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A COMPARATIVE STUDY OF NITROGEN EXCRETION IN SOME AMPHIBIA AND REPTILES

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Abstract—1. The nitrogenous excretion of twelve species of Amphibia and two species of Crocodilia was studied. The urinary content of ammonia, urea, uric acid, and other nitrogenous compounds was determined.

2. Of the Amphibia, completely aquatic forms (all members of the Family Pipidae: *Ambystoma mexicanum*) excrete more ammonia than urea. Partly terrestrial forms excrete mainly urea.

3. The two species of Crocodilia excrete mainly ammonia, some uric acid, and a little urea.

INTRODUCTION

DELAUNAY (1931) first pointed out that the nature of the main nitrogenous waste product in an animal, namely ammonia, urea, or uric acid, is dependent on the environment in which the animal lives. Ammonia, being highly toxic, is the main nitrogenous excretory product only in aquatic animals, which are able to dispose of it rapidly, owing to a large and constant flow of water through them. In terrestrial animals, with a restricted water supply, harmful accumulation of ammonia is prevented by its elaboration into the non-toxic urea or uric acid. As Needham (1931) points out, however, synthesis of urea and uric acid is wasteful both of organic carbon and of energy, and is therefore disadvantageous except where necessitated by water shortage.

Most Amphibia are terrestrial for at least some part of their lives. The work of several authors suggests that they are typically ureotelic. Urea-excreting species include *Rana pipiens* (Van der Heyde, 1921), *R. esculenta* (Przylecki, Opienska, & Giedroyc, 1922), *R. temporaria* (Munro, 1939), *Bufo bufo*, and *Triturus vulgaris* (Munro, 1953). Smith (1929) states that the excretion of *Rana catesbeiana* is very variable, but that ammonia usually predominates. Completely aquatic species, such as *Xenopus laevis* and *Ambystoma mexicanum* (larval), appear to be ammoniotelic (Munro, 1953).

Among the Reptiles, the Squamata are uricotelic (Needham, 1931). The Chelonia have been investigated by Moyle (1949). She finds an interesting correlation of nitrogen excretion and environment. Aquatic species excrete approximately equal amounts of ammonia and urea. Terrestrial forms are ureotelic if preferring damp surroundings, and uricotelic if living in dry localities. Among the Crocodilia,

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Alligator mississippiensis is ammoniotelic (Hopping, 1923; Coulson, Hernandez & Brazda, 1950). Kahlil & Haggag (1958) found that *Crocodylus niloticus* excretes its waste nitrogen partly as a fluid, containing predominantly ammonia, and partly as a solid deposit, containing mainly uric acid. Their approximate calculations suggest that in the over-all excretion uric acid predominates.

It was considered desirable to carry out a more comprehensive investigation of a large number of species of Amphibia, in an attempt to correlate nitrogen excretion with environment. Twelve species of Anura and Urodela, living in

TABLE 1—EXPERIMENTAL ANIMALS AND THEIR HABITATS

Species	Habitat (outside the breeding season)
Cl. AMPHIBIA	
Ord. URODELA	
Salamandra salamandra	terrestrial
Triturus cristatus	terrestrial; can live in water
Ambystoma mexicanum (neotenic larva)	aquatic
Ord. ANURA	
Fam. Pipidae	
Xenopus laevis	aquatic
Xenopus tropicalis	aquatic
Hymenochirus sp. (*boettgeri* ?)	aquatic
Pipa pipa	aquatic
Fam. Ranidae	
Rana esculenta	semi-aquatic
Rana temporaria	terrestrial
Fam. Hylidae	
Hyla arborea	terrestrial, living in trees
Fam. Bufonidae	
Bufo bufo	terrestrial, preferring dry environment
Bufo calamita	terrestrial, preferring dry environment
Cl. REPTILIA, Ord. CROCODILIA	
Crocodylus niloticus	aquatic, but able to leave water
Caiman crocodilus	aquatic, but able to leave water

various habitats, from dry terrestrial to completely aquatic, were chosen for investigation. Special attention was paid to species which have reverted to an aquatic mode of life, particularly the Family Pipidae, which includes *Xenopus*, in order to ascertain to what extent they have returned to ammonia excretion. Two species of Crocodilia have also been investigated.*

MATERIALS AND METHODS

Maintenance of animals. The species investigated, together with their natural habitats, are shown in Table 1. All Amphibia were kept in glass aquaria. *Ambystoma*

* Experiments on the terrestrial Amphibia and Crocodilia were carried out by M.M.C. and E.B. (Cragg, 1953). The Pipidae were subsequently investigated by J.B.B. and E.B.

mexicanum, *Triturus cristatus*, and the Pipidae were kept in water about 6 in. deep. The *Rana* species and *Hyla arborea* were kept in one inch of water, with stones and twigs projecting above the water level. *Salamandra salamandra* and the two *Bufo* species were kept on damp sand.

The *Xenopus* species and *Ambystoma* were fed twice a week on chopped liver or heart. *Hymenochirus* were fed on *Tubifex* placed in their tank twice a week. The other species of Amphibia were all fed on earthworms and blowflies three times a week.

The crocodiles were not kept in the laboratory, their urine samples being collected in the Reptile House of the Zoological Society of London.

Collection of urine from Amphibia. Before each experiment, the animals' bladders were emptied. Urination occurred on handling, or on massaging the abdomen. The animals were then washed, dried, and weighed. 24-hr urine samples were collected by one of the methods described below. Usually, several conspecific animals were used at once, and their urines were pooled.

Method 1, for semi-terrestrial Amphibia. The animals were placed in a crystallizing dish provided with a glass tube at the bottom, through which the urine drained into a centrifuge tube. The tube contained a drop of dilute sulphuric acid, to prevent loss of ammonia and bacterial decomposition of urea. The crystallizing dish was covered with a tightly fixed perforated sheet of paper. The dish and centrifuge tube were covered by a bell jar. Air was drawn into the bell jar through dilute sulphuric acid, and out through a 2 per cent solution of boric acid, to trap any ammonia which had volatilized. The atmosphere inside the bell jar was kept moist by the presence of pieces of wet cotton wool. After 24 hr, the animals' bladders were again emptied, and both the dish and the animals were washed, the washings draining into the centrifuge tube. The urine and washings were centrifuged to remove any solid material, and made up to a suitable volume.

Method 2, for aquatic Amphibia (*Ambystoma*, *Triturus*, and the Pipidae). The animals were kept in a covered dish containing just sufficient water to cover them. A few drops of a 0·02 per cent solution of terramycin (intramuscular, Chas. Pfizer & Co., Inc., New York) were added to the water, to prevent bacterial decomposition of urea (Balinsky & Baldwin, 1961). Originally the container was kept under the bell jar, but as no ammonia was collected in the boric acid trap, this procedure was later omitted. After 24 hr, the animals and the surrounding fluid were poured into a Büchner funnel, the fluid draining into a container beneath. The animals were washed in the funnel. The fluid thus collected was centrifuged, brought to pH 1–2 with 10 N sulphuric acid, and made up to a suitable volume.

Both methods were used for some excretion measurements in *Xenopus laevis*, *Triturus cristatus*, *Salamandra salamandra*, and *Bufo calamita*, and yielded similar results. The results obtained by the two methods are, therefore, comparable.

Collection of urine from Crocodilia. One animal at a time was placed in a perspex cylinder with a perforated brass plate screwed on at each end. The cylinder was fixed at an angle of about 20° to the horizontal. A baffle of perspex was inserted about an inch from the lower end, with an outlet tube just above it. The urine thus

drained along the side of the cylinder, was stopped by the baffle, and flowed via the drainage tube into a small flask containing toluene.

As noted by Khalil and Haggag (1958), the urine sample consists of a colourless liquid and a solid white deposit. It was diluted with distilled water, adjusted to pH 7–8 with dilute hydrochloric acid, and brought to the boil, all the white solid material going into solution. The fluid was filtered, and made up to a suitable volume.

Aliquots of the urine samples collected by the above methods were taken for assay of ammonia, urea, amino acids, uric acid, creatinine, creatine, allantoin, and total non-protein nitrogen.

Analytical methods

Ammonia nitrogen was determined by distillation in Markham's (1943) apparatus, the distillate being collected in a 2 per cent solution of boric acid containing the indicator of Conway & O'Malley (1942). In later experiments, Russell's (1944) colorimetric method was used, with modified reagents (Balinsky & Baldwin, 1961).

Urea nitrogen was estimated by hydrolysing to ammonia with urease, contained in an extract of soya bean meal (Krebs & Henseleit, 1932). The ammonia nitrogen was measured as described above. Urea nitrogen was given by the difference between the ammonia nitrogen before and after treatment with soya bean extract. A blank determination with soya bean extract alone was also carried out, and the result subtracted from the experimentally determined amount of urea nitrogen. In later experiments, the colorimetric method of Archibald (1945), with slightly modified reagents (Balinsky & Baldwin, 1961), was used.

Amino acid nitrogen was converted to ammonia by treatment with ninhydrin (Sobel, Hirschman & Besman, 1945) and calculated from the difference between ammonia content before and after treatment with ninhydrin. Urea also yields some ammonia by hydrolysis under these conditions. The amount of hydrolysis was determined using standard amounts of urea. Knowing the amount of urea present in the sample, the appropriate correction was applied to the amino acid determination.

Uric acid was measured by the method of Dresel & Moyle (1950).

Creatinine was determined by Folin's picrate method as described by Borsook (1935).

Creatine was converted to creatinine by boiling with picric acid (Cole, 1953), and determined as creatinine.

Allantoin was estimated by the method of Young & Conway (1942).

Total non-protein nitrogen. Protein was precipitated from the urine sample (8 volumes) with 10 per cent sodium tungstate (1 volume) and 0·7 N sulphuric acid (1 volume). The total non-protein nitrogen was estimated by digesting for 6 hours with 2 ml of concentrated sulphuric acid and the catalyst of Chibnall, Rees & Williams (1943). The ammonia was then estimated in Markham's apparatus as described above.

RESULTS

Amphibia

No ammonia was collected in the boric acid traps. This is in agreement with the findings of Przylecki *et al.* (1922), indicating that no ammonia is lost to the atmosphere through the skin or lungs of Amphibia.

The results of the ammonia, urea, and total non-protein nitrogen measurements are shown in Table 2. It will be seen that urea is the predominant nitrogenous

TABLE 2—PARTITION OF NITROGEN EXCRETED BY TWELVE SPECIES OF AMPHIBIA

The figures quoted are mean values, followed by standard deviations in brackets.

Species	No. of experiments	Total non-protein N (μg/g body wt./24 hr)	Urea N (μg/g body wt./24 hr)	Ammonia N (μg/g body wt./24 hr)	Ammonia N as percentage of ammonia N + urea N	
					Mean	Standard error of mean, ±
Salamandra salamandra	5	76·6 (30·7)	62·7 (20·2)	3·4 (2·6)	4·70 (3·23)	1·44
Triturus cristatus	6	84·5 (49·1)	67·3 (42·1)	2·8 (1·9)	4·05 (1·87)	1·65
Ambystoma mexicanum	8	174·8 (55·9)	49·4 (21·1)	74·3(30·6)	61·94 (9·94)	3·51
Xenopus laevis	82	—	107·3 (77·9)	165·2(68·0)	62·23(11·95)	2·63
Xenopus tropicalis	6	—	80·2 (33·8)	128·2(36·3)	61·67(15·74)	6·43
Hymenochirus sp.	6	—	28·5 (19·5)	96·2(15·3)	78·03(12·61)	5·15
Pipa pipa	3	—	7·5 (1·6)	101·3(40·7)	92·53 (2·78)	1·66
Rana esculenta	7	63·6 (23·9)	48·9 (21·8)	4·5 (1·5)	9·43 (3·75)	1·42
Rana temporaria	6	118·7 (60·4)	92·7 (44·6)	8·8 (5·7)	8·24 (2·10)	0·86
Hyla arborea	8	174·3 (54·3)	149·9 (49·8)	7·1 (3·3)	4·60 (1·40)	0·50
Bufo bufo	7	117·6(111·2)	106·3(102·2)	6·2 (3·4)	4·77 (1·25)	0·47
Bufo calamita	9	98·9 (62·1)	85·7 (53·9)	5·8 (5·7)	5·72 (2·31)	0·77

waste product in *Salamandra salamandra*, *Triturus cristatus*, *Rana esculenta*, *R. temporaria*, *Hyla arborea*, *Bufo bufo*, and *B. calamita*. The percentage of ammonia in the Ranidae is higher than that of the other ureotelic Anura. The difference in all cases is significant at the 5 per cent level.

Ambystoma mexicanum and all the Pipidae investigated are ammoniotelic. The urea excretion of *Xenopus* species is still considerable. *Pipa pipa* and *Hymenochirus* sp. excrete the least amount of urea. In general, the percentages of ammonia are lower than those obtained by Munro (1939, 1953). It is interesting to note the high values of standard deviation in the percentage of urea in the excretion of *Xenopus*, *Hymenochirus*, and *Ambystoma mexicanum*. It seems that great variability exists in the relative amounts of the two waste products in these species. A similar variability was observed in *Ambystoma mexicanum* by Munro (1953), and in the lungfish *Protopterus aethiopicus* by Smith (1930). It appears to occur whenever the two waste products are excreted in comparable amounts.

The results of determinations of other nitrogenous waste products are shown in Table 3. It will be seen that, though amino acids are excreted by some of the species in significant amounts, they do not appear to form a major nitrogenous waste product in Amphibia. Uric acid, creatine, and creatinine are all excreted in very small amounts. Incomplete conversion of creatine to creatinine is indicated by the fact that there is always more of the former than of the latter. Both compounds are present, however, in all species investigated. Zwartenstein (1929) was able to

TABLE 3—MINOR NITROGENOUS WASTE PRODUCTS OF AMPHIBIA

Species	Mean percentage of total non-protein nitrogen excreted as			
	Amino acids	Uric acid	Creatinine	Creatine
Salamandra salamandra	0·0	—	—	—
Triturus cristatus	0·3	0·2	0·5	1·3
Ambystoma mexicanum	3·8	—	—	—
Xenopus laevis	2·8	0·15	0·2	0·3
Rana esculenta	5·8	0·05	—	—
Rana temporaria	3·8	0·1	—	—
Hyla arborea	0·0	0·25	—	—
Bufo bufo	0·1	—	—	—
Bufo calamita	0·6	0·45	0·2	0·5

detect only creatine in the urine of *Xenopus laevis*. After allowing for the colour produced in the determination by uric acid, no excretion of allantoin could be detected. This is in agreement with the presence of allantoinase in Amphibia (Brunel, 1937).

TABLE 4—PARTITION OF NITROGEN EXCRETED BY TWO SPECIES OF CROCODILIA

The figures quoted are mean values, followed by the standard errors of the means, with standard deviations in brackets underneath.

Species	No. of expts.	Percentage of total non-protein nitrogen excreted as		
		Ammonia	Uric acid	Urea
Crocodylus niloticus	7	66·2 ± 3·49 (9·24)	20·8 ± 3·47 (9·18)	4·5 ± 1·06 (2·80)
Caiman crocodilus	6	52·5 ± 5·21 (12·74)	27·3 ± 5·88 (14·41)	5·9 ± 1·30 (3·18)

Crocodilia

The urine of *Crocodylus niloticus* contained 1·80–2·25 mg of non-protein nitrogen per millilitre, and that of *Caiman crocodilus* 1·34–2·14 mg. Table 4 shows the partition of nitrogen in the two species. Ammonia is the most important waste product, followed by uric acid and then by urea. The relative amounts of ammonia and uric acid show considerable variability.

The urine of both species has a pH of 8–9. No amino acid nitrogen could be detected. A few estimations of other non-protein constituents were made. Creatine and creatinine nitrogen varies from 0·5 to 3 per cent of the total, the former being the larger fraction. The allantoin nitrogen is 1–2 per cent of the total in *Crocodylus*, and 3–4 per cent in *Caiman*.

DISCUSSION

The object of the present investigation was to determine to what extent the nitrogenous waste products are correlated with the environment of each species. The results certainly emphasize the importance of the environment. Among the Amphibia, which are generally mainly terrestrial and ureotelic, completely aquatic forms are ammoniotelic. Ammoniotelism is found in the Pipidae among the Anura, and in *Ambystoma mexicanum* among the Urodela.

On morphological grounds, it is assumed that the Pipidae are derived from more terrestrial ancestors, and hence it appears that ammoniotelism is a secondary acquisition, probably evolved soon after the return to water of the primitive Pipidae. In the neotenic *Ambystoma mexicanum*, ammoniotelism is merely a larval character, retained by the adult because it does not normally undergo metamorphosis. When metamorphosis is induced, ureotelism also results (Munro, 1953).

It seems probable that ammonia excretion has adaptive value to these animals. The most likely explanation of this is that of Needham (1931), namely that urea synthesis constitutes a waste of carbon and energy. Another possible advantage of ammoniotelism is that it aids the retention of univalent cations by exchange of these for ammonium ion in the kidney tubules (Coulson *et al.*, 1950).

It should be noted that ammoniotelism is restricted to completely aquatic Amphibia. Species such as *Rana esculenta* and *Triturus cristatus*, which may spend much of their time in water even outside the breeding season, produce mainly urea. In other words, in Amphibia which live partly in and partly out of water, nitrogen excretion is fitted for the terrestrial mode of life. Ureotelic species kept in water (urine collected by Method 2) continue to excrete urea in aquatic surroundings. It appears, therefore, that the nature of the predominant waste product in these species is determined by adaptations of enzymic mechanisms rather than by the direct effect of the environment.

A relatively greater percentage of ammonia is excreted by the *Rana* species. While *Rana temporaria* is considerably less aquatic than *R. esculenta*, it might be significant that the Genus *Rana* is, in general, much more dependent on water than, say, *Bufo*, and much less likely to be exposed to serious dehydration. A less complete conversion of ammonia to urea than is necessary in *Bufo* might thus be tolerated in *Rana*. It is also interesting that, among the Pipidae, the smallest amount of urea is excreted by the most specialized forms, *Hymenochirus* and *Pipa*.

The present results for *Crocodylus niloticus* and *Caiman crocodilus* suggest that, in keeping with their predominantly aquatic habitat, both species are ammoniotelic, resembling in this *Alligator mississippiensis* (Hopping, 1923; Coulson *et al.*, 1950).

The approximate calculations of Khalil & Haggag (1958) suggest a higher percentage of uric acid in the excreta of *Crocodylus niloticus* than indicated by the present experiments. As these authors observe that the ratio of liquid urine to solid deposit appears to be very variable, differences in the percentage of uric acid might well be reflections of varying relative amounts of solid and liquid excreted. Differences of this kind might well be conditioned by the environment in which the experimental animals were kept. Unfortunately, it is not clear under what conditions the experimental animals of Khalil & Haggag were living. The specimens used in the present investigation were taken from the terraria of the Reptile House, where they were able to spend some of their time submerged in water.

The present results are in agreement with Delaunay's (1931) generalization that aquatic animals are ammoniotelic. This generalization appears to hold even for species which belong to predominantly ureotelic or uricotelic groups. Secondarily aquatic forms appear to have reverted to ammoniotelism when they abandoned their terrestrial mode of life.

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