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THE COLOURS OF *OPHIOCOMINA NIGRA* (ABILDGAARD)

II. THE OCCURRENCE OF MELANIN AND FLUORESCENT PIGMENTS

By A. R. FONTAINE

Department of Zoology and Comparative Anatomy, Oxford*

(Plates I–II and Text-figs. 1–3)

Although the dark colours of ophiuroids have been attributed to melanin pigmentation (Verne, 1926; Fox, 1953, p. 222; Fox & Scheer, 1941), the chemical properties of the dark pigment have not hitherto been examined. I have therefore investigated the dark pigment of *Ophiocomina nigra* (Abildgaard), and shown that it should be regarded as a true melanin. Associated with the melanin-containing cells are fluorescent pigments, which, it is suggested, are involved in the process of melanogenesis. An enzyme system is also demonstrated which may control melanogenesis.

The colour phases of *O. nigra* have already been described (Fontaine, 1962), and the same colour nomenclature has been used again here to describe the experimental animals. The experimental work was carried out at Oxford, the ophiuroids being sent alive from Plymouth and maintained in closed-circulation marine aquaria.

THE ANATOMY OF THE MELANOCYTE

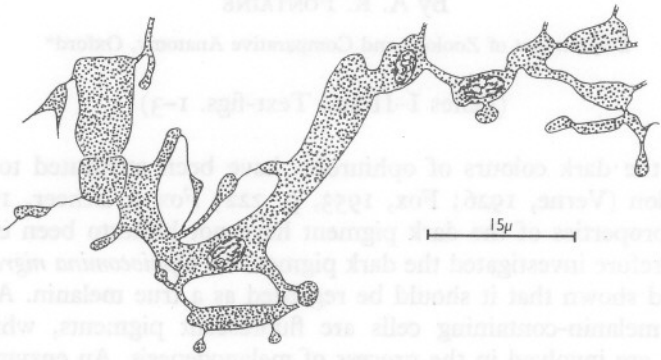
Excised pieces of the integument were mounted in sea water and examined microscopically by reflected or transmitted light. These excised pieces could be sealed with 'Vaseline' in a hanging drop in a cavity-slide and kept alive for approximately 24 h. Studies on living material were supplemented by examination of 8 μ paraffin or doubly embedded sections of tissues fixed for 6–8 h in Zenker's or Helly's fluids. The tissues were decalcified in 15% ethylene–diamine–tetra-acetic acid buffered at pH 7·3, and cleared in toluene. The sections were usually examined unstained or else lightly stained with toluidine blue.

The dark pigment occurs in the form of coarse granules about 0·5–0·7 μ in diameter, very dark brown in black or dark-brown forms, and fawn coloured in light-brown individuals and in light-coloured areas of dark individuals.

* Present address: Department of Biology, Victoria College, Victoria, B.C., Canada.

The granules occur densely packed in arborescent cells, which vary in shape depending upon the framework of the particular ossicle in which they are lodged.

The ossicles of *O. nigra*, like those of all echinoderms, consist of a three-dimensional lattice of calcareous elements. Spaces between the struts are filled with tissue, and cells of various sorts, including the melanocytes, are thus contained in tube-like channels ramifying throughout the ossicles. The long axes of the cells run roughly parallel to the axes of the lattice.

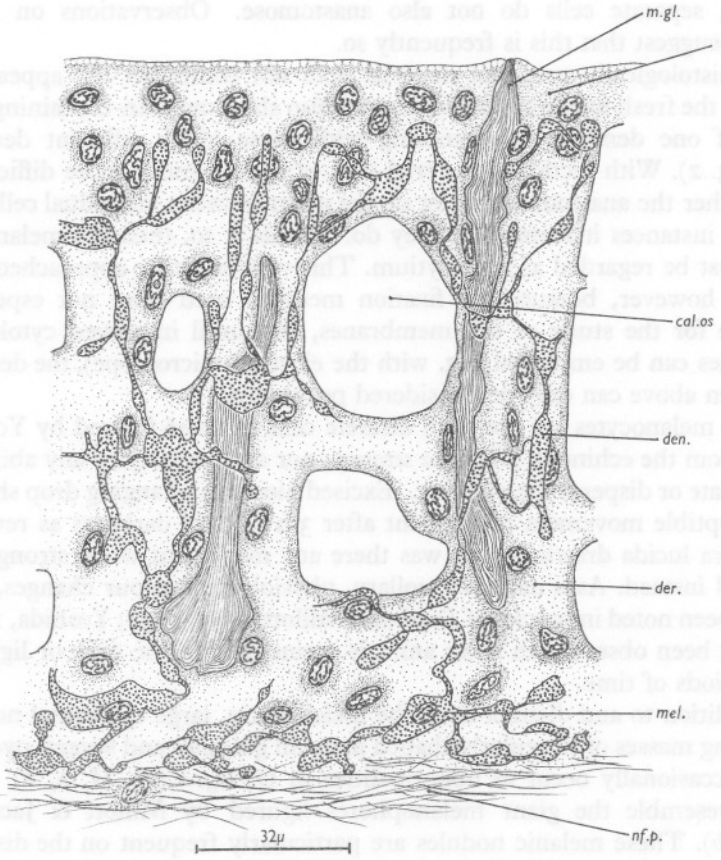


Text-fig. 1. Melanocyte of *O. nigra*, to show appearance of cell-body region; the dendrites have been omitted. Camera lucida drawing, transverse section of upper arm plate.

My use of the term *melanocyte* for these cells may need justification. Gordon (1953, in preface) discusses the terminology of melanin-containing cells distinguishing between melanocytes (melanin-producing and -containing cells) and melanophores (pigment effector cells). Because the available evidence indicates that the pigment cells of *O. nigra* are not effector cells, it seems preferable to call them melanocytes although there is no evidence at present that melanin is produced directly within them.

The cell bodies of the melanocytes are usually located in a layer beneath the ossicles. The cell bodies are of irregular shape and variable in size (Text-fig. 1), but are generally somewhat flattened in a plane parallel to the adjacent epidermis, and contain a central, ovoid nucleus about 5–6 μ in length. From the cell body radiate a number of dendrites which pass laterally and then through the ossicle towards the surface of the integument coming to lie just beneath or penetrating the lower portion of the epidermis and often spreading out parallel to it (Text-fig. 2). A dendrite consists of a principal shaft with smaller branches springing irregularly from it. The shaft and its branches consist of nodes of densely packed granules strung together by thin protoplasmic strands containing granules often in a single, bead-like row. Sac-like processes full of melanin granules arise irregularly from the finer branches. Owing to its convolutions, it is difficult to measure the length of a single dendrite, but some

exceed 60 μ. The dendrites pursue a tortuous course through the interstices of the ossicle. Viewed from above (Pl. I, B, C), the dendrites are seen in channels within the ossicle, from which they spread out radially beneath the epidermis on reaching the outer surface of the ossicle.



Text-fig. 2. Tangential section, upper arm plate of *O. nigra*, showing melanocyte cell bodies forming the pigment layer of the dermis and the ramification of their dendrites through the calcareous layer. Note the apparent syncytial nature of the melanocytes. *cal.os.*, calcareous ossicle (decalcified); *den.*, dendrite of melanocyte; *der.*, dermal tissue; *epid.*, epidermis; *mel.*, melanocyte cell body in pigment layer; *m.gl.*, mucous gland; *nf.p.*, neurofibrillar plexus. Camera lucida drawing.

Small masses of pigment granules are frequently found in the epidermis which do not have any apparent connexion with a melanocyte. They are presumably derived from the latter by clasmatosis. These isolated masses of granules are more frequent in the outer epidermal layer.

The most unusual feature of these cells is that the minor processes of the

dendrites appear to run into and become continuous with the processes of adjacent dendrites (Pl. I, D). Such anastomoses are frequent and cause the appearance in any field of view of a dense and confused network of melanin-containing processes (Pl. I, A). The dendrites ramify so widely and are so densely crowded that it is difficult to determine whether the processes of adjacent, separate cells do not also anastomose. Observations on living material suggest that this is frequently so.

The histologically prepared material does not contradict the appearance given by the fresh material. These sections also show melanin-containing processes of one dendrite anastomosing with those of an adjacent dendrite (Text-fig. 2). With sectioned material it is, of course, much more difficult to tell whether the anastomoses do or do not occur between individual cells, but in many instances it seems that they do. If this is so, then the melanocyte layer must be regarded as a syncytium. This view must be approached with caution, however, because the fixation methods used were not especially adequate for the study of cell membranes, and until improved cytological techniques can be employed (e.g. with the electron microscope), the description given above can only be considered provisional.

These melanocytes are not such discrete cells as those figured by Yoshida (1956) from the echinoid, *Diadema setosum*, nor do they display any ability to concentrate or disperse the pigment. Excised pieces in a hanging drop showed no perceptible movement of pigment after 3 h in total darkness as revealed by camera lucida drawings, nor was there any movement when strong light was used instead. As a natural corollary, physiological colour changes, such as have been noted in species of *Diadema* (Millott, 1950, 1954; Yoshida, 1956), have not been observed in adult animals maintained in the dark or light for long periods of time.

In addition to and distinct from the melanocytes, large epidermal nodules containing masses of irregularly shaped melanin granules and several pycnotic nuclei occasionally occur as abnormalities in *O. nigra* (Pl. II, A, B). They closely resemble the giant melanophores figured by Millott & Jacobson (1952*a*, *b*). These melanic nodules are particularly frequent on the disk and between the spines of animals kept for a long time in aquaria: they may well be melanotic tumours arising at sites of infection.

Amoebocytes containing scattered melanin granules are frequently found in the dermal connective tissue layer.

NATURE OF THE DARK PIGMENT

Investigation of the properties of the dark pigment was carried out by histochemical studies on fixed and sectioned material and by extraction of the pigment and subsequent study of the crude extract.

Properties of melanins

The most prominent characters used for the histochemical diagnosis of melanins are (1) insolubility, (2) ready bleaching by oxidizing agents and (3) direct reduction of alkaline solutions of silver nitrate. These characteristics are shared with a number of minor dark pigments, not necessarily tyrosine derivatives (e.g. chromaffin and argentaffin substances, some haem derivatives and lipo-pigments), which can be distinguished on the basis of tests more or less specific for their respective class. In any case, these minor pigments, some of which may be artifacts, contribute nothing to the external pigmentation of an animal.

However, melanins can be easily confused with a major class of pigments derived from tryptophane which are responsible for visible dark pigmentation of the integument of a number of animals. These pigments were called ommochromes by Becker (1941). Being polyphenols the ommochromes can have the reducing properties of melanins and also the inertness of melanins, particularly when the ommochrome is in a highly polymerized state and has been subjected to some fixation or dehydration process (Goodwin & Srisukh, 1950). It is obvious, therefore, that confusion is liable to occur when the identification of an inert, dark pigment is based solely on traditional histochemical methods.

The chemical and physical properties of ommochromes were described by Becker (1941, 1942) whose account has been supplemented and corrected by Butenandt *et al.* in a continuing series of papers beginning in 1954.

Some properties by which ommochromes may be distinguished from melanins are summarized below:

(1) Ready solubility in acid alcohols at room temperature; also soluble in formic acid, dimethylformamide, and dilute alkalis, giving red solutions.

(2) Soluble without change in mineral acids (except HNO_3) dislaying highly coloured solutions.

(3) Red fluorescence in UV light in acid solution.

(4) Some, but not all, ommochromes show redox properties. Usually they are dark when reduced, light when oxidized. Melanins are dark when oxidized, light when reduced.

(5) Ommochrome granules are usually violet-brown when viewed microscopically; melanin granules never show violet shades.

So far, ommochromes have been detected in the integuments of a wide variety of arthropods and several molluscs (Forrest, 1959). It is probable that they are widely distributed in invertebrates but as yet have not been recognized as such in many groups.

Extraction of melanin

Two methods of extracting the brown pigment were employed.

Method 1. Finely ground tissues were allowed to cytolize in distilled water overnight. The intensely dark-brown suspension was pipetted off and centrifuged until most of the pigment had settled out. This was washed several times with acetone to remove traces of lipid pigments, with ethanol to remove other coloured substances, and finally with water to remove the organic solvents. It was then studied while moist or after drying over phosphorus pentoxide. This method is based on that of Millott & Jacobson (1952*b*).

Alternatively the suspension was filtered off and treated as before. In either case the resulting preparation of crude pigment was black-brown.

Method 2. The standard methods for extracting melanins (Fox, 1953) involve solution in strong and usually hot alkali. In this study, a relatively mild method was employed. Finely ground tissues were extracted with 0·2% NaOH for 12 h at room temperature. As the density of pigment in solution rose, the solution changed colour from greenish-black to dark red-brown. After filtration to remove suspended, non-pigment matter, the solution was brought to pH 5·5 with glacial acetic acid, precipitating the pigment as dark-brown flocks. If concentrated HCl were used instead, the precipitate was red-brown. After filtration the precipitate was redissolved in alkali, filtered and reprecipitated in order to effect some further purification. The final product was washed and treated as described above. The resulting preparation was dark brown-black or, if HCl had been used, red-brown.

Extraction methods for other pigments

Finely ground tissue was extracted to exhaustion with acetone to remove lipid pigments. This treatment yielded an orange solution (Fraction I) fluorescing faint blue-green in UV light and subsequently shown to contain only carotenoid pigments (see page 19).

The tissue mash was then extracted to exhaustion with 80% aqueous ethanol to remove lyophilic pigments. This yielded a pale yellow solution (Fraction II) fluorescing brilliant greenish-blue in UV light and subsequently shown to contain riboflavin and traces of Fraction III (see page 20).

A dark yellow solution (Fraction III) fluorescing a very brilliant blue in UV light was obtained by extracting the mash to exhaustion with 5% HCl in ethanol. Nothing further was extracted by using acid methanol subsequently. Fraction III should have contained any ommochromes that were present in the tissues. However, none was detected. This fraction was shown to contain two probable pteridine pigments (see page 21).

After all these extractions, the tissue mash was still brown in colour. When it was examined microscopically, the pigment could still be seen in the tissue fragments as brown granules. This dark pigment was now soluble with difficulty in cold 0·2% NaOH, but could be completely extracted in 1 h with hot alkali, yielding a red-brown solution similar to that previously described. From these observations, it may be concluded that the dark pigment is not an ommochrome. This relative insolubility in alkali subsequent to the alcoholic extractions is probably due to fixation of the tissue proteins by the alcohol, and further suggests that the native pigment occurs as a pigment-protein complex.

PROPERTIES OF THE EXTRACTED DARK PIGMENT

Solubility properties

Portions of the extracted pigment were placed in stoppered test-tubes with 5 ml. of the solvent where they remained for up to 1 week. The solvents and results are shown in Table 1.

TABLE 1. SOLUBILITY PROPERTIES OF EXTRACTED DARK PIGMENT

Solvent	Extract: Method 1	Extract: Method 2	Colour of solution
Water	−*	−*	.
Methanol	−	−	.
Ethanol	−	−	.
Benzene	−	−	.
Diethyl ether	−	−*	.
Acetone	−	−	.
Pyridine	−*	−*	.
Ethylene chlorhydrin	−	−	.
10% urea, cold	−	−	.
0·1 N-HCl	−	−	.
Conc. HCl	++	++	Red-brown
Conc. H_2SO_4	+	++	Red-brown
0·1 N-HNO_3	bleached	bleached	.
Conc. HNO_3	bleached	bleached	.
Abs. formic acid	−	+	Red-brown
5% HCl in methanol	−	+	Brown
2 N-H_2SO_4 at 80° C	−	+	Red-brown
0·2% NaOH	++	++	Green-black

(−) indicates insoluble.
(+) indicates soluble with difficulty.
(++) indicates readily soluble.
(*) indicates occasional suspensions; i.e. pigment settled out of solution within 24 h leaving the solvent perfectly clear.

These solubilities are similar to those of typical melanins. It is noteworthy that the pigment extracted by alkali is distinctly more soluble, suggesting that a considerable amount of alkaline hydrolysis has taken place despite the mild methods used. In concentrated HNO_3 the pigment showed a colour sequence before bleaching similar to that described by Millott & Jacobson (1952 *b*); that is, black → red-brown → red → colourless.

Ethylene chlorohydrin (Lea, 1945) has been described as a solvent for melanin, but later work has not confirmed this claim for native melanins (Taft, 1949; Millott & Jacobson, 1952*b*). The melanin of *O. nigra* is also insoluble in this solvent; it was not soluble in cold 10% urea although this also has been described as a solvent for dopa-, *Sepia*-, and adrenalin melanins (Lea, 1954). The insolubility in both urea and ethylene chlorhydrin may be due to the ophiuroid melanin not being completely removed from a protein component to which it is complexed in the tissues.

Effect of oxidizing agents.

The following oxidizing agents effected complete bleaching of the pigment within 48 h: 0·1 % potassium permanganate solution: dilute 'Milton' (a commercial preparation containing sodium hypochlorite); 1 % chromic acid; hydrogen peroxide (20 vol.); saturated bromine water. These reactions are characteristic of melanins (Fox, 1953).

Fluorescence. A portion of the wet precipitate taken up in distilled water showed a faint yellow-green fluorescence in UV light. This was not altered by rendering the suspension either acid or alkaline. Solutions of the pigment in 0·2 % NaOH also gave a faint yellow-green fluorescence. When dissolved in concentrated HCl or H_2SO_4, the pigment gave no fluorescence at all. These observations agree with its being a melanin (Fox, 1953). Ommochromes would have fluoresced red in acid solution (Butenandt *et al.*, 1954).

Reduction reaction. The addition of sodium hydrosulphite to a suspension of the pigment in water quickly changed the dark brown to a fawn colour. The subsequent addition of sodium nitrite, or H_2O_2, and shaking in air restored most of the colour, although it was never quite so dark as the original sample. This readily reversible reduction is characteristic of melanins (Figge, 1939, 1940).

Other chemical reactions. Ehrlich's reaction for the indole nucleus was strongly positive, while the aldehyde reaction for tryptophane was negative. A suspension of the pigment in water gave a persistent yellow-green colour with $FeCl_3$, indicating the presence of *o*-dihydroxyphenols (Fox & Pantin, 1941).

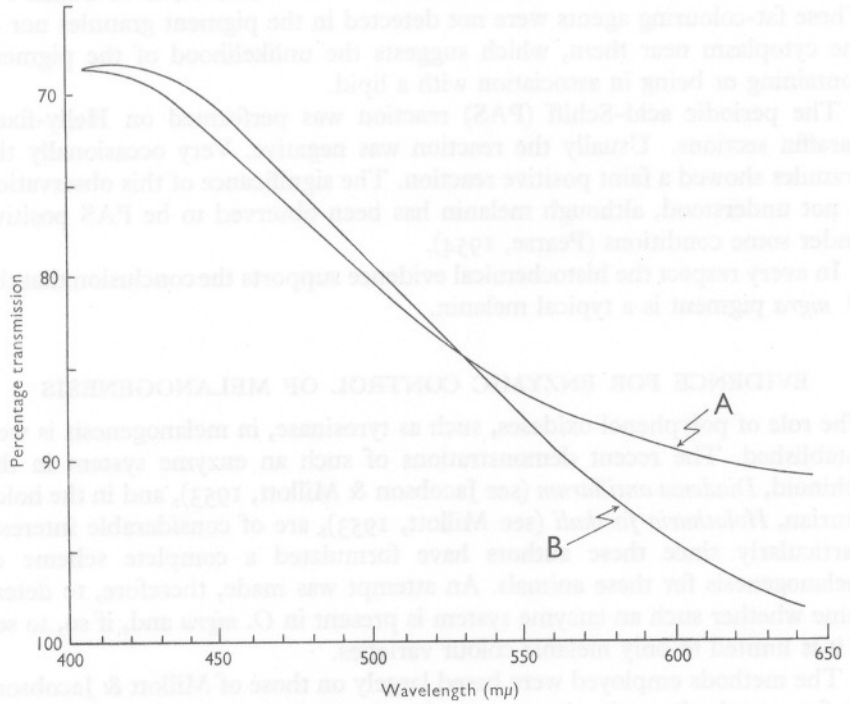
Absorption spectrum. The visible light absorption spectrum of the ophiuroid pigment dissolved in 0·2 % NaOH is shown in Text-fig. 3, along with that of synthetic dopa-melanin similarly dissolved for comparison. The dopa-melanin was prepared by auto-oxidation of a 0·0005 M solution of DL-3,4-dihydroxyphenylalanine, buffered at pH 6·8, for 48 h at 37° C (Nickerson, 1946). The two curves show similar characteristics and, in turn, are similar to Nickerson's curve (1946, fig. 2) of the absorption spectrum of fully oxidized synthetic dopa-melanin.

HISTOCHEMICAL REACTIONS OF THE DARK PIGMENT

The basis for the histochemical identification of melanins has already been discussed in this report and the limitations of the techniques pointed out. Despite these limitations a histochemical survey supplements the observations upon extracts. In a study of this sort upon a newly described pigment, it is particularly valuable in that it provides a wider area for comparison with other, better-known pigments.

To test the solubility of the ophiuroid pigment, paraffin sections of tissues

fixed in Bouin's or Helly's fluids were treated with the same solvents outlined in Table 1. Subsequent microscopic examination showed that none of the solvents acted upon the pigment, except 0·2 % NaOH which effected its slow dissolution. This solubility behaviour is characteristic of a melanin. The difficulty of solution in alkali is, as mentioned previously, probably due to fixation of a protein 'skeleton' to which the native pigment is attached.



Text-fig. 3. Absorption spectra of melanins dissolved in 0·2 % NaOH at approximately similar concentrations. A. *O. nigra* melanin. B. Synthetic dopa-melanin.

Similarly prepared tissues were exposed to the oxidants listed on page 16. All of them effected bleaching within 24 h, except H_2O_2 which required 48 h.

Sections were placed in water containing a trace of sodium hydrosulphite. Within a minute the dark-brown pigment had been reduced to a fawn-coloured state. Addition of sodium nitrite, H_2O_2 or shaking in air did not oxidize the fawn substance back to its original colour. This is unlike the results obtained with the extracts, which could be reversibly reduced easily and repeatedly. Why the fixed and sectioned material does not behave similarly is not clear. However, the reduction to a lighter coloured state is a distinct characteristic of melanin (Figge, 1939, 1940; cf. Pearse, 1954, p. 343).

Helly-fixed paraffin sections were treated with Masson–Fontana's solution in the dark for 24 h (Lison, 1953, p. 423). The pigment granules reduced the silver nitrate to metallic silver so that the granules appeared black upon examination. This is diagnostic of pigments with reducing properties, such as melanin, but it is not an exclusive characteristic of this pigment.

Frozen sections were cut of tissues fixed in formol/saline and embedded in gelatine. These sections were then stained with Sudan black or Sudan IV. These fat-colouring agents were not detected in the pigment granules nor in the cytoplasm near them, which suggests the unlikelihood of the pigment containing or being in association with a lipid.

The periodic acid-Schiff (PAS) reaction was performed on Helly-fixed paraffin sections. Usually the reaction was negative. Very occasionally the granules showed a faint positive reaction. The significance of this observation is not understood, although melanin has been observed to be PAS positive under some conditions (Pearse, 1954).

In every respect the histochemical evidence supports the conclusion that the *O. nigra* pigment is a typical melanin.

EVIDENCE FOR ENZYMIC CONTROL OF MELANOGENESIS

The role of polyphenol oxidases, such as tyrosinase, in melanogenesis is well established. The recent demonstrations of such an enzyme system in the echinoid, *Diadema antillarum* (see Jacobson & Millott, 1953), and in the holothurian, *Holothuria forskali* (see Millott, 1953), are of considerable interest, particularly since these authors have formulated a complete scheme of melanogenesis for these animals. An attempt was made, therefore, to determine whether such an enzyme system is present in *O. nigra* and, if so, to see if it is limited to only melanic colour varieties.

The methods employed were based largely on those of Millott & Jacobson. Unfortunately the coelomic spaces and coelomic fluid available from *O. nigra* are insufficient to allow performance of experiments comparable to those carried out with *Diadema* and *Holothuria*. The study was limited, consequently, to whole tissue extracts as used by Fox & Pantin (1941) in their study of *Metridium senile*.

Treated separately, the arms of a number of both melanic (P 1) and non-melanic (P 4) *O. nigra* were cut free from the disk and finely ground with acid-washed sand in Sørenson buffer at pH 7·4. The amount of buffer used was equivalent to 10 ml./g. wet weight of tissue. After filtration to remove debris, the extract was yellowish and opalescent. Aliquots were placed in test-tubes and treated as described in Table 2. After the expiration of 2, 8, and 12 h at room temperature the colour of the various tubes was recorded. The experiments were repeated several times using from two to six animals at a time.

When filtered off the dark pigments formed *in vitro* exhibited the same properties as the native melanin already described from *O. nigra*.

The production of this dark pigment is evidently an oxidation process as evidenced by its inhibition in a nitrogen atmosphere and acceleration by bubbling through of air. It was also noted that the darkening proceeded from the top of the tube downward. The inhibition after boiling and by cyanide indicate control of the oxidation by an oxidizing enzyme(s). This enzyme(s) must be distinct from the cytochrome oxidase system since sodium azide is

TABLE 2. COLOUR CHANGES EXHIBITED BY AQUEOUS EXTRACTS OF *O. NIGRA* (MINUS INTESTINE) TREATMENT AT pH 7·4

	Colour variety					
	P1 (melanic)			P4 (non-melanic)		
	Time elapsed in hours					
Treatment of the extract	2	8	12	2	8	12
Extract only	y-br	br	bl	y-br	br	Dark br
Extract boiled	No change			No change		
Extract in an atmosphere of nitrogen	No change			No change		
Extract plus air bubbled through	br	bl	—	br	Dark br	—
Extract irradiated with UV light	br	bl (after 4 h)		br	Dark br (after 4 h)	
Extract plus 0·0003 M-NaCN	No change			No change		
Extract plus 0·002 M-NaN_3	y	br	bl	y	br	Dark br

y = yellow, br = brown, bl = black.

known to be an inhibitor of this system. Acceleration of the reaction by UV irradiation is in accord with the reaction being controlled by a phenolase system. In summary the evidence suggests that melanogenesis in *O. nigra* is controlled by an oxidase system probably similar to the phenolase system described for other echinoderms. In this case the enzyme system is present in non-melanic as well as melanic colour phases.

NATURE OF THE FLUORESCENT PIGMENTS

The methods by which the fluorescent pigments were extracted are described on page 14.

Fraction I

This acetone-soluble fraction gave a blue-green colour with concentrated H_2SO_4 and a blue colour with the Carr–Price reagent. It was negative to the murexide reaction and fluoresced blue-green in UV light. It was not reduced by sodium hydrosulphite. These reactions indicate that Fraction I was

composed of carotenoid pigments. Further studies showed the presence of seven carotenoid pigments which will form the subject of a separate account.

Fraction II

This ethanol-soluble fraction gave no carotenoid colour reactions. It was reduced to a leuco-compound by hydrosulphite, regaining its colour with shaking in air. It was negative to the murexide reaction. The original extract fluoresced brilliant bluish-green in UV light. Permanganate oxidation did not decolorize the pigment in solution, but the solution now fluoresced yellow-green, indicating that some fluorescent component had been oxidized. When a portion of the original extract was passed through an alumina column, a single pale yellow band of pigment was adsorbed near the top of the column. This band fluoresced yellow-green in UV light. The solvent continuing down the column showed a faint blue fluorescence in UV light although it appeared colourless in daylight. Because it occurred in such minute amounts and was permanganate-labile, this blue-fluorescing substance is believed to represent traces of the similar pigment detected in quantity in Fraction III.

The yellow-green fluorescing band adsorbed to the alumina could be easily eluted with further aqueous ethanol. Its fluorescence, ability to withstand permanganate oxidation and reversible redox reaction suggest that this pigment might be riboflavin. In order to confirm this, further analysis was made according to the methods of Busnel & Drilhon (1948). Four ophiuroids with intestine removed (11·95 g wet weight) were finely ground and extracted with 150 ml. of 55% methanol (pH 5·5) in the dark at 37° C. for 48 h. At the end of that time the dirty yellow supernatant was decanted off and centrifuged for 15 min to remove suspended impurities. The resulting yellow, opalescent solution was freed from lipids by shaking with chloroform for 2 h. The solution was now clear, deep yellow and fluoresced brilliant blue-green in UV light.

The blue-fluorescing contaminant was removed by the permanganate oxidation technique of Eeckelen & Emmerie (1935). Fifteen ml. each of glacial acetic acid and saturated $KMnO_4$ were added to the extract and left to react in the dark for 10 min. At the end of that time the excess permanganate was destroyed by addition of drops of H_2O_2, leaving a pale yellow solution. This solution at pH 5·0 fluoresced a bright yellow-green in UV light. Raising the pH to 11·0 with NaOH diminished the fluorescence to a dull green. Traces of hydrosulphite reduced the solution to a colourless state, but addition of H_2O_2 restored the colour. A portion of the purified extract was brought to pH 11·0 with NaOH and placed before an UV light source for 1 h. Following irradiation, the extract was acidified to pH 5·0 with acetic acid and shaken with an equal volume of chloroform. Most of the pigment migrated into the chloroform layer where it fluoresced dull green in UV light. This is

a sensitive test for riboflavin (Roth, 1950), which is converted to a chloroform-soluble derivative, lumiflavin, when exposed to visible or particularly UV light in alkaline solution.

The visible light absorption spectrum of the purified extract in 55% methanol revealed a broad peak centring at 440 mμ and a lesser, ill-defined maximum at about 475–480 mμ. Neutral aqueous solutions of pure riboflavin show visible light absorption maxima at 445 mμ and 475 mμ (Sebrell & Harris, 1954, p. 307).

All these observations point to the yellow-green fluorescent pigment of Fraction II as being riboflavin. Especially characteristic are the fluorescence reactions, stability to permanganate oxidation and conversion to a chloroform-soluble form after irradiation in alkaline solution.

Fraction III

The pigments detected in this extract in no way resembled ommochromes which, if present, should have been extracted by acid alcohol. The original extract was negative to the Voisenet–Rhodes aldehyde reaction for tryptophane and to the Ehrlich reaction for indole. It gave no colour reactions for carotenoids. It was slowly reduced to a colourless state by hydrosulphite, regaining the colour by shaking in air or adding H_2O_2. Permanganate oxidation destroyed all the pigment leaving a perfectly clear solution. The original extract fluoresced a very brilliant blue with tints of green in UV light. When the solution was passed through an alumina column, a dark violet band (in daylight) was strongly adsorbed at the top of the column. This band fluoresced yellow-green in UV light. The liquid passing through the column was pale yellow in daylight, but fluoresced brilliant light blue in UV light. It is probably the same blue-fluorescing substance detected in trace amounts in Fraction II. Neither of these pigments gave a positive murexide reaction and Fraction III was discarded.

These reactions suggest the presence of at least two fluorescent pigments, the properties of which approximate to pigments of the pteridine type; one belonging to the pteridine group exhibiting blue-violet fluorescence; the second belonging to the pteridine group exhibiting yellow-green fluorescence (Ziegler–Günder, 1956). The negative murexide reaction does not positively contradict this conclusion, however, since some pteridines are known not to give the murexide reaction, while others give it only weakly (Gates, 1947). The identification can only be considered tentative until further studies, especially of UV absorption spectra, can be made. A summary of the reactions of all three fractions is given in Table 3.

TABLE 3. SUMMARY OF THE REACTIONS GIVEN BY EXTRACTS OF THE FLUORESCENT PIGMENTS

	Fraction	Solvent	Carotenoid colour reactions	Hydro-sulphite	Murexide reaction	Per-manganate oxidation	UV fluorescence	Miscellaneous reactions	Diagnosis
I		Acetone	+	Not reduced	–		Faint blue-green		Carotenoids
II	Original extract	Aqueous ethanol	–	Reversibly reduced	–	Partially stable	Brilliant blue-green; yellow-green after $KmnO_4$ oxid.	—	—
	Pigment A	Aqueous ethanol	–	Reversibly reduced	–	Stable	Yellow-green (pH 5); dull green (pH 11)	Converted to chloroform-soluble form by irrad. in UV light; absorption maxima 440; 475–480 mμ (ethanol)	Riboflavin
	Pigment B	Aqueous ethanol	–	Reversibly reduced	–	Unstable	Light blue	—	Trace of III C below
III	Original extract	5 % HCl in absolute ethanol	–	Reversibly reduced	–	Unstable	Brilliant blue-green	Negative to aldehyde reaction for tryptophane, and Ehrlich reaction for indole	—
	Pigment C	5 % HCl in absolute ethanol	–	Reversibly reduced	–	Unstable	Yellow-green	Violet in daylight	? Pteridine
	Pigment D	5 % HCl in absolute ethanol	–	Reversibly reduced	–	Unstable	Brilliant light blue	Yellow in daylight	? Pteridine

RESULTS OF FLUORESCENCE MICROSCOPY

An attempt was made to locate the cytological site of the fluorescent pigments described in the previous section by means of fluorescence microscopy. A preliminary survey showed that the fluorescent pigments were restricted to the visibly pigmented portions of the integument. No appreciable fluorescence was detected in any other tissues, except the intestines of well-fed animals.[1]

The light source used was a mercury vapour lamp fitted with a Wood's-type filter (Ilford No. 828). A 3 cm thick filter of ammoniacal copper sulphate was interposed between lamp and microscope to eliminate some visible red light transmitted by the UV filter. Observations were carried out in a dark room upon freshly excised pieces of tissue mounted in sea water, using a 4 mm achromatic objective and a $\times 14$ ocular fitted with a Wratten G 15 filter. A second ocular without a yellow filter was used to interpret colour value but the first was used for prolonged observation.

Verne & Busnel (1942) have shown that protein-bound pteridines are released and become demonstrable under the fluorescence microscope by the addition of dilute alkalis and sometimes dilute acids. Similarly protein-bound riboflavin is activated by dilute acids (Fontaine & Busnel, 1937). Upon addition of drops of reagent to fresh tissues, these authors have observed the diffusion of these fluorescent pigments from their cytological site; the identity of the pigments was confirmed by conventional analytical methods. Until the reagents have been added, no fluorescence is detectable.

When viewed under the fluorescence microscope, a slice of the integument of a dark brown (P 1) *O. nigra* showed a pale blue general background fluorescence, probably due to tissue lipids, against which the melanin showed up distinctly as dark, non-fluorescing granules. The addition of a drop of 10% NaOH instantly released an intense bluish-green fluorescence which appeared to arise from directly within the melanocyte. This fluorescent material slowly diffused from the cell into the adjacent tissues, gradually imparting its fluorescence to the whole tissue slice. This fluorescent material was fairly photostable, showing no 'fatigue' after 20 min of irradiation. After about 30 min, however, there was a gradual diminution in intensity and all fluorescence was extinguished within two hours of irradiation.

When a drop of 30% acetic acid was added to a fresh preparation, a somewhat more intense greenish-yellow fluorescence was released. This material diffused apparently from the melanocyte throughout the adjacent tissue in a similar manner. It was distinctly more photo-labile than the previously

[1] In healthy *O. nigra* the intestine is dark green in daylight. The green pigment is soluble in ethanol and displays a brilliant red fluorescence in UV light. Under the fluorescence microscope, it fluoresces red on addition of either dilute acids or alkalis. It is probably phaeophorbides, products of chlorophyll taken in with food. Animals starved for 4–6 weeks gradually lost the green pigment and the red fluorescence proportionately.

described pigment and it exhibited signs of 'fatigue' within 10 min of its release, disappearing entirely within 30 min.

If, after adding alkali to a preparation and observing the blue-green fluorescence, drops of acid were added subsequently, a green-yellow fluorescence of the usual intensity was produced. Evidently the fluorescence evoked by one reagent is distinct from that produced by the other reagent. This indicates that each reagent activates separate and distinct substances. The differences in photostability also support this conclusion.

The acid-released fluorescence is interpreted as representing mainly riboflavin (detected previously by chemical methods) for these reasons: the yellow-green fluorescence, its release under acid conditions, its relative photo-lability. The alkali-released fluorescence is interpreted as representing mainly the (?) pteridine pigments detected previously because of the blue-green fluorescence, the activation by alkali, and the relative photo-stability. It is quite likely that either reagent activates some of both riboflavin and the (?) pteridines but the observations suggest that a preponderance of only one pigment type is liberated by a specific reagent.

The small size of the melanocyte dendrites and their dense concentration made observation difficult; the difficulty was enhanced by the necessity of limiting observation to a dry 4 mm objective. Nevertheless, there is little doubt that after adding either reagent the fluorescent materials diffused directly out of the melanocytes. It must be emphasized that there are no visibly pigmented cells in the integument when viewed by ordinary light other than the melanocytes (and lipocytes which were conclusively shown to contain carotenoids; unpublished observations). Thus, there are no 'pterinophores' nor a 'pterin-layer' such as Ziegler–Günder (1956) has described in the integument of certain melanic Amphibia. It is highly probable that the melanocyte of *O. nigra* is another example of a 'polychromatic melanocyte' (Busnel, 1942).

The description of fluorescence given above applies to the integument of a dark brown (P 1) *O. nigra*. However, it was observed that the intensity of fluorescence varied according to the oxidation state and abundance of melanin in the integument of differently coloured individuals and on differently coloured parts of the same individual. Specimens of different colour classes were observed, and the differences in intensity of fluorescence between individuals of different colour class and between differently coloured parts of their bodies are recorded in Table 4. The scoring is based on visual estimates of the relative intensity of fluorescence and is intended to be roughly quantitative in so far as such a subjective method is valid.

These observations indicate certain strong relationships between melanization and the occurrence of fluorescent pigments. The acid-released pigment has been attributed to riboflavin for reasons outlined above. In all the colour phases in which melanin occurs (P 1–P 3), there is a considerable amount of

riboflavin present also, as judged by fluorescence intensity. In the red-orange or non-melanic phase (P4), on the other hand, the fluorescence attributed to riboflavin drops off sharply. Evidently there is a strong positive correlation between these two pigments.

The alkali-released fluorescence has been attributed to (?) pteridines in a discussion above. The data recorded in Table 4 show that the occurrence of melanin in the light-coloured (i.e. partially reduced) state is accompanied by a marked increase in the (?) pteridine pigment concentration. Thus, there is

TABLE 4. VARIATIONS IN INTENSITY OF FLUORESCENCE OF THE INTEGUMENT OF *OPHIOCOMINA NIGRA*

Colour form	No. of individuals examined	Area of integument	Degree of melaniza-tion	Intensity of fluorescence* 30 % acetic acid	10 % NaOH
P1 (very dark brown to black)	5	Dorsal arm plate	Intense	+++	++
		Ventral arm plate	Intense	+++	++
		Disk, aboral	Intense	+++	++
		Disk, aboral from pale central spot	Very light	+++	+++
P2 (lighter brown)	6	Dorsal arm plate	Moderate	+++	++
		Ventral arm plate	Light	+++	+++
		Disk, aboral	Moderate	+++	++
		Disk, aboral from pale central spot	Very light	+++	+++
P3 (fawn)	2	Dorsal arm plate	Light	+++	+++
		Ventral arm plate	Light	+++	+++
		Disk, aboral	Light	+++	+++
P4 (red-orange and non-melanic)	5	Dorsal arm plate	Trace	+	o
		Ventral arm plate	Trace	+	+
		Disk, aboral	Trace	+	o
		Disk, aboral from pale central spot	Trace	+	+
(No P5 was available)					

* +++ intense, ++ moderate, +faint, o none detectable.

an increase in the intensity of alkali-released fluorescence in the fawn-coloured disk patterns of otherwise dark animals; there is a similar increase in alkali-released fluorescence shown by the entire integument of uniformly fawn-coloured (P3) animals. The melanin-less (P4) colour phase displays very little fluorescence attributable to (?) pteridines.

Three conclusions seem valid. First, the presence of melanin in either the oxidized or reduced form is accompanied by relatively large concentrations of riboflavin and lesser amounts of (?) pteridines. Secondly, the occurrence of melanin in its fawn or reduced form is accompanied by relatively large concentrations of (?) pteridine pigments as well as the usual amount of riboflavin. Thirdly non-melanic individuals have very low concentrations of fluorescent pigments in general.

DISCUSSION

The dark integumentary pigment of *Ophiomyxa pentagona* is contained in arborescent cells which have been figured by Hamann (1889) and Reichensperger (1908). No descriptions were given except for Hamann's notation that these cells are widely ramified. He also noted pigment granules in amoebocytes within the dermis. So far as I can ascertain, these are the sole accounts of dark pigment-containing cells in the Ophiuroidea. The account given here of the melanocytes of *O. nigra* is therefore unique.

It has already been emphasized (p. 12) that the syncytial appearance of the *O. nigra* melanocytes should be considered tentative until better cytological methods can be applied. Caution seems necessary in this instance because the literature holds many examples of 'syncytial' pigment cells which were later shown to be discrete cellular units. Nevertheless, observations on living and sectioned tissues do show what must be interpreted as anastomoses between dendrites of the same and adjacent cells.

Whether or not the cells are syncytial, the general structure and arrangement of the melanocytes of *O. nigra* are unlike any integumentary pigment cell previously described from the Echinodermata. Millott (1954) and Yoshida (1956) have shown that the melanin of two species of the echinoid, *Diadema*, occurs in pigment effector cells. In the holothurians, *Thyone* and *Holothuria*, melanin occurs extracellularly as dense masses of granules between the epidermal cells (Millott, 1950, 1953). It is evident that the structural arrangements for retaining integumentary melanin are remarkably diverse throughout the phylum.

The properties of the *O. nigra* pigment indicate fairly conclusively that it is a melanin. In particular, its ready solubility in dilute alkali distinguishes it as a 'phaeomelanin' (Görnitz, 1923). Many of the properties of the *O. nigra* melanin are identical with the melanin which Millott & Jacobson (1952*a*, *b*) describe from *Diadema antillarum*. Unfortunately they did not test the alkali solubility of this pigment. On the other hand, the melanin of *Holothuria forskali* is only slightly soluble in normal NaOH (Millott, 1953) and has other properties somewhat different from the *O. nigra* melanin.

That melanic and non-melanic forms of *O. nigra* have similar enzyme systems potentially capable of controlling melanogenesis raises some interesting points. The evidence suggests that *in vivo* melanin formation in non-melanic colour phases is prevented by a physiological inhibitor. Why this inhibition should be removed by the *in vitro* experiments is not understood. It is noteworthy that regenerating arm-tips of non-melanic animals kept in aquaria are melanic, their dark-brown contrasting sharply with the red-orange of the rest of the body. This is unlikely to be due to activation of the enzyme system by the bright light in the aquaria since two non-melanic (M4) *O. nigra* dredged from 38 m at Millport were found to have melanized regenerating arm-tips.

Evidently under conditions of wound-healing and repair, the normal inhibitor of melanogenesis in non-melanic animals is overcome or absent. The situation in *O. nigra* parallels that of *Metridium senile* in which pure white and other non-melanic colour forms were found to possess 'tyrosinase' systems (Fox & Pantin, 1941), from which the authors postulate physiological inhibition.

The discovery in *O. nigra* of fluorescent pigments with distinct redox properties and the further demonstration of their cytological association with melanocytes provides another example of the close metabolic interrelations of these pigments. As has been noted in various poikilotherm vertebrates and some invertebrates (Ziegler–Günder, 1956), the presence of melanin is almost invariably associated with the presence of riboflavin and other fluorescent redox pigments frequently shown to be pteridines. The results of fluorescence microscopy show that in *O. nigra* the presence of melanin is associated with relatively large concentrations of fluorescent redox pigments, particularly of riboflavin. Conversely, only trace amounts of these pigments are detectable in non-melanic integuments. It is also interesting that the occurrence of melanin in its fawn-coloured or reduced state is correlated with an increase in the (?) pteridine pigment concentration. These findings closely parallel those of Busnel & Drilhon (1948) who discovered an analogous situation in the hypodermis of a number of brachyuran Crustacea. Polonovski *et al.* (1950) and Isaka (1952) have demonstrated that pteridine pigments can act as inhibitors of melanogenesis *in vitro*. Riboflavin, on the other hand, acts antagonistically; i.e. it catalyzes melanogenesis (Fontaine & Busnel, 1938; Isaka, 1952). Galston & Baker (1949), however, present evidence that riboflavin inhibits tyrosinase in the presence of strong light, leading Fox (1953, p. 287) to suggest that riboflavin controls rather than accelerates photostimulated melanogenesis. Although the ultimate role of these fluorescent pigments has yet to be elucidated, they are undoubtedly intimately involved in the process of melanogenesis through their redox properties.

According to the scheme of melanogenesis envisaged in the echinoid, *Diadema*, by Jacobson & Millott (1953), melanin formation takes place in amoebocytes when these cells leave the coelomic fluid (which has a low redox potential) and migrate into tissues with a more favourable redox balance. Melanin granules thus formed in amoebocytes are deposited in the melanophores. The evidence of these authors suggests that oxidation-reduction potential is a factor of primary consequence in the melanogenetic process of *Diadema*. They have suggested that quinone pigments may be the agents responsible for redox balance in the tissue of this sea-urchin.

In *O. nigra*, the demonstration of non-quinonoid fluorescent pigments in association with the melanocytes provides a possible though different redox mechanism for control of melanogenesis. The differential distribution of these redox pigments in various parts of the same individual and between different individuals could account for the presence or absence of melanin, its relative

concentrations or oxidation state in various parts of the epidermis of single animals, or between individuals of different colour class. If such a redox mechanism is in action, as the results of fluorescence microscopy suggest, then melanin would not be formed within the melanocytes (or at least not deposited in the melanocytes if future work shows that the amoebocytes are the elaborators of the pigment) where the integument contains only low concentrations of riboflavin. Such a mechanism would provide a physiological explanation for the occurrence of melanic and non-melanic individuals. Further, melanin would occur only in the reduced or fawn-coloured state in those integuments or portions of integuments which are relatively rich in (?) pteridines as well as riboflavin. This would provide an explanation for the appearance of colour patterns on otherwise dark animals and of generally fawn-coloured, melanic individuals.

From the available evidence, it is suggested that in *O. nigra* melanogenesis is inhibited by the absence of fluorescent redox pigments, particularly of riboflavin, and that these pigments must necessarily be present for melanin to be formed at all. Once the process of melanogenesis has been initiated, the final oxidation state and, of course, the visible colour of the melanin pigment is controlled by a balance between the relative amounts of riboflavin and a second set of fluorescent redox pigments tentatively considered to be pteridines. What controls the differential distribution of the fluorescent pigments remains to be discovered. Whatever the mechanisms may ultimately prove to be, this hypothesis provides a useful framework for further investigation.

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SUMMARY

The ophiuroid, *Ophiocomina nigra*, displays a colour variation ranging from black to yellow-orange. The pigment responsible for the dark colours has been studied histochemically and by means of chemical techniques applied to extracts. The solubility, oxidation-reduction, fluorescence and other chemical properties of this pigment indicate that it is a true melanin. The visible colour of the melanin depends upon its oxidation state in the integument. Fully oxidized pigment is dark brown; partially reduced pigment is fawn coloured.

The anatomy of the melanin-containing cells is described. These are dendritic cells, originating subepidermally and ramifying widely throughout the calcareous ossicles of the exoskeleton until they reach the epidermis. The most unusual feature of these cells is that they apparently form a syncytium.

They are not pigment effector cells. An enzyme system possibly controlling melanogenesis is demonstrated as occurring in both melanic and non-melanic colour phases.

Three fluorescent redox pigments were detected in association with the melanin and, by means of fluorescence microscopy, were shown to be located directly within the melanocytes. These pigments were extracted and some of their properties examined. One of them, soluble in aqueous alcohol, is identified as riboflavin. Two other pigments, soluble in acidic alcohol, are tentatively identified as pteridines.

Melanic animals are shown to contain large concentrations of fluorescent pigments, particularly of riboflavin, whereas non-melanic forms have very little of these pigments. It is also demonstrated that the occurrence of melanin in its reduced state in fawn-coloured areas of the integument of otherwise dark animals is correlated with a marked increase in the pteridine concentration in those areas. It is suggested that the fluorescent pigments are involved in melanogenesis and that their absence inhibits the process. This results in non-melanic colour phases. It is also suggested that the differential distribution of the presumed pteridine pigments is reponsible for the production of lighter colour patterns on melanic individuals.

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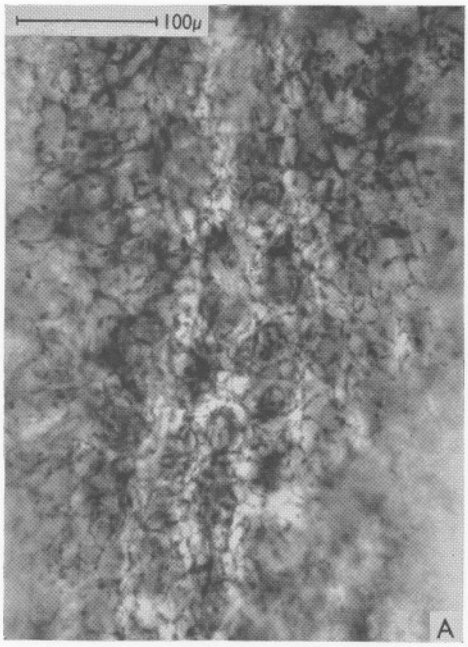

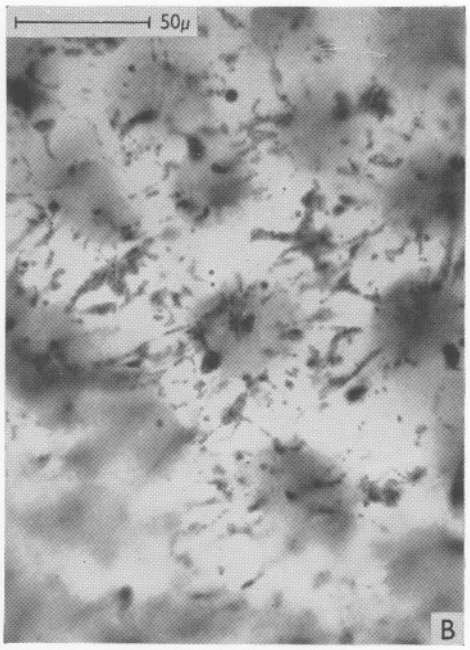

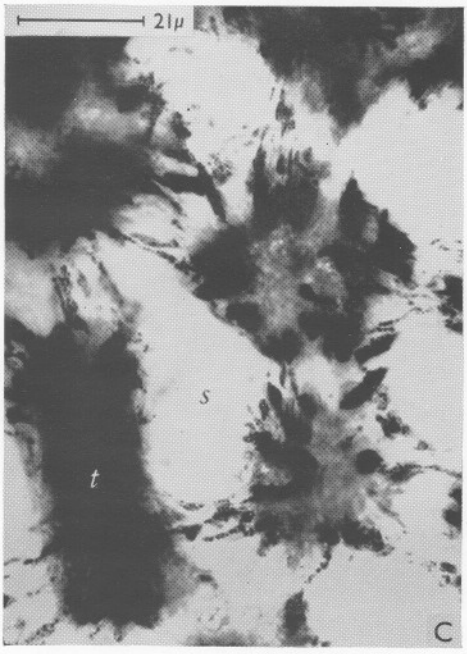

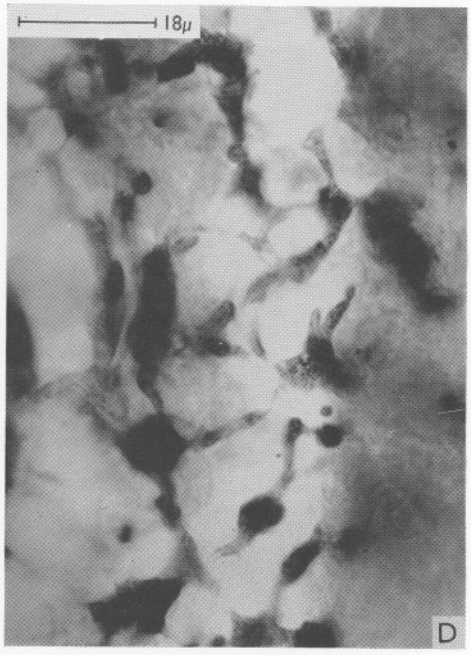

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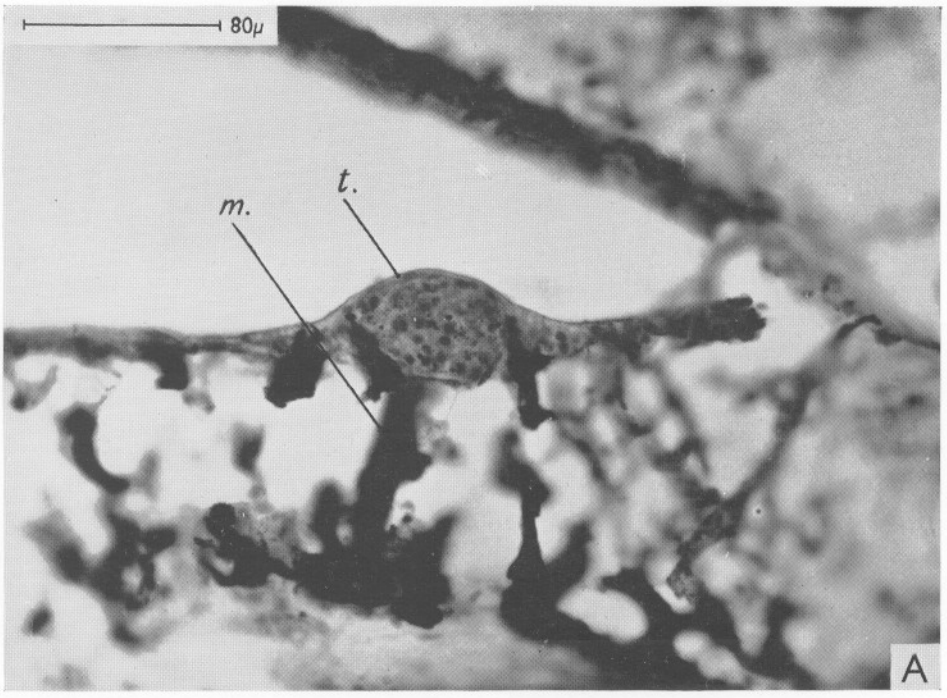
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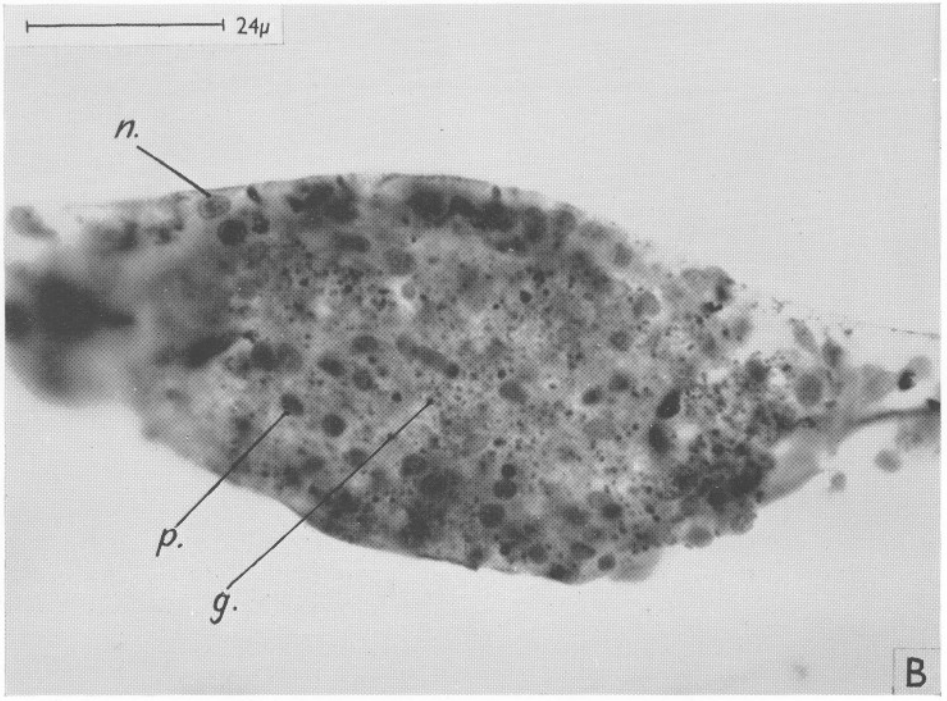
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EXPLANATION OF PLATES I-II

PLATE I

Photomicrographs of living melanocytes at the base of an arm spine of *O. nigra*.

A. Low-power surface view, showing the confused mass of dendrites within and under the epidermis; the ossicle supporting the spine shows as a white reticulated structure.

B. Medium-power surface view, focused just below the epidermis. The lattice-like nature of the ossicle is apparent. Note that the dendrites run through the interstices of the lattice and then spread out radially in a plane parallel to the surface.

C. High-power view, focused down in the interior of the ossicle, showing that the dendrites send out horizontal branches as well as perpendicular branches through the calcareous layer.

D. High-power view focused beneath the epidermis to show anastomosis of dendritic branches.

s., calcareous strut of ossicle; *t*, tissue contained in the interstices of ossicle.

PLATE II

Supposed melanotic tumours in the epidermis of *O. nigra*.

A. Low-power photomicrograph of a melanotic nodule occurring between arm spines.

B. Medium-power view to show cytological detail.

g., melanin granules; *m.*, mucous gland; *n.*, nucleus of epidermal cell; *p.*, pycnotic nucleus within the tumour; *t.*, supposed melanotic tumour.