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ON THE DIVERSE COLOURS OF *NEREIS DIVERSICOLOR*

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(Text-figs. 1 and 2)

The variable colour of the polychaete *Nereis diversicolor* has been known since O. F. Müller described the species as 'Die bunte Nereide' in 1771. Most of the worms belonging to this species appear to be orange or brown in colour, though close examination reveals that some green pigment is invariably present. However, others may be found which are predominantly green in appearance, and a few which are completely green and appear to lack brown or orange pigments in the epidermis.

Several previous authors have given attention to this. Mendthal (1889) considered the green colour of some worms to be due to a diet of green algae. McIntosh (1910) rejected this idea, and suggested that the green colour was due to 'pale greenish ova'. However, it may be pointed out that males are also green, and in our experience the oocytes of this species are colourless or have only a pale straw colour. Various other writers have commented on the variability of the colour of this animal without contributing to the elucidation of the problem, Thomas (1930) alone has attempted an analysis of the different pigments. He concluded that the green pigment was probably a porphyrin, possibly a modified chlorophyll. Herpin (1923, 1925) and Dehorne (1925), as well as Thomas (1930) and Dales (1950), have drawn attention to the rather different appearance of the two sexes when mature. Hempelmann's paper (1939) on the chromatophores of *Platynereis dumerilii* has little bearing on the present problem.

Most of the chemical work described here has been done on worms from Plymouth in 1953 and 1954, but observations on variation of colour have been made mainly at Chalkwell, Essex, over the last five or six years, and some chemical analyses have also been made on worms from this locality.

Identification of the pigments

In one extract, twenty-five whole worms were placed, without grinding, in absolute methanol 19 parts and concentrated sulphuric acid 1 part, overnight in the ice chest. The following day the deep-green extract was filtered through glass wool into a separating funnel, and diluted with an equal volume of water.

The mixture was then extracted with one-third of the volume of chloroform. The chloroform hypophase was dark brown, and the aqueous epiphase was bright green. The hypophase was transferred to another funnel and washed with 2 % NaCl and then with water, forming 'chloroform extract A'. The aqueous epiphase was again extracted with chloroform, and this time the hypophase was bluish green and the epiphase pale green. The hypophase was removed and washed as before, filtered through chloroform-soaked paper, and evaporated to dryness *in vacuo*. A greenish residue remained which was used for subsequent tests; this was 'chloroform extract B'.

Chloroform Extract A

Extract A was evaporated to dryness *in vacuo* and redissolved in dry chloroform. The solution was chromatographed on ungraded Brockmann's alumina packed in chloroform, and then exhibited the following bands:

Band 1. A dark green band at the top of the column; non-fluorescent.

Band 2. A narrow yellow band; also non-fluorescent.

Band 3. A reddish brown band which travelled rapidly down the column and passed out. This was strongly red-fluorescent and gave the following spectrum (Hartridge Reversion spectroscope):

	I	II	III	IV
	660–680	600–620	590–565	520– mμ
Centres	670	610	579·5	(520)

Bands 1 and 2 could not be eluted by any of the usual solvents or combinations of solvents, and were finally eluted by ether-acetic acid 5:1 mixture, giving a green solution with no fluorescence and very indeterminate spectrum. The pigment had the extraordinarily high HCl-number of 36.

Band 3 was further chromatographed on MgO grade III (Nicholas, 1951), packed in chloroform, and displayed the following bands:

Band A2. A dark green band at the top; non-fluorescent.

Band B2. A brown band, also non-fluorescent, separated from the upper band by a gap.

Band C2. An apparently colourless wide band, red-fluorescent, passing down and out of the column very readily.

Band A2 (Fig. 1)

This band when cut off and eluted with methanol displayed the clear spectrum of biliverdin:

I	II	
639	390	mμ

and when acidified with HCl there was a shift of the bands to:

I	II	
678	375	mμ

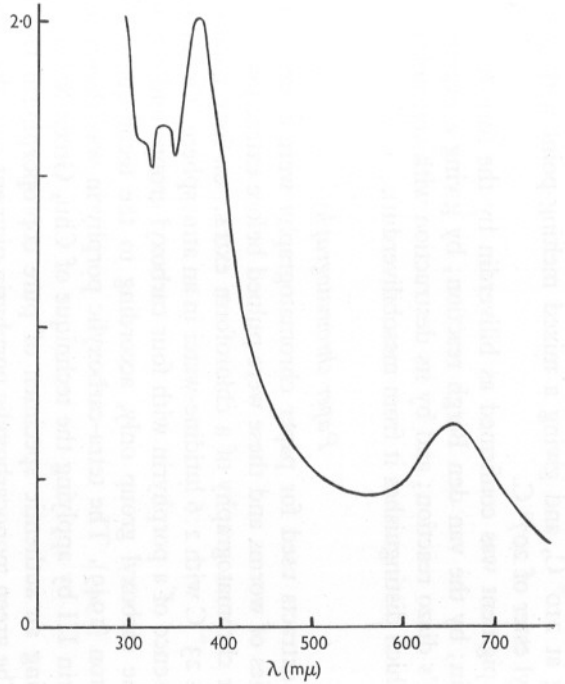

Fig. 1. Absorption curve of an acetic acid extract of the body-wall of entirely green worms. Vertical scale in arbitrary (Unicam spectrophotometer) units.

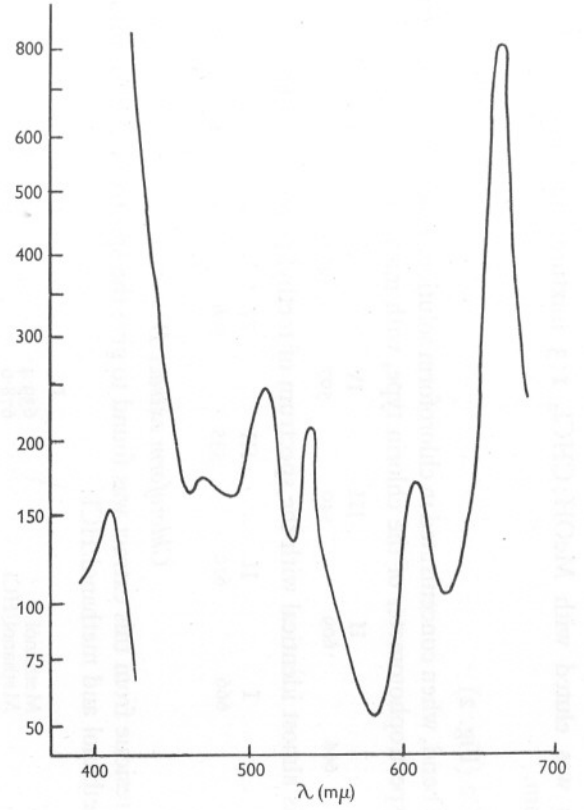

Fig. 2. Absorption curve of a chloroform extract of whole worms. Vertical scale in $E_{1cm.} \times 10^3$.

Band B2

This was eluted with MeOH:$CHCl_3$ 1:3 mixture, but gave no clear spectrum.

Band C2 (Fig. 2)

This band, when concentrated in chloroform solution, gave a clear spectrum in the spectrophotometer of the chlorin type, with maxima at

I	II	III	IV	V	Sorêt
666	609	540	507	(468)	414 mμ

which is almost identical with the spectrum of methyl phaeophorbide-*a*:

I	II	III	IV	Sorêt
666	610	535	506	412 mμ

Chloroform extract B

The residue from this extract was found to give the spectrum of biliverdin with methanol and methanol/HCl:

	I	II
Methanol	639·4	391·2 mμ
Methanol/HCl	678·6	375·4 mμ

The acid-number was 1.

The solution of the pigment in methanol/HCl was allowed to stand for 36 h and then extracted with chloroform in the usual way, followed by crystallization from chloroform-methanol. Fine blue-green needles were obtained, melting at 210° C, and giving a mixed melting-point with pure biliverdin dimethyl ester of 207° C.

The pigment was confirmed as biliverdin by the formation of zinc bilipurpurin; by the van den Bergh reaction; by giving a negative result with Ehrlich's diazo reaction; and by its destruction with concentrated sulphuric acid (which distinguishes it from mesobiliverdin).

Paper chromatography

The extracts used for paper chromatography were obtained from larger quantities of worms and these were pulped before extraction.

Paper chromatography of a chloroform extract on long papers (Kennedy, 1953) at 23° C with 2:6 lutidine-water in an atmosphere of ammonia revealed the presence of a porphyrin with four carboxyl groups and a green pigment with one carboxyl group only, according to the technique of Nicholas & Rimington (1949). The tetra-carboxylic porphyrin was shown to be coproporphyrin III by applying the technique of Chu, Green & Chu (1951), and employing an authentic specimen of pure coproporphyrin III tetramethyl ester. The green monocarboxylic porphyrin pigment gave the same spectrum as that obtained from the band C2 already mentioned. Since the extract

from which C2 was obtained was made by using methanol and concentrated sulphuric acid, the pigment of band C2 would be an ester. From its solubility in boiling absolute ethanol, and its acid-number of 16, the pigment appeared to be methyl phaeophorbide-*a* only. Hydrolysis of the ester and formation of derivatives—phylloerythrin, phaeoporphyrin-a_5 and phytorhodin-*g*—confirmed that this was so. Extraction of the ether solution of the pigment with 0·2% sodium bicarbonate was without result, proving the absence of phaeophorbide-*b* (Willstätter & Stoll, 1913). That the pigment is present in the worm as the free phaeophorbide-*a* is shown by the formation of the spot of R_F 0·94 on the long-paper chromatogram of worm extracts made in chloroform only without any esterification.

As compared with the quantity of biliverdin, phaeophorbide-*a* is present in small amounts and may indeed be restricted to the gut. Worms thus owe their green appearance to biliverdin, while the yellow, brown and orange pigments also present in the epidermis in most worms are carotenoids.

Distribution of the Pigments in the Body

In most worms biliverdin occurs in the form of minute granules 1–2 μ in diameter scattered through the epithelial cells immediately under the cuticle. The pigment is deposited mainly along the borders of the blood capillaries, and is therefore most dense on the dorsal side of the body and in the parapodia; but it also occurs at the bases of the parapodia and between the segments. The appearance of the worms suggests that there is less biliverdin in the most anterior part of the body on the dorsal side, and while this seems to be so, the darker appearance is due mainly to the much larger amounts of carotenoids in this region. This darker appearance of the anterior region of the body may have some significance in relation to survival of these worms which protrude from the burrows while browsing on the surface mud. Biliverdin does not usually occur in any quantity on the ventral side of the body except in the parapodia, but depositions are found in the pygidium, and under the cuticle lining the proboscis. However, the intestine and the coelomic cells are also usually faintly green and contain small quantities of similar granules of biliverdin, and in females which have wholly or partially spawned, the gut and the coelomic cells may present a vivid green appearance. All worms possess at least some biliverdin though its presence may be masked by yellow, orange or brown carotenoid pigments.

Some individuals may appear entirely green, biliverdin alone being represented in the epidermis. These worms are always either ripe males with free sperm in the coelom, ripe females, or females which have spawned. This does not mean that females with an orange appearance are not sexually mature or do not spawn. Such green individuals, whether male or female, owe their striking appearance not only to a disappearance of the orange and brown carotenoids from the skin, but also to an increase in the amount of biliverdin in the body.

On the other hand, slight variations in the 'greenness' of worms which are not breeding seem to be due to variations in the quantity of carotenoids, worms appearing greener when they have relatively small amounts, and more orange when they have relatively greater quantities of carotenoids. On the other hand, apart from the breeding worms, there seems to be little variation in the amount of biliverdin deposited in the epidermis throughout most of the year.

Seasonal Changes in Colour

In the Chalkwell population during the early spring of 1949, nearly all the worms assumed a predominantly green appearance by the end of February when the main spawning occurred (Dales, 1950). It was naturally concluded that this colour change was in some way related with the maturation of the gametes. This is now known to be only partly true, since subsequent experience has shown that the same population in other years, and populations which have been seen elsewhere at the time of spawning, may have only a relatively small number of green individuals. Other observers have also reported the rather variable occurrence of green specimens, which as a general rule constitute only a small proportion of any population. During the summer such green individuals are definitely rare. In the early spring of 1951, 1952, and 1954 there were few green worms at Chalkwell, but in the winter and spring of 1952–53 there was again a much larger proportion of worms with a green appearance, but by no means as many as in 1948–49.

Discussion

Breeding takes place in mid-February in the *Nereis diversicolor* population at Chalkwell, but only a very small proportion of the females spawn successfully owing to the relative scarcity of the males and the fact that spawning takes place only in the presence of the male. All males with mature sperm appear a bright green owing to the complete extraction of the orange and brown pigments and also to an increase in the relative amount of biliverdin, as already mentioned. This was shown to be so by the much greater amount of pigment which could be extracted per unit weight of such worms as compared with non-breeding animals. This increase in the total quantity of biliverdin is almost certainly associated with a reduction in the quantity of haemoglobin (in the sense of Keilin & Hartree, 1951). As the males reach maturity their tissues undergo phagocytosis, the body-wall muscles are eroded away, making the worm extremely fragile. As this process continues the amount of haemoglobin decreases; there is often barely enough blood to fill the dorsal vessel in the fully mature male. This suggests that the biliverdin is indeed derived from the worm's own haemoglobin.

On the other hand, females when apparently mature at the time of spawning

are more variable in appearance; some are predominantly green, but all at this time have quantities of orange or brown pigments as well as the biliverdin in the body-wall. Later, however, those females which have spawned lose their orange and brown pigments and become as green as mature males. Males soon die when they have emitted their sperm. Females that have spawned often live for several weeks after spawning. Females rarely spawn so completely that one or two oocytes are not left in the coelom enabling their sex to be determined with certainty, and all such females undergo a phagocytosis of the tissues during or after spawning, accompanied by a loss of carotenoids and a greatly increased destruction of the blood haemoglobin with a corresponding increase in the quantity of biliverdin in the body. In the female the process of phagocytosis seems to have got out of step with the spawning, and this rather anomalous situation may be related in some way to the loss of the epitokous phase in this species. In unspawned females the oocytes are eventually broken down by the coelomic cells, being converted into a milky mass which might be mistaken for sperm. Soon afterwards quantities of orange pigments appear in the body-wall. These worms live throughout the following summer and may double their length, but it seems unlikely that they have a second opportunity to spawn (see Dales, 1951). There is no doubt that the green female individuals are those which have spawned, even if an appreciable number of oocytes remains in the coelom. Spawning takes place in the burrows and the worms are remarkably sedentary, at least at this time of year. For the first 8 weeks at least the larvae develop in the parent burrow and are just visible to the naked eye when about 6 weeks old. Larvae are invariably found in burrows occupied by a spawned or partially spawned green female, or in empty burrows, but never in burrows occupied by orange worms in which the oocytes are being absorbed. The explanation of these colour differences may be simply a consequence of sexually mature worms ceasing to feed. Worms that spawn subsequently draw on their carotenoids and even on their muscular tissues and become green and fragile. Worms that do not spawn, on the other hand, have a large reserve supply of food in the form of their own oocytes, and when these are absorbed the worms become even more orange in appearance than before. If the carotenoids form part of food reserves, the occurrence of greater numbers of green individuals in winters with early cold periods (1948–49, 1952–53) could be due to the earlier depletion of these stores. On the other hand, the carotenoids may be utilized directly in the production of the gametes, but if this is so it is surprising that though the carotenoids disappear in the male at maturity, they may not disappear in the female until after spawning. Incidentally, the greater number of green individuals in the early spring of 1949 was certainly not due to a larger proportion of males or spawning females in that year. Counts to determine the sex ratio in early February 1949 showed that less than 10% of the total population were males, and this proportion has

not varied materially in subsequent years. The proportion of spawned females has also remained at approximately the same value.

Although it is difficult to observe or measure changes in the quantity of epidermal biliverdin in adults which already have variable amounts of green, orange and brown pigments, it was thought that some indication of whether the biliverdin was derived from ingested blood or not could be solved by rearing worms from the larval stage on different substances. Young worms were collected in large numbers from the burrows in early May when the average age was estimated at about 8 weeks. At this time there are about ten to twelve chaetigerous segments and they are completely colourless. These young worms were reared on (1) dried and powdered nettle leaves alone, (2) dried slaughterhouse blood alone, and (3) a dried concentrated aquarium fish-food ('Brosiam') containing negligible amounts of chlorophyll or haemoglobin. The young *Nereis* seemed to be sufficiently omnivorous to thrive well on each of these substances for at least long enough for the present purpose. The water in each culture dish was replaced daily with filtered sea water to ensure that the worms were in fact feeding on the diet provided. The haemoglobin appeared in the blood when the young worms were about 9 weeks old, and the other pigments appeared soon after. The orange and brown pigments first made their appearance as granules in stellate chromatophores concentrated in the anterior region and arranged as a double row down the body on the dorsal side. The biliverdin appeared at about the same time, and from the first was more uniformly scattered through the epidermal cells on the dorsal side. All the young worms were similarly pigmented after several weeks' growth on these different foods; those fed entirely on nettle leaves, and those on dried blood, had much the same quantity of epidermal biliverdin as those fed on the dried fish-food.

Thus there is little doubt that the biliverdin is formed by the breakdown of the haemoglobin of the blood. This takes place along the margins of the vessels, mainly between the capillaries on the dorsal side of the body around the proboscis and in the pygidium. Bloch-Raphaël (1939) concluded, in her review of the seat of haemoglobin synthesis and breakdown in polychaetes, that in nereids these processes probably take place in the body-wall and around the proboscis, and that the bile pigments are excreted into the gut. This view agrees with the present observations on *N. diversicolor*. The granules of biliverdin may gradually be removed from the epidermis by the coelomic cells and conveyed to the gut, the cells travelling down the septa and oblique muscles. Coelomic cells loaded with biliverdin, often in granules 4–5 μ across, can be seen in these positions in spawned females. When the rate of haemoglobin breakdown is increased as in ripe males and spawned females, the rate of elimination is not increased in proportion, so that the pigment accumulates in the body.

SUMMARY

The variable colour of *Nereis diversicolor* is due to variations in the proportion of green, orange and brown pigments. The orange and brown pigments are mainly carotenoids; the green colour is due to biliverdin.

Phaeophorbide-*a* and coproporphyrin III also occur, but both these pigments may be restricted to the gut wall; biliverdin occurs both in the wall of the gut, and in the epidermis and coelomic cells.

The biliverdin is formed by the breakdown of the haemoglobin of the blood.

Haemoglobin-breakdown takes place in the epidermis on the dorsal side of the body, in the epithelial tissue surrounding the proboscis and in the pygidium. Granules of biliverdin are probably removed by the coelomic cells and conveyed to the gut into which they are excreted.

In ripe males, and in females during and after spawning, phagocytosis of the tissues is accompanied by an increased haemoglobin-breakdown with a corresponding accumulation of biliverdin in the body. The green appearance is due not only to an increased amount of biliverdin, but also to a complete extraction of carotenoids from the body-wall.

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