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OBSERVATIONS ON THE SKIN PIGMENT AND AMOEBOCYTES, AND THE OCCURRENCE OF PHENOLASES IN THE COELOMIC FLUID OF *HOLOTHURIA FORSKALI* DELLE CHIAJE

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(Text-fig. 1)

Black or brown pigments are common among echinoderms and certain of them have been described as melanins—for example by Briot (1906) in unspecified holothurians; Crozier (1915) in *Holothuria captiva* and *H. surinamensis*; Verne (1926) in *H. forskali*; Cornil, Mosinger & Calen (1935*a*) in *H. forskali* and *H. tubulosa;* and Millott (1950) in *Thyone briareus*. The evidence for such statements has not always been made clear, but in certain instances, for example, in *Thyone* (Millott, 1950, and unpublished observations), other holothurians (Briot, 1906), and in the echinoid *Diadema* (Millott & Jacobson, 1951; 1952 *a*), such pigments have been shown to possess the chemical and histochemical characteristics of melanin. The fact that the black skin pigment of *Holothuria forskali* also shows the characteristics of melanin has been mentioned in a preliminary note (Millott, 1952).

In *Diadema* it was also shown that the pigment arose within the amoebocytes of the coelomic fluid by a process of oxidation involving an enzyme system with the characteristics of phenolases (Millott & Jacobson, 1952*b*).

In view of the frequent association of melanin production with such enzymes, the importance of these observations is obvious, and it is therefore appropriate to extend them to other echinoderms. This is all the more so, because studies on echinoderm pigmentation have been relatively few, and most of these concern echinochrome or allied pigments, and because the origin of melanin in other forms has proved so difficult to elucidate. Despite extensive investigation, the process of melanogenesis is still a controversial matter, and there is thus good reason to extend observations on the occurrence of the pigment, and to glean all indications as to how it is produced.

As reported previously (Millott, 1952), *Holothuria forskali* with its abundant black pigment forms a very suitable object for study, and in this essentially introductory and incomplete account evidence will be produced showing that the black skin pigment has the properties of melanin and that, as in *Diadema*, its presence is coupled with the existence of a well-defined phenolase system,

distinct from the cytochrome-cytochrome oxidase system. The amoebocytes of the coelomic fluid appear to play a vital part in the formation and distribution of the pigment.

The Properties of the Skin Pigment

Unfortunately the precise chemical nature of melanin is unknown. Indeed the term would appear to refer to a group of pigments rather than to a specific substance. Specific tests are therefore non-existent, but it is permissible to identify a pigment, provisionally at least, as a melanin, if it has the following characteristics (Lison, 1936): (i) if it occurs in the form of black, brown, or yellow granules; (ii) if it shows extreme resistance to solvents; (iii) if it is decolorized by oxidizing agents; (iv) if it reduces directly ammoniacal solutions of silver nitrate.

As indicated below, the black body wall pigment of *Holothuria forskali* shows these characteristics.

Mode of Occurrence

The body-wall pigment exists in the form of black granules scattered throughout the body wall, sparsely or in irregular patches, which are most numerous in the epidermis and subepidermal connective tissue. The granules are present inside cells as well as in intercellular spaces (see p. 534).

Solubility

The following simple method was used to test the solubility of the pigment.

The densely pigmented superficial layer of the body wall was stripped off and shaken with distilled water. A dark greenish yellow pigment dissolved and the solution was decanted off. The residue was shaken with distilled water and the whole filtered. The precipitate, which formed a dark brown film on the filter-paper, was extracted repeatedly with various solvents. The final filtrate from each solvent was evaporated to dryness on a water-bath to detect small amounts of pigment which might have dissolved. All other operations were carried out at laboratory temperature.

The results are shown below.

Solvent	Remarks	Solvent	Remarks
Water	Slightly soluble	Carbon disulphide	Insoluble
Ethanol 90%	Insoluble	Pyridine	Slightly soluble
Acetone	Insoluble	1·0 N-HCl	Slightly soluble
Benzene	Insoluble	1·0 N-H_2SO_4	Insoluble
Ether	Insoluble	1·0 N-HNO_3	Insoluble
Petrol ether	Insoluble	1·0N (approx.)-NaOH	Slightly soluble
Chloroform	Insoluble		

Where the pigment proved slightly soluble, the residue obtained by evaporation to dryness was extracted repeatedly with the same solvents. Its behaviour towards all solvents was precisely the same as the original precipitate, thus showing that the latter was not a mixture of similarly coloured substances, nor changed by the action of the solvents.

In general, therefore, the behaviour of the pigment towards solvents resembles that of melanin.

Bleaching by Oxidizing Agents

Small pieces of superficial body wall measuring about 1·5 by 0·5 cm. were subjected to the action of the following oxidizing agents, known to decolorize melanin (Lison, 1936). The results are indicated below:

Agent	Remarks
Chlorine	Pigment bleached completely in 12 hr.
Bromine water	Pigment bleached completely in 12 hr.
Hydrogen peroxide (about 12 vol.)	Black pigment changed to red in 12 hr. Prolonged action of agent led to no further change
2% chromic acid	Bleached to pale brown in 10 hr.
Potassium permanganate followed by oxalic acid	Bleached completely in 1 hr. after addition of oxalic acid

The pigment thus behaves like a melanin.

The Argentaffine Reaction

Paraffin sections of the body wall, 10 μ thick, were subjected to Masson's technique for the argentaffine reaction (see Lison, 1936), and counterstained in Orange G.

The granular body-wall pigment under study readily reduced ammoniacal silver nitrate.

Thus the black pigment of the body wall of *Holothuria forskali* shows clearly the characteristics of melanin.

The Coelomic Fluid

The Amoebocytes

Suspended in the coelomic fluid are numerous amoeboid cells, which in general features resemble those previously described in a wide variety of echinoderms (see Geddes, 1879; Cuénot, 1891; McClendon, 1912; Kindred, 1921, 1924, 1926; Dawson, 1933; and Ohuye, 1938).

When examined alive, or after fixation in Bouin, their most striking feature is the large number of cytoplasmic inclusions, certain of which are especially interesting, taking the form of spheroids (Fig. 1 A and B, *sph.*), which may be colourless, yellow or brown, bearing on their surfaces, deep brown, or black granules.

The appearance of granules of black pigment in association with cytoplasmic spheroids, at once recalls the condition observed in the amoebocytes of *Diadema*, where it has been shown that melanin appears in association with similar spheroids (Millott & Jacobson, 1952*b*).

Formation and Darkening of the Coagulum

When removed from the animal and exposed to air, the coelomic fluid slowly forms a sparse white stringy clot, which, after about 12 hr. at laboratory temperatures, breaks up into isolated pale brown globules frequently flecked with black. After a further 12 hr. the entire globule turns black.

Examination by microscope shows that the clot is formed of free and compacted amoebocytes.

In coelomic fluid which has been exposed for several hours, many spheroids (both brown and colourless) lie free in the clot following disorganization of many of the amoebocytes, and it is to the brown spheroids and the associated granules that the colour of the clot is due.

As far as can be judged from appearances, the pigment granules at the surface of the spheroids tend to amalgamate, forming densely brown patches around the spheroids, from which brown colour seems to spread into the spheroid (Fig. 1A, *b.p.*). This process occurs progressively, and, though the spheroids of many amoebocytes remain colourless, most become brown on continued exposure, until after 2 days the coagulum is seen to consist of a dense network of brown strands formed by the spheroids and debris of disorganized amoebocytes, with isolated, intact and apparently healthy, amoebocytes in the interstices.

Such appearances at once recall the changes seen to occur in the coelomic fluid of *Diadema* (Millott & Jacobson, 1952*b*), where, however, the clot is much more extensive and darkens more rapidly. It is especially significant, however, that here, as in *Diadema*, the darkening involves both spheroids and the granular pigment at their surfaces.

Although it was not possible in the time available to show directly by chemical or histochemical means, as in *Diadema*, that the pigment formed in the amoebocytes is a melanin, that it probably is becomes clear from histological preparations, in which it can be seen that the black pigment of the skin (which as we have already seen has the properties of melanin) is undoubtedly derived from the coelomic amoebocytes (see below).

Such a conclusion is fully supported by the reaction of the amoebocytes to ammoniacal silver solutions, for, after using Masson's technique for the argentaffine reaction, the pigment granules and many of the associated spheroids blackened intensely.

Histological Evidence of the Derivation of Skin Pigment from the Amoebocytes

Methods

Small pieces of body wall were fixed in Bouin's fluid for 24 hr. at laboratory temperature. Sections 10 μ thick were cut in wax and stained in safranin and light green.

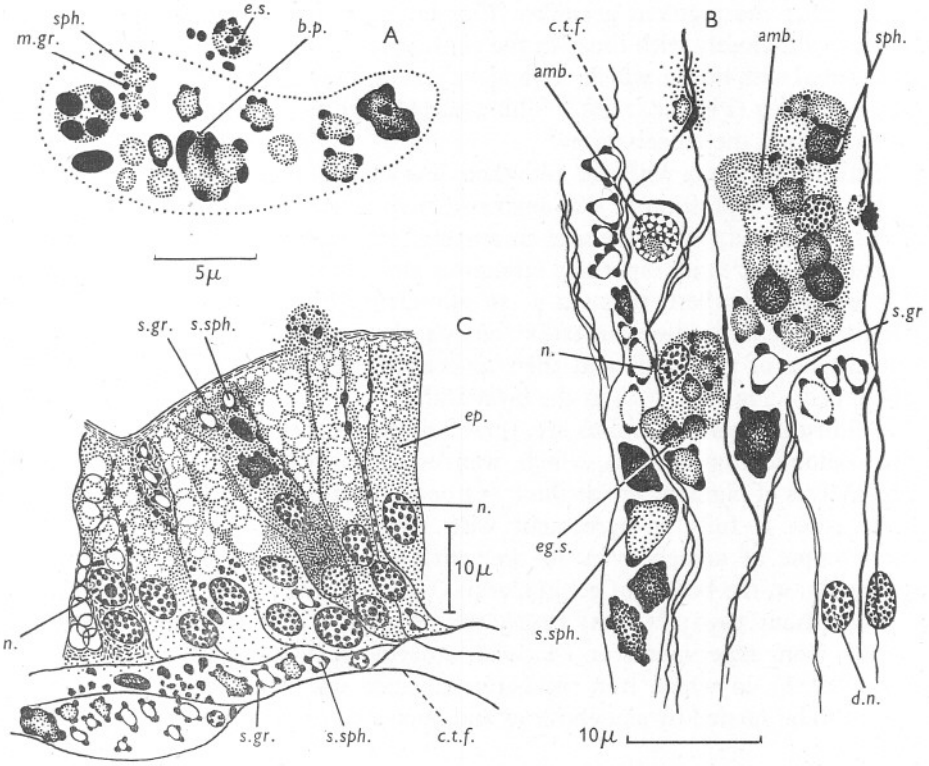

Fig. 1A. Living amoebocyte, from the coagulum formed in the coelomic fluid of *Holothuria forskali*, sketched after exposure of coelomic fluid in an open dish for 20 hr. at laboratory temperature and redrawn. B. Portion of the subepithelial zone of the body wall of *H. forskali*, showing amoebocytes and melanin deposits. From a preparation fixed in Bouin's fluid and stained in safranin and light green. C. Part of a section through the superficial region of the body wall of *H. forskali*, showing melanin deposits in epithelial and subepithelial zones. Fixed Bouin's fluid, stained by Masson's method for the argentaffine reaction (see p. 531), counterstained in Orange G.

amb. amoebocyte.
b.p. brown pigment spreading into spheroid (see p. 532).
c.t.f. connective tissue fibre.
d.n. isolated nucleus (?) from disintegrated amoebocyte.
eg.s. spheroid apparently being egested by amoebocyte.
ep. epithelial cell.
e.s. spheroid eliminated from amoebocyte with granules of pigment.
m.gr. melanin granule.
n. nucleus.
s.gr. melanin granule in body wall.
sph. spheroid in amoebocyte.
s.sph. spheroid in body wall (?) eliminated from amoebocyte.

Detailed examination of the deposits of black or brown pigment in the skin shows that the pigment granules (Fig. 1C, *s.gr.*), in size, form and colour, correspond closely with those in the amoebocytes, and further, that most are associated with bodies which, in range of size and appearance, closely resemble the spheroids (Fig. 1C, *s.sph.*). This clearly suggests that the skin pigment is derived from the amoebocytes.

Fully in harmony with this are other clear signs of participation of amoebocytes in the deposition of skin pigment, such as the constant presence in all parts of the body wall of numerous amoebocytes indistinguishable from those in the coelomic fluid, especially numerous in the loose connective tissue below the epidermis where pigment is so abundant (Fig. 1B). Again there are frequent signs of disorganization among the amoebocytes, and occasional indications of spheroids and their associated pigment being cast out from these cells (Fig. 1B, *eg.s.*) in the body wall.

All histological indications are, therefore, that the pigment is derived from the coelomic amoebocytes, which, wandering into the body wall, unburden themselves of pigment by casting it out or by disintegration. Such a tentative conclusion is fully in agreement with many previous indications of the importance of amoebocytes in the carriage of pigments and metabolites generally, in the bodies of echinoderms (Cuénot, 1891; List, 1897; Kindred, 1926; Lison, 1930; Millott, 1950), and especially with the more revealing results from experiments on *Diadema*, reported previously (Millott & Jacobson, 1952*b*), in which iron saccharate injected into the coelomic fluid was found to be carried by amoebocytes and deposited in the melanophores of the skin.

Again, the histological evidence of breakdown of the amoebocytes in the body wall, with liberation of their contained pigment, parallels the same phenomenon observed to occur in the living amoebocytes of the coagulum (p. 532).

It is interesting to note, in passing, that Cornil *et al.* (1935*b*), working on *Holothuria forskali* and *H. tubulosa*, observed the frequent association of pigment deposits in the body wall with amoebocytes and the appearance of cellular disintegration, and, further, believed that the matter eliminated was re-utilized by the amoebocytes. '...et l'on peut penser que les amas de désintégration sont susceptibles d'être réutilisés par le travail amibocytaire. L'appareil pigmentaire serait ainsi soumis de façon constante à un processus de destruction (pigmentolyse) et de néoformation (pigmentogenèse).'

Finally, the participation of coelomic amoebocytes in melanogenesis is supported by the discovery of a well-defined enzyme system in these cells, which brings about the oxidation of both mono- and poly-phenols, and is distinct from the cytochrome-cytochrome oxidase system. Such enzymes have been shown to be associated with melanogenesis in a wide variety of forms.

The Phenolase System in the Coelomic Fluid

Simple colour reactions, based on those previously employed by Raper (1932) and Pugh & Raper (1927), were used to reveal the presence of phenolases, coelomic fluid being added to buffered phenolic substrates, oxidation being indicated by darkening of the solutions. The experimental solutions were made up as follows: 5·0 c.c. coelomic fluid (freshly withdrawn and well shaken to disperse the coagulum) + 5·0 c.c. 0·06 M Sørensen buffer (mostly at pH values ranging between 6·5 and 7·0), containing 0·1–0·2 % of the phenolic substrate + a few drops of toluol or chloroform (as an antiseptic).

In all instances experiments were accompanied by controls in which the coelomic fluid had been boiled and cooled before use. Both experiments and controls, frequently inspected and gently agitated, were incubated at 24° C. The controls were especially necessary in view of the tendency of some phenolic substrates to auto-oxidize, and the fact that the coagulum itself darkens on exposure. Usually a little detergent was added both to experiments and controls (see below).

These experiments showed that the coelomic fluid possesses the power to oxidize phenols such as 'dopa', pyrocatechol, L-tyrosine, pyrogallol and cresol (mixed isomers) with glycine. The characteristic coloured oxidation products always appeared in the initially colourless solutions, those containing 'dopa' or tyrosine becoming grey or black, pyrocatechol reddish brown, pyrogallol brown, whilst those containing a mixture of cresols and glycine became scarlet or orange. The controls, with boiled coelomic fluid, showed either no change, or where the substrates tended to auto-oxidize, relatively little change.

It is noteworthy that detergents, such as 'Cetavlon' (Imperial Chemicals (Pharmaceuticals) Ltd.) and saponin, exerted a marked accelerating effect on oxidation. Without detergent, oxidation of the phenolic substrates occurred only slowly, requiring at least 12 hr. at pH 7·0 and 24° C. to bring about a distinct colour difference between experiment and control. After adding a little detergent pronounced darkening of the experimental solutions as compared with the controls could be observed within 3 hr. at the same temperature, pH, and with the same substrate concentrations.

Thus it is clear that the capacity of the coelomic fluid to oxidize phenols depends on a heat-labile factor with marked surface action. However, it is not yet clear whether this is an enzyme system or not, since such oxidations can be brought about by heat-labile factors such as copper-protein complexes that are not usually regarded as enzymes (see Bhagvat & Richter, 1938).

Two features of the reactions, however, namely their sensitivity to potassium cyanide and pH, indicate the participation of enzymes. Thus oxidation of L-tyrosine and pyrocatechol at pH 7·3 is strongly inhibited by concentrations of potassium cyanide as low as 0·0002 M, and the oxidation of tyrosine occurs

most readily in solutions buffered within the pH range 6·0–7·5 at 24° C., falling off rapidly on either side.

Owing to the limitations of the method employed, it was pointless to attempt the determination of a precise optimum; nevertheless it is clear that the range of activity corresponds with that of tyrosinase (Raper, 1928).

It now remains to be discovered whether the enzyme system indicated is to be found in the coelomic fluid, or in the suspended amoeboid cells. That it is present only in the cells can be shown by separating them by centrifuge, boiling the remaining fluid, and after cooling, reconstituting the coelomic fluid by replacing them in the fluid. The reconstituted fluid thus formed shows an undiminished power to oxidize phenolic substrates, and thus the heat-labile factor I believe to be an enzyme system, must be present in the amoebocytes, and not in the fluid.

Such experiments have a further significance in showing that there is no heat-labile factor in the fluid inhibiting oxidation, as was found in *Diadema* (Millott & Jacobson, 1951), since no increased oxidizing power resulted from the treatment.

The existence of an enzyme system in the amoebocytes capable of oxidizing phenolic substrates at once leads us to suspect as responsible, in some measure at least, the widely distributed cytochrome-cytochrome oxidase system. However, the fact that mono-phenols such as L-tyrosine are oxidized shows that this system cannot be entirely responsible. The sensitivity of the cytochrome-cytochrome oxidase system to sodium azide at pH 7·3, and to acetone, shown by Keilin (1936) and Keilin & Hartree (1938), enables us to eliminate this system as responsible in any significant measure for the effects described here, for the oxidation of polyphenols such as catechol was not affected either by 0·002 M sodium azide at pH 7·3, or by acetone.

There are thus clear indications that the coelomic amoebocytes of *Holothuria forskali* contain an enzyme system of the phenolase type commonly associated with melanogenesis.

It is now appropriate to point out certain peculiar features in the system. First, thiourea exerted but slight inhibitory effect, at a concentration of 0·001 M, on the oxidation of tyrosine at pH 6·5 and 24° C.; and no discernible effect, at a concentration of 0·0005 M, on the oxidation of pyrocatechol at pH 7·3. Phenolases are characteristically sensitive to substances binding copper ions, so that this is an unexpected property of the system. Secondly, in some preliminary experiments, it was found that irradiation with ultraviolet light exerted but a small and inconstant effect: 5 min. irradiation of coelomic fluid by means of a 'Hanover' mercury arc, sometimes increased (but only slightly) its power to oxidize L-tyrosine at pH 6·5. Irradiation for longer periods diminished it. This too was unexpected, as similar treatment of the coelomic fluid of *Diadema* was found to produce the normal effect and activate the enzyme system (Millott & Jacobson, 1952*b*). These

findings are advanced with due caution, however, as it is considered that more experiments are necessary, especially to eliminate possible heating effects.

Discussion

The foregoing investigation, though regrettably incomplete, is adequate to confirm previous indications of the occurrence of melanin in *Holothuria forskali*, and to show that the occurrence of melanin in this form is coupled with the presence of an active phenolase system. The association is noteworthy in view of many indications of the participation of such enzymes in melanogenesis in a wide variety of animal forms, but especially because of their importance in the echinoid *Diadema*. Thus in both echinoderms the enzyme system occurs in the histologically similar coelomic amoebocytes, which in both produce a black pigment with the characteristics of melanin. Further, it is most significant that the pigment appears in the amoebocytes of both, in association with cytoplasmic elements, the spheroids.

The association of granules of melanin with spheroids is remarkably constant, not only within the amoebocytes, but also in the skin (Fig. 1 c), and the ubiquity of the association suggests that the spheroids are involved in pigment formation, a suggestion which is fully supported by the histological evidence brought forward on p. 534. This is most significant, for in *Diadema*, where the same association prevails, there is evidence that the spheroids and their immediate surroundings are regions of high redox potential, a factor known to be important in melanogenesis (Figge, 1940; Dawes, 1941; Schuppli, 1950).

The enzymic activity of the coagulum formed in the coelomic fluid, in its capacity to bring about oxidation of both mono- and poly-phenols, sensitivity to cyanide, pH range and insensitivity to sodium azide and acetone, resembles that of tyrosinase, an enzyme that has been widely associated with melanogenesis. In the absence of more precise experiments, it is clearly unprofitable to speculate as to whether one or more enzymes are involved, a matter that has proved difficult to decide even with refined techniques (see Dennell, 1947; Lison, 1936; Greenstein, 1948; Lerner & Fitzpatrick, 1950; Schaaf, 1950; Kertész, 1951). It is for this reason that I have at all times preferred to refer to an 'enzyme system' rather than to use more committal terms.

The indications of the participation of cytoplasmic elements such as the spheroids in melanogenesis are interesting in relation to the controversy as to whether melanin is of nuclear or cytoplasmic origin (see Verne, 1926; Meirowsky, Freeman & Fischer, 1950; Meirowsky & Freeman, 1951; etc.), but it should be borne in mind that the relevant evidence cited in this investigation is largely histological, and can be advanced only as an indication, and the possible origin of some of the melanin at least, from the nucleus, has been by no means excluded. Clearly further work is required, but it may be

noted in passing, that it is doubtful whether the distinction is worthy of much emphasis in the light of modern conceptions of cellular metabolism.

Finally, it must be emphasized that the incomplete information obtained makes it unprofitable to attempt to formulate a conception of the process of melanogenesis in *Holothuria*, as was done with *Diadema* (Millott & Jacobson, 1952*b*), but it must be admitted that, so far, the processes seem remarkably similar, and further comparison must necessarily await further work.

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Summary

The black body-wall pigment of *Holothuria forskali* shows the characteristics of melanin.

From histological evidence it appears that the pigment is formed in association with the amoebocytes of the coelomic fluid, which eliminate the pigment in the body wall.

The amoebocytes contain a phenolase system, distinct from the cytochrome-cytochrome oxidase system, with the properties of tyrosinase.

The relation of these findings to those of a preceding and more complete investigation into melanogenesis in *Diadema* is discussed.

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