Purification and recovery of AP during the various steps of isolation

Fraction	Protein (μg)	AP activity (U)	AP specific activity* (U · mg protein^{-1})	Yield (%)
Crude homogenate	25000	25.2	1	100
Butanol extract	3250	30.2	9.3	120
190,000 × g supernatant	1545	25.7	16.6	102
Active fractions of G 2000 SW ($0.06 < K_{av} < 0.21$)	180	21.8	121.3	87

Protein was determined according to Lowry et al.[9] and AP activity according to Wöltgens et al.[8]. *This column also represents the purification factor.

This results in AP quantities sufficient to raise antibodies for future ultrastructural localization of AP. The purity of AP obtained in the present study is the highest reported from crude tooth homogenates. Molecular weights reported for AP from dental tissue from other species than the Syrian hamster, range from 55,000 to 240,000[4-6, 11]. Especially since different techniques are employed, some of them not being the most appropriate for this purpose, it is difficult to compare our value of 50,200 with the previous reported ones. Whether di- or polymer forms are involved and whether this is due to the butanol treatment, is not clear. The observed pI of 3.7 is very low compared with values for most mammalian AP's, although values around and below 4 have been reported[4, 12]. Comparison with other dental tissue AP is not possible, since no other pI values are available. The fact that in gel permeation as well as in chromatofocussing only 1 AP peak is found, strongly suggests that in hamster molars one, or several very similar forms occur. This means that the mesenchymal dentine-producing tissue and the epithelial enamel-producing tissue make use of the same AP isoenzyme.

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Freeze tolerance in the frog, *Rana sylvatica*

K.B. Storey[1]

Institute of Biochemistry and Department of Biology, Carleton University, Ottawa (Ontario, Canada K1S 5B6), 3 October 1983

Summary. Wood frogs survive extracellular freezing at moderate subzero temperatures (−4 °C) for at least 11 days. Freezing survival is aided by the accumulation of high concentrations of glucose as a cryoprotectant in blood and tissues. Glucose production was accompanied by a rapid decline in liver, but not muscle, glycogen levels suggesting that liver is the organ controlling cryoprotectant synthesis.
Key words. Rana sylvatica; frog, freeze tolerance; cryoprotectant synthesis; glycogen levels, liver; glucose levels, cryoprotectant.

A natural tolerance of extracellular freezing during overwintering has recently been reported for several species of terrestrial frogs[2, 3]. While freeze tolerance occurs quite widely amongst terrestrial insect groups[4, 5] these frogs are the only known vertebrate animals which survive freezing. As such they present us with what may be the optimal model system for studying problems related to tissue and organ cryopreservation in mammals. The present report discusses our initial investigations of the biochemical adaptations for freeze tolerance in the wood frog, *Rana sylvatica.*

Materials and methods. Specimens of *R. sylvatica* were collected during September 1982 from woodlands around Ottawa. Frogs were held in the laboratory at 23 °C for 3 weeks and were fed crickets. Feeding was then discontinued and animals were transferred to a cold room at 3 °C (range 1–4 °C) and held for up to 12 weeks. Frogs were held in plastic boxes with damp sphagnum moss to maintain humidity. Animals were sampled at intervals over the course of this cold acclimation to monitor cryoprotectant production. Cold acclimated frogs were then transferred to an incubator at 3 °C and temperature was lowered 1 °C per day until −4 °C was reached.

Results and discussion. Freezing occurred between −2° and −3 °C (Schmid[2] reported a supercooling point of −1.9 °C for *R. sylvatica*). Cold acclimated animals survived freezing at −4 °C for at least 11 days. External evidence of freezing included stiff and brittle limbs, solid abdomens and opaque eyes. Internally ice crystals were found surrounding the leg muscles and a solid mass of ice filled the abdominal cavity. Organs were surrounded by ice but were not frozen themselves. Heart beat and breathing were not observed. Animals could survive repeated freeze/thaw cycles; when thawed limb movement returned within 12–24 h at 3 °C.

The percentage of b.wt as ice was estimated by the method of Schmid[2]. Briefly, frozen frogs are transferred to an insulated container with 20 ml of water at 20 °C and allowed to thaw. The cooling of the surrounding water is measured and is compared to the cooling values of comparable weights of ice or water at −4 °C. The results showed an average of 47.8 ± 2.5%

of b.wt as ice (n = 6). Schmid[2] reported a lower amount, 35%, for *Hyla versicolor*.

The accumulation of cryoprotectants is an important adaptation for survival amongst cold hardy invertebrates[4,5]. Glycerol is the most common cryoprotectant but other polyols and various sugars also occur in some species. Schmid[2] reported 0.3 M glycerol in urine and muscle samples of the gray tree frog, *H. versicolor*. Cryoprotectants were measured in blood samples of warm and cold acclimated frogs and in those frozen at −4 °C for 4 days. Glucose is the cryoprotectant accumulated by *R. sylvatica* (table). Only minor amounts of glycerol were produced and sorbitol, fructose and mannose were not found. Amounts of glucose of up to 325 µmol/ml were found in blood with an average of 185 µmol/ml (3.33 g%). Glucose accumulated only in frogs which had experienced subzero temperatures. No glucose accumulation was found in cold acclimated frogs even after 12 weeks at 3 °C. Lack of an anticipatory response, as is found for cryoprotectant synthesis in many insect species[4,5], suggests that frogs synthesize cryoprotectant only when immediately threatened with freezing temperatures. Wood frogs hibernate under forest leaf litter; with a deep snow covering most may never experience subzero temperatures. However, if temperatures at hibernation sites do drop below 0 °C the frogs can quickly turn on their cryopreservation mechanisms. Glucose is perhaps an optimal cryoprotectant to use in this regard. Synthesis from glycogen requires only 3 enzymatic steps (phosphorylase, phosphoglucomutase, glucose-6-phosphatase) which are normally present in all tissues.

Effect of low temperature acclimation and freezing exposure on glucose and glycogen metabolism in the wood frog, *R. sylvatica*

		Control	Cold acclimated	Freezing exposure
		Blood metabolites (µmol/ml)		
Glucose		2.4 ± 0.3	2.0 ± 0.3	185.0 ± 39.7
Glycerol		0.1 ± 0.03	0.1 ± 0.05	1.1 ± 0.2
		n = 9	n = 7	n = 6
		Tissue metabolites (µmol/g wet weight)		
Liver:	Glycogen	904.3 ± 59.8	738.3 ± 75.0	100.7 ± 45.5
	Glucose	3.3 ± 0.6	4.1 ± 1.3	344.4 ± 34.7
Muscle:	Glycogen	48.5 ± 4.6	36.4 ± 8.3	38.1 ± 9.3
	Glucose	1.1 ± 0.2	0.8 ± 0.1	26.0 ± 4.1
		n = 5	n = 5	n = 6

Results are means ± SEM with n as shown. Control frogs were sampled after 3 weeks at 23 °C. Cold acclimated frogs were sampled at intervals between 1 and 12 weeks at 3 °C; no significant differences were found with time. Freezing exposed frogs underwent a 1 °C per day decrease in temperature from 3 to −4 °C followed by 4 days frozen at −4 °C. Frogs were killed by double pithing. Blood samples were removed from the severed aorta using a heparinized capillary tube. Tissues were rapidly dissected out and frozen in liquid nitrogen. Perchloric acid extracts of blood and tissues were prepared and metabolites were measured enzymatically as described by Storey and Storey[6,7]. Glycogen is quantitated as glucose units.

To further investigate cryoprotectant metabolism in *R. sylvatica* tissue glycogen and glucose levels were measured (table). Liver of *R. sylvatica* contained large amounts of glycogen; accumulation of glycogen during autumn months has been well documented for other anuran species as a preparation for winter survival[8,9]. Glycogen content of the liver decreased somewhat over the 12 weeks of cold acclimation, no doubt supporting basal metabolism. Liver glycogen content dropped dramatically, however, over the 8 days of exposure to subzero temperature. The corresponding rise in liver glucose levels indicates that liver glycogen reserves are converted to glucose. Muscle glycogen reserves were not affected by freezing exposure despite an increase in muscle glucose levels. This suggests that liver glycogen may be the source of all glucose (tissue and blood) produced with glucose distributed from liver to all other tissues via the blood. The 638 µmol/g wet weight decrease in liver glycogen (measured as glucose units) more than accounts for the 340 µmol/g rise in liver glucose levels. Although muscle glucose levels were elevated in freezing exposed animals, they were not in equilibrium with blood glucose concentration. This may perhaps be due to a restriction of blood flow to the extremities when subzero temperatures are encountered and/or an early freezing of extremities.

The present study shows that wood frogs can rapidly alter their intermediary metabolism when faced with freezing temperatures and initiate a rapid synthesis of glucose for use as a cryoprotectant. The accumulation of high levels of glucose in animals which have been exposed to freezing suggests major alterations in the mechanisms of control of blood glucose levels by pancreatic hormones (insulin, glucagon) in the freeze tolerant animal.

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Fluid and protein clearance in the rat endometrium. Part I: Ultrastructural proof of the absence of an intrinsic lymphatic system from the rat endometrium

J.M. Lauweryns[1] and F.J. Cornillie

Katholieke Universiteit te Leuven, School of Medicine, Laboratory of Histopathology, 12 Minderbroedersstraat, B-3000 Leuven (Belgium), 5 December 1983

Summary. An integrated histological and ultrastructural study of the endometrial microcirculation in rats reveals that lymphatic capillaries are absent from the superficial uterine mucosa. Blood capillaries are fenestrated, and their basement membrane may be poorly developed.

Key words. Rat, uterus; rat, endometrium; uterus, rat; microcirculation, endometrial; clearance, non-lymphatic; fluid-drainage physiology; lymph drainage; capillaries, lymphatic.