J. mar. biol. Ass. U.K. (1959) 38, 27–32
Printed in Great Britain

A PORPHYRIN PIGMENT IN THE INTEGUMENT OF *ARION ATER* (L.)

By G. Y. KENNEDY

Cancer Research Unit, University of Sheffield

MacMunn (1886) extracted a porphyrin from the integument of the starfish *Asterias rubens*—then known as *Uraster rubens*—and he considered that this and similar pigments which he had obtained from the slug *Arion empiricorum* and the coelenterates *Flabellum variabile* and *Fungia symmetrica* were identical with the haematoporphyrin of Hoppe-Seyler (1871), which was then the only porphyrin known. Dhéré & Baumeler (1928*a*) confirmed the presence of a porphyrin in *Arion empiricorum*, but did not specify which porphyrin they had found. Kennedy & Vevers (1953), working at the Plymouth Laboratory of the Marine Biological Association, found that the pigment of the integument of *Asterias rubens* L. was protoporphyrin, and in a further survey of the porphyrins of marine invertebrates (Kennedy & Vevers, 1954), they examined the integuments of two molluscs, *Aplysia punctata* Cuvier and *Duvaucelia plebeia* (Johnston), and showed that each contained uroporphyrin I. This pigment was also found by Kennedy & Vevers (1956) to be present in the integument of the tectibranch mollusc *Akera bullata* (O. F. Müller). The present paper describes the isolation and identification of the porphyrin from the integument of the black garden slug *Arion ater* (L.). *Arion empiricorum* was a synonym introduced by Férussac, Férussac & Deshayes (1819–51) to cover the numerous colour and pattern varieties of the slug more generally known as *Arion ater*. The work appears here since it is a continuation of investigations of mollusc pigments initiated in the Plymouth Laboratory, the results of which have already been published in this *Journal*.

The writer is very grateful to Mr O. Thornburn of the Cancer Research Unit, University of Sheffield, for collecting the specimens of *Arion ater* for this work.

METHOD

Twenty-seven large specimens of the black *Arion* were collected from Lodge Moor, near Sheffield, and killed by chloroform vapour. The very viscous yellow slime was removed with a cloth and the integuments dissected off. The internal surfaces of the integuments were scraped clean and the tissue chopped with scissors into 500 ml. of a mixture of absolute methanol 19 parts and concentrated sulphuric acid 1 part. The extraction was allowed to proceed overnight at room temperature, and the residue then filtered off and

re-extracted for 4 h with a further 500 ml. of the methanol-sulphuric acid mixture. The extracts were pooled, diluted with an equal volume of water, cooled to 5° C, and extracted with chloroform until the hypophases were no longer fluorescent. The chloroform extract was washed with 2% sodium chloride twice, followed by six washes with distilled water, dried roughly by filtration through chloroform-soaked paper, and concentrated *in vacuo* at 60° C.

The extract was dark brown and red-fluorescent, and obviously contained a great deal of porphyrin. A sample diluted with chloroform gave the following spectrum (Hartridge):

626·7 572·0 537·3 500·7 mμ.

This spectrum very closely resembles that given by uroporphyrin.

CHROMATOGRAPHY

Column chromatography

The chloroform extract of the pigment was evaporated to dryness and the residue redissolved in fresh dry chloroform. The solution was passed down a column of magnesium oxide grade III (Nicholas, 1951), packed in chloroform, and the chromatogram developed with chloroform containing 0·5, 1·0 and 2·0% methanol successively. A brownish very red-fluorescent band formed rapidly and moved down the column on adding the 2·0% methanol in chloroform mixture. The dark brown non-fluorescent band at the top of the column did not move, and was discarded. The red-fluorescent band was collected, filtered, and the methanol washed out with water. This band gave a spectrum (Hartridge)

626·1 570·5 536·4 501·4 mμ (in $CHCl_3$).

No shift of the bands was observed on substituting authentic uroporphyrin I for the *Arion* pigment in the spectroscope. The spectrophotometer (Unicam) readings were:

626 570 536 501 mμ (in $CHCl_3$).

Fischer (1926) obtained a spectrum for uroporphyrin:

626·2 570·5 537·3 500·8 mμ (in $CHCl_3$).

No other bands were formed on the column.

Paper chromatography

Long paper. A sample of the *Arion* pigment ester was hydrolysed by allowing it to stand in contact with concentrated hydrochloric acid at room temperature overnight, and the acid was removed by standing solution over solid potassium hydroxide in a vacuum desiccator. The free porphyrin was dissolved in a

little 2:6-lutidine and spotted on strips of Whatman No. 4 filter-paper 50 cm long and 4 cm wide in the apparatus devised by the writer (1953). Authentic uroporphyrin I, coproporphyrin I and mesoporphyrin IX were used as markers in both adjacent and mixed spots, and the chromatograms were run at 23° C with 2:6-lutidine:water (5:3) in an atmosphere of ammonia for 12 h. The *Arion* pigment formed one spot only corresponding with the one given by the uroporphyrin marker at R_F 0·15. The spot of 'pseudo-uroporphyrin' described by Falk, Dresel, Benson & Knight (1956) was not observed.

Separation of isomers. The esterified pigment was examined by the technique of Falk & Benson (1953) for the separation of uroporphyrins I and III. Uroporphyrin I was the only isomer detected, without trace of uroporphyrin III.

Decarboxylation

The *Arion* pigment was heated in solution in 1% (w/v) hydrochloric acid in a sealed tube for 3 h at 180° C, and the contents chromatographed by the technique of Chu, Green & Chu (1951) using coproporphyrins I and III and uroporphyrin I as markers. The *Arion* pigment gave one spot only corresponding with coproporphyrin I.

Melting-point

The *Arion* pigment ester was crystallized from chloroform-methanol, and recrystallized. The melting-point, determined in a Gallenkamp brass-bobbin apparatus was 292·2° C (uncorrected). The following melting-points for uroporphyrin I have been recorded:

293° C	Fischer & Orth, 1937, pp. 501–2
284° C	Granick & Gilder, 1947
290° C	Fischer, quoted by Carrié, 1936
293° C	Rimington & Miles, 1951

The evidence presented leads to the conclusion that the porphyrin of the integument of *Arion ater* is uroporphyrin I.

EXAMINATION OF OTHER COLOURED FORMS OF *ARION*

Specimens of the other coloured forms of *Arion* were collected, and the integuments examined in the same way for uroporphyrin. The amount of porphyrin in each individual was roughly estimated by direct visual comparison with the same volume of an extract from a black slug in a fluorimeter. The results were rather striking, in that the amount of porphyrin present was directly proportional to the amount of dark pigment in the integument; thus, in the brown animals, there was less porphyrin than in the black; in the red, less than in the brown, and so on, until in the pale grey integuments there was no porphyrin at all.

DISCUSSION

The identification of uroporphyrin I in the integument of *Arion ater* provides yet another example of the occurrence of this pigment in molluscs (Kennedy & Vevers, 1954, 1956). The associated black pigment, which appears to be melanin, is clearly present as a protection against the effects of light upon the animal, since uroporphyrin is known to cause photosensitivity (Fischer & Zerweck, 1924; Schreus & Carrié, 1931; Macgregor, Nicholas & Rimington, 1952), and in those individuals which have little or no associated pigment, there is no uroporphyrin in the integument. It seems also that the red, brown and orange pigments occurring in the integuments of the various coloured forms of *A. ater* are protective in this way, since, as the colour becomes paler, the amount of porphyrin decreases. Perhaps these red, brown and orange pigments are stages in the formation of melanin, each of which is sufficient to protect the animal according to the amount of uroporphyrin present. (The writer has not been able to examine the integument of *A. rufus*, in which the red pigment called 'rufine' by Dhéré & Baumeler (1928*b*) appears.)

Specimens of the land snail *Cepaea nemoralis*, which had brown and yellow or brown and pink striped shells with grey integuments, had no uroporphyrin at all in either the shell or the soft parts.

Kennedy & Vevers (1954) and Kennedy & Dales (1958) have discussed the occurrence of porphyrins in invertebrates, and the significance of associated pigments in protecting the animals against photosensitivity.

A further interesting point is provided by the sea-cucumber, *Holothuria forskali* Delle Chiaje, which has two pigments in the integument, a melanin and a yellow pigment with a very intense green fluorescence, visible even in daylight. The animal is black all over, except for the ventral surface, which is yellow, and if it is turned with the yellow ventrum towards the light, the animal immediately begins to turn back so that the yellow part is concealed. This suggests that the yellow pigment photosensitizes the animal, and that the black melanin protects that portion of the integument which is normally exposed to light. The yellow pigment may act as an orientating mechanism, as suggested by Crozier (1914). Grassé (1948, p. 91) wrote: 'La surface dorsale tout entière est photoréceptive; quand on fait tomber en un point quelconque une lumière ponctiforme, la peau se déprime à l'endroit touché; presque toutes les espèces ont un phototropisme négatif et fuient la lumière d'une fenêtre; le passage d'une ombre devant un animal épanoui le fait se contracter; il y a sans doute un rapport entre cette sensibilité à la lumière et la présence dans les téguments d'un pigment jaunâtre à belle fluorescence verte.' In some experiments carried out by the writer (unpublished) the pigment behaved in many ways like a flavin. The integument of *Cucumaria normani* Pace when exposed to light darkens and becomes black.

Kennedy & Dales (1958) have suggested that there is some parallel between the occurrence of free porphyrins in invertebrates and of these pigments in human porphyrias. In molluscs, if free uroporphyrin I is present, it occurs in the shell if there is one, or in the integument if there is not. The shell and the integument are both mantle products, and this is another example of the great affinity of uroporphyrin for tissues with a high calcium content.

SUMMARY

The porphyrin of the integument of *Arion ater* (L.) has been identified as uroporphyrin I. Chromatographic behaviour, reactions and melting-point are described. In the coloured forms of *A. ater*, the amount of porphyrin is shown to be directly proportional to that of the associated melanoid pigment. Reasons for this are suggested.

REFERENCES

CARRIÉ, C., 1936. *Die Porphyrine.* Leipzig: Georg Thieme.

CHU, T. C., GREEN, A. A. & CHU, E. J., 1951. Microchromatography of porphyrin esters. *J. biol. Chem.*, Vol. 190, pp. 643–6.

CROZIER, W. J., 1914. The orientation of a holothurian by light. *Amer. J. Physiol.*, Vol. 36, pp. 8–20.

DHÉRÉ, Ch. & BAUMELER, Ch., 1928*a*. Sur la porphyrine tégumentaire de l'*Arion empiricorum*. *C.R. Soc. Biol., Paris*, T. 99, pp. 726–8.

—— 1928*b*. Sur la rufine, pigment tégumentaire de l'*Arion rufus*. *C.R. Soc. Biol., Paris*, T. 99, pp. 492–6.

FALK, J. E. & BENSON, A., 1953. Separation of uroporphyrin esters I and III by paper chromatography. *Biochem. J.*, Vol. 55, pp. 101–4.

FALK, J. E., DRESEL, E. I. B., BENSON, A. & KNIGHT, B. C., 1956. Studies on the Biosynthesis of Blood Pigments: 4. The nature of the porphyrins formed on incubation of chicken erythrocyte preparations with glycine, δ-aminolaevulinic acid or porphobilinogen. *Biochem. J.*, Vol. 63, pp. 87–94.

FÉRUSSAC, J. B. L. d'A., BARON DE, FÉRUSSAC, A. E. J. P. J. F. d'A. DE & DESHAYES, G. P., 1819–51. *Histoire naturelle des Mollusques terrestres et fluviatiles.* 4 Tomes et Atlas. Paris.

FISCHER, H., 1926. *Abderhaldens Handbuch der biologischen Arbeitsmethoden*, Abt. 1, Tl. 2, p. 169; Abt. 4, Tl. 5, p. 536.

FISCHER, H. & ORTH, H., 1937. *Die Chemie des Pyrrols*, Bd. 2. Leipzig: Akad. Verlag.

FISCHER, H. & ZERWECK, W., 1924. Über den Harnfarbstoff. *Hoppe-Seyl. Z.*, Bd. 137, pp. 176–241.

GRANICK, S. & GILDER, H., 1947. Distribution, structure and properties of tetrapyrroles. *Advanc. Enzymol.*, Vol. 7, pp. 305–57.

GRASSÉ, P. P., 1948. *Traité de Zoologie*, T. 11. Paris: Masson et Cie.

HOPPE-SEYLER, F., 1871. *Medizin-chemische Untersuchungen.* Heft 1–4.

KENNEDY, G. Y., 1953. Partition paper chromatography of the porphyrins. *Scand. J. clin. Lab. Invest.*, Vol. 5, pp. 281–4.

KENNEDY, G. Y. & DALES, R. P., 1958. The function of the heart-body in Polychaetes. *J. mar. biol. Ass. U.K.*, Vol. 37, pp. 15–31.

KENNEDY, G. Y. & VEVERS, H. G., 1953. The biology of *Asterias rubens* L. V. A porphyrin pigment in the integument. *J. mar. biol. Ass. U.K.*, Vol. 32, pp. 235–47.

KENNEDY, G. Y. & VEVERS, H. G., 1954. The occurrence of porphyrins in certain marine invertebrates. *J. mar. biol. Ass. U.K.*, Vol. 33, pp. 663–76.

—— 1956. Porphyrin pigments in the tectibranch mollusc *Akera bullata* O. F. Müller. *J. mar. biol. Ass. U.K.*, Vol. 35, pp. 35–9.

MACGREGOR, A. G., NICHOLAS, R. E. H. & RIMINGTON, C., 1952. Porphyria cutanea tarda. *Arch. intern. Med.*, Vol. 90, pp. 483–504.

MACMUNN, C. A., 1886. On the presence of haematoporphyrin in the integument of certain invertebrates. *J. Physiol.*, Vol. 7, pp. 240–52.

NICHOLAS, R. E. H., 1951. *Biochem. J.*, Vol. 48, pp. 309–13.

RIMINGTON, C. & MILES, P. A., 1951. A study of the porphyrins excreted in the urine by a case of congenital porphyria. *Biochem. J.*, Vol. 50, pp. 202–6.

SCHREUS, H. & CARRIÉ, C., 1931. Beobachtungen bei einem Fall von kongenitaler Porphyrie. *Dermat. Z.*, Bd. 62, pp. 347–62.