II. ON THE DEVELOPMENT OF THE BLACK MARKINGS ON THE WINGS OF *PIERIS BRASSICAE.*

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The formation of black animal pigments or melanins has been shown by Biedermann [1898], Gortner [1910, 1911, 1] and other investigators to be due to the oxidising action of an enzyme upon a colourless chromogen. The chromogen is probably tyrosine or a closely allied substance and the enzyme a tyrosinase.

In order to investigate the factors which determine the formation of pattern, and if possible to extend our knowledge of the mechanism of melanin production, a study was made of the black wing-markings of the common large white cabbage butterfly (*Pieris brassicae*). Hopkins [1895] has already shown that the chalky white pigment deposited in the scales of these insects gives the murexide test and is in fact uric acid. He has also shown that the yellow pigment which occurs on the under-sides of the wings of *Pieris brassicae,* and which constitutes the sole pigment of the wings of *Gonepteryx rhamni,* gives the murexide test and is a derivative—probably an oxidation product—of uric acid.

The development of the black wing-markings of *P. brassicae,* which differ slightly in the male and female, has been observed by Urech [1892], but his account is chiefly descriptive and does not deal with the cause or chemistry of their production.

Von Fürth [1904] and Roques [1909] have shown that the haemolymph of certain pupae is rich in tyrosinase, and the former has described a tyrosinase obtained from the pupae of *Deilephila elpenor* similar in most respects to that mentioned in this paper.

For the material of the present study a number of pupae of *Pieris*

brassicae were obtained. These have a greyish green colour which remains until a short time before the emergence of the complete insect in May. A few days before this takes place the white wing-pigment makes its appearance through the chitin of the pupa-case, and a little later pale spots can be observed on the wing. If the pupa is dissected at this stage the markings do not appear black but yellowish, like spots of grease upon white paper. These markings begin to darken quickly and just before emergence become deep black. During this transition the chitinous case of the pupa becomes definitely separated from the body of the insect, so that the latter can be dimly seen lying within its semi-transparent sheath. It is probably during this period that sufficient air finds access to the wing to cause the oxidation in the black areas to take place. When fully developed this black pigment is deposited in minute granules within the chitin of the wing scales and is similar in appearance and solubility to the melanin of hair [Onslow, 1915] and other structures. It can be partially extracted by prolonged boiling in dilute alkali. It is soluble in weak alkalies, somewhat less soluble in weak acids (N/20 HCl) and insoluble in strong acids and saturated ammonium sulphate solution.

Experimental.

Several pupae were chloroformed just before the wing-markings began to blacken, and the haemolymph was extracted by grinding them in an agate mortar with a little kieselguhr and chloroform water. After filtration, the pale green slightly opalescent fluid obtained was made faintly alkaline to litmus with very dilute sodium carbonate solution and tubes were prepared each containing 2 cc. of the fluid. The following substances were then added to the tubes which were plugged with cotton-wool and kept at room temperature for a period of 12 hours.

Experiment I.

No.	Contents	Appearance after 12 hours
1	Haemolymph	Deep black at the surface
2	,, +tyrosine	,, ,, ,,
3	,, +p-cresol	Blood-red
4	,, (boiled)	Unchanged

Since a deep black ring was formed at the surface of the fluid without the addition of tyrosine it is clear that the haemolymph was not only rich in tyrosinase but also in chromogen. It was found absolutely necessary to

keep the tubes at a temperature of 20° or less, for at higher temperatures formation of the pigment was inhibited. This observation is in agreement with that of von Fürth [1904] who found that a temperature of 30° completely inhibited the reaction, whereas tubes kept in the ice-chest were not affected.

It is interesting to note that in vertebrates the action of the tyrosinase is accelerated by a temperature of 36°. This difference might be accounted for by assuming that the organic peroxide constituent of the oxidase of insects is completely destroyed at a temperature of 30°. Unfortunately the author has not yet been able to test this hypothesis.

In order to ascertain whether any chromogen was present in the scales, the fore-wings were dissected from a number of pupae. The members of each pair of wings are distinguished in the following experiment by the letters *A* and *B*, *B* always serving as a control. The bodies of these pupae were utilised for the preparation of the haemolymph in the manner already described. Each wing was placed in a watch-glass containing the following solutions in which they remained for 12 hours at room temperature.

Experiment II.

No. of wing	Contents of watch-glass	Appearance after 12 hours
Pair 1 A_1	Normal saline	+
Pair 1 B_1 (boiled)	„	−
Pair 2 A_2	Haemolymph	+ +
Pair 2 B_2 (boiled)	„	+ +
Pair 3 A_3 „	„	+ +
Pair 3 B_3 „	„ (boiled)	−
Pair 4 A_4	Saturated tyrosine solution	+ + +
Pair 4 B_4 (boiled)	„ „ „	−

\+ indicates slight darkening of the markings.
\+ + „ considerable darkening of the markings.
\+ + + „ blackening of the entire wing.
− „ no change.

It appears from this experiment that both the wing and the haemolymph carry the two factors necessary for melanin production, since it is only when both the wing and the haemolymph are boiled that no darkening takes place (B_3). It is probable that the haemolymph which contains both chromogen and ferment adheres to the surface of the wing and may even be present in the wing nervures. When the tyrosinase of the body lymph comes into contact with the chromogen present in the wing-markings it oxidises this more than it does the rest of the wing. A fourth pair of wings was prepared,

in order to determine whether the localisation which causes the formation of pattern is due to

(1) the restriction of the tyrosinase to the markings and the distribution of the chromogen over the entire wing, or

(2) the restriction to the black markings of the chromogen alone.

That it is the chromogen which is restricted to the black areas and the tyrosinase which is present over the whole surface is clear, for when the wing is placed in a tyrosine solution it becomes black all over, but when it is placed in a tyrosinase solution the darkening is restricted to the markings. This is similar to the case of the markings on the elytron of the Periodical *Cicada* described by Gortner [1911, 2]. Owing to the fact that the haemolymph contains a quantity of chromogen the contrast between the markings and the rest of the wing is somewhat obscured by the general darkening. On microscopical examination however the pigment of the markings can be seen within the chitin of the scales, whereas the pigment deposited on the white portions of the wing lies in irregular masses on the surface of the scales. Fortunately however it was possible to make the reaction far more marked by precipitating the tyrosinase from the haemolymph with ammonium sulphate solution and by very thoroughly washing the precipitate with the same fluid so as to remove the last traces of chromogen. If the precipitate is now dried between filter papers and re-dissolved in an equivalent amount of 0·05 % sodium carbonate solution, a fluid containing tyrosinase is obtained free from chromogen.

An attempt was made to substitute the tyrosinase prepared from the skins of young black rabbits [Onslow, 1915] but the result was not successful, owing no doubt to the difference in the tyrosinase of warm-blooded animals.

Summary.

The black markings on the wings of *Pieris brassicae* are caused by the oxidation of a colourless chromogen by a tyrosinase. This ferment is supplied from the body-lymph of the pupa, possibly by means of the wing-nervures, to the chromogen which has previously been deposited in the areas destined to become black. The form of the markings is determined by the localisation of the chromogen to these areas. The oxidation takes place just before the emergence of the fully-developed insect and as soon as the atmospheric oxygen has access to the surface of the wing.

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