Adaptations of metabolism for freeze tolerance in the gray tree frog, *Hyla versicolor*

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Biochemical adaptations allowing the natural survival of extracellular freezing were examined in the gray tree frog, *Hyla versicolor*. Laboratory-reared immature adults froze between −1 and −1.5°C and survived 5 days of freezing at −2°C as well as repeated rapid bouts of freeze–thaw. Measurements of ice content showed 41.5% of total body water frozen. Glycerol accumulated as the cryoprotectant in sexually mature adult *H. versicolor* (423 μmol/mL in blood) while both glycerol and glucose accumulated in immature adults (16.3 ± 6.8 and 25.9 ± 11.6 μmol/mL in blood, respectively). Cryoprotectant synthesis was freezing stimulated only and did not occur over long-term cold acclimation at 0 to 1°C. Cryoprotectant synthesis was correlated with a 203% increase in liver total phosphorylase activity and an increase in phosphorylase *a* content from 40 to 60%. Activities of 15 other enzymes of intermediary metabolism were determined in liver and leg muscle; activities of most enzymes increased with freezing exposure as did soluble protein content. Survival of freezing depends upon anaerobic mechanisms of energy production in tissues. Frogs frozen at −2°C accumulated lactate in liver and muscle. Energy charge dropped in both tissues and the creatine phosphate reserves of muscle were depleted.

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Les adaptations biochimiques qui permettent de survivre au gel extracellulaire ont fait l'objet d'une étude chez la rainette, *Hyla versicolor*. Des adultes encore immatures élevés en laboratoire gèlent entre −1 et −1,5°C et peuvent survivre à 5 jours de gel à −2°C ou encore à des périodes répétées de gel et de dégel. La mesure du contenu en glace a révélé que le gel affecte 41,5% du contenu hydrique total. Le glycérol s'accumule et sert de substance cryoprotectrice chez les individus à maturité sexuelle (423 μmol/mL sang), alors que le glycérol et le glucose sont tous deux utilisés par les individus immatures (16,3 ± 6,8 et 25,9 ± 11,6 μmol/mL, respectivement). La synthèse des substances cryoprotectrices n'est stimulée que par un gel et elle ne se fait pas au cours d'acclimatations à long terme au froid (0–1°C). La synthèse des substances cryoprotectrices est reliée à une augmentation de 203% de l'activité de la phosphorylase hépatique totale et à une augmentation de l'ordre de 40 à 60% du contenu en phosphorylase *a*. L'activité de 15 autres enzymes du métabolisme intermédiaire a été déterminée dans le foie et les muscles de la patte; l'activité de la plupart des enzymes augmente au cours d'un gel, le contenu en protéines solubles également. La survie au gel dépend de mécanismes anaérobiques de production d'énergie dans les tissus. Les rainettes gardées à −2°C accumulent du lactate dans le foie et les muscles. Le contenu énergétique des deux tissus baisse et les réserves de phosphocréatine musculaires diminuent.

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Introduction

The ability to withstand freezing of extracellular fluids during winter hibernation and diapause is found amongst a large number of insect species as well as other types of invertebrates (Block 1982; Danks 1978; Ring 1980). Recently, the first report of freeze tolerance in vertebrate animals was made; Schmid (1982) found that three species of terrestrial frogs, the gray tree frog, *Hyla versicolor*, the spring peeper, *Hyla crucifer* and the wood frog, *Rana sylvatica*, survived freezing. Freezing occured at about −2°C and animals survived for several days at −6°C. For *H. versicolor*, 34% of total body water, equivalent to extracellular water, was frozen. Frogs did not survive freezing at −30°C but the ability to survive freezing at moderate subzero temperatures is probably sufficient to ensure successful overwintering at hibernation sites under forest leaf litter. Freeze tolerance in *H. versicolor* was associated with the presence of high concentrations of glycerol, acting as a cryoprotectant, in tissues and urine (Schmid 1982).

The present report details our initial investigations of the biochemical adaptations for freeze tolerance in *H. versicolor*. The data show that sexually mature adult *H. versicolor* accumulate high concentrations of glycerol as a cryoprotectant while immature adults produce lesser amounts of both glycerol and glucose. Synthesis is in direct response to exposure to subzero temperature with no anticipatory synthesis of cryoprotectants during cold acclimation at slightly higher temperatures. Survival of freezing appears to depend upon the ability of tissues to generate energy via anaerobic (glycolytic) means. Parallel studies of freeze tolerance in *R. sylvatica* are reported elsewhere (Storey 1984; Storey and Storey 1984).

Materials and methods

Animals and chemicals

Laboratory-reared gray tree frogs, *Hyla versicolor*, were obtained (between mid-August and late September) as newly metamorphosed adults which had been raised in the laboratory from eggs. Frogs were initially fed a diet of *Drosophila*, later fed a diet of crickets, and were raised over a period of several weeks to a size averaging 1.46 ± 0.12 g. Animals collected from outdoor populations were obtained during early October and held in the laboratory at 3°C for at least 8 weeks before use.

Biochemicals and coupling enzymes were from Boehringer Mannheim Corp. or Sigma Chemical Co.

Animal experiments

Experiments with laboratory-reared sexually immature adults were performed during November to January, a time when outdoor populations face natural freezing exposures. Frogs were placed in plastic boxes with lids with a generous supply of damp sphagnum moss to maintain humidity. Feeding was stopped and animals were transferred from room temperature to an incubator at 12°C and held for 1 week. The temperature of the incubator was then lowered 1°C/day until 0°C was reached followed by 2 weeks at 0°C. Temperature was then lowered 0.5°C/day until −2°C was reached followed by 5 days frozen at −2°C. Animals were sampled at intervals throughout this acclimation; remaining animals were then returned to 3°C to thaw and were held at 3°C for 4 weeks before sampling.

TABLE 1. Tissue distribution of glycogen, glucose, and glycerol after freezing exposure in three specimens of *H. versicolor* collected from outdoor populations in autumn

	Days frozen	Tissue	Glycogen[a]	Glucose[b]	Glycerol[b]
Immature adult	2	Liver	26.8	9.6	6.6
		Leg muscle	12.1	3.1	8.0
		Heart	137.0	17.6	6.2
		Kidney	16.0	5.1	5.1
		Blood	—	5.7	7.3
Immature adult	9	Liver	5.3	26.3	12.9
		Leg muscle	2.7	1.5	10.8
		Heart	99.1	29.1	19.2
		Kidney	16.0	—	25.6
		Blood	—	7.8	15.8
Mature male	14	Liver	342.6	16.6	311.7
		Leg muscle	14.1	5.5	244.5
		Kidney	0	12.2	363.2
		Brain	56.3	17.1	464.8
		Lung	11.1	6.3	134.3
		Testes	1.2	2.6	5.7
		Stomach	2.4	9.4	254.5
		Intestine	11.7	7.1	204.5
		Abdominal muscle	13.0	4.4	217.6
		Skin	2.0	7.1	238.8
		Blood	—	6.8	422.5

[a]Glycogen is expressed as glucose units.
[b]Metabolite levels are given in micromoles per gram wet weight for tissues and micromoles per millilitre for blood.

In all cases, frogs were killed by double pithing. Animals were rapidly dissected open and a blood sample was removed from the severed aorta using a heparinized capillary tube. Blood volume was recorded and the sample was mixed with 180 μL of ice-cold 8% perchloric acid containing 1 m*M* EDTA. Tissues were then rapidly dissected out, frozen in liquid nitrogen and stored at −80°C until use. Six frozen frogs were dissected at −2°C (with no blood sampled) to preserve tissue metabolite levels in the frozen animals; all other frogs were dissected on ice.

Frogs collected from outdoor populations were moved from 3°C to an incubator at −2.5°C. Animals froze after 2−7 h and were sampled, as described above, after 2−14 days at −2.5°C.

Percentage of body water as ice

The ice content of frozen frogs was determined as outlined by Schmid (1982) and Storey (1984).

Metabolite measurements

Preparation of perchloric acid extracts of blood and tissues for metabolite assay was performed as described by Storey and Storey (1984). Metabolites were measured in coupled enzyme assays (Storey and Storey 1984).

Enzyme activities

Frozen tissues were homogenized (1:5, w/v) in 50 m*M* imidazole buffer, pH 7.5, containing 100 m*M* NaF, 5 m*M* EDTA, 5 m*M* EGTA, 0.1 m*M* phenylmethylsulphonyl fluoride, and 15 m*M* 2-mercaptoethanol. Homogenates were centrigfuged at 15 600 × *g* at 4°C for 5 min using an Eppendorf centrifuge and the resulting supernatant was used for the determination of enzyme activities. All enzyme activities were measured at 23°C. Optimal assay conditions for frog liver and muscle enzymes are described in Storey and Storey (1984). Soluble protein was determined by the method of Bradford (1976) using the prepared reagent from Bio-Rad Laboratories.

Results

Freezing

Freezing of laboratory-reared sexually immature adult *H. versicolor* began at about −1.0 to −1.5°C. When frozen at −2°C frogs had stiff limbs and solid abdomens. When dissected open, a large mass of ice filled the abdominal cavity and ice crystals were found surrounding the leg muscles. Internal organs were surrounded or imbedded in ice but were not themselves frozen. No heart beat or breathing were observed and bleeding did not occur.

Ice content of frogs frozen at −2°C for 5 days was estimated to be 41.5 ± 2.4% (n = 4) of total body water, suggesting freezing of extracellular water only. This value was somewhat less than that determined for *R. sylvatica* (48%), but greater than the 35% reported by Schmid (1982) for *H. versicolor*.

After freezing at −2°C for 5 days, some of these immature adults were returned to 3°C. Five out of six animals survived the freezing exposure, all showed normal behaviour (breathing, normal sitting posture, hopping when prodded) within 2 days of the return to 3°C, and all were alive and appeared healthy 4 weeks later when they were sacrificed. The adult *H. versicolor* survived 14 days of freezing at −2.5°C. Individual frogs required between 4 and 24 h of thawing at 3°C before they responded to pinching with vigorous limb movement but breathing and gulping were seen earlier. Immature adults also survived rapid cycles of freeze−thaw: two cycles of freezing at −4°C for 30 min followed by thawing at room temperature (approx. 20°C) for 1 h. Thawed frogs breathed normally, took a normal sitting posture, and hopped across the bench when poked.

TABLE 2. Blood metabolite levels in *H. versicolor*: comparison of control animals with animals frozen at −2°C and animals returned to 3°C after freezing exposure

	Concentration (μmol/mL)		Glucose/glycerol ratio
	Glucose	Glycerol	
Control (n=8)	1.46±0.52	0.10±0.02	14.6
Frozen	16.9	14.7	1.15
	7.7	5.6	1.38
	19.0	9.0	2.11
	60.0	36.0	1.66
Average	25.9±11.6	16.3±6.8	
Recovery	36.1	10.6	3.4
	12.6	15.4	0.82
	4.1	7.6	0.54
	6.3	41.6	0.15
Average	14.8±7.33	18.8±7.76	

NOTE: Control and average values are means ± SEM. Acclimation schedule was as described in Materials and methods. As no alterations in either blood or tissue metabolite levels were found at any acclimation temperature 0°C or above, all animals sampled at these temperatures have been combined in computing control levels. After 2.5 weeks at 0°C, temperature was further lowered 0.5°C/day until −2°C was reached and internal freezing occurred. After 5 days at −2°C, frozen frogs were sampled. Remaining animals were then returned to 3°C and were held for 4 weeks before sampling.

Cryoprotectants

As first noted by Schmid (1982), mature adult *H. versicolor* accumulate high amounts of glycerol when frozen (Table 1). Levels in blood measured 422.5 μmol/mL while tissue levels ranged from 134.3 μmoL/g wet weight for lung to 464.8 μmoL/g for brain. The exception to this high glycerol content was testes, with levels of only 5.7 μmoL/g; similar very low cryoprotectant levels have been found for eggs and ovary of *R. sylvatica* (K. B. Storey, unpublished observations). Sexually immature adults differed from mature adults in showing much lower concentrations of glycerol in blood and tissues. This occurred both with animals captured from outdoors (Table 1) and laboratory-reared animals (Tables 2, 3). In addition to glycerol, however, these frogs had elevated levels of glucose in blood and tissues although total cryoprotectant levels were not as high as in the mature adults. Levels of glycerol and glucose in blood of immature frogs were as high as 42 and 60 μmol/mL, respectively. Other potential cryoprotectants (sorbitol, fructose, mannose) were not detected in either blood or tissues.

Table 2 shows the levels of glycerol and glucose in blood of laboratory-reared immature adults under different conditions. Low-temperature acclimation of frogs using a 1°C/day decrease in temperature from 12 to 0°C followed by 2 weeks at 0°C (range, 0−1°C) (see Materials and methods) did not affect the blood levels of either compound; results for all animals sampled during this acclimation have therefore been pooled as control values in Table 2. Levels of glucose and glycerol are low in control animals; glucose concentration is similar to that found in blood of other frogs (Farrar and Dupre 1983; Storey and Storey 1984). Exposure to subzero temperatures (0.5°C/day decrease from 0 to −2°C followed by 5 days at −2°C), however, raised blood glucose and glycerol levels to 25.9 ± 11.6 and 16.3 ± 6.8 μmol/mL, respectively. Cryoprotectant concentrations varied quite widely between individual frozen frogs, but levels in each individual were closely linked; glucose content always exceeding that of glycerol up to a maximum of 2.1 fold. After thawing and 4 weeks recovery at 3°C, overall levels of cryoprotectants were still elevated.

TABLE 3. Levels of some intermediary metabolites in liver of *H. versicolor*: comparison of control animals with animals frozen at −2°C and animals returned to 3°C after freezing exposure

	Concentration (μmol/g wet weight)		
	Control	Frozen	Recovery
Glycogen[a]	340.7±58.2	354.6±57.6	174.2±50.6
Glucose	2.16±0.23	66.3±10.8	11.8±3.20
Glycerol	0.95±0.30	10.4±3.04	17.7±8.13
Lactate	0.76±0.27	10.3±3.04	1.54±0.54
ATP	1.75±0.14	0.74±0.17	0.95±0.13
ADP	0.57±0.09	1.25±0.08	0.79±0.09
AMP	0.08±0.03	0.89±0.23	0.32±0.09
Total adenylates	2.40	2.88	2.16
Energy charge[b]	0.85	0.48	0.66

NOTE: Results are means ± SEM; n = 6 for controls, n = 3 for frozen, and n = 4 for recovery.

[a]Glycogen is expressed as micromoles of glucose.

[b]Energy charge is defined as (ATP + 1/2 ADP)/(ATP + ADP + AMP).

TABLE 4. Levels of some intermediary metabolites in leg muscle of *H. versicolor*: comparison of control animals with animals frozen at −2°C and animals returned to 3°C after freezing exposure

	Concentration (μmol/g wet weight)		
	Control	Frozen	Recovery
Glycogen[a]	11.7±2.06	7.75±1.63	10.1±0.49
Glucose	1.15±0.31	5.69±2.60	4.43±1.14
Glycerol	0	11.8±2.83	16.4±7.33
Lactate	3.18±0.61	4.52±0.90	3.38±0.96
Succinate	0.52±0.05	0.89±0.51	0.42±0.03
Creatine-P	16.7±3.86	9.25±0.80	9.93±2.70
Creatine	15.3±1.11	18.3±1.66	22.9±5.36
ATP	4.51±0.25	4.43±0.12	4.43±0.29
ADP	0.66±0.05	1.45±0.56	1.20±0.38
AMP	0.11±0.02	0.50±0.23	0.23±0.05
Total adenylates	5.28	6.38	5.86
Energy charge	0.92	0.82	0.87

NOTE: Results are means ± SEM; n = 5 for control, n = 3 for frozen, and n = 4 for recovery.

[a]Glycogen is expressed as micromoles of glucose.

However, in three out of four instances, glycerol levels exceeded those of glucose and the glucose/glycerol ratios were much more variable than in the frozen animals.

Tissue levels of cryoprotectants in laboratory-reared immature adults were measured in liver and leg muscle (Tables 3, 4). Levels of both glucose and glycerol were again low in control animals; glycerol was not detected in leg muscle. Concentrations of both cryoprotectants rose in freezing exposed animals as was also seen in immature adults collected outdoors (Table 1). Glycerol content of laboratory-reared animals was 10−11 μmol/g wet weight in both tissues, the same level as in blood. Glucose levels in leg muscle were only about 1/3 of those in blood, while glucose in liver, at 66 μmol/g, was 3 times greater than blood levels. Glucose concentrations in frozen animals do not appear, therefore, to be in equilibrium in tissues and blood in contrast to the findings for glycerol. Levels of glucose/glycerol in the liver of frozen animals were 87.9/4.6, 54.7/14.9, and 56.4/11.6 and, in the muscle, glucose/glucerol levels were 2.23/6.57, 4.04/12.5, and 10.8/16.3 μmol/mL. Thus, glucose content exceeded that of glycerol in liver of all individuals while the opposite was true

TABLE 5. Maximal activities of enzymes in liver and leg muscle of control *H. versicolor* versus animals exposed to subzero (−2°C) temperatures

Enzyme	Liver		Leg muscle	
	Control	Freezing exposed	Control	Freezing exposed
Phosphorylase *a*	0.74±0.02	3.34±0.28	6.79±0.64	3.04±0.34
Total phosphorylase	1.85±0.06	5.61±0.27	9.84±0.48	12.6±0.85
Hexokinase	0.28±0.06	1.04±0.48	0.39±0.07	0.50±0.11
Glucokinase	2.26±0.11	3.14±0.12		
Phosphofructokinase	2.05±0.14	3.62±0.16	14.6±1.15	16.6±0.25
Glycerol-3-P dehydrogenase	24.3±3.00	36.1±13.3	14.7±1.35	20.3±4.10
Pyruvate kinase	43.5±0.60	78.1±30.8	132.0±2.40	214.4±36.5
Lactate dehydrogenase	25.2±1.00	47.3±9.40	227.3±36.8	316.8±44.3
Citrate synthase	5.95±0.84	9.90±1.80	15.7±0.65	17.3±0.02
Malate dehydrogenase	239.7±10.0	390.4±10.6	225.2±1.50	294.2±13.9
Glucose-6-phosphatase	3.35±0.12	2.54±0.22	5.48±0.37	9.07±0.53
Glucose-6-P dehydrogenase	6.00±1.65	10.0±1.51	0.28±0.01	0.35±0.06
6-Phosphogluconate dehydrogenase	2.44±0.20	6.30±0.70	0	0
Fructose-1,6-bisphosphatase	0.90±0.06	1.95±0.18	0.14±0.02	0.06±0.01
NADP-isocitrate dehydrogenase	14.4±2.20	23.7±0.90	9.18±0.04	10.9±0.85
3-Hydroxyacyl-CoA dehydrogenase	0.14±0.02	0.33±0.05	0.16±0.01	0.14±0.02
Glutamate dehydrogenase	95.3±5.30	207.7±22.7	0.98±0.36	1.17±0.20
Soluble protein	139±13	209±19	66.4±7.6	80.4±4.9

NOTE: Enzyme activities were measured under optimal substrate conditions (Storey and Storey 1984). Results are given as micromoles of substrate utilized per minute per gram wet weight ($\bar{x}$ ± SEM, n = 3). Control versus freezing-exposed frogs were obtained as described in Table 1.

of muscle. Levels of glucose and glycerol remained high in thawed animals after 4 weeks at 3°C. Glycerol levels were again similar in both tissues and blood. Glucose content of liver had decreased and was similar to that of blood while leg muscle still had glucose concentrations about 1/3 of those in blood.

Freezing exposure and tissue metabolite concentrations

Glycogen content of the liver of laboratory-reared immature adults was high (Table 3) as it is in liver of other freeze-tolerant frogs (Storey and Storey 1984) and of other frogs during winter (Farrar and Dupre 1983; Schlaghecke and Blum 1978). Freezing exposure, with its attendant cryoprotectant production, did not significantly alter liver glycogen content. However, glycogen contents of individual livers were highly variable, while cryoprotectant levels were quite low so a precursor–product relationship (as occurs for liver glycogen and cryoprotectant in *R. sylvatica*; Storey and Storey 1984) cannot be ruled out. Glycogen content of liver of the adult *H. versicolor* was similarly high (Table 1), but that of immature adults from outdoor populations was very low and this may account for the low cryoprotectant levels of these animals. Levels of glycogen in liver of laboratory-reared animals were significantly lower in animals examined 4 weeks after freezing exposure (Table 3); this may partially reflect glycogen utilized to maintain basal metabolism at 3°C. Glycogen content of leg muscle (Table 4) was quite low and remained relatively constant in the three groups of frogs. The elevated levels of glucose and glycerol in leg muscle in freezing-exposed frogs could not, therefore, be derived from the catabolism of endogenous glycogen.

Freezing exposure resulted in a significant increase in lactate levels in liver (Table 3), indicating the use of anaerobic glycolysis for energy production. A smaller elevation of lactate was found in leg muscle of frozen animals (Table 4). In both tissues, lactate decreased to control levels again when frogs were returned to 3°C.

Adenylate levels in liver of frozen frogs also indicated an energy stressed state; energy charge decreased from 0.85 in control liver to 0.48 in liver from frozen animals (Table 3). Phosphagen content was extremely low in frog liver, total creatine + creatine phosphate being 0.6 μmol/g or less. Apparently then, phosphagen provides no significant energy reserve for liver. Adenylate energy charge in leg muscle also decreased in the frozen animals but substantial creatine phosphate reserves were also depleted to provide energy during freezing (Table 4).

Enzyme activities

Table 5 shows the activities of 16 enzymes in *H. versicolor* liver and muscle representing the pathways of glycolysis, tricarboxylic acid cycle, gluconeogenesis, pentose phosphate cycle, fatty oxidation, and amino acid oxidation. Activities in control animals are compared with those in animals with freezing exposure. Activities of most enzymes in both tissues increased in animals which had received freezing exposure. Most enzymes in liver increased in activity by 49–88%; this was similar to the 50% increase in total soluble protein found in liver. Six enzymes showed higher percentage increases, in particular the activities of phosphorylase and hexokinase increased by 203 and 271%, respectively. The percentage of phosphorylase in the active *a* form also increased from 40% in control to 60% in freezing-exposed frogs.

The activities of most enzymes in leg muscle increased in the freezing-exposed frogs compared with controls. The percentage increase ranged from 0 to 74% (average = 26%) with an overall increase in soluble protein content of 21%. The activity of phosphorylase rose only 28% with a strong decrease in the percentage of phosphorylase *a* from 69% in control to 24% in

freezing-exposed animals.

Discussion

Hyla versicolor can withstand freezing at moderate subzero temperatures. Our studies, like those of Schmid (1982), found a high survival rate for immature adults after 5 days of freezing, while the adult *H. versicolor* survived 14 days of freezing. The animals also survived repeated bouts of freeze–thaw over short (1–2 h) time courses.

Schmid (1982) reported 0.3 *M* glycerol in urine and muscle of an *H. versicolor* specimen collected from the natural environment in midwinter. This agrees well with our findings for adult *H. versicolor*. Our results for immature frogs, however, showed much lower levels of glycerol produced during exposure to subzero temperatures and also showed some glucose synthesis as well. Glucose was identified as the sole cryoprotectant in another freeze-tolerant frog, *Rana sylvatica*; using an acclimation regimen similar to that used in the present study, *R. sylvatica* accumulated amounts of glucose as high as 325 μmol/mL in blood (Storey and Storey 1984). The reasons for the difference in amounts and types of cryoprotectants produced by sexually mature versus immature *H. versicolor* are not known at present; perhaps adults and juveniles differ in available glycogen stores for overwintering or in the triggering mechanism(s) used to stimulate cryoprotectant synthesis. Certainly the animals differ in winter experience; after metamorphosing in late summer, *H. versicolor* go through two winter seasons before breeding in their second spring. Laboratory-reared animals (and probably also the immature adults captured outdoors) were facing their first freezing exposure, therefore, while mature adults had already faced at least two winters. Long-term cold acclimation at 0–1°C failed to trigger an anticipatory synthesis of cryoprotectant by the frogs. This was also true of our studies of *R. sylvatica* (Storey and Storey 1984), but anticipatory production of cryoprotectants is a key adaptation of freeze-tolerant insects (Storey and Storey 1983). Synthesis in frogs is only triggered by direct exposure to subzero temperature, a system which would allow animals in the natural environment to avoid the conversion of stored fuel reserves into cryoprotectants during winters when the temperature in the microenvironment of the hibernation site did not fall below 0°C. Once synthesized, cryoprotectants persist in the animals as our measurements made 4 weeks after freezing exposure demonstrate. However, even though synthesis had been triggered by subzero exposure, production did not continue when animals were returned to 3°C. This suggests a very precise temperature control over the mechanisms of cryoprotectant synthesis; similar tight temperature control and temperature triggering is found for cryoprotectant synthesis in insects (Storey and Storey 1983).

The site of cryoprotectant synthesis in *H. versicolor* is probably the liver as it is in *R. sylvatica* (Storey and Storey 1984). Liver had a high glycogen content while most other tissues had very low glycogen contents (Table 1). In addition, freezing resulted in a strong activation of liver phosphorylase activity (a 203% increase in total phosphorylase and an increase in phosphorylase *a* content from 40 to 60%) compared with controls. Muscle did not show the same freezing activation of phosphorylase. The relative levels of glucose in tissues (liver > blood > muscle) of frozen animals also suggest the liver as the site of synthesis. Stronger evidence for this proposal was found in our studies of *R. sylvatica*; the rise in tissue and blood glucose was inversely proportional to the decrease in liver glycogen content while glycogen content of all other tissues was low and constant during exposure to subzero temperatures (Storey and Storey 1984).

During freezing exposure, extracellular fluids and blood are frozen. Observation shows that breathing and heart beat are stopped. Individual tissues are therefore isolated in an ischaemic and anoxic state and must rely upon the anaerobic degradation of endogenous substrates for the supply of cellular energy. The anoxia tolerance of tissues, particularly those such as brain, probably determines the length of time which the animals can survive in the frozen state. Freezing exposure resulted in an accumulation of lactate in liver and leg muscle of *H. versicolor* with the greatest accumulation in liver. Similar results were found for frozen *R. sylvatica* (Storey and Storey 1984). Energy demands in liver during freezing appear to be met by the use of anaerobic glycolysis. However, metabolism in the frozen animals is still energy limited as evidenced by the drop in adenylate energy charge in liver from 0.85 in the control state to 0.48 after 5 days at −2°C. The requirements of basal metabolism in muscle during freezing are met both from anaerobic glycolysis and from the utilization of the stores of creatine phosphate in muscle with creatine phosphate contributing the larger portion of energy when expressed in terms of ATP equivalents (7.4 μmol ATP/g wet weight from creatine phosphate compared with 2.0 μmol ATP/g from lactate production).

Freezing exposure resulted in increased activities of most enzymes in liver and muscle. In most instances, the increase in activity was similar to the percentage increase in soluble protein (50% in liver and 21% in muscle expressed per gram wet weight) found in the tissues. This suggests that tissues respond to freezing in one of two ways: (*i*) dehydration of cells to minimize the content of freezable "bulk" water resulting in increased enzyme activities or protein content expressed per gram wet weight or (*ii*) a nonspecific increase in cellular protein content which raises cellular osmotic pressure and aids in the prevention of cellular dehydration during extracellular freezing. Both of these strategies have been demonstrated in freezing-tolerant plant and animal systems (Ring 1980; Siminovitch 1981), although the first is the most likely. Major synthesis in the energy-stressed frozen state seems unlikely; however, further studies on this topic are needed. Several enzymes in liver, but none in muscle, showed very large percentage increases in activity in freezing-exposed animals, suggesting specific alterations in the levels of these enzymes. These included phosphorylase, which is well-known to be cold activated in cold-hardy insect species (Zieger *et al.* 1979). The increase in glutamate dehydrogenase activity may suggest increased synthesis of amino acids during freezing exposure as is known for some freeze-tolerant insects (Storey *et al.* 1981).

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