Osmotic and freezing tolerance in spermatozoa of freeze-tolerant and -intolerant frogs
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Freeze-thaw injury in erythrocytes of the freeze-tolerant wood frog, *Rana sylvatica*.

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Costanzo, Jon P., and Richard E. Lee, Jr. 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.—Erythrocytes from the freeze-tolerant wood frog (*Rana sylvatica*) were subjected to in vitro tests of freeze tolerance, cryoprotection, and osmotic fragility. The responses of cells from frogs acclimated to 4 or 15°C were similar. Erythrocytes that were frozen in saline hemolyzed at -4°C or lower. The addition of high concentrations (150 and 1,500 mM) of glucose or glycerol, cryoprotectants produced naturally by freeze-tolerant frogs, significantly reduced cell injury at -8°C but concentrations of 1.5 or 15 mM were ineffective. Hemolysis was reduced by 94% with 1,500 mM glycerol and by 84% with 1,500 mM glucose; thus glycerol was the more effective cryoprotectant. Mean fragility values 15°C were similar. Erythrocytes that were frozen in saline were 1,938 and 49 mosM, respectively. Survival in freeze tolerance and cryoprotection experiments was comparable for erythrocytes from frogs and humans, suggesting that these cells may respond similarly to freezing-related stresses. However, the breadth of osmotic tolerance, standardized for differences in isoncoticity, was greater for frog erythrocytes than for human erythrocytes. Our data suggest that erythrocytes from *R. sylvatica* are adequately protected by glucose under natural conditions of freezing and thawing.

SEVERAL SPECIES of temperate-zone vertebrates survive extensive and repeated freezing of their tissues under ecologically relevant conditions. For example, some frogs tolerate up to 65% of their body water as ice and endure prolonged freezing at temperatures as low as -8°C (see Ref. 19 for review). Ice is probably restricted to extracellular compartments under natural (i.e., slow) rates of cooling, because it is generally believed that intracellular ice formation is lethal (13).

Physiological studies of natural freeze tolerance in frogs have investigated the time course of ice formation (5, 6), organ desiccation rates (8), metabolism in the frozen state (19), dynamics of cardiac function (7), seasonal variations in tolerance (6, 18), and behavioral correlates of recovery processes (8). The gross physiological stresses associated with body freezing include a massive redistribution of water in cellular (13) and organ (8) body compartments, tissue anoxia owing to ischemia (7, 19), and perturbations in the behavior of macromolecules and membrane structures (11). These stresses presumably are mitigated by glucose and/or glycerol, cryoprotectants rapidly mobilized from the liver in direct response to ice formation.

To date, no studies of freeze-tolerant vertebrates have examined the responses of blood constituents to freezing and thawing. This is surprising because the heritage of cryobiology is founded in research aimed at preserving frozen human erythrocytes. Furthermore, cryoprotectants effective in reducing freezing damage to human erythrocytes are produced naturally in freeze-tolerant frogs. Our current objective was to adapt classic experiments on human erythrocytes (9, 10, 15) to determine the responses of erythrocytes from the freeze-tolerant wood frog (*Rana sylvatica*) in tests of freeze tolerance, cryoprotection, and osmotic stress. We replicated these experiments using human cells for comparative purposes. Because the body temperature of *R. sylvatica* during late winter is variable, and temperature may strongly influence membrane behavior, we tested cells from frogs acclimated to different thermal regimens.

MATERIALS AND METHODS

Specimens and sample preparation. Male wood frogs were collected from breeding ponds in Adams County, southern Ohio, during March 4–12, 1989. Previous study showed that frogs collected from this population in late winter are fully freeze tolerant (e.g., Ref. 5). Frogs [mean mass 12.6 ± 0.3 (SE) g; n = 58] were kept in plastic boxes on damp moss and exposed to 4 or 15°C for 20 ± 2 days (range 10–72 days) in total darkness before bleeding. Drinking water was continuously available, but food was withheld. Erythrocytes from five individuals in each acclimation group were used in each experiment. We repeated experiments on human erythrocytes from five healthy adults (2 males, 3 females).

Whole blood (250 µl) was collected from the severed aorta of double-pithed frogs (or by finger puncture) and suspended in 1,500 µl heparinized isotonic phosphate-buffered saline [PBS; 230 mosM, pH 7.4 for frogs (21); 300 mosM, pH 7.4 for humans (12)]. Erythrocytes were washed using gentle aspiration, centrifuged, decanted, and resuspended to a final hematocrit of 3–5% in 1,500–1,800 µl nonheparinized PBS. Cell suspensions (contained in polypropylene microcentrifuge tubes) were prepared separately for each specimen. All centrifugation was done at low speed (2,000 g) for 5 min to reduce the possibility of mechanical damage to cells (9).

Measurements of erythrocyte survival. Erythrocyte suspensions were centrifuged to isolate cellular material,
and the hemoglobin concentration of the supernatant was measured as cyanmethemoglobin using a spectrophotometer (procedure no. 525, Sigma Chemical, St. Louis, MO). Because frog samples had very low hemoglobin concentrations, we modified the procedure by increasing the ratio of sample to reagent volume by a factor of 10. Percent hemolysis was calculated by dividing the absorbance of the treatment sample by that of a 100% hemolysis standard, which was produced by freezing a separate aliquot of the suspension to \(-15^\circ\text{C}\) for 2 h. We used freezing to ensure complete hemolysis because incubation in distilled water may fail to release all hemoglobin (e.g., Ref. 21).

**Freeze tolerance.** Aliquots (300 μl) of cell suspension were incubated for 30 min at 4 (control), 0, -2, -4, -6, or \(-8^\circ\text{C}\). Pilot tests using a thermodouple thermometer indicated that target temperatures were attained within this time frame. Samples were inspected visually to ensure that freezing occurred. When necessary, ice nucleation was induced by a brief external application of aerosol coolant (Histofreeze, Fisher Scientific, Pittsburgh, PA). Erythrocyte survival was measured after passive thawing at 4°C.

**Cryoprotection.** Aliquots (200 μl) of cell suspension were centrifuged, decanted, and resuspended in a 1.5, 15, or 1,500 mM solution of glucose or glycerol. Cryoprotectant solutions were made by dissolving solute in PBS; concentrations bracketed those produced in freeze-tolerant frogs during freezing. The lowest concentration (1.5 mM) was similar to plasma glucose levels reported for unfrozen *R. sylvatica* (19). Packed cells and cryoprotectant solutions were cooled to 0°C before mixing, and the resulting suspensions were kept on ice until tested. All samples were frozen for 30 min at \(-8^\circ\text{C}\) and thawed passively at 4°C before erythrocyte survival was measured. The (untreated) suspension frozen to \(-8^\circ\text{C}\) in the freeze-tolerance experiment served as a control. A separate aliquot, resuspended in PBS and frozen to \(-15^\circ\text{C}\), provided a 100% hemolysis standard.

**Osmotic stress.** Aliquots (200 μl) of cell suspension were centrifuged, decanted, and resuspended in saline in one of the following concentrations: isotonic (control) or 900, 1,200, 1,500, 1,800, or 2,100 mosM. Hypertonic solutions were derived from PBS brought to the desired osmolality with NaCl. After a 30-min incubation at 24°C, samples were centrifuged, decanted, and resuspended with gentle aspiration in PBS. Samples were again incubated and then assayed for erythrocyte survival. A separate aliquot, twice resuspended in PBS and frozen to \(-15^\circ\text{C}\), provided the 100% hemolysis standard.

We measured the hypotonic fragility of erythrocytes with a commercially obtained kit (Unopette Test 5930, Becton Dickinson, Rutherford, NJ). Whole blood (20 μl) was added to individual reservoirs containing 4.98 ml PBS in concentrations ranging from 300 to 103 mosM. We diluted our PBS to prepare 68 and 34 mosM solutions for testing frog cells. Suspensions were incubated for 30 min at 24°C and centrifuged, and the absorbance of the supernatant was measured at 540 nm. We froze the kit’s 100% hemolysis standard (whole blood incubated in 0% NaCl) to \(-15^\circ\text{C}\) to ensure complete hemolysis. Corpuscular fragility values were determined for each sample by fitting a curve to the hemolysis data and estimating the NaCl concentrations (hypertonic and hypotonic) yielding 50% cell survival.

**Statistical analyses.** Erythrocyte survival was compared between cold- and warm-acclimated frogs (acclimation group \(\times\) treatment variable) and between frogs and humans (species \(\times\) treatment variable), using two-factor analyses of variance. Where appropriate, paired means for each treatment were compared using Student’s \(t\) tests for independent samples. Mean survival of cells incubated in equivalent concentrations of glucose and glycerol was compared using dependent-measures \(t\) tests. All statistical procedures involving percentage data were performed using square root-arcsine transformed values. Significance was judged at \(P \leq 0.05\).

**RESULTS**

**Effect of acclimation temperature.** Mean survival of wood frog erythrocytes in freeze tolerance, cryoprotection, and osmotic stress tests differed significantly among treatments within both 4 and 15°C groups. Acclimation temperature was a significant factor only in the glycerol-cryoprotection experiment; however, this effect was limited to the 1,500 mM treatment. With 1,500 mM glycerol, mean cell survival for cold-acclimated frogs (96.3 ± 0.4%) was significantly \((t = 2.9, P = 0.020)\) greater than that for warm-acclimated (93.1 ± 1.1%) frogs. Overall, the responses of cells from frogs in the two acclimation groups were similar; thus these data sets were combined in subsequent analyses.

**Freeze tolerance and cryoprotection.** No hemolysis in the control samples (incubated at 4°C) was detected. Significant \((F = 87.2, P < 0.001)\) hemolysis of untreated frog cells was observed only after freezing to \(-4^\circ\text{C}\) or below. About two-thirds of the cells survived freezing at \(-6^\circ\text{C}\), but only 14% remained intact after freezing at \(-8^\circ\text{C}\) (Fig. 1). The patterns of survival for frog and human erythrocytes were not statistically different \((F = 0.1, P = 0.759; \text{Fig. 1})\).

Survival of frog cells frozen to \(-8^\circ\text{C}\) was significantly greater in the presence of glucose \((F = 130.8, P < 0.001)\) or glycerol \((F = 165.3, P < 0.001)\). Similarly, both agents increased the survival of frozen human cells (glucose \(F = 6.8, P = 0.004\); glycerol \(F = 53.6, P < 0.001\)). Concentrations of 150 and 1,500 mM glucose or glycerol inhib-

**FIG. 1.** Survival of erythrocytes from wood frogs (*Rana sylvatica*; \(n = 10\)) and humans \((n = 5)\) after a 30-min exposure to low temperature. Samples exposed to subzero temperatures were frozen. Values are sample means ± SE.
ited hemolysis, but 1.5 and 15 mM solutions had no cryoprotective effect (Fig. 2). The survival of frog cells was 86% with 1,500 mM glucose and 95% with 1,500 mM glycerol; these results contrast sharply with the 14% value obtained for cells frozen in PBS.

Survival patterns of frog and human cells frozen in glycerol solutions were similar (F = 3.2, P = 0.080), but for cells frozen in glucose solutions they differed significantly (F = 9.9, P = 0.003). With 1,500 mM glucose, frog cells better tolerated freezing (Fig. 2). Although sufficient quantities of both agents markedly reduced hemolysis, equivalent concentrations of glycerol provided superior cryoprotection (Table 1).

Osmotic stress. Frog erythrocytes survived exposure to a wide range of osmotic concentrations; substantial hemolysis occurred only in NaCl solutions <68 or ≥900 mosM. Mean fragility values in hypertonic saline were 49 ± 2 and 145 ± 2 mosM, respectively. Because a given NaCl concentration represents disproportionate osmotic stresses between the species owing to differences in isotonicity, comparisons were based upon standardized fragility values. Wood frog erythrocytes had the greater range of osmotic tolerance (Fig. 3).

**DISCUSSION**

Little is known of the effect of thermal acclimation on physiological responses of erythrocytes from vertebrate ectotherms. In the present investigation, frogs were acclimated to 4 or 15°C, temperatures commonly encountered by *R. sylvatica* during late winter, before their erythrocytes were subjected to tests of freeze tolerance, cryoprotection, and osmotic stress. Generally, erythrocyte responses to freezing and thawing stresses did not differ between the acclimation groups. Our data for 15°C-acclimated frogs suggest that the tolerance of erythrocytes to freezing and thawing stresses is not contingent upon preconditioning via prolonged exposure to low temperature (e.g., 4°C). This response seems advantageous in *R. sylvatica* because environmental temperatures in southern Ohio during March are variable and may change abruptly.

Our experiments involved frogs collected only during late winter; thus our results do not necessarily represent responses of erythrocytes sampled at other times. Additionally, the rapid freezing (and thawing) protocols used in the present study undoubtedly induced cell injury more extensive than that occurring in frogs frozen and thawed under natural conditions. Furthermore, we caution that cell damage was gauged solely on the basis of hemolysis, which indicates catastrophic membrane failure; subhemolytic cryoinjury was not measured.

In the present study, in vitro freezing in the absence of cryoprotectant caused substantial injury to *R. sylvatica* erythrocytes. Cryoinjury results from membrane failure associated with harmful changes in electrolyte concentration, reduction of cell volume below some critical threshold, or structural changes in cell membranes (9–11, 15). Mechanical damage occurs as cells become compressed in gaps among ice masses (13) and membrane plasticity is reduced at low temperature (20). Clearly these stresses are interrelated and may be applied in concert; Pegg (17) discusses the problematic difficulties in designing experiments to separate their effects.

**TABLE 1. Survival of wood frog (Rana sylvatica) erythrocytes frozen in glucose and glycerol solutions**

<table>
<thead>
<tr>
<th>Cryoprotectant Concentration (mM)</th>
<th>Erythrocyte Survival, %</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Glycerol</td>
</tr>
<tr>
<td>1.5</td>
<td>9.6±0.3</td>
<td>11.5±1.3</td>
</tr>
<tr>
<td>15</td>
<td>12.1±1.5</td>
<td>12.0±2.3</td>
</tr>
<tr>
<td>150</td>
<td>35.6±3.6</td>
<td>60.7±5.0</td>
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<tr>
<td>1,500</td>
<td>86.1±2.7</td>
<td>94.7±0.8</td>
</tr>
</tbody>
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Values are means ± SE; n = 10 erythrocyte preparations. Erythrocyte survival was measured after a 30-min freeze to −8°C and passive thawing at 4°C. P values indicate probability that differences between means for glucose and glycerol treatments differ statistically.

**FIG. 1. Survival of erythrocytes from wood frogs (n = 10) and humans (n = 5) after freezing to −8°C for 30 min and passive thawing at 4°C in relation to glycerol (A) and glucose (B) concentrations of suspension medium. Values are sample means ± SE. *P < 0.05 between species.**

**FIG. 2. Survival of erythrocytes from wood frogs (n = 10) and humans (n = 5) after a 30-min incubation at 24°C in various NaCl solutions. Data were standardized to account for species differences in isotonicity; unity represents 230 mosM for wood frog cells and 300 mosM for human cells. Curves were drawn by interpolation.**
cytes was hitherto lacking, the responses of human erythrocytes to freeze-thaw stresses are well studied. Our results for cryoprotection of human erythrocytes are in general agreement with published data (9, 15). Loveclough (10) reported relatively higher survival of human erythrocytes in 1,500 mM glycerol; however, he incubated cells in the suspension medium before freezing them. In contrast, we froze cell suspensions immediately after adding cryoprotectant because, in freeze-tolerant frogs, cryoprotectants are manufactured only at the onset of freezing (19). Comparisons of the responses of frog and human erythrocytes are instructive but should be made carefully because these cells differ markedly in form (erythrocytes from R. sylvatica are nucleated and larger than those of humans) and physiology.

The relationship between temperature and survival was comparable for erythrocytes of wood frogs and humans, suggesting that, despite their inherent differences, these cells may respond similarly to freezing-related stresses. Interestingly, carbohydrates that reduce freezing damage to human cells are naturally produced in large quantities (up to 500 mM) by freeze-tolerant frogs: glucose in R. sylvatica, Pseudacris triseriata, and Hyla crucifer and glycerol in Hyla versicolor (5, 18, 19). Both glucose and glycerol provided significant protection for R. sylvatica cells frozen in vitro. Our hemoysis data for cells frozen in 1,500 mM glucose (14%) and 1,500 mM glycerol (5%) potentially reflect some injury occurring before freezing, as the one-step addition of cryoprotectant produced an acute osmotic stress. Osmotic injury in these tests was not measured; however, it was probably negligible because the initial stress was not severe and cryoprotectants rapidly permeate cell membranes. Furthermore, exposure of erythrocytes to greater stress (e.g., 2,000 mosM NaCl, a nonpenetrating solute) results in only <4% hemolysis (9).

The mechanisms by which solute additives, such as glucose and glycerol, protect wood frog erythrocytes from cryoinjury are unknown, although they may be surmised from studies on mammalian cells. Because glycerol permeates cells, it serves colligatively to reduce the amount of ice formed at any given temperature, thereby lessening “solution effects.” Glycerol also stabilizes membrane structures (11) and buffers cells from physical contact with intracellular ice (1). Glucose, generally regarded a relatively poor cryoprotectant of mammalian cells, likely is inferior because it is semipermeable (10). Thus the disparate survival of wood frog and human erythrocytes frozen in 1,500 mM glucose may stem from differences in glucose permeability, which varies considerably among taxa (3). Also glucose may function extracellularly to protect erythrocytes of R. sylvatica (14). Clearly, the mechanisms of cryoprotection in this species require further study.

Earlier studies investigated in vitro cryoprotection of tissues and cells of R. sylvatica. For example, 250 mM glucose (but not glycerol) protects ventricle strips frozen to −5°C, and 400 mM glucose protects isolated hepatocyte frozen to −4°C (19). Our erythrocytes were frozen to −8°C, a treatment causing extensive hemolysis of unprotected cells, to better evaluate cryoprotective potential. Judging from our results, typical plasma glucose levels in frozen R. sylvatica seem sufficient to fully protect erythrocytes at temperatures commonly encountered during winter, e.g., −2 to −4°C (19). Interestingly, our results show that glycerol was the relatively superior cryoprotectant, although it is not the primary one produced by R. sylvatica. Nevertheless, the use of glucose (rather than glycerol) by most freeze-tolerant frogs confers several metabolic advantages (19).

Studies of the behavior of erythrocytes in hypertonic and hypotonic solutions are pertinent to cryobiology because freezing and thawing result in a progressive concentration and subsequent dilution of extracellular solutes. In the present study, erythrocytes better tolerate the hypertonic stress induced by freezing than that established in osmotic fragility tests. For example, survival of frog erythrocytes frozen to −4°C (which produces ~2,150 mosM in the unfrozen fraction) was 94%, whereas survival of cells incubated in 2,100 mosM at 24°C was 43%. Erythrocytes exposed to hypertonic salt solutions become fragile (9), and some additional injury may have occurred during centrifugation. However, the greater survival in the freezing experiment probably resulted because osmotic stress was applied more gradually, giving cells a longer period to adjust to hypertonic conditions.

Wood frog erythrocytes were exceptionally resistant to both high and low NaCl concentrations and, at both extremes, more hardy than human cells. Differences in protocol aside, our results for human erythrocytes generally accord with those obtained in previous studies of hypertonic (9, 15) and hypotonic (12, 16) fragility. Little is known about erythrocyte resistance to osmotic stress in vertebrate ectotherms. However, hypotonic fragility ranges from 133 mosM in the goldfish, Carassius auratus (2), to 40 mosM in the aquatic frog, Xenopus laevis (22). Our mean value for R. sylvatica, 49 mosM, is within this range.

Our fragility data for wood frog and human erythrocytes suggest marked dissimilarities in osmotic tolerances that undoubtedly reflect inherent differences in cell morphology and physiology (e.g., Ref. 4). Although a detailed analysis of these differences is beyond the scope of the present study, the comparatively greater breadth of osmotic tolerance in R. sylvatica cells seems adaptive. Water economy in R. sylvatica (and other terrestrial anurans) fluctuates with environmental conditions; hence, the osmotic concentration of body fluids is not rigidly controlled, particularly at low temperatures. Undoubtedly, the wide span of osmotic tolerance in this species also confers an important resistance to stresses associated with freezing and thawing.

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