CLXXXV. PROBLEMS OF NITROGEN CATABOLISM IN INVERTEBRATES.

IV. THE SYNTHESIS OF URIC ACID IN *HELIX POMATIA*.

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IN the third paper of this series [Baldwin, 1935] it was pointed out that there exists among gastropods a marked correlation between the presence of arginase and the production of uric acid. Both arginase and uric acid are abundantly present in terrestrial forms, and Needham [1935] believes that the production of uric acid as the main nitrogenous end-product is an adaptation to terrestrial life, just as it is believed to be in the insects, birds and some reptiles. Whether uricotelic metabolism and the possession of arginase are independent or linked concomitants of the adaptation to terrestrial habitat is not clear, but the fact that the correlation holds even for such apparently aberrant cases as that of *Arion* suggests that arginase may actually participate in the synthesis of uric acid.

In all animals hitherto examined a high arginase activity goes hand in hand with a cyclical synthesis of urea [Krebs and Henseleit, 1932; Manderscheid, 1933]. The arginase content of the hepatopancreas of *Helix pomatia* is actually of the same order as that of the most active of mammalian livers [Baldwin, 1935], and by analogy it might be suggested that in *Helix*, as in the mammals, arginase is primarily concerned with the synthesis of urea. Baldwin and Needham [1934] attempted to demonstrate such a synthesis on the part of surviving slices of the hepatopancreas and were unsuccessful, but their negative results would be explicable if urea were in some way removed as fast as it was formed. This possibility remains open; destruction of the urea by a urease seems however to be ruled out since no evidence could be found for the presence even of traces of that enzyme in the hepatopancreas.

Now Wiener [1902] has suggested that, in the case of the bird, uric acid may arise from urea according to the following scheme:

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This view received a little experimental support from the perfusion experiments of Meyer and Jaffé [1877], Wiener [1902] himself, and Izar [1911]. Friedmann and Mandel [1908] however obtained negative results, and in more recent times the perfusion method has yielded only negative results in the hands of Russo and Cuscuna [1931] and of Cuscuna [1934]. Benzinger and Krebs [1933] and Schuler and Reindel [1933] have obtained negative results with the tissue slice technique and the subject has also been extensively studied by workers of the Italian school. Urea was injected into birds (hens, geese, pigeons), the following substances being orally administered at the same time: malonic, tartronic and lactic acids [Clementi, 1929]; pyruvic and propionic acids [Torrise and Torrise, 1931]; dialuric acid [Torrise, 1932]; glycerol and glyceric acid [Biondi, 1932]; tartronic, lactic, barbituric and dialuric acids [Russo, 1933; 1934]. In no case was any significant increase in the output of uric acid recorded, while in some of the experiments the urea was quantitatively recovered. Clementi [1930] concluded that only ammonium carbonate has any influence on the uric acid excretion, while that of urea is only affected by arginine [Clementi, 1932], and Needham *et al.* [1935] have shown that this is true also for the chick embryo. There can thus be no doubt that Wiener's scheme is entirely inapplicable to the avian organism, if only because the avian liver is unable to synthesise urea [Manderscheid, 1933].

Schuler and Reindel [1933] have found that in the Columbidae the liver and the kidney act successively in the production of uric acid, the liver producing a non-purine precursor which the kidney then converts into uric acid. Baldwin and Needham [1934] made use of this fact to find out whether the synthesis of uric acid follows the same path in the snail as in the bird, and concluded that the respective mechanisms must be quite different. Consequently there is no *a priori* reason for believing that Wiener's scheme, inadmissible as it may be for the case of the bird, is necessarily excluded in that of the snail. Wolf [1933] in fact claims to have shown that a hepatopancreas *brei* prepared from *Helix pomatia* is able to produce uric acid from urea under certain conditions, but his technique and results are open to serious criticism [Baldwin and Needham, 1934].

The present paper reports the results obtained in a study designed to test the possibility that urea may be convertible into uric acid in *Helix pomatia*, since, if this were the case, it would go far towards explaining the observed correlation between the presence of arginase and a uricotelic metabolism in the gastropods. The results are necessarily of a preliminary nature; the main problems of technique have been solved and a tentative working hypothesis is put forward. Further experiments will be carried out as soon as the material again becomes available.

EXPERIMENTS.

The method employed consisted essentially in allowing surviving slices of the hepatopancreas of well-fed specimens of *Helix pomatia* to act upon a solution containing possible precursors and seeing whether any more uric acid was produced than in control experiments. In order to find the rate of synthesis it was necessary to determine also the preformed uric acid content of a further sample of the slices.

Some experiments were first done in which no attempt was made to extract uric acid from the tissue itself. The slices were simply suspended in a suitable Ringer solution to which galactose had been added and shaken under standard conditions for 5 hours. The experimental slices received in addition 0.3–3 mg. urea or the same quantity of urea and tartronic acid, but these substances were of course omitted from the controls. After shaking the flasks were

placed on a boiling water-bath, the proteins precipitated by adding uranium acetate and the fluids filtered. Uric acid was then estimated in the filtrates and the following results were obtained:

Substance added	No. of Exps	mg. uric acid synth. per g./hr.	Increase	% Increase
Controls	—	0.0327	—	—
Urea and tartronic acid	4	0.0686	0.0359	109
Urea alone	3	0.0465	0.0138	42
Tartronic acid alone	3	0.0413	0.0086	26

Although only very approximate, these data served to show the desirability of pursuing the question.

Two main difficulties were encountered in developing the technique. In the first place the cells of the hepatopancreas contain solid concretions of uric acid (see Strohl's review in Winterstein [1914]) and it was necessary to find a method for extracting and estimating this intracellular uric acid. Simple procedures such as grinding with sand, extraction with lithium carbonate *etc.* proved unsatisfactory and the method finally adopted was the following. The tissue, usually about 1 g. moist, was placed in a small mortar and thoroughly ground with two drops of octyl alcohol; sand was added and the grinding repeated. Then 5–10 ml. of Benedict and Hitchcock's [1915] phosphate mixture were added, the whole well mixed and the sand and fragments of tissue allowed to settle. The supernatant suspension was decanted and the residue again ground up, treated with phosphate mixture, again allowed to settle and the supernatant again decanted. The residue was ground a third time and finally washed into the combined supernatants with more of the phosphate mixture. About 25 ml. of the latter were used altogether. The whole mixture was digested on a boiling water-bath for 25 mins., 7 ml. of 1.55 % uranium acetate were then added to precipitate proteins and the digestion was continued for a further 5 mins. The whole was now filtered into a measuring cylinder, the precipitate being returned to the flask and digested for 10 mins. with a further 10 ml. of phosphate mixture, again filtered and finally washed on the filter-paper with 5 ml. of phosphate mixture. In this way crystal-clear yellow filtrates were obtained which contained practically the whole of the uric acid. Samples were taken for the estimation of uric acid by the method of Folin [1930; 1933]; the standard solution always contained 0.02 mg. uric acid and the comparisons were effected with a Klett top-reading colorimeter.

The second difficulty arose in preparing Ringer solutions in which any appreciable synthesis of urea from urea and tartronic acid could be demonstrated. The results described above were obtained using a Ringer having the composition described by Baldwin and Needham [1934], but when new solutions of the same composition were prepared the "extra" synthesis resulting from the addition of urea and tartronic acid seldom exceeded 10–20 % of the "basal" synthesis which was always found, even in the controls. Following a hint previously given by Prof. H. Cardot the solutions were next made up in freshly prepared glass-distilled water and a marked improvement was found at once. It is probable that the ordinary distilled water of the laboratory contains traces of copper but that this had been precipitated from the solution used in the first experiments, since this had been made up some weeks before it was actually used. Two Ringer solutions were used; one, containing bicarbonate, was used as a medium for the suspension and shaking of the tissue slices, while the second, which was unbuffered, served to wash the tissues. Both had a calculated freezing-point depression of 0.31° and were therefore isotonic with the blood of fed

waking snails [Duval, 1930; Kamada, 1933], and the composition, given in Table I, followed that recommended by Bernard and Bonnet [1930].

Table I.

	g./litre	
	Ringer I	Ringer II
NaCl	1.16	2.84
NaHCO ₃	2.43	—
KCl	0.344	0.344
CaCl ₂ , 6H ₂ O	1.75	1.75
MgCl ₂ , 6H ₂ O	2.56	2.56

Ringer I was saturated with CO₂ before use and Ringer II with oxygen. The p_H of the bicarbonate Ringer when equilibrated with 5 % CO₂ at 28° was 7.7 which is the same as that of the haemolymph of well-fed waking snails [Baldwin and Needham, 1934], and the suitability of these solutions was shown by the fact that isolated hearts of *Helix pomatia* could be maintained in an actively contractile state for periods of at least 20 hours, *i.e.* at least four times as long as the projected experiments.

Urea and tartronic acid were added in the form of a solution containing 30 mg. of these substances per ml. and previously neutralised. Fresh solutions were frequently made up since a heavy growth of bacteria or mould usually appeared in a day or two.

The following procedure was adopted in all the experiments. Slices were prepared from the hepatopancreases of 5–7 well-fed snails. They were washed three times with Ringer II, divided into four approximately equal portions, freed from surplus water and weighed. Meanwhile four flasks of the type used by Krebs [1933] had each received 10 ml. of Ringer I and 100 mg. galactose [see Baldwin and Needham, 1934]. A gas mixture containing 5 % CO₂ and 95 % O₂ was passed through in order to bring the p_H to the required value, the tissue was dropped in and the flasks were placed in the thermostatic bath, which was electrically maintained at 28°. The flasks were usually shaken for 5 hours in all, the gas mixture being passed in for the first 20–30 mins. One flask was retained for the determination of the preformed uric acid content of the slices, a second served as a control and the other two received the substance or substances the effects of which were to be tested. At the end of the period of shaking the slices were removed from the flasks by means of forceps and ground up *etc.* in the manner already described, the extracts being added to the suspension fluid.

It should be mentioned in passing that some fragmentation of the tissue always took place as a result of shaking, although the greatest care was taken to ensure smooth running of the shaking machine. Consequently it is likely that the observed rates of “extra” synthesis were smaller than those obtaining *in vivo*.

RESULTS.

The protocol of Table II gives data obtained in a typical experiment. By “extra” uric acid is meant the amount formed in the experimental flasks over and above that found in the controls. The amounts of urea and tartronic acid added were usually 3 mg. each, which are equivalent to about 4.2 mg. uric acid, so that the yields shown in the protocol (“extra” uric acid) represent 5.2 and 4.4 % of the theoretical yields. These, though small, are certainly significant since in some experiments where the theoretical yields were only 0.3 mg. uric acid, the actual yields were still about the same, 0.15 mg., representing about 50 % in this case. It is probable that the tissue, working at its full capacity, can synthesise

Table II.

Apparatus	1	2	3	4
ml. Ringer I	10	10	10	10
mg. galactose	100	100	100	100
mg. urea added	—	3	3	—
mg. tartronic acid added	—	3	3	—
mg. uric acid found	0.625	0.728	0.632	0.374
Therefore mg. uric acid/g. tissue	0.790	1.010	0.974	0.448
Therefore mg. uric acid synth./g.	0.342	0.562	0.526	—
mg. "extra" uric acid/g. in all	—	0.220	0.184	—
mg. uric acid synth./g./hr. = R	0.0662	0.1020	0.0956	—
% increase in R	—	62	54	—

about 0.15 mg. uric acid per g. in 5 hours from urea and tartronic acid, and that it is impossible to force the pace simply by adding more of the precursors. This point is important since the production of uric acid from urea and tartronic acid is known to take place *in vitro* under suitable conditions [Wiener, 1902], but it is evident that the change observed in the present experiments is associated with the presence of the tissue and is not merely an uncatalysed reaction taking place *in vitro*.

It seems likely that the "basal" synthesis which was observed in the controls is due to xanthine oxidase. That enzyme is known to be present in the hepatopancreas of *Helix* [Przylecki, 1926; Baldwin and Needham, 1934] as are its substrates also [Wolf, 1933]. Furthermore it was found that this basal synthesis persisted even when the Ringer solutions were unsuitable for the demonstration of any "extra" synthesis, that it appears to be independent of p_H over a range of about 6–8 (within the limits of experimental error), that it continues even in *brei* and is not completely abolished by $M/10$ NaCN. An attempt was made to demonstrate the presence of xanthine oxidase by means of the methylene blue technique, but as blanks carried out on unwashed *brei* already had a reduction time of many hours it is not surprising that this attempt failed. The hepatopancreases of 10 snails were finely ground, extracted several times with Ringer II and the extract centrifuged. The centrifugate was then saturated with ammonium sulphate and filtered at the pump. Concentrated solutions of the precipitate were tested for xanthine oxidase by the methylene blue method but the reduction time again ran into many hours. Further attempts will be made to concentrate the enzyme, but meanwhile it is not unreasonable, in view of the existing evidence, to attribute the basal synthesis to the operation of the xanthine-xanthine oxidase system.

The results obtained in experiments in which urea (U) and tartronic acid (T) were added are summarised in Table III and, in graphical form, in Fig. 1. A glance at Fig. 1 shows that there is considerable variation in the rates of synthesis, and on comparison with Table III it will be seen that the variation is greatest in the experiments in which the preformed uric acid is greatest. The extra synthesis is determined as the difference between the final uric acid contents of experimental samples and the corresponding controls, and the basal synthesis as the difference between the initial and final uric acid contents of separate controlling samples. Consequently in comparing the rates of synthesis in control and experimental samples we involve a cumulative error which may be very large on account of the initial errors due to sampling, and these may themselves be large.

Table III shows that the preformed uric acid may vary from 0.1 to 1 mg. per g. tissue, and each sample here represented 6 snails on the average. The indi-

vidual variation must probably be still greater. Some determinations were made in order to evaluate the probable error due to sampling and the results are

Fig. 1. Rates of synthesis of uric acid from urea and tartronic acid. (The numbers refer to the actual experiments and A to the averages of the various groups. Note that the addition of urea alone has no appreciable effect.)

Table III.

Exp. no.	Substance added	Preformed uric acid, mg./g.	mg. extra uric acid/g.	mg. uric acid synth./g./hr.	
				Control	Experiment
13	3 mg. U and T	0.150	0.216 0.150	0.0618	0.1050 0.0918
34	„	0.448	0.220 0.164	0.0622	0.1020 0.0956
35	„	0.312	0.128 0.070	0.0676	0.0932 0.0816
36	„	0.459	0.090	0.0502	0.0682
41	„	0.198	0.074 0.111	0.0482	0.0630 0.0704
Averages (U and T)		0.313	0.136	0.0580	0.0856
37	3 mg. U	0.379	-0.054 0.110	0.0696	0.0588 0.0916
39	„	1.020	-0.010 0.050	0.0360	0.0340 0.0460
42	„	1.080	0.170 -0.110	0.0520	0.0860 0.0300
Averages (U)		0.826	0.026	0.0525	0.0577
		Controls U and T U			
mg. extra urid acid/g. in 5 hours		—	0.136	0.026	
		0.0580	0.0856	—	
mg. uric acid synth./g./hour = R		0.0525	—	0.0577	
Increase in R		—	0.0276	0.0052	
% increase in R		—	47.5	9.9	

given in Table IV. Each set of determinations was made on the mixed slices obtained from 6 snails and it was found that the greatest error is probably within 10 %, the mean variation being ± 5 %.

Table IV.

Exp. no.	mg. uric acid per g. slices	% diff. from group average
21	0.0978	-2.5
	0.1020	+1.7
	0.0930	-7.3
	0.1085	+8.2
22	0.100	-6.5
	0.109	+1.9
	0.112	+4.7
	0.106	-1.1
23	0.123	+3.4
	0.124	+4.2
	0.110	-7.6
	0.121	+1.7

Mean variation from group average = $\pm 5\%$.

In order to reduce the sampling error as far as possible the snails used in any given experiment were taken from a batch which had been awakened and kept in a uniform manner for the same length of time, but beyond this no standardisation was possible, and it was not feasible to reduce the sampling error any further. To work on only one snail, assuming that the uric acid concretions are uniformly distributed, would have made the scale of operations too small, while the alternative procedure of using a large number of snails was rejected since the preparation of slices from only 6 animals usually took at least $1\frac{1}{2}$ hours and longer waiting would certainly have been detrimental to the tissues. It was therefore necessary to accept the results as they came and to average as many data as could be obtained.

Although there is in individual cases a considerable range of variation between the rates of synthesis in the controls the average results for the two sets of controls are in excellent agreement and it is therefore unlikely that the rates of extra synthesis are seriously in error. At least five times as much uric acid was produced, on the average, when tartronic acid was present as when urea was present alone, and the results are still more striking if we compare the rates of synthesis with and without tartronic acid with the average rate of all the controls instead of with the group averages as in Table III. We then get the following:

	R	% increase in control
Controls	0.0553	—
Urea alone	0.0577	4.3
Urea and tartronic acid	0.0856	55.0

We do not know whether tartronic acid is a normal metabolite or whether it is replaceable by other 3-carbon compounds such as lactic acid. It is proposed to study these questions in future experiments. But in any case there can be no doubt that urea and tartronic acid are together convertible into uric acid by surviving slices of the hepatopancreas of *Helix pomatia*.

Such an arrangement would probably be a very convenient one from the point of view of the snail itself. Activity is associated, as Howes and Wells [1934] have shown, with high water content, and probably therefore with a flow of water through the body. If urea is synthesised from the products of protein catabolism it can be passively washed out of the body, even if no process of active secretion is brought into play, and Baldwin and Needham [1934] have already suggested that "diuresis" may play an important part in the elimination

of urea from the body. When the water current ceases the animal either aestivates or hibernates, according to the conditions, and becomes in effect a closed system reminiscent of the cleidoic egg. Urea can no longer be washed out of the body and its conversion into uric acid would obviate a twofold toxæmia; first, simple uræmia—and to judge by the data given by Wolf [1933] there is actually no great accumulation of urea during hibernation—and secondly the ammonæmia which might be produced by the action of the nephridial urease [Przylecki, 1922; Baldwin and Needham, 1934] upon accumulated urea.

As a working hypothesis, then, it seems not unreasonable to suppose that urea is synthesised in *Helix* by means of the ornithine cycle, which of course involves arginase, and is then further converted more or less completely into uric acid, probably by way of dialuric acid, the degree of conversion depending upon the condition of the animal. Such an hypothesis is in no way excluded by any of the known facts, it explains the observed correlation between arginase and uricotelic metabolism in the gastropods, and it appears to stand the test of biological fitness. Further work is necessary to establish the truth or otherwise of the hypothesis in the special case of *Helix* before its applicability to the gastropods as a whole can be considered, and further experiments will be carried out as soon as the material again becomes available.

SUMMARY.

Urea and tartronic acid are together convertible into uric acid under the influence of surviving slices of the hepatopancreas of *Helix pomatia*. The significance of this finding is discussed in the text.

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