J. Mar. biol. Ass. U.K. (1956) 35, 35–39
Printed in Great Britain

PORPHYRIN PIGMENTS IN THE TECTIBRANCH MOLLUSC *AKERA BULLATA* O. F. MÜLLER

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The sudden appearance of *Akera bullata* in the static water tanks of H.M. Dockyard, Devonport, provided specimens for the investigation of pigments. Morton & Holme (1955) have given an account of certain aspects of the biology of this interesting tectibranch mollusc.

Fifteen fresh specimens were minced in the Waring blender, and the resulting material was treated in the following ways.

Method 1

A portion of the minced material was extracted with absolute methanol, and the extract filtered. The green solution was intensely red-fluorescent in ultra-violet light, and was submitted to long-paper chromatography (Kennedy, 1953). The solvent phases were 2:6-lutidine (5 parts) and water (3 parts), and the chromatograms were run at 23° C in an atmosphere of ammonia.

When the chromatograms were viewed by ultra-violet light, they revealed a spot, red-fluorescent, with R_F value 0·95, indicating a monocarboxyl porphyrin compound such as phaeophorbide. There was another spot with R_F value 1·0 (i.e. travelling with the solvent), which was red, and had a blue fluorescence. This was most probably carotenoid with fat.

The main purpose of this preliminary experiment was to determine the presence or absence of unchanged chlorophyll in the animal. The fact that no red-fluorescent spot with an R_F value 1·0 was found, indicated the absence of this pigment.

Method 2

A portion was extracted with a mixture of methanol and concentrated sulphuric acid (19:1) and allowed to stand overnight in the ice-chest. It was then diluted with an equal volume of water, and the pigmented material was extracted with Analar chloroform, giving an extract which was intensely fluorescent when viewed in ultra-violet light. This extract was roughly dried by passing it through chloroform-soaked paper, and evaporated to dryness *in vacuo*. The residue was redissolved in dried Analar chloroform, and chromatographed on magnesium oxide grade III (Nicholas, 1951). A deep

red, red-fluorescent, band appeared on development with chloroform which passed slowly down the column and was collected as Fraction A (Kennedy, 1953). A deep green, non-fluorescent, band remained on the column and could not be eluted with graded chloroform/methanol mixtures.

Fraction A was evaporated to dryness *in vacuo*. A portion of the residue was dissolved in Analar pyridine, and the remainder in Analar chloroform. Spectrophotometric examination of the pyridine solution gave the following absorption maxima:

I	II	III	IV	Soret band
626	572	536	502	405·5 mμ

A specimen of uroporphyrin I, in pyridine, gave the following maxima:

627	571	536	501	406 mμ

Fraction A was, therefore, considered to be uroporphyrin. Examination of this fraction by the ascending paper chromatography methods of Chu, Green & Chu (1951) and of Falk & Benson (1953) showed that the uroporphyrin was present as isomer I.

The deep-green non-fluorescent material remaining on the column was eluted with ethyl acetate, which was later removed *in vacuo*. The residue was re-dissolved in dry chlorofrom, and chromatographed on alumina (B.D.H. chromatographic grade). Two bands were obtained, the top one green, and the lower one yellow. The green band was entirely non-fluorescent. The yellow band was slightly yellow-fluorescent, suggesting carotenoids, and gradually passed through the column to give a solution with an indeterminate spectrum.

On further development with chloroform the whole column became green-blue and the washings were colourless. After many attempts the green-blue pigment was finally eluted with a mixture of *n*-butanol and formic acid (50:0·5). The formic acid was removed from the eluate with water, using the smallest volumes possible, and adding compensatory volumes of *n*-butanol to avoid losing pigment. The butanol was then removed *in vacuo*. The pigment residue could then be dissolved in chloroform, and was flocculated with light petroleum (b.p. 40–60° C), giving a dark-green product. This was dried *in vacuo*, and a portion redissolved in chloroform gave the following maxima (in the Hartridge reversion spectroscope):

I	I	III	IV	
655·3	604·0	554·2	465·5 downwards	mμ

A sample dissolved in toluene and examined in the Unicam spectrophotometer gave the following maxima:

660	559	427	mμ

This pigment gave none of the typical bile pigment reactions. The absorption curve, together with the absence of fluorescence, suggested that the pigment might be a metal complex of a chlorophyll derivative.

Metals may frequently be removed from metalloporphyrins by treatment with acids or with hydrazine, and therefore the green pigment was warmed with concentrated hydrochloric acid on a water-bath, and driven into ether by adding saturated potassium acetate. The pigment was unchanged, which suggests the probable absence of zinc, divalent tin and silver. Treatment with concentrated sulphuric acid produced some red fluorescence which rapidly disappeared, due undoubtedly to the destruction of the pigment. Concentrated sulphuric acid is known to remove copper, divalent iron, nickel and cobalt. It is therefore possible that one of these metals could be present.

On dissolving the green pigment in propionic acid and boiling with hydrazine for several minutes, the mixture gradually acquired a fine red fluorescence (when viewed with ultra-violet light) and gave an absorption spectrum (Hartridge reversion spectroscope) consisting of one very strong band at 662 mμ and two weak bands at 540 and 509 mμ respectively. This indicates that the pigment could be a metal derivative of phaeophorbide *a*.

A chemical test for metals was kindly carried out by Mr F. A. J. Armstrong. The pigment gave 30% residue after ignition, and a very strong reaction for copper. There was a trace of iron, but no nickel.

A sample of phaeophorbide *a*, prepared from chlorophyll *a*, was dissolved in boiling ethanol, and while boiling, freshly precipitated copper powder was added, the whole operation being conducted under ultra-violet light. The intense red-fluorescence of the phaeophorbide disappeared almost at once, and, after boiling for a further 60 sec, and examination in chloroform in the Hartridge Reversion Spectroscope, the spectrum was found to be:

I	II	III	IV	
656·0	604·0	554·0	465	mμ

This agrees well with the spectrum of the non-fluorescent pigment in chloroform.

A sample of the ethanolic solution was evaporated to dryness and dissolved in toluene, and the spectrum examined in the Hartridge Spectroscope:

661 560 427 mμ

Thus it is reasonable to deduce that the two pigments—natural and synthetic —are identical, that is, the non-fluorescent green pigment of *Akera* is a copper co-ordination complex of phaeophorbide *a*.

Method 3

The second portion of the original fresh minced material was extracted with a mixture of ether and acetic acid (5:1), and gave a green solution which was intensely red fluorescent. The acetic acid was washed out with water containing 0·05% of Teepol detergent; this was used to avoid emulsification (A. S. C. Lawrence, personal communication, 1953). The remaining ether

solution was extracted successively with 1, 8 and 15%, w/v, hydrochloric acid. The 1 and 8% acids extracted no fluorescent material, thus indicating the absence of coproporphyrin and protoporphyrin. The 15% hydrochloric acid extract was, however, deep green-blue, and intensely red-fluorescent, suggesting the presence of free phaeophorbides, which have HCl numbers of 15 (phaeophorbide *a*) and 19·5 (phaeophorbide *b*) (Willstätter & Stoll, 1913). The whole ether solution, therefore, was completely extracted with 15% hydrochloric acid (uroporphyrin is not extracted by the ether/acetic acid method, and therefore remains in the original minced material).

The 15% hydrochloric acid extract was repeatedly shaken with chloroform, and the deep-blue hypophases pooled. The chloroform solution was washed with water and roughly dried by passing through chloroform-soaked paper, and evaporated to dryness *in vacuo*. An iridescent blue-black residue was obtained, which was re-dissolved in dry peroxide-free ether and fractionated with 0·2% aqueous sodium bicarbonate. Phaeophorbide *b*, being slightly more acidic than phaeophorbide *a*, is extracted quantitatively from a mixture of the two by 0·2% aqueous sodium bicarbonate (Willstätter & Stoll, 1913). No fluorescent hypophases, however, were obtained, indicating the presence of phaeophorbide *a* only. The ether solution was evaporated *in vacuo* and the residue redissolved in Analar chloroform. Examined in the Hartridge reversion spectroscope this solution gave the following absorption maxima:

I	II	III	IV	
666·6	606·7	539·2	507·3	mμ

This is clearly phaeophorbide *a*.

Discussion

Kennedy & Vevers (1954) found uroporphyrin I in the integument of *Aplysia punctata*, and it is noteworthy, though not surprising, that the same porphyrin was isolated from *Akera*, a related form.

It is curious that only phaeophorbide *a* was detected, in view of the fact that *Akera*—like *Aplysia*—grazes on *Ulva* (Morton & Holme, 1955) which contains chlorophyll *a* and *b*. Dales & Kennedy (1954) reported a similar result from *Nereis diversicolor*. Both chlorophylls *a* and *b* must be ingested by the animals, and it is very difficult to account for the absence of the *b* degradation component. The reason may lie, as far as *Akera* is concerned, in the fact that phaeophorbide *b* is difficultly soluble in ether, and, if present originally in very small amounts, as is most likely (the ratio of chlorophyll *a* to chlorophyll *b* is often as high as 3:1), the pigment may have been washed out in the process of removal of the acetic acid. This explanation is not really satisfactory, and in some further work the full reason will be sought.

The presence of a copper complex of phaeophorbide *a* is of great interest. Some molluscs are known to concentrate copper from the sea, and of course

the respiratory pigment haemocyanin, which is a copper-polypeptide, occurs in the cephalopod and gastropod molluscs, and in the arthropods. However, there is no doubt that the green non-fluorescent pigment isolated from *Akera*, is a copper phaeophorbide. That this is not an artifact, caused by the co-ordination of copper ions with phaeophorbide in the course of extraction, is supported by the fact that free phaeophorbide also occurs, whereas the affinity of copper for porphyrin pigments is so great that if free copper were present in the animal, *all* the phaeophorbide would have become changed to the metal complex. The reagents used were, of course, beyond reproach, but the same thing would have been true had they contained copper salts or impurities—*all* the phaeophorbide would have formed the copper derivative. In some further work on the pigments of *Aplysia*, to be completed in the near future, J. E. Morton and G. Y. Kennedy will show that a similar pigment occurs in that animal.

SUMMARY

Some pigments of *Akera bullata* (O. F. Müller) are described with methods for their isolation. The pigment of the integument is uroporphyrin I, and from the viscera phaeophorbide *a* together with its non-fluorescent copper complex were isolated. Some points of interest concerning these pigments are discussed.

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