J. Exp. Biol. (1965), **42**, 299–305
Printed in Great Britain

NITROGEN EXCRETION IN ASCIDIACEA

II. STORAGE EXCRETION AND THE URICOLYTIC ENZYME SYSTEM*

BY IVAN GOODBODY
Department of Zoology, University of the West Indies, Jamaica

(*Received* 24 *July* 1964)

INTRODUCTION

In an earlier paper (Goodbody, 1957) I have drawn attention to the fact that ascidians, like most aquatic invertebrates, excrete the bulk of their nitrogenous wastes in the form of ammonia. Nevertheless, many ascidian species store substantial quantities of nitrogenous substances, usually purines or related compounds, within the tissues of their bodies and this led many of the earlier workers in the field to believe that this was the principal method of excretion in these animals.

Storage excretion probably occurs in all species of ascidians and may take several forms. The majority have granule-containing blood cells termed nephrocytes in which the inclusion is believed to be a purine. In the family Ascidiidae numerous small vesicles develop around the alimentary canal and in each vesicle there is a single concretion bathed in vesicular fluid. In some Styelidae and Pyuridae similar small concretions develop within the tissues of the animal, notably in the mantle, around the alimentary canal and in the subendostylar region. In Molgulidae there is a single large concretion enclosed in a renal sac adjacent to the pericardium. For a full review of the occurrence of these concretions see Azéma (1937) and Goodbody (1954).

The concretions have been variously described as containing purines, uric acid and carbonates. Kupffer (1872) first described concretions containing uric acid in *Ascidia complanata*. (This species is unidentifiable in later literature, see Herdman (1891)); Kupffer's identification of uric acid was based on a murexide test. Hertwig (1871), working with *Phallusia mammillata*,† found that the concretions were partly soluble in acids with an intense evolution of gas, a small residue remaining; he considered that the concretions were largely composed of carbonate. Roule (1884) also records the presence of urates and carbonates in the concretions of Phallusidae but does not say how the results were obtained (Roule's Phallusidae corresponds to the families Corellidae, Ascidiidae and Hypobythiidae of Berrill's (1950) classification). Azéma (1928) was unable to obtain certain results with the murexide test in Ascidiidae because of the difficulty of separating the concretions from the tissues, but in a later paper he regards guanine as being the main constituent of the concretions of *Ascidia pellucida*.

In the Molgulidae Lacaze-Duthiers (1874, 1877) identified uric acid in the concretion of *Molgula manhattensis* and refers to the dark colour at the centre of the

* Contribution No. 361 from the Bermuda Biological Station.

† Throughout this paper nomenclature of Old World species follows Berrill (1950) except in the case of *Microcosmus sulcatus* Coquebert, synonyms for which are found in Harant & Vernières (1933). Nomenclature of New World species follows Van Name (1945).

concretion as being due to inorganic material. Azéma (1937) also identified uric acid in the concretion of *M. manhattensis* and found it had an ash content of about 16 % of the total weight of the concretion. Das (1948) made a further analysis of the concretion of *M. manhattensis* and records it as containing uric acid, xanthine, guanine and creatinine.

In *Polycarpa pomaria* Roule (1885) records a positive murexide reaction for granules surrounding the intestinal region and concludes that they are uric acid. Sulima (1914) made a quantitative determination of purines and uric acid in *Microcosmus sulcatus* and obtained values of 0·2 % uric acid and 0·3 % purines (calculated as xanthine) in the whole body tissues. Azéma (1928) also identified xanthine in *M. claudicans* and *M. sulcatus*. Finally Karrer, Manunta & Schwyzer (1948) found that the extracted concretions of *M. sulcatus* contained uric acid (59·6 %), 2-amino-6-8-dihydroxy purine (39·6 %) and xanthopterine (0·8 %).

It is clear from the foregoing review that purines in some form or other, and notably uric acid, occur in the renal concretions and granules of at least some species of ascidian. The present paper contributes some new information about the concretions in Ascidiidae and also attempts to demonstrate that storage excretion results from a deficiency in the uricolytic enzyme system. Publication of this material has been delayed because of the difficulties and uncertainties in proving the absence of certain enzymes; as others are now entering this field of research it seems desirable to present the available information.

THE RENAL CONCRETIONS

Renal concretions from four species of ascidian have been examined and their uric acid content has been determined. *Ascidiella aspersa* (Müller), *Ascidia nigra* Savigny and *Phallusia mammillata* (Cuvier) all belong to the family Ascidiidae and have numerous small concretions each enclosed in a separate vesicle. In *Molgula manhattensis* (DeKay) there is a single large concretion in a renal sac; this concretion is readily dissected out whole. The concretions of the Ascidiidae may also be obtained free of tissue by the following method. The vesicular tissue is carefully dissected away from the alimentary canal and frozen solid. Repeated thawing and refreezing coupled with gentle grinding or trituration during thawing breaks up the cellular vesicles and frees the concretions. The latter are relatively heavy and by repeated washings in distilled water the tissue debris can be decanted while the concretions sediment to the bottom of the container.

Experimental procedure

(*a*) Uric acid was extracted from the concretions by the method of Needham (1935) using the phosphate mixture of Benedict & Hitchcock (1915). The uric acid was then determined colorimetrically by the method of Benedict & Franke (1922).

(*b*) The method of Markham & Smith (1949) was used for chromatographic analysis of the concretions of *A. aspersa* and *M. manhattensis*.

Ascidiella aspersa

I have obtained no clear evidence for the presence of uric acid or purines in quantity in samples of concretions from this species. The bulk of the concretion is composed of calcium carbonate. Murexide tests for uric acid give negative results and in chroma-

tograms uric acid is shown to be present below the 2 % level; the chromatograms show no trace of other purines. Colorimetric determination of the uric acid demonstrates it to be present below the 0·5 % level. Micro-Kjeldahl determinations give a value of 0·1 % nitrogen in the concretion. On heating at 45° C. there is a loss of 25 % by weight. As there is so little nitrogen present this loss must be due to water and carbohydrates or fat. Fat is known to accumulate in the vesicles of some ascidians and may account for the loss on heating.

Unlike the concretions from the other species studied those of *A. aspersa* dissolve readily and rapidly in dilute acids with intense evolution of gas. They are insoluble in alkali. A spectrographic analysis carried out for me by Dr D. J. Swaine showed that calcium was the major cation and carbonate the major anion present in samples, although there were traces of other cations and small quantities of sulphate and phosphate. An X-ray examination carried out by Dr W. Mitchell showed that calcite was present in the concretions.

It is clear that the renal concretions in the samples of *A. aspersa* studied do not contain significant quantities of uric acid or other nitrogenous wastes and that they are principally composed of calcium carbonate in the form of calcite.

Ascidia nigra *and* Phallusia mammillata

As the concretions in both these species are similar thay may be treated together. The results of several analyses are given in Table 1. It will be noted that in both species more than half the weight of the concretion may be accounted for as uric acid. The remaining fraction of the material is still undetermined but two important observations may be made. Uric acid contains 33·5 % nitrogen: on this basis the nitrogen of the uric acid in *A. nigra* would account for 18·3 % of the weight of the concretion. The total nitrogen is only 19·14 % and we may therefore safely conclude that no other nitrogenous waste product of importance is contained within the concretion. Similarly, in *P. mammillata* the nitrogen content is consistent with the view that uric acid is the only nitrogenous product of significance in the concretion.

Table 1. *Composition of renal concretions in* Ascidia nigra *and* Phallusia mammillata

(Values are given as a percentage of the total weight of concretion.)

	A. nigra	*P. mammillata*
Uric acid*	55	62·5
Nitrogen†	19·14	22·61
Total carbon†	28·65	25·79
Organic carbon‡	27·9	24·75
Carbonate‡	0·5	0·5
Calcium‡	4·7	5·6

* Uric acid determined personally.
† Analyses by Pascher of Hamburg.
‡ Analyses carried out by E.-L. Böhm and T. Goreau.

In view of the quantity of calcium carbonate in the concretions of *Ascidiella aspersa* it seemed that the remaining portion of the concretions of *Ascidia nigra* and *P. mammillata* might also be calcium carbonate. However, the analyses made by Bohm and Goreau show that carbonate is present only in insignificant traces but that there are

small amounts of calcium. Possibly the calcium is bound to uric acid in the form of calcium urate.

Molgula manhattensis

Previous analyses were made by Azéma (1937) and Das (1948). Das recorded the presence of xanthine, guanine and uric acid in the concretion. I have been able to detect only uric acid on chromatograms and this has been determined colorimetrically as comprising 47 % of the total weight of the concretion.

THE URICOLYTIC ENZYME SYSTEM

In view of the large quantities of ammonia excreted by some ascidians (Goodbody, 1957) it is reasonable to assume that storage excretion, where it occurs, is the result of incomplete purine metabolism resulting from a deficiency in the enzyme system. There is only one previous record of an attempt to identify uricolytic enzymes in an ascidian. This was Przylecki's (1926) work on *Ascidia mentula* in which he considered that both uricase and xanthine oxidase were present. I have given reasons elsewhere (Goodbody, 1954) for doubting his conclusions on uricase although xanthine oxidase is probably present.

Table 2. *List of enzyme systems investigated in different species of ascidians, showing those systems found to be present*

(Ac = allantoicase; An = allantoinase; U = uricase; Ur = urease; X = xanthine oxidase.)

Species	Enzyme systems tested	Enzyme systems present
Perophora bermudensis	Ac, X	—
Clavelina oblonga	Ac, X	—
C. lepadiformis	Ac, An, U	—
C. picta	An, U, X	—
Polyclinum aurantium	Ac, An	—
Ecteinascidia conklini	X	—
E. turbinata	Ac, An, U, X	—
Asciduella aspersa	Ac, An, U, Ur, X	—
Ascidia nigra	Ac, An, U, X	X
A. curvata	X	X
Eudistoma olivaceum	U, X	—
Botryllus planus	U, X	—
Polyandrocarpa tincta	X	—
Styela partita	X	—
Polycarpa obtecta	A, U, X	U, X
Microcosmus exasperatus	U, X	—
Herdmania momus	X	X
Pyura vittata	U, X	X
Ciona intestinalis	Ac, An, U, Ur, X	X
Molgula manhattensis	Ac, An, U, X	X

Using the methods of Florkin & Duchâteau (1941, 1943) I have tested whole animals and portions of the tissues of a wide variety of ascidian species for xanthine oxidase, uricase and to a smaller extent allantoinase, allantoicase and urease. In Table 2 is given a list of all species tested, together with the enzyme systems examined in each one. From this whole list only uricase and xanthine oxidase have ever been

identified for certain in any species. Uricase has only been identified in *Polycarpa obtecta*; this in itself is curious as this is one of the styelid species which accumulate uric acid in their tissues. Xanthine oxidase has been identified in seven species (*Ascidia nigra*, *A. curvata*, *Polycarpa obtecta*, *Herdmania momus*, *Pyura vittata*, *Ciona intestinalis* and *Molgula manhattensis*). With the exception of *Ciona intestinalis* all of these are species which are known to accumulate uric acid in vesicles or tissue spaces. On the other hand, all of the enzymes are absent from *Ascidiella aspersa* in which I have demonstrated that the concretions are composed of calcium carbonate, and they are also absent from *Microcosmus exasperatus* which accumulates large quantities of yellow granules, probably xanthine.

CONCLUSIONS

I have already stated that the results from tests for uricolytic enzymes are equivocal because of the difficulties of proving a negative result. Nevertheless, we are now in a position to make some general statements concerning storage excretion and purine metabolism. The present work confirms that uric acid is the principal stored product in *Ascidia nigra*, *Phallusia mammillata* and *Molgula manhattensis*; there is also sufficient evidence quoted in the introduction to show that the same condition exists in several other species, while in others, particularly *Microcosmus polymorphus*, purines are accumulated. In addition I have identified uric acid in the tissues of *Polycarpa obtecta*, *Pyura vittata* and *Herdmania momus* and have extracted as much as 1·5 mg. of uric acid from the subendostylar region of a large specimen of *H. momus*. The accumulation of purines and uric acid in some form appears, then, to be a general phenomenon in ascidians.

If the conclusion in my first paper (Goodbody, 1957) is correct, that protein metabolism is ammonotelic, then these purine accumulations must be specially synthesized for a particular purpose or be the end product of nucleo-protein and purine metabolism. The latter is the more reasonable hypothesis and is supported by the experiments on enzymes. With the exception of *Polycarpa obtecta*, which nevertheless accumulates uric acid, uricase is absent in all species tested. With the exception of *Ciona intestinalis* xanthine oxidase was only found in species known to accumulate uric acid. The logical conclusion is that ascidians accumulate and store purine bases and uric acid because they do not possess the requisite enzymes for metabolizing them further. The question now arises as to why this condition should exist; most other animals with an ammonotelic protein metabolism also break down their purine bases to ammonia, and the condition found in ascidians is exceptional. It is not possible at present to answer this question but it raises another; is it possible that these stored products play some significant role in the life of the animal? In this connexion it is worth recording that in Jamaica I have found *Herdmania momus* occurring in two forms; usually the test is firm, and there are numerous calcareous spicules and well-developed atrial muscles, but occasional specimens are found in which the test is soft, and spicules and musculature are poorly developed. Concretions of uric acid are more abundant in the former than in the latter. Furthermore, Hertwig (1871) found that the concretions of *Phallusia mammillata* were partially soluble in acids with intense evolution of gas, thus suggesting the presence of carbonates. In the present

work no carbonates were detected in concretions of this species. Is it possible that physiological phases occur in which the purines are utilized or accumulated? This would seem to be the next most profitable line of research to follow in this field.

SUMMARY

1. The evidence for the occurrence of storage excretion in ascidians is reviewed. Most species probably store uric acid or purine bases in some form.

2. The renal concretions of *Ascidia nigra* and *Phallusia mammillata* contain 50–60% uric acid, the remainder of the concretion is unidentified but is non-nitrogenous and is not calcium carbonate. In *Ascidiella aspersa* the concretion is predominantly composed of calcium carbonate and there is no significant quantity of uric acid or purine base.

3. Uric acid is also identified in *Molgula manhattensis*, *Polycarpa obtecta*, *Pyura vittata* and *Herdmania momus*.

4. Storage excretion probably results from a deficiency in the uricolytic enzyme system. It is concluded that while protein metabolism is ammonotelic, purine metabolism is uricotelic or xanthotelic.

The early part of this work was carried out at the Department of Natural History in the University of Aberdeen, and it was later extended at the University of the West Indies and at the Bermuda Biological Station. I am grateful to colleagues in both Aberdeen and Jamaica for advice and criticism, to Drs D. J. Swaine and W. Mitchell of the Macaulay Institute in Aberdeen for analyses mentioned in the text and to Dr T. F. Goreau and Mr E.-L. Böhm of the University of the West Indies for analyses of carbonate and calcium in *A. nigra* and *P. mammillata*. The work in Bermuda was supported in part by grant no. G 797 from the National Science Foundation of America and by a travel grant from the Carnegie Foundation administered by the University College of the West Indies. The work was also supported in part by a grant from the Nuffield Foundation.

REFERENCES

AZÉMA, M. (1928). Quelques aspects de l'excrétion chez les Ascidies. *C.R. Ass. Anat.* **23**, 14–25.

AZÉMA, M. (1937). Recherches sur le sang et l'excrétion chez les Ascidies. *Ann. Inst. océanogr. Monaco*, **17**, 1–150.

BENEDICT, S. R. & FRANKE, E. (1922). A method for the direct determination of uric acid in urine. *J. Biol. Chem.* **52**, 387–91.

BENEDICT, S. R. & HITCHCOCK, E. H. (1915). On the colorimetric estimation of uric acid in urine. *J. Biol. Chem.* **20**, 619–27.

BERRILL, N. J. (1950). *The Tunicata*. London.

DAS, S. M. (1948). The physiology of excretion in *Molgula* (Tunicata, Ascidiacea). *Biol. Bull., Woods Hole*, **95**, 307–19.

FLORKIN, M. & DUCHÂTEAU, G. (1941). Sur la distribution zoologique de la xanthine-oxydase *Bull. Acad. Belg. Cl. Sci.* **27**, 174–8.

FLORKIN, M. & DUCHÂTEAU, G. (1943). Les formes du système enzymatique de l'uricolyse et l'évolution du catabolisme purique chez les animaux. *Arch. int. Physiol.* **53**, 267–307.

GOODBODY, I. (1954). Nitrogen excretion in Ascidiacea. Ph.D. Thesis, Aberdeen.

GOODBODY, I. (1957). Nitrogen excretion in Ascidiacea. I. Excretion of ammonia and total non-protein nitrogen. *J. Exp. Biol.* **34**, 297–305.

HARANT, H. & VERNIÈRES, P. (1933). *Faune de France*. **27**. *Tuniciers. Fascicule* 1: *Ascidies*. Paris.

HERDMAN, W. A. (1891). A revised classification of the Tunicata, with definitions of the orders, suborders, families, subfamilies and genera, and analytical keys to the species. *J. Linn. Soc.* (*Zool.*), **23**, 558–652.

HERTWIG, C. W. T. R. (1871). Beiträge zur Kenntnis des Baues der Ascidien. *Jena. Z. Naturw.* **7**, 74–102.

KARRER, P., MANUNTA, C., & SCHWYZER, R. (1948). Über ein vorkommen von Purinen und eines Pterins in einer Ascidienart (*Microcosmus polymorphus*). *Helv. chim. acta* **31**, (5), 1214–18.

KUPFFER, C. (1872). Zur Entwicklung der einfachen Ascidien. *Arch. mikr. Anat.* **8**, 358–96.

LACAZE-DUTHIERS, H. (1874). Les Ascidies simples des côtes de France. Pt. 1. *Arch. Zool. exp. gén.* (Series I), **3**, 119–74, 257–330.

LACAZE-DUTHIERS, M. (1877). Les Ascidies simples des côtes de France. Pt. 2. *Arch. Zool. exp. gén.* (Series I), **6**, 457–675.

MARKHAM, R. & SMITH, J. D. (1949). Chromatographic studies of nucleic acids. I. A technique for the identification and estimation of purine and pyrimidine bases, nucleosides and related substances. *Biochem. J.* **45**, 294–8.

NEEDHAM, J. (1935). Problems of nitrogen catabolism in invertebrates. II. Correlation between uricotelic metabolism and habitat in the phylum Mollusca. *Biochem. J.* **29**, (1) 238–51.

PRYZLECKI, ST. J. (1926). La répartition et la dégradation de l'acide urique chez les invertébrés. *Arch. int. Physiol.* **27**, 157–202.

ROULE, L. (1884). Recherches sur les Ascidies simples des côtes de Provence (Phallusiadées). *Ann. Mus. Hist. nat. Marseille.* **2**, Memoir no. 1, 1–270.

ROULE, L. (1885). Recherches sur les Ascidies simple des côtes de Provence. *Ann. Sci. nat. Zool.* (Series 6), **20**, 1–229.

SULIMA, A. (1914). Beiträge zur Kenntnis des Harnsaurestoffwechsels niederer Tiere. *Z. Biol.* **63**, 223–44.

VAN NAME, W. G. (1945). The North and South American Ascidians. *Bull. Amer. Mus. Nat. Hist.* **84**, 1–476.