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Glycolysis and energetics in organs of hibernating mice (*Zapus hudsonius*)

Kenneth B. Storey and David A. Kelly

Abstract: Hibernation-induced changes in the concentrations of glycolytic intermediates, creatine phosphate, and adenylates were monitored in brain and skeletal muscle of the meadow jumping mouse, *Zapus hudsonius*, after both short (24 h) and long (5–7 d) periods of hibernation. Levels of hexose phosphates were greatly reduced in both organs after 24 h of hibernation, suggesting strong suppression of carbohydrate catabolism early in hibernation and indicating enzymatic regulation at the level of carbohydrate input into glycolysis. Both organs showed large changes in energy status during hibernation. Creatine phosphate content declined progressively over time to levels in 5–7 d hibernating animals that were 50 and 60% of control values in brain and muscle, respectively. Total adenylates and ATP levels also fell sharply during hibernation, but the net effect of changing adenylate levels on energy charge was minimal; energy charge was 0.94 in both organs of control animals and fell to 0.88–0.90 in hibernating animals. The overall reduction in the pool sizes of phosphagen and adenylates in organs of hibernating animals may be one factor involved in metabolic suppression during hibernation.

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K.B. Storey[1] **and D.A. Kelly.**[2] Institute of Biochemistry and Department of Biology, Carleton University, Ottawa, ON K1S 5B6, Canada.

[1] Author to whom all correspondence should be addressed.
[2] Present address: Faculty of Kinesiology, The University of Western Ontario, London, ON N6A 5C1, Canada.

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Résumé : L'hibernation entraîne des changements dans les concentrations des intermédiaires de la glycolyse, du phosphate de créatine et des adénylates; ces changements ont été mesurés dans le cerveau et le muscle squelettique chez des Souris-sauteuses des champs, *Zapus hudsonius*, après de courtes (24 h) et de longues (5−7 jours) périodes d'hibernation. Les concentrations des phosphates d'hexoses étaient beaucoup plus faibles dans les deux types d'organes après 24 h d'hibernation, ce qui semble indiquer une forte inhibition du catabolisme des hydrates de carbone dès le début de l'hibernation et l'intervention d'un système de contrôle enzymatique qui règle l'influx des hydrates de carbone nécessaires à la glycolyse. Les deux types d'organes ont subi d'importants changements de leur statut énergétique au cours de l'hibernation. Le contenu en phosphate de créatine a diminué progressivement jusqu'à des concentrations égales à celles qui prévalaient chez les animaux en hibernation depuis 5−7 jours, concentrations égales à 50% des concentrations témoins dans le cerveau et à 60% des concentrations témoins dans le tissu musculaire. Les concentrations totales d'adénylates et les concentrations d'ATP ont également diminué brusquement pendant l'hibernation mais l'effet net des changements des concentrations d'adénylate sur la charge énergétique était minimal; la charge énergétique était de 0,94 dans les deux types d'organes chez les animaux témoins et de 0,88−0,90 chez les animaux en hibernation. La réduction globale des pools de phosphagène et d'adénylates dans les organes des animaux en hibernation constitue peut-être un mécanisme de suppression métabolique au cours de l'hibernation.
[Traduit par la Rédaction]

Introduction

To survive the winter many small mammals hibernate. By abandoning homeothermy and sharply suppressing metabolic rate, often to levels less than 5% of the euthermic resting rate, body fuel reserves can be stretched to last over many months of dormancy (Wang 1987). Metabolic suppression is particulary strong in species with small body mass, such as mice and bats. For example, within the genus of jumping mice *Zapus*, Q_{10} values for oxygen consumption between the euthermic and hibernating states range from 3 to 4, suggesting strong specific suppression of metabolism over and above the effects of reduced body temperature on metabolic rate (which should give a Q_{10} value of about 2) (Geiser 1988). Meadow jumping mice, *Zapus hudsonius*, are among the smallest of the profound hibernators, species that do not eat during the periodic arousals that interrupt the hibernating season. Body mass doubles (to about 25−35 g) during a 2-week fattening period prior to hibernation, and this supports *Z. hudsonius* for the approximately 7 months of the year spent in hibernation (Whitaker 1972).

Numerous metabolic adjustments accompany hibernation; some mediate metabolic arrest, some deal with the large change in body temperature, and others regulate fuel use or waste product accumulation. Fat becomes the primary fuel, ketosis occurs, gluconeogenesis from amino acids or glycerol can provide carbohydrate, and net contents of glycogen and glucose are generally spared. Contributing to metabolic arrest is the specific suppression of the activities of various regulatory enzymes and metabolic processes (particularly mitochondrial functions) (Yacoe 1983; Fedotcheva et al. 1985; Gehnrich and Aprille 1988; Storey 1989; Brooks and Storey 1992), often facilitated by a respiratory acidosis brought on by CO_2 retention (Malan 1988; Nestler 1990). In *Z. hudsonius* changes in the properties of three glycogenolytic enzymes (glycogen phosphorylase, phosphofructokinase, pyruvate kinase) in liver were all consistent with the conversion of the enzymes to less active forms in the hibernating animal, the mechanism being reversible protein phosphorylation (Storey 1987*a*). Selected changes in these enzymes also occurred in other organs (Storey 1987*b*). Furthermore, the enzyme responsible for carbohydrate entry into the tricarboxylic acid cycle of the mitochondria, pyruvate dehydrogenase, was markedly affected in all organs tested. The percentage of enzyme in the active *a* form fell from 15% in heart and 29% in kidney of euthermic *Z. hudsonius* to less than 1% in both organs of hibernating mice (Storey 1989).

Thus, enzymatic changes accompanying hibernation in *Z. hudsonius* organs were generally consistent with both overall metabolic suppression and specific inhibition of carbohydrate catabolism, especially in liver. Changes in pathway flux can also be indicated by changes in the levels of intermediates in the pathway in response to an imposed stress or alteration of physiological state. The present study, therefore, continues an analysis of metabolic regulation during hibernation by monitoring changes in the levels of glycolytic intermediates and high-energy compounds (creatine phosphate, adenylates) in two organs of *Z. hudsonius* at different stages of hibernation. Metabolic status early in hibernation (after 1 d) is compared with that towards the midpoint (5−7 d) of a hibernation bout. The duration of torpor bouts in *Z. hudsonius* is probably about the same as those of the western species *Zapus princeps*; these start at about 5 d in the early autumn, quickly rise to over 20 d in midwinter, and fall to 5−10 d in spring (French 1988). The results for glycolytic intermediates confirm the suppression of carbohydrate catabolism during hibernation. Analysis of cellular energetics showed an important new result: both creatine phosphate and total adenylate levels were suppressed during hibernation, although energy charge was preserved. This suggests that the overall metabolic rate depression of hibernation may also be served by a general reduction in the availability of high-energy compounds in cells that may restrict or lower the rates of ATP-requiring metabolic processes.

Materials and methods

Meadow jumping mice, *Zapus hudsonius*, were collected by livetrapping in northern New York State by Dr. A. French and were held in laboratory colonies at the State University of New York, Binghamton, for 2−3 months before use. The animals were housed in glass aquaria, with individuals held

Table 1. Concentrations (nmol/g wet mass) of glycolytic intermediates in brain and skeletal muscle of euthermic and hibernating *Zapus hudsonius*.

	Brain			Skeletal muscle		
		Hibernating			Hibernating	
	Euthermic	24 h	5–7 d	Euthermic	24 h	5–7 d
Glucose-6-P	190±16	81±4*a*	201±11*b*	1258±85	105±10*a*	635±31*ab*
Fructose-6-P	53±4	12±1*a*	26±2*ab*	135±6	21±4*a*	144±8*b*
Fructose-1,6-P_2	87±9	7±1*a*	32±7*ab*	19±2	30±8	39±7
Glyceraldehyde-3-P	12±1	10±1	41±8*ab*	12±1	37±2*a*	22±3*ab*
Phosphoenolpyruvate	31±3	47±3*a*	21±1*ab*	35±2	31±2	24±4
Pyruvate	262±20	162±18*a*	168±5*a*	181±8	136±6*a*	123±12*a*

NOTE: Values are given as means ± SE; $n = 5$ animals under each condition. Values followed by *a* are significantly different from the corresponding euthermic value using the Student–Newman–Keuls test (two-tailed) ($P < 0.05$) and those followed by *b* are significantly different from the corresponding value for 24-h hibernating animals.

separately in plastic bottles on nesting beds of sphagnum moss to simulate hibernacula. The animals were given an ad libitum diet of sunflower seeds, Purina rabbit chow, and rolled oats. The temperature was 23°C, and natural daylight conditions were simulated with adjustments made once per week to reflect changing outdoor conditions.

To induce hibernation, mice were moved to a cold room with a diurnal temperature cycle between 12 and 6°C. Water and rabbit chow were provided but other food was withdrawn. Most animals entered hibernation within 24 h after the imposition of dietary restriction. Three groups of animals were maintained: control (euthermic, maintained at 23°C, and sampled the same day as the short-term hibernators), short-term hibernators (sampled 24 h after entry into hibernation), and long-term hibernators (sampled 5–7 d after entry into hibernation) (variation in the length of torpor was due to different entry times of individual animals).

All mice were killed by decapitation, their heads falling immediately into a container of liquid nitrogen. Skeletal muscle from the thighs was then quickly dissected out and freeze-clamped using tongs cooled in liquid nitrogen. At a later time the frozen brain was removed from the cranium, the procedure being carried out under liquid nitrogen.

Samples of frozen tissue were ground to a powder under liquid nitrogen using a mortar and pestle. Frozen tissue powder was then transferred to preweighed 15-mL polypropylene centrifuge tubes chilled on dry ice. The tubes were quickly weighed a second time and the mass of tissue powder was obtained by subtraction. Tubes were then transferred one at a time to a dry ice – methanol bath at −8°C. Five volumes (w/v) of 6% perchloric acid containing 1 mM EDTA (also chilled at −8°C) were rapidly added and the tissue powder was immediately homogenized using a Polytron PT10 homogenizer. Homogenates were centrifuged at 6000 × *g* for 15 min at 5°C to pellet protein, and a measured portion of the acid supernatant was then removed and transferred to a second centrifuge tube. The supernatant was neutralized by the addition of a solution containing 3 M KOH, 0.4 M Tris base, and 0.3 M KCl. Precipitated $KClO_4$ was then removed by a second centrifugation and the neutral supernatant was removed. One aliquot was removed for immediate assay of pyruvate and phosphoenolpyruvate (PEP), and the remainder was separated into two portions and frozen at −80°C for subsequent assays of other metabolites.

Metabolites were quantified using a Turner 430 spectrofluorometer and the coupled enzyme assays of Lowry and Passonneau (1972). Data were analyzed using one-way analysis of variance followed by the Student–Newman–Keuls test (two-tailed).

Results

Table 1 shows the concentrations of glycolytic intermediates in brain and skeletal muscle of *Z. hudsonius* and the effects of a short (24 h) or long (5–7 d) period of continuous hibernation on the levels of these compounds. Levels of hexose phosphates, glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P), were high in the brain of control mice but fell after 24 h of hibernation to 43 and 23% of control values. After longer hibernation, levels of both rose again, G6P returning to control levels, whereas F6P rose to 50% of initial control values. Fructose-1,6-bisphosphate (FBP) showed a similar pattern, dropping to 8% after 24 h and then rising to 37% of initial control values after 5–7 d. Glyceraldehyde-3-phosphate (GAP) levels were unchanged in brain after 24 h but increased 3.4-fold in longer term hibernators. PEP levels increased by 50% after 24 h but then fell to 70% of control levels after longer hibernation. Pyruvate behaved oppositely, decreasing to 62% of control after 24 h and remaining suppressed with longer hibernation.

In skeletal muscle, G6P and F6P levels also fell after 24 h of hibernation, reaching only 8 and 16% of the corresponding control values. After longer hibernation, levels of both compounds rose again, G6P reaching 50% of control values, whereas F6P content returned to control levels. FBP responded oppositely, rising by 58% after 24 h and remaining elevated in the long-term hibernators. GAP increased 3-fold in short-term hibernators but decreased with longer term hibernation. PEP levels did not change significantly during hibernation, but pyruvate levels fell significantly during hibernation, to 75 and 68% of control values after 24 h and 5–7 d of hibernation, respectively.

Table 2 shows the changes in creatine phosphate (CrP) and adenylate concentrations in *Z. hudsonius* brain and skele-

Table 2. Concentrations (μmol/g wet mass) of adenylates and creatine phosphate in brain and skeletal muscle of euthermic and hibernating *Zapus hudsonius*.

	Brain			Skeletal muscle		
		Hibernating			Hibernating	
	Euthermic	24 h	5–7 d	Euthermic	24 h	5–7 d
Creatine phosphate	6.48±0.59	4.95±0.45*a*	3.48±0.26*ab*	8.14±0.22	5.12±0.29*a*	4.81±0.57*a*
ATP	3.97±0.38	3.36±0.20	2.36±0.03*ab*	7.22±0.76	1.57±0.33*a*	3.13±0.25*ab*
ADP	0.56±0.03	0.20±0.04*a*	0.40±0.08*b*	0.83±0.02	0.28±0.07*a*	0.49±0.08*ab*
AMP	0.05±0.002	0.05±0.01	0.04±0.005	0.05±0.002	0.10±0.02	0.15±0.04*a*
Total adenylates	4.53±0.40	3.61±0.26*a*	2.80±0.12*a*	8.10±0.78	1.95±0.40*a*	3.77±0.38*ab*
Energy charge	0.94±0.01	0.96±0.01	0.91±0.01*ab*	0.94±0.01	0.88±0.01*a*	0.90±0.01

NOTE: For details see Table 1.

tal muscle during hibernation. In brain, CrP content decreased significantly after 24 h hibernation to 76% of control values and continued to decrease to 54% of controls with longer hibernation. Brain ATP content remained constant after 24 h but decreased to 60% of control after longer hibernation. Brain ADP content had decreased significantly after 24 h, but AMP content did not change during hibernation. Paralleling the changes in ATP content, the total adenylate pool fell during hibernation. However, energy charge, defined as [ATP + 1/2 ADP] / [ATP + ADP + AMP], was maintained at high values during short-term hibernation but fell slightly with longer hibernation.

Skeletal muscle CrP levels also decreased during hibernation, falling to 63 and 59% of control values in 24-h and 5- to 7-d hibernators, respectively. ATP content was strongly reduced to 22% of controls in 24-h hibernators, but increased again with longer hibernation, rising to 43% of control values. ADP and AMP contents followed the same pattern as ATP. The net effect was a large decrease in total adenylate content during hibernation but a relatively small decrease in energy charge from 0.94 in controls to 0.88–0.90 in hibernating animals.

Discussion

The analysis of changes in glycolytic intermediates in *Z. hudsonius* organs was consistent in showing a sharp decrease in the contents of hexose phosphates in both brain and skeletal muscle early in hibernation (24 h) (Table 1). This is consistent with other instances of aerobic metabolic arrest, such as in estivating snails (Churchill and Storey 1989), and opposite to the typical response seen in many systems when glycolysis is activated by different stresses (Lowry et al. 1964; Parkhouse et al. 1988; Storey 1987*c*; Kelly and Storey 1988). These results suggest that carbohydrate utilization by both brain and skeletal muscle is strongly reduced during the transition to the hypometabolic state. For skeletal muscle, reduced glycolytic flux is also linked to a switch to lipid as the primary fuel of metabolism during hibernation. Other authors have reported carbohydrate sparing during hibernation or daily torpor, measured by changes in RQ values or the oxidation rates of specific substrates (Wang 1987; Nestler 1990, 1992), and the present results show that this is directly reflected in changes in the levels of intermediates in the glycolytic pathway of carbohydrate degradation. The prominent changes in hexose phosphates, the initial metabolites of glycolysis, in *Z. hudsonius*, also suggest that regulation of carbohydrate catabolism is controlled at the level of carbon input into the system, probably by regulating glycogen breakdown (via control of glycogen phosphorylase) and (or) glucose utilization (via control of hexokinase or plasma membrane glucose transporters). A previous study showed that glycogen phosphorylase activity is reduced in *Z. hudsonius* brain during hibernation; the percentage of phosphorylase *a* decreased from 57% in euthermic controls to 25% in 5–7 d hibernating animals (24-h hibernators were not tested) (Storey 1987*b*). The parallel changes in F6P and FBP levels, the substrate and product of phosphofructokinase, in brain of 24 h hibernating animals indicated that this enzyme was not regulating carbohydrate flux during hibernation, although this is a key site of glycolytic regulation under situations of energy stress in mouse brain (such as during ischemia; Lowry et al. 1964). Brain also showed evidence of regulation at the pyruvate kinase reaction in 24 h hibernating animals; PEP levels rose and pyruvate levels dropped in comparison with controls, a situation consistent with an additional inhibitory block on glycolysis at this enzyme locus. However, unlike the phosphorylation-mediated control of brain glycogen phosphorylase, the possible mechanism of pyruvate kinase control is not clear, for an initial analysis found the enzyme to be of the nonallosteric type with no change in enzyme kinetic parameters detected between control and 5- to 7-d hibernators (Storey 1987*b*). Although levels of most metabolites in *Z. hudsonius* brain had increased after 5–7 d compared with the situation at 24 h, levels were generally still reduced compared with controls, and this suggests a continuing state of reduced carbohydrate catabolism with long-term hibernation.

The changes in glycolytic intermediates in skeletal muscle indicated a similar pattern of change in carbohydrate metabolism in muscle, as occurred in brain during hibernation. The outstanding feature of short-term hibernation was the strong suppression of hexose phosphate levels, indicative of a restriction on carbohydrate input to glycolysis. El Hachimi et al. (1990) also found a decrease in skeletal muscle G6P and F6P contents to less than 10% of control values in the hibernating jerboa. As in brain, regulatory control at the phosphofructokinase locus was not indicated, but the decrease in pyruvate levels could again suggest inhibitory control at the pyruvate kinase locus. Over the longer term (5–7 d),

concentrations of hexose phosphates increased (as in brain) but levels of other intermediates remained reduced. The significant rise in F6P in 5–7 d hibernating animals compared with 24-h hibernators with no change in FBP levels provides an indication that inhibitory control at the phosphofructokinase locus, although not important in the short term, may be important in sustaining glycolytic flux at a low level over long-term hibernation.

Brain and skeletal muscle underwent large changes in energy status during hibernation in *Z. hudsonius*. Both organs showed a substantial decrease in the levels of creatine phosphate and ATP during hibernation. Phosphagen levels decreased over time, falling to 50–60% of control values in both organs in long-term hibernators. ATP decreased progressively over time in brain, but the very strong decrease in ATP in muscle of 24-h hibernating mice (to only 22% of control values) was partially reversed as hibernation time was extended. The loss of ATP was not accompanied by opposite increases in ADP or AMP, so the total adenylate pool followed the same pattern; in 5- to 7-d hibernators the total adenylate pool was only 62% of the corresponding control values in brain and 47% in skeletal muscle. The fall in total adenylates implicates the action of the enzyme AMP deaminase, which catabolizes AMP to IMP and ammonium ion. The enzyme has an important function in removing AMP in many systems, particularly working muscle, and its action has the following consequences: (*i*) it helps push the myokinase reaction towards ATP generation, (*ii*) it helps stabilize an elevated energy charge, (*iii*) it supplies allosteric effectors, and (*iv*) it removes some of the protons that accumulate under acidotic situations (such as during anaerobic glycolysis or perhaps also under the respiratory acidosis of hibernation) via the synthesis of ammonium ion (Mommsen and Hochachka 1988). In *Z. hudsonius* the result of AMP removal from the adenylate pool was that energy charge was maintained at high values (0.88–0.96 compared with 0.94 for controls) in the organs from hibernating animals. This could be valuable in helping to maintain the hypometabolic state, for in other instances where ATP is depleted (e.g., muscle exercise, hypoxia–ischemia), the concomitant sharp increase in AMP is a key signal that activates catabolic pathways (particularly glycolysis via AMP activation of phosphofructokinase) to increase ATP synthesis, a situation that is clearly disadvantageous during hibernation, except perhaps during the arousal period.

Although the study by El Hachimi et al. (1990) showed no change in skeletal muscle ATP content and a doubling of the ATP/AMP ratio during hibernation in the jerboa, several other authors have reported a decrease in the ATP content of mitochondria or erythrocytes from hibernating mammals. The total ATP + ADP content was reduced to one-fifth in mitochondria from liver of hibernating versus active ground squirrels (Bronnikov et al. 1990), whereas Gehnrich and Aprille (1988) reported a 30% reduction in intramitochondrial adenine nucleotides during hibernation. Doherty et al. (1993) showed that erythrocytes from hibernating ground squirrels contained about 50% of the ATP content of cells from euthermic animals. Furthermore, cold storage of blood from euthermic animals resulted in a decrease in ATP levels to values comparable to those of hibernators, whereas cold storage of hibernator blood did not alter ATP levels. Hypothermia disrupts energy metabolism in nonhibernators (Hochachka 1986), but hibernators may exploit low temperature induced suppression of adenylate and CrP levels as part of the transition to the hypometabolic state. Thus, a net reduction in the overall cellular pools of high-energy compounds (CrP, ATP) may be part of the mechanism of metabolic suppression in hibernation. Aprille (1988) reviewed the strong evidence which shows that changes in the mitochondrial adenylate pool size can lead to selective regulation of metabolic activities that have adenine nucleotide dependent steps localized in the mitochondria, such as gluconeogenesis and urea biosynthesis. In newborn rabbit liver, for example, the mitochondrial matrix adenylate pool, the rate of pyruvate carboxylation (by ATP-dependent pyruvate carboxylase in the matrix) by isolated mitochondria, and the rate of gluconeogenesis from lactate by isolated hepatocytes all rose in parallel over several hours post partum, but metabolic activities could be suppressed again by depleting mitochondria of adenylates. Furthermore, the state 3 respiration rate is also sensitive to matrix adenylate pool size. Thus, one way to facilitate metabolic rate depression by hibernating mammals may be to alter the activities of the mitochondrial membrane ATP-Mg/P_i transporters to modify ATP-Mg uptake–release and via the consequent change in the net mitochondrial adenylate pool size, suppress the rates of ATP generation and ATP-dependent reactions in the organelle (Aprille 1988). However, in the nonhibernating mammalian species studied to date, the changes in mitochondrial adenylate pool size are usually caused by a redistribution of adenylates between mitochondria and cytoplasm with no net change in overall tissue adenylate levels (Aprille 1988). Hibernators may take the process one step further with a net decrease in total cellular adenylate levels that could help to sustain long-term metabolic suppression in all subcellular compartments at low body temperatures.

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