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Cryoprotection by urea in a terrestrially hibernating frog

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Summary

The role of urea as a balancing osmolyte in osmotic adaptation is well known, but this 'waste product' also has myriad other functions in diverse taxa. We report that urea plays an important, previously undocumented role in freezing tolerance of the wood frog (*Rana sylvatica*), a northern woodland species that hibernates terrestrially in sites where dehydration and freezing may occur. Wood frogs inhabiting an outdoor enclosure accumulated urea to 65 mmol l^{-1} in autumn and early winter, when soil moisture was scarce, but subsequently urea levels fell to ~2 mmol l^{-1} as the availability of environmental water increased. Laboratory experiments showed that hibernating *R. sylvatica* can accumulate at least 90 mmol l^{-1} urea under relatively dry, warm conditions. During experimental freezing, frogs synthesized glucose but did not accumulate additional urea. Nevertheless, the concentrations of urea and glucose in some tissues were similar. We tested urea's efficacy as a cryoprotectant by measuring lysis and lactate dehydrogenase (LDH) leakage in samples of *R. sylvatica* erythrocytes frozen/thawed in the presence of physiological levels of urea or other osmolytes. In conferring protection against freeze/thaw damage, urea was comparable to glycerol and as good as or better than glucose, cryoprotectants found in freeze-tolerant frogs and other animals. Urea treatment also improved the viability of intact tissues frozen *in vitro*, as demonstrated by post-thaw measures of metabolic activity and LDH leakage. Collectively, our findings suggest that urea functions both as an osmoprotectant and a cryoprotectant in terrestrially hibernating amphibians.

Key words: amphibian, freeze tolerance, osmolyte, hibernation, *Rana sylvatica,* wood frog, cryoprotection.

Introduction

Osmoconformers adapt to osmotic stress by accumulating one or more 'compatible solutes', organic osmolytes that in high concentration are benign to cellular functions. Organisms acquire these osmolytes most economically by exploiting metabolic end-products (Hochachka and Somero, 2002), and, therefore, urea has become a major balancing osmolyte in diverse ureogenic species (Griffith, 1991). Amphibians accumulate urea when exposed to low water potential, a response that aids in maintaining proper hydration during saline adaptation and estivation (Jørgensen, 1997; Shpun et al., 1992). Urea might also be important in amphibian hibernation, although relatively little is known about the winter physiology and water balance of these animals (Pinder et al., 1992).

Several species of temperate frogs overwinter beneath forest duff, where they may be exposed to dehydrating conditions and subzero temperatures, which they can survive by virtue of their profound tolerances to dehydration (Hillyard, 1999) and somatic freezing (Schmid, 1982). Notably, the wood frog (*Rana sylvatica* LeConte), which ranges further north than any other anuran, recovers from severe dehydration (Churchill and Storey, 1993) and survives the freezing of up to 70% of its body water at temperatures between –4 and –6°C (Storey and Storey, 2004). Freeze tolerance in amphibians is supported by a host of molecular, biochemical and physiological responses that provide protection against the stresses associated with the freezing and thawing of tissues. Foremost among these is an accumulation of newly synthesized carbohydrate (glucose in *R. sylvatica*; glycerol and/or glucose in hylid tree frogs) over the first few hours of freezing. These permeable osmolytes, or 'cryoprotectants', colligatively lower the freezable fraction of body water and reduce cell dehydration and shrinkage, thereby limiting osmotic and mechanical injury to membranes and other cellular structures. In addition, cryoprotectants safeguard cellular functions by stabilizing intracellular proteins (Carpenter and Crowe, 1988; Mazur, 1984). Glucose, purportedly the sole cryoprotectant in *R. sylvatica*, contributes to freezing survival at cell, organ and whole-animal levels of organization (Costanzo et al., 1995).

Cryoprotectants employed by freeze-tolerant organisms constitute a diverse array of organic compounds, although they all share certain attributes, including low molecular mass, high solubility and permeability, stability, ready availability, and compatibility with macromolecules (Storey and Storey, 2004). In principle, urea accumulating in response to water deficit could also serve a cryoprotective function in hibernating amphibians, but this contention has not been tested. Here, we provide evidence that overwintering *R. sylvatica* can

accumulate substantial quantities of urea and that this osmolyte protects cells and tissues from freeze/thaw injury.

Materials and methods

Experimental animals and acclimatization regimen

Male wood frogs were collected in February from a breeding congregation in southern Ohio. They were kept in a 48-m^2 pen at the Ecology Research Center (39.5°N, 84.7°W), Miami University, from spring through early autumn. Located in a mature deciduous woodlot, the pen provided woodland herbaceous cover and cool, moist conditions similar to those found in the species' natural habitat. Frogs had access to a pool of water and were fed crickets 2–3 times each week. This diet was supplemented with various insects, which were attracted to a 'black light' hung in the pen. In late October, feeding was suspended and the frogs, on the verge of dormancy, were recaptured by hand. Some were brought to the laboratory and kept in simulated hibernation until used in experiments. Except as specifically noted, these frogs were placed inside covered, opaque boxes containing wet moss and held in darkness at 4°C.

Other frogs were permitted to overwinter in an open-air enclosure located in the same deciduous woodlot. This 10-m^2 arena was circumscribed by a 1.25-m high wall of plastic mesh. In order to prevent frogs from escaping, the lower edge of the mesh extended 10 cm below ground. The arena contained a small pool and natural cover, such as woodland forbs, a few small shrubs, and detritus from nearby deciduous trees. A data logger (model CR-10; Campbell Scientific, Logan, UT, USA) and sensors permitted us to monitor a suite of environmental variables (solar radiation, wind speed, relative humidity, air temperature, soil temperature, soil moisture, leaf litter moisture) as well as operative environmental temperatures (T_e) of 'active' and 'hibernating' frogs. These T_e models were made from painted metal castings of a small frog and were placed on the ground surface or in a shallow depression in the soil beneath leaf litter, respectively.

Tissue sampling and osmolyte assays

Several experiments required us to measure osmolyte concentrations in the blood or organs and to collect erythrocytes or organ samples for use in cryoprotection experiments. Frogs were double-pithed and blood was immediately drawn from an incision in the aortic trunk (or collected from the ventricle of frozen frogs) into heparinized capillary tubes. The tubes were centrifuged (2000 ***g***, 5 min) and the plasma was reserved on ice for analysis. Carcasses were promptly dissected, and various organs, or portions of organs, were excised and blotted free of surface moisture. In some experiments, samples were placed in ice-cold phosphate-buffered saline (PBS; in g l^{-1}; 6.10 NaCl, 0.15 KCl, 0.88 Na_2HPO_4, 0.15 KH_2PO_4; 230 mosmol kg^{-1}, pH 7.4 at 23°C) and reserved for later use. In order to assay osmolytes, deproteinized tissue extracts were prepared by homogenizing pre-weighed samples in $HClO_4$ and neutralizing the supernatants with KOH. Glucose and urea in tissue extracts and in blood plasma were assayed using glucose oxidase (no. 510; Sigma, St Louis, MO, USA) and urease (no. 736; Sigma) procedures, respectively. Plasma osmolality was determined by vapor-pressure osmometry (model 5500; Wescor, Logan, UT, USA).

Osmolytes in winter frogs

We examined seasonal changes in osmolyte levels and hydroosmotic balance in *R. sylvatica* inhabiting the outdoor enclosure by sampling animals at intervals from November to April. Frogs wore a thin waistband of polyethylene tubing to which a passive integrative transponder (PIT) tag was attached. Systematically sweeping a portable reader (model LID 500; Trovan Ltd, North Humberside, UK) over the ground permitted us to locate frogs hidden beneath snow and/or leaf litter. Captured frogs were transported under refrigeration to the laboratory and immediately assayed for plasma osmolality and for plasma levels of urea and glucose. We estimated the body water content of each frog by thoroughly drying its carcass in a 65°C oven and determining the mass of water that had evaporated. Our primary objective in this experiment was to examine the dynamic physiological state of overwintering frogs in relation to prevailing microenvironmental conditions.

In the laboratory, we investigated the association between environmental moisture and urea concentration by manipulating conditions to which frogs were exposed during simulated hibernation. Frogs were held in opaque plastic boxes on a thick substratum of absorbent moss. The boxes were closed with a loosely fitting lid that excluded ambient light and permitted exchange of respiratory gases whilst also minimizing water loss. Initially, frogs were held at 4°C on fully hydrated moss. After habituating to these conditions for ~6 weeks, some of the frogs were killed and assayed for plasma urea. We then reduced the amount of moisture available to the remaining frogs by wringing most of the water from the moss. Frogs were held at 4°C on this substratum for 10 days or several weeks before additional animals were assayed. The box was then transferred to a 10°C incubator, and the remaining frogs were sampled 3 days later.

Osmolytes in frozen/thawed frogs

We compared levels of urea and glucose in laboratory-held frogs sampled directly from their boxes (control) with those subjected to somatic freezing. These experiments were performed using frogs rendered moderately hyperuremic by keeping them for 10 days on slightly damp moss. The experimental freezing protocol exposed frogs to conditions mimicking a natural chilling episode (Costanzo et al., 1991a). Briefly, each frog was outfitted with a copper/constantan thermocouple placed against its abdomen and cooled inside a 50 ml polypropylene centrifuge tube submerged in a refrigerated ethanol bath (model 2095; Forma Scientific, Marietta, OH, USA). During cooling, body temperature was recorded on a multichannel data logger (model OM-500; Omega, Stamford, CT, USA). Once they had reached –0.5°C, freezing was initiated by applying small ice crystals to their

skin. Subsequently, the frogs were cooled to an equilibrium temperature of –2.5°C over the next 24 h, during which time approximately 65% of their body water would have frozen (Layne and Lee, 1987). Portions of the liver, small intestine and muscle from one forelimb (flexor carpi) and hindlimb (gracilis) were rapidly excised from fully frozen frogs (*N*=5), and also from control frogs (*N*=5), and assayed for glucose and urea (see below). Replicate sets of organ samples were weighed to the nearest 0.1 mg and thoroughly dried in a 65°C oven. They were reweighed and tissue water content was determined from the change in mass.

Cell cryoprotection by urea

Following Costanzo and Lee (1991), we tested the cryoprotective efficacy of urea by comparing the viability of *R. sylvatica* erythrocytes frozen/thawed in the absence or presence of urea. Cells were washed twice in PBS and resuspended in PBS (control) or PBS containing 40 or 80 mmol l^{-1} urea. Cell suspensions (3–5% hematocrit) were divided among multiple 80-μl samples held in 0.5-ml microcentrifuge tubes and incubated for 45 min on ice. Next, the samples were placed individually in glass tubes immersed in a refrigerated ethanol bath, chilled to –1°C and inoculated by briefly applying aerosol coolant to the exterior of the microcentrifuge tube. After the suspensions began to freeze, they were held at the target temperature, –4 or –6°C, for 30 min.

Cell viability was assessed after passively thawing the samples at 4°C. Cell suspensions were centrifuged (2000 ***g***, 3 min), and the hemoglobin concentration of the supernatant, an index of hemolytic damage, was assayed (no. 525, Sigma) as cyanmethemoglobin. Sample absorbances were referenced to that of a total hemolysis standard produced by freezing a separate aliquot of suspension for 2 h at –80°C. Supernatants were also assayed (TOX-7; Sigma) for the cytoplasmic enzyme lactate dehydrogenase (LDH), whose concentration in the suspension medium indexed membrane damage. Preliminary trials showed that unfrozen erythrocytes tolerated exposure to at least 80 mmol l^{-1} urea without leaking LDH and at least 200 mmol l^{-1} urea without lysing.

We also compared the cryoprotective efficacy of urea with that of glucose and glycerol, cryoprotectants found in freeze-tolerant frogs. This experiment was carried out as described above, except that the suspensions were held on ice for 4 h before being frozen. The longer incubation period was needed to permit the intracellular glucose concentration to attain equilibrium (Brooks et al., 1999).

Organ cryoprotection by urea

In two separate experiments, we examined urea's efficacy in cryoprotecting *R. sylvatica* organs, comparing indices of cryoinjury in samples frozen/thawed *in vitro* after incubation in the presence or absence of urea. Preliminary tests were undertaken to confirm that exogenous urea readily permeates *R. sylvatica* tissues. The intact heart, kidneys and 25–50 mg portions of liver and leg muscle (gastrocnemius) were dissected from freshly killed frogs, rinsed several times in ice-cold PBS and placed in individual 0.5-ml centrifuge tubes containing 80 mmol l^{-1} urea in 250 μl of PBS. After incubating on ice for 45 min, the samples were removed and gently blotted to remove surface moisture. The heart was bisected sagittally, and the samples of liver and gastrocnemius were divided into equal portions. We measured urea concentrations in one set of the samples and in an entire kidney. The other set and the remaining kidney were dried in a 65°C oven, and tissue water content was determined from the change in mass upon drying. Tissue urea concentrations were calculated as μmol g^{-1} dry tissue.

In one experiment, the heart, both kidneys and a portion of the liver were dissected from double-pithed frogs, thoroughly rinsed with ice-cold PBS, and placed in individual 0.5-ml centrifuge tubes containing 250 μl of PBS (control) or PBS containing 80 mmol l^{-1} urea. Samples were incubated on ice for 45 min before being frozen for 45 min at –4°C and passively thawed at 4°C, as per the erythrocyte experiments.

Viability of the frozen/thawed organ samples was inferred from metabolic activity, which was assessed using the non-toxic, oxidation–reduction indicator dye alamarBlue (Alamar Biosciences, Sacramento, CA, USA). This assay, which yields a colorimetric change in proportion to cellular respiration, has been used to evaluate cell viability following hypothermic exposure (Acker and McGann, 2002; Cook et al., 1995). Organ samples were transferred 60 min after thawing to a 1.5-ml centrifuge tube containing 90 μl alamarBlue diluted (1:10) with PBS, or PBS containing 80 mmol l^{-1} urea, and incubated at 15°C with gentle orbital agitation for 120 min. We then decanted and centrifuged (700 ***g***, 3 min) the medium, transferred 0.75 ml of the cell-free supernatant to a cuvette and read the absorbance at 570 and 600 nm using a spectrophotometer. Organ samples were thoroughly dried in a 65°C oven, and reduction rates were standardized to dry tissue mass.

Control experiments showed that no reduction occurred in dye solution alone or in dye solution containing heat-denatured tissue. However, preliminary tests suggested that urea treatment reduced metabolic activity in certain organs, so we more thoroughly investigated this phenomenon. We conducted an experiment similar to that described above, except that the organ samples were not frozen before being assayed and, in order to improve statistical power, we compared urea-treated and control samples harvested from the same frog. The heart was bisected sagittally, and pieces of the excised liver and gastrocnemius were cubed (~1 mm^3) and apportioned into two lots, thus permitting differential treatment of the same organs. Each lot, plus one intact kidney, was rinsed with several changes of ice-cold PBS and then placed in indicator dye diluted with PBS (control) or PBS containing 80 mmol l^{-1} urea. Samples were incubated at 15°C for 60 min (heart, gastrocnemius) or 150 min (kidney, heart) before being assayed for metabolic activity, as described above. Results of this experiment were used to correct the organ cryoprotection data for any reduction in metabolic activity purely attributed

to urea treatment. In a companion experiment, we tested the specificity of the metabolic inhibition by substituting 80 mmol l^{-1} glycerol for urea.

In a second experiment, the sagittally bisected heart, 25–50 mg portions of liver and gastrocnemius, and both kidneys were thoroughly rinsed with ice-cold PBS, incubated in PBS (control), or PBS containing 80 mmol l^{-1} urea, and then frozen and thawed following procedures used in the first experiment. Thawed samples were blotted gently, placed in 1 ml fresh medium (PBS with or without urea, as appropriate) and incubated at 4°C with gentle orbital agitation for 30 min. After removing the samples, we centrifuged the incubation medium (12 000 ***g***, 4 min) in order to sediment any cellular debris. The concentration of LDH in the supernatant was measured (as above) using a standard curve prepared from purified enzyme. Organ samples were dried in a 65°C oven so that LDH leakage could be normalized to dry tissue mass.

Statistical inferences

Sample means were compared using Student's *t*-tests or analysis of variance (ANOVA) followed by Bonferroni multiple comparisons tests. The nonparametric Wilcoxan Signed Rank test was substituted where the data did not meet the assumptions of parametric tests. Analyses involving percentage data were performed on values after arcsine/square-root transformation. Significance of statistical analyses was accepted at P<0.05. Mean values are reported ± S.E.M.

Results

Osmolytes in hibernating and freezing frogs

We sampled frogs inhabiting the outdoor enclosure on six occasions between 1 November and early April (Fig. 1). Body water content was lowest in autumn and early winter, when the soil was relatively dry, but increased during winter, coincident with a rise in soil moisture, reaching its peak in April. Plasma osmolality tended to vary inversely with body water content and to track changes in plasma concentrations of urea and glucose. Urea levels varied seasonally, being maximal in winter (~50 mmol l^{-1}; individual values to 65 mmol l^{-1}) and falling to 2 mmol l^{-1} in April (Fig. 1D). Glucose levels also varied during the experiment; however, unlike the case with urea, glycemia was not associated with hydration state or environmental moisture availability. Rather, hyperglycemia appeared to be linked to transient bouts of somatic freezing that occurred sporadically throughout winter (Fig. 1).

Uremia in laboratory-hibernating frogs was associated with the hydric and thermal conditions to which the frogs were exposed. Frogs maintained at 4°C on fully hydrated moss had relatively low plasma urea levels (3.9±1.3 mmol l^{-1}; N=9). By contrast, frogs held on the (wrung-out) damp moss at 4°C had accumulated urea (14.0±1.4 mmol l^{-1}; N=5) within 10 days and became strongly hyperuremic (58.5±4.8 mmol l^{-1}; N=2) within several weeks. Plasma urea levels rose to 92.3±3.5 mmol l^{-1} (N=3) when these frogs were exposed to 10°C for 3 days.

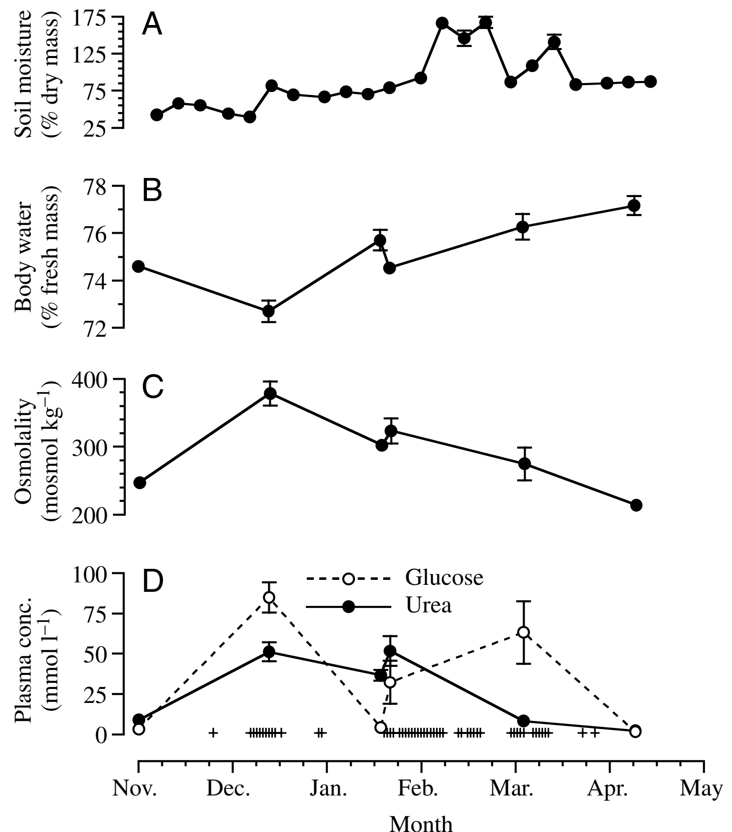

Fig. 1. Seasonal variation in soil moisture (A) and its relationship to body water content (B), plasma osmolality (C) and plasma osmolyte concentrations (D) in *R. sylvatica* inhabiting a semi-natural, outdoor enclosure. Data presented in A are weekly means ± S.E.M. of values recorded at 6-h intervals. Data presented in B–D are means ± S.E.M. (N=3–6 frogs per group); all response variables varied significantly (ANOVA, P<0.0001). In D, '+' denotes each day that frogs were likely to be frozen, given that T_e of a hibernating frog model was ⩽–0.4°C, the approximate equilibrium freezing/melting point of *R. sylvatica* tissues.

Effect of somatic freezing on urea and glucose levels

Somatic freezing induced the characteristic hyperglycemic response in *R. sylvatica*, as plasma glucose concentration in the frozen frogs (77.4±16.6 mmol l^{-1}; N=5) was markedly higher (P<0.002) than that in unfrozen controls (3.6±0.4; N=5). Because the organs of frozen frogs contained 11.3% (flexor carpi) to 51.4% (heart) less water than the organs of unfrozen controls (ANOVA; P<0.05; see Lee et al., 1992), it was necessary to express osmolyte concentrations as μmol g^{-1} dry tissue in order to allow comparisons between the groups. Urea concentration did not change with freezing in most organs; however, glucose concentrations were up to 35-fold higher in frozen frogs as compared with unfrozen controls (Table 1). In frozen frogs, the liver and gut contained considerably more glucose than urea. By contrast, in the case

Table 1. *Concentrations of urea and glucose (μmol g^{-1} dry tissue) in organs of unfrozen and frozen* Rana sylvatica

	Urea	Glucose	*P*
Unfrozen			
Liver	48.1±5.2	26.7±5.1	0.049
Gut	63.8±4.4	9.0±1.7	<0.001
Flexor carpi	56.5±3.8	6.5±1.4	<0.001
Gracilis	47.6±3.8	6.6±1.8	<0.001
Frozen			
Liver	56.5±3.2	946.0±82.6*	<0.001
Gut	66.9±6.1	174.0±20.6*	0.004
Flexor carpi	45.2±8.0	49.5±5.7*	0.453
Gracilis	28.1±5.2*	51.9±10.2*	0.006

Values are means ± S.E.M.; *N*=5 replicates per group.

Means within each row were compared using dependent-measures Student's *t*-tests; *P* as indicated. Asterisk denotes that the value differed significantly (unpaired Student's *t*-test; *P*<0.05) from the corresponding mean for unfrozen frogs.

of skeletal muscles, which accumulated relatively little glucose during freezing, concentrations of both glucose and urea were ~50 μmol g^{-1} (Table 1).

Cryoprotection of erythrocytes

Not unexpectedly, *R. sylvatica* erythrocytes tolerated freezing at –4°C better (LDH leakage, *P*<0.0001; hemolysis, *P*<0.0001) than they tolerated exposure to –6°C (Fig. 2). Viability of frozen/thawed erythrocytes was markedly increased (LDH leakage, *P*=0.0001; hemolysis, *P*=0.0001) in the presence of urea in the incubation medium, as urea treatment reduced cryohemolysis by 70% in the samples frozen at –4°C and by 33% in the samples frozen at –6°C. Results with 40 mmol l^{-1} urea were indistinguishable (*P*>0.05) from those obtained with 80 mmol l^{-1} urea (Fig. 2).

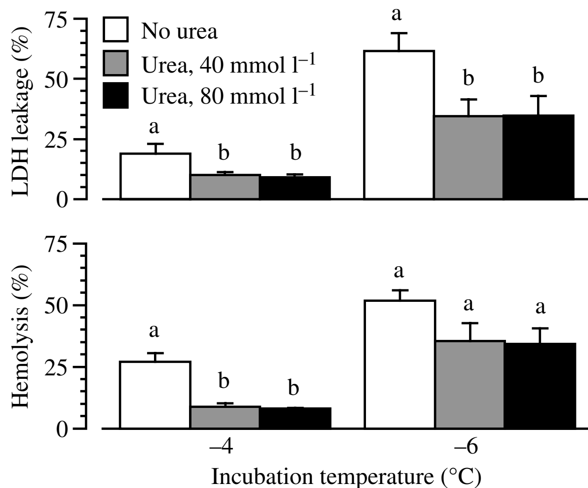

Fig. 2. Cryoinjury to *R. sylvatica* erythrocytes frozen at –4 or –6°C in PBS (no urea) or PBS containing 40 mmol l^{-1} urea or 80 mmol l^{-1} urea. Damage was assessed as the percentage of total LDH leaking from cells and as the percentage of cells lysing. Within temperature-treatment groups, mean values (± S.E.M., *N*=6–7 replicates per group) identified by different letters differed significantly (repeated-measures ANOVA/Bonferroni; *P*<0.05).

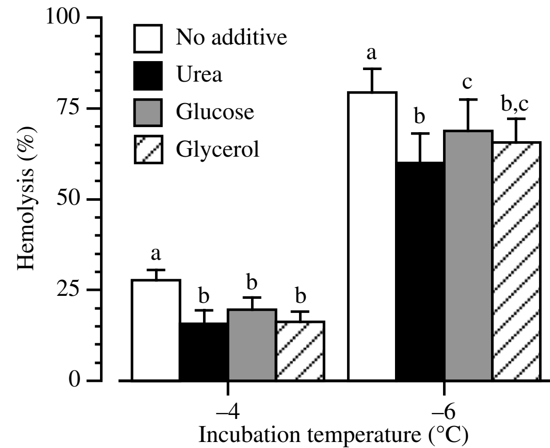

Fig. 3. Cryoinjury to *R. sylvatica* erythrocytes frozen at –4 or –6°C in PBS (no additive) or PBS containing 40 mmol l^{-1} urea, 40 mmol l^{-1} glucose or 40 mmol l^{-1} glycerol. Damage was assessed as the percentage of cells lysing. Within temperature-treatment groups, mean values (± S.E.M., *N*=7 replicates per group) identified by different letters differed significantly (repeated-measures ANOVA/Bonferroni; *P*<0.05).

Rates of cryohemolysis for erythrocytes incubated in PBS, or PBS containing 40 mmol l^{-1} urea, 40 mmol l^{-1} glucose or 40 mmol l^{-1} glycerol, varied both by exposure temperature (*P*<0.0001) and by incubation medium (*P*<0.0001). All three osmolytes reduced cell damage in samples frozen at –4 or –6°C (Fig. 3). The level of cryoprotection afforded by urea was equal to that of glycerol, a well-known cryoprotectant. Furthermore, cryoprotection by urea was equal or superior to that of glucose.

Organ cryoprotection

Our preliminary experiment confirmed that *R. sylvatica* organs readily take up exogenous urea from an incubation medium. Urea concentrations in the samples treated with 80 mmol l^{-1} urea ranged from 200 to 300 μmol g^{-1} dry tissue

Table 2. *Water content and urea concentration in* Rana sylvatica *organs after incubation with 80 mmol l^{-1} urea*

	Water content (% fresh mass)	Urea concentration (μmol g^{-1} dry tissue)	Urea concentration (mmol l^{-1})
Heart	83.2±0.5^{a}	233.2±25.1^{a,b}	46.7±4.8^{a}
Liver	76.4±0.4^{b}	246.6±12.8^{a,b}	76.3±4.2^{b}
Kidney	81.2±0.1^{c}	299.3±27.2^{a}	69.6±6.6^{b}
Gastrocnemius	82.8±0.3^{a}	204.0±6.8^{b}	42.2±1.2^{a}

Values are means ± S.E.M.; *N*=6 replicates per group.

Within each column, means identified by different letters are statistically different (ANOVA/Bonferroni; *P*<0.05).

Table 3. *Indices of metabolic activity in* Rana sylvatica *organs incubated in PBS or PBS containing 80 mmol l^{-1} urea*

	No urea	Urea	*P*
Heart	3.6±0.5	3.8±1.4	0.593
Liver	4.9±0.4	3.6±0.3	0.002
Kidney	4.1±0.2	4.0±0.3	0.593
Gastrocnemius	5.7±0.4	4.9±0.4	0.021

Values are means ± S.E.M.; *N*=6 (heart, kidney) or *N*=12 (liver, gastrocnemius) replicates per group.

Metabolic index is the percent reduction of indicator dye per milligram dry tissue per hour at 15°C.

Within each row, means were compared using paired Student's *t*-test. Statistical significance is taken at the level of *P*<0.05.

(Table 2) and were generally 4–6-fold higher than in control frogs (see Table 1). Therefore, any difference in freeze/thaw viability of urea-treated organs can be ascribed to elevated tissue levels of this osmolyte.

Control experiments also showed that urea treatment strongly influenced metabolic activity in some of the organs we studied. Reduction of the indicator dye was highest in gastrocnemius and lowest in heart (Table 3). Exposure to 80 mmol l^{-1} urea had no effect on respiration in the heart and kidney but reduced metabolic activity by 15.3% in the gastrocnemius and by 25.4% in the liver. In a separate experiment, substituting 80 mmol l^{-1} glycerol for urea had no effect on respiration, as metabolic indices for the glycerol-treated samples were statistically indistinguishable (paired Student's *t*-test, *N*=3) from control values for the heart (*P*=0.33), liver (*P*=0.16), kidney (*P*=0.30) and gastrocnemius (*P*=0.98).

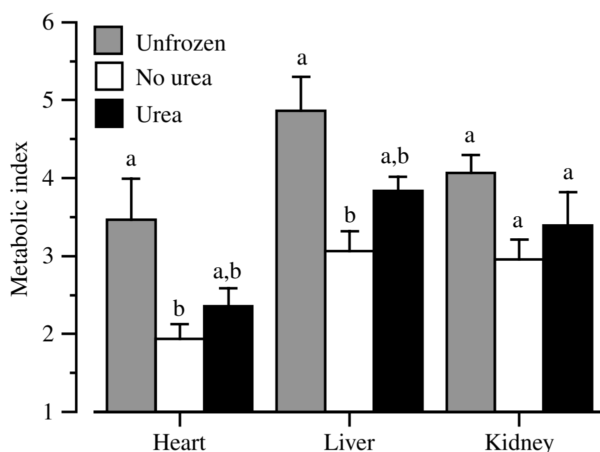

Fig. 4. Post-thaw viability of *R. sylvatica* organ samples incubated in PBS (no urea) or PBS containing 80 mmol l^{-1} urea and frozen at –4°C. Control samples were incubated in PBS but not frozen/thawed. Metabolic index is the percent reduction of indicator dye per milligram dry tissue per hour at 15°C. Mean values (± S.E.M., *N*=6–12 replicates per group) identified by different letters differed significantly (ANOVA/Bonferroni; *P*<0.05).

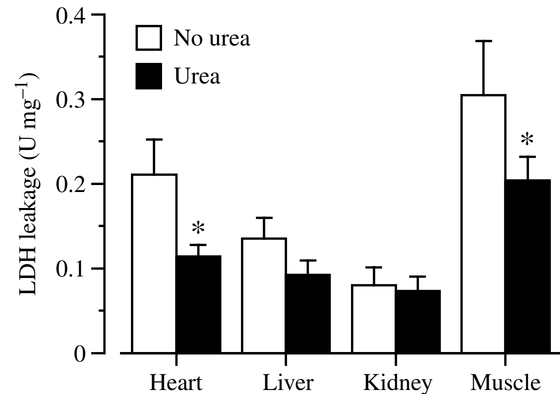

Fig. 5. Cryoinjury to *R. sylvatica* organ samples incubated in PBS (no urea) or PBS containing 80 mmol l^{-1} urea and frozen at –4°C. Damage was assessed from LDH leakage from frozen/thawed samples; values were standardized to sample dry mass. Asterisk denotes that the mean value (± S.E.M., *N*=8 replicates per group) for urea-treated samples differed significantly (Wilcoxan signed-rank test; *P*<0.05) from the mean for samples tested in the absence of urea.

Organs were metabolically active after *in vitro* freezing at –4°C, although their respiration rates were nominally lower than those of unfrozen organs. The drop was relatively minor (and statistically nonsignificant, *P*>0.05) for the urea-treated samples as compared with the control samples (Fig. 4). The kidney appeared innately resistant to cryoinjury, as neither urea-treated nor control samples had respiration rates that differed significantly from unfrozen samples.

These findings were corroborated by the experiment in which organ cryoinjury was assessed from LDH leakage. Except for the kidney, which again proved especially tolerant to freezing/thawing, urea-treated organs exhibited less leakage than samples frozen without urea (Fig. 5). With the heart and gastrocnemius, the difference was robust; however, with the liver it lacked statistical significance.

Discussion

Amphibians regulate intracellular levels of urea by modulating its production, reabsorption and excretion (Katz and Hoffman, 1990; Shoemaker et al., 1992). Generally, they accumulate urea during osmotic stress and therefore readily tolerate hyperuremia (Bentley, 1966; Griffith, 1991; Katz, 1992; Scheer and Markel, 1962; Shpun et al., 1992). Studies of the role of urea in amphibian water economy have focused on saline adaptation and estivation (see review by Jørgensen, 1997) but have neglected hibernation, perhaps because little attention has been paid to the winter physiology of these animals. The few relevant studies (e.g. Nielsen and Jørgensen, 1990; Sinsch, 1991) have involved aquatic hibernators, which have little need to retain urea, so the general significance of this osmolyte in aiding water balance during winter is as yet undocumented. Nevertheless, urea accumulation is probably common in terrestrial hibernators, which may face osmotic stress because soil moisture is seasonally minimal in autumn

and subsequent freezing of the soil further reduces its water potential (Spaans and Baker, 1996). Additionally, in amphibians, cold exposure promotes urea retention by strongly depressing excretion rates (Hong, 1957; Jørgensen, 1950; Miller et al., 1968; Nielsen and Jørgensen, 1990; Schmidt-Nielsen and Forster, 1954).

In *R. sylvatica* inhabiting an outdoor enclosure, plasma urea, which was 25-fold higher in early winter than in spring, generally tracked seasonal changes in soil moisture, suggesting that this osmolyte is important in maintaining hydro-osmotic balance (Fig. 1). In early autumn, urea levels were only slightly elevated despite the soil being very dry; however, during periods of moderate weather, the frogs became active and may have hydrated from dew collecting on fallen leaves. Results of this field study are bolstered by reports of hyperuremia in winter in *R. sylvatica* (Layne and Rice, 2003) and the freeze-tolerant tree frog *Hyla versicolor* (35–40 mmol l^{-1}; 60 mmol l^{-1} in some individuals; J. R. Layne, Jr, unpublished) and by our laboratory experiments demonstrating that urea can accumulate to at least 90 mmol l^{-1} in cold-acclimated frogs. This response would be especially sensitive to warm, dry conditions, which would both accelerate urea synthesis and stimulate its retention. In nature, urea accumulation in hibernating *R. sylvatica* is probably promoted by their preference for overwintering in relatively dry, upland habitats (Regosin et al., 2003).

Urea: an unlikely cryoprotectant?

Organic solutes commonly have overlapping roles in osmoprotection, cryptobiosis and freeze tolerance (Somero and Yancey, 1997; Storey, 1997; Yancey, 2001). Stresses imposed by desiccation and freezing are similar. When tissues freeze, solvent is lost to ice forming in extracellular spaces, and intracellular structures become exposed to increasing ionic strength and crowding. Cryoprotective osmolytes defend cell water volume and also fortify membranes and intracellular macromolecules against ionic and osmotic perturbations. Stability, low molecular mass, high water solubility and permeability are characteristics that render certain organic osmolytes, including urea, well-suited as cryoprotectants. Accordingly, urea functioning as an osmoprotectant during autumn and early winter could, in principle, also protect frogs from the damaging effects of subzero temperatures that occur later in hibernation.

On the other hand, it is commonly understood that urea has deleterious effects on protein stability and function. Unlike compatible osmolytes, which are preferentially excluded from the protein surface, thereby favoring its folded state (Arakawa and Timasheff, 1985; Carpenter and Crowe, 1988; Timasheff, 1992), urea can preferentially bind to proteins, dehydrating their exposed surfaces and promoting unfolding (Creighton, 1991; Wu and Wang, 1999; Zou et al., 1998). However, because interactions between such 'micromolecules' and macromolecules are strongly governed by their physicochemical environment (Somero and Yancey, 1997; Timasheff and Xie, 2003), dynamics observed in artificial urea/enzyme systems may not be germane *in vivo*. Furthermore, protein destabilization generally occurs with urea in high (i.e. molar) concentrations that probably greatly exceed those in *R. sylvatica* tissues; in fact, in modest concentrations, urea may be less perturbing than some compatible osmolytes (e.g. Yancey and Burg, 1990).

To our knowledge, urea has not been reported as a natural cryoprotectant in any organism, yet amphibians readily tolerate urea in concentrations that could provide significant protection against freeze/thaw injury. We tested this hypothesis using the erythrocyte suspension as a model system because earlier studies had characterized the responses of these cells to freeze/thaw and osmotic stresses (Costanzo and Lee, 1991; Costanzo et al., 1993) and also had demonstrated the modest protection that urea afforded human erythrocytes (Doebbler and Rinfret, 1962). In our experiments, physiological concentrations of urea improved the viability of *R. sylvatica* erythrocytes frozen/thawed at temperatures of ecological relevance to the species (Figs 2, 3). The apparent absence of a concentration-dependent effect suggests that urea's action is not purely colligative but also involves protection of macromolecules and cellular structures (Carpenter and Crowe, 1988; Mazur, 1984). Recent findings (Bhuyan, 2002; Kumar et al., 2004) of protein renaturation in the presence of urea at relatively low concentrations support this notion.

Comparing the results presented in Figs 2 and 3 suggests that erythrocyte tolerance to freeze/thawing varied between experiments. The heightened sensitivity of cells apparent in Fig. 3 could have resulted because this work was done in late winter, after *R. sylvatica* usually arouses from hibernation. Seasonal variation in tolerance to osmotic and freeze/thaw stress can potentially stem from changes in the structure and chemical composition of the plasma membrane. An ongoing study suggests that cholesterol levels in the erythrocyte membrane, which have profound effects on fluidity and thermal adaptation, vary seasonally in the hatchling painted turtle, *Chrysemys picta*, another cold-hardy species (M. R. Polin, J. P. Costanzo and R. E. Lee, unpublished).

Assessing post-thaw viability of *R. sylvatica* organs from rates of metabolic activity or LDH leakage demonstrated that urea markedly improved freezing tolerance of intact tissues. Cardiac muscle was particularly amenable to cryoprotection by urea. In preliminary studies, we found that isolated *R. sylvatica* hearts frozen for 30 min at –4°C spontaneously resumed autonomic contractions upon thawing when the incubation medium (PBS) contained 100 mmol l^{-1} urea (N=2) but not when urea was absent (N=2). We observed no discernable benefit of urea treatment with kidney, but for unknown reasons this organ proved particularly resistant to cryoinjury under the conditions of our experiment. Urea treatment of liver tissue curbed freeze/thaw injury, as thawed samples exhibited no appreciable reduction in metabolic activity. Urea treatment also reduced hepatocyte membrane damage (indexed by LDH leakage) in six of the eight experimental replicates; overall, however, the effect lacked statistical significance. We suspected that in some cases the protection afforded liver by

urea was masked by the presence of high concentrations of glucose, which also reduces LDH leakage from frozen/thawed hepatocytes (Storey and Mommsen, 1994). This could have occurred only if our *in vitro* liver samples had autogenerated copious glucose during the experiment. We confirmed this supposition by assaying portions of *R. sylvatica* liver immediately upon dissection and after incubation in 80 mmol l^{-1} urea and subsequent freezing at –4°C. In one trial, liver glucose concentration increased with freezing, from 13 to 56 μmol g^{-1}, and in another it rose from 19 to 47 μmol g^{-1}. By contrast, glucose levels in gastrocnemius, ~5 μmol g^{-1}, were unchanged. The results for liver were probably confounded by the presence of a second cryoprotectant; however, on the whole, our data marshal sound evidence that urea cryopreserves both isolated cells and intact tissues.

In some experiments the viability of tissues following experimental freezing/thawing was assessed using an index of metabolic activity. Our preliminary experiments showed that physiological levels of urea markedly reduced aerobic respiration in some tissues. Although incidental to the focus of our present report, this result merits consideration. In principle, elevated urea could induce metabolic depression in overwintering frogs if, as has been postulated for various estivating organisms, transition between arousal and dormancy is modulated by shifts between the active/inactive states of key regulatory enzymes in response to changes in urea concentration, pH and temperature (Somero, 1986; Withers and Guppy, 1996; Yancey et al., 1982). The relatively low levels of urea found in *R. sylvatica* tissues might be generally nonperturbing and yet inhibitory to certain key enzymes (e.g. phosphofructokinase; Hand and Somero, 1982). Because urea's effects are strongly potentiated by low pH (e.g. Hand and Somero, 1982) and dormant frogs are acidotic (Pinder et al., 1992), hyperuremia, acidosis and cold may be potent, synergistic effectors of hypometabolism in overwintering *R. sylvatica*. The few available data suggest that hyperuremic amphibians do not accumulate methylamines to levels that might counteract the urea inhibition (Pinder et al., 1992; Wray and Wilkie, 1995; Withers and Guppy, 1996), however additional study is needed before definitive conclusions can be drawn.

The urea-hypometabolism hypothesis garners support from *in vitro* studies of enzyme kinetics (Cowan and Storey, 2002; Grundy and Storey, 1994; Stewart et al., 2000) but heretofore was not demonstrated at higher levels of organization. Our present findings should be regarded as tentative until confirmed through additional research. However, microrespirometry experiments have shown that treatment with 80 mmol l^{-1} urea reduces oxygen consumption in *R. sylvatica* liver and skeletal muscle by ~33%, whereas increasing the osmolality without adding urea has no effect (T. J. Muir, J. P. Costanzo and R. E. Lee, unpublished). Curiously, in the present study, urea treatment suppressed *in vitro* metabolism in *R. sylvatica* liver and muscle but not in heart or kidney. Urea sensitivity of enzyme systems varies among tissues (Cowan and Storey, 2002) and, indeed, tissues differ in their capacity for metabolic depression (Boutilier and St-Pierre, 2002; Flanigan et al., 1991). A marked hypometabolism in only the liver and skeletal muscle could profoundly reduce energy consumption inasmuch as these organs constitute >50% of the total tissue mass. Additional study is needed to assess the depth of metabolic depression in intact, hyperuremic frogs.

Urea and glucose: a dual cryoprotectant system?

The freezing-induced mobilization of carbohydrate cryoprotectant (glucose in *R. sylvatica*; glycerol and/or glucose in some tree frogs) has long been touted as the hallmark physiological adaptation in amphibian freeze tolerance. Our present findings indicate that urea accumulated before freezing can contribute to freezing survival, raising the possibility that freeze-tolerant frogs rely on more than one class of cryoprotective agent. Moreover, in the case of *R. sylvatica*, one could reasonably argue that urea plays a key role in reducing freeze/thaw injury.

Given that equimolar solutions of urea and glucose have the same equilibrium freezing/melting point, as a colligative cryoprotectant the value of either depends solely on its concentration within the tissues. Glucose accumulates in tissues during somatic freezing whereas urea does not (Table 1; see also Layne and Rice, 2003). Nevertheless, in some circumstances, frozen frogs could have as much (or more) urea as glucose inside their cells. Frogs often generate only modest amounts of glucose during freezing (Table 4). This statement contradicts the conventional wisdom that freezing *R. sylvatica* amass 0.3–0.5 mol l^{-1} glucose; however, it is important to realize that these often-cited values are extremes and are uncharacteristic of the glycemic response. In fact, not only has the popular and scientific literature exaggerated the glycemic response, but, because tissues dehydrate profoundly while freezing (Lee et al., 1992), the common convention of reporting metabolite concentrations as per-unit-mass of fresh tissue has grossly overestimated the capacity for *de novo* glucose synthesis. Glucose synthetic capacity apparently varies with age, sex, body size, cooling rate and myriad other factors. In particular, frogs can achieve markedly higher glycemic levels in autumn, when glycogen is abundant, than they can in spring (Costanzo and Lee, 1993; Layne, 1995; Storey and Storey, 1987), and glucose production capacity may be unusually well-developed in northern populations (Layne, 1995). The literature attests that the blood glucose concentrations achieved during freezing by temperate *R. sylvatica* are typically <100 mmol l^{-1} in autumn and <25 mmol l^{-1} by the end of winter (Table 4). Under conducive environmental conditions, blood urea levels could be as high or higher.

Another important consideration is that cryoprotectant must actually enter cells in order to exert its full effect. Whereas virtually all cells are laden with urea before freezing commences, glucose must be synthesized in the liver, circulated throughout the body and transported into cells while the tissues are freezing. Because freezing is initiated where the body contacts ice in the environment, peripheral organs, such

Table 4. *Plasma glucose concentrations in* Rana sylvatica *subjected to experimental freezing*

Origin	Season	Glucose (mmol l^{-1})	Reference
Ontario, Canada	Autumn	214±16 (6)[a]	Storey and Storey (1984)
		185±40 (6)[a]	Storey and Storey (1984)
		181±16 (5)[a]	Storey and Storey (1985)
		411±60 (3)[b]	Storey and Storey (1986a)
		257 (3)[a]	Storey and Storey (1986b)
		171±26 (3)[a,c]	Storey (1987)
		118±7 (9)	Storey et al. (1992)
		30±6 (4)[d]	Churchill and Storey (1993)
	Spring	57±13 (3)[e]	Storey and Storey (1987)
		10±4 (4)[d]	Churchill and Storey (1993)
		83±16 (10)	Hemmings and Storey (1994)
		69±9 (7)	Storey and Storey (1996)
N.W.T., Canada	Spring	60±19 (4-6)[f]	Costanzo et al. (1999)
Ohio, USA	Spring	15±0.4 (5)	Layne and Lee (1987)
		15±2 (10)[g]	Costanzo et al. (1991a)
		18±5 (5)[h]	Costanzo et al. (1991b)
		145±50 (3)[d]	Costanzo et al. (1997)
		24±16 (12)	Layne et al. (1996)
		50±11 (3)[d]	Irwin et al. (2003)
Pennsylvania, USA	Autumn	93±8 (3)	Layne (1995)
	Spring	6±1 (4)	Layne (1995)
	Summer	3±1 (4)	Layne (1995)
New York, USA	Winter	88±30 (9)	Layne et al. (1996)

Data are reported for experiments in which frogs (adults, unless otherwise indicated) probably attained equilibrium ice content and were sampled after brief thawing. Means ± S.E.M. (*N*). [a]juveniles; [b]females; [c]sampled after two cycles of freezing; [d]blood was collected from still-frozen animals; [e]highest of the means reported for several freezing regimens; [f]sampled 14 h after thawing at 5°C; [g]slowly cooled frogs; [h]administered isotonic saline before freezing.

as the skin and skeletal muscles, quickly become isolated from the blood supply and thus accumulate little glucose (Rubinsky et al., 1994; Storey and Storey, 1988). Glucose uptake by muscle fibers is further hampered by their low permeability (King et al., 1993; Storey and Storey, 2004), and nervous tissues also accumulate relatively little glucose with freezing (Costanzo et al., 1992; Kling et al., 1994). As demonstrated in our experiment (Table 1), even in moderately hyperuremic frogs, cells in some tissues could contain as much urea as glucose because equilibrium levels of the former are attained in advance of freezing.

Our assertion about urea's relative importance as a cryoprotectant draws additional support from findings that urea was as good or better than glucose at reducing freeze/thaw injury to *R. sylvatica* erythrocytes (Fig. 3). Arguably, this result could simply reflect differential permeabilities and intracellular concentrations of the two solutes. However, this was probably not the case because glucose quickly penetrates *R. sylvatica* erythrocytes and is not metabolized at the low temperature used in our experiments (Brooks et al., 1999). Rather, this finding suggests that urea is more effective than glucose in increasing the fraction of unfreezeable cell water and/or in preserving the integrity of macromolecules and cellular structures. Caution should be used in interpreting the results of *in vitro* experiments, which cannot accurately replicate *in vivo* conditions. In the live frog, for example, both osmolytes could be present and working in concert. We found no evidence for synergism in cryoprotective efficacy when we tested erythrocytes preincubated with 40 mmol l^{-1} urea and 40 mmol l^{-1} glucose (data not shown), although undoubtedly the colligative effects in reducing ice content would be additive.

Evolutionary perspectives

Long known as a balancing osmolyte, urea also serves myriad other physiological functions in diverse animal taxa (Withers, 1998). Our present findings not only suggest that urea plays a key, previously undocumented role in amphibian cold hardiness but they also identify a novel class of natural cryoprotectant. In freeze-tolerant frogs, both dehydration and somatic freezing initiate molecular events that increase glycemia, suggesting that amphibian cryoprotectant systems derive from rudimentary mechanisms of water conservation (Storey and Storey, 2004). Among amphibians, osmotic stress universally stimulates urea retention, which defends against cellular dehydration. Our contention that urea is a key cryoprotective agent in *R. sylvatica* (and probably other taxa) supports the tenet of overlapping adaptations in cold hardiness

and dehydration tolerance in ectothermic animals (Churchill and Storey, 1993; Ring and Danks, 1994). In addition, finding a cryoprotective role for urea in *R. sylvatica* resolves the apparent enigma of why osmolytes in freezing adaptation should be exclusively carbohydrates, whereas organisms facing other osmotic stresses use a mixture of osmolytes, often of different classes (Yancey, 2001).

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