THE COLOURS OF *OPHIOCOMINA NIGRA* (ABILDGAARD)

III. CAROTENOID PIGMENTS

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(Text-figs. 1 and 2)

The remarkable colour variation of the ophiuroid, *Ophiocomina nigra* (Abild-gaard), has already been discussed (Fontaine, 1962*a*). The black and brown colours of the integument are shown to be due to a melanic pigment contained in specialized melanocytes (Fontaine, 1962*b*). The carotenoid pigments producing the red to yellow colours of the integument are now considered.

Although carotenoids have been detected in several ophiuroids (Fox, 1953), the only intensive analysis of their pigments is that of Fox & Scheer (1941) who studied three species from the southern coast of California. They found a preponderance of oxygen-containing carotenoids (xanthophylls and acidic carotenoids) in these species including several new pigments. Carotenoids were detected qualitatively in *O. nigra* from the Swedish coast by Lönnberg (1931, 1934) by means of the Carr–Price and other colour tests. He also separated alcoholic extracts into epiphasic and hypophasic fractions. Spectroscopic examination (Lönnberg & Hellström, 1931) revealed pigments with absorption spectra similar to a carotene and to xanthophyll (lutein). These authors did not attempt to separate the pigments chromatographically.

ANATOMY AND HISTOCHEMISTRY OF THE LIPOCYTES

The carotenoid pigments of *O. nigra* are located subepidermally in specialized lipocytes, unlike the situation in the asteroids in which the pigment occurs in unspecialized epidermal cells (Vevers, 1952). The lipocytes were examined in living animals by excising pieces of integument and mounting them on a cavity slide in a hanging drop of sea water. Attempts at fixation and staining by the usual lipid techniques introduced some serious artifacts, therefore the following description is limited to what can be observed in living cells with the aid of the phase-contrast microscope.

The carotenoid-containing cells of *O. nigra* are dendritic and somewhat similar in structure to the melanocytes described previously. They are, however, more compact and have less tendency to branch. Their cell bodies

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contain a single, ovoid nucleus, 4–5 μ long, appearing as a clear space in the highly coloured background. The cell bodies occur about 10 μ deeper in the subepidermal layer than do those of the melanocytes, forming a distinct layer of lipocytes beneath the melanocyte layer. The dendritic processes of the lipocytes do not approach as close to the body surface as do those of the melanocytes and never penetrate the epidermis. The result is that the melanocytic dendrites tend to overlie the lipocytic dendrites, causing the lipocytes to be obscured in melanic animals. The influence of this arrangement on colour and its variation is discussed later. The lipocyte dendrites are thicker and more uniform in diameter throughout their length than the melanocyte dendrites and are often up to 50 μ in length. They do not appear to be syncytial and, in this respect, are in sharp contrast with the melanocytes.

The pigment in the lipocytes sometimes appears to be in solution, but is far more often seen in the form of amorphous granules. Occasionally both occur together. In specimens which have been preserved by boiling and freezing (see page 37), the pigment occurs entirely in the granular form. Small pieces of living and frozen-boiled tissue were extracted for 24 h with *n*-hexane. The solvent was barely coloured at the end of that time. Subsequent microscopic examination showed that the pigment was still present in the lipocytes. Since *n*-hexane is a solvent which does not denature protein, it may be concluded that the carotenoids occur chiefly in the form of a protein-complex within the cells.

The lipocytes are either red-orange or yellow in colour, both sorts usually being found together in an individual. There is a tendency for the lipocytes to be more densely concentrated on the aboral surfaces. As with the melanocytes, there is no evidence that the lipocytes are pigment effector organs. There was no detectable pigment migration or cell movement after individuals or tissues in hanging-drops were placed in strong light or in the dark for several hours.

The cells identified as lipocytes could be shown to contain carotenoids by means of histochemical tests applied to fresh material. The pigment of the lipocytes was insoluble in water, dilute acids and alkalis, and formalin. It was soluble in ethanol and methanol, but not directly so in the non-polar organic solvents. The pigment could be easily transferred into these solvents, however, from aqueous alcohol. The pigment was readily oxidized and bleached by 1% chromic acid. Drops of concentrated sulphuric acid applied to the tissue produced a strong green-blue colour in the lipocytes. Treating the tissue with iodine-potassium iodide solution produced a dark-violet colour in the lipocytes. The Carr–Price reaction did not give satisfactory results with fresh tissues because of their water content. Extracts of the pigment in chloroform gave a strong positive reaction (green-blue), however. These histochemical reactions are all characteristic of carotenoid pigments (Lison, 1953).

MICROANATOMY OF COLOUR VARIATION

With reference to the colour variation of *O. nigra*, it has been demonstrated (Fontaine, 1962 *b*) that the black and brown colours are due to melanin, lighter browns being due either to decreasing density of melanization or to the oxidation state of the pigment. The presence of the carotenoid-containing lipocytes confers the red, orange and yellow colours to the integument. Generally, the lightest colour phases are completely non-melanic, all of the colour which they display being due to the variously coloured lipocytes present in the integument. Intermediate colour phases, i.e. red-brown individuals, etc., have a normal density of lipocytes with, in addition, a variable density of melanocytes overlying them. There is a subtle difference in the tone and range of colours between the ophiuroids of the Plymouth and Millport populations, as a result of differential distribution of the various types of pigment cells.

Plymouth

The lightest colour phases among the Plymouth specimens (P4 and P5) display glossy, clear colours and have no melanocytes anywhere on the body. Variations in colour of these non-melanic phases are due to the relative proportions of the red-orange and yellow lipocytes exclusively. For example, the redder the animal, the greater the number of red-orange lipocytes there are in proportion to yellow lipocytes. The same description applies to differences in colour between oral and aboral surfaces of the same individual. Intermediate colour phases (P3) of this population have a lipocyte density similar to the non-melanic phases, but these lighter colours are darkened by the presence of a variable number of melanocytes in addition. The darkness of an individual depends upon the degree to which the lipocytes are obscured by the melanocytes. A similar condition holds for the dark-brown (P2) phase, except that in this form the relative number of lipocytes is apparently decreased. This results in a very dark animal with only minor tints of lighter coloration. The very darkest (P1) phase is remarkable in that it possesses very few lipocytes, most of which are to be found near the bases of the arm spines. This general lack of lipocytes means that practically all colour is conferred by the melanocytes in this phase.

Millport

In contrast to the Plymouth population, there is a remarkable preponderance of red-orange lipocytes in the Millport animals, with relatively few yellow lipocytes scattered among them. In the non-melanic (M4 and (M5) phases, the numerical dominance of the red-orange lipocytes confers the overall dull redness which they characteristically display. As usual, intermediate (M3) and (M2) phases have the underlying carotenoid pigments partially masked by the overlying melanin. The darkest (M1) phase differs

markedly from its Plymouth counterpart in that there is an apparently undiminished number of lipocytes present under the dense concentration of melanocytes. This preponderance of red-orange lipocytes is responsible for the general drab colours of Millport animals. The relatively high lipocyte density modifies the dark colour of the highly melanic Millport phases, resulting in the reddish tints of even the darkest animals and contrasting so sharply with the glossy black and brown Plymouth ophiuroids. This difference is independent of the distribution and density of the melanocytes.

The difference in the number of lipocytes between colour classes of the two populations are shown quantitatively on page 43.

ANALYTICAL METHODS

Standard methods for the separation and identification of carotenoid pigments are summarized by Karrer & Jucker (1950). Methods especially applicable to animal carotenoids have been recently reviewed by Fox (1953). Strain (1938) provides valuable technical information for dealing with xanthophyllic material. The methods used during the present work are set out briefly below.

Aerial oxidation was prevented by use of vacuum distillation for the concentration of solvents and by performing as many operations as possible in an atmosphere of nitrogen. Extractions were performed in a vessel with a tightly fitting ground glass stopper coated with high-vacuum silicone grease. Nitrogen was bubbled through the extracting solvents and the atmosphere above displaced with nitrogen before fitting the stopper. The space above solutions was always filled with nitrogen before storage. Chromatograms were run with gentle pressure from a nitrogen cylinder.

To minimize light-induced isomerizations solutions of pigments were stored in the dark. Manipulations were performed under a hood transmitting little but red light, to which carotenoids are practically insensitive. The effective part of the hood was made of double thicknesses of red 'cellophane', the light transmission of which is very low in the range 400–580 mμ.

Carotenoid extractions are usually carried out with some organic solvent which will denature and precipitate proteins, thus breaking down any protein-carotenoid complexes. For this reason the alcohols or acetone are normally employed. Acetone has a serious disadvantage in that it tends to form emulsions when diluted with water from which it is difficult to remove the pigment. This tendency is exaggerated if appreciable amounts of non-pigment lipids are present. Preliminary experiments with O. *nigra* tissues showed that acetone emulsions nearly always occurred upon dilution with water preparatory to transferring the pigment to petroleum ether. For that reason, the extraction mixture of Fisher, Kon & Thompson (1952) was employed. The proportions of this extraction mixture, per gramme of tissue, are:

$1\frac{1}{2}$ ml. of distilled water,
3 ml. of absolute ethanol,
10 ml. of petroleum ether, B.P. 40–60° C.

The aqueous ethanol denatures protein, liberating bound carotenoids which migrate into the petroleum ether layer, thus saving one step in the analytical scheme. Similarly

the non-lipid pigments, are preferentially soluble in the aqueous ethanol and are effectively separated from the beginning.

The adsorbents used were alumina (Savory & Moore, Brockmann standardized) for chromatography of epiphasic pigments, and calcium carbonate (B.D.H. 'Analar') for hypophasic pigments.

All solvents were of 'Analar' grade, except absolute ethanol which was industrial spirit (75·2° proof), and di-ethyl ether ('Anaesthetic grade' with a very low peroxide content). Absorption spectra were read in a 'Unicam' S.P. 350 diffraction grating spectrophotometer, using carbon disulphide as solvent.

It proved impossible to obtain any individual pigment in the crystalline state, chiefly through insufficient material. Identifications of individual pigments, in so far as possible, have therefore been made from absorption spectra, chromatographic and phase behaviour, and colour reactions.

PIGMENTS OF THE MILLPORT POPULATION

The animals collected at Millport were preserved there by dipping each in boiling sea water for a minute (which coagulates the protein and fixes the carotenoids *in situ*) followed by storage in deep-freeze (Fisher, Kon & Thompson, 1952, 1955).

Representatives of all the colour classes (M 1–M 5) were used in order to give a cross-section of all the possible pigments. The arms of the animals were cut off close to the disk and the disks discarded. Thus no gonadal pigments or gut 'enterochlorophylls' were present. A total of thirty-two animals were used, representing 45·98 g wet weight of tissue (arms only). The tissue was finely ground in a mortar with acid-washed silver sand and extracted for 24 h with the ethanol/petroleum ether mixture previously described. At the end of that time, the petroleum ether layer was coloured dark orange-yellow. The aqueous ethanol was pale yellow, the colour being due mostly to the lyophilic pigments. After decantation and filtering, the petroleum ether extract of crude carotenoid pigments was partitioned against 90 % methanol. Nearly all the pigment migrated into the hypophase, colouring it orange-yellow. The epiphase retained comparatively little pigment and was coloured pale yellow.

Colour reactions

Epiphase

The following colour reactions of the epiphasic pigments were noted:

Reagent	Solvent	Colour
Conc. H_2SO_4	Diethyl ether	Interphase blue-green, persistent
Conc. HNO_3	Diethyl ether	Transient blue, fading rapidly
25 % HCl	Diethyl ether	No coloration, even after prolonged shaking
Carr–Price	Chloroform	Green-blue, moderately intense

Method 1. One half of the epiphase was concentrated *in vacuo* and chromatographed on an alumina column. The chromatogram was developed with 0·5 % methanol in petroleum ether resulting in a rapid separation of two bands. Continued long develop-

ment revealed no tendency to splitting of the bands, suggesting single substances. Band I (uppermost) was orange-yellow and somewhat broader than Band II. Band II (lower) was red-orange, quite narrow and clear cut.

Development was stopped after 1 h, the bands dissected apart, and re-chromatographed to effect further purification. After eluting with diethyl ether, the pigments were transferred into CS_2 and their absorption spectra read (see Table 1).

Method 2. The remaining half of the epiphase was saponified by allowing it to stand overnight mixed with an equal volume of 25 % KOH in methanol. After saponification, sufficient water was added to make a final concentration of 90 % methanol, resulting in a biphasic system. While the epiphase was coloured pale yellow, a red precipitate gathered at the interface, indicating the soap of an acid carotenoid. The hypophase was colourless and was discarded.

After drying over anhydrous Na_2SO_4, the epiphase was concentrated *in vacuo* and chromatographed on an alumina column. A single orange-yellow band corresponding in position to Band I appeared. After elution with diethyl ether, it was transferred into CS_2 and its absorption spectrum read (see Table 1).

The interface dissolved readily in petroleum ether containing 1 % acetic acid. After several washes with water to remove excess acid, the solution was dried over anhydrous Na_2SO_4 and chromatographed on calcium carbonate. A single, sharp red-orange band, representing Band II, appeared. This fraction could not be eluted at all with any of the usual solvents. In subsequent analyses, this fraction was not re-chromatographed but was transferred directly into CS_2 and its absorption spectrum read (Table 1).

TABLE 1. ABSORPTION MAXIMA OF EPIPHASIC PIGMENTS FROM MILLPORT *OPHIOCOMINA* (SOLVENT, CS_2)

Band	Method 1	Method 2
I	485, 448 mμ	485, 448 mμ
II	500 mμ	500 mμ

Identifications

Of the two epiphasic pigments, only one remained epiphasic following saponification, indicating that it was a carotene. Its absorption maxima (485, 448 mμ) suggest that it is β-carotene (Vevers, 1952; Karrer & Jucker, 1950, p. 135.)

The second pigment is an ester of an acidic carotenoid as indicated by its soap formation during saponification and its solubility in acidic petroleum ether. Its smooth absorption spectrum with a peak at 500 mμ (before and after saponification), and its position on the chromatogram below β-carotene suggest mytiloxanthin or a closely similar pigment occurring as an ester in the tissue (Scheer, 1940).

Hypophase

Colour reactions

The following colour reactions of the hypophasic pigments were noted:

Reagent	Solvent	Colour
Conc. H_2SO_4	Diethyl ether	Blue-green, persistent
Conc. HNO_3	Diethyl ether	Instant bleaching
25 % HCl	Diethyl ether	No coloration of acid layer
Carr–Price	Chloroform	Moderate green-blue

Method 1. The hypophase was taken into petroleum ether from aqueous ethanol by dilution and dried over anhydrous Na_2SO_4. One half of this solution was concentrated *in vacuo* and chromatographed on calcium carbonate, developing with 10 % benzene in petroleum ether. After 3 h development, five bands had separated out.

Band I (uppermost) was yellow and adsorbed close to the top of the column. Band II was red-orange and very broad. Adsorbed close below it was a broad orange-red band (III), below which was a narrow red band (IV). Band V (lowest) was pink, forming an indistinct narrow zone well separated from the others and lying near the bottom of the column. This fraction degenerated very rapidly and its spectrum was never determined.

Bands I–IV were dissected out from one another, eluted with absolute ethanol, transferred into petroleum ether, and re-chromatographed separately to effect further purification. During this second chromatography Band III split into two substances, an upper orange-red band (III) and a lower red band (III*a*). Band III*a* was too small in amount to get a reading of its absorption spectrum. It probably represents an isomer or other derivative of III.

The absorption maxima in CS_2 of the principal bands of the hypophase are given in Table 2.

TABLE 2. ABSORPTION MAXIMA OF HYPOPHASIC PIGMENTS FROM MILLPORT *OPHIOCOMINA* (SOLVENT, CS_2)

Band	Method 1	Method 2
I	537, 493, 460 mμ	537, 493, 460 mμ
II	500 mμ	500 mμ
III	505 mμ	505 mμ
III*a*	Insufficient for a reading	Insufficient for a reading
IV	512 mμ	512 mμ
V	Degenerated before reading	Degenerated before reading

Method 2. The remaining half of the hypophase pigments were saponified as described previously and then partitioned between petroleum ether and 90 % methanol. Following saponification the hypophase was coloured deep orange-yellow indicating the presence of free xanthophylls, while a red precipitate at the interface indicated the presence of one or more acidic carotenoids. The epiphase remained colourless or, at most, showed faint traces of colour.

The interface precipitate was collected and dissolved in petroleum ether containing 1 % glacial acetic acid. After washing out the acid and drying the solution, a portion of it was chromatographed on calcium carbonate. Long development with 25 % benzene in petroleum ether revealed only a single broad red-orange band similar in position to Band II already described. This fraction could not be eluted with the usual solvents. A second portion was dissolved directly in CS_2 and the absorption spectrum read (see Table 2). It undoubtedly is identical with Band II detected by Method 1.

The hypophase was chromatographed on a calcium carbonate column. Four bands appeared on long development corresponding to Bands I, III, IV and V as described previously. The absorption maxima of these fractions is recorded in Table 2.

Identifications

Band II is an acidic carotenoid as indicated by its behaviour after saponification. Its absorption maximum suggests that it is a free form of the mytiloxanthin or a similar pigment which was detected previously in the esterified form.

The remaining pigments are not rendered acidic by saponification and represent free forms of xanthophylls. The absorption maxima of Band I approximate those of rubixanthin or gazaniaxanthin (Karrer & Jucker, 1950). However, both these substances are mono-hydroxy-xanthophylls, a class of carotenoid not detected in preliminary experiments by partitioning with 96 % methanol. Moreover, the position of Band I at the top of the column is most unlikely for a mono-hydroxy-compound. The identification of Band I is in doubt, therefore, and it may be a new xanthophyll.

Band III seems most likely to represent the new xanthophyll which Fox & Scheer (1941) encountered in the California ophiuroid, *Ophiothrix spiculata*. This band could also be compared with a new xanthophyll (maximum, 506 mμ) found by these authors in the 'melanic' ophiuroid, *Ophiopteris papillosa*.

The single absorption maximum of Band IV approximates that of the acidic carotenoid, astaxanthin, in its derivative form of astacene. Since Band IV is not rendered acidic upon saponification, it cannot be astaxanthin. Haematoxanthin shows a single broad maximum at 513 mμ, but it is an epiphasic pigment of unknown structure. The identity of Band IV is doubtful, therefore, but it is probably a new xanthophyll.

PIGMENTS OF THE PLYMOUTH POPULATION

Extracts of the pigments of Plymouth animals were made from fresh material sent alive from Plymouth to Oxford. Two analyses were made on a total of eleven animals; wet weight of tissue (arms only), 19·03 g. A full range of colour forms (P 1–P 5) was used to ensure a representative cross-section of the population. The analytical methods used were the same as described previously.

As with the Millport material, there was a preponderance of hypophasic pigment. Before saponification, the epiphase revealed two bands during chromatography: an upper orange-yellow band (I) with absorption maxima at 485, 448 mμ; and a lower red-orange band (II) with a maximum at 500 mμ. After saponification Band I remained epiphasic; Band II was collected as a red precipitate at the interface. Its properties were identical with those of Band II described from the Millport material. It is obvious that the epiphasic pigments of the Plymouth and Millport animals are identical; that is, the epiphase pigments of both populations are composed of β-carotene and an acidic carotenoid which resembles mytiloxanthin.

Chromatography of the hypophasic pigments before saponification revealed the presence of only four bands. These bands were similar in colour and position to their numerical counterparts from Millport. They also had similar absorption maxima, as follows: Band I: 537, 493, 460 mμ; Band III: 505 mμ; Band II: 500 mμ; Band IV: 512 mμ. After saponification, Bands I, III and IV remained hypophasic, whereas Band II displayed acidic properties and behaviour similar to its Millport counterpart. Evidently the hypophasic

pigments of both Plymouth and Millport animals are qualitatively identical, with two minor differences. In the Plymouth animals, there is no representation of Bands III*a* and V. It was suggested previously that III*a* may be an isomer or degenerative product of III, and it is possible that it did not form under the conditions of these analyses. Band V was extremely minute and its absence from the Plymouth material would make no appreciable difference to the visible coloration of the Plymouth animals.

A more important factor from the standpoint of any visible colour differences between the populations is the relative quantity of each type of pigment present. In both populations Bands III and IV are quantitatively similar, as judged by the width and intensity of colour of the pigment band

TABLE 3. CAROTENOID PIGMENTS DETECTED IN
OPHIOCOMINA NIGRA

| | Band | Colour | Absorption maxima (mμ) | Quantitative estimates | | Diagnosis |
				Millport population	Plymouth population	
Epiphasic pigments	I	Orange-yellow	485, 448	+ +	+ +	β-carotene
	II	Red-orange	500	+	+	Ester of acid carotenoid similar to mytiloxanthin
Hypophasic pigments	I	Yellow	537, 493, 460	+ +	+ + +	? New xanthophyll
	II	Red-orange	500	+ + + +	+ +	Free form of acid carotenoid similar to mytiloxanthin
	III	Orange-red	505	+ + + +	+ + + +	? Xanthophyll of Fox & Scheer (1941)
	III*a*	Red	—	Trace	None	Artifact
	IV	Red	512	+	+	? New xanthophyll
	V	Pink	—	Trace	None	Artifact

on the columns. Of all the pigments Band III is the most prominent constituent in both cases, but the proportions between Bands I and II are very different. In the Millport animals Band II is very much wider and more prominent than Band I. Band II, in fact, rivals Band III in width and intensity of colour. In the Plymouth animals, conversely, Band I is most prominent, being slightly less wide than Band III.

A summary of the pigments detected in both populations is given in Table 3.

THE VERTEBRAL PIGMENT SPOTS

In the course of dissecting fresh specimens of *O. nigra*, carotenoid-containing cells quite different from those previously described as lipocytes were encountered. These cells must be taken into consideration since their contents contributed to the analyses just described. They are located in the aboral region of each vertebral ossicle, occurring in two large distal and two smaller proximal patches on either side of the mid-dorsal region (Fig. 1 A and B). If

the upper arm plates of fresh specimens are dissected off under a stereo-microscope, these vertebral pigment spots appear as bright red-orange patches lying just under the arm plate. Their colour begins to fade very quickly when they are exposed to intense illumination, suggesting that they are remarkably
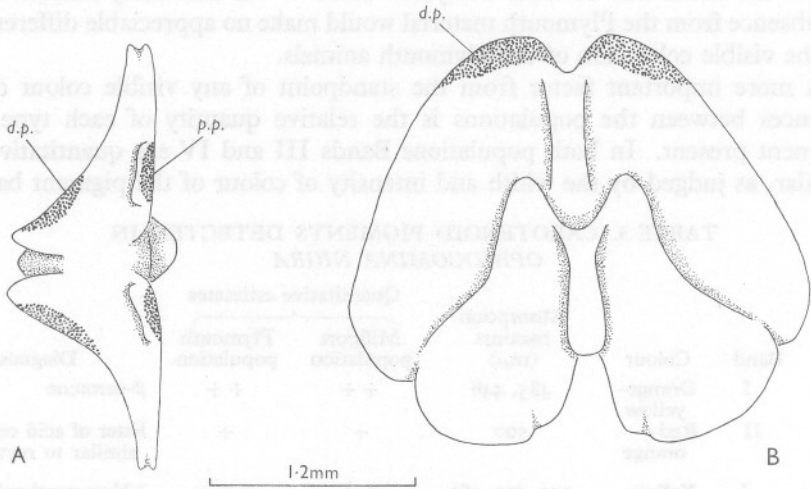

Fig. 1. Vertebral ossicle of *O. nigra* to show the disposition of the vertebral pigment spots. A, View of aboral surface. B, View of distal face. *d.p.*, distal pigment spot; *p.p.*, proximal pigment spot.
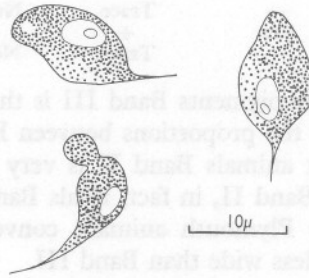

Fig. 2. Carotenoid-containing cells from the vertebral pigment spots. Camera lucida drawings with phase-contrast microscope.

sensitive to light and/or aerial oxidation. The pigment gives the histochemical reactions of carotenoids.

Tiny patches of these areas were dissected out, mounted in sea water and examined by means of the phase-contrast microscope. The cells (Fig. 2) are roughly ovoid, most having one end drawn out into a fine filament, the termination of which was invisible. Exclusive of the filament, the cells are approximately 10μ long. The nucleus is ovoid, about 4μ long, and contains

a nucleolus. The cytoplasm is filled with brilliant orange-red granules less than $1\,\mu$ in diameter. These granules are discrete and do not seem to be liquid droplets. In moribund cells they exhibit marked Brownian movement. The cytoplasmic background is colourless and occasionally vacuoles are present.

Gelatin-embedded, frozen sections were cut of material fixed in formol-calcium, and coloured with Sudan black B. Under these conditions no granules could be observed. Instead the cytoplasm was filled with a number of black-coloured droplets of various sizes.

The pigments in the vertebral pigment spots were analyzed from six Millport animals. This material had been preserved as usual by boiling and storage in deep-freeze. While still frozen (and very brittle in consequence) the upper and lateral arm plates were dissected off along most of the length of each arm revealing the vertebral pigment spots. The frozen state facilitates this otherwise tedious manipulation. With a fine blade the upper surface of the vertebrae was shaved off. This material contained the pigment spots plus small amounts of muscle, ossicle and connective tissue. About 900 pigment spots were removed this way.

The tissue was extracted in absolute ethanol and subjected to the analytical scheme already described. The pigments were entirely epiphasic and, upon chromatography, revealed two bands similar in position and appearance to the epiphasic pigments detected in both populations. Band I was orange-yellow, epiphasic after saponification, and showed an absorption maximum at $485\,\mathrm{m}\mu$ and an ill-defined peak at $445\text{--}450\,\mathrm{m}\mu$. Band II was red-orange, revealed acidic properties upon saponification and showed an absorption maximum at $500\,\mathrm{m}\mu$. It can be concluded that the vertebral pigment spots contain β-carotene and esters of the acid carotenoid similar to mytiloxanthin. It is probable that a large proportion, if not all, of the epiphasic pigments detected previously in extracts of whole arms is derived from the pigments of the vertebral pigment spots.

QUANTITATIVE VARIATIONS

The quantitative variations of total pigment between colour classes of a population and between corresponding classes of different populations were studied from simultaneous extractions of the following classes of animals: P1, P4, M1, M3 and M5. There were unfortunately no P3 and P5 animals available at the time of this experiment.

A method of direct measurement by weight was thought to be sufficiently sensitive to detect gross changes in total carotenoids without the need of photometric measurements involving the calibration of an instrument with a 'standard' series of some known carotenoid. β-carotene, the conventional standard, occurred in such small quantities that its measurement would be practically useless, and the other pigments are not available as pure substances.

The weights determined, therefore, are those of total carotenoids plus an indeterminate amount of colourless oil which is assumed to be constant between the colour classes. This is perhaps a dangerous assumption, but Fox & Pantin (1941) have shown that the total lipid content (exclusive of carotenoids) of *Metridium senile* remains constant between its various colour forms. Scheer (1940) has shown that a similar situation exists in *Mytilus californianus*.

To make a measurement the animals were dried on filter paper, the arms cut off and the wet weight (arms only) determined to the nearest milligram. After grinding the tissue, extraction was conducted for exactly 12 h using the methods previously described. The volumes of solvents were calculated and scrupulously measured. After extraction, exactly one-half the volume of the petroleum ether layer was pipetted into a tared flask. The solution was evaporated to dryness *in vacuo* and the flask with its oily residue placed in a dessicator over P_2O_5 for 1 h to remove traces of moisture. The flask was weighed to the nearest tenth of a milligram, and the total oils per gramme of tissue calculated. The results are shown in Table 4 and discussed on p. 46.

TABLE 4. COMPARISON OF THE WEIGHT OF TOTAL OILS IN DIFFERENT COLOUR PHASES OF *OPHIOCOMINA NIGRA*

Population	Colour class	No. of animals	Wet weight of tissue (g)	Total oils in petroleum ether (g)	Total oils, mg/g of tissue
Plymouth	P1	3	5·070	0·0250	4·9
	P4	2	3·275	0·0258	7·8
Millport	M1	4	5·195	0·0418	8·0
	M3	4	3·845	0·0308	8·0
	M5	3	3·660	0·0310	8·5

DISCUSSION

The carotenoids of *O. nigra* are mainly free xanthophylls and an acidic carotenoid. This agrees with the pattern which Fox & Scheer (1941) found in the three Californian species which they investigated. The presence in *O. nigra* of the xanthophyll with a maximum at 505 mμ, first detected by Fox & Scheer, together with two other xanthophylls whose spectra do not approach any known pigments, supports the suggestion (Fox, 1953, p. 106) that there are probably specific carotenoid pigments associated with each class of echinoderm.

Fox (1953, pp. 105–6) has called attention to the fact that the asteroids and ophiuroids investigated so far store more oxygen-containing carotenoids (xanthophylls and acid carotenoids) than do the other echinoderm classes. He supposes this to be due to their carnivorous habits (animal food being carotenoid rich in comparison to mud or detritus), basing this assumption on the classification of feeding mechanisms proposed by Yonge (1928). In view

of recent studies (Vevers, 1956; Fontaine, unpublished) in which it is shown that detritus and phytoplankton can and probably do form the major part of the diet of *O. nigra* and some other ophiuroids and asteroids, these animals should really be classed as omnivores. Fox's alternative suggestion that the stelleroid group metabolizes the hydrocarbon carotenes more rapidly than the oxygen-containing compounds seems, therefore, to be more likely.

The discovery of β-carotene in *O. nigra* is noteworthy since, being an important vitamin A precursor, it raises the possibility of *O. nigra* being of some importance in the economy of the sea by serving as a food source of provitamin A for other animals. The detection of β-carotene confirms the findings of Lönnberg & Hellström (1931). On the other hand, no trace was found by me of xanthophyll (lutein) which was detected by these authors. This anomaly may very well be due to racial or food-selection differences, but inasmuch as the pigments of the Scandinavian animals were not separated chromatographically before spectroscopy they should be re-investigated.

The function of the vertebral pigment spots is unknown. Their marked sensitivity to light and aerial oxidation, and their anatomical position under a light screen in the form of the pigmented integument of the upper arm plate suggests that they may play a role in photo-reception or be associated with a photo-receptor element. The photo-receptor pigments of the asteroid *Marthasterias glacialis* have been shown to be β-carotene and astaxanthin ester (Millott & Vevers, 1955). The vertebral pigment spots of *O. nigra* contain β-carotene also but not astaxanthin; rather, a pigment resembling mytiloxanthin ester. On the available evidence it is not possible to ascribe a photo-receptor function to the vertebral pigment spots. It may be that these spots serve as a storage organ for these pigments which may have a specific metabolic role as yet unknown. It would be interesting to see if vertebral pigment spots occur in other ophiuroid species and, if so, which pigments are present.

The relationships between population colour differences and carotenoid pigmentation are extremely interesting. In view of the considerable difference in tone between the Millport and Plymouth populations, it is remarkable that there is so little qualitative difference between their respective pigments. With the exception of two minor constituents, Bands III*a* and V from Millport animals, the kinds of pigment are identical in each population.

A great deal of the inter-population colour difference is due to the relative concentrations of the pigments forming Bands I and II. It has been noted that the orange-red xanthophyll forming Band III is the most abundant carotenoid in both populations. In Millport animals, however, Band II (a red-orange acid carotenoid) occurs in far greater concentration than does Band I (a yellow xanthophyll). In Plymouth animals, the converse is true. Presumably this yellow xanthophyll is the predominant constituent of the yellow lipocytes seen under the microscope. Its comparative abundance in extracts from Plymouth ophiuroids agrees well with the histological picture

in which many yellow lipocytes are apparent in Plymouth specimens but relatively few in Millport animals. The darkest Millport ophiuroids possess an abundance of lipocytes as well as of melanocytes, whereas the darkest Plymouth animals have remarkably few lipocytes. This difference accounts for the glossy blacks of melanic Plymouth animals in contrast to the dull reddish appearance of melanic Millport ophiuroids.

The quantitative data support this observation. Non-melanic (P4) individuals have about one and a half times more total oils than do melanic (P1) animals. In contrast, the difference in total oil content between melanic (M1), intermediate (M3) and non-melanic (M5) animals is negligible. All Millport animals, therefore, including the darkest, have a high total oil content comparable to that of the non-melanic Plymouth animals. If the assumption that non-pigment lipids are constant between colour classes is correct, then these figures can be interpreted as representing differences in carotenoid concentration. Thus, the carotenoid content of Millport animals remains approximately constant between members of the most widely divergent colour classes. By contrast, in Plymouth animals there is wide divergence of carotenoid content between the extremes of colour, with non-melanic forms having very low values.

SUMMARY

The red to orange-yellow colours of the ophiuroid, *Ophiocomina nigra*, are due to carotenoid pigments contained in specialized lipocytes. These cells are dendritic, with their cell bodies located in the dermis below a melanocyte layer and with dendrites approaching, but not penetrating, the epidermis. The lipocytes are either yellow or orange-red.

Five principal pigments were detected in addition to two minor constituents, at least one of which may be an artifact. These major pigments are: β-carotene, an acid carotenoid similar to mytiloxanthin, and four xanthophylls of uncertain identity.

New organs termed vertebral pigment spots are present on the aboral surfaces of the vertebral ossicles. The cells of these organs contain β-carotene and esters of the acidic carotenoid. Their function is unknown, but they are notably sensitive to light and/or aerial oxidation.

The pigments of populations of brittle-stars from Millport and Plymouth are compared. The populations show marked colour differences, although within each population there is a continuous variation of colour from black to

orange-yellow. Qualitatively there is no distinction between the pigments of the populations. There is, however, a preponderance of a red-orange acid carotenoid in the Millport population and of a yellow xanthophyll in the Plymouth population. These quantitative differences account for some of the inter-population colour variation. A second factor is the general lack of lipocytes in the darkest individuals from Plymouth. The darkest Millport animals possess a full complement of lipocytes. The presence of these lipocytes in dark Millport animals is responsible for their reddish tones. These histological observations are supplemented by some quantitative data.

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