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DYNAMICS OF BODY WATER DURING FREEZING AND THAWING IN A FREEZE-TOLERANT FROG (*RANA SYLVATICA*)

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Abstract—1. Calorimetric analyses showed that wood frogs (*Rana sylvatica*) frozen to −2.5°C contained 7.8 g ice, as 65.4% of the body water had frozen.

2. During 24 h of freezing, water content decreased in liver (58.9%), intestine (58.6%) and skeletal muscle (22–36%). Complete rehydration during thawing at 3.5°C required from 3 to >48 h, depending on the organ.

3. Because organs dehydrate, increases in tissue metabolite concentrations associated with freezing, if calculated on a per wet-weight basis, may be greatly exaggerated.

4. Reversible organ dehydration during freezing may enhance freeze tolerance of *R. sylvatica* by concentrating cryoprotectant and reducing cryoinjury to tissues.

Key Word Index: Cryobiology; freeze tolerance; frogs; organ dehydration; ice content; *Rana sylvatica*

INTRODUCTION

Among the vertebrates only a few species of amphibians and reptiles are known to survive extensive ice formation within their body fluids (Schmid, 1982; Storey, 1990; Costanzo and Claussen, 1990; Costanzo *et al.*, 1992a). One of these, the wood frog (*Rana sylvatica*), survives the freezing of more than 60% of its body water (Layne and Lee, 1987) for periods of at least 2 wk (Storey, 1990). Dissection of a frozen wood frog reveals that ice accumulates within the coelomic cavity and beneath the skin (Storey and Storey, 1984; Layne and Lee, 1987). The specific source of this water and the mechanism of its transport to these compartments have yet to be identified. In any regard, the redistribution of this water represents a problem of tissue dehydration.

Cryobiologists have long understood that cells dehydrate when animals freeze at high subzero temperatures (e.g. −5 to −15°C; Mazur, 1984). Ice forming in extracellular fluids excludes solutes from the growing ice lattice, with the resulting elevation of osmotic pressures withdraws water from cells. Excessive water loss injures cells owing to the concentration of specific solutes to toxic levels, changes in pH, or shrinkage below some critical volume (Meryman, 1971; Mazur, 1984); it also perturbs homeostatic mechanisms of organs and organ systems.

On the contrary, a partial dehydration of organs seems beneficial because it mollifies the mechanical stress from ice forming with tissues (Rubinsky and Pegg, 1988). Some work on wood frogs supports this idea. For example, *R. sylvatica* readily survives slow cooling to −2.5°C but succumbs if cooling is rapid (Costanzo *et al.*, 1991), in part because rapid cooling inhibits organ dehydration (Costanzo *et al.*, 1992b). The present study further examined the tissue dehydration response in *R. sylvatica* by establishing the magnitude and time course of organ dehydration and rehydration during freezing and thawing, respectively.

MATERIALS AND METHODS

Study specimens

Adult, male wood frogs were collected from breeding ponds in upland deciduous woodlands in Adams County, Ohio during late winter 1988 and 1991. The frogs were fasted and maintained in an environmental chamber (4.0 ± 0.5°C) for 1–5 wk prior to experimentation. Procedures detailed by Layne and Lee (1987) and Costanzo *et al.* (1991) were used to freeze the frogs. Briefly, each frog was placed in a 50-ml plastic centrifuge tube, fitted with a surface thermocouple probe, and cooled in a refrigerated bath (−2.5°C). The onset of ice formation (indicated by a recorded exotherm) was induced in supercooled frogs by lightly applying aerosol coolant to the tube's exterior.

Ice contents of frozen frogs

Ice contents of 12 frogs (14.6 ± 0.3 g) frozen to −2.5°C for 24 h were determined using an established method (cf. Layne and Lee, 1987, 1991) for whole-body calorimetry; this involves calorimetric analyses of wet mass, dry mass, and corresponding specific heats (0.999 cal/g and 0.22 cal/g, respectively). An estimate of the melting point of body fluids (−0.5°C; Layne and Lee, 1987) was used in the calculations. Body water content was determined for 9 additional frogs (mean mass ±1 SEM = 14.0 ± 0.4 g), taken from the environmental chamber (4°C) and killed by double-pithing, by drying carcasses at 65°C to constant mass. Ice content was

expressed both in terms of absolute mass and fraction of total body water frozen.

Organ water content during freezing and thawing

Water contents of organs from frogs (12.8 ± 0.4 g; $n = 31$) were sampled unfrozen (time zero) and at 9 intervals during a 24-h freeze to −2.5°C and a subsequent 48-h interval initiated at the onset of thawing at 3.5°C. Freezing or thawing frogs were removed from their tubes (whereas unfrozen frogs were taken directly from the environmental chamber), killed by double-pithing, and dissected. Portions (<200 mg) of the following organs were excised: intestine (2–3 sections), liver (2–3 lobes), and skeletal muscle. Samples (1–3) from the latter were taken from both gastrocnemius muscles and one gracilis major. Surface moisture was removed from each sample by lightly blotting and centrifuging them on filter paper for 5 min at 1000 rpm. Samples were subsequently dried to constant mass; their initial water content, determined on the basis of mass lost upon drying, was expressed as g water/g dry mass. Data for replicate samples were averaged to produce a single value for each frog.

Statistical procedures

Means (reported ± 1 SE) were compared statistically using analysis of variance (ANOVA). Fisher LSD tests were used, where appropriate, to distinguish among sample means. Analyses involving percentage data were conducted on square-root, arcsine-transformed values. Significance was judged at $P < 0.05$.

RESULTS

Ice contents of frozen frogs

Body water content in 9 unfrozen frogs averaged 11.3 ± 0.3 g, representing 80.4% of the fresh body mass. Calorimetric analysis of 12 frogs frozen to −2.5°C predicted that 7.8 ± 0.2 g ice was present, an amount equivalent to 65.4 ± 1.3% of the total body water.

Organ water content during freezing and thawing

Mean water contents of organs from unfrozen frogs served as references in calculating changes in organ water contents during freezing and thawing. These values differed significantly ($F = 22.7$, $P < 0.001$), ranging from 3.26 ± 0.03 g water/g dry mass in the liver to 4.40 ± 0.18 g/g dry mass in the intestine (Fig. 1).

Generally, the water contents of organs sampled at four intervals decreased during freezing (Fig. 1). The mean values differed statistically for the intestine ($F = 39.3$, $P < 0.001$), liver ($F = 22.4$, $P < 0.001$), and gastrocnemius ($F = 4.7$, $P < 0.021$) indicating that a progressive dehydration of these organs occurred during freezing. The trend was similar for the gracilis major, but the sample means were not statistically different ($F = 3.3$, $P < 0.059$). By 24 h of freezing, the liver and intestine had lost about 58% of their initial water, whereas decreases in the gastrocnemius and gracilis major were 36.4 and 22.8%, respectively (Fig. 1). Further inspection of Fig. 1 shows that water vacated organs at different rates during freezing. Of

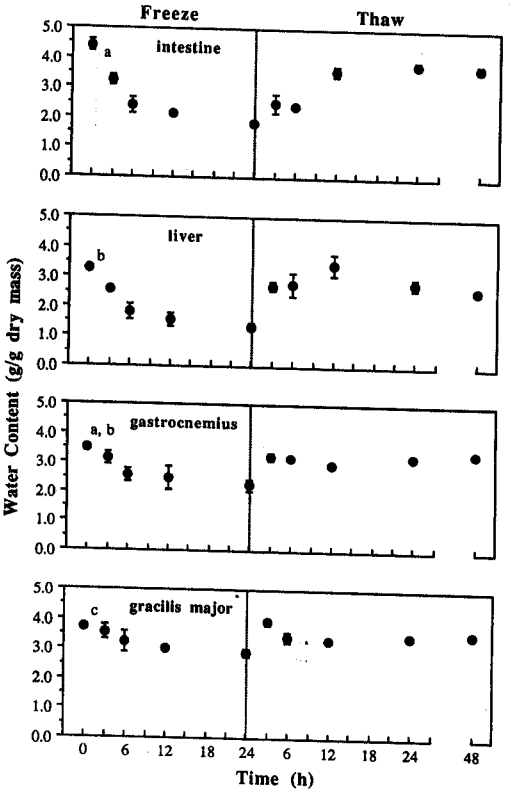

Fig. 1. Time-course of dehydration and rehydration of intestine, liver, gastrocnemius, and gracilis major from adult male *R. sylvatica* during a 24-h freeze to −2.5°C and a subsequent 48-h thaw at 3.5°C. Data points represent means ± SEM ($n = 3$–4 frogs). Means at time zero were compared using the Fisher least significant difference test; points identified by different letters were statistically distinguishable at $P < 0.05$.

the total amount of water ultimately lost during freezing, 37 and 45% had vacated liver and intestine, respectively, within the first 3 h, whereas the skeletal muscles had lost only 20–25% by this time.

Water contents of organs from thawing frogs progressively increased over time (Fig. 1). Mean values obtained at five sample intervals differed significantly for the intestine ($F = 29.0$, $P < 0.001$), liver ($F = 8.7$, $P < 0.001$), gastrocnemius ($F = 13.4$, $P < 0.001$), and gracilis major ($F = 8.4$, $P = 0.001$). Rehydration was complete (i.e. organ water content no longer differed statistically from the corresponding unfrozen value) in the gracilis major by 3 h, in the liver by 6 h, and in the gastrocnemius by 48 h of thawing. The water content in the rehydrating intestine peaked at 3.82 ± 0.11 g water/g dry mass at 24 h of thawing, but this value was still significantly ($F = 7.7$, $P = 0.050$) lower than that for unfrozen controls, 4.40 ± 0.18 g/g. Thus, this organ regained about 87% of its initial water content but did not fully rehydrate within the 48-h thaw period.

DISCUSSION

Our data for whole-body water and ice contents of Ohio *R. sylvatica* are in close agreement with those reported by Layne and Lee (1987). They indicate that

about two-thirds of the total body water forms ice in frogs frozen to −2.5°C, a conspicuous quantity being sequestered in the coelomic cavity and in spaces between the integument and musculature (Storey and Storey, 1984; Layne and Lee, 1987). Our data on the dynamics of organ water during freezing and thawing indicate the probable source of this water.

All organs examined in this study dehydrated substantially during freezing. Most of the water ultimately lost vacated organs within the first 12 h of freezing, a period corresponding with rapid ice formation and progressive decline in cardiovascular function (Layne and Lee, 1987; Layne *et al.*, 1989). By 24 h of freezing, both liver and intestine had lost on average 58% of their initial water. In contrast, dehydration was less pronounced in the skeletal muscles, probably because in these organs ice forms more rapidly (and tissue perfusion ceases sooner) owing to their peripheral location.

Water vacating organs during freezing must eventually be replaced before homeostasis is renewed. Our data show that water is rapidly regained by liver and skeletal muscle during thawing, although complete rehydration of the intestine may require >48 h. The former organs are critical for metabolic and locomotory functions, but the digestive tract is of lesser immediate importance because cold-exposed frogs are aphagic. Interestingly, our time-course for rehydration of the skeletal muscles is consistent with Layne and First's (1991) finding that muscle function returns within 24 h of thawing.

The precise mechanism effecting the redistribution of water in freezing and thawing frogs remains unknown; however, the cardiovascular system is likely involved. Remarkably cardiac function persists for many hours during freezing and resumes shortly after thawing commences (Layne *et al.*, 1989; Layne and First, 1991). This tolerance probably facilitates the dehydration–rehydration response. Accordingly, rapid cooling inhibits organ dehydration presumably because it hastens cardiovascular failure (Costanzo *et al.*, 1992b). Clearly the mechanism of water redistribution, as well as the specific origin of the tissue fluid (i.e. extracellular or intracellular), require further study.

The magnitude of the organ dehydration response suggests that measurements of tissue metabolites (routinely expressed on a wet-mass basis) reported in previous studies may seriously overestimate freezing-induced responses. For example, Storey and Storey (1984) reported a 70-fold increase in liver glucose (from 5.5 μmol/g wet mass to 387.8 μmol/g wet mass) during freezing of *R. sylvatica*. However, recalculating their data on the basis of liver dry mass (assuming 3.26 g water/g in unfrozen frogs and 1.34 g water/g in frozen frogs, as per the present study) yields glucose concentrations of 23.5 μmol/g in unfrozen frogs and 908.2 μmol/g in frozen frogs, indicating a substantially more modest increase (39-fold) during freezing. Failure to account for changes in tissue hydration state during freezing may therefore complicate such analyses.

The reversible dehydration of organs during freezing would be beneficial to *R. sylvatica* if cryoprotectant (glucose) becomes concentrated within frozen tissues and diluted upon thawing. Furthermore, tissue dehydration may substantially reduce cryoinjury. For example, Rubinsky and Pegg (1988) suggested that cryoinjury to human organs is a consequence of mechanical damage due to the growth of ice within tissues. Using low temperature scanning electron microscopy, they demonstrated that ice preferentially forms and propagates within the vascular system. Solute rejected from growing ice crystals increases the osmotic pressure of the unfrozen fluid within vessels, withdrawing water from adjacent cells. These events in turn lead to additional ice growth that excessively expands and ultimately destroys the vasculature. We believe that *R. sylvatica* (and possibly other freeze-tolerant vertebrates) avoids mechanical cryoinjury by evacuating water from organs and sequestering it as ice innocuously within subcutaneous, coelomic, and other body compartments.

It is notable that wood frog organs recover from severe dehydration during freezing. All freeze-tolerant anurans overwinter in terrestrial hibernacula (Schmid, 1982) and, not unexpectedly, these frogs have a greater desiccation tolerance than do aquatic, freeze-intolerant species (Schmid, 1965). The implication of this correlation is that physiological mechanisms conferring desiccation tolerance also enhance the capacity for freeze tolerance. The significance of this principle extends to clinical applications in which cells are dehydrated prior to being cryopreserved (e.g. Farrant, 1980). Currently is it not possible to cryopreserve mammalian organs. However, a programmed dehydration in advance of freezing may enhance their potential for cryopreservation. Recent success with frozen rat heart explants support this notion (Banker *et al.*, 1991) and provides new insight into the development of protocols for the cryopreservation of human tissues and organs.

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