CLXXXVI. PROBLEMS OF NITROGEN CATABOLISM IN INVERTEBRATES.

I. THE SNAIL (*HELIX POMATIA*).

BY ERNEST BALDWIN¹ AND JOSEPH NEEDHAM.

From the Biochemical Department, Cambridge.

(Received June 19th, 1934.)

I. INTRODUCTION.

IN the livers of animals having a ureotelic metabolism, as Clementi [1915; 1918] has pointed out, the enzyme arginase is present, whereas those which have a uricotelic metabolism do not possess it. This principle, though valid for members of the vertebrate phylum, has not as yet been successfully extended to the invertebrates owing to certain serious difficulties. Firstly, arginase seems to be rare in its occurrence among such animals (further work upon this distribution is now in progress); secondly, few invertebrates excrete notable quantities of urea. Clementi [1918] has examined the hepatopancreas of *Astacus fluviatilis* and *Helix pomatia* and the whole bodies of termite larvae, while Hunter and Dauphinee [1924] have further studied the caecae of a starfish (*Pisaster*), the hepatopancreas of *Cancer* and the digestive glands of a clam (*Saxidomus*). All these organs, with an exception in the case of *Helix*, proved to contain no detectable amounts of arginase. Perhaps this is not surprising, since many invertebrates rely upon argininephosphoric acid for their muscular activity [Meyerhof and Lohmann, 1928; Meyerhof, 1928; Needham *et al.*, 1932], so that high concentrations of arginase might be incompatible with the efficiency of the muscle.

For comparison with these results the findings of Delaunay [1927] are of great interest. The following figures, taken from that author, express the amounts of urea-N excreted by various invertebrates as percentages of the total nitrogenous excretion:

Invertebrates	AQUATIC ...	...	Crustacea, <i>e.g. Maia</i>	} Average 7-8 %
			Cephalopoda, <i>e.g. Sepia</i>	
			Echinodermata, <i>e.g. Echinus</i>	
	TERRESTRIAL	...	Annelida, <i>e.g. Lumbricus</i>	16 %
			Mollusca, <i>e.g. Helix</i>	22 %
			Arion	71 %
Vertebrates	...	...	Mammalia	90 %

There is thus a striking correlation between the presence of arginase in the terrestrial pulmonate gastropods and the fact that they appear to excrete a relatively large percentage of their nitrogen in the form of urea.

The enzyme arginase has long been known to hydrolyse arginine into urea and ornithine, and for many years our knowledge of its functions went no further. Birds, for example, obtain the ornithine used for their detoxication processes from arginine by means of the arginase which is present in their kidneys. Clementi [1932] has found that the whole of the small amount of urea excreted

¹ Senior 1851 Student.

by hens comes from ingested arginine, presumably by the action of arginase. But recently, thanks to the work of Krebs and Henseleit [1932], new light has been thrown on the importance of arginase [Krebs, 1934]. In the livers of mammals arginase functions, not merely as a single enzyme of high specificity performing a specific task, but as one of an organised group of enzymes by whose successive action a continuous urea production is brought about, thus:

- (i) arginine $\rightarrow$ ornithine + urea;
- (ii) ornithine + CO_2 + $\text{NH}_3 \rightarrow$ citrullin;
- (iii) citrullin + $\text{NH}_3 \rightarrow$ arginine.

The system is a cyclical one and its action depends both upon the integrity of the cell structure and the concomitant processes of oxidation. The urea production of amphibian, reptilian and human foetal livers has also been studied by Manderscheid [1933] and found to follow the same lines.

It seemed to us desirable, in view of these interesting facts, to attempt to discover the origin and the mode of formation of the urea excreted by the invertebrates and to begin this study by selecting the edible snail, *Helix pomatia*, as our material. On account of its greater relative urea excretion the slug might have been preferable but we found that, unlike the snail, it is difficult to keep in the laboratory, easily suffering lethal infection by bacteria. The snail, however, is known to be highly resistant to such invasion [Gatenby and Duthie, 1932; Gatenby and Hill, 1934] and furthermore, the amount of information already available is much greater than in the case of the slug.

But several difficulties are inherent in this material. Firstly, according to Delaunay [1927; 1931] the snail excretes some 20 % of the total nitrogen in the form of urea, but this total is itself small. Hesse [1910] states that the rate of excretion corresponds to 3.85 mg. total N per kg. (presumably excluding the shell) per hour at 17.5°, which is equivalent to about 3.6 mg. total N per snail per day under our conditions. Also, the "total" value taken by Delaunay did not represent the true total, as some uric acid was neglected in his estimations, being insoluble. The total daily output of urea was therefore very small. We found that the average excretion of a fully-fed snail is about 0.4 mg. per day; the highest value found was 3.92 mg. in the case of a snail which had just woken from hibernation and eaten prior to the collection of its excreta. The basal level, reached after 5 days' starvation, is only about 0.04 mg. of urea per day. A delicate method of estimation was therefore necessary, and this was found in that of Krebs and Henseleit [1932] which we employed with no modification save that the estimations were performed at 28° instead of 37°. Some preliminary determinations gave theoretical yields.

The second difficulty was more fundamental and more troublesome. Snail tissues, according to Przyłeki [1922], contain urease, a finding which we were able to confirm. Albrecht too [1920] found urease, and also urea, in the tissues of four marine molluscs, including one gastropod. Consequently, in attempting to find conditions under which urea formation might proceed *in vitro*, we always encountered the difficulty that urea might be broken down as well as synthesised. Fortunately no urease was demonstrably present in the hepatopancreas, as we shall see, and we were therefore able to perform many experiments with this tissue.

II. EXPERIMENTAL ANIMALS AND METHODS.

Our snails were obtained in the hibernating state from dealers. In order to waken them they were placed, epiphragm downwards, in a Petri dish containing a layer of water about 1 cm. deep. This was put in a wire cage kept inside a

special glass-fronted incubator maintained at 23–27° and 95 % humidity. Waking usually followed within 24 hours, when the snails crawled away to feed upon fresh cabbage which was kept in the cage and replaced as often as necessary.

When injection and starvation experiments were carried out the animals were kept in crystallising dishes covered by Petri dishes, the latter having previously been drilled and short glass tubes cemented over the holes. A slow stream of air could thus be passed through the dishes. A little water was placed in the bottom of each dish to keep the atmosphere moist and to aid in the collection of the excreta, the dishes themselves being kept in the incubator.

Two principal experimental methods were employed, firstly the tissue-slice technique, and secondly the injection of various substances. In the latter case, the substance to be injected was dissolved in sterile water, usually 1 ml., and injected by means of a sterile hypodermic syringe into the posterior haemocoel, passing the needle from the side of the apical end of the shell. With practice this could be accomplished with great ease, and the animals often did not even withdraw into the shell during the injection.

For tissue-slice experiments the organs were rapidly removed from the animals, care being taken to avoid contamination with slime. Adherent tissues, blood vessels *etc.* were removed and the organs gently dried on filter-paper. The slices were cut with a sharp razor blade. The hepatopancreas proved to be an exceedingly fragile tissue in the slice form, and during 3 or 4 hours' shaking about a half fragmented. The nephridium ("kidney" or organ of Bojanus [1818]), a thin walled, hollow structure, contained spherical concretions of uric acid [Strohl, 1914; Cooke, 1895]; these were removed as far as possible, and the organ was then sliced transversely. After slicing, the tissues were washed three times, for 3 minutes each time, in a special Ringer solution. After removing the excess of the washing solution, samples of the tissues were weighed out on the torsion balance; on account of the extensive fragmentation it was useless to weigh at the end of the experiment. For the same reason, in attempting to obtain a synthesis of urea *in vitro*, we did not shake the tissues in the manometer-flasks as may be done, for example, with rat-liver and kidney slices. They were instead suspended in the appropriate solutions in small conical flasks through which a special gas mixture could be passed while they were being shaken in the bath. Before taking an aliquot portion for manometric estimation, the fluid was usually filtered through glass wool. The bath was electrically maintained at 28° throughout.

Most of the work was done using a Ringer solution buffered with bicarbonate in equilibrium with the standard Warburg gas mixture (95 % O₂, 5 % CO₂). The composition of the Ringer solution followed that recommended by Bernard and Bonnet [1930]:

					g./litre
NaCl	...	...	...	...	5.87
KCl	...	...	...	...	0.73
CaCl ₂ , 6H ₂ O	...	...	...	...	3.96
MgCl ₂ , 6H ₂ O	...	...	...	...	5.62
NaHCO ₃	...	...	...	...	1.87
KHCO ₃	...	...	...	...	0.22

The freezing-point depression of this solution is $\Delta -0.74^\circ$. The terrestrial gastropods are exceptional in that they periodically undergo wide fluctuations in the tonicity of their internal medium; thus Arvanitaki and Cardot [1932] show that the osmotic pressure of *Helix* blood may decrease from $\Delta -0.47^\circ$ to

$\Delta -0.2^\circ$ after a short period of rain. Kamada, too, [1933] has demonstrated a similar difference between the body fluids of hibernating and active snails by means of vapour pressure measurements. It is therefore necessary to dilute the above Ringer-bicarbonate solution to the degree indicated by freezing-point measurements on the haemolymph of the animals in question, adding sufficient bicarbonate to make the final bicarbonate concentration $2.5 \cdot 10^{-2} M$. This concentration, suitable for mammals, seems to be not unsuitable for the snail. The gas phase in equilibrium with native *Helix* blood contains 2.9 vols. % CO_2 (as compared with 2.5 vols. % in the case of Warburg's mammalian solution). By the blood-gas pump method 6 vols. % CO_2 are found, and this is comparable with the values given in the older literature for snail blood (see *Tab. Biol.* 3, 408). By complete acidification the total CO_2 (including bicarbonate) is found to be roughly 45 vols. %, a value not far removed from the 55 vols. % of mammalian sera.

The following determinations showed that the above Ringer-bicarbonate should be diluted with its own volume of water.

	p_{H}	Δ
Ringer-bicarbonate	7.5	0.74
Snail blood (asleep) <i>H. pomatia</i>	8.03	0.37
„ „ <i>H. aspersa</i>	—	0.40
„ (waked and fed) <i>H. pomatia</i>	7.75	—
Hepatopancreas (hibernation)	6.87	—
„ (active)	6.10	—
Organ of Bojanus (hibernation)	6.92	—
„ (active)	6.60	—
Gut contents (hibernation)	5.73	—
„ (active)	5.51	—

CO_2 was passed through to saturate the solution and the slight precipitate allowed to settle. The tissue p_{H} was determined, following Meyerhof [1930], by grinding with sand at 0° and making electrometric measurements. It appears that waking from hibernation is associated with a slight change of the tissue p_{H} toward the acid side.

III. OPTIMUM CONDITIONS.

We began our investigations by studying the conditions for the optimum survival of the tissues. From 50 to 250 mg. of the moist slices of the hepatopancreas and the nephridium were taken, and their respiration was examined at 28° . This temperature is not excessive for molluscan tissues, as Chapheau has shown [1932]. The first experiments were carried out in Ringer buffered at p_{H} 7.4 with phosphate, manometer-cups of the Cremer type being employed and provision being made for the absorption of CO_2 [see Dixon, 1934]. The oxygen uptake of the hepatopancreas was linear in all cases, that of the nephridium slightly irregular. The addition of glucose stimulated the respiration considerably, that of galactose still more, while lactate had no effect:

	% increase in O_2 consumption
Controls	—
Lactate	6.4
Glucose	45
Galactose	79

In the presence of the sugars practically linear oxygen uptakes were obtained over 2–3 hours or even longer, in this case with both tissues.

We next used the indirect "improved method" of Warburg [1924] in order to compare the effects of phosphate and bicarbonate as buffering agents. Here we allowed 10–15 minutes for equilibration with the gas mixture, but after closing the taps, slight outputs of gas were observed although the gas mixture was warmed and moistened by means of coils and a bubbler immersed in the bath. The output ceased, however, after about 30 minutes and the experiments were continued for 2 hours, the oxygen uptakes being calculated over the whole period. The initial rises we attribute to glycolysis.

Our results for the hepatopancreas are summarised in Table I, which shows that bicarbonate is undoubtedly preferable to phosphate as a buffering medium.

Table I.

Medium (p_H 7.4)	$\mu\text{l. O}_2/\text{g. wet wt./hour}$		Q_{O_2}	
	Phosphate	Bicarbonate	Phosphate	Bicarbonate
Water	470	913	– 2.35	– 4.56
1 % glucose	682	900	– 3.41	– 4.50
1 % galactose	809	1043	– 4.04	– 5.21

These Q_{O_2} values are of the same order as those obtained by Chapheau [1932] in his work on another mollusc, the oyster.

It will be observed that glucose had no effect upon the respiration in bicarbonate, galactose however producing a very marked increase. This fact is of great interest in view of May's discovery [1931] that the principal polysaccharide of the hepatopancreas of *Helix* consists of "sinistrin" which, while resembling glycogen in many respects, is laevorotatory and yields only galactose on hydrolysis. Glycogen was also present, but in the eggs only galactogen was found [May, 1932].

IV. THE PRESENCE OF UREASE.

We tested the hepatopancreas and nephridium of *Helix* for urease in two different ways. In the first place tissue-slices were shaken with a known amount of urea in Ringer buffered at p_H 5 with acetate. The shaking was carried out in the cups of the manometers and a preparation of jackbean urease tipped in from a side-bulb after suitable intervals of time. Several manometers were used for each tissue and were equilibrated in the bath. The taps were then turned and one cup was tipped; the other cups at hourly intervals later. Each cup received 0.4 mg. urea, corresponding to 148 $\mu\text{l. CO}_2$, at the beginning of the experiment.

Two samples of the hepatopancreas evolved 140 and 145 $\mu\text{l.}$ respectively, being tipped at zero time. In a third manometer a very slight uptake of gas was recorded during 2 hours' shaking, followed by the production of 141 $\mu\text{l.}$ on tipping, while the fourth of the series, tipped after 4 hours, produced 141 $\mu\text{l.}$ of gas. There was thus no evolution of CO_2 during the experiment, and a quantitative recovery of the added urea (as CO_2) at the end; it is therefore evident that the hepatopancreas contains no significant amount of urease.

The nephridium, however, gave very different results. One cup, tipped at zero time, gave 180 $\mu\text{l.}$, 32 $\mu\text{l.}$ in excess of the theoretical amount. A second cup put out 124 $\mu\text{l.}$ during 2 hours' shaking, but on tipping a further 157 $\mu\text{l.}$ were evolved—a total excess of 133 $\mu\text{l.}$, apparently not involving the disappearance of any of the urea initially added. A third manometer was shaken for 4 hours, and this produced only 25 $\mu\text{l.}$ before tipping, and 144 $\mu\text{l.}$ when tipped, a perfectly quantitative recovery in this case. The interpretation of these results is difficult. Were it not for the fact that the added urea is quantitatively recovered it could

be suggested that the nephridium contains urease. As the results are so erratic it can scarcely be supposed that the pre-tipping gas production is due to pre-formed urea being attacked by kidney urease, since the tissue was thoroughly washed before being taken for the experiments. On attempting to repeat these results we found outputs of 0, 6 and 16 μ l. before tipping, and 148, 147 and 143 μ l. after tipping, the flasks being tipped after 0, 1 and 2 hours respectively.

It occurred to us that the "extra CO_2 " could be accounted for if one supposed that urea was being produced in some way during the shaking, and broken down by a urease which was in close proximity to the synthesising enzymes but which was for some reason, *e.g.* low tissue permeability, not accessible to added urea. Yet even if this were the case, some detectable amount of the added urea would surely be attacked in 4 hours. Two further experiments threw no new light on the question. In these we took kidney slices and followed their CO_2 output. After an hour and a half, urea was added to one manometer and to the other a uric acid suspension obtained by carefully washing and centrifuging the milky contents of the kidney itself. In neither case was there any divergence from the previous curve. The one common factor in all these experiments was that added urea appeared not to be attacked by the kidney tissue. In view of the ease with which the jackbean enzyme can be extracted with water we decided to see whether the kidney urease could also be so extracted, for it was just possible that the thorough washing of the tissue in the previous experiments might remove the enzyme. Even so, the gas production before tipping would still be unexplained, since even glycolysis is scarcely a probable cause.

For extracting the enzyme we took, in each of two experiments, 6 nephridia and 3 hepatopancreases. Each batch of tissue was finely ground with sand and 2 cc. of Ringer solution, the mass being kept overnight in the ice-chest. Next day the solutions were centrifuged and the centrifugates treated with 1/20 volume of acetate buffer at p_{H} 5 and again centrifuged after standing for 1 hour. Samples were now taken in the side-bulbs of the manometric flasks and added, after equilibration, to varying quantities of urea. After 7–8 hours' shaking the following volume changes had taken place in the different flasks:

	Hepatopancreas μ l.	Nephridium μ l.
Exp. 1	-0.7	+14.5
	-2.9	+ 6.5
	-2.8	+12.2
	—	+ 6.5
	—	+ 7.4
Exp. 2	+8.8	+17.3
	+0.7	+13.3
	+0.0	+15.0
	—	+ 7.4
	—	+ 9.0
Averages	+0.5	+10.9

The figures for the hepatopancreas, together with the previous results, show that urease is entirely absent from that organ. The nephridium, however, undoubtedly contains urease [see also Przyłęcki, 1922], but the amount is certainly very small. One whole nephridium, it appears, is capable of breaking down about 0.1 mg of urea in 24 hours, or, if we suppose that only 10 % of the tissue enzyme can be extracted by our method, 1 mg. in 24 hours. This being so it is not surprising that we failed to detect it in the earlier experiments. But, small though this amount may be, the daily rate of urea output by the intact snail is of the same order.

V. THE SOURCE OF THE EXCRETED UREA.

The following sources suggest themselves:

- (a) Exogenous urea.
- (b) Exogenous arginine.
- (c) Synthetic urea: (i) ornithine cycle; (ii) some other mechanism.
- (d) Urea from uricolysis.

The evidence for each of these will now be considered, first that from *in vitro* experiments and then that from the injections. On account of the slow rate of urea formation in the intact snail, long-continued shaking was necessary in the slice experiments, but the risk of bacterial infection was lessened by the immunity referred to above.

(i) *Experiments in vitro.*(a) *Exogenous urea.*

Fosse [1912; 1913; 1916] has detected small quantities of urea in plant tissues; thus germinating peas yielded 640 mg. of urea per kg., while urea was also obtained from cabbage and from endives, 10 kg. of the latter being necessary for the demonstration. Unfortunately, no figures were given for the urea content of cabbage, but it must probably be very small. We ourselves prepared an extract from 5.6 g. cabbage by maceration with sand and water, boiling, filtering and evaporating. Urea was then estimated in aliquot portions and a value of approximately 1 mg. per 100 g. wet weight of cabbage was found. Now according to Stahl [1889] an average specimen of *H. pomatia* eats about 1.5 g. of cabbage daily, corresponding to 0.015 mg. urea, whereas a fully-fed snail excretes at least 0.4 mg. urea daily. Hence the ingested urea is only about one-fortieth of that excreted.

(b) *Exogenous arginine.*

We have already noticed that the presence of arginase has been demonstrated in the hepatopancreas of *Helix* by Clementi [1918]. We were able to confirm this and to show, moreover, that the enzyme is also present in the nephridium. The method employed was firstly to shake slices of the organs for 2½ hours with arginine in Ringer-bicarbonate at p_H 7.4 containing galactose at 28° and then after removal of the tissue and bringing the fluid to p_H 5 to determine the urea content of an aliquot part; secondly to prepare a "brei" of the organ and to incubate it with arginine in phosphate buffer of p_H 8.5 at 28°, afterwards proceeding as before. In both cases it was found that a considerable amount of urea was formed by hepatopancreas and nephridium. The activities, calculated in the "Arginaseeinheiten" of Edlbacher [1925; 1929], as modified by Krebs and Henseleit [1932], were as follows:

		Arginase units μ l. urea CO ₂ made/ mg. dry wt. tiss. $\times$ hrs.
Present work:		
"Brei" at 28°:	hepatopancreas	20.2
Slices at 28°:	hepatopancreas	1.3
	nephridium	7.1
Edlbacher:		
"Brei" at 37°:	bird-liver	3
	bird-kidney	36
	mammalian liver	4000
	mammalian kidney	40
	mammalian spleen, pancreas, heart etc.	0

The values for the snail are therefore of the same order as those for avian tissues and for mammalian organs other than liver. The general level at which we had to work may be gauged from the fact that the average arginase content of mammalian liver is about two thousand times that of gastropod tissues, even when the latter are studied in the form of a "brei" near the optimum p_H of the enzyme. For use in arguments about the state of affairs in the intact snail, the results with tissue-slices are preferable. That the arginase activity found is more than sufficient to account for the urea produced by the snail in normal life is shown by the following calculation. The maximum urea formation from the arginine added to the slices would have given 1410 μ l., that actually observed gave 550 μ l., while the expected yield, assuming that the urea excreted by the intact snail was derived solely from arginine under the influence of a uniformly distributed arginase, would have been 4 μ l. Thus even if the enzyme were confined to the hepatopancreas and nephridium, it would still be able to account for the normal urea output of the body, since the former organ alone could produce in one day 14 mg. urea instead of the 0.4 mg. actually observed. This conclusion, however, involves at least three assumptions, (i) that the amount of substrate is not a limiting factor, (ii) that the mechanisms for supplying the substrate to the enzyme are not inefficient, and (iii) that the activity of the urease is not considerable.

Having thus satisfied ourselves of the presence of arginase in the snail tissues we were in a position to compare the ingestion of arginine with the excretion of urea. The latter was found by placing fully-fed snails in the cages already described for 24 hours. The excreta were collected and evaporated, urea being then estimated in aliquot portions. As the p_H of the water containing the excreta was ordinarily about 8.0, no destruction of urea can have taken place during the evaporation, while controls showed that not more than 10 % of the urea was destroyed by bacterial action during 24 hours. The results varied from 0.1 to 1.8 mg. per snail per day, the average figure from 22 snails being 0.4 mg. per snail per day. Now we have seen that, on the average, a snail eats 1.5 g. cabbage per day, with a protein content of 4 % of the wet weight [König, 1903]. Vegetable proteins contain on the average some 14 % of arginine [Osborne, 1924], and hence the daily intake of arginine must be at least 8.5 mg. per snail, which is equivalent to about 2.4 mg. urea, *i.e.* about six times the amount excreted.

It thus appears that the ingested arginine could amply account for the urea excreted if the urease activity of the snail is set aside. Przyłęcki [1922] found that at 37° 100 g. of snail tissue ("whole snail") can break down 76.8 mg. urea in 24 hours. The average weight of an individual is 25 g. [Künkel, 1916] so that at 28° the urease could break down 9.6 mg. in 24 hours. If we suppose that the urease normally works at its full capacity, the amount of urea produced daily must be equal to the amount excreted *plus* that broken down, *viz.* 0.4 + 9.6 = 10 mg. for the production of which 30 mg. of arginine would be required; less than one-third of the urea formed could now be accounted for by ingested arginine.

The position is therefore difficult. Of the activity and the efficiency of the urease *in vivo* we know nothing, and all calculations have to be based upon very rough average figures. It is possible to say, if we accept Przyłęcki's figures, that the limiting values for urea production are about 10 mg. per day (urease inactive) and 0.4 mg. per day (urease fully active), while the ingested arginine can account for about 2.5 mg. per day.

Thus the possibility of a synthesis cannot be said to be excluded. But the

hypothesis that arginine is the sole precursor of the urea formed gains some weight from a consideration of the magnitudes involved. The following data for the hen are calculated from those given by Clementi [1932], who clearly showed the correctness of this hypothesis in the case of the bird.

	Weight g.	mg. urea excreted per day			
		Total		Per kg.	
		Fed	Starving	Fed	Starving
Hen	2500	60	15	24	6
Snail	20	0.4	0.04	20	2

It will be seen that our figures for the snail are, weight for weight, of the same order. And as we have seen, the arginase activity of snail tissues closely resembles that of the birds.

If, however, we accept the view that ingested arginine is the sole source of urea in *Helix*, we are bound to bear in mind the case of the slug, *Arion*, where the urea amounts, according to Delaunay's figures, to 71 % of the total nitrogen excreted. We feel, however, that much uric acid may possibly have escaped Delaunay's analyses (see p. 1373), and that a re-examination of the nitrogen-partition of the excreta of terrestrial Stylommatophora other than *Helix* is demanded. It is of interest here that even in mammalian tissues arginine may be the sole precursor of urea in some conditions, *e.g.* autolysis (placenta: Salaskin *et al.* [1932]; liver: Solovjev and Mardarshev [1932]).

(c) *Synthetic urea.*

We next proceeded to investigate the possibility of a synthesis of urea from ammonia, whether by the ornithine cycle or by some other process. The technique consisted in shaking the organ slices, in the manner described above, with bicarbonate-Ringer to which ammonia (as NH_4Cl) and galactose had been added, aliquot portions of the final fluid being taken for urea estimations.

Neither the hepatopancreas nor the nephridium produced any urea under the conditions employed (which are summarised in Table II) nor was any urea

Table II.

Tissue	mg. wet weight	ml. Ringer bicarbonate	ml. 10 % galactose	NH_3 added mg./100 ml.	Ornithine added mg./100 ml.	Time hours
Hepatopancreas	698	10	0.2	15.9	—	3
Hepatopancreas	594	5.0	0.1	31.8	—	4
Nephridium	328	2.5	0.05	31.8	—	4
Hepatopancreas*	264	2.5	0.05	31.8	—	4
Hepatopancreas	582	2.5	0.05	31.8	—	4
Nephridium	111					
Hepatopancreas	743	5.0	0.1	31.8	20.0	4
Nephridium	140	2.5	0.05	31.8	20.0	4
Hepatopancreas	448	2.5	0.05	31.8	20.0	4
Nephridium	111					
Hepatopancreas†	1630	4.0	0.08	31.8	20.0	3½
Nephridium†	384	2.5	0.05	31.8	20.0	3½
Heart†	51	1.0	0.02	31.8	20.0	3½
Hepatopancreas	1360	4.0	0.08	31.8	20.0	3½
Nephridium	485	2.5	0.05	31.8	20.0	3½
Heart	37	1.0	0.02	31.8	20.0	3½
Foot muscle	1061	4.0	0.08	31.8	20.0	4
Gut wall	1053	2.5	0.05	31.8	20.0	4

* *Helix aspersa*—otherwise *H. pomatia* was always used.

† Organs taken from hibernating snails.

formed when the nephridium and the hepatopancreas were allowed to co-operate. The addition of ornithine had no effect.

Q_H (μ l. urea CO_2 per mg. dry weight of tissue per hour) is here never greater than 10^{-2} ; this is within the limits of experimental error and compares strikingly with the values of 1–15 found by Krebs and Henseleit [1932] on mammalian liver. Yet the Q_{O_2} of the gastropod "liver," about -5 at 28° , is quite comparable with that of mammalian liver, -6 to -14 at 37° [see Krebs, 1933].

In no case was any significant positive pressure set up in the manometer as a result of tipping in the urease. But all these experiments assumed some localisation of urea production. The rate of urea production in the intact snail is very small, as we have seen, and if urea synthesis were carried on more or less uniformly throughout the animal, then the magnitude of the effect to be expected might be within the limits of experimental error. We therefore performed another experiment in which the hepatopancreas was considered as an aliquot part of the whole body.

6.2 g. of liver slices were prepared from 10 snails and shaken with bicarbonate-Ringer, 0.2 ml. 10 % glucose, 0.5 ml. 1 % NH_4Cl and 0.08 ml. 10 % ornithine, for 6 hours at 28° under the usual conditions. The tissue remaining intact was removed and the fluid deproteinised, boiled, filtered and evaporated to 2.06 ml., the whole of which was taken for a urea estimation. In this case the maximum possible urea formation from the added ammonia was $1065 \mu\text{l.}$, and that expected if the hepatopancreas behaved as an aliquot part of the body, $15\text{--}60 \mu\text{l.}$ Only $2\text{--}3 \mu\text{l.}$ were found, a volume within the limits of experimental error. Contributory evidence confirming this was obtained in subsequent experiments.

Hence we can only conclude that the hepatopancreas is certainly neither the main seat, nor a seat, of synthetic urea formation. Nor is urea formation confined to the gut wall, heart or foot muscle. To what extent the nephridium can be held responsible it is difficult to say on account of the fact that it contains urease. Definite evidence might be obtainable by using a specific inhibitor for that enzyme and repeating in its presence experiments upon the foregoing lines, but up to the present there is no evidence for the synthesis of urea from ammonia in any of the organs examined.

(d) *Uricolysis.*

The existence of enzyme systems capable of hydrolysing uric acid to give urea has been demonstrated in a number of animals by Przyłęcki [1925], Fosse *et al.* [1930] and Stransky [1933], and we have investigated the possibility that a similar mechanism might be present in the snail. The slice technique was again employed, and experiments were performed in bicarbonate buffer as well as in 0.05 % NaOH as used by Stransky. The conditions are summarised in Table III, and none of the experiments gave any evidence of urea formation.

Table III.

Tissue	mg. wet weight	Buffer	p_H	Time hours
Hepatopancreas	2078	HCO_3^-	7.4	3
Gut wall	1122	HCO_3^-	7.4	3
Hepatopancreas	2910	0.05 % NaOH	9.5	4
Nephridium	605	0.05 % NaOH	9.5	4
Gut wall	1561	0.05 % NaOH	9.5	4

Although with an acetone powder, prepared from frog-liver according to the directions of Stransky [1933], we obtained yields of only up to 15 % urea from

added uric acid under comparable conditions, we calculated that if the uricolytic activity of the snail tissues were comparable with that of frog-liver powder, the resulting urea production should have been easily detectable.

The finding by Wolf [1933] of traces of allantoin in the nephridium of *Helix* at all times of the year might be taken to indicate the presence of uricase in the animal, were it not for the fact that allantoin has been shown to exist almost universally in plants [Fosse and co-workers, 1929-34].

It is of interest in this connection that Albrecht [1920] found uricase to be absent from the alimentary tract of all the four molluscs examined by him. The enzyme has been reported as existing in the hepatopancreas of *Aplysia* by Sulima [1914] but his evidence only consisted of slight decreases in the uric acid content of tissue "brei" after 8 hours' incubation.

(ii) *Experiments in vivo.*

The foregoing experiments led to the discovery of no substance other than arginine as the precursor of the excreted urea. The following experiments were designed to supplement and extend those carried out *in vitro*, since it was possible that the conditions employed in the latter experiments were not suitable for demonstrating a synthesis of urea.

To commence this work we took four pairs of snails and starved them, urea estimations being performed each day upon their excreta. Fig. 1 shows the rate of excretion expressed as mg. urea per snail per day, plotted against the period of starvation. It will be seen that after 6 days a basal level of about 0.04 mg. per snail per day is reached, and similar results were obtained as often as the experiment was repeated.

Fig. 1. The numbers on the curves refer to the number of snails used in the experiments.

It was originally our intention to feed the starving snails with filter-paper impregnated with a mixture of amino-acids, since the animals will readily ingest a great variety of substances, including even plasticine [Clements *et al.*, 1933]. We therefore made up a mixture of glycine, alanine, leucine, tyrosine, phenylalanine, tryptophan and glutamic and aspartic acids, arginine however being omitted. Unfortunately the animals refused to touch this food and we had therefore to use the injection technique already described. We had hoped to find, in the

proposed feeding experiments, that the starvation level of excretion would not be exceeded unless arginine were added to the diet; in the injection experiments, therefore, we decided to inject small doses of as many amino-acids as were sufficiently soluble and to compare the subsequent urea output with that following the administration of comparable amounts of arginine. We also tried the effect of giving various amounts of urea, with interesting results.

Fig. 2.

● Urea. ○ Arginine. △ Glycine.

Fig. 3.

□ Alanine. △ Glycine. ◇ Valine. ⊗ Glutamic acid.
 ◆ Aspartic acid. ▽ Tryptophan.

The snails were injected and kept individually or in groups of two or three under the conditions previously described and the excreta collected and worked up. As a rule, no further excess urea was excreted after the first 24 hours. Our data are given in Figs. 2 and 3, and are summarised in Table IV.

Table IV.

Compound injected	No. of exps. done	Total mg. N injected in all exps. = N	Total mg. N excreted in all exps. = E	Basal value mg. N = B	$\frac{E - B}{N} \cdot 100$
Arginine	14	96.4	4.45	0.28	4.3
Urea	10	192	52.2	0.20	27
Alanine	16	77.0	0.67	0.32	0.45
Glycine	5	32.6	0.86	0.10	2.3
Valine	3	14.6	0.07	0.06	0.07
Glutamic acid	1	1.62	0.014	0.02	0.0
Aspartic acid	1	0.84	0.018	0.02	0.0
Tryptophan	1	6.04	0.224	0.02	3.3

It will be seen that when due allowance has been made for a basal excretion of 0.02 mg. urea-N per snail per day, 4 % of the arginine-N injected is excreted as urea. That some arginine either leaked out or was excreted unchanged was shown by the fact that a positive Weber-Sakaguchi reaction [Weber, 1930] was obtainable on a deproteinised sample of the excreta. Of urea injected, 27 % was recovered as such. The fate of the rest we have not elucidated; some urea, presumably, must have been broken up by the tissue-urease, and on the basis of Przyłęcki's data we were able to calculate that from 5-6 mg. could be so decomposed under our conditions, and more than this would probably be broken up in the intact snail. In Exp. 13, 15 mg. of urea were injected, and 3 mg. were recovered in the first 24 hours, and it is not unreasonable to suppose that the rest may have been broken down by the animal. Przyłęcki's figures were obtained with a "brei" whereas our "enzyme preparation" consisted of the intact animal. Now Przyłęcki has suggested that urease is used by the snail for controlling the p_H of the blood, urine *etc.*, but we have found that doses of 25 mg. of urea already begin to show a definitely toxic effect; greater doses invariably resulted in the death of the animal. We have just seen that, by excretion and by enzymic hydrolysis, the snail can probably dispose of about 15 mg. of urea. It seems possible to us that the urease acts as a kind of detoxication mechanism, preventing the accumulation of urea in the animal. This suggestion is supported by a consideration of the tolerance towards substances other than urea.

About 20 mg. of arginine may be given and above this a toxic effect is noticed, whereas 50 mg. alanine, 38 mg. glycine and 55 mg. valine were administered without ill effect. One experiment, in which 44 mg. tryptophan were given, led to a curious result, for on the following day the entire contents of the nephridium had been extruded as though this amino-acid had a violently "diuretic" effect. The animal itself was perfectly well, however, and no trace of unchanged tryptophan could be found in the excreta.

Broadly considered, the results of Table IV show that arginine alone among the amino-acids leads to any considerable increase in the output of urea—about 4 % of the injected N. The case of glycine, where 2 % of the N administered was excreted as urea, owes its apparent importance to a single experiment (Exp. 40) as Fig. 3 shows, where 10 % of the N injected was eliminated as urea. Valine and glutamic and aspartic acids led to no excess urea excretion whatever. As regards tryptophan, only one experiment was performed; this compound has a violent diuretic effect, and much of the urea excreted could have come from the blood. According to Delaunay [1931] waking snail blood contains about 0.025 mg. urea N per ml., and one specimen of *Helix* contains some 4 ml. of blood [Cardot and Troussier, 1929] while the tissues generally contain more urea [Wolf, 1933; Delaunay, 1931], so that the urea excreted in this case,

0.2 mg., could be amply accounted for. Alanine evoked a slight excess excretion several times, but in general no urea seems to be formed from it.

But turning to examine individual cases (Fig. 3) we see that the mean basal level, about 0.02 mg. urea-N per snail per day, is frequently exceeded. This is, of course, to be expected on account of individual variation, but sometimes the basal level is exceeded to a very marked extent, *e.g.* in Exp. 35 where 30 mg. alanine led to the excretion of 0.167 mg. urea-N, a recovery of 3.6 %, which approaches the mean figure of 4.3 % found for arginine. Other high results will be seen in Fig. 3. In such cases as these, where the basal level is exceeded by as much as ten times, it is difficult to attribute the results to individual idiosyncrasy, and, indeed, these increases were absolutely erratic and not repeatable with any given snail.

It was observed that the exceptional rises were always greater when the injection was the first to which the animals had been subjected, and it occurred to us that perhaps carbohydrate might play an important part in any supposed synthesis of urea, as Krebs and Henseleit [1932] have shown to be the case in the synthesis performed by the mammalian liver. If this were so, progressive starvation might lead to diminution of carbohydrate reserves and correspondingly less urea production. The general failure to produce any urea from injected amino-acids would also be explicable on this hypothesis.

To test this we took 7 snails, previously starved till the basal rate of excretion was reached, gave these injections of ornithine, each snail receiving 36.7 mg. (=5 mg. N) and examined their excreta individually. Fig. 4 shows

Fig. 4.

the resulting curves for the snails which survived throughout, the broken line representing the average of all the data. Two injections of ornithine were given, the excess urea output being less for the second than for the first. After this, only three snails were still alive, and these received 50 mg. glucose in addition to the usual ornithine injection. A great increase resulted and persisted for a

second 24 hours. Now whether ornithine functions here by virtue of its potential relationship with arginine, or simply as an amino-acid was not determinable by these experiments, but they suggested a new possibility. Urea might be synthesised, not from ammonia as such, but from the amino-group of the amino-acid, by a synthetic system immediately contiguous with the deaminating system, and we therefore attempted to obtain such a synthesis *in vitro*.

Hepatopankreas slices were shaken with bicarbonate-Ringer, glucose, ornithine and alanine, urea being estimated in the final fluid after removal of the tissue. The following quantities were used:

No.	mg. tissue	Time hours	ml. Ringer	ml. 10 % glucose	ml. 10 % ornithine	ml. alanine (16.4 mg./ml.)
1	3310	6	2.0	0.1	0.04	0.25
2	6380	7	3.0	0.15	0.04	0.3
3	7600	6½	3.0	0.15	0.06	0.3

In the first of these experiments we obtained a total CO₂ output of 38 μ l. but the subsequent experiments did not confirm even this small value.

Since no positive evidence seemed to be obtainable from this source we turned again to *in vivo* experiments, attempting now to decide whether or not ornithine was involved as a catalyst in the urea production previously noticed. Starving snails each received 32 mg. alanine and 10 mg. glucose, while one-half of the animals received 5 mg. ornithine in addition. Those receiving the ornithine yielded more urea than the controls and so, when the effect of this injection had passed off, the controls received the alanine *plus* glucose *plus* ornithine, the former experimental animals now becoming the controls. There was no corresponding change over in the urea excretion. A second set of injections, in which 10 mg. glucose instead of 5 mg. were given, gave exactly the same results; whichever set of snails gave the higher results in the beginning again gave high results with subsequent injections.

It is noteworthy, however, that from a given group of snails receiving alanine a greater response was obtained when carbohydrate was given than when the amino-acid was given alone.

We have therefore to conclude that, although the ingested arginine is usually sufficient to account for the urea excreted, there is also a possibility that some urea may arise from the amino-nitrogen of ingested amino-acids, without the intervention of the ornithine cycle.

VI. THE SOURCE OF URIC ACID.

Although added ammonia leads to no urea formation we have found that uric acid is produced when ammonia is added to hepatopankreas slices shaken in bicarbonate-Ringer under our usual conditions. It was impossible to perform similar experiments upon the nephridium on account of the large amount of intracellular uric acid which is present in it, but the hepatopankreas, after thorough washing, contained only a small amount. Final estimations were made by the colorimetric method of Benedict and Franke [1922], using a Leitz compensating colorimeter, since the samples, after deproteination with acetic acid, gave green solutions with Benedict's reagent. In some experiments another difficulty was encountered in that the colours tended to fade seriously in the unknown solutions. We did not succeed in preventing this and were obliged to put the solutions into the colorimeter-cups as soon as possible after mixing.

The following data summarise the results:

Exp.	g. hepatopankreas	Time hours	mg. uric acid expected from ammonia added	mg. found	Percentage yield
1	0.6 (hibernation)	4	1.98	1.9	96
2	4.5 (hibernation)	6	3.5	2.0	57
3	3.36 (active and feeding)	7	3.5	1.2	34

It may be concluded that an almost quantitative formation of uric acid from ammonia may take place. But this cannot always be demonstrated; thus in another experiment, hepatopankreas slices incubated 6 hours with 31.8 mg. per 100 g. of ammonia gave 0.197 mg. per g. of uric acid, as opposed to 0.317 mg. produced by slices to which no ammonia had been added. It is probable that the hepatopankreas of starving animals (as in this case) is highly susceptible to the toxic action of ammonia, as has been found for the liver of the starving hen by Benzinger and Krebs [1933].

The quantity of uric acid in the nephridium is striking. At the end of hibernation the average content of a number of the kidneys was 32 mg. uric acid each, and as the average weight of a nephridium is 240 mg. the organ contains 131 mg. per g. wet weight and 660 mg. per g. dry. In other series of experiments we obtained values of 720 and 810 mg. per g. dry weight, so that it may be said that approximately three-quarters of the dry weight of the organ is uric acid. A brilliant murexide reaction is readily obtained from the nephridial contents. Marchal [1889] got 7 mg. uric acid from the nephridium or organ of *Bojanus*, but as he isolated it for weighing, his losses must have been considerable. Indeed, all the earlier knowledge of the nitrogen excretion of terrestrial gastropods, beginning with the work of Jacobson [1820, 1, 2; see Strohl, 1914; Wolf, 1933] could have led to the belief that they were primarily uricotelic. Unfortunately the experiments of the last-named author, which purport to bring the proof of this, do not carry much conviction. The amounts of the substances which he added to the hepatopankreas *in vitro* were out of all proportion to the amounts of uric acid formed. Thus 500 mg. urea with 5 g. hepatopankreas gave an increase of only 1.16 mg. uric acid in a typical experiment of 3½ hours' duration. The yield was never more than 1 %. As the hepatopankreas was in the form of a "brei" saturated with chloroform, which Krebs and Henseleit [1932] have shown to possess very restricted physiological properties, and as the samples of untreated "brei" show a variation of uric acid content of about the same order as the assumed new formations, it is difficult to escape the conclusion that the increases may be well within the experimental error. Quite similar remarks apply to the attempt by Sulima [1914] to demonstrate the synthesis of uric acid in the hepatopankreas "brei" of the opisthobranchiate, *Aplysia*.

We have attempted to penetrate further into the mechanism of uric acid formation in the snail by making use of pigeon tissues. It has recently been independently demonstrated by Schuler and Reindel [1933] and by Benzinger and Krebs [1933] that in the Columbidae there is a dissociation of the function of uric acid formation in such a way as to render necessary the collaboration of two organs, the liver and the kidney. Although the liver of the pigeon is unable to make uric acid itself from ammonia, it liberates into the blood-stream or into physiological salt solution a precursor-substance, which is converted by the kidney into uric acid. The fundamental experiment upon which this conclusion is based is that liver and kidney slices together, or kidney slices shaken in Ringer previously occupied by liver slices, produce much more (3 or 4

times) uric acid than either alone. As the xanthine oxidase content of the two tissues is quite similar, the phenomenon cannot be accounted for by the hypothesis of purine oxidation, for the uric acid formed should then be the sum of the two quotas formed in isolation. We have therefore attempted to ascertain whether the hepatopancreas of the snail contains any precursor substance which the pigeon-kidney can turn into uric acid, or conversely, whether the precursor known to be present in the pigeon-liver can be turned into uric acid by the snail-hepatopancreas, account being taken of purine oxidations. In this way it was hoped to discover whether the mechanism of uric acid formation is or is not identical in the bird and the gastropod. Schuler and Reindel [1933] have shown that the reaction which produces the precursor in the pigeon-liver is still functional in Willstätter acetone-ether powder preparations. We therefore prepared powders from pigeon-liver and from snail-hepatopancreas in the manner described by them.

For deproteinising the experimental mixtures after their period in the bath at 28° or 37° Rona-Kleinmann's 1.55 % uranyl acetate was used, and the uric acid was estimated by the method of Folin [1930; 1933]. The results are shown in Table V.

Table V.

All values in mg. uric acid per g. tissue or powder.

Exp. no.	Description	Initial	Hours shaking	Final	Difference
1	Snail-hepatopancreas powder alone	1.04	2	0.94	-0.1
	Pigeon-kidney slices alone	—	3	{ 0.353 0.391	—
	Pigeon-kidney slices in the centrifugate of snail powder and Ringer	—	3	—	{ +0.32 (Pg) +1.37 (Sn)
		{ 1.63 1.48 1.68	—	—	—
2	Snail-hepatopancreas powder alone	—	2	3.28	+1.69
	Snail-hepatopancreas powder <i>plus</i> milk xanthine oxidase	—	2	3.28	+1.69
	Pigeon-kidney slices alone	—	3	0.536	—
	Pigeon-kidney slices in the centrifugate of snail powder and Ringer	—	3	—	{ +1.13 (Pg) +1.00 (Sn)
		—	3	—	—
3	Pigeon-kidney slices alone	—	3	0.346	—
	Do. <i>plus</i> hypoxanthine (enough to make 3.15 mg. uric acid)	—	3	2.23	+1.884
	Do. <i>plus</i> xanthine (enough to make 3.15 mg. uric acid)	—	3	2.13	+1.784
4	Pigeon-liver powder alone	0.156	—	—	—
	Snail-hepatopancreas slices alone	0.091	4	0.147	+0.056
	Snail-hepatopancreas slices in the centrifugate of pigeon powder and Ringer	—	4	—	{ +0.065 (Pg) +0.025 (Sn)

N.B. The final values for the double experiments cannot be expressed in this way; the differences however are calculated both per g. of pigeon and per g. of snail, whether tissue or powder, as is indicated above by the letters (Pg) and (Sn).

The first experiment made it clear that the snail-hepatopancreas powder forms no uric acid when shaken alone in bicarbonate-Ringer solution, analogous to pigeon-liver powder. Pigeon-kidney slices, however, when shaken for 3 hours in Ringer solution previously in contact for 2 hours with snail-hepatopancreas powder, showed an increased formation of uric acid over the control kidney slices amounting to 1.37 mg. per g. snail powder. The same conclusion was reached in Exp. 2, where the increase was of quite the same order. But the possibility remained that the increase might be due, not to any precursor of

synthetic uric acid, but to the action of pigeon-kidney xanthine oxidase on substrates contained in the snail-hepatopancreas powder. That such substrates exist to a considerable extent in the latter was shown in another part of Exp. 2, where a preparation of xanthine oxidase from milk (by the method of Dixon and Kodama [1926]) was allowed to act on the powder. A uric acid formation of 1.69 mg. per g. powder was observed. The xanthine oxidase of the pigeon-kidney, however, might be of a much lower order of activity than the milk preparation, in which case the increases would have to be regarded as synthetic rather than oxidative. Exp. 3 demonstrated that this was not the case, and that the xanthine oxidase of the pigeon-kidney slices was capable of producing from excess substrate in equivalent time a quantity of uric acid of the same order as the apparent syntheses previously observed.

The same conclusions were more directly reached in the converse experiment (4) where snail-hepatopancreas slices were allowed to act upon Ringer solution in which pigeon-liver powder had been shaken, *i.e.* a mixture known to contain the precursor for avian uric acid. The results were quite negative. Whereas the apparent syntheses of Exps. 1 and 2 had been 36.5 and 33 % of the final values respectively, the apparent synthesis was here only 10 % of the final value, a figure too near to the limits of experimental error to be significant.

Summing up, then, it may be said that the hepatopancreas powder of the snail contains no precursor which the pigeon-kidney under the best conditions can turn into uric acid, apart from an ample supply of the substrates for the xanthine oxidase of the bird. Conversely, the precursor substance known to be present in and to be formed by pigeon-liver powder cannot be turned into uric acid at a detectable rate by the snail-hepatopancreas. Gastropod-hepatopancreas and avian kidney do not, therefore, produce more uric acid by synthesis together than alone, and the conclusion is reached that the formation of uric acid must proceed along a different path in the two phyla. Convergence in its chemical aspects appears not to imply the possession of a common mechanism.

The question then arises as to whether xanthine oxidase could be essential to the whole formation of uric acid by the snail. If the purine ring were synthesised at an earlier stage, with the formation of the less oxidised compounds, such as guanine and adenine, xanthine oxidase would deal, not only with the ammonia ultimately derived from nuclein catabolism, but also with that derived from the deamination of amino-acids. An active xanthine oxidase would therefore be required in the gastropod-hepatopancreas. The literature contains very little information on this matter, but Mendel and Wells [1909] failed to find the enzyme in the hepatopancreas of the marine gastropod, *Sycotypus canaliculatus*, although some uric acid was present (5.6 mg. per 100 g. wet weight). In the crustacean *Homarus*, xanthine oxidase was absent from all the tissues and blood, according to Morgan [1926]. It was therefore striking that in experiments designed to test its presence in the hepatopancreas of *Helix*, the result was convincingly positive.

	mg. hepato- pancreas taken	Hours' shaking	mg./g. in 6 hours	Difference
<i>Slices</i>				
No addition	555	6	0.317	—
plus hypoxanthine	605	6	0.597	+ 0.280
plus xanthine	760	6	0.626	+ 0.309
<i>Powder</i>				
No addition	188	4½	1.38	—
plus hypoxanthine	200	4½	1.37	0.0
plus xanthine	200	4½	1.28	0.0

(Sufficient hypoxanthine or xanthine was added to make 3.15 mg. uric acid.)

The enzyme had disappeared from the Willstätter powder, a result not surprising in view of the relative instability of the tissue-xanthine oxidases.

Further experiments on the mode of synthesis of uric acid, both in birds and gastropods, will be of great interest, and we hope to continue our work as the material becomes available. Comparison of the nitrogen catabolism of the different classes of molluscs (gastropods, lamellibranchs, cephalopods) will have particular value. Lamellibranchiate molluscs show no notable accumulation or excretion of uric acid, and their nephridial concretions are chiefly composed of calcium phosphate; "Haben wir bei den Gastropoden vor allem Harnsäure als Ausscheidungsprodukt der Emunktorien kennen gelernt, so werden wir finden, das bei den Muscheln diese Substanz als Excretionsstoff bedeutend, ja fast ganz zurücktritt, und dagegen hier positive Harnstoffbefunde zu verzeichnen sind" says Strohl [1914]. This conclusion, supported by much qualitative literature (in which perhaps the best papers are those of Letellier [1887; 1891] and Marchal [1889]), is especially significant when we remember that the Lamellibranchs are exclusively aquatic, whereas about 50 % of the Gastropod species are terrestrial (*cf.* the classification of Needham [1929]). What is the source of lamellibranch urea? Does it originate from a cyclical synthetic mechanism, or is it in all invertebrates derived from arginine alone? And of what nature is the nitrogen excretion of the purely aquatic gastropods?

SUMMARY.

1. An account is given of experiments designed to elucidate the sources and mode of formation of the excreted urea and uric acid in a terrestrial gastropod, the snail *Helix pomatia*. Manometric technique, using tissue slices and metabolic experiments on the intact animal, were the two main lines of approach. The peculiar difficulties inherent in this material are described in the text.

2. Only one-fortieth part of the excreted urea can be derived from the urea of the normal diet.

3. From *in vitro* experiments no evidence could be obtained of any mechanism synthesising urea from the products of deamination.

4. In general, the arginine of the diet appears to be responsible for the excreted urea. It is noteworthy that this is also the case in another terrestrial class, the birds.

5. *In vivo* experiments, however, show that additional urea excretion may follow the injection of amino-acids, especially if carbohydrate be administered simultaneously. This fact may indicate the existence of a synthetic mechanism, but its interpretation in terms of "diuresis" is at least equally plausible.

6. The discovery of arginase in *Helix* by previous workers has been confirmed. It is of much evolutionary interest that a portion of the ornithine cycle exists already in some invertebrates.

7. The discovery of urease in *Helix* by previous workers has also been confirmed.

8. The uricotelic character of the metabolism of this gastropod is supported by new *in vitro* experiments. But the hepatopancreas of the snail contains no precursor which the pigeon-kidney can convert into uric acid, apart from an ample supply of substrates for xanthine oxidase. Conversely, the precursor substance known to be present in and to be formed by pigeon-liver cannot be turned into uric acid by the snail-hepatopancreas. Thus the formation of uric acid from the ammonia of protein deamination probably follows different courses in the two phyla.

9. Xanthine oxidase is shown to be present in the hepatopancreas of the snail, though hitherto unrecorded from molluscs or crustaceans. It is suggested that this enzyme may be on the main path of formation of molluscan uric acid, whether derived ultimately from nuclein breakdown or from protein deamination. The catabolism of nucleoprotein alone cannot account for the quantities of uric acid present in the nephridium and from time to time excreted.

10. If the molluscan hepatopancreas can be homologised with the liver Clementi's rule does not apply outside the vertebrate phylum, since in *Helix* arginase and uricotelic metabolism coexist. On the other hand, Przyłęcki's rule, that uricotelic organisms never contain uricase, receives new support.

The thanks of the authors are due to Prof. H. Cardot for his frequent and generous counsel on the physiology of molluscs, to Dr G. Millikan for his estimations of the CO₂ of the haemolymph, to Mr N. G. Heatley for electrometric p_H determinations, to Mr James Shepherd for his cryoscopic measurements, and to Dr David Green. One of us (E. B.) is indebted to the Royal Commission for the Exhibition of 1851 for a Senior Studentship during the tenure of which this work was done. Its expenses were partly defrayed by a Government Grant from the Royal Society.

REFERENCES.

- Albrecht (1920). *J. Biol. Chem.* **45**, 395.
 Arvanitaki and Cardot (1932). *J. Physiol. Path. Gen.* **30**, 577.
 Benedict and Franke (1922). *J. Biol. Chem.* **52**, 386.
 Benzinger and Krebs (1933). *Klin. Woch.* **12**, 1206.
 Bernard and Bonnet (1930). *Compt. Rend. Soc. Biol.* **103**, 1119.
 Bojanus (1818). Oken's Isis.
 Cardot and Troussier (1929). *Compt. Rend. Soc. Biol.* **103**, 71.
 Chapheau (1932). Thesis, Bordeaux, and *Bull. Sta. Biol. d'Arcachon*.
 Clementi (1915). *Arch. Fisiol.* **13**, 189.
 — (1918). *Rend. R. Acad. Lincei*, **27**, 299.
 — (1932). *Proc. XIV Congr. Internaz. Fisiol.* Rome, 24.
 Clements, Howes and Wells (1933). *Nature*, **131**, 330.
 Cooke (1895). *Camb. Nat. Hist. Series*, **3**, 242.
 Delaunay (1927). *Trav. Sta. Biol. d'Arcachon*, 1.
 — (1931). *Biol. Rev.* **6**, 265.
 Dixon (1934). *Manometric methods*. (Camb. Univ. Press.)
 — and Kodama (1926). *Biochem. J.* **20**, 1104.
 Edlbacher (1925). *Z. physiol. Chem.* **148**, 273.
 — (1929). *Z. physiol. Chem.* **185**, 1.
 Folin (1930). *J. Biol. Chem.* **86**, 179.
 — (1933). *J. Biol. Chem.* **101**, 111.
 Fosse (1912). *Compt. Rend. Acad. Sci.* **155**, 851.
 — (1913). *Compt. Rend. Acad. Sci.* **156**, 1938.
 — (1916). *Ann. Chim.* (9), **6**, 13 and 155.
 — Brunel and de Graeve (1930). *Compt. Rend. Acad. Sci.* **190**, 79.
 — and co-workers (1929–34). *Compt. Rend. Acad. Sci.* **188 ff.**
 Gatenby and Duthie (1932). *J. Roy. Micr. Soc.* **52**.
 — and Hill (1934). *Quart. J. Micr. Soc.* **76**, 331.
 Hesse (1910). *Z. allg. Physiol.* **10**, 273.
 Hunter and Dauphinee (1924). *Proc. Roy. Soc. Lond.* B **97**, 227.
 Jacobson (1820, 1). *Deutsch. Arch. Physiol.* **6**.
 — (1820, 2). *J. Physique*, **91**.
 Kamada (1933). *J. Exp. Biol.* **10**, 75.
 König (1903). *Chem. Nahr.-Genussm.* **1**, 788. (Springer, Berlin.)

- Krebs (1933). In Oppenheimer's Handbuch der Biochemie, **1**, 863.
— (1934). *Ergebn. der Enzymforschung*, **3**, 247 ff.
— and Henseleit (1932). *Z. physiol. Chem.* **210**, 33.
Küinkel (1916). Thesis, Heidelberg.
Letellier (1887). *Arch. Zool. Exp.* (ser. 2), **5**, bis 2.
— (1891). *Compt. Rend. Acad. Sci.* **112**, .
Manderscheid (1933). *Biochem. Z.* **263**, 245.
Marchal (1889). *Mém. Soc. Zool. Franç.* **3**, 31.
May (1931). *Z. Biol.* **91**, 215.
— (1932). *Z. Biol.* **92**, 319 and 325.
Mendel and Wells (1909). *Amer. J. Physiol.* **24**, 170.
Meyerhof (1930). *Die Chemischen Vorgänge im Muskel.* (Springer, Berlin.)
— (1928). *Arch. Sci. Biol.* **12**, 536.
— and Lohmann (1928). *Biochem. Z.* **196**, 22.
Morgan (1926). *Biochem. J.* **20**, 1282.
Needham, D. M., Needham, J., Baldwin and Yudkin (1932). *Proc. Roy. Soc. Lond.*
 B 110, 260.
Needham, J. (1929). *Sci. Progr.* **23**, 633.
Osborne (1924). *The vegetable proteins*, 2nd edition. (Longmans, Green and Co.,
 London.)
Przyłęcki (1922). *Arch. Int. Physiol.* **20**, 103.
— (1925). *Arch. Int. Physiol.* **24**, 317.
Salaskin, Solovjev and Tjukov (1932). *Z. physiol. Chem.* **205**, 1.
Schuler and Reindel (1933). *Z. physiol. Chem.* **221**, 209, 232.
Solovjev and Mardashev (1932). *Z. physiol. Chem.* **205**, 1.
Stahl (1889). *Jen. Z. Naturwiss.* **22**.
Stransky (1933). *Biochem. Z.* **266**, 287.
Strohl (1914). In Winterstein's Handbuch der vergl. Physiol. **2**, 443.
Sulima (1914). *Z. Biol.* **63**, 223.
Warburg (1924). *Biochem. Z.* **152**, 51.
Weber (1930). *J. Biol. Chem.* **86**, 217.
Wolf (1933). *Z. vergl. Physiol.* **19**, 1.