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Comparative Studies of Waste Nitrogen Metabolism in Amphibia

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Munro (1953) first showed that the fully aquatic South African clawed toad, *Xenopus laevis*, excretes ammonia as its main nitrogenous waste product. More typical frogs, such as *Rana temporaria*, even when given an abundant water supply, produce mainly urea (Munro, 1939). A comparative study of nitrogen excretion in a number of species of frogs (Cragg, Balinsky & Baldwin, 1961) showed that, although the majority are ureotelic, all members of the aquatic family Pipidae, to which *Xenopus* belongs, produce large amounts of ammonia.

In an attempt to explain the ammoniotelism of *Xenopus*, it was decided to investigate the levels of activity of some of the enzymes involved in the formation of each of the two waste products in *X. laevis*, as compared with those of a more typical frog. Urea synthesis in the liver of *Rana esculenta* was first demonstrated by Manderscheid (1933), and shown to resemble the corresponding mammalian process. Brown & Cohen (1960) have demonstrated the presence of all the Krebs urea-cycle enzymes in several species of Amphibia. The activities of all these enzymes increase during

metamorphosis of the tadpole, at the same time as it becomes ureotelic (Dolphin & Frieden, 1955; Brown, Brown & Cohen, 1959).

Very little is known of the mode of ammonia formation in frogs. The work of Balinsky & Baldwin (1961) suggests that in *Xenopus* the process occurs in the kidney. In mammals, it is generally assumed that urinary ammonia is formed mainly by the hydrolysis of glutamine (Van Slyke *et al.* 1943), though other mechanisms have been suggested (Madison & Seldin, 1958), including L-amino acid and glycine oxidases, and glutamic dehydrogenase.

The present paper describes a comparative study of arginase in the liver, and deamination in the kidney of *X. laevis* and *R. temporaria*. A preliminary report of some of the results has already been published (Balinsky, 1960).

MATERIALS AND METHODS

Experimental animals. The species used for the experiments were *X. laevis* and *R. temporaria*. The former, all males, were kept in water in glass aquaria, which were cleaned out frequently. They were fed once a week on chopped liver. The *Rana* specimens used were freshly collected. They were kept for a short time in glass tanks, in half an inch of water. They were not fed, but used for experiments soon after arrival.

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Liver arginase activity. Frog liver was blended in 14 mm-manganese sulphate in an M.S.E. top-drive blender. Dilutions of 1 g. wet wt. to 400–1000 ml. of manganese sulphate were found to be suitable for the determinations. Unless otherwise stated, the reaction mixture contained 0.3 ml. of tissue preparation, and 2.2 ml. of 0.27 M-arginine monohydrochloride, previously adjusted to pH 9.55 at 37° with sodium hydroxide solution. The final substrate concentration (0.238 M) was thus slightly below saturation level. A higher concentration of arginine was not used because of inhibition by high arginine concentrations of the colour reaction used for assay. (As will be seen below, a linear plot of activity against weight of tissue present was obtained under the present conditions.) The incubation period was 5 min. at 37°. The reaction was stopped by addition of 0.5 ml. of 30% (w/v) metaphosphoric acid and the amount of urea formed was assayed by the method of Van Slyke & Archibald (1946), as modified by Folley & Greenbaum (1948a).

Kidney deamination. The rates of ammonia formation from various amino acids and amides by the kidney of frogs were measured by the following procedure. As the kidney of frogs is too soft for slicing, the preparation used was a brei, obtained by slitting the connective tissue sheath around the kidney, and scraping out the kidney tissue with a scalpel. Brei (20–60 mg.) was weighed out on a torsion balance. Two or three such samples were taken from each animal, one always being used as control.

The suspension medium contained 0.1 M-sodium chloride, 1.35 mM-calcium chloride, 8 mM-disodium hydrogen phosphate, and 2 mM-potassium dihydrogen phosphate. It had a pH of 7.4 at room temperature. Each brei sample was suspended in 0.6 ml. of suspension medium. Incubation was carried out under an atmosphere of oxygen, Warburg flasks being used for convenience. The side arms contained 0.4 ml. of 125 mM-substrate, dissolved in the same suspension medium. The flasks were attached to their manometers, placed in a water bath at 37°, and oxygen was passed through them for 3 min. The contents of the side arms were then tipped, and the incubation was carried on, with shaking, for 2 hr. In control determinations, the contents of the side arms were only tipped in after the 2 hr. incubation period.

The reaction was stopped, and the protein precipitated by addition of 0.5 ml. each of equivalent solutions of zinc sulphate and barium hydroxide (Nelson, 1944). The precipitate was removed by centrifuging, and 1 ml. of the supernatant was used for determination of ammonia content.

Ammonia was determined by the colorimetric method of Russell (1944), as modified by Balinsky & Baldwin (1961). The high concentrations of amino acids, present as substrates in the reaction mixture, were frequently found to interfere with the colour produced by Russell's method, giving a colour in the absence of ammonia, and causing the colour to fade on incubation in the boiling-water bath. With many amino acids, direct ammonia determination on the deproteinized reaction mixture was still possible if standards and blanks, containing the same concentrations of amino acids, were run concurrently with the experimental samples, and if the time of incubation in the boiling water bath was reduced to 1 min. In the presence of glycine, serine and lysine, however, direct ammonia determination was not possible. In the presence of these amino acids,

Russell's method was employed in conjunction with the Conway microdiffusion technique, as described by Balinsky & Baldwin (1961), except that diffusion was carried out overnight at room temperature.

The control flasks contained about 10–15 μ moles of ammonia/gram wet wt. of tissue. This represents preformed ammonia as well as that produced from endogenous substrates during the incubation period.

The amount of ammonia per gram wet weight found in the experimental flasks, less that in the corresponding controls, was taken as a measure of the overall rate of deamination. The amount of amino acids deaminated in 2 hr. was usually less than 5%, and always less than 10%, of the total amino acid present in the reaction mixture. The resulting small decrease in amino acid concentration was neglected when preparing standards for direct ammonia determination.

Chromatography of amino acids in blood. Blood samples (0.5 ml.) were obtained as described by Balinsky & Baldwin (1961), and deproteinized by addition of 10 vol. of absolute ethanol. The amino acids present in the sample were separated by two-dimensional paper chromatography as described by Smith (1960), the solvent-extraction technique being used to remove inorganic salts. An amount of extract equivalent to 150 μ l. of blood was applied to each sheet of paper.

RESULTS

Arginase. Some properties of frog arginase were examined, with preparations of the liver of *R. temporaria*. The enzyme was found to be fully activated if the suspension medium contained 12 mM-manganese sulphate. Such a suspension retained full activity after an hour at room temperature. Fig. 1 shows the effect of adjusting the pH

Fig. 1. Effect of varying pH on arginase activity in extracts of the liver of *Rana temporaria*. The incubation mixture consisted of 0.3 ml. of tissue extract in 14 mM-manganese sulphate, and 2.2 ml. of 0.27 M-arginine, previously adjusted to various pH values. The incubation was carried out at 37° for 5 min.

of the substrate to various values by addition of sodium hydroxide solution. The pH was measured with a glass electrode at 37°. The optimum pH under the present assay conditions lay near 9.55. The effect of varying substrate concentration is shown in Fig. 2. Maximum activity was not reached below a substrate concentration of about 0.3 M.

Fig. 3 shows the results of two experiments in which the concentration of tissue suspension was varied. The plot of activity against weight of tissue was linear. A non-linear plot was obtained by Brown & Cohen (1959).

Table 1 shows a comparison of arginase activity in *R. temporaria* and *X. laevis*. The arginase activity of *Xenopus* liver, instead of being smaller

than that of *Rana* as expected, is actually greater. This holds whether activity is measured per gram of liver or per gram of body weight. The arginase activity per unit body weight of *Rana temporaria* females is about 64 % of that of the males. This is in agreement with the findings of Edlbacher & Röthler (1925) in mammals. In the present experiments, this difference is evidently due to smaller relative liver size in females, as no difference is found in activity per unit liver weight. In Edlbacher & Röthler's experiments, difference in activity per unit weight of liver was indicated (Baldwin, 1936).

The actual levels of arginase activity per gram wet weight of liver are very high. They are higher

Fig. 2. Effect of varying substrate concentration on arginase activity in extracts of the liver of *Rana temporaria*. The incubation mixture consisted of 0.3 ml. of tissue extract in 14 mM-manganese sulphate, and 2.2 ml. of arginine solution of various concentrations, giving final molarities as indicated. The arginine solutions were adjusted to pH 9.55. The incubation was carried out at 37° for 5 min.

Fig. 3. Effect of varying the concentration of tissue in the extract of liver from *Rana temporaria* on the arginase activity of the extract. The incubation mixture consisted of 0.3 ml. of tissue extract in 14 mM-manganese sulphate, and 2.2 ml. of 0.27 M-arginine at pH 9.55. The incubation was carried out at 37° for 5 min. ○, Expt. no. 1; ●, Expt. no. 2.

Table 1. Arginase activity in the liver of *Xenopus laevis* and *Rana temporaria*

The livers were blended in 14 mM-manganese sulphate. The incubation mixture consisted of 0.3 ml. of tissue suspension (1 g. of liver/400–1000 ml.) and 2.2 ml. of 0.27 M-arginine at pH 9.55. The incubation was carried out at 37° for 5 min. Arginase activities are given as mean values ± S.E.M. with S.D. in parentheses.

Species	No. of expts.	Arginase activity	
		(μmoles of urea formed/min./mg. wet wt. of liver)	(μmoles of urea formed/min./g. body wt.)
<i>Xenopus laevis</i>	18	3.09 ± 0.26 (1.12)	140.0 ± 9.7 (41.2)
<i>Rana temporaria</i> : males	9	2.16 ± 0.14 (0.43)	83.9 ± 4.3 (13.0)
<i>R. temporaria</i> : females	4	2.21 ± 0.14 (0.28)	53.1 ± 6.7 (13.3)

Table 2. *Deamination of amino acids and amides by kidney breis of Xenopus laevis and Rana temporaria*

The incubation mixture contained 0.1 M-sodium chloride, 1.35 mM-calcium chloride, 8 mM-disodium hydrogen phosphate, 2 mM-potassium dihydrogen phosphate and 50 mM-substrate. The pH was 7.4. Incubation was carried out under oxygen at 37° for 2 hr.

Substrate	<i>Xenopus laevis</i>		<i>Rana temporaria</i>	
	No. of expts.	Mean ammonia formation* (μ moles/g. wet wt./2 hr.)	No. of expts.	Mean ammonia formation* (μ moles/g. wet wt./2 hr.)
L-Glutamine	6	109.1 $\pm$ 11.9 (29.1)	8	86.9 $\pm$ 12.1 (34.1)
L-Alanine	14	28.3 $\pm$ 1.9 (7.3)	13	15.8 $\pm$ 1.8 (6.6)
L-Leucine	3	15.9	2	7.2
L-Serine	2	20.2	2	3.1
L-Glutamic acid	2	13.5	2	3.6
L-Lysine	2	2.2	2	5.8
Glycine	2	17.4	2	5.0
D-Alanine	2	140.5	2	382.5
Urea	2	0.0	—	—

* Means for L-glutamine and L-alanine are followed by S.E.M., with S.D. in parentheses.

than those for rat liver under similar assay conditions (Folley & Greenbaum, 1948b), and several times as high as those found in various Amphibia by Brown & Cohen (1960).

Deamination in the kidney. A qualitative chromatographic analysis of *Xenopus* blood indicated the presence of the following amino acids: glycine, alanine, valine, leucine, isoleucine, serine, threonine, methionine, lysine, glutamic acid, glutamine and proline. By far the most prominent spot was that of alanine. Glutamine, quantitatively the most important amino acid in human blood (Spector, 1956), was present in smaller and somewhat variable amounts.

A number of the above-mentioned amino acids were used as substrates in the measurement of deamination by kidney breis of *R. temporaria* and *X. laevis*. The results are shown in Table 2. The rate of hydrolysis of L-glutamine in *Xenopus* is only slightly higher than in *Rana*. On the other hand, many L-amino acids are deaminated much more rapidly by *Xenopus* than by *Rana*. L-Lysine is deaminated slowly by both species, as is the case in mammalian tissues. The D-amino acid oxidase of *Rana* is even more active than that of *Xenopus*. Lastly, there appears to be no urease present in *Xenopus* kidney.

Incubation of *Xenopus* kidney brei with L-alanine as substrate in an atmosphere of nitrogen instead of oxygen, produced an 80% inhibition of ammonia production. An 84% inhibition resulted on saturation of the incubation mixture with octan-2-ol.

DISCUSSION

The above results show that the properties of *Rana*-liver arginase are very similar to those of rat-liver arginase (Folley & Greenbaum, 1948a).

In their assays of arginase activity, Brown & Cohen (1959, 1960) used a substrate concentration (12.5 mM) well below the optimum level for crude arginase (see Fig. 2). This is probably the reason why the levels of arginase activity reported by them (Brown & Cohen, 1960) are lower than those found in the present experiments. It also follows that the reaction Brown & Cohen were studying was of first order in arginase. These authors are therefore probably right in suggesting (Brown & Cohen, 1959) that assay under first-order kinetics was responsible for their observation that the amount of urea formed after a fixed time did not vary linearly with protein concentration.

The present results indicate that ammonia can be formed from a large number of amino acids in the kidney of *Xenopus*. From the diversity of substrates which will form ammonia, it seems likely that a number of different deaminating systems are present there. The extent to which each substrate participates in ammonia formation *in vivo*, however, is difficult to assess, especially as some enzymes might be less active than others in the tissue preparation used. The L-amino acid oxidases, for instance, are known to lose activity on dilution (Krebs, 1935). In addition, some reactions may be promoted *in vivo* by the removal of the reaction product, ammonia, in the stream of glomerular filtrate flowing through the kidney tubules.

The large amount of alanine present in *Xenopus* blood, together with its considerable rate of deamination, suggests that it might be at least as important an ammonia precursor as glutamine. Coulson & Hernandez (1959) consider alanine and glycine as the main precursors of ammonia in the ammoniotelic *Alligator mississippiensis*. They do not regard glutamine as an important precursor.

Although such speculations must remain very tentative, a comparison of the deamination of each substrate under the same conditions in different species appears to be more reliable.

It seems that *X. laevis* kidney possesses a more active system for L-amino acid deamination than *R. temporaria*. The difference is unlikely to be due to the nutritional state of the animals, as the D-amino acid oxidase in *Rana* is extremely active, and also because the rate of protein metabolism in starving frogs is high (Przylecki & Karczewski, 1923).

Ammoniotelism in *Xenopus* could be due to less active urea synthesis, to more rapid ammonia formation, or to a combination of both factors. There is no shortage of arginase in *Xenopus*. Experiments to test the activity of the other Krebs urea-cycle enzymes in *Xenopus* are in progress, and their activity does appear to be smaller (Silver & Balinsky, 1961). The greater rate of L-amino acid deamination might well be an equally important factor in determining the percentage of ammonia excreted by that animal.

SUMMARY

1. Some properties of arginase in the liver of *Rana temporaria* were studied. The crude enzyme has a pH optimum of 9.55, is activated by manganese ions, and shows maximum activity only at substrate concentrations of about 0.3M. These properties are similar to those of rat-liver arginase.

2. Arginase activity in the liver of *Xenopus laevis* is even higher than in *Rana temporaria*, whether expressed in units per gram of liver or per gram body weight.

3. Kidney glutaminase activity in *Xenopus* is only slightly higher than in *Rana*. Many L-amino acids are deaminated much more rapidly in *Xenopus* kidney than in that of *Rana*. Kidney D-amino acid oxidase activity in *Rana* is higher than in *Xenopus*.

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