Metabolic responses to dehydration by liver of the wood frog, *Rana sylvatica*

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The metabolic responses by the liver to the evaporative loss of up to 60% of total body water were quantified in spring-collected wood frogs, *Rana sylvatica*, a freeze-tolerant species. Dehydration stimulated rapid hyperglycemia, liver glucose levels rising 3.8-fold to 90 nmol/mg protein (9.9 µmol/g wet mass) by the time that 10% of total body water had been lost. Glucose accumulation occurred at the expense of liver glycogen reserves, which fell over the course of dehydration, and was supported by a 5.8-fold increase in the activity of glycogen phosphorylase α in the liver, made up of increases in both the total phosphorylase activity expressed and the percentage of the enzyme in the active form. Analysis of changes in the levels of glycolytic intermediates in the liver over the course of dehydration showed sharp increases in glucose-6-phosphate and fructose-6-phosphate during the period of active glucose synthesis but no change in the levels of fructose-1,6-bisphosphate or triose phosphates. This indicated that an inhibitory block on glycolysis at the phosphofructokinase reaction helped to promote the diversion of glycogenolysis into glucose export. When water loss exceeded 10%, cellular energetics were affected; ATP levels fell progressively between 25 and 60% dehydration, but a concomitant drop in the total adenylate pool held the energy charge stable at 0.7–0.8 up to 35% dehydration. At extreme dehydration (50 and 60%), metabolic indicators of hypoxia stress appeared in the liver: lactate accumulated and the energy charge fell. The data show that a primary response to whole-body dehydration in wood frogs is the activation of liver glucose synthesis and this suggests that the production of glucose as a cryoprotectant during freezing in this species is probably derived from a pre-existing amphibian volume-regulatory response to dehydration.


Les réactions métaboliques du foie à la suite de la perte par évaporation de près de 60% du contenu hydrique total ont été quantifiées chez des Grenouilles des bois (*Rana sylvatica*), une espèce tolérante au gel, capturées au printemps. La déshydratation provoque une hyperglycémie rapide au cours de laquelle les concentrations de glucose hépatique augmentent par un facteur de 3.8 jusqu’à 90 nmol/mg protéine (9.9 µmol/g masse fraîche) après une perte de seulement 10% du contenu hydrique total. L’accumulation de glucose se fait aux dépens des réserves de glycogène hépatique qui diminuent au cours de la déshydratation et provient aussi de l’augmentation par un facteur de 5,8 de l’activité de la glycérophosphorylase a dans le foie, augmentation tributaire des augmentations de l’activité totale exprimée de la phosphorylase et du pourcentage de l’enzyme sous forme active. L’analyse des changements dans les concentrations des intermédiaires de la glycolyse dans le foie au cours de la déshydratation a mis en lumière des augmentations importantes du glucose-6-phosphate et du fructose-6-phosphate durant la période de synthèse active du glucose, mais n’a pas démontré de changement dans les concentrations de fructose-1,6-bisphosphate ou des trioses phosphates. Il semble donc qu’un agent inhibiteur de la glycolyse au moment de la réaction de la phosphofructokinase serve à enrayer la glycéronéolysè pour favoriser l’excrétion de glucose. Lorsque la perte d’eau excède 10%, la dynamique énergétique est affectée au niveau cellulaire : les concentrations d’ATP baissent progressivement entre 25 et 60% de déshydratation, mais une baisse concomitante du pool total d’adénylate maintient la charge énergétique entre 0,7 et 0,8 jusqu’à 35% de déshydratation. À un degré de déshydratation supérieur (50 et 60%) les indicateurs métaboliques du stress hypoxique se manifestent dans le foie : le lactate s’accumule et la charge énergétique diminue. Les résultats démontrent que la principale réaction à une déshydratation de tout le corps chez la Grenouille des bois consiste en l’activation de la synthèse du glucose hépatique, ce qui semble indiquer que la production du glucose comme cryoprotecteur à l’occasion d’un gel chez cette espèce provient probablement d’une réponse amphibienne préexistante de contrôle du volume à la suite d’une déshydratation.

**Introduction**

Evaporative water loss is a major concern for all amphibians on land and most are subject to high rates of dehydration. Numerous strategies (behavioural, physiological, and metabolic) have evolved to help reduce water loss, particularly in arboreal or fossorial species. Furthermore, the dehydration tolerance of different species has been shown to correlate positively with their degree of terrestriality (Shoemaker 1992). Previous studies have indicated that ranid frogs generally tolerate the loss of 30–37% of initial body mass or 38–50% of total body water (Thorson and Sviha 1943; Hillman 1980). In a recent study we found that the wood frog, *Rana sylvatica*, a freeze-tolerant species (Schmid 1982), also exhibited excellent dehydration tolerance; autumn-collected animals readily tolerated the loss of 50% of total body water, whereas spring-collected frogs tolerated 60% dehydration in experiments conducted at 5°C (Churchill and Storey 1993).

One of the responses to dehydration by *R. sylvatica* was an activation of liver glycogenolysis leading to a rapid elevation of glucose in the liver, blood, and other organs (Churchill and Storey 1993). This response to dehydration at 5°C was virtually identical with the well-described accumulation of glucose as a cryoprotectant in response to freezing in this species (Storey and Storey 1984, 1992), elevated glucose levels serving to lessen the extent of cellular and organ water loss during freezing (Storey et al. 1992; Costanzo et al. 1993). Indeed, dehydration and freezing both have the effect

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of reducing cell volume, the one being caused by a net loss of water from the body whereas the other is caused by the sequestration of up to 65% of total body water as extracellular and extra-organ ice (Lee et al. 1992; Churchill and Storey 1993; Storey and Storey 1993). We proposed, therefore, that the cryoprotectant response to freezing in selected anurans evolved out of a pre-existing hyperglycemic response by amphibians to dehydration. The results of new studies of the effects of dehydration in the semiaquatic leopard frog Rana pipiens support this proposal, for this freeze-intolerant frog also shows a rapid hyperglycemia, based on liver glycogenolysis, in response to dehydration (Churchill and Storey 1994).

However, the magnitude of the response is much lower in R. pipiens than in R. sylvatica; maximum glucose levels in the liver of autumn-collected frogs, dehydrated until 50% of total body water was lost, were only 20 μmol/g wet mass in the leopard frog versus 127 μmol/g wet mass in the wood frog (Churchill and Storey 1993, 1994).

The present study further examines the metabolic responses of R. sylvatica liver to dehydration. In particular we analyze the regulatory control over liver glycogenolysis that is responsible for dehydration-induced glucose export and the effects of dehydration on organ energetics.

Materials and methods

Chemicals and animals

All biochemicals were purchased from Boehringer Mannheim Corp., Montréal, Quebec, or Sigma Chemical Co., St. Louis, Missouri. Male wood frogs were collected from breeding ponds in the Ottawa region in early to mid-April 1990. Animals were held at 5°C in a moist environment without food for at least 1 week before use. Immediately before they were assigned to different treatment groups, all frogs were induced to void their bladder by placing gentle pressure on the abdomen.

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 (each weighing 5–8 g) were then placed on top of the sponge in each desiccator and the desiccator was placed in the 5°C incubator. The ratio of desiccant mass to frog mass was chosen to give a rate of loss of 0.5–1% of total body water per hour. Frogs were briefly removed from the incubator approximately every 12 h (to minimize handling) and weighed to determine water loss; this interval was reduced to 2 h when the animals’ mass had fallen to within 5% of the targeted value for that sample group. The percent change in body water content was calculated as

\[ \% \text{ change} = \left( \frac{(M_f - M_i)/(M_i \times BW_{Ci})}{} \right) \times 100 \]

where \( M_i \) is initial body mass, \( M_f \) is mass during experimental dehydration, and \( BW_{Ci} \) is the initial body water content of frogs prior to dehydration in grams of H_2O per gram of body mass. The initial body water content of wood frogs was 80.8 ± 1.2%, calculated from the initial and final masses of control frogs (bladder voided) that were killed and then dried to a constant mass for 72 h at 80°C.

Since previous tests had shown that spring-collected R. sylvatica survived the loss of 60% of total body water (Churchill and Storey 1993), the experimental dehydration course was set up to sample groups of animals at 10, 25, 35, 50, or 60% of initial body water had been lost; the actual measured values for each of these experimental groups was 12.0 ± 0.2, 25.9 ± 1.1, 37.9 ± 0.8, 51.9 ± 1.5, and 61.1 ± 1.3% of total body water lost. A sixth group was first dehydrated until 63.8 ± 0.4% of total body water was lost and then allowed to regain water over 24 h to a final mean of 4.6 ± 0.4% less than initial control values. Figure 1 shows the effect of progressive whole-animal dehydration on the levels of glucose, glycogen, and lactate in the liver of these frogs. Because dehydration causes some loss of organ water, metabolite concentrations are expressed per milligram of tissue protein, since protein content (mg/g dry mass) remained stable with dehydration (Churchill and Storey 1993). Water loss stimulated a rapid increase in liver glucose content. Levels had risen 3.8-fold, from 24 to 90 nmol/mg protein, by the time that frogs had lost 10% of their body water; these values were equivalent to 2.6 and 9.9 μmol/g wet mass. Glucose levels remained elevated throughout the remainder of the dehydration course and high levels still persisted in the liver after animals had been rehydrated for 24 h. Liver glycogen stores were 2000 nmol/mg protein (in glucose units) in control frogs, a value equivalent to 160 μmol/g wet mass (Fig. 1B). Glycogen was depleted as dehydration progressed, declining to a low of 250 nmol/mg when frogs were 50% dehydrated. Lactate levels remained stable over the course of dehydration until 50% of total body water had been lost. At 60% dehydration, however, lactate had risen 1.6-fold to 75 nmol/mg, equivalent to 8.1 μmol/g wet mass.

Metabolite measurements

Perchloric acid extracts of liver were prepared as described by Storey and Storey (1984), with an aliquot of each homogenate removed before centrifugation for glycogen determination by the method of Keppler and Decker (1974). Portions of the neutralized extract were used immediately for the measurement of pyruvate, phosphoenolpyruvate (PEP), creatine phosphate, ATP, ADP, and AMP levels, and the remainder was frozen at –80°C for subsequent assays of other metabolites. Metabolites were quantified spectrofluorometrically by the coupled enzyme assays of Lowry and Passonneau (1972), except for fructose-2,6-bisphosphate (F2,6BP), which was extracted and assayed as described by van Schaftingen (1984).

Glycogen phosphorylase

Liver extracts were prepared as described by Storey and Storey (1984) and samples of the well-mixed homogenate (without centrifugation) were used for enzyme assay. Assay conditions for phosphorylase a and total phosphorylase activities were as described by Storey and Storey (1984), with the use of 4 mg/mL glycogen and the absence versus presence of 1.6 mM AMP, respectively.

Protein determinations

Total protein in the perchloric acid-precipitated pellet was dissolved in NaOH and then quantified by the Coomassie Blue dye-binding method using the Bio-Rad prepared reagent and a standard of bovine gamma globulin.

Data analysis

All data are expressed per milligram of liver protein and reported as means ± SE, n = 4. Statistical analysis was performed using one-way analysis of variance followed by Dunnett’s test (two-tailed).

Results

Spring-collected wood frogs exposed to experimental dehydration were sampled when approximately 10, 25, 35, 50, or 60% of initial body water had been lost; the actual measured values for each of these experimental groups was 12.0 ± 0.2, 25.9 ± 1.1, 37.9 ± 0.8, 51.9 ± 1.5, and 61.1 ± 1.3% of total body water lost. A sixth group was first dehydrated until 63.8 ± 0.4% of total body water was lost and then allowed to regain water over 24 h to a final mean of 4.6 ± 0.4% less than initial control values. Figure 1 shows the effect of progressive whole-animal dehydration on the levels of glucose, glycogen, and lactate in the liver of these frogs. Because dehydration causes some loss of organ water, metabolite concentrations are expressed per milligram of tissue protein, since protein content (mg/g dry mass) remained stable with dehydration (Churchill and Storey 1993). Water loss stimulated a rapid increase in liver glucose content. Levels had risen 3.8-fold, from 24 to 90 nmol/mg protein, by the time that frogs had lost 10% of their body water; these values were equivalent to 2.6 and 9.9 μmol/g wet mass. Glucose levels remained elevated throughout the remainder of the dehydration course and high levels still persisted in the liver after animals had been rehydrated for 24 h. Liver glycogen stores were 2000 nmol/mg protein (in glucose units) in control frogs, a value equivalent to 160 μmol/g wet mass (Fig. 1B). Glycogen was depleted as dehydration progressed, declining to a low of 250 nmol/mg when frogs were 50% dehydrated. Lactate levels remained stable over the course of dehydration until 50% of total body water had been lost. At 60% dehydration, however, lactate had risen 1.6-fold to 75 nmol/mg, equivalent to 8.1 μmol/g wet mass.
The effects of dehydration on the levels of glycolytic intermediates in the liver are shown in Fig. 2. Glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) concentrations were increased sharply by 10% dehydration, reaching 9.9 and 1.95 nmol/mg protein, respectively. G6P levels remained stable up to 50% dehydration and then decreased sharply at 60% dehydration. However, F6P levels fell continuously during dehydration. In contrast to the changes in F6P, levels of fructose-1,6-bisphosphate (F1,6P) remained constant throughout most of the dehydration course but increased 2-fold at 60% dehydration. The patterns of changes in the substrate (F6P) and product (F1,6P) of phosphofructokinase (PFK) were consistent with inhibition of the enzyme as an immediate response to dehydration (F6P increased and F1,6P remained constant) followed by activation of the enzyme when dehydration became extreme (at 60% dehydration F6P decreased and F1,6P increased). After rehydration, levels of all three hexose phosphates in liver were very low. Figure 2C shows that the levels of the triose phosphates, dihydroxyacetone phosphate + glyceraldehyde-3-phosphate, remained constant over the entire dehydration time course. However, PEP and pyruvate showed distinct patterns of change during dehydration. At 10% dehydration, levels of both had increased significantly, 2.0- and 3.4-fold, respectively (Fig. 2D). Pyruvate remained elevated over the remainder of the dehydration time course, but the PEP concentration dropped in 50 and 60% dehydrated frogs. Upon rehydration PEP increased again whereas pyruvate decreased.

The effect of dehydration on the activity of glycogen phosphorylase and the levels of the key PFK activator, F2,6P2, are shown in Table 1 for liver from control and 25 and 50% dehydrated frogs. Dehydration resulted in a significant increase in the percentage of glycogen phosphorylase in the active a form as well as an increase in the total units of enzyme activity (U) in liver; at 50% dehydration, both of these parameters had increased about 2.5-fold. In combination, these two effects gave activities of the a form of 0.037, 0.124, and 0.215 U/mg, respectively, a net 3.4-fold increase at 25% dehydration and a 5.8-fold increase at 50% dehydration. Expressed in terms of tissue wet mass, these values correspond to liver phosphorylase a activities of 3.1 U/g wet mass for controls and 20.0 U/g wet mass at 50% dehydration. The liver content of F2,6P2 also changed in response to dehydration. Content of the bisphosphate in liver of 25% dehydrated frogs was highly variable but F2,6P2 decreased significantly in the 50% dehydrated frogs.

The effects of dehydration on adenylates and energetics in the wood frog liver are shown in Fig. 3. ATP and total adenylate contents had begun to fall by 25% dehydration and continued to decline with subsequent dehydration; mean ATP content in the liver of 60% dehydrated frogs was only 4.7 nmol/mg compared with the control value of 28.1 nmol/mg. Concentrations of ADP and AMP were largely unchanged over the course of dehydration. The energy charge remained high and constant up to 35% dehydration (control values were 0.81 ± 0.02) (Fig. 3C) but then began to fall, decreasing to a low of 0.47 ± 0.07 at 60% dehydration. The liver contained very low levels of creatine phosphate, as is typical of this organ; amounts ranged from 2 to 6 nmol/mg and showed no obvious pattern of change during dehydration (data not shown). After 24 h rehydration, ATP levels had increased significantly, rising to about 50% of control values, but the energy charge had returned to near control values at 0.73 ± 0.01.

Discussion

Whole-organ dehydration rapidly stimulates metabolic responses by the liver of Rana sylvatica. Glycogenolysis was rapidly activated, as evidenced by manyfold increases in liver G6P and glucose in frogs that had lost only 10% of total body water (Figs. 1, 2). This activation occurred without disruption of organ energetics (Fig. 3) and without a significant change in liver water content; thus, for spring-collected frogs at 60%
dehydration, liver water content was $1.32 \pm 0.19$ g water/g dry mass, not significantly different from the control value of $1.64 \pm 0.06$ g water/g dry mass (Churchill and Storey 1993). The stimulus for the activation of glycogenolysis in liver cannot, therefore, be a direct change in liver cell volume due to dehydration but is probably mediated by hormonal action. Indeed, the comparable activation of glucose output from the \textit{R. sylvatica} liver as a response to freezing is antagonized by the administration of propranolol, which suggests that cryoprotectant synthesis is under control by hormones that stimulate \beta-adrenergic receptors (Storey and Storey 1993). Thus, dehydration may be detected at some peripheral site, possibly as a cell-volume change in skin cells, and this may in turn send a signal that triggers liver glycogenolysis.

The hyperglycemic response to dehydration by these spring-collected frogs resulted in a 3.8-fold increase in liver glucose content to a maximum of 90 nmol/mg protein or 9.9 \mu mol/g wet mass. Blood glucose also rose to 10.5 \mu mol/g wet mass and glucose was delivered to other organs, the glucose content increasing 2.6- to 7.4-fold in the kidney, heart, and brain (Churchill and Storey 1993). Similarly, the dehydration-stimulated glucose accumulation in leopard frog (\textit{R. pipiens}) organs reached a maximum of only 20 \mu mol/g wet mass in the liver, with lower values in other organs (Churchill and Storey 1993). In both of these cases, the net accumulation of glucose in organs was not of a magnitude great enough to substantially contribute to the colligative regulation of cell volume during dehydration. However, another potential use for dehydration-induced hyperglycemia can be proposed: to provide fermentative fuel to organs when dehydration begins to compromise aerobic metabolism. As anurans lose water, plasma volume decreases and blood viscosity increases; both factors increase stress on the heart and reduce the perfusion of peripheral organs. Indeed, cardiovascular collapse is a primary cause of dehydrational death in amphibians (Shoemaker 1992). As dehydration reaches high values, a reduction in whole-animal oxygen consumption and an accumulation of lactate in organs is seen, indicating hypoxia stress due to impaired perfusion (Hillman 1978, 1987). Elevated liver lactate levels were seen in the present study when dehydration reached 60% and have also been reported as the result of dehydration stress in other species (Hillman 1978, 1987; Churchill and Storey 1993). By elevating glucose levels as an early response to dehydration, organs may be prepared to deal with extended periods of hypoxia if desiccation continues unabated. Furthermore, it is apparent from Fig. 1B that the consumption of liver glycogen is considerably greater than the corresponding rise in glucose in the liver (Fig. 1A) or other organs (Churchill and Storey 1993).
Regulatory controls on the process of dehydration-induced liver glycogenolysis can be deduced from the present data. Glycogenolysis is promoted by activation of glycogen phosphorylase activity resulting from increases in both the total enzyme activity expressed (U/mg) and the percentage of enzyme activity in the active a form (Table 1). This resulted in a net 5.8-fold increase in phosphorylase a activity in liver of 50% dehydrated frogs. Changes in both the total activity of liver phosphorylase and the percentage in the a form are similarly stimulated by freezing in R. sylvatica (Storey and Storey 1992). The activation of phosphorylase had undoubtedly occurred well before dehydration reached 10% of total body water, for both G6P and glucose levels in the liver were well elevated by that time. Activation of liver glycogenolysis for the purpose of glucose export requires a concomitant inhibition of endogenous routes of G6P catabolism in the liver in order to promote export. The effects of dehydration on the levels of liver glycolytic intermediates clearly indicate that inhibitory control on glycolysis occurs at the PFK reaction. Thus, whereas levels of the PFK substrate (F6P) rose in parallel with the increase in G6P, concentrations of the PFK product, F1,6P2, did not change (Fig. 2) and this indicated that glycolytic flux was regulated at this locus. The lack of change in the triose phosphates (whose concentrations are equilibrated with that of F1,6P2 via the enzyme aldolase) further support this. When dehydration reached 60%, however, regulation at the PFK locus changed; F6P (and G6P) levels decreased and the F1,6P2 level increased, indicating activation of flux through PFK at this time. This corresponds to the elevation of liver lactate levels also seen at this time and is consistent with a dehydration-induced hypoxic stress requiring the activation of fermentative energy production in the liver. Changes in the potent allosteric activator of PFK, F2,6P2, were also consistent with hypoxia stress affecting the glycolytic rate at 60% dehydration. Hypoxia/anoxia typically suppresses F2,6P2, a signal promoting the anabolic use of carbohydrate, in the liver, leaving PFK responsive to regulation by organ energetics (Hue and Rider 1987).

Although changes in glycolytic intermediates and lactate suggested that dehydration-induced hypoxia stress occurred only when dehydration became severe, the data for liver adenylates indicated that energy status was perturbed by even low levels of dehydration. ATP levels were significantly depressed at 25% dehydration, but because of a concomitant decrease in total adenylates in the liver, the energy charge was maintained at a constant level through the intermediate levels of dehydration, and decreased only after 50% of total body water was lost (Fig. 3). The fall in total adenylates implicates the action of AMP-deaminase, which converts AMP to IMP; removal of AMP from the adenylate pool helps to maintain a high energy charge for as long as possible and promotes the adenylate kinase (2 ADP $\rightarrow$ ATP + AMP) reaction. ATP levels in the R. sylvatica liver also fell during the early hours of freezing exposure (although again the energy charge was maintained), during the time when glucose export from the liver is high, but subsequently stabilized during long-term freezing (Storey and Storey 1986). Perhaps reducing the total adenylate pool, but maintaining the energy charge at a constant level, is a mechanism associated with metabolic arrest, a lowering of the overall metabolic potential to extend long-term survival under stress conditions.

In summary, then, the data show that evaporative water loss rapidly initiates a hyperglycemic response by the R. sylvatica liver and that this response is regulated by activation of glycogen phosphorylase coupled with an inhibitory block on glycolysis at the PFK locus. Although liver ATP levels fall at intermediate levels of dehydration, metabolic conditions indicative of hypoxia stress due to dehydration (decreased energy charge, lactate accumulation) do not appear until dehydration is extreme and nearing the lethal threshold. Coupled with recent similar studies of dehydration effects on the metabolism of a freeze-intolerant frog (R. pipiens)
on autumn-collected *R. sylvatica* that are primed for freezing-induced cryoprotectant synthesis (Churchill and Storey 1993, 1994), the results of the present study further support the proposal that the cryoprotectant response of freeze-tolerant frogs probably grew out of a more primitive hyperglycemic response by anurans to dehydration.

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