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Freeze Tolerance in Animals

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I. INTRODUCTION

For terrestrial ectotherms inhabiting temperate and polar regions of the earth, the subzero temperatures of winter pose a severe threat, since life processes in nature are incompatible with intracellular ice. Behavioral strategies allow some species to elude subzero exposure. For example, Monarch butterflies migrate to Mexico, various frog and turtle species choose underwater hibernation, and toads may dig one meter down into the earth to remain below the frost line. For many species, however, survival of the subzero temperatures of winter requires physiological and biochemical adaptations conferring cold hardiness.

At temperatures below the freezing point (FP) of body fluids, water exists in a metastable state, the probability of spontaneous nucleation increasing as temperature further decreases. The FP of body fluids of most animals (in the absence of cryoprotective measures) ranges from about −0.5°C for terrestrial animals to −1.7°C for marine animals adapted to full strength (1,000 mosmol) seawater. For survival at lower temperatures, cold-hardy ectotherms have one of two options. *1*) In freeze tolerance, ice formation in extracellular fluid compartments is tolerated, the resulting freeze concentration of body fluids leaving no undercooled compartment and stabilizing the intracellular environment against nucleation. *2*) In freeze avoidance, ice formation within the body is lethal and is avoided by deep depression of the supercooling point (SCP) of body fluids.

The present review focuses on freeze tolerance as a strategy of winter survival in animals. Our bias is toward the biochemistry of natural freeze tolerance; other authors provide more detailed reviews of phylogenetic, ecological, and physiological considerations (1, 2, 8, 9, 77, 93, 102, 113, 129, 187). To begin with, however, brief reviews of chilling and freezing injury in nonhardy systems and of the principles and adaptations involved in freeze tolerance versus freeze avoidance are instructive.

II. COLD HARDENING, FREEZE TOLERANCE, AND FREEZE AVOIDANCE

A. Chill and Freezing Injury

Injuries to nonhardy cells as a result of exposure to subzero temperatures are of two types: chilling injury and freezing injury. Injuries from chilling or hypothermia have as their basis the effects of low temperature on the physical properties of molecules and on rate processes (44, 66). These include *1*) temperature effects on the conformation, orientation, and mobility of lipids in membranes with effects on transmembrane diffusion and transport, binding of membrane-associated proteins, and the function of membrane-associated metabolic pathways (62); *2*) differential temperature effects on the various classes of weak bond interactions resulting in disruption

of the higher orders of protein structure and affecting both individual protein/enzyme function as well as integrated function of enzymes in a pathway, formation of multienzyme complexes, and enzyme associations with structural proteins (18, 55, 158); *3*) changes in dielectric permittivity and ionic activities (for water, changes in the dissociation constant, pK_W, with temperature change increase cellular pH as temperature decreases); and *4*) rate effects resulting from differential temperature coefficients for various processes and disrupting cellular functions including diffusion processes, active and carrier-mediated transport, activities of individual enzyme, and flux through metabolic pathways. Overall, low-temperature injuries are caused by the disruption of metabolic regulation, particularly cellular energetics, which, when too severe or prolonged, lead to irreversible and lethal metabolic imbalances.

Injuries from freezing include physical damage by ice crystals and the structural/metabolic consequences of freeze concentration (the removal of pure water into ice concentrates solutes in remaining liquid compartments). Both of these wreak havoc with subcellular structure and metabolic compartmentation and combine to make intracellular freezing lethal. These same factors are also potentially injurious when freezing is confined to extracellular compartments. Structural damage from ice (intensified when recrystallization occurs) includes distortion and deformation of cells trapped in minute channels of unfrozen extracellular solution, disruption of cell-cell connections, and damage to capillaries (92). Freeze concentration during extracellular ice formation can result in *1*) osmotic shock caused by the rapid redistribution of water and solutes across the cell membrane, *2*) a reduction in cell volume caused by water outflow which, when a critical minimum cell volume is exceeded, damages membrane structure, and *3*) dehydration and elevated solute levels (especially ions) in remaining liquid compartments that may have injurious effects on membrane or protein structure/function and subcellular organization (44, 92). The physical properties of molecules are also altered in freeze-concentrated solutions, and these can affect metabolic regulation during cooling/warming or during long-term storage at moderate subzero temperatures (44). In addition, metabolic damage may occur in the frozen state, since ice in extracellular fluid spaces imposes an ischemic and anoxic state on cells.

The key factors in lethality during extracellular freezing are still debated. Various hypotheses have been made. *1*) Lovelock (86) proposed that lethal injury resulted from extremely high levels of electrolytes produced by freeze concentration. Experimental evidence now argues against this for both freeze-tolerant animals (173) and cryopreserved cells (92). *2*) Meryman (94) proposed that cells can shrink only to a critical cell volume with further osmotic stress being irreversibly damaging. The often-observed lethal limit of ~65% of total water as ice for both freeze-tolerant animals (81, 173, 187) and cryopreserved mammalian cells (94) supports this view. *3*) Mazur (92) argues that the unfrozen fraction of extracellular fluid is the key factor and

not the composition of the extracellular solution or the extent of cell shrinkage during freezing. For single cells in solution, his data are persuasive, for photomicrographs show the consequence of a reduced unfrozen fraction: cells trapped and deformed in minute channels of unfrozen solution.

B. Cold Hardening

Adaptations for cold hardiness can be placed into two groups, i.e., those that sustain metabolic regulation and homeostasis at low temperature and those that preserve cell structure/function against the consequences of internal ice formation (143). Both freeze-tolerant and freeze-sensitive species must address the problems of maintaining homeostasis at low temperatures with adaptations that compensate for potential chilling injuries. This may require some alterations to membrane or protein composition to shift the optimal temperature range of cellular processes to a lower setting and to reset the metabolic balance between ATP-utilizing, ATP-producing, and passive processes in the cell for low temperature function. However, extensive compensatory adaptations that would preserve "normal" metabolic functions (growth, feeding, reproduction) at subzero temperatures may not be needed for species that spend the winter in a dormant or diapause state. Indeed, the differential effects of low temperature on metabolic processes may, in fact, be exploited to radically alter metabolism at low temperature. Adaptations that preserve cell structure/function with respect to the injurious consequences of ice formation are those that confer either freeze tolerance or freeze avoidance.

C. Freeze Tolerance

The phenomenon of freeze tolerance in living animals under natural climatic conditions refers to the freezing of extracellular water only. The adaptive processes that give rise to freeze tolerance are such as to render cytoplasmic water unfreezable. A few reports of the natural occurrence of intracellular ice exist but are now viewed as questionable. Only under exceptional circumstances in the laboratory, with the use of ultrafast rates ($100–600°C \cdot min^{-1}$) of freezing (producing minute ice crystals) and thawing (preventing recrystallization), has survivable intracellular freezing of single cells in suspension been demonstrated (92).

The requirements for success in natural freeze tolerance include the following: *1*) ice must be confined to extracellular compartments, and physical damage from ice crystals must be minimized; *2*) the rate of freezing must be slow and controlled to limit osmotic stress on cells, allow time for the redistribution of ions and other soluble metabolites between extra- and intracellular spaces, and permit controlled shrinkage of the cell membrane; *3*) cell volume reduction and dehydration beyond an injurious limit must be

avoided; and *4*) cells must survive and maintain homeostasis in the face of long-term ischemia imposed by the surrounding extracellular ice. Freezing in nature must also recognize environmental realities. Temperature change is most often only a few degrees per hour so rates of freezing are consequently slow. Inoculative freezing by environmental ice may occur. Multiple cycles of freezing and thawing must be survivable over a winter. Freezing is also at moderate subzero temperatures (generally −5 to −50°C), temperatures at which cells are still metabolically active and the process of ice recrystallization readily occurs. Nonetheless, a stable physical and metabolic state must be maintained, sometimes for weeks, in the frozen state.

A number of adaptations supporting successful freeze tolerance have been identified, and our discussion of these occupies the bulk of this article. Briefly, they include *1*) the use of specific ice-nucleating proteins to induce and control extracellular ice formation and the probable role of thermal hysteresis proteins in preventing recrystallization; *2*) adaptations for cell volume regulation and for stabilizing subcellular components against the consequences of freeze concentration, e.g., the accumulation of low-molecular-weight cryoprotectants for colligative action in cell volume regulation and stabilization of proteinacous components, mechanisms to stabilize membrane structure, and mechanisms that increase the content of unfreezable water in cells; and *3*) alterations to cellular metabolism to retain viability in the subzero and/or frozen state such as metabolic depression, a good capacity for anaerobiosis, and changes in membrane composition and enzyme type to allow low-temperature function.

D. Freeze Avoidance

Animals choosing the freeze-avoidance strategy must deal with the ever-present threat of lethal freezing below the FP of body fluids. Freezing prevention is achieved by two mechanisms: inhibition of ice nucleation (undercooling) and colligative depression of FP (44). Whole animal SCPs are depressed to a level such that spontaneous nucleation should not occur within the normal winter temperature experience of each species. For terrestrial arthropods, SCPs may range to −40°C or even lower (113, 132, 187). Adaptations supporting freeze avoidance include *1*) the elimination of potential ice nucleators, including shielding from inoculative freezing by environmental ice and the seasonally active removal or masking of endogenous nucleators (104, 121, 132, 283); *2*) the addition of specific thermal hysteresis (antifreeze) proteins to body fluids to inhibit the growth of embryo ice crystals (37); *3*) the accumulation of high concentrations of low-molecular-weight cryoprotectants [glycerol levels ranging from 15 to 25% of fresh weight are not uncommon (112, 132)] to provide colligative FP and SCP depression (185, 187); and *4*) dehydration to reduce the content of freezable water and elevate the effective concentration of cryoprotectants (112, 113). More extensive reviews of the

freeze-avoidance strategy of winter hardiness in animals have been published by other authors (2, 17, 37, 113, 132, 187).

III. PHYLOGENY OF FREEZE TOLERANCE

A tolerance for ice in extracellular fluid spaces forms part of the overwintering strategy of individual animal species from diverse phylogenetic groups. Included are many species of terrestrial insects (9, 13, 17, 33, 50, 82, 93, 95, 113, 114, 123); some intertidal bivalves, gastropods, barnacles, annelids, and nematodes (1, 17, 41, 102); and several species of frogs that hibernate on land (125, 144, 156). Freeze tolerance also occurs among Rotifera, Nematoda, and Tardigrada, although in these microfauna freezing survival is associated with encysted and anhydrobiotic forms (17, 32).

This review concentrates on freeze tolerance as it occurs in the three major groups of macrofauna: marine invertebrates, insects, and frogs. General characteristics of freeze tolerance in these groups are summarized below.

A. Marine Invertebrates

During winter, life in the intertidal zone can impose twice-daily exposures to subzero air temperatures on the invertebrate inhabitants. The inability to cope with such exposures appears to be the reason for the very low diversity of fauna in the intertidal zone of both Arctic and Antarctic regions and the predominance of a select group of cold-hardy species in subpolar regions (1). Such species, particularly sessile forms, are predominantly freeze tolerant. Selected species of barnacles (*Balanus balanoides*), bivalves (*Mytilus edulis, Modiolus demissus, Cardium edule, Venus mercenaria*), and gastropods (*Littorina littorea, Nassarius obsoletus, Acmaea digitalis, Melampus bidentatus*) have received the most study (26, 27, 29, 64, 102, 118, 173), whereas limited information is available for many other species (1, 76, 101, 107, 110, 161).

Cold hardiness in marine invertebrates must accommodate the facts of life and physiology in the intertidal environment, and, as such, freeze tolerance is the logical strategy. Animals cannot avoid intimate contact with seawater or inoculative freezing across semipermeable membranes but they can make use of the latent heat of freezing of trapped seawater (the volume of seawater in the mantle cavity may be 10 times that of tissue water) as a thermal buffer to maintain a stable body temperature (−2 to −3°C) during low-tide aerial exposures (173). Marine invertebrates are osmoconformers, and, as such, neither dehydration nor the accumulation of high concentrations of low-molecular-weight cryoprotectants can be used to improve freeze tolerance or allow freeze avoidance. However, cells are well adapted for dealing with osmotic stresses and have mechanisms of cell volume regulation that can be put to effective use in dealing with freeze concentration. On the

other hand, a freeze-avoidance strategy is apparently difficult to achieve in the intertidal environment, and, indeed, only one good example of this has been reported. The Antarctic limpet, *Patinigera polaris*, secretes a water-impermeable mucus containing glycoprotein thermal hysteresis factors that allow the animal to undercool to −10°C (53).

Previous reviewers provide more detailed accounts of the characteristics of freeze tolerance in individual species (1, 77, 102, 161). Animals undercool only a few degrees below the FP of body fluids (−1.7°C in full strength seawater) (1, 5, 84, 173). Ice forms only in extracellular spaces (76). Freezing survival is moderate; although 50% lethal dose (LD_{50}) temperatures as low as −18.6°C have been recorded (29), survival for times of 24 h or more at stable, maximal ice contents is generally limited to −8 to −11°C (41, 103, 118, 173). Winter animals can tolerate 65–80% of total body water as ice (1, 84, 102).

1. Factors influencing freezing survival of marine invertebrates

I) SALINITY. Acclimation to higher salinities improves freeze tolerance via colligative effects (1, 102).

II) TEMPERATURE. Acclimation to low temperature improves freeze tolerance in some species (1).

III) ANAEROBIOSIS. Marine invertebrates are excellent faculative anaerobes; conditions that induce anaerobiosis (aerial exposure, hypoxic water, valves clamped shut, acclimation to temperatures below 10°C) all improve freeze tolerance in a noncolligative manner (102). The molecular basis of this effect has not been explored but may be related to various metabolic changes during anaerobiosis, e.g., elevated Ca^{2+}, alanine, and succinate concentrations, decreased pH of body fluids, the initiation of anaerobic routes of ATP production, and anoxia-induced metabolic rate depression (102, 145).

IV) SEASONALITY AND GEOGRAPHIC VARIATION. Many species show a greater freeze tolerance in winter than in summer; this includes a tolerance for higher tissue ice content and a reduced lower lethal temperature (1, 29, 84). Animals from northern latitudes are more tolerant than those from southern, even when southern animals are given long exposure to northern climates (64, 102).

V) MICROHABITAT. The thermal inertia of rocks to which animals attach or an insulating cover of ice can keep microhabitat temperatures significantly above ambient temperatures, at least during short-term aerial exposures.

2. Adaptive strategies for freeze tolerance in the marine environment

I) ICE-NUCLEATING PROTEINS (INP). Seasonally occurring INPs induce and control extracellular ice formation during winter months; their presence has been demonstrated in *M. edulis* and *M. bidentatus* (5, 61, 84).

II) THERMAL HYSTERESIS PROTEINS. Antifreeze glycoproteins have been identified in *M. edulis* (162).

III) METABOLIC DEPRESSION AND ANAEROBIOSIS. Metabolic rate depression is clearly implicated as key to freezing survival. *1*) Winter dormancy is common among intertidal animals (64); *2*) low temperatures induce a switch to anaerobic metabolism [e.g., Q_{10} for O_2 consumption in *M. demissus* was 22 between 4 and 0°C (99)]; and *3*) anoxia induces metabolic rate depression (to levels 5–10% of aerobic rates; 145) and also improves freeze tolerance (1, 102).

IV) CRYOPROTECTANTS. Specific low-molecular-weight cryoprotectants are not accumulated; amounts of glycerol produced in *M. bidentatus* (3 μmol/g fresh wt; 84) and *B. balanoides* (1 mM; 26) are insignificant compared with cellular osmolarity (1,000 mosmol) in full-strength seawater. The absence of cryoprotectants is probably the reason that lower lethal temperatures are in the range of −10 to −15°C, compared with values that may exceed −50°C for polyol-containing freeze-tolerant insects.

V) IONS. A primary site of freezing damage in marine invertebrates appears to be injury to membrane structure and function, resulting during cell shrinkage. Murphy (102) demonstrated a Ca^{2+} dependency to freeze tolerance in *M. demissus*; conditions that improved freeze tolerance (anoxia, acclimation to 5 or 0°C) elevated blood Ca^{2+} content, and transfer from low- to high-Ca^{2+} seawater increased freeze tolerance. The mechanism of the Ca^{2+} effect appears to be interaction with the cell membrane to stabilize structure/function.

VI) MEMBRANES. Winter cold tolerance in barnacles may be aided by selected changes in the composition of membrane lipids (27, 164).

B. Insects

Many terrestrial insects are freeze tolerant, most commonly members of the Coleoptera, Diptera, Hymenoptera, and Lepidoptera (9, 13, 17, 33, 50, 82, 92, 113, 187). Examples of freeze tolerance can be found at all life stages. Various species whose development extends over more than 1 yr are freeze tolerant in more than one life stage (114) or in more than one larval instar (36); this strategy is particularly important for polar species that require more than one summer season to reach maturity.

Freeze tolerance is most highly developed in the Insecta. Freezing survival to −25 to −30°C is common, and a number of Arctic species have lower lethal temperatures of −55 or even −70°C (95, 114, 115). Pretreatment with freezing at −30°C or prior dessication can also allow some species to survive exposure to liquid nitrogen (2, 65). Long-term survival in a frozen state is geared to withstand a normal winter season; e.g., larvae of *Eurosta solidaginis* readily survived 12 wk of freezing at −16°C (138) but not 1 yr frozen (J. Storey, unpublished results). Survival while frozen depends on ATP production via fermentative pathways and the net depletion of adenylate and phos-

phagen reserves (138). Extracellular freezing is initiated, in most forms, at temperatures above −10°C, precipitated by the action of INPs (35, 186). However, Ring (115) lists several species that have extremely low SCPs and are also freeze tolerant; *Pytho deplanatus*, with a SCP of −54°C is the most extreme example. Some species show seasonal changes in SCP, higher in winter and lower in summer (98, 185), whereas others show constant high SCPs throughout the year (34, 39, 40). This variable behavior derives from the species-specific interaction between polyols and INPs in determining SCP. Low-molecular-weight carbohydrates are accumulated as cryoprotectants in most, but not all (50, 115), species. Glycerol is the most common, sorbitol occurs frequently, and ribitol, threitol, erythritol, trehalose, and sucrose have also been identified (9, 10, 33, 39, 40, 49, 50, 95, 113–115, 122, 185, 187). Freeze-tolerant insects frequently use a dual or multiple cryoprotectant system, glycerol and sorbitol being the most common combination (34, 35, 50, 97, 98).

1. Critical elements of freeze tolerance in insects

I) ICE-NUCLEATING PROTEINS. Hemolymph INPs are employed to initiate extracellular ice formation at relatively high subzero temperatures, allowing a controlled and slow reduction of cell volume and freeze concentration of cellular fluids.

II) THERMAL HYSTERESIS PROTEINS. These have been identified in some instances and appear to function in preventing recrystallization of extracellular ice.

III) CRYOPROTECTANTS. Polyhydric alcohols (sometimes sugars) are accumulated, provide colligative freezing point depression, and act to limit freeze concentration and stabilize subcellular structure and function. Fat body glycogen reserves fuel synthesis; production is most often keyed to temperature and/or developmental triggers.

IV) WATER CONTENT. Dehydration does not occur but the content of unfreezable water increases due to the actions of polyols and changes in high-molecular-weight glycogen/protein components.

V) SURVIVAL IN THE FROZEN STATE. Long-term survival is enhanced by a good anoxia tolerance and by metabolic rate depression, often diapause related.

VI) MEMBRANES. Structural stability during freeze concentration probably results from the use of trehalose and proline as protectants.

C. Terrestrially Hibernating Frogs

Four species of North American frogs that hibernate on land tolerate extracellular freezing. These are the wood frog *Rana sylvatica*, the gray tree frog *Hyla versicolor*, the spring peeper *Hyla crucifer*, and the chorus frog

Pseudacris triseriata (125, 144, 156). *Rana sylvatica* has an exceptional range that extends north of the Arctic circle. Natural freeze tolerance was first specifically documented by Schmid (125) (for *R. sylvatica* and *H. versicolor*) but can also be inferred from earlier studies on *R. sylvatica* (85) and *P. triseriata* (88). Two related species from British Columbia, *Rana aurora* and *Hyla regilla,* did not tolerate freezing (K. Storey, unpublished observations). Freeze tolerance does not extend to terrestrially hibernating toads and salamanders (156) or to aquatic hibernating frogs (125).

The limits on freezing survival for terrestrial frogs are quite narrow but sufficient for species survival in the protected hibernation sites chosen. Animals supercool to −2 or −3°C (80, 88, 125, 156, 157). Frogs from northern populations (Ontario and Minnesota) survive freezing exposures at temperatures of −6 to −8°C (125, 144); frogs sampled in the spring from Ohio populations are tolerant of only −3°C (80). Minimal winter temperatures measured at typical hibernation sites (at the soil surface under leaf litter and snow) are −4 to −7°C, even in Arctic sites (88, 96, 125) so the survival limits of these frogs appear to be well matched to the winter microenvironment. Long-term survival of freezing is good. Animals of all four species survived 3 days frozen at −3°C (156). Adult female *R. sylvatica* readily survived 13 days of freezing at −2.5°C, and survival was 50% for immature adults after 11 days at −6°C (144).

The physical characteristics of freezing include stiff limbs, ice crystals under the skin and interspersed with skeletal muscles, a mass of ice filling the abdominal cavity and surrounding all organs, no breathing, no heartbeat, and no bleeding when the aorta is severed. Organs such as liver and heart appear pale (as when perfused with saline) and are apparently so because of the withdrawal of blood. Large amounts of blood appear to be pooled and frozen in distended large vessels above the heart. Indeed, body fluids sampled from limbs just after thawing have hematocrit values of only 4%, compared with 24% for blood of control animals (G. McDonald and K. Storey, unpublished results). When frogs are thawed, heartbeat is the first noticeable sign of life, followed by gulping, then by a return of muscle tension in the limbs, and finally voluntary motor movements. Leg movements in response to pinching are observed between 4 and 24 h when frogs are thawed at 3°C and between 30 and 60 min when frogs of 2-g size are thawed at 23°C.

1. Adaptive strategies for freeze tolerance in frogs

I) CRYOPROTECTANTS. *Hyla versicolor* accumulates glycerol, whereas the other three species are novel in their use of glucose as a cryoprotectant (156). Synthesis is based on the catabolism of liver glycogen and is triggered only by ice nucleation in the body (139). Winter preparations do not include anticipatory cryoprotectant synthesis.

II) ICE-NUCLEATING AGENTS. The presence of specific INPs in extracellular fluids of frogs has not been tested. However, removal of nonspecific

nucleators may promote freezing survival: freeze tolerance is lost when frogs begin feeding in the spring (157).

III) WATER CONTENT. Whole body dehydration is rapidly lethal to frozen frogs. Animals frozen in direct contact with air (instead of in a thick mat of damp sphagnum moss) are "freeze dried" and dead within a few days. Since frog skin is highly water permeable, an important strategy for winter survival must be the choice of well-protected and humid hibernation sites.

IV) METABOLISM IN THE FROZEN STATE. Cessation of breathing, heartbeat, and blood flow places individual tissues and organs in an ischemic state during freezing. Survival is based on a good anaerobic potential and the use of endogenous energy (creatine phosphate, ATP) and fuel (glycogen, possibly amino acids) reserves with lactate and alanine accumulating as end products (137, 154, 155).

V) SPRING FREEZE TOLERANCE. Tolerance persists for all four species when frogs emerge from winter hibernation and move to breeding ponds but declines sharply later in the spring once animals begin to feed (157). The ability to synthesize cryoprotectants is progressively lost after emergence (but does not appear to impair freeze tolerance); for example, blood glucose in *R. sylvatica* was 56 and 14.6 μmol/ml after 2 days of freezing exposure in spring frogs from Ontario and Ohio populations, respectively (80, 157), compared with levels of $\sim$260 μmol/ml in identically treated autumn individuals (155). The reduced capacity for cryoprotectant synthesis in the spring has two causes: *1*) maximal rates of synthesis are reduced, and *2*) liver glycogen reserves are committed to other functions, probably to fuel animal activities at the breeding ponds (157).

IV. ICE AND WATER

A. Water Content

A reduction in total body water content of freeze-tolerant animals during winter hardening could increase the relative amount of unfreezable versus freezable water, reduce the effects of freeze concentration on cells, and improve freeze tolerance. Indeed, several studies have shown that dessication and freeze tolerance are positively correlated in plants (108), whereas various dessication-tolerant animals (e.g., *Artemia*, nematodes) are fully freeze tolerant when frozen at low water contents (32, 106). In addition, dehydration is an integral part of the freeze-avoidance strategy of cold hardiness among insects (112, 115).

For freeze-tolerant animals, however, a reduction in total body water content is not a part of cold hardening. For marine invertebrates, dehydration is clearly impossible, whereas for frogs, net water loss is dangerous: animals frozen without a protective covering of damp moss freeeze dry and die within a few days. Quantitative data are available only for insects, and

these show constant total body water during both cold hardening and over time in the frozen state. For *Eurosta solidaginis* larvae water content was 60–68% of fresh weight over the outdoor winter season and also during laboratory acclimations at reasonable relative humidities (81, 117). Constant water contents were also found for two Arctic species, *Pytho americanus* (61%) and *Xylophagus* sp. (64%), over a course of subzero temperature acclimation and recovery (115). Apparent dehydrations of freeze-tolerant Arctic insects during cold acclimation reported in an earlier study now appear to be artifacts of the freeze-drying technique used: the short drying times used did not remove all extractable water from animals with high polyol contents (114).

B. Ice Content

Ice content in the frozen state has been estimated by a number of authors. The most common technique is calorimetry (125, 173, 191), often modified to take into account the specific heats of wet and dry masses (32, 81). Cold-hardy insects and marine invertebrates generally tolerate ~65% of total body water as ice with levels as high as 90% reported (2, 29, 81, 84, 101, 126, 173, 190). Available estimates for frogs are somewhat lower, 35% ice for *H. versicolor* frozen at −6°C (125), 48% for autumn *R. sylvatica* at −4°C (144), and 56–65% for spring *R. sylvatica* frozen at −2 to −3°C (80).

Present data suggest that adaptive strategies with respect to ice content are of two kinds. For most species, the maximum ice content that is survivable is a constant, and cold hardening involves mechanisms to depress the temperature at which this content is reached. Thus freezing injury occurs at an ice content of 64–67% in muscle tissues of *V. mercenaria, M. edulis* (173), and in Ohio and New York populations of *E. solidaginis* (81) but the temperatures at which this ice content is reached are −6, −10, −22.5, and −27.5°C, respectively. Similarly, lethal temperatures for three intertidal *Littorina* species ranged between −16 and −20°C but lethal ice content was 82% in all cases (101). Parenthetically, studies on *M. edulis* appear to rule out high salt concentrations as a mechanism of freezing injury; Williams (173) found that the freezing limit was 64% ice for animals adapted to both 50 and 150% seawater despite widely different intracellular salt concentrations in the frozen state (e.g., K^+ levels were 189 and 490 mM, respectively, in the two states). A few species take a second strategy in dealing with ice content. These increase the maximum ice content that is survivable. Murphy (100) found that intertidal snails, compared with subtidal species, tolerated greater tissue ice contents.

Seasonal differences in the lethal freezing temperature for a single species are largely due to differences in the maximal amounts of tissue ice tolerated (100); the same is true of cold- versus warm-acclimated insects (180). Lethal amounts of ice also accumulate at higher temperatures in sum-

mer months (29, 84); e.g., the intertidal gastropod, *M. bidentatus*, had 75% of body water as ice at the winter lethal temperature for 50% (LT_{50}), −13°C, but only 3% ice at the summer LT_{50}, −5°C (84). The much reduced tolerance to freezing in summer is probably the result of intracellular nucleation.

The process of ice formation has been monitored for freeze-tolerant insect and frog species (80, 81). Maximal ice formation in *E. solidaginis* larvae exposed to −23°C proceeded in two phases and required at least 48 h (despite nucleation and freezing exotherm events that were over within 5 min). Over the first 6 h, ice formed at a rate of 7.8%/h; subsequently the rate decreased to 0.4%/h to reach a final content of 64% of total body water (81). In *R. sylvatica*, maximal ice formation required ~24 h for 14-g frogs held at −2 to −3°C; the half time was 6.5 h, and the rate of ice formation was linear at 2.9%/h (80). These data clearly demonstrate that physiological ice formation is a gradual process in freeze-tolerant animals, one that undoubtedly allows ample time for cell volume regulation as the freezing stress is applied.

C. Unfreezable Water

A significant portion of water in cells is unfreezable. This includes water that is intimately associated with structural components, e.g., involved in the ternary structure of proteins, hydration shells around solutes, and water lining membrane pores. In addition, in freeze-concentrated solutions at low temperatures, viscosity becomes so great and diffusion rates so low that further ice crystal growth is functionally prevented and impossible to detect over physiological time spans. Such water is often termed bound water, although Franks (44) cautions that "unfreezable water is the manifestation of a purely kinetic phenomenon, not to be confused with an equilibrium binding process."

How much unfreezable water exists in cells? Estimates for a number of active cell systems indicate an average of 20% (range 10–30%) unfreezable water (89, 92, 106), whereas various dessication-tolerant systems (seeds, nematodes, *Artemia*) can be dried to a level of 0.1 g/g dry wt (23). For freeze-tolerant animals, Williams (173) estimated 20% osmotically inactive water in *M. edulis*, whereas Zachariassen et al. (191) found 25% osmotically inactive water in *Eleodes blanchardi* beetles [freeze injury, however, occurred at ice contents <65% total body water (190)].

An increase in the amount of unfreezable water in cells is a key adaptive strategy for freeze tolerance. Cold hardiness in plants is correlated with an increased content of unfreezable or bound water (83). The same is true in insects; bound water content of 20,000-*g* supernatant fractions of *E. solidaginis* larvae increased by 328% during cold acclimation from 22 to −30°C (148). The molecular basis of this phenomenon was attributable to both low-molecular-weight, dialyzable (chiefly polyols) and high-molecular-weight, nondialyzable (protein, glycogen) soluble components (142, 148).

Overall, the increased content of bound or unfreezable water in fully cold-hardened cells may lead to a situation, near the lower lethal temperature, in which body water exists in only two forms, ice and unfreezable water.

V. ICE-NUCLEATING AND THERMAL HYSTERESIS PROTEINS

A. Ice-Nucleating Proteins

Reviews by Zachariassen (186, 187), Duman (35), and Duman et al. (37) provide more extensive discussion of the roles of hemolymph INPs in freeze-tolerant insects. First documented by Zachariassen and Hammel (189), specific, winter-active ice-nucleating activity in hemolymph has now been documented in many freeze-tolerant species including both insects (34, 36, 39, 40, 131, 185, 188) and intertidal mollusks (5, 61).

Specific INPs in extracellular compartments produce a controlled and noninjurious freezing in freeze-tolerant species. By their actions *1*) ice is confined to extracellular compartments; *2*) nucleation occurs at high subzero temperatures (above −10°C); *3*) undercooling, the difference between hemolymph FP and whole animal SCP, is minimized (usually to <2°C); *4*) the temperature at which ice formation occurs is stabilized; *5*) rates of ice formation can be kept low; *6*) transmembrane osmotic stress is applied gradually as the temperature falls; and *7*) ice formation is initiated at multiple extracellular sites, producing many small crystals. Physical and osmotic damage to cells is minimized or prevented, and freeze concentration, membrane deformation, and ischemia are applied gradually. The potential for nonspecific nucleation in intracellular or gut compartments is also eliminated, since, at equilibrium, no supercooled compartments remain.

Ice-nucleating agents in both freeze-tolerant insects and mollusks are proteinaceous (34, 38, 40, 61, 131, 189). Larvae of the cranefly, *Tipula trivittata*, have both protein and lipoprotein nucleators in hemolymph (39), both lipid and protein components being essential to the ice-nucleating function.

The INP from *Vespula maculata* has been purified (38). The protein has a molecular weight of 74,000 and an amino acid composition that includes 20% glutamate/glutamine, 12% serine, and 11% threonine. The hydrophilic nature of the protein may be the key to its nucleating ability, the structure of the protein perhaps providing site(s) that order water molecules into embryo crystal(s) and thereby reduce the energy barrier to nucleation (38). Zachariassen (187) suggests that nucleating ability also depends on interactions between molecules of INP. Dilutions of hemolymph lead to a progressive reduction in nucleating ability with activity lost at dilutions of 10^2 for gastropods (61) and 10^5–10^6 for insects (39, 188). Apparently, then, nucleation does not result from seeding by a single INP molecule but requires multiple INPs perhaps existing within some critical distance of each other.

B. Thermal Hysteresis Proteins

Thermal hysteresis proteins (THP) occur widely in freeze-avoiding insects where they lower hemolymph FP and SCP [but not melting point (MP)] in a noncolligative manner (35, 37, 187). They appear to be the key to successful freeze avoidance. Their occurrence has also been reported in freeze-tolerant forms (two species of insects and one intertidal bivalve) coexisting with INPs (5, 34, 36, 162). Until recently, the presence of two such agents with apparently contradictory functions was puzzling. Duman (35) initially suggested that THPs found their use in early autumn or spring, times when the species involved were not freeze tolerant. However, a more plausible role for THPs has recently been advanced. Knight and Duman (79) have shown that the THPs of *Dendroides canadensis* are extremely efficient at inhibiting ice recrystallization. The actions of multiple INPs in hemolymph result in the formation of many small ice crystals during freezing. However, small crystals are thermodynamically unstable and, over time in the frozen state, recrystallize into larger crystals, which have the potential to do physical damage (44). Rates of recrystallization are temperature dependent, and the phenomenon is particularly damaging for materials stored at temperatures above −60°C (92). Thus freeze-tolerant animals undoubtedly require some mechanism of inhibiting or limiting ice recrystallization to promote long-term survival in a frozen state. Such is, apparently, the function of THPs.

C. Regulation of Synthesis

Very little is known about the environmental triggers and biochemical regulatory mechanisms involved in the production of INPs. Zachariassen et al. (188) demonstrated that INP production in *E. solidaginis* was triggered by 5°C exposure. Triggering in other species is unknown except that in most cases INPs appear in hemolymph in autumn and disappear in spring (5, 35, 61, 186). The proteinaceous nature of nucleating agents suggests that both transcriptional and translational events are probably involved in regulating synthesis and, as such, synthesis may be subject to hormonal or developmental influences. The content of nonspecific nucleators in *E. solidaginis* from a freeze-avoiding Texas population appears to be influenced by juvenile hormone deprivation and replacement (16) but no studies have yet examined similar influences on INP production in freeze-tolerant insects.

Regulation of THP synthesis has received thorough attention from Duman and co-workers (35, 37, 38), working with both freeze-tolerant and freeze-avoiding insects. Photoperiod, temperature, thermoperiod, circadian rhythm, and hormonal controls all interact to control THP synthesis in freeze-tolerant larvae of *D. canadensis* (34, 67, 68, 70, 71). A photoperiod trigger of THP synthesis occurs between light:dark (LD) 11:13 and 10:14 as day length is shortened at 20°C (67). This trigger is modulated by temperature, however; at 10°C, a long photoperiod (LD 16:8) could not prevent THP

synthesis (67). Thermoperiod stimulates synthesis in the absence of photoperiod cues; cycles of 8 h at 25°C:16 h at 17°C induced the production of THPs under either constant dark or constant light conditions, whereas a 16:8 temperature cycle did not (71). Administration of juvenile hormone (JH) under long-day (LD 16:8) and high-temperature (20°C) conditions elevated THP levels, whereas treatment with an anti-JH compound, precocene II, under short-day (LD 8:16) conditions prevented the normal synthesis of THPs (68). Thus interacting controls by photoperiod and temperature, each perhaps modulating hormonal secretions, achieve a precise synthesis of THPs matched to the particular weather conditions of each autumn.

The capacity to alter overwintering behavior from a freeze-tolerance to a freeze-avoidance strategy has recently been reported for two beetle species (36, 69). Hemolymph INPs were lost with the switch to freeze-avoidance behavior but THPs were present throughout. These examples appear to challenge any thoughts that major biochemical adjustments are needed to confer freeze tolerance in a multicellular system.

VI. CRYOPROTECTANT METABOLISM IN INSECTS

A. Molecular Actions of Cryoprotectants

Zachariassen (187) summarizes the experimental findings on the role of polyols in the determination of SCP depression and ice content in freeze-tolerant insects and concludes that the cryoprotective effects are those expected from the colligative properties of polyols. Thus in freeze-tolerant forms the effect of polyols on hemolymph SCPs, acting in the presence of hemolymph INPs, is simply equivalent to the MP depression. The effect of cryoprotectants on ice content in the frozen state is, similarly, equivalent to a reduction of the amount of ice in a purely colligative manner.

In a freeze-tolerant system the major role of cryoprotectants is in limiting cell volume changes with freezing such that freeze concentration beyond a critical cell volume does not occur within the normal subzero temperature experience of each species or population. Prior to freezing, cryoprotectants are in equilibrium between intra- and extracellular fluids (150). During freezing, as pure water is drawn into extracellular ice crystals, cryoprotectant concentration rises in the remaining aqueous phase. Effects of the presence of cryoprotectants in the system, compared with a system without cryoprotectants at the same subzero temperature, include *1*) a reduced osmotic outflow of water from cells and, therefore, reduced cell dehydration and cell volume changes; *2*) a reduced freeze-concentration effect: the percent change in solute concentration of the unfrozen fraction, after freezing is complete, is lower when initial solute concentrations are high; *3*) lower electrolyte concentrations; and *4*) a greater unfrozen fraction of extracellular solution (44, 92).

Not all freeze-tolerant animals produce cryoprotectants, and cryoprotectants are not compulsory for freeze tolerance. Low-molecular-weight

cryoprotectants are not present in freeze-tolerant marine invertebrates (26, 84), have not been identified in numerous insect species (both freeze tolerant and freeze avoiding; 50, 132), and are produced in much reduced amounts in spring frogs (80, 157). Animals appear to be able to survive freezing without cryoprotectants when freezing exposures are at mild subzero temperatures and when the resulting freeze concentration does not stress cells beyond the critical cell volume. Thus Hansen (50) found a strong correlation between glycerol concentration and the lowest subzero temperature tolerated by various species, species without cryoprotectant surviving freezing at only −8 to −16°C.

Although various low-molecular-weight solutes could serve the colligative functions described above, the choice of polyhydric alcohols and sugars as natural cryoprotectants may be related to other factors.

1) Since few sugars or polyols will crystallize spontaneously from aqueous solutions, the content of unfreezable water is stabilized (44).

2) Polyhydric alcohols stabilize the native state of proteins by maintaining the hydration shell under conditions of lowered water activity (30). Actions of polyols also extend to direct hydrogen bonding with polar residues on proteins when water content is very low (24). In addition, glycerol, even at molar concentrations, resembles water almost exactly in its ability to stabilize and maintain hydrophobic interactions. The protective actions of polyols on enzyme structure, and thus function, are also well known from their ability to inhibit thermal denaturation of proteins (6, 43, 46). Thus the presence of polyols in freeze-tolerant insects may be key to preserving homeostasis in the frozen state by stabilizing individual enzymes/proteins as well as macromolecular interactions (e.g., multienzyme complexes, enzyme associations with subcellular structural components) against the potential denaturing effects of subzero temperatures and low water content.

3) Naturally occurring polyols are relatively nontoxic to cells at the high concentrations required. They are both chemically inert (i.e., do not show nonenzymatic chemical reactions with cellular components) and biochemically inert (they are "dead-end" products, not intermediates in metabolism). Polyols are compatible solutes, causing very little perturbation of the activities or kinetic properties of enzymes, even at molar levels (66).

4) Natural cryoprotectants are freely penetrating to provide protection to all cells and readily equilibrate across the cell membrane as freeze concentration proceeds.

5) Polyols may help to limit physical damage from ice; Baust (8) has shown that ice crystals grown in a glycerol solution are blunted.

B. Carbohydrate Cryoprotectants

Low-molecular-weight carbohydrates as cryoprotectants are a well-known feature of insect cold hardiness. Overall, glycerol (a C_3) is the most common cryoprotectant nd is widely utilized by both freeze-tolerant and freeze-avoiding species (33, 50, 95, 113, 114, 132). Other cryoprotectants re-

ported in freeze-tolerant insects include the polyols sorbitol (C_6), ribitol (C_5), erythritol and threitol (both C_4), as well as C_{12} sugars (trehalose, sucrose) (10, 34, 36, 39, 49, 50, 97, 98, 140). C_6 sugars (glucose, fructose) sometimes appear in low levels but their role as cryoprotectants is questionable.

Single cryoprotectant systems occur in some freeze-tolerant insects [e.g., glycerol (14, 40, 114), sorbitol (39), ribitol (49)], but dual cryoprotectant systems also occur frequently [e.g., glycerol and sorbitol (34, 36, 98) or sorbitol and threitol (97)]. Trehalose is the major cryoprotectant in some species (3, 57) but more often occurs as one component of a multiple cryoprotectant system (98, 132) where it appears to have a distinct function in membrane protection (see sect. VIII*A*).

Typically cryoprotectant production begins in the early autumn, builds to a plateau level over midwinter, and then falls in early spring (15, 165). Where dual cryoprotectant (glycerol and sorbitol) systems occur, glycerol production begins earlier and the pool is maintained longer than sorbitol (Fig. 4) (34, 98). Where sorbitol and threitol are the pair, sorbitol is produced first (97). Geographic variations in weather conditions may modify the timing of polyol production. Thus, compared with Canadian populations, New York populations of *E. solidaginis* showed several weeks' delay in the accumulation of both glycerol and sorbitol and, oppositely, lost polyols earlier in spring (98, 140).

C. Pathways of Polyol Synthesis

The metabolic pathways for synthesis of glycerol, sorbitol, and trehalose are shown in Figure 1. Biosynthesis of all carbohydrate cryoprotectants uses glycogen as the substrate; this has now been well documented for several species (45, 57, 112, 114, 127, 136, 149, 165, 177, 180). Carbon stoichiometry is upheld, the sum of cryoprotectants produced representing over 90% of the glycogen carbon lost (45, 57, 112, 136). Earlier suggestions that triglyceride reserves could be utilized for glycerol biosynthesis (98) have not held up in tested species (177). In fact, both total glyceride and total protein reserves often remain constant during autumn cold hardening and throughout the winter (52, 112, 140).

Glycerol or sorbitol biosynthesis proceeds in two steps (dehydrogenase and phosphatase reactions) from C_3 or C_6 intermediates of glycolysis; the simplicity of synthesis of these two compounds may account for their widespread use in nature. For sorbitol biosynthesis, precursor-product relationships have clearly documented the synthetic pathway; induction of sorbitol synthesis by an abrupt switch from 13 to 3°C produced sequential rises in glucose 6-phosphate (Glc-6-*P*), glucose, and sorbitol levels in *E. solidaginis* larvae after 1, 2, and 24 h, respectively (136). This clearly demonstrated that the route of sorbitol synthesis from glycogen is via glycogen phosphorylase, phosphoglucomutase, glucose-6-phosphatase, and polyol dehydrogenase (Fig.

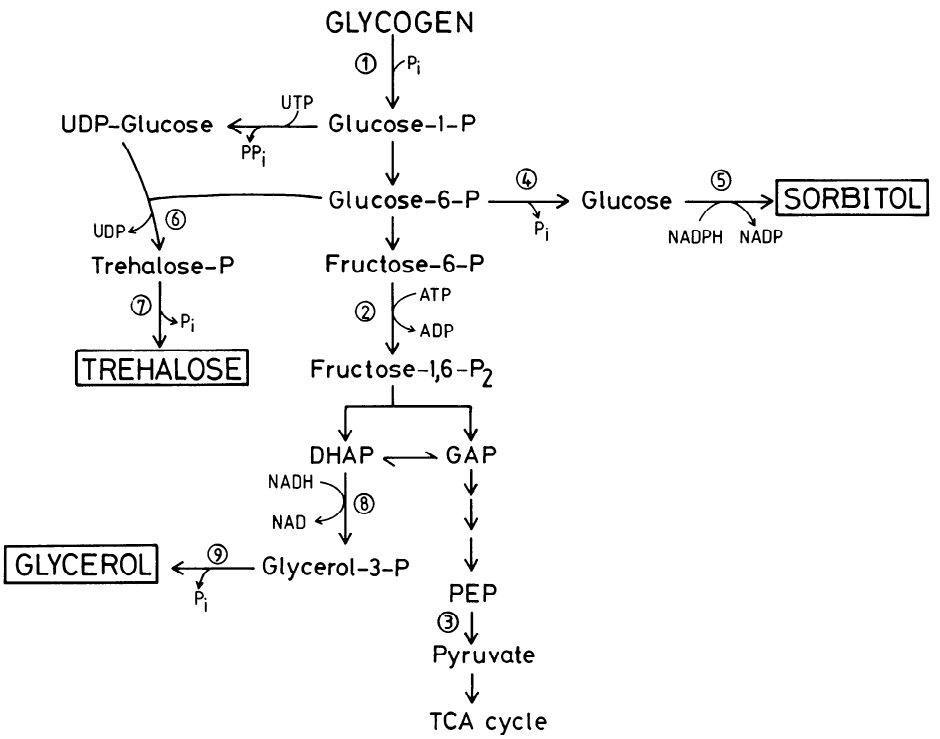


FIG. 1. Synthesis of major cryoprotectants in insects: glycerol, sorbitol, and trehalose. Enzymes are *1*) glycogen phosphorylase, *2*) phosphofructokinase, *3*) pyruvate kinase, *4*) glucose-6-phosphatase, *5*) polyol dehydrogenase, *6*) trehalose-6-phosphate synthase, *7*) trehalose-6-phosphatase, *8*) glycerol-3-phosphate dehydrogenase, *9*) glycerol-3-phosphatase. TCA, tricarboxylic acid cycle; DHAP, dihydroxyacetonephosphate; GAP, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate.

1). Similarly, glycerol synthesis via glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase was indicated by an accumulation of glycerol 3-phosphate (glycerol-3-*P*) when active synthesis was blocked by an ambient temperature drop below 5°C (149).

Synthesis of other polyols makes use of the broadly specific sugar phosphate phosphatases and NADP-dependent polyol dehydrogenases found in insects (21, 22, 75, 159) to convert sugar phosphates into polyols. C_4 and C_5 polyols are derived from pentose phosphate cycle intermediates. Species-specific molecular mechanisms must be involved in directing the catabolism of glycogen toward the appropriate sugar phosphate and perhaps also in promoting the accumulation of the requisite C_4 or C_5 sugar, since insect polyol dehydrogenases typically have high K_m values for sugars (21, 159). The cryoprotectants accumulated by *Belgica antarctica* appeared to be influenced by the mixture of dietary sugars available (10).

D. Provision of Reducing Equivalents and ATP

1. Provision of NADH and NADPH

Synthesis of all polyols requires reducing power in the form of NADPH or NADH. Available experimental evidence indicates that the pentose phosphate cycle is the source of these reducing equivalents (74, 170, 178). A recent suggestion by Hochachka and Somero (66) that NADH generated by the tricarboxylic acid cycle could be used for polyol biosynthesis does not hold up to scrutiny.

The stoichiometry of the pentose phosphate cycle can be written as follows

$$6C_6 + 6NADP^+ \rightarrow 4C_6 + 2C_3 + 6CO_2 + 12NADPH + 12H^+ + P_i$$

We can calculate, therefore, that sufficient reducing equivalents for glycerol or sorbitol synthesis would result when 86 or 46%, respectively, of carbon is processed around the cycle before entering the polyol pool (assuming NADPH:NAD transhydrogenase activity is available to support glycerol-3-phosphate dehydrogenase function and that C_3 products of the cycle can be reconverted to C_6 during sorbitol synthesis). The resulting efficiency of carbon conversion from glycogen to glycerol is 84%: synthesis of 12 mol glycerol (from $6C_6$) is supported by the oxidation of 1.13 mol of C_6 (1 mol lost in the pentose phosphate cycle and 0.13 mol C_6 used to generate the 5 mol ATP required by the phosphofructokinase reaction). For sorbitol synthesis, which has no ATP requirement, the conversion efficiency is higher, at 92%. These amounts agree closely with measured carbon stoichiometries for polyol synthesis in vivo (45, 136, 149).

Experimental studies have demonstrated that pentose phosphate cycle activity is increased, relative to carbon flow through glycolysis, during low-temperature-induced polyol synthesis (74, 170, 178). Catabolism of [1-^{14}C] versus [6-^{14}C]glucose (C-1 carbon is lost in the pentose phosphate cycle, C-6 is lost only in the tricarboxylic acid cycle) was compared at high and low temperatures. Low-temperature exposure, stimulating polyol synthesis, had two effects. *1*) The relative amount of $^{14}CO_2$ released from C-1-labeled glucose increased greatly (e.g., C-1:C-6 of expired CO_2 was 2:1 at 2°C and 1:1 at 22°C in *Protophormia terranovae* and 11:1 after 8 h at 5°C versus 2:1 at 15°C in *E. solidaginis*). *2*) Incorporation of ^{14}C into polyol pools was much higher from C-6- versus C-1-labeled glucose (a 10:1 ratio over the first 2 days of glycerol synthesis in *P. terranovae*, up to 2:1 for glycerol or sorbitol synthesis in *E. solidaginis*) (170, 178). These effects did not occur during cold exposure of a nontolerant species, *Musca domestica* (C-1:C-6 ratio in $^{14}CO_2$ was 1:1 at both 22 and 2°C) (178).

2. Provision of ATP

Synthesis of glycerol requires ATP input at the phosphofructokinase reaction. For this reason, glycerol synthesis should be optimal under aerobic conditions and may be severely limited if low oxygen conditions prevail. Energetic considerations may thus be one reason for the typical pattern of anticipatory synthesis of glycerol during autumn months and may also be the reason why sorbitol came into use as a cryoprotectant. Although it is a less efficient use of carbon, production of C_6 sorbitol, which has no ATP requirement, can go forward under oxygen-limited conditions. Such an argument explains the pattern of polyol accumulation in diapause eggs of *Bombyx mori;* these experience an increasingly oxygen-limited state as the serosal cuticle is formed and preferentially accumulate sorbitol over glycerol during diapause initiation (45, 133).

We recently tested the capacity of *E. solidaginis* larvae to produce cryoprotectants under a N_2 gas atmosphere. Production of glycerol was reduced to 57% of the amounts accumulated by aerobic controls but the shortfall in cryoprotectant was made up by sorbitol production with the result that total hydroxyl equivalents (11) under anoxic conditions were fully 93% of aerobic values (K. Storey and J. Storey, unpublished results). However, anoxic polyol synthesis required an 18% greater consumption of glycogen to support anaerobic ATP production.

E. Enzymatic Regulation of Polyol Synthesis

In terms of carbon flow, polyol synthesis is an enormously important event in the cold hardening of insects. Total polyol content generally reaches at least 0.5 M in freeze-tolerant forms and ~2 M in freeze-avoiding forms and utilizes almost the entire glycogen reserve of the animal (45, 112, 140). Strict regulation must, therefore, be applied to efficiently channel carbon into polyol pools.

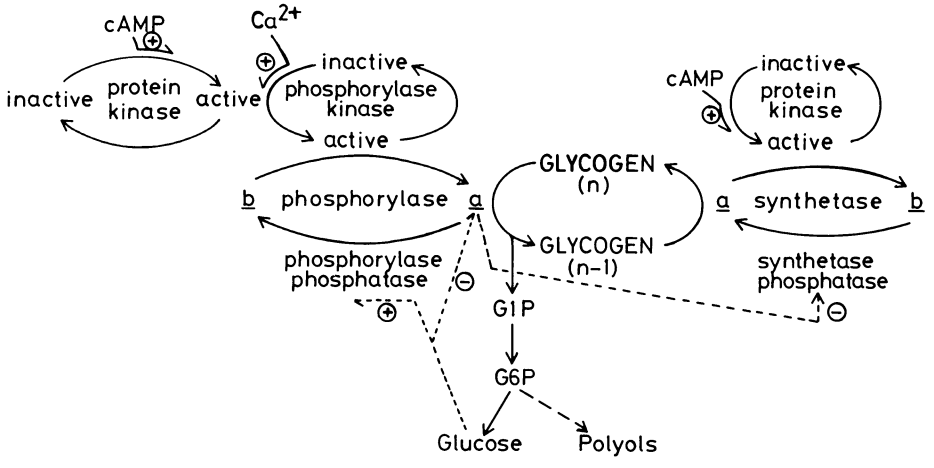
Polyol synthesis takes place in the fat body of insects. Preparations by fat body for overwintering include an accumulation of glycogen reserves (112, 140) and an enhancement of the catalytic potential of the top portion of glycolysis to deal with the high carbon flux of polyol synthesis. Maximal activities of glycogen phosphorylase, hexokinase, and phosphofructokinase are increased, whereas enzyme activities in the lower portion of glycolysis are unchanged (153). Activities of specific dehydrogenases and phosphatases involved in polyol synthesis are also increased (153, 166, 176). However, changes in the isozymic forms of enzymes do not appear to be required (152).

1. Glycogen phosphorylase

The regulation of glycogen phosphorylase is a prime example of direct temperature control over polyol synthesis. The enzyme in fat body of many

insect species is cold activated, including both cold-hardy [*Hyalophora cecropia, Philosamia cynthia, E. solidaginis* (58, 153, 192)] and non-cold-hardy [locusts, crickets (192)] species. The enzyme is also subject to normal cyclic AMP (cAMP)- and Ca^{2+}-stimulated hormonal regulation and shows shock activation (192).

The regulatory control of glycogen phosphorylase in animals is shown in Figure 2. Cold activation of fat body phosphorylase takes the form of a rapid conversion of the inactive *b* form of the enzyme to the active *a* form. The percentage of enzyme in the *a* form rises from ~10% in fat body of control animals at 20–25°C to 40–80% within ~2 h at 2–4°C (58, 192). Cold activation of phosphorylase occurs equally well in isolated fat bodies (192), demonstrating that hormonal or neuronal mediation is not required. As in other animals, the proportions of active and inactive forms of the enzyme are controlled by the actions of phosphorylase kinase and phosphorylase phosphatase, mediating the phosphorylation and dephosphorylation of the enzyme, respectively. Differential temperature effects on the activities of these two enzymes provide the key to low-temperature activation of phosphorylase (56, 60). Both enzymes are active in fat body at warm (25°C) temperature, their



NOVEL REGULATORY FEATURES IN COLD HARDY ANIMALS

Insects:	0–5°C Inactivation of phosphorylase phosphatase
Frogs:	Freezing stimulated increase in total phoshorylase Loss of homeostatic control by glucose?

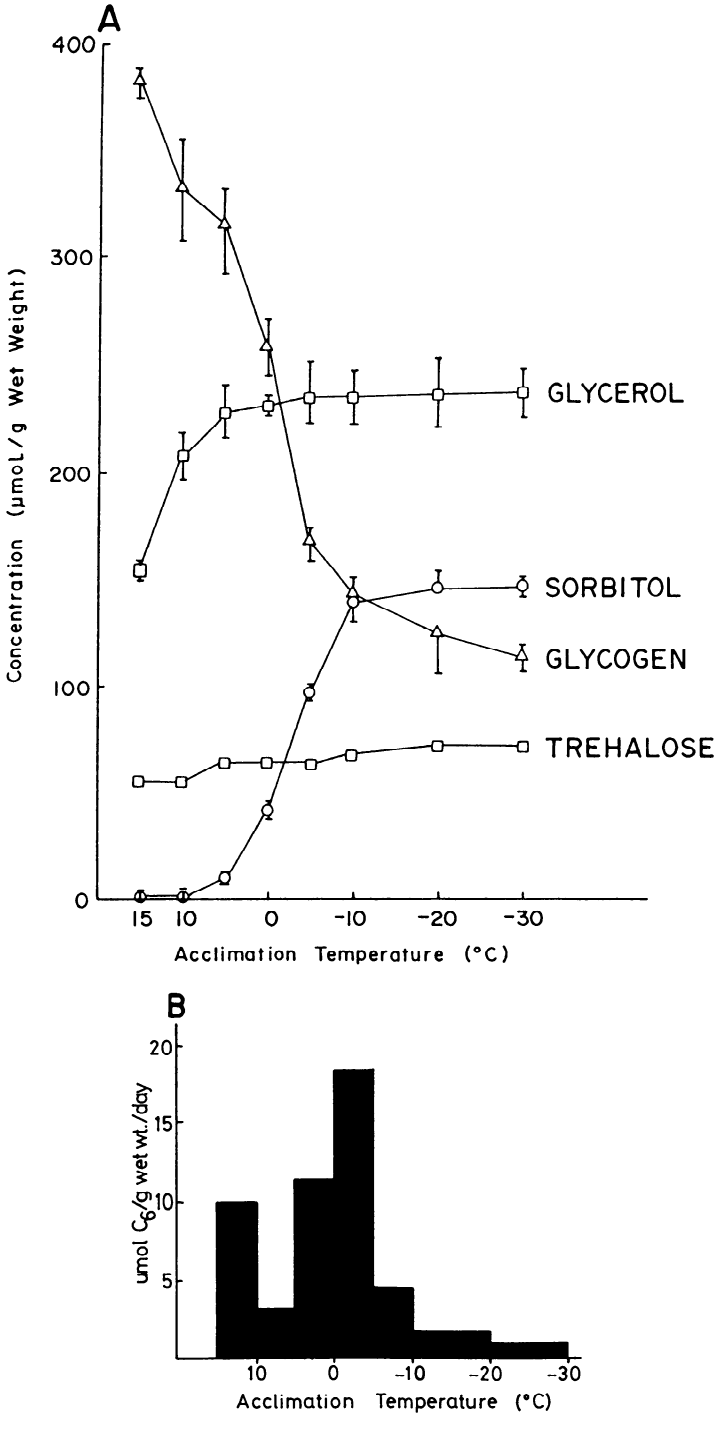
FIG. 2. Control of glycogen metabolism and molecular mechanisms of glucose homeostasis in animals.

activities balanced to maintain a low percent of phosphorylase *a*. At low temperature (0°C), phosphorylase kinase is not activated [the enzyme shows a normal Arrhenius plot with activation energy (E_a) = 11 kcal/mol and equivalent K_m values for phosphorylase *b* at 30 and 0°C (4)] but activity persists. Phosphatase activity, however, drops precipitously at low temperature (56). The result is that kinase activity greatly exceeds phosphatase activity at low temperature, leading to a net activation of glycogen phosphorylase over time brought about by simple temperature effects on the enzymes involved. Indeed, the effect was readily demonstrated in vitro with mixtures of the three enzymes from fat body of *P. cynthia;* phosphorylase *a* content rapidly increased in mixtures incubated at 0°C, whereas at 25°C there was little net change in phosphorylase *a* content (56).

The temperature range for cold activation of glycogen phosphorylase is similar between species. Ziegler et al. (192) incubated silk moth fat bodies in vitro; the percent phosphorylase *a* remained constant between 30 and 15°C, was increased slightly at 10°C, and then rose 1.6- and 2.5-fold at 5 and 0°C, respectively. On the basis of changes in carbon flow, phosphorylase activation in other species must follow a similar temperature-sensitive pattern, initiated at ~5°C and maximal between 0 and −5°C (Fig. 3) (78, 142).

2. Phosphofructokinase

Low-temperature activation of glycogen phosphorylase initiates carbon flow but cannot determine the resulting product (as noted above, trehalose, sorbitol, and glycerol are at least three possibilities). For this, regulation at other sites is needed. To some extent the product formed will be determined by the enzyme complement present in individual species, each product depending on the presence of unique enzymes (e.g., glycerol-3-phosphatase, polyol dehydrogenase, trehalose synthase). Species that accumulate trehalose or sorbitol in response during cold hardening require carbon to be diverted from glycolysis at the C_6 level. Tight regulation of 6-phosphofructokinase (PFK) must be applied to allow a nearly quantitative conversion of glycogen to these products. Modulation of the maximal activity of PFK in fat body is one mechanism involved: e.g., activity of PFK was 20-fold higher in fat bodies of two species of glycerol-accumulating insects than in two species of trehalose-accumulating insects (59). However, a number of species produce both glycerol and sorbitol as cryoprotectants so a differential regulation of PFK must occur to individually control the synthesis of each product. In *E. solidaginis* such regulation must account for the synthesis of glycerol (requiring PFK activity) at high temperatures and sorbitol synthesis (requiring PFK inactivation) at low temperatures (Fig. 3) (136, 149). Similarly, diapause eggs of *B. mori* accumulate equal amounts of glycerol and sorbitol when incubated at 25°C, but at 5 or 1°C sorbitol accumulation is about threefold higher than glycerol (45).


A
400
300
200
100
0
Concentration (µmol/g Wet Weight)
GLYCEROL
SORBITOL
GLYCOGEN
TREHALOSE
15 10 0 -10 -20 -30
Acclimation Temperature (°C)
B
20
15
10
5
µmol C6/g wet wt./day
10 0 -10 -20 -30
Acclimation Temperature (°C)

Kinetic studies of PFK from *E. solidaginis* have shown the factors involved in depressing enzyme function at low temperatures (141). Enzyme maximal activity is extremely sensitive to low temperature; Arrhenius plots are linear but have a very steep slope (E_a = 19,800 cal/mol), producing a Q_{10} of 3.6. By contrast, beef heart PFK shows a Q_{10} of 2. Low temperature also has adverse effects on enzyme kinetic properties: affinity for fructose-6 phosphate decreases and activators (AMP, fructose-2,6-bisphosphate) are less effective. In addition, the enzyme is subject to inhibitions by glycerol-3-*P* and sorbitol, both of which accumulate at low temperature (149), whereas levels of the potent activator, fructose-2,6-bisphosphate, decline by 50% within 24 h in cold-exposed larvae (K. Storey, unpublished data). Net effects of temperature and modulators (at physiological levels) on *E. solidaginis* PFK in vitro result in a 20-fold decrease in affinity for fructose-6-phosphate at low, compared with high temperature ($S_{0.5}$ = 0.54 mM at 25°C and 10.2 mM at 5°C) (141).

3. Other enzymatic controls

For species that produce sorbitol, carbon flow is largely directed by low-temperature activation of glycogen phosphorylase coupled to low-temperature inactivation of PFK. Additional regulatory factors may include the following. *1*) Glucose-6-phosphatase shows a higher affinity for substrate at low temperature (K_m for Glc-6-*P* decreases by 30% at 3 vs. 22°C) (142); and *2*) polyol dehydrogenase activity increases during low-temperature acclimation (153), and function of the high K_m enzyme (159) is promoted by elevated glucose levels in cold-exposed animals (136).

Species that produce glycerol require a cold active PFK and additional controls to divert the major percentage of carbon flow out of glycolysis at the level of dihydroxyacetone-phosphate (Fig. 1) (3–4% of hexose phosphates must be fully oxidized to provide the ATP required by PFK). Controls might act at various enzyme sites including the following.

I) GLYCEROL-3-PHOSPHATE DEHYDROGENASE. Activity of the enzyme rises dramatically during winter diapause (166). Low temperature also increases enzyme substrate affinity for dihydroxyacetone-phosphate (K_m is 0.48 at 22°C to 0.18 mM at 0°C) in glycerol-producing forms (176).

II) GLYCEROL-3-*P* OXIDATION. Glycerol-3-*P* diversion into glycerol synthesis would be facilitated by a low-temperature inhibition of alternative routes

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FIG. 3. *A*: accumulation of polyols and sugars as a function of decreasing temperature, 1°C/day from 15 to −30°C, in larvae of goldenrod gall fly *Eurosta solidaginis*. Glycerol is accumulated at higher temperatures and synthesis stops below 5°C. Sorbitol synthesis is triggered at 3–5°C with maximal production between 0 and −5°C. Glycogen is expressed in glucosyl units. *B*: data for glycogen replotted to show rates of glycogen breakdown versus temperature. [*A*: from Storey et al. (149). *B*: from Storey (142).]

of glycerol-3-*P* catabolism, namely oxidation by mitochondria. However, neither mitochondrial glycerol-3-*P* oxidase nor cytochrome oxidase showed unusual properties (Q_{10} values ~2), which would suggest specific inhibition of glycerol-3-*P* oxidation at low temperature (176).

III) GLYCEROL-3-PHOSPHATASE. Activity was present in slightly higher (25–35%) amounts in hibernating versus nonhibernating *P. terranovae* but there was no indication of cold activation (Q_{10} = 2.1) of the enzyme (176). In *Chilo suppressalis,* however, activity of acid phosphatase (utilizing glycerol-3-*P* as substrate) was significantly higher in diapausing versus nondiapausing or postdiapause larvae, and a strong correlation between acid phosphatase activity and glycerol levels was seen over the winter (169).

IV) PYRUVATE KINASE. Inhibitory control of glycolysis at this locus could route flux toward glycerol synthesis. Wood et al. (176) found no evidence for specific low-temperature inactivation of pyruvate kinase in *P. terranovae.* However, temperature effects on the kinetic properties of *E. solidaginis* pyruvate kinase would decrease enzyme activity at low temperature: at 10 compared with 23°C, affinity for substrates (phosphoenolpyruvate and ADP) is decreased, inhibition by alanine increases, and activation by fructose-1,6-bisphosphate is reduced (H. MacDonald and K. Storey, unpublished data).

F. Triggering of Polyol Synthesis

Since ambient weather conditions can be extremely variable from day to day or year to year, it is important for cold hardening to have an anticipatory component so that animals are physiologically and biochemically prepared to withstand freezing exposures before these occur naturally. Triggering of cryoprotectant synthesis can take a variety of forms. Animals can respond directly to environmental cues (temperature, photoperiod, humidity, food availability) or responses can be to a greater or lesser degree cued to endogenous factors (hormones, development, diapause).

1. Preparatory measures for polyol synthesis

As prerequisites to polyol synthesis, a variety of metabolic adjustments are needed, occurring over the weeks before polyol production begins. For example, fat body glycogen reserves must be built up and all the requisite enzymes must be put in place (requiring changes in the activities of various constitutive enzymes, de novo induction of synthesis of others). Also required may be the removal of feedback regulatory mechanisms that would normally function in fat body to limit glycogenolysis. Virtually no studies have examined the triggering and regulation of preparatory measures directly, but a few studies have examined the appearance of the capacity for polyol synthesis.

Nordin et al. (105) have recently provided excellent evidence of the role of development in programming cells to respond to cold stress with glycerol synthesis. They demonstrated that only 5th instar of *Ostrinia nubilalis* were capable of glycerol biosynthesis when exposed to 5°C. The capacity was lacking in 2nd, 3rd, and 4th instar larvae (even diapause-bound 4th instars). In this species, then, the developmental transition to the 5th instar is key to setting in place the capacity for glycerol biosynthesis. Similarly, only the 3rd instar of *E. solidaginis* (the overwintering form) appears to be capable of glycerol synthesis (98). Such a system, putting the biochemical machinery for polyol synthesis in place as a consequence of the molt to the overwintering form (probably mediated via hormonal signals), may be widely applicable to the many species that overwinter in only one developmental stage. For insects that are multivoltine or that can overwinter in more than one life stage (114), additional controls must be added to induce the capacity for polyol synthesis at the appropriate time. These controls may include photoperiod and temperatures cues, often acting at an earlier life stage and probably translated into hormonal signals (160, 172).

Since many species overwinter in a diapause state and polyol synthesis frequently begins concurrent with diapause initiation (160), the capacity for polyol synthesis might be induced as one of the preparations for diapause. Such a system operates for the embryonic diapause of silkworm eggs. The actions of diapause hormone on the ovaries of females during egg development stimulate glycogen accumulation and a hypertrophic status in diapause-bound eggs and prepare the eggs to begin polyol accumulation concurrent with diapause initiation (182). In other species, however, development of the capacity for polyol synthesis is not linked to diapause preparations. Thus both diapausing and nondiapausing 5th instar larvae of *O. nubilalis* and pupae of *Papilio machaon* have the capacity to produce glycerol in response to cold exposure (105, 127).

2. Temperature triggering of polyol production

For vast numbers of insect species the immediate trigger initiating polyol production (provided the biosynthesic capacity is in place) is low temperature (15, 57, 105, 116, 127, 136, 177, 192). Laboratory experiments have shown that the trigger temperature is most often in the range between 0 and 5°C (116, 142, 192) with maximal rates of synthesis at somewhat lower temperatures, 0 to −5°C (Fig. 3) (12, 51, 78, 116, 142). Animals in nature probably respond not just to a trigger temperature but to a thermoperiod cue (e.g., some threshold length of time at or below the trigger temperature) to key polyol production to the average thermal regime. For example, sorbitol accumulation in a field population of *E. solidaginis* was not initiated in response to individual nights (during October) with minimal temperatures below 3°C [the laboratory-determined trigger (116)], but began only in re-

sponse to long-term chilling (6 consecutive days with daily average temperatures below 5°C in mid-November) (Fig. 4) (140). Low temperature is the simplest and most direct signal of impending winter, and temperature trig-

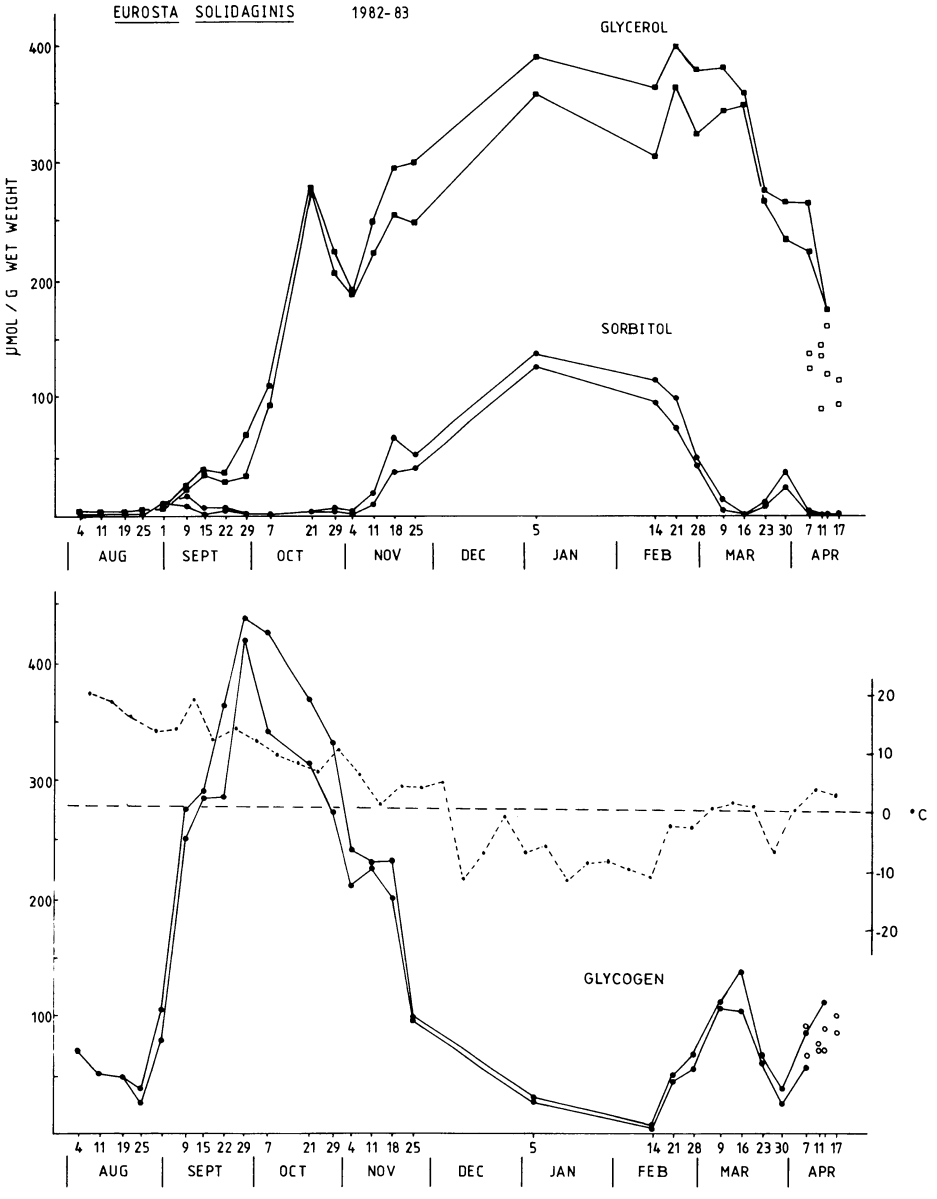

FIG. 4. Profiles of glycerol, sorbitol, and glycogen (in glucosyl units) levels in *Eurosta solidaginis* larvae over a winter season. Mean weekly temperatures (● - - - ●) are also plotted. Except for 1st 3 sampling points, data shown are values from 2 samples, 2 or more larvae pooled per sample. Open symbols in April represent pupae. [From Storey and Storey (140).]

gering of polyol synthesis in the 5 to −5°C range allows cryoprotectants to be put in place before first freezing exposures begin (most freeze-tolerant insects supercool to −5 to −10°C).

The mechanism of low-temperature triggering of polyol synthesis appears to be direct temperature effects on the fat body enzymes involved in polyol synthesis (see sect. VI*E*) without a need for nervous or hormonal mediation. In support of this, Shimada (127) found no differences in glycerol production and glycogen utilization, stimulated by 5°C exposure, in intact versus decerebrate diapausing pupae of *P. machaon*. Wood and Nordin (177) showed that decapitated *P. terranovae* were fully capable of low-temperature-induced glycerol accumulation whereas head or thorax ligations of *O. nubilalis* larvae, in either diapause or nondiapause 5th instars, could not prevent glycerol accumulation at 5°C (105). Ziegler et al. (192) showed that cold exposure activated glycogen phosphorylase in debrained *H. cecropia* pupae and also in isolated fat bodies in vitro.

3. Photoperiod and other environmental cues

Although the most common cue, a low-temperature trigger of polyol synthesis does not suit the needs of all species. For example, to achieve a differential accumulation of two cryoprotectants, synthesis of one can be tied to a low-temperature trigger but production of the other must respond to another cue.

Photoperiod cues a variety of seasonal responses in cold-hardy insects, including winter diapause (124, 160) and THP synthesis (34, 35, 67). There is little evidence, however, of photoperiod involvement in triggering polyol synthesis but also, admittedly, little specific study of photoperiod effects. Photoperiod regimes (at 20°C) that stimulated THP synthesis in *D. canadensis* did not stimulate polyol production (67). Short day (10:14 LD) exposure at 25°C induced diapause in *C. suppressalis* but did not stimulate glycerol synthesis (166). However, Young and Block (184) found an influence of short photoperiod, low temperature, and low relative humidity on glycerol accumulation in *Alaskozetes antarcticus* (a freeze-avoiding mite).

Changes in water and food supplies over the autumn months can also provide insects with cues to the approach of winter. For gall insects such cues are particularly crucial, since maturation of the larvae (accumulating fuel and water reserves to supply both winter needs as well as renewed development in spring) must be complete before frost kills the host plant. Cues taken from the status of the gall appear to affect glycerol synthesis in *E. solidaginis* (sorbitol synthesis in this species uses the 0–5°C trigger). High rates of glycerol production were correlated with the attainment of maximal larval weight, suggesting that maturation of the larvae and/or the food/water status of the gall may influence glycerol accumulation (140). Rojas et al. (117) suggested that gall water content is key to triggering glycerol synthesis. The

larvae do not, themselves, dehydrate during cold hardening but water content of the goldenrod gall drops rapidly (from 65–70% to 15–20%) over the late autumn. Glycerol levels in larvae rose, correlated with the dehydration of the gall. In addition, at any given time, larvae taken from still-living "green" galls had lower glycerol content than those taken from "dry" galls. However, further studies designed to test the effects of experimental manipulation of environmental water content on glycerol synthesis were inconclusive (117). Studies on *A. antarcticus*, however, have shown strong correlations between relative humidity and glycerol accumulation (184).

4. Hormonal, developmental, and diapause triggers

Studies with ligated or debrained animals or isolated fat bodies have indicated that hormonal mediation is not involved in the mechanism of low-temperature triggering of polyol synthesis in many species. Direct studies of the actions of added hormone(s) on polyol synthesis are few, however. Juvenile hormone stimulated THP synthesis in *D. canadensis* but did not induce polyol accumulation (68). For other species, however, hormones have an effect on polyol production (although the mechanism of this effect is not known). Tsumuki and Kanehisa (168) found that both JH and JH analogues stimulated glycerol production in *C. suppressalis* larvae held at 15 or 25°C, whereas application of ecdysone led to reduced glycerol levels. Synthetic JH also elevated glycerol levels at 4°C but ecdysone had no effect at low temperature. Hamilton et al. (48) compared the effects of hormone deprivation (head ligation), JH replacement, and treatment with an antijuvenoid (precocene II) on glycerol and sorbitol levels in *E. solidaginis*. Although results were mixed, the data suggest some hormonal involvement in polyol biosynthesis; glycerol levels, for example, were substantially reduced after head ligation. Further studies are required to establish the extent of hormonal involvement in cryoprotectant metabolism.

Although developmental transitions are certainly involved in providing the fat body with the biosynthetic machinery for polyol production, they have not yet been implicated in directly activating polyol production. Thus 5th instar larvae of *O. nubilalis* develop the capacity for glycerol synthesis but this is expressed only in response to a low-temperature cue (105). Third instar *E. solidaginis* are capable of polyol biosynthesis but glycerol production appears to be linked to the food/water status of the host plant, whereas sorbitol synthesis is triggered by exposure to 0–5°C.

Polyol synthesis is correlated with diapause initiation in a number of species (160, 165). In some instances the link is obligatory; eggs of *B. mori*, for example, begin polyol accumulation concurrent with diapause initiation, irrespective of ambient temperature (45). For other species, the correlation occurring in nature does not necessarily imply cause and effect. Diapause initiated by short-day photoperiod at warm temperatures is not accompanied

by glycerol production in at least three species (105, 127, 165); synthesis in the diapausing animal begins, instead, in response to chilling (105, 127) or other trigger.

G. Regulation of Cryoprotectant Catabolism

A number of species have the capacity to repeatedly accumulate or clear cryoprotectants (glycerol, sorbitol, threitol, trehalose) in response to successive cold or warm exposures (51, 57, 97, 116, 140). This capacity relies on the quantitative reconversion of cryoprotectants to glycogen during warming (57, 136, 140). Other species appear capable of only a one-time synthesis of glycerol and respond to warming with either *1*) no change in glycerol content (136) or *2*) an irreversible loss of glycerol (15).

The handling of polyol pools in the spring or on diapause termination is also species specific. Sorbitol and trehalose are quantitatively reconverted to glycogen (Fig. 4) (45, 57, 140, 180) but the fate of glycerol is variable. Glycerol clearance in *E. solidaginis* and *B. mori* does not result in glycogen synthesis (Fig. 4) (45, 140, 180). In *Epiblema scudderiana* only ~20% of glycerol carbon reappears as glycogen (112). In postdiapausing *C. suppressalis*, [^{14}C]glycerol has three fates: glycogen, trehalose, and CO_2 (167). Carbon dioxide production may result from one of two uses of glycerol, complete oxidation of glycerol as an aerobic fuel or partial oxidation followed by incorporation of acetyl-CoA into fatty acid biosynthesis. The partitioning of glycerol carbon into different uses during spring dehardening is undoubtedly species specific and probably regulated by hormonal controls.

The regulation of polyol catabolism requires the presence of key enzymes. Glycerol catabolism is impossible without the presence of glycerol kinase (glycerol + ATP → glycerol-3-*P* + ADP). The absence of this enzyme in cold-acclimated *E. solidaginis* maintains glycerol in an inert pool over the winter (153). Sorbitol catabolism depends on an active sorbitol dehydrogenase (sorbitol + NAD^+ → fructose + NADH + H^+) (136, 181). In *B. mori* the enzyme is induced during diapause termination (181), whereas in *E. solidaginis* it is present throughout the winter (153), allowing temperature-dependent interconversions between sorbitol and glycogen. Reconversion of cryoprotectants to glycogen requires a glycogen synthase in fat body that is warm activated (Fig. 2). In *P. cynthia* pupae glycogen synthase is activated in response to a temperature change from 2 to 25°C. Two mechanisms are involved: *1*) the percentage of glycogen synthase in the active *a* form rises from 10 to 45% within 12 h, and *2*) total synthase activity in fat body doubles (58). Concomitantly, glycogen phosphorylase *a* content falls from >90% to 1–2% of total. Warm activation of glycogen synthase is also apparent in fat bodies incubated in vitro (58), indicating that covalent modification of the enzyme can be triggered by temperature change alone. The molecular basis of this effect in cold-hardy insects has not yet been studied but, in an elegant

and simple manner, the reciprocal control of glycogen phosphorylase and synthase may result from temperature effects on a single enzyme: a warm active/cold inactive glycogen phosphorylase phosphatase (see sect. VI*E*). Thus, in mammalian systems, the key factor in synthase control is phosphorylase *a* inhibition of glycogen synthase phosphatase (Fig. 2) (63). Warm-temperature inactivation of phosphorylase (due to the resumption of phosphorylase phosphatase activity) would, therefore, remove the inhibition of synthase phosphatase, promote the conversion of glycogen synthase *b* to the active *a* form, and stimulate of glycogen synthesis.

H. Role of Dual Cryoprotectant Systems

Multiple cryoprotectant systems occur in a variety of cold-hardy insects, both freeze-tolerant and freeze-avoiding forms. Glycerol and sorbitol are the most common combination, although glycerol-mannitol, sorbitol-threitol, and combinations of three or more compounds occur in some instances (34, 97, 98, 132). The dual cryoprotectant systems that have been examined all reveal individual patterns of accumulation and loss for the two compounds (e.g., Fig. 4), demonstrating the presence of individual triggers and regulatory mechanisms. The added complexity required to individually regulate two compounds, when many other species rely solely on glycerol for cryoprotection, suggests that a dual cryoprotectant system must have evolved for important reasons.

1. Anticipatory component

Differential synthesis of glycerol in early autumn and sorbitol in late autumn gives an anticipatory component (glycerol) to cryoprotection to meet unexpected early autumn cold exposures, whereas total polyol levels can remain relatively low until prolonged cold exposure induces the second component (sorbitol) (136).

2. Variable versus invariable cryoprotection

Because sorbitol can be repeatedly interconverted with glycogen, it provides a variable cryoprotection to match winter temperature variations. Glycerol, however, provides an invariable cryoprotection as the polyol can only be removed when glycerol kinase is induced in spring (136).

3. Fuel reserves

During the spring loss of cold hardiness, sorbitol is reconverted to glycogen to provide the fat body glycogen pool, which sustains further develop-

ment (Fig. 4). Since glycerol is not reconverted to glycogen (it is probably oxidized as an aerobic fuel or converted to lipid), the use of dual polyols allows the insect to predetermine the relative amounts of fuel reserves that will result in spring (140). This is probably particularly important for species that cannot replace carbohydrate supplies by spring feeding.

4. ATP requirements

Glycerol synthesis is the more efficient use of carbohydrate reserves (in terms of the colligative result) but synthesis requires energy input in the form of ATP at the PFK reaction. In some species, mitochondrial ATP synthesis may be limiting at low temperature, in a hypoxic-anoxic environment, or after diapause initiation. Sorbitol synthesis, requiring no ATP input, would then replace glycerol.

5. Flexibility

Individual compounds (glycerol, sorbitol, trehalose, glucose) may be differentially suited to stabilizing or regulating individual cellular components. A dual or multiple cryoprotectant system provides a greater degree of flexibility for structural preservation and maintenance of metabolic integrity during freezing.

VII. CRYOPROTECTANT METABOLISM IN FREEZE-TOLERANT FROGS

A. Patterns of Cryoprotectant Accumulation and Loss

Freezing exposure leads to a rapid accumulation of glucose in tissues of *R. sylvatica*, *H. crucifer*, and *P. triseriata* and to glycerol accumulation in *H. versicolor* (125, 137, 144, 154, 156). Synthesis of cryoprotectants utilizes the large reserves of glycogen (~1,000 μmol/g wet wt as glucose or 180 mg/g) found in liver, levels at least 10-fold greater than glycogen reserves of other organs. Production and distribution of glucose have been examined in detail in *R. sylvatica*. Rates of glucose production by liver can exceed 20 $\mu mol \cdot g^{-1} \cdot h^{-1}$ at −2.5°C with a half time to maximal levels in liver of only 5 h (155). Cryoprotectant is released from liver and distributed to other tissues via the blood. Maximal levels of cryoprotectant are reached in 24–48 h, correlated with the attainment of maximal ice content and the cessation of blood circulation.

Distinct tissue patterns to the distribution of glucose are found in all species (e.g., Fig. 5 for *R. sylvatica*) (137, 147, 154–156). Central tissues (liver, heart, brain, kidney) show the highest contents (75–100% of blood levels); others such as lung, stomach, and intestine have intermediate amounts

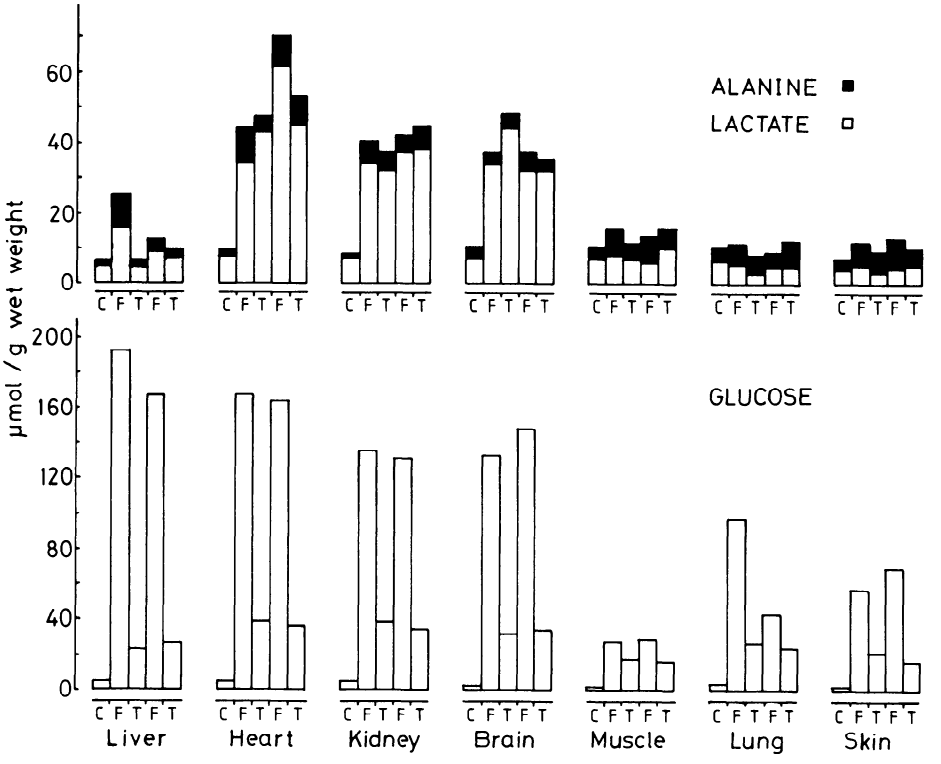

FIG. 5. Glucose, lactate, and alanine contents of wood frog, *Rana sylvatica*, tissues over consecutive freezing (at −2.5°C) and thawing (at 3°C) exposures, each 2 days in duration. C, 3°C control; F, frozen; T, thawed. Muscle is leg skeletal muscle; skin is from ventral abdomen. Values for liver glycogen over the cycles were 980 ± 90 μmol glucosyl units/g wet wt for controls and 285 ± 30, 760 ± 136, 327 ± 40, 724 ± 22 μmol/g over succeeding freeze/thaw exposures. [Modified from Storey (147).]

(~50% of blood levels); whereas a third group (skeletal muscles, skin, gonads) has contents <35% of blood levels (147). This pattern appears to result from circulatory changes during freezing, since cryoprotectants become more evenly distributed shortly after thawing (137, 147).

When frogs are thawed, clearance of cryoprotectant begins immediately. Glucose is returned to the liver and restored as glycogen. The process requires a longer time than does synthesis; in one study, half times for glucose clearance ranged from 1.9 days in liver to 8 days in leg muscle (155). Repeated freeze-thaw exposures elicit the same pattern and time course of glucose distribution (Fig. 5).

Freeze-tolerant frogs exhibit neither the anticipatory synthesis of cryoprotectant nor the permanent winter reserve of cryoprotectant that characterizes cold hardiness in insects. With each freezing exposure, glucose is produced and distributed, and with each thaw, glucose is cleared and restored

as liver glycogen. Such a system appears to be related to the particular use of glucose as the cryoprotectant and may derive from several factors: *1*) the metabolic costs of cryoprotectant synthesis/degradation are avoided if freezing does not occur; *2*) rates of glucose synthesis are high enough and rates of ice formation are low enough that maximal cryoprotectant levels can be attained during the course of freezing without a requirement for anticipatory synthesis; *3*) high glucose can probably not be maintained after thawing as blood sugar will become subject to regulation by insulin; and *4*) the toxic effects of high glucose, typical of diabetes, could manifest themselves if cryoprotectant were maintained in the thawed state.

B. Triggering

Triggering of cryoprotectant synthesis in frogs is unique in responding only to the initiation of ice formation in the body. No anticipatory response is seen, and no triggering or modulation by environmental factors (e.g., low temperature acclimation) has been found in *R. sylvatica* (139, 155, 156) or *H. versicolor* (137). Ice nucleation as the trigger was demonstrated by two experimental findings: *1*) when held at a constant −1.2°C, frogs that froze had high blood glucose (>150 μmol/ml), whereas those that remained in a supercooled state had blood glucose levels equivalent to control frogs at 3°C (<2 μmol/ml) (139); and *2*) when cooling/freezing of individual frogs was monitored, blood and liver glucose remained constant during cooling to the supercooling point but increased 3.3- and 6.6-fold, respectively, within 5 min after the appearance of the freezing exotherm (Fig. 6) (139).

The stimulus for cryoprotectant synthesis, initiation of ice formation, occurs in the body extremities and skin, whereas the response, activation of glycogen breakdown, occurs in the liver. Obviously, an intermediate signal must be involved to translate the physical event of ice formation into a metabolic activation in the liver. This signal is undoubtedly nervous or hormonal and could be an adaptation of the catecholamine-mediated "fight or flight" response.

C. Regulation of Glucose Synthesis in Liver

The synthetic pathway for glucose is short, involving three enzymes, i.e., glycogen phosphorylase, phosphoglucomutase, and glucose-6-phosphatase (Fig. 1). Although glucose-6-phosphatase activity increases 2.5-fold in liver of frozen frogs (154), control of synthesis rests largely with the regulation of glycogen phosphorylase. Unlike the situation in cold-hardy insects, phosphorylase in frogs is not cold activated. The enzyme responds instead to the initiation of ice formation in the body and shows a unique regulatory component. In addition to the conversion of an inactive, dephosphorylated *b* form to an active, phosphorylated *a* form, enzyme activation is also facilitated by a

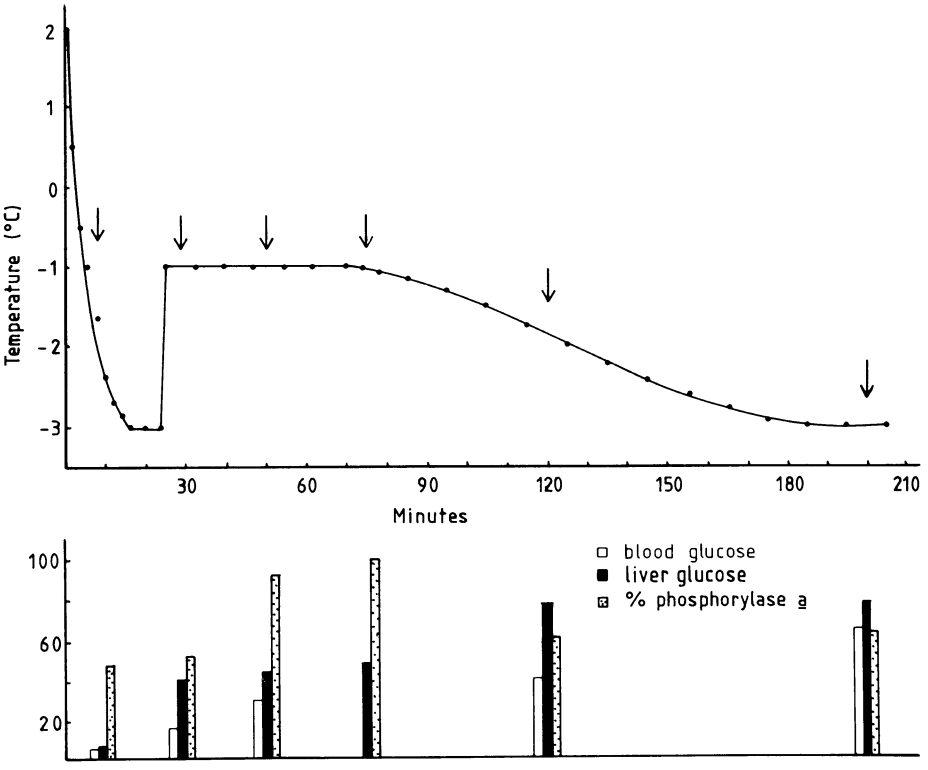

FIG. 6. Triggering of cryoprotectant glucose synthesis in *Rana sylvatica* by initiation of ice formation. *Top*: a composite curve for chilling and freezing of wood frogs at a constant −3°C. Except for initial sample during chilling, ↓ indicates sampling time relative to appearance of freezing exotherm. *Bottom*: values for liver and blood glucose concentrations and percentage of glycogen phosphorylase in active *a* form at different sampling times. Values on *y*-axis are in μmol/g wet wt for liver glucose, μmol/ml for blood glucose, and % for phosphorylase *a*. [Modified from Storey and Storey (139).]

large increase in the total phosphorylase (*a* + *b*) content measurable (137, 139, 146, 154). The immediate response to freezing is a rapid increase in the percentage of existing enzyme in the *a* form, initiated within 2 min and complete in 30–60 min. On a slower time course, however, the total activity of phosphorylase in liver increases, first apparent by 30 min and remaining maximal for at least 3 h before declining to an intermediate level over the long term in the frozen state (Fig. 7). Maximal rates of glucose synthesis are correlated with the rise in total phosphorylase content. Overall, these two mechanisms allow an extremely sensitive regulation of phosphorylase *a* expression; at peak levels, phosphorylase *a* content in liver of freezing-exposed frogs is 7- to 13-fold greater than control (3°C) values, whereas during thawing (active glucose reconversion to glycogen) phosphorylase *a* content can be depressed as much as 100-fold (Fig. 7) (146, 154). The molecular mechanisms

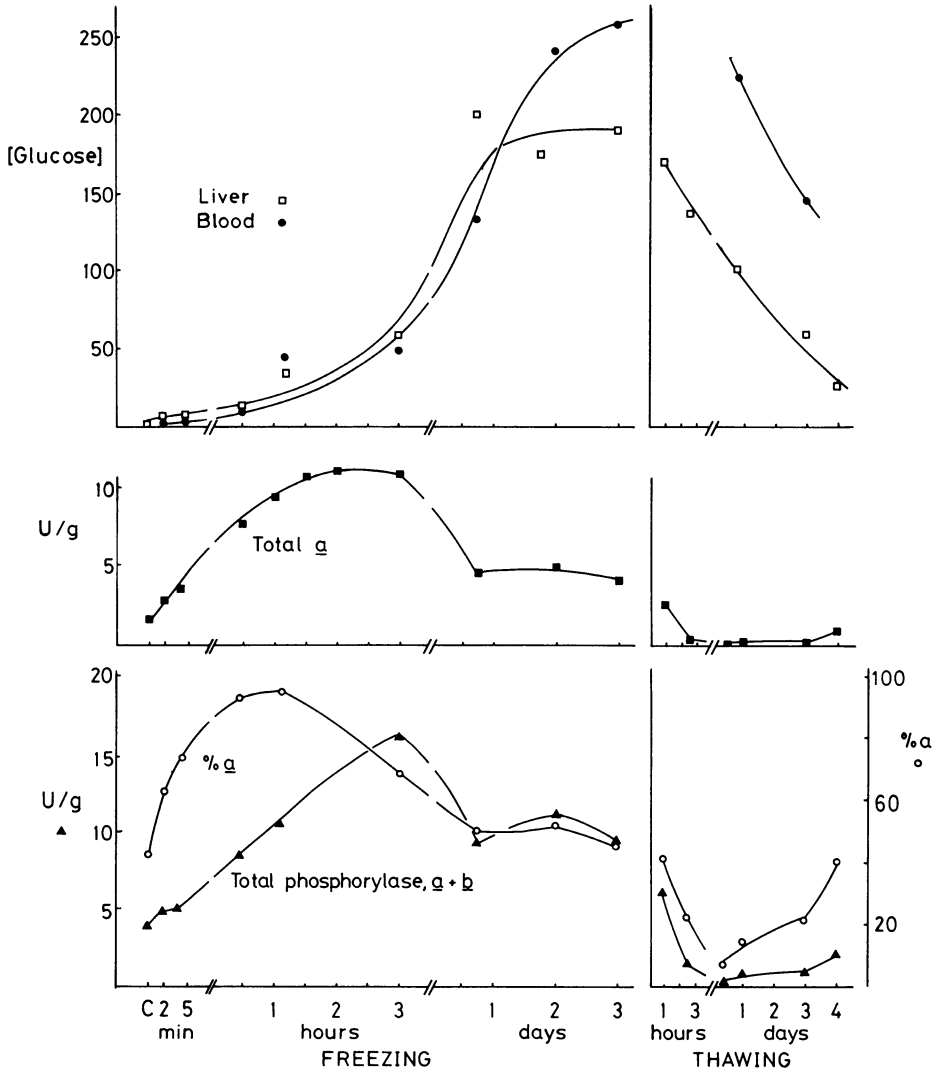

FIG. 7. Modulation of glycogen phosphorylase activity in liver of *Rana sylvatica* over a course of freezing at −3°C (timed from appearance of freezing exotherm) and thawing at 3°C. Activities are expressed in units/g wet wt. *Top*: liver (μmol/g wet wt) and blood (μmol/ml) glucose levels.

behind this novel regulatory control are under investigation. Preliminary results include: *1*) the observations are not artifacts of changes in glycogen phosphorylase partitioning between soluble (supernatant) and particulate (pellet) fractions of the cell; *2*) both the percent *a* and total phosphorylase activity can be increased in vitro by the addition of cAMP and phosphorylase kinase; and *3*) the effect appears to be caused by the presence of a second

inactive form of the enzyme whose activity cannot, like that of phosphorylase *b*, be expressed in the presence of AMP.

D. Glucose Homeostasis in Vertebrates and Its Perturbation in Frogs

Blood glucose levels in vertebrates are normally strictly regulated, within narrow limits, by homeostatic and hormonal controls acting predominantly on the rates of glycogen synthesis versus glycogen breakdown in liver (63). Glycogen synthesis in liver is rapidly activated during dietary intake of sugar; conversely, glycogen breakdown fuels the demands of other tissues for glucose during periods of fasting or stress. The extreme hyperglycemia that arises during freezing exposure in frogs must result from major changes to the molecular and hormonal mechanisms controlling glucose/glycogen metabolism in frog liver, changes that only come into play under freezing conditions.

Hormonal controls on blood glucose levels are well known. Glucose homeostasis during alternating periods of feeding and fasting is regulated by the pancreatic hormones, insulin decreasing and glucagon increasing blood glucose levels. Catecholamines stimulate stress-related hyperglycemia, particularly as part of the fight or flight arousal. The very rapid activation of glucose output from *R. sylvatica* liver in response to freezing (significant changes within 5 min) strongly suggests that hormone stimulation underlies the hyperglycemic response. Harri and Lindgren (54) have observed that epinephrine readily stimulates hyperglycemia in cold (5°C), but not warm (25°C), acclimated *Rana temporaria*, indicating that an increased sensitivity to catecholamine action is part of anuran adaptation to cold. Such an increased sensitivity to catecholamine action may underlie the activation of glycogenolysis in response to freezing. However, preliminary measurements of catecholamines in *R. sylvatica* have found no changes in epinephrine, norepinephrine, dopamine, or serotonin in frozen versus control frogs (S. Perry and K. Storey, unpublished data). Normal pancreatic controls over blood glucose must, undoubtedly, also be perturbed during freezing by mechanisms that could include *1*) an enhanced effect of catecholamines in suppressing insulin secretion from the pancreas, *2*) a depleted supply of insulin in the pancreas during winter hibernation, *3*) differential low-temperature effects on the synthesis and/or secretion of insulin and glucagon in frog pancreas, and *4*) physical isolation of the pancreas due to vasoconstriction or extracellular freezing preventing hormone secretion. Again, however, preliminary data show no significant changes from control levels in the amounts of glucagon in blood during short and long freezing exposures (K. Storey, unpublished data).

The regulation of cryoprotectant levels probably also involves altered homeostatic controls over glycogen phosphorylase and glycogen synthase in liver of freeze-tolerant frogs. The normal response of vertebrate liver to rising glucose is an inactivation of glycogen phosphorylase, an activation of

glycogen synthase, and a rapid net synthesis of glycogen. The molecular controls underlying this are as follows (Fig. 2): *1*) glycogen phosphorylase *a* is allosterically inhibited by glucose; *2*) phosphorylase phosphatase is activated by glucose (this is not an allosteric effect but arises because a glucose-phosphorylase *a* complex is a better substrate for phosphatase action) and rapidly reduces phosphorylase *a* content; *3*) glycogen synthase phosphatase is inhibited by phosphorylase *a* and becomes active once the inhibitor is removed; and *4*) active synthase phosphatase converts glycogen synthase *b* to the active *a* form (63). Obviously, under freezing conditions in *R. sylvatica* liver, this chain of events must be broken to allow the massive glucose buildup during freezing. A novel control cascade may exist and awaits study. Control mechanisms involved in the freezing-stimulated production of glucose must, however, be reversible, since normal glucose homeostasis returns during thawing. One possible explanation for the observed events could be the loss of glucose inhibition of phosphorylase *a* or glucose activation of phosphorylase phosphatase in winter frogs; this would allow uncontrolled glucose accumulation without phosphorylase inactivation or a subsequent activation of synthase.

E. Control of Glycolysis During Cryoprotectant Synthesis

Activation of liver phosphorylase in frogs initiates the high rates of glycogenolysis needed for cryoprotectant synthesis but inhibitory regulation must also be applied to glycolysis to channel carbon flow into glucose. Measurements of changes in the levels of glycolytic intermediates over the initial minutes of freezing in *R. sylvatica* liver indicated that PFK is the inhibitory locus involved (levels of its substrate, fructose-6-phosphate, rose 10-fold but no change was found in content of product, fructose-1,6-bisphosphate; 146). The molecular mechanisms responsible for inhibition of PFK are not yet clear. Possible enzyme control by covalent modification was evaluated but changes in K_m fructose 6-phosphate, I_{50} ATP, and K_m fructose 2,6-bisphosphate over the initial minutes of freezing were all consistent with a dephosphorylation, and therefore activation, of liver PFK (146). Levels of fructose 2,6-bisphosphate in liver were also assessed but content of the PFK activator did not change over the initial minutes (up to 70 min) of freezing-stimulated glucose synthesis (146). However, over the long term (2 days of freezing exposure) fructose 2,6-bisphosphate content of liver was strongly depressed (147). This may be key to a general metabolic depression in the frozen state; such a role for fructose 2,6-bisphosphate occurs in other instances of facultative hypometabolism (145).

F. Advantages of Glucose as a Cryoprotectant in Vertebrates

Glucose has a central and extremely important role in the metabolism of vertebrate cells. It is the transportable form of carbohydrate, the substrate

for glycogen synthesis in all tissues, and the major oxidative fuel for tissues such as brain. Under normal conditions, glucose levels in blood and tissues are strictly regulated by the actions of the pancreatic hormones. Glucose must have key advantages for the freezing protection of frog cells to justify the maintenance of an extreme hyperglycemia [blood glucose in freezing-exposed mature female *R. sylvatica* can exceed 500 μmol/ml, or 9 g/100 ml, compared with 1–5 μmol/ml in unfrozen controls (144)] and the development of mechanisms for overriding normal homeostatic controls on glucose levels. At least three roles for glucose during freezing can be proposed: *1*) a true cryoprotective function, limiting freeze concentration of the cell and stabilizing subcellular structures and proteins, *2*) a role as a fermentable fuel to sustain organs in the anoxic and ischemic state imposed by the freezing of blood, and *3*) a function as a metabolic depressant, perhaps as part of an inducible hypometabolism in the frozen state. In support of this last proposal, we have recently noted that high glucose (0.5–2.0 M) inhibits selected metabolic pathways in isolated *R. sylvatica* hepatocytes at low temperatures; inhibition is removed when glucose is washed out (K. Storey and T. Mommsen, unpublished data).

Additional advantages to the use of glucose as a cryoprotectant are related to its metabolism. Mechanisms for the very rapid activation of glucose synthesis, via hormonal stimulation and cAMP and Ca^{2+}-mediated phosphorylation cascades acting on glycogen phosphorylase, are constitutive in vertebrate liver, as is the synthetic pathway. Ultrahigh accumulations of glucose for cryoprotection can be quite easily achieved by *1*) modification of existing regulatory mechanisms (under neural or hormonal control) to respond to the stimulus of ice nucleation, and *2*) inhibition or blocking of the natural homeostatic mechanisms, which put upper limits on tissue and blood glucose contents. Glucose synthesis is also energetically inexpensive [no ATP or NAD(P)H input is required] so synthesis can proceed under energy-restricted, hypoxic/anoxic conditions such as occur during freezing. In addition, glucose can be readily and quantitatively reconverted to glycogen regulated by natural hormonal mechanisms.

G. Cryopreservation of Vertebrate Organs

The increasing use of organ transplant therapy as a clinical tool in the treatment of human illnesses requires techniques for the long-term storage of organs at subzero temperatures. Experimentation to date has largely failed in attempts at freezing preservation of viable mammalian organs (72). Studies of natural freeze tolerance in the frog bring a unique approach to the subject. As a vertebrate, the frog has organ systems equivalent to those of mammals and has developed natural solutions to all the problems of organ freezing: control of extracellular ice formation, regulation of cell volume and freeze concentration of intracellular fluids, use of cryoprotectants, and organ

survival in the ischemic frozen state. Frog organ systems provide the optimal models for studies of *1*) freezing injury, *2*) organ-specific adaptations for freezing survival, *3*) the protective effects of cryoprotectants, both physical and metabolic, and *4*) development of techniques for artificial cryopreservation of organs in vitro.

Studies of organ-specific freezing preservation in the frog have begun with two in vitro model systems, ventricle strips and isolated hepatocytes. Freezing effects on muscle performance have been studied using ventricle strips from *R. sylvatica* and *Rana pipiens* (a freeze-intolerant frog; 20). Ventricle strips from *R. sylvatica* readily survived freezing exposure (−5°C for 1 h) and regained contractility equivalent to that of control, unfrozen strips when frozen in the presence of 250 mM glucose added to the incubation bath. In the absence of glucose or with the substitution of 250 mM glycerol, however, physical function was not regained after freezing. Ventricle strips from *R. pipiens* did not survive freezing under any conditions. The experiments show that freeze tolerance is a property of individual tissues, even when isolated from the whole animal, and that the natural cryoprotectant, glucose, is key to the preservation of cell structure and function during freezing. Other workers have utilized frog (presumably *R. pipiens*) hearts as a model for organ cryopreservation and produced limited recovery of function of tissue pieces, sinus venosus, and atria after stepwise cooling to a final −78°C in the presence of ethylene glycol (87, 111). Thus frog heart is obviously a very promising model system for organ cryopreservation studies; studies on a freeze-tolerant heart from a freeze-tolerant animal and employing a natural cryoprotectant will be of key importance in developing methodologies for the cryopreservation of mammalian hearts. Miller and Dehlinger (96) have also reported that isolated gastrocnemius muscle preparations from *R. sylvatica* regained some excitability and contractility after brief freezing (30 min) at −3 to −5°C; presumably, restoration of skeletal muscle function after freezing would also benefit from the presence of glucose as a cryoprotectant.

Isolated hepatocytes of *R. sylvatica* show a high rate of freezing survival at −4°C in the absence of cryoprotectant and a 100% freeze tolerance when frozen in the presence of 400 mM added glucose (survival assayed by trypan blue staining, lactate dehydrogenase leakage, and rates of urea synthesis; K. Storey and T. Mommsen, unpublished data). An increase in glucose content to 1 M allowed freezing survival at −20°C. Again, the key role of glucose in the freeze protection of vertebrate cells is apparent, a potentially valuable lesson for the artificial cryopreservation of mammalian tissues and organs.

VIII. MEMBRANE, PROTEIN, MITOCHONDRIAL, AND SOLUTE ADAPTATIONS FOR COLD HARDINESS

Macromolecular structure and function in freeze-tolerant animals are subject to two stresses: low temperature and freezing. Potential injuries due to chilling and freezing are summarized in section II*A*. Adaptations for freeze

tolerance must include both mechanisms that stabilize and protect subcellular structures from the stresses of freezing and mechanisms that preserve and integrate essential metabolic functions to maintain homeostasis over a wide subzero temperature range (143).

To date, most studies of cold acclimation in ectotherms have focused on the responses involved during acclimation to a constant low (above 0°C) temperature by animals that must compensate to preserve full physiological function at low temperature. The actual situation for many overwintering ectotherms is quite different. First, animals may face wide temperature variations on a daily basis and may be best served by a flexible metabolic makeup, one with a high degree of temperature insensitivity in both structural and functional properties of proteins and membranes so that homeostasis can be maintained over a wide subzero temperature range. Winter hardening may involve only minor changes to membrane or protein composition to reset optimal function to an overall lower ambient temperature range. Second, many cold-hardy animals spend the winter in a dormant state (often cold induced) in which many metabolic functions are shut down. Rather than compensate for low-temperature effects on metabolism, low temperature may, in fact, be exploited to alter metabolism by *1*) inactivating nonessential processes as part of a winter dormancy, and *2*) triggering specific preservation adaptations including production of polyols, INPs, and THPs (143). What adjustments are required may arise not so much from macromolecular restructuring as from a temperature-dependent interplay between macromolecules and stabilizing agents (polyols, trehalose, proline) that are introduced as cryoprotectants.

A. Membrane Adaptations

1. Low-temperature function

Membrane adaptations involved in cold acclimation have been extensively reviewed by other authors (28, 62, 163). Because of the key importance of membrane functions to life processes, a primary adaptation to temperature change in ectotherms is membrane restructuring by which lipids of appropriate physical properties are matched to the prevailing thermal environment. This homeoviscous adaptation results in membranes with physical properties (fluidity, unit cell dimensions, order parameters) largely independent of acclimation temperature and physiological processes (permeability, enzyme and transport activities, receptor and neural functions) that are temperature compensated (62).

The only evaluation of homeoviscous adaptation in the cold hardening of a freeze-tolerant animal involves the barnacle *Balanus balanoides* (27, 164). Although Cook and Gabbott (27) reported an increase in the degree of unsaturation of body lipids during the winter months, a more extensive study by

Tooke and Holland (164) found little indication of homeoviscous adaptation. Neither the total phospholipid nor neutral lipid fractions showed a seasonal change in the unsaturation index, although within the phospholipid fraction a small increase in total saturated fatty acids and a small decrease in monounsaturated fatty acids were noted from winter to summer.

2. Freezing preservation

Seasonal changes in membrane lipid composition are related to the maintenance of membrane function at low temperature but do not influence freeze tolerance (44). However, damage to membranes is a primary cause of freezing injury and arises from the physical stresses on bilayer structure when cells shrink and deform under osmotic stress as well as from damage by extracellular ice crystals. Direct studies of the mechanisms of membrane preservation supporting freeze tolerance in animals are lacking but these have been extensively studied in freeze-tolerant plants (44, 134) and in relevant animal model systems (30, 31, 119). Three adaptive strategies are known. *1*) Changes to the plasma membrane or associated protein components inhibit the propagation of ice crystals across the membrane. Thus isolated protoplasts from hardened plants are better able to resist seeding by extracellular ice than are cells from tender plants (134). *2*) Stress on the membrane during freezing-induced cell volume reduction is relieved by the removal of membrane material, the mechanisms of removal (exocytosis in hardened plant protoplasts) and storage (a stabilized bilayer) of deleted material being such as to allow ready reincorporation during thawing (134, 174). Although this mechanism operates in plants, there is no evidence for the removal of membrane material during freezing-induced shrinkage of mammalian cells (92). *3*) Membrane structure is stabilized by low-molecular-weight protectants: trehalose and proline (30, 31, 119). Both trehalose and proline effectively stabilize artificial membranes or membranes from nontolerant species in the face of dehydration or freezing stresses (30, 119). Trehalose occurs naturally as a membrane protectant in dessication-tolerant species (yeast, fungal spores, *Artemia* cysts, nematodes) (31), whereas proline is used in halophytic and cold-hardy plants (135, 183). Both compounds increase in concentration during cold hardening in freeze-tolerant insects (91, 98, 149, 171, 187), suggesting that the use of trehalose and proline as membrane protectants is probably also key to natural freeze tolerance in animals. Thus two functional classes of low-molecular-weight cryoprotectants occur in freeze-tolerant animals: polyols regulate dehydration/freeze concentration and stabilize protein structure/function, whereas trehalose and/or proline stabilize membrane structure.

The actions of trehalose, and to a lesser extent proline, have been well studied, although the molecular mechanisms underlying their effects are not completely understood. Trehalose inhibits the phase transitions associated

with drying or freezing of biological membranes (31, 119). Both compounds effectively inhibit freezing-induced fusion between synthetic phospholipid vesicles (31), and both preserve structure and metabolic function of sarcoplasmic reticulum during drying or freezing (30, 119). The molecular mechanism involved in trehalose action appears to be the ability of the disaccharide to form hydrogen bonds with the polar head groups of phospholipids (or with the hydration sphere centered around them) and, in doing so, spreads phospholipid monolayers and inhibits the transition to gel phase brought about by reduced water content (119, 120). The molecular action of proline may involve both effects on the hydration layer surrounding phospholipids as well as intercalation between phospholipid head groups (120).

The stabilizing effects of trehalose on membrane structure are specific and significantly greater than those of other di- and monosaccharides (including glucose) and polyhydric alcohols (175). Indeed, glycerol is not only a poor stabilizer of membrane structure but can induce membrane fusion at high temperature and high concentration, hence the requirement to rapidly remove cryoprotectant after thawing of preserved materials.

B. Protein Adaptations

1. Low-temperature function

Freeze-tolerant animals, like all cold-hardy ectotherms, must maintain a basic structural and functional integrity of cellular proteins over a wide range of low temperatures despite the potentially disruptive effects of low temperature on weak bond interactions (affecting protein structure, as well as protein:ligand, protein:protein, and protein:membrane associations) and on rate processes (see sect. II*A*). Capacity and compensatory adaptations are involved in adjusting protein complement during cold acclimation. These include *1*) production of new protein isoforms geared for optimal function at low temperature, and *2*) changes in the amounts of proteins/enzymes present, via altered rates of synthesis or degradation, to serve low-temperature requirements (73, 109, 128).

For freeze-tolerant animals there is no indication of extensive alterations to cellular proteins during cold hardening. Present information shows no changes in either soluble or total protein content of tissues (137, 149, 154), no changes in isozymic forms of numerous enzymes in insects (152) or frog liver or skeletal muscle (K. Storey, unpublished data), and only modest changes in enzyme activities with low-temperature acclimation or freeze/thaw exposure (137, 153, 154).

Instead, alterations to protein composition during cold hardening appear to be extremely selective. Specific proteins, key to freeze tolerance, are induced (e.g., INPs, THPs) or altered in activity (e.g., key enzymes of cryoprotectant synthesis) (37, 153, 154). Changes to the regulatory properties of

selected enzymes (glycogen phosphorylase, PFK) serve specific low-temperature functions (56, 141). For the majority of enzymes/proteins, however, functional requirements at low temperature can probably be met without requiring restructuring or changes in amounts of proteins. Three strategies may be involved: *1*) the use of protein designs that have a high degree of temperature insensitivity (due to careful balancing of weak bond contributions) to permit structural stability and functional integrity over a wide temperature range (66), *2*) the use of stabilizing compounds (polyols) to counteract denaturing effects of low temperature and stabilize the native state of proteins at low temperature (6, 43, 46), and *3*) a low-temperature dormancy that may not only accommodate reversible cold-induced conformational changes in nonessential proteins but even rely on such changes for a reduction and/or inactivation of many cellular processes in the hypometabolic state.

2. Freezing preservation

Studies of freezing effects on enzymes in vitro show adverse effects on structure and/or function (including extreme deviation from Arrhenius behavior) due to low water activity, high solute levels in freeze-concentrated solutions, changing solute composition as eutectic points are exceeded, restricted diffusion of substrates, products, and effectors, and altered microenvironmental pH (42, 83). The extent to which these factors might affect protein structure and/or function during freezing in vivo in freeze-tolerant animals is debatable. Low water activity as a stress can probably be ruled out; cell dehydration during natural freezing does not approach the levels at which hydration could influence enzyme function (90), and, indeed, a considerable amount of freezable water remains in tissues of freeze-tolerant animals at the LT_{50} (101, 191). Changes in intracellular solute concentration and composition as a result of freeze concentration are also not likely to be substantial enough to harm protein structure in vivo, particularly in a cellular environment that contains additional stabilizing influences: polyols, high protein concentration, and protein associations with subcellular structures. In addition, intracellular pH appears to conform to alphastat regulation even in the frozen state (151), indicating that the effects of freeze concentration are not great enough to interfere with intracellular buffering or, consequently, perturb the charge state on intracellular proteins.

The obvious fact that freeze-tolerant animals can undergo many cycles of freezing and thawing over a winter indicates that low temperature, ice formation, and the water-solute changes that are a consequence of freeze concentration have no irreversible effects on the conformation and/or function of intra- or extracellular proteins in the frozen state, at least at temperatures above the lower lethal limit. During freezing, however, reversible alterations to protein structure, protein-protein, or protein-membrane associa-

tions, enzyme kinetic properties, or the availability of enzyme substrates/effectors may occur, and these may limit or alter the activity of certain enzymes, pathways, and organelles in the frozen state. The only adaptive strategy presently known to be involved in preserving the structure and/or function of cellular proteins during freezing is the proliferation of polyhydric alcohols and sugars during cold hardening (6, 30, 43). However, future studies may uncover additional mechanisms of protein stabilization or additional proteinaceous factors (besides INPs or THPs) involved in natural freeze tolerance. For example, cold shock to spinach leaves produced rapid changes in translatable RNA, leading to several new protein products; this was correlated with improved freeze tolerance (47). Comparable studies with freeze-tolerant animals should examine the role of gene expression and protein synthesis in response to both short-term cold or freezing shock and long-term cold hardening to identify new protein products associated with freezing survival.

C. Mitochondrial Adaptations

Mitochondria from freeze-tolerant animals should show two classes of adaptations: those that allow optimal mitochondrial function at low temperature and those that preserve the organelles against the stresses of freeze concentration. Low-temperature acclimation of ectotherms is known to include changes in mitochondial numbers to alter oxidative potential (128). Adaptation can also involve metabolic changes to allow improved mitochondrial function at low temperature; thus studies of Q_{10} effects on the state 3 oxidation of substrates show that respiratory rates are less affected by low temperature in cold-tolerant versus nontolerant insects (179) and in cold-acclimated versus warm-acclimated individuals of a single species (7). Such adaptation for low-temperature function may derive from alterations to either membrane or protein components of the mitochondria. Low-temperature function is also promoted by a shift in the optimal pH for mitochondrial function to a higher value in cold-acclimated insects (7), reflecting the higher intracellular pH environment at low temperature [e.g., intracellular pH in *E. solidaginis* rises from 6.8 at 15°C to 7.3 at −12°C (151)].

Mitochondria from cold- versus warm-acclimated *E. solidaginis* larvae show different patterns of substrate utilization, suggesting adaptive changes in fuel use for the winter season. Mitochondria from cold-acclimated larvae did not oxidize lipid at 1°C (but did at 20°C) and showed an enhanced preference for proline over carbohydrate as a substrate at low temperature (7). Substrate preferences of mitochondria from warm-acclimated larvae, however, changed little at high versus low temperature. The results suggest that proline is the preferred aerobic fuel for winter survival and are consistent with observed patterns of fuel reserves over the winter. Thus proline is accumulated during cold hardening (149), glycogen is committed to other

uses (cryoprotectant synthesis, a fermentable substrate for the frozen state) in the overwintering animal (138, 149), and lipid reserves remain constant over the winter (140).

With respect to freezing stress, mitochondria from cold-acclimated larvae showed a higher optimal salt (KCl) concentration for mitochondrial function and a broader KCl range over which high respiratory control ratios were maintained than did mitochondria from warm-acclimated larvae (7). This indicates that adaptative changes are made that maintain structural and functional integrity in an environment of higher osmolytes such as occurs in the presence of high polyols or in the freeze-concentrated frozen state.

D. Solute Changes in Cold Hardening

Changes in hemolymph osmolarity as a result of cold hardening in freeze-tolerant insects can be quantitatively accounted for by changes in polyol contents (39, 150, 171, 185), indicating that no additional compounds, as yet unidentified, are involved in the colligative aspects of cryoprotection.

An increase in the free amino acid pool size (by 30–45%) occurs during cold hardening in freeze-tolerant insects, caused mainly by elevated levels of proline, alanine, and glutamate/glutamine (91, 149, 150, 171). This has little quantitative effect on the total pool of osmolytes but proline and alanine have specific roles (see sects. VIII*A* and IX*A*). Cold acclimation of *E. solidaginis* larvae resulted in a relative increase in the pool size of neutral amino acids in hemolymph and a relative decrease in the pool size of charged amino acids (150).

Levels of inorganic ions in freeze-tolerant animals have been reported by several authors but no consistent patterns emerge. The intertidal bivalve, *M. demissus*, showed 25 and 7% increases in blood Ca^{2+} and Mg^{2+} concentrations, respectively, as a result of low-temperature acclimation but no changes in Na^+ or K^+ content (99). Freeze tolerance improved when animals were moved from seawater with artificially low Ca^{2+} to water with normal Ca^{2+} levels. This improvement was linked to Ca^{2+} binding to cell membranes (102). Among insects, cold acclimation of *E. solidaginis* larvae produced a twofold increase in hemolymph Mg^{2+} content, slightly higher Na^+ and Ca^{2+} contents, and a slightly lower K^+ content (150). Results for *Upis ceramboides* were essentially the opposite: hemolymph Ca^{2+}, Mg^{2+}, and Na^+ decreased substantially and K^+ increased in winter, compared with summer, beetles (95). Levels of hemolymph ions in *T. trivittata* did not change over the winter (39).

IX. LIFE IN THE SLOW LANE: THE FROZEN STATE

Extracellular freezing isolates the individual cells of tissues and organs, blocking the delivery of oxygen and exogenous fuels, preventing waste re-

moval, and requiring cells to rely on endogenous fuels and energy reserves for survival. Circulation ceases and an ischemic state prevails. Scholander et al. (126) showed this effect of freezing on Arctic chironomid larvae; Q_{10} for respiration rose from 2–4 to 20–50 between 0 and −5°C. Survival over the long term in the frozen state depends on the low metabolic rates that are a consequence of subzero temperature and two metabolic adaptations: *1*) a good anoxia tolerance, and *2*) metabolic rate depression.

A. Anoxia Tolerance

Freeze-tolerant animals may face days, weeks, or even months of freezing during a winter and must have an anaerobic capacity that can support this. Adult *R. sylvatica* readily survive at least 2 wk of freezing (144), whereas various studies on insects have shown freezing survival of 5–12 wk (115, 138).

Long-term life without oxygen requires adequate supplies of fermentable substrates, pathways for fermentative energy production, and, in many cases, mechanisms to reduce energy demand during anoxia. Freeze-tolerant animals begin the winter with large reserves of glycogen; most of this is used for cryoprotectant synthesis but the remainder supports glycolytic energy production during freezing (138, 155). Freeze-tolerant frogs retain substantial glycogen reserves in all organs (although only liver produces cryoprotectant), and these fuel organ-specific metabolism during freezing-induced ischemia (155). Fermentable amino acids may also be substrates. Alanine is accumulated in various species of insects and frogs at subzero temperature and/or in the frozen state (Figs. 5, 8) (25, 91, 130, 155). This end product is typically the sink for amino groups released during the catabolism of longer-chain amino acids, and its production indicates anaerobic amino acid or protein catabolism coupled to carbohydrate fermentation. In frog skeletal muscle, for example, alanine accumulation during freezing is balanced by a reduction in levels of aspartate, glutamate, and glutamine (155).

Energy production in the frozen state relies mainly on anaerobic glycolysis with lactate and alanine accumulating in both insects and frogs. A more specialized anaerobic metabolism, coupling the fermentation of glycogen and aspartate and producing alanine, succinate, and propionate as end products, allows intertidal marine invertebrates to deal with daily cycles of oxygen deprivation during aerial exposures (66); the role of these fermentative pathways in freezing survival in marine species should be investigated.

Metabolism in the frozen state has been characterized for *E. solidaginis* as an insect model (138, 149) and for *R. sylvatica* as a frog model (147, 155). Studies in both animals showed that the early minutes and hours of freezing [recall that maximal ice formation requires at least 24 h (80, 81)] had no effect on the measured levels of adenylates and phosphagen, indicating that there is no stressful energetic cost to freezing itself (138, 155). This was confirmed by ^{31}P-nuclear magnetic resonance analysis of metabolism in vivo in *E. soli-*

daginis; no abrupt alterations in the signals from phosphagen or adenylates were seen during the freezing process (151).

Eurosta solidaginis larvae are able to survive at least 12 wk of freezing at −16°C (Fig. 8) (138). Energy metabolism showed little disruption (only a 25% reduction in arginine phosphate) over the first week of freezing, a time period representative of the length of most outdoor freezing exposures (140). After

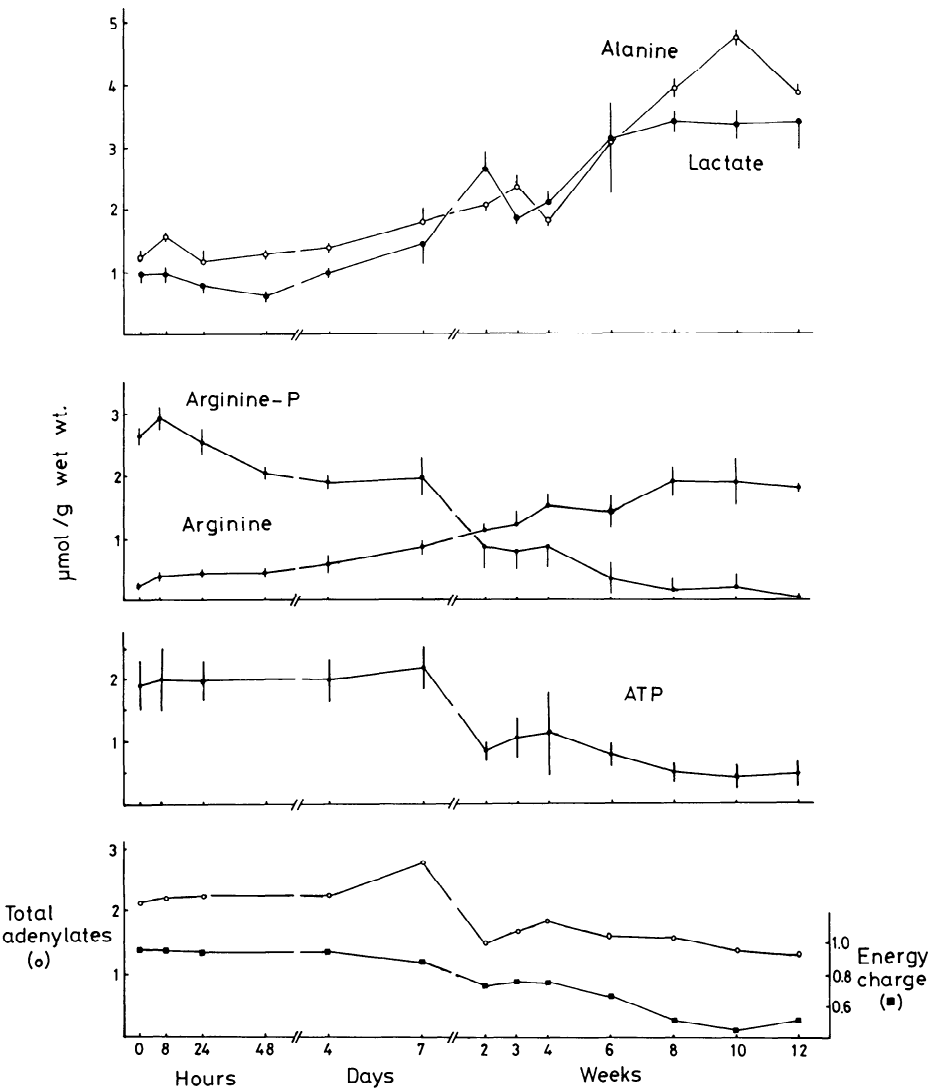

FIG. 8. Metabolism in frozen state in *Eurosta solidaginis* larvae held at −16°C. Energy charge is defined as ([ATP] + ½[ADP])/([ATP] + [ADP] + [AMP]). [Modified from Storey and Storey (138).]

12 wk of freezing, energy stress was severe (despite fermentative ATP production with lactate and alanine accumulating) with phosphagen reserves reduced to <5% of initial amounts and an adenylate energy charge of 0.5. Nonetheless, this stress was not lethal and, after hawing, recovery was rapid and larvae continued development, leading to an 86% hatch rate of adults (138).

Wood frogs also showed good anoxia tolerance over both single and multiple freezing exposures (Fig. 5) (147, 154, 155). Energy charge and total adenylates remained high and constant in tissues such as skeletal muscle over several days of freezing supported by anaerobic glycolysis and creatine phosphate hydrolysis. Liver, which lacks phosphagen reserves, showed a decline in ATP content over time but energy charge was maintained at a high level by a drop in total adenylate content. Again, as in insects, energy stress during freezing was readily reversible when animals were thawed (155). Anaerobic glycolysis in the frozen state showed tissue-specific differences in frogs. Heart produced only lactate, liver accumulated equal amounts of lactate and alanine, and skeletal muscle produced alanine in amounts 4.5-fold greater than lactate (155). Such differences may highlight metabolic function during freezing. The heart continues to circulate blood and cryoprotectant to internal organs well after extremities are frozen and the abdominal cavity filled with ice; this increasingly hypoxic exercise is associated with the accumulation of large amounts of lactate. Skeletal muscle, however, does no work during freezing episodes and shows a response suited to long-term ischemia, i.e., accumulation of alanine, a neutral end product that will not perturb intracellular acid-base balance.

B. Metabolic Rate Depression

The ability to dramatically reduce metabolic rate and enter a hypometabolic state is used as a survival strategy by many species of animals in the face of harsh environmental conditions. Metabolic depression to levels 5–10% of normoxic rates allows facultative anaerobes (such as intertidal invertebrates) to maintain energy balance during anoxia without the need for glycolytic activation (i.e., no Pasteur effect occurs; 145). Limited endogenous fuel reserves are conserved for long-term survival by reducing and rebalancing ATP-utilizing, ATP-producing, and passive processes in the cell to produce a new homeostasis in the hypometabolic state. Metabolic rate depression is a feature of diapause, dormancy, and hibernation and, as such, is key to winter survival for many animals (145). In fact, for many amphibian, reptile, and insect species a period of winter dormancy or diapause is compulsory.

For the freeze-tolerant animal, a hypometabolic state during overwintering has the advantage of greatly improving the chances for survival of the ischemic conditions of the frozen state. Winter dormancy and low-temperature-induced depression of oxygen consumption are common in marine mollusks (1); for example, Q_{10} for oxygen consumption in *M. demissus* was 22

between 4 and 0°C (99, 102). Murphy (102) has shown that animals in such a hypometabolic state have a much higher rate of freezing survival: freeze tolerance was greater for anaerobic versus aerobic and for low- versus high-temperature-acclimated animals. Thus the metabolic depression accompanying winter dormancy and/or induced by the switch to anaerobic metabolism at low temperature facilitates freezing survival. Metabolic depression is undoubtedly also a key factor in the freezing survival of many insects that overwinter in a diapause state. Diapause can lower aerobic metabolic rate by up to 20-fold (172). Diapause in silkworm eggs is, in fact, associated with a reduction of oxygen permeability of the egg membrane, producing a hypoxic state and leading to metabolic rate depression and the polyol accumulation characteristic of diapause (133). The preexisting hypometabolic state allows an easy transition to the oxygen-restricted frozen state.

The biochemical mechanisms regulating metabolic depression have only recently come under study (145). Broad-based mechanisms are needed to coordinate the rates of numerous cellular processes and pathways and produce a new equilibrium in the hypometabolic state. Rates of ATP-producing, ATP-consuming, and passive processes must all be balanced.

To date, we know of three biochemical mechanisms that serve to coordinate metabolic depression (145). All three should be fully investigated with respect to metabolic depression in freeze-tolerant animals.

1. *Enzyme regulation by phosphorylation and dephosphorylation reactions (covalent modification)*

Key enzymes of glycolysis are regulated in this manner to reduce glycolytic flux in tissues of marine mollusks and goldfish during anaerobiosis and during hibernation in mice.

2. *Enzyme and pathway regulation by association or dissociation of enzymes into active complexes bound to subcellular particles*

Lowered glycolytic activity during anaerobiosis in marine mollusks is associated with a decrease in the percentage of glycolytic enzymes bound to the particulate fraction (presumably in active glycolytic particles), whereas glycolytic activation in mammalian systems elicits the opposite response.

3. *Control of carbohydrate usage via fructose-2,6-bisphosphate regulation of PFK*

Fructose-2,6-bisphosphate controls PFK with respect to the carbohydrate requirements of anabolic metabolism. Reduced levels of the activator in the depressed state restrict PFK activity, limit the anabolic use of carbohydrates, and preserve carbohydrates for energy metabolism alone. Fruc-

tose-2,6-bisphosphate content in *R. sylvatica* liver drops 5- to 10-fold with each freezing exposure (147).

C. Nuclear Magnetic Resonance Analysis of Metabolism In Vivo

Nuclear magnetic resonance is an extremely effective technique for examining metabolism noninvasively in a living animal. ^{13}C-nuclear magnetic resonance can be used to investigate the make up of carbon compounds; in *E. solidaginis* larvae, for example, this analysis showed glycerol and sorbitol as the major carbohydrate components as well as a variety of lipid components, including one major type of monounsaturated fatty acid (19). ^{1}H-nuclear magnetic resonance should be useful for examining the state of water in frozen biological systems but, in practice to date, the quantitation and interpretation of H_2O signals are very difficult in systems containing high polyols.

^{31}P-nuclear magnetic resonance detects resonances from adenylates, phosphagen, inorganic phosphate, and sugar phosphates and is widely used to monitor changes in energy status and cellular pH (via the chemical shift of the P_i peak) occurring as a result of experimental manipulation in living cells, tissues, or whole animals. The technique was applied to an examination of freezing and thawing of intact *E. solidaginis* larvae over the range −30 to +25°C (151). Analysis revealed that intracellular pH followed alphastat regulation ($\Delta pH/\Delta T = -0.0185°C^{-1}$) over a wide range, −12 to +15°C. This included the frozen state, suggesting that the effects of freeze concentration on the pH of buffered solutions that are demonstrable in vitro (44) do not occur in vivo. The intracellular pH environment of cold-hardy animals at subzero temperatures and/or in the frozen state appears, therefore, to be one that would maintain constancy in protein structure and function.

^{31}P-nuclear magnetic resonance revealed no abrupt alterations to the spectra of phosphorylated compounds at the point of extracellular freezing (∼−8°C). However, temperature change had dramatic effects on the peak intensities of ATP and arginine phosphate (relative to P_i) in vivo but not in model solutions of standards (151). Peak intensity of the β-peak of ATP dropped from 64 to 2% of the peak intensity of P_i with a temperature decrease from 15 to −20°C; that of arginine phosphate dropped from 78 to 11%. The effect was completely reversed with rewarming. The conclusion to be drawn is that these molecules are increasingly restricted in their rotational freedom in the cell in vivo as temperature declines, perhaps because of increased binding to subcellular components. This could have profound consequences for cellular metabolism and its regulation in the frozen state, for alterations in the bound versus free amounts of these compounds can change regulatory parameters such as phosphorylation potential and energy charge as well as affect the allosteric control of individual enzymes.

Thus we have evidence that cellular metabolism is changed not only by

rate effects in the subzero or frozen state but also by physical effects on solutes that alter their associations with macromolecules and structures. In the frozen state, a large percentage of cellular water is held in an unfreezable state, solutes appear to be increasingly restricted or bound, and diffusion rates decline dramatically. All probably contribute to producing a hypometabolic state.

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