Glucose loading prevents freezing injury in rapidly cooled wood frogs

JON P. COSTANZO, RICHARD E. LEE, JR., AND MICHAEL F. WRIGHT
Department of Zoology, Miami University, Oxford, Ohio 45056

COSTANZO, JON P., RICHARD E. LEE, JR., AND MICHAEL F. WRIGHT. *Glucose loading prevents freezing injury in rapidly cooled wood frogs.* Am. J. Physiol. 261 (Regulatory Integrative Comp. Physiol. 30): R1549–R1553, 1991.—The wood frog (*Rana sylvatica*) is the most commonly studied of ten species of freeze-tolerant vertebrates. Under natural (i.e., slow) rates of cooling, freezing initiates the production of the cryoprotectant glucose, which is mobilized from the liver and distributed to tissues throughout the body. Rapid cooling during freezing is injurious to wood frogs, probably because cryoprotectant production and mobilization are inhibited. To test this hypothesis, we investigated whether rapid-cooling injury is reduced if exogenous glucose is experimentally introduced to tissues before freezing. Glucose-loaded and control (saline-injected) wood frogs were rapidly cooled during freezing to −2.5°C and subsequently assayed for injury at both cellular (erythrocyte) and neuromuscular (behavioral reflex) levels. Rapid cooling produced substantial hemolysis in control frogs, but erythrocyte injury was significantly reduced in glucose-loaded frogs. Similarly, neuromuscular injury was significantly higher in control frogs than in glucose-loaded frogs. These findings suggest that rapid-cooling injury results from an inadequate production and distribution of endogenous glucose during freezing. Furthermore, the inverse relationship between the degree of freezing injury and the quantity of exogenous glucose present strongly implicates glucose as a cryoprotectant in *R. sylvatica*.

freeze tolerance; vertebrate; cryoprotectant; erythrocyte; hemolysis

NEARLY A DECADE has elapsed since Schmid (14) first conclusively demonstrated and recognized freeze tolerance as an overwintering adaptation in vertebrates. Currently, freeze tolerance is known in five species of frogs [*Rana sylvatica*, *Pseudacris triseriata*, *Hyla crucifer*, *Hyla versicolor*, and *Hyla chrysoscelis* (10, 16)], one salamander [*Hynobius keyserlingi* (1)], one snake [*Thamnophis sirtalis* (4)], and three turtles [*Chrysemys picta*, *Terrapene carolina*, and *Terrapene ornata* (5, 7, 11, 17)]. Both snapping turtles [*Chelydra serpentina* (7)] and wall lizards [*Podarcis muralis* (3)] survive nominal freezing, but the adaptive value of this capacity is questionable.

With most freeze-tolerant frogs, freezing initiates a synthesis and mobilization of the cryoprotectant glucose. In the wood frog (*R. sylvatica*), for example, the concentration of glucose in the liver increases rapidly during freezing. Within minutes after the onset of ice formation, elevated glucose is also detected in the blood and other tissues (15). Ultimately, glucose concentrations in the blood of Canadian *R. sylvatica* average 200–250 μmol/ml (18), but those in frogs from Ohio, near the southern extent of the species' range, are typically only 15 μmol/ml (8, 12). These concentrations were determined for thawed tissues; effective glucose levels must be substantially higher in frozen tissues, since accumulating ice reduces the solvent volume, thereby concentrating solutes.

Storey and Storey (16) ably discussed the advantages of using glucose as a cryoprotectant. Aside from its obvious role in cellular metabolism, glucose is rapidly produced, easily transported and, in high concentrations, may induce hypometabolism. Furthermore, glucose is a critical substrate for anaerobic energy production; this is an important benefit, since freezing tissues ultimately become ischemic.

Few studies of freeze-tolerant vertebrates have examined the specific mechanisms by which glucose functions as a cryoprotectant. Working with *R. sylvatica*, Storey and Storey (16) determined that elevated glucose protects hepatocytes frozen in vitro, and Canty et al. (2) established that glucose (but not glycerol) protects myocardium preparations from freezing injury. Recently, Costanzo and Lee (6) demonstrated that both glucose and glycerol are effective cryoprotectants of wood frog erythrocytes and concluded that natural blood levels are sufficient to protect these cells during natural freezing episodes. These investigations provided important evidence for in vitro cryoprotection, yet studies of glucose's cryoprotective capacity in intact vertebrates are lacking.

We recently determined that freeze tolerance in *R. sylvatica* depends on slow cooling during the freezing bout. Cooling at high rates (e.g., −1.2°C/h) results in injury that is avoided when frogs are cooled more slowly (e.g., −0.2°C/h) during freezing to −2.5°C (8). Through subsequent study (9) we determined that rapid cooling reduces glucose production in the liver by about one-half; consequently, other tissues (heart, muscle, eye, brain) show only modest or no increase in cryoprotectant during freezing. Glucose mobilization probably is hampered because rapid cooling hastens cardiovascular failure that, during slow cooling, is deferred for 20 h or more (13).

Although these earlier studies (8, 9) showed that rapid cooling results in freezing injury and much reduced cryoprotectant levels, a causal relationship between these responses was not established. Therefore, we tested the hypothesis that cryoinjury in rapidly cooled *R. sylvatica* occurs because insufficient glucose is produced and distributed to tissues during freezing. Our investigation was

based on the premise that injury should be reduced if glucose is experimentally introduced to tissues before freezing. We used a novel approach involving the administration of exogenous glucose to increase cryoprotectant levels in wood frogs. We report that glucose loading before freezing reduced rapid-cooling injury, both at cellular and neuromuscular levels.

MATERIALS AND METHODS

Specimens. Male wood frogs (*R. sylvatica*) were collected from breeding ponds in Adams County in southern Ohio during early February 1990. Frogs collected at this time are fully freeze tolerant (8, 9, 12). The frogs were maintained in laboratory cages containing damp moss, fasted, and exposed to 4°C in total darkness to simulate winter conditions. All frogs were acclimated for at least 8 wk and were in good health before use.

Glucose loading. Our preliminary studies showed that wood frogs administered a volume (up to 10% of body mass) of 1.4 M glucose survived with no apparent injury. Thus we judged glucose loading to be a reasonable experimental approach in the present investigation. Frogs (mean mass ± SE = 13.5 ± 0.3 g; $n = 45$) were randomly assigned to one of three groups and administered isotonic (115 mM) phosphate-buffered saline containing glucose in one of the following concentrations: 0 (control), 190, or 650 mM. Using a 27-gauge needle, we injected the dorsal lymph pad of each frog with a volume equivalent to 6.7% of its body mass. After the injections, frogs remained in darkened cages at 4°C for 2.5 h before further experimentation. The glucose concentrations of the injection media were calculated to produce target plasma concentrations of 15 and 50 μmol/ml glucose, values within the range of endogenously produced concentrations reported for frozen *R. sylvatica* (12, 15). The calculations assumed that the body water content was 80% of fresh mass, glucose would be homogeneously distributed among body fluid compartments, and basal glucose levels were uniformly 1.0 μmol/ml (12).

Freezing protocol. Ten frogs in each group were rapidly cooled during freezing to −2.5°C, a normally injurious treatment (cf. Ref. 8). Concomitantly, the five frogs remaining in each group were assayed to characterize prefreeze tissue chemistry (see below). Frogs to be frozen were placed inside a 50-ml plastic centrifuge tube, equilibrated to 0°C in an ice bath, and submerged in a precooled ethanol bath (RTE 210, Neslab Instruments). A thermocouple probe, placed against the abdomen of each frog and shielded from the tube wall with foam insulation, provided a continuous temperature recording on a multichannel data logger (OM500, Omega Engineering).

After the frogs supercooled to between −1.0°C and −1.5°C, ice nucleation (verified for each frog by a recorded exotherm) was induced by lightly applying aerosol coolant to the tube's exterior. Frogs subsequently required 4.8 ± 0.2 h ($n = 30$) to reach −2.5°C; they were kept at this temperature an additional 1–1.5 h to allow the entire body to reach thermoequilibrium. Mean cooling rate during freezing, calculated from temperature recordings in the interval −1°C to −2°C, was −1.2 ± 0.1°C/h ($n = 30$). Subsequently, frogs were transferred to a cold room (4°C) and allowed to thaw for 2 h. Tissue analyses were conducted on one-half of the thawed frogs in each group; the remaining individuals were used to determine recovery period, an index of neuromuscular injury (see below).

Tissue analyses. Frogs were quickly killed by double-pithing before being dissected on ice. Blood was collected from the aorta in heparinized microcapillary tubes and centrifuged; the plasma was saved (−80°C) for glucose, osmolality, and hemoglobin determinations. A portion of the liver (~100 mg) was immediately excised, lightly blotted, weighed, and homogenized in 1.0 ml ice-cold phosphate-buffered saline (115 mM, pH 7.4). Homogenates were centrifuged at 2,000 *g*; extracts were rapidly frozen (−80°C) for use in glucose assays. Glucose was measured using an enzymatic, spectrophotometric method for deproteinized samples (procedure no. 510, Sigma, St. Louis, MO). Osmotic concentration in plasma was measured using a vapor pressure osmometer (model 5500, Wescor).

Indexes of rapid-cooling injury. Because hemoglobin escapes from critically damaged erythrocytes, we gauged in vivo red blood cell injury by the quantity of free hemoglobin measured in plasma. Plasma samples collected from thawed frogs were assayed for hemoglobin using a spectrophotometric method (procedure no. 525, Sigma). An index of neuromuscular injury was based on the time required for intact frogs to regain the righting reflex. Once thawed, frogs were removed from their tubes, placed individually in plastic containers on a damp paper substrate, and monitored daily for the ability to right within 2 s of being placed on their backs. Frogs were kept for 7 days at 4°C, but thereafter exposed to 22°C. Individuals were assigned a recovery score corresponding to the date the righting reflex was first demonstrated. Those failing to recover within a 14-day period were assigned a score of 14.

Statistical analyses. Mean values for plasma and liver glucose concentration, and plasma osmolality were compared statistically between treatments (prefreeze vs. postfreeze) and among control, 190 mM glucose-injection, and 650 mM glucose-injection groups using two-factor ANOVAs. Where appropriate, Fisher's least significant difference tests were employed to statistically separate means within treatments. Correlation analysis was used to relate plasma glucose and hemoglobin concentrations. Mean hemoglobin concentrations and mean recovery scores were compared using one-factor ANOVAs. Log-transformed data were used in all parametric procedures. Significance was judged at $P < 0.05$. Values are reported as means ± SE.

RESULTS

Effects of glucose administration: unfrozen frogs. Tissue analyses were conducted on five unfrozen frogs in each group to establish baseline characteristics after the injections (Table 1). Control (saline-injected) frogs had mean plasma and liver glucose concentrations of 1.4 μmol/ml and 11.4 μmol/g, respectively. Frogs injected with 190 mM glucose and 650 mM glucose had relatively higher mean plasma concentrations (16.0 and 53.9 μmol/

TABLE 1. *Analyses of tissues from wood frogs (Rana sylvatica) administered saline, 190 mM glucose, or 650 mM glucose and sampled before or after freezing to −2.5°C*

	Liver Glucose, μmol/g	Plasma Glucose, μmol/ml	Plasma Osmolality, mosmol/kg
	Prefreeze		
Control (saline)	11.4±1.9*	1.4±0.3*	249±3.7*
Glucose			
190 mM	12.7±1.0*	16.0±3.9†	257±2.7*†
650 mM	26.8±3.9†	53.9±7.8‡	267±4.5†
	Postfreeze		
Control (saline)	50.2±15.2*	17.9±4.7*	268±4.9*
Glucose			
190 mM	93.9±7.4†	37.5±6.6†	279±3.6*†
650 mM	71.7±6.6*†	71.7±5.7‡	283±4.7†
	P		
Between treatments	<0.001	<0.001	<0.001
Among groups	0.016	<0.001	0.002

Values are means ± SE; $n = 5$ frogs/group. Comparisons between treatments (prefreeze vs. postfreeze) and among injection groups were made using 2-factor analyses of variance. Within each treatment, sample means for injection groups were compared using Fisher least significant difference tests; means identified by different symbols were statistically distinguishable.

ml, respectively) that approximated the respective target values, 15 and 50 μmol/ml; they also had elevated mean liver concentrations (Table 1). Glucose loading significantly increased plasma osmolality (Table 1).

Effect of freezing on glucose-loaded frogs. Glucose concentrations in frozen frogs were significantly higher than in their unfrozen counterparts (Table 1), with maximal increases approaching 7.4-fold in liver (190 mM glucose group) and 12.8-fold (control group) in plasma. Glucose concentrations in the plasma and livers of frozen frogs reflected the quantity of glucose injected before freezing, although liver values were highly variable within injection groups (Table 1). The plasma osmolality of frozen frogs was significantly elevated over their unfrozen counterparts and depended on the quantity of glucose injected before freezing (Table 1).

Rapid-cooling injury. Freezing injury to erythrocytes was determined on the basis of hemoglobin concentration in plasma. Mean hemoglobin concentrations were highest in the control group (12.8 ± 3.4 mg/ml), intermediate in the 190 mM glucose group (8.5 ± 1.3 mg/ml), and lowest in the 650 mM glucose group (1.9 ± 0.5 mg/ml); these differences were highly significant ($F = 16.4$, df = 14, $P < 0.001$). Hemoglobin was not detected in the plasma of any unfrozen frogs.

Neuromuscular injury attributed to freezing was assessed on the basis of the recovery score, the time required to regain a critical behavioral reflex. The mean recovery score was 10.6 ± 1.7 days for the control group, 8.6 ± 1.9 days for the 190 mM glucose group, and 3.2 ± 1.1 days for the 650 mM glucose group; these means differed significantly ($F = 6.1$, df = 14, $P = 0.015$). Frogs in the 650 mM glucose group recovered most rapidly, with one individual regaining the righting reflex within 1 day of thawing. Three additional frogs had recovered by the fourth day, and recovery of all frogs in this group occurred within 8 days. In contrast, none of frogs in the control group recovered until 6 days after thawing, and two failed to recover within the course of the experiment. Ultimately, one frog in the 190 mM glucose group and two frogs in the control group died.

DISCUSSION

In the present study, glucose loading proved an effective experimental manipulation for evaluating mechanisms of freeze tolerance in *R. sylvatica.* When sampled 2.5 h after the administration of glucose, concentrations of glucose in plasma and (to a lesser extent) liver were elevated above those of control frogs. The plasma osmotic concentration was elevated in glucose-loaded frogs, but the increase was not proportional to the quantity of exogenous glucose; the reason for this incongruence is unknown. We assume that frogs used in the freezing trials were similarly rendered hyperglycemic and elevated glucose was present in their tissues at the onset of freezing. Thus the reduction in rapid-cooling injury in glucose-loaded frogs is attributed to the presence of exogenous glucose during freezing.

Under natural (i.e., slow cooling) conditions, freezing of *R. sylvatica* is accompanied by an endogenous production of glucose in liver and its distribution to other tissues (15). However, the rapid-cooling protocol used in the present study inhibits glucose production and mobilization in *R. sylvatica* (9). For example, in rapidly cooled frogs liver glucose was elevated to 22.7 μmol/g, only about one-half the concentration (43.7 μmol/g) produced in slowly cooled frogs. Furthermore, no increase in glucose concentration occurred in peripherally located organs (e.g., skeletal muscle, eye) during rapid cooling, whereas significant increases resulted with slow cooling. Glucose mobilization is inhibited by rapid cooling probably because it accelerates cardiovascular failure (9). In the present study, endogenous glucose was produced despite our use of the rapid-cooling protocol (Table 1); however, probably much of this quantity was synthesized during thawing rather than freezing (9). Because little endogenously produced glucose was present during the freezing bout, the responses of the frogs primarily depended on the glucose and osmotic concentrations measured in frogs before freezing.

Interestingly, the increase in plasma glucose (range, 16.5–21.5 μmol/ml; see Table 1) was nearly consistent among injection groups and, therefore, independent of prefreeze glucose concentration. Thus the process of glucose production probably lacks stringent regulation (e.g., via a negative-feedback system). The precise mechanism triggering cryoprotectant synthesis has yet to be determined, although Storey (18) suggested it may be an exaggeration of the vertebrate "fight or flight" response, perhaps stimulated by the action of catecholamines in liver. Our present results seem consistent with this notion.

Assuming that rapid cooling is injurious primarily because glucose is inadequately produced and distributed to tissues during freezing, injury should be mitigated if cryoprotectant levels are experimentally elevated in tissues before freezing. We chose two response variables

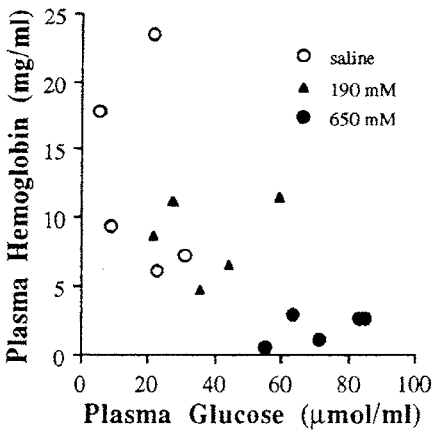

FIG. 1. Relationship between plasma concentrations of glucose and hemoglobin in wood frogs (*Rana sylvatica*) administered saline, 190 mM glucose, or 650 mM glucose before rapid cooling during freezing to −2.5°C. The correlation was significant ($r^2 = 0.44$, $F = 10.2$, df = 14, $P = 0.007$).

(erythrocyte injury, neuromuscular injury) with which to test this hypothesis. Because glucose loading reduced rapid-cooling injury for both criteria, our data strongly support this hypothesis.

Glucose adequately protects wood frog erythrocytes frozen in vitro at ecologically relevant temperatures (6). Hemolysis is prevented during slow cooling, since sufficient glucose is carried in the blood. Little cryoprotectant was present in our rapidly cooled control frogs, consequently in vivo cryoinjury to erythrocytes was substantial. Glucose loading significantly reduced cryohemolysis, with the severity of cell injury contingent on the quantity of glucose administered. Accordingly, plasma concentrations of hemoglobin and glucose in individual frogs were strongly correlated (Fig. 1), firmly attesting to the cryoprotective capacity of glucose.

Rapid cooling impairs neuromuscular function, thus delaying behavioral recovery after freezing (8). Criteria for freezing recovery of vertebrates, which include apparent well-being (12, 17), simple neuromuscular reflexes (8, 15), feeding ability (3, 5, 9), and reproductive capacity (4), have not been standardized. We chose the righting reflex as our recovery criterion because this behavior rigorously requires coordinated neuromuscular function. Slowly cooled *R. sylvatica* typically recovers neuromuscular reflexes and normal behaviors within 24 h of thawing (12), whereas rapidly cooled frogs require substantially longer periods (8). The debilitating effect of rapid cooling is evident in the present study, yet the administration of exogenous glucose before freezing greatly reduced neuromuscular injury. Furthermore, the differential recovery periods among the treatment groups supports the notion that glucose functions as a cryoprotectant in *R. sylvatica*.

Observations incidental to our study's design showed that mortality occurred in the control group (2 individuals) and in the 160 mM glucose group (1 individual), but all frogs in the 650 mM glucose group survived. Interestingly, three control frogs survived freezing (under unnaturally harsh conditions) despite apparently low levels of cryoprotectant. Hyperglycemia clearly maximizes freeze tolerance capacity in *R. sylvatica*, but high concentrations of cryoprotectant may not be essential for freezing survival under ecologically relevant conditions.

Our results clearly demonstrated that rapid-cooling injury was mitigated by the administration of exogenous glucose before freezing. We therefore conclude that rapid cooling is damaging because insufficient endogenous cryoprotectant is produced and distributed to tissues during freezing. Furthermore, the severity of the injury at both cellular and neuromuscular levels depended on the quantity of glucose present, thus providing additional direct evidence that glucose functions as a cryoprotectant in *R. sylvatica*.

We thank Rocky Knauf and Ted Easterling for granting access to their properties to collect frogs. Chris Chabot and Janet Strong-Gunderson provided critical reviews of the manuscript.

This work was supported by National Heart, Lung, and Blood Institute Grant 1 R15 HL-40535-01 to R. E. Lee and by the Ohio Board of Regents Research Challenge Grant, Miami University, to R. E. Lee.

Address for reprint requests: J. P. Costanzo, Dept. of Zoology, Miami University, Oxford, OH 45056.

Received 8 March 1991; accepted in final form 25 July 1991.

REFERENCES

1. BERMAN, D. I., A. N. LEIRIKH, AND E. I. MIKHAILOVA. Winter hibernation of the Siberian salamander *Hynobius keyserlingi*. *J. Evol. Biochem. Physiol.* 1984: 323–327, 1984.
2. CANTY, A., W. R. DRIEDZIC, AND K. B. STOREY. Freeze tolerance of isolated ventricle strips of the wood frog, *Rana sylvatica*. *Cryo Lett.* 7: 81–86, 1986.
3. CLAUSSEN, D. L., M. D. TOWNSLEY, AND R. G. BAUSCH. Supercooling and freeze tolerance in the European wall lizard, *Podarcis muralis*, with a revisional history of the discovery of freeze-tolerance in vertebrates. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 160: 137–143, 1990.
4. COSTANZO, J. P., D. L. CLAUSSEN, AND R. E. LEE. Natural freeze tolerance in a reptile. *Cryo Lett.* 9: 380–385, 1988.
5. COSTANZO, J. P., AND D. L. CLAUSSEN. Natural freeze tolerance in the terrestrial turtle, *Terrapene carolina*. *J. Exp. Zool.* 254: 228–232, 1990
6. COSTANZO, J. P., AND R. E. LEE. Freeze-thaw injury in erythrocytes of the freeze-tolerant wood frog, *Rana sylvatica*. *Am. J. Physiol.* 261 (*Regulatory Integrative Comp. Physiol.* 30): R1346–R1350, 1991.
7. COSTANZO, J. P., M. F. WRIGHT, AND R. E. LEE. Freeze tolerance and intolerance in hatchling turtles (Abstract). *Cryobiology* 27: 678, 1990.
8. COSTANZO, J. P., R. E. LEE, AND M. F. WRIGHT. Effect of cooling rate on the survival of frozen wood frogs, *Rana sylvatica*. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 161: 225–229, 1991.
9. COSTANZO, J. P., R. E. LEE, AND M. F. WRIGHT. Cooling rate influences cryoprotectant distribution and organ dehydration in freezing wood frogs. *J. Exp. Zool.* In press.
10. COSTANZO, J. P., M. F. WRIGHT, AND R. E. LEE. Freeze tolerance as an overwintering adaptation in Copes' grey treefrog (*Hyla chrysoscelis*). *Copeia*. In press.
11. JOHNSON, B. B. *Freeze Tolerance in the Adult Painted Turtle, Chrysemys picta, and the Effect of Freezing on the Cardiac Cycle* (Masters thesis). Oxford, OH: Miami Univ., 1990.
12. LAYNE, J. R., AND R. E. LEE. Freeze tolerance and the dynamics of ice formation in wood frogs (*Rana sylvatica*) from southern Ohio. *Can. J. Zool.* 65: 2062–2065, 1987.
13. LAYNE, J. R., R. E. LEE, AND T. L. HEIL. Freezing-induced changes in the heart rate of wood frogs (*Rana sylvatica*). *Am. J. Physiol.* 257 (*Regulatory Integrative Comp. Physiol.* 26): R1046–R1049, 1989.
14. SCHMID, W. D. Survival of frogs in low temperature. *Science Wash.*

DC 215: 697–698, 1982.
15. STOREY, K. B., AND J. M. STOREY. Biochemical adaptation for freezing tolerance in the wood frog, *Rana sylvatica*. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 155: 29–36, 1984.
16. STOREY, K. B., AND J. M. STOREY. Freeze tolerance in animals. *Physiol. Rev.* 68: 27–84, 1988.
17. STOREY, K. B., J. M. STOREY, S. P. J. BROOKS, T. A. CHURCHILL, AND R. J. BROOKS. Hatchling turtles survive freezing during winter hibernation. *Proc. Natl. Acad. Sci. USA* 85: 8350–8354, 1988.
18. STOREY, K. B. Life in a frozen state: adaptive strategies for natural freeze tolerance in amphibians and reptiles. *Am. J. Physiol.* 258 (*Regulatory Integrative Comp. Physiol.* 27): R559–R568, 1990.