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ORGAN-SPECIFIC CONTROL OF GLYCOLYSIS IN
TWO MODEL SYSTEMS OF FACULTATIVE
HYPOMETABOLISM: THE DIVING TURTLE AND
THE HIBERNATING MOUSE

by David A. Kelly

A thesis submitted to the Faculty of
Graduate Studies and Research in partial
fulfillment of the requirements for the
degree of Master of Science in Biology

Department of Biology
Carleton University
Ottawa, Ontario
July, 1988

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ISBN 0-315-46297-3

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Graduate Studies and Research acceptance of
the thesis

ORGAN-SPECIFIC CONTROL OF GLYCOLYSIS IN TWO
MODEL SYSTEMS OF HYPOMETABOLISM: THE DIVING
TURTLE AND THE HIBERNATING MOUSE

submitted by David A. Kelly, B.Sc. in partial
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of Master of Science in Biology

Thesis Supervisor

Chairman, Department of Biology

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Abstract:

Patterns of glycolytic control were investigated using metabolite crossover studies in two model system vertebrates displaying a faculty for hypometabolism - the hibernator Zapus hudsonius (the meadow jumping mouse) and the diving freshwater turtle Pseudemys scripta.

Thigh muscle from the hibernator showed decreased carbon flux through glycolysis after 24 hours of torpor. Creatine phosphate was utilized in thigh muscle during the first 24 hours of hibernation. ATP showed a drop after 24 hours to 22% of normothermic levels. Brain tissue from the hibernator showed decreased carbon flux through glycolysis after 24 hours of torpor and a strong inhibition of PFK and PK after 24 hours of torpor. Creatine phosphate levels in brain showed a continuous decline over 7 days.

In the turtle, red muscle and heart showed an activation of PFK after one hour, which in heart was reversed after five hours. Brain also showed inhibition of PFK after five hours. Brain, heart, red muscle and white muscle all showed an activation of PK after one hour, which was reversed after five hours in brain, heart and red muscle. Liver showed strong inhibition of PFK within one hour of submergence. Creatine phosphate was utilized in heart, red and white muscle and liver.

ACKNOWLEDGEMENTS

I would deign to acknowledge my debt to Dr. Ken Storey for his almost limitless tolerance of my admittedly irregular habits and for his guidance and advice - not all of which was scientific, but all of which was certainly memorable; to his wife Jan for her instructive criticism in the design and execution of the experiments detailed herein; to Sandra Walters and Dr. Steve Brooks for their technical advice and for cheerfully and repeatedly performing the necessarily odious task of keeping me current on where to find everything; and to Ajoy, Ross, John and Tom for helping to create an entertaining environment in which to work, play and debate (not necesarily in that order). Special thanks to my thesis advisors, Dr. George Carmody and Dr. Tom Moon for their helpful suggestions regarding the design and performance of these experiments and their analysis and criticism of the results. I would like to thank the OC Transpo Co. for steadfastly providing me with an excuse every morning I arrived late. Lastly, my parents and brothers Tom and Steve deserve special thanks for their encouragement and support.

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LIST OF ABBREVIATIONS

α -GPDH	-	α -glycerophosphate dehydrogenase
ADP	-	adenosine diphosphate
AMP	-	adenosine monophosphate
ATP	-	adenosine triphosphate
CoA	-	coenzyme A
DHAP	-	dihydroxyacetone phosphate
EDTA	-	ethylene diamine tetraacetic acid
FDP	-	fructose-1,6-diphosphate
F-2,6-P ₂	-	fructose-2,6-bisphosphate
F6P	-	fructose-6-phosphate
G3P	-	glycero-3-phosphate
G6P	-	glucose-6-phosphate
G6PDH	-	glucose-6-phosphate dehydrogenase
HHNS	-	hypothalamo-hypophyseal neurosecretory system
HK	-	hexokinase
LDH	-	lactate dehydrogenase
μ mol/g	-	micromoles per gram wet weight
μ l	-	microlitres
μ M	-	micromolar
mM	-	millimolar
NAD ⁺	-	nicotinamide adenine dinucleotide
NADH	-	nicotinamide adenine dinucleotide (reduced)

n mol/g	-	nanomoles per gram wet weight
PCA	-	perchloric acid
PEP	-	phospho(enol)pyruvate
PFK	-	phosphofructokinase
PGI	-	phosphoglucose isomerase
PK	-	pyruvate kinase
R-L	-	right-to-left
s.e.m.	-	standard error of the mean
TIM	-	triose phosphate isomerase
tris	-	tris (hydroxymethyl) amino methane
U	-	units of enzyme activity
V	-	volume

Chapter 1. Introduction:

Metabolic Depression

Depression of basal metabolic rate and entry into a hypometabolic state - characterized by suspension of many normal physiological functions - is a commonplace response among many organisms to a variety of environmental cues. Anaerobiosis in marine invertebrates, goldfish and diving turtles in response to limited oxygen availability; hibernation in mammals and diapause in insects in response to reduced temperature; and estivation in lungfish in response to a lack of water are all examples of such depressed metabolism strategies. The physiological and metabolic consequences of these diverse strategies are largely similar. Many physical processes such as feeding and movement are curtailed, and various physiological functions - including heart rate and breathing or ventilation rate - are reduced. Non-essential cellular functions such as anabolism are reduced or suspended, while essential metabolic functions are brought into a new balance. However, extensive enzymatic replacement and reorganization does not take place, ensuring that a rapid return to the normal metabolic state can be achieved. The molecular mechanisms controlling metabolic depression are

conserved across phylogenetic lines in a fashion similar to metabolic activation (as in, for example, muscular exertion).

The key to survival in a hypometabolic state is the coordination of cellular processes. Studies of mammalian organs subjected to hypothermia or ischemia (Siesjo, 1981; Hansen, 1985; Hochachka, 1986) have suggested that irreversible damage occurs when the balance between energy production, energy utilization and passive processes in the cell is disrupted. Ca^{2+} channels can become activated, and this leads to uncontrolled influx of Ca^{2+} into the cell (Hochachka, 1986). Various Ca^{2+} -stimulated disruptions follow, the most severe of which is membrane destruction by Ca^{2+} -activated phospholipases. Irreversible cell damage ensues quickly; in mammalian brain, for example, a few minutes of ischemia leads to permanent loss of organ function (Lowry and Passonneau, 1964).

Regulatory controls used for metabolic depression - in addition to the requirement that they be readily coordinated - must be susceptible to rapid initiation and easy reversal, applicable to many cellular processes and somehow responsive to external stimuli, perhaps via second messengers. Control must be "above" the level of allosteric regulation, so that overall coordination of cellular activities may be achieved, and yet "below" the level of genetic expression and protein

synthesis to allow for rapid transitions into and out of the hypometabolic state.

The primary purpose of entering into a hypometabolic state is to conserve fuels and energy reserves under conditions of environmental stress of unpredictable duration. Calculations of the energy which would be utilized by the ground squirrel to remain euthermic throughout the winter season reveal that hibernation allows a saving of 88% of this figure (Wang, 1978). The bivalve mollusc Mytilus edulis can reduce its anoxic metabolic rate to levels 5 to 10% of the normoxic rate (Famme et al., 1981; Shick et al., 1983). Diving turtles under hypoxic and anoxic conditions also reduce their metabolic rate to as little as 10 to 15% of their standard aerobic rate (Jackson, 1968). Estivating lungfish can decrease their oxygen consumption by 50 to 85% (Swan et al., 1968; Lahiri et al., 1970). Similarly, adult insects undergoing diapause and hibernation show respective 4-fold and 20-fold metabolic decreases compared to nondiapause or nonhibernating adults; hibernating insects show this metabolic drop compared to nonhibernating adults at the same temperature (Wigglesworth, 1972).

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Part A: Investigation of Glycolytic Control in the
Hibernator Zapus hudsonius by Crossover Analysis

The environmental stress which a hibernating animal must endure is winter. Cold and a shortage of food are incompatible with maintaining the high metabolic rate and high body temperature of small mammals. Thus, the hibernating mammal undergoes an extreme reduction in metabolic rate (accompanied by a reduction in body temperature to near ambient temperature) and enters into a state of torpor. Dormancy is punctuated by numerous periodic arousals during which the mammal elevates its metabolic rate and body temperature to the homeothermic level and becomes active once again. The duration and frequency of periodic arousals vary with species (Jameson, 1981). These arousals are energetically very expensive and represent the greatest proportion of energy used during the hibernation period, a figure often as high as 80% (Muchlinski and Rybak, 1978). These arousals, however, likely serve an important metabolic or physiological function (perhaps clearance of noxious metabolic products, such as ketone bodies and urea) (Galster and Morrison, 1975; Pengelley and Fisher, 1961).

It is generally accepted that the primary fuel utilized during both torpor and arousal by a hibernating mammal is triglyceride fat (Tashima et al., 1970). The animals

prepare for winter by accumulating substantial amounts of fat, often increasing body mass by one third (Neumann and Cade, 1964). In the absence of dietary carbohydrate intake, the only processes available to fulfill the animal's requirement for carbohydrate are glycogenolysis and gluconeogenesis from protein (Zimmerman, 1982). [The glycerol moiety of triglyceride fat can enter the gluconeogenic pathway as glycerol-3-phosphate; but, since only one glucose molecule can be formed per two triglyceride molecules hydrolyzed, this process can only sustain very moderate glucose utilization (Stryer, 1981).] During starvation in mammals, muscle breakdown is prevented by inhibiting glucose utilization in many tissues (Robinson and Williamson, 1980); although many studies have shown less of body protein over the course of hibernation (Bintz, 1985), it has been suggested that massive depletion of muscle protein and an attendant accumulation of nitrogenous wastes during torpor can be greatly diminished by directly inhibiting protein catabolism (Zimmerman, 1982).

Evidence is accumulating that specific suppression of glucose utilization occurs in various tissues in hibernators, enhancing the reduction in glucose demand already present in these metabolically depressed tissues. Thus, glucose mobilization can be suppressed, but in a coordinated fashion which does not result in disruption of glucose homeostasis (that is, matching of substrate

availability to substrate requirements). Should glucose homeostasis become disrupted, ultimately a gap occurs between ATP requirements and ATP-generating capacities which leads to a loss of Ca^{2+} ion regulation and cell death by the mechanism detailed earlier.

The so-called "glucose sparing effect of ketone" - a phenomenon observed in fasting mammals in which elevated blood ketone levels inhibit glucose uptake and utilization by many tissues - may be responsible for much of the observed drop in glucose demand in tissues of hibernators (Robinson and Williamson, 1980). Accelerated lipolysis results in a release of free fatty acids into the bloodstream and their uptake by the liver where they are converted to acetylcoenzyme A (CoA) by β -oxidation. A drop in CoA concentration leads to condensation of two acetyl CoA fragments to form ketone bodies (acetoacetate and β -hydroxybutyrate) (Stryer, 1981). Studies have shown reduced uptake of glucose in the presence of ketone by heart and pectoralis muscle taken from hibernating Belding's squirrels (Spermophilus beldingi); ketone showed no inhibition of glucose uptake by muscles from fed or fasted squirrels (Krulowicz, 1985). Glucose utilization by muscles decreased over a six-day fast, but the reduction was independent of ketone concentration (Krulowicz, 1985).

A suggested mechanism explaining how ketone inhibits glucose oxidation in rat hearts is as follows. Cellular

acetyl CoA increases due to ketone body metabolism and this leads to increased intracellular citrate, a PFK inhibitor. The resulting accumulation of fructose-6-phosphate (F6P) leads to an accumulation of glucose-6-phosphate (G6P), since the two are held in equilibrium. High G6P inhibits hexokinase and this results in a buildup of glucose within the cell, which causes glucose uptake by the cell to decline (Randle et al., 1964).

Increased acetyl CoA can also inhibit pyruvate dehydrogenase, preventing pyruvate entry into the tricarboxylic acid (TCA) cycle and leading to pyruvate buildup, which in turn stimulates lactate production. Lactate would then be released to the bloodstream and transported to the liver as a gluconeogenic substrate (Robinson and Williamson, 1980). However, work on S. beldingi muscles indicates that lactate release does not figure importantly in the glucose sparing effect of ketone (Krulowicz, 1985). In fact, work on the little brown bat (Myotis lucifugus) showed that lactate dehydrogenase (LDH) activity decreases during hibernation in pectoralis major muscle, liver and heart, leading to reduced carbon flux to lactate during hibernation (Moon, 1978). Additionally, it is worth noting that LDH activity in these same tissues was not found to have changed in the big brown bat (Eptesicus fuscus) throughout hibernation and arousal, perhaps

- indicating that larger hibernators do not show decreased LDH activity during hibernation (Fonda et al., 1983).

The mechanisms suggested by researchers which control entry into torpor and reduction of body temperature are many and varied. This phenomenon is one of the least understood in mammalogy. Since hibernation requires profound adjustments in all physiological systems, a variety of integrated neurological processes are involved. For instance, in the hypothalamo-hypophyseal neurosecretory system (HHNS), cells in various species of bats, mice and squirrels contain more material which is stainable using Gormori's stain in hibernation than during the active summer period (Jasinski, 1970; Legait, et al., 1970; Polenov and Yurisova, 1976). This appears to indicate a lower activity of the HHNS during hibernation. Unfortunately, the functional role played by hypothalamic neurosecretory centers in the regulation of the endocrine systems during hibernation has not been well elucidated (Hudson and Wang, 1979). However, it is known that neurohumors such as serotonin (5-HT), norepinephrine (NE) and acetylcholine (ACh) can profoundly affect hypothalamic control of body temperature (Jansky and Novotna, 1976; Jansky, 1978) and this has led to speculation that a lowering of the hypothalamic set point and peripheral vasodilation promote the drop in body temperature (Snapp and Heller, 1981).

Important regulatory hormones which become altered during hibernation include insulin and corticosteroids. Both free and bound insulin decrease during entry into hibernation in the hedgehog; this suggests adaptive preparation for hibernation and may be linked to reduced glucose demand during torpor (Laurila and Suomalainen, 1974). Similarly, a decrease in pancreatic beta cell activity was suggested as the cause of the 350% increase in blood sugar levels in hibernating bats, Eptesicus fuscus (Hinkley and Burton, 1974). In the hibernating muskrat, there appears to be an association between 11-hydroxycorticosteroids and arousal, the fluctuation regulated by endogenous rhythms of the pituitary-adrenal system (Hudson and Wang, 1979). In the hibernating and aroused garden dormouse and hedgehog, however, the 17-hydroxycorticosteroid levels were measured and found to be virtually the same (Boulouard, 1972). This suggests that corticosteroid secretion is closely regulated in hibernators and also that if they perform a specific function during hibernation, that role differs from species to species (Wang and Hudson, 1978).

When looking for a physiological mechanism which stimulates entry into torpor and reduction of body temperature, it is necessary to differentiate between changes due to low body temperature and those changes which precede the hibernating state and show an active, or at

least permissive, role in preparation for the hibernating state (Hudson and Wang, 1978). In other words, careful differentiation must be made between causes and consequences of hibernation. Studies on the relationship between respiratory acidosis and the "relative acidosis" (an increase in pH which is lower than the estimated pH increase due to dissociated protein imidazole groups) observed during hibernation led some researchers to conclude that acidosis precedes lowering of the hypothalamic set point which leads to the reduction in body temperature associated with hibernation (Wunnenberg and Baltruschat, 1982; Malan et al., 1985; Malan, 1988). However, more recent examinations of such data and related work have led to the conclusion that "relative acidosis" is less a mechanism promoting a drop in body temperature and entry into torpor than a consequence of this phenomenon (Geiser, 1988).

Whatever the cause of entry into torpor and the temperature decrease associated with mammalian hibernation, one of the most contentious questions related to hibernation is whether the metabolic depression associated with this phenomenon is due strictly to temperature effects or whether an actual physiological mechanism is required to effect metabolic inhibition. Recent findings indicate that the metabolic reduction shown by larger hibernators can for the most part be explained by temperature effects, whereas small hibernators appear to require metabolic inhibition in

addition to temperature effects in order to sufficiently reduce energy requirements during torpor (Geiser, 1988). This partially resolves a pivotal disagreement in hibernation studies. On one side, Malan (1986) reports finding that the Q_{10} (that is, the rate change over a 10°C temperature change) of the oxygen consumption rates observed when switching from normothermia to hibernation is considerably greater (3.6) than the Q_{10} of mammalian biological reactions in general (2 to 3), suggesting that the low values of metabolism observed during hibernation cannot be explained by temperature effects alone. In contrast, groups whose Q_{10} measurements fell into the "normal" biological range of 2 to 3, claimed that temperature alone explained the reduction of metabolism during torpor (Snapp and Heller, 1981). Further clarification in this area might be provided by a study which scrutinized the glycolytic pathway during hibernation in a small hibernator for evidence of metabolic inhibition.

Glycolytic depression is often both a consequence and an indicator of overall metabolic depression, and scrutiny of the glycolytic pathway should produce some information regarding the role of carbohydrate metabolism in hibernation. Evidence to support the assumption of selectively depressed glycolysis - as opposed to glycolytic depression as a consequence of reduced mammalian body temperature alone - includes sparing of glucose by ketones

(Krilowicz, 1985). The fact that hibernating mammals become ketotic suggests greater comparative fatty acid oxidation than carbohydrate oxidation, so that glycolysis may be limiting anaplerotic carbohydrate input into the TCA cycle as oxaloacetate (Stryer, 1981). Further evidence to support selective depression of glycolysis includes a finding that during hibernation, investigators observed a substantial reduction in glycogen phosphorylase in cardiac muscle and an almost complete obliteration of glycogen phosphorylase activity in liver of the Arctic ground squirrel, Citellus undulatus (Hannon and Vaughan, 1960). Calculations of glucose turnover, using the findings of Tashima et al. (1970), also show a selective depression of glucose utilization. The rate of utilization by the hibernating ground squirrel, Citellus lateralis, appears to be 60-fold less than the active rate; this reduction is greater than the temperature decrease alone would indicate. Assuming a Q_{10} effect in the "normal" range of 2 to 3, the utilization rate would be expected to decrease merely 10- to 20-fold.

The hibernator specifically examined in this investigation was the meadow jumping mouse, Zapus hudsonius, of the family Zapodidae and a close relative of the much studied Napeozapus insignis. Zapus hudsonius inhabits southeastern Canada and northeastern U.S., hibernating 7 to 8 months of the year and emerging in late April or May. The dietary staples of the species include seeds, fruit and

fungi, sometimes supplemented by insect material (Sheldon, 1934; Whitaker, 1972; Collins and Cameron, 1984). Zapus hudsonius prepares for hibernation strictly by accumulation of body fat (beginning about two weeks before entry into torpor), often increasing its weight by one-third; no evidence of hibernacular food storage or other behaviour which would suggest feeding during arousal. Thus, Zapus hudsonius is thought to be a non-feeding hibernator (Sheldon, 1934; Neumann and Cade, 1964). Also important to the choice of Zapus hudsonius as test-hibernator is its great activity under normothermic conditions (adults are capable of hops of 1 to 2m in length and 0.3 to 0.6m in height) coupled with its small size (adults display a tailless body length of 80 to 100mm and a mass of 17 to 26g) guarantees a high normothermic mass-specific metabolic rate (Sheldon, 1934; Whitaker, 1972) - one which would be expected to require selective inhibition as opposed to merely Q_{10} effects to reduce overall metabolism in the hibernating state. Bower and Cade (1966) examined this high mass-specific metabolic rate using 11 mice (Napeozapus insignis and Zapus hudsonius) of average mass 21.6g at 31°C and determined it to be $1800\text{mm}^3 (\text{O}_2)/\text{hr/g}$ body weight on average. Zapodids were also found to have a low evaporative water loss rate (3mg/hr/g), likely an adaptation for hibernation (Bower and Cade, 1966), which will ensure little

change in body mass over 5 to 7 day hibernation due to sources other than fuel use.

Examinations of enzyme pathway control can be made using "crossover analysis". The crossover theorem states that in a generalized first order monomolecular reaction (for example, $A \rightarrow B$), the net rightward reaction rate V is some function of the concentrations of A and B ($V = f([A]/[B])$) such that it increases with increasing $[A]$ and decreases with increasing $[B]$. The generalized monomolecular reaction applies to a variety of detailed reaction mechanisms, and the only requirement is that the monotonic properties prevail (Williamson, 1970). Based on the generalized rate laws, the following logic describes consistent and faulty relationships between reaction flux and reactant concentration for the generalized monomolecular reaction.

Figure 1. Crossover logic for a generalized monomolecular reaction: A B

V	[A]	[B]	
+	+	+	
+	+	-	
+	-	+	"fault"
-	+	+	
-	-	-	
-	+	-	"fault"

+ indicates measured value increases

- indicates measured value decreases

"fault" indicates an inconsistency between flux and reactant concentration

(Williamson, 1970)

Thus, if the measured changes in flux and reactant concentration are experimentally inconsistent, then the generalized rate law is incomplete and some other agent is affecting reaction rate (Williamson, 1970). When applying the crossover theorem to an enzymatic pathway, one examines substrate and product concentrations pertaining to each enzyme in order to locate "faults", sites of enzyme activation (decreases in substrate concentration and increases in product concentration) or enzyme inactivation (increases in substrate concentration and decreases in product concentration).

Control of the glycolytic pathway is generally exerted at the irreversible sites: phosphofructokinase (PFK) and/or pyruvate kinase (PK) (Stryer, 1981). Consequently, the levels of the substrates (F6P and PEP respectively) and products (FDP and pyruvate respectively) of these enzymes were assayed under conditions of normothermia and short (24 hour) and long (5 to 7 day) term hibernation to determine whether these enzymes were subject to selective control over the course of hibernation. As well, assays were made of levels of G6P - an indicator of glucose flux through the pathway, primarily from glycogenolysis and glucose transport - and of levels of G3P, a product of the equilibrium enzyme aldolase (which breaks down FDP), and a further indicator of PFK modulation.

Part B. Organ-Specific Control of Glycolysis in the
Anoxic Turtle *Pseudemys scripta*

Displays of long term anoxia survival among several species of freshwater turtles have been well-documented. *Pseudemys scripta*, for instance, has shown the capacity to survive anaerobic dives for up to 2 weeks at 16 to 18°C (Robin et al., 1964). *Chrysemys picta belli* is capable of survival under anoxic conditions in cold water (3°C) for periods as long as 12 to 18 weeks (Ultsch and Jackson, 1982). A list of metabolic adaptations which support such facultative anaerobiosis includes: (Kelly and Storey, 1988)

1. maintenance of substantial reserves of fermentative substrate (glycogen), particularly in liver;
2. high glycolytic capacity in all organs;
3. tolerance for strong changes in pH of body fluids, coupled with sufficient buffering capacity to handle very high levels of accumulated lactate;
4. mechanisms for depression of overall metabolic rate.

The adaptation for metabolic rate depression is the key to long term survival of anoxia, since fermentative glycolytic catabolism of carbohydrates to lactate (the only anaerobic metabolic strategy available to vertebrates) is costly in terms of the substrate it requires (glycogen), is inefficient in terms of its energy yield, (2 as opposed to 36

ATP per unit glucose yielded by aerobic metabolism), and generates an end product which is physiologically toxic in high concentration (lactate). During the aerobic to anaerobic transition, overall metabolic rate becomes sharply reduced in concert with an overall drop in energy demand by various behaviours and physiological processes, allowing for prolonged anoxia survival. In turtles, anoxic metabolic rate is typically only 10 to 20% of the corresponding aerobic metabolic rate, measured at the same body temperature (Jackson, 1968; Caligiuri, 1981; Herbert and Jackson, 1985).

Traditional thought maintains that the great anaerobic capacity of freshwater turtles (Belkin, 1963) is an adaptation for routine diving. However, recent investigations have shown that undisturbed freshwater turtles making several routine dives over a five day period remain aerobic, do not rely on glycolysis and do not accumulate lactate (Gatten, 1981; Gatten, 1987). Thus, over short terms of submergence (routine dives of a few minutes) the turtle does not make an immediate metabolic shift to anaerobiosis. Instead, there is a two phase response to declining oxygen tension. Initially, all normal physiological functions are maintained and the aerobic rate of ATP generation is sustained. Large shifts in the oxygen tension of blood are routine occurrences in turtles, and so these animals possess such highly adaptive mechanisms as

redistribution of blood flow to regulate internal oxygen delivery to vital organs (heart and brain, primarily) under a wide variety of blood oxygen tensions (Lutz et al., 1985; Wood et al., 1987). Over the physiological range of oxygen tensions, mitochondrial respiration has been found to be oxygen dependent such that (when ATP concentrations and consumption rates are maintained constant) any declines in ATP production due to reduced arterial oxygen tension are offset by increased anaerobic glycolysis and creatine phosphate utilization (Lutz et al., 1985). Once physiological oxygen tension drops below a critical threshold (approximately 20 torr), metabolic depression is triggered and the turtle enters the second phase. The rates of all metabolic processes drop in order to conserve fuel and energy reserves for survival through indeterminate periods of submergence.

Investigations employing direct calorimetry designed to establish the timing of metabolic depression and anaerobiosis in the freshwater turtle Pseudemys scripta at 24°C have revealed that no metabolic depression occurs during the first 20 to 25 minutes of submergence despite declining arterial oxygen tension. Between 25 and 45 minutes of submergence, metabolic rate showed a depression to approximately 40% of the aerobic rate while available oxygen reserves were essentially depleted. This represents the severely hypoxic condition. For the remainder of the

dive, the rate of metabolism gradually declined to a new, low and constant level (approximately 10 to 15% of the aerobic rate) (Jackson, 1968). Throughout long term anaerobiosis, blood and tissue oxygen content drops to low and constant values of approximately 5% of the aerobic levels (Lutz et al., 1985). Lung and plasma CO_2 tensions rise progressively over the course of anoxia, exceeding 100 torr at the end of six hours of submergence (Robin et al., 1981).

Although the cause of metabolic depression during submergence has not yet been established, a few theories have been advanced. The hypothesis that lowered body oxygen triggers metabolic depression is supported by the observations that pre-dive ventilation with pure oxygen greatly delays the onset of metabolic depression and that the decline in metabolic rate coincides with arterial oxygen tension decreasing below a critical threshold such that available oxygen reserves are becoming depleted (Jackson, 1968). Another prevailing theory suggests that metabolic depression in freshwater turtles during submergence is a consequence of increases in blood CO_2 and lactate content and attendant acidosis. Possible support for this theory comes in the fact that the pacemaker enzyme of glycolysis is pH sensitive in many species; lowering the pH of tissues below 7.2 causes a huge drop in PFK activity (Lewiston et al., 1972). [Arterial plasma pH was observed to fall from

8.0 during normoxia to below 7.2 after extended anoxia (12 weeks at 3°C) in Crysemys picta belli (Jackson and Heisler, 1983).] The theory of acidosis control of entry in anaerobiosis in diving turtles is also supported by work on brain slices from Pseudemys scripta which showed a progressive decrease in lactate generation with decreasing pH (Lewiston et al., 1972).

Among vertebrates, it has generally been established that aerobic metabolism is essential for survival of the organism. Even such cells as muscle, which are capable of sustaining an oxygen debt, can only maintain cellular integrity for a short time on anaerobic glycolysis alone (Robin et al., 1964).

Cardiovascular reorganization - an important adaptation for conservation of oxygen reserves during anaerobiosis in turtle - is effected by intracardiac shunts. Reptiles such as the turtle possess a three-chambered heart, and intracardiac shunts are present as normal perfusion pathways. The consequences of these shunts upon gas transport are predictable; right-to-left (R-L) shunts reduce arterial oxygen saturation and both R-L and L-R shunts cause inequalities in pulmonary and systemic flows (Wood et al., 1987). R-L shunts also provide pulmonary by-pass during diving (Millen et al., 1964) which increases systemic flow and acts as a "CO₂ shunt" past the lungs, favoring oxygen uptake by diminishing the rate of acidification of arterial

blood and favoring oxygen delivery by increasing the rate of acidification of tissues (White, 1978). The mixing of arterial and venous blood, as observed in a reptile with a three-chambered heart, means that the partial pressure of oxygen in the mixture becomes uncoupled from ventilation and lung oxygen tension. R-L shunts cause arterial oxygen tension to become a dependent variable of arterial oxygen saturation and blood oxygen affinity. Consequently, arterial oxygen tension varies with body temperature and blood pH, and these factors can induce shifts of the oxygen dissociation curve, which lead to systemic reorganization of delivery of arterial oxygen to tissues (Rossoff et al., 1980). Blood flow to extremities is also restricted, conserving oxygen for the central core (Wood et al., 1987).

However, mere reorganization of cardiovascular flow to conserve oxygen reserves for heart and brain does not fully account for the long periods of anoxia which can be withstood by freshwater turtle. The hearts of anoxia intolerant vertebrates (such as mammals) are highly aerobic organs which are incapable of surviving an extended interruption in the flow of oxygen. The hearts of freshwater turtles, on the other hand, can develop almost 50% of aerobic tension after one hour of anoxia (Bing et al., 1972). Turtle hearts possess a number of specific properties which account for their high level of anoxia tolerance. One such adaptation is the large store of ATP

and creatine phosphate. Both compounds are utilized as energy sources during the hypoxia to anoxia transition phase (Clark and Miller, 1973). Levels of creatine phosphate and ATP are significantly higher than those in liver and brain (Clark and Miller, 1973). Additionally, although turtle hearts have a lower concentration of glycogen phosphorylase than mammalian hearts, they display a percentage of active glycogen phosphorylase a which is eight times that of mammalian heart (in order to facilitate rapid initiation of glycolysis during hypoxia) (McNeill et al., 1971). A finding of further significance is that turtle heart glycolysis at the PFK locus appears not to be inhibited by ATP; rather, the regulation of this enzyme is assumed to be the responsibility of creatine phosphate (Storey and Hochachka, 1974a). During anaerobiosis, the drop in creatine phosphate levels increases the activity of PFK and this results in rapid glycolysis (Gatten, 1987). Turtle heart PK, in contrast to heart PK in mammals, has a low pH optimum and strongly inhibited by ATP. Consequently, turtle heart PK functions optimally under anaerobic conditions when lactate levels are high and ATP levels are low (Storey and Hochachka, 1974b). Also unlike PK in mammalian heart, PK activity in turtle heart is strongly enhanced by FDP (Storey and Hochachka, 1974b); considering its regulatory effect upon both PK and PFK, FDP is of great importance to glycolytic rate control in turtle heart during anaerobiosis.

As in heart, brain of the freshwater turtle requires more than mere reorganization of blood flow with its attendant conservation of oxygen reserves to survive extended anoxia. Specific adaptations in turtle brain give a demonstration of successful coordination of depression of rates of ATP-producing, ATP-utilizing and passive processes (principally, ion leakage through membrane channels).

Studies have shown that, despite a drop in ATP production to approximately one-fifth of aerobic rates, turtle brain retains excitability and ion homeostasis over many hours of anoxia (Lutz et al., 1984; Lutz et al., 1985); in contrast, these parameters fail rapidly within minutes when mammalian brain is subject to anoxia. Of course, one must always keep in perspective the fact that turtle brains have metabolic rates almost 50 times lower than rat brains (Lutz et al., 1984).

Investigations of the glycolytic capacity of Pseudemys scripta brain show that a significantly ($p < 0.05$) greater fraction of ATP generation is carried out by glycolysis under both aerobic and anaerobic conditions than in rat brain. Additionally, turtle operates close to glycolytic capacity even under aerobic conditions (24%, compared to a maximum of 28% of total aerobic ATP generating capacity which can be achieved strictly by anaerobic glycolysis). This increased brain glycolytic capacity is thought to be an adaptation for short term routine diving, while a reduction

in the energy requirements of brain and other cells represents the long term anoxic survival strategy (Robin et al., 1979).

Fermentative catabolism of glucose to lactate is widely recognized as the process used to sustain anaerobiosis in the turtle. Creatine phosphate reserves contribute substantially to ATP needs during the hypoxia to anoxia transition period, but are often largely depleted within a few hours of submergence (Clark and Miller, 1973; Lutz et al., 1985). Other pathways' contributions to ATP production during anaerobiosis (such as amino acid fermentation or carbohydrate conversion to alternative end products) are minor (Penney, 1974). Turtle organs contain large stores of glycogen which can fuel energy needs over long excursions. Cardiac glycogen becomes exhausted after three hours of submergence, although brain glycogen appears not to be mobilized during the first several hours of submergence. Liver glycogen shows a progressive fall throughout anaerobiosis, accompanied by a progressive rise in blood glucose. Mobilization of liver glycogen appears to be the process which provides much of the fuel for longer anoxic excursions (Clark and Miller, 1973).

Since glycolysis represents the key ATP producing pathway during anaerobiosis in the submerged turtle, the response of glycolysis to declining arterial oxygen tension is an important avenue of study. Consequently, organ-

specific glycolytic control was investigated in a number of different organs and tissues (brain, heart, white muscle, red muscle and liver) in the aerobic, severely hypoxic (one hour of submergence) and depressed anoxic (five hours of submergence) states.

The experimental methods employed to examine glycolytic control in submerged turtle were those used to make the same examination in the hibernator: metabolite crossover assays of the levels of the substrates (F6P and PEP respectively) and products (FDP and pyruvate respectively) of the irreversible glycolytic pathway enzymes - phosphofructokinase (PFK) and pyruvate kinase (PK) (Stryer, 1981). These enzyme products and substrates were assayed under conditions of normoxia, severe hypoxia (one hour of submergence) and depressed anoxia (five hours of submergence) to determine whether these enzymes were subject to selective control over the course of anaerobiosis. As well, assays were made of levels of G6P - an indicator of glucose flux through the pathway, primarily from glycogenolysis and glucose transport - and of levels of G3P, a product of the equilibrium enzyme aldolase (which breaks down FDP), and a further indicator of PFK modulation.

Chapter 2. Materials and Methods:

Part A. Collection and Isolation of Tissues from the Hibernator *Zapus hudsonius*

Collection and Treatment of Animals:

Meadow jumping mice (*Zapus hudsonius*) were collected by live trapping in southern Ontario and northern New York State and were held in laboratory colonies at York University, Toronto and State University of New York at Binghamton for 2 to 3 months before use.

The animals were housed in glass aquaria, with individuals held separately in plastic bottles on nesting beds of sphagnum moss to simulate hibernacula. The animals were maintained on an ad libitum diet of sunflower seeds, Purina rabbit chow and rolled oats. Natural daylight was simulated, and adjustments were made once per week to mimic outdoor lighting conditions.

To induce hibernation, mice were moved to a darkened cold room whose temperature was cycled daily between 12°C and 6°C. Water and rabbit chow remained available ad libitum, while other food was withdrawn, following the protocol detailed in Collins and Cameron (1984). Most

animals entered hibernation within 24 hours of the imposition of dietary restriction.

Three groupings of animals were maintained: a control group (n=5), a short term group which was allowed to hibernate for 24 hours (n=5) and a longer term group which hibernated for 5 to 7 days (n=5). Sawdust was placed on the backs of hibernating mammals to determine whether arousal had occurred before sampling. Aroused animals - those which had shaken off the sawdust - were not sampled.

Collection of tissues:

The animals were killed by decapitation, and their heads were immediately frozen whole in liquid nitrogen. Thigh muscle was then quickly dissected out and freeze-clamped to liquid nitrogen temperatures. Later under liquid nitrogen, brains were carefully dissected from the whole frozen heads. All tissues were stored at -80°C until use.

Equipment:

All pH measurements were made using a Corning Model 125 pH meter. Solutions were prepared in deionized, distilled water, purified by the Millipore Milli-Q water purification system. Tissue samples were homogenized using a Polytron PT/10 homogenizer. The Sorvall RC 5-B Superspeed Refrigerated Centrifuge with the SS-34 rotor was used for all centrifugations.

Assays to determine glycolytic intermediate, adenylate and creatine phosphate concentrations for both thigh and brain of Zapus hudsonius were performed on the Turner Model 430 spectrofluorometer. A freeze clamping device was used for quick-freezing dissected tissues.

Part B. Collection and Isolation of Tissues from the
Anoxic Turtle *Pseudemys scripta*

Collection and Treatment of Animals:

Specimens of the painted turtle, *Pseudemys scripta*, were purchased from Boreal Canada, and held in recirculating aerated fresh water at 15°C without feeding for three weeks prior to use. Experiments were carried out upon black-shelled colour morphs only.

Isolation of Tissues:

Control and aerobic animals were sampled directly from the holding tank. To impose anoxia, animals were placed in a large container of fresh water through which nitrogen gas was continuously bubbled. Specimens of one group were removed from this "anoxia tank" after sixty minutes, while specimens of the second group were removed after five hours of anoxia. The heads and shells of *Pseudemys scripta* specimens were removed and samples of brain tissue and red and white muscle tissue were rapidly dissected out, blotted, and frozen in liquid nitrogen. [Red and white muscle samples were obtained from the region of the pectoral girdle after separation of the plastron from the shell.] Hearts and livers were rapidly dissected out and quick-frozen using a metal freeze clamping device which had been previously

chilled in liquid nitrogen. Tissues were stored at -80°C until used.

Equipment for Fluorometric and Spectrophotometric
Measurement of Metabolite Concentrations:

Assays to determine glycolytic intermediate concentrations for all tissues in Pseudemys scripta other than liver were performed on either the Turner Model 430 spectrofluorometer or the Aminco-Bowman Model 4-8208 spectrofluorometer. Assays for lactate levels in all tissues were performed on the Gilford Instruments Model 240 spectrophotometer. Assays for adenylate and creatine phosphate levels for all tissues and for glycolytic intermediate levels for liver were performed using the Pye Unicam SP 8-100 UV/visible spectrophotometer.

Part C. List of Chemical Suppliers and Experimental
Protocols for Fluorometric and Spectrophotometric
Intermediate Metabolite Assays

Chemicals:

All experimental chemicals used were laboratory grade,
and are listed below according to supplying company:

Allied Chemicals Ltd.

Magnesium Sulfate

Boehringer Mannheim

ADP

α -glycerophosphate dehydrogenase

Glucose-6-phosphate dehydrogenase

Lactate dehydrogenase

Myokinase

NAD⁺

NADH

Triose phosphate isomerase

J.T. Baker Chemical Co.

Perchloric acid

Potassium chloride

Potassium hydroxide

Potassium phosphate, dibasic

Potassium phosphate, monobasic

Sigma Chemical Co.

Aldolase

AMP

ATP

Creatine phosphate

DHAP

EDTA

Fructose-1,6-phosphate

Glucose

Glucose-6-phosphate

Hexokinase

Phospho(enol)pyruvate

Phosphoglucose isomerase

Pyruvate

Pyruvate kinase

Tris base

Sample Preparation:

Frozen tissues were ground to powder under liquid nitrogen using a mortar and pestle and were then transferred to sterile, preweighed polypropylene centrifuge tubes stored on dry ice. The tubes were weighed a second time to determine tissue weight. They were then transferred one at a time to a -8°C dry ice / methanol bath. Five volumes (w/v) of 6% perchloric acid (PCA) containing 1mM EDTA (chilled at -8°C) were quickly added. The powdered tissue was rapidly homogenized using a Polytron PT 10 homogenizer. Homogenates were centrifuged at 6000g for 15 minutes at 5°C to pellet the precipitated protein. The acid supernatant was removed, transferred to a second centrifuge tube and neutralized by the addition of 3M KOH / 0.4M tris / 0.3M KCl. KClO_4 was precipitated by a second centrifugation at 6000g for 12 minutes. Neutralized extracts were stored at -20°C until analysis (Williamson and Corkey, 1969).

Fluorometric Measurement of Metabolite Concentrations:

Concentrations of creatine phosphate and all adenylates and glycolytic intermediate metabolites for thigh muscle and brain tissue extracts were determined fluorometrically using pyridine nucleotide coupled enzyme assays. In each metabolite assay, the amount of pyridine nucleotide oxidized or reduced is exactly proportional to the amount of reacted substrate. For each assay, to a cuvette containing 0.8mL of

the appropriate reagent mixture, a volume of extract was added plus distilled / deionized water to a total volume of 1mL. After mixing, initial fluorescence was recorded and then reactions were begun by the addition of enzyme. Once reactions were judged to have proceeded to completion, a final fluorescence was recorded. Blanks (containing no extract) were run together with extract samples for each metabolite measured. A standard series (of unknown NADH concentrations) was run for each assay and from this a standard curve relating NADH concentration to fluorescence was obtained.

Changes in extract sample fluorescence were compared to the NADH standard curve to determine the amount of NADH which had been used or produced in the cuvette. The amount of pyridine nucleotide reacted could then be calculated from the formula (1) below.

$$\mu \text{ moles} = \text{conc.} \times \text{vol.} \quad (1)$$

Conc. is defined as the pyridine nucleotide concentration as determined from the standard curve and vol. is defined as the total cuvette volume. Change in fluorescence of the pyridine nucleotide was measured on the Turner Model 430 spectrofluorometer.

To determine the concentration of the metabolite of interest in the tissue, the following equation was used:

$$\mu \text{ mol/g} = \mu \text{ moles} \times \frac{V_5}{V_4} \times \frac{V_2 + V_3}{V_2} \times \frac{V_1 + w}{w}$$

where: μ moles = number of micromoles of metabolite present

$V_5 = 1000 \mu\text{L}$

V_4 = volume of sample added to cuvette, in μL

V_3 = volume of KOH solution required to neutralize acid supernatant, in mL

V_2 = volume of acid supernatant recovered, in mL

V_1 = volume of perchloric acid solution required to homogenize powdered tissue, in mL

w = tissue wet weight

Spectrophotometric Measurement of Metabolite

Concentrations:

Determinations of metabolite concentrations using a spectrophotometer differed from fluorometric determinations only in that initial and final absorbances at 340nm were recorded and net absorbance was compared to a standard curve of NADH absorbance at 340nm to obtain the value for the amount of metabolite present in the cuvette in μ moles.

ATP and Creatine Phosphate

To a cuvette containing buffer (50 μM tris-HCl, pH 8.1), NAD^+ (50 μM), MgSO_4 (1 mM), glucose (100 μM), glucose-6-phosphate dehydrogenase (G6PDH, 0.03 U) and neutralized extract, hexokinase (HK, 0.28 U) is added, and the change in fluorescence due to ATP utilization is recorded. Then creatine kinase (CrK, 1.0 U) and ADP (100 μM) is added and the change in fluorescence due to creatine phosphate utilization is recorded.

For the spectrophotometric assay, the cuvette must contain 500 μM NAD^+ and 1 mM glucose. To the cuvette, 500 μM ADP must be added (Lowry and Passonneau, 1972).

ADP and AMP:

To a cuvette containing buffer (50 mM potassium phosphate, pH 7.0), NADH (10 μM), MgSO_4 (2 mM), ATP (100 μM), phospho(enol)pyruvate (PEP, 300 μM), lactate dehydrogenase (LDH, 0.4 U), and neutralized extract, pyruvate kinase (PK,

0.6 U) is added and the fluorescence change due to ADP utilization is recorded. Then myokinase (MK, 0.36 U) is added and the fluorescence change due to AMP utilization is recorded.

For the spectrophotometric assay, the cuvette must contain 150 μM NADH (Lowry and Passonneau, 1972).

G6P and F6P

F6P ---> G6P (PGI)

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

To a cuvette containing buffer (50 μM tris-HCl, pH 8.1), NAD⁺ (100 μM), MgSO₄ (1 mM), and neutralized extract, glucose-6-phosphate dehydrogenase (G6PDH, 0.02 U) is added and the change in fluorescence due to G6P utilization is recorded. Then phosphoglucose isomerase (PGI, 0.35 U) is added and the change in fluorescence due to F6P utilization is recorded.

For the spectrophotometric assay, the cuvette must contain 500 μM NAD⁺. An amount of phosphoglucose isomerase equal to 0.50 U must be added to the cuvette (Lowry and Passonneau, 1972).

G3P and FDP

To a cuvette containing buffer (50 mM potassium phosphate, pH 7.0), NADH (10 μM), α -glycerophosphate dehydrogenase (α -GPDH, 0.20 U) and neutralized extract, triose phosphate isomerase (TIM, 2.4 U) is added and the fluorescence change due to G3P utilization is recorded. Then aldolase (0.05 U) is added and the fluorescence change due to FDP utilization is recorded.

For the spectrophotometric assay, the cuvette must contain 150 μM NADH (Lowry and Passonneau, 1972).

Pyruvate and PEP

To a cuvette containing buffer (50 mM potassium phosphate, pH 7.0), NADH (10 μM), MgSO_4 (2mM), ADP (200 μM), and neutralized extract, lactate dehydrogenase (LDH, 0.4 U) is added and the fluorescence change due to pyruvate utilization is recorded. Then pyruvate kinase (PK, 0.6 U) is added and the fluorescence change due to PEP utilization is recorded.

For the spectrophotometric assay, the cuvette must contain 150 μ M NADH (Lowry and Passonneau, 1972).

Lactate: (Spectrophotometric Assay Only)

$\text{lactate} + \text{NAD}^+ \longrightarrow \text{pyruvate} + \text{NADH} + \text{H}^+$

(LDH)

$\text{pyruvate} + \text{hydrazine} \longrightarrow \text{pyruvate hydrazone}$

To a cuvette containing buffer (190 mM hydrazine, 10 mM HCl, pH 9.6), NAD^+ (2.0 mM) and neutralized extract, bovine heart lactate dehydrogenase (LDH, 10U) is added and the absorbance change due to lactate utilization is recorded (Lowry and Passonneau, 1972).

Chapter 3. Results:

Part A. Investigation of Glycolytic Control in the Hibernator *Zapus hudsonius* by Crossover Analysis

Crossover Experiments: Thigh Muscle from *Zapus hudsonius*

TABLE 1. Concentrations of glycolytic intermediate metabolites in control, short term and longer term hibernated *Zapus hudsonius* thigh muscle.

Metabolite	Control (nmol/g)	24 hours (nmol/g)	5 to 7 days (nmol/g)
G6P	1258 ± 85	105 ± 10 ^a	635 ± 131 ^b
F6P	135 ± 6	21 ± 4 ^a	144 ± 8 ^b
FDP	19 ± 2	30 ± 8	39 ± 7
G3P	12 ± 1	37 ± 2 ^a	22 ± 3 ^{bb}
PEP	35 ± 2	31 ± 2	24 ± 4
Pyruvate	181 ± 8	136 ± 6 ^a	123 ± 12

^a Significant difference from the corresponding control value, (p < 0.01).

^b Significant difference from the 24 hour value, (p < 0.01).

^{bb} Significant difference from the corresponding 24 hour value, (p < 0.05).

Table 1 summarizes observed changes in the concentrations of selected glycolytic intermediate metabolites in the thigh muscle of Zapus hudsonius during short term (24 hour) and longer term (5 to 7 day) hibernation. Metabolite concentrations were measured using pyridine nucleotide-linked enzyme assays as detailed in Materials and Methods.

After the first day of hibernation, metabolite concentrations were compared to control values using Student's t-test to determine level of significance. Significant decreases ($p < 0.01$) were observed in G6P, F6P and pyruvate levels, while a significant increase ($p < 0.01$) was observed in G3P levels. Decreases in hexose phosphate concentrations (G6P and F6P) suggest a reduction of carbon flux through glycolysis, perhaps linked to depressed glycogenolysis. Levels of FDP and PEP did not show significant changes. The crossover theorem suggests possible activation of the glycolytic pacemaker enzyme phosphofructokinase (PFK) by the decrease in substrate concentration (F6P) and the accompanying increase in the concentration of PFK product (FDP), as well as a metabolite (G3P) held in equilibrium with FDP.

After an extended period of hibernation (5 to 7 days), metabolite concentrations were compared to short term values using Student's t-test to determine level of significance. Significant increases ($p < 0.01$) were observed in G6P and

F6P levels, while a significant decrease ($p < 0.05$) was observed in the level of G3P. No significant changes appeared in the levels of FDP, PEP or pyruvate. The crossover theorem suggests possible inhibition of PFK based on the increase in F6P and the decrease in G3P.

TABLE 2. Concentrations of adenylates and creatine phosphate in control, short term and longer term hibernated Zapus hudsonius thigh muscle.

Metabolite	Control ($\mu\text{mol/g}$)	24 hours ($\mu\text{mol/g}$)	5 to 7 days ($\mu\text{mol/g}$)
Cr-P	8.14 ± 0.22	5.12 ± 0.29^a	4.81 ± 0.57
ATP	7.22 ± 0.76	1.57 ± 0.33^a	3.13 ± 0.25^b
ADP	0.83 ± 0.02	0.28 ± 0.07^a	0.49 ± 0.08
AMP	0.047 ± 0.002	0.10 ± 0.02^{aa}	0.15 ± 0.04

^a Significant difference from the corresponding control value, ($p < 0.01$).

^{aa} Significant difference from the corresponding control value, ($p < 0.05$).

^b Significant difference from the 24 hour value, ($p < 0.01$).

Table 2 summarizes changes observed in the concentrations of adenylates and creatine phosphate in thigh muscle throughout short and longer term hibernation of Zapus hudsonius.

After one day of hibernation, concentrations of these metabolites were compared to control values. Significant decreases ($p < 0.01$) were observed in ATP, ADP and creatine phosphate levels and significant increases ($p < 0.05$) were observed in AMP levels compared to control values. These changes were reflected in an energy charge decrease from 0.94 to 0.83.

Metabolite concentrations measured after extended hibernation were compared to short term values. A significant increase ($p < 0.01$) was observed in ATP concentration. However, no significant changes in ADP, AMP or creatine phosphate were evinced. These results were reflected in a slight energy charge increase from 0.83 to 0.86, which suggested at least partial re-establishment adenylate level stability.

Crossover Experiments: Brain Tissue from *Zapus*hudsonius

TABLE 3. Concentrations of glycolytic intermediate metabolites in control, short term and longer term hibernated *Zapus hudsonius* brain tissue.

Metabolite	Control (nmol/g)	24 hours (nmol/g)	5 to 7 days (nmol/g)
G6P	190 ± 16	81 ± 4 ^a	201 ± 11 ^b
F6P	53 ± 4	12 ± 1 ^a	26 ± 2 ^b
FDP	87 ± 9	7 ± 1 ^a	32 ± 7 ^{bb}
G3P	12 ± 1	10 ± 1	41 ± 8 ^{bb}
PEP	31 ± 3	47 ± 3 ^a	22 ± 1 ^b
Pyruvate	262 ± 20	162 ± 18 ^{aa}	168 ± 5

^a Significant difference from the corresponding control value, (p < 0.01).

^{aa} Significant difference from the corresponding control value, (p < 0.05).

^b Significant difference from the 24 hour value, (p < 0.01).

^{bb} Significant difference from the corresponding 24 hour value, (p < 0.05).

After the first day of hibernation, glycolytic intermediate metabolite concentrations in brain (Table 3) were compared to control values. Significant decreases ($p < 0.01$) were observed over this period in G6P and F6P levels. Decreases in the concentrations of these hexose phosphates suggest a reduction of carbon flux through glycolysis, perhaps linked to depressed glycogenolysis. An enormous decrease from 87 ± 9 nmol/g to 7 ± 1 nmol/g wet weight was also observed in FDP levels and was shown to be statistically significant ($p < 0.01$). The crossover theorem suggest that this decrease in product concentration might indicate inhibition of PFK. No significant change was observed in G3P concentration. However, a significant increase ($p < 0.01$) was observed in PEP levels, accompanied by a significant decrease ($p < 0.05$) in pyruvate concentration. The crossover theorem suggest that this increase in substrate concentration, accompanied by a decrease in product concentration might indicate inhibition of the irreversible enzyme pyruvate kinase (PK).

Metabolite levels measured after extended hibernation were compared to short term values and significant increases ($p < 0.01$) were observed over this period in G6P and F6P levels. Significant increases ($p < 0.05$) were observed in levels of FDP and G3P. A significant decrease ($p < 0.01$) in PEP levels was observed over this period, although it was accompanied by no significant change in pyruvate levels.

TABLE 4. Concentrations of adenylates and creatine phosphate in control, short term and longer term hibernated Zapus hudsonius brain tissue.

Metabolite	Control ($\mu\text{mol/g}$)	24 hours ($\mu\text{mol/g}$)	5 to 7 days ($\mu\text{mol/g}$)
Cr-P	6.48 ± 0.59	4.95 ± 0.45^a	3.48 ± 0.26^{bb}
ATP	3.97 ± 0.38	3.36 ± 0.20^a	2.36 ± 0.03^b
ADP	0.56 ± 0.03	0.20 ± 0.04^a	0.40 ± 0.08
AMP	0.048 ± 0.002	0.05 ± 0.01	0.044 ± 0.005

^a Significant difference from the corresponding control value, ($p < 0.01$).

^b Significant difference from the 24 hour value, ($p < 0.01$).

^{bb} Significant difference from the corresponding 24 hour value, ($p < 0.05$).

After one day of hibernation, significant increases ($p < 0.01$) were observed in brain ATP, ADP and creatine phosphate levels compared to control values (Table 4). No significant changes were observed in AMP levels. These changes were reflected in a very slight energy charge increase from 0.92 to 0.95.

A significant decrease ($p < 0.01$) was observed in ATP concentration after 5 to 7 days. However, no significant changes in ADP or AMP appeared. These results were reflected in a moderate energy charge decrease from 0.95 to 0.90.

Part B. Investigation of Glycolytic Control in the
Anoxic Turtle *Pseudemys scripta* by Crossover Analysis

The periods of submergence in anoxic water at 18°C (1 hour and 5 hours) were chosen based on previous time-course studies of anoxia-induced metabolic depression which showed a reduction in metabolic rate to 10% of aerobic rate after 5 hours and a stabilization of energetic state (Jackson, 1968). Adjustments to glycolytic rate control would be fully expressed by this time. The earlier (1 hour) sampling time was chosen to focus on the transition period occurring after exhaustion of body oxygen reserves, during which metabolic rate is rapidly falling (Jackson, 1968; Lutz et al., 1985).

Crossover Experiments: *Pseudemys scripta* adenylates

TABLE 5. Concentrations of creatine phosphate and adenylates (with totals) and energy charge in heart muscle of 1 hour and 5 hour submerged *Pseudemys scripta*.

Metabolite	Control ($\mu\text{mol/g}$)	1 hour ($\mu\text{mol/g}$)	5 hours ($\mu\text{mol/g}$)
Cr-P	1.70 $\pm$ 0.07	1.72 $\pm$ 0.03	1.69 $\pm$ 0.04
ATP	3.53 $\pm$ 0.05	2.86 $\pm$ 0.05 ^{aa}	4.31 $\pm$ 0.26 ^b
ADP	0.54 $\pm$ 0.05	0.35 $\pm$ 0.05 ^{aa}	0.48 $\pm$ 0.04
AMP	0.10 $\pm$ 0.007	0.07 $\pm$ 0.004 ^a	0.07 $\pm$ 0.01
Energy charge	0.91	0.93	0.94
Total adenylates	4.17	3.28	4.86

^a Significant difference from the corresponding control value, ($p < 0.01$).

^{aa} Significant difference from the corresponding control value, ($p < 0.05$).

^b Significant difference from the 1 hour value, ($p < 0.01$).

TABLE 6. Concentrations of creatine phosphate and adenylates (with totals) and energy charge in brain tissue of 1 hour and 5 hour submerged *Pseudemys scripta*.

Metabolite	Control ($\mu\text{mol/g}$)	1 hour ($\mu\text{mol/g}$)	5 hours ($\mu\text{mol/g}$)
Cr-P	1.60 ± 0.11	1.92 ± 0.19	3.75 ± 0.42^b
ATP	2.41 ± 0.29	1.78 ± 0.21^a	4.28 ± 0.49^b
ADP	0.53 ± 0.14	0.34 ± 0.05^a	0.90 ± 0.10^b
AMP	0.27 ± 0.03	0.06 ± 0.01^a	0.11 ± 0.01^b
Energy charge	0.83	0.89	0.89
Total adenylates	3.21	2.18	5.29

^a Significant difference from the corresponding control value, ($p < 0.01$).

^{aa} Significant difference from the corresponding control value, ($p < 0.05$).

^b Significant difference from the 1 hour value, ($p < 0.01$).

TABLE 7. Concentrations of creatine phosphate and adenylates (with totals) and energy charge in red muscle of 1 hour and 5 hour submerged *Pseudemys scripta*.

Metabolite	Control ($\mu\text{mol/g}$)	1 hour ($\mu\text{mol/g}$)	5 hours ($\mu\text{mol/g}$)
Cr-P	6.28 ± 0.58	3.65 ± 0.20^a	8.54 ± 0.17^b
ATP	3.71 ± 0.07	3.26 ± 0.08^a	4.12 ± 0.34^{bb}
ADP	0.63 ± 0.02	0.48 ± 0.02^a	0.47 ± 0.07
AMP	0.11 ± 0.01	0.03 ± 0.01^a	0.04 ± 0.007
Energy charge	0.90	0.93	0.94
Total adenylates	4.45	3.77	4.63

^a Significant difference from the corresponding control value, ($p < 0.01$).

^b Significant difference from the corresponding 1 hour value, ($p < 0.01$).

^{bb} Significant difference from the corresponding 1 hour value, ($p < 0.05$).

TABLE 8. Concentrations of creatine phosphate and adenylates (with totals) and energy charge in white muscle of 1 hour and 5 hour submerged *Pseudemys scripta*.

Metabolite	Control ($\mu\text{mol/g}$)	1 hour ($\mu\text{mol/g}$)	5 hours ($\mu\text{mol/g}$)
Cr-P	5.56 ± 0.27	4.91 ± 0.21	2.49 ± 0.14^b
ATP	4.25 ± 0.15	3.21 ± 0.15^{aa}	4.92 ± 0.83
ADP	0.66 ± 0.04	0.36 ± 0.01^a	0.45 ± 0.02^b
AMP	0.06 ± 0.006	0.03 ± 0.007^a	0.04 ± 0.007
Energy charge	0.92	0.94	0.98
Total adenylates	4.97	3.60	5.47

^a Significant difference from the corresponding control value, ($p < 0.01$).

^{aa} Significant difference from the corresponding control value, ($p < 0.05$).

^b Significant difference from the 1 hour value, ($p < 0.01$).

TABLE 9. Concentrations of creatine phosphate and adenylates (with totals) and energy charge in liver of 1 hour and 5 hour submerged *Pseudemys scripta*.

Metabolite	Control ($\mu\text{mol/g}$)	1 hour ($\mu\text{mol/g}$)	5 hours ($\mu\text{mol/g}$)
Cr-P	2.94 ± 0.56	2.86 ± 0.34	4.35 ± 0.12^b
ATP	2.86 ± 0.20	2.43 ± 0.13^{aa}	3.03 ± 0.05^b
ADP	2.02 ± 0.22	1.41 ± 0.11^{aa}	2.00 ± 0.27^b
AMP	0.26 ± 0.06	0.47 ± 0.02^a	0.75 ± 0.05^b
Energy charge	0.75	0.73	0.70
Total adenylates	5.14	4.31	5.78

^a Significant difference from the corresponding control value, ($p < 0.01$).

^{aa} Significant difference from the corresponding control value, ($p < 0.05$).

^b Significant difference from the 1 hour value, ($p < 0.01$).

Tables 5 through 9 show creatine phosphate levels, adenylate levels and their totals and energy charge values for the five tissue groups in Pseudemys scripta after submergence in anoxic water for 1 and 5 hours, as well as aerobic control values for these. Short term (1 hour) anoxia resulted in a drop in ATP content in all tissues. Energy charge, however, was unaffected by these changes and remained essentially constant throughout submergence. The important change appeared in total adenylate content, which temporarily decreased in all tissues. Such decreases ranged from 68% (in brain) to 85% (in red muscle) of total adenylate levels in aerobic control tissues. After 5 hours of anoxia, total adenylate content had essentially returned to control levels in all tissues.

Crossover Experiments: End Product Accumulation

TABLE 10. Concentrations of glycolytic end products (lactate) in all tissues and organs over the time course of submerged anoxia in Pseudemys scripta.

Organ or Tissue	Control ($\mu\text{mol/g}$)	1 hour ($\mu\text{mol/g}$)	5 hours ($\mu\text{mol/g}$)
Heart	7.5 $\pm$ 0.48	5.9 $\pm$ 0.65	19.5 $\pm$ 0.88 ^b
Brain	8.6 $\pm$ 0.23	14.2 $\pm$ 0.91 ^a	33.2 $\pm$ 2.35 ^b
Liver	8.2 $\pm$ 0.15	17.8 $\pm$ 0.82 ^a	20.2 $\pm$ 0.52 ^b
Red Muscle	8.3 $\pm$ 0.29	11.3 $\pm$ 0.87	25.2 $\pm$ 0.88 ^b
White Muscle	9.4 $\pm$ 0.32	13.3 $\pm$ 0.40 ^a	19.8 $\pm$ 1.61 ^b

^a Significant difference from the corresponding control value, (p < 0.01).

^b Significant difference from the 1 hour value, (p < 0.01).

TABLE 11. Accumulation rate of glycolytic end products (lactate) in all tissues and organs over the time course of submerged anoxia in Pseudemys scripta.

Organ or Tissue	Total Lactate Formed (5 hours) ($\mu\text{mol/g}$)	Rate of Increase	
		0-1 hour ($\mu\text{mol/g/h}$)	1-5 hours ($\mu\text{mol/g/h}$)
Heart	12.0	-	3.4
Brain	24.6	5.6	4.7
Liver	12.0	9.6	0.6
Red Muscle	16.9	3.0	3.5
White Muscle	10.4	3.9	1.6

Tables 10 and 11 show the accumulation of ~~lactate~~ as the end-product of glycolysis over the period of submergence. The concentration of lactate in all tissues and organs examined increased during anoxia; however, total lactate accumulated over the 5 hour period varied widely from 10.4 $\mu\text{mol/g}$ wet weight in white muscle to 24.6 $\mu\text{mol/g}$ in brain. The rates of lactate accumulation between 0 and 1 hour of anoxia and 1 and 5 hours of anoxia are also shown. Brain showed the highest and most nearly constant rates of lactate accumulation in the two time periods, followed closely by red muscle in these respects. Both liver and white muscle showed sharp drops in lactate accumulation in the later hours. For liver this drop was dramatic; the calculated rate of accumulation between 1 and 5 hours was only 6% of the value calculated between 0 and 1 hours. Such results quantify changes in anoxic metabolic rates specific to particular organs and tissues; as well, comparisons can be made between observed metabolic rates of examined organs and tissues determined by this method, in the time frame over which metabolic depression occurs. Additionally, the absolute magnitude of anoxia-induced metabolic depressions can be observed. Based on lactate accumulation in brain and liver, metabolic rates between 1 and 5 hours of anoxia can be seen to differ by 8 fold.

Tables 12 through 16 show the effects of anoxia on the levels of glycolytic intermediates in the selected tissues and organs of Pseudemys scripta. Student's t-test was used to determine the level of statistical significance of differences between control aerobic glycolytic intermediate concentrations and one hour anoxic values for these, and between one and five hour anoxic values.

TABLE 12. Concentrations of glycolytic intermediate metabolites in heart tissue of control, 1 hour and 5 hour submerged *Pseudemys scripta*.

Metabolite	Control (nmol/g)	1 hour (nmol/g)	5 hours (nmol/g)
G6P	190 $\pm$ 11	207 $\pm$ 10	195 $\pm$ 12
F6P	35 $\pm$ 2	35 $\pm$ 3	16 $\pm$ 1 ^b
FDP	76 $\pm$ 8	146 $\pm$ 14 ^a	63 $\pm$ 3 ^b
G3P	23 $\pm$ 1	94 $\pm$ 8 ^a	101 $\pm$ 4
PEP	17 $\pm$ 2	< 10	60 $\pm$ 3
Pyruvate	108 $\pm$ 2	333 $\pm$ 23 ^a	217 $\pm$ 16 ^b

^a Significant difference from the corresponding control value, (p < 0.01).

^b Significant difference from the 1 hour value, (p < 0.01).

TABLE 13. Concentrations of glycolytic intermediate metabolites in brain tissue of control, 1 hour and 5 hour submerged *Pseudemys scripta*.

Metabolite	Control (nmol/g)	1 hour (nmol/g)	5 hours (nmol/g)
G6P	109 ± 5	109 ± 6	115 ± 7
F6P	30 ± 1	16 ± 2 ^a	13 ± 1
FDP	131 ± 6	100 ± 5 ^a	52 ± 7 ^b
G3P	38 ± 2	83 ± 8 ^a	17 ± 1 ^b
PEP	32 ± 1	< 10	238 ± 12 ^b
Pyruvate	124 ± 11	551 ± 22 ^a	561 ± 46

^a Significant difference from the corresponding control value, (p < 0.01).

^b Significant difference from the 1 hour value, (p < 0.01).

TABLE 14. Concentrations of glycolytic intermediate metabolites in red muscle of control, 1 hour and 5 hour submerged Pseudemys scripta.

Metabolite	Control (nmol/g)	1 hour (nmol/g)	5 hours (nmol/g)
G6P	233 ± 19	854 ± 38 ^a	493 ± 30 ^b
F6P	52 ± 2	133 ± 2 ^a	34 ± 3 ^b
FDP	75 ± 22	258 ± 23 ^a	111 ± 26 ^b
G3P	17 ± 1	116 ± 9 ^a	81 ± 2 ^b
PEP	20 ± 1	< 10	72 ± 2
Pyruvate	148 ± 22	534 ± 26 ^a	212 ± 11 ^b

^a Significant difference from the corresponding control value, (p < 0.01).

^b Significant difference from the 1 hour value, (p < 0.01).

TABLE 15. Concentrations of glycolytic intermediate metabolites in white muscle of control, 1 hour and 5 hour submerged *Pseudemys scripta*.

Metabolite	Control (nmol/g)	1 hour (nmol/g)	5 hours (nmol/g)
G6P	458 ± 61	1267 ± 90 ^a	667 ± 64 ^b
F6P	80 ± 11	225 ± 20 ^a	27 ± 5 ^b
FDP	186 ± 6	205 ± 21	144 ± 17 ^{bb}
G3P	20 ± 3	61 ± 5 ^a	35 ± 3 ^b
PEP	27 ± 1	< 10	< 10
Pyruvate	148 ± 16	216 ± 12 ^a	211 ± 13

^a Significant difference from the corresponding control value, ($p < 0.01$).

^b Significant difference from the 1 hour value, ($p < 0.01$).

^{bb} Significant difference from the 1 hour value, ($p < 0.05$).

TABLE 16. Concentrations of glycolytic intermediate metabolites in liver tissue of control, 1 hour and 5 hour submerged *Pseudemys scripta*.

Metabolite	Control (nmol/g)	1 hour (nmol/g)	5 hours (nmol/g)
G6P	232 ± 17	323 ± 20 ^a	516 ± 28 ^b
F6P	34 ± 5	53 ± 4 ^{aa}	47 ± 20
FDP	74 ± 2	122 ± 6 ^a	83 ± 10 ^b
G3P	145 ± 7	147 ± 7	95 ± 7 ^b
PEP	166 ± 5	228 ± 60 ^a	231 ± 16
Pyruvate	524 ± 35	457 ± 33	904 ± 97 ^b

^a Significant difference from the corresponding control value, ($p < 0.01$).

^{aa} Significant difference from the corresponding control value, ($p < 0.05$).

^b Significant difference from the 1 hour value, ($p < 0.01$).

Observed changes in glycolytic intermediates in heart (summarized in Table 12) after one hour of submergence in the anoxic water suggested activation of both PFK and PK. The concentrations of the products of these enzymes, FDP and pyruvate, increased significantly ($p < 0.01$), whereas the levels of the enzyme substrates either remained constant (F6P) or decreased (PEP). Levels of G3P are held in equilibrium with FDP by the enzyme aldolase, and so also represent an indicator of PFK product pool size. The significant rise ($p < 0.01$) in G3P concentration further suggested PFK activation after 1 hour of anoxia.

After five hours of anoxia, however, a significant drop ($p < 0.01$) in FDP coupled with a decline in levels of F6P suggested inhibition of PFK. At the PK locus, levels of PEP rose while levels of pyruvate fell to a significantly lower level ($p < 0.01$), indicating enzyme inhibition.

Metabolite changes in Pseudemys scripta brain (summarized in Table 13) displayed a similar pattern of PK control. After one hour, levels of substrate were reduced while product levels increased dramatically ($p < 0.01$) relative to controls, indicating PK activation. After 5 hours, PEP levels rose sharply and indicated that PK had become inhibited. At the PFK locus, enzyme activation was suggested by a significant reduction ($p < 0.01$) in substrate (F6P) concentration after 1 hour of anoxia. Concentrations of total PFK products (FDP and G3P) do not change

dramatically, although FDP itself decreases while G3P increases. This finding supports apparent PFK activation. The significant decrease ($p < 0.01$) in both PFK products after 5 hours of anoxia in brain indicates inhibition of PFK.

Observed glycolytic changes in red muscle (summarized in Table 14) indicate overall glycolytic activation during the first hour of anoxia. G6P concentrations rose 3.7 fold, suggesting enhancement of glycogenolysis; a concomitant rise in F6P levels was also observed. Activation of PFK was indicated by respective 3.4 and 6.8 fold increases in PFK products FDP and G3P. At the PK locus, PEP concentration was reduced while pyruvate concentration increased, indicating a similar enzyme activation.

After 5 hours the situation had become reversed. Levels of G6P, F6P, FDP, and G3P were all sharply reduced, indicating decreases in both carbon input into glycolysis and PFK activity. An increase in PEP and a decrease in pyruvate concentrations suggested similar enzyme inhibition at the PK locus. All of these changes, at 1 hour and 5 hours inclusive, showed great statistical significance ($p < 0.01$).

White muscle (Table 15) showed the same significant increases ($p < 0.01$) in hexose phosphate concentrations after 1 hour of anoxia as were observed in red muscle, indicative of increased delivery of carbon to glycolysis and

likely associated with increased glycogenolysis. Although FDP concentration did not show a significant change over 1 hour of anoxia. Decreases in PEP levels, accompanied by significantly increased ($p < 0.01$) levels of pyruvate indicate enzyme activation after 1 hour of anoxia at the PK locus.

Metabolite concentrations at the PK locus remained unchanged between 1 and 5 hours of anoxia. However, after 5 hours of anoxia, the concentrations of all other metabolites were significantly diminished, indicating (as in red muscle) a reduction in both carbon input to glycolysis and PFK activity.

Table 16 summarizes responses of liver glycolytic metabolites to anoxia. These results were markedly different from those of the other tissues and organs examined. G6P concentration increased 1.4 fold after 1 hour and 2.2 fold after 5 hours; this continuing rise is likely linked to the role of liver glycogenolysis in supplying glucose substrate to other organs during anoxia.

At the PFK locus, significant increases in all substrates and products indicate perhaps a slight activation of the enzyme at 1 hour. After 5 hours, significant decreases in enzyme products (FDP and G3P) suggest a subsequent PFK inhibition after 5 hours. A significant rise ($p < 0.01$) in PEP concentration suggests PK inhibition

during early anoxia. This appeared to become reversed by 5 hours.

It appears worth noting that for all tissues, the precision on the reported values of metabolite concentrations worsened as the imposed environmental stress (anoxia) became more extreme; that is, variances were observed to increase from control to one hour to five hours of anoxia.

Chapter 4. Discussion:

Mechanisms Controlling Facultative Metabolic Depression

Hibernation and anaerobiosis are both examples of metabolic strategies possessed by organisms to allow for the survival of the organism under conditions ordinarily incompatible with life. Since organisms utilizing either of these strategies need the capacity for repeated entries into and reversals out of hypometabolism. [Hibernating small mammals must undergo periods of torpor followed by periods of arousal throughout the winter, while facultative anaerobes experience fluctuations in oxygen availability, which result in oscillation between lipid aerobic catabolism and carbohydrate anaerobiosis.] Thus, as discussed in earlier sections, regulatory controls for such depressed metabolism strategies must have the following characteristics:

1. rapid initiation
2. ease of reversal
3. application to many cellular processes
4. ready coordination and integration
5. responsiveness to external stimuli (Storey, 1988).

The key to survival under hypometabolism is coordinated control of cellular processes throughout the organism.

Control must, in general, be "above" the level of allosteric control to ensure wide application of regulatory mechanisms, and "below" the level of gene expression to facilitate rapid transition into and out of the hypometabolic state (Storey, 1988).

One cellular process requiring modulation in the hypometabolic state is glycolysis; a known metabolic mechanism capable of exerting selective control over glycolysis and fulfilling the criteria above is one involving the concentration of the compound fructose-2,6-bisphosphate (F-2,6-P₂), a potent activator of the glycolytic pacemaker, phosphofructokinase (PFK).

Figure 2. Fructose-2,6-bisphosphate metabolism. F-2,6-P₂ activates phosphofructokinase and inhibits fructose-1,6-bisphosphatase. Synthesis and degradation of the compound is controlled by a single enzyme that displays 6-phosphofructo-2-kinase (PFK-II) activity in its phosphorylated form and fructose-2,6-bisphosphatase (FBPase-II) activity in its dephosphorylated form (Hue, 1983; Storey, 1988).

In mammals, various triggers (such as starvation, anoxia, changes in glucose availability, and many hormones) can alter F-2,6-P₂ concentration, focusing the effects of these diverse stimuli on the glycolytic pacemaker (Hue, 1983). Production of F-2,6-P₂ is by the phosphoprotein enzyme 6-phosphofructo-2-kinase and is subject to regulation by cAMP and Ca²⁺-calmodulin dependent covalent modification. Cellular F-2,6-P₂ content decreases as a result of starvation, ischemia or anoxia (Hue, 1984) and appears to act as an indicator of glucose abundance. The use of carbohydrate for biosynthetic purposes during times of plentiful energy is modulated by F-2,6-P₂ by its regulatory effect on PFK; as well, F-2,6-P₂ content drops when carbohydrate must be conserved. Thus, one might expect to find F-2,6-P₂ decreased in the hypometabolic state as a mechanism to conserve carbohydrate by diminishing glycolysis (Storey, 1988).

Investigations of the effects of altering F-2,6-P₂ concentration have revealed the following general trends:

1. removal of F-2,6-P₂ leaves PFK (and glycolytic rate) responsive solely to cellular energetic conditions (via ATP and AMP allosteric effects);
2. largely diminishing F-2,6-P₂ results in loss of the strong synergistic interaction between this compound and AMP, leading to a strong depression of PFK activity (and thus, glycolytic rate);

3. reduction of F-2,6-P₂ content leads to a depression of carbohydrate utilization for purposes other than ATP generation (such as biosynthesis). Thus, F-2,6-P₂ features significantly in depressed metabolism schemes, particularly those which must include reduction of glycolytic rate and restriction of carbohydrate in processes which generate no ATP (Storey, 1988).

Another well-known mechanism by which metabolism can be depressed is covalent modification by phosphorylation or dephosphorylation of protein. This mechanism has the advantages of being capable of producing major changes in the activities of a wide diversity of enzymes and other proteins quickly, and of being already in place (that is, the kinases and phosphatases which carry out phosphorylation and dephosphorylation are already regulated by intracellular messengers, such as cAMP and Ca²⁺) in all animal cells (Storey, 1988).

Both PFK and pyruvate kinase (PK) are subject to regulation by covalent phosphorylation. Phosphorylation, for instance, was found to reduce the activity of both these enzymes during anaerobiosis in the whelk (Busycotypus canaliculatum), overriding the allosteric control by adenylate, phosphagen and inorganic phosphate levels. The decrease in PFK activity in this case was also associated with a massive decrease in F-2,6-P₂ concentration (Storey, 1985). Treatment of the preparation of anoxic enzyme

variants with alkaline phosphatase also restored aerobic enzyme kinetics and thus provided support for the theory that covalent phosphorylation was responsible for the alteration in enzyme activity in this particular model system (Storey, 1984). A list of examples of other rate-limiting enzymes subject to control by reversible phosphorylation includes glycogen synthetase, triglyceride lipase, pyruvate dehydrogenase and acetyl CoA carboxylase (Cohen, 1980). Thus, this mechanism can be employed to depress the activities of multiple metabolic pathways, making it an effective and convenient means for whole tissue or even whole organism metabolic reorganization in response to an external stress. Work currently in progress on covalent modification of ion-specific membrane channels to reduce ion flux down concentration gradients in line with reduced energy availability and consequent reduced capacities of ATP-driven ion pumps in the various hypometabolic states is clarifying how metabolic and metabolically driven processes are reorganized and coordinated under such conditions (Storey, 1988).

Part A. Investigation of Glycolytic Control in the
Hibernator *Zapus hudsonius* by Crossover Analysis

Physiological adjustments involved in hibernation in mammals include regulation and coordination of a profound metabolic depression resulting from a lowering of the hypothalamic set point by some as-yet-undetermined mechanism. Such adjustments are necessary for the reduction of body temperature and entry into torpor to allow for long term survival under conditions of reduced ambient temperature and food availability. Other physiological adjustments include establishment and maintenance of a homeostasis with respect to fuels, water and waste products in a closed system (Storey, 1987).

In order to avoid the destructive effects of hypothermia (primarily uncontrolled Ca^{2+} influx and attendant Ca^{2+} stimulated disruptions, resulting in loss of cell function and often cell death), hibernators must be able to bring all physiological processes to a new equilibrium at the lower temperature (Hochachka, 1986).

Although Q_{10} effects at the hibernating body temperature will reduce the rates of all metabolic processes and may be the only factor involved in the rate reduction of many reactions (such as equilibrium enzyme reactions), many

metabolic processes may require specific modulation. A list of such processes could include:

1. initiation of metabolic rate depression by targeting of specific sites for inactivation,
2. reorganization of metabolism for the hibernating state,
3. closing down of processes unnecessary to survival during hibernation,
4. integration and balancing of cellular processes in the hibernating state. Selective inhibition of glycolysis, if present in the hibernator, could be a key regulatory step in many of these processes by limiting glycogen and glucose utilization and carbohydrate input into the tricarboxylic acid (TCA) cycle.

The decreased hexose phosphate (G6P and F6P) levels observed in short term (24 hour) hibernator thigh appear to indicate reduced carbon flux through glycolysis, linked perhaps to depressed glycogenolysis and glucose uptake. After longer term (5 to 7 day) hibernation, significant increases in hexose phosphate content might seem to indicate that carbon flux through glycolysis was being increased. Thus, it appears that the process of mobilization of fuel stores via glycogenolysis is not closed down during hibernation. This correlates with the finding that Zapus hudsonius skeletal muscle glycogen phosphorylase a content is not diminished during hibernation (Storey, 1987).

However, reduction of glucose mobilization rate over hibernation may be simply a Q_{10} effect (that is, not selectively inhibited), with the result that a marginal imbalance develops between glucose mobilization and glycolysis, leading to a buildup of hexose phosphates between 24 hours and 5 to 7 days of hibernation.

Short term hibernator thigh showed slight activation of PFK, followed by strong inhibition of this enzyme after hibernation for 5 to 7 days. This transient increase in enzyme activity followed by an eventual decrease suggests selective modification of the enzyme in response to the changing role of carbohydrate metabolism in the overall metabolic strategy of the hibernator. The short term activation of PFK might be linked to the process of reorganization and integration of overall metabolism toward primarily lipid oxidation (although its actual role at this early stage in hibernation is as yet unclear). The long term depression of PFK activity is a result of neither covalent phosphorylation nor a decrease in the important PFK activator F-2,6-P₂ (Storey, 1987). As well, the kinetic parameters of PFK with regard to its activators (AMP and F-2,6-P₂) and inhibitors (ATP and citrate) do not change in skeletal muscle of Zapus hudsonius over 5 to 8 days of hibernation (Storey, 1987).

One possible explanation of the mechanism for inhibition of PFK in hibernation comes from Hand and Somero

(1983), who made comparisons between normothermic skeletal muscle PFK and skeletal muscle PFK in hibernating ground squirrels (Citellus beecheyi). They proposed that the low temperature and relative acidosis of the hibernating state led to conversion of active tetrameric PFK into its inactive dimeric form. Another mechanism to explain how PFK may become inhibited is linked to glucose sparing by ketone. Metabolism of ketone bodies leads to a buildup of acetyl CoA, which in turn leads to an increase in intracellular citrate, a PFK inhibitor (Randle et al., 1964; Krilowicz, 1985). [A large increase in intracellular citrate concentration would have an inhibitory effect upon PFK, since the kinetic parameters of this enzyme with respect to citrate do not change in hibernation.]

Because of the lack of significant change throughout hibernation in PEP concentration, there appears to be no modulation of pyruvate kinase (PK) activity during hibernation. The significant drop in pyruvate concentration over short term hibernation could be associated with the reorganization of metabolism toward lipid oxidation, or may merely be a consequence of reduced carbon flux through the glycolytic pathway. In any case, no selective modulation of PK activity was expected, since no phosphorylation inactivation of this enzyme in hibernating Zapus hudsonius skeletal muscle was found in an earlier study (Storey, 1987). PK from Zapus hudsonius was found to be of the non-

allosteric M-type; this means that the kinetic parameters of this enzyme are unaffected by either allosteric activators (FDP) or inhibitors (L-alanine) (Storey, 1987). This finding was not at all unusual, since non-allosteric M-type PK is typical of mammalian skeletal muscle (Engstrom, 1978).

Hibernation shows a strong utilization of creatine phosphate in Zapus hudsonius thigh during the first 24 hours, but no creatine phosphate utilization between 24 hours and 5 to 7 days. Thus, one might speculate that creatine phosphate is used to partially fuel the metabolic reorganization occurring in the earliest stages of hypometabolism; creatine phosphate is thereafter conserved. ATP levels, on the other hand, drop during the first 24 hours of hibernation to 22% of control levels, but then later rebound back to higher level, 43% of control, after 5 to 7 days. Short term ATP depletion coincides with depression of glycogenolysis, and may be linked to reduced delivery of exogenous substrate during early hypometabolism. Longer term increases in ATP likely indicate the attainment of some new level of metabolic stability in deep hibernation. Interestingly, there is no huge increase in ADP concentration associated with the large ATP depletion. ADP levels actually decline to 34% of their control values after 24 hours and rebound to 59% of their control values after 5 to 7 days. AMP levels show a slight increase after 24 hours and a further increase after 5 to 7 days, but such increases,

by no means account for the adenylate bases turned over in ATP utilization. Thus, overall adenylate levels were observed to sharply decline after 24 hours and to show only partial recovery after 5 to 7 days. Large buildups in AMP content may have been forestalled by action of the enzyme AMP deaminase. It is reasonable that the hibernator should want to avoid large buildups of AMP and ADP in view of their strong activating effects upon glycolytic and TCA cycle enzymes. [AMP activates PFK and pyruvate dehydrogenase, while ADP activates isocitrate dehydrogenase.] Since the hibernator is undergoing metabolic depression, such activations would be undesirable, as they would tend to oppose this process.

Strong decreases in hexose phosphate levels after 24 hours of hibernation appear to indicate decreased delivery of glucose to glycolysis in the brain by this juncture, linked perhaps to depressed glycogenolysis and glucose uptake. After 5 to 7 days of hibernation, significant increases in hexose phosphate content were observed. At first glance this might seem to indicate an increase in delivery of glucose to glycolysis, perhaps by increased glycogen mobilization, a result which disagrees with the finding that the percentage of glycogen phosphorylase in the active, phosphorylated a form decreases from 57% in control Zapus hudsonius to 25% after 5 to 8 days of hibernation (Storey, 1987). The probable function of this inactivation

is to conserve glycogen reserves in brain at a time when exogenous substrate availability is changing and brain metabolism is becoming increasingly more dependent upon ketones (Kr̩ilowicz, 1985). However, if depression of glycogenolysis by decreasing phosphorylase a content is not strictly coordinated with depression of glycolysis, then a slight imbalance between glucose mobilization and glycolysis results, leading to an apparent buildup in hexose phosphate levels between 24 hours and 5 to 7 days of hibernation.

Short term hibernator brain showed such enormous decreases in FDP content that strong, selective inhibition of PFK seems to be indicated. The significant increases observed in FDP and G3P content after 5 to 7 days are likely not an indication of reversal of metabolic depression by reactivation of PFK but rather are a consequence of the buildup in hexose phosphate content between short and longer term hibernation, by mass-action effect. [The inhibition of PFK during hibernation is, of course, not total. The buildup in hexose phosphate content introduced to the pathway an imbalance in the levels of total glycolytics which led to increased concentrations of all upper glycolytics by a trickle-down effect.] The observed depression of PFK activity was not a result of covalent phosphorylation - a process known to effect glycolytic enzymes in hibernating Zapus hudsonius brains only in isolated cases (Storey, 1987). The kinetic parameters of

Zapus hudsonius brain PFK - with regard to activators (AMP and F-2,6-P₂) and inhibitors - also show no apparent changes after 5 to 8 days of hibernation. However, F-2,6-P₂ content in brain decreases to merely 72% of its control level, and so it is possible that this mechanism may be responsible for the selective inhibition of PFK observed (Storey, 1987). As well, it is reasonable to assume that hibernation leads to increased uptake of ketone bodies by the brain, since hibernation leads to elevated blood ketone body levels, and brain ketone body uptake increases when blood ketone levels increase (under such conditions as starvation) (Robinson and Williamson, 1980). Thus, the glucose sparing effect of ketone bodies may be at least partially responsible for the observed inhibition of PFK, by a mechanism similar to that shown in thigh.

After 24 hours of hibernation, a strong selective inhibition of PK was observed in Zapus hudsonius brain. Although the level of PEP had dropped significantly after 5 to 7 days, because it was accompanied by no significant increase in pyruvate level, this finding does not appear to be indicative of a reversal of metabolic depression by reactivation of PK. Instead, this finding may indicate a slight shift in the kinetics of any one (or perhaps several) of a number of equilibrium enzymes between aldolase and pyruvate kinase along the glycolytic pathway. Such a shift could result from differential (that is, non-uniform) Q₁₀

effects on the various equilibrium enzymes, and might require several days to display evidence of its existence. Part of the increase observed in the FDP and G3P levels between 24 hours and 5 to 7 days could also be due to such a putative shift, in addition to any increases resulting from a buildup in hexose phosphate levels. The mechanism responsible for this strong inhibition of PK is unknown. Zapus hudsonius brain PK showed no phosphorylation deactivation during hibernation in an earlier study (Storey, 1987); as well, Zapus hudsonius brain PK - similar to PK in thigh - was found to be of the non-allosteric M-type typical of mammalian brain. Thus, this enzyme's kinetic parameters are unaffected by either allosteric activators or inhibitors (Engstrom, 1978).

Hibernation shows a strong utilization of creatine phosphate in Zapus hudsonius brain during the first 24 hours, and continued creatine phosphate utilization between 24 hours and 5 to 7 days (at a much reduced rate). Thus, one might speculate that creatine phosphate is used to partially fuel the metabolic reorganization occurring in the earliest stages of hypometabolism; creatine phosphate is thereafter conserved. ATP levels, on the other hand, do not show a significant drop during the first 24 hours of hibernation with respect to control levels, but then later decrease to 59% of control values. Longer term depletion of ATP may indicate the effects of diminished glucose delivery

and the changeover to ketone metabolism in the brain during deep hibernation. ADP levels were again observed to actually decline to 36% of their control values after 24 hours and rebound to 71% of their control values after 5 to 7 days. AMP levels show a slight increase after 24 hours (although no further significant increase appears after 5 to 7 days), but such an increase by no means accounts for the adenylate bases turned over in ATP utilization. Thus, overall adenylate levels were observed to slightly decline after 24 hours and to show further decline after 5 to 7 days. As in hibernating *Zapus hudsonius* thigh muscle, large buildups in AMP content may have been forestalled by action of the enzyme AMP deaminase.

The observed differences in the patterns of regulation of glycolytic depression between *Zapus hudsonius* brain and thigh muscle indicate differences in the metabolic makeup of these two very different tissue types and also differences in the physiological roles of each under both normothermic and hypometabolic conditions. For instance, in deep hibernation, the brain (along with the lungs, heart and liver) receives a substantial portion of the circulating blood, while delivery of blood to skeletal muscle - especially to skeletal muscle in the extremities - is sharply curtailed (Behrisch et al., 1981). This suggests that the brain perhaps undergoes metabolic depression to a less severe degree than does thigh muscle; this also

suggests that some parts of the brain may retain some role during deep hibernation in the maintenance of and arousal from the hypometabolic state - as neurosecretory organs (Hudson and Wang, 1979) and perhaps even in the detection and response to environmental stimuli (Heller and Kilduff, 1985). Such speculation is supported by studies which report increases in some hypothalamic neurosecretions in deep hibernation over corresponding euthermic values. For instance, the garden dormouse shows increased somatostatin levels after hibernation for several days at 6°C (Richoux and Dubois, 1976). On the other hand, skeletal muscular contraction has little apparent function during hibernation (other than shivering thermogenesis during arousal), and so it is essential that skeletal muscle basal metabolism be depressed to a great degree in deep hibernation for the sake of fuel conservation.

Other considerations to make when analyzing the differences between the pattern of metabolic depression in Zapus hudsonius thigh and the pattern observed in brain is the fact that under both normothermic and hypometabolic conditions, these two tissues utilize different fuels. Under normothermic conditions, brain takes up and utilizes glucose preferentially, while skeletal muscle primarily oxidizes triglyceride fat and utilizes some glucose; during hibernation, both the brain and thigh muscle begin to take up and utilize ketone bodies, while turning down

glycolysis and glycogenolysis. However, thigh muscle fulfills many of the metabolic requirements of hibernation by utilizing triglyceride fat, while brain still does not. Because of these differences in fuel utilization patterns, it appears likely that the two different tissues would each execute different sequences of metabolic reorganization steps during entry into hibernation, thus yielding the different patterns of regulation of depression of glycolysis observed.

Glycolytic control at the PFK locus in the hibernator appears to be carried out in an organ-specific fashion, since F-2,6-P₂ content changes in some organs (brain, fat pad and heart) but does not change in others (skeletal muscle and liver) (Storey, 1987).

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Part B. Organ-Specific Control of Glycolysis in the
Anoxic Turtle *Pseudemys scripta*

As mentioned earlier, the turtle species *Pseudemys scripta* displays an enormous faculty for anaerobiosis, surviving submergence at 16 to 18°C for up to two weeks (Robin et al., 1964). Fermentative metabolism of carbohydrate to lactate is the primary means of ATP generation during such a period of submergence. Although creatine phosphate reserves are an important contributor to the energy needs of the organism during the hypoxic transition phase, they are largely depleted within a few hours of anoxia (Clark and Miller, 1973; Lutz et al., 1984). Examinations of ATP production by other pathways - for example, amino acid fermentation or carbohydrate conversion to end products other than lactate - show such contributions to be minor (Penney, 1974). Large glycogen reserves found within various organs, plus glucose supplied from liver glycogen reserves represent a sufficiently plentiful fuel source for lengthy anoxic excursions (McDougal et al., 1968; Penney and Shemerdiak, 1973).

In the turtle, a two-phase response to declining stored oxygen reserves takes place. Initially, all normal functions are maintained and a constant rate of ATP generation is sustained. This initial phase is exhibited in

most routine dives by turtles, which are considered aerobic because blood lactate does not appear to accumulate even over an extended testing period (5 days) containing many such dives (Gatten, 1981). Since large changes in blood oxygenation occur routinely in these animals, turtles possess a number of highly sensitive mechanisms to regulate internal oxygen supplies, such as redistribution of blood flow during the diving period (Lutz et al., 1985, Wood et al., 1987). Throughout the physiological range of oxygen tensions, mitochondrial respiration is oxygen dependent such that (when ATP consumption rates and concentrations are maintained) declines in ATP production due to reduced arterial oxygen tension are offset by increased anaerobic glycolysis and creatine phosphate utilization (Lutz et al., 1985). However, when arterial oxygen tension drops below a critical threshold (around 20 torr), the turtle enters the second phase. Metabolic depression is triggered, causing a drop in the rates of all metabolic processes which is designed to conserve energy and fuel reserves during anoxic excursions of indeterminate length (Caligiuri et al., 1981; Lutz et al., 1985). Since glycolysis represents the key ATP generating pathway during anaerobiosis in the submerged turtle, the response of glycolysis to declining arterial oxygen tension is an important avenue of study. Accordingly, organ-specific glycolytic control was investigated in a number of different organs and tissues

(brain, heart, red muscle, white muscle and liver) in the aerobic, severely hypoxic (1 hour of submergence) and depressed anoxic (5 hours of submergence) states.

The choices of the particular period lengths of submerged anoxia above were not made arbitrarily. Studies have shown that turtle (Pseudemys scripta elegans) brains become fully anoxic after 30 minutes of respiration with 100% nitrogen (Lutz et al., 1985) and that available reserves of oxygen become depleted after 45 minutes of submergence at 24°C (Jackson, 1968). Thus, after one full hour of submergence, the aerobic to anaerobic transition is complete and the turtle is fully anoxic. After 4 complete hours of submergence at 24°C, metabolism displayed evidence of depression to a final low and constant level (approximately 10% of the aerobic metabolic rate) (Jackson, 1968). Hence, the 5 hour anoxic turtles were chosen as representative of this later, fully depressed state.

A rough overview of the degree of tissue-specific depression of metabolic rate achieved during anoxia can be gained by examination of the rates of lactate accumulation in each tissue and organ. For instance, total lactate accumulation by brain over 5 hours of submergence was twice that of liver - a finding which agrees with the work of Clark and Miller (1973) - indicating perhaps that the rate of anaerobic glycolysis is higher in brain than in liver. As well, the timing of such metabolic depressions is shown

by lactate accumulation data. For example, the average rate of lactate production in liver showed a 16 fold decrease after the first hour of anoxia, indicating that a profound metabolic depression occurs very early in this particular organ. After one hour, white muscle also showed decreased lactate accumulation (by 2.4 fold), indicating metabolic depression in the later hours, although the rate of lactate accumulation in red muscle and brain does not display marked change. This finding for brain disagrees with the findings of Lutz et al. (1984), who concluded that after two hours of anoxia, lactate production in brain had dropped to 20% of the corresponding rate during the hypoxic transition phase. However, it must be noted that their experimental protocol involved inspiration of 100% nitrogen to induce anaerobiosis rather than submergence. During submergence, profound modifications are made to the turtle's blood flow patterns to conserve dwindling oxygen supplies for the brain (Bentley et al., 1983); thus, the brain may remain largely aerobic many minutes longer than the 30 minutes predicted by Lutz et al. (1985), and may make the transition to the anoxic state more gradually than predicted, yielding a lower value for lactate accumulation rate in the first hour of submergence in the natural state. It must be noted as well that although massive reorganizations of blood flow patterns occur during submergence (Bentley et al., 1983),

cardiovascular function persists (in a much altered form) (Wood et al., 1987).

Of course, since lactate is freely exchangeable between tissues and blood, any conclusions drawn from lactate accumulation rate data as regards degree and timing of metabolic depression must be approached with caution and supported by other independent findings.

The pattern of organ-specific regulation of glycolysis during anoxia in the turtle was consistent for four of the five tissues and organs examined - brain, heart, red muscle and white muscle. [Liver proved to be the exception, and since specific targeting of organs with major anabolic roles during entry into the hypometabolic state to eliminate non-essential anabolism is observed in many organisms with a capacity for facultative hypometabolism (Storey, 1988), this was not entirely unexpected.] The pattern of concentration changes in glycolytic intermediates for the four organs and tissues mentioned above was consistent with an activation of glycolysis after one hour of submergence and an inhibition of glycolysis by five hours of submergence. Such findings agree with predictions based on metabolic rate measurements made at similar sampling times (Jackson, 1968; Herbert and Jackson, 1985; Lutz et al., 1985).

In two organs, heart and red skeletal muscle, facilitation of glycolysis after one hour of submergence is the result of activation at the key glycolytic regulatory

loci: PFK and PK. Such activation was shown in both of these tissue types by massive increases in the product concentrations of the two enzymes (FDP and pyruvate, respectively), which indicated - according to the crossover theorem (Williamson, 1970) - activation with flux control. For heart, the finding that PFK showed a rapid, transient activation over the short term (which reversed itself as anoxia was prolonged) agreed with results from experiments examining the response of isolated turtle hearts to decreased oxygen tension in the perfusate (Penney and Shemerdiak, 1973). The large increases in hexose phosphate levels observed in both red and white muscle after one hour of submergence suggest activation of muscle glycogen phosphorylase. Brain and white muscle also showed glycolytic activation after one hour of submergence. A strong activation was observed at the PK locus in each, but modulation of PFK activity was less evident. The decrease in F6P (despite constant G6P) coupled with the 2.2 fold rise in G3P content suggests regulatory control in brain at either the PFK or aldolase locus. For white muscle the key regulatory loci involved in modulating glycolytic rate as conditions become increasingly hypoxic appear to be PK and glycogen phosphorylase alone (Kelly and Storey, 1988).

A possible explanation for the different patterns of activation of glycolytic enzymes displayed by heart and red muscle, and white muscle and brain after one hour of

submergence stems from a difference in preferred aerobic fuel. Brain and white muscle predominantly utilize carbohydrate for aerobic ATP generation, and so the transition from aerobiosis to anaerobiosis merely involves modulation of already-active glycolytic processes. Aerobic metabolism in heart and red muscle, however, is typically powered by lipid oxidation. Hence, the transition through hypoxia to anoxia necessitates a switchover from lipid to carbohydrate as the metabolic fuel and necessitates activation of the glycolytic pathway - repressed during lipid oxidation, probably by citrate inhibition of PFK (Stryer, 1981). The switchover to carbohydrate fermentation during submergence would therefore be expected to require PFK activation in these two tissue types. [The mechanism of PFK activation would naturally include removal of citrate inhibition.]

After submergence for five hours, these same four organs and tissues all displayed evidence of distinct reduction of glycolytic rate. Metabolite concentration changes (PEP increased, pyruvate decreased) in heart, brain and red muscle indicated strong inhibition at the PK locus. In addition, the lack of significant change in F6P levels, coupled with significant decreases (compared to one hour values) in FDP and G3P after five hours of submergence in brain suggested overall reduced flux through glycolysis and a regulatory site at PFK. Both red and white muscle showed

significant drops in G6P after five hours of anoxia, suggesting that overall flux through glycolysis had become reduced, perhaps by a decrease in blood glucose delivery to glycolysis. [Investigation of glycogen phosphorylase a activity in these two tissue types by Brooks and Storey (1988a) showed no significant change between aerobic levels and those observed after five hours of submergence.] Decreased FDP, coupled with a minor decrease in F6P in heart suggests inhibition at the PFK locus after five hours and may indicate a regulatory site at this locus. The observed lack of change in levels of G6P is an interesting result. It has been widely established that both creatine phosphate and glycogen are essentially depleted by the five hour mark of anoxia in the turtle (Clark and Miller, 1973), leading to a significant decrease in heart glycogen phosphorylase a content (Brooks and Storey, 1988a). Thus, after five hours of submergence, glucose delivery to the glycolytic pathway in heart has switched over entirely from glycogenolysis to glucose uptake, and glucose flux through glycolysis has decreased with no buildup or depletion of G6P - apparently indicating tight coordination of glycogenolysis and glucose uptake and perhaps of glycolysis as well. In contrast to the other three organs and tissues, metabolite changes in white muscle indicated no change in the status of either the PK or PFK loci between one and five hours, suggesting that

metabolic regulation in this tissue is "above" the level of glycolysis, perhaps at the level of glucose uptake.

Examination of metabolic responses of glycolysis in liver are complicated by its function as the organ supplying blood glucose to all other organs as the fermentative substrate. Not only is more than half of liver glycogen content mobilized within the first three hours of anoxia, but also liver is the only organ which indicates a continuing drop in glycogen content at times exceeding three hours (Clark and Miller, 1973). Glycogen in other organs apparently fuels anaerobiosis during early anoxia only, while long term steady state anaerobiosis is fueled by utilization of glucose supplied exogenously by the blood from liver glycogen reserves. In mammalian liver, the various hormones (glucagon and adrenaline, for instance) and physiological conditions (such as starvation) which stimulate glucose release to the blood from glycogen reserves also lead to inhibition of liver glycolysis to prevent futile oxidation of hexose phosphates and enhance glucose export. Such inhibition of liver glycolysis in mammals is exerted at the PK and PFK loci via covalent modification (phosphorylation) to produce less active forms of these enzymes (Engstrom, 1978; Sakakibara and Uyeda, 1983). Similarly, the increased PEP coupled with decreased pyruvate in the liver samples indicates inhibitory control of glycolysis at the PK locus after one hour of submergence.

[The increases in G6P, F6P and FDP could have resulted from increased glycogenolysis, which would lead to increased hexose phosphate availability. In any case, the PFK activation after one hour of submergence observed in heart and red muscle appeared to be absent in liver.] After five hours of submergence, a continuing state of reduced flux through glycolysis was suggested by observed decreases in F6P, FDP and G3P levels, despite the continuing increase in G6P levels. Interestingly, PK from liver showed a slight activation after five hours of anoxia in both the present study and in enzyme kinetics experiments (Brooks and Storey, 1988a), although the purpose of such an activation is as yet unclear.

The organ-specific glycolytic control seen in the submerged turtle may represent a typical pattern for facultative anaerobiosis. The initial activation of glycolysis appears to be a reasonable effect of the early hypoxic condition if one recalls that oxygen reserves are exhausted after 45 minutes of anoxia (Jackson, 1968) and that carbohydrates represent (essentially) the sole fuel for anaerobiosis (Penney, 1974). As well, the strict dependence of anoxic turtle heart upon glycolysis is demonstrated by its sensitivity to iodoacetate, an inhibitor of anaerobic glycolysis. When a dose of iodoacetate (not lethal in air) is administered to a turtle, it rapidly dies when placed in water (Bing et al., 1972). Thus, after one hour of anoxia,

fermentation of carbohydrates to lactate is responsible for virtually all ATP production; the efficiency of this process is 2 ATP per glucose oxidized or one-eighteenth of the efficiency of aerobic glucose oxidation. Overall metabolic rate after one hour, however, has only been depressed to one-fifth of the aerobic metabolic rate (Jackson, 1968). Activation of glycolysis, one might conclude, would therefore be necessary to make up this difference between ATP generation and ATP utilization. After about 3.5 hours, the anoxic metabolic rate in the turtle has reached steady state condition at about one-tenth of the aerobic rate (Jackson, 1968), and so, this initial glycolytic activation has become reversed (along with all other physiological and metabolic adaptations) in order to conserve carbohydrate fuels for long term anaerobiosis.

Possible molecular mechanisms underlying the control of PFK and PK during submergence could include regulation via substrate availability, allosteric regulation, covalent modification of enzymes, pH effects on enzyme activity and changes in enzyme binding associations with the subcellular particulate fraction (Storey, 1988). Adenylate and creatine phosphate data in this study seem to indicate that allosteric regulation in glycolytic control during anoxia is restricted to the early transition phase through hypoxia to anoxia. Creatine phosphate control of PFK may be a key factor, especially since turtle PFK is relatively insensitive to ATP

regulation but is markedly sensitive to inhibition by high creatine phosphate content (Storey and Hochachka, 1974). Because it is utilized to a large extent early in anoxia (Clark and Miller, 1973; Lutz et al., 1984), while ATP stores are only moderately depleted (McDougal et al., 1968; Clark and Miller, 1973), creatine phosphate depletion could be partially responsible for the PFK activation observed after one hour of submergence. It should be noted that the transient ATP decrease observed in this investigation during the first hour of anoxia did not translate into a large increase in the content of the potent PFK activator, AMP, but instead leads to a decrease in total adenylate content (perhaps by action of the enzyme AMP deaminase). Another mechanism of metabolic control worth examining in anoxic turtles is regulation by F-2,6-P₂ content. The levels of this compound showed no significant change after five hours of anoxia in liver, brain or white muscle; however, F-2,6-P₂ content decreased significantly in heart ($p < 0.05$) and increased significantly in red muscle ($p < 0.05$) after five hours of submergence (Brooks and Storey, 1988a). In heart, the decrease in F-2,6-P₂ levels may reflect a shift in PFK control from F-2,6-P₂ to energy charge (adenylates) so that glycolytic rate is linked to energy demand in the heart; the increase in F-2,6-P₂ content in red muscle may have resulted from the large increase in hexose phosphate content after one hour of anoxia which may have exerted a mass action

effect upon the production of F-2,6-P₂ and led to partial PFK activation at this point of submergence (Brooks and Storey, 1988a). The fact that F-2,6-P₂ content changed during anoxia in heart and red muscle (whose preferred aerobic fuel is lipid) and not in other tissues and organs may reflect some role in lipid to carbohydrate conversion during the hypoxia to anoxia transition phase, although such a putative role remains to be determined.

Kinetic studies made upon enzyme preparations from Pseudemys scripta showed no significant kinetic changes in five hour anoxic liver or red muscle PFK, but showed a significant ($p < 0.05$) activation of heart and white muscle PFK (Brooks and Storey, 1988a). This finding did not contradict those of the present study for any tissues other than heart; the activation of white muscle PFK may indicate activation of this enzyme during the hypoxia to anoxia transition phase followed by displacement of the responsibility for regulation of glycolytic rate from PFK to the (membrane) mechanisms of glucose uptake (Brooks and Storey, 1988a). No physiological rationale for the kinetic changes in heart PFK has been advanced (Brooks and Storey, 1988a), although one possible explanation might involve increasing the sensitivity of heart metabolic rate to energetic conditions within the organism by increasing the sensitivity of heart PFK to ATP concentration. In a related study, brain PFK from five hour submerged Pseudemys scripta

was found to have increased sensitivity to citrate, indicating perhaps allosteric inhibition by this compound during long term submergence (Brooks and Storey, 1988b).

Another mechanism by which various anoxia tolerant species modulate glycolytic rate during hypometabolism is a reduction in glycolytic enzyme binding to the subcellular particulate fraction (Storey, 1988). Reduced glycolytic flux is associated with a percentage of glycolytic enzymes bound to the glycolytic complex. However, much work remains to be accomplished before a clear picture of the importance of this mechanism to anaerobiosis in the submerged turtle will emerge.

Additionally, a thorough investigation of regulation (especially rate regulation) of glucose uptake by the cell membranes of the various tissues examined - heart, brain, red muscle and white muscle - must be made to confirm or contradict the speculations about the "level" of cellular glycolytic control made in this study.

Summary:

The patterns of glycolytic control were investigated using metabolite crossover studies in two model systems: vertebrates displaying a faculty for hypometabolism - the hibernator Zapus hudsonius (the meadow jumping mouse) and the diving freshwater turtle Pseudemys scripta.

Metabolic depression in these organisms was discovered to follow a particular overall scheme. The rate of delivery of substrate to the glycolytic pathway diminished; the rate-limiting enzymes of glycolysis (PFK and PK) were selectively inhibited in an organ specific fashion; delivery of glycolytic end-product (pyruvate) to the TCA cycle was decreased in the hibernator and switched off entirely in the turtle.

Thigh muscle from the hibernator showed selective glycolytic modulation at the PFK locus (increased activity of PFK after 24 hours of torpor, followed by inhibition of PFK activity after 5 to 7 days) and no modulation of PK during hibernation whatsoever. Brain tissue from the hibernator showed decreased carbon flux through glycolysis after 24 hours of torpor and a strong inhibition of PFK and PK after 24 hours of torpor. No reversal of this inhibition of glycolytic enzymes was observed in later hibernation.

Thus, the supposition of Geiser (1988) - that small mammal

hibernators must selectively depress their basal metabolism in order to sufficiently reduce energy requirements to survive hibernation - was supported by the findings of this experiment.

In the turtle, rates of accumulation of lactate by brain, heart, red muscle, white muscle and liver after one hour and five hours of submergence in nitrogen-bubbled water were taken as an indication of organ-specific depression of overall metabolic rate in the severely hypoxic and depressed anoxic states. Evidence of organ-specific modulation of glycolytic rate was also obtained. Red muscle and heart showed an activation of PFK after one hour, which in heart was reversed after five hours. Brain also showed inhibition of PFK after five hours. Brain, heart, red muscle and white muscle all showed an activation of PK after one hour, which was reversed after five hours in brain, heart and red muscle. The pattern of short term activation of the rate-limiting enzymes of glycolysis was interpreted as indicating an initial acceleration of anabolic processes in order to overcome the huge drop in energetic efficiency encountered when switching from aerobic to fermentative metabolism. The longer term inhibition of glycolytic rate-limiting enzymes was consistent with previous findings which indicated that metabolic processes are selectively depressed in diving turtles so that they may survive anoxic excursions of indeterminate length. Liver showed strong inhibition of PFK

(and therefore of glycolytic flux) within one hour of submergence, consistent with the demands of its role as primary supplier of glucose to organs during extended anaerobiosis via glycogenolysis and glucose export.

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