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GLUCOSE CONCENTRATION REGULATES FREEZE TOLERANCE IN THE WOOD FROG *RANA SYLVATICA*

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Summary

In spring, the lowest temperature during freezing that can be survived by wood frogs (*Rana sylvatica*) from southern Ohio is approximately −3 °C. We investigated whether the thermal limit of freeze tolerance in these frogs is regulated by tissue levels of glucose, a putative cryoprotectant that is distributed to tissues during freezing. Frogs receiving exogenous glucose injections prior to freezing showed dose-dependent increases in glucose within the heart, liver, skeletal muscle and blood. Tissue glucose concentrations were further elevated during freezing by the production of endogenous glucose. Most glucose-loaded frogs survived freezing to −5 °C, whereas all control (saline-injected) frogs succumbed. Further, we investigated some mechanisms by which glucose might function as a cryoprotectant in *R. sylvatica.* Organ dehydration, a normal, beneficial response that reduces freezing injury to tissues, occurred independently of tissue glucose concentrations. However, elevated glucose levels reduced both body ice content and *in vivo* erythrocyte injury. These results not only provided conclusive evidence for glucose's cryoprotective role in *R. sylvatica*, but also revealed that tissue glucose level is a critical determinant of freeze tolerance capacity in this species.

Introduction

Freeze tolerance refers to an organism's ability to survive an extensive freezing of body fluids under thermal and temporal conditions of ecological significance to the species. In the terrestrially hibernating wood frog *Rana sylvatica*, freeze tolerance is an important adaptation that promotes winter survival (Schmid, 1982).

The physiology of vertebrate freeze tolerance has received considerable attention over the last decade. Early studies (e.g. Storey and Storey, 1984) determined that the onset of freezing triggers a mobilization of glucose from liver glycogen; the glucose becomes distributed throughout the body. Based on this correlation, it was suggested that glucose protected the animal from cryoinjury. Subsequent studies of *in vitro* responses of tissues and cells from *R. sylvatica* have generally supported this hypothesis. For example, Canty *et al.* (1986) demonstrated that isolated ventricle preparations tolerated freezing only if the suspension medium contained glucose. More recently, Costanzo and Lee (1991) determined that glucose concentrations of 150 mmol l^{-1} reduced freezing injury to

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erythrocytes, whereas concentrations of 15mmol l^{-1} were ineffective. By extrapolating their findings to intact frogs, they concluded that the high blood glucose levels (e.g. 250mmol l^{-1}; Storey and Storey, 1984) attained during freezing sufficiently protect erythrocytes from cryoinjury.

In the sole *in vivo* test of the cryoprotectant hypothesis, Costanzo *et al.* (1991*a*) mitigated the injury associated with rapid cooling (i.e. 1degree h^{-1}; Costanzo *et al.* 1991*b*) by augmenting the glucose levels, *via* glucose injections, of wood frogs prior freezing. They subsequently confirmed that rapid cooling inhibits the synthesis and distribution of glucose (Costanzo *et al.* 1992*a*).

Although the aforementioned studies imply that an elevated glucose level enhances freezing survival in *R. sylvatica*, a direct relationship between tissue glucose concentration and freeze tolerance capacity at the organismal level has not yet been established. Towards this end, we studied *R. sylvatica* from southern Ohio, which following hibernation accumulate relatively small quantities of glucose during freezing and tolerate freezing only at modest body temperatures (approximately $-3\,^{\circ}C$; Layne and Lee, 1987). Our plan was to enhance the freeze tolerance capacity of these frogs by introducing exogenous glucose to supplement natural levels of cryoprotectant. Additionally, we investigated several mechanisms by which elevated glucose levels could promote freeze tolerance. First, cryoinjury to cells might be minimized owing to both colligative and specific actions of glucose (Mazur, 1984; Karow, 1991). Second, by depressing the melting point, elevated glucose levels might reduce total body ice content, a critical determinant of freezing survival (Storey and Storey, 1988). Lastly, hyperglycemia could facilitate organ dehydration during freezing, a beneficial response that reduces cryoinjury to tissues (Lee *et al.* 1992).

Materials and methods

Specimens

Male wood frogs (*R. sylvatica* Le Conte) were collected during February 1991 from breeding ponds in Adams and Scioto Counties, southern Ohio, USA. These frogs readily tolerate freezing at -2.5 to $-3\,^{\circ}C$, but do not recover following exposure to lower body temperatures (e.g. $-5\,^{\circ}C$; Layne and Lee, 1987). All specimens were kept for at least 4 weeks in cages containing damp moss, fasted, and exposed to $4\,^{\circ}C$ in total darkness prior to testing. This regimen promotes their cold tolerance and ensures retention of freeze tolerance (e.g. Costanzo *et al.* 1991*a*,*b*).

Glucose loading

Frogs, randomly assigned to one of three groups, were administered isotonic (115mmol l^{-1}) saline containing glucose in one of the following concentrations: 0 mmol l^{-1} (control), 650mmol l^{-1} or 1500mmol l^{-1}. Following Costanzo *et al.* (1991*a*), the dose (volume, ml=6.7% of body mass) was injected into the dorsal lymph pad using a 27-gauge needle. After receiving the injections, frogs remained in darkened cages at $4\,^{\circ}C$ for 2h prior to further experimentation.

Freezing protocol

Frogs were placed individually within plastic tubes and instrumented with a thermocouple probe positioned against the abdomen (Costanzo *et al.* 1991*a*,*b*). The probe was used in conjunction with a multichannel data logger (OM500, Omega Engineering, Inc.) which made continuous temperature recordings during cooling and freezing. The frogs were cooled by placing the tubes in an ethanol bath. After each specimen had supercooled to approximately −1.0˚C, freezing (verified by a recorded exotherm) was initiated by applying a small ice crystal to the frog's skin. Subsequent cooling to −5˚C required 24–35h.

Freezing survival

Survival rates were determined for frozen frogs (*N*=10 per treatment group) after they had been passively thawed (24h exposure at 4˚C) and transferred to individual cages containing a substratum of damp absorbent paper. Specimens were periodically examined for general health and tested for the righting reflex, a rigorous survival criterion that requires coordinated neuromuscular function (Costanzo *et al.* 1991*b*). Only those frogs meeting the criterion within 1 week of thawing were judged to have survived the freezing episode.

Tissue analyses

Additional frozen frogs (*N*=6 per treatment group) were used in determinations of tissue glucose concentrations and hydration state. Equal numbers of unfrozen frogs, taken directly from their cages at 4˚C, served as controls. All frogs were killed by double-pithing, and rapidly dissected on ice. Blood sampled from the ventricle was collected in heparinized microcapillary tubes and centrifuged at 2000***g***. The resulting plasma was used to determine glucose (see below), osmotic and hemoglobin concentrations. Plasma osmolality was measured using a vapor pressure osmometer (model 5500, Wescor). Free hemoglobin in plasma was measured using a cyanmethemoglobin procedure (no. 525, Sigma Chemical Co.).

The heart and portions (approximately 80mg) of liver and gracilis major (skeletal muscle) were bisected, blotted free of surface moisture, and weighed to 0.1mg. Half of the tissue samples were assayed for glucose; these were rapidly homogenized in 10 volumes of ice-cold perchloric acid (7%, w/v) and centrifuged at 2000***g*.** The deproteinized extracts were neutralized with KOH and assayed using a glucose oxidase procedure (no. 510, Sigma Chemical Co.). Water content of the remaining samples, expressed as a percentage of fresh mass, was calculated from the weight lost during drying at 65˚C.

Calorimetry

Calorimetric procedures detailed by Layne and Lee (1990) were used to estimate the percentage of total body water that was frozen. Frozen frogs (*N*=9 per treatment group) were individually tested in a calorimeter containing 150ml of distilled water. We calculated the mass of ice in each frog (reflected by the change in water temperature)

using the following constants: specific heat of water=4.179 J g^{-1}; specific heat of dry matter, determined calorimetrically=0.88 J g^{-1}; mean body water content, determined by drying carcasses to constant mass=80.8% of fresh mass. Tissue melting point, calculated from mean plasma osmolality (−1.86°C per 1000mosmol), was specific to each treatment group (see Results).

Statistical analyses

The proportions of the treatment groups surviving freezing were compared using Fisher's exact tests. Means comparisons (analyses of variance, ANOVA; Fisher's least significant difference tests) followed Sokal and Rohlf (1973). All statistical procedures involving percentage data were performed using square-root-arcsine transformed values. Significance was judged at $P \leqslant 0.05$.

Results

Freezing survival

None of the frogs in the saline (control) group survived freezing at −5 °C, whereas four in the 650mmol l^{-1} group and eight in the 1500mmol l^{-1} group ultimately recovered. Because mean values for body mass, cooling rate, freeze duration and equilibrium body temperature for the three treatment groups were statistically indistinguishable (Table 1), the differences in survival rates were ascribed to the glucose treatment.

Nine of the ten control frogs died within the first 24h of the recovery period. The remaining specimen, although breathing and slightly responsive to tactile stimulation, generally lacked muscle tone and soon died. In contrast, only one frog in the 650mmol l^{-1} group died during the 7 day observation period. This individual, and five others, never regained muscle tone. All frogs in this group incurred vascular injury (evidenced by a conspicuous dermal hematoma on the thighs and abdomen and marked redness of the eyes) and demonstrated impaired neuromuscular function. However, these conditions were transient in the four specimens that ultimately recovered; these individuals required 3–6 days to recover near-normal body postures. None of the frogs in

Table 1. *Freezing variables and survival data for wood frogs (*Rana sylvatica*) administered saline, 650mmol l^{-1} glucose or 1500mmol l^{-1} glucose, and frozen at −5.0°C*

	Saline	650mmol l^{-1} glucose	1500mmol l^{-1} glucose	*F*	*P*
Body mass (g)	14.7±0.3	16.2±0.5	15.8±0.6	1.9	0.168
Body temperature (°C)	−4.96±0.03	−4.90±0.02	−4.97±0.02	2.3	0.115
Cooling rate (degrees h^{-1})	−0.21±0.01	−0.20±0.01	−0.19±0.01	1.3	0.282
Freeze duration (h)	28.1±0.7	30.2±1.8	30.2±1.8	0.7	0.524
Number surviving/number tested	0/10	4/10	8/10		

Values are means ± S.E.M.; *N*=10 frogs per group.
Statistical data pertain to comparisons of means within rows.
The proportion of surviving frogs differed significantly (χ^2=13.3; *P*=0.001) among injection groups.

the 1500mmol l^{-1} group died, although two, despite having regained limb-retraction reflexes, ultimately failed to meet the righting-reflex recovery criterion. The remaining eight frogs recovered normal body postures and reflex behavior within 3–4 days.

Tissue analyses

The loading treatment significantly elevated glucose concentrations in the heart, liver, muscle and blood of unfrozen frogs in a dose-dependent manner (Table 2). Similarly, glucose concentrations in frozen frogs were highest in the 1500mmol l^{-1} group, intermediate in the 650mmol l^{-1} group and lowest in the saline group (Table 2). Glucose levels were substantially higher in the frozen, *versus* unfrozen, counterparts, indicating that frogs had mobilized endogenous glucose during freezing. Therefore, tissue glucose concentrations in the frozen specimens more accurately represent operant cryoprotectant levels. As a result of the glucose injections, plasma osmotic concentrations of unfrozen frogs in the saline, 650mmol l^{-1} and 1500mmol l^{-1} groups (257±2, 268±2 and 299±4mosmol kg^{-1}, respectively) differed significantly (F=50.3, P<0.001). Although this trend prevailed in the frozen frogs (318±11, 370±24, and 408±52mosmol kg^{-1}), the differences among the means were not statistically significant (F=1.8, P=0.207), perhaps owing to large sample variances.

Dynamics of tissue water

Freezing significantly lowered the mean water content in the hearts and livers of frogs in all treatment groups (two-factor ANOVA; heart, F=100.7, P<0.001; liver, F=55.9, P<0.001). The water lost during freezing, expressed as a percentage of prefreeze levels, ranged from 13.6 to 16.6% in the heart and from 12.1 to 24.5% in the liver (Table 3); however, these changes did not differ among treatment groups (heart, F=0.1, P=0.93; liver, F=2.4, P=0.11). Skeletal muscle also dehydrated significantly during freezing

Table 2. *Glucose concentrations in organs (μmol g^{-1}) and blood (μmol ml^{-1}) from wood frogs (*Rana sylvatica*) administered saline, 650mmol l^{-1} glucose or 1500mmol l^{-1} glucose, and sampled before or after freezing at –5.0°C*

	Saline	650mmol l^{-1} glucose	1500mmol l^{-1} glucose	*F*	*P*
Before freezing					
Heart	0.7±0.1[a]	32.8±4.8[b]	60.5±3.9[c]	69.9	<0.001
Liver	3.8±1.4[a]	31.9±3.7[b]	49.7±2.5[c]	74.5	<0.001
Muscle	2.2±0.5[a]	7.3±1.6[b]	18.3±1.8[c]	33.7	<0.001
Plasma	1.4±0.1[a]	49.3±8.2[b]	78.2±6.5[c]	41.1	<0.001
After freezing					
Heart	11.8±5.8[a]	81.8±14.1[a,b]	145.8±37.9[b]	8.1	0.004
Liver	17.3±6.5[a]	198.8±57.9[b]	241.8±40.2[b]	8.5	0.003
Muscle	5.3±1.3[a]	17.0±4.2[a,b]	29.1±5.7[b]	8.1	0.004
Plasma	17.9±6.2[a]	75.2±15.4[b]	86.8±8.9[b]	13.5	<0.001

Values are means ± S.E.M.; N=6 frogs per group.
Within rows, means identified by different superscripts were statistically distinguishable.

Table 3. *Water content (percentage of fresh mass) of organs from wood frogs (*Rana sylvatica*) administered saline, 650mmol l^{-1} glucose or 1500mmol l^{-1} glucose, and sampled before or after being frozen at –5.0°C*

	Prefreeze	Postfreeze	*F*	*P*
Heart				
Saline	83.1±0.6	70.8±2.4	25.4	<0.001
650mmol l^{-1} glucose	83.5±0.5	69.6±2.1	45.1	<0.001
1500mmol l^{-1} glucose	82.8±0.3	71.5±2.0	41.2	<0.001
Liver				
Saline	74.2±0.5	56.0±1.1	224.4	<0.001
650mmol l^{-1} glucose	76.3±1.0	58.2±4.3	16.9	0.002
1500mmol l^{-1} glucose	75.1±1.0	66.0±3.9	7.0	0.023
Muscle				
Saline	80.0±0.2	78.4±0.7	5.3	0.044
650mmol l^{-1} glucose	79.7±0.2	71.6±1.5	29.0	<0.001
1500mmol l^{-1} glucose	76.3±0.6	75.0±1.1	0.3	0.574

Values are means ± S.E.M.; *N*=6 frogs per group.
Statistical data pertain to comparisons of means within rows.

(*F*=27.6, *P*<0.001), although in this organ mean water content was also dependent on treatment group (*F*=11.3, *P*<0.001). *Post-hoc* analyses (one-factor ANOVA) showed that the group means for muscle of unfrozen frogs were non-uniform (*F*=29.2, *P*<0.001), an effect that confounded the statistical interpretation of these results. Nevertheless, inspection of the data revealed no dose-dependent trends in water loss from muscle during freezing (Table 3).

Body ice content

Calorimetric equations required estimates of body water content and tissue melting point. Because the body water content of frogs in the saline, 650mmol l^{-1} and 1500mmol l^{-1} groups (*N*=9, 5 and 9, respectively) were statistically indistinguishable (*F*=2.84, *P*=0.084), the pooled mean, 80.8±0.3% (*N*=23), was used in the calculations. Tissue melting points were −0.59°C, −0.69°C and −0.76°C for frogs in the saline, 650mmol l^{-1} and 1500mmol l^{-1} groups, respectively.

Because the frogs in all treatment groups were matched for body size (*F*=0.1, *P*=0.887) and frozen at a similar equilibrium body temperature (*F*=0.5, *P*=0.644), differences in body ice contents were directly attributable to the injection treatment. Generally, body ice content was inversely related to the quantity of glucose administered (Table 4).

Cryoinjury to erythrocytes

The degree of catastrophic cryoinjury to the erythrocytes of frozen frogs was gauged by the concentration of hemoglobin in plasma, which differed significantly (*F*=7.8; *P*=0.005) among frogs in the saline (68.5±11.2mg ml^{-1}), 650mmol l^{-1} (42.1±6.0mg ml^{-1}) and 1500mmol l^{-1} (25.8±3.9mg ml^{-1}) groups. Hemoglobin was not detected in the plasma of any unfrozen frog.

Table 4. *Estimates of ice content and unfreezable water fraction for wood frogs (*Rana sylvatica*) administered saline, 650mmol l^{-1} glucose or 1500mmol l^{-1} glucose, and frozen at –5.0°C*

	Saline	650mmol l^{-1} glucose	1500mmol l^{-1} glucose
Body ice content (percentage of total body water)			
Empirical (calorimetric) estimate	76.3±1.5[a]	73.2±2.2[a]	69.7±1.8[b]
Model estimate	88.2	86.2	84.8
Unfreezable body water (percentage of total body water)	11.9	13.0	15.1

Mean values (±S.E.M.; *N*=9 frogs per group) identified by different superscripts were statistically distinguishable.

Modeled ice content was derived from equation 2 of Claussen and Costanzo (1990) and hypothetically assumes that all body water is freezable.

Recovery from freezing stress

The eight surviving frogs in the 1500mmol l^{-1} group were killed and assayed on the eighth day of the recovery period. They had levels of plasma glucose (6.7±1.3 μmol ml^{-1}), osmolality (259±3mosmol kg^{-1}) and hemoglobin (2.8±0.7mg ml^{-1}) that were lower (F=106.4, P<0.001; F=11.1, P<0.001; F=44.4, P=0.006, respectively) than levels in their frozen counterparts, but quite similar to levels in the unfrozen frogs (1.4±0.1 μmol ml^{-1}; 257±2mosmol kg^{-1}; 0mg ml^{-1}, respectively). Therefore, the effects of both the glucose injection and the freezing episode were considerably diminished, but not completely ameliorated, by the time the frogs were sampled.

Discussion

All of the ten species of vertebrates considered ‘freeze tolerant’ normally experience subzero body temperatures in nature, and are thus adapted to survive an extensive freezing of their body fluids. Inter- and intraspecific differences exist in the freeze tolerance capacity of these animals, which, in one ecological context, is delimited by the minimum body temperature tolerated during freezing. Other factors being equal, a lower critical temperature broadens an individual’s freeze tolerance capacity, thus increasing the probability of winter survival.

Low temperature is a primary constraint on freeze tolerance capacity inasmuch as it promotes (lethal) intracellular freezing and increases body ice content and the associated osmotic stress to cells and tissues (Mazur, 1984). Cryoprotectant production is one physiological strategy employed by certain animals to overcome these effects. The present study not only provided conclusive evidence that glucose has a cryoprotective role in *R. sylvatica*, but also suggested that freeze tolerance capacity in this species is governed by tissue glucose levels.

Glucose enhances freezing survival

The lowest body temperature survived by spring-collected *R. sylvatica* from southern

Ohio is approximately −3 °C (Layne and Lee, 1987). Not unexpectedly, our control frogs failed to recover following freezing at −5 °C. However, specimens receiving glucose supplements prior to freezing had markedly improved survival rates which, owing to the careful control of potentially confounding factors (i.e. body size, cooling rate, freeze duration and body temperature), can be directly attributed to the cryoprotective action of this compound. Frogs rendered hyperglycemic *via* glucose injections clearly mobilized glucose during freezing (Table 2). Thus, the mechanism of cryoprotectant production apparently functions independently of extant glucose levels. Accordingly, Costanzo *et al.* (1991*a*) concluded that the cryoprotectant system of *R. sylvatica* lacks a negative feedback mechanism.

The blood glucose level in control frogs after freezing, 18 μmol ml^{-1}, was comparable to values reported earlier (Costanzo *et al.* 1991*a*,*b*; Layne and Lee, 1987) for spring-collected *R. sylvatica* from southern Ohio. This level of cryoprotectant permits frogs to survive freezing at −2.5°C, but, as determined in the present study, not at −5 °C. In contrast, eight of the ten frogs administered 1500 mmol l^{-1} glucose tolerated freezing at −5 °C. These specimens had glucose levels in plasma and liver of 87 μmol ml^{-1} and 242 μmol g^{-1}, respectively. However, because the survival rate for these frogs was less than 100%, these glucose concentrations may represent near-threshold levels required to tolerate freezing at this temperature. Interestingly, the glucose levels associated with the survival of our frogs were comparable to those attained during freezing by autumn-collected *R. sylvatica* from a more northerly population that naturally survives freezing at −5 °C (Storey and Storey, 1984).

Because hepatic glycogen is the primary source of cryoprotectant in *R. sylvatica* (Storey and Storey, 1984), the size of the glycogen reserve is a major determinant of the quantity of glucose produced (Costanzo and Lee, 1993). Temperate-region anurans metabolize stored carbohydrate during winter and therefore commonly have lower glycogen reserves in spring than in autumn (e.g. Jungreis and Hooper, 1970; Koskela and Pasanen, 1975; Smith, 1950). Those inhabiting northerly regions may initiate hibernation with relatively larger glycogen reserves than more southerly conspecifics (see Costanzo and Lee, 1993). Judging from the results of the present study, it seems reasonable that both seasonal (Layne and Lee, 1989; Schmid, 1982; Storey and Storey, 1988) and geographic (Costanzo *et al.* 1992*b*; Layne and Lee, 1987) variability in anuran freeze tolerance chiefly stem from differential capacities for cryoprotectant production.

Having demonstrated the critical role of glucose in promoting freeze tolerance in *R. sylvatica*, we conducted additional experiments to elucidate the physiological basis for this relationship. Cryoprotectants commonly function by depressing the melting point of body fluids, which reduces the ice content and associated osmotic stress at a given body temperature (Mazur, 1984). They may also exert 'specific effects,' such as stabilizing macromolecules and cell membranes (Karow, 1991). Additionally, high glucose levels might facilitate other physiological responses to freezing stress, such as the evacuation of water from organs. By implementing our glucose-loading protocol, we investigated these potential effects.

Organ dehydration during freezing

During freezing, organs of *R. sylvatica* lose up to half of their water, which becomes sequestered, as ice, within the coelomic cavity and beneath the skin (Lee *et al.* 1992). This response reduces the mechanical injury to tissues caused by excessive ice formation (Rubinsky and Pegg, 1988) and thus represents a major physiological adaptation promoting freeze tolerance in *R. sylvatica*. The mechanism effecting the withdrawal and redistribution of tissue water during freezing remains to be identified (Costanzo *et al.* 1992*a*; Lee *et al.* 1992).

The present data do not support the hypothesis that hyperglycemia regulates organ dehydration in a concentration-dependent manner. Overall, the degree of organ dehydration was uniform among the treatment groups and in good accord with values published earlier (e.g. Costanzo *et al.* 1992*a*). Although the mechanism effecting organ dehydration appears to operate independently of glucose concentration, our experimental design permitted only tests of concentration-dependent responses. Thus, we cannot dismiss the possibility that even modest hyperglycemia (such as that achieved by the control frogs) facilitates organ dehydration during freezing.

Body ice content

About 65% of the total body water freezes when *R. sylvatica* is cooled to -2.5°C (Layne and Lee, 1987; Lee *et al.* 1992). This level approximates to the reported maximum survivable limit for many species (e.g. Storey and Storey, 1988). However, the results for our glucose-loaded frogs, many of which survived freezing at -5°C, suggest that the critical ice content in *R. sylvatica* is closer to 70% than 65%. The demise of our saline-injected frogs was undoubtedly related to their relatively higher ice contents (mean 76%).

Our finding that body ice content was inversely related to tissue glucose concentration in *R. sylvatica* represents a convincing demonstration of the role of increased solute concentration in reducing body ice content. Interestingly, however, the production of glucose accounted for no more than 27% of the increase in plasma osmolality associated with freezing. Costanzo and Lee (1993) reported similar results for spring-collected *R. sylvatica* and estimated that plasma osmolality increased by $32\,\mathrm{mosmol\,kg^{-1}}$ during freezing as a result of the addition of non-glucose osmolites.

Cryoprotectants, such as glucose, may also limit ice formation by increasing the fraction of water that is 'bound,' or nonfreezable, by virtue of its close association with membrane structures and macromolecules. A model of the colligative properties of solutions, equilibrium melting point and body temperature (Claussen and Costanzo, 1990) was used to derive ice contents for our frogs, assuming (hypothetically) that all body water is freezable. Not unexpectedly, these values were higher than our calorimetric estimates of body ice contents (Table 4). The mathematical difference between the modeled and empirical values represents the fraction of unfreezable water. Interestingly, the group means (12–15%) increased in proportion to tissue glucose concentration. Some cryoprotectants indeed function by increasing the fraction of unfreezable water (Storey and Storey, 1988). However, given our limited data, for *R. sylvatica* this assertion remains tentative.

Freezing survival of erythrocytes

In an *in vitro* study of erythrocytes from *R. sylvatica*, Costanzo and Lee (1991) determined that glucose markedly enhances the tolerance of these cells to stresses associated with freezing and thawing. Subsequent *in vivo* tests of erythrocyte freezing survival have yielded similar results (Costanzo *et al.* 1991*a*). In the present investigation, the level of erythrocyte cryoinjury was inversely related to plasma glucose concentration. Our results thus bolster the hypothesis that glucose mitigates freezing injury at the cellular level.

Some hemolysis occurred during survivable freezing episodes in the present and previous (Costanzo *et al.* 1991*a*) studies. Consequently, thawed frogs are somewhat anemic, although this condition may not necessarily compromise their oxygen delivery system (e.g. Pitkin, 1987). The hemolysis apparent in these frogs nevertheless asserts that sublethal cell damage occurs during survivable freezing episodes. Conceivably, the level of such injury determines the time required to re-establish homeostasis and to recover normal neurobehavioral function. For example, recovery is usually rapid following moderate freezing exposures (Layne and First, 1991), but is deferred subsequent to more stressful freezing episodes, such as those involving rapid cooling (Costanzo *et al.* 1991*a*,*b*) or, in the present study, exposure to low temperature. Qualitatively, the time course for recovery in our *R. sylvatica* was inversely related to glucose levels, a result that further supports the cryoprotective role of this compound.

In addition to the repair of sublethal cellular injury, recovery from the freezing episode must also include the re-establishment of normal carbohydrate levels and osmotic balance, since marked deviations in these variables induce physiological stress. Our data revealed that hyperglycemia and hyperosmolality prevailed even 8 days after thawing. The physiological consequences associated with mechanisms promoting freezing survival are potentially significant and merit further study.

In the present study, the thermal limit of freeze tolerance in wood frogs was regulated by tissue levels of the cryoprotectant glucose. Organ dehydration, a beneficial response that reduces mechanical injury to tissues during freezing, occurred independently of tissue glucose concentrations, but elevated glucose levels directly mitigated cryoinjury by reducing the body ice content and cellular injury. Glucose loading proved an effective experimental protocol for the study of freeze tolerance.

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