CCXLIII. NITROGEN EXCRETION AND ARGINASE ACTIVITY DURING AMPHIBIAN DEVELOPMENT

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MUCH evidence has accumulated to show that ammonia and urea excretion is characteristic of aquatic and amphibian life, and uric acid of terrestrial life [Needham, 1930; Delauney, 1935]. An ontogenetic sequence similar to the above has been shown to occur during the development of the chick [Needham, 1925]. During ontogenesis, at one stage 90 % of the N excretion is present as NH_3 . On the other hand, examination of recent data for partition of urinary N, compiled by Needham [1930], shows that in no aquatic animal investigated is a purely ammoniacal urine excreted.

Clementi [1914] has pointed out that the enzyme arginase is present in the livers of ureotelic animals, but absent where the metabolism is uricotelic, while Krebs & Henseleit [1932] have shown how this enzyme fits into a cyclic mechanism for urea production by the liver. But the factors controlling the balance of NH_3 and urea in the excretion of vertebrates other than mammals have not received much attention.

The questions arise: are there certain aquatic types amongst the vertebrates whose excretion is neither predominantly ureotelic nor uricotelic but almost exclusively ammoniacal? Are there specific environmental conditions causing the excretion of a predominantly ammoniacal urine? Is arginase also absent from the livers of those types? It was believed that a determination of the NH_3 and urea production during development and metamorphosis of the frog might be illuminating.

In this paper data are given relating to the N partition, the intensity of N excretion under various experimental conditions, and the arginase activity of the liver at various stages in the development of *Rana temporaria*.

Material and methods

Tadpoles were collected late in March from a loch in the neighbourhood. They were at the stage where external gills were just disappearing. Batches of approximately 500–1000 were found densely crowded in isolated pools in the weed fringing the water. From the uniformity of size and development it was assumed that each group had developed from a single batch of eggs. During development they were fed on ant pupae and kept at about 17°.

For the estimation of excreted NH_3 and urea, batches of 5–50 tadpoles, according to the size of the individuals and the duration of the experiments, were kept in evaporating basins, in volumes of water varying from 15 to 50 ml. Some of the first experiments were done with the tadpoles immersed in tap water or distilled water, but it was believed that excreted NH_3 might be lost from such fluid and all later experiments were done with fluid very lightly buffered with

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phosphate at pH 6.5. Estimations were also made of free NH_3 and urea- NH_3 in the excretion of tadpoles kept at five different pH ranging from 6 to 8. The ratio of free NH_3 to total NH_3 was found to be the same at each pH , viz. 92 : 100.

Ammonia was determined by the Conway-Byrne [1933] diffusion method. As the ordinary Conway unit was too small for the volumes of fluid here determined, pyrex petri dishes of 4 in. diameter with ground glass rims were used. In the centre of each was placed a shallow watch glass to hold 1 ml. 0.01 N HCl . The unit was sealed by means of a glass plate smeared with vaseline. Ammonia was liberated by saturated K_2CO_3 solution. The unit was left for 18 hr. to allow diffusion of the NH_3 into the standard acid, which was subsequently titrated with 0.0025 N $NaOH$, using Tashiro's indicator. Repeated estimations with standard solutions of NH_4Cl , with concentrations of NH_3 in the units varying between 0.017 and 0.136 mg., gave results of an accuracy not less than 95%. The same method was used for the estimation of excreted urea. A similar degree of accuracy was obtained when urease, freed as far as possible of NH_3 by shaking with permutit, was used. Urea was estimated in the arginase experiments by the manometric method of Krebs & Henseleit [1932].

For convenience in presentation of the Tables, the development has been divided into a number of arbitrary stages as follows: I. Hindlimbs absent. II. Hindlimbs just visible. III. Hindlimbs $\frac{3}{4}$ grown. IV. Hindlimbs almost fully grown and freely movable. V. Hindlimbs fully grown. Forelimbs fully grown but still covered. Intestine whitish and actively shortening. VI. One forelimb emerged. VII. Both forelimbs emerged. Tail still fully developed. VIII. Tail atrophying. IX. Tailless.

EXPERIMENTAL

Table I shows the effects of starvation, larval weight and developmental stage on the N excretion. The NH_3 and urea excretions calculated per g. wet

Table I. *Effects of size and starvation on N excretion of developing frogs. Temperature 17.5°*

	Average individual weight in mg.	Developmental stage	ml. 0.0025 N $NaOH$		Total output per g. animal per 24 hr.	% as free NH_3
			Free NH_3	Urea NH_3		
Direct from food; exp. over 20 hr.	310	II-III	3.10	0.50	5.3	86
	315	IV	2.30	0.51	5.4	82
	300	V	1.45	0.69	5.3	67
	176	VIII	0.52	2.10	8.0	25
Starved 24 hr.; exp. over 8 hr.	30	I	0.73	0.08	5.5	90
	56	I	1.19	0.13	5.7	90
	104	II	2.02	0.27	5.4	88
	210	III	1.59	0.21	4.6	81
Starved 48 hr.; exp. over 20 hr.	40	I	1.48	0.15	4.9	91
	75	I	1.15	0.19	3.3	85
	250	III	1.49	0.16	3.5	90
	210	V	1.25	0.69	4.5	64
Free NH_3 + Urea NH_3						
Starved 72 hr.; exp. over 20 hr.	205	II-III	1.42		3.6	—
	200	IV	1.23		3.7	—
	194	V	0.36		2.6	—
Starved 96 hr.; exp. over 20 hr.	200	II-III	1.35		3.2	—
	190	IV-V	1.01		2.5	—

weight of animal are much the same in both small and young tadpoles as in the metamorphic stages. This is the case both in fed animals, growing and presumably still absorbing food, and in starved animals. Prolonged starvation, however, has the effect of reducing considerably the N output in all tadpole stages. The sudden increase in N output associated with stage VII, i.e. where both forelimbs have emerged, is very definite and amounts to an increase of at least 50 %. It is not possible at present to give the reason for this sudden change in intensity of metabolism.

Table II. *Effect of temperature on ammonia and urea production of different developmental stages*

Temperature	Developmental stage		No. of animals	ml. 0.0025 N NaOH per g. animal per 24 hr.	% as NH ₃
	Beginning	End			
4°	II-III	II-III	7	1.51	90
	IV	IV	5	1.49	74
	V	3 at V 1 at VI	4	1.60	68
	VII	VII	5	3.00	32
	VII-VIII	VII-VIII	4	8.5	19
17°	II-III	II-III	6	5.4	85
	IV	IV	5	5.9	83
	V	1 at V 1 at VI 2 at VII	4	5.9	64
	VII-VIII	VII-VIII	4	8.5	19
	VII-VIII	VII-VIII	4	8.5	19
28°	II-III	II-III	6	10.3	89
	V	4 at V 1 at VI	5	10.8	70
	VII-VIII	VII-VIII	4	8.5	19
	VII-VIII	No survival	5	—	—

The effect of rise in temperature is to increase markedly the excretion of NH₃ and urea (Table II). Again it is emphasized that at all stages at similar temperatures the output of NH₃ and urea per g. of larvae gives a total of the same value.

As shown by Fig. 1, the Q_{10} is about the same for all tadpole stages. Between 4° and 14° it has the value of 2.8 and between 14° and 24° of approximately 2. The curves are of the same type, slightly convex to the abscissa. There is also a suggestion that the N excretion at stage V is slightly greater at all temperatures than that at stage II-III.

Again a pronounced increase in N excretion associated with the development of stage VII is apparent at each temperature. The curve also approaches very near to a straight line. Data [Pryzlecki *et al.* 1922] for the total NH₃ and urea excretion by the adult frog are also shown in the graph. It is noticeable that the points for froglet and adult frog form practically straight lines running almost parallel with one another, that for the froglet being at a slightly higher level. The temperature characteristics as regards N output are therefore different in the tadpole and frog.

There was no survival of froglets kept at 25° and 28° for 24 hr. The highest survival temperature over 24 hr. was 21°. This is in striking contrast to the experiments on tadpoles which are still healthy after 24 hr. at 28° and on adult frogs (Pryzlecki) where survival was obtained after 24 hr. at 31°.

Estimation of the N excretion of tadpoles at stage I gave the rather surprising result, that of the total amount of NH₃ liberated by treatment with

urease and K_2CO_3 , at least 90% was present as free ammonia and 10% was combined as urea. It was at first thought that this very high NH_3 content might be due to bacterial decomposition of urea occurring during the experiment. After an experimental period of 20 hr. a good deal of excrement had undoubtedly been shed into the fluid. To test this, two experimental dishes were set up, each containing the same number of tadpoles at a similar developmental stage. To the fluid in one dish was added a small concentration of urea. After an experimental period of 20 hr. estimation of free NH_3 in both dishes gave similar values. This experiment also ruled out the possibility of decomposition of urea by K_2CO_3 during the relatively prolonged estimation period.

In a second experiment tadpoles were starved over a period of 6 days, new fluid being put in each day, and immediately after the experimental period analysed for NH_3 and urea. On continued starvation excrement ceases to appear in the fluid, yet even on the sixth day of starvation free NH_3 still formed 90% of the total.

Experiments similar to the above were done on adult frogs, with which a free NH_3 percentage in no case higher than 12% was obtained. The experiments summarized in Table III were done with the object of determining at what stage

Table III

Developmental stage	Duration of stage	% present as free ammonia
I	60 days	92
II	15 "	84
III	15 "	84
IV	5 "	—
V	5 "	68
VI	10-20 hr.	—
VII	1-2 days	54
VII-VIII	1 day	29
VIII	1 "	26
IX	—	12

Fig. 1. Effect of temperature on the combined NH_3 and urea production at different stages of development.

or stages in development the change to predominantly urea excretion occurred, whether it was a gradual change over or an abrupt transition. It must be noted

that in spite of the use of tadpoles from the same batch, a very great variation occurred in the time required for individual animals to attain particular developmental stages. This applies more particularly up to stage IV. There are also great variations in the individual weight of animals from the same batch at similar stages and it is invariably found that a weight loss of almost 50 % occurs at metamorphosis. Accordingly, the durations of stages given in the table are necessarily approximations.

The results show that NH_3 production forms rather above 90 % of the N output in animals about 40 days old. The value falls slowly during the next month to about 85 % and during the next 10 days, at a period concomitant with rapid growth of the forelimbs and shortening of the intestine, to 68 %. During the next 4 days and beginning during the 10–20 hr. associated with emergence of both forelimbs there is a decrease in NH_3 of about 40 %, and a correspondingly great increase in urea production. During the next 3 days and paralleling the atrophy of the tail there is a rather slower decrease in NH_3 from 29 to 12 %, which is approximately the adult level.

Arginase activity of liver tissue during development

Livers were dissected out under a binocular microscope. To prevent any disturbing factor due to inactivation of arginase in the tissues during preparation a rotational procedure was adopted for the dissections. Liver was removed from one animal at stage III, one animal at stage V, one at stage VII, then back to the first stage. Tissue immediately after dissection was dropped into ice-cold frog Ringer solution. Dissection of 20 animals occupied 20 min. The combined livers for each stage were then placed on filter paper to remove excess water and quickly weighed. Arginase was extracted by grinding with quartz sand in a small volume of distilled water [Baldwin, 1935] for 5 min. and making the volume in each case up to 8 ml. Centrifuging for 5 min. brought down the sand and organic debris and a fairly clear yellowish fluid was obtained. 3 ml. of the fluid from each stage were added to boiling tubes containing 1 ml. glycine buffer of pH 9.8 and 1 ml. of a solution containing 40 mg. pure arginine. Adequate controls of brei and chemicals for urea content were made as shown below in the protocol for one experiment. The tubes were placed in a thermostat at 28° for 90 min.

Table IV

Exp. no.	Developmental stage	Liver wt. g.	No. of animals	Total body wt. g.	Liver wt. : body wt.
I	III	0.0091	8	0.982	1 : 100
	V	0.0412	6	1.393	1 : 30
	VII	0.0280	6	0.903	1 : 30

Table V

Exp. no.	Manometer no.	1	2	3	4	5	6	7
I	Manometer contents	Brei III + arginine	Temp. control	Arginine	Brei VII	Brei V + arginine	Brei VII + arginine	Brei V
	ml. taken for estimat.	2	—	—	2	2	2	2
	$\mu\text{l. CO}_2$ evolved	+6	0	+1	+1.5	+42	+106	+1.5
	$\mu\text{l. urea-CO}_2$ per mg. liver	4.1	—	—	—	9.8	30	—

No suggestion is made that the figures express the true proportionality of the arginase activities of the tissues. It was thought sufficient to show whether or not an increased activity of the enzyme was apparent during progressive developmental stages. The urea production during incubation therefore is expressed as $\mu\text{l. CO}_2$ per mg. wet weight of tissue. Table VI shows that there is an increasing activity of enzyme, that the activity is very small in the early stages increasing only slowly up to stage V and that it increases very greatly during the short period of 2-3 days covering stages V-VII.

Table VI

Exp. no.	$\mu\text{l. urea-CO}_2$ at 28° per mg. wet weight of liver		
	Stage III	Stage V	Stage VII
I	4.1	9.8	30
II	2.5	7.4	23.5
III	1.4	6.0	20.2

Actually there are some indications that the increase in arginase activity is even greater than is suggested by the figures given. An experiment was set up to determine the arginase concentration and activity relationship in froglet liver, during 90 min. at 28°. The curve obtained was at no part a straight line and fell off very rapidly at the higher concentrations which were just outside the range of the experiments quoted. Other experiments to determine the time/activity relationship showed a diminution in arginolytic activity before the end of the first hour at 28°. In order to determine the true proportionality of the increase it would be necessary to decrease greatly the incubation time as suggested by Baldwin [1935]. This would require the preparation of a greater amount of tissue in order to be sure of accuracy in estimation of the early stages, but would simultaneously involve a longer period of preparation.

DISCUSSION

Experiments on the nitrogen excretion of frog larvae have previously been done by Bialascewicz & Mincovna [1921]. They state that the N excretion is almost equally divided between urea and NH_3 in tadpoles up to 300 hr. old and this value is maintained from the earliest developmental stages. Unfortunately no experiments were done during the work here described on such early stages. The earliest used which just follow on loss of the external gills have a urea excretion of less than 10%. It will be necessary to repeat these early experiments since a N excretion fluctuating thus would be remarkable.

It is necessary to consider first why the tadpole excretes a predominantly ammoniacal urine. Needham [1930] suggests that "The great disadvantage of NH_3 excretion is that a constant supply of acid is required to neutralize it". That the larvae survive is justification for saying that NH_3 excretion is possible under the conditions. One of the main considerations is that the larvae are living in a medium whose osmotic pressure is far lower than that of their blood. Therefore water is being absorbed and a large urine flow necessarily ensues, which would both wash the NH_3 thoroughly out of the kidney and also keep the concentration low at all times. Yet it probably still holds that an equivalent of acid is required for the excreted NH_3 . It would be of importance to determine the nature of this acid in order to see whether it is really waste or valuable substance necessarily lost. Even if acid is lost it may be compensated for by the fact that the energy used in production of NH_3 is much smaller than that involved in urea formation, as pointed out by Needham [1930].

For comparison with the tadpole experiments, estimation was made of the N partition in the excretion of starved eels. Free NH_3 formed 90% of the total NH_3 liberated on addition of urease and potassium carbonate. Manderschied [1933] did not find the ornithine cycle present in the livers of two fresh-water teleosts, from which it might be concluded that urea in the excreted fluid is minimal, and confined to that arising from purine breakdown. Experiments with surviving slices of liver from the eels used above showed neither arginase activity nor evidence of the ornithine cycle. These experiments suggest that the ornithine cycle and probably arginase is absent from the livers of fresh-water fishes, but more data must be collected before this can be concluded. On the other hand, as Hunter & Dauphinee [1924] have shown, arginase is present in the livers of all salt-water fishes so far investigated.

Taking into account these facts, it seems that, as a method of getting rid of waste N, NH_3 excretion is more efficient than urea excretion for the tadpole with its typically piscine characters; and also, even although the animal is in the larval state, the lack of arginase in the liver is not due to larval simplicity of structure, but is essentially an established state for fresh-water existence. This state is only upset when its efficiency is impaired by a new level in development being reached, viz. for land existence. That this is the case is suggested firstly by the relative abruptness of the transition, and secondly by the fact that the initiation of this change is coincident with the period when the gills are shrivelling and the forelimbs are just piercing the gill coverings, and thus giving rise to a functionally active terrestrial animal.

Looking at the above results as a whole, it is significant that there are changes occurring practically at the same time in

- (1) the ratio of excreted urea- NH_3 : free NH_3 ,
- (2) the total urea + NH_3 excreted,
- (3) the arginase activity of the liver,
- (4) the weight of the liver.

Fig. 2. The proportion of NH_3 to urea in the excretion at different stages during development. The relative arginase activity of the liver is shown at corresponding stages.

The histogram (Fig. 2) shows how the arginase activity although only expressed in the roughest proportionality corresponds strikingly with the

changing rate of urea excretion. The increase in urea is accompanied by a decrease in NH_3 and it can be inferred that the NH_3 which is disappearing is built up into urea. As no chemical mechanism other than the ornithine cycle, involving arginase activity, is known which can produce urea from NH_3 , it can also be inferred that this mechanism is being brought into play in a very short period of time.

The parallelism does not hold so well for the corresponding changes in liver weight and nitrogen output. The liver increases rapidly in size from a one-lobed to a three-lobed structure during the period preceding the rapid metamorphic changes associated with emergence of forelimbs, and it has already reached a maximum size before there is any great increase in apparent arginase activity. The increase in size, however, is interesting in that it must be associated with an increase in cell division as the extra lobes could not be formed otherwise. Whether the increase in cell number is related to increase in enzyme formation is open to speculation. Sohngen & Coolhaas [1924] have stated that galactozymase formation is accompanied by an increase in cell count.

Finally, one cannot but be impressed by the parallelism which exists between the degree of thyroid liberation at various stages and the changes listed above. The investigations of many workers have made it clear that "there is an accumulation of colloid during the early development of the hindlegs followed by active colloid discharge resulting in the rapid body changes of metamorphosis such as shrinkage of the gills, intestine and tail, and growth of forelimbs" [Allen, 1938]. It is possible that liberation of thyroid substance which has been proved to be the stimulus for the structural changes also exerts a direct effect on the N metabolism of the liver. Terroine & Bonnet [1936] find that injection of thyroxine into the pig increases the total N output. The fractions responsible for the increase are urea and creatine. Lanczos & Mansfeld [1939] find that injection of thyrotropic hormone into the adult frog increases the N metabolism. Edlbacher & Schuler [1932], on the other hand, tried the effect of thyroxine on mammalian arginase. Activity of the enzyme was found to be inhibited by about 30%. All these experiments suggest that one way or another thyroid has some effect on N metabolism.

It is intended to repeat the above experiments on axolotls fed with thyroid substance.

SUMMARY

The total of NH_3 and urea excretion in tadpoles has a similar value in small and large animals at all stages of development including the metamorphic period. The N output is markedly increased in the froglet and adult frog.

Rise of temperature increases greatly the NH_3 and urea excretion in all tadpole stages and in froglet and frog.

A rapid change over from predominantly NH_3 excretion to predominantly urea excretion is shown to occur during the metamorphic crisis.

An increase in liver arginase occurs at the same time as the above transformation, the most rapid and greatest increase being during active metamorphosis.

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REFERENCES

- Allen (1938). *Biol. Rev.* **13**, 1.
Baldwin (1935). *Biochem. J.* **29**, 252.
Bialasiewicz & Mincovna (1921). *Trav. Lab. Physiol. l'Institut Nencki*, **1**, 11.
Clementi (1914). *R.C. Accad. Lincei*, **23**, 612.
Conway & Byrne (1933). *Biochem. J.* **27**, 430.
Delauney (1931). *Biol. Rev.* **6**, 3.
Edlbacher & Schuler (1932). *Hoppe-Seyl. Z.* **206**, 78.
Hunter & Dauphinee (1924). *Proc. Roy. Soc. B*, **97**, 227.
Krebs & Henseleit (1932). *Hoppe-Seyl. Z.* **210**, 33.
Lanczos & Mansfeld (1939). *Arch. exp. Path. Pharmac.* **191**, 430.
Manderscheid (1933). *Biochem. Z.* **263**, 245.
Needham (1925). *J. exp. Biol.* **3**, 189.
—— (1930). *Chemical Embryology*, Vol. 2. Cambridge University Press.
Przylecki, Opienska, Giedroyc (1922). *Arch. internat. Physiol.* **20**, 207.
Sohngen & Coolhaas (1924). *J. Bact.* **9**, 131.
Terroine & Bonnet (1936). *Arch. internat. Physiol.* **44**, 265.