NITROGENOUS EXCRETION OF AMPHIPODS AND ISOPODS

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(*Received* 17 *February* 1950)

INTRODUCTION

It is generally agreed that the most primitive type of nitrogenous excretory product, arising directly from the α-amino-N of proteins by deamination, is ammonia. In an extensive survey of the invertebrates, Delaunay (1927) was able to account for more than 50% of the total non-protein nitrogen (N.P.N.) excreted in terms of ammonia in a large number of unrelated aquatic species, including the sipunculid, leech, crayfish, crab and cuttlefish. Delaunay showed further that the nitrogenous excretion of terrestrial invertebrates is closely analogous to that of terrestrial vertebrates, in that the predominant end-product may be either urea or uric acid. He suggested that the synthesis of these more complex molecules should be regarded as an adaptive mechanism for the detoxication of ammonia, necessitated by a reduced circulation of water through the body: 'Les processus d'uréogénèse et d'uricogénèse... représentent des mecanismes chimiques d'adaptation qui évitent l'accumulation de corps ammoniacaux toxiques dans le milieu intérieur et sont ainsi nécessaires pour la vie terrestre' (Delaunay, 1934). This concept of a correlation between environmental conditions and the nature of the main end-product of nitrogen metabolism has found much support, as is shown, for example, in a recent comprehensive review by Florkin (1945).

Adaptations to terrestrial conditions have been acquired independently in several phyla, including the annelids, molluscs, arthropods and chordates, and in all cases so far studied ammonia is replaced at least in part by some less toxic end-product, usually urea, uric acid or even guanine. In order to obtain a closer insight into the correlation of the nitrogenous excretion with habitat, it is particularly relevant to study those groups which morphologically are closely related and yet distributed over a variety of habitats. Interesting results have been obtained in this connexion for the chelonian reptiles and gastropods. In the case of the Chelonia, Moyle (1949) was able to demonstrate a biochemical separation into three groups closely corresponding to three types of environment in which these reptiles are found. In the excreta of semi-terrestrial species urea predominates, whereas the desert forms are essentially uricotelic, although the excretion of urea has not been entirely suppressed. The secondarily aquatic forms, on the other hand, have to some extent reverted towards ammonotelism, urea and ammonia being produced in approximately equal quantities.

The problem of nitrogen katabolism in gastropods (Needham, 1935) was investigated indirectly by a study of the uric acid content of the nephridia and other tissues on the supposition that a uricotelic metabolism would be associated with some retention of this end-product. It was found that the uric acid content is high in terrestrial as opposed to aquatic forms, and although significant amounts of uric acid were present in a few aquatic species, in most of these a terrestrial ancestry can be traced on morphological grounds.

The class Crustacea is primarily aquatic, but certain representatives of the Isopoda and Amphipoda inhabit different levels of the sea-shore while others, the woodlice, have established themselves on land. Since the nitrogenous excretion of these animals appears to have received no attention so far, if was felt that a detailed study of this small and comparatively uniform group was particularly desirable. A survey of the nitrogenous excretion of a variety of species of amphipods and isopods, including marine, fresh-water and terrestrial forms, was therefore undertaken.

Table 1. *Description of amphipod and isopod species investigated*

Species	Order	Habitat	Locality	Experimental medium
Gammarus locusta (1)	A	ML	Near low-water mark among weeds and under stones	100 % SW
Marinogammarus marinus (2)	A	ML and E	In association with *Gammarus locusta*, also higher up on the shore and in estuaries	80 % SW
Marinogammarus pirloti (2)	A	ML	Near high-tide mark, sometimes in small streams where conditions alternate between the salinity of undiluted sea water and practically pure fresh water. Also found in large clusters under completely exposed stones	100 % SW, 60 % SW and 30 % SW
Gammarus zaddachi (1)	A	E	In upper regions of estuaries; able to tolerate very low salinities	30 % SW
Orchestia sp. (shore-hopper)	A	ST	At high-water mark, under weeds and stones	100 % SW
Ligia oceanica (shore-slater)	I	ST	Just above high-water mark in rock crevices; sometimes in association with terrestrial woodlice	100 % SW
Oniscus asellus (3)	I	T	The most primitive of the terrestrial isopods, restricted to damp environments; most frequently beneath bark of rotting wood	—
Porcellio laevis (3)	I	T	Dampish environments, usually among vegetable rubbish near domestic dwellings	—
Armadillidium vulgare (3) (common pill wood-louse)	I	T	Under wood and stones; able to withstand drier habitats than any other of the species studied, possibly due to its ability of rolling up in a ball	—
Gammarus pulex	A	FW	In slow-moving streams	Tap water
Asellus aquaticus	I	FW	In mud and weed of slow-moving streams and stagnant ponds	Tap water

For species nomenclature see (1) Spooner (1947), (2) Sexton & Spooner (1940), (3) Webb & Sillem (1906). Habitats are represented by ML, marine littoral; E, estuarine; ST, semi-terrestrial; T, terrestrial; FW, fresh water. SW denotes sea water; in the case of the semi-terrestrial species this was used for moistening the dish only. A, I, denote amphipod and isopod respectively.

MATERIAL

Since large numbers of animals were required in order to obtain sufficient amounts of nitrogenous excreta for analysis, it was possible to study only the most commonly occurring species. The marine littoral and estuarine forms were collected in the neighbourhood of Plymouth, while the terrestrial and fresh-water species were obtained in Cambridgeshire. A description of the species studied is given in Table 1.

ANALYSIS OF EXCRETA

Collection of excreta

Freshly collected animals were used whenever possible, in order to avoid the problems of feeding and keeping the animals in a healthy condition in the laboratory. The excreta were usually collected for one or two consecutive periods of 24 hr., during which time the animals received no food.

The aquatic species were kept in a cool, dark place in enamel dishes covered with lids and containing shallow water of the composition indicated in Table 1. Where sea water was used, this was previously passed through a bacterial filter-candle; micro-Kjeldahl determinations showed that it was then entirely free from nitrogenous substances. At the end of each experiment the water was filtered to remove the faeces, moulted skins and other debris. In several cases the solid matter thus removed was extracted and the extracts tested for uric acid, the most insoluble nitrogenous end-product likely to be encountered. Consistently negative results were, however, obtained, and it seemed improbable, therefore, that any nitrogenous excretory matter was removed by this preliminary filtration. The filtrate, usually 200–250 ml., was acidified to about pH 4 (acid to bromcresol green) and then concentrated on a boiling water-bath to a final volume of 20–50 ml. All the substances estimated were found to be stable to this treatment with the exception of uric acid, which is destroyed completely. For estimations of this compound it was therefore necessary to concentrate a separate small portion of the original sample under reduced pressure. Preliminary experiments showed that uric acid can be totally recovered if a sample of 50 ml. is concentrated to 5 ml. provided the temperature of the water-bath does not exceed 60° C.

The semi-terrestrial forms proved rather more difficult to handle. Nicholls (1931) appears to have kept *Ligia* alive during several weeks of total submersion, but in our own experiments casualties were rather high even during 24 hr. This may have been due to the inevitable crowding. The animals were therefore kept in moistened enamel dishes covered with dark lids, and washed before and after the experiment. The washings of the dish and animals after the experiment were collected for analysis. Preliminary experiments, in which the animals were placed in a large conical flask with a circulation of air passing through, showed that no volatile ammonia is lost in this way. In the case of *Orchestia*, the shore-hopper, the difficulties of keeping the animals alive during the experiment proved even greater. Complete submersion invariably resulted in a high mortality, and it was difficult to prevent

the animals from escaping if kept in any other way. Even when they were placed in a moist breffet with a few clean stones the casualties often amounted to 20%. The most successful method, which, unfortunately, was adopted only in the last few experiments, was to line the bottom of the breffet with several layers of damp muslin and a few clean stones. The animals, stones and muslin were washed thoroughly at the end of the experiment.

The terrestrial species were kept in glass dishes inside closed containers through which air was circulated. Before entry into the dish the air was bubbled through dilute sulphuric acid to remove any volatile bases, and after exit through a solution of boric acid to trap any volatile ammonia produced during the experiment. It was found that some 10–30% of the total ammonia excreted was usually given off in a volatile form. Before the experiment the animals were washed and dried on filter paper. They were again washed after the experiment in order to collect any adhering excretory material. The solid faecal pellets were scraped out of the dish, ground up with very dilute hydrochloric acid in a mortar, mixed with the washings of the dish and animals, brought rapidly to the boil, cooled and filtered. A control experiment showed that no uric acid is lost in this way. The filtrate was concentrated if necessary.

All samples of excreta which were not analysed immediately were stored in the refrigerator after addition of a few drops of chloroform. However, the possibility of bacterial involvement during the course of the experiments is a factor to be reckoned with but difficult to allow for. Since the experiments were conducted at relatively low temperatures and for a period of not more than 1 day, we do not consider that bacterial interference can have appreciably altered the true nitrogen partition data.

Analytical methods

Protein precipitation was carried out by the method of Folin & Wu (1919). Care was taken to ensure that the final pH of the solution was more acid than pH 2·8 (Merrill, 1924).

Ammonia N was estimated by steam distillation in Markham's apparatus (1942). The distillate was collected in a 2% (w/v) boric acid solution containing the mixed indicator recommended by Conway & O'Malley (1942) and subsequently titrated with N/70-HCl. This method is not entirely specific for ammonia, since any other volatile bases present will be included in the estimation. The only other volatile base which might reasonably be expected in this material is trimethylamine, particularly as Norris & Benoit (1945) have demonstrated the apparently universal occurrence among members of the Crustacea of trimethylamine oxide. However, the peculiarly repugnant smell of trimethylamine was never observed, and we believe that the volatile N was, in fact, ammonia throughout and refer to it as such.

Total N was estimated by the micro-Kjeldahl method. Samples were incinerated for 4 hr. with 2 ml. conc. H_2SO_4 and the catalyst of Chibnall, Rees & Williams (1943), and the ammonia thus formed was estimated by distillation in Markham's apparatus (1942). When high concentrations of salt were present in the sample, care had to be taken to remove the HCl by vigorous boiling in the presence of conc. H_2SO_4, and rather more conc. H_2SO_4 was usually added in these cases. Even so, it was sometimes

found that a little HCl distilled over into the boric acid, and it was then necessary to redistill the distillate.

Urea N was estimated as ammonia by steam distillation in Markham's apparatus (1942) after incubation with the urease extract of Cole (1937) at about pH 5 (purple coloration with 3 drops bromcresol green and 4 drops methyl red indicator solutions). In the presence of high concentrations of salt the urease tended to become inactivated, and in such cases an equal volume of water was added to the sample prior to incubation.

Amino-acid N was determined by treatment with ninhydrin at pH 2·5 according to the method of Sobel, Hirschman & Besman (1945). The ammonia thus liberated was steam distilled as described above instead of being removed by aeration, as recommended by Sobel *et al.* (1945).

Uric acid was estimated colorimetrically by the method of Brown (1945), with slight modifications. The colour development appears to vary markedly with temperature and with the volume of the solution used for the estimation. The method finally adopted was therefore as follows. The sample was measured into a 25 ml. volumetric flask, and distilled water was added if necessary to obtain a final volume of 5 ml., 2 ml. each of the cyanide and urea solutions, followed by 1 ml. of the phosphotungstic acid reagent, were then added as described by Brown (1945). The solution was left in an incubator at 37° C. for 1 hr. and then made up to volume. The maximum colour development is thereby attained, and the colour remains stable for several hours. Readings were taken with a photoelectric colorimeter, using a red filter (Chance OR/2 at Plymouth and Ilford 608 at Cambridge). An approximately straight-line relationship was found to hold for values from 0·00 to 0·02 mg. uric acid, and care was taken to work within this range. The calibration curve was made using Folin's standard uric acid solution (1930).

Guanine and xanthine were precipitated with silver sulphate by the method of Gulland, Jordan & Threlfall (1947) and subsequently estimated colorimetrically with the phenol reagent of Folin (1927) as used by Hitchings (1941).

Allantoin was estimated by the method of Young & Conway (1942). Uric acid gives a similar colour reaction amounting to one-eighth of that obtained with an equivalent weight of allantoin. We therefore ignored the traces of colour which sometimes occurred with samples where uric acid was known to be present.

Results

The total soluble N.P.N. varied over a wide range in the course of the experiments. In general, the results of experiments yielding less than 1 mg. N.P.N. were discarded, since it was felt that partition data based on such small totals could not be relied upon in view of the experimental errors inherent in the analytical methods employed. In addition, we regard as unreliable all results obtained from experiments involving a high mortality, since in such cases a considerable proportion of the soluble N.P.N. is in all probability due to the disintegration and partial autolysis of dead organisms. It was found, for example, that a high percentage of casualties was often associated with exceptionally high values for amino-acid N. However, when dealing with large

populations, a few casualties are almost inevitable, but in most of the results which are presented here the deaths amounted to less than 1% and never more than 10% of the total.

The results are shown in Tables 2, 3 and 4, according to the respective habitats of the species. For comparative interest the wet weight of animals used in each experiment is recorded together with the total soluble N.P.N. obtained. In

Table 2. *Nitrogen partition in excreta of marine littoral and estuarine species*

Species	Exp. no.	Time (hr.)	Weight (g.)	Total N (mg.)	% NH_3-N	% Amino-acid N	% Urea-N	% Uric acid N	% Non-dialysable N	% Total N accounted
Gammarus locusta (A)	1	24	11	4·7	91	8	0	0	4	103
	2	41	11	9·3	74	5	3	0	2	84
	3	22	12	4·6	67	7	0	—	4	78
	4	24	11	6·8	87	7	0	—	3	97
	Av.	28	11	6·4	80	7	1	0	3	91
Marinogammarus marinus (A)	1	24	34	4·2	68	4	0	0	18	90
	2	28	19	2·0	81	4	0	—	0	85
	3	27	11	1·1	64	8	0	—	10	82
	4	21	11	1·4	75	4	0	—	4	83
	5	21	19	2·0	87	4	0	—	7	98
	Av.	24	19	2·1	75	5	0	0	8	88
Marinogammarus pirloti (A)	1	23	24	13·1	79	2	0	0	1	82
	2	23	30	11·7	92	1	0	—	3	96
	3	23	20	3·9	92	0	0	—	2	94
	4	23	18	3·2	86	4	0	—	2	92
	5	23	36	7·4	81	9	0	0	2	92
	6	24	24	8·6	90	0	0	—	3	93
	7	24	20	3·4	82	0	0	—	2	84
	8	24	18	4·1	96	0	0	—	2	98
	9	24	36	7·1	82	0	0	—	3	85
	Av.	23	25	6·9	87	2	0	0	2	91
Gammarus zaddachi (A)	1	26	11	4·1	88	2	4	—	4	98
	2	21	11	4·7	88	0	0	—	1	89
	3	21	8	5·0	73	6	0	—	4	83
	4	24	8	6·7	84	4	0	0	4	92
	Av.	23	10	5·1	83	3	1	0	3	91
Orchestia sp. (A)	1	22	34	6·5	51	26	3	0	7	87
	2	28	33	3·4	91	7	0	0	9	107
	3	25	30	5·5	62	12	0	—	6	80
	4	24	38	6·8	78	6	0	—	9	93
	5	21	38	6·6	66	6	0	—	11	83
	Av.	24	35	5·8	70	11	1	0	8	90
Ligia oceanica (I)	1	28	68	18·8	79	9	0	0	3	90
	2	24	91	9·2	58	13	0	—	6	77
	3	28	71	10·1	83	10	0	—	3	96
	4	25	30	4·8	89	5	0	—	3	97
	5	26	56	6·6	97	4	0	—	—	101
	6	23	54	3·1	73	3	0	—	—	76
	7	24	55	8·5	99	0	0	—	—	99
	Av.	25	61	8·7	83	6	0	0	4	91

addition the total non-dialysable N was estimated in most cases. This was done by dialysing a deproteinized aliquot for 24–48 hr. against running tap water and subsequent determination of the total N of the sac contents. This analysis was included since it seemed possible that a large proportion of the total N might be present in the form of products arising from incomplete digestion of the food proteins and partial autolysis of dead specimens. The results showed, however, that the percentage of non-dialysable N was usually relatively low, and a further investigation of the

nature of this fraction was therefore abandoned; there is in any case no reason at present to believe that this fraction contains compounds which could be regarded as true end-products of metabolism.

Guanine and allantoin were absent throughout and are therefore not included in the tables.

Marine littoral and estuarine species (Table 2). A very uniform type of nitrogenous excretion was revealed by these forms. About 90% of the total N.P.N. was accounted for in the six species studied, and the predominant nitrogenous constituent was ammonia in every case, the average values for each species ranging from 70 to 87% of the total N.P.N. The amino-acid N was variable but on the whole was lower in the aquatic than in the semi-terrestrial species, although even in the latter the values seldom exceeded 10%. Traces of urea were occasionally found, but it must be pointed out that the methods used for the estimations of urea and amino-acids are subject to rather large experimental errors; both depend on a relatively small titration over and above the sum of that due to volatile N in the sample and the

Table 3. *Nitrogen partition in excreta of terrestrial species*

Species	Exp. no.	Time (hr.)	Weight (g)	Total N (mg.)	% NH_3-N	% Amino-acid N	% Urea-N	% Uric acid N	% Non-dialysable N	% Total N accounted for
Oniscus asellus (I)	1	24	38	1·8	44	12	0	5	14	75
	2	26	60	0·9	50	0	0	4	38	92
	Av.	25	49	1·4	47	6	0	5	26	84
Porcellio laevis (I)	1	26	50	2·0	58	2	0	2	10	72
	2	24	50	1 3	56	0	0	6	20	82
	Av.	25	50	1·7	57	1	0	4	15	77
Armadillidium vulgare (I)	1	24	50	2·0	59	0	0	4	—	63
	2	24	57	2 6	51	11	0	15	—	77
	3	26	58	2·1	55	7	2	6	—	70
	Av.	25	55	2·2	55	6	1	8	—	70

urease or ninhydrin blanks, and these are relatively large. We therefore feel justified in concluding that urea is at most a trivial end-product in these species.

Uric acid was present only in traces and never exceeded 0·1% of the total N.P.N.

Terrestrial species (Table 3). These proved the most difficult to handle, since the total amount of N.P.N. found in relation to the weight of animals used was always small. Roughly 50 g. woodlice were required to obtain 1–2 mg. total soluble N.P.N. Consequently it was possible to do only a few experiments, and these are not altogether conclusive. The N.P.N. recovered amounted to about 80% of the total, or 65% if the non-dialysable N is ignored. No obvious difference was observed in the behaviour of the three species studied. Roughly 50% of the N.P.N. was in the form of ammonia. Amino-acids were present in small concentrations only. Urea was absent throughout; the trace found in *Armadillidium vulgare* is within the limits of the probable experimental error. Some uric acid was always found, but this accounted for only 5–10% of the total N.P.N.

Fresh-water species (Table 4). The major excretory product was again found to be ammonia in both the fresh-water species studied, but an interesting deviation from

the other aquatic species described was discovered in this group. *Gammarus pulex*, which structurally is closely related to the marine and brackish *Gammarus* species, consistently produced small amounts of urea. The average value amounted to only 10% of the total N.P.N., but is almost certainly significant nevertheless. Uric acid, however, was entirely absent. In *Asellus aquaticus*, on the other hand, urea was never detected, but with this species some uric acid was always observed, although the mean of all experiments is only 5% of the total N.P.N. However, owing to the great sensitivity of the method for uric acid determination, this value is considerably more reliable than those which depend upon the steam distillation of ammonia.

Table 4. *Nitrogen partition in excreta of fresh-water species*

Species	Exp. no.	Time (hr.)	Weight (g)	Total N (mg.)	% NH_3-N	% Amino-acid N	% Urea-N	% Uric acid N	% Non-dialysable N	% Total N accounted for
Gammarus pulex (A)	1	24	19	3·4	73	0	9	0	20	102
	2	25	18	5·1	69	0	6	0	13	88
	3	25	17	4·7	82	2	5	0	7	96
	4	25	19	5·8	66	2	6	0	10	84
	5	24	19	3 3	58	6	15	0	12	91
	6	24	20	4·1	71	10	10	0	7	98
	Av.	25	19	4 4	70	3	9	0	12	93
Asellus aquaticus (I)	1	24	9	3·6	66	4	0	9	—	79
	2	24	6	1·9	71	11	0	4	—	86
	3	24	11	2·1	55	3	0	5	—	63
	4	25	16	3·4	60	21	0	4	—	85
	5	23	16	2·9	56	10	0	1	13	80
	Av.	24	12	2·8	62	10	0	5	13	79

URIC ACID CONTENT OF WHOLE ANIMALS

Introduction

In view of the unexpected discovery that even in the terrestrial species the major excretory component is ammonia, it was decided to follow Needham's (1935) technique of extracting uric acid from whole animals, in order to discover whether a significant retention of uric acid occurs in any of the species studied. Such a retention might lead to false conclusions with regard to the nature of the main end-product of nitrogen metabolism.

Methods

The procedure adopted for the extraction of uric acid was as follows. Approximately 5 g. animals were ground up in a mortar with a little clean sand. Phosphate mixture (Benedict & Hitchcock, 1915) was then added and the whole thoroughly ground, transferred to a beaker, boiled gently for exactly 5 min. and then cooled rapidly. After centrifuging, the precipitate was once more ground with hot phosphate mixture, boiled for 3 min., cooled and centrifuged. The combined supernatants were diluted to 70 ml. 7 ml. of this extract were then made up to 10 ml. after the addition of 1 ml. each of the protein precipitants, resulting in a final concentration of 5 g. wet weight of tissue in 100 ml. After centrifuging off the precipitate, samples were taken for uric acid determinations. These proportions were adhered

to as far as possible, though in some cases greater dilutions were used as noted in the table.

Several experiments were carried out in order to check the reliability of this procedure:

(1) *Completeness of extraction.* The residues remaining after the extraction of 5 g. *Armadillidium vulgare* were ground up once more with hot phosphate mixture, boiled, cooled and centrifuged. The uric acid present in this second extract amounted to only 1% of that in the first.

(2) *Specificity of colour reaction.* Throughout the literature a general dissatisfaction is expressed with respect to the specificity of methods for the determination of uric acid which depend upon direct colour development in extracts of body fluids or tissues. It was therefore decided to investigate first of all the amount of residual colour remaining after incubation of the extract with urico-oxidase, prepared according to the method of Block & Geib (1947). This treatment resulted in complete removal of chromogenic substances in extracts of *Asellus aquaticus*, *Porcellio laevis* and *Oniscus asellus*. A small amount of residual colour was obtained with *Armadillidium vulgare*, but this was always less than 10% of the total chromogenic material and may have been due to incomplete oxidation of uric acid by the enzyme.

(3) *Recovery of uric acid.* Preliminary experiments were designed to investigate the effect of the presence of deproteinized tissue extracts on colour development with the phosphotungstic acid reagent. Thus 1 ml. of a deproteinized extract of *Oniscus asellus* gave a reading equivalent to 0·004 mg. uric acid. When 0·01 mg. uric acid was added, the reading obtained was equivalent to 0·014 mg. indicating that the intensity of colour had been neither enhanced nor inhibited appreciably. The average recovery from three such experiments using 1 ml. extract was 98%. With 2 ml. extract the average recovery in two experiments was 86%, indicating that some inhibition of colour development occurs if samples larger than 1 ml. are employed.

Finally, uric acid was added before the beginning of the extraction. Thus 5 g. *Gammarus pulex* were ground up in a mortar, and before continuing with the usual extraction procedure 1 ml. of Folin's standard solution (1930), containing 1 mg. uric acid, was added. Previous experiments had shown that the uric acid content of *G. pulex* amounts to 0·06 mg./g. wet weight of tissue, and the result was therefore calculated on the assumption that 100% recovery is equivalent to 1·3 mg. uric acid. In actual fact 1·2 mg. uric acid was extracted, giving a recovery of 92%. In a second experiment using *Oniscus asellus* the uric acid recovery amounted to only 79%. These results do, however, indicate that, in spite of its instability to heat, only a small proportion of uric acid is lost during the extraction procedure.

For the purposes of the present investigation the accuracy of the method seemed entirely sufficient, since it was desired merely to ascertain whether large differences of uric acid content exist between the various species.

Results

Table 5 shows that the results were remarkably uniform for the marine littoral and estuarine species, varying from 0·06 to 0·1 mg. uric acid/g. wet weight of tissue.

A similarly low uric acid content is found in one terrestrial species, *Oniscus asellus*, and in one fresh-water species, *Gammarus pulex*. On the other hand, *Armadillidium vulgare* contains approximately ten times as much uric acid as *Oniscus asellus*. The position of *Porcellio laevis* is a little uncertain, but on the whole it appears to conform with the group in which the uric acid content is low. However, the fresh-water hog slater, *Asellus aquaticus*, contains at least five times as much uric acid as *Armadillidium vulgare* and approximately one hundred times as much as its fresh-water associate, *Gammarus pulex*.

Table 5. *Uric acid content of whole animals*

Species	Habitat	Weight (g.)	No.	Approximate size	Final vol. extract (ml.)	Uric acid content mg./g. wet weight of tissue
Marinogammarus marinus (A)	ML	5·5	—	Random	100	0·07
		6·3	—	Random	100	0·07
Marinogammarus pirloti (A)	ML	3·6	—	Random	100	0·10
		2·7	—	Random	100	0·10
Orchestia sp. (A)	ST	5·1	—	Random	100	0·08
		5·5	—	Random	100	0·08
Ligia oceanica (I)	ST	5·1	—	Random	100	0·08
		5·2	—	Random	100	0·06
Oniscus asellus (I)	T	5·0	—	Random	100	0·08
		5·5	—	Random	100	0·09
		5·2	78	Random	100	0·09
Porcellio laevis (I)	T	5·0	—	Random	100	0·22
		5·1	83	Random	100	0·06
		5·0	70	Random	100	0·09
		2·5	17	Large (1·5–1·7 cm.)	50	0·13
Armadillidium vulgare (I)	T	5·1	—	Random	100	0·9
		2·7	45	Random	50	0·7
		4·9	—	Relatively small	100	0·7
		4·5	—	Relatively large	100	1·2
		0·8	182	Very small (0·3–0·5 cm.)	50	0·4
		1·1	7	Very large (1·3–1·5 cm.)	100	1·3
Gammarus pulex (A)	FW	5·4	—	Random	100	0·06
		5·2	—	Random	100	0·06
Asellus aquaticus (I)	FW	1·9	—	Random	40	6·5
		1·1	60	Random	100	8·1
		1·2	70	Random	100	6·7
		0·5	75	Small (max. length 0·7 cm.)	50	6·3
		0·5	18	Large (*c.* 1 cm.)	50	6·1
		0·5	19	Large (*c.* 1 cm.)	50	6·3

There appears to be no accumulation of uric acid with age in *Asellus aquaticus*, although more data are necessary in order to clarify this point. Some increase in uric acid content with size was, however, observed in *Armadillidium vulgare*, but when compared per unit body weight, the largest animals studied contained only three times the quantity extracted from the very smallest. The ratio for the average weights of the individuals in the two size groups is 1:36, which implies that the

protein turn-over involved in the building up of the body tissues of the larger animals must be very considerable. It would appear, then, that the amounts of uric acid retained in *A. vulgare* are relatively insignificant, and it cannot therefore be supposed that the nitrogen metabolism is essentially uricotelic even in this terrestrial species.

DISCUSSION

The six species studied at Plymouth were selected from a variety of habitats on the sea-shore and in estuaries, but, nevertheless, a very close similarity in the nitrogen excretion of these forms was evident. The nitrogen partition data leave no doubt that all are essentially ammonotelic. It therefore appears that even semi-terrestrial species, such as, for example, *Ligia oceanica*, which is frequently to be found in exposed and sunny places well above high-tide mark, have become adapted to this mode of life without concomitant modifications of the primitive ammonotelic type of metabolism.

Turning to the two fresh-water species we find that ammonia is again the major nitrogenous component of the excreta, but here either urea or uric acid occurs as a minor constituent. The amounts excreted (some 10% urea for *Gammarus pulex* and 5% uric acid for *Asellus aquaticus*) are relatively small, and it seems probable, therefore, that these components originate from the breakdown of purines rather than synthetically from the α-amino-N of proteins. If this were the case, it might be conceived that there exists among fresh-water organisms a tendency towards the loss of one or more of the enzymes involved in complete uricolysis, and that this modification has reached a more advanced stage in *A. aquaticus* than in *Gammarus pulex*.

The major excretory component of the woodlice proved also to be ammonia, amounting to some 50% of the total N.P.N. Considerably higher figures (70–90% NH_3-N) were obtained with the semi-terrestrial and aquatic species, but we believe that this difference is apparent rather than real and due in large part to the difference in the procedures adopted for the collection of the excreta. It seems highly probable that the more thorough extraction of the faecal pellets in the case of the terrestrial forms resulted in the appearance of a higher proportion of nitrogenous extractives derived from incompletely digested food materials. Thus it is perhaps significant that the non-dialysable fraction was markedly higher for the terrestrial species.

If, therefore, the nitrogen partition data of the excreta alone are considered, the terrestrial Isopoda must without doubt be regarded as ammonotelic, although small amounts of uric acid (5–10%) were present among the excreta. Now Needham (1935) has pointed out that uric acid excretion in certain snails is sporadic and occurs in large quantities at infrequent intervals. In such a case the analysis of the excreta, especially if these are collected over short periods of time only, might lead to misleading conclusions with regard to the identity of the major excretory component. However, even if such an erratic excretion of uric acid were typical also of the woodlice, it is very improbable that a major nitrogenous excretory product would be masked when large populations, rather than single individuals, are investigated in the course of a 24 hr. experiment, especially if, as in the present work, several such experiments

are performed. It must therefore be concluded that uric acid constitutes only a minor excretory product in terrestrial isopods and might again conceivably arise as the result of a loss of uricolytic enzymes, in particular of urico-oxidase.

A very striking difference was observed between the terrestrial species on the one hand and the semi-terrestrial and aquatic on the other, in the total amount of nitrogen excreted. Table 6 summarizes the mean values of mg. N.P.N./10 g./24 hr. excreted, for all experiments of each of the species studied. The variability of the results is considerable, but this is to be expected, since the level of nitrogen excretion must presumably depend largely upon the state of nutrition of the animals when collected, and some seasonal variation may also be involved. Table 6 does, however, show clearly that the total N.P.N. is very much smaller in the terrestrial than in any of the other species studied. It seems possible, therefore, that the woodlice have become adapted to a terrestrial mode of life, not by elaborating a non-toxic nitrogenous end-product, but rather by means of a general sparing or suppression of protein metabolism. Such a metabolic adaptation would be analogous to that found in the cleidoic egg (Needham, 1942).

Table 6. *Mean values for total N.P.N. excreted*

The figures in brackets denote the lowest and highest values respectively, which were obtained in individual experiments. For habitat abbreviations see Table 1.

Species	Habitat	No. of experiments	mg. N/10 g./24 hr.
Gammarus locusta (A)	ML	4	4·9 (4·2–6·2)
Marinogammarus marinus (A)	ML and E	5	1·1 (0·8–1·5)
Marinogammarus pirloti (A)	ML	9	2·9 (1·7–5·7)
Gammarus zaddachi (A)	E	4	6·0 (3·4–8·4)
Orchestia sp. (A)	ST	6	2·0 (0·8–3·4)
Ligia oceanica (I)	ST	9	1·3 (0·6–2·4)
Oniscus asellus (I)	T	2	0·3 (0·1–0·5)
Porcellio laevis (I)	T	2	0·3 (0·3–0·4)
Armadillidium vulgare (I)	T	3	0·4 (0·3–0·4)
Gammarus pulex (A)	FW	6	2·3 (1·7–2·9)
Asellus aquaticus (I)	FW	5	2·6 (1·9–2·9)

The relatively low level of nitrogen excretion in the terrestrial species might, however, be due to a partial retention of the nitrogenous end-products. It was for this reason that we investigated the uric acid contents of several of the species at our disposal. As expected, the marine littoral and estuarine species revealed a uniformly low uric acid content, ranging from 0·06 to 0·1 mg./g. wet weight of tissue, but when we turned to the terrestrial isopods interesting differences were observed. The uric acid content of *Oniscus asellus* falls within the low range obtained with the semi-terrestrial, marine and estuarine species. Whereas the values for *Porcellio laevis* are rather more variable, they are scarcely higher than those for *Oniscus asellus*, but *Armadillidium vulgare* contains about ten times as much uric acid as *Oniscus asellus*.

Considerations of morphological adaptations to aerial respiration indicate that *Oniscus* is the most primitive genus among terrestrial isopods, and there is no doubt that *Armadillidium* is the best adapted for a terrestrial existence. *Porcellio* occupies

an intermediate position, and it is conceivable that other species of this genus might have given rather higher uric acid values, for *P. laevis* itself frequents exceptionally damp habitats. Then again Waloff (1941) has demonstrated in a comparative study of the humidity reactions of *Oniscus asellus*, *Porcellio scaber* and *Armadillidium vulgare*, that the resistance to desiccation is greatest in *Armadillidium* and least in *Oniscus*.

An interesting correlation is therefore to be found between the degree of morphological and physiological adaptation to a terrestrial mode of life and the uric acid content of the body tissues. It seems more plausible, however, to attribute this increased uric acid retention in the more xerophilous species to a reduced rate of excretion, rather than to a fundamental difference in metabolism. Even for *Armadillidium vulgare*, the amounts of uric acid accumulated in the course of a lifetime are too small to suggest that ammonotelism has here been replaced by uricotelism. Our results indicate that the amount of uric acid accumulating in the course of one year is at most 0·3 mg. uric acid N/g. fresh weight of tissue. If we assume that the ammonia and uric acid excretion is roughly constant throughout the year, we can calculate that the total uric acid N excreted and stored in one year amounts to not more than one-fifth of the ammonia-N excreted during the same period of time.

If then a higher uric acid content is associated with more complete adaptation to terrestrial conditions, one would anticipate a uniformly low uric acid content in aquatic species in general. This is the case for the marine littoral and estuarine species as well as one of the two fresh-water species examined here, namely, the amphipod, *Gammarus pulex*. But in our other fresh-water form, the isopod, *Asellus aquaticus*, we found 5–7 times as much uric acid as in *Armadillidium vulgare*, and it is difficult to understand this extraordinary retention, in particular as there is apparently little or no increase with age. Indeed, it seems doubtful whether we are justified in the conclusion that uric acid in this species is derived solely from purines. It would therefore seem worth while to search for a uricogenic mechanism in *Asellus aquaticus*. If it is found that this species is capable of synthesizing uric acid *de novo*, it would then be desirable to investigate also the terrestrial Isopoda in order to decide whether these forms also possess a weak uricogenic system.

It is of interest to compare our data for the uric acid content of the tissues with those obtained by Needham (1935) using gastropods. Our values for *Armadillidium vulgare* are closely similar to those for some typically terrestrial pulmonates, as, for example, *Helix aspersa* and *Helicella itala*, although others, such as *H. virgata* and *Cochlicella acuta*, may contain three times as much and *Helix pomatia* even more than this. Furthermore, the uric acid content of *Asellus aquaticus* is greater than that of any of the gastropods studied by Needham (1935) with the exception of *Cyclostoma elegans* and possibly also *Helix pomatia*. It is known that *Cyclostoma* possesses 'concrementary glands' which contain dense deposits of uric acid (Meyer, 1925), and perhaps it is significant that large groups of cells filled with white 'urinary concrements' are characteristic also of *Asellus aquaticus* (Zenker, 1854; Němec, 1896). In both cases there appears to exist a specialized site for the storage of excretory substances which is separate from the excretory organs.

It seems, then, that our values for the uric acid content of isopods are similar to

Needham's (1935) data for gastropods, and it becomes evident that the whole problem of nitrogen metabolism in this latter group requires reinvestigation in order to decide whether Needham's assumption, that a uricotelic metabolism as indicated by a uric acid retention in the tissues, is in fact justified. In particular, it seems desirable to study the nitrogen partition of the excreta over long periods of time so as to determine whether ammonia is perhaps a major end-product of nitrogen metabolism even in some of the more xerophilous gastropods.

There is no doubt that uric acid is a metabolic end-product both in the terrestrial isopods and in *A. aquaticus*, but only future work can decide upon its origin. From the data here presented we incline to the view that the occurrence of uric acid in these isopods is due solely to a loss of the uricolytic enzymes, and that, in fact, all the species examined are essentially ammonotelic. We have not, however, eliminated the possibility that the terrestrial species might store some nitrogenous end-product other than uric acid, such as, for example, guanine. However, no trace of this was detected in the faecal pellets, and there exists no morphological evidence for the presence of any obvious organ of storage in the terrestrial Isopoda.

The embryonic development of the terrestrial Isopoda takes place inside a brood pouch, the latter being filled with fluid which is utilized to increase the volume of the developing young (Verhoeff, 1920). Ammonotelism in this group would then be in agreement with Needham's (1929) generalization: 'The main nitrogenous excretory product of an animal depends upon the conditions under which the embryos live, ammonia and urea being associated with aquatic prenatal life, and uric acid being associated with terrestrial prenatal life.' It is, however, very uncertain whether this generalization can in fact be applied to the invertebrates, since our knowledge with regard to the degree of cleidoicity in embryonic development is very deficient in most cases. The eggs of many insects, for example, possess specialized structures adapted for water absorption (Wigglesworth, 1939).

The occurrence of ammonotelism among semi-terrestrial and terrestrial species raises the problem of the degree of toxicity of ammonia in invertebrates. Sumner (1937) has shown that ammonia is indeed highly toxic for mammals and birds, the lethal level being reached with a blood concentration of 5 mg./100 ml., and Conway & Cooke (1939) came to the conclusion that ammonia, if present at all, does not exceed concentrations of 1 part in 10 million in the blood of the fowl, rabbit and man respectively. Similarly, Florkin (1943) found that the blood of certain poikilothermic vertebrates and also some insects (Florkin & Frappez, 1940) is almost free from detectable ammonia. This is not, however, the case for some molluscs and crustaceans, values ranging from 0·6 to 1·9 mg. NH_3-N/100 ml. having been observed in the snail, lobster and crayfish (Florkin & Renwart, 1939; Florkin & Frappez, 1940). Furthermore, according to Morgulis (1923), a single specimen of the prawn, *Panulirus argus*, can survive an injection of as much as 0·5 g. ammonium sulphate, and blow-fly larvae, as is well known, can tolerate exposures to relatively high concentrations of ammonia (Brown, 1938). It is clear that a more detailed study of the extent of the toxicity of ammonia towards invertebrates would be most valuable, and, in view of our results, determinations of the blood ammonia concentrations of *Ligia*

and the terrestrial Isopoda should be of interest. For it might be argued that the lower invertebrates are less susceptible to the toxic effects of ammonia than the more highly evolved insects and vertebrates, and that it is for this reason that ammonotelism is a possibility even among terrestrial representatives of the lower invertebrates.

SUMMARY

1. The nitrogen excretion of eleven species of amphipods and isopods, including marine, fresh-water and terrestrial forms, has been studied.

2. All species are essentially ammonotelic, since more than 50% of the total soluble N.P.N. of the excreta was present in the form of ammonia throughout.

3. The level of nitrogen excretion is appreciably lower in the terrestrial species than in any of the others, indicating that, in this group, adaptation to terrestrial conditions has been attended by a general suppression of nitrogen metabolism rather than by a transformation of ammonia to other, less toxic products.

4. Some 5–10% of the total soluble N.P.N. was present as urea in the case of the fresh-water amphipod, *Gammarus pulex*, and as uric acid in the terrestrial isopods as well as the fresh-water isopod, *Asellus aquaticus*. It is suggested that these minor excretory components might originate from purines as a result of the loss of one or more of the uricolytic enzymes.

5. In association with the excretion of uric acid some retention of this insoluble compound usually occurs, and it was found that among the terrestrial species the amount so stored parallels the degree of morphological and physiological adaptation to terrestrial conditions. The greatest accumulation of uric acid was, however, observed in the fresh-water species, *A. aquaticus*, and although such a storage cannot necessarily be taken as evidence for a partially uricogenic metabolism, this possibility must be borne in mind.

We wish to thank Prof. E. Baldwin for his continual interest and advice, and the Department of Scientific and Industrial Research for grants during the tenure of which this work was carried out. We are particularly grateful to Dr F. S. Russell, F.R.S., and the staff of the Marine Biological Laboratory, Plymouth, for putting the facilities of the laboratory at our disposal and for the most helpful co-operation in the collection and identification of the material. Our thanks are also due to Mr G. Drury and Mr J. F. Henderson, of the Zoological Laboratory, Cambridge, for assistance in the collection of the fresh-water species, and we are especially indebted to Dr Sidnie Harding, F.R.S., for valuable advice on zoological matters.

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