UREA BIOSYNTHESIS IN VARIOUS ANIMAL SPECIES AND IN THE METAMORPHOSING TADPOLE

by

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GENERAL INTRODUCTION AND OBJECTIVES OF THE STUDY

Since Krebs' introduction in 1932 of the theory of urea synthesis by enzymatic processes (3), extensive investigations of the biochemical mechanisms involved have been carried out. Definition of the enzymatic steps has been resolved through studies conducted primarily on tissues of mammalian species. There is valuable information to be gained, however, from an understanding of a biochemical phenomenon such as this as it occurs throughout the animal and plant kingdoms. For years it has been known that urea is excreted by a large number of animals, but is a relatively insignificant excretory substance in a host of other species. This observed variation is generally thought to be due to the presence of a functioning urea cycle in those animals which excrete urea as the principal nitrogenous waste product. To our knowledge this has not been confirmed by a systematic study of the enzymes of urea synthesis in a wide selection of animal species.

It is the purpose of this work to extend the investigation of the mechanisms of urea synthesis to a number of vertebrate species, and to study especially the urea enzymes in a representative amphibian. By means of these studies we have attempted to add perspective to the understanding of urea synthesis and nitrogen metabolism in animal life.

The investigation consists of two separate studies. The first is a comparative study of representatives from the phylum vertebrata. The activities of three enzyme systems, ornithine transcarbamylase, carbamyl phosphate synthetase, and carbamyl phosphate-aspartate transcarbamylase are estimated in a number of different species. In the second study the appearance of a functioning urea cycle in metamorphosing Rana catesbeiana tadpoles is investigated.

A REVIEW OF THE UREA CYCLE AS CURRENTLY UNDERSTOOD

The synthesis of urea from ammonium ions has been known since 1874 (1,2). It was not until 1932, however, when Krebs and Henseleit applied the tissue slice technique (3), making in vitro studies possible, that detailed analysis of the enzymatic system of urea formation began. Previous studies had been carried out largely on the intact organism or in perfused liver. Krebs and Henseliet demonstrated that ornithine and arginine act as intermediates in the synthesis of urea (3,4), and later citrulline (5) was proved to be similarly involved (6-8). Cohen then introduced methods for studying urea synthesis in tissue homogenates (9), and the synthesis of citrulline from ornithine (10-14) and arginine from citrulline (15-19) was demonstrated.

Upon further study, the reaction ornithine $\longrightarrow$ citrulline was shown to involve a glutamyl derivative (20-28) and could be divided into two enzymatic steps:

(1) $CO_2 + NH_3 + ATP$ (+ glutamyl derivative) $\rightarrow$ intermediate

(2) Intermediate + ornithine $\longrightarrow$ citrulline.

The intermediate in animal systems has since been shown to be carbamyl phosphate (29-31), an energy-rich compound from which the carbamyl group is conveyed to the δ-amino group of ornithine through a "group transfer." It is known that the first step in urea synthesis in animals is the formation

of carbamyl phosphate in a reaction catalyzed by the enzyme carbamyl phosphate synthetase. Studies with the purified enzymes have demonstrated also that an N-acyl derivative of carbamyl glutamate, ATP and magnesium ions are required as cofactors (25,26).

The second step in the synthesis of citrulline involves transfer of the carbamyl group of carbamyl phosphate to ornithine in the reaction:

Ornithine + carbamyl phosphate → citrulline + H_3PO_4.

The enzyme, ornithine transcarbamylase, catalyzes the reaction (21-27,32,38).

The production of arginine from citrulline has been found by Ratner and her group (34-42) to consist of two steps:

(1) Citrulline + aspartate + ATP $\overset{Mg^{++}}{=}$ argininosuccinate + AMP + PP_i

(2) Argininosuccinate = arginine + fumarate.

The first step of this reaction is catalyzed by a "condensing" enzyme, referred to as argininosuccinate synthetase, and the second by a "cleavage" enzyme. The overall step is referred to as arginine synthetase.

The enzyme arginase has been recognized for many years and is known to be a catalyst in the final reaction of the urea cycle (44-47):

Arginine + H_2O → ornithine + urea.

The current concept of the enzymatic method by which urea is synthesized from ammonia and carbon dioxide in mammalian liver is presented schematically in Figure 1. Net result of the overall reaction is the production of one mole of urea from two moles of ammonia and one mole of carbon dioxide at the expense of three moles of ATP.

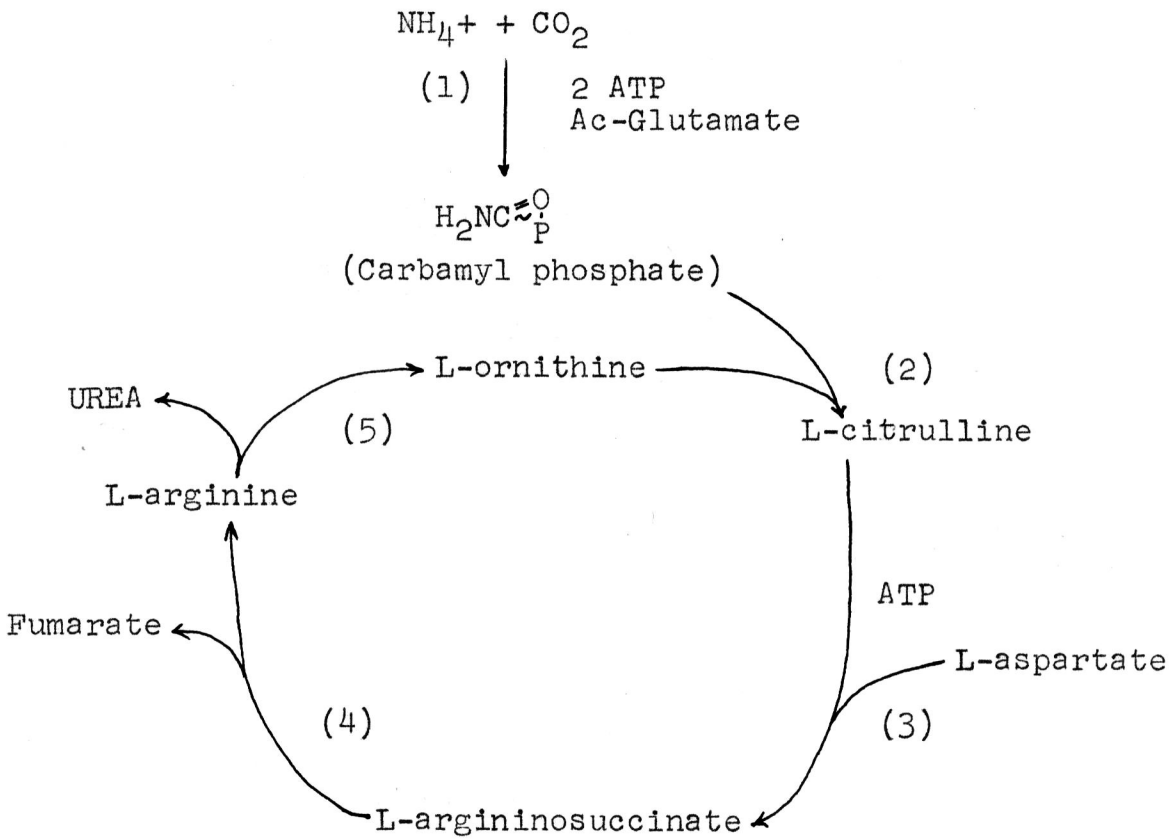

Fig. 1. Krebs urea cycle. Enzymes involved are: (1) Carbamyl phosphate synthetase, (2) ornithine transcarbamylase, (3) argininosuccinate synthetase, (4) argininosuccinate cleavage enzyme, and (5) arginase.

A STUDY OF THE UREA CYCLE IN VARIOUS ANIMAL SPECIES

A. Comparative Aspects of the Biosynthesis of Urea

In association with the evolutionary processes by which primitive marine life invaded fresh water and dry land, there probably occurred numerous biochemical modifications (49). It is supposed by many (48-50) that one of these is the mechanism by which animal organisms dispose of the waste products of nitrogen metabolism. One of three forms of nitrogenous waste -- ammonia, uric acid, or urea -- is the chief excretory product of nearly all animals (48). On this basis animal life can be grouped into ammonotelic, uricotelic, and ureotelic forms. The choice of the excretory product seems to be determined by the amount of water in the organism's environment available for disposal of this excretory substance. Baldwin (48) states that "the conversion of ammonia to other products is an indispensable adaptation to limitation of the availability of water."

As a general rule, those animals whose habitat is basically aquatic can safely excrete ammonia. Those whose environment is not aquatic excrete less toxic substances, usually urea or uric acid. Among the invertebrates the aquatic members excrete ammonia, and the terrestrial members excrete uric acid. Ureotelism seems not to have developed. The terrestrial vertebrates generally are ureotelic (amphibia

and mammals) or uricotelic (saurian reptiles and birds). The marine elasmobranchs are ureotelic. Marine teleosts are known to excrete considerable trimethylamine oxide $(CH_3)_3N \rightarrow O$ (48).

Needham (50,52) contends that the choice between ureotelism and uricotelism is determined by the conditions under which embryonic development takes place. His studies of the nitrogen excretion of some chelonian reptiles and chick embryo (52) suggested that this may be true.

Clementi (57) showed in 1914 that arginase occurs in high concentrations in the livers of ureotelic animals, but is present in traces at most in the livers of those which are uricotelic (Table I). Hunter and Dauphinee (58) later studied the arginase content of various tissues, demonstrating the presence of arginase in the livers of teleosts, though they are ammonotelic, and its presence in many tissues of the elasmobranch fishes. An increase in arginase activity in relation to increasing urea excretion in metamorphosing tadpoles has been observed (51,55). Several workers (51,55,56) have also investigated the effect of thyroid-stimulated, precocious metamorphosis on anurans and urodeles (frogs and toads). Barch (56) reports that the metabolic response to thyroxin precedes the morphological response.

Except for these studies on arginase activity, there has been little investigation into the differences existing

within the tissues of various animals which might account for the differences in excretory product. The presence of arginase in tissues of ureotelic animals is suggestive evidence that these species possess a functioning Krebs urea cycle. It is necessary, however, to demonstrate also that other urea cycle enzymes are present in ureotelic species in order to be reasonably sure that the urea produced is, in fact, synthesized by this enzymatic mechanism. In the following study we have estimated the activity of two urea cycle enzymes, ornithine transcarbamylase and carbamyl phosphate synthetase in the livers of several representatives from the phylum vertebrata and in the arthropod, Limulus. We have then shown a correlation between the presence of these two enzymes and the chief excretory product of the species studied.

In addition to investigating the occurrence of the two urea cycle enzymes, OTC and CPS*, the activity of carbamyl phosphate-aspartate transcarbamylase was also estimated (Table II). Aspartate transcarbamylase catalyzes the reaction:

Carbamyl phosphate + aspartic acid → carbamyl aspartic acid.

Carbamyl aspartate has been shown to be a precursor of orotic acid, and thus of pyrimidines, in animal tissues (61-63) and

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* Ornithine transcarbamylase and carbamyl phosphate synthetase may be referred to hereafter as OTC and CPS, respectively.

in bacteria (60-62). The enzyme, carbamyl aspartate synthetase, occurs in various tissues of many animals (65,66), micro-organisms (59,64,66-68), and plants (68). The wide distribution of this enzyme suggests that carbamyl aspartate is synthesized in most tissues and may be the initial reaction in the synthesis of pyrimidines.

B. Experimental Methods

All animals used in the study were normal and healthy. No dietary restrictions were placed on them. Except for Squalus sucklii, the fish were obtained from the Department of Zoology, University of Wisconsin. Squalus liver was obtained from Marine Biological Laboratory, Woods Hole, Massachusetts.* The frogs and turtles were purchased from a commercial source; all other animals were supplied by various departments of the University of Wisconsin. The monkeys were hepatectomized under ether anesthesia. All other animals were stunned and decapitated, and the livers removed and chilled on ice immediately. Weighed portions of the livers were then taken for assay of the three enzymes studied.

1. Carbamyl Phosphate Synthetase Assay

The liver was homogenized in chilled isotonic KCl (one part wet weight liver to 9 parts KCl) in the Waring Blender or glass homogenizer. An acetone-dried, washed residue was

* Squalus liver was frozen on dry ice immediately upon removal from the living animal and enzyme assays were carried out approximately 24 hours later.

prepared (70,71). Carbamyl phosphate was prepared after the method of Lipmann (29). The remainder of the assay was carried out by Leo M. Hall and Russell Johnson according to the method of Hall et al. (69). The quantity of liver protein was determined by the method of Lowry (73), and the activity of CPS expressed as uM citrulline produced per mgm. liver protein per 15 minutes incubation.

2. Ornithine Transcarbamylase Assay

Assay of this enzyme was carried out according to a modification of the technique described by Burnett and Cohen (32). A 10% homogenate of a portion of the liver was prepared with chilled double-distilled water. The homogenate was allowed to dialyze overnight at 2°C against two changes of double-distilled water and then centrifuged at 2500g for 30 minutes. The supernatant was diluted 1:10 with double-distilled water, and an aliquot* was incubated with an assay system consisting of: 20uM L-ornithine, pH 8; 20 uM carbamyl phosphate (29); and 100 uM $NaHCO_3$. The final volume was adjusted to 2.0 ml. with double-distilled water and the mixture incubated at 38°C. for 30 minutes. In addition to the tubes containing the fractions to be assayed, the following controls were included: (1) an incubation mixture with water replacing the homogenates,

* 0.1 ml. to 0.2 ml. was found to be a satisfactory amount for rat liver assay. Larger aliquots were taken for assay of livers with lower ornithine transcarbamylase activity.

(2) a "zero time" deproteinized mixture, and (3) a tube containing boiled homogenate. After deproteinization with 5 ml. 0.5M perchloric acid, citrulline was estimated by the method of Archibald (74). Protein was determined by the Lowry method (73), and activity of ornithine transcarbamylase expressed as uM citrulline produced per mgm. liver protein per minute incubation.

3. Aspartate Transcarbamylase Assay

The supernatant of the KCl homogenate prepared for CPS assay was dialyzed overnight against two changes of double-distilled water. This extract was then assayed for aspartate transcarbamylase activity by Dr. Geo. Burnett according to the method of Lowenstein and Cohen (33). Activity of aspartate transcarbamylase was expressed as counts per minute per mgm. protein per minute of incubation.

In Table II are summarized the results of these studies.

Subsequent to completing the above investigation, improved methods were devised for extraction and assay of the urea cycle enzymes. In light of this work we were obligated to establish the validity of our earlier studies. In a number of the species previously studied the activities of OTC and CPS were re-evaluated using the newer procedures. A 10% homogenate of the liver was prepared with a 0.1% cetyl trimethyl ammonium bromide (CTAB) solution in a glass Erway homogenizer. The homogenates were then centrifuged in the

refrigerated centrifuge (5°C.) at 4000 RPM for 15 min. From the pellet a second extraction was made in the same manner. The two supernatants were combined and various aliquots taken for enzyme assay.

The assay system for ornithine transcarbamylase consisted of 20uM L-ornithine, pH 8.0; 90uM glycyl-glycine buffer, pH 8.0; 20 uM dilithium carbamyl phosphate; liver extract*; and double-distilled water to a final volume of 2.0 ml. The remainder of the assay was carried out as previously outlined.

In the assay of carbamyl phosphate synthetase a "cocktail" was prepared consisting of: 1 part ATP 0.1M, pH 7; 1 part NH_4HCO_3 1.0M; 1 part L-ornithine 0.1M, pH 7; 1 part acetyl glutamate 0.1M, pH 7; and 2 parts $MgSO_4$ 0.1M. The cocktail was gassed with CO_2 to pH 6 to 8 immediately before use. The assay system included 0.3 ml. cocktail, 1 to 2 units partially purified beef liver ornithine transcarbamylase (32), liver extract**, and double-distilled water to a final volume of 1.0 ml. Following a 15-minute incubation, the system was deproteinized with 5 ml. 0.5M perchloric acid and citrulline estimated according to the method of Archibald (74).

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* 0.2 ml. of a 1:10 dilution of the extract was a satisfactory amount for the rat liver assay. Up to 50 times this amount was used in the assay of fish liver.

** 0.1 ml. of undiluted extract was an appropriate amount for the rat liver assay. Up to six times this amount was used in the assay of fish liver.

It should be pointed out that all enzyme assays carried out in this study were conducted under conditions thought to be optimal for in vitro rat liver enzyme systems. Whether these conditions are optimal also for the other species studied has not been determined.

As can be seen from Table II, exceptionally high levels of aspartate transcarbamylase activity were found in gar pike and bowfin liver. Confirmatory studies were then carried out on these species using a CTAB extraction. A portion of liver tissue was homogenized in 0.1% CTAB (10% homogenate). The homogenates were centrifuged in the Spinco centrifuge for 45 minutes at 30,000 RPM. The supernatants were decanted off and 0.5 ml. of the undiluted enzyme preparation added to the following system:

1.5 ml. 0.04M Carbamyl phosphate
1.5 ml. 0.1M Aspartic acid
1.4 ml. 0.5M 2,2' Iminodiethanol buffer, pH 9.2
1.5 ml. Double-distilled water.

This mixture was incubated for 15 minutes in the water bath at 37°C. without shaking. The reaction was deproteinized by the addition of 1.5 ml. perchloric acid (1.8N) containing 0.7M silver acetate. The precipitate was spun down and 6.0 ml. of the supernatant passed through 2 gm. Dowex (50w X 8 200-400 mesh, hydrogen form). This was followed by two 2.0 ml. washes of double-distilled water. Three ml. were taken for color determination. Color was developed by the method of Koritz and Cohen (75).

By use of this procedure no activity of aspartate transcarbamylase could be demonstrated. It is difficult to account for the difference in the activity of this enzyme demonstrated by these two methods, and it is clear that further study is necessary to clarify this disagreement in findings.

C. Discussion

In Table III a comparison is made of CPS and OTC activity when CTAB and KCl or water extracts of liver were studied. In the livers of those species in which neither CPS nor OTC activity could be demonstrated in a KCl or water extract none was found either in CTAB homogenates. Somewhat higher levels of activity of these enzymes were found in the livers of ureotelic species when the CTAB rather than KCl or water method of extraction was employed. Values obtained by the use of both methods were of about the same order of magnitude, however. It is believed that CTAB inhibits ATPase (76), and this may account for the slightly higher values obtained by use of this method. Studies carried out with the CTAB extracts probably, then, confirm the results previously obtained (Table II).

In Table II is tabulated the data concerning activity of OTC, CPS, and aspartate transcarbamylase in the livers of various species. It can be seen that in none of the animals known to excrete principally ammonia, could activity of either OTC or CPS be demonstrated. To our knowledge, the

chief excretory product of Limulus and the Holostei has not been determined. It would seem most reasonable, though, that these aquatic animals also excrete ammonia, and, as was anticipated, in neither of these species could OTC or CPS activity be demonstrated. The absence of OTC and CPS activity in the ammonotelic species strongly indicates that these animals do not have a functioning urea cycle and are, thus, not biochemically outfitted to synthesize urea from ammonia and carbon dioxide. On the other hand, in all species which are known to be ureotelic, except Squalus sucklii (dogfish), activity of the two urea cycle enzymes, OTC and CPS, was clearly demonstrated. In Squalus, OTC, but no CPS activity, was found. It is to be remembered, however, that assays of Squalus liver were carried out on homogenates of previously frozen whole liver. G. Brown (78) and Baldwin (77) have since reported that they were unable to demonstrate CPS activity in liver homogenates of other elasmobranchs. Baldwin's study, like our own, was conducted on acetone-dried powder preparations of liver, while Brown assayed water extracts of KCl-washed liver mitochondria.

These failures to demonstrate CPS activity in elasmobranch liver are evidence, but only in a negative sense, that the elasmobranch may synthesize carbamyl phosphate by a mechanism which differs from that of other ureotelic animals. It is more likely, that the enzyme which catalyzes carbamyl phosphate synthesis in these fish possesses properties which are not the same as those of fellow ureoteles.

This enzyme system might well, then, escape detection by our assay procedures. The possibility exists also that acetyl glutamate is not the cofactor normally involved in this step of urea synthesis in the elasmobranch.

It is worth noting at this time that the marine elasmobranch is an extremely interesting animal from a biochemical viewpoint. Most marine vertebrates face a serious threat to life from an unfavorable osmotic pressure gradient between their own tissue and the sea water in which they live. Whereas the blood of these fishes contains about 1% dissolved salts, sea water has about 3% (48). There is, then, a continual force tending to drive water out of the animal across its semi-permeable gill membranes. This force is compensated for by an elaborate mechanism for water retention. The marine elasmobranch on the other hand, has solved this problem quite satisfactorily by synthesizing urea and by maintaining a concentration of about 2-2.5% of this substance in its blood (48). With a blood urea level of this magnitude the osmotic pressure gradient is reversed and water is actually forced into the animal's circulatory system. Urea synthesized in excess of that needed to maintain this satisfactory osmotic pressure relation is excreted. The elasmobranch, thus, accomplishes two goals by the single biochemical mechanism of urea synthesis. It is no longer faced with desiccation by loss of body water to its environment, and equally important, it is protected against death from ammonia toxicity.

That activity of the urea cycle enzymes studied is present in the livers of ureotelic, but not ammonotelic animals, is presumptive evidence that the synthesis of urea is dependent upon a functioning Krebs enzymatic urea cycle. Those species, except Squalus sucklii, which do not possess adequate activity of OTC and CPS apparently lack mechanisms essential to the synthesis of urea. All those animals in whom enzyme activity could be demonstrated do, indeed, synthesize and excrete urea. What then are the implications of these findings? As has previously been discussed, there is reason to believe that with the invasion of dry land, animal life developed means of detoxifying ammonia and thus avoided toxemia from its own nitrogenous waste. In the case of the terrestrial vertebrates this was accomplished chiefly by the adoption of ureotelism. Since the synthesis of urea is apparently dependent upon a functioning enzymatic cycle, it seems apparent that ureotelic species have acquired enzymatic processes which are not present in the more primitive ammonotelic fishes. It is reasonable to assume that as the need arose for the manufacture and excretion of less toxic excretory products, there appeared in terrestrial vertebrates enzymatic steps by which ammonia could be converted to urea. Moreover, these enzymatic steps are evidently those of the Krebs ornithine-citrulline cycle.

From Table II it can be seen that carbamyl phosphate-aspartate transcarbamylase activity was found to be present in all species except the arthropod, Limulus. The finding of activity of this enzyme in all vertebrates studied is in agreement with the work of other investigators (see p.10 who have found it to be wide-spread in nature.

As mentioned previously, Clementi had demonstrated years ago that arginase occurs in high concentrations in the livers of ureotelic animals but in traces only in those species which are uricotelic (57). In Table I a comparison of Clementi's work with our present study is made. Although identical species were not studied in each instance, members of the same class can be compared. Except in the case of the Elasmobranchii and Teleostei, CPS and OTC activity were found to exist in the same classes of animals in which Clementi had demonstrated the presence of arginase. We, of course, found no CPS activity in elasmobranch livers, and neither CPS nor OTC activity was found to be present in teleost livers. Any urea excreted by these fishes probably is produced by the action of arginase on ingested arginine and not by means of the urea cycle.

By extraction both with isotonic KCl and CTAB (Table III) frog liver was found to have unusually high levels of CPS activity. On the basis of this finding Marshall, Metzenberg, and Cohen (79) subsequently obtained CPS in a high state of purity from the livers of R. catesbeiana.

TABLE I

Comparison of the Distribution of Arginase* to the Activity of Ornithine Transcarbamylase and Carbamyl Phosphate Synthetase in the Livers of Vertebrates

Animal		Presence of Arginase	Ornithine Transcarb-amylase +	Carbamyl Phosphate Synthetase++
Class	Species**			
Mammalia	Guinea pig	+	.482	.495
	Rat	+	.941	.727
	Monkey	+	.968	.321
Reptilia				
Chelonia (Turtle)		+	.807	.807
Amphibia				
Frog		+	.809	2.03
Pisces				
Elasmobranchii	Torpedo ocellata	+		
	Raia clavata	+		
	Squalus sucklii		1.35	0
Teleostei		+	0	0

* As determined by Clementi (57).
** Similar species within a class were not studied in some cases.
\+ uM citrulline/mgm protein/min.
++ uM citrulline/mgm protein/15 min.

TABLE II

Enzyme Activity in Livers of Various Animals

	Excretion mechanism A=ammonotelic U=ureotelic	No. of animals assayed	Ornithine transcar-bamylase*	Aspartic transcar-bamylase**	Carbamyl phosphate syn-thetase***
ARTHROPODA (Crustacea)					
King crab (*Limulus* sp.)	?	1	0	0	0
VERTEBRATA					
Class Chondrichthyes					
Order Elasmobranchii					
Shark dogfish (*Squalus sucklii*)	U	1	1.35	1	0 +
Class Osteichthyes					
Order Holostei					
Gar pike (*Lepidosteus osseus oxyurus*)	?	5	0	169	0
Bowfin (Mudfish) (*Amia calva*)	?	1	0	204	0
Order Teleostei					
Perch (*Perca flavescens*)	A	15	0	36	0
Carp (*Cyprinus carpio*)	A	1	0	37	0
Brown trout (*Salmo trutta fario*)	A	4	0	141	0
Class Amphibia					
Order Anura					
Bullfrog (*Rana catesbeiana*)	U	6	.809	4	2.03
Leopard frog (*Rana pipiens*)	U	6	.767	2	2.46
Green frog (*Rana clamitan*)	U	6	.570	2	-

TABLE II (continued)

Animal	Excretion mechanism A=ammonotelic U=ureotelic	No. of animals assayed	Ornithine transcarbamylase*	Aspartic transcarbamylase**	Carbamyl phosphate synthetase***
Class Reptilia					
Order Chelonia (Testudinata)					
Turtle (Chrysemys)	U	6	.807	6	.807
Class Mammalia					
Subclass Marsupialia					
Opossum (Didelphis virginiana)	U	1	.858	13	.142
Order Rodentia					
Porcupine (Erethizon dorsatum)	U	2	.300	12	.409
Guinea pig (Cavia sp.)	U	3	.482	2	.495
Ground squirrel	U	8	1.08	11	.407
Rat	U	3	.941	35	.767
Order Lagomorpha					
Rabbit (Oryctolagus sp.)	U	5	.326	3	.243
Order Chiroptera					
Bat	U	1	1.88	-	-
Order Carnivora					
Cat (Felis domesticus)	U	2	1.10	1.5	.909
Order Primates					
Monkey (Macaca rhesus)	U	2	.968	2	.321

+ Only a single specimen was available, and results were inconclusive.
* uM citrulline/mgm. protein/minute.
** Counts per minute/mgm.protein/minute.
*** uM citrulline/mgm. protein/15 minutes.

TABLE III

Comparison of the Activity of Carbamyl Phosphate Synthetase and Ornithine Transcarbamylase in the Livers of Various Animals as Determined by Two Different Methods.

Animal	Carbamyl Phosphate Synthetase* KCl Extract	Carbamyl Phosphate Synthetase* CTAB Extract	Ornithine Transcar-bamylase** H_2O Extract	Ornithine Transcar-bamylase** CTAB Extract
Carp	0	0	0	0
Brown trout	0	0	0	0
Gar pike	0	0	0	0
Bowfin	0	0	0	0
Bull frog	2.0	2.8	.81	1.8
Guinea pig	.50	1.1	.48	.60
Rabbit	.24	.38	.33	.37
Rat	.73	1.8	.94	3.5

* Activity expressed as uM citrulline/mgm protein/15 minutes.
** Activity expressed as uM citrulline/mgm protein/minute.

A STUDY OF THE BIOSYNTHESIS OF UREA IN METAMORPHOSING TADPOLES

A. Introduction

Several studies have shown that the chief excretory end product of nitrogen metabolism in tadpoles is ammonia, while in frogs it is urea (51,54-56,80,82). Dolphin (51) and Munro (55,82) have demonstrated in metamorphosing tadpoles an increase in arginase activity occurring in association with increasing urea excretion. These findings suggest that the Krebs urea cycle may be involved in this transformation from ammonotelism to ureotelism. No conclusive evidence has been presented, however, to show that introduction of the enzymatic urea cycle is, in fact, the mechanism by which the tadpole comes to excrete urea.

In this study we have attempted to demonstrate introduction of the urea cycle in the metamorphosing tadpole liver and to correlate this biochemical modification with the changes occurring in its morphological characteristics. Levels of the enzyme activities at various stages of development were estimated in order to determine whether they are sufficient to account for the rate of urea synthesis in the intact animal.

In investigating this problem we have performed the following experiments:

(1) Determination of the quantities of ammonia and urea excreted by tadpoles in various stages of development.

(2) Classification of the tadpoles according to the stages of development of the larvae.

(3) Assay of individual livers of metamorphosing tadpoles.

(4) Studies of the nature of induction of the urea cycle during metamorphosis.

B. Experimental Methods

Tadpoles of the bullfrog Rana catesbeiana (var. Wisconsin, California, and North Carolina) were studied. These were obtained from a commercial source.

1. Classification of the Stages of Development

The tadpoles were classified on the basis of external morphology according to the classification of Taylor and Kollros (83),who have defined 25 larval stages in Rana pipiens. We found that, in general, this method of classification also applies to R. catesbeiana. Some of the features of this classification are summarized in Table IV. Another satisfactory criterion of measuring larval development is the ratio of hind limb to tail length. In our earlier studies this method only was used as a method of classification. It is closely correlated with the Stages defined by Kollros and Taylor as can be seen from the graph in Fig. 2. Earlier experiments can be correlated with later

ones by reference to this graph.

2. Assay Procedures

CTAB extraction and OTC and CPS activity determinations were carried out on individual livers as described previously in the comparative study. Arginase activity was estimated by the following method: 0.5 ml. of appropriately-diluted liver extract (10% CTAB homogenate) was incubated with a system containing 0.5 ml. 0.05M arginine, pH 9.5; 0.5 ml. 0.001M $MnCl_2$; and 0.5 ml. 0.1M glycine buffer, pH 9.5. Incubation was carried out for 30 minutes at 38°C., and the system deproteinized with 5 ml. 0.5M perchloric acid. The precipitate was spun down by a 5-minute centrifugation in the clinical centrifuge. Five ml. of the supernatant was then heated in a water bath (100°C. for 60 minutes) with 2.0 ml. of digestion mixture (3 volumes syrupy H_3PO_4 and 1 volume concentrated H_2SO_4 with 1 ml. $CuSO_4$ per liter of acid) and 0.4 ml. isonitrosopropiophenone. Urea was estimated colorimetrically.

Arginine synthetase activity of CTAB homogenates was determined by a modification of the Ratner method (84). Estimations of the liver nitrogen were made according to the method of Lowry (73).

3. Measurement of Ammonia and Urea Excretion

Tadpoles were placed in beakers containing phosphate buffer (0.01M, pH 6.5), and 24 hours later the solution was

analyzed for ammonia and urea by the method of Munro (82).

C. Nitrogen Excretion by Tadpoles

The percent of total nitrogen (urea N + ammonia N) excreted as urea N was determined in R. catesbeiana (Fig. 4). It can be seen that urea N comprises only about 10% of the total N excreted until the tadpole has reached a stage in development where the ratio hind limb/tail length is about 1 (Stage XIX). At this Stage a marked increase in the relative amount of urea N excretion occurs, and by the time Stage XXII (R=1.4) is reached, over 50% of the total N is excreted as urea N. Taylor and Kollros state that the onset of true metamorphosis occurs at Stage XVIII, just prior to the Stage at which marked elevation in urea output is noted to occur. It should be pointed out that the total N excreted by the tadpoles ranged from 1.4 to 2.8 mg. per 24 hours, and the animals at the ends of the scale excreted 1.94 and 1.95 mg. N/day. This indicates that there was an actual shift in the proportion of excretory N disposed of as urea N, and not the introduction of an additional method of excretion. These levels of excretion are comparable to those obtained by Munro (82) in his studies on thyroid-stimulated, precocious Rana temporaria tadpoles.

D. Activity of Urea Cycle Enzymes in Metamorphosing Rana catesbeiana Tadpoles

Definition of Units -- One unit corresponds to the production of one mole of product per hour of incubation under assay conditions. The value units/g. represents μmoles of product produced per hour per gram wet weight of liver.

1. Carbamyl Phosphate Synthetase

Figure 6 illustrates the increase in CPS activity when plotted against the developmental Stage of tadpoles. Activity of the enzyme is plotted both as units/g. liver and in specific activity (units/mgm. liver protein/hour incubation) with essentially the same type of curve being produced. At about Stage XVIII, the onset of true metamorphosis, a sharp rise in CPS activity occurs. The activity of the enzyme increases from 17.4 units/g. at Stage X to over 500 units/g. at Stage XXIV, while the specific activity increases from 0.76 to 9.2. This data indicates that there is actually an increase in the quantity of the enzyme as metamorphosis takes place.

2. Ornithine Transcarbamylase

In Table VI is data from a representative experiment, which shows an increase in OTC activity similar to that observed with CPS. OTC activity, expressed as units/g. also rises sharply at about Stage XVIII, and increases from 1260 units/g. at Stage X to 5660 units/g. at Stage XXIV. The

activity of this enzyme is, however, considerably higher than that of CPS at all stages of development. For example, at Stage X the OTC level is 1260 units/g., while at the same Stage there is negligible CPS activity. Prior to the onset of metamorphosis OTC level is at about 1000-2000 units/g., which is approximately one-fourth to one-third that found in a young frog.

3. Arginase

The activity of arginase likewise rises sharply at about the time of true metamorphosis. Again, this is true whether activity is expressed as units/g. or as specific activity. In a typical experiment values of specific activity increased from 17.4 at Stage X to 66 at Stage XXIV, while the units/g. increased from 1040 to 3970 through these Stages. These findings are in agreement with those of other investigators (51,55).

4. Arginine Synthetase

Assays of the two enzymes ("condensing" and "cleavage" enzymes), involved in the reaction

citrulline + aspartate→arginine + fumarate

were carried out in a combined procedure. (These two enzymes will hence be referred to as arginine synthetase) Under conditions of this experiment, little or no activity of this system could be demonstrated in tadpoles prior to Stage XX. With the maximum amount of extract permitted by our assay

procedure and with an hour incubation, only 0 to 2 units/g. could be demonstrated. Beyond Stage XX, however, the activity becomes appreciable (> 5 units). Estimation of arginine synthetase was carried out on a group of tadpoles rapidly developing toward the frog Stage (Stage XX to XXIV). When activity of this enzyme system was plotted against Stages of tadpole development there was a suggestion, but no convincing evidence, that activity increased as morphologic development progressed. The values of arginine synthetase activity (units/g.) were then plotted against the ratio of hind limb to tail length (Fig. 5). From this graph it is apparent that the level of enzyme activity does, indeed, show an increase with increasing maturity of the tadpole, corresponding to the changes observed with the other enzymes studied. A comparison of these two methods of plotting enzyme activity illustrated to us that the ratio of hind limb to tail length is a more sensitive criterion for classifying morphologic development in tadpoles whose gross external characteristics are quite similar.

The combined data from several experiments conducted over a period of a few months with metamorphosing tadpoles has been compiled in Fig. 7. The levels of enzyme activity at various Stages are expressed as percent of activity of the enzyme at Stage XXIV. The dark line represents urea excretion expressed in per cent of urea excreted at Stage XXIV. The activity values are averages compiled

from several experiments using R. catesbeiana of different varieties and conducted during different seasons. Thus it is not surprising that the bar graph does not present a particularly smooth curve. In individual experiments, however, more uniform results were obtained.

Rate-Limiting Enzyme of the Urea Cycle

The rate-limiting enzyme, as determined from our experiments, appears to be within the arginine synthetase system (Table V). Since the system is known to consist of the so-called condensing and cleavage enzymes, one cannot say, on the basis of our studies, which of these enzymes is actually rate-limiting. In studies with mammalian tissue, Ratner et al. (84) have found that the cleavage enzyme usually is rate-limiting in crude extracts. Using argininosuccinate as substrate, Brown and Cohen (85) have since shown that in R. catesbeiana tadpoles the condensing enzyme appears to be the rate-limiting component.

Even though the arginine synthetase system is the rate-limiting factor, and its activity is low as determined by our assay methods, its activity is of sufficient magnitude to account for the quantity of urea excreted by the tadpole at each of the various stages. R. catesbeiana tadpoles in pre-metamorphosis stages, weighing about 5 gm. were found to excrete approximately 0.2 µmoles of urea per hour. Livers of animals of this size weigh about 300 mgm. Thus,

approximately 0.7 μmoles of urea were produced per hour per g. liver by these tadpoles. If the urea cycle is operating in these animals, one unit of the rate-limiting enzyme should suffice to support this rate of urea synthesis via the urea cycle. This corresponds to about the greatest activity of the arginine synthetase system that we could demonstrate.

Whether a functionally complete urea cycle needs to be invoked in order to account for the quantity of urea excreted by pre-metamorphic tadpoles is not certain. The activity of arginase alone on ingested arginine or arginine synthesized by intestinal flora may be sufficient to achieve this level of urea output (about 10% of the excretory N). Interestingly, arginase has been found in all larval tadpoles studied regardless of stage of development.

It can also be seen from Table V that the relative activities of the urea cycle enzymes in tadpole liver, whether expressed as units/g. or specific activity are: ornithine transcarbamylase > arginase > carbamyl phosphate synthetase > arginine synthetase.

It should be pointed out before considering further the significance of findings in this investigation that estimations of enzyme activity in vitro do not always reflect accurately the levels occurring in vivo. As in the comparative study, assays conducted in this investigation were performed under conditions which are believed to be

optimal for rat liver enzymes. Whether these conditions are also optimal for the tadpole and frog is not yet known, and for this reason the quantitative values derived from these experiments may not in all cases accurately reflect the actual enzyme activity.

E. Evolutionary and Other Implications of this Study

We might briefly review some of the evolutionary aspects of nitrogen excretion. Dry land was apparently colonized by descendents of animal organisms which had originally inhabited the sea (49). As is true of other evolutionary phenomena, this colonization probably took place through a long series of stepwise modifications. Animal life gradually became adapted to intermediate zones of environments which were less-and-less similar to the sea, e.g., that zone separating sea from fresh water (estuarine zone), that separating dry land from sea (littoral zone), as well as marsh and swamp areas (86,87). It was most essential that throughout these adaptations the animals maintained within themselves an environment in which life could continue. This required that, in addition to other modifications, the progeny of marine organisms necessarily acquired a new means for disposing of nitrogenous waste.

In most animals amino acids are deaminated by oxidative enzymes with the production of ammonia--a substance highly toxic to animal tissue. Among aquatic forms ammonia simply diffuses into the vast reservoir of water with which they

are in intimate contact. The inhabitants of more-or-less terrestrial environments, however, are faced with the serious problem of ammonia toxicity. They must convert ammonia to a less toxic form(s) which can be retained within the body for some time before being excreted. Among the adult amphibia this problem is solved by excretion of urea, a product which, in addition to its relatively low toxicity, is highly soluble in water. Two main evolutionary lines probably proceeded from the amphibians. The first of these, the mammals, continued to practice ureotelism, while the second, most reptiles and birds, adopted uricotelism. Between these two groups lies a fascinating third group, the chelonian reptiles (turtles and tortoises). Among the members of this group are aquatic, semi-aquatic, and wholly terrestrial species. The semi-aquatic forms are essentially ureotelic. The wholly aquatic animals, although retaining significant ureotelism, also excrete considerable ammonia, while uricotelism is favored by the wholly terrestrial species.

Needham (53) explains this difference in excretory product among the higher vertebrates on differences in mode of reproduction. Ureotelic metabolism is associated with viviparity and uricotelism with development of a cleidoic egg. The cleidoic eggs of most reptiles and birds develop under a marked limitation of water. If they were to excrete urea, the amount of urea which would accumulate by the end

of development would produce uremia and cellular death because of disturbed osmotic relations. These animals produce, instead, uric acid, which is relatively insoluble as well as of low toxicity. Uric acid precipitates out in the allantois, and thus causes no disturbance of osmotic pressure. In mammals the manufacture of uric acid is not necessary. The mammalian embryo is in connection with the maternal blood stream. Hence, it can dispose of urea by way of the maternal kidney rapidly enough to prevent uremia.

The Dipnoi, or lung fishes, give us another example of how the mechanism of excretion is related to the amount of water available for disposal of waste. These tropical fish inhabit swamps and rivers. During the seasons when adequate supplies of water are available they excrete ammonia. When, however, the streams dry up they lie dormant in cacoon-like structures in mud. During this time urea is produced, and as the streams again refill, these fish unload considerable quantities of stored urea (48).

According to the theory of biological recapitulation, an embryo in the course of its development, passes through stages which resemble the embryo from which it has resolved. Needham (50) has presented evidence to suggest that there is a biochemical recapitulation as well as the well-known morphologic recapitulation. He has studied the developing chick embryo, and has shown that in ontogenetic sequence it excretes ammonia, then urea, and finally uric acid -- the

characteristic excretory products of the fish, amphibia, and reptile, respectively. However, there is evidence that the urea formed derives from arginine and not from biosynthesis (72). In the tadpole we have what might better be considered a biochemical recapitulation as the ammonotelic tadpole larva develops into its adult ureotelic forms.

From our findings in this study it seems clear that the transition from ammonotelism to ureotelism in the tadpole is closely correlated with a marked increase in levels of activity of the urea cycle enzymes. The onset of excretion of urea in significant quantities is evidently dependent upon the introduction of a functioning urea cycle. Significantly, the introduction of the cycle occurs precisely at that point in development when the tadpole becomes morphologically outfitted for terrestrial life. At exactly this stage, when abandonment of ammonotelism is imperative if life on land is to be possible, the mechanisms for production of a waste product of low toxicity appear. By means of the urea cycle ammonia can now be detoxified by the synthesis of urea.

The question is now raised, what accounts for the increases in enzyme activity? Is this truly an enhanced synthesis de novo of these enzymes at the onset of metamorphosis? We feel that it is. Other possibilities, which are in need of investigation, do exist, however. For example, (1) activators or inhibitors may exist which are produced at different stages of larval development and which

specifically influence the catalytic properties of the enzymes, or (2) the actual catalytic and other physiochemical properties of the pre-metamorphic and post-metamorphic enzymes may not be identical.

CONCLUSIONS

A comparative study is made of activities of enzymes of the Krebs urea cycle in various ammonotelic and ureotelic vertebrate species. Ornithine transcarbamylase and carbamyl phosphate synthetase activity was demonstrated in all ureotelic species, with the exception of Squalus sucklii (dogfish). In Squalus no carbamyl phosphate synthetase activity was found in homogenates prepared from previously frozen liver. The activity of neither of these enzymes could be demonstrated in ammonotelic species.

The appearance of urea cycle enzymes in the liver of Rana catesbeiana tadpoles undergoing metamorphosis was also studied. Activities of all four enzymes assayed increase dramatically during that stage in development at which the tadpole becomes morphologically suited for terrestrial life and when, consequently, ammonotelism is abandoned in favor of ureotelism.

These findings are further evidence that the synthesis of urea is contingent upon the presence of a functioning Krebs urea cycle. From an evolutionary viewpoint, a functioning urea cycle appears to have evolved as a means of adaptation to life in non-aquatic environments.

TABLE IV

Stages in Larval Development of Rana catesbeiana

[After Taylor and Kollros (83)]

General designation	Stage number	Synopsis of characteristics
Limb bud stages	I-V	Length of bud increases from slight elevation to 2 X diameter.
Paddle stages	VI-X	Flattening of limb bud to complete indentation between toes; 5th toe web directed to 3rd toe.
Foot stages (pre-metamorphic stages)	XI-XVII	5th toe web reaches prehalix; nasolachrymal duct appears; proximal, middle, and distal toe pads appear.
Metamorphic stages	XVIII-XXV	Cloacal tail-piece disappears; front legs appear; larval mouth still present; labial fringes complete, angle of mouth tends toward posterior margin of eyeball, rapid decrease of tail length; tympanic cartilage ring perceptible; tail absent.

TABLE V

Activities of the Urea Cycle Enzymes in Liver of *Rana catesbeiana* Tadpoles at Various Stages of Development (CPS=carbamyl phosphate synthetase, OTC= ornithine transcarbamylase, AS=arginine synthetase, A=arginase.)

	Specific Activity				Units/g. liver			
Stage	CPS	OTC	AS	A	CPS	OTC	AS	A
X	.19	37	0	23	4.4	1680	0	1040
XIV	.29	26	0	18	12	1080	0	726
XVI	.31	44	0	17	17	2360	0	888
XVIII	.47	54	0	17	24	1940	0	714
XX	.77	50	.29	20	49	3120	10	1250
XXII	7.4	89	.17	40	100	5115	7.2	2195
XXIV	7.7	84	.30	44	127	5500	13	2920

TABLE VI

Ornithine Transcarbamylase Activity in Rana catesbeiana Tadpoles. Data Derived from a Representative Assay.

Stage	Body wt. g.	Liver wt. g.	Protein incub. ug.	System	Citrulline produced µmoles	Units per g. liver*
X	11.7	0.330	40.4	complete	0.65	1260
				-ornithine	0.23	
				-CP	0	
				(boiled)	0.30	
XX	23.0	0.735	40.8	complete	0.91	2770
				-ornithine	0.07	
				-CP	0	
				(boiled)	0.14	
XXIV	19.2	0.900	66.0	complete	1.85	5660
				-ornithine	0.14	
				-CP	0	
				(boiled)	0.28	

* Corrected for values obtained with boiled enzyme extracts.

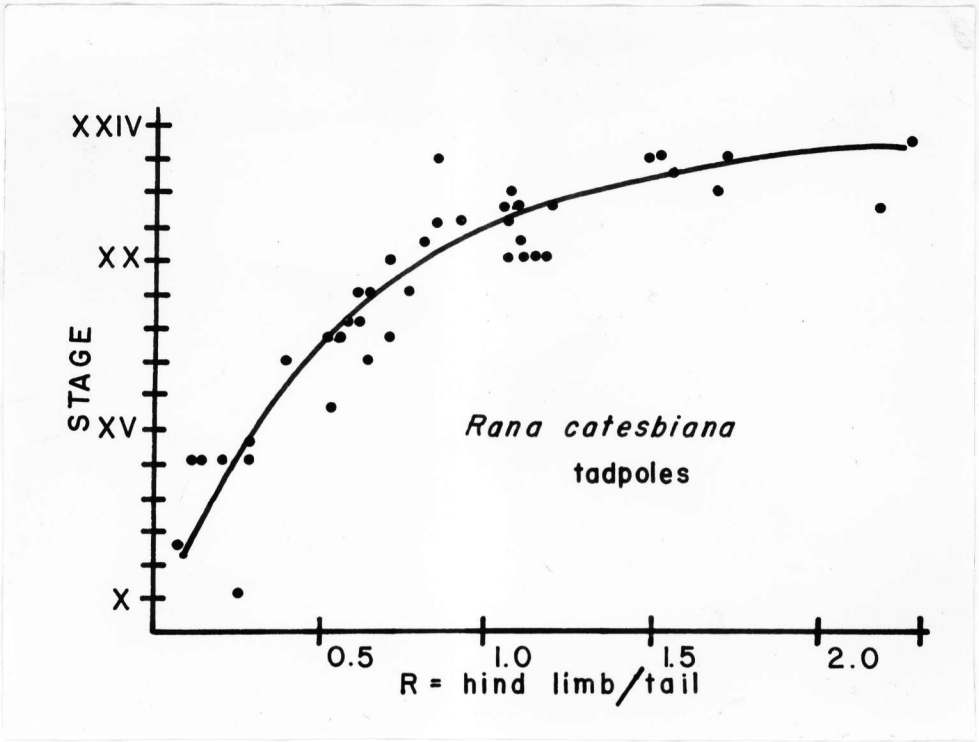

Fig. 2. The correlation of Stage of tadpole development with ratio of hind limb to tail length in Rana catesbeiana.

Fig. 3. Stages of development of Rana catesbeiana tadpoles. Top to bottom, left to right: Stages X, XVIII, XX 1/2, XXIII, XXIV.

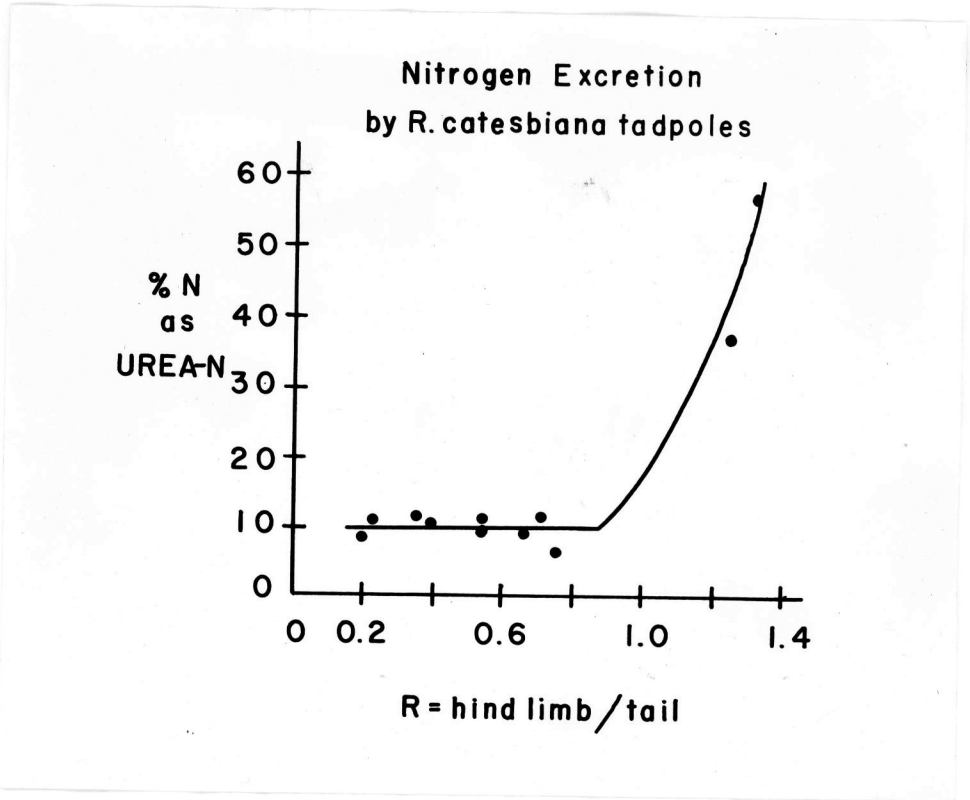

Fig. 4. Nitrogen excretion of Rana catesbeiana tadpoles. The percent of total nitrogen (ammonia N + urea N) excreted as urea nitrogen.

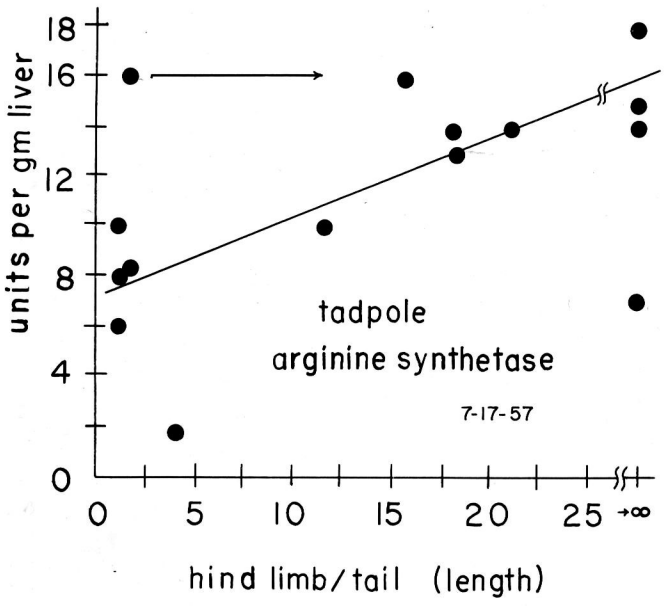

Fig. 5. Activity of tadpole arginine synthetase (units/g.) as plotted against the ratio of hind limb to tail length in Rana catesbeiana tadpoles.

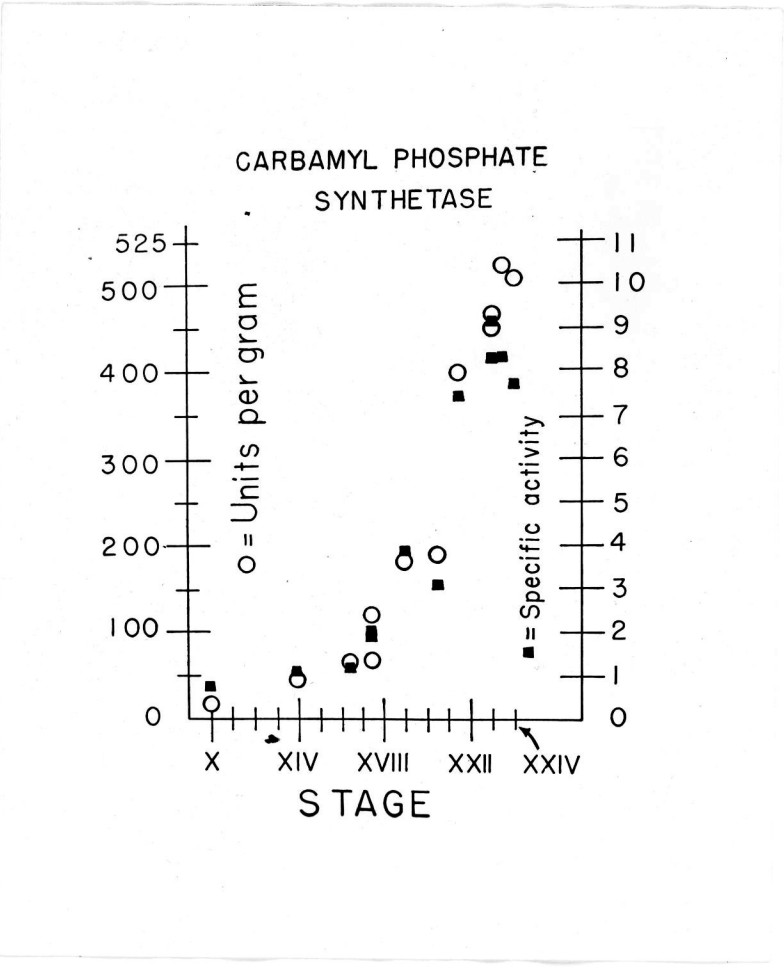

Fig. 6. Increase in carbamyl phosphate synthetase activity, expressed as units/gram and specific activity, in metamorphosing Rana catesbeiana tadpoles.

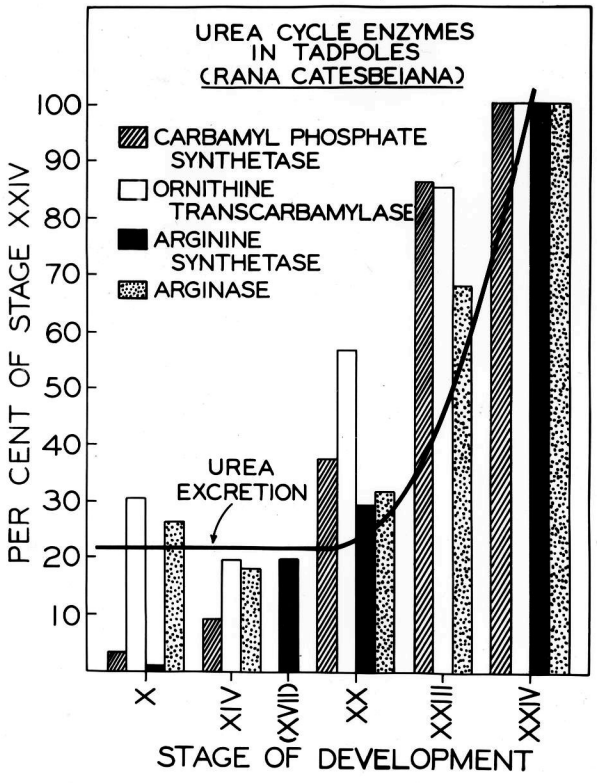

Fig. 7. Activity of urea cycle enzymes in Rana catesbeiana tadpoles during various stages of development expressed as percent of activity at Stage XXIV. Urea excretion (dark line) expressed as percent of urea excreted at Stage XXIV.

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