Dehydration tolerance in wood frogs: a new perspective on development of amphibian freeze tolerance

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Churchill, Thomas A., and Kenneth B. Storey. Dehydration tolerance in wood frogs: a new perspective on development of amphibian freeze tolerance. *Am. J. Physiol.* 265 (*Regulatory Integrative Comp. Physiol.* 34): R1324–R1332, 1993.—Wood frogs, *Rana sylvatica*, tolerate the loss of 50–60% of total body water during experimental dehydration. The rate of water loss for unprotected frogs is the same whether animals are frozen (at −2°C) or unfrozen (at 1°C) but is greatly reduced when frogs are frozen under a protective layer of moss. Dehydrational death could occur in as little as 7–9 days for unprotected animals; this indicates the importance for winter survival of selecting well-protected and damp hibernation sites. Prior dehydration affected the cooling and freezing properties of frogs, reducing supercooling point and the amount of ice formed after 24 h at −2°C and acting synergistically with freezing exposure in stimulating cryoprotectant synthesis. Analysis of the effects of controlled dehydration at 5°C showed that changes in body water content alone (without freezing) stimulated liver glycogenolysis and the export of high concentrations of glucose into blood and other organs. Autumn-collected frogs dehydrated to 50% of total body water lost showed glucose levels of 165–1,409 nmol/mg protein in different organs, increases of 9- to 313-fold compared with control values and reaching final levels very similar to those induced by freezing exposure. The data support the proposal that various adaptations for natural freeze tolerance may have been derived from preexisting mechanisms for dealing with water stress in amphibians and that cell volume change may be one of the signals involved in triggering and sustaining molecular adaptations (e.g., cryoprotectant output) that support freezing survival.

anuran biochemistry; cryoprotectants; cell volume regulation

TO SUCCESSFULLY EXPLOIT terrestrial habitats, amphibians must overcome their susceptibility to evaporative water loss. As a group, amphibians have highly water-permeable skins. Typical water loss rates are 4.7–9.2 $\mu l \cdot cm^{-2} \cdot h^{-1}$ at 20°C (17), which can lead to a loss of total body weight of 6–9%/day (9). For most anurans, the critical activity point (the level of dehydration at which the animal can no longer right itself when placed on its back) is reached when 30–45% of initial body weight has been lost; the critical activity point is generally higher for species living in dry environments compared with those in wetter habitats (9, 20). This susceptibility to evaporative water loss has kept many species restricted to aquatic or very damp terrestrial environments, but others have developed effective mechanisms for reducing water loss across the skin or increasing cutaneous water uptake (4, 20). Furthermore, at a physiological level, amphibians have developed the highest tolerances among vertebrates for variation in the osmolality and ionic composition of body fluids (11). Indeed, this tolerance is seen not only as a passive response to environmental stress (desiccation or exposure to hypersaline water) but also as active changes in body fluid osmolality initiated by the animal itself, such as the accumulation of 300–400 mM urea in the body fluids of estivating toads or the production of similar concentrations of glucose or glycerol by freeze-tolerant frogs (16).

Freeze tolerance, the ability to endure the freezing of extracellular body fluids, occurs in a small group of amphibian and reptile species that hibernate in cold terrestrial environments (for review see Refs. 23, 28). For cells, the consequences of extracellular freezing have many similarities to the effects of desiccation because both stresses effectively remove cell water, increase the osmolality and ionic strength of the cytoplasm, and decrease cell volume. During freezing this is caused by the sequestration of up to 65% of total body water as extracellular and extraorgan ice, whereas during dehydration this is due to a net water loss from the body. We reasoned, therefore, that the physiological and biochemical responses to freezing and dehydration might be similar. Freeze tolerance may include an elaboration of various inherent amphibian adaptations that accommodate wide variations in body and cell water contents, and, conversely, mechanisms employed for freeze tolerance may also be expressed during dehydration.

The present study examines the effects of dehydration on the wood frog, *Rana sylvatica*, the most extensively studied of the freeze-tolerant frogs (for review see Refs. 23, 28). We analyze the limits of body water loss, the corresponding changes in organ water contents, the effects of dehydration on some metabolic responses, and the effect of hydration state on parameters of freezing with a further comparison of seasonal (spring vs. fall) differences in these parameters.

METHODS AND MATERIALS

Chemicals and animals. All chemicals and biochemicals were purchased from Boehringer Mannheim, Montreal, Quebec, Canada, or Sigma Chemical, St. Louis, MO. Wood frogs were collected from the Ottawa region in early to mid-April 1990 for spring experiments or early October 1990 for autumn experiments. Animals were acclimated at 5°C in a moist environment without food for at least 1 (spring frogs) or 4 wk (autumn frogs) before experimentation. Immediately before frogs were assigned to different treatment groups, all animals were induced to void their bladder water by placing gentle pressure on the abdomen.

Effects of damp and dry environments on water loss by spring frogs. Experiments were designed to evaluate the effects of microhabitat selection on frog water loss in both the liquid and frozen states. To create a damp environment, frogs were placed in a closed plastic container on a bed of damp sphagnum moss and covered with moist paper towel. To create a dry environment, frogs were blotted with paper towel and then placed in covered plastic containers and covered with dry paper towel. Containers were then placed in incubators set at either 1° or −2.0°C. The paper towels covering frogs held under damp conditions at 1°C were checked daily and remoistened several times during the experiment. Frogs that were given freezing exposure

began to freeze within ~1 h at −2°C. By the first 6-h sampling point, frogs in the dry environment were already quite solidly frozen, whereas those in the damp environment were partially frozen and could still move slightly; by 24 h all frogs were solidly frozen. At intervals (2–3 times/day), frogs were briefly removed from the incubators and body weights of individuals (identified by a numbered band of tape on a hind leg) quickly measured; weight changes were assumed to be due to water loss or gain. The initial body water content (g H_2O/g body mass) of frogs was obtained from initial and final weight measurements on control frogs (n = 4) that were double pithed and then dried to a constant weight at 80°C for 72 h.

Experimental dehydration protocol. Silica-gel desiccant (400 g) was placed in the bottom of glass vacuum desiccators and covered with a piece of sponge 1–2 cm thick. Five frogs (5–8 g each) were then placed on top of the sponge in each desiccator, and the desiccator was placed in an incubator at 5°C. The stopcock on top of the desiccator was left open so that oxygen availability would not be impeded. The ratio of desiccant to frog weight was chosen to give a rate of total body water loss of 0.5–1%/h. Frogs were briefly removed at 10- to 20-h intervals (to minimize handling) and weighed to determine water loss. Frogs were treated until 25 or 50–55% dehydration was reached. Both spring and autumn frogs were tested. The percentage change in body water content was calculated as

$$\%\text{change} = [(W_i - W_d)/(W_i \times \text{BWC}_i)] \times 100$$

where W_i is initial body weight, W_d is weight during experimental dehydration, and BWC_i is initial body water content of frogs before dehydration in grams H_2O per gram body mass.

Supercooling points, freezing, and percent ice determinations. Groups of control and 25 or 50–55% dehydrated frogs were subjected to supercooling point determinations, 24 h freezing at −2°C, and then percent ice determinations as follows. Individual frogs were placed on a pad of paper towel centered over a Yellow Springs Instruments telethermometer probe that came in contact with the abdomen. Frog and thermistor were secured with a band of masking tape, and then animals were placed in an incubator set at −2.0°C. Frogs were then allowed to cool to −2.0°C (approximate rate −0.5°C/min), and body surface temperature was recorded with a linear chart recorder. If no exotherm was recorded before body surface temperature reached −2°C, incubator temperature was lowered to −4°C and frogs were allowed to cool further; in some cases, a further temperature adjustment to −6°C was needed. Nucleation was observed as an abrupt exotherm after the frogs had reached their supercooling points. A maximum of four frogs was frozen at one time; in no instance did more than 20 min elapse between nucleation of the first and last animals. As soon as supercooling points had been recorded for all frogs, incubator temperature was readjusted (if needed) to −2.0 ± 0.1°C, and frogs were allowed to freeze for 24 h at this temperature.

After freezing exposure, the ice content of individual frogs was measured using the calorimetry method of Lee and Lewis (13) and the following experimentally measured parameters (mean ± SE): F factor for the calorimeter 1.03 ± 0.01 and specific heat of the dry mass 0.195 ± 0.038 cal·g^{-1}·°C^{-1}. The body water content of frogs was 80.8 ± 1.2% of body mass for control frogs, 61.1 (fall) and 60.3% (spring) for 25% dehydrated frogs, and 35.1 (fall) and 38.9% (spring) for 50–55% dehydrated frogs. Immediately after thawing in the calorimeter, animals were killed by pithing and then cut open ventrally. Because the heart had not yet resumed beating, blood was collected after the heart was excised and placed on tinfoil; one of the atria was snipped, and the heart was gently squeezed to expel the blood. This was quickly collected with a capillary pipette, transferred to an Eppendorf microcentrifuge tube, and immediately frozen for subsequent measurement of metabolite levels.

Spring and fall dehydration time courses. Initial dehydration tests, performed as described above, indicated that wood frogs could endure the loss of 50 (autumn) or 60% (spring) of total body water; animals rehydrated over ~24 h when placed in a dish of water at 5°C and showed normal appearance and reflexes when examined 1 day or 1 wk later. Therefore a time-course experiment to monitor metabolic responses to dehydration by spring frogs at 5°C was set up with sampling of frogs at five intervals up to 60% dehydration; in addition, another group of frogs was dehydrated to 50% total water loss and then rehydrated to approximately the initial body water content. To rehydrate frogs at 5°C, animals were placed in a plastic container with ~2 cm distilled water in the bottom; initial tests showed that individual animals required 12–24 h for complete rehydration (as determined by body weight gain), but all were sampled after 24 h. In experiments with autumn frogs, animals were sampled after 25 or 50% dehydration at 5°C as well as after 50% dehydration followed by either partial (to 25%) or full rehydration. In both experiments, frogs were briefly removed from the 5°C incubator approximately every 12 h and weighed; when calculated water loss (or regain) was within 5% of the targeted sampling point for an individual group, these frogs were then weighed every 2 h until the target was reached. In all cases, control frogs were sampled immediately after removal from the 5°C incubator.

Tissue sampling and metabolite measurements. All frogs were killed by pithing. Blood was generally collected by exposing the heart, snipping one of the atria, and sampling with a capillary micropipette with care taken to avoid collection of any blood that had been diluted by extraorgan water. For some severely dehydrated frogs with very weak blood flow, blood was collected as described for frogs that were frozen at −2°C. Blood samples were transferred to Eppendorf microcentrifuge tubes, capped, and frozen; sample weight was determined by weighing tubes before and after the addition of blood. Organ samples were then quickly excised and frozen in liquid nitrogen. Samples were transferred to −80°C until processing.

Perchloric acid extracts of tissue samples were prepared as described previously by Storey and Storey (25); in some cases, an aliquot of the well-mixed homogenate was removed before centrifugation for the determination of glycogen. Neutralized extracts were frozen at −80°C until they were used for metabolite assays. All metabolites were measured in coupled enzyme assays using an Amico-Bowman spectrofluorometer with an excitation wavelength of 340 nm and an emission wavelength of 460 nm. Glycogen determination was performed as described by Keppler and Decker (12). Glucose and lactate were measured as described by Lowry and Passonneau (14); glycerol was assayed as described by Eggstein and Kuhlmann (5).

Ratio of wet weight to dry weight and protein determinations. Organ water contents were determined from initial and final weight measurements of tissue samples that were dried to a constant weight at 80°C for 72 h and are expressed as grams water per gram dry weight. To determine organ protein content, the acid-precipitated pellets obtained from perchloric acid extractions were resuspended in NaOH and then total protein measured by the Coomassie Brilliant Blue dye-binding method of Bradford (2) using the Bio-Rad prepared reagent and a standard of bovine γ-globulin.

Data and statistics. Data are presented as means ± SE with replicates of at least n = 4. Statistical analysis was performed using Student's t test or Dunnett's test.

RESULTS

Effect of damp or dry environments on water loss by frogs. Because frozen frogs cannot move and probably

also cannot take up water from their frozen surroundings, they could be unable to redress any net loss of body water over what could be weeks of continuous freezing during winter hibernation. It seems likely, then, that microhabitat selection, the choice of a moist and protected site, would be a key factor for successful hibernation of freeze-tolerant frogs. To experimentally assess the importance of habitat water content on the body water content of wood frogs, we monitored changes in the body water content of frogs held under damp (closed container with a bed of damp sphagnum moss and a covering of damp paper towel) vs. dry (closed dry container covered with dry paper towel) conditions and either held at 1°C or frozen at −2°C. Figure 1 shows changes in body water content (relative to initial water content) for four groups of spring-collected frogs. Frogs held at 1°C under damp conditions showed no dehydration over 160 h of monitoring; indeed, this group showed a significant water gain over time, with a final water content of 113.4 ± 5.8% of the initial value. By contrast, frogs frozen at −2°C under the same conditions showed a small water loss over time, with a final water content of 97.5 ± 1.4% of the initial value. However, frogs held in dry containers showed steady decreases in body mass over time that yielded a final body water content after 160 h of only 49.5 ± 1.7 and 52.6 ± 2.0% of the initial water content at 1 and −2°C, respectively. The pattern of water loss for both frozen and unfrozen frogs was virtually identical, with the calculated rate of total body water loss averaging 0.32%/h.

Effect of dehydration on supercooling ability and ice content. Both spring and autumn frogs were experimentally dehydrated over desiccant to ~25 or 50–55% of total body water lost; the rate of body water loss in this situation was 0.5–1.0%/h. The body surface temperature of individual frogs was then monitored during cooling below 0°C to determine supercooling point (SCP; also called crystallization temperature) and rebound temperature (T_r; the elevated body temperature observed after the freezing exotherm; Table 1). Control frogs in both the spring and autumn did not appear to supercool. No freezing exotherm was recorded for these animals; they cooled until body temperature reached the freezing point, and then temperature stabilized while freezing progressed. However, this freezing temperature was significantly lower for the autumn vs. spring frogs, a mean of −0.80 vs. −0.18°C. When frogs were experimentally dehydrated before subzero exposure, supercooling was observed, and SCP decreased as the extent of dehydration increased. T_r values followed the same pattern. In addition, both SCP and T_r values were generally lower for autumn-collected frogs than for spring animals; e.g., the mean SCP of autumn frogs was −4.8°C compared with −3.4°C for comparably treated spring frogs.

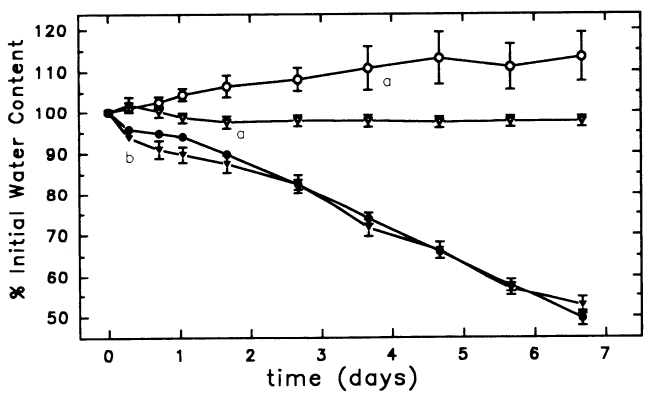

Fig. 1. Effect of exposure to damp or dry environments on body water content of spring-collected *R. sylvatica* at 1°C or frozen at −2°C. Data are means ± SE and are expressed relative to initial water content of frogs, which is set at 100%; $n = 4$. ○, 1°C damp; ▽, −2°C damp; ●, 1°C dry; ▼, −2°C dry. Damp conditions consisted of a closed container with frogs placed on a bed of damp sphagnum moss and covered by damp paper towel, and dry conditions consisted of a closed dry container with frogs covered by dry paper towel. [a] Data for this and all longer time points are significantly different from initial water content of frogs in damp environments (2-way analysis of variance without replicates followed by 2-tailed Dunnett's test, $P < 0.05$). [b] $P < 0.01$ for frogs in dry environments.

Table 1. *Effects of dehydration on supercooling point and rebound temperature of R. sylvatica*

%Dehydrated	Spring		Autumn	
	SCP, °C	T_r, °C	SCP, °C	T_r, °C
0		−0.18±0.02		−0.80±0.04*
25	−2.4±0.3	−0.4±0.1†	−2.6±0.5	−1.4±0.1*†
55	−3.4±0.2‡	−1.2±0.1†	−4.8±0.1*‡	−3.0±0.3*†

Values are means ± SE; $n = 4$ frogs/group. For frogs that did not show supercooling, rebound temperature (T_r) is effectively freezing point. Actual values for mean loss of total body water by the frogs were 25.6 ± 1.0 and 52.4 ± 1.9% for spring frogs and 24.2 ± 1.0 and 56.1 ± 3.2% for autumn frogs. SCP, supercooling point. * Significantly different from corresponding value for spring frogs (2-tailed Student's t test, $P < 0.005$). † Significantly different from corresponding value for 0% dehydrated frogs (2-tailed Dunnett's test, $P < 0.05$). ‡ Significantly different from corresponding value for 25% dehydrated frogs (2-tailed Student's t test, $P < 0.05$).

Ice content of frogs after 24 h freezing at −2.0°C also depended on the dehydration state of the animals. The greater the prior dehydration of the frogs, the lower the amount of ice formed (as a percentage of remaining body water; Table 2). Spring frogs also accumulated significantly more ice at all levels of dehydration than did the corresponding group of autumn frogs. Thus ice contents of spring frogs fell from 61.3% of total body water for control animals to 35.3% for 55% dehydrated, whereas the corresponding values for autumn frogs were 48.9 and 20.5%, respectively.

Blood samples taken from these frogs after thawing in the calorimeter showed that freezing at −2°C stimulated a large increase in blood glucose concentration in autumn animals and a lesser increase in spring frogs (Table 3; control values for dehydrated but unfrozen frogs can be

Table 2. *Effects of dehydration on ice content of R. sylvatica after 24 h freezing at −2°C*

%Dehydrated	%Total Body Water as Ice	
	Spring	Autumn
0	61.3±2.0	48.9±0.9*
25	57.6±3.5†	47.0±2.5*†
55	35.3±3.6†	20.5±2.3*†

Values are means ± SE; $n = 4$ frogs/group. * Significantly different from corresponding value for spring frogs (2-tailed Student's t test, $P < 0.05$). † Significantly different from corresponding value for 0% dehydrated frogs (2-tailed Dunnett's test, $P < 0.01$).

Table 3. *Effect of dehydration and freezing exposure at −2°C for 24 h on blood glucose and lactate levels in R. sylvatica*

%Dehydrated	Glucose, μmol/g blood		Lactate, μmol/g blood	
	Spring	Autumn	Spring	Autumn
0	10±4	29±6*	12±2	8±2
25	31±20	65±24	17±4	13±3
55	24±19	208±32*†	36±6†	37±10†

Values are means ± SE; $n = 4$ frogs/group. * Significantly different from corresponding values for spring frogs (2-tailed Student's t test, $P < 0.05$). † Significantly different from corresponding values for 0% dehydrated frogs (2-tailed Dunnett's test, $P < 0.05$).

Table 4. *Effect of dehydration at 5°C on levels of glucose and lactate in blood of spring R. sylvatica*

%Dehydrated	Glucose, μmol/g blood	Lactate, μmol/g blood
0	1.85±0.28	1.70±0.53
5	3.90±0.74	5.42±0.28
10	5.37±0.77	2.40±0.52
25	3.05±0.58	2.13±0.22
35	5.44±0.72	7.47±2.72*
60	10.5±3.5*	14.4±3.96*
Rehydrated	4.24±0.1*	1.01±0.01

Values are means ± SE; $n = 4$ frogs/group. For rehydration, frogs were replaced in water for 24 h at 5°C; 100% rehydration occurred within 12–24 h for different individuals. * Significantly different from corresponding glucose or lactate value for control 0% dehydrated frogs (2-tailed Dunnett's test, $P < 0.05$).

found in Table 4). This seasonal difference in glucose output in response to freezing has been documented previously (25, 26). Prior dehydration before freezing resulted in significantly greater levels of blood glucose in autumn frogs compared with 0% dehydrated animals; a similar trend was seen for spring frogs, but individual variation in blood glucose content was very high. A portion of this increase could be attributed to the concentrating effect that water loss would have on blood metabolite levels, but, as illustrated by the 7.2-fold greater level of blood glucose in 50% compared with 0% dehydrated autumn frogs, dehydration and freezing clearly also had synergistic interactions on glucose output. Freezing exposure also resulted in an increase in blood lactate levels, and, again, an interaction between dehydration and freezing was noted; lactate levels were 3- and 4.5-fold higher in blood of 50% dehydrated spring and autumn frogs, respectively, compared with 0% dehydrated frogs.

Effect of progressive dehydration on organ water, protein, and metabolite contents of spring frogs. To investigate the effects of dehydration itself on metabolic events in frog organs, spring frogs were dehydrated over desiccant at 5°C and sampled at set intervals when they had lost ~5, 10, 25, 35, 50, and 60% of their total body water. Actual measured values for the mean percentage of total body water lost by frogs at the different sampling points were 6.2 ± 0.5, 12.0 ± 0.2, 25.9 ± 1.1, 37.9 ± 0.8, 51.9 ± 1.5, and 61.1 ± 1.3%, respectively. In addition, another group of frogs was submitted to 60% dehydration (measured value 63.8 ± 0.4%) and then allowed to rehydrate in a container of water over 24 h, reaching a final water content equivalent to 4.6 ± 0.4% dehydrated.

Figure 2 shows the effect of whole animal dehydration on the water and protein contents of the liver of spring frogs. Despite the very large changes in total body water content during dehydration, the water content of the liver showed no significant change. At 60% dehydration, liver water content was 1.32 ± 0.19 g water/g dry wt, compared with a control value of 1.64 ± 0.06 g water/g dry wt.

The protein content of liver (mg protein/g dry wt) also showed no significant change over the dehydration time course (Fig. 2). Because the levels of protein were constant in the dehydrated liver (and other organs), we subsequently chose to express metabolite levels as nanomoles per milligram protein, rather than the more commonly used micromoles per gram wet weight to eliminate the effect of any possible changes in organ water content on metabolite concentrations.

Figure 3 shows the levels of glucose and lactate in six organs of these spring frogs, comparing the values for control and 60% dehydrated frogs. Control levels of glucose in organs ranged from 10 nmol/mg protein in gut and brain to 67 nmol/mg protein in heart (Fig. 3*A*). As a result of dehydration, glucose levels increased significantly in four organs, with the highest level 205 nmol/mg in heart. Brain showed the largest relative increase (7.4-fold) in glucose content, and increases in liver, kidney, and heart were 2.6-, 2.6-, and 3.1-fold, respectively. Glycerol content was also assayed in all organs, but levels were very low and unchanged under all conditions.

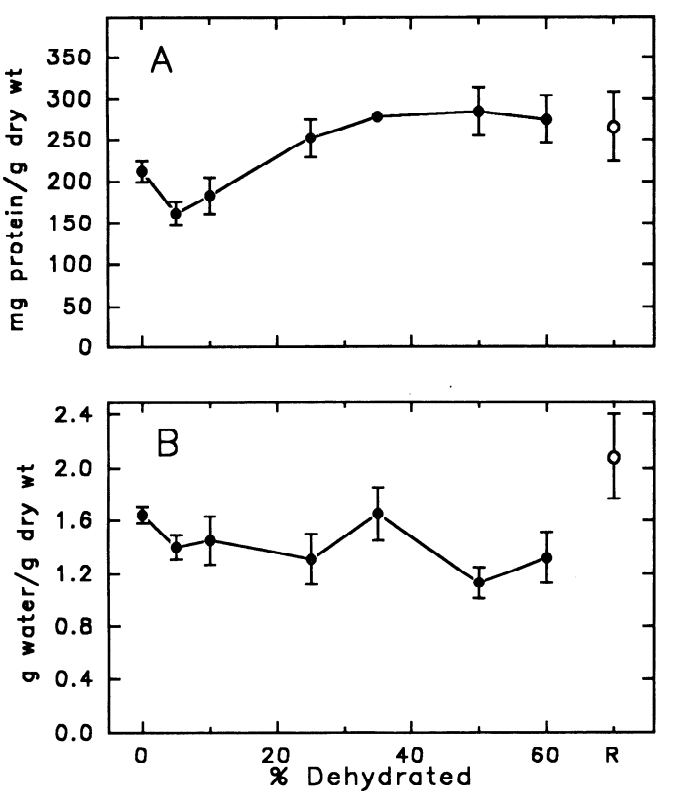

Fig. 2. Effect of whole animal dehydration on protein (*A*) and water (*B*) contents of spring *R. sylvatica* liver. Data are means ± SE; $n = 4$. ○, frogs dehydrated to 60% total body water lost and then rehydrated (R) for 24 h.

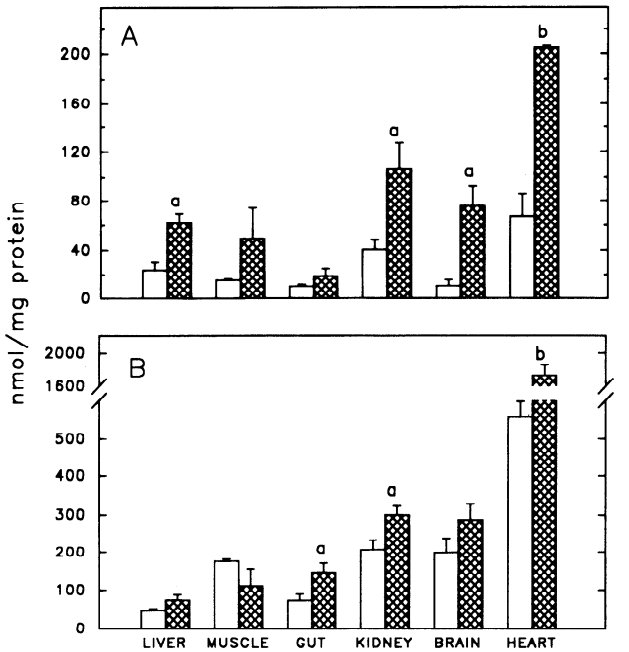

Fig. 3. Effect of dehydration on levels of glucose (*A*) and lactate (*B*) in 6 organs of spring *R. sylvatica*. Data are means ± SE; $n = 4$ for control 0 (□) and 60% dehydrated (▩) frogs. Gut combines stomach and intestine. Significantly different from corresponding control: [a] $P < 0.05$; [b] $P < 0.005$.

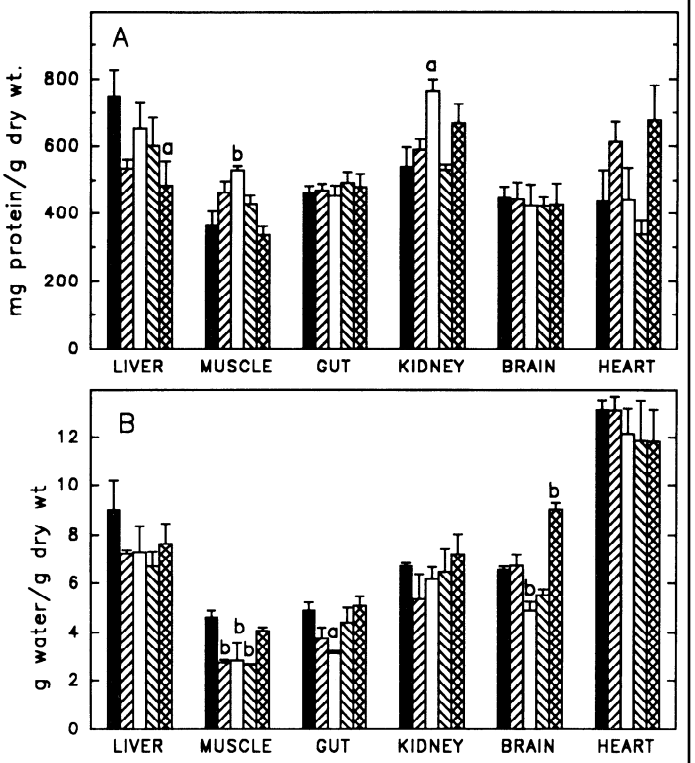

Fig. 4. Effect of changes in total body water content on protein (*A*) and water (*B*) contents of 6 organs of autumn *R. sylvatica*. Data are means ± SE; $n = 4$. Bars are as follows: ■, control 0% dehydrated; ▨, 25% dehydrated (measured value for mean water loss 28.5 ± 0.1%); □, 50% dehydrated (49.1 ± 2.3%); ▧, rehydrated to 25% (21.7 ± 1.5% after dehydration to 47.9 ± 1.7% water lost); ▩, rehydrated to 0% (7.4 ± 1.2% dehydrated after 50.1 ± 1.7% water lost). Significantly different from corresponding control value: [a] $P < 0.05$; [b] $P < 0.005$.

The loss of 60% of total body water also resulted in a significant increase in lactate levels in three organs of the frogs (Fig. 3*B*). Lactate in gut, kidney, and brain rose by twofold or less (equal to an increase of ~10 μmol/g wet wt in each organ). Heart, however, showed a very large increase in tissue lactate from 556 to 1,724 nmol/mg protein, equivalent to an increase of 25 μmol/g wet wt.

Table 4 shows the levels of glucose and lactate in the blood of these frogs over the course of dehydration (expressed as μmol/g blood). Blood levels of both metabolites rose with progressive dehydration, with amounts at 60% dehydrated 5.7-fold higher for blood glucose and 8.5-fold higher for lactate than control values. After 24 h of rehydration, frogs had fully cleared the lactate load, whereas glucose levels were reduced but still higher than control values.

Dehydration and organ metabolic responses by autumn frogs. Because the freeze tolerance of wood frogs is much greater in the autumn and winter than in the spring, and because freezing places a dehydration stress on cells and organs, we reasoned that the dehydration tolerance and/or the metabolic responses to dehydration by wood frogs might also vary seasonally. Therefore dehydration experiments were also performed using autumn-collected frogs comparing five groups: controls, frogs dehydrated to 25 and 50% loss of total body water, and 50% dehydrated frogs that were then rehydrated either partially (to the 25% dehydrated level) or fully. Figure 4 shows the water and protein of organs over the course of experimental dehydration and rehydration. Although the measured whole body water losses were 28.5 ± 0.1 and 49.1 ± 2.3% of total water, respectively, for the two dehydrated groups, organ water loss was much smaller. Indeed, water contents of three organs (liver, heart, and kidney), expressed as grams water per gram dry weight, were unchanged during dehydration and rehydration. Water contents of other organs fell progressively with increasing dehydration, but water was regained (with a significant overshoot in brain) during the rehydration period (Fig. 4*B*).

Figure 4*A* shows the effect of whole animal dehydration on the protein contents of the six organs. Brain, gut, and heart showed no significant change in protein content expressed as milligrams per gram dry weight over the experimental course. Muscle and kidney showed a trend for higher protein levels in the dehydrated tissue that declined again during rehydration. Protein in liver showed a significant decrease over control values (750 ± 74 mg/g dry wt) only in the fully rehydrated animals (485 ± 70 mg/g dry wt).

The effects of dehydration and rehydration on glucose and lactate levels in organs of autumn *R. sylvatica* are shown in Fig. 5. Control levels of glucose in organs ranged from 3 to 35 nmol/mg protein. Dehydration stimulated a strong increase in glucose content of all organs that resulted in maximal levels 9- to 313-fold higher than the corresponding control values. Glucose levels rose progressively with increasing dehydration in all organs and remained high in the partially rehydrated frogs. Except in muscle and kidney, however, organ glucose levels were strongly reduced in the fully rehydrated frogs. Maximal amounts of glucose in gut, muscle, and kidney ranged from 165 to 404 nmol/mg; these corresponded to values of

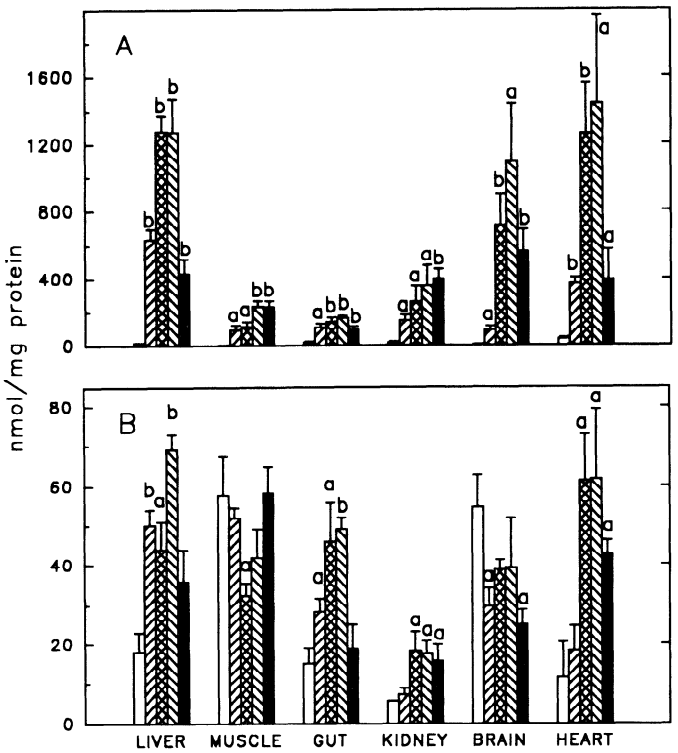

Fig. 5. Effect of changes in total body water content on glucose (*A*) and lactate (*B*) contents of 6 organs of autumn *R. sylvatica*. Data are means ± SE; $n = 4$. Bars are as follows: □, control 0% dehydrated; ▨, 25% dehydrated; ▩, 50% dehydrated; ▧, rehydrated to 25% (after dehydration to 50% water lost); ■, rehydrated to 0%. Significantly different from corresponding control value: [a] $P < 0.05$; [b] $P < 0.005$.

16–35 μmol/g wet wt. Glucose accumulation in brain, heart, and liver was much higher, peaking at 1,092, 1,409, and 1,263 nmol/mg protein, respectively; these values corresponded to 72, 43, and 127 μmol/g wet wt, respectively. Lactate levels also rose significantly during dehydration in four organs, with peak values for the 50% dehydrated or the partial rehydration conditions (Fig. 5*B*). In most cases lactate content, like glucose, was reduced in the fully rehydrated frogs. Lactate accumulation was greatest in heart and liver, with net increases of 50.5 and 51 nmol/mg protein, respectively. Oppositely, skeletal muscle and brain showed decreases in lactate content during animal dehydration; these reversed themselves in muscle after rehydration. Maximal lactate levels in muscle, gut, and liver corresponded to lactate concentrations of 5.2–5.6 μmol/g wet wt.

Dehydration effects on the liver glycogen pool were also quantified because this pool is the source of cryoprotectant glucose production in freeze-tolerant frogs. Glycogen stores in liver of autumn control frogs were 18 ± 0.1 μmol glucosyl units/mg protein, equivalent to 1,216 ± 39 μmol/g wet wt (Table 5). Dehydration to 50% loss of body water resulted in a significant decrease in liver glycogen to 15.5 μmol/mg protein, and glycogen levels were even lower in the partially rehydrated frogs. However, fully rehydrated animals had resynthesized the liver glycogen pool.

Table 5. *Glycogen content of liver of autumn R. sylvatica during whole animal dehydration and rehydration*

	Glycogen, μmol/mg protein
0% dehydrated	18.0±0.1
25% dehydrated	32.7±0.1
50% dehydrated	15.5±1.0*
Rehydrated to 25%	11.8±1.3*
Rehydrated to 0%	20.7±0.3*

Values are means ± SE; $n = 4$ frogs/group. * Significantly different from corresponding value for 0% dehydrated frogs (2-tailed Dunnett's test, $P < 0.05$).

DISCUSSION

Amphibians are highly susceptible to cutaneous water loss and display numerous adaptations for dealing with wide variations in the osmolality of body fluids and for regulating body water content (4, 20). Such adaptations are particularly well developed among estivating, arboreal, or euryhaline species. In many ways freezing is another variant of water stress but with cell water lost, instead of to the outside environment, to extracellular and extraorgan ice. Quite probably, therefore, some of the amphibian adaptations for freeze tolerance may have evolved out of preexisting mechanisms for dealing with variations in body water content.

Our initial interest was to examine water relations in frozen frogs. In nature, frogs might have to endure several weeks of continuous freezing, and during this time frozen frogs would predictably have no way to actively change the water balance of their bodies. We wondered, then, whether frozen animals would show any differences in the rates of cutaneous water loss compared with unfrozen frogs and whether insulation provided by the environment would be a factor in freezing survival. The results shown in Fig. 1 indicate, as we predicted, that frozen frogs cannot take up water from their frozen environment. Hence the relative humidity and insulation of their surroundings would be key factors in determining survival in the frozen state. When frogs were frozen under a thick insulating layer of damp moss, water loss was minor: only 2.5% of total body water was lost after nearly 7 days (0.016%/h). However, without this insulation frozen frogs lost water at a 20-fold higher rate, with an average loss of 0.32%/h or 7.7%/day. Unfrozen frogs in dry containers at 1°C showed the same rate of water loss, and this value is within the range established for other anuran species (9). Thus there appears to be no specific adaptation aimed at retarding cutaneous water loss in frozen animals. Of note, however, is the crouched posture that is always adopted by freeze-tolerant frogs when they freeze: limbs are pulled in tight to the body, digits are tucked under the body, and head is lowered to the ground (for photo, see Ref. 26). This is the water-conserving posture that is displayed by all anurans when under water stress (20); indeed, the unfrozen wood frogs in the 1°C dry environment also displayed this posture. This posture helps to cut down evaporative water loss, especially for animals in moving air (20), and for the frozen frog it would help to minimize the rate of water loss whatever the microenvironment of the hibernation site.

However, the most critical factor for long-term survival while frozen is undoubtedly the protection offered by the

hibernation site. Our study found that *R. sylvatica* could endure the loss of 50–60% of total body water or 40–48% of total body mass. This percentage is within the vital limit range established for numerous terrestrial and semiterrestrial anurans (20, 29). Wood frogs could, therefore, survive for ~7–8 days in a dry hibernaculum and even longer with a large reserve of bladder water. However, because hibernation can last for 6 mo, it is clear that frogs could not survive the winter if the hibernation site dried out. Overall, then, these results stress the importance of microhabitat selection for winter survival; a moist and protected hibernation site is undoubtedly crucial for overwintering success.

Body water content had a very pronounced effect on the cooling and freezing characteristics of the frogs. Both SCP and T_r decreased significantly as percent dehydration increased. This is not unexpected, considering that a decrease in body water content means an automatic increase in the osmolality of remaining body fluids. Furthermore, supercooling may be further promoted in dehydrated frogs by a lack of moisture on the outer body surface of the animal that would greatly reduce the possibility of nucleation occurring as the result of inoculation by surface ice. Prior dehydration also resulted in a reduced amount of body ice accumulated after 24 h freezing at −2°C (Table 2), a result that was again expected based on the freezing properties of solutions (6). Interestingly, SCP, T_r, and percent ice values were lower in almost all instances in autumn-collected frogs than in corresponding spring frogs. This could not be attributed to a difference in the initial body water content, which averaged 80.8% of body weight for controls of both groups. Thus there may be seasonal adjustments in one or more factors that affect the cooling properties of frogs just as the much higher levels of cryoprotectants synthesized by autumn compared with spring frogs are undoubtedly the key factor in the lower ice contents exhibited by autumn animals.

Freezing stimulated an increase in the levels of both glucose and lactate in blood of control and dehydrated frogs in both autumn and spring animals. Freezing-stimulated glycogenolysis in liver with glucose efflux and delivery as a cryoprotectant to all other organs has been extensively studied in *R. sylvatica* (21, 22, 25). What is intriguing about the results in Table 3, however, is that levels of both metabolites varied as a function of the extent of animal dehydration; glucose levels were 7.2-fold and lactate levels 4.6-fold higher in the blood of 55% dehydrated autumn frogs than in animals not dehydrated before freezing exposure. The same trend, although less pronounced, was seen in the spring frog data. It appears, therefore, that freezing and dehydration have synergistic effects in stimulating cryoprotectant synthesis. This result raised the very important question of whether cryoprotectant biosynthesis could be stimulated by dehydration alone. Could the trigger for cryoprotectant production be not freezing per se but perceived cell volume changes resulting from extracellular ice formation?

To analyze the metabolic effects of dehydration itself on wood frogs, we subjected groups of both spring and autumn frogs to controlled dehydration over silica gel desiccant at 5°C. Spring frogs responded to dehydration with pronounced increases in glucose levels in blood and four organs; blood glucose was 5.7-fold higher and organ glucose levels were 2.6- to 7.4-fold higher in 60% dehydrated frogs than in 0% controls (Table 4 and Fig. 3), providing good evidence that glucose synthesis and output are linked to water stress and cell volume regulation. Our previous studies (25, 26), as well as the data of Table 3, have shown that the magnitude of the glucose output response to freezing is much greater in the autumn than in the spring, for reasons that include the much larger reserves of liver glycogen in autumn frogs as well as apparent changes to both enzymes and glucose transporters to favor cryoprotectant synthesis in the autumn or winter. Similarly, we found that the magnitude of the glucose output response to dehydration was also much greater in autumn animals. Controlled dehydration of autumn frogs resulted in a strong increase in the glucose content of all organs, with maximum levels that ranged from 9- to 313-fold higher than the corresponding control values. Glucose levels rose progressively with increasing degree of dehydration in all organs and remained high in the partially rehydrated frogs. Except in muscle and kidney, however, organ glucose levels were strongly reduced in the fully rehydrated frogs. The production of high levels of glucose in response to dehydration was correlated with a decrease in liver glycogen reserves (which was reversed in the fully rehydrated animals; Table 5), indicating, as is also the case during freezing, that liver glycogen is the carbohydrate reserve that supports glucose synthesis.

The data show, therefore, that this glucose accumulation response to dehydration at 5°C is virtually the same as the cryoprotectant response of wood frogs to freezing (22, 25). This suggests a strong link between amphibian desiccation tolerance and freeze tolerance and may also indicate that the cryoprotectant biosynthesis response in frogs may have originated as a metabolic response to changes in cell volume. Perhaps changes in the water content of receptor cells (most likely on the skin), whether due to freezing or dehydration, provide the primary signal that triggers liver glucose output. It is unlikely that volume changes to liver cells themselves could be the immediate trigger for glucose biosynthesis for three reasons: *1*) cryoprotectant biosynthesis in liver is activated within 5 min after freezing begins (21), a time when whole body ice content is still negligible; *2*) even when whole animal dehydration rose to 50% of total body water lost, liver water content was not affected; and *3*) freezing-induced cryoprotectant output is inhibited by injections of propranolol, a β-adrenergic antagonist (28), suggesting that liver glycogenolysis is triggered by an external hormone signal that is mediated by intracellular cyclic AMP levels.

However, cryomicroscopic analysis has shown that liver cell volumes clearly decrease during freezing as cell water is sequestered as ice in vascular (24) and extraorgan spaces. Such direct changes to liver cell volumes during freezing may enhance cryoprotectant output (recall the evidence of synergism between dehydration and freezing from Table 3) and/or be important in sustaining the extremely high levels of organ glucose (100–300 mM) found

in frozen animals. In this regard, new studies with rat liver are very intriguing. Work by Dahl et al. (3) has documented the opposing effects on liver cell volume caused by insulin (cell swelling) and glucagon (cell shrinkage) and linked the regulation of proteolysis by these hormones to their effects on cell volume. Glucagon is also well known to stimulate glycogenolysis in vertebrate liver (including *R. sylvatica*; Ref. 15), mediated via adenosine 3′,5′-cyclic monophosphate activation of glycogen phosphorylase. Another study has shown that incubation of hepatocytes under conditions that increase their volume (e.g., addition of amino acids or hyposmotic media) stimulates glycogen synthase activity, and hence glycogen storage, and that this effect is antagonized by the addition of glucagon (1). Thus changes in liver cell volumes during freezing and thawing could well be expected to facilitate cryoprotectant synthesis vs. clearance; the mechanism of this could be volume-stimulated changes in the activities of protein kinases and protein phosphatases to alter the activity states of glycogen phosphorylase vs. glycogen synthetase. In line with this, the data in Fig. 5 for organ glucose levels and Table 5 for liver glycogen show that glucose remained elevated in frogs during rehydration from the 50 to 25% water loss value but that the sugar was cleared during further rehydration. This suggests that there may be a critical cell volume associated with the activation of liver glycogen synthesis.

The present study also provides other important data about the physiological and biochemical effects of dehydration on wood frogs. Figure 4 shows that organ water content is highly defended when the whole frog is subjected to desiccation. Thus, although total body water dropped by 25 or 50%, organs experienced low or in some cases (liver, heart, and kidney) no significant water loss. The greatest change in organ water content was found in leg skeletal muscle, a result not unexpected for a peripheral tissue; indeed, when organ water content was calculated as the percentage of total organ weight, this amounted to a decrease from 82 ± 0.9% water in skeletal muscle of control frogs to 71.4 ± 3.5% water in 50% dehydrated animals. However, the water content of internal organs, particularly those that would be crucial for dehydration survival and recovery (the heart for circulation, the kidney for ion balance) was closely controlled as the animal dehydrated. Preferential maintenance of organ water contents during anuran dehydration has been described previously (9, 18). Because organ water content is defended, the bulk of the water loss during dehydration must have come from extraorgan and plasma fluid spaces (bladders were evacuated before experiments began). This was clearly evident upon dissection of dehydrated *R. sylvatica* because the liquid that is normally present in the abdominal cavity was clearly gone and blood was extremely viscous. However, upon placement of dehydrated animals in containers of water, the original body weight could be regained within ~24 h, including a complete restoration of organ water content in most cases (Figs. 2 and 4).

In general, dehydration had no effect on organ protein contents, but some changes were noted. Kidney and skeletal muscle both showed elevated protein contents in the 50% dehydrated frogs, perhaps suggesting some specific protein synthesis by these organs in response to dehydration; however, this was reversed upon rehydration. Liver in spring frogs showed no variation in protein content over the course of dehydration and recovery (Fig. 2), but the protein content of fully rehydrated liver from autumn frogs was significantly lower than the original control value. It can also be noted that the data in Figs. 2 and 4 show distinct seasonal differences in the protein and water contents of liver. Seasonal cycles in the reserves of protein, glycogen, and lipid are well known in anurans as preparations for hibernation and reproduction, with the liver and fat body being the primary storage sites of these reserves (see Ref. 16 for summary). The present data show that liver protein content is two- to threefold higher in autumn compared with spring and that glycogen content is up to 10-fold higher in liver of autumn wood frogs (25, 27). Both glycogen and protein are highly hydrated molecules, and because of this, major seasonal changes in the glycogen and protein composition of frog liver would, of necessity, result in accompanying changes to the water content of liver cells.

Finally, dehydration resulted in substantial increases in lactate levels in several organs, most prominently heart (Figs. 3 and 5). Blood lactate levels in spring frogs first showed a significant elevation over control values when dehydration reached 35%, and lactate increased further at 60% dehydration (Table 3); similarly, heart lactate was low at 25% dehydration but increased by threefold at 50% dehydration (Fig. 5). Lactate levels similarly remain low in other species during dehydration up to 20–30% of body mass lost, but when dehydration rises to near the critical activity point, lactate levels increase sharply (7, 8). Lactate accumulation by *R. sylvatica* heart probably reflects the increased work load placed on the heart by the increase in viscosity and decrease in blood volume that occur during dehydration; to support this work load, ATP production via anaerobic glycolysis is activated. Indeed, cardiovascular collapse due to hyperviscosity and hypovolemia is cited as one of the principal causes of dehydrational death in amphibians (20). Furthermore, for these same reasons, severe dehydrational stress (near the critical activity point) compromises circulatory oxygen delivery to other organs and is accompanied by a decline in maximal rates of whole animal oxygen consumption and a rise in whole animal lactate levels (8, 10); hence, lactate accumulation by other organs is explainable as a consequence of dehydration-induced hypoxic stress.

In summary, then, we suggest that freeze tolerance and dehydration tolerance are linked in the wood frog and show that key metabolic responses to freezing (e.g., glucose output as a cryoprotectant) are also elicited as a response to dehydration. We propose that various elements of natural freeze tolerance in amphibians may have grown out of preexisting capacities for enduring wide variation in body water content and osmolality and that cell volume changes may be important signals involved in triggering the expression of various freeze-tolerance adaptations including cryoprotectant synthesis. Indeed, it is becoming clear from various recent studies (summarized in Ref. 30) that changes in cell volume can have many

important metabolic effects on cells and that the actions of various extracellular stimuli may be exerted in whole or in part by their actions in altering cell volumes.

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