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**METABOLIC BIOCHEMISTRY OF FREEZE TOLERANCE IN
VERTEBRATES**

by

THOMAS ALLEN CHURCHILL

B.Sc. (Hons.) Carleton University, 1988

A thesis submitted to the Faculty of
Graduate Studies and Research in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
in the department
of BIOLOGY

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submitted by

Thomas Allen Churchill, B.Sc.

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for the degree of Doctor of Philosophy

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ABSTRACT

A unique group of amphibians and reptiles has developed complex adaptations that enable them to survive freezing. This thesis investigates: i) cryoprotectant synthesis in freeze tolerant frogs, ii) freeze tolerance in a newly identified freeze tolerant vertebrate, the garter snake, iii) metabolic responses elicited by other stresses (anoxia and dehydration) in the garter snake and two frog species.

Investigation of cryoprotectant synthesis in spring frogs, Pseudacris crucifer, revealed large amounts of glucose produced during freezing; approximately 0.1 M. Changes in the levels of glycolytic intermediates indicated that an activation of glycogen phosphorylase and phosphofructokinase (PFK) inhibition directed glycolytic flux to cryoprotectant synthesis. Tissue glucose distribution was much lower than in fall animals. These results suggested seasonal variation in glucose transport mechanisms.

A similar investigation of cryoprotectant synthesis in spring, Hyla versicolor showed a maintenance of regulatory enzyme controls at glycogen phosphorylase and PFK directing glycogen carbon to cryoprotectant. Only glucose was synthesized as a cryoprotectant; quantities of glycerol (the major cryoprotectant of winter H. versicolor) showed no increase. The amount of cryoprotectant produced was directly correlated to glycogen content in the liver.

Investigation of freeze tolerance in garter snakes revealed that these snakes were only partially freeze tolerant. Survival of brief freezing exposures (5-10 h at -2.5°C ; 30-50 % ice) was possible. Two amino acids, glutamate and taurine, were implicated as possible cryoprotective agents. Comparison of the metabolic responses (adenylate levels, anaerobic endproducts, glycolytic flux) to freezing in garter snakes were similar to those elicited by anoxia.

Dehydration timecourses were investigated in two freeze tolerant frog species, Rana sylvatica and Pseudacris crucifer. Even though whole body water contents

dropped by 50-60 %, individual tissues exhibited little or no change in water content. There were many similarities between the metabolic responses to dehydration and those to freezing. The most remarkable similarity between freezing and dehydration was the accumulation of glucose, presumably acting as a cellular protectant; quantities in liver rose to 127 and 220 $\mu\text{mol/g}$ in R. sylvatica and P. crucifer, respectively.

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LIST OF ABBREVIATIONS

% **a** - percent glycogen phosphorylase in active **a** form

α -KG - alpha ketoglutarate

a + b - total glycogen phosphorylase units:

active **a** form plus inactive **b** form

ATP, ADP, AMP - adenosine tri-, di-, and monophosphate

CAP - critical activity point

CoA - coenzyme A

Cr-P - creatine phosphate

CrK - creatine kinase

DHAP - dihydroxyacetonephosphate

E.C. - energy charge = $(ATP + ADP/2)/(ATP + ADP + AMP)$

EDTA - ethylenediamine tetraacetate

EGTA - ethyleneglycol bis(β -aminoethyl ether)
tetraacetate

F1,6P₂ - fructose 1,6 bisphosphate

F2,6P₂ - fructose 2,6 bisphosphate

F6P - fructose 6 phosphate

Fpt. - freezing point

G1P - glucose 1 phosphate

G3P - glycerol 3 phosphate

G6P - glucose 6 phosphate

G6PDH - glucose 6 phosphate dehydrogenase

GAP - glyceraldehyde 3 phosphate

GK - glycerokinase

GOT - glutamate-oxaloacetate transaminase

GPT - glutamate-pyruvate transaminase

HK - hexokinase

HPLC - high performance liquid chromatography

I₅₀ - concentration of inhibitor reducing V_{max} by 1/2

INF - ice nucleating factor

K_a - concentration of activator that produces 1/2 V_{max}

K_m - Michaelis-Menten constant

M6P - mannose-6-phosphate

MDH - malate dehydrogenase

MgSO₄ - magnesium sulphate

**NAD⁺, NADH - oxidized and reduced forms of nicotinamide
adenine dinucleotide**

PEP - phosphoenolpyruvate

PFK - phosphofructokinase, catalyzes F6P $\rightarrow$ F1,6P₂

PFK II - phosphofructokinase 2, catalyzes F6P $\rightarrow$ F2,6P₂

PGI - phosphoglucomutase

PK - pyruvate kinase

PMI - phosphomannose isomerase

PGM - phosphoglucose isomerase

PMSF - phenylmethylsulfonyl fluoride

PPi - pyrophosphate

PPi-PFK - pyrophosphate-linked phosphofructokinase

Q₁₀ - the effect of a 10 °C change on reaction rate

S_{cp}t. - supercooling point

SDH - sorbitol dehydrogenase

STK - succinyl thiokinase

THF - thermal hysteresis factor

Tr - rebound temperature

Vmax - maximal enzyme velocity

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CHAPTER 1

GENERAL INTRODUCTION

One of the most dramatic environmental stresses that ectothermic animals must endure is the harsh temperatures of winter. Temperatures of a Canadian winter can drop as low as -30°C to -40°C during extreme cold spells (Macartney et al., 1989). Basic cold avoidance tactics are sufficient protection against freezing for some animals. Frogs and toads often seek refuge by burrowing into rotting logs by covering themselves with leaves and other debris on the forest floor; when snow falls, an additional 'blanket' of protection is obtained. Several toad species such as Bufo bufo avoid low winter temperatures by digging down into the earth below the frost line away from environmental exposure (Storey and Storey, 1988). Some animals totally avoid the harsh winter conditions by moving to warmer climates. Many bird species migrate to southern climes near the Tropic of Cancer or the Equator (Storey and Storey, 1988). Nevertheless, many animals remain in the sub-zero environment of the north and these must undergo physiological and biochemical changes in order to successfully deal with freezing temperatures. The supercooling points of amphibians and reptiles are commonly in the -6 to -9°C range (Paukstis and Shuman, 1989) and this allows most hibernating vertebrates to avoid freezing. Tolerance of freezing occurs widely among insects and various types of intertidal marine invertebrates, however, this overwintering strategy is unusual in vertebrate animals (Aarset, 1982; Murphy, 1983; Zachariassen, 1985; Storey and Storey, 1992). There are to date only five species of terrestrial frogs, one species of salamander, five species (including subspecies) of turtle, and one species of snake that are known to survive whole body freezing: the wood frog, Rana sylvatica; the grey tree frog, Hyla versicolor; Cope's tree frog, Hyla chrysoscelis; the chorus frog, Pseudacris triseriata; the spring peeper, Pseudacris crucifer; the salamander, Hynobius keyserlingi; and the painted turtle, Chrysemys picta, including two subspecies, the midland subspecies C. p. marginata and the western subspecies C. p. bellii; the eastern box turtle, Terrapene carolina carolina; the western box turtle,

Terrapene ornata; the red eared slider, *Trachemys scripta elegans*; and the garter snake, *Thamnophis sirtalis*. With the exception of the garter snake, and the red eared slider, all of these animals can successfully tolerate the accumulation of 50 - 60 % of body water as ice and temperatures as low as -10°C for short periods of time; survival dramatically increases if temperatures are maintained at -1 to -4°C (Storey and Storey, 1992).

Ice Formation and Cryoprotectants

Except for a few specialized cases, the formation of intracellular ice is lethal due to membrane penetration and the destruction of subcellular microcompartmentation from the large impinging ice crystals (McGann et al., 1988). Control of intracellular and extracellular ice formation is essential to survival of a freeze tolerant organism. Typical cytoplasmic osmotic concentrations can range from 200 mM to 400 mM, contributed by salts, total metabolite pools, and proteins. The net colligative effect of these compounds are not significant in reducing the freezing point and/or supercooling point of the intracellular matrix. Equilibrium freezing points of body fluids are about -0.5°C in terrestrial animals and can reach -1.7°C in marine fishes (Storey and Storey, 1988). Adaptive pressures targeted at these animals have resulted in the capacity for some to synthesize additional polyhydrated carbon based compounds within the cytoplasm. Most cryoprotectants found in nature are 3 or 6 carbon sugars or their corresponding sugar alcohols; freeze tolerant insects commonly synthesize glycerol and/or sorbitol, although glucose accumulation occurs in most of the freeze tolerant frogs (Duman, 1982; Zachariassen, 1985; Storey and Storey, 1988). There are two basic types of protective action by which these cryoprotectants operate. Membrane stabilization has been shown to be effected through binding of trehalose (and the amino acid, proline) to the hydrophilic headgroups of phospholipids (Carpenter and Crowe, 1988). Carbohydrate cryoprotectants protect cellular components as a result of

colligative effects (Zachariassen, 1985). Carbohydrate cryoprotectants are useful for freeze tolerant (and freeze avoiding) animals due to their negative effect on intracellular ice formation. Freeze tolerant marine invertebrates (periwinkles, molluscs, barnacles) show no indication of carbohydrate cryoprotectant production. However, it is believed that these marine invertebrates rely on high total amino acid levels and the mobilization of Ca^{++} from shell calcium carbonate in order to stabilize membrane structures and intracellular proteins; key amino acids implicated are proline, glutamate, aspartate, and taurine (Murphy, 1983). The freeze tolerant turtle, *C. p. marginata*, also relies on some of these amino acids, taurine and glutamate, as well as increases in total amino acid pools to survive 24 h in the frozen state (Storey et al., 1988). Another important functional characteristic of polyhydroxylated molecules is their structural similarity to water. Due to the presence of polyhydroxyl groups, intracellular water undergoes hydrogen bonding with the cryoprotectants. The net effect of this type of weak bonding influences a reduction in the degree of intracellular dehydration that typically occurs during a freezing episode (Carpenter and Crowe, 1988). The polyols are capable of stabilizing proteins, by acting as water does under normal conditions, by forming a hydration shell around the hydrophilic regions of the polypeptide. The naturally synthesized carbohydrate cryoprotectants are non-toxic even at high concentrations (eg. 2 - 4 molar). Chemical inertness is an essential property which must be recognized; these polyols do not participate in any irreversible chemical reactions with cellular compounds or structures (Zachariassen, 1985). Most cryoprotectants are products of 'dead end' pathways and therefore are not directly exposed to the regulatory effects of a dynamic biochemical pathway. For example, glycolysis can respond to other metabolic requirements such as the maintenance of energy levels without interference from allosteric effects by high polyol concentrations.

During the initial stages of freezing, there is an efflux of intracellular water; as initial ice formation occurs extracellularly, salt concentrations also increase, thereby

increasing the difference in osmotic potential. As the intracellular water content declines, the internal osmotic concentration rises significantly (Rubinsky, 1987). Both structural and functional qualities of the enzyme proteins in the cytoplasm have been shown to be dramatically affected by high salt concentrations; ionic interactions between salts and amino acid subunits of the proteins can readily denature the enzyme by favoring an inactive conformation of the protein. Additionally, as water exits the cytoplasm, the hydration shells of the enzymes are also adversely affected due to the lack of available water and this can injure the functional characteristics of the proteins.

Ice Nucleating Proteins and Thermal Hysteresis Proteins

In addition to the production of cryoprotectants, there are several protein factors, ice-nucleating factors (INF) and thermal hysteresis factors (THF), which permit a controlled freezing process, thus minimizing the destructive formation of large ice crystals (Duman, 1982). These special classes of proteins nucleate ice extracellular ice formation at high subzero temperatures (INF) and then bind to the expanding outer edges of the crystals (THF) so that growth is halted before any damage is possible. Only two of the freeze tolerant vertebrates (*R. sylvatica* and *C. picta*) are known to possess these proteins (Storey et al., 1991; Wolanczyk et al., 1990). However, the other species are suspect since high supercooling points are found in some, and others synthesize only small quantities of protective sugars, but are equally well suited to survival of freezing episodes.

Anoxia during Whole Body Ice Formation

During freezing exposures all heart activity and blood flow is stopped, thus ischemic conditions prevail. Tolerance to anoxia is another key factor in determining survival of the frozen state (Storey and Storey, 1988). Even sensitive tissues, such as the brain, must be able to generate ATP anaerobically using endogenous substrates

only. In addition, the tissues must be able to withstand substantial increases in lactate, accompanied by alterations in the acid-base balance within the tissue. Levels as high as 12, 31, 35 $\mu\text{mol/g}$ in liver, heart, and kidney have been found in frogs frozen for 4 days (Storey, 1987). During a freezing stress, energy requirements imposed by a gradual stoppage of blood flow and gas exchange must be fulfilled. As the organism freezes, metabolism is forced to rely on glycolysis; anaerobic endproducts lactate and alanine accumulate in order to maintain the ATP pool (Lutz et al., 1985; Storey and Storey, 1984). An initial attempt to keep ATP levels high by rapid endproduct synthesis is usually successful, but after longer term freezing episodes the organism must cope with dramatic declines in ATP and energy charge (Atkinson, 1977) levels (Storey and Storey, 1986). After 24 h freezing at -4°C , *R. sylvatica* accumulated 15 - 30 $\mu\text{mol/g}$ lactate in liver, kidney, and brain (Storey, 1987). However after 3 successive freeze-thaw cycles, energy charge dropped to 0.34 in *R. sylvatica* liver (Storey and Storey, 1985). Four of the five species of freeze tolerant frogs (at least) exhibit such a tolerance and survival rates of 100 % are not uncommon (Storey and Storey, 1986, 1988).

Metabolic Adaptations of Freeze Tolerant Animals

Maintenance of glycolytic control during freezing is essential for effectively coordinating cryoprotectant synthesis as well as for the production of sufficient ATP for cellular functions such as ion pumping and other metabolic processes. Q_{10} effects alone will drastically reduce enzyme activity; a decline from 20°C to 0°C could result in a 4 fold reduction in enzyme activity. As well, large increases in cryoprotectant, coupled with increased salt concentrations as a result of dehydration, leads to a cytoplasm with high viscosity and high ionic strength (Carpenter and Crowe, 1988). Simple diffusion of the enzymes and substrates is greatly reduced and additional ionic interactions with sensitive amino acid subunits may adversely affect

activity of proteins. Thus, metabolic processes would be limited in this respect even if flux through glycolysis was, otherwise, fully activated. Regulation of the glycolytic pathway is carried out by several key enzyme control points; generally, phosphofructokinase (PFK), pyruvate kinase (PK), and glycogen phosphorylase are implicated in this role. In order to successfully achieve cryoprotectant synthesis, efficient activation/inactivation cues must be in operation. Triggering cryoprotectant synthesis in *R. sylvatica* occurs in response to ice nucleation at the skin (Storey and Storey, 1985). Hormonal messengers probably signal the phosphorylation/activation of glycogen phosphorylase (% a, and total a + b units increase). In concert with a presumed protein kinase mediated phosphorylation of liver PFK, resulting in inhibition of the glycolytic pathway, large quantities of glucose accumulate (Storey and Storey, 1985). Protein kinase cascade sequences are known to operate on all three regulatory enzymes (Stryer, 1981). The resulting phosphorylation/dephosphorylation of the target enzyme can affect an activation or inactivation depending on enzyme and regulatory role. Secondary level effects such as those by allosteric effectors can dramatically increase or decrease enzyme activity. Metabolites that exhibit influence on PK are fructose-1,6-bisphosphate (activator), alanine and ATP (inhibitors); those affecting PFK include ATP and citrate (inhibitors), F2,6P₂ and AMP (activators) (Stryer, 1981; Schaftingen, 1984). Increased glucose levels usually imply that F2,6P₂ levels also increase either as a result of mass action or via concerted hormonal activation of PFK II (catalyzing $F6P + ATP \rightarrow F2,6P_2$). Modification of PFK II by means of a protein kinase phosphorylation can indirectly inhibit glycolytic flux through PFK I in an effort to produce glucose as cryoprotectant in the liver. Glucose output from mammalian liver is hormonally regulated by glucagon or catecholamine stimulation that phosphorylates and activates glycogen phosphorylase and phosphorylates and inactivates PFK I, PFK II, and PK. This system has been extensively studied in mammals and is probably in operation in freeze tolerant animals.

The studies presented in this thesis elucidate some of the metabolic adaptations that exist in this unique group of vertebrates so that whole body freezing can be tolerated and survival is ensured.

CHAPTER 2

GENERAL MATERIALS AND METHODS

Chemicals and biochemicals

All chemicals and biochemicals were purchased from Sigma Chemical Co., Boehringer Mannheim Corp., or J.T. Baker Chemical Co.

General freezing protocol

For freezing exposure, each animal was placed on a pad of paper toweling with the ventral side centered over a thermistor. Animal and thermistor were secured in place with masking tape, and the animal was placed in an incubator set at a given subzero temperature (range ± 0.1 °C). Thermistors were connected to a Yellow Springs Instrument telethermometer, which in turn was connected to a Kipp and Zonen chart recorder. Body surface temperature was recorded over time as animals cooled over 20 - 60 min to their supercooling points. As body temperature neared the supercooling point, temperature recordings of each individual were made with a multichannel telethermometer every 10 - 15 s. If body temperature reached incubator temperature without nucleation occurring, the temperature of the incubator was then lowered in -0.5 °C increments and body temperature was allowed to fall until it again equalled incubator temperature or until nucleation was observed. Nucleation was indicated by an exotherm recorded on the chart paper. Supercooling point is taken as the lowest temperature recorded before nucleation; the body temperature measured within 1 min post exotherm was recorded as the rebound temperature. After the appearance of the freezing exotherm, air temperature of the incubator was raised (if necessary) to the desired exposure temperature and held there for the remainder of the freezing exposure. The lengths of all freezing exposures were timed from the appearance of the exotherm.

Some of the following experiments (specifically the garter snake experiments, chapter 5 and 6) did not use animals that were frozen by spontaneous nucleation; instead the freezing period was initiated by cutaneous ice nucleation by touching

("seeding") the animal with a cold metal rod (-196 °C) followed by incubation at a subzero temperature higher than their supercooling point but lower than the rebound temperature for the specified period of time.

Calorimetry

Percent body water as ice was determined by thawing the frozen animals in a calorimeter filled with water. Calorimetry was performed as outlined by Lee and Lewis, 1985, using the following experimentally determined factors:

i) S_d - specific heat of the dry mass (cal/g/°C)

ii) F factor for the calorimeter

Calculations were based on the mean values of these parameters without incorporating their associated error values and may therefore underestimate the variance associated with the reported percentage ice determinations. The full equation for the '% body water as ice' is as follows:

$$\% \text{ body water as ice} = (W_i * 100) / (W_s * PW)$$

$$W_i = \frac{(F * W_w * S_w * (T_i - T_0) + (T_s - T_0) * (W_d * S_d + W_s * PW * S_w))}{((T_s - T_0) * S_w + Q + S_i * (M_p - T_s) + S_w * (T_f - M_p))}$$

where:

W_i = weight of ice

F = factor for calorimeter representing deviation

from an ideal calorimeter (range of 1.03 - 1.20)

W_w = weight of water + animal water in the calorimeter

S_w = specific heat capacity of water (1.000 cal/g/°C)

T_i = initial temperature of water in calorimeter

T_f = final temperature of water in calorimeter

T_s = temperature of animal prior to thawing

W_d = weight of dry mass of sample

S_d = specific heat capacity of dry mass

(0.15 - 0.35 cal/g/°C)

W_s = weight of animal

PW = per cent water of animal

Q = latent heat of fusion of water (79.7 cal/g/°C)

S_i = specific heat capacity of ice (0.500 cal/g/°C)

M_p = melting point of animal (-0.2 to -0.5 °C)

F factor for the calorimeter can be determined by melting standard amounts of ice (frozen at set temperatures) in the calorimeter. The **F** factor is a measure of the deviation from an ideal calorimeter, that is, no heat taken up from the environment and no heat released. The above equation for the weight of ice in an unknown sample can be rearranged to determine an unknown **F** value.

Thus,

$$F = \frac{(W_i \cdot S_i \cdot T_s + W_i \cdot S_w \cdot T_f + W_i \cdot Q)}{(W_w \cdot S_w \cdot (T_i - T_f))}$$

As well, the specific heat of the dry mass of the animal can be determined by drying a dead animal to a constant weight at 80 °C and cooling the weighed amount of dry tissue to a subzero temperature. Then calorimetry is performed with the dry mass and **S_d** can be determined.

$$S_d = \frac{(F \cdot W_w \cdot S_w \cdot (T_i - T_f))}{(W_d \cdot (T_f - T_s))}$$

General dehydration protocol

Animals were dehydrated at 5 °C over desiccant (Silica Gel) at a rate of 0.5 - 1 % of total body water lost/hour. Direct contact with the desiccant was prevented by placing a layer of 1-2 cm sponge between desiccant and frogs. 'Per cent dehydrated' was determined by the following equation:

% DEHYDRATED = % of Total body water lost due to dehydration

$$= [wt_i - wt_d] / [wt_i * \% H_2O_i]$$

wt_i = weight of frog prior to dehydration

wt_d = weight of frog after dehydration

$\% H_2O_i$ = water content of frog prior to dehydration

Percent H₂O Content was obtained by initial and final weight measurements of frogs that were killed and then dried to constant weight (72 h at 80 °C); this ranged from 80 - 85 %. Frogs were rehydrated by placing the dehydrated animal in water (not covering the head); complete rehydration took approximately 12 - 24 h, although frogs were sacrificed at 24 h to eliminate any individual differences.

Tissue Preparation for Metabolite Measurements

After exposure to the respective stress, the animals were immediately killed by decapitation and pithing and the selected organs were excised and frozen in liquid nitrogen. Organ samples were then transferred to a -80 °C freezer for long-term storage and were used for the measurement of metabolite and enzymes.

Preparation of samples for metabolite assays was as follows. Tissue samples were weighed out in polypropylene test tubes and then stored on dry ice until processed. Five volumes of 6 % perchloric acid/1 mM EDTA were then added and the tissue was quickly homogenized by means of a sonicating Polytron homogenizer. At this point, 20 μ l of the acid homogenate was withdrawn and transferred to 0.50 ml of 100 mM sodium acetate for subsequent glycogen determination. The remaining homogenate was centrifuged at 27 000 g for 20 min at 0 - 4 $^{\circ}$ C in order to precipitate the denatured protein. The supernatant was transferred to a second tube and then neutralized by the addition of 3 N KOH/0.4 M KCl/0.3 M Tris. The potassium perchlorate (KClO_4) was removed by centrifugation at 27 000 g for 20 min at 0 - 4 $^{\circ}$ C. Aliquots of the neutralized extract were used for pyruvate, PEP, creatine phosphate, ATP, ADP, AMP assays which were performed immediately after the extraction process due to their instability. The remaining neutralized extract was then frozen at - 80 $^{\circ}$ C and subsequently used as the source for metabolite assays. Metabolite determinations of alanine, G6P, F6P, G1P, PEP, pyruvate, L-lactate, creatine phosphate (with creatine kinase being substituted for arginine kinase in the arginine phosphate assay), ATP, ADP, AMP, DHAP, GAP, F1,6P₂, glucose, fructose, and mannose were performed as described by Lowry and Passonneau (1972). Glycerol was determined enzymatically as described by Eggstein and Kuhlmann (1974) and sorbitol as described by Bergmeyer et al. (1974).

Glycogen determination

The pH of the acetate/homogenate solution was altered to pH = 4.8 with 30 μ l of 0.1 M K_2CO_3 . Amyloglucosidase from *Aspergillus niger* was added to each homogenate solution and incubated overnight (24 h at 55 $^{\circ}$ C) to allow the degradation of the glycogen to glucose (Keppler and Decker, 1974). Four milliliters of 50 mM Tris buffer pH = 8.0 were added to the acetate/homogenate solution upon completion

of the conversion of glycogen to glucose. The total glycogen was then assayed as glucose via the standard enzymatic glucose assay as described by Lowry and Passonneau (1972).

Glycogen levels are reported in terms of glucose units in $\mu\text{mol/g}$ wet wt.; $1000 \mu\text{mol/g}$ wet wt. = 180 mg/g wet wt.

Metabolite Assays

All metabolite assays used are based on the oxidation or reduction of the purine nucleotide NAD(H) monitored either spectrophotometrically at 340 nm or fluorometrically at 460 nm. The assays were performed by mixing approximately 1 ml of the respective reaction mixture and an aliquot of the sample extract in a cuvette. An initial absorbance or fluorescence reading was recorded and the specified enzyme was added to initiate the reaction. After a sufficient amount of time, as determined by standard amounts of the metabolite, a final reading was recorded. 'Blanks' which contained only added enzyme and no sample extract were also carried out. The amount of NAD(H) converted in the cuvette and thus the amount of the metabolite was determined by the following equation which is a rearrangement of Beer's Law:

$$A = \epsilon * b * c$$

where A = absorbance

ϵ = molar extinction coefficient ($\text{ml}/\mu\text{mol}/\text{cm}$);

for NADH $\epsilon = 6.22 \text{ ml}/\mu\text{mol}/\text{cm}$

b = light path (usually 1 cm)

c = concentration ($\mu\text{mol}/\text{ml}$)

Therefore, $\mu\text{mol} = (A * \text{ml}) / \epsilon$

All absorbances were measured on a Pye Unicam PU-8610A or SP8-100 spectrophotometer at 340 nm; fluorescences were measured on an Aminco-Bowman Spectrophotofluorometer (Excitation wavelength = 340 nm, Emission wavelength = 460 nm). For the fluorometric assays, a standard curve of NADH 0 - 50 nmol/ml (μM) was used for the extrapolation of sample fluorescence to determine the unknown μmol amount of the metabolite in the cuvette. The original concentrations of the metabolites in the tissue sample were calculated and expressed in $\mu\text{mol/g}$ wet weight by means of the following equation:

$$\mu\text{mol/g wet wt.} = \mu\text{mol} * (V_1 + W)/W * (V_2 + V_3)/V_2 * V_5/V_4$$

where μmol = μmoles of metabolite in cuvette

W = wet weight of tissue (g)

V_1 = volume of perchloric acid solution used to
homogenate the tissue (ml)

V_2 = volume of acid extract supernatant (ml)

V_3 = volume of KOH solution required to
neutralize the acid supernatant (ml)

V_4 = volume of sample in cuvette (μl)

V_5 = 1000 μl

The Assays

ADP and AMP

Pyruvate kinase, (PK, 0.6 U) was added to a cuvette containing buffer (50 mM Tris, pH = 7.0), NADH (150 μ M), MgSO₄ (5 mM), KCl (50 mM), ATP (100 μ M), PEP (300 μ M), lactate dehydrogenase (LDH, 0.4 U), neutralized extract, and the O.D. change due to ADP utilization was recorded. Then myokinase (MK, 0.36 U) was added and the O.D. change due to AMP utilization was recorded.

L-Alanine

Alanine + α -KG -----> pyruvate + glutamate (GPT)

pyruvate + NADH + H⁺ ----> lactate + NAD⁺ + H₂O (LDH)

Glutamate-pyruvate transaminase (GPT, 5.0 U) was added to a cuvette containing buffer (50 mM Tris, pH = 8.0), NADH (150 μ M), α -ketoglutarate (α -KG, 200 μ M), lactate dehydrogenase (LDH, 5 U), neutralized extract and the O.D. change due to alanine utilization was recorded.

L-Aspartate

Aspartate + α -KG ----> oxaloacetate + glutamate (GOT)

oxaloacetate + NADH + H⁺ ---> malate + NAD⁺ + H₂O (MDH)

Glutamate-oxaloacetate transaminase (GOT, 1.8 U) was added to a cuvette containing buffer (50 mM Tris, pH = 7.0), NADH (150 μ M), α -ketoglutarate (α -KG, 200 μ M), malate dehydrogenase (MDH, 1.4 U), neutralized extract and the O.D. change due to aspartate utilization was recorded.

ATP and creatine phosphate

Cr-P + ADP -----> creatine + ATP (CrK)

ATP + glucose -----> ADP + G6P (HK)

G6P + NAD⁺ ---> 6-P-gluconolactone + NADH + H⁺ (G6PDH)

Hexokinase (HK, 0.3 U) was added to a cuvette containing buffer (50 mM Tris, pH = 8.0), NAD^+ (500 μM), MgSO_4 (5 mM), glucose-6-phosphate dehydrogenase (G6PDH, 0.1 U), neutralized extract and the change in O.D. due to ATP utilization was recorded. Then creatine kinase (CrK, 1 U) and ADP (500 μM) were added and the change in O.D. due to creatine phosphate (creatine-P) was recorded.

DHAP, GAP, and F1,6P₂

Glycerophosphate dehydrogenase (GPDH, 0.1 U) was added to a cuvette containing buffer (50 mM Tris, pH = 7.0), NADH (100 μM , neutralized extract and the change in O.D. due to DHAP utilization was recorded. Then triosephosphate isomerase (TPI, 1.2 U) was added and the change in O.D. due to GAP utilization was recorded. Finally, aldolase (0.05 U) was added and the change in O.D. due to F1,6P₂ utilization was recorded. Each of the three reactions is allowed to run to completion before the enzyme for the next assay was added.

Glucose, Fructose, and Mannose

Hexokinase (HK, 0.3 U) was added to a cuvette containing buffer (50 mM Tris, pH = 8.0), NAD^+ (500 μM), MgSO_4 (5 mM), ATP (500 μM), glucose-6-phosphate

dehydrogenase (G6PDH, 0.1 U), neutralized extract and the change in O.D. due to glucose utilization was recorded. Then phosphoglucose isomerase (PGI, 0.5 U) was added and the change in O.D. due to fructose utilization was recorded. Then phosphomannose isomerase (PMI, 0.5 U) was added and the change in O.D. due to mannose utilization was recorded.

G6P, F6P, and G1P

Glucose-6-phosphate dehydrogenase (G6PDH, 0.1 U) was added to a cuvette containing buffer (50 mM Tris, pH = 8.0), NAD^+ (500 μM), MgSO_4 (5 mM), neutralized extract and the change in O.D. due to G6P utilization was recorded. Then phosphoglucose isomerase (PGI, 0.5 U) was added and the change in O.D. due to F6P utilization was recorded. Then phosphoglucose mutase (PGM, 0.5 U) was added and the change in O.D. due to G1P utilization was recorded.

Glycerol

Glycerol kinase (GK, 1.0 U) was added to a cuvette containing buffer (50 mM Tris, pH = 7.0), NADH (150 μM), PEP (0.5 mM), ATP (0.5 mM), MgSO_4 (5 mM), KCl (5 mM), lactate dehydrogenase (LDH, 0.2 U), pyruvate kinase (PK, 0.3 U), neutralized extract and the change in O.D. due to glycerol utilization was recorded.

L-Lactate

L-Lactate dehydrogenase (LDH, 5 U) from bovine heart was added to a cuvette containing buffer (200 mM hydrazine-HCl, pH = 9.6), NAD^+ (0.15 mM), neutralized extract and the change in O.D. due to lactate utilization was recorded.

PEP and pyruvate

Lactate dehydrogenase (LDH, 0.2 U) was added to a cuvette containing buffer (50 mM Tris, pH = 7.0), NADH (150 μM), ADP (0.2 mM), MgSO_4 (5 mM), KCl (50 mM), neutralized extract and the change in O.D. due to pyruvate utilization was recorded. Then pyruvate kinase (PK, 0.2 U) was added and the change in O.D. due to PEP utilization was recorded.

D-Sorbitol

Sorbitol dehydrogenase (SDH, 1 U) was added to a cuvette containing buffer (50 mM Tris, pH = 8.0), NAD^+ (2 mM), neutralized extract and the change in O.D. due to sorbitol utilization was recorded.

Succinate

Succinyl-thiokinase (STK, 0.1 U) was added to a cuvette containing buffer (50 mM Tris, pH = 7.0), NADH (150 μ M), ATP (0.3 mM), Coenzyme A (CoA, 0.3 mM), PEP (0.3 mM), MgSO_4 (5 mM), KCl (50 mM), neutralized extract and the change in O.D. due to succinate utilization was recorded.

Amino acid determinations

For the analysis of free amino acid levels, frozen tissue samples were homogenized 1:10 w/v in 0.5 % (w/v) sulfosalicylic acid and then centrifuged at 6000 g in an IEC benchtop centrifuge. Amino acids were analyzed in aliquots of the supernatant using a Waters high-performance liquid chromatograph (HPLC) after precolumn derivatization with ortho-phthalaldehyde (Roth, 1971). Amino acid standards were used to determine retention times and peak integration values.

Fructose-2,6-bisphosphate Determination

Fructose 2,6 bisphosphate determinations were performed as described by van Schaftingen (1984). Approximately 50 - 100 mg frozen tissue samples were weighed into polypropylene test tubes. The samples were then stored on dry ice until processing. Hot NaOH (20 vol of 100 mM at 80 $^{\circ}\text{C}$) was added and then the sample was homogenized. The alkaline homogenates were then incubated in an 80 $^{\circ}\text{C}$ water bath for 10 min. The homogenates were spun at 10 000 g for 5 min and the supernatant was pipetted off and retained for subsequent assays. A control was prepared for each time point by adjusting the pH of the homogenate to pH = 2 and then incubating for 10 min, at which time the pH was readjusted to pH = 10. All samples were then placed on ice until the F2,6P₂ assays were performed.

The Assay

An appropriate aliquot of the sample was added to a 1 ml cuvette containing buffer (50 mM Tris-acetate, pH = 8.0), Mg-acetate (2 mM), F6P (1 mM), sodium pyrophosphate (0.5 mM), NADH (0.2 mM), aldolase (0.3 U), triosephosphate isomerase (0.3 U), glycerophosphate dehydrogenase (1.2 U), and pyrophosphate dependent phosphofructokinase from potato tubers (PPi-PFK, 0.005 U), and the change in O.D. was recorded over a period of several minutes on a Pye Unicam SP8-100 chart recording spectrophotometer. The linear rate obtained was converted into μmole of NADH utilized/unit time and compared to a plot of PPi-PFK velocity versus activator (F2,6P₂) concentration constructed with standard amounts. A linear form of the curve {a Hill plot: $1/(\text{velocity})$ vs. $1/(\text{activator})$ } was used to estimate the concentration of F2,6P₂ in the samples.

Glycogen phosphorylase a and b

Frozen tissues (with a few crystals of PMSF) were weighed and homogenized 1:5 w/v in ice cold 50 mM imidazole-HCl buffer (pH = 7.5) containing 15 mM 2-mercaptoethanol, 100 mM NaF, 5 mM EGTA, and 5 mM EDTA, using an Ultra-Turrax homogenizer. Optimal assay conditions for glycogen phosphorylase in the glycolytic direction were 50 mM phosphate buffer (pH = 7.0), 4 mg/ml dialyzed glycogen, 10 μM glucose-1,6-diphosphate, 0.5 mM NADP⁺, 0.25 mM EDTA, 15 mM MgSO₄, and excess dialyzed phosphoglucomutase and G6PDH in a final volume of 1 ml. Phosphorylase activity was determined using a well suspended homogenate

(ie. not centrifuged) in the absence (phosphorylase a) and presence (phosphorylase a+b) of 1.6 mM AMP.

Tissue Wet weight/Dry weight and Protein Determinations

Tissue water content was expressed in terms of "grams water/gram dry weight" and was determined by initial and final weights of a portion of tissue that was dried to a constant weight at 80 °C (for 72 h).

Total acid precipitated protein in pellets obtained from perchloric acid extractions were resuspended in 0.2 N NaOH and protein was measured with commercial BioRad protein reaction mixture by comparison of sample absorbance at 595 nm to a series of Bovine Gamma Globulin protein standards using a Pye Unicam PU8610 UV/vis spectrometer by Coomassie blue dye-binding method using the BioRad prepared reagent.

Data Calculations and Statistical Analysis

All values reported are mean $\pm$ S.E.M. Statistical significance was determined by the Student's t-test. Correlation coefficients for straight lines fit to data were determined using linear regression analysis (1st order regression).

CHAPTER 3
GLYCOLYTIC REGULATION DURING CRYOPROTECTANT SYNTHESIS IN
SPRING PEEPERS Pseudacris crucifer

SUMMARY

Analysis of changes in the levels of glycolytic intermediates in response to freezing in organs of Pseudacris crucifer was used to investigate the regulatory control of cryoprotectant synthesis. Cryoprotectant (glucose) levels increased 5 fold within the first 2 min of freezing; maximal rates of synthesis in the liver reached 600 $\mu\text{M}/\text{day}$. An apparent cold activation of glycogen phosphorylase was indicated by rises in G1P and G6P. Changes in the substrate and products of phosphofructokinase (PFK) (F6P and F1,6P₂) revealed an inhibition of glycolysis at this point.

Energy stress during freezing was minimal; however, after 4 h freezing the energy charge (Atkinson, 1977) had dropped to 0.67 and final lactate levels showed an 2 fold increase. As assay of tissue cryoprotectant levels from frogs frozen for 36 h revealed that liver glucose had reached $141 \pm 5 \mu\text{mol/g}$. Glucose distribution throughout the rest of the body showed preferential accumulation in the internal organs (ie. intestine, heart, kidney); values ranged from 12 - 36 $\mu\text{mol/g}$ wet wt.

INTRODUCTION

P. crucifer is one of the five freeze tolerant frog species that accumulates glucose (or glycerol) as cryoprotectants. *P. crucifer* can tolerate 5-7 days at -6.0°C with as much as 35.6 % body water as ice (Schmid, 1982). Fall animals of this species are capable of synthesizing large quantities of glucose as cryoprotectant. Liver glucose levels after 2 d frozen at -3.0°C rose to $228\text{ }\mu\text{mol/g}$; whereas levels of glucose in other body tissues range from $86\text{ }\mu\text{mol/g}$ in brain to $175\text{ }\mu\text{mol/g}$ in blood and heart (Storey and Storey, 1986). In early spring (early April), survival rates are identical to fall values (100 % for frogs frozen at -2.5°C for 4 d) and the accumulation of cryoprotectant is still possible (Storey and Storey, 1987a). However, 6 weeks later (mid-May) survival rates drop dramatically (25 % for frogs frozen at -2.5°C for 15 h) and glucose levels remain below $5\text{ }\mu\text{mol/g}$. This transition from fully capable frogs in the winter to spring/summer frogs that exhibit no cryoprotectant response presumably involves the disappearance of biochemical mechanisms permitting glucose production.

The present study examines the glycolytic control involved in the rapid phase of glucose synthesis in response to freezing in the spring peeper, *P. crucifer*. This metabolic control was determined by means of a glycolytic crossover study. Variations in glycolytic flux were detected by comparison of freezing-stimulated changes in metabolite levels to control values. Spring-collected animals were used and the results, when compared with previous studies of autumn animals, also revealed key seasonal differences in the efficient transport of cryoprotectant from the liver in this species.

MATERIALS AND METHODS

Frogs, *P. crucifer* were collected from the wild by a local supplier in the Ottawa (Canada) region in early April. Mean animal weights ($\pm$ SEM) were: males = 1.90

± 0.05 g; females = 2.30 ± 0.15 g. The animals were acclimated at 3°C for 3-4 days prior to experimentation; the date of experimentation was April 16-20, 1988.

Frogs from a southern population were collected in Florida, U.S.A. and shipped to our lab in late May, 1990. Animals were allowed to acclimate in damp moss at 5°C for several days prior to experimentation.

Preparation of Experimental Animals

The experiment investigated metabolic responses over a time course of freezing. Animals were taped to a paper towel with a YSI telethermometer probe taped under the abdomen and then placed in a -4.0°C incubator (Florida frogs were frozen at -2.0°C). The telethermometer was wired to a chart recorder for clearer viewing of the cooling process. Nucleation was observed as an abrupt exotherm after the animals had reached their supercooling points, $\text{Scpt.} = -2.2 \pm 0.1^{\circ}\text{C}$; $\text{Fpt.} = 0.8 \pm 0.1^{\circ}\text{C}$. The liver and heart were sampled over a 36 h post-nucleation time course. Other tissues, leg muscle, kidney, intestine, skin, abdominal muscle, and eggs were sampled from control animals and at 36 h post nucleation. For survival tests, frogs were frozen for the respective times and then returned to damp moss at 5°C for recovery. Sampling included immediate excision of the desired tissue and rapid freezing in liquid nitrogen. Frozen tissues were stored in a -80°C freezer until tissue processing.

Tissue Extraction and Metabolite Assays

Perchloric acid extracts and metabolite assays were performed as described in chapter 2.

RESULTS

Spring *P. crucifer* from Canada that had been given freezing exposure for a brief period (1/2 h) showed no signs of internal ice crystal formation. In addition, the blood was still fluid and the heart maintained its pumping activity, although the legs were slightly stiff. At 1 h post-exotherm, animals had significant amounts of ice in the visceral cavity, with large crystals surrounding the liver and heart; the blood was frozen and the heart, inactive. Ten out of ten frogs survived 4 h frozen but, four out of four frogs did not survive 36 h frozen followed by 24 h thaw; tissues were sampled from 36 h frozen animals for maximal cryoprotectant levels.

A similar pattern of ice formation was found in a population of southern *P. crucifer* from Florida, U.S.A. when frozen at -2.0°C . Ice content increased gradually within the first several hours, but eventually plateaued out at equilibrium ice content (Fig. 1).

In the spring Ottawa frogs, cryoprotectant (glucose) production by liver was initiated shortly after ice nucleation (Fig. 2). Glucose levels increased 5 fold within the first 2 min post exotherm; quantities levelled off at $5\text{ }\mu\text{mol/g}$ wet wt. until 15 min, and then subsequently rose to $25\text{ }\mu\text{mol/g}$ at 4 h. The data from 15-60 min reflected glucose production rates in the liver of 0.60 M/day . This rate of synthesis continued until levels plateaued at $141 \pm 5\text{ }\mu\text{mol/g}$ in the liver. Glycerol was measured in the animals as an alternative cryoprotectant, but no significant quantities were found; levels were $< 1.5\text{ }\mu\text{mol/g}$ wet wt. in liver.

Glycogen in liver showed no change from control values over 36 h freezing exposure; a range of $600\text{--}700\text{ }\mu\text{mol/g}$ wet wt. was maintained throughout (Fig. 3). Although glucose levels increased to $141\text{ }\mu\text{mol/g}$ wet wt., small differences in glycogen quantities as a result of glycogenolysis would be absorbed by the inherent variability of the animals. Nevertheless, indicative of increased glycogenolysis and corresponding to

FIG. 1. Effects of freezing on ice formation and survival in spring frogs from a Florida population of Pseudacris crucifer. Data are means $\pm$ SEM, n = 4. Percent survival was determined on an arbitrary scale of 1 - 5. 1 point was awarded for each of 5 characteristics that assessed recovery from freezing. The presence of these characteristics (and receiving of a point) is awarded for recovery from freezing. These characteristics were: 1) eye response to touch, 2) leg response to touch, 3) bloating due to water retention, 4) righting ability, 5) hop response.

FIG. 2. Effect of freezing on levels of glucose in liver of spring *Pseudacris crucifer*. Data are means $\pm$ SEM, n = 4. a, b-Significantly different from the corresponding control values (C), $P < 0.05$, $P < 0.005$, respectively. Control animals were taken from the 5 °C incubator in which the frogs were acclimated. Sampling points were timed from the freezing exotherm.

FIG. 3. Effects of freezing on levels of glycogen in liver of spring Pseudacris crucifer. Data are means $\pm$ SEM, n = 4. Control animals were taken from the 5 °C incubator in which the frogs were acclimated.

the increase in glucose level between 15 min - 4 h post exotherm, the G1P pool increased significantly from immeasurably low control quantities; values doubled within the 15 min- 30 min time interval (Fig. 4a). Activities of the enzyme, glycogen phosphorylase, revealed a 2 fold increase in % α form of the enzyme from 51 % to 99 % (control vs. 1-4 h) (Fig. 4b). As well, the total activity of glycogen phosphorylase ($\alpha + \beta$) increased 65 % over control values. The other hexose phosphates, G6P and F6P, increased initially (levels of both sugar phosphates tripled within the first 15 min) (Fig. 5a, 5b). By the time glucose levels had reached a plateau at 4 h, levels of both G6P and F6P had begun to decline.

In comparing the two key metabolites linked to the major glycolytic control point, phosphofructokinase (PFK), a distinct glycolytic crossover was observed at the 15 min time point. Corresponding to the 3 fold rise in F6P levels, F1,6P₂ dropped 55 % from control values at 15 min (Fig. 5b, 5c). By using the ratio of F1,6P₂ : F6P as an indication of flux through PFK, an obvious 'shutting down' of the enzyme was observed; the ratio dropped 84 % from a control value of 1.05 to a subsequent value of 0.16. Upon examination of the two phosphates DHAP and GAP, there was no correlation with the block at PFK. DHAP levels increased in a linear manner by 2 fold until a maximum was attained at 15 min; values levelled off at subsequent times and returned to control values at 4 h. GAP quantities showed no clear pattern as erratic fluctuations were exhibited (Fig. 6a).

The second regulatory point that is suspect in controlling glycolytic flux is the pyruvate kinase (PK) locus. The two adjacent metabolites, PEP and pyruvate, showed no initial relative changes (Fig. 6b, 6c); the control ratio for Pyr/PEP was 1.3. However, at the 15 min- 30 min time interval, this ratio jumped to 2.1, possibly indicative of an activation of PK. But, closer examination of these metabolite levels revealed that only PEP varied in concentration; a 30 % decrease after 30 min post exotherm.

FIG. 4. Effects of freezing on (A) levels of glucose-1-phosphate and (B) glycogen phosphorylase % a, a + b, and units a in liver of spring *Pseudacris crucifer*. Data are means $\pm$ SEM, n = 4. a, b -Significantly different from the corresponding control values, $P < 0.05$, $P < 0.005$, respectively. Control animals were taken from the 5 $^{\circ}\text{C}$ incubator in which the frogs were acclimated. For glucose-1-phosphate, C represents control animals; for glycogen phosphorylase, solid bars (■) represent control animals, rising left-hatched bars (▧) represent 1-4 h frozen frogs. Also shown for comparison is data for phosphorylase in 7 h frozen frogs, *R. sylvatica* represent by diagonal crosshatched bars (▨) (data for *R. sylvatica* was adapted from Storey and Storey, 1985).

FIG. 5. Effects of freezing on levels of (A) glucose-6-phosphate, (B) fructose-6-phosphate, and (C) fructose-1,6-bisphosphate in liver of spring Pseudacris crucifer. Data are means $\pm$ SEM, n = 4. a, b -Significantly different from the corresponding control values (C), $P < 0.05$, $P < 0.005$, respectively. Control animals were taken from the 5 °C incubator in which the frogs were acclimated.

FIG. 6. Effects of freezing on levels of (A) dihydroxyacetone-phosphate + glyceraldehyde-3-phosphate, (B) phosphoenolpyruvate, and (C) pyruvate in liver of spring Pseudacris crucifer. Data are means $\pm$ SEM, n = 4. a, b -Significantly different from the corresponding control values (C), $P < 0.05$, $P < 0.005$, respectively. Control animals were taken from the 5 °C incubator in which the frogs were acclimated.

The anaerobic endproduct, lactate, showed a significant increase over the 36 h time course, but in terms of absolute quantity, the rise was slight; 2 fold over the initial 4 h, and another 2 fold from 4 - 36 h (Fig. 7).

The stress imposed on the frogs by whole body freezing was adequately dealt with from an "energetics" viewpoint. Due to the maintenance of high levels of the high energy phosphate, ATP and low quantities of ADP and AMP (Fig. 8), energy charge values [Energy Charge = $(\text{ATP} + 1/2 \text{ ADP}) / (\text{ATP} + \text{ADP} + \text{AMP})$] (Atkinson, 1977) stabilized in the range 0.90 - 0.86 in liver throughout most of the 4 h time course (Fig. 9). However, at 4 h post exotherm, ATP dropped slightly (by 20 %). Correspondingly, ADP and AMP increased rapidly; 3 fold and 6 fold control values, respectively. This was reflected in the energy charge, the E.C. dropping to 0.67 by 4 h and to 0.43 by 36 h. Total adenylate quantities remained unaltered throughout the 36 h period (Fig. 9).

The heart typically reflects metabolite levels in the blood. Glucose accumulation in the heart followed an identical trend as was found in the liver (Fig. 10). Although absolute quantities were approximately 30 % of those in the liver, the increase in concentration that started at 15 min post exotherm was similar in proportion.

Final glucose distribution in other tissues at 36 h post exotherm showed increases in cryoprotectant, with rises ranging 15 - 70 fold greater than initial control values; absolute quantities ranged from 5 $\mu\text{mol/g}$ wet wt. in eggs to 36 $\mu\text{mol/g}$ wet wt. in heart (Fig. 11). Leg muscle, kidney, abdominal muscle, intestine, and skin showed intermediate values of 7 - 15 $\mu\text{mol/g}$ wet wt..

DISCUSSION

The rapid response of *P. crucifer* to ice nucleation in the skin has been previously documented by Storey and Storey (1986). Formation of subcutaneous ice

FIG. 7. Effects of freezing on levels of lactate in liver of spring Pseudacris crucifer. Data are means $\pm$ SEM, n = 4. a, b-Significantly different from the corresponding control values (C), $P < 0.05$, $P < 0.005$, respectively. Control animals were taken from the 5 °C incubator in which the frogs were acclimated.

FIG. 8. Effects of freezing on levels of adenylates: ATP (open circles), ADP (closed circles), AMP (open squares) in liver of spring Pseudacris crucifer. Data are means $\pm$ SEM, n = 4. a, b-Significantly different from the corresponding control values (C), $P < 0.05$, $P < 0.005$, respectively. Control animals were taken from the 5 °C incubator in which the frogs were acclimated.

FIG. 9. Effects of freezing on total adenylate levels [ATP + ADP + AMP] (open circles) and energy charge values [(ATP + 1/2 ADP)/(ATP + ADP + AMP)] (closed circles) in liver of spring Pseudacris crucifer. Data are means $\pm$ SEM, n = 4. b- Significantly different from the corresponding control values (C), P < 0.005. Control animals were taken from the 5 °C incubator in which the frogs were acclimated.

FIG. 10. Effects of freezing on levels of glucose in the heart of spring Pseudacris crucifer. Data are means $\pm$ SEM, n = 4. a, b-Significantly different from the corresponding control values (C), $P < 0.05$, $P < 0.005$, respectively. Control animals were taken from the 5 °C incubator in which the frogs were acclimated.

FIG. 11. Effects of freezing on levels of glucose in seven tissues of spring Pseudacris crucifer. Data are means $\pm$ SEM, n = 4. a, b-Significantly different from the corresponding control values, P < 0.05, P < 0.005, respectively. (open bars) represent control animals that were taken from the 5 °C incubator in which the frogs were acclimated; (hatched bars) represent 36 h frozen frogs.

crystals in the limbs is thought to trigger a signal, either chemical or neural, so that glycolytic carbon within the liver is suddenly directed solely towards cryoprotectant synthesis. This hypothesis is supported by the present findings; within 15 min post exotherm, rapid cryoprotectant synthesis was underway with a maximum rate of 0.60 M/day. A previous study, investigating glycolytic control during cryoprotectant production in R. sylvatica, reported similar values for glucose accumulation; maximal production rates were 0.72 M/day (comparable to the present findings) (Storey, 1987a). The glucose is then rapidly distributed to other organs of the body via the blood (Storey, 1984, 1987b).

Glucose accumulation in the heart gave indication that simultaneous uptake of the cryoprotectant by the blood and vital organs. Storey (1987a) found excellent correlation between liver glucose and blood glucose levels in R. sylvatica. In the present study, final glucose distribution in the tissues after 36 h freezing exhibited a general trend of minor glucose uptake; quantities remained below 50 $\mu\text{mol/g}$ (even in heart). In a previous study performed by Storey and Storey (1987a), spring P. crucifer had high survival rates (100 % for frogs frozen at -2.5°C for 4 d) and high cryoprotectant levels (39 - 197 $\mu\text{mol/g}$ glucose). The investigation involved freezing the frogs for a much longer time (4 d) that could lead to higher glucose production. Additionally, glycogen levels in the liver were very high (955 $\mu\text{mol/g}$ as glucose), thus supplying the necessary carbon substrate for cryoprotectant synthesis. Reduced accumulation of cryoprotectant found in the present study was also found in another group of P. crucifer in the study performed by Storey and Storey (1987a). After 70 - 100 min post exotherm, glucose levels in the blood were only 4.1 $\mu\text{mol/g}$. Although this time of freezing was relatively short, only frogs with this short a freezing exposure survived. In groups of frogs frozen for 2 h - 3 h, only one from each group of 4 or 5 animals survived. This reduced survival time may be directly related to the absence of large quantities of glucose being produced as a cryoprotectant. There are other factors

such as the presence of nucleating proteins and anoxia tolerance that must also be considered in the reduced survival in these spring animals, but a major contributing factor to reduced spring survival appears to be low cryoprotectant levels.

Storey and Storey (1987) have established that tolerance to freezing disappears rapidly upon spring emergence. Along with this decreased tolerance to freezing, cryoprotectant levels also drop dramatically. Over the initial few weeks of spring emergence, glycogen quantities have been shown to drop dramatically; 610 $\mu\text{mol/g}$ to 283 $\mu\text{mol/g}$ over a 3 week period (Storey and Storey, 1987). However, liver glycogen levels in these spring animals were high (520-840 $\mu\text{mol/g}$ as glucose), therefore, the carbohydrate reserve for cryoprotectant synthesis does not seem to be limiting. The key reason for decreased freezing survival in the present study using spring animals appears to be glucose transport. Glucose levels were high in the liver (140 $\mu\text{mol/g}$), but when heart and other tissues are examined glucose was much lower (maximum 40 $\mu\text{mol/g}$ in heart) here suggesting that capacity for glucose transport out of the liver might decrease in the spring and therefore limit cryoprotectant distribution. Although this study is not conclusive in this respect, our initial conclusion suggests that one of the primary factors for limited spring freeze tolerance may be attributed to a reduction in the number of glucose transporters. A reduction in glucose transporters either between the liver and the blood or between the blood and the individual target tissues would explain the reduced efficiency of cryoprotectant accumulation.

The main purpose of this study was to investigate glycolytic control during rapid cryoprotectant synthesis. The activation of glycogenolysis was apparent by the 2 fold increase in % α form of glycogen phosphorylase and the 65 % rise in total phosphorylase activity. Consequently, both G1P and G6P levels increased rapidly, although the corresponding drop in glycogen was not observed. The lack of change in glycogen quantities was not unexpected since absolute concentrations of glucose were quantitatively small compared with amounts of glycogen. Previous findings in R.

sylvatica showed an identical activation of glycogen phosphorylase (Fig. 4). In R. sylvatica, activation of cryoprotectant synthesis was also indicated by rises in G1P and G6P quantities as well as an activation of phosphorylase; glycogen phosphorylase % α increased 80 % and total enzyme quantity also increased 3 fold (Storey and Storey, 1984).

An inhibition of glycolytic flux at the PFK locus was defined by the drop in F1,6P₂ levels and the sudden rise in F6P experienced within the first 10 min of the time course. Storey and Storey (1984) found similar changes in metabolite levels that were associated with PFK in fall R. sylvatica. F6P levels increased and F1,6P₂ levels decreased, thus indicating that PFK was in an inactive state.

The Pyr/PEP crossover point revealed no change in flux through the pyruvate kinase locus; pyruvate maintained constant levels. However, the slight decline in PEP quantities was attributed to the inactivation of PFK.

Effectively directing carbon to cryoprotectant synthesis must be rapid in frogs once blood is frozen, because of the speed of whole body freezing. Within several hours post-exotherm, distribution of cryoprotectants from the liver to body tissues will become impossible. In this system, glycogen phosphorylase and PFK acted in opposition, thereby effectively reshunting glycolytic carbon into glucose synthesis. The quantity of active phosphorylase units was dramatically elevated by both increasing % α and total phosphorylase amounts, thus a 3 fold rise in the activity of the α form was achieved. Phosphorylation cascade sequences are well known for both glycogen phosphorylase and PFK, linking extracellular hormonal cues to a rapid amplification of protein kinase and protein phosphatase activities and causing an activation of one enzyme and an inhibition of the other. Glycolytic flux is thereby easily and efficiently redirected in favor of glucose production.

In addition to this primary regulatory regime, it is obvious that secondary level controls have been subject to modification. During liver gluconeogenesis, as glucose

concentrations rise, glucose molecules increasingly bind to the glycogen phosphorylase enzyme, thus forming a reversible complex. This glucose-phosphorylase α complex provides a higher affinity substrate for phosphorylase phosphatase than does the phosphorylase α alone. Therefore, once glucose levels are high, dephosphorylation of phosphorylase α would be promoted (Barford, 1991). A mechanism to prevent or circumvent this could be expected in order to allow the accumulation of very high glucose for cryoprotectant purposes.

The maintenance of cellular energy levels are extremely vital at low temperatures, especially in the frozen state. All adenylates maintained control values throughout most of the freezing time course; this was reflected in a constant and high energy charge (E.C. = 0.90). During the initial stages of freezing, the blood is still fluid and internal organs are able to continue to function. The frog can still maintain breathing activity and with the lungs and heart still in operation, oxygen is able to reach the non-peripheral tissues. The formation of whole body ice eventually renders the animal anaerobic, since the exchange and transport of gas via the circulatory system is impossible once the animal is fully frozen. The effects of whole body ice formation were apparent at the final stages of the time course; at 4 h the energy charge had dropped to 0.67 and ADP and AMP levels showed dramatic increases. In addition, lactate levels rose 2 fold by 4 h freezing. This observed imbalance between energy supply and demand was irreversible under the present stress conditions as E.C. values dropped further to 0.43 at 36 h freezing.

In summary, all of the essential aspects contributing to a well developed freeze tolerance were present in spring *P. crucifer* with the exception of one key factor. The levels of glycogen stores in the liver, the site of cryoprotectant synthesis, were comparable to autumn quantities. The triggering of cryoprotectant production, as well as the effective enzyme regulatory controls required for directed glucose synthesis in

the liver were also present. The reduced distribution of glucose to other body tissues implicated a possible loss of glucose transporters in these spring animals.

CHAPTER 4
SEASONAL DEPENDENCE OF FREEZE TOLERANCE IN THE GREY TREE
FROG, Hyla versicolor.

SUMMARY

Glycolytic regulatory controls involved in cryoprotectant (glucose and glycerol) synthesis were examined in spring Hyla versicolor. Cryoprotectant analysis showed a minor accumulation of glucose, however the lack of directed carbon flow resulted in no accumulation of the primary cryoprotectant, glycerol. A positive correlation between glycogen and glucose levels was established ($r = 0.937$). The key metabolic control points, phosphofructokinase (PFK) and glycogen phosphorylase, were implicated in regulating glucose accumulation. Changes in PFK substrate and product suggested inhibition of the enzyme; fructose-6-phosphate increased 6 fold and fructose-1,6-bisphosphate remained essentially at control levels throughout a 4 h period of freezing exposure. Glycogen phosphorylase metabolite, glucose-1-phosphate, increased 2 - 3 fold over control values during the first 2 min of freezing. The anaerobic endproduct, lactate, increased rapidly within the first 2 min of freezing; final quantities measured $2.2 \mu\text{mol/g}$. Adenylate levels (ATP, ADP, AMP) maintained control proportions throughout the freezing episode, with the exception of the 4 h point; energy charged dropped from 0.78 to 0.56.

INTRODUCTION

The grey tree frog, Hyla versicolor, is one of 5 known species of terrestrially hibernating frogs that tolerate freezing. (Storey and Storey, 1985, 1986, 1987, 1988). Unlike the other 4 species, H. versicolor uses glycerol as its cryoprotectant. During freezing episodes, subcutaneous ice formation in the limbs triggers glycerol synthesis in the liver to supply vital tissues with cryoprotectant (Storey and Storey, 1986, 1987; Storey and Storey, 1985; Storey, 1984, 1987b). Rapid distribution of this low molecular weight polyol via the blood enables the organism to effectively tolerate week-long periods of extreme cold (Storey and Storey, 1985). Previous studies have demonstrated that animals collected in the fall are fully capable of surviving long freezing exposures (ie. high cryoprotectant levels and high freezing survival rates), whereas spring animals are much less suited to endure freezing stress (low cryoprotectant levels and reduced survival) (Storey and Storey, 1987). As well, glycolytic regulation during cryoprotectant synthesis has been established for R. sylvatica: large increases in glycogen phosphorylase activity coupled with inhibition of phosphofructokinase redirects carbon to favor glucose accumulation (Storey, 1987a; Storey and Storey, 1984). The goal of this study was to examine glycerol synthesis and the regulation of metabolic steps involved in its production in H. versicolor.

MATERIALS AND METHODS

Animals and Chemicals

Grey tree frogs of the species H. versicolor, were obtained from a local supplier who obtained the animals from the Ottawa region in May, 1988. Average animal weights were 7.41 ± 0.23 g. The animals were acclimated at 3 °C for 1-2 weeks prior to experimentation.

Preparation of Experimental Animals

The experiment investigated metabolic changes over a time course of freezing at -4.0°C . Animals were taped to a paper towel with a YSI telethermometer probe taped to the abdomen and then placed in a -4.0°C incubator (the telethermometer was wired to a chart recorder for clearer viewing of the cooling process). Animals were allowed to cool until nucleation was observed as an abrupt exotherm after the animals had reached their supercooling points. After a timed interval, frogs were quickly removed, pithed and livers were excised and frozen in liquid nitrogen. Frozen tissue was stored in a -80°C freezer until tissue processing. Control livers were taken from frogs acclimated at 3°C .

Tissue Preparation and Metabolite Assays

At each time point, four liver samples ($n=4$) were extracted for metabolites via the perchloric acid method described in chapter 2. Aliquots of the neutralized extract were used immediately for pyruvate, ATP, ADP, AMP assays. The neutralized extract was then frozen at -80°C and used as the source for subsequent metabolite assays. All assays were performed as described in chapter 2.

RESULTS

Fig. 12 shows the effect of freezing on glucose, glycerol, and glycogen in liver of H. versicolor. Upon nucleation, only one of the two cryoprotectants that are known to accumulate in this species showed any increase. Although glycerol levels maintained control values throughout the freezing episode, glucose increased 2.4 fold over the first 2 min (Fig. 12a, 12b). However, quantities of this sugar at 4 h were very small in

FIG. 12. Effects of freezing on levels of (A) glucose, (B) glycerol, and (C) glycogen in liver of spring *Hyla versicolor*. Data are means $\pm$ SEM, n = 4. a, b -Significantly different from the corresponding control values (C), $P < 0.05$, $P < 0.005$, respectively. Control animals were taken from the 5 °C incubator in which the frogs were acclimated.

terms of cryoprotectant capacity; maximum levels in liver reached only $11.3 \mu\text{mol/g}$. Glycogen stores may have decreased (although not statistically significant) over the 4 h freezing period from $98 \mu\text{mol/g}$ to $34 \mu\text{mol/g}$ (Fig. 12c). A positive correlation ($r = 0.937$; $p < 0.0005$) was found to exist between glycogen stores and glucose production using 0.5, 1, and 4 h values (Fig. 13); additional points from *P. crucifer* from chapter 3 were included in the graph but not in the correlation test.

A rapid activation of glycogenolysis was apparent in liver over the early minutes of freezing exposure by increases in both G1P and G6P; levels rose 3 fold and 2 fold over the first 2 min (Fig. 14 and 15). Maximal values were 10 fold and 7 fold higher than control values, respectively. Examination of glycogen phosphorylase revealed an increase in total ($a + b$) and active (% a) phosphorylase units (Fig. 14). The active a form showed a 3 fold increase (30 % to 94 %) in response to freezing, whereas total phosphorylase increased 2 fold (1.83 IU to 3.93 IU/g); net units of the active a form rose by 6.7 fold compared with control liver.

Changes in the substrate (F6P) and product (F1,6P₂) of PFK indicated an inhibition of glycolytic flux over the early minutes of freezing exposure (Fig. 15b, c). Thus, F6P rose 6 fold and F1,6P₂ showed an initial drop and by 60 min, rose only 2 fold. Triose phosphates, dihydroxyacetone-phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP), exhibited minor variations from control values; DHAP increased slightly, GAP remained at control levels (Fig. 16). Levels of both the substrate (PEP) and product (pyruvate) of pyruvate kinase (PK) increased; PEP and pyruvate levels both doubled (Fig. 16b, c).

The anaerobic endproduct, lactate, showed a dramatic increase over the initial portion of the 4 h freezing duration. Within 2 min levels were elevated almost 4 fold; subsequent levels gradually peaked at $2.2 \mu\text{mol/g}$ (Fig. 17).

Adenylate levels, ATP, ADP, and AMP, showed little variation from control values over the early portion of the time course; ATP quantities remained high at 2.0 -

FIG. 13. Correlation between glycogen levels and glucose production in response to freezing in spring Hyla versicolor and Pseudacris crucifer (from Chapter 1.). Data represent individual animals. r - correlation coefficient for Hyla versicolor data is 0.937.

FIG. 14. Effect of freezing on the (A) levels of glucose-1-phosphate and (B) glycogen phosphorylase % a, total a + b, and units a in liver of spring Hyla versicolor. Data are means $\pm$ SEM, n = 4. a, b -Significantly different from the corresponding control values, $P < 0.05$, $P < 0.005$, respectively. Control animals were taken from the 5 °C incubator in which the frogs were acclimated. For glucose-1-phosphate, C represents control animals; for glycogen phosphorylase (B), open bars ( ) represent control animals and hatched bars ( ) represent 4 h frozen frogs.

FIG. 15. Effect of freezing on levels of (A) glucose-6-phosphate, (B) fructose-6-phosphate, and (C) fructose-1,6-bisphosphate in liver of spring Hyla versicolor. Data are means $\pm$ SEM, n = 4. a, b -Significantly different from the corresponding control values (C), $P < 0.05$, $P < 0.005$, respectively. Control animals were taken from the 5 °C incubator in which the frogs were acclimated.

FIG. 16. Effects of freezing on levels of (A) dihydroxyacetone phosphate + glyceraldehyde-3-phosphate, (B) phosphoenolpyruvate, and (C) pyruvate in liver of spring Hyla versicolor. Data are means $\pm$ SEM, n = 4. a, b -Significantly different from the corresponding control values (C), $P < 0.05$, $P < 0.005$, respectively. Control animals were taken from the 5 °C incubator in which the frogs were acclimated.

FIG. 17. Effects of freezing on levels of lactate in liver of spring Hyla versicolor. Data are means $\pm$ SEM, n = 4. a, b -Significantly different from the corresponding control values (C), $P < 0.05$, $P < 0.005$, respectively. Control animals were taken from the 5 °C incubator in which the frogs were acclimated.

2.5 $\mu\text{mol/g}$ (Fig. 18a, 18b, 18c). The maintenance of adenylate levels was reflected in a constant and high energy charge $\{E.C. = (\text{ATP} + 1/2 \text{ADP})/(\text{ATP} + \text{ADP} + \text{AMP})\}$ (Atkinson, 1977); E.C. values were approximately 0.78 with very small variation over the first 60 min (Fig. 19b). However after 4 h freezing, E.C. dropped to 0.56 ± 0.01 indicating an energy stress on liver by that time; ATP decreased 2 fold, ADP and AMP rose 2 fold. Total adenylate quantities however remained constant throughout (Fig. 19a).

DISCUSSION

Cryoprotectant levels in *R. sylvatica* has been well documented in animals collected in autumn (Storey and Storey, 1986, 1987, 1988). As well, glycolytic regulation favoring glucose synthesis has also been examined (Storey, 1987a; Storey and Storey, 1984). Typical cryoprotectant quantities range from 500 to 1000 $\mu\text{mol/g}$ wet wt. in these previous studies (Storey, 1987a; Storey and Storey, 1984). During a typical freezing stress, ice forms extracellularly in the tissues of the frog. Intracellular water is gradually pulled out of the cytoplasm as a result of the increasing osmotic pressure due to the sequestration of extracellular water as ice (Clegg, 1981). In addition to the danger of ice crystal penetration and destruction of the cell membrane, this secondary stress on the cell and its contents must be minimized to enable a successful survival of the freezing episode. The production of large quantities of low molecular weight polyhydroxylated compounds aids in maintenance of intracellular water (Storey and Storey, 1988; Clegg, 1981). These cryoprotectants reversibly bind to existing water in the cytoplasm via hydrogen bonds that occurs due to the abundance of hydroxyl groups on these sugars (Storey and Storey, 1988; Churchill and Storey, 1989). Enzyme structure/function may also be affected by the removal of water. However, the polyols stabilize the protein's hydration shell, which would otherwise be

FIG. 18. Effects of freezing on levels of (A) ATP, (B) ADP, and (C) AMP in liver of spring Hyla versicolor. Data are means $\pm$ SEM, n = 4. a, b -Significantly different from the corresponding control values (C), $P < 0.05$, $P < 0.005$, respectively. Control animals were taken from the 5 °C incubator in which the frogs were acclimated.

FIG. 19. Effects of freezing on (A) Total Adenylate levels [ATP + ADP + AMP] and (B) Energy Charge values $[(\text{ATP} + 1/2 \text{ ADP})/(\text{ATP} + \text{ADP} + \text{AMP})]$ in liver of spring Hyla versicolor. Data are means $\pm$ SEM, n = 4. b -Significantly different from the corresponding control values (C), $P < 0.005$, respectively. Control animals were taken from the 5 °C incubator in which the frogs were acclimated.

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adversely affected by the lack of water (Clegg, 1981). These select few freeze tolerant animals, as well as many insect species, have adapted this metabolic strategy of directing carbon flow to cryoprotectant sugars; commonly glucose, glycerol, and sorbitol (Storey and Storey, 1988; Zachariassen, 1985). Before any freezing episode can occur, glycogen stores must be in high quantities for subsequent cryoprotectant production. It is not unusual for glycogen levels to reach 1000 - 1200 $\mu\text{mol/g}$, thus carbohydrate reserves are excellent for sugar synthesis (Storey, 1987a; Churchill and Storey, 1989a, b). In several studies, in addition to the present investigation, glycogen levels in frogs collected in the spring have been found to be much lower (5 - 100 $\mu\text{mol/g}$) (Storey and Storey, 1987). During emergence from the winter period of hibernation the activity of frogs changes. This is the time for attracting mates by constant croaking and subsequent mating activities.

The synthesis of cryoprotectant in large quantities must be controlled by a well defined series of regulatory control points which enable carbon to be directed solely to cryoprotectant (Storey and Storey, 1988; Zachariassen, 1985). Present findings indicate that this synthesis is regulated at both glycogen phosphorylase and PFK loci. Freezing exposure resulted in a rapid initiation of glycogenolysis, shown by an increase in % α (from 30 % to 97 %), as well as an increase in total phosphorylase units; thereby elevating active α units 10 fold. These effects targeted at phosphorylase were also evident in 2 fold increases in G1P, G6P, and F6P metabolite levels. A concomitant inhibition occurring at the PFK control site, as was indicated by the simultaneous increase in F6P and decrease in F1,6P₂ levels, respectively, permitted carbon flow in favor of cryoprotectant, glucose. Although glucose levels were quantitatively low, insufficient glycogen levels may provide the explanation for the poor cryoprotectant production in response to freezing in these spring animals. Previous studies examining the persistence of freeze tolerance in spring *H. versicolor* have shown similar low cryoprotectant levels, as well as poor survival rates; glycerol

levels only reached 100 $\mu\text{mol/g}$ and 4/4 frogs died at 4 h freezing at -4°C (Storey and Storey, 1987). Storey and Storey (1987) attributed the minimal rates of synthesis to low stores of glycogen and preferential directioning of those stores to other, more essential fates.

The production of glycerol and glucose as cryoprotectant in a 4 : 1 ratio (400 : 100 $\mu\text{mol/g}$) has been well established in several previous investigations examining autumn H. versicolor in our laboratory (Storey and Storey, 1984). The lack of any glycerol synthesis in this study was significant in terms of seasonal differences between fall and spring cues. There are, from our extensive study of overwintering in both insects and frogs, presumably only two known possible mechanisms by which fall H. versicolor are able to activate the synthesis of molar amounts of glycerol (Storey, 1987a; Churchill and Storey, 1989a). The first mechanism involves the phosphorylation/dephosphorylation of pyruvate kinase (PK) (Storey, 1987a). A concerted activation of glycogen phosphorylase, as was seen in the present study, would increase glycogenolysis and increase carbon flow through glycolysis to permit glycerol synthesis. The second mechanism may act independently or in conjunction with a phosphorylation of pyruvate kinase. One recent study investigating glycolytic control in a freeze avoiding insect that produces glycerol as cryoprotectant, indicated that glycerol synthesis was accomplished by means of a cold activation of the enzyme, glycerol-3-phosphate dehydrogenase (Churchill and Storey, 1989a). Such an activation would also be consistent with glycerol synthesis in this frog species. Whether the first or second mechanism is appropriate in this case, there is a distinct lack of glycerol synthesizing machinery in spring animals, as was indicated by low glycerol levels and no relative changes in PK metabolites, PEP and pyruvate. As well, in either mechanism, PFK must not be inhibited as was seen in this spring study. It may be that the block at PFK was an attempt to increase glucose levels as cryoprotectant but glucose levels remained low. Mechanisms of activating and inactivating PFK (by

phosphorylating and dephosphorylating the enzyme) via protein kinases/phosphatases may be involved and require further investigation. Metabolism may be geared towards other, more timely fates since cryoprotectant production in springtime would be wasteful with respect to essential carbon stores, as well as the energy required to synthesize sugars such as glycerol. Developmental and seasonal cues may also be implicated.

The energy stress during a freezing episode is reflected well in the energy charge value (Atkinson, 1977). In spring frogs, E.C. maintained a constant value of 0.78 throughout the first hour, however subsequent freezing exposure resulted in a dramatic decline in ATP levels (and increases in ADP and AMP) which corresponded to an E.C. value of 0.56. Survival of extremely low E.C. values is possible in several species, including the freeze tolerant frogs; R. sylvatica that had been frozen repeatedly for 1 week exposures recovered even though E.C. fell to 0.34 after the final cycle of freezing (Storey, 1987b; Storey and Storey, 1985). In the present study the anaerobic endproduct, lactate, accumulated 3-4 fold over the first few minutes freezing, possibly indicating a glycolytic activation as a means of maintaining ATP levels and keeping E.C. from dropping.

CHAPTER 5
FREEZING SURVIVAL IN THE COMMON GARTER SNAKE
Thamnophis sirtalis parietalis

SUMMARY

Survival and % ice were determined in the common garter snake, Thamnophis sirtalis parietalis, frozen over a 48 h time course at -2.5°C ; the snakes were only partially freeze tolerant with 50 - 90 % survival at 5 - 10 h. Subsequent metabolite analyses that were performed on tissues from 5 h frozen snakes (liver, kidney, muscle, intestine, brain, eggs, lung, heart) established that the sugars commonly found as cryoprotectants (glucose, glycerol, sorbitol, fructose, and mannose) are not produced in this species. Furthermore, even though maximal ice formation at these temperatures was found to be lethal, exposure to brief periods of freezing that may be encountered in underground dens can be tolerated.

INTRODUCTION

The garter snake is the most northerly distributed reptile in North America (60°N); the range extends up to James Bay in eastern Canada and to northern Alberta in western Canada (Behler, 1979; Cook, 1984). Northern populations migrate considerable distances from summer locations to communal den sites where they hibernate in large numbers, since suitable den sites are scarce. This availability of den sites is believed to limit distribution of garter snakes in the more northerly climates. Communal den temperatures in northern Alberta have been measured at positive near-zero values, but never below freezing, even though outside air temperatures drop to as low as -25 to -35 °C (Macartney et al., 1989). Other vacant dens have shown dramatically low temperatures, comparable to ambient air temperatures (-30 °C). Although den temperatures may maintain plus-zero values throughout most of the winter, garter snakes may have to deal with autumn or spring frosts before and after hibernation which can cause short bouts of freezing, particularly if the animals come in contact with environmental ice.

There are two overwintering strategies possible to escape the detrimental effects of freezing: freeze avoidance and freeze tolerance (Storey and Storey, 1988b and c). Previous literature presumed that all terrestrially hibernating reptiles avoid freezing by whole body supercooling; supercooling points of reptiles are -6 to -8 °C (Paukstis and Shuman, 1989; Storey et al., 1988a). However, because freeze tolerance has recently been well documented for several ectothermic vertebrates (four species of frogs and one reptile) that overwinter at or near the soil surface (Storey and Storey, 1985b, 1986, 1987c, 1988b, c; Storey et al., 1988a; Storey, 1987a, b; Storey and Storey, 1984a, 1985a), a closer examination of the possibility of freeze tolerance in garter snakes seemed necessary in establishing the diversity of the freeze tolerance phenomenon. One study by Constanzo et al. (1988), gave some initial evidence of a limited freeze

tolerance in a more southerly population (Wisconsin) of garter snakes; the snakes survived brief exposure of high subzero temperatures (48 h at -0.75°C) and accumulated no more than 36 % body weight as ice. The present study investigates the freeze survival of garter snakes, *T. s. parietalis*, from a more northern population (Manitoba) in order to assess maximal tolerable % ice, survival, and possible cryoprotectants.

MATERIALS AND METHODS

Animals and Chemicals

Snakes of the species *T. s. parietalis* were obtained from Manitoba, Canada. Average animal weights were: males = 16.4 ± 0.6 g, $n = 25$; females = 64 ± 9 g, $n = 18$. The animals were acclimated at 3°C for at least 2 - 3 weeks prior to experimentation.

Preparation of Experimental Animals

Ice formation was investigated over a 48 h period of exposure at -2.5°C . Snakes were placed in large styrofoam cups with a YSI telethermometer probe taped to the abdomen and were then placed in a -2.5°C incubator. Snakes were allowed to cool to -2.5°C and then freezing was initiated by cutaneous ice nucleation by touching the snake with a cold metal rod (-196°C) followed by incubation at $-2.5 \pm 0.2^{\circ}\text{C}$ for the specified periods of time. Nucleation was observed as an exotherm after the animals had reached temperatures below freezing points, but above supercooling points. $T_r = -0.8 \pm 0.1^{\circ}\text{C}$, $n = 9$; $\text{Scpt.} = -5.5 \pm 0.3^{\circ}\text{C}$ (fall values). After freezing for periods of time up to 48 h, % ice determinations were performed by the calorimetry method outlined by Lee et al. (1985) and as described in chapter 2; survival rates were also determined. Tissues were sampled by immediate excision of the respective tissues

and quickly freezing in liquid nitrogen. Tissues were stored at -80°C until processing. Control animals were killed immediately after removal from the 3°C incubator.

Tissue Preparation and Metabolite Assays

At each time point, four liver samples ($n=4$) were extracted for metabolites via the perchloric acid method as described in chapter 2. All assays were performed as described in chapter 2.

RESULTS

Snakes were investigated for the ability to supercool. Fall animals were cooled from 15°C to 3°C over 2 weeks and then held at 3°C for 2 - 3 weeks. Fall snakes exhibited substantial supercooling capacity down to -5.5°C . Snakes were held from autumn until January/February at 3°C . Tests of these winter snakes for continued supercooling ability found that winter animals possessed minimal supercooling ability; three out of four did not supercool and froze at $-0.8 \pm 0.1^{\circ}\text{C}$ (Table 1). Skin permeability was tested by freezing animals in containers with damp moss. This was tested in order to determine whether or not inoculative freezing due to ice propagation across the skin is a factor in the snakes' natural environment. Freezing points were similar to those of snakes without contact with external moisture; values were -0.3°C with only one incident of supercooling observed.

Ice formation in garter snakes and subsequent survival rates was assessed over a 48 h time course of freezing exposure (at -2.5°C). Half maximal ice content of approximately 35 % occurred after 2.5 h. Average ice content after 24 h was 59.5 % and after 48 h average ice content was 70 % (Fig. 20). Average water content was determined to be 70.5 ± 0.7 % total body weight. Survival over the freezing period

Table 1. Supercooling points (S_{cp}.) and rebound temperatures (Tr) of garter snakes during freezing.

	S_{cp}.	Tr
Dry snakes:		
Fall	-5.5 ± 0.3 °C (4)	-0.8 ± 0.1 °C (3)
Winter	-1.0 °C (1)	-0.8 ± 0.2 °C (4)
Seeded at		
-2.5 ± 0.2 °C:	----	-0.8 ± 0.1 °C (9)
In damp moss:		
Winter	-1.2 °C (1)	-0.3 ± 0.1 °C (4)

Data are means $\pm$ SEM. Values in brackets represent number of animals assessed. Supercooling points that are $n = 1$ were taken from 4 snakes that were frozen; the other 3 snakes exhibited no supercooling.

FIG. 20. Ice formation and survival of freezing by garter snakes held at -2.5°C . Time is measured from the appearance of the freezing exotherm. The percentage of the total body water as ice (o) is shown for individual snakes. The bars show the percentage of individuals that survived. The number of individuals tested for survival was five at 3 or 5 h and four at all other times.

ICE FORMATION IN THAMNOPHIS SIRTALIS

maintained 100 % within the first 2-5 h (30-50 % ice), but quickly dropped off with the formation of a further 5-10 % body ice; snakes frozen for 10 h showed 50 % survival but after 24 h freezing, none recovered (Fig. 20). Eighteen out of twenty snakes that did survive the freezing episodes exhibited signs of life almost immediately after thawing in the calorimeter. Their tails twitched in response to pinching and they were able to right themselves when positioned on their back. Most of the snakes were capable of moving across the lab bench when perturbed. Several snakes that had been exposed to longer times of freezing and that had greater ice contents, required longer recovery times of approximately 1-2 d.

At 5 h, freezing was not complete with respect to internal organs. Although the snakes were rigid, reflecting outer skin and muscle ice formation, blood near the core of the animals was still fluid and the hearts maintained their pumping activity. As well, most visceral organs were not frozen and could be easily excised. The skin of the animals turned from dark greenish-black to a bluish colour when frozen; the eyes also turned milky at 5 - 10 h.

Changes in the levels of putative cryoprotectants and glycogen were measured in organs of snakes in control vs. 5 h freeze exposed animals. Metabolite measurements showed only several changes from control levels depending on the tissue type (Table 2). Liver glucose increased 4 fold during the 5 h freezing episode. No significant amounts of carbohydrate cryoprotectant were found, although glycerol dropped in eggs, kidney, liver, heart; the decline in quantities found in eggs was the only change that showed statistical significance. Sorbitol, fructose, and mannose were also measured but values in all organs were $< 0.55 \mu\text{mol/g}$ (Table 2). Amino acid analyses of all tissues showed large quantities of glutamate and taurine; other amino acids varied to a minor extent, depending on the tissue (Table 3).

Table 2. Cryoprotectant levels in garter snakes 5 h frozen.

	<u>control</u>	<u>frozen</u>
	liver	
glycogen	148 ± 34	233 ± 51
glucose	0.840 ± 0.260	3.430 ± 0.720 ^a
glycerol	1.110 ± 0.220	0.790 ± 0.050
	muscle	
glycogen	54 ± 9	75 ± 9
glucose	0.147 ± 0.063	0.189 ± 0.028
glycerol	0.500 ± 0.040	0.540 ± 0.060
	kidney	
glycogen	44 ± 3	67 ± 7
glucose	0.644 ± 0.257	0.752 ± 0.080
glycerol	1.090 ± 0.290	0.690 ± 0.140
	intestine	
glycogen	15 ± 3	9.2 ± 2.8
glucose	0.450 ± 0.142	0.510 ± 0.070
glycerol	0.980 ± 0.170	0.710 ± 0.120
	lung	
glycogen	6.1 ± 2.2	16.0 ± 2.7
glucose	0.439 ± 0.187	0.454 ± 0.077
glycerol	1.750 ± 0.220	1.330 ± 0.110
	heart	
glycogen	69 ± 20	71 ± 3
glucose	0.336 ± 0.057	0.318 ± 0.095
glycerol	1.450 ± 0.300	0.960 ± 0.120
	brain	
glycogen	58 ± 7	91 ± 20
glucose	0.327 ± 0.209	0.321 ± 0.161
	eggs	
glycogen	63 ± 4	76 ± 13
glucose	0.581 ± 0.175	0.416 ± 0.150
glycerol	1.590 ± 0.320	0.960 ± 0.190 ^a

Data are means ± S.E.M., n = 4. Values are expressed as $\mu\text{mol/g}$. ^a - Significantly different from control values; P < 0.05. Sorbitol, fructose, and mannose levels were < 0.55 $\mu\text{mol/g}$ in all tissues.

Table 3. Levels of amino acids in organs of garter snakes.

Amino acid concentrations (nmol/g wet weight)				
	Liver		Muscle	
	Control	Frozen	Control	Frozen
Aspartate +				
Asparagine	901 ± 205	1143 ± 41 ₄ *	664 ± 287	719 ± 533
Glutamate	1071 ± 44	794 ± 48	571 ± 276	714 ± 439
Serine	255 ± 109	233 ± 12*	675 ± 550	302 ± 105
Glutamine	1928 ± 205	808 ± 118*	3918 ± 3622	3272 ± 881
Glycine	72 ± 9	50 ± 10	284 ± 243	173 ± 83
Threonine	349 ± 292	702 ± 125	152 ± 60	105 ± 42
Arginine	350 ± 306	384 ± 40	5355 ± 5207	2856 ± 2121*
Taurine	17896 ± 1412	14137 ± 603	18061 ± 4330	7890 ± 2654
Alanine	231 ± 30	483 ± 117	533 ± 132	621 ± 79
GABA	228 ± 112	111 ± 8	38 ± 7	1598 ± 1468
Tyrosine	59 ± 35	132 ± 23	141 ± 141	135 ± 31
AABA	109 ± 48	37 ± 3	82 ± 24	71 ± 2
Valine + NH ₄ ⁺	3323 ± 1893	2425 ± 222	2816 ± 3387	4514 ± 1329
Phenylalanine	4 ± 4	8 ± 8	25 ± 25	18 ± 9
Isoleucine	23 ± 3	32 ± 8	6 ± 6	15 ± 15
Leucine	22 ± 14	70 ± 27	14 ± 7	21 ± 21
Lysine	41 ± 23	60 ± 6	56 ± 47	58 ± 20
Total	26862	21609	33391	23082

DISCUSSION

In the present study, fall snakes were found to have supercooling points of -5.5°C whereas winter snakes largely lacked this ability; only one snake showed supercooling to -1.0°C , while three others froze at the freezing point of body fluids, -0.8°C . Garter snakes in Manitoba spend at least 7 months of the year underground in dens hibernating. The capacity for supercooling in the fall gives the snakes a mechanism of avoiding ice formation when temperatures fall below the freezing point of body fluids. This adaptation may be sufficient for dealing with occasional overnight frosts when snakes are above ground and migrating towards appropriate den sites. Supercooling points of some reptiles and amphibians have been recorded as low as -6 to -8°C . However, supercooling in snakes is short term and nucleation occurs within minutes; the only alternative for the snakes is freezing tolerance.

During brief periods of freezing (1 - 5 h), movement was still possible; stiff, rigid motion was observed in all of the larger snakes (20 - 100 g). Environmental studies have shown that hibernating snakes can move between layers in the den depending on ambient temperatures; as temperatures drop to near zero values, animals move to warmer areas (Macartney et al., 1989). If the snakes are prematurely nucleated by the presence of environmental ice, there will be a short lag time before whole body freezing occurs thus permitting time for movement to the warm sections of the den.

In the spring, emergence from hibernacula is a response to warmer spring temperatures, however, evening temperatures may drop to sub-zero values thereby subjecting any emerged snakes to overnight bouts of freezing (Macartney et al., 1989). The apparent freeze tolerance in garter snakes presently documented would be limited to such brief periods of freezing. The amounts of ice tolerable by garter snakes (35 - 40 % body water as ice) are much lower than the amounts tolerated by the freeze

tolerant frogs and even the turtles (Storey and Storey, 1988). Garter snakes can only survive for a few hours at -2.5°C compared to at least 11 days for painted turtle hatchlings in laboratory conditions (in subterranean nests these turtles can survive months frozen) and 14 days for wood frogs (Storey and Storey, 1988).

Cryoprotectant production is a common aspect of freeze tolerance in animals (Zachariassen, 1985; Storey and Storey, 1988c). However, not all organisms that exhibit freeze tolerance show this metabolic adaptation. Several marine molluscs along with the one species of freeze tolerant turtle do not produce any polyhydroxylated compounds; glucose, glycerol, sorbitol, etc. (Storey et al., 1988a; Murphy, 1983; Aarset, 1982). It is believed that the marine invertebrates rely on molar levels of amino acids and the mobilization of Ca^{+2} from shell calcium carbonate in order to stabilize membrane structures and intracellular proteins; key amino acids implicated are proline, glutamate, aspartate, and taurine (Murphy, 1983). The freeze tolerant turtle, *C. p. marginata*, also relies on several of these amino acids, taurine and glutamate, as well as increases in total amino acid pools to survive 24 h in the frozen state (Storey et al., 1988a). In this study, glutamate levels remained low during freezing ($0.5 - 2.5 \mu\text{mol/g}$), whereas taurine levels were more substantial; ranging from $8 - 24 \mu\text{mol/g}$. The present study found no synthesis of carbohydrate cryoprotectant in the common garter snake; although there were minor increases in glycerol and glucose in some tissues, they were not significant in terms of cryoprotectant capacity. These snakes maintained substantial glycogen supplies ($150 - 250 \mu\text{mol/g}$ in liver) and therefore low cryoprotectant cannot be attributed to poor glycogen reserves. Nevertheless, the snakes were clearly able to survive a 4-7 h freezing episode with 55 - 65 % total body water as ice (Fig. 20).

CHAPTER 6

ANOXIA TOLERANCE PERMITS SURVIVAL OF BRIEF EXPOSURE TO FREEZING IN THE COMMON GARTER SNAKE

Thamnophis sirtalis parietalis

SUMMARY

Garter snakes, *Thamnophis sirtalis parietalis*, have been reported to be partially freeze tolerant (Chapter 3). The similarity of the metabolic responses to freezing stress and to those of anoxia stress is examined in the present investigation. Metabolite analyses were performed on tissues from snakes exposed to 5 h freezing and 5 h anoxia (liver, muscle, heart, kidney, lung, intestine, brain, eggs) to determine any relationship between the two stresses. Adenylate and energy charge (E.C.) (Atkinson, 1977) levels were not dramatically affected under either of the stresses in most of the organs; E.C. values maintained a range of 0.80 - 0.86. However, muscle and intestine E.C. values dropped to 0.36 and 0.58 during freezing. Anaerobic endproducts, lactate, alanine, and succinate, accumulated in all of the tissues, however increases were greater in anoxic snakes than frozen animals. Lactate production varied depending on tissue type and stress; brain and muscle levels reached 10 $\mu\text{mol/g}$, whereas kidney and eggs show no changes. Changes in the substrates and products of phosphofructokinase (PFK) and pyruvate kinase (PK) generally revealed an inhibition of glycolysis at PFK during freezing. Breakdown of endogenous liver glycogen effectively supplied the other tissues with carbohydrate substrate via blood glucose. After 5 h anoxia, however, an activation of PFK was generally effected, although PK was implicated in kidney and heart. It was established that although these snakes may be partially freeze tolerant, their tolerance of anoxia may be the determining factor in surviving short term freezing.

INTRODUCTION

The garter snake, *Thamnophis sirtalis parietalis*, is the most northerly distributed reptile species in North America able to survive short term exposure to freezing. Their tolerance to freezing episodes is limited with 100 % recovery extending to 3 h at -2.5 °C (80 % at 5 h freezing exposure) (previous chapter). Tolerable ice contents reach as much as 40 % of total body water as ice at 3 h and even 50 % at 5 - 8 h. The relevance of this limited freeze tolerance in the snakes' natural environment is questionable since garter snakes preferentially select appropriate denning sites where temperatures rarely reach subzero values. However, further investigation of the partial freeze tolerance exhibited in garter snakes may provide insights into the development of freeze tolerance in vertebrates.

During a typical freezing exposure, all heart activity and blood flow is stopped, thus anaerobic conditions prevail within. Tolerance to anoxia is another key factor in determining survival of the frozen state. Even sensitive tissues, such as the brain, must be able to withstand substantial periods of oxygen lack with their accompanying increases in lactate and alterations in acid-base balance within the tissue. Levels as high as 12, 31, 35 $\mu\text{mol/g}$ in liver, heart, and kidney have been found in frogs frozen for 4 days (Storey, 1987b). Freeze tolerant frogs are capable of surviving up to 5 d anoxia at 5 °C in laboratory conditions (Churchill and Storey, unpublished data); survival times are presumably much longer (months) in a natural environment since they survive whole body freezing throughout the entire winter months. As well, garter snakes exhibit a similar tolerance to anoxia under laboratory conditions. Snakes are capable of surviving up to 48 h anoxia at 5 °C by shutting down metabolic flux through glycolysis and producing substantial amounts of lactate in order to replenish ATP stores (Churchill and Storey, unpublished data). With any freezing stress there is a component of anoxic stress involved. It may be that in this lower vertebrate, its

tolerance to anoxia may make an important contribution to creating a partial freeze tolerance. If this is the case, then, development of the freeze tolerance phenomenon, in lower vertebrates, may be linked to a tolerance to anoxia.

In the present study, metabolite analyses were performed on all tissues in frozen and anoxic snakes in order to determine any metabolic similarities between freezing and anoxia.

MATERIALS AND METHODS

Animals and Chemicals

Garter snakes, *T. s. parietalis*, were obtained from Manitoba, Canada. The animals were collected in early autumn and acclimated at 3 °C for at least 2 - 3 weeks prior to experimentation.

Preparation of Experimental Animals

A 5 h freezing episode was compared to a 5 h period of anoxia in terms of metabolite analyses. Frozen snakes were prepared as in Chapter 5.

A second group of animals were made anoxic by flushing a glass jar with a gaseous mixture of 2.5 % CO₂/97.5 % N₂ for 10 - 15 min at 6 °C. Tissues were sampled after 5 h freezing or 5 h anoxic exposure by immediate excision of the respective tissues and quickly freezing in liquid nitrogen. Tissues were stored at -80 °C until processing. Control animals were killed immediately after removal from the 3 °C incubator.

Tissue Preparation and Metabolite Assays

At each time point, four liver samples ($n=4$) were extracted for metabolites via the perchloric acid method as described in chapter 2. Aliquots of the neutralized extract were used immediately for pyruvate, ATP, ADP, AMP assays. The neutralized extract was then frozen at -80°C and used as the source for subsequent metabolite assays. All assays were performed as described in chapter 2.

RESULTS

Survival

Twelve out of twelve snakes survived 5 h anoxia and ten out of ten snakes survived 48 h anoxia exposure; assessment of survival was determined as recovery of normal movement and response to probing immediately following removal from anoxia and as well as 24 h post-removal (kept at 5°C). However, four out of four snakes did not recover from 96 h anoxia; the snakes were dead prior to removal from the jars.

Adenylates and Energy Stress

Metabolite analyses of 5 h frozen snakes showed similarity with respect to 5 h anoxic animals, however, the freezing stress was seen to be unique in several aspects. Adenylates generally maintained control values during the 5 h freezing and consequently the energy charge was kept high in most of the organs (kidney, lung, heart, brain, eggs); E.C.'s were 0.80 - 0.86 (Fig. 21). However, the stress of freezing was more apparent in the remaining three organs, liver, muscle, intestine, in which changes in ATP, ADP, and AMP corresponded to decreases in E.C. (ATP dropped, ADP and AMP increased or decreased less) (Table 4); E.C. fell dramatically from a range of 0.70 - 0.83 to 0.53 - 0.61 (Fig. 21). After 5 h anoxia, energy charge was maintained in all tissues except liver, intestine, and kidney. Similar to the freezing

FIG. 21. Energy Charge values $[(ATP + 1/2 ADP)/(ATP + ADP + AMP)]$, in organs of garter snakes. Conditions are: , aerobic, control snakes at 3 °C; , 5 h freezing exposure at -2.5 °C; , 5 h anoxia exposure at 6 °C. ^a - Significantly different from the corresponding control value by the Student's t-test, $P < 0.01$; ^b - $P < 0.05$.

Table 4. Levels of adenylates in garter snake organs under freezing or anoxia stresses.

	ATP	ADP nmol/g wet weight	AMP
Liver			
Control	3710 ± 180	1160 ± 410	359 ± 139
Frozen	1630 ± 430 ^a	2420 ± 370 ^b	796 ± 42 ^b
Anoxic	1410 ± 160 ^a	575 ± 46	193 ± 12
Skeletal muscle			
Control	489 ± 70	450 ± 17	50 ± 12
Frozen	160 ± 70 ^a	202 ± 57 ^a	124 ± 72
Anoxic	220 ± 50 ^b	162 ± 20 ^a	33 ± 3
Heart			
Control	1760 ± 140	324 ± 32	161 ± 26
Frozen	1940 ± 474	589 ± 67	154 ± 33
Anoxic	1700 ± 270	400 ± 85	97 ± 37
Brain			
Control	460 ± 10	208 ± 47	243 ± 9
Frozen	265 ± 124	135 ± 64	141 ± 41 ^b
Anoxic	520 ± 50	346 ± 28 ^b	147 ± 25 ^a
Kidney			
Control	1100 ± 60	174 ± 35	123 ± 27
Frozen	1010 ± 90	215 ± 87	141 ± 9
Anoxic	770 ± 100 ^b	364 ± 59 ^b	137 ± 16
Lung			
Control	980 ± 100	353 ± 79	166 ± 48
Frozen	1060 ± 110	305 ± 34	142 ± 11
Anoxic	1170 ± 80	290 ± 56	196 ± 20
Intestine			
Control	690 ± 40	386 ± 35	160 ± 11
Frozen	410 ± 40 ^a	284 ± 10 ^b	228 ± 32 ^b
Anoxic	530 ± 90	550 ± 47 ^b	346 ± 19 ^a

Data are means ± SEM, n = 4 animals for each condition. ^a - Significantly different from the corresponding control value using the Student's t-test, P < 0.01; ^b - P < 0.05.

exposure, intestine showed a dramatic decline in E.C. from 0.74 to 0.56 in anoxia, whereas liver and kidney were affected to a lesser extent; final E.C.'s were 0.78 and 0.75, respectively. Under this stress, liver total adenylates dropped to 50 % of control quantities, thereby buffering the effect of ATP decreases on E.C. (Fig. 21 and 22).

Anaerobic Endproducts

Anaerobic endproduct accumulation showed variable increases, depending upon type of stress and tissue (Table 5). In all organs, freezing exposure resulted in fewer endproducts than the anoxic stress. In a few cases, where anaerobic endproducts were seen to increase during 5 h freezing, a greater accumulation occurred in the same organ after 5 h anoxia. In liver, the freezing exposure resulted in an increase in alanine and succinate; 109 % and 74 %, respectively. After 5 h anoxia, lactate as well showed an increase; liver lactate rose from 4.9 to 5.5 $\mu\text{mol/g}$. In either stress, kidney showed increases in only alanine; net accumulation during anoxia were 4 fold greater than in freezing and changes in aspartate levels accounted for 100 % of the alanine produced. Similarly, lactate increases in heart and brain were greater in anoxia than freezing; during the anoxic stress, succinate increased by 123 % over control levels in heart. Lactate, alanine, and succinate increased in lung and intestine only during the exposure to anoxia; whereas muscle exhibited increases in lactate and alanine. Eggs revealed no elevation of any of the endproducts measured. Aspartate, being the probable source of succinate (and alanine) by means of a transaminase reaction with pyruvate, generally decreased in the organs in which succinate rose (liver, heart, lung, intestine, kidney) as well as muscle, where succinate decreased during freezing and anoxia.

FIG. 22. Total adenylates (ATP + ADP + AMP), in organs of garter snakes.

Conditions are: , aerobic, control snakes at 3 °C; , 5 h freezing exposure at -2.5 °C; , 5 h anoxia exposure at 6 °C. ^a - Significantly different from the corresponding control value by the Student's t-test, P < 0.01.

Table 5. Levels of anaerobic end products and aspartate in garter snake organs under freezing or anoxia stresses.

	Lactate	L-Alanine $\mu\text{mol/g wet weight}$	Succinate	L-Aspartate
Liver				
Control	4.88 ± 0.54	0.23 ± 0.03	0.34 ± 0.05	1.40 ± 0.22
Frozen	4.57 ± 0.18	0.48 ± 0.12	0.59 ± 0.07^b	1.62 ± 0.14
Anoxic	5.51 ± 0.34	0.72 ± 0.21^b	0.49 ± 0.10	1.04 ± 0.09
Skeletal muscle				
Control	6.15 ± 0.10	0.53 ± 0.13	0.27 ± 0.03	0.56 ± 0.05
Frozen	4.69 ± 0.57^b	0.62 ± 0.08	0.24 ± 0.06	0.34 ± 0.04^a
Anoxic	7.98 ± 0.81^b	0.91 ± 0.30	0.23 ± 0.04	0.20 ± 0.07^a
Heart				
Control	4.13 ± 0.30	0.43 ± 0.14	0.22 ± 0.05	1.54 ± 0.33
Frozen	6.62 ± 0.75^b	0.61 ± 0.10	0.34 ± 0.05	2.11 ± 0.18
Anoxic	7.30 ± 0.92^b	0.65 ± 0.08	0.49 ± 0.05^a	0.37 ± 0.08^a
Brain				
Control	6.06 ± 1.45	0.40 ± 0.12	1.12 ± 0.15	n.d.
Frozen	9.49 ± 0.91^b	0.61 ± 0.20	1.03 ± 0.31	n.d.
Anoxic	10.7 ± 0.85^b	0.56 ± 0.16	0.89 ± 0.10	n.d.
Kidney				
Control	5.18 ± 0.18	0.33 ± 0.04	0.52 ± 0.17	1.39 ± 0.30
Frozen	5.22 ± 0.40	0.55 ± 0.05^a	0.46 ± 0.12	1.13 ± 0.13
Anoxic	5.42 ± 0.63	1.22 ± 0.36^b	0.64 ± 0.05	0.34 ± 0.11^a
Lung				
Control	4.07 ± 0.07	0.26 ± 0.06	0.23 ± 0.01	1.28 ± 0.23
Frozen	4.44 ± 0.19	0.24 ± 0.01	0.23 ± 0.02	1.50 ± 0.18
Anoxic	5.50 ± 0.54^b	0.45 ± 0.05^b	0.57 ± 0.08^a	0.48 ± 0.13^a
Intestine				
Control	3.98 ± 0.41	0.45 ± 0.09	0.50 ± 0.11	1.73 ± 0.16
Frozen	4.02 ± 0.13	0.19 ± 0.03^b	0.44 ± 0.20	1.88 ± 0.27
Anoxic	5.49 ± 0.58^b	0.70 ± 0.18	1.96 ± 0.05^a	0.83 ± 0.12^b
Eggs				
Control	3.78 ± 0.51	1.32 ± 0.20	0.43 ± 0.15	2.83 ± 0.12
Frozen	3.45 ± 0.13	1.64 ± 0.01	0.23 ± 0.06	2.58 ± 0.10

Data are means $\pm$ SEM, $n = 4$ animals for each condition.

^a - Significantly different from the corresponding control value using the Student's t -test, $P < 0.01$; ^b - $P < 0.05$.

Glycolytic Regulation: Changes in Metabolite Levels

Liver

Table 6 shows glucose levels rose 4 fold in response to the freezing. Both % α and total glycogen phosphorylase showed no significant changes from control values, although % α maintained a high percentage at 85 % during exposure to freezing (Table 6). Glycogen levels, however, showed no changes from control quantities.

Accompanying the increase in liver glucose, corresponding increases in the hexose phosphate pool also occurred (G6P and F6P both increased 3 - 4 fold) (Table 7). Liver metabolite levels in frozen snakes clearly revealed an activation of glycogenolysis.

Levels of substrate (F6P) and product (F1,6P₂) of phosphofructokinase (PFK) exhibited an increase of 400 % and no change from control levels, respectively (Table 7). This indicated an apparent inhibition at this key regulatory control enzyme. No significant changes were observed in the pyruvate kinase substrate (PEP) and product (pyruvate), thus suggesting no role in regulatory control.

During exposure to anoxia, glucose levels showed an increase of 2 fold (Table 6). However, % α phosphorylase dropped to 20 %, apparently in contradiction to an active state of glycogenolysis (Table 6). Accompanying the increase in glucose levels, quantities of hexose phosphates, G6P and F6P exhibited increases (Table 7). Although F1,6P₂ levels increased slightly (2 fold), F6P levels rose dramatically over control values (7 fold) (Table 7). Thus, PFK showed an inhibition similar to the frozen snakes. However, a 2 fold increase in the key activator of PFK, F2,6P₂, from 3.85 to 8.13 nmol/g did not correspond with metabolite changes indicating PFK inactivation (Table 8). Increases in the potent allosteric effector molecule may have been solely due to the mass action effects of high F6P on PFK 2; F6P levels may have risen greater than the K_m for PFK 2, which catalyses the phosphorylation of F6P to yield F2,6P₂.

Table 6. Glycogen metabolism in garter snake organs under freezing or anoxia stresses.

	Glycogen $\mu\text{mol/g}$	Glucose $\mu\text{mol/g}$	Glycogen phosphorylase U/g wet wt.	% a
Liver				
Control	148 $\pm$ 34	0.84 $\pm$ 0.26	0.92 $\pm$ 0.17	76 $\pm$ 5
Frozen	233 $\pm$ 51	3.43 $\pm$ 0.72 ^a	1.44 $\pm$ 0.22	85 $\pm$ 22
Anoxic	166 $\pm$ 46	1.85 $\pm$ 0.41 ^b	1.17 $\pm$ 0.14	20 $\pm$ 4 ^a
Skeletal muscle				
Control	54 $\pm$ 9	0.15 $\pm$ 0.06	21.7 $\pm$ 0.9	41 $\pm$ 6
Frozen	75 $\pm$ 9	0.19 $\pm$ 0.03	20.7 $\pm$ 1.0	46 $\pm$ 8
Anoxic	86 $\pm$ 10 ^b	0.45 $\pm$ 0.05 ^a	18.2 $\pm$ 2.4	18 $\pm$ 7 ^b
Heart				
Control	69 $\pm$ 20	0.34 $\pm$ 0.06	0.36 $\pm$ 0.07	16 $\pm$ 3
Frozen	71 $\pm$ 3	0.32 $\pm$ 0.10	0.71 $\pm$ 0.16 ^b	82 $\pm$ 6 ^a
Anoxic	76 $\pm$ 7	0.53 $\pm$ 0.10	1.85 $\pm$ 0.50 ^b	6 $\pm$ 2 ^b
Brain				
Control	58 $\pm$ 7	0.33 $\pm$ 0.21	4.35 $\pm$ 0.44	78 $\pm$ 5
Frozen	91 $\pm$ 20	0.32 $\pm$ 0.16	4.23 $\pm$ 0.30	81 $\pm$ 4
Anoxic	86 $\pm$ 6 ^b	0.59 $\pm$ 0.17	4.89 $\pm$ 0.55	64 $\pm$ 5 ^b
Kidney				
Control	44 $\pm$ 4	0.64 $\pm$ 0.26	0.51 $\pm$ 0.05	37 $\pm$ 5
Frozen	67 $\pm$ 7 ^b	0.75 $\pm$ 0.08	0.56 $\pm$ 0.02	41 $\pm$ 9
Anoxic	33 $\pm$ 7	0.56 $\pm$ 0.11	0.90 $\pm$ 0.11 ^b	20 $\pm$ 3 ^b
Lung				
Control	6 $\pm$ 2	0.44 $\pm$ 0.19	0.26 $\pm$ 0.03	37 $\pm$ 8
Frozen	16 $\pm$ 3 ^b	0.45 $\pm$ 0.08	0.25 $\pm$ 0.02	35 $\pm$ 5
Anoxic	8 $\pm$ 3	0.34 $\pm$ 0.10	0.65 $\pm$ 0.07 ^a	18 $\pm$ 5
Intestine				
Control	15 $\pm$ 3	0.45 $\pm$ 0.14	0.60 $\pm$ 0.06	21 $\pm$ 5
Frozen	9 $\pm$ 3	0.51 $\pm$ 0.07	0.46 $\pm$ 0.09	35 $\pm$ 16
Anoxic	9 $\pm$ 4	0.21 $\pm$ 0.06	1.27 $\pm$ 0.17 ^a	20 $\pm$ 4
Eggs				
Control	63 $\pm$ 4	0.58 $\pm$ 0.18	1.48 $\pm$ 0.36	31 $\pm$ 3
Frozen	76 $\pm$ 13	0.42 $\pm$ 0.15	1.38 $\pm$ 0.33	39 $\pm$ 3

Data are means $\pm$ SEM, n = 4 animals for each condition. Glycogen is expressed in glucose units. ^a - Significantly different from the corresponding control value using the Student's t-test, P < 0.01; ^b - P < 0.05.

Table 7. Glycolytic intermediates in garter snake organs under freezing or anoxia stresses.

	G6P	F6P	F1,6P₂ nmol/g wet weight	GAP + DHAP	PEP	PYR
Liver						
Control	18 ± 2	7 ± 3	56 ± 15	333 ± 67	96 ± 20	286 ± 52
Frozen	68 ± 14 ^a	31 ± 7 ^a	76 ± 5	359 ± 20	107 ± 22	179 ± 20
Anoxic	61 ± 6 ^a	49 ± 18 ^b	97 ± 4 ^b	233 ± 63	150 ± 31	247 ± 46
Skeletal muscle						
Control	254 ± 68	121 ± 32	73 ± 10	230 ± 45	145 ± 33	170 ± 11
Frozen	728 ± 84 ^a	210 ± 35	14 ± 3 ^a	277 ± 30	74 ± 42	149 ± 7
Anoxic	345 ± 72	185 ± 78	53 ± 15	314 ± 41	162 ± 54	88 ± 10 ^a
Heart						
Control	102 ± 17	< 5	16 ± 6	29 ± 6	111 ± 40	91
Frozen	138 ± 35	< 5	14 ± 3	28 ± 4	105 ± 12	117 ± 46
Anoxic	155 ± 30	< 5	11 ± 3	31 ± 9	127 ± 46	200 ± 11
Kidney						
Control	14 ± 10	8	< 10	388 ± 160	262 ± 73	146 ± 13
Frozen	31 ± 11	8 ± 2	< 10	297 ± 79	246 ± 120	68 ± 14 ^b
Anoxic	8	4 ± 2	< 10	163 ± 31	75 ± 14 ^b	82 ± 16 ^b
Lung						
Control	48 ± 6	14 ± 3	120 ± 8	195 ± 27	97 ± 25	99 ± 18
Frozen	94 ± 13 ^a	17 ± 2	117 ± 50	178 ± 24	169 ± 80	102 ± 13
Anoxic	47 ± 2	11 ± 2	79 ± 23	218 ± 38	99 ± 31	91 ± 17
Intestine						
Control	19 ± 4	6 ± 2	147 ± 89	163 ± 44	181 ± 22	129 ± 31
Frozen	22 ± 8	9 ± 2	56 ± 24	138 ± 22	194 ± 54	77 ± 13
Anoxic	47 ± 7 ^a	5 ± 3	264 ± 57	211 ± 34	164 ± 17	100 ± 23

Data are means ± SEM, n = 4, animals for each condition. ^a - Significantly different from the corresponding control value using the Student's t-test, P < 0.01; ^b - P < 0.05.

Table 8. Levels of fructose-2,6-P₂ in garter snake organs.

	Control	Frozen	Anoxic
	nmol/g wet weight		
Liver	3.85 ± 1.12	3.82 ± 0.82	8.13 ± 3.23 ^a
Skeletal muscle	2.18 ± 0.28	2.26 ± 0.30	1.87 ± 0.51
Heart	0.21 ± 0.01	0.23 ± 0.06	0.10 ± 0.01 ^b
Brain	5.51 ± 0.79	5.58 ± 1.80	4.04 ± 0.22
Kidney	0.73 ± 0.11	0.62 ± 0.15	1.22 ± 0.33
Lung	1.33 ± 0.35	0.36 ± 0.07 ^a	2.14 ± 0.27
Intestine	0.56 ± 0.06	1.34 ± 0.25 ^a	0.76 ± 0.27

Data are means ± SEM, n = 4 animals. ^a - Significantly different from the corresponding control value, P < 0.05; ^b - P < 0.005.

Again, no significant changes were observed in PK crossover metabolites, PEP and pyruvate (Table 7).

Muscle

Exposure to freezing revealed no increase in breakdown of internal glycogen stores; glycogen levels remained constant and glucose levels showed no increases (Table 6). As well, % α and total phosphorylase showed no changes from control values (Table 6). F6P levels showed no significant changes from control values during freezing; F1,6P₂, however, dropped to 20 % control values (Table 7). Since G6P and F6P are interrelated by the equilibrium enzyme, phosphoglucose isomerase, the dramatic 3 fold increase in G6P may be interpreted as indicating an inhibition of PFK. Effector levels of F2,6P₂ were not able to account for the decrease in flux through PFK; levels maintained control values (Table 8). No alteration in PK activity was detected; pyruvate concentrations did not change (Table 7).

Glycogenolytic activity dropped as was evident from the decrease in % α phosphorylase from 41 % to 18 % (Table 6). However, glucose concentrations doubled, most likely as a result of glucose mobilization from the liver (Table 6). F6P and F1,6P₂ quantities showed no significant changes from control values suggesting no change in PFK activity (Table 7). Pyruvate levels dropped to 50 % of control values and PEP showed no changes, which suggests a possible inhibition of PK. The anaerobic endproduct, lactate, increased 4.5 $\mu\text{mol/g}$ presumably in order to maintain ATP quantities (Table 5).

Heart

During freezing no regulatory control was seen to be effected by either PFK or PK loci since metabolite levels did not change (Table 7). However, there was an activation of glycogen phosphorylase in terms of both % α and total phosphorylase

units; % α increased from 16 to 82 % and total units doubled to 0.71 IU/g (Table 6). The net effect on units of α was a 10 fold increase in activity.

During anoxia, no control was detected at PFK even though F2,6P₂ levels dropped 50 % (Table 8). Glycogen phosphorylase % α decreased to 38 % control values but because of the compensatory effect of total phosphorylase units, net active α units actually increased; total phosphorylase rose 5 fold (Table 6). Regulation of glycolysis was evident as an activation of PK, pyruvate doubled whereas PEP remained constant (Table 7).

Brain

Glycogen phosphorylase activity maintained high control values during both stresses, freezing and anoxia. The % α maintained control values during the freezing bout at 81 % (Table 6). After the 5 h exposure to anoxia % α values dropped to 64 % (Table 6). F2,6P₂ presumably effected rapid flux through PFK by establishing high effector levels throughout the duration of the stresses; levels were 5.5 nmol/g among the highest organ levels of this effector (Table 8).

Kidney

Control of glycolysis in the kidney was not clearly defined in either the freezing or anoxic stress (Table 6 and 7). G6P and F6P levels showed no significant increases after 5 h freezing (Table 7). Glycogen phosphorylase showed no signs of activation in either % α or total phosphorylase units (Table 6). No regulatory control was apparent at PFK as was evident by a lack of change in F6P and F1,6P₂ contents (Table 7). During freezing, a distinct change was detected in a drop in pyruvate levels to 50 % control values. Although no increase in PEP quantities was observed, as would be indicative of an inhibition of PK, it may be possible that PK was inactivated and the effects were only detected through the depletion of pyruvate concentrations (Table 7).

During anoxia, G6P and F6P levels decreased by at least 50 % (Table 7). Glycogen phosphorylase changed in terms of both % α and total phosphorylase units, however, there was no net decrease in active α units; % α dropped by 50 % and total units increased by 80 % (Table 6). No change was observed in F1,6P₂ or in F2,6P₂ levels (Table 7 and 8). However, the dramatic decline in PEP levels might reflect an activation of PK, which would correlate well with the observed depletion of triose phosphate and hexose phosphate reserves.

Lung

During 5 h freezing, F1,6P₂ showed no changes from controls, G6P increased significantly by 200 % and F6P showed no significant increase (Table 7). These metabolite changes suggest that PFK was inactivated in 5 h frozen snakes. Hexose phosphate accumulation was not correlated with increased glycogenolysis, since % α and total phosphorylase units remained unaltered from control values (Table 6). Additionally, F2,6P₂ levels revealed a dramatic decline from 1.33 to 0.36 nmol/g (Table 8). PEP and pyruvate quantities remained unchanged (Table 7).

During exposure to 5 h anoxia, there were no significant changes in % α as values ranged from 18 - 37 %. Total phosphorylase units increased by 2.5 fold over control levels; levels at 5 h anoxia were 0.65 U/g (Table 6). No significant changes were apparent in activities of either PFK or PK, since substrate and product levels remained unchanged from control values (Table 7). As well, F2,6P₂ levels did not change (Table 8).

Intestine

Regulation of glycolysis during the freezing episode was not apparent at either PFK or PK. PFK substrate and product, F6P and F1,6P₂, did not exhibit any significant changes (Table 7). DHAP + GAP values showed no change from the

control value (Table 7). F2,6P₂ levels rose 2.4 fold rise (Table 8) which might suggest an activating effect on PFK. PK substrate (PEP) and product (pyruvate) levels indicated no change in PK from control activity (Table 7).

The effects of anoxia consisted of a 2 fold activation of total phosphorylase units, % α remained unaltered at 20 % (Table 6). This increase in breakdown of internal glycogen stores was evident by a concomitant 2.5 fold elevation in G6P quantities (Table 7). PFK substrate and product levels showed no significant changes from control values (Table 7). Levels of the potent allosteric effector, F2,6P₂, were not significantly different from control values (Table 8). Again, metabolite changes indicated that there was no control elicited by PK during anoxia.

Eggs

During freezing, no change in either % α or total phosphorylase units was detected (Table 6).

DISCUSSION

During a freezing stress, energy requirements imposed by a gradual stoppage of blood flow and gas exchange must be fulfilled. As the organism freezes, metabolism is forced to be rerouted to glycolysis; anaerobic endproducts lactate, and sometimes alanine and succinate, accumulate in order to maintain the ATP pool (Lutz et al., 1985; Storey and Storey, 1984). An initial attempt to keep ATP levels high by rapid endproduct synthesis is usually successful, but after longer term freezing episodes the organism must cope with dramatic declines in ATP and energy charge levels (Storey and Storey, 1986). After 24 h freezing at -4 °C, *R. sylvatica* accumulated 15 - 30 μ mol/g lactate in liver, kidney, and brain (Storey, 1987b). However after 3 successive

freeze-thaw cycles, energy charge dropped to 0.34 in *R. sylvatica* (Storey and Storey, 1985). All four species of freeze tolerant frogs exhibit such a tolerance and survival rates of 100 % are not uncommon (Storey and Storey, 1986, 1988). In the present study, all tissues of garter snakes with the exception of muscle and intestine demonstrated a maintenance of adenylate levels and hence, energy charge during freezing. The maintenance of the energy pools observed after 5 h total anoxia reflected the anaerobic capacity of garter snakes which would undoubtedly be experienced during entry into the frozen state. Indeed, the fact that ten out of ten snakes survived 48 h anoxia demonstrated the capability of garter snakes to tolerate anoxia. Anaerobic endproducts, lactate, alanine, and succinate, accumulated to varying degrees in each of the eight organs. Anoxia brought about greater accumulation as would be expected since the frozen snakes would not have been fully anoxic during the entire 5 h. The dramatic drop in energy charge in frozen muscle was most likely due to structural damage as a result of ice formation, and not anaerobic conditions since 5 h anoxic muscle showed high ATP and E.C. values. Intestine, however, was unable to maintain high adenylate and E.C. values during either stress. Diving turtles are well known for their extreme tolerance of month-long periods of anoxia during long hibernating dives (Kelly and Storey, 1988). The liver has been shown to experience dramatic declines in energy state after 15 hours anoxia (at 22 °C) [survival increases to 4 months at 3 °C]; a subsequent resumption of an aerobic environment restores ATP and E.C. levels (Clark and Miller, 1973).

Crossover theory (Williamson, 1970) states that if an enzyme is regulating flux through a metabolic pathway, metabolite levels of substrate and product will change relative to control levels. For example, if the regulating enzyme is activated, then substrate levels will decrease and levels of product of that reaction will increase. Conversely, if the enzyme is inactivated, levels of substrate and product will increase

and decrease, respectively. An analogy is that of a dam blocking the flow of a river; if the dam (enzyme) blocks flow, then the water (substrate) will rise on the upstream side of the dam etc. A crossover plot is a diagram with metabolite levels plotted relative to control values during a particular stress; control levels are represented on the diagram as a horizontal line at 100 %. The metabolites along the metabolic pathway (glycolysis in this case) are placed in order that they occur along that pathway (ie. for glycolysis: G6P, F6P, F1,6P₂, DHAP + GAP, PEP, pyruvate) and the relative values are connected. At any point along the plot where the line joining relative metabolite levels intersects the 100 % control line, is termed a "crossover" point and implicates the enzyme that catalyzes those metabolites as being regulatory either by activation or inhibition.

The crossover analyses revealed general patterns in glycolytic control with minor variations in some tissues of snakes. In most of the organs, the freezing episode induced a general shut-down of the glycolytic pathway (except in heart which exhibited increased flux) effected primarily at PFK, although PK may have been the regulating enzyme in several of the tissues (kidney and heart). Liver glycolytic flux was directed to the production of glucose via a concerted effort of an increase in glycogen breakdown and a blockage of metabolism at PFK. Glucose mobilization from the liver permits organs lacking in metabolic substrate to take up glucose from the blood once plasma sugar levels have increased. Metabolism in the heart, however, did not initially rely on liver glucose production and was controlled solely by the increase in glycogenolysis as was strongly suggested by the 10 fold rise in units of α .

Crossover diagrams for garter snake tissues are shown in Fig. 23 - 28 and help visualize any metabolic regulation that may be present during either stress. Anoxic liver revealed PFK inactivation similar to frozen snakes, along with a dramatic decline in phosphorylase activity thereby effecting its gluconeogenic role (Fig. 23). Also at

FIG. 23. Cross-over diagram showing percentage changes in the levels of glycolytic intermediates in liver of garter snakes after 5 h freezing exposure (○), or 5 h anoxia exposure (●), expressed as a percentage of the corresponding control value.

FIG. 24. Cross-over diagram showing percentage changes in the levels of glycolytic intermediates in muscle of garter snakes after 5 h freezing exposure (○) or 5 h anoxia exposure (●), expressed as a percentage of the corresponding control value.

Crossover Diagram: Liver ⁹⁷

Crossover Diagram: Muscle

this time in liver, total adenylates had dropped to 50 % control values; E.C. was 0.78. The data presented agree with the theory that an active state of glycogenolysis had been established in order to temporarily aid in restoration of energy stores in other tissues. However prolonged anoxia induced conservation of liver carbohydrate as was suggested by the drop in phosphorylase activity. Liver metabolism required restoration of endogenous energy stores at this time. Subsequent reversal of the inactivated status of PFK, presumably via increasing levels of the potent activator molecule, F2,6P₂, would predictably effect regeneration of ATP and phosphagen levels (Storey, 1988). The crossover diagram showed the possible inhibition of PFK, however some of the metabolite data had large errors associated with them, thus making the crossovers in muscle less reliable (Fig. 24). The crossover plots for kidney, lung, and intestine either showed no crossovers or had extremely large errors associated with the metabolite data, thus making the crossovers unreliable (Fig. 25, 26, 27). The heart utilized an additional activation of PK, along with maintenance of increased glycogen phosphorylase activity in order to maintain function of the organ during anoxia (Fig. 28). Brain was unaffected throughout either stress; both glycogen phosphorylase and F2,6P₂ levels strongly suggested a high basal activity of glycolysis which would not be unexpected since maintenance of this vital organ is essential to the homeostatic function of the whole animal.

It is apparent, now, that freezing in this species of snake is very similar to an anoxic stress; although formation of ice in tissues may lead to unique structural damage, as was suggested by the drop in muscle adenylates during freezing but not during 5 h anoxia. Whether garter snakes will actually encounter brief freezing episodes in their natural habitat is a fundamental question in establishing whether this partial freeze tolerance is an adaptive strategy or if it is a result of an inherent anoxia tolerance. Den temperatures over winter have been shown to reach near-freezing

FIG. 25. Cross-over diagram showing percentage changes in the levels of glycolytic intermediates in kidney of garter snakes after 5 h freezing exposure (○) or 5 h anoxia exposure (●), expressed as a percentage of the corresponding control value.

FIG. 26. Cross-over diagram showing percentage changes in the levels of glycolytic intermediates in lung of garter snakes after 5 h freezing exposure (○) or 5 h anoxia exposure (●), expressed as a percentage of the corresponding control value.

Crossover Diagrams: Kidney

Crossover Diagram: Lung

FIG. 27. Cross-over diagram showing percentage changes in the levels of glycolytic intermediates in intestine of garter snakes after 5 h freezing exposure (○) or 5 h anoxia exposure (●), expressed as a percentage of the corresponding control value.

FIG. 28. Cross-over diagram showing percentage changes in the levels of glycolytic intermediates in heart of garter snakes after 5 h freezing exposure (○) or 5 h anoxia exposure (●), expressed as a percentage of the corresponding control value.

Crossover Diagrams: Intestine

Crossover Diagram: Heart

values at the onset of winter, however, no sub-zero temperatures were recorded throughout the entire hibernating period (Macartney et al., 1989). Adjacent uninhabited dens experienced extreme freezing temperatures; values as low as -30°C have been documented (Macartney et al., 1989). The ability of these snakes to select appropriate denning sites must be investigated in order to determine if there is some mechanism by which snakes can choose dens in which bouts of freezing will be minimized.

Previous studies have documented the widespread occurrence of anoxia tolerance in reptiles (Belkin, 1963). Constanzo (1989) recently reported that garter snakes can successfully overwinter for 165 days under water at 5°C . Garter snakes are capable of extracting oxygen from the water through their skin (Constanzo, 1989); when oxygen tensions in the pool of water eventually fall below a critical level, a severe hypoxic condition will occur. It may be a well developed anoxia tolerance that allows the snakes to survive brief periods of freezing.

CHAPTER 7
THE EFFECTS OF DEHYDRATION IN THE FREEZE TOLERANT WOOD
FROG
Rana sylvatica

SUMMARY

This study investigated the metabolic changes involved in and elicited by exposure to whole body dehydration. Desiccation limits for the wood frog, Rana sylvatica, were > 50 % total body water lost for fall animals and > 60 % dehydrated for spring animals. There was no difference in desiccation rates between frogs that were frozen at -2.0 °C and these were held at 1 °C. However, frogs kept in damp moss and exposed to similar dehydrating conditions exhibited no desiccation. Changes in protein and water contents during dehydration revealed patterns for individual tissues that indicated greater desiccation in peripheral tissues such as muscle. For example, muscle lost 10.6 % of tissue water at 50 % whole body dehydration. Vital organs/tissues (liver, gut, kidney, brain, heart) exhibited minor decreases in tissue water; values dropped by 0.8 - 7.6 % tissue water.

Upon exposure to dehydration, glucose levels increased dramatically in spring and fall frogs; up to 127 $\mu\text{mol/g}$ in liver! Glucose accumulation in the liver was followed by rapid distribution to all other tissues; peak levels ranged from 16 - 72 $\mu\text{mol/g}$. This mobilization of glucose is extremely similar to the metabolic responses elicited by freezing episodes in R. sylvatica. I hypothesize that the glucose accumulated in response to dehydration is used as a desiccation protectant to minimize tissue dehydration and to maximize survival in this species of frog.

INTRODUCTION

In order to successfully exploit terrestrial habitats, amphibians must overcome their susceptibility to evaporative water loss. Because amphibians are ectothermic and due to their highly vascularized skin, water loss can be 4 - 5 times as rapid at 30 °C than at 5 °C (Schmidt-Nielsen and Forster, 1954; Porter, 1972). General desiccation rates for amphibians are typically $4.7 - 9.2 \mu\text{l}/\text{cm}^2/\text{hour}$ at 20 °C (Porter, 1972) which can eventually lead to the loss of approximately 6 - 9 % total body weight lost per day (Hillman, 1980). Problems are encountered in the animal during the entire process of dehydration. As the skin begins to dry, electrolyte exchange as well as oxygen uptake is hindered (Duellman and Trueb, 1986; Hillman, 1978; Shoemaker, 1964; Sinsch, 1991). As plasma water content drops, blood viscosity increases dramatically and eventually blood/tissue gas exchange is not possible. At extreme dehydration, the heart rate slows down and stops, thus preventing any possible blood flow.

The 'critical activity point' (CAP) [defined as the point of dehydration at which the animal can no longer right itself when placed on its back] ranges between 30 % - 45 % initial body weight lost for frogs (Hillman, 1980); the CAP for salamanders is slightly lower at 20 % - 40 % (Ray, 1958). Generally, amphibians that inhabit the driest environment have the highest vital limits of water loss and conversely, amphibians that inhabit the wettest environments have the lowest limits. Therefore, terrestrial amphibians usually have a 'vital limit of body water loss' of 10 - 20 % higher than aquatic amphibians (Hoar, 1986).

The species of concern in this chapter, *R. sylvatica*, is also one of a small class of vertebrate animals that are naturally freeze tolerant (for review see Storey and Storey, 1988). The amphibians in this group can successfully tolerate the accumulation of 50 - 60 % of body water as ice at temperatures from -1 °C to -10 °C (Storey and Storey, 1988; Storey and Storey, 1984). The control of extracellular ice formation is

essential to the survival of the animal. Typical tissue osmotic concentrations of 200 - 400 mM contributed by salts, metabolite pools, and proteins have little net colligative effect on reducing freezing or supercooling points. Thus, *R. sylvatica* has developed the adaptation of synthesizing hundred millimolar amounts of glucose as a cryoprotectant. The production of cryoprotectant in response to freezing is vital to minimize the osmotic shock that the cell experiences as ice forms extracellularly and to allow a controlled formation of ice which is more easily survivable. Since dehydration puts the same water stress (osmotic) on cells as does freezing, the question arises, to what extent do the metabolic responses to dehydration mimic those to freezing? Are responses to freezing really elicited by osmotic stresses on cells? Or, are they an exaggeration of metabolic responses to dehydration already present?

In the present study, I examine the responses to a dehydration stress by the organs of the freeze tolerant wood frog, *R. sylvatica* in both spring and fall animals. Physiological parameters and biochemical aspects are investigated with respect to dehydration limit, protein/water content, % tissue water loss, and endproduct accumulation.

MATERIALS AND METHODS

Chemicals and Animals

Frogs of the species *R. sylvatica* were collected from the Ottawa, Ontario region in early/mid-April, 1990 for the spring experiments. Frogs for fall experiments were collected in early-October, 1990. Animals were acclimated at 5 °C in a moist environment without food for at least 1 week (for spring frogs) and 4 weeks (for fall frogs) prior to experimentation.

Experimental Design and Protocol

Factors Influencing Dehydration in Spring *Rana sylvatica*: Effects of Damp/Dry Environments

In order to examine the effect of microhabitat selection on animal desiccation, frogs were exposed to damp and dry environments at a low above zero temperature (1 °C) where body fluids remained liquid, as well as at a subzero temperature (-2.0 °C) at which temperature body fluids were frozen. A dry environment was simulated by drying off the frogs and placing them in a plastic container covered with dry paper towelling. A damp environment was simulated by placing frogs on damp sphagnum moss and covering them with moist paper towelling which was moistened as required daily; this was only done for the +1.0 °C-damp frogs since the paper towelling for this group dried out periodically, presumably due to evaporation. Four frogs (spring animals) were placed in each of the 4 experimental groups: +1.0 °C-damp, +1.0 °C-dry, -2.0 °C-damp, -2.0 °C-dry and individual animal weights were measured 2-3 times daily. The frogs subjected to -2.0 °C temperatures were frozen as outlined in 'Freezing Protocol' (below). Based on previous trials with frogs of similar body weight it is estimated that freezing began within 1 h at -2.0 °C. The frogs in the dry environment were frozen solid by the 6 hour timepoint, however, the frogs in the damp environment were still moving slightly at 6 hour although they did indeed have ice formed in their peripheral tissues; these frogs were solid by the 24 hour timepoint. Results were recorded in terms of '% initial water content' vs. time.

Dehydration Protocol

To experimentally assess the effects of whole body water loss on the metabolic responses of frogs, frogs were subjected to controlled dehydration over silica gel desiccant. The frogs were dehydrated as outlined in chapter 2 until approximately 25 % or 50 % dehydration (total body water lost) was reached. 'Percent dehydrated' was determined as in chapter 2. Both spring and autumn frogs were tested.

Supercooling points, freezing and percent ice determinations

Ice content of control, 25 % dehydrated, and 50 % dehydrated frogs (spring and fall animals) was determined after 24 h frozen at -2.0°C . The freezing procedure used was as follows. Animals were taped to a paper towel with a YSI telethermometer probe taped in contact with the abdomen; they were then placed in a -2.0°C incubator and cooled at an approximate rate of $0.5 - 1^{\circ}\text{C}/\text{min}$ until body temperature reached -2.0°C (initial incubator temperature). If no exotherm was recorded before body surface temperature reached -2.0°C , then incubator temperature was further lowered to -4.0°C and frogs were allowed to cool further; in some cases a further temperature adjustment to -6.0°C was needed. Nucleation was observed as an abrupt exotherm after the animals had reached their supercooling points. As soon as the frogs had nucleated, incubator temperature was raised to $-2.0 \pm 0.1^{\circ}\text{C}$. A maximum of four frogs were frozen at one time; all frogs nucleated within 20 min of each other. As soon as supercooling points had been recorded for all frogs, incubator temperature was raised to $-2.0 \pm 0.1^{\circ}\text{C}$ and frogs were allowed to freeze for 24 h at this temperature.

Ice content for each respective group of dehydrated frogs was measured by using the calorimetry method outlined by Lee et al. (1985) and described in chapter 2. Calorimetric parameters were as follows [average $\pm$ SEM]: F factor (for the calorimeter) = 1.03 ± 0.01 ; Sd (Specific Heat of Dry Mass) = 0.195 ± 0.038

cal/g/°C; % H₂O Content: i) control frogs = 80.8 ± 1.8 %, ii) 61.1 % (fall)/60.3 % (spring), iii) 35.1 % (fall)/38.9 % (spring). Per cent initial water content was obtained by taking weight measurements of control frogs, double pithing them and then drying their wet masses to constant weights in an oven at 80 °C for 72 h. Immediately following thawing in the calorimeter, animals were killed by pithing, cut open ventrally, and a blood sample was collected using a microcapillary tube after severing the aorta above the heart. Blood samples were immediately frozen for subsequent measurement of metabolite levels.

Spring and Fall Dehydration Timecourses

Initial survival tests that were performed indicated that the survival limit was reached at values greater than 50 % (fall) and 60 % (spring) of total body water lost using dehydration over silica gel desiccant as described above; survival was 100 % at both points of extreme dehydration. Therefore, the dehydration timecourse experiments monitored metabolic responses to dehydration at intervals up to 50 % (fall experiments) and 60 % (spring experiments) dehydration. Experiments with spring frogs sampled animals at approximately 5 %, 10 %, 25 %, 35 %, 50 %, 60 % dehydration, and one group that was dehydrated to approximately 50 % dehydrated and then rehydrated to approximately initial body water content. All of these animals were repeatedly weighed to monitor water loss approximately every 12 - 24 h until within 5 % of the targeted point of dehydration and then the frogs were weighed every 2 hours. The fall timecourse involved points at 25 %, 50 % dehydrated, two groups were dehydrated to 50 % and then either partially or fully rehydrated. Frogs were rehydrated by placing the dehydrated animal in distilled water (not covering the head); complete rehydration took approximately 12 - 24 h, although frogs were sacrificed at 24 h to ensure that all had fully rehydrated. All experiments were performed at 5 °C.

Tissue Preparation and Metabolite Measurements

All frogs were killed by single pithing. Blood was collected by exposing the heart and snipping one of the atria; blood was collected with a capillary micropipette with care taken to avoid collection of any blood that had been diluted by extra-organ water. 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 in chapter 2.

RESULTS

Factors Influencing Dehydration in Spring *Rana sylvatica*

To assess the effects of microhabitat selection on dehydration in situ, spring frogs were subjected to 4 different experimental regimes (Fig. 29). In both the damp and dry environments, the additional parameter of freezing showed at best a minimal effect on the rate of dehydration. The frogs kept in damp moss at 1°C exhibited no dehydration; this group of frogs actually absorbed water from the microenvironment. Values plateaued at about 110 h and eventually peaked at 160 h with a final value of 110.8 ± 4.7 % initial water. Frogs frozen at -2°C in damp moss revealed only minor water loss and only dropped by 2.6 % initial water at the lowest point (112 h). The two groups of frogs kept in dry containers at -2°C and 1°C exhibited virtually identical dehydration rates. The rate of dehydration for these two groups was approximately 0.32 % total body water loss per hour. Final '% water loss' values at

FIG. 29. Effect of exposure to damp or dry environments on the body water content of Rana sylvatica at 1°C or frozen at -2°C. Data are means $\pm$ SEM, n = 4 and are expressed relative to the initial water content of the frogs. Conditions are: ○, 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 toweling, whereas dry conditions consisted of a closed dry container with frogs covered by dry paper toweling. * -Significantly different compared to the 1 °C/dry frog group, $P < 0.05$. c -Significantly different compared to the -2 °C/damp frog group. a,b -Both dry groups of frogs at 1 °C and -2 °C significantly different compared to their respective damp group of frogs: $P < 0.05$, $P < 0.005$, respectively.

160 h were $51.0 \pm 1.8 \%$ and $47.9 \pm 2.0 \%$ for '1 °C; dry' and '-2 °C; dry', respectively. Survival was 100 % at all points of dehydration.

Supercooling Points and Rebound Temperatures of Dehydrated Frogs

Frogs exposed to no dehydration, exhibited no supercooling either in spring or fall (Table 9). However, rebound temperatures (T_r) were markedly different, with fall T_r 's at -0.80 ± 0.04 °C, 0.62 °C below spring values; the rebound temperature is defined as the temperature to which the body returns to immediately following the nucleation event. However, when animals were dehydrated, they supercooled markedly before freezing. At 25 % dehydrated, scpt. values both in the spring and fall, were -2.5 °C (average). At 55 % however, the fall scpt. at -4.8 ± 0.1 °C, was 1.4 degrees lower than spring values (at -3.4 ± 0.2 °C). Rebound temperatures at 25 % and 55 % dehydrated, were substantially lower (1.0 - 1.8 °C lower) in fall animals; fall T_r 's were -1.4 °C at 25 % and -3.0 °C at 50 % dehydrated.

Ice Content of Dehydrated Frogs

Fall frogs frozen at -2.0 °C accumulated 48.9 % of total body water as ice over 24 h, whereas spring animals frozen under the same condition accumulated substantially more at 61.3 % (Table 10). Dehydrated fall frogs contained 11 - 15 % less ice at 25 % and 55 % dehydrated as well; a final value of $20.5 \pm 2.3 \%$ body water as ice accumulated in fall frogs at 50 % dehydration.

Blood Metabolites in Dehydrated and Dehydrated/Frozen Frogs

Table 11 shows glucose and lactate levels in the blood of spring frogs subjected to a dehydration time course over desiccant. Control levels of glucose and lactate were 1.85 and 1.70 $\mu\text{mol/g}$ wet wt., respectively. Upon mild dehydration (5 %), glucose and lactate rose 2 - 3 fold, and at the dehydration limit (60 %), blood values peaked at

Table 9.
Effects of dehydration on supercooling point and rebound temperature (Tr) in Rana sylvatica.

% Dehydrated	Spring		Fall	
	Scpt. (°C)	Tr (°C)	Scpt. (°C)	Tr (°C)
0 %	--	-0.18 ± 0.02	--	-0.80 ± 0.04*
25 %	-2.4 ± 0.3	-0.4 ± 0.1 ^a	-2.6 ± 0.5	-1.4 ± 0.1 ^{b,*}
55 %	-3.4 ± 0.2 ^a	-1.2 ± 0.1 ^b	-4.8 ± 0.1 ^{b,*}	-3.0 ± 0.3 ^{b,*}

Data are means ± SEM, n = 4. a, b - Significantly different from control values of the respective season, P < 0.05, P < 0.005. In the case of Scpt., 55 % dehydrated values were compared to 25 % dehydrated animals, since no supercooling occurred in control frogs. * - Fall animals were significantly different compared to corresponding spring values, P < 0.001.

Table 10.

Effects of dehydration on ice content in Rana sylvatica after 24h at -2.0 °C.

% Dehydrated	% Ice Content	
	Spring	Fall
0 %	61.3 ± 2.0	48.9 ± 0.9*
25 %	57.6 ± 3.5	47.0 ± 2.5*
50 %	35.3 ± 3.6 ^b	20.5 ± 2.3 ^{b,*}

Data are means ± SEM, n = 4. a, b - Significantly different from control values of the respective season, P < 0.01, P < 0.005. * - Fall animals were significantly different from corresponding spring frogs, P < 0.005.

10.5 and 20.3 $\mu\text{mol/g}$ wet wt., respectively. Rehydrated frogs cleared accumulated blood lactate back down to control levels, whereas glucose levels only dropped slightly to $11.3 \pm 7.1 \mu\text{mol/g}$ wet wt..

The effect of freezing and dehydration on blood glucose and lactate is shown in Table 12. Freezing significantly increased blood glucose, as shown previously; control values were $10 \pm 4 \mu\text{mol/g}$ for spring frozen compared with 1.85 for unfrozen frogs (Table 11). Frogs that had been subjected to 24 h freezing at -2.0°C at various states of dehydration, showed dramatic increases in blood glucose and lactate accumulation (Table 12). Lactate levels in both the spring and fall were similar at all three points of dehydration (0 %, 25 %, and 55 %) when compared between spring and fall animals. However, at 55 % dehydration lactate in both spring and fall frogs increased by 3-5 fold over control values. Spring glucose levels were high, ranging from 10 - 31 $\mu\text{mol/g}$ wet wt., but not nearly as high as fall animals similarly exposed. Frozen fall frogs at 0 % and 25 % dehydrated showed substantial increases compared to their spring counterparts, they exhibited minor glucose synthesis. However, the combination of freezing and 55 % dehydration elicited a major buildup of glucose of $208 \pm 32 \mu\text{mol/g}$ wet wt. (Table 12).

Water Content and Metabolism in Dehydrated *Rana sylvatica*

Fig. 30a and 30b shows glucose and lactate accumulation in a variety of spring *R. sylvatica* tissues. Control glucose levels ranged from 10 nmol/mg protein in gut and brain to 67 nmol/mg protein in heart (Fig. 30a). In response to 60 % dehydration, levels increased significantly in four organs to a range of 19 - 205 nmol/mg in gut and heart, respectively. In terms of the largest increase in absolute quantity, liver glucose rose 5 fold to 62 nmol/mg (15 $\mu\text{mol/g}$ wet wt.).

Lactate levels in the liver, increased from 47 to 74 nmol/mg (Fig. 30b); although this increase was not statistically significant. Muscle lactate dropped slightly

Table 11.
Effects of dehydration on blood glucose and lactate in spring *Rana sylvatica*.

% Dehydrated	Glucose	Lactate
	($\mu\text{mol/g wet wt.}$)	
0 %	1.85 ± 0.28	1.70 ± 0.53
5 %	3.90 ± 0.74^a	5.42 ± 0.28^b
10 %	5.37 ± 0.77^b	2.40 ± 0.52
25 %	3.05 ± 0.58	2.13 ± 0.22
35 %	6.88 ± 2.42^a	7.47 ± 2.72^a
60 %	10.5 ± 3.5^a	20.3 ± 6.6^a
Rehydrated	11.3 ± 7.1	1.01 ± 0.01

Data are means $\pm$ SEM, $n = 4$. a, b - Significantly different from the corresponding control value, $P < 0.05$, $P < 0.005$.

Table 12.

Effects of freezing (24 hr at -2.0°C) and dehydration on blood glucose and lactate levels.

% Dehydrated	Glucose ($\mu\text{mol/g wet wt.}$)		Lactate	
	Spring	Fall	Spring	Fall
0 %	10 ± 4	$29 \pm 6^*$	12 ± 2	8 ± 2
25 %	31 ± 20	65 ± 24	17 ± 4	13 ± 3
55 %	24 ± 19	$208 \pm 32^{b,**}$	36 ± 6^b	37 ± 10^a

Data are means $\pm$ SEM, $n = 4$. a, b - Significantly different from control values of the respective season, $P < 0.05$, $P < 0.005$. *, ** - Fall animals were significantly different compared to their corresponding spring values, $P < 0.05$, $P < 0.005$.

FIG. 30. Effect of dehydration on the levels of (A) glucose and (B) lactate in six organs of spring *R. sylvatica*. Data are means $\pm$ SEM, n = 4 for control (open bars) and 60 % dehydrated (hatched bars) frogs. a, b - Significantly different from the corresponding control, P < 0.05; P < 0.005.

by 37 % to a final value of 111 nmol/mg, however this decrease was not statistically significant. Gut, kidney, and brain rose from a range of 74 - 206 nmol/mg to 146 - 299 nmol/mg protein at 60 % dehydration; this was a consistent increase of about 10 μ mol/g wet wt. for all three tissues. The heart showed the largest absolute increase (25 μ mol/g wet wt.) from 556 to 1724 nmol/mg.

Protein content in spring frog liver dropped (although not statistically significant) initially from control levels of 212 to 148 mg protein/g dry wt. at 5 % dehydrated (Fig. 31a). However protein content increased between 10 % - 40 % and plateaued out at 40 % - 60 % dehydrated with a final value of 275 ± 29 mg protein/g dry wt. Water content dropped slightly from control levels of 1.64 g water/g dry wt., with a low of 1.13 g water/g dry wt. occurring at 50 % dehydrated (Fig. 31b). Rehydrated frog water content was 27 % higher than initial control quantities but was not significantly different from control values. Only protein and water contents at 35 % and 50 % dehydrated showed statistical significant differences from controls.

Generally, fall protein and water levels were much higher than their spring counterparts (Fig. 32a and b). Muscle and kidney protein levels rose gradually, over the 2 dehydration points (25 % and 50 %), by 40 - 45 % to reach levels of 530 and 760 mg protein/g dry wt., respectively. Upon full rehydration, protein levels dropped back down to near control values. Gut and brain showed no changes from initial levels of 420 - 490 mg protein/g dry wt. Protein levels in the heart jumped erratically; initially by 77 % (at 25 % dehydrated) and then down to control quantities. Levels for 'rehydrated to 0 %' hearts jumped back up to 155 % control levels. Liver levels dropped from control values of 750 mg protein/g dry wt. by approximately 30 %, and then remained there throughout the rest of the dehydration-rehydration experiment.

Fall water content of liver, muscle, and gut followed a well defined pattern of gradual water loss during the dehydration timepoints and gradual water gain in the tissue during the rehydration points (Fig. 32b). Levels dropped by 32 - 55 % at 50 %

FIG. 31. Effect of whole animal dehydration on the (A) protein and (B) water contents of spring *R. sylvatica* liver. Data are means $\pm$ SEM, n = 4. Open symbols (R) represent frogs dehydrated to 60 % total body water lost and then rehydrated for 24 h. a, b -Significantly different from the corresponding control (0 % dehydration), $P < 0.05$; $P < 0.005$. Actual measured values for mean water loss by experimental 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 %. Rehydrated frogs first lost 63.8 ± 0.4 % of total body water, then regained water over 24 h to a final mean 4.6 ± 0.4 % dehydrated.

FIG. 32. Effect of changes in total body water content on the (A) protein and (B) water contents of six organs of autumn *R. sylvatica*. Data are means $\pm$ SEM, $n = 4$. Bars are (with actual measured values for mean water loss in brackets): , control 0 % dehydrated; , 25 % dehydrated (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 dehydration to 50.1 ± 1.7 % water lost). a, b -Significantly different from the corresponding control value, $P < 0.05$; $P < 0.005$.

dehydrated and re-established themselves upon complete rehydration at 88 - 103 % control values. Heart showed no detectable fluctuations in water content and kidney dropped only initially by 30 % and then resumed control levels. Dehydration in brain appeared to be slightly delayed by not showing a decrease in water content until '50 % dehydrated' (and at 'rehydrated to 25 %'); at 'rehydrated to 0 %', quantities rose to 137 % control values (Fig. 32b).

Although protein and water (g/g dry wt.) contents appeared to reveal major fluctuations, an assay of percent water in individual organs reflected minor changes (Table 13). Control % water ranged from 82.0 % in muscle to 93.9 % in heart. At 25 % dehydrated, all tissues except brain and heart showed decreases in % water; liver, gut, and kidney dropped by 3.1 - 5.1 percentage points, whereas muscle decreased by 8.9 %. At 50 % dehydrated, liver, gut, and muscle decreased an additional 0.4 - 2.8 %. Brain showed an abrupt drop of 4.0 %, whereas kidney showed indications of rehydration as levels rose to control quantities. Overall, kidney and heart showed a net change in percent water that was insignificant. All other tissues (liver, brain, gut, muscle) declined by 3.5, 4.0, 7.6, 10.6 %, respectively.

As a frog dehydrates, the effect on whole body water is severe, as the animal drops to half of its initial body weight at 60 % total body water lost. Thus it must be considered that the levels of metabolites in individual organs may artificially increase due to tissue water loss. In order to detect any metabolic changes that may influence metabolite levels, all metabolite concentrations were expressed in terms of "per mg protein".

Whereas dehydrated spring frogs showed large increases in tissue glucose (and lactate), dehydrated fall frogs exhibited glucose accumulation in all tissues assayed but comparatively minor fluctuations in lactate levels (Fig. 33). Control glucose levels ranged from 3 - 35 nmol/mg protein; these rose with dehydration with peak levels usually obtained at 'rehydrated to 25 %', although muscle and kidney peaked at the

Table 13.

Variation in percent tissue weight that is water during whole animal dehydration in Rana sylvatica

	Percent Water in Tissues		
	Control	25 %	50 %
Liver	90.9 $\pm$ 0.7	87.8 $\pm$ 0.4 ^b	87.4 $\pm$ 1.2 ^a
Muscle	82.0 $\pm$ 0.9	73.1 $\pm$ 0.7 ^b	71.4 $\pm$ 3.5 ^a
Gut	83.0 $\pm$ 0.8	78.2 $\pm$ 1.8 ^a	75.4 $\pm$ 0.7 ^b
Kidney	88.1 $\pm$ 1.0	83.0 $\pm$ 2.1 ^a	87.3 $\pm$ 1.3
Brain	86.8 $\pm$ 0.2	86.8 $\pm$ 0.8	82.8 $\pm$ 1.1 ^a
Heart	93.9 $\pm$ 0.8	93.4 $\pm$ 0.5	92.2 $\pm$ 0.6

Data are means $\pm$ SEM, n = 4. a, b - Significantly different from the corresponding control value, P < 0.05, P < 0.005.

FIG. 33. Effect of changes in total body water content on the glucose and lactate contents of six organs of autumn *R. sylvatica*. Data are means $\pm$ SEM, n = 4. Bars are: , control 0 % dehydrated; , 25 % dehydrated; , 50 % dehydrated; , rehydrated to 25 % (after dehydration to 50 % water lost); , rehydrated to 0 %. a, b -Significantly different from the corresponding control value, P < 0.05; P < 0.005.

'rehydrated to 0 %' point. Maximal quantities for gut, muscle, and kidney ranged from 165 - 404 nmol/mg; corresponding wet weight values were 16 - 35 μ mol/g wet wt. for these tissues. However, brain, heart, and liver showed dramatic increases of 1092, 1409, and 1263 nmol/mg. These corresponded to final glucose values of 72, 43, and 127 μ mol/g wet wt. Overall, glucose quantities rose 9 - 313 fold in all 6 tissues.

Except for brain and muscle, lactate levels increased in all tissues (fig. 33). In those tissues that did exhibit increases, lactate levels peaked at '50 % dehydrated' or '25 % rehydrated' timepoints and then, dropped slightly at the final timepoint, 'rehydrated to 0 %'. The largest increases occurred in heart and liver; control values were 11.5 and 18.0 nmol/mg and rose to 62 and 69 nmol/mg, respectively. In terms of wet weight measurements, peak levels occurred in muscle, gut, and liver; quantities reached 5.2 - 5.6 μ mol/g wet wt. Overall, lactate levels ranged from 7.1 to 69 nmol/mg (0.2 to 6.49 μ mol/g wet wt.) and regardless of whether the changes were increases or decreases, all changes were significant ($p < 0.05$) by at least the '50 % dehydrated' point.

An assay of liver glycogen (as glucose) in fall *R. sylvatica* revealed the likely source of glucose carbon, as glycogen stores declined from 18.0 to 15.5 μ mol/mg at '50 % dehydrated' ($p < 0.05$) and subsequently dropped to 11.8 μ mol/mg ($p < 0.005$) at 'rehydrated to 25 %' (Table 14). In terms of absolute quantities, this was a decrease of 298 μ mol/g wet wt. glycogen as glucose; quantities dropped from control values of 1216 ± 39 μ mol/g wet wt.

DISCUSSION

Microhabitat selection is a critical aspect of terrestrial life and as well, winter hibernation. In a natural environment, however, the motive force for desiccation presumably is not as strong, although air currents may dramatically increase

Table 14.
Effects of dehydration and rehydration on liver glycogen in fall Rana sylvatica.

% Dehydrated	Glycogen ($\mu\text{mol}/\text{mg}$ protein)
0 %	18.0 ± 0.1
25 %	19.6 ± 0.1
50 %	15.5 ± 1.0^a
Rehydrated to 25 %	11.8 ± 1.3^b
Rehydrated to 0 %	20.7 ± 0.3

Data are means $\pm$ SEM, $n = 4$. a, b - Significantly different from the corresponding control value, $P < 0.05$, $P < 0.005$.

evaporative water loss. Upon examining the factors influencing dehydration in spring *R. sylvatica*, it is apparent that as long as the animal maintains a moderately damp microenvironment evaporative water loss does not present a significant problem. This moist environment may typically be found in leaf litter on the forest floor, under long grass, or under the cover of rotten logs which can afford protection by way of shielding from air currents, cooler temperatures (ie. avoiding direct sunlight), and additional moisture absorbed from the ground or previous rains. This study was first approached the point of view that there may be considerable water loss during freezing in a dry environment and as well, whether frozen frogs would lose water differently than thawed ones. However, the rate of sublimation is apparently identical to the rate of evaporation in these frogs as was indicated by no significant differences in dehydration slope of dry/frozen frogs versus dry/1 °C frogs in Fig. 29.

As soon as breeding in spring is over these frogs migrate to the woods where they spend all summer enduring the terrestrial environment. As air temperatures drop in the fall and early winter the frogs seek refuge in the debris on the forest floor. Once hidden, the frogs remain quiet until they naturally freeze and stay in a state of 'suspended animation' throughout the winter. It is the time when the frogs find a suitably damp overwintering microenvironment that these animals are subject to changes in water balance. Entry into the frozen state involves whole body supercooling and then sudden nucleation and subsequent ice formation up to 65 % body water as ice. Several questions arise addressing the influences of dehydration on the actual supercooling event and the freezing process. What effect do different hydration states (water balance) have on cooling and freezing behaviour, since animals would undoubtedly be in less than perfect states of water balance when freezing occurs? As well, dehydration puts the same water stress (osmotic) on cells as does freezing. Therefore, to what extent do the metabolic responses to dehydration mimic those to freezing?

No supercooling was apparent in control (fully hydrated) frogs, however the 'rebound temperature' (T_r) dropped by 0.62°C . As the degree of dehydration increased from 25 to 55 %, both the supercooling point and T_r dropped to a low of -4.8°C and -3.0°C in fall frogs, respectively. Comparing spring to fall frozen frogs at 25 % and 55 % dehydration, the T_r was 1.0 to 2.8°C lower in the fall; the supercooling point followed a similar trend dropping 0.2 to 1.4°C lower. The decrease in T_r and Scpt. temperatures over the 0 %, 25 %, 55 % points was attributable to the dramatic decrease in total body water. In a fully hydrated state, a frog has much more interstitial water and specifically, more water at the site of nucleation, near the skin. As the amount of body water increases, the probability of spontaneous nucleation also increases. It is not uncommon for frogs kept in water under laboratory conditions (ie. fully hydrated) to show no supercooling capacity and to simply freeze at high subzero temperatures (-0.0 to -0.5°C). In addition to the primary factor of whole body water loss increasing supercooling point and rebound temperature depression, the accumulation of glucose also influences the depression of these two freezing parameters. Fall frogs 55 % dehydrated had $127\text{ }\mu\text{mol/g}$ glucose in the liver (Fig. 33), however it is unlikely that levels of blood glucose had increased this much. Levels of glucose in the heart are typically representative of blood glucose levels; at 50 % dehydration, glucose levels had increased by $< 30\text{ }\mu\text{mol/g}$. The equation describing the colligative effect of solutes on freezing point depression is $T = K \cdot M$; where T represents the fpt. depression, M is the molarity of solutes in solution, and K is the fpt. depression constant (1.86 for water). However, this equation is only applicable to dilute solutions ($< 20\text{ mM}$), and at higher concentrations the freezing point depression depends upon the solute; at higher concentrations, the solute dependent freezing point depression is determined empirically. For glucose the presence of 1 M solutes decrease the fpt. by approximately 2.17°C ; this value deviates from the calculated value of 1.86°C . According to the basic freezing point

depression equation, an increase in blood glucose of $30 \mu\text{mol/g}$ (34 mM) only depresses the fpt. by $0.063 ^\circ\text{C}$. Thus, colligative effects on sept. and fpt. depression in this experiment are minor at best.

As a frog dehydrates, water evaporates rapidly via the highly vascularized skin that has evolved over evolutionary time in aid of gas and electrolyte exchange (Duellman and Trueb, 1986; Hillman, 1978). A type of cascade event of desiccation follows with the outer muscle giving up tissue water to the even drier skin. As well, the visceral cavity is filled with water that bathes the internal organs. Upon dissection of dehydrated frogs this water was clearly missing, likely as a source of water to maintain the hydrated state of the tissues, that would otherwise rapidly dehydrate. Protein content and water content revealed this drop in tissue water in most of the six tissues. Heart, kidney, and brain showed the smallest changes (or none at all); these vital organs carry out specific functions that are critical to the whole system. Any dramatic increase in protein content or decrease in water content during dehydration occurred in liver, muscle, and gut. Generally, if the water content dropped, then the concentration of protein increased. Liver protein content dropped to a new level approximately 30 % below control values of $750 \text{ mg protein/g dry wt.}$ in the fall; whereas water content was seen to decline to similar proportions. Even though, protein and water content were observed to exhibit dramatic changes, an overview of 'tissue per cent water' showed that only minor decreases in water content as a percentage of total weight. This was primarily due to the loss of water in the visceral cavity (and in the skin) and not the actual desiccation of the tissues themselves. A combined loss of water from the free water inside the abdominal space and a decrease in blood water must have been dramatic in order to effect such a drastic reduction in total body weight. There are serious implications for the whole organism when critical tissues such as skin and heart/blood rely on at least a moderately hydrated state in order to function.

The transition of vertebrates from water to land habitats involved, in evolutionary terms, the disappearance of internal gill filaments (in fish) and the generation of a highly vascularized skin. This skin has evolved presumably to take the place of the gills that were required for gas (O_2/CO_2) exchange and electrolyte balance (Na^+ , K^+ , Cl^- , NH_4^+) in an aqueous environment (Duellman and Trueb, 1986). However, due to the nature of a terrestrial environment, evaporative water loss from this extremely permeable dermis is a major problem in maintaining any functional characteristics that are key to homeostasis of the whole animal. However, it is impossible to completely avoid a dehydration stress since desiccation of the frog starts immediately upon exposure to air; any air currents would obviously increase the rate of dehydration dramatically. Therefore, it is not surprising that amphibians have developed several physiological and biochemical adaptations in order to reduce the rate of dehydration either by directly preventing skin/air contact or by a series of metabolic adjustments (Gatten, 1987; Katz, 1989; Loveridge and Withers, 1981; Shoemaker, 1964).

Perhaps the most unusual metabolic adjustment to dehydration in *R. sylvatica* discovered in this investigation is the accumulation of glucose in all tissues of the frog. First of all, the dramatic triggering of glycogen breakdown resulted in a rise of 127 $\mu\text{mol/g}$ wet wt. glucose in the liver! Since this increase in liver glucose only accounted for 43 % of the decline in glycogen stores, presumably the increase in blood glucose would implicate mobilization of liver glucose to the blood and subsequently, to the other tissues/organs. Although the glucose distributed by the liver may act (at least partially) as glycolytic substrate for the other tissues, there may be a more novel reason for the accumulation of the hexose sugar. This accumulation of glucose is similar to the metabolic responses elicited by freezing episodes in *R. sylvatica* (Storey and Storey, 1984, 1988). During the initial stages of freezing, liver glycogenolysis is activated upon nucleation and within minutes liver glucose levels, followed by blood glucose

levels are observed to rise (Storey and Storey, 1984, 1988). After 24 h frozen, these frogs have mobilized hundred millimolar quantities of glucose to all tissues. It is widely accepted that during these freezing episodes, the sugars that accumulate act as cryoprotective agents in a colligative manner by structuring hydration shells around cellular proteins and membrane structures. Although further investigation of this phenomenon during dehydration is required before reaching any conclusions about the nature of this glucose accumulation, I hypothesize that glucose is used as a cellular protective agent during dehydration in order to minimize tissue dehydration and to ensure survival of this frog.

CHAPTER 8
REGULATORY CONTROL OF GLYCOLYSIS DURING DEHYDRATION IN
THE FREEZE TOLERANT WOOD FROG
Rana sylvatica

SUMMARY

The previous study (see Chapter 7) documented the accumulation of glucose, up to 127 $\mu\text{mol/g}$ wet wt., in response to dehydration episodes to 50 - 60 % dehydrated. The effects of dehydration at 5 $^{\circ}\text{C}$ on glucose synthesis and the metabolic regulation involved in production of this desiccation protectant were investigated. The dramatic increase in glucose levels was also observed in the spring frogs dehydrated in this study; quantities rose 4 fold within the first 5 % dehydration. Although maximum levels did not rise greater than 11 $\mu\text{mol/g}$ wet wt., low glycogen quantities (commonly found in spring frogs) were implicated in reduced desiccation protectant synthesis. Increased glycogenolysis was accomplished via a 2.5 fold increase in the active a form of glycogen phosphorylase and a 2.3 fold increase in total a + b units. Using 'crossover theory' (Williamson, 1970), glycolytic metabolites directly linked to the key regulatory enzyme, phosphofructokinase (PFK), reflected an inhibition of PFK; G6P rose 8 fold and F6P increased 3 fold within the first 5 % dehydration, whereas F1,6P₂ levels showed no changes throughout the timecourse. Levels of the potent allosteric effector molecule, F2,6P₂, showed no positive correlation with the inactivation of PFK. There was no indication of regulation of glycolysis at the pyruvate kinase (PK) locus since metabolites associated with PK, phosphoenolpyruvate (PEP) and pyruvate, both exhibited increases over the initial portion of the dehydration experiment. The stress of the dehydration exposure on the animals was reflected in a gradually declining energy charge value $[(\text{ATP} + \text{ADP}/2)/(\text{ATP} + \text{ADP} + \text{AMP})]$ (Atkinson, 1977) from a control value of 0.81 to 0.47 at 60 % dehydration. However, upon rehydration, the E.C. value resumed near control values.

INTRODUCTION

Evaporative water loss is of utmost importance for amphibians. There are two basic strategies possible to escape the detrimental effects of dehydration: avoidance or tolerance. Behavioral adaptations are able to combat this stress, however complete avoidance (ie. living in an aqueous environment all of the time) is not practical since one of the basic characteristics of many amphibians is an aquatic and a terrestrial lifestyle. There are both physiological, as well as biochemical adaptations for desiccation tolerance in all anuran species to some degree (Hoar, 1986). Most amphibians, and all frogs, possess substantial tolerance to whole body dehydration; limits of water loss range from 30 % to 60 % of total body water (Ray, 1958; Porter, 1972). However, amphibians that inhabit the driest environment generally have the highest vital limits of water loss and conversely, amphibians that inhabit the wettest environments have the lowest limits (Hillman, 1980; Ray, 1958). Thus, terrestrial amphibians generally possess dehydration limits that are on average 10 - 20 % higher than their aquatic counterparts (Hoar, 1986).

The wood frog, Rana sylvatica, can tolerate the loss of 60 % of total body water and following recovery in water for 10 - 20 hours will resume its initial hydration state with no apparent adverse effects (Chapter 7). During a typical bout of dehydration, R. sylvatica accumulates large quantities of glucose in the liver (up to 130 $\mu\text{mol/g}$) and all other tissues in order to combat the process of desiccation in a colligative manner (Chapter 7).

As well, the wood frog, R. sylvatica, is one of six amphibian species that is naturally freeze tolerant. Tolerance of up to 60 % ice is achieved by means of the production of glucose which is used as cryoprotectant (Storey and Storey, 1988). Large quantities of this common blood sugar help minimize the osmotic shock that the cell experiences as ice forms extracellularly (Storey and Storey, 1992).

Glycolytic regulation within the liver has been determined for the wood frog, R. sylvatica during the period of glucose synthesis (Storey, 1987). R. sylvatica activates glycogenolysis by varying the proportions of glycogen phosphorylase (a and b forms) to favor the active a form; this occurs in conjunction with a rapid rise in total phosphorylase quantities and an inhibition of phosphofructokinase which permits the redirection of glycolytic carbon to favor glucose accumulation (Storey, 1987; Storey and Storey, 1984). Since the biochemical adaptations in response to freezing are exhibited during dehydration in the same species (glucose accumulation), it is important to investigate the metabolic control involved during dehydration.

The present study examines the glycolytic control involved in the rapid phase of glucose production in response to dehydration in the wood frog, R. sylvatica. This metabolic control was determined by means of a glycolytic crossover study in which variations in glycolytic flux were detected by comparison of metabolite levels to control values.

MATERIALS AND METHODS

Chemicals and Animals

Frogs of the species R. sylvatica were collected from the Ottawa, Ontario region in early/mid-April, 1990. Animals were acclimated at 5 °C in a moist environment without food for at least 1 week prior to experimentation.

Experimental Design and Protocol

The frogs were dehydrated as outlined in chapter 2 until 25 % or 50 % dehydration was reached. 'Percent dehydrated' was determined as in chapter 2. Both spring and autumn frogs were tested. The dehydration timecourse involved points at 5

%, 10 %, 25 %, 35 %, 50 %, 60 % dehydration, and one group that was dehydrated to approximately 50 % dehydrated and then rehydrated to approximately initial body water content.

Tissue Preparation and Metabolite Measurements

Tissues were sampled at the respective timepoints by quickly pithing the frogs and then, immediately excising the tissues and freezing them in liquid nitrogen. Tissues were stored at -80 °C until processing. Control animals were killed immediately after removal from the 5 °C incubator.

At each dehydration point, four samples (n=4) of each respective tissue were extracted for metabolites via the perchloric acid method described in chapter 2.

RESULTS

In Fig. 34a, control liver glucose levels were 23.7 nmol/mg protein. Within the first 5 % dehydrated, levels jumped up 4 fold. Quantities at 60 % dehydration were 62 nmol/mg, which in terms of wet weight levels is 8.1 μ mol/g wet wt.; values increased slightly but with greater variability upon rehydration. Liver glycogen stores fluctuated erratically within 5 - 10 % dehydration; control values were 2.0 μ mol/mg (160 μ mol/g wet wt.) (Fig. 34b). The decline in glycogen stores in the liver corresponded well with the rise in liver, as well as muscle, glucose levels. At 25 % dehydration and subsequent points, glycogen values dropped to 0.25 μ mol/mg (35 μ mol/g wet wt.). Lactate quantities rose rapidly (2 fold) at 5 % dehydration from control levels of 47.4 nmol/mg (Fig. 34c). Levels then dropped back down to near control values until 60 % dehydration, at which point levels increased to 74.4 nmol/mg. In terms of wet weight values, lactate levels never increased above 9.75 μ mol/g wet wt.

Glucose accumulation in muscle paralleled that of liver glucose production (Fig. 35a). Values doubled within the first 5 % dehydration and subsequently levelled off at approximately 25 - 35 nmol/mg. At 60 % dehydration, a high of 49.4 nmol/mg (9.8 μ mol/g wet wt.) was reached, although levels varied dramatically at this last point of dehydration; upon rehydration glucose levels fell back to control values. Lactate levels in muscle dropped by 30 % within the first 10 % dehydration; and further fell to 85 nmol/mg from 10 to 35 % dehydration (Fig. 35b). Quantities remained low upon rehydration.

Levels of metabolites at phosphofructokinase (PFK), [fructose-6-phosphate (F6P), and fructose-1,6-bisphosphate (F1,6P₂)] reflected an inhibition at this key regulatory enzyme of glycolysis (Fig. 36b). Hexose phosphates, G6P and F6P, increased immediately upon 5 % dehydration, by 2.1 and 2.5 fold, respectively; peak values were 17.3 and 3.0 nmol/mg. Although F6P dropped down below control values at 35 % dehydration, G6P plateaued out at 9.5 nmol/mg (4.5 X control levels) until the final point of dehydration (60 % dehydrated) after which point levels fell to control values. At 60 % dehydration, F1,6P₂ rose 2 fold; combined with the decreases in G6P and F6P, this was indicative of an activation of PFK. At the final point, rehydrated to 0 %, F1,6P₂ dropped back to control levels and G6P and F6P remained low.

The combined levels of triose phosphates, dihydroxyacetone-phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP), maintained control levels throughout the entire dehydration timecourse as well as after rehydration (Fig. 36c). Levels of metabolites at the enzyme pyruvate kinase (PK), phosphoenolpyruvate (PEP) and pyruvate, both increased immediately upon initial dehydration; values rose by 2.1 and 5.3 fold, respectively (Fig. 36d); in terms of absolute quantities, both PEP and pyruvate rose by approximately 0.5 nmol/mg. Quantities levelled off between 10 % and 35 % dehydration for both PEP and pyruvate at approximately 0.9 and 0.6 nmol/mg.

FIG. 34. Effect of dehydration on the levels of (A) glucose, (B) glycogen, and (C) lactate in the liver of spring Rana sylvatica. Data are means $\pm$ SEM, n = 4. Open symbols (R) represent frogs dehydrated to 60 % total body water lost and then rehydrated for 24 h. a, b -Significantly different from the corresponding control (0 % dehydration), $P < 0.05$, $P < 0.005$, respectively. Actual measured values for mean water loss by experimental 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 %, 61.1 ± 1.3 %. Rehydrated frogs first lost 63.8 ± 0.4 % of total body water, then regained water over 24 h to a final mean of 4.6 ± 0.4 %.

FIG. 35. Effect of dehydration on the levels of (A) glucose and (B) lactate in muscle of spring Rana sylvatica. Data are means $\pm$ SEM, n = 4. Open symbols (R) represent frogs dehydrated to 60 % total body water lost and then rehydrated for 24 h. a, b -Significantly different from the corresponding control (0 % dehydration), $P < 0.05$, $P < 0.005$, respectively.

FIG. 36. Effect of dehydration on the levels of (A) glucose-6-phosphate, (B) fructose-6-phosphate (circles) and fructose-1,6-bisphosphate (squares), (C) dihydroxyacetone phosphate + glyceraldehyde-3-phosphate, (D) phosphoenolpyruvate (circles) and pyruvate (squares) in the liver of spring Rana sylvatica. Data are means $\pm$ SEM, n = 4. Open symbols (R) represent frogs dehydrated to 60 % total body water lost and then rehydrated for 24 h. a, b -Significantly different from the corresponding control (0 % dehydration), $P < 0.05$, $P < 0.005$, respectively.

Pyruvate maintained elevated levels until rehydration, at which point values dropped to 0.24 nmol/mg. PEP, however, dropped to 1/2 control values at 50 % and 60 % dehydration; upon rehydration, PEP jumped to 0.62 nmol/mg.

Examination of glycogen phosphorylase showed dramatic changes that can be attributed to an increase in metabolic flux (Table 15). Glycogen phosphorylase *a* form increased to 54.0 % at 25 % dehydration from control values of 30.9 ± 5.7 % ($P < 0.05$). At 50 % dehydration, % *a* form rose to 76.9 %. Total units *a* + *b* rose from control levels of 0.12 IU/mg protein to 0.23 IU/mg ($P < 0.05$) within 25 % dehydration. At 50 % dehydration, values increased to 0.28 ± 0.05 IU/mg, yielding a net increase of *a* units of 5.8 fold from 0.037 to 0.215 IU/mg. In terms of "wet weight" levels, *a* units increased from a control value of 3.1 to 30.0 IU/g wet wt. at 50 % dehydration. As well, levels of the potent allosteric effector molecule of PFK, F2,6P₂, varied dramatically with control levels at 4.98 μ mol/mg protein and then jumping up to 9.57 μ mol/mg at 25 % dehydrated. The error on this value was 34 %, and since the variability in this activator's quantity was high, the increase was not significant. However, metabolites associated with the PFK locus indicated an inhibition at 35 % dehydration (F6P increased, F1,6P₂ remained at control levels). At 50 % dehydration, F2,6P₂ dropped to 45 % of control values ($p < 0.05$). Metabolites indicated an activation of PFK, in contradiction to the drop in F2,6P₂ levels that would otherwise induce an inactivation of this enzyme.

ATP levels were generally not significantly different from control values (28.1 nmol/mg) until 25 % dehydration. There was an initial increase in ATP at 5 % dehydration, however levels quickly declined to near control or less than control values upon subsequent dehydration exposure. Levels gradually declined in a linear fashion until reaching a low of 4.7 nmol/mg at 60 % dehydration (Fig. 37a). Upon rehydration, ATP levels increased 3.5 fold. Total adenylate values followed an identical pattern as ATP quantities, dropping gradually until 60 % dehydration and then

Table 15.
Regulatory control of liver glycolysis in spring *Rana sylvatica* during dehydration.

	Glycogen % _a	Phosphorylase Total (IU/mg)	F-2,6-P ₂ (pmol/mg)
C	30.9 ± 5.7	0.12 ± 0.03	4.98 ± 0.65
25 %	54.0 ± 5.0 ^a	0.23 ± 0.03 ^a	9.57 ± 3.29
50 %	76.9 ± 1.3 ^b	0.28 ± 0.05 ^a	2.22 ± 0.54 ^a

Data are means ± SEM, n = 4. a, b - Significantly different from the corresponding control value, P < 0.05, P < 0.005.

jumping up when rehydrated (Fig. 37a). ADP and AMP levels increased initially by 1.5 and 2 fold until 25 % dehydration at which point, levels dropped slightly by 29 % and 38 %, respectively and remained at these values even during rehydration (Fig. 37b). Creatine phosphate levels (Fig. 37c) exhibited an initial increase, although values had extremely large errors at 5 % and 10 % dehydration points (5.1 ± 1.8 and 6.5 ± 2.3 nmol/mg). At 35 % dehydration the increase in creatine phosphate levels was significant to $p < 0.05$. At the two final points of dehydration (50 % and 60 %), levels of this high energy phosphate dropped to 1.76 nmol/mg. During rehydration, levels resumed control values. Overall creatine phosphate levels were very low in liver, only about 10 % of ATP levels. Energy charge $[(ATP + 1/2 ADP)/(ATP + ADP + AMP)]$ values dropped slightly (but not significantly) between 5 - 25 % dehydration from control values of 0.81 ± 0.02 (fig 37d). Subsequently, values decreased between 35 - 60 % dehydration from 0.72 to 0.47. Upon rehydration, energy charge levels resumed near control values at 0.73 ± 0.01 .

DISCUSSION

Recently I have documented the accumulation of a common blood sugar that may act as a protective agent to combat the adverse effects of dehydration exposure in the wood frog, *R. sylvatica* (Chapter 7). As well, the glycolytic regulation that is key in glucose production in *R. sylvatica* has been examined in frozen frogs (Storey, 1987; Storey and Storey, 1984), therefore comparison to metabolic responses elicited by dehydration are of particular interest. The production of glucose as a cellular protectant during freezing exposure in extremely large quantities is controlled by a well defined series of regulatory control points that permit the directing of glycolytic carbon to glucose (Storey and Storey, 1990). The present findings clearly show that glycolysis is regulated at two enzyme loci, glycogen phosphorylase and phosphofructokinase

FIG. 37. Effect of dehydration on the levels of (A) ATP (circles) and Total Adenylates [ATP + ADP + AMP] (squares), (B) ADP (circles) and AMP (squares), (C) creatine phosphate, (D) Energy Charge $[(ATP + 1/2 ADP)/(ATP + ADP + AMP)]$ in the liver of spring Rana sylvatica. Data are means $\pm$ SEM, n = 4. Open symbols (R) represent frogs dehydrated to 60 % total body water lost and then rehydrated for 24 h. a, b -Significantly different from the corresponding control (0 % dehydration), P < 0.05, P < 0.005, respectively.

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(PFK). The activation of glycogenolysis was seen as a sudden rise in phosphorylase % α (from 31 % to 54 %). Additionally, total units of phosphorylase enzyme increased by two fold; the phosphorylase activation was also evident in 2 fold increases in glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) metabolite levels. An inhibition of the key regulatory control enzyme, PFK also occurred concomitantly as was indicated by the simultaneous increase and no change from control values in F6P and F1,6P₂ levels, respectively. This allowed the directing of carbon flow to glucose as the cellular protectant. Levels of the positive allosteric effector molecule, fructose-2,6-bisphosphate (F2,6P₂), did not correlate with the initial inactivation of PFK at 25 % dehydration; quantities doubled, although the error was large and this increase was not statistically significant. Conversely, at 50 % dehydration F2,6P₂ levels dropped to 1/2 control values ($p < 0.05$). Again this fluctuation in quantities of this potent effector molecule did not correlate with the apparent slight activation of PFK as indicated by changes in F6P and F1,6P₂ levels; F6P fell dramatically and F1,6P₂ rose by 2 fold at 60 % dehydration. These apparent contradictory trends in allosteric effector levels and PFK activity, may simply be as a consequence of high F6P levels. PFK may be inactivated at 25 % dehydration by means of other levels of enzyme control such as a phosphorylation mechanism which would override lower levels of enzyme regulation. If this was the case, high F6P levels would direct enzyme synthesis of F2,6P₂ in a direction favoring the potent activator, so even though F2,6P₂ levels would rise they would do so as an indirect result of an already inactivated PFK. The converse would also be consistent with the drop in F2,6P₂ levels in spite of the slight activation of PFK.

During periods of extreme and/or prolonged cold exposure, cellular defence from dehydration is essential when extracellular ice formation occurs (Clegg, 1981). As ice crystals form, intracellular water is gradually 'sucked' out of the cytoplasm as a consequence of the increasing osmotic pressure, which in turn is as a result of

sequestration of extracellular water as ice (Clegg, 1981; Storey et al., 1992). This osmotic stress on the cell and its contents must be minimized to enable a successful survival of the freezing episode. The synthesis of large quantities of low molecular weight polyhydroxylated sugars aids in the retention of intracellular water (Storey and Storey, 1988, 1992; Clegg, 1981).

Although the accumulation of glucose in response to dehydration was quantitatively low, insufficient glycogen levels may provide explanation for poor 'desiccation protectant' production. Previous studies examining the persistence of freeze tolerance in spring frogs have shown similar low cryoprotectant levels, as well as poor survival rates (Storey and Storey, 1987). Therefore the low glucose values can be attributed to low glycogen stores and to the preferential redirecting of existing stores to other fates such as muscle work used in spring activities (ie. croaking and mating); typical fall glycogen levels range from 700 - 1000 $\mu\text{mol/g}$ (in glucose units) (Chapter 7; Storey, 1987). There are several studies performed on spring frogs that have been found to have extremely low levels of glycogen (5 - 175 $\mu\text{mol/g}$) and consequently synthesize low levels of glucose during freezing and dehydration (Storey and Storey, 1987; Chapter 7). The activity level of frogs changes during the spring emergence from hibernation over the winter. Therefore, it would seem that glycogen levels are seasonally dependent and that glucose accumulation in response to dehydration may also be similarly influenced. This correlation between glucose accumulation and available glycogen is confirmed in chapter 7 which documents the accumulation of glucose used as a protective agent during whole body dehydration in *R. sylvatica*.

Energy requirements are extremely vital at low temperatures in ectotherms, especially in a severely dehydrated state. In this experiment all adenylates maintained control values throughout the initial part of the dehydration time course; this was apparent by the constant and high energy charge value in liver between control and 25 % dehydration (E.C. = 0.81). However, during the latter portion of the period of

dehydration, the energy charge rapidly dropped from 0.72 to 0.47; ADP and AMP levels did not show any increases that usually accompany a decline in E.C., but ATP (as well as total adenylate quantities) dropped dramatically. The energy stress during the dehydration episode is reflected well in the energy charge value. Survival of extremely low E.C. values is possible in many lower vertebrates, including R. sylvatica; frogs of this species have easily recovered from E.C. values of 0.34 during stressed conditions (Storey, 1987; Storey and Storey, 1985).

There seem to be many similar metabolic responses involved in both dehydration and freezing, but the two stresses are not identical (ie. ice formation within the actual tissue versus complete removal of water), although they may have developed over similar evolutionary lines. There appears not to be any increased desiccation resistance in species that tolerate extreme changes in cell volume during freezing. Many terrestrial frog species readily survive at least 40 - 50 % dehydration and yet are not able to survive freezing (Hoar, 1986; Storey and Storey, 1988). The transition from water to land and consequently the environmental stresses encountered in a terrestrial habitat, presumably developed biochemical adaptations that help combat any adverse conditions that are encountered in this new lifestyle. It may be that the metabolic responses to freezing are an exaggeration of pre-existing mechanisms for desiccation tolerance.

CHAPTER 9
GLUCOSE PRODUCTION PRODUCTION IN RESPONSE TO DEHYDRATION
IN THE TREE FROG
Pseudacris crucifer

SUMMARY

In this study the effects of dehydration at 5 °C on glucose synthesis and the metabolic regulation involved in the production of this desiccation protectant were investigated. Spring frogs, *Pseudacris crucifer*, exhibited moderate increases in glucose (13.6 - 42.2 $\mu\text{mol/g}$ wet wt.) and lactate levels (15 - 50 $\mu\text{mol/g}$) in response to 55 % dehydration. However, in fall animals exposed to a timecourse of up to 50 % total body water loss, most tissues showed an increase to a range of 4.6 - 36.8 $\mu\text{mol/g}$ wet wt. glucose; whereas liver glucose rose to 220 $\mu\text{mol/g}$ wet wt. Glycogen stores were presumably the source of glucose carbon since levels dropped by 560 $\mu\text{mol/g}$ wet wt. glycogen as glucose. Protein content varied slightly for most tissues remaining in a range of 300 - 750 mg protein/g dry wt., although this was largely due to varying water content of the individual tissues. Using 'crossover theory' (Williamson, 1970) to examine regulatory control of glycolysis, a 4 fold increase in fructose-6-phosphate combined with no change in fructose-1,6-bisphosphate levels over the first 35 % dehydration indicated an apparent inhibition of phosphofructokinase (PFK). During this time, adenylate (ATP, ADP, AMP) quantities maintained control values. Energy charge values $[(\text{ATP} + \text{ADP}/2)/(\text{ATP} + \text{ADP} + \text{AMP})]$ (Atkinson, 1977) reflected the maintenance of cellular energy stores over 35 % dehydration with values holding at 0.82 - 0.86. However, by 50 % dehydration ATP levels had dropped to < 20 % control values, and AMP had increased 2 fold. Metabolites associated with PFK indicated an inhibition of the enzyme, presumably in an attempt to replenish ATP pools. Nevertheless, energy charge values dropped to 0.42 reflecting the limit of a tolerable desiccation stress.

INTRODUCTION

The spring peeper, P. crucifer, is one of six amphibian species that is naturally freeze tolerant. As with R. sylvatica, tolerance of up to 60 % ice is achieved by means of the production of glucose which is used as cryoprotectant (Storey and Storey, 1988, 1992); cryoprotectant synthesis in P. crucifer was previously documented in chapter 1. This polyhydroxylated sugar helps minimize osmotic shock that the cell undergoes.

Chapters 7 and 8 document the unique metabolic strategy of directing carbon flow to glucose to act as a cellular protectant during periods of whole body water loss. During a period of relatively rapid dehydration (0.5 - 1.0 % per hour), R. sylvatica accumulated large quantities of glucose in the liver (up to 130 $\mu\text{mol/g}$) (Chapter 7). R. sylvatica has been shown to tolerate a maximum of 60 % total body water dehydrated. Full recovery is accomplished by submersing the severely desiccated frog in distilled water; resumption of the initial hydration state can be achieved in 10 - 20 hours (Chapter 7 and 8). Since P. crucifer has been extensively studied with respect only to its freeze tolerant capacities in the past, an investigation and comparison of the metabolic responses elicited by dehydration present a particularly novel interest in this second frog species.

The present study investigates dehydration tolerance in the spring peeper, P. crucifer, with respect to changes in tissue water, the phenomenon of glucose accumulation in response to desiccation, and any regulatory control of flux through glycolysis that may occur during these alterations.

MATERIALS AND METHODS

Chemicals and Animals

Frogs of the species P. crucifer were collected from the Ottawa, Ontario region in early/mid-April, 1990 for spring experiments. Frogs for fall experiments were collected in early-October, 1990. Animals were acclimated at 5 °C in a moist environment without food for at least 1 week (for spring frogs) and 4 weeks (for fall frogs) prior to experimentation. Average percent water content for combined fall and spring frogs was $82.3 \pm 1.3 \%$.

Initial survival tests that were performed indicated that the survival limit occurred at 55 % dehydration, therefore the spring dehydration experiments used 55 % dehydrated frogs for the initial assay of glucose and lactate accumulation in the liver. For the spring dehydration timecourse, as well as fall experiments however, 50 % dehydration was used as the final point. The spring dehydration timecourse involved points at 10 %, 25 %, 35 %, 50 % dehydration (at 5 °C). Fall experiments involved frogs 50 % dehydrated (at 5 °C). However, for glucose levels, protein content, and water content an additional group of frogs first dehydrated to 45 % water loss, then rehydrated to the initial water content. Frogs were rehydrated by placing the dehydrated animal in water (not covering the head); complete rehydration took approximately 12 - 24 h, although frogs were sacrificed at 24 h to eliminate any differences in frogs due to varying times of rehydration. Percent dehydrated was determined from the equation given in chapter 2.

Tissue Preparation and Metabolite Measurements

Tissues were sampled at the respective timepoints by quickly pithing the frogs and then, immediately excising the tissues and freezing them in liquid nitrogen. Tissues were stored at -80°C until processing. Control animals were killed immediately after removal from the 5°C incubator.

At each dehydration point, four samples ($n=4$) of each respective tissue were extracted for metabolites via the perchloric acid method as described by chapter 2.

RESULTS

Spring *P. crucifer* exhibited significant increases in glucose in 4 different tissues due to the stress of losing 55 % of total body water (Fig. 38a). Control glucose levels varied between 16.2 - 161.6 nmol/mg protein; corresponding wet weight values were 1.2 - 9.5 $\mu\text{mol/g}$ wet wt. Muscle, kidney, heart, and liver exhibited significant increases to final values of 101.5 - 246.7 nmol/mg [equivalent to 13.6 (N.S.), 18.2 ($p < 0.05$), 18.7 ($p < 0.05$), 42.2 ($p < 0.05$) $\mu\text{mol/g}$ wet wt., respectively]. However, glucose levels were not affected in either brain and gut. Lactate levels in three of the six tissues exhibited no changes from control levels; heart, gut, and brain all had average lactate values of 535, 92, and 81 nmol/mg (43, 12, and 7 $\mu\text{mol/g}$ wet wt.), respectively (Fig. 38b). Liver was the only tissue that showed a significant increase from control values of 42.3 nmol/mg to 47.7 nmol/mg at 55 % dehydration (an increase of 78 % to a final value of 8.7 $\mu\text{mol/g}$ wet wt.). Muscle and kidney however, both exhibited dramatic decreases on dehydration. Muscle lactate dropped from 216 nmol/mg (15 $\mu\text{mol/g}$ wet wt.) to 58 % of the control level ($p < 0.05$); kidney lactate fell from control values of 1340 nmol/mg (75.4 $\mu\text{mol/g}$ wet wt.) to 539 nmol/mg (40 $\mu\text{mol/g}$ wet wt.) at 55 % dehydration ($p < 0.005$).

FIG. 38. Effect of dehydration on levels of (A) glucose and (B) lactate in tissues of spring Pseudacris crucifer. Data are means $\pm$ SEM, n = 4. Bars are , control 0 % dehydrated; , 55 % dehydrated. a, b -Significantly different from corresponding control value by Student's t test, P < 0.05; P < 0.005, respectively. Actual measured value for mean water loss by experimental frogs at 55 % dehydrated was 54.5 ± 1.9 %.

Due to the nature of the dehydration stress, protein levels, as well as water content in liver were also measured in a separate dehydration timecourse (Fig. 39a and 39b). Protein content in spring liver showed no alterations from control levels of 326 ± 41 mg protein/g dry weight (Fig. 39a). Water content for these animals however, did show deviations from control levels (Fig. 39b). Control values of 1.84 ± 0.28 g water/g dry wt. dropped to 1.03 g water/g dry wt. upon initial dehydration at 10 % dehydration ($p < 0.05$). At 25 % dehydration, levels increased to 1.36 g/g ($p < 0.1$ with respect to control values); and by 35 %, values had resumed control quantities at 1.56 ± 0.30 g water/g dry wt. So, there was an initial water loss that the liver then counteracted even though the whole body was losing water.

Experiments with fall frogs, revealed various changes in protein and water contents in individual tissues throughout the dehydration timecourse (Fig. 40a and 40b). Protein levels in liver and heart exhibited no changes from control values of 550 ± 100 and 434 ± 28 mg protein/g dry wt., respectively as a function of 50 % dehydration. Kidney showed no initial changes at 50 % dehydration; however, upon rehydration to 0 %, protein content dropped by 40 % compared with control values ($p < 0.05$). Gut showed an increase in protein content at 50 % dehydration ($p < 0.05$). During rehydration, levels returned to control values, whereas muscle protein rose to greater than control levels (130 % of control) ($p < 0.05$). Water content in muscle, gut, and kidney all showed no change in water content when dehydrated. Upon rehydration, water content in kidney rose to 50 % greater than control values ($p < 0.05$); muscle and gut showed no changes upon rehydration. Heart and brain water content dropped by 57 % ($p < 0.05$) and 35 % ($p < 0.005$) during dehydration and resumed control levels upon rehydration. Liver water content decreased by 48 % ($p < 0.005$) when dehydrated to 50 %; rehydration resulted in an increase to near control water content. Although there were significant fluctuations in protein and water content in the 6 tissues during dehydration, when '% water in tissue' was assessed only

FIG. 39. Effect of dehydration on (A) protein content and (B) water content in liver of spring Pseudacris crucifer. Data are means $\pm$ SEM, n = 4. a -Significantly different from corresponding control value by Student's t test, P < 0.025. Actual measured values for mean water loss by experimental frogs at the different sampling points were 11.8 ± 1.5 %, 25.2 ± 1.2 %, 36 ± 2.9 %, 51.3 ± 1.8 %.

FIG. 40. Effect of dehydration on (A) protein content and (B) water content in tissues of fall *Pseudacris crucifer*. Data are means $\pm$ SEM, $n = 4$. Bars are , control 0 % dehydrated; , 50 % dehydrated; , rehydrated to 0 %. a, b -Significantly different from corresponding control value by Student's t test, $P < 0.05$, $P < 0.005$, respectively. Actual measured value for mean water loss by experimental frogs at 50 % dehydrated was 48.4 ± 0.5 %. Rehydrated frogs first lost 43.9 ± 2.6 % of total body water, then regained water over 24 h. to a final mean of 0.2 ± 3.5 %.

liver and muscle showed decreases and the 4 other tissues remained at control levels at 50 % dehydration (Table 16). Liver water as a % of total liver weight dropped from 89.3 % to 81.1 %; a decrease of only 8.2 % ($p < 0.005$). Muscle exhibited the largest decrease of the two tissues, falling by 15.5 % to a final value of 61.2 ± 4.7 % water ($p < 0.05$).

Dehydration in fall frogs had similar effects to those seen on spring animals, however, the increase in tissue glucose levels was much greater than their spring counterparts (Fig. 41a). Control glucose quantities ranged from 0.6 nmol/mg in muscle to 45.3 nmol/mg in kidney; corresponding wet wt. values were 0.04 - 2.55 $\mu\text{mol/g}$ wet wt., respectively. Liver control glucose values were 22 ± 4 nmol/mg (1.2 $\mu\text{mol/g}$ wet wt.). At 50 % dehydration, levels of the hexose jumped dramatically to 36 - 352 nmol/mg (4.6 - 36.8 $\mu\text{mol/g}$ wet wt.) for most tissues. Glucose in the liver, however, increased to 2690 nmol/mg protein, corresponding to 220 $\mu\text{mol/g}$ wet wt. ($p < 0.005$). Upon rehydration, the only decrease in glucose levels occurred in the liver which dropped to 1440 nmol/mg (76 $\mu\text{mol/g}$ wet wt.). All other tissue quantities remained high and even increased; small increases were seen to occur in heart and gut (by 10 % and 16 %, respectively) ($p < 0.005$ in both tissues), although glucose in kidney and brain rose by 215 ± 5 % in both tissues ($p < 0.1$ and $P < 0.01$). All of the tissues except for muscle and kidney, showed significant changes in quantities of lactic acid when dehydrated to 50 % (Fig. 41b). Lactate in muscle and kidney exhibited no significant increase at 50 % dehydration (Fig. 41b). Control values for all tissues ranged between 6.54 - 47.6 nmol/mg (0.59 - 2.68 $\mu\text{mol/g}$ wet wt.). Upon exposure to 50 % dehydration, lactate levels in the tissues that did show increases rose to 43 - 105 nmol/mg ($p < 0.01 - 0.005$); corresponding wet weight values for all tissues except for liver and heart had a range of 4.2 - 6.5 $\mu\text{mol/g}$ wet weight. Control liver and heart wet weight values at 1.15 and 1.39, respectively, rose dramatically to

Table 16.
Effects of Whole Animal Dehydration on Tissue Percent Water in fall Pseudacris
crucifer.

	Percent Water in Tissues	
	Control	50 % dehydrated
Liver	89.3 $\pm$ 0.8	81.1 $\pm$ 1.9 ^b
Muscle	76.7 $\pm$ 0.8	61.2 $\pm$ 4.7 ^a
Intestine	85.4 $\pm$ 4.4	84.6 $\pm$ 5.7
Kidney	96.2 $\pm$ 2.6	94.4 $\pm$ 3.5
Brain	92.8 $\pm$ 2.6	92.1 $\pm$ 3.4
Heart	93.2 $\pm$ 2.8	93.6 $\pm$ 5.8

Data are means $\pm$ SEM, n = 4. a, b - Significantly different from the corresponding control value, P < 0.05, P < 0.005.

FIG. 41. Effect of dehydration on levels of (A) glucose and (B) lactate in tissues of fall Pseudacris crucifer. Data are means $\pm$ SEM, n = 4. Bars are , control 0 % dehydrated; , 50 % dehydrated; , rehydrated to 0 %. a, b -Significantly different from corresponding control value by Student's t test, P < 0.05, P < 0.005, respectively.

8.64 and 9.83 $\mu\text{mol/g}$ wet wt.; an increase of at least 7 fold ($p < 0.01$ and $p < 0.05$, respectively).

Glycogen stores in liver of dehydrated fall frogs exhibited decreases which correlated well with the accumulation of glucose in the liver as well as the other tissues (Table 17). Control quantities of glycogen were 24.4 $\mu\text{mol/mg}$ protein and upon exposure to 50 % dehydration, levels dropped to 16.6 $\mu\text{mol/mg}$ ($p < 0.05$). In terms of a decrease in wet weight quantities, this was a difference of a total of 560 $\mu\text{mol/g}$ wet wt. glycogen (as glucose).

The glucose synthesis response to dehydration and the corresponding metabolic regulation involved in liver was further examined in dehydrated spring *P. crucifer*. Glucose levels doubled within the first 10 % dehydration; values jumped from 27.6 nmol/mg to 53.1 nmol/mg ($p < 0.01$) (Fig. 42a). Quantities continued to rise until a value of 295 nmol/mg was obtained at 50 % dehydration; corresponding wet weight values began at 3 $\mu\text{mol/g}$ wet wt. for control and concluded the dehydration timecourse at 53.7 $\mu\text{mol/g}$ wet wt. Although glycogen quantities did exhibit declining levels, values are not significantly different than control until 50 % dehydration where values dropped from control levels of 3.53 $\mu\text{mol/mg}$ protein to 0.76 $\mu\text{mol/mg}$ ($p < 0.01$) (Fig. 42b). This was a net decrease of 375 $\mu\text{mol/g}$ wet wt. glycogen (as glucose) from 515 $\mu\text{mol/g}$ to 140 $\mu\text{mol/g}$. Lactate values rose initially from 42.3 nmol/mg to 50.4 nmol/mg ($p < 0.05$) at 10 % dehydration (Fig. 42c). Corresponding wet weight values jumped from 4.86 $\mu\text{mol/g}$ wet wt. to 8.02 $\mu\text{mol/g}$; values remained high for the full duration of the dehydration timecourse; levels at 50 % dehydration were 47.8 nmol/mg.

Glucose levels in the muscle of spring frogs paralleled the increases seen in the liver (Fig. 43a). Within 10 % dehydration, glucose values increased 4 fold from control values of 16.2 nmol/mg to 66.4 nmol/mg ($p < 0.005$). Glucose levels increased 6 fold over the entire 50 % dehydration ($p < 0.05$); wet weight values

Table 17.
Effects of dehydration on glycogen stores in liver of fall Pseudacris crucifer.

% Dehydrated	Glycogen
0 %	24.4 $\pm$ 0.5
50 %	16.6 $\pm$ 1.8 ^b
Rehydrated	15.2 $\pm$ 3.1 ^a

Data are expressed in $\mu\text{mol/mg}$ protein; means $\pm$ SEM, $n = 4$. a, b - Significantly different from the corresponding control value, $P < 0.05$, $P < 0.005$.

FIG. 42. Effect of dehydration on levels of (A) glucose, (B) glycogen, and (C) lactate in liver of spring Pseudacris crucifer. Data are means $\pm$ SEM, n = 4. a, b - Significantly different from corresponding control value by Student's t test, P < 0.05, P < 0.01, respectively.

FIG. 43. Effect of dehydration on levels of (A) glucose and (B) lactate in muscle of spring Pseudacris crucifer. Data are means $\pm$ SEM, n = 4. a, b -Significantly different from corresponding control value by Student's t test, $P < 0.05$, $P < 0.005$, respectively.

increased from 1.18 $\mu\text{mol/g}$ to 13.5 $\mu\text{mol/g}$. Lactate in the muscle decreased during dehydration (Fig. 43b). Within 10 % dehydration, lactate levels dropped to 57 % control values ($p < 0.005$); in terms of wet weight values, levels fell by 5.1 $\mu\text{mol/g}$ from 15.7 $\mu\text{mol/g}$. Levels remained low throughout the duration of the dehydration timecourse.

Liver metabolites associated with the key regulatory enzymes, phosphofructokinase (PFK) and pyruvate kinase (PK), gave some indication of how glycolysis is regulated during dehydration (Fig. 44a-d). G6P rose by nearly 5 fold as frogs dehydrated to 35 % water loss ($p < 0.005$) (Fig. 44a). However, at 50 % dehydration, G6P fell dramatically to 30 % control values ($p < 0.05$). F6P paralleled G6P changes and rose 2 fold within the first 10 % dehydration ($p < 0.05$) and continued to increase an additional 1.75 fold until levels peaked at 35 % dehydration ($p < 0.005$) (Fig. 44b). Paralleling the fall in G6P at 50 % dehydration, F6P decreased to 16 % of control levels ($p < 0.005$). F1,6P₂ exhibited no fluctuations at all until 50 % dehydration, at which point levels jumped by at least 5 fold ($p < 0.005$) (Fig. 44b). Combined levels of the triose phosphates, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (DHAP and GAP), showed no changes from control levels (Fig. 44c). Metabolites associated with pyruvate kinase, PEP and pyruvate, exhibited no significant fluctuations until 50 % dehydration at which point pyruvate increased 2 fold ($p < 0.1$) (Fig. 44d).

Energy status in the liver during the dehydration stress remained stable until the final timepoint, 50 % dehydration (Fig. 45a-d). ATP, ADP, and AMP all maintained control levels until 50 % dehydration; then, ATP plummeted to 15 % of the control value ($p < 0.005$), ADP dropped by 40 % ($p < 0.05$), and AMP jumped 2 fold ($p < 0.005$) (Fig. 45a and b). Total adenylate quantities also dropped at 50 % dehydration to one-half of control values (Fig. 45a). ATP and total adenylate wet weight values were all less than 5 $\mu\text{mol/g}$. The drop in levels of high energy adenylates, combined

FIG. 44. Effect of dehydration on levels of (A) glucose-6-phosphate, (B) fructose-6-phosphate (filled circles) and fructose 1,6-bisphosphate (open circles), (C) dihydroxyacetone phosphate + glyceraldehyde-3-phosphate, (D) phosphoenolpyruvate (open circles) and pyruvate (filled circles) in the liver of spring Pseudacris crucifer. Data are means $\pm$ SEM, $n = 4$. a, b -Significantly different from corresponding control value by Student's t test, $P < 0.05$, $P < 0.005$, respectively.

FIG. 45. Effect of dehydration on levels of (A) ATP (circles) and Total Adenylates [ATP + ADP + AMP] (squares), (B) ADP (circles) and AMP (squares), (C) creatine phosphate, (D) Energy Charge $[(\text{ATP} + 1/2 \text{ADP})/(\text{ATP} + \text{ADP} + \text{AMP})]$ in the liver of spring Pseudacris crucifer. Data are means $\pm$ SEM, n = 4. a, b - Significantly different from corresponding control value by Student's t test, $P < 0.05$, $P < 0.005$, respectively.

with the dramatic drop in total adenylates resulted in a 50 % decrease in energy charge value from 0.86 to 0.42 ($p < 0.005$) (Fig. 45d). Creatine phosphate levels exhibited an initial drop from control levels at 10 % and 25 % dehydration ($p < 0.05$ at both points), however, levels returned to control values at 35 % and 50 % dehydration (Fig. 45c); wet weight values never rose above $0.25 \mu\text{mol/g}$.

DISCUSSION

Previous studies performed in our laboratory have demonstrated that if a fully hydrated frog is kept even in slightly damp moss, minimal amounts of body water are lost due to evaporation (chapter 7). In a natural environment, this type of supplemental water and protective covering is provided by the debris of fallen trees: leaf litter and rotting logs. Additionally, matted grasses and twigs help prevent dry air currents from increasing the rate of evaporation of any surface moisture on the forest floor. If the frog does dehydrate slightly in spite of these precautions, water uptake across the skin, especially in specialized areas on the rear of the animal, may be obtained from ground water or water trapped in organic debris. Since *P. crucifer* is a tree frog, a large proportion of their terrestrial life will be spent in the branches and crooks of shrubs and trees. In the spring time, flooded ponds provide excellent sites for tree frog habitation, specifically the laying and hatching of egg masses. I have often seen *P. crucifer*, as well as, several other species of tree frogs, seated in the notches of extensively branched trees or small shrubs that lie isolated in ponds. Not only do the branch notches protect the small frogs from evaporative water loss, but also from any potential predation that may occur in the early spring when food is at a premium as animals start to emerge from their state of winter torpor. Unfortunately these tree frogs are relatively small in comparison to most other types of frogs and in general, amphibians. This fact increases the probability of evaporative water loss, since a small size (1-3 cm

and 1-3 g) dictates a high surface area : volume ratio. Thus, dehydration is a crucial consideration for this species in particular as it attempts to fill a terrestrial niche. In preparation for the cold autumn and winter weather, amphibians either migrate towards ponds so that they can remain unfrozen underneath the ice during the winter or hide several centimeters below the surface of the forest floor. As the weather gets cooler and the air becomes dryer, amphibians must find suitable protected overwintering sites or else P. crucifer and other amphibians will be exposed to dangerously high degrees of desiccation and cold. This study was approached from the perspective that there may be considerable water lost when living in a dry environment. This would be especially true for a species of tree frog that lives in direct exposure to air in its habitat in the branches of trees. Hence, due to its lifestyle, P. crucifer (and more generally all tree frogs) would experience the most dramatic loss of water during daily living of any terrestro-aquatic amphibian (Duellman and Trueb, 1986; Hoar, 1986).

Once a frog exits its aquatic environment and is exposed to air, the process of dehydration starts immediately; any air convection would increase the rate of desiccation dramatically. This transition of lifestyle brought about (in evolutionary terms) the generation of an extensively vascularized outer skin (and lungs) so that the function carried out by the pre-existing gill filaments was maintained. However, this new adaptation (a highly vascularized skin) must contend with a harsh terrestrial climate). Rates of water transpiration are 5 fold greater at 30 °C than at 5 °C (Schmidt-Nielsen and Forster, 1954; Porter, 1972). Typical rates of desiccation for anuran are approximately $7.0 \mu\text{l}/\text{cm}^2/\text{hour}$ at room temperature (20 °C) (Porter, 1972). Animals, particularly amphibians, that have rates of evaporation as high as these are extremely subject to severe daily water loss; up to 9 % total body weight lost per day (Hillman, 1980).

As the dehydration episode proceeds in a frog, the outer muscle and skin desiccate first in an extremely sharp osmotic gradient from a fully hydrated tissue to the

dry air. Consequently, organs inside the visceral cavity are sequentially exposed to a direct osmotic stress since the outer muscle is adjacent to the internal tissues. However, as shown in Table 16, the percent water in the individual tissues of fall frogs did not vary to the degree that occurred for the whole body (ie. 50 % dehydrated). Only two of the six tissues/organs exhibited significant decreases in water content; at 50 % dehydration liver and muscle water content dropped by 8.2 % ($p < 0.005$) and 16.5 % ($p < 0.05$), respectively. Kidney, brain, heart, and gut all maintained a range of % water values of 84 - 96 % water. Therefore the only water remaining that could account for the loss of whole body water was the large amount of visceral water that surrounds the internal organs. Protein contents of organs remained constant; control levels ranged from 3.5 - 12 g water/g dry wt. with liver values at 8.5 g water/g dry wt. Spring frogs exhibited no change in protein content in the liver in terms of 'per gram dry weight'. Water content, however, dropped initially by 44 % from control values of 1.84 g water /g dry wt. at 10 % dehydration and then returned to their initial state at 25 % - 50 % dehydration. The dramatic difference in control water content values between spring (1.84 g/g) and fall animals (8.5 g/g) can be attributed simply to the large difference in glycogen quantities. Storey et al. (1981) concluded that in Eurosta solidaginis, an insect larva containing large amounts of glycogen, a substantial quantity of water was accounted for by being bound by the glycogen molecules present. Brittain and Geddes (1978) reported values for glycogen binding water of 0.8 to 9.0 g water/g carbohydrate depending on the size of the glycogen molecules present. Indeed, for every gram of glycogen placed into solution, approximately 5 grams of water is required for solubilization (personal observation).

The development of physiological and biochemical adaptations as an evolutionary response to repeated dehydration episodes is not unexpected since the adaptations to the terrestrial environment are fundamental characteristics of amphibians. It was recently discovered that the wood frog, R. sylvatica, when exposed to

moderately dry environments synthesizes large amounts of blood glucose (chapter 7 and 8). Liver glucose levels show significant increases within 5 % dehydration and after 55 % dehydration, glucose levels rise to a maximum of 130 $\mu\text{mol/g}$ wet wt. (chapter 7). Glucose in such large quantities may act as a protective agent against the direct effects of cellular dehydration, similar to the action of other compounds that build up in hundred millimolar quantities in animals that undergo severe osmotic stress (eg. urea, glycerol, sorbitol, salts) (Shoemaker, 1964; Katz, 1989; Loveridge and Withers, 1981). In this experiment, *P. crucifer* demonstrated a similar biochemical adaptation of glucose accumulation in response to whole body desiccation; peak levels reached 220 $\mu\text{mol/g}$ wet wt. in liver at 50 % dehydration!

In order to efficiently direct glycogen carbon to glucose via the glycolytic pathway, there must be several key regulatory enzyme control points that respond in such a way to prevent glucose catabolism (ie. to lactate production and to the TCA cycle) (Storey and Storey, 1988, 1992). The present findings clearly show that glycolysis is regulated at two enzyme loci, glycogen phosphorylase and phosphofructokinase (PFK). The activation of glycogenolysis was seen as a sudden rise in G6P levels and over time as glucose levels increased, liver glycogen decreased. An inhibition of the key regulatory control enzyme, phosphofructokinase (PFK) was also apparent as suggested by an increase in F6P and no change in F1,6P₂ levels. Further enzyme kinetic studies must be performed, however, involving glycogen phosphorylase and PFK in order to determine whether or not kinetic changes in these key enzymes can be attributed to a phosphorylation/dephosphorylation mechanism (Boyer and Krebs, 1987).

In the previous dehydration investigations involving *R. sylvatica*, an identical pattern of changes in metabolite levels and glycogen phosphorylase kinetics indicated an activation of glycogenolysis and a block in glycolysis (chapter 8). The activation of glycogenolysis was apparent as increases in both % a and total a + b units contributing

to an overall increase in active α units of 3.4 fold. The block of glycolysis occurred at PFK as indicated by metabolite changes; F6P exhibited significant 2 fold increases within the first 10 % dehydration, F1,6P₂ levels maintained control values. Although the alterations in metabolite levels were essentially identical to those observed in frozen P. crucifer (chapter 3) further kinetic studies must be undertaken in order to determine the exact mechanisms involved.

CHAPTER 10
GENERAL DISCUSSION

The phenomenon of natural freeze tolerance occurs widely among many insect and marine invertebrate species, however, freeze tolerance among vertebrate animals has only been documented in five species of terrestrially hibernating frogs, one species of salamander, several turtle species, and one species of snake (Storey and Storey, 1992). There are several factors which play key roles in the successful survival of freezing exposure in this unique group of vertebrate animals and even though freeze tolerant frogs have been extensively investigated with respect to tolerable ice content, cryoprotectant metabolism, and nucleating proteins, there are many aspects of their metabolic adaptations that remain to be uncovered. Conversely, tolerable limits of ice content and the metabolic responses of freeze tolerant reptiles (turtles and snake) are largely unknown and present a new system to which all of the previously obtained principles for freeze tolerance in vertebrates must be re-examined and tested.

This thesis carried out investigations on freeze tolerant frogs to further elucidate aspects of the mobilization of carbohydrate cryoprotectant in the liver and the distribution of these sugars to the tissues in two species (chapters 3 and 4). In spring frogs, the tolerance to freezing exposures immediately following spring thaw is comparable to the tolerance expressed in fall/winter animals (Storey and Storey, 1987). However, frogs tested several weeks later have a markedly reduced freezing tolerance. This reduced tolerance in spring animals has been linked to the reduced capacity for cryoprotectant (glucose and/or glycerol) synthesis. In chapter 3, tree frogs, *P. crucifer*, exhibited a reduced tolerance to freezing and yet the regulatory controls of glycolysis that direct glycogen carbon to cryoprotectant (glucose) were still in operation. A combined effect of increased glycogen breakdown and a blockage of the glycolytic pathway at phosphofructokinase ensured that carbon flux through glycolysis was effectively directed from glycogen to cryoprotectant glucose. The final cryoprotectant level in the liver was comparable to those found in the liver of similarly exposed fall frogs (Storey and Storey, 1987). However, even though glucose

distribution throughout the rest of the body showed a preference to the vital organs, levels of cryoprotectant were much lower in the tissues of these spring animals than in comparable fall frogs. This difference in the distribution of glucose to body tissues between fall and spring frogs appears to implicate the disappearance or reduction of glucose transport mechanisms in spring animals. Preliminary studies have demonstrated that fall wood frogs possess much greater glucose transporting activity in the liver than does a freeze intolerant frog, Rana pipiens (King P. and Storey K., preliminary data). The question arises: Does the number of transporter proteins increase? or, Are there two different seasonal transporter proteins, one for fall/winter cryoprotectant mobilization and one for 'normal' metabolic activity in the spring/summer? Further investigation of this aspect of freeze tolerance is required in order to provide answers to any of these questions.

It has been suggested that the reduced capacity for carbohydrate cryoprotectant production in spring frogs may be as a result of low liver glycogen contents (Storey and Storey, 1992). The frogs rely on endogenous food stores throughout the winter as they remain in a frozen state and upon thawing in the spring, mating activity commences immediately without any feeding activity in between. Thus, glycogen stores in the liver fall to extremely low values at some point in spring as a result. In chapter 4, the effects of freezing exposure on cryoprotectant synthesis in spring Hyla versicolor was investigated with respect to low levels of liver glycogen. H. versicolor is different from the other freeze tolerant frog species in that instead of utilizing glucose as sole cryoprotectant, it makes use of a combination of glycerol and glucose in a ratio of 400:100 $\mu\text{mol/g}$ for fall animals (Storey and Storey, 1984). However, spring animals showed no accumulation of glycerol. Instead regulatory controls directed glycolytic carbon from modest glycogen stores to glucose. Glycogen levels were much lower than in fall animals (typically 1000-1200 $\mu\text{mol/g}$) or even early spring frogs (P. crucifer in chapter 3 had 600-700 $\mu\text{mol/g}$). A correlation test between "glucose

produced" versus "glycogen content" demonstrated that there was positive correlation, $r = 0.937$. Therefore, glycogen stores must be fully capacitated in the fall, in order to successfully overwinter in a frozen state with sufficient cryoprotectant. In spring *H. versicolor* (and in the other freeze tolerant frogs), glucose is preferentially synthesized as a cryoprotectant. The reason for this may be that glucose is more readily interconvertible with glycogen than is glycerol when utilized as cryoprotectant. Once glycerol is synthesized as cryoprotectant, it has been observed that only 20 % of the glycerol carbon can be reconverted back to glycogen (Churchill and Storey, 1989b). In *Hyla versicolor*, there appears to be a seasonal adaptation that allows the production of glucose as the sole cryoprotectant in the spring. Thus, the synthesis of glucose would protect the frogs from mild and brief periods of freezing exposure that may occur in early spring. And when thawing occurs, glucose carbon can then be reconverted into glycogen as the subsequent source of glycolytic substrate for energy production, since endogenous food stores are at a premium in the spring. Additionally, it would seem that the biochemical adaptation of producing glucose as the sole cryoprotectant in *P. crucifer*, *R. sylvatica*, and *P. triseriata* is advantageous since the restoration of glycogen stores during periods of thawing is ensured.

The discovery of additional species of lower vertebrates to add to the select group of freeze tolerant vertebrates is extremely important. The investigation of the adaptations in recently discovered freeze tolerant animals (specifically garter snakes) provides an interesting comparison to the principles already established in the freeze tolerant frogs (chapters 5 and 6). As well, these studies may help generate a pattern of evolutionary development with respect to adaptations to the cold. Both the turtles and the garter snake appear to have lost the capacity for, or completely avoided, the synthesis of carbohydrate cryoprotectant (although small quantities of glucose may provide minor protection in these reptilian species). This is not to say that there are no forms of cryoprotectant present in these animals. Not only have elevations of single

amino acids been linked to the cryoprotection of in vitro systems, but it has also been suggested that specific combinations of amino acids act synergistically to protect cells and tissues from freezing injury (Loomis et al., 1988; Loomis, 1987). The transition of cryoprotection systems from what has been largely thought of as a one component system in the frogs to a multi-faceted system involving small amounts of carbohydrate protectants along with combinations of amino acids will help elucidate the principles of freezing tolerance in animals moving up the evolutionary pathway.

Other stresses that can occur in a natural environment are at least partially reflected during bouts of freezing. Dehydration as a result of extracellular ice formation and the state of anoxia during whole body freezing are both prime examples of such stresses. Comparison of the metabolic responses involved in freezing to those of other stresses, specifically anoxia (chapter 6) and dehydration (chapters 7, 8, and 9) distinguishes the metabolic adaptations already present as a result of their tolerance to anoxia and dehydration and the adaptations that develop as a result of freezing. The comparison of metabolic responses to freezing and anoxia in garter snakes (chapter 6) determined that freezing stress elicits similar responses to an anoxic stress. Even though the formation of ice in tissues may lead to unique structural damage that does not obviously occur during anoxia, it may be that the well developed adaptations for anoxia tolerance already in operation may confer a tolerance to freezing, at least for short periods. As well, there are dramatic similarities between the metabolic responses to freezing and dehydration, especially with respect to the accumulation of large amounts of glucose (127 and 220 $\mu\text{mol/g}$ in R. sylvatica and P. crucifer, respectively) and the glycolytic regulation involved in the mobilization this protective molecule (chapters 7, 8, and 9). Although dehydration and freezing stresses are obviously not identical stresses, there are extremely similar metabolic responses that may be indicative of the development of these responses along similar evolutionary lines.

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