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A COMPARATIVE STUDY OF LACTATE DEHYDROGENASE ACTIVITY IN DIVING AND NONDIVING REPTILES

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ABSTRACT

The properties of lactate dehydrogenase (LDH) were examined in two snake species and one turtle species. The snakes used in the study were the semi-aquatic *Nerodia rhombifera* and the terrestrial Elaphe obsoleta, while *Pseudemys scripta* represented the turtle species. Our purpose was to compare the LDH activity of nondiving reptiles *(Nerodia* and *Elaphe)* with that of the well established diver *Pseudemys*. The kinetic properties of LDH and its susceptibility to inhibition by elevated pyruvate concentrations were investigated in the brain and heart of the three species. Brain and heart were chosen because they are highly aerobic tissues and therefore should be quite sensitive to anoxia.

In both tissues the LDH activity of the snakes was higher than that of *Pseudemys* at pyruvate concentrations ranging between .03 mM and .50 mM. The Km values of the snakes were lower than those of *Pseudemys* in both tissues suggesting a greater enzyme-substrate affinity in the snake tissues. The Vmax values were higher in snake brain and heart than in turtle indicating a faster conversion of substrate to product in the snake tissues.

Brain LDH activity was reduced by high pyruvate concentrations to an equal extent in the three species. *Nerodia* heart LDH showed the greatest susceptibility to substrate inhibition while heart LDH activity was equally inhibited in *Elaphe* and *Pseudemys*.

The results indicate that the LDH of *Pseudemys* is no better adapted to anaerobic conditions than that of *Nerodia* and *Elaphe*.

INTRODUCTION

Certain representatives of all the vertebrate classes have the ability to remain submerged for extended periods of time. These animals are commonly referred to as diving animals. The diving mammals and birds have been studied most extensively while the diving reptiles have received relatively less attention. Of the three classes it appears, however, that the diving reptiles may be best adapted to an underwater existence.

The diving capabilities of sea snakes have been well documented (Graham, 1974; Heatwole and Seymour, 1975; Heatwole, 1976). The ability of snakes to survive submergence is not restricted to those forms that are strictly aquatic. Baeyens et al. (1980) found that both the semi-aquatic diamondback water snake, *Nerodia rhombifera,* and the terrestrial black rat snake *Elaphe obsoleta,* could tolerate total submergence for periods of approximately one hour. The turtles are, however, the most remarkable reptilian divers. Fresh water turtles of the family *Emydidae* can tolerate apneic periods of several hours at summer water temperatures (Burggren and Shelton, 1979; Lucey and House, 1977; Penney, 1974) while at winter water temperatures they can remain continuously submerged for periods of four to six months (Musacchia, 1959; Ultsch and Jackson, 1982).

The ability of reptiles to remain submerged has been variously attributed to such circulatory adaptations as a pronounced bradycardia and a preferential shunting of blood away from anaerobic tissues to the brain and heart, a refractoriness of the respiratory centers to carbon dioxide, and increased storage capacities for oxygen in lungs and blood (Belkin, 1968; Gatten, 1980; Schmidt-Nielsen, 1983). A more important factor in the survival of turtles encountering a limited oxygen supply is their ability to liberate energy through anaerobic metabolism (Jackson, 1968). This is due in part to special properties of brain, heart and muscle enzymes to generate ATP by anaerobic means (Lutz et al., 1978; Simon et al., 1979; Storey and Hochachka, 1974).

The present study focuses on the glycolytic enzyme lactate dehydrogenase (LDH) derived from brain and heart of three reptilian species. The two snakes, *N. rhombifera* and *E. obsoleta* were chosen because of their ability to tolerate extended periods of submergence (Baeyens et al., 1980). For comparative purposes, we also chose the red eared turtle, *Pseudemys scripta,* because of its well established reputation as an outstanding reptilian diver (Belkin, 1968; Caligiuri et al., 1981; Robin et al., 1964). The Michaelis-Menten kinetics of LDH and its susceptibility to substrate inhibition were compared in the three species. It was hoped that examining the properties of LDH would lead to an explanation of the differences in anaerobic threshold between the snakes and turtle.

MATERIALS AND METHODS

N. rhombifera (55-63 cm snout vent length [SVL]) were collected from minnow ponds in Lonoke County, Arkansas. *E. obsoleta* (80-105 cm SVL) were collected from wooded areas in Pulaski County, Arkansas. *P. scripta* were obtained from commercial dealers. All animals were maintained at room temperature (20-25 °C) and were allowed at least 30 days to acclimate to captive conditions before they were used for experimentation.

Enzyme activity was examined in heart and brain of the three species. Five individuals of each species were used for the enzyme analyses. After determining that there was no individual variation in enzyme activity for a specific tissue within a species, the results obtained for the five individuals of that species were pooled and compared with the results obtained from the other two species. Enzyme activity was expressed as a change in absorbancy/min/mg protein. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. The differences between means of enzyme activity, Km values and Vmax values were analyzed by Student's t-tests.

Preparation of Tissue Extracts

The animals were killed by cervical dislocation and samples of heart and brain were immediately dissected free from the animal. After the

tissue samples were weighed to the nearest mg, they were homogenized in 0.1 M phosphate buffer (pH 7.4) in a ratio of 1 g of tissue to 7 ml of buffer. Following homogenization the samples were centrifuged at 2445 g for 40 min. The clear supernatant was then stored frozen at −80 C. Before enzyme analysis the supernatant was further diluted in a 1:9 ratio with phosphate buffer.

LDH Activity Determinations

Enzyme activity was measured by following the oxidation of NADH to NAD^+ at 340 nm with a Varian dual beam recording spectrophotometer (model DMS90). The assay mixture consisted of 2.8 ml of 0.1 M phosphate buffer (pH 7.4), 100 µl of 30 mM sodium pyruvate, and 100 µl of NADH. The reaction was initiated by adding 10 µl of the tissue preparation to the mixture in a cuvette. The decrease in absorbancy was recorded over a 5 min period.

To examine the Michaelis-Menten kinetics of LDH the pyruvate concentrations of the reaction mixture were varied between .03 mM and .50 mM. The mean of three measurements at each substrate concentration was recorded. Apparent Km and Vmax values were calculated from Lineweaver-Burk plots.

Substrate Inhibition Studies

To determine the susceptibility of LDH to inhibition by pyruvate, the pyruvate concentration of the reaction mixture in the cuvette was varied between .16 mM and 6.6 mM. Three measurements were made at each substrate concentration and the results were averaged.

Table 1. Brain and heart LDH activity in *N. rhombifera, E. obsoleta* and *P. scripta*. LDH activity is expressed as change in absorbancy/min/mg protein.

SPECIES	BRAIN	HEART
N. rhombifera	18.792 ± 0.373 (15)*	37.180 ± 0.534 (15)**
E. obsoleta	15.056 ± 0.220 (15)***	28,830 ± 0.201 (15)****
P. scripta	14.041 ± 0.219 (15)	26.045 ± 0.232 (15)

Mean ± S.E. (N)

*Significantly different from E. obsoleta and P. scripta brain (P<0.001)

**Significantly different from E. obsoleta and P. scripta heart (P<0.001)

***Significantly different from P. scripta brain (P <0.05)

****Significantly different from P. scripta heart (P <0.05)

RESULTS

Kinetic Studies

Table 1 gives the activities of brain and heart LDH in the three species when measured at a pyruvate concentration of 1 mM. In each species the LDH activity was greater in heart than brain. In both tissues the activities were greatest in *Nerodia* followed by *Elaphe* with *Pseudemys* having the lowest activity.

The Lineweaver-Burk plot for brain LDH is shown in Fig. 1. Throughout the range of pyruvate concentrations the activity of *Nerodia* is highest while *Pseudemys* is lowest with *Elaphe* being intermediate. *Nerodia* also had the highest Vmax value and *Pseudemys* had the lowest of the three species. The apparent Km value of *Nerodia* was the lowest of the three species while that of *Pseudemys* was the highest.

The results were similar for heart with *Nerodia* having the highest activity throughout the range of substrate concentrations utilized, *Pseudemys* having the lowest and *Elaphe* showing intermediate activity (Fig. 2). The Vmax values were greater in the two snakes than in *Pseudemys*, while the Km value of *Pseudemys* was greater than that of either snake. The results of the kinetic studies are summarized in Table 2.

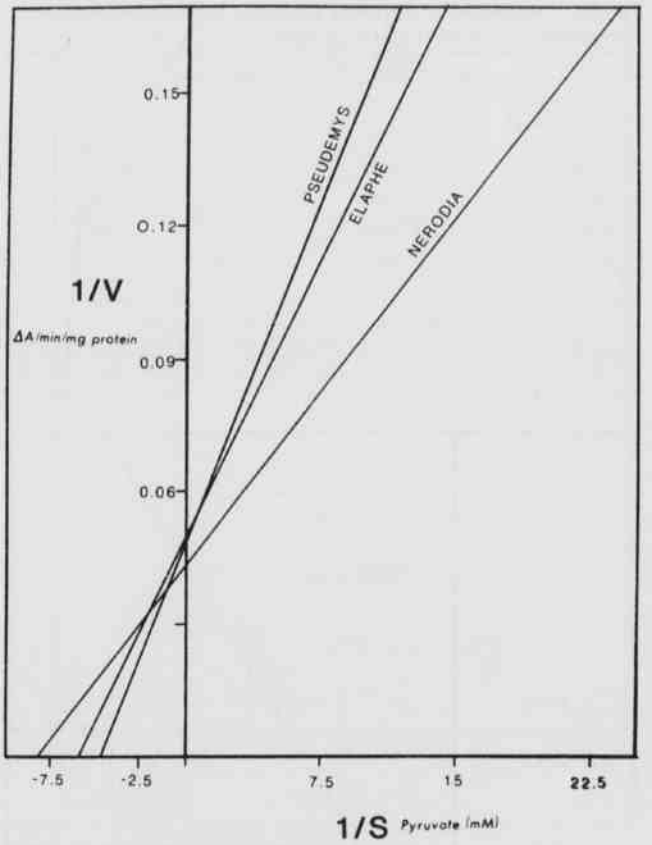

Figure 1. Lineweaver-Burk plot of brain LDH activity in *N. rhombifera, E. obsoleta* and *P. scripta*.

Substrate Inhibition Studies

Highest brain LDH activity occurred at a 1.3 mM pyruvate concentration in the three species (Fig. 3). At higher substrate concentrations there was a progressive and equal reduction in enzyme activity in each species. The highest activity of heart LDH occurred at a substrate concentration of 1.7 mM in the three species (Fig. 4). *Nerodia* heart LDH was the most sensitive to substrate inhibition, while the degree of substrate inhibition was approximately equal in *Elaphe* and *Pseudemys*.

DISCUSSION

A number of studies have suggested that aquatic gas exchange may be important in extending the survival time of forcibly submerged reptiles (Belkin, 1963; Belkin, 1968; Girgis, 1961). In *P. scripta*, however, there appears to be no evidence for gas exchange during a dive (Belkin, 1963; Robin et al., 1964). Likewise, in an unpublished experiment conducted in our laboratory, the dive times of *N. rhombifera* and *E. obsoleta* could not be extended by increasing the oxygen tension of the water, suggesting the improbability of aquatic gas exchange in these two species.

The physiological responses of reptiles to submergence have been well documented (Andersen, 1966; Astrup et al., 1977; Belkin, 1961; Berkson, 1966). These responses may extend dive times for short periods but they are incapable of sustaining an animal for hours or days in an anaerobic state. The ability of such outstanding reptilian divers as *Pseudemys* to remain submerged for extended periods must in large part be attributed to various biochemical adaptations. In this regard, it has been suggested

Table 2. Summary of the brain and heart LDH kinetic data in *N. rhombifera, E. obsoleta* and *P. scripta.*

SPECIES	TISSUE	Vmax	Km
N. rhombifera	Brain	23.0	0.121
E. obsoleta	Brain	20.0	0.167
P. scripta	Brain	20.6*	0.215**
N. rhombifera	Heart	42.8	0.142
E. obsoleta	Heart	35.3	0.128
P. scripta	Heart	25.2***	0.167****

*Significantly different from N. rhombifera brain ($P < 0.05$)
**Significantly different from N. rhombifera and E. obsoleta brain ($P < 0.05$)
***Significantly different from N. rhombifera and E. obsoleta heart ($P < 0.05$)
****Significantly different from N. rhombifera and E. obsoleta heart ($P < 0.05$)

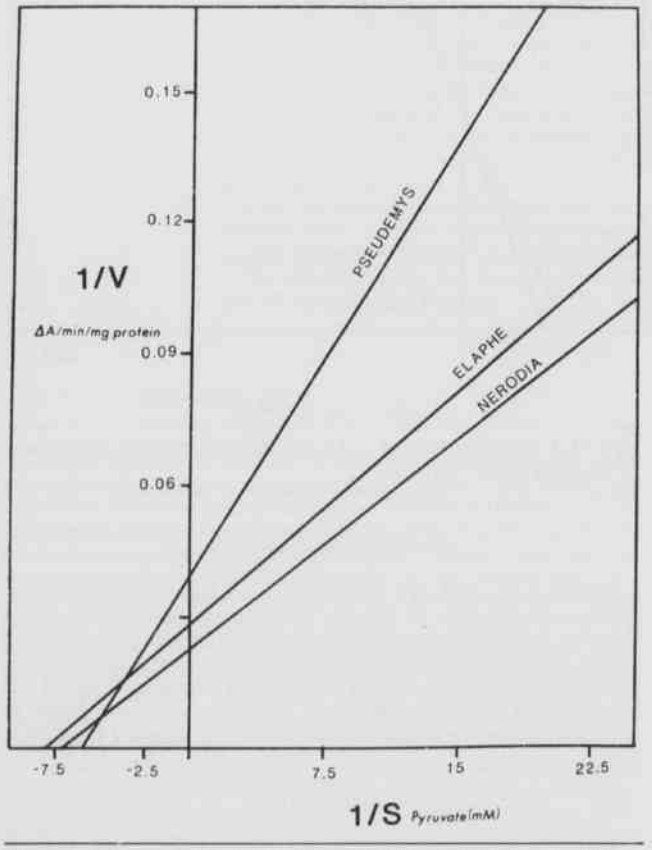

Figure 2. Lineweaver-Burk plot of heart LDH activity in *N. rhombifera, E. obsoleta* and *P. scripta.*

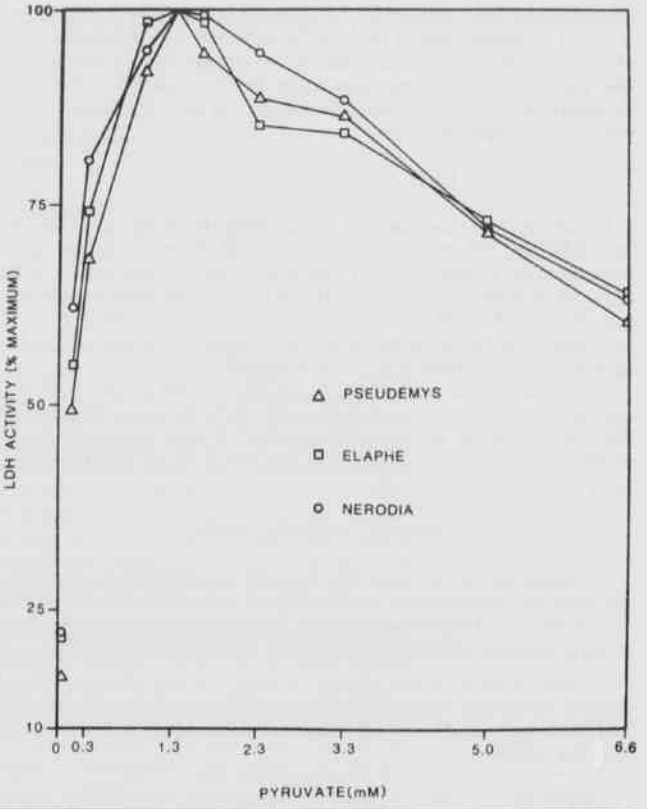

Figure 3. Substrate inhibition plot of brain LDH activity in *N. rhombifera, E. obsoleta* and *P. scripta.*

that anaerobic glycolysis may be of particular importance. Belkin (1962) found that treating the loggerhead musk turtle, *Sternothaerus minor,* with iodoacetate resulted in a significant decrease in survival time when subjected to an oxygen free atmosphere as compared to untreated controls. Similarly, blocking the glycolytic pathway in the river cooter, *P. concinna,* permits indefinite survival in air but renders the animal incapable of prolonged diving (Belkin, 1961). An increased reliance on glycolysis during diving in reptiles is also indicated by the high levels of lactic acid which accumulate in the blood following the dive (Berkson, 1966; Johlin and Moreland, 1933; Robin et al., 1981).

Lactic dehydrogenase may play a key role in prolonging anaerobic survival in turtles. Altman and Robin (1969) suggested that LDH may be an important determinant of the diving ability of *P. scripta* because of its unusually high resistance to substrate inhibition. Miller and Hale (1968) measured the LDH activity of *P. scripta* brain and found it to be much higher than that of rat brain. They attributed the ability of the turtle brain to withstand long periods of anaerobiosis to be in part due to the elevated LDH activity. Even if LDH is not the rate limiting enzyme of glycolysis its continued activity under anaerobic conditions is essential in order to generate NAD^+ so that glycolysis can be sustained.

We found that brain and heart LDH activity was greater in *Nerodia* and *Elaphe* than in *Pseudemys* at pyruvate concentrations ranging from 0.03 mM to 0.50 mM. In both tissues the Km values of the snakes were lower than those of Pseudemys suggesting a greater affinity of LDH for pyruvate in the snakes. In addition, the higher Vmax values of the snake brain and heart suggest a faster conversion of substrate to product when the enzyme is saturated with substrate. Since *Pseudemys* is clearly a better diver than either snake, there does not appear to be a positive correlation between LDH activity and the ability to withstand anaerobic conditions in these three species. In a similar study, no correlation could be established between LDH activity and diving ability in marine mammals (Castellini and Somero, 1981).

Most vertebrates possess two genes for LDH that make similar but not identical polypeptides called H and M (Kaplin et al., 1968). In embryonic tissue both genes are equally active resulting in the formation of five different isozymes (M_4, M_3H, M_2H_2, MH_3, and H_4). As embryonic tissue differentiates the relative amounts of the M and H forms change.

An important factor determining the distribution of the LDH isozymes is the availability of oxygen (Hochachka and Somero, 1973; Hochachka, 1975; Kaplin et al., 1968). Skeletal muscle, which is dependent on anaerobic metabolism for energy production during strenuous bouts

of activity, has LDH composed of high proportions of M subunits. This is of adaptive value in an anaerobic tissue because the M subunits continue to remain active, converting pyruvate to lactate, even in the presence of high substrate levels. In contrast, tissues which are more dependent on aerobic metabolism for energy production have LDH with greater proportions of H type subunits. These isozymes are characteristically more susceptible to inhibition by increased levels of substrate. The heightened sensitivity to substrate inhibition of H monomers is important because it allows the channeling of pyruvate into the Kreb's cycle rather than its conversion to lactate during aerobic conditions.

The capability of prolonged anaerobic survival has been attributed to the presence of particular combinations of LDH isozymes in various tissues of diving reptiles. Miller and Hale (1968) compared LDH activity in brain, heart and skeletal muscle of the albino rat and *P. scripta*. They calculated the ratio of M- and H- subunits by measuring the relative enzyme activities in high and low concentrations of pyruvate. The proportion of M-LDH in brain and heart was found to be much higher in the turtle than in the rat. Furthermore, the proportion of M-LDH is similar in brain, heart and skeletal muscle of the turtle and the proportion of M-LDH in these three tissues is also similar to the proportion of M-LDH in mammalian skeletal muscle. Since mammalian skeletal muscle is highly adapted to anaerobic conditions, they concluded that the turtle tissues may be similarly adapted.

Altman and Robin, (1969) found that LDH from both heart and skeletal muscle of *P. scripta* has similar proportions of H and M subunits. The composition of the LDH is so similar that changes in the pH of the electrophoresis media caused the LDH to appear homogenous in the two tissues. Of equal importance, the pyruvate inhibition patterns of LDH from heart and skeletal muscle extracts were virtually identical, indicating heart LDH can maintain high levels of activity even in the presence of increased substrate concentrations. In a related study, the properties of the LDH M and H subunits were examined in a marine turtle *Caretta caretta* (Baldwin and Gyuris, 1983). The M_4 and H_4 isozymes were purified from skeletal muscle and liver respectively, and their kinetic properties were measured in the presence of varying substrate concentrations. The LDH H_4 activity progressively fell with increasing concentrations of pyruvate, being reduced to 44% of maximal activity at the highest concentration of pyruvate (10 mM). In contrast the M_4 activity was completely insensitive to substrate concentration. Due to the sensitivity of the H_4 isozyme to substrate inhibition the authors concluded that the kinetic properties of the M and H monomers of *C. caretta* were clearly different from those of *P. scripta*.

For diving animals, which are heavily dependent on glycolysis for energy production during anaerobic conditions, having LDH with a high M subunit activity would be an adaptive advantage because of its ability to remain functional in the presence of high substrate concentrations. Thus, even tissues like brain and heart, which normally derive most of their energy aerobically, could remain functional by shifting their energy production to anaerobic glycolysis during a dive.

In comparing the LDH activity of brain and heart in *Nerodia, Elaphe,* and *Pseudemys* we could find no evidence that the enzyme derived from *Pseudemys* was particularly resistant to substrate inhibition. Brain LDH activity was equally inhibited by high substrate concentrations in all three species. The LDH activity of *Pseudemys* heart was no more resistant to substrate inhibition than that of *Elaphe* heart. Thus, in terms of susceptibility to substrate inhibition the LDH of *Pseudemys* brain and heart is no better adapted to anaerobic conditions than that of the two snakes. These results are somewhat surprising since *Pseudemys* is capable of sustaining a dive for a much longer period of time than the snakes and therefore must accumulate higher concentrations of substrates in the tissues.

In conclusion, based on total enzyme activity, Michaelis-Menten kinetics and susceptibility to pyruvate inhibition the LDH of *Pseudemys* brain and heart appears to be no better adapted to anaerobic conditions than that of *Nerodia* or Elaphe.

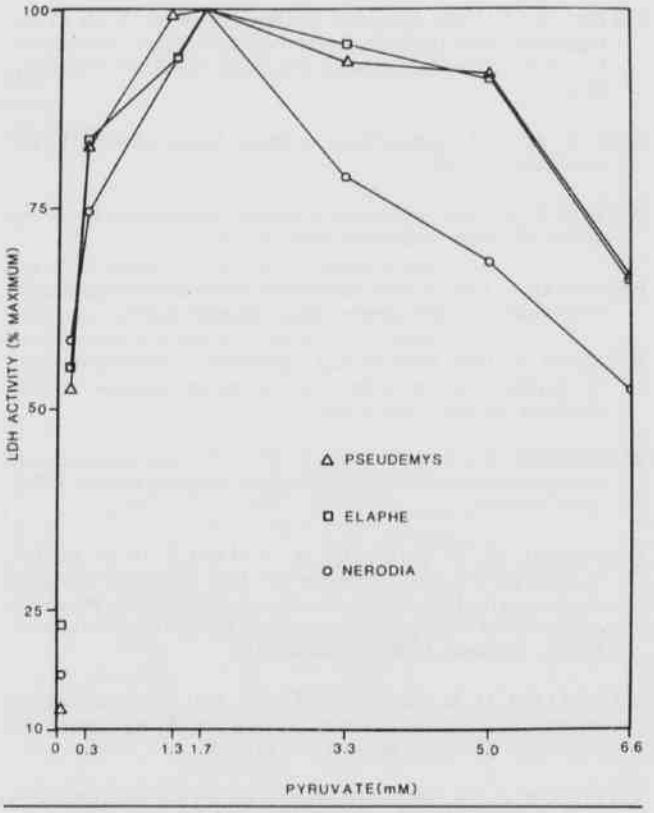

Figure 4. Substrate inhibition plot of heart LDH activity in *N. rhombifera, E. obsoleta* and *P. scripta*.

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