ADAPTATIONS OF THE AMPHIBIAN ORNITHINE-UREA CYCLE ENZYME, ARGINASE

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This study was initiated in order to investigate possible metabolic adaptations involving the amphibian ornithine-urea cycle enzyme, arginase. Several statements can be made concerning natural arginase activities in species from different habitats and the effect of the environmental parameters, water availability and temperature, on arginase activity. The possible role of kidney arginase in active urea transport has been examined.

Natural variations in the activities of kidney and liver arginase in seven species of anurans (Rana septentrionalis, R. clamitans, R. sylvatica, R. pipiens, Hyla versicolor, H. chrysoscelis, and Bufo americanus) from Minnesota were studied. It was found that aquatic species had significantly higher natural activities of kidney arginase than terrestrial species. There appeared to be no correlation between natural activities of liver arginase and habitat preference in these species.

It was observed that both water availability and temperature affected arginase activity. Controlled desiccation seemed to increase the activity of both kidney and liver arginase in R. sylvatica. R. pipiens and B. americanus showed an increase in only kidney arginase activity when desiccated. There was no significant increase in either kidney or liver arginase activity when R. clamitans and R. septentrionalis were desiccated. Injection of antidiuretic hormone caused no change in the activities of kidney or liver arginase in R. septentrionalis, R. clamitans and B. americanus.

The data collected seem to indicate that temperature may affect arginase activity in two ways. First, winter R. pipiens were found to have lower kidney and liver arginase Michaelis constants than summer R. pipiens, however the maximum velocity did not vary with season. This temperature effect could be reproduced in the laboratory, for it was found that holding R. catesbeiana at 7° C. for 14 days lowered the Km of both liver and kidney arginase. Second, incubation performed at 2° C. resulted in a lower value for liver and kidney arginase Km (in R. pipiens and in R. catesbeiana) than incubation performed at 25° C. It seems apparent that there exist both an immediate adaptive response to lowered temperature and a long term decrease in Km due to seasonal trends of lower temperature.

The drug probenecid inhibits the active transport of urea. The possible effects of this drug on kidney arginase were investigated. Incubation of R. pipiens liver and kidney arginase in the presence of probenecid resulted in decreased enzyme activity. Probenecid had no effect on bovine arginase activity. These results yield indirect evidence that kidney arginase is involved in active urea transport.

William D. Schmid

Introduction

A very important phenomenon observed in all living systems is the ability of organisms to adapt to changes in their surroundings. Adaptations can occur at many levels of organization. Although many adaptations are readily noticed at the gross morphological level (for example industrial melanism in moths), other adaptations at the molecular level, which are not readily observed, are probably also very important to living organisms. Goldstein (1970) makes the following statement concerning molecular or biochemical adaptations. "The adaptation of animals to environmental changes is one of the central problems in biology today. The mechanisms by which these adaptations take place involve complex interactions of biochemical and physiological systems with environmental factors."

One obvious method of adaptation at the molecular level is change in the enzyme systems of an organism. Knox and Greengard (1964) point out that enzymes in animal cells change in response to physiological conditions and have coined a phrase, "enzyme physiology," to describe the study of these changes. Knox and Greengard (1964) describe the basis for this study well when they state, "the behavior of an enzyme is often different in different species, in different tissues, and even in the same tissue at different times, because of the changing metabolic state of the cells."

A perusal of the scientific literature has yielded several examples of changes in enzyme systems that are produced by environmental factors. Perhaps the classic example of environmental adaptation at the molecular level is that the addition of a simple sugar to the culture medium in which a micro-organism has

been growing will result in the induction of synthesis of enzymes necessary for the metabolism of this substrate. It has been found that dietary changes will produce changes in the enzyme systems of higher organisms. The amount of protein in the diet of rats (Mandelstam, 1952 and Millman, 1951) and monkeys (Nuzum, 1971) affected levels of hepatic ornithine-urea cycle enzymes. The activities of enzymes in canine pancreatic juice can be modified by diet (Behrman, 1969). Finally, Balnave (1969) reported that acetyl-Co A carboxylase and citrate cleavage enzyme activities were reduced when the amount of fat in a chicken's diet was increased.

There are examples of several other environmental factors that modify enzyme activities. The levels of monoamine oxidase were modified by photoperiod in the quail hypothalamus (Follett, 1969) and in the brain of the lizard Sceloporus occidentalis (Quay, 1970). Circadian changes in the level of acetyl-cholinesterase from the brains of house crickets (Acheta domesticus L.) were observed by Cymborowski (1970). Environmental osmotic changes can also modify enzyme levels. Oide (1970) found that the activity of alkaline phosphatase was three times higher in sea-water-adapted eels than in fresh-water-adapted eels. Temperature and water balance seemed to affect the enzymes involved in urea synthesis in the earthworm Lumbricus terrestris (Tillinghast, 1969). Finally, Schoffeniels (1963) found that the activity of ornithine-urea cycle enzymes can be modified by the ionic conditions of the environment.

It is apparent that evidence exists that indicates environmental factors affect enzyme activities in several different animals. In order to initiate a study of this phenomenon one would

have to select a specific aspect of the problem so that a plan of research could be designed. A fruitful approach might be the study of enzymes involved in nitrogen metabolism in animals. Gordon (1970) states that "nitrogen metabolism has come to be considered one of the most sensitive physiological systems in its responses to environmental changes." He further reports that the ability for shifts in nitrogen metabolism can be observed in the teleost fishes Periophthalmus sabrinus (the mudskipper) and Sicyases sanguineus (the clingfish). Emerson (1969) found that in several euryhaline invertebrates ammonia increased when the animals were transferred from 100% sea water to 50% sea water. It was found that temperature and water availability affected both the amount and type (urea or ammonia) of nitrogenous waste excreted by the earthworm, Lumbricus terrestris (Tillinghast, 1969). Hill (1969) reported that if the amount of protein in the diet of the tuatara, Sphenodon punctatus, is increased the amount of urea excreted increases.

One specific enzyme system involved in nitrogen metabolism is the ornithine-urea cycle. The enzymes in this system are involved in the conversion of ammonia to urea. It has previously been mentioned that diet (Mandelstam, 1952; Millman, 1951; and Nuzum, 1971) and temperature and water availability (Tillinghast, 1969) are environmental factors that modify levels of urea cycle enzymes. One particular enzyme, arginase, has been observed to vary with diet in rat liver (Ashida, 1961 and Schimke, 1962) and in the livers of teleost fishes (Cvancara, 1965). Finally, Mora, et al. (1965a) state that carbamyl phosphate synthetase, ornithine transcarbamylase,

and arginine-synthetase vary similarly under different conditions, suggesting that these enzymes are under the same regulatory mechanism.

In order to initiate a study of metabolic adaptations involving urea cycle enzymes one must select a group of organisms that may exhibit these adaptations. The amphibia may well be such a group. Balinsky (1970) has stated that the ability for shifts in nitrogen metabolism, in response to environmental changes, is well developed in the amphibia. Amphibians exist in a wide range of habitats (from completely aquatic to completely terrestrial) and show different methods of nitrogen excretion (Schmid, 1968). Changes in urea cycle enzymes may be the mechanism that mediates changes in modes of nitrogen excretion. Cohen (1970) has pointed out that Rana catesbeiana tadpoles increase the activity of ornithine-urea cycle enzymes during metamorphosis as they switch from ammonotelism to ureotelism. These changes may be adaptive both in animals that exist naturally in different habitats with regard to water availability, and in situations where there are environmental changes (such as drought) that produce differences in water availability. Further, amphibians from Minnesota are exposed to a wide range of environmental temperatures during the course of a year. Somero (1969a) points out that aquatic poikilotherms "exhibit similar metabolic rates at widely different habitat (body) temperatures." One could look for metabolic adaptations in urea cycle enzymes that would buffer against temperature fluctuations.

Somero (1971) has devised three categories into which

biochemical adaptations can be classified. This classification system depends upon the time course of the adaptive change. First, there are changes that occur on an evolutionary time scale. These metabolic changes occur very slowly over many generations. Secondly, there may be seasonal shifts in an organism's biochemistry. Finally, there may be immediate responses in an organism's biochemistry to environmental factors. In this study, it was thought amphibian urea cycle enzymes could be investigated to see if all three of these classes of biochemical adaptations are exhibited. Firstly, one could look for natural variations of enzymes between different species to see if there are any differences or similarities in levels of activity. Possible correlations with habitat preference could be investigated. Such similarities or differences may also shed some light on genetic or evolutionary differences between species. Second, seasonal fluctuations of enzymes in response to seasonal temperature changes can be investigated. Lastly, one could look for immediate shifts in enzyme levels in response to such environmental factors as dehydration.

It has been suggested that shifts in levels of enzymes, in response to environmental changes, may have adaptive value for organisms. Consequently I have proposed that a fruitful method of investigating this phenomenon would be to look specifically at amphibian urea cycle enzymes. In the present study it was deemed desirable to limit the investigation solely to the enzyme arginase (L-arginine ureohydrolase, EC 3.5.3.1). The reason for this limitation is twofold. First, it was chosen for matters of convenience because the assay technique (devised by Brown, 1959)

for the enzyme is relatively easy to perform. Second, and more important, arginase has been implicated in the active transport of urea in the kidney tubules of several species of amphibia (Forster, 1954 and Schmidt-Nielsen, 1963). One could, therefore, investigate both the role of arginase in the active transport of urea and changes in the active transport process that are due to environmental influences on the enzyme-mediated system. Needless to say, these enzyme changes may be highly adaptive to the organism exhibiting them.

The investigations upon which this study is based are divided into five sections, which will appear in the following order: (1) assay technique, (2) natural variations in levels of arginase activity, (3) response to dehydration, (4) response to temperature, and (5) observations of the role of arginase in active urea transport.

Technique

In order to investigate meaningfully possible metabolic adaptations involving the amphibian urea cycle enzyme arginase, a dependable assay procedure had to be developed. Arginase catalyzes the following reaction:

$$\text{arginine} + H_2O \xrightarrow[Mn^{++}]{\text{arginase}} \text{urea} + \text{ornithine}$$

The assay measures the amount of urea produced over a given time interval in the presence of a known amount of enzyme and substrate (arginine). The assay procedure used here is a modification of the technique reported by Brown (1959).

Preparation of Homogenate

Freshly excised tissue to be assayed (liver or kidney) was gently blotted on "Kimwipes disposable wipers," weighed on a Sartorius balance, and homogenized in 5 ml of ice cold 0.1% cetyltrimethylammonium bromide, CTB (Eastman Organic Chemicals, Rochester, N.Y.) solution in a glass homogenizer. Homogenation was accomplished by hand with the homogenizer placed in an ice bucket. The homogenate is transferred to a chilled cellulose nitrate centrifuge tube and centrifuged for 15 minutes at 4000 X g at 3° C. in a Sorvall RC2-B superspeed refrigerated centrifuge. The supernatant is decanted into a chilled test tube and placed on ice. The pellet is resuspended in 5 more ml of ice cold CTB and centrifuged again for 15 minutes at 4000 X g at 3° C. This supernatant is added to the test tube containing the first supernatant and the pellet is

discarded. Aliquots of the pooled supernatant are then used for arginase assays.

Incubation

Two different incubation techniques were used. In the experiments to determine natural variations in arginase activities the reaction vessel used was a test tube. Various aliquots of the supernatant were added to test tubes containing 0.5 ml of 0.85 M L-arginine (Sigma Chemical Co., St. Louis) and different amounts of a 1.0 mM $MnSO_4$ solution to yield a total volume of 2.0 ml. The tubes were placed in a "Porta Temp" water bath (Precision Scientific Co.) and the reaction was allowed to proceed for 15 minutes at 25° C. and then stopped by adding 2.0 ml of 85% acetic acid.

In the kinetic determinations 125 ml Erlenmeyer flasks were used as reaction vessels. Different amounts of arginine and Mn^{++} ion were used to provide various substrate concentrations. One ml of supernatant was added to each flask to produce a total volume of 50 ml in the flasks. The flasks were placed in a "Gyrotory Shaker" (Model G-76, New Brunswick Scientific Co.) and incubation was accomplished at 25 $\pm$ 1° C. At various times (0, 7, 15 minutes) 2.0 ml aliquots were withdrawn from each flask and added to a test tube containing 2.0 ml of 85% acetic acid.

Urea Analysis

Urea analysis was accomplished using the indicator alpha-isonitrosopropiophenone (Sigma Chemical Co., St. Louis) which complexes with urea to yield a red color. The indicator diacetyl monoxime was tried and found to be less sensitive than alpha-

isonitrosopropiophenone. Two ml of a sulfuric-phosphoric acid mixture (90 ml of concentrated H_2SO_4 and 270 ml of concentrated H_3PO_4 diluted to a total volume of 1 L) were added to each test tube containing 2.0 ml of the reaction solution and 2.0 ml of acetic acid. Two-tenths ml of an alpha-isonitrosopropiophenone solution (4 gm in 100 ml of 95% ethanol) was then added to each test tube to yield a total volume of 6.2 ml. The test tubes were stoppered with marbles and placed in a boiling water bath for 1 hour and 45 minutes. This incubation was done in total darkness. Absorbancy was measured at 540 nanometers using a Klett-Summerson photoelectric colorimeter. Standard urea solutions were mixed and used to determine a standard curve. The standard curve is presented in Fig. 1.

Fig. 1. Standard curve for alpha-isonitrosopropiophenone.

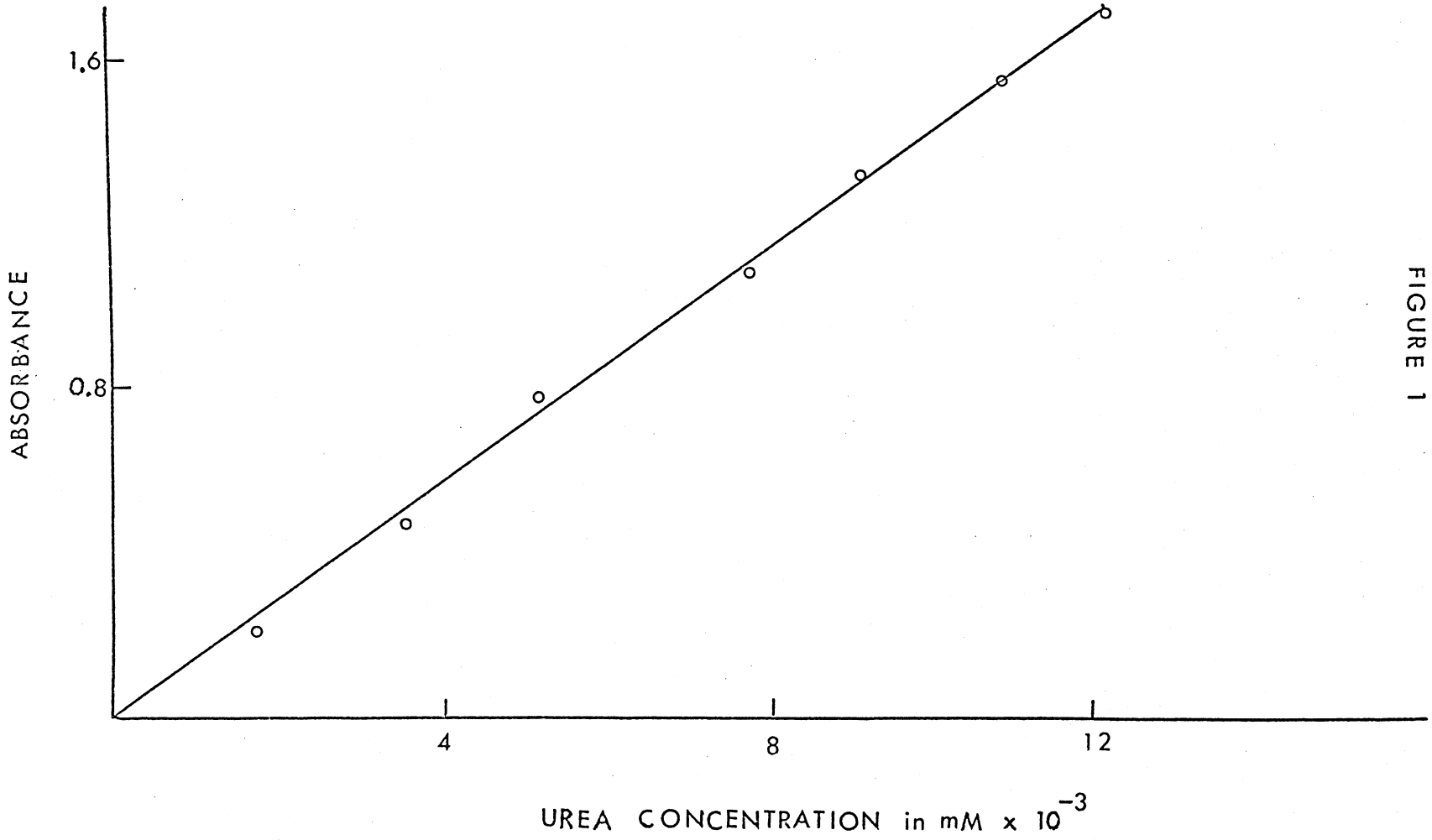


FIGURE 1

Enzyme Units

Levels of arginase activity are expressed as mM of urea produced per time interval. Specific activities are expressed as units of arginase per mg tissue wet weight or units per mg protein. Protein was determined using the method of Lowry (1951). Bovine serum albumin was used to determine a standard curve.

Kinetic Data

Michaelis constants (Km) and maximum velocities (Vmax) were estimated by plotting initial velocities vs. initial velocity/substrate concentrations according to Dowd and Riggs (1965). A more complete discussion of the kinetic analysis is given in the appendix.

It has been stated that modifications of Brown's technique (1959) were used in the assay procedure used in this study. These modifications involve the temperature of the assay mixture and the use of buffers. Brown states that the enzymes should be pre-incubated at 50° C. for 30 minutes in the presence of Mn^{++} ion. It was found (see Table 1) that pre-incubation for 30 minutes at 25° C. reduced enzyme activity.

Table 1. Units of Rana pipiens Kidney Arginase Activity (1 Unit = 1.0 µgm urea/30 min./1.0 ml Supernatant)

Pre-incubation	No Pre-incubation
184.1	517.8
244.5	517.8
201.4	549.8

Because of these data, it was decided that the enzyme would not be pre-incubated. Besides pre-incubating at 50° C., Brown (1959) indicated that the reaction mixture should be incubated at 30° C. Because this study was performed on amphibians, animals whose body temperatures probably rarely exceed 30° C., it was thought more accurate to incubate the reaction mixture at 25° C. In this way the biochemical nature of the enzyme in the test tube would more closely approximate its nature in the animal.

Along with temperature another physical factor that affects enzyme catalyzed reactions is the hydrogen ion concentration or pH. The maximum activity of both kidney and liver arginase in amphibians was found to occur at a pH of 9.5 (Figures 2 and 3). The arginine solution used in the assay procedure is mixed so that its pH is 9.5. Arginine, like all amino acids, has buffering properties and it was found that the arginine in the reaction vessel maintained the pH of the reaction mixture at 9.5. Therefore the reaction mixture was not buffered with sodium glycinate buffer as Brown (1959) had done.

It should be mentioned that the enzyme preparation produced by this particular technique is not purified. Since the purpose of this study was to investigate possible metabolic adaptations of the urea cycle enzyme arginase that may be important to the biology of the animal rather than the biochemistry of the enzyme arginase, it was felt that it would be acceptable to work with a crude enzyme preparation. Obviously, further studies that would delve more deeply into the biochemical phenomena involved in this problem of metabolic adaptations would necessitate a purification procedure.

Fig. 2. pH *vs.* activity curve for *Rana catesbeiana* kidney arginase.

Fig. 3. pH *vs.* activity curve for *Rana catesbeiana* liver arginase.

FIGURE 2

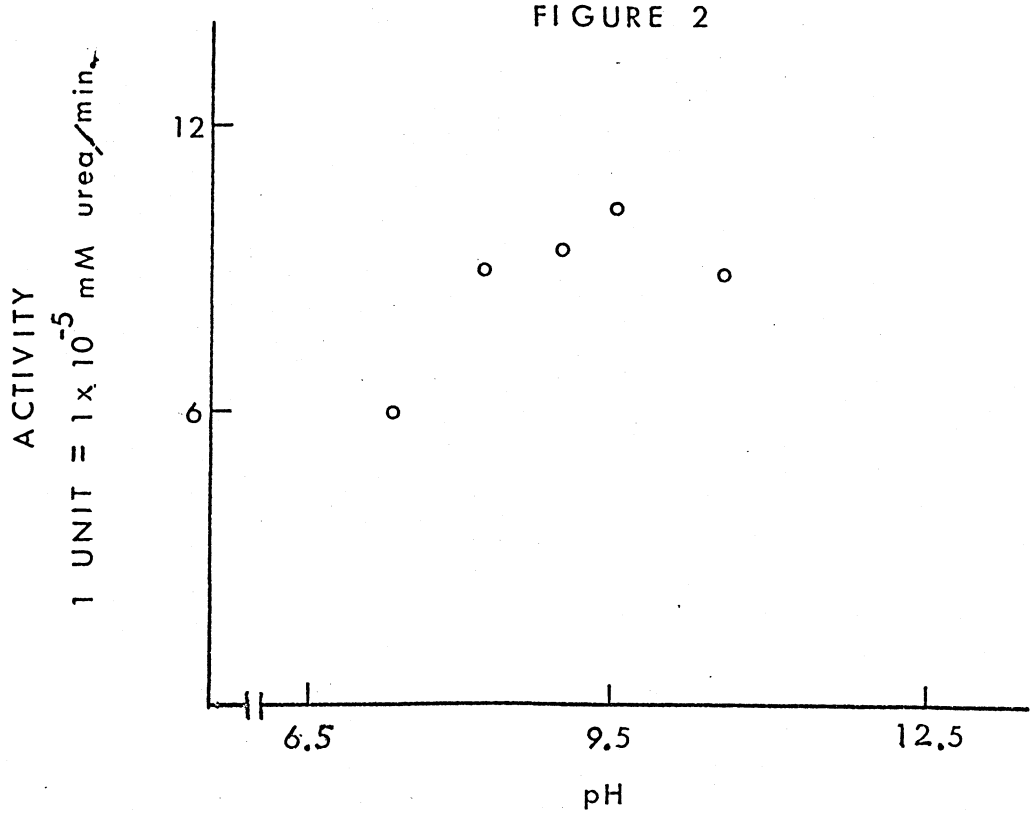
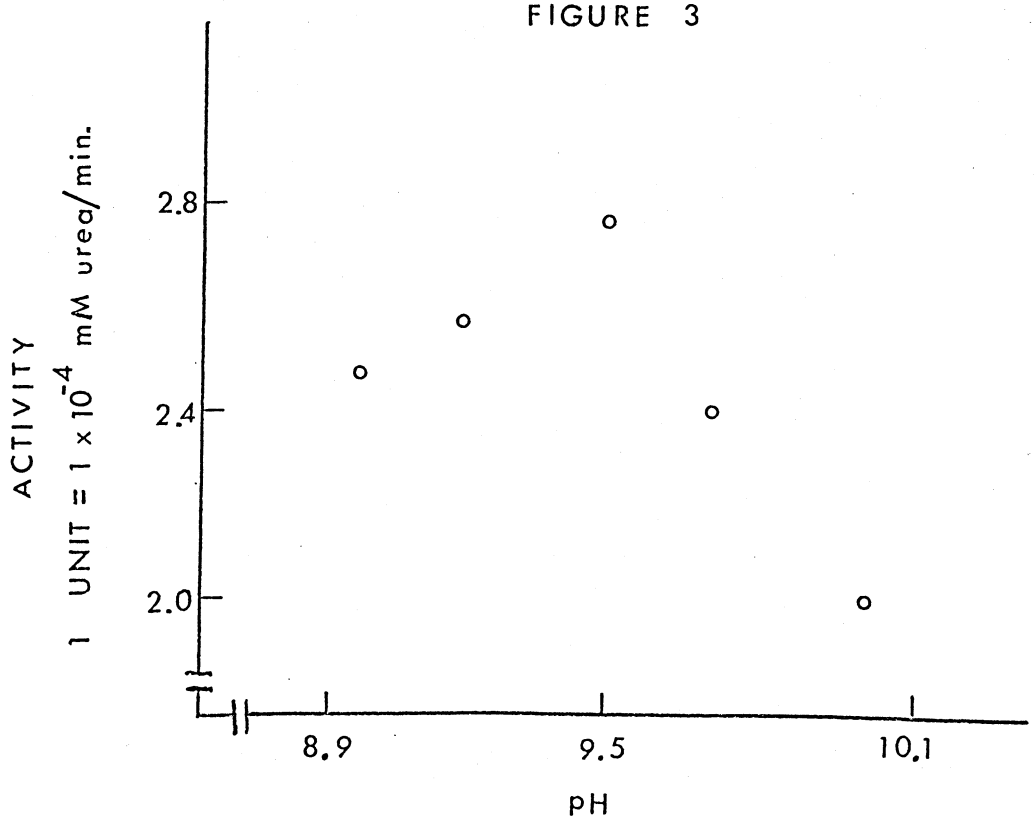


FIGURE 3



Natural Variations

Introduction

Insight into possible metabolic adaptations involving the urea cycle enzyme arginase may be gained by investigating possible variations of the level of this enzyme in animals from different habitats. In Minnesota, amphibians exist in a wide range of habitats with regard to water availability. If different levels of urea cycle enzymes are adaptively valuable to amphibians, one could discover this by looking at natural variations in the levels of arginase among species from different habitats. One would look for possible correlations between levels of enzyme activity and the amount of water available in the particular habitat in which the species is most often found. Such correlations would yield evidence, albeit indirect, that there may be adaptive value in regulating arginase activity.

Carlisky (1970) has reported values for arginase activity in the kidneys and livers of nine species of amphibians. His data indicate that aquatic species have higher levels of kidney arginase than terrestrial species. There appeared to be no correlation between liver arginase activity and habitat preference in these amphibians.

Before discussion of the materials and methods section, mention must be made of the relative merits of collecting your own specimens as opposed to obtaining research animals from biological supply houses. Since it is the business of animal suppliers to have animals available when prospective customers want them, they must collect large numbers of animals and then maintain them in their

facility until customers want them. Obviously several variances (the length of time the supplier holds the animal and the conditions under which the animal is held, etc.) are unknown to the investigator who uses supplied animals. Baze (1970) measured hepatic ornithine-urea cycle enzyme activities in several different species of turtles that were both supplied by a biological supply house and collected fresh from the field. He found that there were significant differences in enzyme levels between supplied animals and collected animals. For these reasons I felt it mandatory that only freshly collected animals be used in my study.

Materials and Methods

All of the animals used in this study were collected in the state of Minnesota. The habitat preferences listed with each species are taken from Schmid (1965) and Wright and Wright (1949). The species used are as follows. (1) Rana septentrionalis Baird, the mink frog, is an aquatic frog. Mink frogs were collected in lakes and bogs in Itasca State Park, Clearwater County, Minnesota. (2) Rana clamitans Latreille, the green frog, is also an aquatic frog. The green frogs used in this study were collected by Mr. Donald Beimborn in bogs in the Saint Paul Science Museum Nature Center in Washington County, Minnesota. (3) Rana pipiens Schreber, the leopard frog, is a semiaquatic frog. These amphibians were collected in bogs and lakes in Itasca State Park. (4) Rana sylvatica LeConte, the wood frog, is more terrestrial than the leopard frog. The wood frogs used in this study were collected by Mr. Richard Stasiak in Itasca State Park. (5) Hyla versicolor LeConte, the tree frog, is a semiaquatic arboreal species. These frogs were

collected in ponds in Chisago County, Minnesota where they had gathered to breed. (6) Hyla chrysoscelis Johnson, another tree frog, is also a semiaquatic arboreal species. These animals were collected in breeding ponds in Dakota County, Minnesota. It was impossible for the author to distinguish between the species H. versicolor and H. chrysoscelis. Proper identifications were made by Dr. William D. Schmid. (7) Bufo americanus Holbrook, the American toad, is a terrestrial species. The toads were collected in breeding ponds in Dakota County, Minnesota.

Collected animals were transported to the laboratory and sacrificed within a day or two. Livers and kidneys were removed and if not assayed immediately, placed in amphibian Ringer solution and frozen until the assays could be performed. It was found that tissue could be frozen for two months with no loss of enzyme activity. Kidney and liver were assayed for arginase activity as described in the technique section.

Results

Natural variations in kidney arginase activities are described in Table 2. Fig. 4 expresses the mean values for kidney arginase activities from each species and the 90% confidence intervals about the mean for the values obtained in this study. Table 3 and Fig. 5 show the values for liver arginase activities in each of the seven species of amphibians studied. Again the mean arginase activities and the 90% confidence intervals about the mean values from each species are given in Fig. 3. The data are arranged so that the results range from terrestrial to semiaquatic and finally to aquatic species.

Table 2. Units of Kidney Arginase Activity (1 Unit = 1 x 10^{-4} mM urea/15 min./1.0 mg tissue).

Species	Mean	Std. Dev.	Std. Error	Number
B. americanus	3.03	3.6	1.4	7
H. versicolor	0.41	0.25	0.14	3
H. chrysoscelis	0.5	0.12	0.07	3
R. sylvatica	4.0	2.6	1.5	3
R. pipiens	8.2	4.7	2.4	4
R. clamitans	22.8	3.4	1.7	4
R. septentrionalis	16.3	5.8	2.4	6

Fig. 4. Natural variations in amphibian kidney arginase activities. The horizontal lines represent the mean values for each species and the vertical lines represent 90% confidence limits about the mean. Species investigated are represented as follows: Ba (*Bufo americanus*), Hv (*Hyla versicolor*), Hc (*H. chrysoscelis*), Rsyl (*Rana sylvatica*), Rpip (*R. pipiens*), Rclam (*R. clamitans*), and Rsep (*R. septentrionalis*). The species are arranged in order of habitat preference from terrestrial (Ba) to aquatic (Rsep).

FIGURE 4

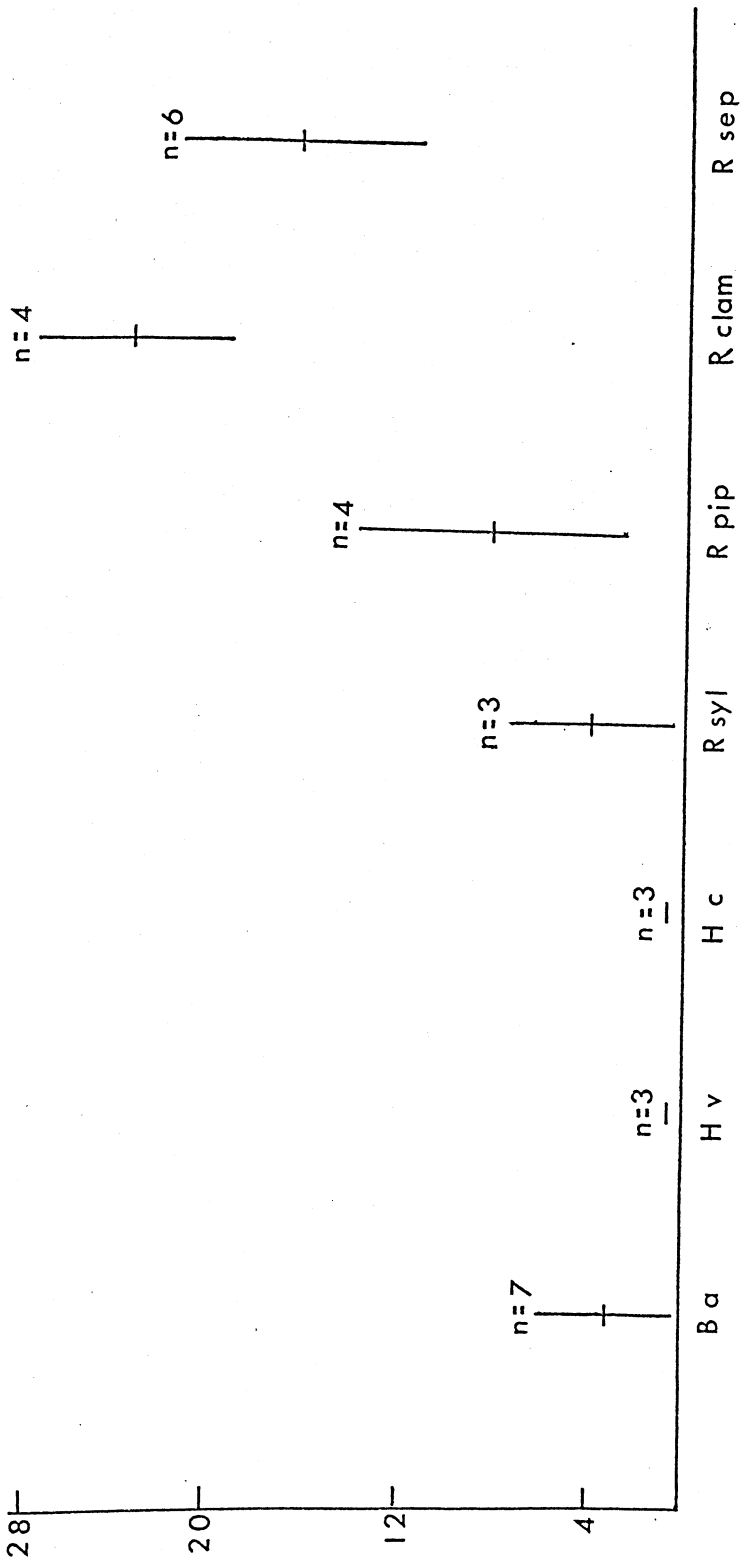
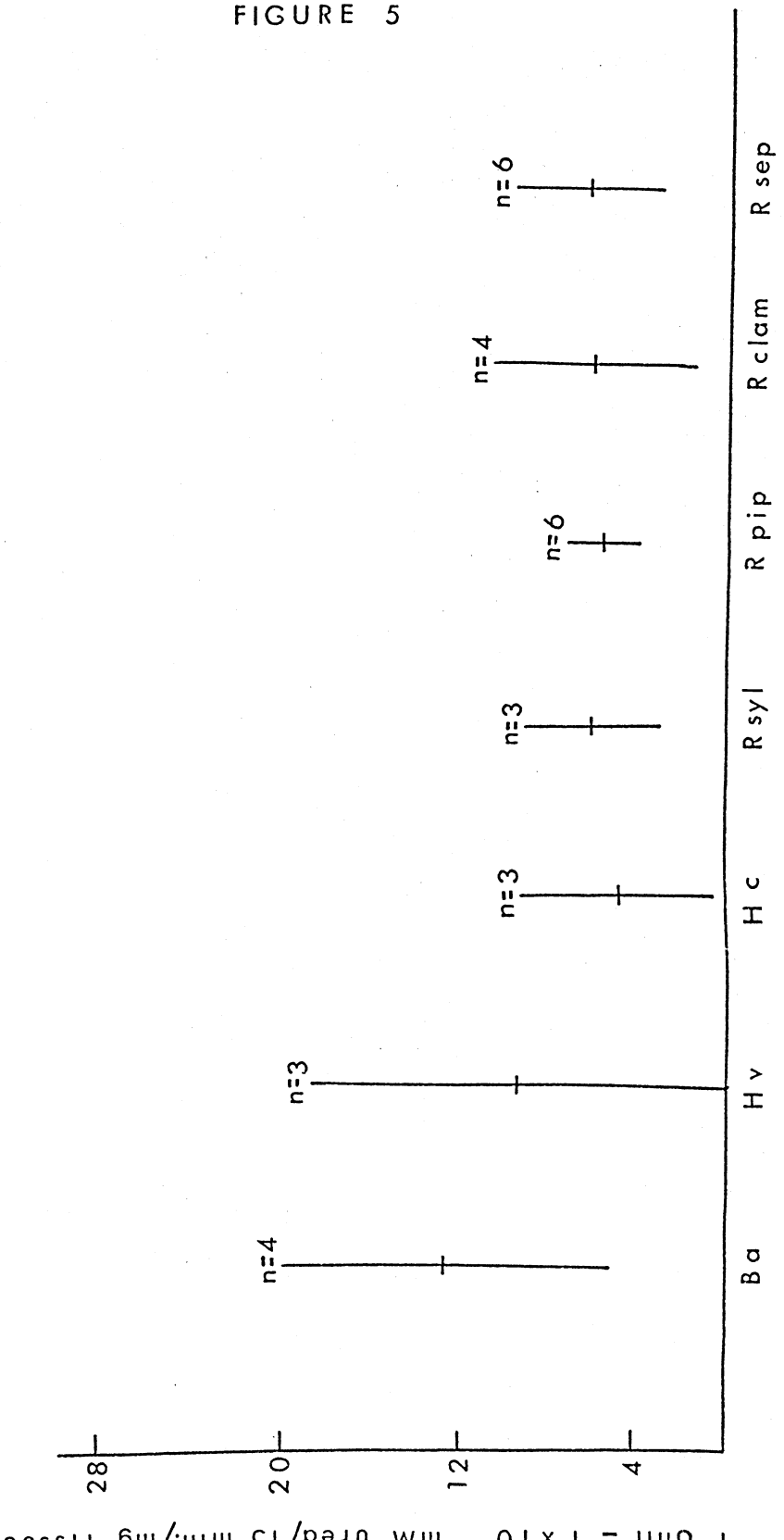

SPECIFIC ACTIVITY
1 Unit = 1 x 10⁻⁴ mM urea/15 min./mg tissue
28
20
12
4
n=7
n=3
n=3
n=3
n=4
n=4
n=6
Ba
Hv
Hc
Rsyl
Rpip
Rclam
Rsep
SPECIES

Table 3. Units of Liver Arginase Activity (1 Unit = 1 x 10^{-3} mM urea/15 min./1.0 mg tissue).

Species	Mean	Std. Dev.	Std. Error	Number
B. americanus	12.4	6.2	3.1	4
H. versicolor	9.2	5.7	3.3	3
H. chrysoscelis	5.1	1.5	0.9	3
R. sylvatica	6.1	5.2	3.0	3
R. pipiens	5.8	2.0	0.8	6
R. clamitans	5.2	3.4	1.7	4
R. septentrionalis	6.3	3.9	1.6	6

Fig. 5. Natural variations in amphibian liver arginase activities. The horizontal lines represent the mean values for each species and the vertical lines represent 90% confidence limits about the mean. Species are designated as in Fig. 4.

FIGURE 5


SPECIFIC ACTIVITY
1 Unit = 1 x 10⁻³ mM urea/15 min./mg tissue
28
20
12
4
n=4
n=3
n=3
n=3
n=6
n=4
n=6
Ba
Hv
Hc
Rsyl
R pip
R clam
R sep
SPECIES

Discussion

It appears from the results that amphibian kidney arginase does, in fact, vary with environment. Fig. 4 shows that the averages of kidney arginase from the two aquatic species, R. septentrionalis and R. clamitans, are significantly higher than averages of kidney arginase in any of the more terrestrial species (H. versicolor, H. chrysoscelis, B. americanus and R. sylvatica) at the 90% confidence level. R. pipiens, a semiaquatic species, has kidney arginase levels intermediate between the aquatic species and the terrestrial species. The values for R. pipiens are significantly less than the values for R. clamitans but not R. septentrionalis at the 90% confidence level. Similarly, there is a significant difference between R. pipiens kidney arginase levels and the levels for H. versicolor and H. chrysoscelis but no significant difference between kidney arginase levels in R. pipiens and B. americanus and R. sylvatica at the 90% confidence level.

Since the aquatic species maintain significantly higher levels of kidney arginase than terrestrial species, it appears there may be some adaptive value in being able to regulate the level of this enzyme. Forster (1970) has proposed an hypothesis that could explain this correlation between the level of amphibian kidney arginase and water availability in the environment. Renal arginase may be involved in an active urea transport mechanism. It has already been stated that active urea transport has been demonstrated in the kidneys of aquatic species of amphibians (Forster, 1954; Schmidt-Nielsen, 1954, 1963; and Carlisky, et al., 1968a). Carlisky, et al. (1968c) have reported that terrestrial species of amphibians

do not exhibit active urea transport. Forster (1970) has proposed that aquatic amphibians use the active urea transport system to solve the problem of hydration stress. Fresh-water amphibians that spend a large amount of time in the water would tend to acquire large amounts of water due to an osmotic flow of water from the environment into the animal. Water could be eliminated in the urine by actively transporting urea from the plasma into the kidney tubules. An active transport mechanism would be mandatory since the ratio of urine urea to plasma urea is much higher in aquatic amphibians than in terrestrial amphibians (Schmid, 1968). This would create an osmotic gradient favorable for water flux from plasma to urine. The water could then be eliminated during urination. If arginase is part of the mechanism of this urea pump one would expect higher levels of arginase in the kidneys of those species that have the pump. This is indeed what was found when amphibians from different habitats in Minnesota were analyzed for kidney arginase activity. As the habitat preference of the particular species studied shifts towards more time spent in the water (a more aquatic species), one observes higher levels of kidney arginase.

An examination of the data on liver arginase activity in animals from different habitats yields no correlation between activity and environment. Fig. 2 shows that no species has liver arginase concentrations that are significantly different from any other species at the 90% confidence level. It has been mentioned previously that liver arginase is sensitive to diet (Mandelstam, 1952; Millman, 1951; Ashida, 1961; Schimke, 1962; and Nuzum, 1971).

It would seem that the dietary state of an animal in its natural environment would be highly variable. The levels of arginase shown in Fig. 5 may merely be reflections of differences in nutritional levels. Starved animals may have liver arginase levels that are very different from animals that have just eaten. If one assumes that animals are collected randomly, the range of nutritional states in each species should not be greatly different.

This is what Fig. 5 shows. The mean liver arginase activities are very similar except for B. americanus and H. versicolor which are slightly (but not significantly) higher than the other five species.

One more comment must be made concerning these natural variations in levels of amphibian arginase from animals with different habitat preferences. It would seem that this biochemical adaptation involving higher levels of kidney arginase in aquatic species as compared with terrestrial species, is an example of Somero's (1971) category of changes on an evolutionary time scale. These differences in enzyme levels may be due to natural selection acting for numerous generations as these animals evolved and filled different niches with regard to water availability. Schmid (1965) points out that aquatic species such as R. clamitans and R. septentrionalis cannot exist in terrestrial habitats because their tolerance to desiccation stress is so low. They must, therefore, spend a large amount of time in contact with water and thus are faced with a hydration stress. This proposed ability to use arginase as a means of active urea transport and an adaptation to hydration stress evolved as the organisms evolved and filled their highly aquatic niche. Similarly, Schmid (1965) points out that terrestrial species cannot tolerate

hydration stress. Because these species filled niches which were not aquatic, there existed little adaptive value for an active urea transport system. Since there was little adaptive value for this system, maintaining high levels of kidney arginase would be wasteful. It seems evident, then, that this difference in the levels of kidney arginase, which is observed when aquatic species are compared with terrestrial species, is due to adaptive changes in these animals as they filled different niches.

Dehydration

Introduction

It is evident from the preceding section that the level of kidney arginase in amphibians from different habitats is correlated with the natural availability of water. One could then inquire as to whether arginase levels would change in response to environmental shifts in water availability, such as drought. There may be adaptive value in an amphibian being able to change the level of arginase in response to dehydration stress. One could also speculate as to possible control mechanisms for shifts in enzyme levels which are immediate responses to environmental fluctuations, one of Somero's (1971) categories.

The effect of dehydration on ornithine-urea cycle enzymes has been observed by a few investigators. Goldstein (1970) and McBean (1967) found that if Xenopus laevis are desiccated by exposure to slightly hyperosmotic saline solutions both the rate of urea production and level of carbamyl phosphate synthetase activity were increased. Aestivating Xenopus laevis were found by Balinsky (1970) to have increased levels of ornithine-urea cycle enzymes. Finally, Baze (1970) observed that the urea cycle enzymes of turtles increased when they were dehydrated. Thus, it may prove fruitful to investigate the effect of controlled dehydration on amphibian arginase activity.

An area that may yield insight into the mechanism of possible enzyme changes in response to dehydration is the endocrine system. Somero (1971) suggests that environmental changes that produce changes in the enzyme systems of an organism may be mediated by

the organism's endocrine system. With regard to urea cycle enzymes, Knox (1964) states that both adrenalectomy and hypophysectomy decreased rat liver arginase activities. Since antidiuretic hormone plays an important role in amphibian water balance physiology (Jorgensen, 1950 and Steggerda, 1937), it would seem that one should investigate the possible direct or indirect effects of this hormone on arginase activity.

Materials and Methods

The dehydration experiments were of two kinds. Comparative studies on the effect of controlled desiccation were performed on some of the species mentioned in the natural variation section. The effect of dehydration on the kinetic properties of liver and kidney arginase in R. pipiens was also investigated. Experiments that investigated the effect of antidiuretic hormone on liver and kidney arginase were performed on R. clamitans, R. septentrionalis, and B. americanus.

Comparative Dehydration Studies

The following species were used in dehydration experiments: R. septentrionalis, R. clamitans, R. pipiens, R. sylvatica, and B. americanus. The collection of these animals is discussed in the materials and methods part of the section on natural variations. Animals were placed individually in finger bowls containing a "Kimwipes" towel and a plastic capsule of Drierite ($CaSO_4$, which acted as a desiccant). It was felt that this gentle air desiccation would approximate natural desiccation during times of drought. Animals were randomly sacrificed at approximately 24 hour intervals

and the livers and kidneys removed and placed in amphibian Ringer solution and frozen until they could be assayed for arginase activity. The assay procedure was discussed in the technique section. Per cent body weight lost during desiccation was determined in order to estimate the degree of dehydration; however, it was found that changes in enzyme activity were correlated much more with length of time desiccated.

Kinetic Studies

R. pipiens were collected from breeding ponds in Kandiyohi County and Ottertail County, Minn. The animals were returned to the laboratory and 10-15 animals (hydrated animals) were immediately sacrificed and the livers and kidneys placed in amphibian Ringer solution and frozen. Other animals (10-15) were placed in large glass containers on a thin layer of sand. After 100 (Kandiyohi sample) and 168 (Ottertail sample) hours of dehydration the animals were sacrificed and the livers and kidneys removed and placed in amphibian Ringer solution and frozen. Kinetic data were obtained, as discussed in the technique section, on samples of pooled liver and pooled kidney.

Hormone Experiments

The effect of Pitressin (vasopressin, supplied by Parke, Davis Co.) on kidney and liver arginase activities in R. septentrionalis, R. clamitans and B. americanus was investigated. The collection procedure is described in the natural variation section. Three control animals and three experimental animals of each species were used. Pitressin (1 unit/100 grams) was injected into the dorsal

lymph sac of experimental animals. The animals were then placed in finger bowls on wet paper towels for three hours and then sacrificed and livers and kidneys removed and frozen in amphibian Ringer solution (the technique for hormone injection is discussed by Sawyer, 1950). Assays were performed as described in the technique section.

Results

The results from the comparative experiments on dehydration are given in Tables 4 and 5. The enzyme activities of all desiccated animals were pooled and the mean for the activity values for each group calculated. Student's t statistic was calculated in order to compare the mean activities of hydrated animals with desiccated animals for each species studied. Fig. 6 shows the change in both liver and kidney arginase activities as the length of desiccation time increases for R. sylvatica.

The data from the kinetic studies are presented in Tables 6 and 7. Table 6 gives the Michaelis constant (in mM), maximum velocity (in mM urea/minute), and specific activities (expressed as units/mg tissue and units/mg protein) of pooled kidneys from 10-15 R. pipiens from the Kandiyohi Co. sample and 10-15 animals from the Ottertail Co. sample. The values of these same kinetic properties are presented in Table 7 for liver arginase.

Finally, the results from the hormone experiments are summarized in Table 8. The mean values for liver arginase activities from control and experimental animals are expressed in this table.

Discussion

A perusal of the data in Tables 4 and 5 indicates that

Table 4. Kidney Arginase Activity (1 Unit = 1 mM urea/15 min./1 mg tissue).

	Hydrated			Desiccated			
Species	Mean	Num.	S. E.	Mean	Num.	S. E.	$\bar{X}_{Des.} - \bar{X}_{Hydr.}$
Ba	1.7	6	0.4	5.7	6	1.0	4.0*
Rsyl	4.0	3	1.5	50.7	5	15.2	46.7*
Rpip	8.2	4	2.4	27.2	4	8.4	19.5#
Rclam	22.8	4	1.7	37.2	11	9.0	7.2
Rsep	16.3	6	3.2	17.0	6	9.9	0.7

(Species abbreviations are the same as in Fig. 4)

* = significant at the 95% confidence level

= significant at the 90% confidence level

Table 5. Liver Arginase Activity (1 Unit = 1 mM urea/15 min./1 mg tissue).

	Hydrated			Desiccated			
Species	Mean	Num.	S. E.	Mean	Num.	S. E.	$\bar{X}_{Des.} - \bar{X}_{Hydr.}$
Ba	12.4	4	3.1	9.2	6	2.9	-3.2
Rsyl	6.1	3	3.0	18.9	5	2.6	12.8*
Rpip	5.8	6	0.8	4.8	5	0.6	-1.0
Rclam	5.2	4	1.7	6.7	13	1.7	1.5
Rsep	6.3	6	1.6	4.9	6	1.7	-1.4

(Species abbreviations are the same as in Fig. 4)

* = significant at the 95% confidence level

Fig. 6. Response of R. sylvatica kidney and liver arginase activity to controlled desiccation. The open circles represent values for kidney arginase activity and the closed circles represent values for liver arginase activity.

FIGURE 6

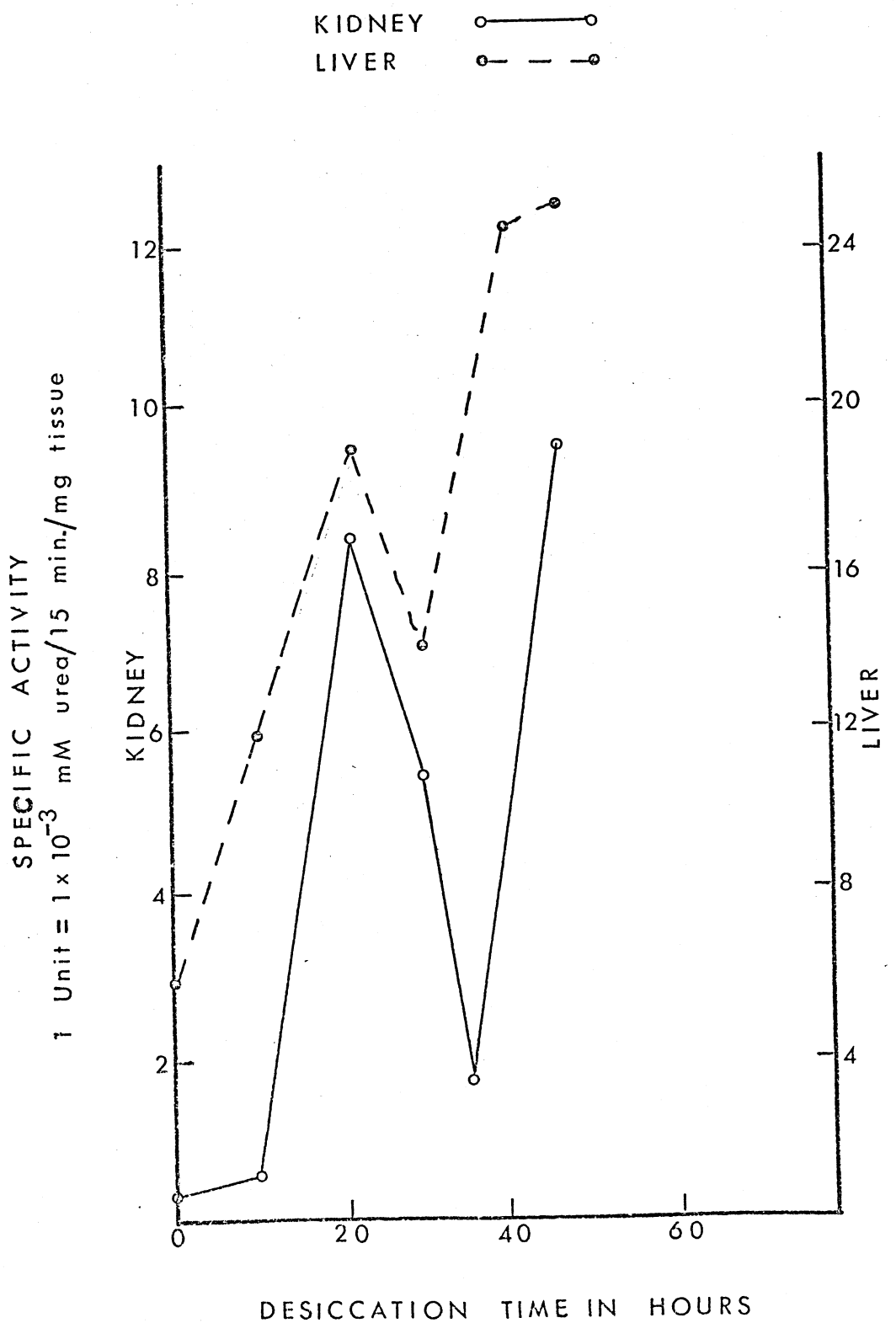

KIDNEY
LIVER
SPECIFIC ACTIVITY
1 Unit = 1 x 10⁻³ mM urea/15 min./mg tissue
KIDNEY
LIVER
12
10
8
6
4
2
24
20
16
12
8
4
0
20
40
60
DESICCATION TIME IN HOURS

Table 6. The effect of dehydration on the kinetic properties and activity of R. pipiens kidney arginase.

	Kandiyohi County Sample	
	Hydrated	Desiccated 100 Hours
Km:	5.9 mM	9.3 mM
Vmax:	5.1×10^{-4} mM urea/ 1 minute	8.3×10^{-4} mM urea/ 1 minute
Sp. Act.:	2.9×10^{-4} mM urea/ 1 min./1 mg prot.	2.8×10^{-4} mM urea/ 1 min./1 mg prot.
	9.1×10^{-6} mM urea/ 1 min./1 mg tissue	1.3×10^{-5} mM urea/ 1 min./1 mg tissue

	Ottertail County Sample	
	Hydrated	Desiccated 168 Hours
Km:	9.7 mM	9.9 mM
Vmax:	6.5×10^{-4} mM urea/ 1 minute	7.8×10^{-4} mM urea/ 1 minute
Sp. Act.:	1.7×10^{-4} mM urea/ 1 min./1 mg prot.	2.3×10^{-4} mM urea/ 1 min./1 mg prot.
	8.2×10^{-6} mM urea/ 1 min./1 mg tissue	1.1×10^{-5} mM urea/ 1 min./1 mg tissue

Table 7. The effect of dehydration on the kinetic properties and activity of R. pipiens liver arginase.

	Kandiyohi County Sample	
	Hydrated	Desiccated 100 Hours
Km:	4.0 mM	4.3 mM
Vmax:	7.5×10^{-4} mM urea/ 1 minute	9.0×10^{-4} mM urea/ 1 minute
Sp. Act.:	2.2×10^{-4} mM urea/ 1 min./1 mg prot.	2.6×10^{-4} mM urea/ 1 min./1 mg prot.
	2.3×10^{-5} mM urea/ 1 min./1 mg tissue	2.5×10^{-5} mM urea/ 1 min./1 mg tissue

	Ottertail County Sample	
	Hydrated	Desiccated 168 Hours
Km:	5.8 mM	6.3 mM
Vmax:	6.1×10^{-4} mM urea/ 1 minute	7.3×10^{-4} mM urea/ 1 minute
Sp. Act.:	2.9×10^{-4} mM urea/ 1 min./1 mg prot.	2.3×10^{-4} mM urea/ 1 min./1 mg prot.
	2.0×10^{-5} mM urea/ 1 min./1 mg tissue	2.3×10^{-5} mM urea/ 1 min./1 mg tissue

Table 8. The effect of antidiuretic hormone on amphibian kidney and liver arginase activity.

Kidney Arginase Activity

1 Unit = 1 x 10^{-4} mM urea/15 min./1 mg tissue

Species	Control	Experimental (1 U./100 g)
B. americanus	3.0 ± 2.3, n = 7	2.5 ± 0.3, n = 2
R. septentrionalis	16.0 ± 10.0, n = 6	12.5 ± 1.5, n = 2

Liver Arginase Activity

1 Unit = 1 x 10^{-3} mM urea/15 min./1 mg tissue

Species	Control	Experimental (1 U./100 g)
R. septentrionalis	5.0 ± 2.5, n = 5	1.5 ± 0.4, n = 2
R. clamitans	12.2 ± 4.9, n = 2	11.5 ± 1.5, n = 2

dehydration results in a significant increase in arginase activity in some species. R. sylvatica shows a significant increase in both kidney and liver arginase at the 95% confidence level when dehydrated. There are no other significant differences between the means of liver arginase activities when hydrated animals are compared with dehydrated animals. Table 4, however, shows that the kidney arginase of dehydrated B. americanus is significantly higher than hydrated animals at the 95% confidence level. R. pipiens also shows an increase in kidney arginase but this difference is significant only at the 90% confidence level. Admittedly these sample sizes are not large and the fact diet affects liver arginase activity may be reasons why significant increases in enzyme activity with dehydration were not observed in other species. Also, values for all animals that were desiccated were used in arriving at a mean value for desiccated animals. The time factor (some animals were desiccated for only a short period of time) may result in lower values for the means than if all animals were desiccated for a long period of time before livers and kidneys were assayed. Since R. sylvatica was the only species which exhibited a significant increase in both liver and kidney arginase, a graph (Fig. 6) of enzyme activity vs. the length of dehydration time is included. This time factor does not enter into the data in Tables 6 and 7, for all animals were desiccated for the same length of time in these experiments.

Further evidence that dehydration causes an increase in arginase activity is expressed in Tables 6 and 7. In every case the maximum velocity of arginase is greater in desiccated animals

than in hydrated animals. Specific activities also indicate that dehydrated animals have higher arginase activities. If specific activity is expressed as units/mg tissue, the values are higher from dehydrated animals in every case. The reason specific activities, when expressed as units/mg prot., from dehydrated animals are not higher than specific activities from hydrated animals in every case may be due to the fact crude tissue extracts were used. There may have been other proteins in the supernatant which interfered with the determination of enzyme concentration.

It appears, then, that amphibians may be able to increase both liver and kidney arginase when faced with dehydration stress. The possible adaptive value of this ability must now be discussed. There may be two major reasons (one involving increases in liver arginase, the other involving increases in kidney arginase) why an increase in arginase activity may be adaptively valuable to amphibians in times of dehydration stress. First, increased liver arginase activity along with an increase in the other enzymes of the ornithine-urea cycle will result in an increased production of urea. This increase may be beneficial to amphibians because an increased concentration of urea circulating in the plasma may act as a buffer against osmotic stress (Brown, 1970). Several authors have reported increases in body urea concentrations during dehydration. Dantzler (1966) reported a slight increase in plasma osmoconcentration when fresh-water turtles were desiccated. Increased levels of plasma urea were noted by McClanahan in spadefoot toads undergoing dehydration. Gordon (1961) reports that the crab-eating frog, R. cancrivora, maintains elevated plasma concentrations of urea so that

it can inhabit environments of high salinity. Increased plasma levels of urea were observed in aestivating Xenopus laevis (Balinsky, 1970) and X. laevis exposed to hyperosmotic solutions (McBean, 1967). Finally, the levels of plasma urea increased when B. viridis (Tercafs, 1962), R. pipiens (Scheer, 1962), and R. temporaria and R. esculenta (Ackrill, 1969) were stressed osmotically.

Another reason that there may be adaptive value in regulating liver arginase activity concerns the fact that the kind of nitrogenous waste excreted by an amphibian is correlated to its environment (Cragg, 1961 and Schmid, 1968). Ammonia is highly toxic, and therefore a large amount of water is necessary for excretion of ammonia. Excretion of ammonia is no problem for aquatic amphibians, which have an unlimited supply of water, but terrestrial amphibians do not have readily accessible water supplies and must therefore convert ammonia to urea, which is much less toxic and can therefore be excreted without the demand for abundant water. However, as Balinsky (1970) points out, the production of urea is metabolically expensive since three molecules of ATP are necessary for every molecule of urea produced. Therefore, it would seem highly adaptive to convert less ammonia to urea (low ornithine-urea cycle enzyme activities) when water is available and thus conserve energy. However, during times of dehydration the ability to convert more ammonia to urea (by increasing the activity of ornithine-urea cycle enzymes) would be beneficial for osmotic reasons.

The second reason there may be adaptive value in being able to increase arginase activity during dehydration stress involves

the fact kidney arginase may be involved in the active transport of urea. Schmidt-Nielsen (1954) points out that during dehydration the glomerular filtration rate in the kidneys of amphibians decreases. Because of this decrease in glomerular filtration rate, the excretion of urea will also decrease. An increase in kidney arginase activity may mediate an increase in the active transport of urea and thus compensate for the decrease in urea excretion due to a lowered glomerular filtration rate. It is interesting to note that Table 4 shows that the terrestrial species (R. sylvatica and B. americanus) exhibit increases in kidney arginase activity during dehydration while the aquatic species (R. septentrionalis and R. clamitans) do not exhibit increases. One would expect that terrestrial species would be much more apt to be exposed to environmental situations that would produce dehydration than aquatic species. It has already been mentioned that Carlisky, et al. (1968c) found that terrestrial species of amphibians did not actively transport urea. However, he did not demonstrate that terrestrial species do not have the ability to actively transport urea under any circumstances. Since arginase is present in the kidneys of terrestrial species, they may have the ability to transport urea actively but use it only when it would be beneficial, such as periods of drought. A third possibility, the involvement of arginase in recovery of bladder water, is discussed in the summary.

It has been suggested that there may be adaptive value in the ability to regulate ornithine-urea cycle enzymes during dehydration. Data have been presented that tend to indicate that amphibians

have the ability to increase the level of arginase activity during dehydration. Comment must now be made on the possibility of a hormonal mechanism of control of this regulatory ability. It was felt that antidiuretic hormone (ADH) may be the means of causing an increase in arginase activity for two reasons. First, it has already been stated that ADH is involved in an amphibian's physiological response to dehydration (ADH increases the permeability of bladder membrane and skin to water). Second, Levinsky (1953) reported that ADH is released by the pituitary in response to dehydration. One could propose, then, that dehydration causes a release of ADH, which then mediates an increase in liver and kidney arginase activities. However, Table 8 shows clearly that in the case of the three species studied (both aquatic, R. septentrionalis and R. clamitans, and terrestrial, B. americanus) ADH did not produce an increase in either kidney or liver arginase activity. This finding does not prove that ADH may not be involved in regulating enzyme activity. ADH may work in conjunction with another hormone (aldosterone for example) or the regulatory mechanism may be highly complex and would therefore not be initiated in a rather simple experiment where only ADH is injected into an animal.

Temperature

Introduction

Variations of amphibian liver and kidney arginase that are responses to water availability have been investigated in the first part of this study. The possible adaptive value of these enzyme variations has been discussed in the preceding sections of this paper. Another environmental factor that may cause amphibians to exhibit biochemical adaptations is temperature. Minnesota has widely different seasonal temperatures. Poikilothermic animals such as amphibians, therefore, will have internal temperatures that change from season to season. Because the rates of chemical reactions are temperature dependent it would seem that metabolic reactions of a poikilotherm would have different rates as the environmental (body) temperature changed. However, Somero (1969a) has pointed out that poikilotherms exhibit similar rates of metabolic reactions at different temperatures. It would seem desirable, then, to investigate the possibility of enzymic adaptations in amphibians from Minnesota. In this way one could discover possible metabolic shifts due to seasonal temperature changes, Somero's (1971) second category of metabolic adaptations.

Materials and Methods

Studies on the effect of temperature on kidney and liver arginase were performed on Rana catesbeiana Catesby, the bullfrog, supplied by J. R. Schettle Biologicals, Inc. (Stillwater, Minn.); purified bovine arginase (obtained from Sigma Chemical Co., St.

Louis); and R. pipiens, collected in Kandiyohi County, Minn.

The experiments performed on R. catesbeiana were the only experiments in this study that were performed on animals that were supplied by a biological supply house. In this experiment one animal was held in water at a temperature of 7° C. for 14 days while another animal was maintained in water at 25° C. After the 14 days both animals were sacrificed and the livers and kidneys removed and placed in amphibian Ringer solution and frozen. Kinetic data were obtained as discussed in the technique section. However, besides incubation at 25° C., crushed ice was added to the "Gyrotory Shaker" so that incubation could also be performed at 2 $\pm$ 1° C.

The purified bovine arginase was mixed with water to yield a solution with a concentration of 1.0 mg/ml. Different amounts of 0.85 M arginine solution and 1.0 mM $MnSO_4$ solution were mixed (the total volume of the mixture was 47.0 ml) to yield solutions with different substrate (arginine) concentrations. These solutions were placed in 125 ml Erlenmeyer flasks. Three ml of the bovine arginase solution (1 mg/ml) were added to the Erlenmeyer flasks yielding a total volume of 50 ml in each flask. Incubation was accomplished, as discussed in the technique section, at 2 $\pm$ 1° C. and at 25 $\pm$ 1° C.

Dr. Robert G. McKinnell informed the author that R. pipiens could be collected throughout the winter at the bottoms of streams, in Kandiyohi County, which were freely flowing for the duration of the winter. Apparently the animals overwinter by congregating under rocks and in the gravel at the bottoms of these streams in

Kandiyohi County. Because of this ability to collect animals in the winter, arginase could be monitored in freshly collected animals throughout the winter, spring and summer seasons. Animals were transported to the laboratory and kept on ice overnight till they could be sacrificed. Livers and kidneys were removed and pooled from 10-15 animals and placed in amphibian Ringer solution and frozen until assays could be performed. Kinetic data were obtained on these pooled samples as discussed in the technique section. Again incubation was performed at 25 $\pm$ 1° C. and at 20 $\pm$ 1° C.

In all cases the parameters estimated were Km, Vmax and specific activity. Km and Vmax were estimated as discussed in the technique section. Specific activities were determined from the flasks with a substrate concentration of 51 mM. Km is expressed in mM, Vmax in mM urea produced/minute, and specific activity as mM urea produced/minute/mg protein or mg tissue.

Results

The data on *R. pipiens* liver and kidney arginase are expressed in Tables 9-12. Table 9 gives the kinetic parameters (Km and Vmax) and the specific activity of kidney arginase from frogs collected during different months of the year. In the experiments performed to obtain the data in Table 9 the incubation temperature was 2 $\pm$ 1° C. Table 10 presents the same data on kidney arginase, however here the incubation was accomplished at 25 $\pm$ 1° C. Tables 11 and 12 give the same parameters (Km, Vmax and specific activity) for liver arginase. In Table 11 incubation was performed at 2 $\pm$ 1° C. and in Table 12 incubation was performed at 25 $\pm$ 1° C.

The data obtained from the experiments on R. catesbeiana are expressed in Tables 13 and 14. In Table 13 the parameters (Km, Vmax and specific activity) for R. catesbeiana kidney arginase are given both for the frog maintained at 7° C. and at 25° C. Data for both incubation temperatures (2 ± 1° C. and 25 ± 1° C.) are also given in Table 13. The same format is used in Table 14 except that these values are for R. catesbeiana liver arginase.

The parameters Km, Vmax and specific activity for bovine arginase are presented in Table 15. These three parameters were also determined at both incubation temperatures, 2 ± 1° C. and 25 ± 1° C.

Discussion

A perusal of the data on the effect of temperature on the kinetic parameters of amphibian arginase indicates both immediate and seasonal changes in the Km with temperature. Tables 9 and 10 indicate that the Km values for kidney arginase increase as the environmental temperature decreases (due to change in season). For example, Table 9 (incubation at 2° C.) indicates that winter samples of kidney arginase have Km values less than 4.0 mM (in February and March the Km is much less than 3.0 mM) whereas in the spring samples the Km increases to almost 4.5 mM (4.3 mM in April and 4.5 mM in May). The Km from the June sample (5.4 mM) is almost double the values obtained in the winter animals. Similarly, Table 10 (where the incubation was performed at 25° C.) also indicates a greater Km in summer animals than winter animals. The December-March samples are all below 6.6 mM, however after April the samples show a Km for the enzyme greater than 8.0 mM.

Table 9. Seasonal change in the kinetic parameters of R. pipiens kidney arginase. Incubation performed at 2° C. ± 1° C.

Date Collected	Km	Vmax	Specific Activity
December	3.5 mM	0.73×10^{-4} mM/min.	1.1×10^{-6} mM/min./mg tissue
February	2.3 "	1.0 "	1.8 "
March	2.1 "	0.74 "	1.7 "
April	4.3 "	2.2 "	2.8 "
May	4.5 "	1.3 "	2.0 "
June	5.4 "	2.1 "	2.9 "

Table 10. Seasonal change in the kinetic parameters of R. pipiens kidney arginase. Incubation performed at 25° C. ± 1° C.

Date Collected	Km	Vmax	Specific Activity
December	4.6 mM	3.1×10^{-4} mM/min.	4.4×10^{-6} mM/min./mg tissue
February	6.6 "	7.2 "	13.0 "
March	6.1 "	3.0 "	7.1 "
April	9.2 "	8.4 "	10.0 "
May	8.9 "	6.0 "	8.7 "
June	8.0 "	5.0 "	7.8 "
July	18.5 "	4.3 "	11.0 "

Table 11. Seasonal change in the kinetic parameters of R. pipiens liver arginase. Incubation performed at 2° C. ± 1° C.

Date Collected	Km	Vmax	Specific Activity
December	2.5 mM	3.7×10^{-4} mM/min.	5.2×10^{-6} mM/min./mg tissue
February	2.5 "	2.8 "	5.4 "
March	2.0 "	1.7 "	5.1 "
April	2.7 "	1.3 "	5.5 "
May	2.4 "	1.7 "	5.0 "
June	3.7 "	1.8 "	3.3 "

Table 12. Seasonal change in the kinetic parameters of R. pipiens liver arginase. Incubation performed at 25° C. ± 1° C.

Date Collected	Km	Vmax	Specific Activity
December	4.8 mM	7.0×10^{-4} mM/min.	12.0×10^{-6} mM/min./mg tissue
February	4.5 "	8.7 "	14.0 "
March	4.6 "	5.6 "	22.0 "
April	4.8 "	3.7 "	14.0 "
May	4.8 "	6.8 "	22.0 "
June	7.0 "	8.0 "	13.0 "
July	8.0 "	12.0 "	17.0 "

Table 13. The effect of temperature on the kinetic parameters of *R. catesbeiana* kidney arginase.

		Incubation Temperature	
		2° C. $\pm$ 1° C.	25° C. $\pm$ 1° C.
Frog	Km:	2.8 mM	3.3 mM
Maintained	Vmax:	2.5 mM/min.	8.0 mM/min.
at 7° C.	Sp. Act.:	0.53×10^{-5} mM/min./mg tissue	1.9×10^{-6} mM/min./mg tissue
Frog	Km:	2.7 mM	7.5 mM
Maintained	Vmax:	2.5 mM/min.	8.1 mM/min.
at 25° C.	Sp. Act.:	0.43×10^{-6} mM/min./mg tissue	1.9×10^{-6} mM/min./mg tissue

Table 14. The effect of temperature on the kinetic parameters of *R. catesbeiana* liver arginase.

		Incubation Temperature	
		2° C. $\pm$ 1° C.	25° C. $\pm$ 1° C.
Frog	Km:	1.5 mM	4.2 mM
Maintained	Vmax:	6.6 mM/min.	15 mM/min.
at 7° C.	Sp. Act.:	1.3×10^{-6} mM/min./mg tissue	5.5×10^{-6} mM/min./mg tissue
Frog	Km:	3.4 mM	5.0 mM
Maintained	Vmax:	15 mM/min.	84 mM/min.
at 25° C.	Sp. Act.:	2.8×10^{-6} mM/min./mg tissue	15×10^{-6} mM/min./mg tissue

Table 15. The effect of temperature on the kinetic parameters of bovine arginase.

	Incubation Temperature	
	2° C. $\pm$ 1° C.	25° C. $\pm$ 1° C.
Km:	6.4 mM	6.7 mM
Vmax:	3.1×10^{-5} mM/min.	2.5×10^{-4} mM/min.
Specific Act.:	1.0×10^{-5} mM/min./ mg protein	7.7×10^{-5} mM/min./ mg protein

Tables 11 and 12 also show an increase in liver arginase Km with change in season (winter to summer). However, the Km for liver arginase does not increase until June. Table 11 (incubation at 2° C.) indicates that in the December-May samples the Km ranged from 2.2-2.8 mM while the June sample had a Km of 3.7 mM. When the assays were performed at 25° C. the Km values for the December-May samples ranged from 4.5-4.8 mM while the June and July samples had Km values of 7.0 and 8.0 mM respectively. There is also a difference in the Km obtained from a sample incubated at 25° C. and the Km from a sample incubated at 2° C. Both kidney and liver arginase show a lower Km when incubated at 2° C. regardless of the season in which the animals were collected.

It is evident that animals collected during the winter months exhibit a lower Km for both liver and kidney arginase. One could inquire, then, if it is possible to produce a lower Km experimentally by exposing amphibians to decreased temperatures in the laboratory. Amphibians were exposed to decreased temperatures in the experiment with R. catesbeiana. Table 13 indicates that exposing R. catesbeiana to 7° C. for 14 days resulted in a decrease in Km (the Km for the frog maintained at 7° C. was 3.3 mM while the Km for the frog maintained at 25° C. was 7.5 mM, when the incubation was performed at 25° C.). Incubation at 2° C. did not result in a decrease in kidney arginase Km when the frog maintained at 7° C. was compared with the frog maintained at 25° C. (the 7° C. animal had a Km of 2.8 mM while the 25° C. animal had a Km of 2.7 mM). Since only one animal was held at 7° C., experimental error in the assay procedure may have resulted in a higher Km for the determination at the incubation

temperature of 2° C. The values for R. catesbeiana liver arginase presented in Table 14, also indicate that maintaining the animal at 7° C. for 14 days resulted in a lowered Km for liver arginase. In this case the liver arginase Km was found to be lower in the frog maintained at 7° C., when incubation was performed both at 2° C. and at 25° C., than in the frog maintained at 25° C. One also observes a decrease in Km when incubation is performed at 2° C. as opposed to incubation at 25° C. in the frog maintained at 25° C. and also in the frog maintained at 7° C. Admittedly the fact only two animals were used in this experiment (and that these animals were supplied by a biological supply house) would suggest one should be cautious in coming to any conclusions about the results. However, it does appear that exposing R. catesbeiana to a lower temperature results in a change (decrease) in the Km of both kidney and liver arginase.

It has been suggested that as the environmental temperature decreases the Km of amphibian liver and kidney arginase also decreases. Somero (1969a) has suggested that there may be adaptive value in this decrease in the Km of an enzyme in poikilotherms in response to lowered temperatures. Since the Km is defined as the substrate concentration at half of the maximum velocity of the enzyme, a lowered Km would result in an increased enzyme-substrate affinity. This would help to alleviate the problem of decreased rates of enzyme catalyzed reactions due to the decrease in environmental (body) temperature that poikilotherms face during the winter. This phenomenon, a lowered Km as the temperature decreases, has been reported by several investigators. Fructose diphosphatase

(Behrisch, 1969) and lactate dehydrogenase (Hochachka, 1968) were analyzed in migrating salmon. Both investigators found that as the salmon encountered lowered environmental temperatures during migration, the Km values for each enzyme decreased. Both lactate dehydrogenase (Somero and Hochachka, 1969) and pyruvate kinase (Somero, 1969b) in the Alaskan King Crab were found to exist in two forms. The two forms of each enzyme differed in Km (a high Km form and a low Km form) and a decrease in Km with lowered temperature was observed in both forms of each enzyme. The flippers of the harbor seal (Phoca vitulina), a homeotherm, were found to be heterothermic. Somero (1970) observed the Km's of lactate dehydrogenase and pyruvate kinase in this extremity and found that as temperature decreased the Km's of both of these enzymes also decreased. Hebb (1969) observed the Km of brain choline acetyl transferase from the goldfish (Carassius auratus) at different temperatures and found that as the temperature decreased the Km for this enzyme also decreased. Finally, Greene (1970) reported that the Km of muscle glyceraldehyde-3-phosphate dehydrogenase in the cold-adapted Antarctic fish (Dissostichus mawsoni) was lower than the Km for the same enzyme from the rabbit.

In the case of amphibian arginase, Forster (1970) has proposed that there may be possible adaptive value in the area of water balance due to the fact arginase Km varies as the temperature changes. Schmidt-Nielsen (1954) found that at lower temperatures both the glomerular filtration rate and the rate of urine flow are markedly decreased but that active transport of urea is maintained in R. clamitans. Forster (1970) has proposed that during the winter frogs compensate for this lowered rate of excretion by

actively transporting urea. If the mechanism of the active transport process is a chemical reaction catalyzed by arginase, lowered temperatures would decrease the rate of the transport process. A lowered arginase Km would compensate for the decrease in reaction rate by producing an increase in enzyme-substrate affinity. Along with this compensation with respect to urea excretion there may be adaptive value in the area of water metabolism. Frogs overwinter in water and are therefore exposed to hydration stress. It has already been proposed that aquatic frogs respond to hydration stress by actively transporting urea from the plasma to the urine, thereby creating an osmotic gradient that is favorable for water flux from plasma to urine. R. pipiens overwintering at the bottom of streams and lakes are exposed to hydration stress. The low temperatures would cause a decrease in the rate of active transport of urea. Again, a lowered kidney arginase Km would compensate for this decreased reaction rate by increasing enzyme-substrate affinity.

It is interesting to note that a definite increase in kidney arginase Km is observed in the samples collected after March. For example, Table 9 shows that the Km's of the December, February and March samples are under 4.0 mM and the Km's of the April, May and June samples are greater than 4.0 mM for kidney arginase. The frogs collected in December, February and March were found in water that had a temperature of 1^{o} C. In April the ice was breaking up in the lakes and frogs were found migrating to breeding ponds. The water in these shallow breeding ponds was found to have temperatures above 10^{o} C. In May frogs could still be found in breeding ponds, which now had temperatures above 20^{o} C. The June

frogs were found foraging for food in grass not far from ponds of water. It seems apparent that as the environmental temperature increases the Km of kidney arginase also increases. Also, during December-March the animals are exposed to hydration stress, while after March the animals are not constantly in the water and therefore are exposed to less of a hydration stress. A lowered kidney arginase Km would, therefore, be highly adaptive during the winter months when the animal is exposed to both hydration stress and lowered temperatures. During the spring and into the summer as the temperature increases and the amount of time spent in contact with water decreases (see Merrell, 1970, for a discussion of migration of R. pipiens), the adaptive value of a low kidney arginase Km would not be so great.

It has been stated that the data (Tables 11 and 12) for R. pipiens liver arginase also show a change in Km with a change in temperature. However, the liver arginase Km is lower in both winter and spring (samples in December through May) than it is in the summer (samples in June and July). It has been proposed that kidney arginase Km increases after March because at this time the environmental temperature increases somewhat and the hydration stress decreases. Liver arginase is not involved in the frog's water balance physiology as much as kidney arginase. However, liver arginase is essential in protein catabolism. During the winter the frogs do not feed (stomachs of frogs collected during the winter were found to be empty). These fasting frogs would probably have to metabolize a fair amount of body protein to maintain bodily functions. Arginase would be essential in order to convert the ammonia produced from the deamination of amino acids

into urea. Lowered environmental temperatures will cause a decrease in the rates of the enzyme catalyzed reactions involved in the metabolism of protein. A decrease in the Km of liver arginase would be advantageous in compensating for the decrease in reaction rate. In the spring (April and May) the temperatures have increased, however the frogs are still fasting (stomachs were empty). The demand for energy is great because the frogs are expending energy in migrating to breeding ponds. A decreased liver arginase Km would help insure an energy supply from catabolism of protein. In June the frogs begin foraging for food (all frogs dissected in the June sample had stomachs full of food). Because of the higher temperatures and the fact the source of metabolic energy now probably comes from insects provided by eating, the adaptive value for a decreased liver arginase Km would be much less. Thus, it would seem beneficial for R. pipiens to have a lower liver arginase Km while fasting (December-May) but not during the summer when they are actively feeding.

It has been suggested that winter R. pipiens have a lower kidney and liver arginase Km than summer R. pipiens. A lower Km was suggested to be adaptively valuable to the animals for reasons of water balance (kidney arginase) and for reasons of diet (liver arginase). The results from the experiment with R. catesbeiana tended to indicate that exposure to lowered temperatures would result in a lowered Km value for both liver and kidney arginase. It seems possible that decreases in environmental temperatures during the fall result in a seasonal shift in the Km of amphibian arginase (the opposite environmental temperature change in the

spring results in an increase in the Km of arginase). Besides this seasonal shift in the Km of arginase, the incubation temperature seemed to affect Km in amphibians. Tables 9 through 12 show that incubation of both liver and kidney arginase at 2° C. resulted in a lower Km than incubation at 25° C. However, Table 15 shows that bovine arginase does not exhibit a lowered Km when incubated at 2° C. as opposed to incubation at 25° C. Since amphibians are poikilotherms, the reaction catalyzed by arginase takes place at about 2° C. during the winter (the environmental temperature to which the animals are exposed). An immediate change in the enzyme (due to temperature) that results in a lower Km would be beneficial to the organism in that daily fluctuations in environmental temperature could be partially compensated for. Since cattle are homeotherms, bovine arginase will always catalyze reactions at approximately the same temperature. Thus, there would be little benefit in an immediate shift in Km with temperature.

Probenecid

Introduction

Throughout this discussion it has been stated that amphibian kidney arginase may be involved in the active transport of urea. Although this has been implied in the literature by Schmidt-Nielsen (1963) and Forster (1970), it has never been conclusively demonstrated. All of the evidence suggesting that renal arginase is involved in the active transport of urea has been indirect. In the past the evidence presented has been to show that the activity of renal arginase is higher in those species which actively transport urea as opposed to those species which do not actively transport urea (Robinson, 1963, Carlisky, 1970, and Carlisky, et al., 1967, 1968a, 1968c). The data I have presented in the section on natural variations in arginase activity in Minnesota amphibians add more weight to the evidence previously presented in that aquatic species (these species may actively transport urea) have higher levels of kidney arginase activity than terrestrial species, (which probably do not normally actively transport urea). More direct evidence that arginase is involved in the active transport of urea would certainly be necessary if this problem is to be solved.

It would seem that if one could demonstrate inhibition of arginase <u>in vitro</u> by a compound that inhibits active urea transport <u>in vivo</u>, one could be more confident in stating that arginase is involved in active transport of urea. Such an inhibitor may be p-(dipropylsulfanyl)-benzoic acid, probenecid:

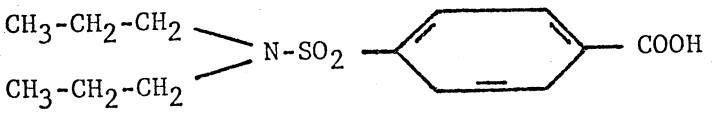


Fig. 7. Probenecid

Beyer (1951) observed the effect of probenecid on renal transport of several substances in the dog. It was found that probenecid reversibly and selectively inhibited the transport mechanism for the tubular secretion of the penicillins, p-aminohippurate and phenol red. The inhibition of active urea transport by probenecid was demonstrated by Forster (1954). It may prove fruitful, then, to investigate the effect of probenecid on amphibian arginase in vitro.

Materials and Methods

Probenecid was supplied by Merck, Sharp and Dohme Inc., West Point, Penna. Beyer (1951) reported that although probenecid is highly insoluble in water, the sodium salt of this compound is soluble in water. If probenecid is mixed with an equivalent amount of sodium bicarbonate or sodium hydroxide the compound will dissolve. At first a solution of probenecid was prepared using sodium hydroxide; however, it was found that the OH^- ion reacted with the Mn^{++} ion in the incubation mixture to form an insoluble precipitate, $Mn(OH)_2$. Therefore the probenecid solution (img/ml) used in this experiment was prepared by using sodium bicarbonate.

R. pipiens collected during late spring in Scott County, Minnesota by Dr. Robert G. McKinnell were held in an aquarium at 25° C. for approximately 2 weeks. The animals were not fed during this time. Approximately 5 animals were killed on the day of the

experiment and their livers and kidneys were removed and pooled. Homogenation, as discussed in the technique section, was performed immediately. Three 125 ml Erlenmeyer flasks were used for kidney arginase and three flasks for liver arginase determinations. Each flask contained 51 mM of arginine, 1 ml of the supernatant of the particular tissue being analyzed and varying amounts of 1.0 mM $MnSO_4$ and the 1 mg/ml probenecid solution (the total volume of the $MnSO_4$-probenecid mixture added to each flask was 46 ml). There was a total volume of 50 ml in each flask. The three flasks contained varying amounts of probenecid (0 mg, 10 mg, and 20 mg). Incubation was performed for 7 minutes at 25° C. in the "Gyrotory Shaker." After 7 minutes 2 ml aliquots were withdrawn from each flask and added to test tubes containing 2 ml of acetic acid. Urea analyses were accomplished as discussed in the technique section.

The effect of probenecid on bovine arginase (Sigma Chemical Co., St. Louis) was also investigated. Bovine arginase was dissolved in water to yield a 1.0 mg/ml solution. Four 125 ml Erlenmeyer flasks were used in this experiment. Each flask contained 51 mM arginine, 3 mg bovine arginase and a mixture of 1.0 mM $MnSO_4$ and 1 mg/ml probenecid solution (the total volume of the mixture was 44 ml). The total volume in each flask was 50 ml. The probenecid concentrations of each flask varied as follows: 0 mg, 10 mg, 20 mg, and 30 mg. Incubation was accomplished for 15 minutes at 25° C. in the "Gyrotory Shaker." After 15 minutes 2 ml aliquots were withdrawn from each flask and transferred to test tubes containing 2 ml of acetic acid. Urea determinations were accomplished as discussed in the technique section.

Specific activities of amphibian kidney and liver arginase and bovine arginase in the presence of varying amounts of probenecid were determined. Specific activities were expressed as mM urea produced/minute/1.0 mg protein.

Results

The results from the experiment on the effect of probenecid on arginase activity are presented in Fig. 8. In this figure a graph of enzyme activity vs. the amount of probenecid in the reaction vessel is depicted.

Discussion

The data expressed in Fig. 8 indicate that the activities of amphibian kidney and liver arginase are depressed in the presence of probenecid. Bovine arginase does not appear to be affected by probenecid. For example, 20 mg of probenecid decreased the activity of kidney arginase by 13 units (29 units for the control and 16 units when 20 mg of probenecid are present) and liver arginase is decreased by 11 units (29 units for the control and 18 units in the presence of 20 mg of probenecid). However, 20 mg of probenecid seem to have no effect on bovine arginase activity.

Since probenecid inhibits the active transport of urea when administered to the animal <u>in vivo</u>, it would seem that this demonstration of the inhibition of amphibian kidney arginase activity, <u>in vitro</u>, suggests that kidney arginase may be involved in the mechanism of active urea transport. However, Fig. 8 also indicates that probenecid inhibits liver arginase. Carlisky, et al. (1968b) has investigated certain properties (pH optima, Km, Km for Mn^{++},

and energy of activation) of amphibian (B. paracnemius, B. arenarum, Phylomedusa sauvagyi, Ceratophis arnata, and R. catesbeiana) renal arginase. Carlisky concluded that amphibian renal arginase is identical to amphibian hepatic arginase. The renal enzyme may be the same as the liver enzyme and therefore probenecid would be expected to inhibit both liver and kidney arginase of amphibians.

Active transport of urea has been demonstrated in only two groups of animals, primarily aquatic amphibians and marine cartilaginous fishes-skates, rays, sharks, and chimaeroids (Forster, 1970). Since the active transport of urea has not been demonstrated in mammals, one would not expect to observe an inhibition of bovine arginase by probenecid. Furthermore, several workers (Mora, et al., 1965b; Hirsch-Kolb, 1968, 1970; Reddy, 1970; and Campbell, 1966) have reported that a wide variety of arginases exist. Mammalian arginase may be quite different from amphibian arginase. Thus, it does not seem unreasonable that probenecid inhibits amphibian arginase while not affecting bovine arginase.

Fig. 8. The effect of probenecid on arginase activity. The open circles represent amphibian liver arginase activities and the closed circles represent amphibian kidney arginase activities. The x's represent purified bovine arginase activities.

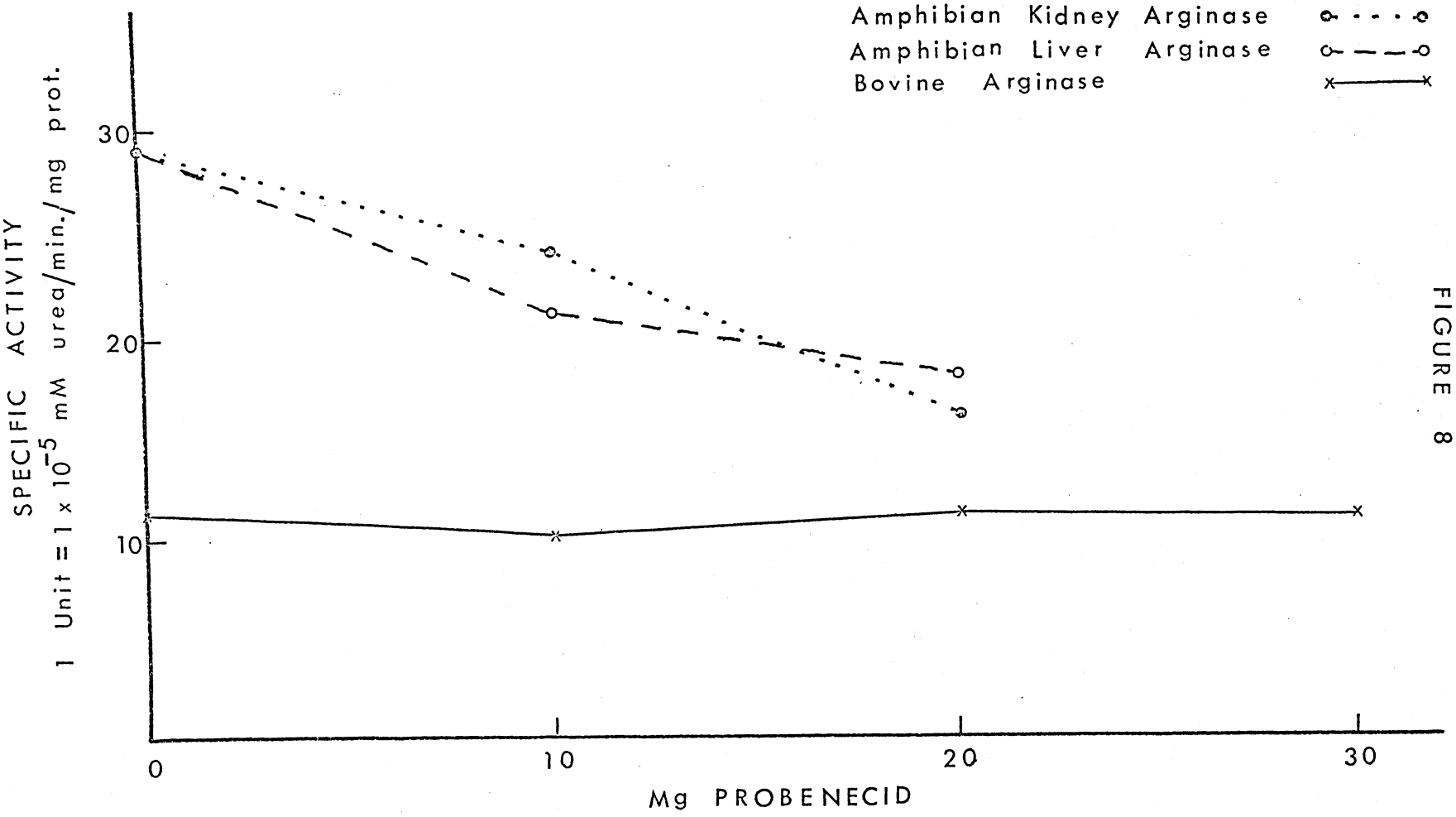

Amphibian Kidney Arginase
Amphibian Liver Arginase
Bovine Arginase
SPECIFIC ACTIVITY
1 Unit = 1 x 10⁻⁵ mM urea/min./mg prot.
30
20
10
0
10
20
30
Mg PROBENECID

FIGURE 8

Summary

This study was initiated in order to investigate possible metabolic adaptations involving the amphibian ornithine-urea cycle enzyme, arginase. Several statements can be made concerning natural arginase activities in species from different habitats and the effect of the environmental parameters, water availability and temperature, on arginase activity. The possible role of kidney arginase in active urea transport has been examined. Finally, one can speculate as to possible avenues for further research that the results gained in this investigation have revealed.

Natural variations in the activities of kidney and liver arginase in seven species of anurans (Rana septentrionalis, R. clamitans, R. sylvatica, R. pipiens, Hyla versicolor, H. chrysoscelis, and Bufo americanus) from Minnesota were studied. It was found that aquatic species had significantly higher natural activities of kidney arginase than terrestrial species. There appeared to be no correlation between natural activities of liver arginase and habitat preference in these species.

It was observed that both water availability and temperature affected arginase activity. Controlled desiccation seemed to increase the activity of both kidney and liver arginase in R. sylvatica. R. pipiens and B. americanus showed an increase in only kidney arginase activity when desiccated. There was no significant increase in either kidney or liver arginase activity when R. clamitans and R. septentrionalis were desiccated. Injection of antidiuretic hormone caused no change in the activities of kidney or liver arginase in R. septentrionalis, R. clamitans and

B. americanus.

The data collected seem to indicate that temperature may affect arginase activity in two ways. First, winter R. pipiens were found to have lower kidney and liver arginase Michaelis constants than summer R. pipiens, however the maximum velocity did not vary with season. This temperature effect could be reproduced in the laboratory, for it was found that holding R. catesbeiana at 7° C. for 14 days lowered the Km of both liver and kidney arginase. Second, incubation performed at 2° C. resulted in a lower value for liver and kidney arginase Km (in R. pipiens and R. catesbeiana) than incubation performed at 25° C. It seems apparent that there exist both an immediate adaptive response to lowered temperature and a long term decrease in Km due to seasonal trends of lower temperature.

The drug probenecid inhibits the active transport of urea. The possible effects of this drug on kidney arginase should be investigated. Incubation of R. pipiens liver and kidney arginase in the presence of probenecid resulted in decreased enzyme activity. Probenecid had no effect on bovine arginase activity. These results yield indirect evidence that kidney arginase is involved in active urea transport.

I feel that there are some interesting aspects to the results gleaned in these studies. One area that may prove fruitful for further research is the increase in kidney arginase activity in terrestrial species when they are desiccated. It has been pointed out that kidney arginase may be involved in an adaptation to hydration stress. One could therefore question why an increase in

kidney arginase activity is observed in terrestrial species, (which rarely are exposed to hydration stress). It may be that kidney arginase is involved in a mechanism for recovery of bladder water in terrestrial amphibians. Ruibal (1962) has pointed out that terrestrial amphibians store water in their bladders. This water can then be reabsorbed during periods of dehydration stress. During such periods of stress amphibians may actively transport urea from plasma to urine. Urea could then passively diffuse from the bladder back into the plasma. This would create an osmotic gradient favorable for water flux from the bladder to plasma. It would seem highly interesting to investigate this area of amphibian water balance physiology.

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Appendix

In performing the kinetic analyses expressed in this paper it was assumed that the reaction catalyzed by arginase is a single substrate-single enzyme reaction that obeys Michaelis-Menten kinetics. Dowd and Riggs (1965) point out that if an enzyme catalyzed reaction follows Michaelis-Menten kinetics, a plot of initial reaction velocity (v) against substrate concentration (s) will yield a rectangular hyperbola of the form:

$$v = \frac{(Vmax)(s)}{Km + s} \qquad (1)$$

The kinetic parameters estimated in this paper were Vmax and Km. Dowd and Riggs (1965) further state that these two parameters can be estimated by plotting the experimental data according to any one of the following three linear transformations of Equation 1.

$$1/v = (1/Vmax) + (Km/Vmax)(1/s) \qquad (2)$$

$$(s/v) = (Km/Vmax) + (1/Vmax)s \qquad (3)$$

$$v = Vmax - Km(v/s) \qquad (4)$$

Traditionally kinetic analyses have made use of Equation 2 (the "double reciprocal" or Lineweaver-Burk plot, 1/v vs. 1/s) in estimating Vmax and Km. However, Dowd and Riggs (1965) demonstrate that this particular method is the least reliable of the three methods. Further, using Equation 4 (plotting v vs. v/s) has an advantage over Equations 2 and 3 because this plot warns the investigator of such deviations from the theoretical relationship, since it tends to exaggerate such deviations. For these reasons I felt it desirable to use Equation 4 (v vs. v/s) rather than the

more traditional Lineweaver-Burk plot (1/v vs. 1/s) or Equation 3 (s/v vs. s) to estimate the kinetic parameters Vmax and Km in this study.

Figures 9 and 10 are examples of v vs. v/s plots. Estimates of Vmax and Km for R. pipiens kidney arginase are given in Fig. 9, while the Km and Vmax of R. pipiens liver arginase are estimated in Fig. 10. Initial velocities were determined by stopping each reaction after 0, 7, and 15 minutes. The amount of urea produced was plotted against the reaction time and found to be linear in all cases. Initial velocities (the slope of the line determined when the amount of urea produced is plotted against reaction time) have units of mM urea/minute. Fig. 11 is a v vs. v/s plot of kidney arginase from R. catesbeiana. The data yielded a deviant value (the closed circle). The same data were used to make a 1/v vs. 1/s plot (Fig. 12). Again the deviant value is a closed circle. It seems clear that the v vs. v/s plot (Fig. 11) clearly indicates the deviant value while the 1/v vs. 1/s plot (Fig. 12) does not warn the investigator of the deviation in this instance.

For the v vs. v/s plots the parameters Km and Vmax are obtained in the following way. The intercept of the plot (estimated from the data points by eyesight) with the ordinate is an estimate of the maximum velocity (Vmax) of the enzyme. The intercept of the plot with the abscissa has the value Vmax/Km. Knowing the value for Vmax it is possible to solve for the Michaelis constant.

All of the kinetic parameters used in this study were estimated in this way.

Fig. 9. v *vs.* v/s plot for *R. pipiens* kidney arginase. Vmax is estimated from the intercept of the plot with the ordinate and Km is estimated from the intercept of the plot with the abscissa.

Fig. 10. v *vs.* v/s plot for *R. pipiens* liver arginase. Vmax and Km are estimated as in Fig. 9.

FIGURE 9

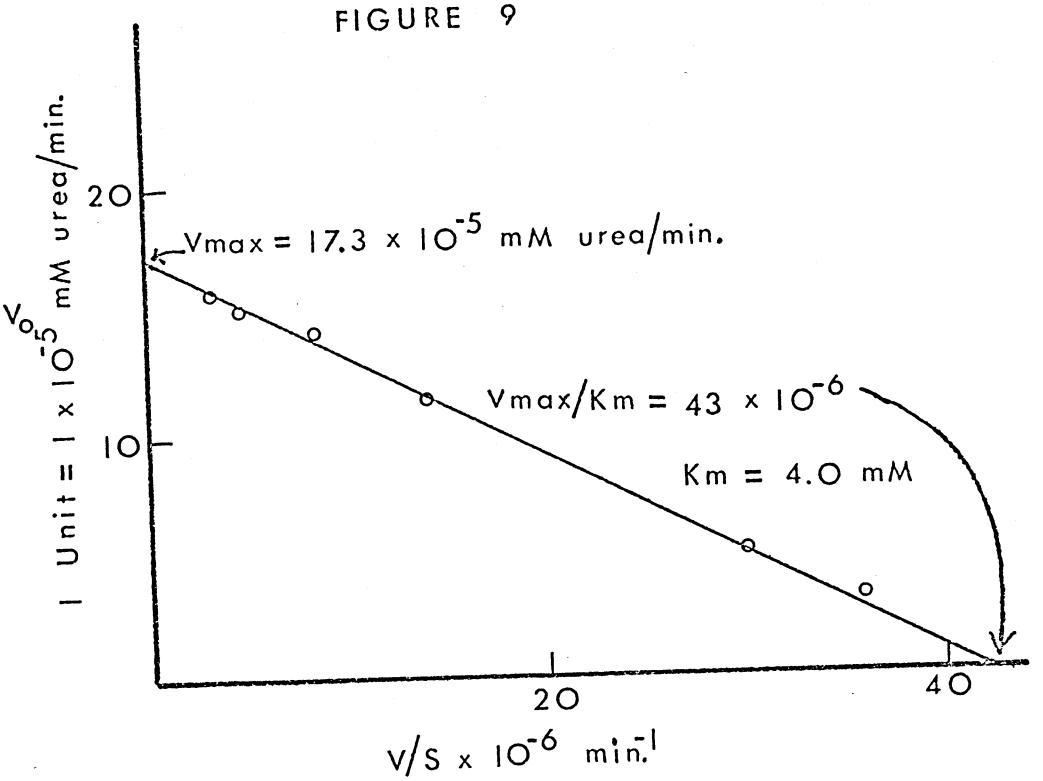

FIGURE 10

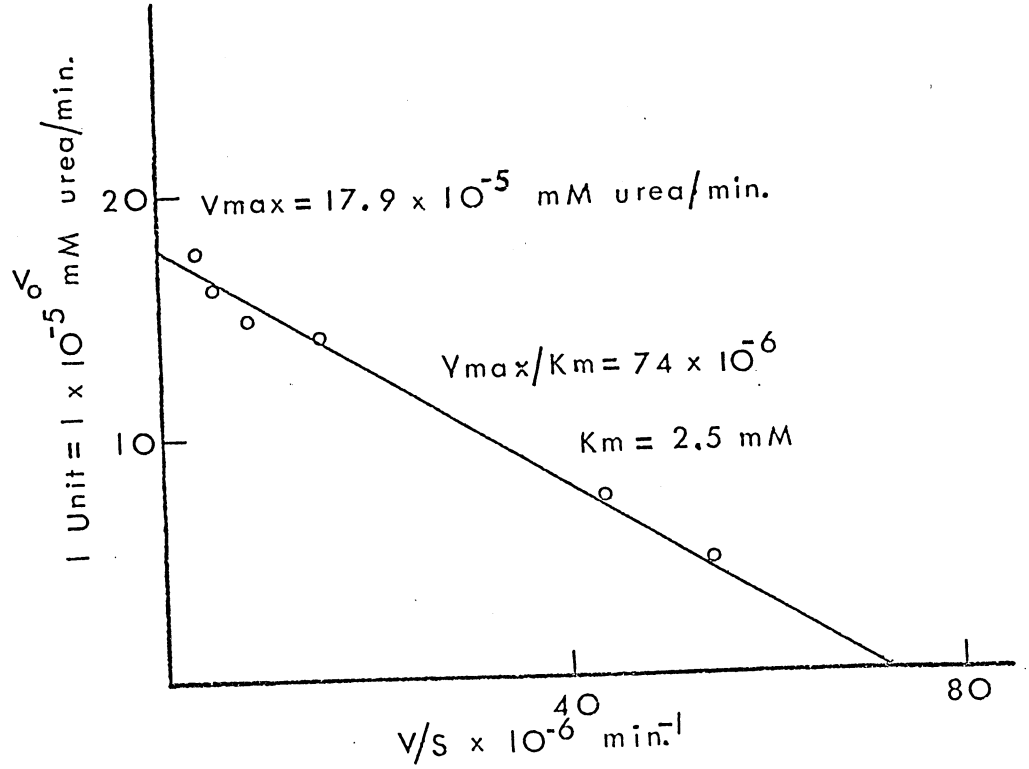

Fig. 11. v vs. v/s plot for R. catesbeiana kidney arginase. Vmax and Km are estimated as in Fig. 9. The closed circle represents a deviant value.

Fig. 12. 1/v vs. 1/s plot of the same data used for Fig. 11 (R. catesbeiana kidney arginase). The closed circle represents the deviant value.

FIGURE 11

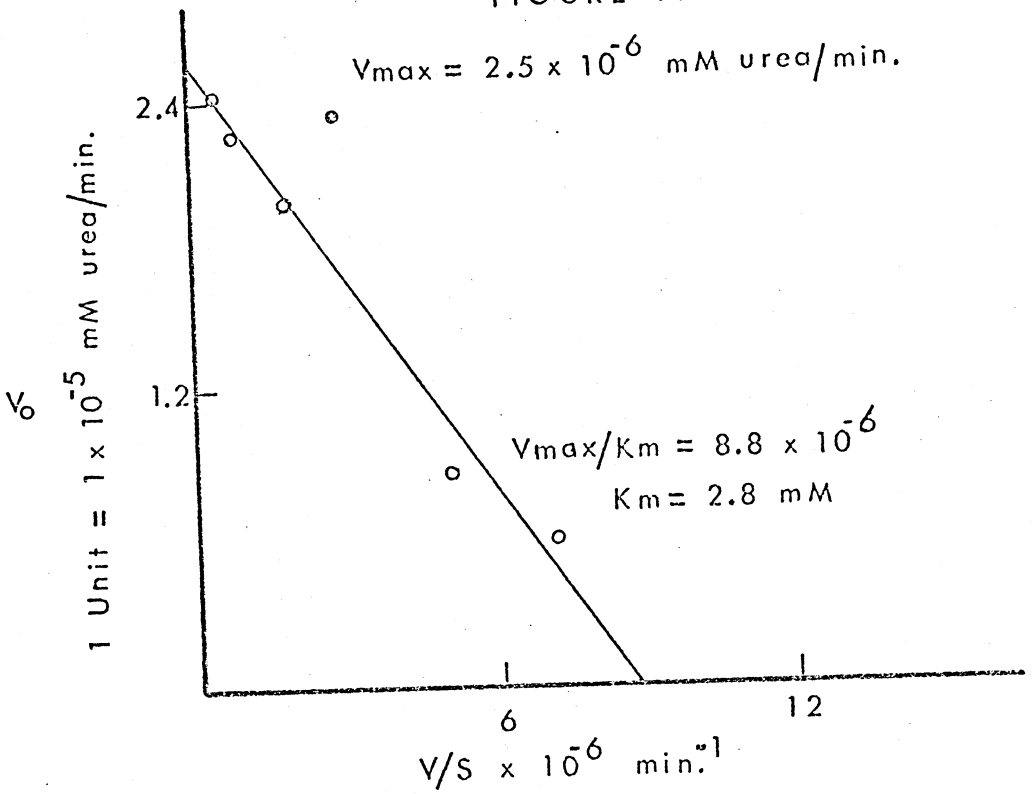

FIGURE 12