions of other proteins in the cold. Most of these genes are constitutive at normal growth temperatures and undergo 2- to 5-fold induction during cold acclimation. However, one class of cold-regulated (*COR*) genes is strongly induced by cold, typically by 50- to 100-fold, and because of this strong induction response they have been the target of many regulatory studies.

COR genes (and a few others) are regulated by the CBF (CRT binding factor) transcription factors (three isoforms are known) that are strongly up-regulated by cold exposure in *Arabidopsis*. This transcription factor binds to the cold-repeat element (CRT) in the 5′ promoter of CBF-responsive genes. Within 15 min of transfer to low temperature, CBF transcripts begin to accumulate, followed after 1 to 2 h by accumulation of transcripts of the CRT-regulated genes. Deletion analysis of the promotors of some of the *COR* genes determined the sequence of the CRT to which CBF binds. The surprising result of this was that the CRT was identical with a drought-responsive element (DRE) that is present in the genes of many other plant species and is regulated by the DREB (DRE binding factor) transcription factors. Thus, two different stresses (freezing, drought), acting via two different transcription factors (CBF, DREB), can activate a common set of genes. As discussed previously in Chapter 14 (see Fig. 14.12), this system illustrates an important principle of biochemical adaptation. Adaptive mechanisms that work well in one situation are often "borrowed" and put to use in organismal responses to a related stress. In this case, with the simple creation of a new transcription factor (CBF), a whole group of preexisting dehydration-responsive genes/proteins could be put to work to aid freeze tolerance and address problems that are common to both stresses (e.g., cell volume reduction, cell water loss, membrane stress). Interestingly, as we will see in the next section, quite a few of the freeze-responsive genes in wood frogs are also induced by dehydration in both freeze-tolerant and freeze-intolerant amphibians, indicating that their origin was also as dehydration-responsive genes.

A recent study has hugely expanded the database on cold-responsive genes in *Arabidopsis* and provides a superb model for the way in which cDNA array screening can be used in studying biochemical adaptation (see Suggested Reading list). Plants grown at 22°C were acutely transferred to 4°C and sampled after 0.5, 1, 4, 8, and 24 h and 7 days. Gene expression at each time point was assessed on gene chips containing 8296 different cDNAs with a 3-fold change in expression considered significant. Gene expression was also assessed in warm-grown plants that constitutively expressed CBF isoform 1, 2, or 3 to identify those genes that were controlled by CBF regulons. Screening found 306 genes that were cold-responsive, 218 that were up-regulated, and 88 that were down-regulated by cold exposure. Only 12% of these genes could be clearly assigned to the CBF regulons, which included subregulons controlled by the RAP2.1 and RAP2.6 transcription factors. Genes for at least 15 other putative or known transcription factors were CBF-independent and were expressed in different time patterns, which indicates the existence of several other regulons that control groups of cold-responsive proteins. Multiple patterns of gene expression were identified. Some genes were activated early, whereas others responded later. Early responders included CBF transcripts that rose within the first hour of cold exposure and were followed within 4 h by increased expression of several *COR* genes. About 50% of genes were only transiently induced (up-regulated at only 1 or 2 time points), whereas others were expressed for longer times, including 64 genes that were expressed over the full 7-day time frame. "Waves" of gene expression occurred with new groups of genes up-regulated at each time point. Cold-responsive genes belonged to many different groups with identified genes encoding proteins involved in transcription regulation, intracellular signaling, sugar metabolism, antioxidant defenses, COR and late embryogenesis proteins that are believed to provide cryoprotection, and many others. Overall, then, the results from this transcriptome screening indicate the highly complex nature of the metabolic responses that make up cold acclimation (and our fractional knowledge about these to date) and also emphasize the existence of regulated patterns of coordinated responses that provide both acute responses that are involved in immediate relief from cold shock and long-term responses that readjust structural and functional components of cells to reestablish homeostasis at low temperature.

The dehydrin and COR proteins in plants deserve some further comment. Dehydrins are induced by environmental stresses that result in cellular dehydration (e.g., drought, high salt, freezing, seed desiccation). At least five different dehydrins are up-regulated by cold exposure in *Arabidopsis*, one of which is also a COR protein. Dehydrins are glycine-rich, hydrophilic, heat-stable proteins that include a characteristic lysine-rich sequence near the C terminal. The polypeptide is believed to form an amphipathic helix that could interact with membrane lipids or with exposed hydrophobic patches of partially denatured proteins in a manner similar to chaperones. They are believed to serve a protective function during stress. Studies with the peach dehydrin PCA60 showed that it protected the activity of lactate dehydrogenase during freeze–thaw cycles *in vitro*, and it also exhibited antifreeze activity as evidenced by thermal hysteresis and effects on ice morphology. Similarly, the COR proteins are not enzymes, but many are very hydrophilic and remain stable upon boiling, which suggests that they act as cryoprotective proteins similar to dehydrins. Overexpression of one *COR* gene, *COR15a*, improved freezing survival by chloroplasts and protoplasts in warm-acclimated trans-

genic plants. Localized in the chloroplast stroma, it is proposed that the 15-kD COR15am mature protein functioned to stabilize membranes by decreasing the propensity of membranes to form deleterious hexagonal II phase lipids upon freeze-induced dehydration. To do this the proteins may alter the intrinsic curvature of the inner membrane of the chloroplast envelope. While these protective proteins in plants seem to play roles in membrane or protein stabilization, a group of cold-shock proteins in bacteria appear to have primary roles in preserving nucleic acid stability (see Text Box 17.4).

Agricultural scientists are very interested in the possibility of using transgenic technology to enhance freeze tolerance or to induce constitutive freeze tolerance in various crop species, thereby reducing the economic cost of frost damage. In one study, for example, *Arabidopsis* were transfected with insect AFP genes (with or without the signal peptide for AFP export). Plants expressed the protein and plants with AFPs in the apoplast fluid froze at significantly lower temperatures than did wild-type plants, especially in the absence of extrinsic nucleation events. However, transgenic lines showed no improvement in freeze tolerance. In other studies, transgenic *Arabidopsis* lines that constitutively express the CBF transcription factor showed the continuous presence of various proteins that are normally cold-inducible. One line showed a level of freeze tolerance comparable to that of the cold-acclimated wild type. However, in another line the transgenic plants were all dwarfs. Hence, there can be a growth penalty for the constitutive maintenance of the many metabolic adaptations needed for freeze tolerance, and this can affect both yield and productivity, characteristics that are generally positively selected in nature and in agriculture. This is probably the reason that frost hardiness is only seasonally expressed in most plants.

Freeze-Induced Gene Expression in Wood Frogs

The frozen state is an ischemic state; as the ice front moves through an organism, freezing extracellular fluid and plasma, the delivery of oxygen and substrates to tissues is cut off. Hence, the transition to the frozen, ischemic state would not be the time to undertake major ATP-expensive biosynthetic projects. Indeed, studies with wood frog heart have shown that a general suppression of transcription and translation takes place and comparable events probably occur in all other organs. To assess the transcriptional capacity of wood frog heart, we used the nuclear run-off assay (see Fig. 14.11 for an example of the technique). The rate of ^{32}P-uridine triphosphate (UTP) incorporation into preinitiated RNA transcripts in fresh nuclei isolated from the hearts of 5°C-acclimated (control) frogs was compared with the rate in frogs frozen for 24 h at −2.5°C. After Trizol isolation of total RNA, the amount of ^{32}P-UTP incorporated by transcripts in nuclei from frozen frogs was only 47% of the amount in control hearts, indicating a substantial suppression of net transcriptional activity during freezing.

TEXT BOX 17.4 BACTERIAL COLD SHOCK PROTEINS

Both heat and cold induce shock responses by organisms. The heat-shock response has been extensively studied, and its prominent feature is the synthesis of protein chaperones that aid protein refolding and act to protect/rescue proteins whose active conformation is disrupted/denatured by heat exposure. A cold-shock response has been characterized in mesophilic bacterial species, such as *Escherichia coli*. A decrease from warm to cold temperature immediately halts growth, primarily because of cold effects on ribosomes that cause a break-up of polysomes into monosomes. However, after a lag period during which cold-shock proteins (Csps) are synthesized, protein translation and bacterial growth resumes. Csps are typically small (~7 kDa) proteins that have roles in gene expression, mRNA folding, transcriptional initiation and regulation, and/or freeze protection. Different bacterial species may show 12 to 37 types of Csps. Some are known cellular proteins whose levels are up-regulated by cold, whereas others are apparently unique to cold shock. CspA, for example, appears to function as an RNA chaperone to guide efficient translation by either (a) preventing formation of secondary structure in mRNA that would make it inaccessible to the translation apparatus or (b) protecting the message from enzymatic degradation. The responses by psychrotolerant and psychrophilic bacteria to cold shock is different. The number of proteins that respond to cold is greater, but their level of expression is weaker than the mesophilic response. This is not unexpected as these species are better prepared constitutively for dealing with low temperature. A subgroup of these proteins, termed cold-acclimation proteins (Caps), is continuously produced during low-temperature growth and may have roles key to life in the cold.

Effects of Csps on freezing survival have been assessed. A single *cspB* deleted mutant of *Bacillus subtilis* showed lower freeze survival than wild-type cells. A freeze-sensitive phenotype of *Lactococcus lactis* was observed upon deletion of the *cspA*, *cspB*, and *cspE* genes, whereas overproduction of the protein products of these genes increased freezing survival. Since some Csps are recognized as nucleic acid binding proteins, they might protect RNA and DNA during the freezing process by binding to these nucleic acids and, hence, increase the survival of the bacterial cells.

Similarly, translational activity in frozen hearts was strongly suppressed as evidenced by the phosphorylation status of the eukaryotic initiation factor 2α alpha (eIF2α); phospho-eIF2α was threefold higher in hearts of frozen frogs compared with unfrozen controls. Thus, against a background of a general suppression of transcription and translation during freezing, the up-regulation of selected genes suggests that they have important roles to play in freezing survival. Some of these are discussed below.

Over the last 8 years, studies in our lab at Carleton University have focused on the discovery and analysis of freeze-responsive genes in the organs of wood frogs. As with many other fields of biology, the recent "explosion" of technique choices for gene discovery have allowed a whole new approach to be taken in identifying the genes/proteins that support freeze tolerance. Where previously we needed to identify a physiological or biochemical trait of freeze-tolerant animals (e.g., cryoprotectant production) and then work backward to elucidate the protein mechanisms underlying it, we can now work in the opposite direction beginning with up-regulated genes and discovering the metabolic and physiological consequences of the expression of these genes. One might think that there would be a lot of overlap in the results of these two approaches but, to date, there has been very little. Indeed, one key result of gene screening technology has been the identification of dozens of genes/proteins, both previously known and novel, that have never before been linked with freeze tolerance (several of these are discussed below). Each of these genes has implicated a previously unsuspected area of cellular metabolism as contributing to freeze tolerance. Using techniques of cDNA library construction and screening and differential display PCR, a variety of freeze-responsive genes have been identified. Note that these studies were done with cDNA isolated from 5°C acclimated frogs as controls and frogs frozen at −2.5°C (usually for 24 h) as the experimental condition and that, therefore, they represent freeze-induced genes, not cold-induced genes as for the *Arabidopsis* studies described above. Freeze-responsive genes that have been confirmed as up-regulated in wood frogs include fibrinogen, mitochondrial membrane proteins [adenosine 5′-diphosphate (ADP)/ATP translocase, the inorganic phosphate carrier, F_1−ATPase subunits 6 and 8], myosin light-chain subunit 2, atrial natriuretic peptide, the glycolytic enzyme phosphoglycerate kinase 1, and several proteins involved with protein synthesis (ribosomal phosphoprotein P0, ribosomal protein L7, and the eukaryotic elongation factors 1 and 2). In addition, three novel proteins (FR10, FR47, Li16) were found.

Very new studies have employed cDNA array screening with 19,000 human cDNA gene chips to further explore freeze-responsive gene expression in wood frogs. Although cross-species reactivity was only 60 to 80% between frog cDNA and human chips, the responses of thousands of genes could still be evaluated. Gene expression changes in nine different organs have been assessed. Results for wood frog heart provide an instructive example. Over 200 genes showed a greater than twofold increase in expression in heart of frozen frogs, compared with 5°C controls. Many of these fell into six general categories: (1) signal transduction: serine/threonine protein kinase, protein phosphatases, and their related regulatory proteins; (2) shock proteins with known or probable chaparone functions such as heat-shock protein 27 and glucose-regulated proteins; (3) membrane transporters including several solute carriers such as the inorganic phosphate carrier and the GLUT4 glucose carrier; (4) genes encoded on the mitochondrial genome; (5) antioxidant defenses including cytochrome P450, aldo-ketose reductases, superoxide dismutase; and (6) genes related to hypoxia/ischemia defense including HIF-1, adenosine receptors, and 5′-nucleotidase. Genes belonging to the last two categories suggest coordinated control during freezing in heart by genes containing either the hypoxia response element or the antioxidant response element. Genes containing these response elements are well-known to be involved in mammalian organ responses to ischemia-reperfusion, which suggests that a substantial part of the heart response to freezing is the implementation of defensive or protective strategies for dealing with long-term ischemia. This makes sense when we remember that heart is one of the last organs to freeze and the first to thaw and that despite ischemic insults to its metabolism, the heart must continue to do physical work as the frog is freezing in order to distribute cryoprotectant and must resume function quickly after thawing in order to reestablish perfusion of other organs as soon as possible. Selected freeze-responsive genes are discussed more fully below.

Fibrinogen Fibrinogen plays an important role in wound repair. This acute-phase plasma protein is synthesized by liver and secreted into the plasma with increased production stimulated by stresses including infection, inflammation, and tissue injury. The protein has two halves, each made of three subunits (Aα, Bβ, and γ) and as the final step in the coagulation cascade, thrombin cleaves near the N termini of the Aα and Bβ chains to release the A and B fibrinopeptides and expose sites for polymerization into the fibrin mesh of a growing blood clot. Screening of a cDNA library made from liver of frozen wood frogs retrieved freeze up-regulated clones with inserts encoding the α and γ subunits of fibrinogen. Northern blots showed that both transcripts were coordinately expressed over a time course of 24 h freezing at −2.5°C followed by 24 h thawing at 5°C; transcript levels rose by more than threefold after 8 h freezing, remained high with longer freezing, and then returned to near control values within 24 h after thawing. In a subsequent study, the β- and γ-fibrinogen subunits were retrieved from screening a liver cDNA library made

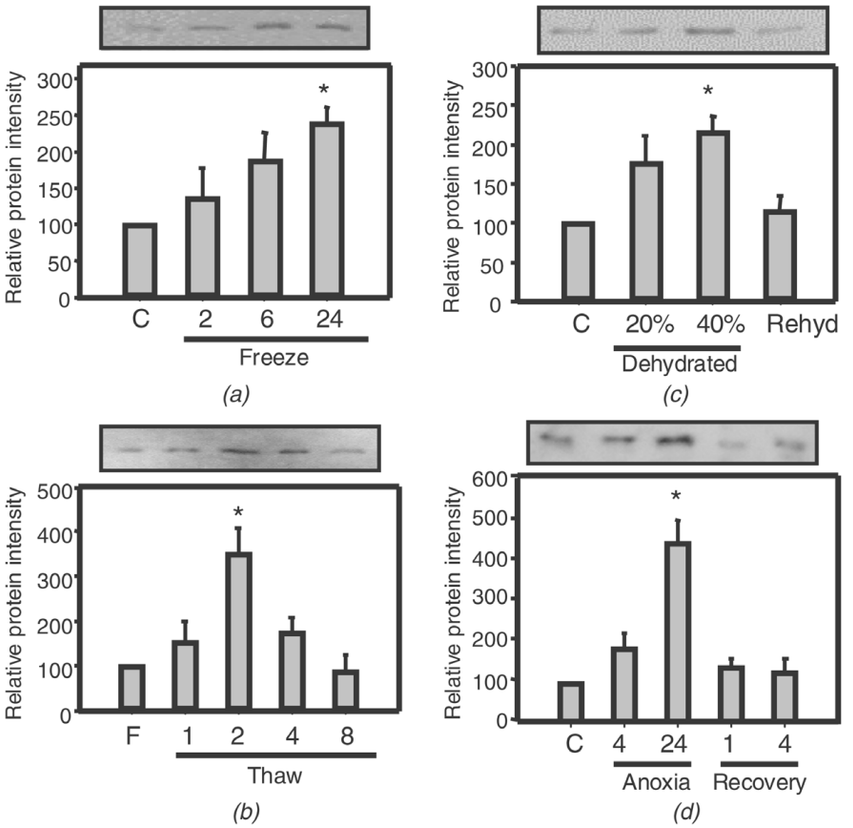

Figure 17.8 Responses of Li16 protein levels in liver of wood frogs, *Rana sylvatica*, under different conditions: (*a*) freezing at −2.5°C for 2, 6, or 24 h, (*b*) thawing at 5°C after 24 h frozen for 1, 2, 4, or 8 h; note that on this graph changes during thawing are expressed relative to protein levels in 24-h frozen (F) frogs, (*c*) dehydration at 5°C to 20 or 40% of total body water lost followed by 24 h rehydration, (*d*) anoxia exposure under a nitrogen gas atmosphere at 5°C for 4 or 24 h followed by aerobic recovery for 1 or 4 h. Example Western blots are shown along with histograms presenting mean values for relative band intensities for $n = 3$ independent trials. C, control at 5°C, *, significantly different from zero time value, $P < 0.01$. See Figure 14.10 for more information about Li16 expression in frozen frogs. [Data from J. D. McNally, S. Wu, C. M. Sturgeon, and K. B. Storey (2002). *FASEB J* **16**:902–904.]

from glucose-loaded wood frogs; glucose loading was used to assess genes that were responsive to the extreme hyperglycemia of the frozen state. Hence, coordinate expression of the three subunits undoubtedly occurs during freezing, as is the well-known response by fibrinogen subunits in other vertebrates. The probable function for fibrinogen up-regulation during freezing is to improve the frog's damage repair capabilities so that any internal bleeding injuries caused by ice crystal damage to the microvasculature can be rapidly addressed when organs thaw. Since the normal mechanisms of interorgan signaling (hormone transport by blood, nervous stimulation) are cut off during freezing and impaired for a considerable time postthaw, an anticipatory up-regulation of damage repair mechanisms makes sense so that newly synthesized clotting proteins can be available immediately upon thawing.

To explore the functions of freeze-responsive genes, we routinely examine the parallel response of each gene to two of the component stresses of freezing: anoxia and dehydration. From these studies, we can gain a good idea of whether freeze-responsive genes are responding to the ischemia or the cellular dehydration components of freezing. Recall from the section on cryoprotectant biosynthesis that glucose production by wood frog liver was stimulated just as strongly by dehydration as by freezing but did not respond to anoxia. Similarly, all freeze-responsive genes that have been extensively characterized to date in wood frogs also respond to either anoxia or dehydration (but not

both). In the case of fibrinogen, transcript levels were elevated under dehydration stress but not in anoxia. This suggests that it is the structural strain on cells that necessitated the need for defenses against bleeding injuries. The endothelial cells lining blood vessels are particularly vulnerable to freezing injury for they face not only volume changes (shrinking during freezing, rapid swelling during thawing) but also the possibility that ice expansion within the lumen of capillaries will cause them physical damage or breach their walls. Indeed, studies of mammalian liver preservation have identified the endothelial cells of the vasculature as primary targets of freezing damage and much more sensitive than the hepatocytes.

Mitochondrial Transporters The mitochondrial inner membrane is highly selective as to which metabolites can enter the organelle; for example, glucose cannot, which leads to the question of how mitochondrial volume is regulated when frog cells dehydrate during freezing! The mitochondrial ADP/ATP transporter (AAT) was one of the first freeze-responsive genes identified in wood frog liver, and recent studies have identified two others, the inorganic phosphate carrier (PiC) and the mitochondrially encoded subunits 6 and 8 of the F_1F_0−ATP synthase. However, transcript levels of two other transporters (the dicarboxylate carrier and the oxoglutarate transporter) did not change during freezing, which indicates that only selected functions need modification to deal with the consequences of freezing. The three freeze-responsive transporters showed somewhat different patterns during freezing. Transcripts of the bicistronic mRNA encoding F_1F_0−ATP synthase subunits 6 and 8 (also known as the β and γ subunits of the F_1−ATPase) were elevated by 7-fold in liver of 24-h frozen frogs and remained high after 24 h thawing at 5°C. *Aat* transcripts reached a peak of 4.5-fold higher than controls after 8 h freezing but declined with longer freezing and during thawing. The levels of *pic* transcripts increased from nearly undetectable amounts in control and 2-h frozen frogs to reach >70-fold higher than control values after 24 h frozen. AAT and PiC protein levels were also quantified; these lagged behind the changes in mRNA transcripts but were ~2-fold and 3.3-fold higher than controls after 24 h frozen. PiC protein returned to near control values by 8 h thawed, whereas AAT was reduced to about 1.5-fold higher than controls after 24 h thawed. The fact that peak protein levels for both genes were reached during freezing suggests that they have a role to play in the survival of liver mitochondria in the frozen state. What that role is remains to be determined, but it may be related to stabilizing mitochondrial energetics under the ischemic conditions during freezing. Indeed, we found that both AAT and ATPase 6/8 also responded strongly to anoxia exposure (but not to dehydration), which further supports the idea that their expression may respond to signals coming from low oxygen or low energetics. However, PiC responded to dehydration stress, which suggests that changes in inorganic phosphate carrying capacity may be needed in order to adjust ionic, osmotic, or volume regulatory parameters between the mitochondrial and cytoplasmic compartments during freeze/thaw-induced changes in cell volume. Indeed, liver lost ~58% of its water during freezing at −2.5°C (when 65% of total body water was converted into ice), and this would cause major changes in ionic strength and osmolality of liver intracellular fluids, necessitating regulatory adjustments of these parameters by the various subcellular organelles.

Novel Proteins To date, screening of wood frog liver has revealed three unidentified freeze-responsive genes that have been named *fr10*, *li16*, and *fr47*. They code for proteins with molecular weights of 10, 14, and 47 kD, respectively. The three proteins show no sequence similarity to any known gene (or reported expressed sequence tag) when subjected to searches of gene banks nor do they appear to be related to each other. Apart from being up-regulated during freezing in liver, the only other shared characteristic that we know of to date is the presence in all three proteins of a hydrophobic region of 21 amino acids in length. In FR10 and Li16 this is an N-terminal region, whereas in FR47 the hydrophobic region is near the C terminus. In both FR10 and Li16 this region contains four leucine and four valine residues. Such hydrophobic regions are not uncommon in proteins and may represent a transmembrane segment.

To characterize the function of an unknown protein, pieces of information can be gathered from a variety of sources (these are summarized in Table 17.4). Also refer to Figure 14.10 for a diagram showing the various techniques that can be used for analysis of a novel gene and its protein product. Some of the approaches that can be used are as follows. First is the characterization of the response by these proteins to the primary stress itself, freezing. Gene transcript levels of all three increased by 3- to 5-fold in liver after 24 h of freezing at −2.5°C, and for FR10 these had returned to control values after 24 h thawed. Antibodies were raised against the C-terminal peptide of Li16 and FR47, and these were then used in Western blotting to assess protein responses to freeze/thaw. Western blots confirmed that both Li16 and FR47 proteins were also elevated in liver of frozen frogs (by 2.4- and 1.6-fold, respectively, after 24 h) (see Fig. 17.8 for Li16). Levels of both proteins continued to increase over the first 2 h when frogs were returned to 5°C to thaw reaching final maxima of 8.4- and 3.5-fold higher than unfrozen controls. However, by 8 h thawed levels had returned to about the same levels as in 24-h frozen frogs (Fig. 17.8). The peak protein level after 2 h of thawing corresponds with a time when only about half of the ice has melted in the frog's body.

TABLE 17.4 Comparison of Properties of Three Novel Proteins That Are Freeze-Responsive in Wood Frog Liver

	FR10	*Li16*	*FR47*
Molecular weight, kD	10	12.8	45.7
Number of amino acids	90	115	390
Sequence characteristics	N-terminal hydrophobic region, aa 1–21, possible nuclear exporting signal	N-terminal hydrophobic region, aa 1–21	Hydrophobic region near C terminal, aa 350–370
Transcript response to stress (relative to unfrozen control = 100)[a]			
24-h freeze (2-h thaw)	320 (110)	370	510
20% dehydration (full rehydration)	↑↑↑ (↑↑)	80	220
24-h anoxia (1-h recovery)	↓↓↓↓ (n.a.)	760	620
Protein response to stress (relative to unfrozen control = 100)[a]			
24-h freeze (2-h thaw)	n.a.	240 (840)	160 (350)
20% dehydration (full rehydration)	n.a.	175 (110)	65 (125)
24-h anoxia (1-h recovery)	n.a.	440 (130)	60 (90)
Transcript response to *in vitro* tissue incubation with second messengers[b]			
Dibutyryl-cAMP	n.a.	n.s.	n.s.
Dibutyryl-cGMP	n.a.	↑↑	n.s.
PMA	n.a	n.s.	↑↑
Organ distribution	All organs tested	Liver, gut, heart	Liver
Genbank accession number	U44831	AF175980	AY100690

[a]Values for liver mRNA transcript and protein levels are relative to levels in controls (set to 100); data show effects of the stress with numbers in brackets for the relative level in recovery (2-h thaw, full rehydration sampled after 24 h, or 1 h back under aerobic conditions); the numbers are mean values from quantification of band densities on 3–5 blots whereas for *FR10* responses to freezing and anoxia, the arrows show results of $n = 1$ trials.
[b]For *in vitro* incubations, liver slices from control frogs were incubated with varying concentrations of second messengers (20–2000 μM db-cAMP or db-cGMP or 2–200 μM PMA) or for varying times (1–10 h) with a set concentration (200 μM) of second messenger. PMA is phorbol 12-myristate 13-acetate. n.a. = not available. n.s. = no significant change.

Source: Data are compiled from Q. Cai and K. B. Storey (1997). *Gene* **198**:305–312; J. D. McNally, S. Wu, C. M. Sturgeon, and K. B. Storey (2002). *FASEB J* **16**:902–904; J. D. McNally, C. M. Sturgeon, and K. B. Storey (2003). *Biochim Biophys Acta* **1625**:183–191.

Organs are still shrunken, and heart beat has not yet resumed so the liver is still under ischemia and volume stresses, but their suppression by 8 h thawed (when heart beat and breathing have resumed and liver appears to be visibly restored to normal size) indicates that they are no longer needed once recovery is well-advanced. Overall, then, this argues for important roles for the three novel proteins in dealing with the stresses associated with freezing.

Next we looked at the organ distribution of the transcripts to give us further hints as to protein role. In this regard the three proteins are very different. Transcripts of *fr47* were found only in liver, *li16* was prominent and strongly up-regulated in liver, heart, and gut whereas *fr10* appeared in all eight organs that we tested. Hence, the functions of FR47 can be interpreted as liver-specific, whereas Li16 is restricted to selected organs (which may share a common characteristic), and FR10 appears to have a universal function. Furthermore, FR47 protein was found in liver of two other freeze-tolerant frogs, *Pseudacris crucifer* and *Hyla versicolor* but not in freeze-intolerant *Rana pipiens* or *Scaphiopus couchii*, which again indicates a definite freeze-specific role.

Our next approach was to find out how these proteins responded to two of the component stresses of freezing—ischemia and cell dehydration. We found that transcripts of *fr10* were strongly up-regulated by dehydration, whereas both *li16* and *fr47* transcripts responded strongly to anoxia exposure. The responsiveness of *fr10* to dehydration and its presence in all organs leads us to think that this protein may have a protective role in dealing with the consequences of freezing on cell membranes, perhaps similar to the functions of the dehydrin or COR proteins in plants. Studies will next be designed to see if the overexpression of FR10 in cell lines from freeze-intolerant organisms can improve their freezing survival. Li16 protein levels rose strongly under anoxia (24 h under N_2 gas at 5°C) to very high levels (Table 17.4), suggesting a function for Li16 in the response to freeze-associated ischemia. Note that a significant response by Li16 to 40% dehydration (Fig. 17.8) is probably related to the hypoxia that occurs at high dehydration levels

when blood viscosity becomes high and circulation is impaired. However, although *fr47* transcripts responded to anoxia, the protein did not. This difference between transcript and protein expression suggests that additional controls are involved in FR47 protein production; an anoxia/ischemia signal may have been "co-opted" to trigger expression of the *fr47* gene during freezing, but the protein is actually produced only when the frog is freezing.

Finally, if we can identify the signal transduction pathway that regulates the expression of a gene, we can get a further idea of the possible metabolic role of the protein or the possible initiating signal, such as a hormone, that may have triggered the signal transduction cascade involved. Recall that freeze-induced cryoprotectant synthesis in frogs was traced to the activation of β-adrenergic receptors and cAMP-dependent protein kinase. To gain similar information about *li16* and *fr47* expression, we incubated liver slices *in vitro* with different second-messengers (Table 17.4). Interestingly, neither gene responded to incubations with the cAMP analog, dibutyryl cAMP, but *li16* transcripts were up-regulated by exposure to dibutyryl cGMP (guanosine 3′5′ cyclic monophosphate). On the other hand, *fr47* transcript levels responded only to phorbol 12-myristate 13-acetate, a stimulator of the Ca^{2+} and phospholipid-dependent protein kinase C. This data provides additional evidence that the two proteins have quite different functions *in vivo* since they respond to different signals.

Furthermore, the results also support the idea that multiple signaling pathways are involved in implementing protective responses during freezing. We now know that protein kinases A, G, and C all participate in mediating different known metabolic responses to freezing and, in addition, we have measured freeze- or thaw-specific changes in the activities of two mitogen-activated proteins kinases: the stress-activated protein kinase (SAPK) and p38 (see Chapter 4 for information on these kinases) in frog organs. This provides strong evidence that natural freezing survival is not just a matter of packing cells with cryoprotectants and hoping for the best (as is still the practice in the pseudoscience field of cryonics) but involves coordinated responses by many aspects of cellular metabolism, triggered and regulated by multiple signal transduction pathways.

To return to the signal transduction pathways implicated in *fr47* and *li16* control, we had a previous indication that the PKC pathway is active in liver during freezing. Analysis of changes in second-messenger levels for both PKA and PKC in liver over the course of a freeze–thaw exposure showed that inositol 1,4,5-trisphosphate (IP_3), an intracellular second messenger of PKC, rose slowly over time during freezing, reaching about 3- and 11-fold higher than control values after 4 or 24 h of freezing. Under anoxia stress, however, IP_3 levels rose by 8-fold within 30 min in frog liver, but IP_3 was much less responsive to dehydration (about 3-fold higher in 20% dehydrated animals). The strong response of IP_3 to anoxia coupled with the graded accumulation during freezing (also seen for *fr47*) suggests that IP_3 accumulation in liver of freezing frogs mirrors the progressive development of anoxia/ischemia as the animal freezes and that one of the actions of IP_3 and PKC is the up-regulation of the *fr47* gene.

We do not have the same correlation with second-messenger levels for *li16*, but the putative link between *li16* and cyclic guanosine 5′-monophosphate (cGMP) fits very well with some of the new data that we have retrieved from cDNA array screening. Array screening indicated putative up-regulation of the adenosine receptor signaling pathway in heart and other organs of frogs during freezing: both A1 and A2 adenosine receptors were up-regulated as well as 5′-nucleotidase, the enzyme that synthesizes adenosine from AMP. Adenosine receptor signaling is mediated intracellularly by cGMP. Recall from Chapter 15 that adenosine accumulates quickly in the brain of anoxia-tolerant turtles when animals are exposed to anoxia and acts via adenosine A1 receptors to suppress the activities of ATP-dependent ion channels as part of the overall metabolic rate depression that ensures anoxia survival. Overexpression of adenosine A1 receptors is also known to increase myocardial tolerance of ischemia in transgenic mice. Hence, taken together, these data give a clear indication that as wood frogs freeze they activate a set of ischemia-protective signals and receptors and suggest that the Li16 protein has a role in this capacity. Overall, then, we have assembled a considerable amount of information about each of the three novel genes and, although we do not yet know the function of their protein products, we are continuing to move forward on several fronts to elucidate their responses and actions.

CONCLUSIONS

The fields of cold hardiness, cryobiology, and freeze tolerance are huge ones and we have only scratched the surface in this chapter with our focus on some of the metabolic and gene expression responses by freeze-tolerant organisms. Principles of biochemical adaptation that we have stated before have emerged again including (a) bending preexisting metabolic capacities to new functions and to new triggers, (b) producing minimum numbers of truly new protein adaptations, (c) employing low-molecular-weight carbohydrate protectants and selected small proteins to stabilize macromolecular structures under stress, (d) modulating regulatory mechanisms and signal transduction pathways to achieve new stress-specific outcomes (such as producing an uninhibited extreme hyperglycemia). Much, much more remains to be learned about natural freezing survival and with the help of amazing new genomics and pro-

teomics technologies, we hope to continue the search for many years to come. Remember, as Albert Einstein once said "Science is the game we play with God to find out what his rules are" and it is, perhaps, the most amazing game around.

SUGGESTED READING

Benson, E., Fuller, B., and Lane, N. (2004). *Life in the Frozen State*. CRC Press, Boca Raton. (Includes as one chapter, K. B. Storey and J. M. Storey, Physiology, biochemistry and molecular biology of vertebrate freeze tolerance: The wood frog). *Up-to-date analysis of all aspects of freezing survival: biophysics of ice and water*, *mechanisms of ice damage*, *natural freeze tolerance in animals and plants*, *medical cryopreservation*, *cryobanking of biological materials*, *vitrification*, *and cell preservation by drying*.

Duman, J. G. (2001). Antifreeze and ice nucleator proteins in terrestrial arthropods. *Ann Rev Physiol* **63**:327–357. *Function*, *structure*, *and regulation of these specialized proteins in insects and other terrestrial invertebrates. See also suggested reading in Chapter 14 for a review on fish antifreeze proteins*.

Fowler, S., and Thomashow, M. F. (2002). *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell* **14**:1675–1690. *Superb example of state-of-the-art usage of cDNA array screening technology for identifying cold-responsive genes that shows how "waves of changes" in gene expression provide both early versus late and transient versus sustained responses during cold acclimation*.

Margesin, R., and Schinner, F. (1999). *Cold-adapted Organisms: Ecology*, *Physiology*, *Enzymology and Molecular Biology*. Springer, Heidelberg. *Multiauthor book dealing with many different topics in natural cold hardiness of animals*, *plants*, *and microorganisms*.

Storey, K. B. (1997). Organic solutes in freezing tolerance. *Comp Biochem Physiol A* **117**:319–326. *Review of the functions and regulation of low-molecular-weight cryoprotectants used by cold-hardy animals*.

Storey, K. B., and Storey, J. M. (1996). Natural freezing survival in animals. *Ann Rev Ecol System* **27**:365–386. *General review emphasizing the types of freeze-tolerant animals and the principles of survival*.

Storey, K. B., and Storey, J. M. (2001). Hibernation: poikilotherms. In *Encyclopedia of Life Sciences*. MacMillan Reference, London. Online at www.els.net. *Easy-to-read review of how animals survive in the cold*.

Storey, K. B., and Storey, J. M. (eds.) (2001). *Cell and Molecular Responses to Stress. Vol. 2: Protein Adaptations and Signal Transduction*. Elsevier, Amsterdam. *Multiauthor book with review on gene expression in animal freezing survival*, *transgenic expression of antifreeze proteins*, *cold-adapted enzymes*, *cold-shock proteins*, *and antioxidant defenses*, *among others*.

Thomashow, D. F. (1999). Plant cold acclimation, freezing tolerance and regulatory mechanisms. *Ann Rev Plant Physiol Plant Mol Biol* **50**:571–599. *A good recent review of the principles of cold hardiness and freeze tolerance in plants touching on membrane protection*, *gene expression*, *and signal transduction*.