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THE BIOLOGY OF *ASTERIAS RUBENS* L.

V. A PORPHYRIN PIGMENT IN THE INTEGUMENT*

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(Text-figs. 1 and 2)

A porphyrin was extracted by MacMunn (1886), working at Plymouth, from the integument of *Asterias rubens* then known as *Uraster rubens*. He extracted whole starfishes with ethanol and ammonia and also with ethanol and sulphuric acid. After diluting his acid-ethanol solution with water he shook the extract with chloroform; the chloroform solution showed two absorption bands, at 607–593 and 566–548 mμ. On evaporation this extract yielded a brown amorphous residue which he found to be soluble in absolute ethanol giving a red solution with the same absorption spectrum. On adding ammonia four absorption bands appeared, at 632–622, 586–566, 548–529 and 516–490 mμ. He considered that this and similar pigments 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. Experimental work has now shown that in other animals examined and found to contain porphyrin this pigment is never present in the form of haematoporphyrin. A reinvestigation has therefore been made of the occurrence of porphyrin in the starfish, *Asterias rubens*.

Certain details of the distribution in the Plymouth area of different colour forms of *A. rubens* have already been recorded (Vevers, 1949). In general, starfishes on the Rame-Eddystone grounds are dark brown or red-brown, but specimens coloured violet, violet-brown, red or even pale pink are not uncommon. In Plymouth Sound, on the other hand, a pure population of bright red starfishes was found in 1948; this population has now been greatly reduced in number but specimens can still be obtained.

In the present work four different colour types were used. The choice of these types was arbitrary, but they were nearly all colours which could be easily recognized in a sample of this species. The colour types used were dark brown, violet-brown and pale, all from the Rame-Eddystone grounds, and red (bright red) from the Plymouth Sound population. The violet-brown specimens were very close to the var. *violacea* of earlier writers.

* A shortened form of this paper was read by one of us (G.Y.K.) at the 2nd International Congress of Biochemistry in Paris 1952.

Chemical study of the pigment was carried out in two parts: (*a*) isolation and identification of the porphyrin, and its preparation in quantity; (*b*) determination of the porphyrin contents per gram of integument in starfishes of different colour groups.

Isolation and Identification of the Porphyrin

Isolation of Crude Free Porphyrin

The aboral integument of *Asterias* was stripped off with scissors, separated carefully from the adherent digestive caeca and both integument and caeca were shaken separately with ether:glacial acetic acid (mixture 5:1) for 2 hr. (Zeile & Rau, 1937). The resulting extracts were washed with distilled water until most of the acetic acid was removed, and extracted with 5% HCl (137 ml. conc. HCl/l.). The acid layer from the integument extract was purple and red-fluorescent in ultra-violet light, and greenish brown and non-fluorescent from the caeca extract.

The red-fluorescent acid extract from the integument was treated with fresh ether and saturated sodium acetate solution added to drive the porphyrin back into the ether. The ether extract was then washed free of salts with distilled water, dried roughly by filtering through ether-soaked paper, and evaporated to dryness.

A violet-red residue remained, which was used for subsequent experiments.

Hartridge Reversion Spectroscope

A little of the pigment dissolved in 'Analar' pyridine gave a spectrum showing the following bands:

I	II	III	IV
632	576	542	507 mμ

corresponding with that of *protoporphyrin.*

Chloroform solutions of the methyl ester of this pigment and of protoporphyrin dimethyl ester (prepared from haemoglobin) gave the following bands:

	I	II	III	IV
Asterias porphyrin methyl ester	630	575	540	507 mμ
Protoporphyrin dimethyl ester	631	575	541	507 mμ

Unicam Spectrophotometer

An absorption spectrum of a solution of the pure pigment in pyridine ('Analar') was recorded with a Unicam S.P. 500 Quartz Spectrophotometer. This spectrum is plotted in Fig. 1, and shows absorption maxima at:

624 568 535 500–506 406 mμ

Acid Test of Chu (1946)

A little of the porphyrin was dissolved in 'Analar' chloroform and 3 drops conc. HCl added. The solution turned *green* suggesting *protoporphyrin.* (Mesoporphyrin gives a purple colour.)

Paper Partition Chromatography

A small amount of the free porphyrin was dissolved in lutidine (a mixture of 2:4- and 2:5-dimethyl pyridines) and partition chromatograms were run on strips of Whatman no. 1 paper $3{\cdot}1 \times 75$ cm. at a temperature of 24° C. in an atmosphere of lutidine, water and ammonia vapour (Nicholas & Rimington, 1949) in a new apparatus for paper chromatography (Kennedy, 1953*b*). Pure protoporphyrin and coproporphyrin were used as markers, both in separate and in mixed spots. Papers were examined by ultra-violet light from an 'Osira' 125 W. black glass lamp after 15–16 hr. chromatography.

With protoporphyrin the *Asterias* porphyrin gave one spot only, and with coproporphyrin, two spots (Fig. 2).

A free *Asterias* porphyrin spotted on paper alone from lutidine solution gave one spot only, with an R_F value of 0·8, indicating the presence of a dicarboxylic porphyrin only, there being no other spots on the paper.

Column Chromatography

A sample of the porphyrin was esterified with methanol saturated with HCl gas at 0° C., and the dimethyl ester was dissolved in 'Analar' chloroform and applied to a column of grade III MgO (Nicholas, 1951) packed in chloroform. The chromatogram was developed by successive mixtures of chloroform: methanol in the proportions 100:0·5, 100:2 and 100:3 (Nicholas, 1951). Elution occurred only with the 100:3 chloroform: methanol mixture, indicating protoporphyrin and not mesoporphyrin or deuteroporphyrin.

Melting-point Determination

After chromatographing three times on grade III magnesium oxide the porphyrin dimethyl ester crystallized from chloroform and methanol in red prisms, melting at 225° C. A mixture of the porphyrin dimethyl ester with pure protoporphyrin dimethyl ester melted at 226° C.

Although Jope & O'Brien (1945) have criticized melting-point determinations as methods of identification of porphyrins, these results would indicate the identity of the *Asterias* porphyrin with protoporphyrin.

Formation of Lead Co-ordination Complexes

Lead co-ordination complexes were prepared from *Asterias* porphyrin, from pure protoporphyrin and from haematoporphyrin. All gave two-banded spectra:

	I	II
Pb—*Asterias* porphyrin	630	583
Pb—Protoporphyrin	630	584
Pb—Haematoporphyrin	620	578

This result indicates the identity of *Asterias* porphyrin with protoporphyrin and distinguishes it from haematoporphyrin.

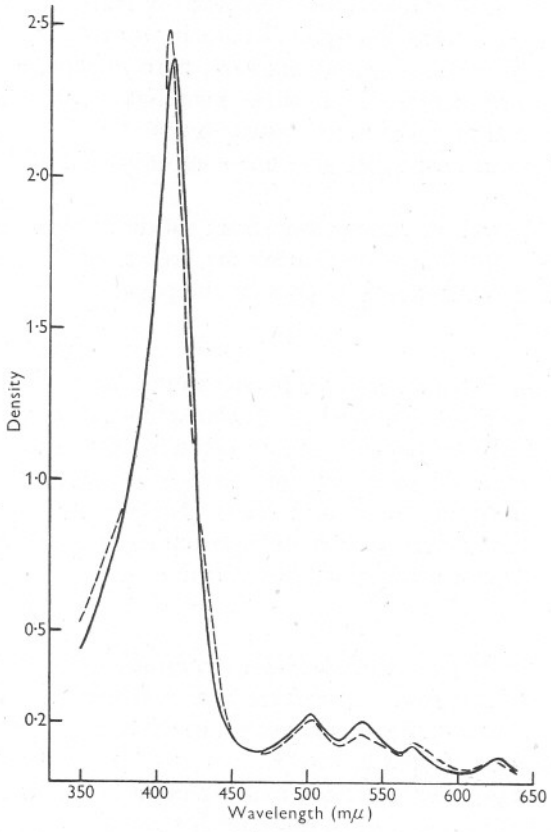


Fig. 1.

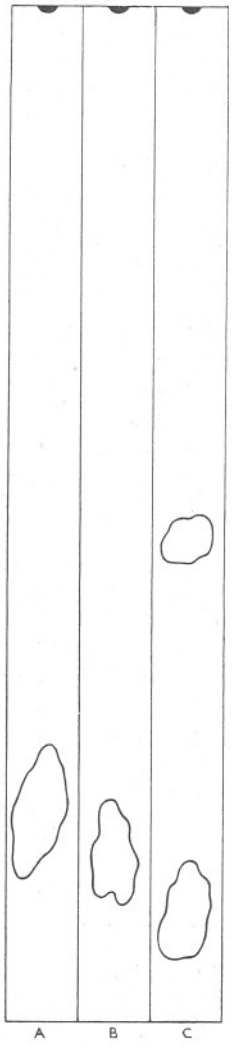


Fig. 2.

Fig. 1. Absorption spectra in pyridine of protoporphyrin (continuous line) and *Asterias* porphyrin (broken line).

Fig. 2. One-dimensional paper chromatograms run for 15–16 hr. at 24° C. in lutidine. The starting-points are marked as black semicircles: A, *Asterias* porphyrin alone; B, *Asterias* porphyrin and free protoporphyrin, mixed spot; C, *Asterias* porphyrin and coproporphyrin, mixed spot.

Conversion to Mesoporphyrin

The *Asterias* porphyrin was reduced according to the method of Rimington (1938). The solid porphyrin was dissolved in 20 ml. of a mixture of glacial acetic acid (7·5 parts) and hydriodic acid (sp.gr. 1·94) (1 part) and boiled for 3 min. After cooling and adding peroxide-free ether, the excess iodine was removed with sodium acetate and sodium sulphite. The ether epiphase gave a spectrum of mesoporphyrin

I	II	III	IV
623	567	528	495 mμ

The ether layer was evaporated and the porphyrin esterified with methanol/HCl at room temperature and extracted with chloroform. This solution was diluted with ether and the porphyrin was taken into 5% (w/v) HCl. The porphyrin was then driven back into the ether with potassium acetate and the ether layer washed, dried by filtering through an ether-soaked paper, and evaporated. The residue was crystallized from chloroform-methanol, and melted at 212–214° C. A mixed melting-point with a specimen of mesoporphyrin IX di-methyl ester (m.p. 215–216° C.) gave 215° C.

This reaction enabled the original *Asterias* porphyrin to be characterized as being of similar constitution to blood pigments derived from aetioporphyrin III, and it could only be protoporphyrin IX, the vinyl groups having been hydrogenated to ethyl groups in mesoporphyrin.

These experiments confirm that *Asterias rubens* integument contains protoporphyrin IX and that the porphyrin is present in the free state.

Chlorophylls in Caeca

Digestive caeca were dissected out of living *Asterias* and ground with acid-washed glass powder in a mortar, and then extracted with 90% methanol. A deep green solution with a marked rosy-red fluorescence was obtained. This solution gave the spectrum of a chlorophyll mixture and was further identified by the formation of various degradation products of chlorophyll. There was no evidence of the presence of a porphyrin in the digestive caeca.

Preparation of *Asterias* Porphyrin

The aboral integument was dissected from a number of large red-brown living *Asterias*. This was scraped free of adhering caeca, washed in sea water, blotted on filter-paper and weighed. The weight of integument as starting material was 548·5 g.

The pooled integument was put through a tissue mincer, and mechanically shaken with 2500 ml. of ether-acetic acid mixture for 5 hr.

The ether-acetic acid mixture was made up as follows: peroxide-free ether ('Analar'), 5 parts; glacial acetic acid ('Analar'), 1 part; then petroleum ether 40–60° C. m.p. ('Analar'), 1 part, added to 9 parts of above mixture.

The petroleum ether helps to avoid emulsification which can be troublesome.

All the following operations were done in very subdued light to minimize the decomposition of the porphyrin.

After shaking, the mixture was allowed to stand overnight in the ice-chest. The following day the mixture was filtered, and the residue re-extracted with a further 1500 ml. of the ether-acetic acid solvent. This was repeated with 1000 ml. lots until the extract was no longer red-fluorescent to ultra-violet light. Even then, the residue was found to be strongly fluorescent due to adsorption of porphyrin on the calcareous spicules of the integument. The residue was therefore shaken with 25% by weight HCl and re-extracted (after neutralization with saturated sodium acetate until Congo-red paper gave a *grey* colour) with 500 ml. lots of ether until the ether-layer was no longer red-fluorescent to ultra-violet light. All the extracts were pooled.

The extracts were first washed with distilled water containing a little sodium acetate, and then with distilled water alone until most of the acetic acid had been removed. The porphyrin was then extracted from the ether solution with 100 ml. portions of 5% by weight HCl until the extracts were no longer fluorescent.

The acid extracts were then pooled in a separating funnel and the porphyrin driven back into 'Analar' ether with saturated sodium acetate. The ether layers were pooled, and washed, first with 2% NaCl, followed by distilled water until washings were no longer acid, and until they no longer gave a precipitate with silver nitrate. During washing a little 'Analar' ether was added from time to time to replace that removed by washing. (Ether dissolves in water to the extent of 7·5% at 20° C.)

All drops of water were shaken down and tapped off, the ether extract filtered through thick paper soaked with ether, into a distilling flask, and reduced to a small volume by distillation *in vacuo*.

The residue was poured into a 500 ml. beaker and evaporated to dryness on the water bath. The porphyrin remaining was rinsed with a little petroleum ether (b.p. 40–60° C.) when cool, and dissolved in the minimum amount of 'Analar' pyridine. This solution was chilled, and dry petroleum ether b.p. 60–80° C. added in the proportion of about 10 volumes with stirring. The porphyrin was precipitated and flocculated by placing the beaker on the hot-water bath until large flocks developed. The beaker was then well covered and, placed in the ice-chest overnight.

The supernatant liquid (if free from flocks) was pipetted off, and the remainder centrifuged. The residue was then washed with petroleum ether (b.p. 40–60° C.) and dried *in vacuo* at room temperature.

The total yield of crude porphyrin by this method was 38·4 mg.

Purification

The dry porphyrin was dissolved in absolute methanol saturated at 0° C. with dry HCl gas, and the mixture allowed to stand in the ice-chest for 48 hr. This esterified the porphyrin.

The solution in methanol/HCl was diluted with a little distilled ice water, chloroform was added with shaking, and the lower layer was tapped off. The whole process was repeated until the lower layer was no longer red-fluorescent. The pooled chloroform layers were washed carefully with 2% NaCl, followed by distilled water, the washings being monitored with ultra-violet light. Washing was continued until the washings were neutral to litmus.

The chloroform extract was filtered through a thick chloroform-soaked paper into a distilling flask, and the extract concentrated to small bulk, then transferred to a porcelain basin and taken to dryness on the water bath.

The dry residue was rinsed, when cool, with petroleum ether (b.p. 40–60° C.) and dissolved in a mixture of chloroform-petroleum ether 1:1, and passed down a column of Brockmann's alumina, packed in chloroform-petroleum ether 1:1 and monitored with ultra-violet light. (A new apparatus for fractional elution chromatography was used, Kennedy, 1953*a*.)

The main portion of the pigment passed through the column, preceded by a blue fluorescent band which was found to be due to fat. At the top of the column, a dark brown pigment remained which was eluted later with chloroform/acetic acid, and gave a greenish fluorescence and two-banded very indistinct spectrum (Eriksen, 1951).

The main bulk of the porphyrin passing through the column was collected and evaporated to dryness in the water bath. The pigment was then dissolved in pure dry chloroform and applied to a column of magnesium oxide grade III packed in chloroform. Development was done by successive mixtures of 0·5:100, 2:100 and 3:100 (v/v) methanol-chloroform (Nicholas, 1951). Only the 3:100 mixture caused elution, indicating the presence of protoporphyrin only.

It may be helpful to include here some details of the crystallization method from chloroform-methanol, a method which often leads to disappointment.

The eluted porphyrin was evaporated to dryness in a porcelain basin on the water bath, and redissolved, on the water bath, in a small volume of dry 'Analar' chloroform. This was allowed to evaporate *slowly* until the effect of 'tears of wine' was apparent on the sides of the basin: i.e. the solvent running back in streaks from a band about 5 mm. above the surface. At this point, 3 volumes—estimated roughly by eye—of *boiling* dry absolute methanol were added with stirring, and crystallization began at once. The basin was covered and set aside in the ice-chest for further crystallization overnight.

The crystals were removed by centrifugation, keeping the temperature as low as possible, and drained by standing the tube on end on a pad of filter-paper. They were then washed with dry petroleum ether (b.p. 40–60° C.).

The mother liquor was evaporated to dryness and the crystallization process repeated.

After two recrystallizations of the mother liquors, the crystals were dried *in vacuo* over P_2O_5 at room temperature. A yield of 33·6 mg. of fine dark red prisms was obtained, melting at 225° C.

It is essential that all solvents should be pure and dry. Methanol was dried by the Mg and I_2 method of Vogel (1948). Chloroform was dried over anhydrous calcium chloride for 72 hr. and then redistilled with a calcium chloride tube attached to the receiver. Petroleum ether, though 'Analar', was dried over calcium chloride and sodium chippings and redistilled.

Determination of Protoporphyrin in *Asterias* Integument

There are difficulties in the determination of protoporphyrin in the integument of *Asterias*, of which the following may be mentioned. (i) The presence of calcium spicules in the integument and consequently the presence of calcium salts during the extraction process is a nuisance when adjusting the pH with sodium acetate. (ii) Protoporphyrin in small concentrations is unstable to light, and the estimation process must be done rapidly in dull light. (iii) Since protoporphyrin has a high acid number, especially in the form of the dimethyl ester, the use of 8% HCl is required in the preparation of the final extracts, and this gives a fluorescence which is less than maximal according to Rimington (1943).

However, it was considered that even an approximate determination of the amount of porphyrin in the various colours of *Asterias* would make a desirable and interesting comparison, and a modified technique was devised which took all the difficulties into consideration and gave reproducible results, using the Rimington fluorimeter (Rimington, 1943).

The fluorimeter was calibrated using dilutions of a freshly prepared standard solution of protoporphyrin made up as described below. The lability of protoporphyrin necessitated fresh standard solutions for each determination.

Standard Solution of Protoporphyrin

1 mg. pure protoporphyrin IX was dissolved in 5 ml. 'Analar' glacial acetic acid and the volume was made up to 100 ml. with 8% HCl (219·2 ml. conc. HCl/l.). This solution contained 10 μg./ml. and was kept in a refrigerator in a black bottle. Dilutions for calibration of the Rimington fluorimeter (for these determinations) were made freshly as required from the solution, with 8% HCl in dim light, but the standard stock solution was not kept for longer than 2 days. Hence, although fluorescence in 8% HCl is not maximal, both standard and unknown solutions in estimations are exactly comparable.

Determinations on each colour group were done separately, each compared with a freshly made up standard of 2 μg./ml. protoporphyrin in 8% HCl.

Method of Determination

All steps were carried out in very subdued light.

(i) 2 g. aboral integument of each colour were weighed out separately, after drying roughly in a standard manner on Whatman No. 1 paper, into wide-mouthed stoppered bottles.

(ii) 100 ml. methanol saturated with HCl gas were added and the whole was stored at 0° C. for 36 hr. in the dark.

(iii) The extracts were filtered into separating funnels and the bottles, residues and papers washed with small volumes of 30 ml. methanol/HCl; the washings being added to the main extracts.

(iv) The extracts were diluted with 1 volume of distilled water and the carotenoids removed by shaking with petroleum ether (b.p. 40°–60° C.) until the *upper* layer was non-fluorescent to ultra-violet light. The upper layers were then discarded.

(v) Peroxide-free ether was then added to the lower layers, followed by saturated sodium acetate (red to congo red), and well shaken. This was repeated until the upper (ether) layers were non-fluorescent to ultra-violet light. The lower layers were discarded.

(vi) The ether extracts were pooled and washed with ice-cold distilled water repeatedly until the washings gave no cloudiness with silver nitrate. All drops were shaken down and tapped off.

(vii) The ether solutions were extracted repeatedly with 2 ml. lots of 8% (w/v) HCl until no further fluorescence was seen in the lower (acid) layer. The lower layers were collected in stoppered measuring cylinders, and the total volume noted. All drops in the funnels were shaken down, and the measuring cylinders were well shaken.

(viii) 5 ml. samples of the acid extract in Eggertz tubes were compared with a 2 μgm./ml. protoporphyrin standard in the Rimington fluorimeter. If the solutions were too strong they were diluted with 8% HCl and the dilution factor noted.

The means of five to ten readings were taken, and the porphyrin values read off from the calibration curve. The results are expressed as μgm. protoporphyrin/g. of integument.

Results

The starfishes were graded by colour as follows: violet-brown, VB; dark brown, DB; red, R; pale, P.

The results of the porphyrin estimations are set out in Table I. Each figure is the mean of three determinations on an extract derived from two or three selected starfishes of the appropriate colour.

TABLE I. PORPHYRIN IN *ASTERIAS*

Given in μgm./g.

	Grade of colour			
Determinations at	VB	DB	R	P
Plymouth	72·0	33·6	24·5	5·5
Sheffield	64·4	49·9	13·0	4·5
Sheffield	70·3	45·2	11·4	—
Sheffield	69·7	47·2	15·2	—

Tests on the Soft Parts of *Chlamys* and *Mytilus*

With a view to tracing the origin of the porphyrin in *Asterias rubens*, the soft parts of *Chlamys* and *Mytilus* were ground with washed sand and extracted with ether-acetic acid mixture. The extract was very faintly blue-fluorescent, but had no red-fluorescence at all. On standing exposed to light in the presence of hydrogen peroxide or quinhydrone no red-fluorescence developed, confirming the absence of a porphyrinogen.

Discussion

The results confirm the earlier evidence of MacMunn that a porphyrin is, in fact, present in the starfish, *Asterias rubens*, but extend his observations to the identification of the pigment as protoporphyrin, and not haematoporphyrin.

The determinations of protoporphyrin given in Table I show that in *A. rubens* integument the amounts per gram of this pigment are graded approximately according to the colour of the starfish. In general the darker starfishes have more protoporphyrin than the lighter ones. The main visible colour of the dark-brown and violet-brown starfishes is not, however, entirely due to the presence of this porphyrin, for these colours are known to be mainly carotenoid (including carotiproteins) (Vevers, 1952). The dark brown or black reticulate pattern has been shown to be a melanin pigment (Vevers, in preparation). At the same time there appears to be some parallelism between the porphyrin content and the colour of the carotenoid pigmentation. The measurements are relative and not absolute; and although there is much variation in pigmentation in *A. rubens*, the range of figures in Table I gives an excellent indication of the order of the porphyrin content in these varying shades of starfish.

The chromatographic results indicate that the protoporphyrin is present in the starfish body in the free state and not as an ester, since it takes up the clear position of protoporphyrin on the paper and does not travel all the way with the mobile phase as do the esters.

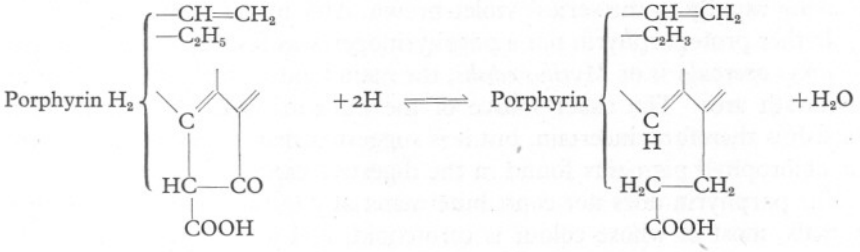
The problem of the origin of the free protoporphyrin in *Asterias* is intriguing. It is entirely absent from the soft parts of the lamellibranchs *Chlamys opercularis* and *Mytilus edulis*, which form the principal food of *Asterias rubens* in the Plymouth area. Uroporphyrin and coproporphyrin are widespread in mollusc shells, but do not occur in the soft parts that alone are eaten by the starfish.

The experiments with the soft parts of *Chlamys* and *Mytilus* have also shown that there is no extractable substance present comparable with a porphyrinogen, which could be converted simply to a porphyrin by oxidation in the starfish. The formation of protoporphyrin in *Asterias* must therefore be more metabolic.

There appear to be three possible sources of the free protoporphyrin: (i) haem enzymes and cytochromes, (ii) chlorophyll of the digestive caeca, (iii) complete synthesis *de novo* from simple substances in the diet.

Haem enzymes and cytochromes are present in the starfish in very small (physiological) amounts, and the amount of protoporphyrin in the integument is vastly greater. No transformation of the porphyrin *c* of cytochrome *c* (haematoporphyrin) to protoporphyrin has yet been demonstrated conclusively.

Chlorophyll is present in the digestive caeca in very large amounts, and could be converted into protoporphyrin by a dismutation, two hydrogen atoms being lost from the nucleus and two from the ethyl side chain. The addition of six hydrogen atoms would bring about opening of the isocyclic ring in chlorophyll and the formation of the second propionic acid side chain of protoporphyrin.



(*After Lemberg & Legge*, 1949.)

The third idea involves complete synthesis of the porphyrin nucleus from glycine, acetate, or α-ketoglutarate as shown by Muir & Neuberger (1950) and Shemin & Rittenberg (1946).

On the whole, the formation from chlorophyll is the most likely source of protoporphyrin in this organism, and will be the subject of future study.

The porphyrin in the integument clearly does not contribute to the colour pattern of the animal, as has been mentioned already. The pigment may therefore be either an excretory by-product formed from chlorophyll, or be concerned in integumentary photoreception, since porphyrins are known to render tissues more sensitive to light.

In the starfish the pigment apparently is only accumulated in the hard leathery integument, as repeated tests have shown it to be entirely absent from the gut, gonads and digestive caeca. Direct ultra-violet examination has also shown that there is no porphyrin pigment in the calcareous spicules embedded in the starfish integument. This is interesting since porphyrin is so frequently associated with calcium compounds, e.g. protoporphyrin in the hen's egg-shell, the porphyrins of mollusc shells (Nicholas & Comfort, 1949), and uroporphyrin in the bones of the squirrel *Sciurus niger*.

SUMMARY

A porphyrin pigment found in *Asterias rubens* has been re-examined and found to be protoporphyrin, and not haematoporphyrin as originally reported by MacMunn. The pigment was identified by spectroscopic and spectrophotometric observations as well as by chemical tests and the use of paper partition and column chromatography. The protoporphyrin is absent from the gut, gonads and digestive caeca, and is present only in the leathery integument.

The porphyrin occurs in the integument as free protoporphyrin; a yield of 33·6 mg. of red crystalline protoporphyrin dimethyl ester was obtained from a sample of 548·5 g. of *Asterias* integument.

The relative amounts of protoporphyrin in different coloured specimens of *A. rubens* were determined by a fluorimetric method. With four well-defined colour types the amount of porphyrin per gram of integument was found to decrease rapidly in the series: violet-brown, dark brown, red and pale.

Neither protoporphyrin nor a porphyrinogen was found in the soft parts of *Chlamys opercularis* or *Mytilus edulis*, the main food animals of *Asterias* in the Plymouth area. The exact source of the integumentary porphyrin in this starfish is therefore uncertain, but it is suggested that it may be derived from the chlorophyll pigments found in the digestive caeca.

The porphyrin does not contribute materially to the colour pattern of the animals, most of whose colour is carotenoid, and it is most likely that it is a by-product of excretion formed from chlorophyll.

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