AN ABSTRACT OF THE THESIS OF

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Title: Comparison of lactate dehydrogenase in simulated hibernating and active room temperature turtles (*Trachemys scripta*)

Abstract approved: David K. Saunders

The Red-eared slider (*Trachemys scripta*) is remarkably tolerant to the anoxic conditions of hibernation. During the winter, these turtles hibernate under a thin layer of mud where the temperature is approximately 5 °C and the oxygen availability is close to zero. During hibernation, the turtles are dependent upon anaerobic glycolysis as the primary source of energy generation. As lactate dehydrogenase (LDH) is a key enzyme of the anaerobic pathway, the possible molecular adaptations of LDH in hibernating turtles were investigated in this study. Ten turtles were placed into simulated hibernation at 5 °C for five months. Control animals were placed in similar conditions at room temperature. Since LDH can exists in the forms of five isozymes, we analyzed LDH isolated from the turtle heart, skeletal muscle, and liver by electrophoresis, followed by densitometric analysis. The densitometry was performed to determine if differences in the percentage of the LDH isozymes in these tissues occurred between hibernating and room temperature turtles. The activity of LDH was also determined at 5 °C and 30 °C for each of the above tissues in both groups of turtles. No statistical differences were found in the mean percentage of LDH isozymes when comparing similar tissues between hibernating and room temperature turtles. However, hibernating turtles had higher LDH

activities in all tissues tested as compared to the room temperature turtles. At 5 °C the mean LDH activity of the hibernating turtle skeletal muscle was 3.5 times greater, heart and liver were 1.75 times greater than that of like tissues in room temperature turtles, while at 30 °C the mean LDH activity in skeletal muscle was 2.0 and liver 1.5 times greater in hibernating turtles, respectively. Thus, it appears that the LDH activity of hibernating turtles, particularly within skeletal muscle, does adapt during hibernation, a feature which contributes to their survival during these prolonged and extreme periods.

COMPARISON OF LACTATE DEHYDROGENASE IN SIMULATED HIBERNATING AND ACTIVE ROOM TEMPERATURE TURTLES (*TRACHEMYS SCRIPTA*)

A Thesis

Submitted to

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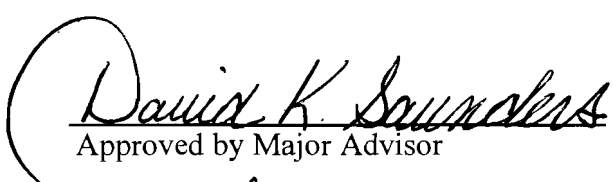
Master of Science

by

Hua Liu

March, 1997



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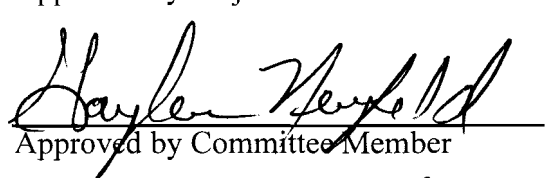

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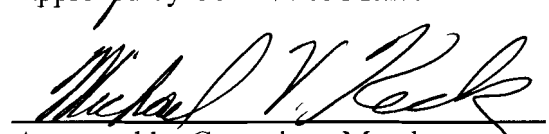

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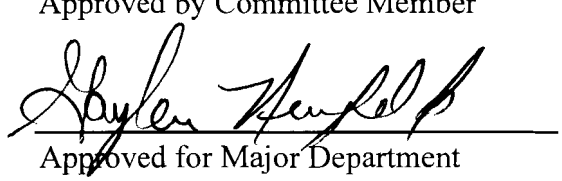

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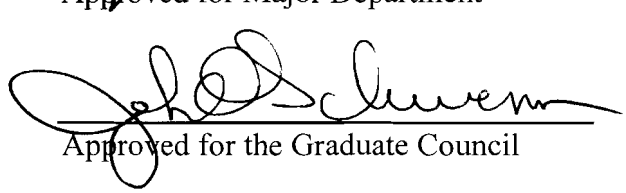

Approved for the Graduate Council

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PREFACE

This thesis was prepared following the publication style of *Physiological Zoology*.

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INTRODUCTION

Trachemys scripta (Red-eared slider) is a fresh-water turtle that lives in North America, a cold temperature area in winter. In late October, when environmental temperatures decline and ice forms over the lakes, these turtles burrow into the bottom mud of lakes and begin hibernation. In mid-March, after the surface ice melts, the turtles become active again. Under the bottom mud of lakes, the temperature is about 5 °C, and the concentration of O_2 is close to zero (Crawford, 1991). These turtles can live in the hypoxic condition for 3 to 5 months and have been shown to live as long as 2 weeks despite total absence of electron transport-chain-mediated oxygen consumption (Robin et al., 1964).

Hibernating turtles in general use several strategies for survival during hibernation. They store lipids, proteins, and glycogen before hibernation (Crawford, 1994) and decrease metabolic rate by about 12-fold (Gatten, 1974), or about 99.4% relative to the aerobic rate at 20 °C, as a result of decreased body temperature, fasting, and hypoxia (Ultsch, 1989). This results in a decrease of heart rate from the normal 70 beats/min to 7~8 beats/min (as low as 5~6 beats/hr for *C. picta* at 3 °C, Herbert and Jackson, 1985) and ceasing of body activities (Gatten, 1974). Further, there is a shift of energy generation from aerobic to anaerobic metabolism (Olson, 1987).

Many studies have provided evidence for the shift from aerobic to anaerobic metabolism during hibernation. Blood PO_2 in painted turtles (*Chrysemys picta*) is almost zero during hibernation, necessitating anaerobic metabolism (Hochachka and Somero, 1984; Hochachka, 1982; Ultsch and Jackson, 1982). During hibernation, the activities of many aerobic enzymes in the mitochondria of the painted turtles are markedly reduced,

such as citrate synthase and pyruvate kinase (Olson, 1987; Kuczenski and Suelter, 1970). Total body lactate concentration of *T. scripta* has been shown to increase 5.8-fold from 6.3 *u*mol/g tissue in active turtles to 36.5 *u*mol/g during hibernation, while pyruvate concentration remained relative constant (Crawford, 1994). Other studies have shown that the plasma lactate concentration of *Chrysemys picta* increases 10-fold from normal values of 20 mM to values as high as 200 mM after 3 months submergence in hibernation (Jackson and Ultsch, 1982). These progressive increases in lactate concentration indicate that there is a remarkable increase in anaerobic metabolism. This especially pronounced ability to liberate energy through anaerobic metabolism in anoxia tolerant turtles may be largely related to some particular molecular and/or kinetic adaptation in the key enzyme of the anaerobic pathway, lactate dehydrogenase (LDH).

Fig. 1 shows the types and kinetic properties of LDH isozymes. These isozymes are tetrameres, combining two subunits, H and M monomers. These monomers can form the various possible combinations of the five isozymes: LDH-1 (H_4); LDH-2 (H_3M); LDH-3 (H_2M_2); LDH-4 (HM_3); and LDH-5 (M_4). H and M subunits display tissue specific distributions due to different kinetic properties. This conclusion is supported by the demonstration that there is a marked difference in the degree of substrate inhibition between purified H_4 and M_4. LDH isozymes are inhibited by the substrates pyruvate and lactate through formation of the LDH-NAD^+-pyruvate and LDH-NADH-lactate complexes (Everse and Kaplan, 1973). The H subunit is the major isozyme in aerobic tissue such as the heart, brain, and kidney because of its high affinity for lactate. Furthermore, the H subunit is strongly inhibited by high pyruvate and lactate

Fig. 1. Types and kinetic properties of LDH isozymes.

Types: LDH-1 (H_4) LDH-2 (H_3M) LDH-3 (H_2M_2)
LDH-4 (HM_3) LDH-5 (M_4)

Inhibition complexes: LDH-NAD^+-pyruvate
LDH-NADH-lactate

Primary function of H and M subunits:

$$\text{Lactate} + NAD^+ \xrightarrow{H} \text{Pyruvate} + NADH$$

$$\text{Pyruvate} + NADH \xrightarrow{M} \text{Lactate} + NAD^+$$

concentrations (Narita and Horiuchi, 1979; Everse and Kaplan, 1973). These properties of the H subunit favor the oxidative pathway in aerobic tissue by maintaining higher pyruvate concentrations for entering into the tricarboxylic acid cycle (TCA) (Everse and Kaplan, 1973; Dawson et al., 1964). Conversely, the M subunit is the principal isozyme in anaerobic tissue such as skeletal muscle because it functions more effectively than the H subunit at high pyruvate concentration and almost no inhibition occurs at high lactate concentrations (up to 200 mM) (Nelia et al., 1974). In skeletal muscle, during burst activity, there is a requirement for the sudden release of energy in the relative absence of oxygen, or when the animal is cut off from environmental oxygen. This energy is supplied by anaerobic glycolysis, which produces large amounts of pyruvate and lactate. Under these conditions, the M subunit is able to function at high substrate concentrations and can support a high level of anaerobic metabolism (Narita and Horiuchi, 1979; Everse and Kaplan, 1973; Dawson et al., 1964).

Although many studies have been performed to investigate temperature and/or hypoxia effects on purified LDH-1 (H_4) and LDH-5 (M_4) of turtles, to date, few studies have analyzed the LDH isozymes in turtle tissue homogenates under normal and hibernating conditions. Therefore, in the present investigation, an attempt has been made to compare the relative percentage of H and M subunits as well as the kinetic properties of LDH isozymes in the heart, skeletal muscle, and liver between active and simulated hibernating turtles. The purpose of this study was to elucidate the possible biochemical adaptation in these tissues of *T. scripta* during hibernation.

MATERIAL AND METHODS

Preparation of Animals

Twenty Red-eared sliders *(Trachemys scripta)* were purchased from Kons Scientific Company, Germantown, Wisconsin. The mean weight of these turtles was 365 g. Turtles were randomly divided into two groups of ten each. The control group (active turtles) was maintained at 26 °C in a basin of water containing rocks so that the turtles could reach the surface for air. During the fifth month of the study, two control animals died. The simulated hibernating group was submerged in a similar basin filled with water and maintained at 5 °C for 5 months. The hibernating group was not fed during this period of submergence. At the end of the submergence all turtles were euthanized with ketamine hydrochloride.

Preparation of Tissue Extracts

Tissue extracts were prepared following the method described by Baldwin and Gyuris (1983). Freshly dissected heart, liver, and skeletal muscle tissues from individual turtles were diced, washed, weighed, and added to ice cold buffer solution (50 mM potassium dibasic phosphate, pH 6.8) at a ratio of 1 g of tissue to 2.5 ml of buffer for heart and skeletal muscle, and 1:2 for liver. Tissues with buffer were homogenized using a Tissue Tearor homogenizer and centrifuged at 10,000 ×g for 30 min at 4 °C. The sediments were discarded, and the supernatants were stored at -20 °C until used for electrophoresis, and the determination of LDH activity and pyruvate inhibition.

Electrophoresis

Tissue supernatants were examined by electrophoresis on cellulose acetate gels to determine the relative percentages of LDH isozymes. My previous studies found the

optimal condition for electrophoresis occurred in a 0.038 M Tris-0.37 M glycine, pH 8, running buffer with extracts being electrophoresed for 2 h at 25 °C (room temperature) at a voltage of 250 V and a constant current of 150 mA (see appendix). Gels were stained for 15 min with a reaction mixture containing 0.05% Tetranitro Blue Tetrazolium, 0.05% Phenazine Methosulfate, 0.1% NAD, 1% lactic acid and 0.2 M Tris-HCl buffer, pH 8 (Angers et al., 1994). After staining, gels were destained in 5% acetic acid and placed in a clearing solution of 40% 1-methyl-2pyrrolidinone for 5 min. Finally, gels were placed on a clean glass slide and put in an oven at 80 °C for 20 min causing the background of the gels to become transparent.

Densitometric Analysis

The relative percentages of LDH isozymes present in tissue extracts were determined by densitometry. The prepared gels were scanned by an Auto Scanner densitometer. The chart paper from densitometer was used to calculate the relative proportions of LDH isozymes with digital imaging techniques using NIH image software on a PowerMac 8500 computer attached to a Flex Cam digital camera.

Measurement of LDH Specific Activity and Pyruvate Inhibition

The specific activity of the LDH isozymes was measured through the direct reaction (pyruvate to lactate) by following the oxidation of NADH at 340 nm with a Milton Roy 301 spectrophotometer in which the cuvette temperatures were controlled at 5 ^{0}C and 30 ^{0}C with a Polytemp circulating refrigerated water bath. The specific activities of LDH in turtle heart, liver, and skeletal muscle were tested with a reagent that contained 0.5 mM NADH, 100 mM sodium phosphate buffer, pH 7.4, and pyruvate concentrations in the

range 0.025-10 mM (Altman and Robin, 1969). LDH specific activity was expressed as *u*mols of NADH oxidized/min/mg protein using 6.22×10^3 as the extinction coefficient of NADH (Kaloustian and Kaplan, 1969). The specific activity was calculated according to the following equation (Narang and Narang, 1974):

$$\text{Specific activity} = \frac{\text{change in o.d./min} \times \text{total reagent mixture volume (ml)}}{6.22 \times \text{mg protein/ml} \times \text{sample volume (ml)}}$$

The extract volume of tissue was 10 *u*l, and the reagent volume was 2 ml. The concentration of enzyme solution was adjusted so that the rate of the reaction was linear over the first 120 seconds and to cause a change of absorbance of NADH at 340 nm less than 0.3 o.d. units per minute. After mixing the tissue homogenate and reagent, the decrease in absorbance was measured at 30 second intervals for 3 min (Narita and Horiuchi, 1979).

Measurement of Protein Concentrations

Protein concentrations were determined with the BioRad method using bovine serum albumin as a standard (Bradford, 1976).

RESULTS

Relative Proportions of LDH Isozymes

The general electrophoretic isozyme patterns of LDH present in the extracts of *T. scripta* heart, liver, and skeletal muscle are shown in Fig. 2. The electrophoretic mobility of the 5 LDH isozymes differs according to their charge. The most anodal band is the LDH-1 (H_4), the most cathodal band is the LDH-5 (M_4), and the intermediate isozyme are in the order of LDH-2 (H_3M), LDH-3 (H_2M_2), and LDH-4 (HM_3) from anode to cathode (Everse and Kaplan, 1973).

Five bands were found in the electrophoresis gel of the heart, representing the five isozymes, LDH 1~5 from anode to cathode. The isozyme patterns observed in liver and skeletal muscle, however, only had 3 bands for most of the extracts. Comparing the migration distance of the isozymes in liver and skeletal muscle with that of heart, it was found that liver contained LDH-2, LDH-4, and LDH-5, while skeletal muscle contained LDH-3, LDH-4, and LDH-5. The diagrammatic results of densitometric scanning for the electrophoretic strips are presented in Fig. 3, which show that the higher the density of isozyme band, the greater the area under the curve.

Table 1 reports the relative percentage of LDH isozyme determined by digital imaging techniques. The greatest concentrations of LDH isozyme in the turtle heart were LDH-2 and LDH-4. For skeletal muscle, LDH-5 had the greatest concentration, while the greatest concentration in the liver was LDH-2. Only one skeletal muscle extract showed an LDH-1 band, four skeletal muscle homogenates had LDH-2 bands and three liver extracts presented LDH-3 bands. The absence of these bands in other relevant tissue extracts may be due to concentrations of these proteins lying below the sensitivity

Fig. 2. General patterns of LDH isozymes present in the extracts of A) skeletal muscle, B) heart, and C) liver of *T. scripta* using cellulose acetate gel electrophoresis. Arrow = point of application.

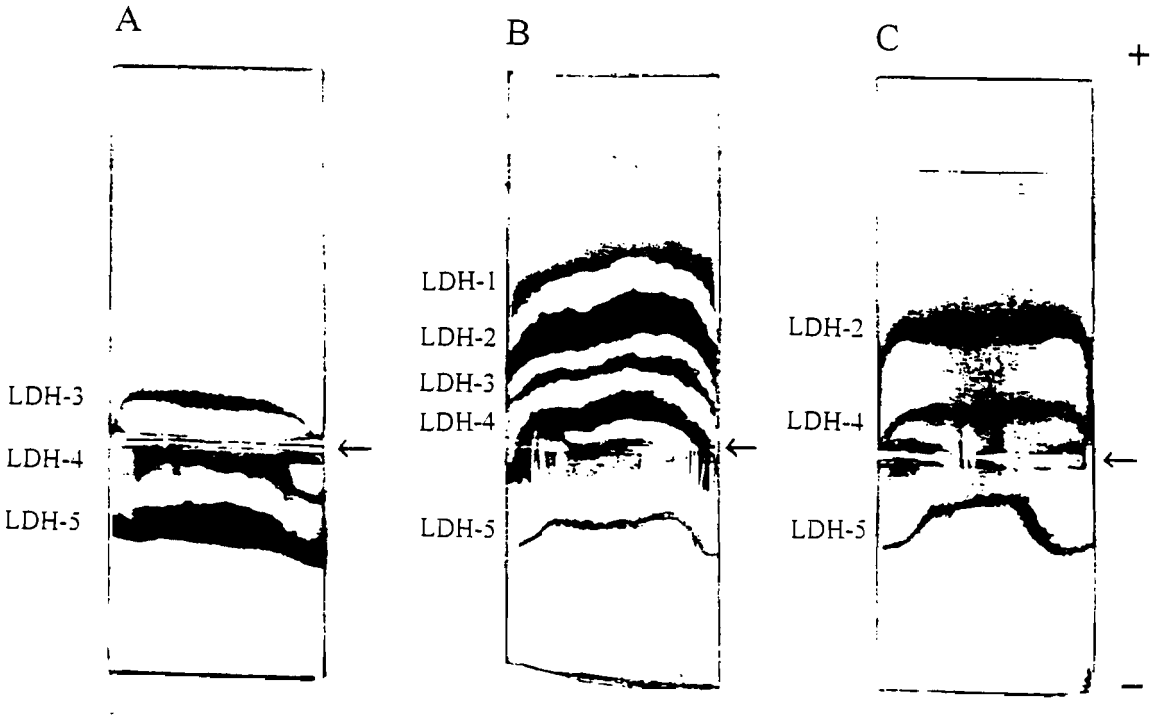
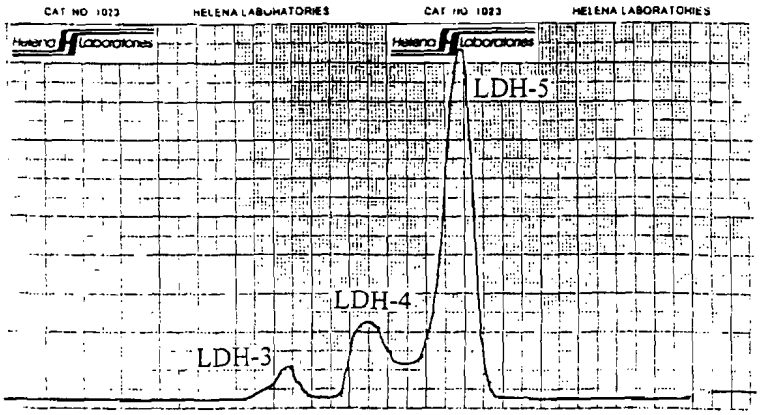
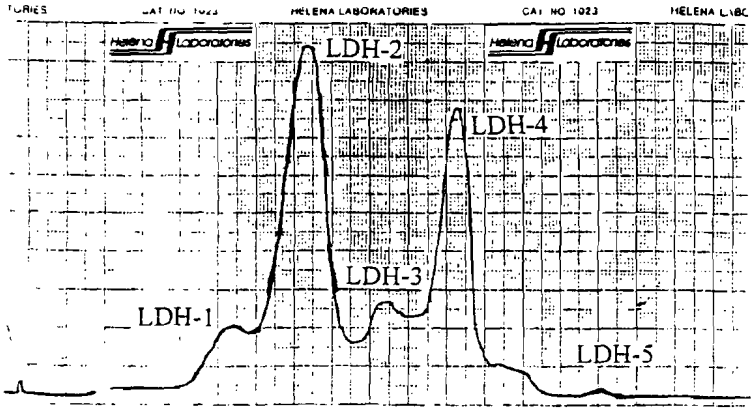

A
B
C
+
LDH-1
LDH-2
LDH-3
LDH-4
LDH-5
LDH-2
LDH-4
LDH-5
LDH-3
LDH-4
LDH-5
−

Fig. 3. Diagrammatic results of desitometric scanning for the electrophoretic strips of A) skeletal muscle, B) heart, and C) liver.

A



B



C

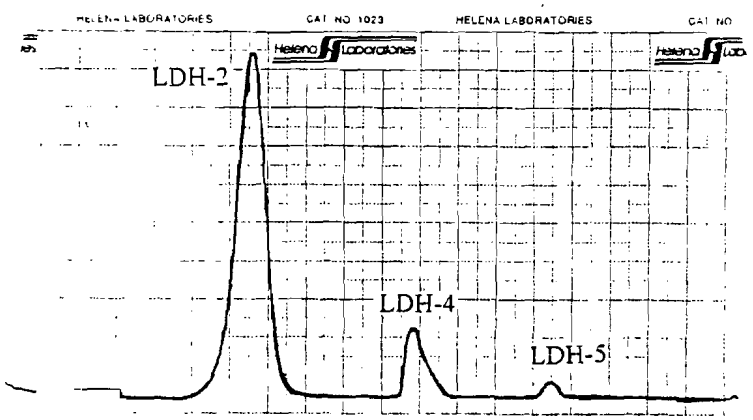


Table 1. Mean (± SE) relative percentage of LDH isozyme in *T.scripta* heart, skeletal muscle, and liver.

	Percentage of each isozyme				
Homogenate	LDH-1	LDH-2	LDH-3	LDH-4	LDH-5
Hibernating turtle:					
Heart.....	5.29 ±1.36	45.40 ±3.21	8.90 ±0.71	39.39 ±3.79	1.02 ±0.25
Skeletal muscle...	---	1.22 ±0.32	9.03 ±1.14	21.29 ±1.87	68.48 ±2.57
Liver.....	---	72.23 ±4.05	1.31 ±0.31	16.88 ±2.42	9.58 ±2.61
Active turtle:					
Heart.....	6.94 ±1.38	48.28 ±4.71	8.07 ±0.94	36.14 ±5.03	0.57 ±0.16
Skeletal muscle...	0.12 ±0.01	0.58 ±0.14	12.41 ±1.16	20.40 ±2.03	66.50 ±2.04
Liver.....	---	82.68 ±4.08	0.58 ±0.06	12.52 ±3.22	4.22 ±0.64

threshold of the staining procedure. The total H and M subunit percentages for each tissue in hibernating and active turtles were calculated from relative percentages of H and M subunits in each isozyme (Table 2). Student's T-test showed that there was no significant difference in the H and M proportions in the heart, skeletal muscle and liver between hibernating and active turtles (for heart $t = 0.7181$, $df = 15$, $P > 0.45$; skeletal muscle $t = 0.8894$, $df = 14$, $P > 0.35$; liver $t = 1.8193$, $df = 15$, $P > 0.08$). These results suggest the relative proportions of H and M subunits in the above tissues in hibernating turtles did not change during the 5 months of simulated hibernation.

LDH Specific Activity and Pyruvate Inhibition

Pyruvate inhibition plots for hibernating and active turtle LDH homogenates of heart, skeletal muscle, and liver assayed at 30 ^{0}C and 5 ^{0}C are shown in Figs. 4, 5, and 6. In each figure, the percentage of peak LDH activity is plotted against various pyruvate concentrations. The temperatures of 30 ^{0}C and 5 ^{0}C were chosen because they represent the average summer and winter temperatures usually recorded in the area inhabited by *T. scripta*.

Pyruvate inhibition plots for both hibernating and active turtle tissue homogenates at 30 ^{0}C showed no inhibition of heart, skeletal muscle, or liver LDH until pyruvate concentrations were raised above 1.0 mM. LDH activity progressively fell as pyruvate concentrations were increased beyond this point. The degrees of inhibition for both hibernating and active turtle homogenates were almost equal in each tissue for a given temperature. At 30 ^{o}C and the highest pyruvate concentration, 10 mM, the activity of LDH from hibernating and active turtles was reduced to 50.25% and 51.35% of peak

Table 2. The total H and M subunit mean (± SE) percentages of LDH in hibernating and active turtle heart, skeletal muscle, and liver.

Homogenate	% total H	% total M
Hibernating turtle:		
Heart.....	53.64 ± 2.33	46.63 ± 2.33
Skeletal muscle...	10.75 ± 1.01	89.25 ± 1.01
Liver.....	59.05 ± 2.65	40.95 ± 2.65
Active turtle:		
Heart.....	56.22 ± 2.78	43.78 ± 2.78
Skeletal muscle...	11.85 ± 0.71	88.15 ± 0.71
Liver.....	65.43 ± 2.21	34.57 ± 2.21

Fig. 4. Effect of pyruvate concentration on the activity of LDH isozymes in the heart of hibernating turtles (n = 10) and active turtles (n = 8) at 30 ^{0}C and 5 ^{0}C. The pyruvate concentrations were 0.025, 0.3, 1.0, 2.0, 3.0, 4.0, 6.0, and 10.0 mM.

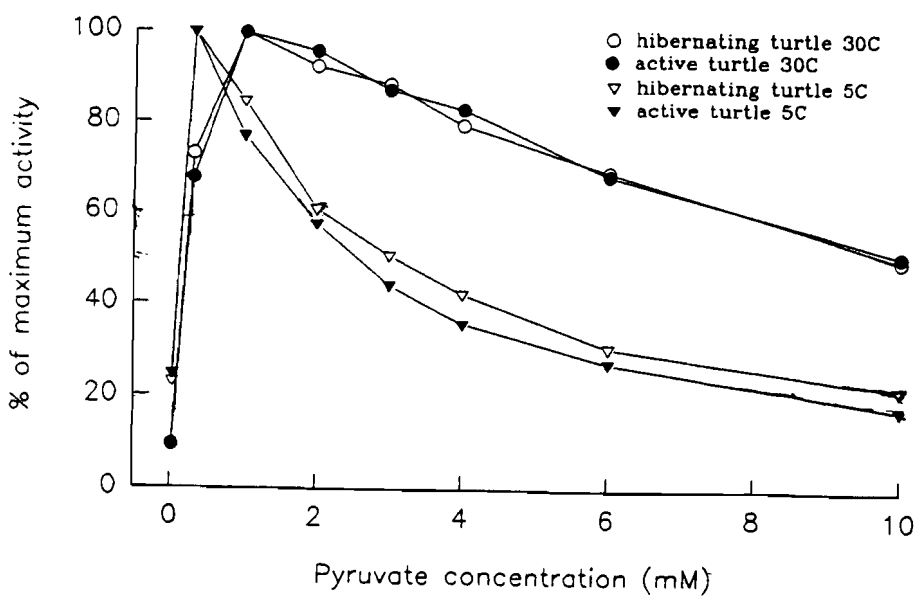

100
80
60
40
20
0
% of maximum activity
0
2
4
6
8
10
Pyruvate concentration (mM)
hibernating turtle 30C
active turtle 30C
hibernating turtle 5C
active turtle 5C

Fig. 5. Effect of pyruvate concentration on the activity of LDH isozymes in the skeletal muscle of hibernating turtles (n = 10) and active turtles (n = 8) at 30 ^{0}C and 5 ^{0}C. The pyruvate concentrations were 0.025, 0.3, 1.0, 2.0, 3.0, 4.0, 6.0, and 10.0 mM.

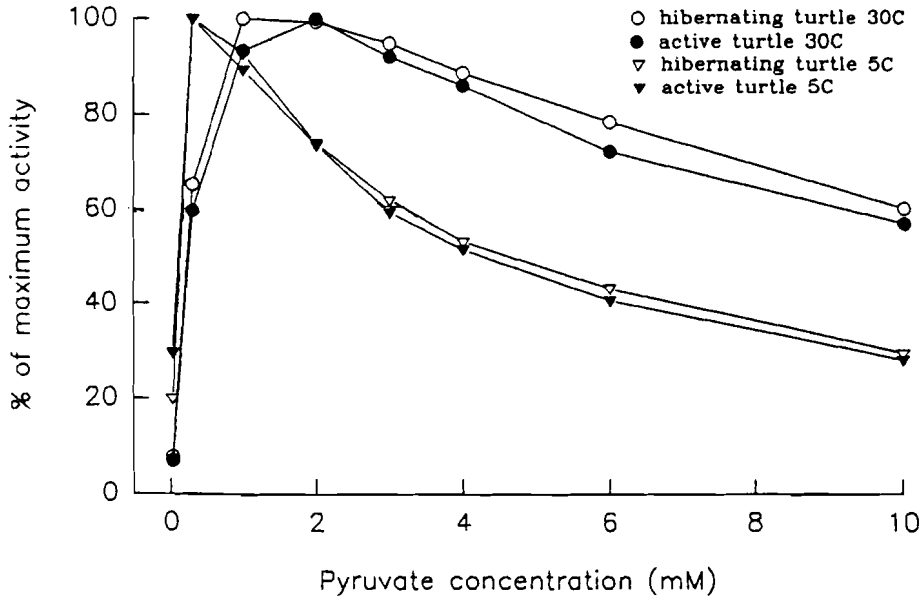
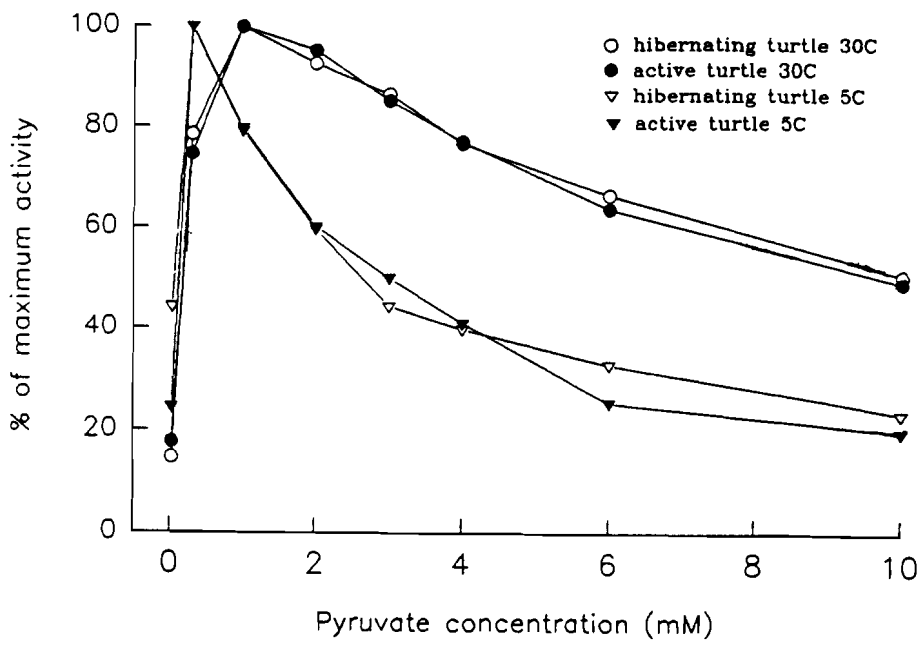

hibernating turtle 30C
active turtle 30C
hibernating turtle 5C
active turtle 5C
% of maximum activity
100
80
60
40
20
0
0
2
4
6
8
10
Pyruvate concentration (mM)

Fig. 6. Effect of pyruvate concentration on the activity of LDH isozymes in the liver of hibernating turtles (n = 10) and active turtles (n = 8) at 30 ^{0}C and 5 ^{0}C. The pyruvate concentrations were 0.025, 0.3, 1.0, 2.0, 3.0, 4.0, 6.0, and 10.0 mM.


100
80
60
40
20
0
% of maximum activity
0
2
4
6
8
10
Pyruvate concentration (mM)
hibernating turtle 30C
active turtle 30C
hibernating turtle 5C
active turtle 5C

activity in the heart, 60.43% and 57.17% in the skeletal muscle, and 50.77% and 49.24% in the liver, respectively.

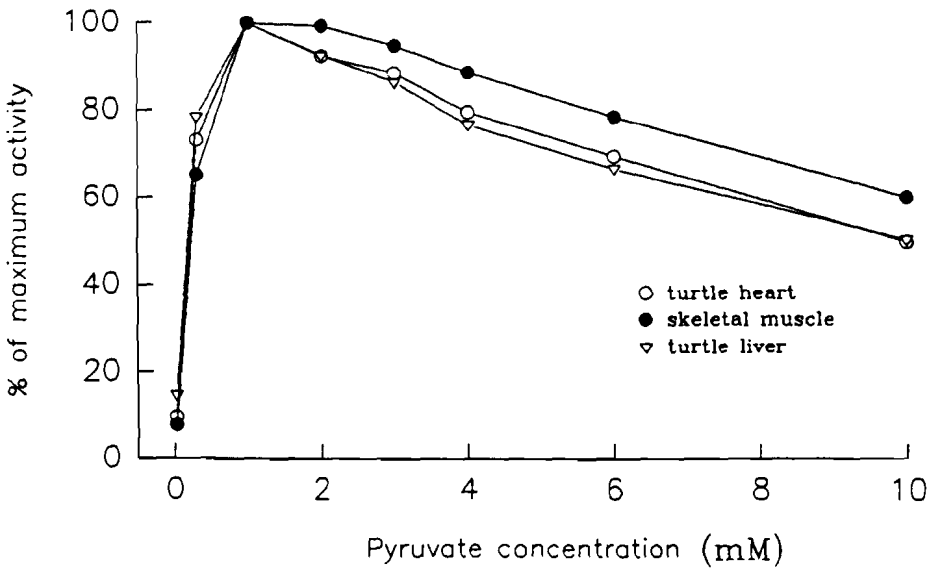
When pyruvate inhibition was assayed at 5 ^{0}C, the LDH activities of all homogenates from hibernating and active turtle heart, skeletal muscle, and liver were not inhibited until the pyruvate concentration exceeded 0.3 mM. Above 0.3 mM pyruvate concentrations, the activity of LDH in the heart, skeletal muscle, and liver was greatly reduced as compared to that seen at 30 ^{0}C. At pyruvate concentrations of 10 mM, the activities of heart LDH from hibernating and active turtles decreased to 21.91% and 17.70% of peak activity, for skeletal muscle the activity decreased to 29.56% and 28.14%, while liver LDH activity decreased to 23.42% and 19.86% of peak activity, respectively.

The above LDH pyruvate inhibition patterns demonstrate: (1) The degree of pyruvate inhibition is significantly affected by the different assay conditions of 30 ^{0}C and 5 ^{0}C. The LDH activity in each tissue is inhibited by pyruvate to a greater extent at 5 ^{0}C than at 30 ^{0}C. (2) The LDH of hibernating and active turtles shows the same sensitivity to pyruvate inhibition when measured at the same temperature. (3) When assayed at 5 ^{0}C, pyruvate inhibition begins at a pyruvate concentration of 0.3 mM, which is less than the level of 1.0 mM measured at 30 ^{0}C, showing that the optimal pyruvate concentration for peak LDH activity decreased in hibernating animals.

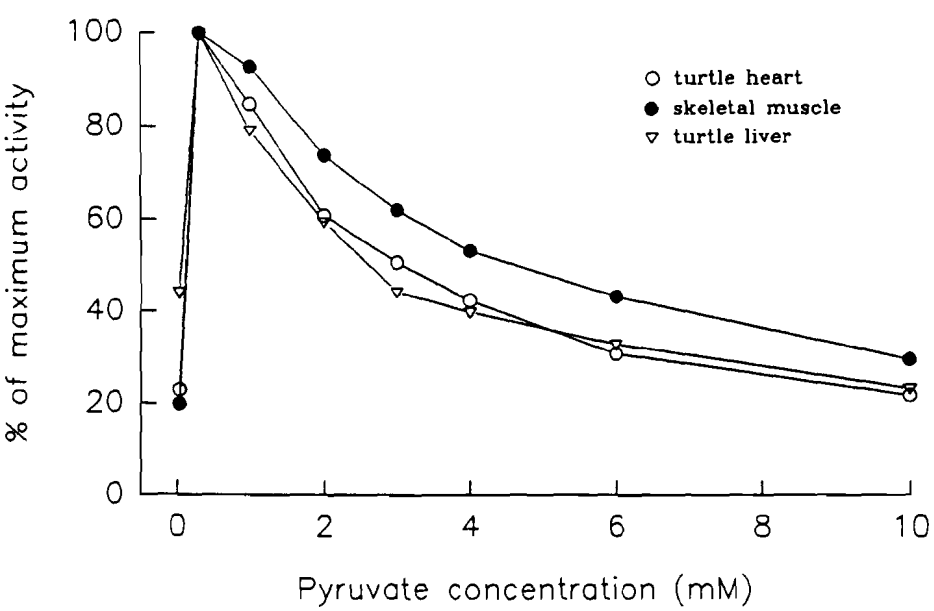
LDH sensitivities to pyruvate inhibition among heart, skeletal muscle, and liver homogenates are shown in Fig. 7. ANOVA (Student-Newman-Keuls method) test for the percentage reduction of peak LDH activity at the highest pyruvate concentration tested (10 mM) showed that heart and liver LDHs were not significantly different ($P > 0.05$) in

Fig. 7. Pyruvate inhibition patterns of LDH in the heart, skeletal muscle, and liver of hibernating turtles at 30 ^{0}C (A) and 5 ^{0}C (B). The LDH pyruvate inhibition patterns of active turtles are similar to that of hibernating turtles for a given tissue.

A


100
80
60
40
20
0
% of maximum activity
0
2
4
6
8
10
Pyruvate concentration (mM)
turtle heart
skeletal muscle
turtle liver

B


100
80
60
40
20
0
% of maximum activity
0
2
4
6
8
10
Pyruvate concentration (mM)
turtle heart
skeletal muscle
turtle liver

the extent of their inhibition by high pyruvate concentration, while the pyruvate inhibition of skeletal muscle LDH was significantly different from that of heart and liver ($P < 0.05$), as skeletal muscle LDH was less inhibited by high pyruvate concentration.

As indicated before, hibernating turtles apparently did not change their LDH sensitivity to pyruvate inhibition as compared to active turtles. However, the LDH activities in all tissues of hibernating turtles are higher than that of active turtles, especially when assayed at 5 ^{0}C (Table 3). Skeletal muscle extracts of hibernating turtles, in particular, showed a greater LDH activity as compared to active turtles. The LDH activities in hibernating turtle skeletal muscle were about 3.5 times (at 5 ^{0}C) and 2 times (at 30 ^{0}C) higher than those found in active turtle muscle. When measured at 5 ^{o}C, the activities of hibernating turtle heart and liver LDH were also significantly different from that of active turtle ($P < 0.05$), with both the tissues having activities about 1.75 times higher in the hibernating turtles.

Table 3. Mean (± SE) LDH maximum activities in heart, skeletal muscle, and liver of hibernating and active turtles assayed at 30 °C and 5 °C. LDH activity is expressed as 1×10^3 μmols of NADH oxidized / min / mg protein.

	LDH activities at 30 °C			LDH activities at 5 °C		
Turtles	Heart	Skeletal muscle	Liver	Heart	Skeletal muscle	Liver
Hibernating turtle	28.26 ±2.55	54.63* ±6.42	4.37* ±0.61	3.77* ±0.49	8.76* ±0.53	0.49* ±0.04
Active turtle	23.14 ±3.04	27.94* ±3.41	2.77* ±0.27	2.14* ±0.25	2.56* ±0.37	0.28* ±0.02

* Significantly different at $P < 0.05$ for like tissues and temperature.

DISCUSSION

The ability of Red-eared sliders to survive long periods of hypoxia during hibernation probably involves several factors, including low metabolic rate, a great ability for anaerobic energy generation, and extraordinary buffer capacities to reduce the metabolic acidosis (Ultsch, 1989; Ultsch and Jackson, 1982; Clark and Miller, 1973). It has been demonstrated that hypoxic survival of turtles depends on energy generated from anaerobic glycolysis. In order to achieve the high anaerobic metabolism, LDH isozymes of hibernating turtles might have some unusual properties. At least two kinds of mechanisms exist to adjust the LDH in response to the specific environmental conditions of hibernation: (1) adjust the level of enzyme types already present in the tissue, a process of evolutionary adaptation, and (2) adjust the function of enzyme variants uniquely suited at particular environmental condition, a short-term acclimation (Hochachka and Somero, 1968).

In this study, after being submerged for 5 months in water at 5 ^{0}C, the hibernating turtles did not show a different relative proportion of H and M subunits as compared to room temperature active turtles. However, the M subunit proportion in heart and skeletal muscle of *T. scripta* is higher than other animals including the loggerhead turtle *(Caretta caretta)* which also hibernates under mud of lakes (Table 4). Having an LDH with a high proportion of M subunits would clearly be advantageous during hibernation because of its ability to remain functional in the presence of high lactate concentrations. The high M/H ratio of *T. scripta* is apparently not only beneficial to hypoxic hibernation but also during active periods. Gatten (1974) found in the summer, *T. scripta* obtained 60~75% of its energy supply from anaerobic glycolysis during a burst of activity, a proportion greater

Table 4. The relative percentages of LDH M subunit in the heart and skeletal muscle of various animals.

	Percentage of total M subunits		
Species	Heart	Skeletal muscle	References
Red-eared slider	46	89	Present study
Loggerhead turtle	29	82	Baldwin & Gyuris, 1983
Rat	22	89	Everse & Kaplan, 1973
Rabbit	0	57	Dawson et al., 1964
Human	--	67	Dawson et al., 1964

than that of other animals undertaking similar activity. The LDH characteristics of *T. scripta* may be regarded as an evolutionary adaptation related to the turtle's ecology and life history.

The results of LDH peak activities showed that skeletal muscle LDH has the highest activity followed by that in the heart and liver. The LDH activity of each tissue in hibernating turtles generally was higher than that in comparable tissues of active turtles. When assayed at 5 ^{0}C, the temperature associated with hibernation sites, the LDH activity of hibernating turtle skeletal muscle is about 3.5 times greater than that of active turtle skeletal muscle. The high LDH activity observed in the skeletal muscle of hibernating turtles may be a factor in the short-term acclimation in response to the low temperatures and hypoxia experienced during hibernation. The increased activity may be related to some activator of LDH and/or the change of LDH catalytic properties. Everse and Kaplan (1973) reported lactate was effective in causing dissociation of the inhibitory LDH-NAD^+-pyruvate complex; thus, an increase in the level of lactate concentration would promote the activation of the enzyme. In the tissues of hibernating turtles, the lactate concentration is much higher than that of active turtles. Therefore, more free enzyme in the tissues of hibernating turtles may explain the higher activity in these animals. Investigations of several poikilotherms have shown LDH affinity for pyruvate varies with temperature and reaches the maximum affinity (minimum K_m) at the normal temperature of the animal's habitat (Narita and Horiuchi, 1979; Hochachka and Somero, 1968). The relatively low K_m value may reflect a particularly strong interaction between pyruvate and active sites of LDH isozymes which may fulfill the need of efficient

anaerobic energy generation during hibernation.

Comparing the pyruvate inhibition pattern in the like tissues, no significant differences were found in the extent of pyruvate inhibition between the LDHs of hibernating and active turtles at 30 °C or 5 °C. Comparing LDH sensitivities of pyruvate inhibition among heart, skeletal muscle, and liver, there was no significant difference between the heart and liver, while the pyruvate inhibition of skeletal muscle LDH was significantly less than that of the heart and liver. These results are consistent with the concept of the H subunit having more sensitivity to pyruvate inhibition than the M subunit (Narita and Horiuchi, 1979). Everse and Kaplan (1973) described the LDH isozyme complex as usually displaying the catalytic properties in proportion to its percentage of M and H subunits present in the molecule. In the present study, there was no significant difference in the proportion of M and H subunits between like tissues of hibernating and active turtles; thus, the pyruvate inhibition patterns were similar between the LDH of hibernating and active turtles in the same tissue at a given temperature. Similarly, the proportions of M and H subunits in heart and liver were similar, so that the extent of pyruvate inhibition is much the same. The LDH of skeletal muscle has a higher proportion of the M subunit than do the LDH enzymes of heart and liver; therefore, the inhibition pattern showed a decrease in sensitivity to pyruvate.

The temperature effect on the LDH pyruvate inhibition shows more inhibition at 5 °C than at 30 °C. This can be explained by the structure of the inhibition complex, LDH-NAD^+-pyruvate being more stable at low temperature. The dissociation constant (K_i) for LDH-NAD^+-pyruvate in crayfish becomes lower as the temperature decreases

(Narita and Horiuchi, 1979). The K_i for the inhibition complex in the snake *Bothrops neuwiedii* showed a 4.6-fold increase from assay temperatures of 10 ^{0}C to 35 ^{0}C (Nelia et al., 1974). The high stability of the inhibition complex at low temperatures may also be suggested by the decreased optimal pyruvate concentration at 5 ^{0}C compared to that at 30 ^{0}C in the present study. Because at low temperatures the connection of LDH-NAD^+-pyruvate is tighter, the number of available LDH active binding sites decreases, so the low pyruvate concentration can saturate LDH active sites. The high stability of the LDH-NAD^+-pyruvate inhibition complex at low temperature seems to reduce the turtle LDH activity during hibernation. However, under physiological conditions in winter, pyruvate concentrations are too low to induce LDH inhibition. Therefore, the affinity of LDH for pyruvate is more important than substrate concentration in determining the reaction velocities (Narita and Horiuchi, 1979; Hochachka and Somero, 1968).

Red-eared sliders are capable of prolonged survival using anaerobic glycolysis as their principal energy source. The present studies indicate that the LDH isozymes of the heart, skeletal muscle, and liver of these animals have unusual properties; a peculiar evolutionary adaptation so that more M subunits are present in their tissues as compared to other animals and an acclimation in response to environmental conditions resulting in high LDH activity in hibernating turtles as compared to room temperature turtles. These properties of LDH in the tissues of Red-eared sliders probably play an important role in these animals' ability to survive prolonged hypoxia.

Although anaerobic glycolysis is beneficial for the basic energy requirement needed

for survival in *T. Scripta,* continuous anaerobic metabolism will result in a build up of lactate causing severe metabolic acidosis which would eventually result in the death of the turtle. Belkin (1963) suggested that the viability of the hypoxic turtle might be primarily related to the extent of metabolic acidosis. Therefore, the long anaerobic survival of turtles like the Red-eared slider should involve other factors in order to prevent excessive acidosis.

One of the most important factors in preventing metabolic acidosis might be the ability to sharply reduce the metabolic rate by 12-fold and enter a hypometabolic state. Evidence exists to demonstrate the importance of low metabolic rate in hypoxia or anoxia survival. When *Chrysemys picta* was submerged in N_2-equilibrated (anoxic) water at 3 ^{0}C, the life of the animal averaged 126 days (Ultsch and Jackson, 1982), while turtles submerged in the same anoxic condition at 15~18 ^{o}C, survived only 2 weeks (Robin et al., 1964). Turtles are poikilotherms and as such, their body temperature decreases with environmental temperature. Thus, at low temperature submergence, these animals have low basal metabolic rates which in turn leads to a decreased lactate accumulation in the anoxic condition. Many other experiments related to the temperature effect on the survival of turtles in hypoxic or anoxic submergence also have shown similar results (Ultsch, 1985; Musacchia, 1959). Further, in their natural environment, turtles exit hibernation in early spring when the water temperature increases and they can swim under the ice so as to reach the air. During this time, the mortality of this turtle is at its highest (Gatten, 1981). This might indicate that when the temperature increases and the turtles becomes active under ice covered hypoxic water, the increased mortality may be

the result of rapidly accumulating lactate levels.

Another important factor to maintain a favorable pH is the strong buffering capacity of the turtle. The plasma carbonic acid-bicarbonate system is the major buffering system in hibernating turtles. In this system, CO_2 generated from the titration of bicarbonate by lactic acid is eliminated from turtle's body through such surfaces as the skin, the cloaca, and the buccopharyngeal membrane (Herbert and Jackson, 1985). Lactate and hydrogen ions are also excreted via the kidneys (Jackson and Silverblatt, 1974). In addition, decreased plasma chloride ion concentration, as well as increased $[Mg^{2+}]$ and $[Ca^{2+}]$ in hypoxic submerged turtle are considered to be buffers with the ability to balance the increase in $[lactate^-]$ which is accomplished by the transportation of these strong ions between plasma and other body compartments (Jackson and Ultsch, 1982; Jackson, 1976; Jackson and Silverblatt, 1974).

In conclusion, Red-eared sliders (*T. scripta)* are several times more tolerant of anoxia than other reptiles. In winter, these turtles survive under the bottom mud of lakes for 3 to 5 months where the PO_2 is close to zero. The extremely high body lactate concentration indicates an important role for anaerobic glycolysis as a principle source of energy generation. The relatively higher proportion of M subunits in turtle tissue as compared to other animals, as well as an increased LDH activity during hibernation, are consistent with the view that it is an LDH adaptation which contributes to the especially pronounced ability to liberate energy through anaerobic metabolism. The sharply decreased metabolic rate and the strong buffering ability of this animal prevents the body lactate concentration reaching the level that would be fatal.

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APPENDIX

This appendix is a description of the special problems in the methods and reagents used in this study.

Animal Preparation

The simulated hibernating group (n = 10) was submerged in a basin of water and maintained at 5 °C for 5 months. No effort made to control/monitor O_2 concentrations in the basin. During the latter half of period of the submergence, turtles did become motionless under water which indicated the turtles were in hibernation. The difference between the simulated hibernating condition and the condition that turtles hibernate in nature is that the experimental hibernating turtles were submerged in open water which contained some dissolved oxygen (D.O.) in contrast to natural hibernating turtles that burrow into the bottom mud under ice covered water where the D.O. is close to zero. A better way to simulate natural hibernation of turtle for future researchers may be to place the turtles under a plastic screen in N_2-equilibrated water column that would prevent turtles from accessing to oxygen.

Electrophoresis

At the beginning of the study, when I performed electrophoresis of turtle extracts on cellulose gel following the method described by Baldwin and Gyuris (1983) (50 mM Tris-maleate buffer, pH 6.7, running for 40 min at a voltage 150 V and a constant current of 150 mA), only one band showed on the gel. The pH and concentration of running buffer as well as the voltage and current applied during electrophoresis have some special effects on the movement of protein on the gel: 1) a change in buffer pH will alter the charges of each isozyme due to their different isoelectric point; 2) a change in the

concentration of buffer would effect on the conductivity of the buffer; 3) altering the voltage and current can change the speed of the protein movement on the gel. Based on these assumptions, several test runs were carried out in which pH, buffer concentrations, voltage, and current were varied. The optimal conditions for electrophoresis were found to involve: a buffer concentration of 0.038 M Tris-0.37 M glycine, pH 8, and extracts being electrophoresed for 2 h at 25 °C with a voltage 250 V and a constant current of 150 mA.

Measurement of LDH Specific Activity

When measuring the LDH specific activity at 5 °C, a layer of fog would form on the surface of the cuvette due to the temperature difference between the solution in the cuvette (5 °C) and the air (room temperature) outside the cuvette. This would affect the readings from spectrophotometer. In order to resolve the problem, I smeared a thin layer of a transparent Prestone Anti-Fog solution on the outside surface of the cuvette, which allowed the wall of the cuvette to remain transparent. The LDH activity of extracts gradually decreased with storage time. The optimal period for measuring LDH activity was within 2 months after preparation of the extracts.

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