Uricolytic Enzymes in Liver of the Dipnoan Protopterus aethiopicus

Abstract. *The enzymes uricase, allantoinase, and allantoicase have been measured in liver preparations of the African lungfish* Protopterus aethiopicus. *The levels for these enzymes in lungfish liver suggest that the amount of urea formed in vivo in* Protopterus *via a uricolytic pathway may be greater than that derived via the ornithine-urea cycle. The operation of a "purine cycle" in lungfish liver is proposed.*

Dipnoi, as a taxonomic group of primitive fishes, stems from the beginning of the Devonian (*1*). The dipnoan lungfish of Africa, *Protopterus aethiopicus*, shares with living genera of Elasmobranchii (sharks, rays) and Amphibia the ability to synthesize urea by two distinct pathways. Members of these vertebrate taxons thus show greater diversity in the biosynthesis of urea than do present-day teleost fishes, reptiles, birds, and mammals. This diversity may well have effected the survival of these ancient lines of vertebrates (*2*). Presumably, their near-cousins on the stem of the vertebrate phylogenetic tree delivered both mechanisms of urea synthesis to immediately higher vertebrates. Deletion of a certain enzyme or of certain enzymes (*3*) or the absence of selection pressures (*4*) obliterated the functional integrity of one or both of the pathways in several vertebrate classes emerging subsequently.

Previously our group presumed (*3*) that the ornithine-urea cycle (*5*) occurred in *Protopterus aethiopicus*, and evidence was provided (*6*) for the occurrence of a relevant enzyme, ornithine carbamoyltransferase (*7*), in liver homogenates of this fish. This observation has been confirmed by Janssens and Cohen (*8*), who reported on the presence of all five enzymes of this metabolic cycle in *Protopterus* liver. Urea can also be formed by *Protopterus* by a second route from purines, that is, through the breakdown of uric acid (*9*). Our present study indicates that in *P. aethiopicus* the formation of urea from uric acid may be quantitatively more important than formation via the ornithine-urea cycle. The question was posed as to the relative contributions of the ornithine-urea cycle and of purine breakdown to the formation of urea in Dipnoi (*6, 10*). Amounts of the uricolytic enzymes uricase (*7*), allantoinase (*7*), and allantoicase (*7*) in *Protopterus* liver were determined in the present experiments to provide a basis for a quantitative evaluation of the rate of urea synthesis by a uricolytic route. A simultaneous report on the evaluation of the contributions of these two pathways to urea formation in Dipnoi is being made by Forster and Goldstein (*11*).

Specimens were obtained live from an import dealer or by direct air shipment from Uganda. The species has been identified as *Protopterus aethiopicus* (*12*). Homogenates of liver in distilled water (10 percent, weight/volume) were prepared with an all-glass hand homogenizer (*13*). An acetone powder of the liver was prepared as described (*14*). Protein in homogenates or in acetone powder extract was determined by the method of Lowry *et al.* (*15*).

Uricase (Fig. 1, step 1) was assayed by a method essentially that of Kalckar (*16*). Liver homogenate was incubated in rectangular, quartz cuvets, at 31°C, with 0.18 μmole of thrice-crystallized uric acid in the presence of 1.10 mmole of glycine sodium buffer, *p*H 9.7; the final volume was 3.0 ml. The decrease in absorbance at 293 mμ as a function of time was measured in a Beckman DU spectrophotometer. Allantoinase (Fig. 1, step 2) was assayed at 37°C (*17*) by the conversion of the product of the reaction, allantoic acid, to glyoxylic acid which is determined spectrophometrically as the 2,4-dinitrophenylhydrazone in alkaline solution.

Allantoicase [Fig. 1, step 3, and 4 (?)] was determined as follows. Homogenate or acetone powder extract was incubated in the presence of 30 μmole of allantoic acid (K and K Laboratories, Jamaica, N.Y.) adjusted to *p*H 7.4; 150 μmole of potassium phosphate buffer, *p*H 7.4; excess lactic dehydrogenase (*7*) (type III, beef heart preparation, Sigma); and 0.30 μmole of reduced nicotinamide adenine dinucleotide (NADH) (Sigma); the final volume was 3.0 ml in quartz cuvets at 25°C. The decrease in absorbance at 340 mμ over that of a control cuvet without allantoic acid was measured in the Beckman DU spectrophotometer as a function of time. Glyoxylic acid, produced by the action of allantoicase on allantoic acid, is reduced by lactic dehydrogenase [presumably by one of its isozymic forms which reduces glyoxylate as well as pyruvate (*18*)]. The rate of

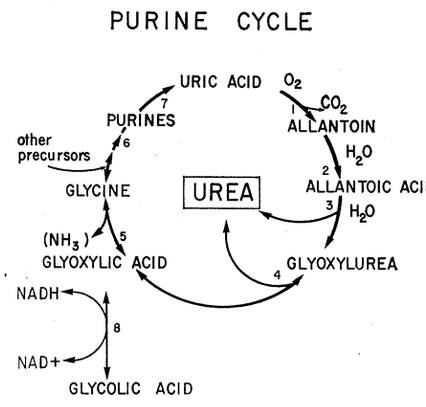

Fig. 1. The purine cycle, showing the uricolytic pathway for the formation of urea.

the reaction is calculated from the linear portion of the curve (typically the 5-minute interval between 3 and 8 minutes). This method may lend itself to a variety of enzymic studies in which glyoxylate is produced. One unit of enzyme represents 1 μmole of substrate converted per minute. The levels of activity are given per gram of liver (wet weight).

The rate of the reaction for uricase with a liver homogenate of *Protopterus* was linear for up to 13 minutes, at which time about 90 percent of the substrate had been converted. The uricase activity was 1.27 units per gram of liver.

In the allantoinase assay the production of allantoic acid was linear with time up to 30 minutes (see Fig. 2), and with amounts of protein up to 212 μg,

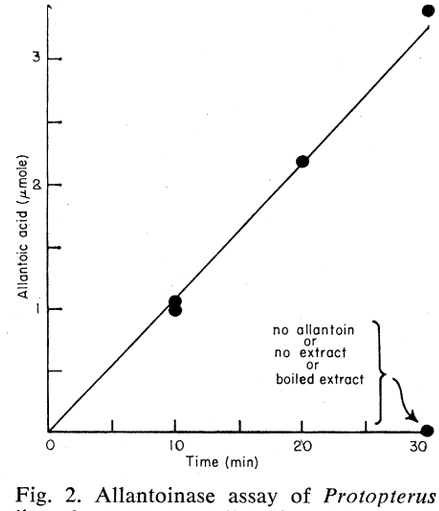

Fig. 2. Allantoinase assay of *Protopterus* liver homogenate. Allantoic acid production plotted against time. System contained 212 μg protein. Water homogenate; temperature, 37°C.

for any incubation tube. There was little or no activity if allantoin or extract was omitted from the reaction mixture or if the extract was boiled (Fig. 2). The level of allantoinase was 6.37 ± 0.07 units per gram of liver.

Assays in triplicate for allantoicase with a water homogenate of liver of a large lungfish (about 4.5 kg) yielded a value (and mean deviation) of 1.25 ± 0.04 units per gram of liver. A 0.1*M* phosphate buffer (*p*H 7.4) extract of liver acetone powder yielded allantoicase that had a specific activity of 0.117 units per milligram of protein.

Uricase and allantoicase (if slight differences in temperature of the incubations are neglected) are about equally rate-limiting in liver (1.27 and 1.25 units per gram of fresh liver, respectively) for the uricolytic pathway. Since two molecules of urea are formed from one molecule of uric acid or allantoic acid, either rate-limiting step (taken individually) would afford the synthesis of about $1.25 \times 2 \cong 2.5$ μmole of urea per minute per gram of liver. On the other hand, Janssens and Cohen (*8*) found a mean value of only 0.11 ± 0.018 units per gram of liver for the rate-limiting enzyme [argininosuccinate synthetase (*7*)] of the ornithine-urea cycle for their specimens of *Protopterus aethiopicus*.

Let us assume that the above enzymically determined rates approximate the relative rates of urea formation in vivo by the two routes of urea formation under consideration. Formation of urea from uric acid would then be approximately $(2.5/0.11) \cong 23$ times that produced by way of the ornithine-urea cycle. Such a comparison of rates along the two pathways should be viewed with some degree of caution, for no information is available on the rate of synthesis in lungfish liver of purines which are precursors of uric acid nor on the concentrations of metabolites that may be converted to urea. But on the basis of enzyme levels of liver pertinent to this discussion, the uricolytic pathway in liver is potentially more active than the ornithine-urea cycle pathway. The contribution of dietary arginine to urea formation and its importance as compared to the other two routes of urea formation is unknown.

In other experiments we have shown that glyoxylic acid in the presence of a homogenate of *Protopterus* liver oxidizes the NADH. This suggests the conversion of glyoxylic acid to glycolic acid (Fig. 1, step 8).

The aforementioned enzymic events and those described by others (*19*) suggest the operation of a purine cycle in *Protopterus* (Fig. 1). It is not known whether step 4 (Fig. 1) in lungfish liver is enzymic, spontaneous, or both of these (*20*). It is, however, a rapid step. It also remains to be demonstrated that the transamination of glyoxylic acid to glycine (step 5 of the proposed cycle) occurs in lungfish liver; however, transamination involving glyoxylic acid does occur elsewhere (*21*). Steps 6 and 7 indicate an overall process representing the conversion of glycine to uric acid (*19*).

These studies were conducted on lungfish in the aquatic habitat. It will be of considerable interest to see what changes, if any, in the urea-synthesizing mechanisms occur during estivation when large amounts of urea are stored by the lungfish. An increased understanding of the intermediary metabolism of these and other extant primitive vertebrates may well provide clues concerning the type of metabolism enjoyed by progenitors of higher vertebrates.

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References and Notes

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2. Many studies have indicated that urea plays physiological roles which may have been of evolutionary significance. In *Protopterus*, urea is stored in the tissues only during estivation in periods of drought, and this storage is thought [H. W. Smith, *From Fish to Philosopher* (Little, Brown, Boston, Massachusetts, 1959)] to be of survival value as a means of preventing ammonia toxicity (ammonia is excreted by the lungfish in the aquatic habitat); in Amphibia, as indicated by studies on *Rana catesbeiana*, the metamorphosing tadpole switches from ammonia to urea excretion with concomitant increases in activity levels of all ornithine-urea cycle enzymes in preparation for a partially terrestrial habitat; also, the ratio of urea nitrogen to ammonia nitrogen of the urine is variable with respect to amphibian species and habitat [see G. W. Brown, Jr., in *Physiology of the Amphibia*, J. A. Moore, Ed., (Academic Press, New York, 1964), p. 1]; in marine sharks and rays, maintenance of a high concentration of urea in the plasma and tissues (1 to 2 percent) is advantageous for osmotic purposes (see Smith); in the marine frog, *Rana cancrivora*, the concentration of urea in the plasma is unusually high compared with that of freshwater species—again pointing to the role of urea in increasing the plasma osmotic pressure in an otherwise hypertonic environment [see M. S. Gordon, K. Schmidt-Nielson, H. M. Kelley, *Fed. Proc.* **20**, 208 (1961); ———, *J. Exp. Biol.* **38**, 659 (1961); K. Schmidt-Nielsen and P. Lee, *J. Exp. Biol.* **39**, 167 (1962)]. All of these observations support the view that urea can serve as an environmental buffering agent in habitat transitions where water economy looms important.
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12. According to the importer (General Biological Supply House, Inc., Chicago), the species was identified from P. H. Greenwood, *The Fishes of Uganda* (Uganda Society, Kampala, 1958). Our African supplier also indicates his specimens are *Protopterus aethiopicus*. X-ray photographs of one of the specimens shipped directly from Uganda showed 38 pairs of ribs (*8*). We are indebted to Dr. M. Schneider for arranging for these photographs and to D. Moore of the Bureau of Commercial Fisheries for their evaluation.
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22. Supported in part by grant GM-9988 from the U.S. Public Health Service. Forster and Goldstein (*11*) kindly forwarded us prior to publication some of their isotopic data obtained in their lungfish experiments.

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