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Comparison between the Type B Red Pigment and Melanin of the Papilionid Butterfly, *Papilio demoleus*

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Abstract The type B reddish brown pigment and melanin in the wings of *Papilio demoleus* were examined for some chemical properties, absorption spectra, and copper content. Both pigments are similar to each other in their solubilities and absorption spectra. In the adsorption on the Dowex 50W column, copper content, the presence or absence of β-alanine and kynurenine in the hydrolysate, and indole reaction, however, both pigments showed marked difference.

Introduction

Ford (1942, 1944a, b) investigated red pigments of the wings of papilionid butterflies and divided them into two types A and B. The type A pigment is the red pigment which is widely distributed in the Lepidoptera. In the Papilionidae, the pigment of this type is present in almost all genera including *Graphium, Troides, Pachliopta, Parnassius, Lühdorphia,* and others. On the other hand, the type B pigment is the reddish brown pigment which is found only in the genera *Papilio, Chilasa,* and *Battus.* Ford investigated several properties and distribution of these red pigments from the standpoint of systematics. From the chemical and biochemical points of view, however, few works have been done.

Umebachi (1962) reported that the type B red pigment-containing scales incorporated ^{14}C-tryptophan, while the type A red pigment did not. Further, Umebachi (1978) extracted and purified the red pigments of both types, and examined for some chemical properties. It was found that the type B red pigment is similar in solubilities and absorption spectra to melanin.

In the prerent paper, the extraction, fractionation, chemical properties, and absorption spectra of the type B red pigment have been investigated in further detail, in comparison with melanin.

Materials and Methods

Materials

Reddish brown scales of the upperside of anal eye spots in the wings of *Papilio demoleus* were scraped and stored. This reddish brown pigment is of type B (Ford, 1944a). Exactly speaking, the type B red pigment is not red but reddish brown. As to melanin, black scales in the wings of the same species were used.

All the butterflies were obtained from Formosa through the Ōkura Biological Institute.

Extraction and fractionation (Fig. 1)

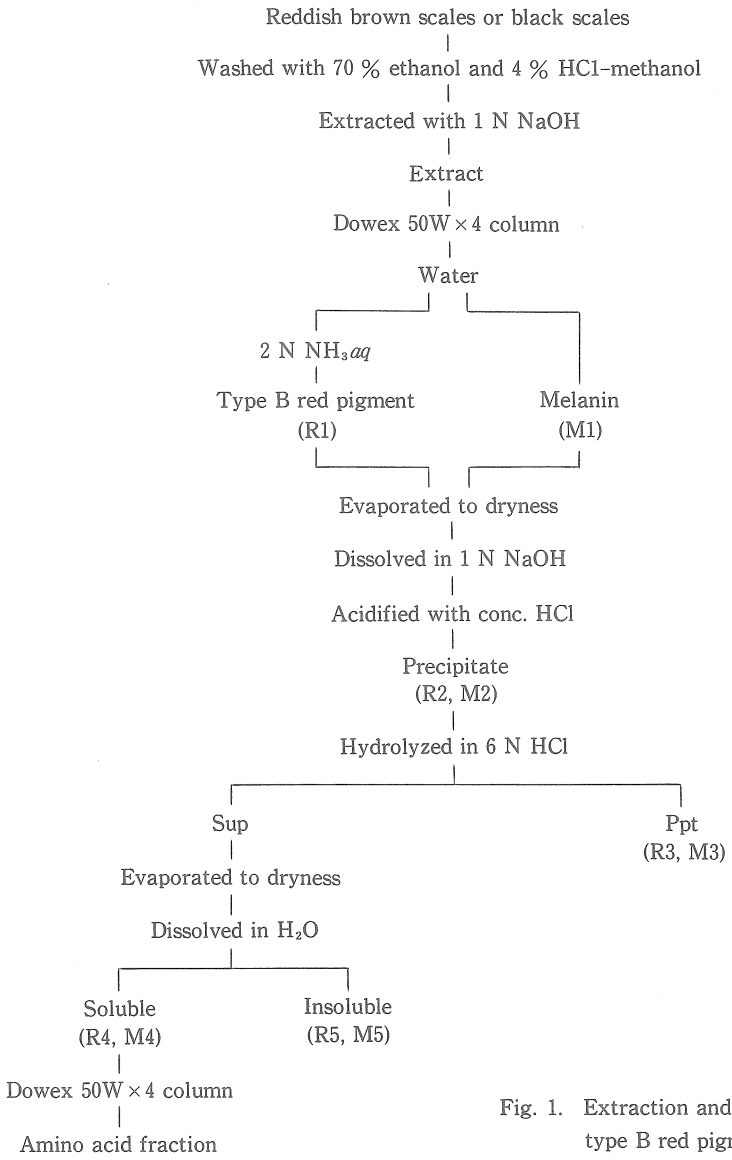

Fig. 1. Extraction and fractionation of the type B red pigment and melanin.

Reddish brown scales or black scales were washed first with 70 % ethanol five times and then with 4 % HCl-methanol five times at room temperature. After that, the scales were washed with 99.5 % ethanol and ethyl ether, and dried.

Next, the scales were treated with 1 N NaOH at room temperature six times. The combined extract was applied on a Dowex 50W×4 column (H^+) (1×13 cm). The column was washed with water, and then elution was performed with 2 N ammonia water. Melanin (dark brown) from the black scales was not adsorbed on the column and was moved down with water. On the other hand, the type B red pigment (brown) was adsorbed on the column and eluted with 2 N ammonia water. The type B pigment moved faster than amino acids (Umebachi, 1978). Anyway, the type B pigment fraction and the melanin fraction thus obtained are called the R1 and M1 fraction respectively in the present paper (Fig. 1).

The pigment fraction (R1 or M1) was evaporated to dryness in a rotary evaporator at 40°C. The residue was washed with water and then dissolved in 1 N NaOH. The pigment solution thus obtained was acidified with conc. HCl and kept in the cold. After standing overnight, the pigment was precipitated by centrifugation. The pigment fraction thus obtained is referred to as the R2 or M2 fraction in the present paper (Fig. 1).

The R2 or M2 fraction was dissolved in 1 N NaOH and again acidified with conc. HCl. The precipitate produced was separated by centrifugation and hydrolyzed under reflux in 6 N HCl at 100°C for 14 to 24 hr. The hydrolysate was centrifuged, and the precipitate was washed with water. The precipitate thus obtained is called R3 or M3. The M3 is melanin itself, whereas the R3 is a small quantity of insoluble substance. On the other hand, the supernatant was evaporated to dryness in a rotary evaporator at 60°C. When the residue was treated with water, some pigment dissolved but some pigment remained insoluble. The extract is referred to as R4 or M4, and the insoluble part is called R5 or M5. In the case of melanin, the M5 fraction was very little. On the other hand, the R5 fraction seemed to contain some of the type B pigment (Fig. 1).

The M4 fraction contained almost no pigment and examined for amino acids. The M4 solution was applied on a Dowex 50W×4 column (H^+) (1×13 cm). After washing with water, the amino acids were eluted with 2 N ammonia water and evaporated to dryness in a rotary evaporator at 40°C. The residue was dissolved in water and submitted to two-dimensional thin-layer chromatography. The R4 fraction contained both a part of the type B pigment (yellowish brown) and amino acids. The amino acids was examined in the same way as mentioned above (Fig. 1).

In the above procedure, a large portion of melanin came to the M3 melanin fraction, whereas most of the type B pigment seemed to come to the R4 and R5 fractions.

Dowex 50W column

A 1×13 cm column of Dowex 50W×4 (H^+) was used. Melanin was not adsorbed on the column. On the other hand, amino acids and the type B pigment were adsorbed on the column and, after washing with water, were eluted with 2 N ammonia water. The type B pigment moved down faster than amino acids.

Thin-layer chromatography

Cellulose thin-layer sheet (Merck No. 5552, 20×20 cm) was used. Two-dimensional chromatography was carried out with 70 % methanol as the first solvent and with a mixture of *n*-butanol--glacial acetic acid-water (12 : 3 : 5) as the second solvent. After development, the chromatogram was inspected under ultraviolet light, and then the ninhydrin test was performed.

Absorption spectra

Absorption spectra of melanin (M2 and M3) and the type B pigment fractions (R2, R3, and R5) were taken in 1 N NaOH in the wave length region of 230 to 800 nm with a Hitachi 240 spectrophotometer.

Atomic absorption spectrophotometry

The melanin fractions (M2 and M3) and the type B pigment fractions (R2, R3, and R5) were dissolved in 1 N NaOH, and their copper contents were determined with a Shimazu 640-13 atomic absorption spectrophotometer (carbon rod atomizer, GF - 2).

Indole test

In order to see whether the pigments contain indole derivatives, the indole reaction with *p*-dimethylaminobenzaldehyde after alkali fusion was performed (Fox and Kuchnow, 1965). This is one of the diagnostic tests for indole melanin.

Results

Adsorption on the Dowex 50W column

As mentioned in the section of methods, the melanin M2 was not adsorbed on the Dowex 50W × 4 column. The dark brown melanin passed through the column without stopping and moved down with water. The purified melanin M3 was not adsorbed on the column either and passed through the column without stopping. As will be mentioned later, the M2 fraction is a protein-bound melanin, whereas the M3 does not contain protein.

On the other hand, the type B pigment R2 was adsorbed on the Dowex 50W column. The brown pigment was not eluted with water but with 2 N ammonia water.

Amino acids in the hydrolysate of R2 and M2 fractions

After the R2 or M2 fraction was hydrolyzed in 6 N HCl, the soluble fraction, R4 or M4 (Fig. 1), was applied on the Dowex 50W × 4 column, and amino acids were eluted with 2 N ammonia water. The amino acid fraction thus obtained was submitted to two-dimensional chromatography.

In the case of the M4, the chromatogram showed at least fifteen ninhydrin-positive spots including leucine, isoleucine, phenylalanine, valine, tyrosine, α-alanine, glutamic acid, threonine, aspartic acid, glycine, serine, histidine, and lysine. Proline might be also present, though it was slight. Beta-alanine was absent or, if any present, it was only a trace. Kynurenine was absent. An unidentified ninhydrin-positive substance was present.

In the case of the R4, too, leucine, isoleucine, phenylalanine, valine, tyrosine, α-alanine, glutamic acid, threonine, aspartic acid, glycine, serine, histidine, and lysine were found. The above-mentioned unidentified ninhydrin-positive substance was also present. Interestingly, considerable amounts of β-alanine and kynurenine were found.

Absorption spectra

The melanin fraction M2 and the purified melanin M3 were dissolved in 1 N NaOH, and their absorption spectra were taken. As shown in Figs. 2A and 2B, the spectra of M2 and M3 were almost the same, and the absorption progressively increased from 800 to 230 nm.

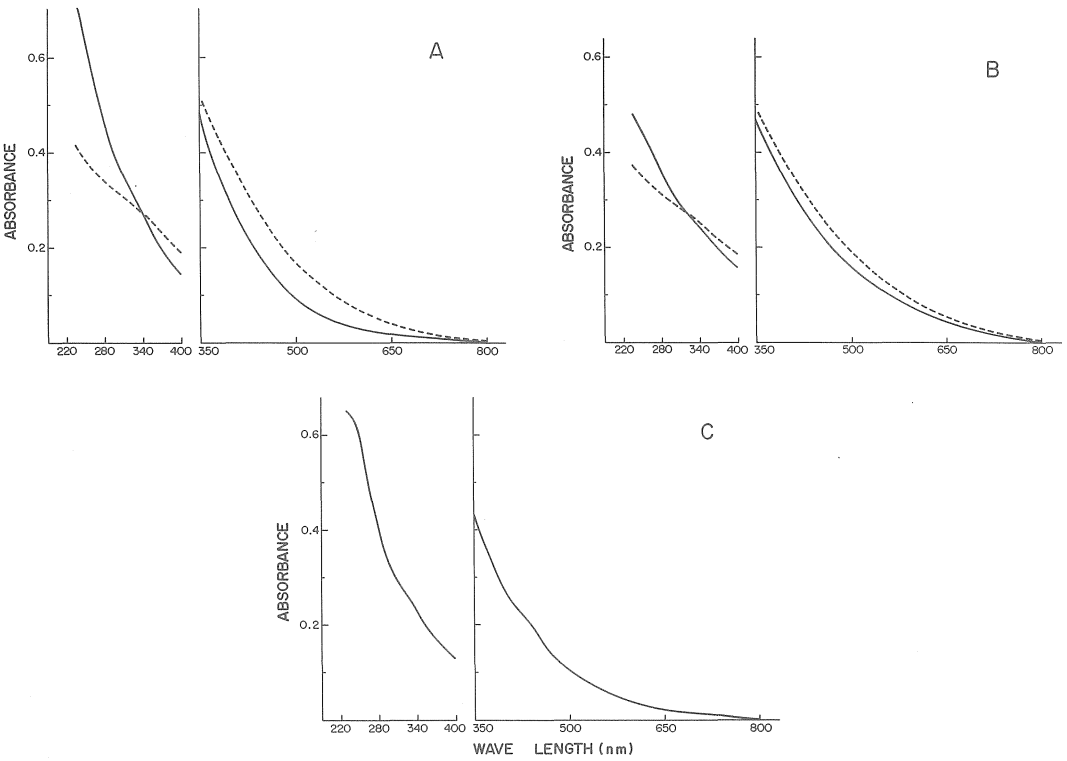

Fig. 2. Absorption spectra of the type B pigment and melanin in 1 N NaOH. A, Fractions R2 (solid line) and M2 (broken line); B, Fractions R3 (solid line) and M3 (broken line); C, Fraction R5.

The absorption spectrum of the type B pigment R2 was also taken in the same way as in the M2 (Fig. 2A). The spectra of both pigments (M2 and R2) were similar to each other in that the absorption progressively increased from 800 to 230 nm. But the increase of absorption in ultraviolet region was much sharper in the R2 than in the M2.

The absorption spectrum of the R3 is given in Fig. 2B, which shows that the spectrum of R3 resembles that of melanin (M1 and M2) rather than that of R2.

The spectrum of the R5 is given in Fig. 2C, which shows that it resembles that of R2. But, in the case of R5, there were two shoulders in the ranges of 420 - 460 and 315 - 355 nm respectively. As to the M5, the quantity was too small to be examined.

Copper content

The melanin fraction M2 showed the presence of copper, and its content was 0.12 μg per mg on an average. As will be mentioned later, the M2 fraction is a protein-bound melanin. On the other hand, the type B pigment fraction R2, which was also a protein -bound pigment, did not show the presence of copper. Even if any present, it will be only a trace.

The pure melanin fraction M3, which did not contain protein, did not show the presence of copper. Furthermore, neither R3 nor R5 fraction contained copper.

Indole test

The indole test after alkali fusion of the purified melanin M3 was always positive. On the other hand, the test to the R3 fraction was negative in some cases and slightly positive in other cases. Moreover, the R5 fraction did not give a clear result to the test.

Discussion

The type B reddish brown pigment of papilionid butterflies is insoluble in HCl-methanol and does not produce 3-hydroxy-kynurenine on acid hydrolysis. The absorption spectrum does not have any peak. These facts clearly show that the type B reddish brown pigment is not ommochrome.

The type B pigment is soluble in 1 N NaOH and its absorption curve progressively increases from 800 to 230 nm. In these respects, the type B pigment is similar to melanin. It is possible that the type B pigment may be formed by polymerization of some phenolic compound. On the other hand, there are some marked differences between the type B pigment and melanin. The protein-bound malanin (M2) contains much copper, whereas the protein-bound type B pigment (R2) does not. Moreover, melanin (M2 and M3) is not adsorbed on the Dowex 50W column, whereas the type B pigment is adsorbed on the column. The latter observation is in accord with a report that melanin acts as a cation-exchanger (White, 1958). Probably melanin is an anion, and the type B pigment is a cation.

From the results of the indole test after alkali fusion, there seems to be no doubt that the black pigment from the black scales is a kind of indole melanin. On the other hand, it is probable that the type B pigment is not melanin. It is interesting that the hydrolysate of the protein-bound type B pigment (R2) contains kynurenine and β-alanine. In this respect, the type B pigment is similar to the yellow pigment (Papiliochrome II) of papilionid butterflies (Umebachi, 1975a, b: Umebachi and Yamashita, 1976, 1977). Papiliochrome II readily decomposes to kynurenine and N-(β-alanyl) noradrenaline (Umebachi, 1975b: Rembold et al., 1978). It is tempting to speculate that the type B reddish brown pigment is a kind of polymer of N-(β-alanyl) DOPAmine derivative, to which kynurenine is attached.

There seems to be no doubt that the melanin fraction M3 is a kind of indole

melanin. On the other hand, it is possible that the R3 fraction obtained by acid -hydrolysis of the type B pigment R2 may be an artifact produced during the hydrolysis. The quantity of the R3 fraction was very small. Perhaps, the type B pigment itself will be obtained only by proteolytic enzyme-digestion of the R2 fraction. Further experiments are being carried out along this line.

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