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Red Pigments in the Wings of Papilionid Butterflies. Extraction and Purification

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Abstract (1) Extraction and purification of the type A and B red pigments in the Papilionidae are described.

(2) The type A red pigment of *Pachliopta aristolochiae* can be extracted with 70% ethanol, and some chemical properties of the crude extract are described. The pigment has proved to be extracted as a protein-bound molecule and not to contain iron. It has been confirmed that the pigment is yellow in acid and red in alkaline solution. The pigment was purified by salting-out and gel filtration. The hydrolysate of the purified protein-bound red pigment thus obtained showed β-alanine besides several other amino acids.

(3) As to the type B pigment, the reddish brown scales of *Papilio demoleus* were used. The scales were washed first with 70% ethanol and then with 4% HCl-methanol. The pigment was extracted with 1 N NaOH and purified with Dowex 50W column. The purified dark brown pigment thus obtained too was a protein-bound molecule. The hydrolysate showed kynurenine and β-alanine besides several other amino acids.

(4) These pigments are discussed in connection with the yellow pigment, Papiliochrome.

Introduction

Yellow pigments in the wings of butterflies belonging to the genus *Papilio* and the subfamily Zerynthiinae are neither pterins nor ommochromes but the pigments which are related to both kynurenine and a DOPAmine derivative (Umebachi, 1961, 1975a,b; Umebachi and Yoshida, 1970). These yellow pigments are a new group of insect pigments and were named Papiliochrome (Umebachi, 1961, 1962b, 1977). Their chemical and physical properties have been investigated in detail mainly using *Papilio xuthus*. The main yellow pigment, Papiliochrome II, of this species readily decomposes to kynurenine and a N-(β-alanyl) DOPAmine derivative (Umebachi, 1975a; Umebachi and Yamashita, 1976, 1977). The latter compound has proved to be N-(β-alanyl) norad-

renaline (Rembold et al., 1978).

As to the red pigments of the wings of papilionid butterflies, Ford (1942, 1944a, b) divided them into two types (A and B). The type A pigment is the red pigment which occurs widely in the Lepidoptera including the Papilionidae. Among the Papilionidae, the red pigment of this type is found in the genera *Graphium, Troides, Pachliopta, Parnassius, Lühdorfia,* and others. On the other hand, the type B is the reddish brown pigment which is found only in the Papilionidae. And moreover, in the family, the pigment of this type is found only in the genera *Papilio, Chilasa,* and *Battus.* Ford investigated these red pigments from the standpoint of systematics and used them for taxonomical studies. But, as to chemical properties of these red pigments, little work has been done. Umebachi (1962a) reported that the type B pigment-contaning scales incorporated ^{14}C-tryptophan, while the type A pigment did not.

The present paper deals with extraction and purification of the red pigment of *Pachliopta aristolochiae** and those of the reddish brown pigment of *Papilio demoleus*. In the previous papers (Umebachi, 1977; Umebachi and Aburano, 1978), the former red pigment was described under the name of R_2 red pigment and the latter pigment, the R_1 red pigment. These red pigments correspond to the type A and type B in Ford's investigation, respectively (Ford, 1942, 1944a, b).

Materials and Methods

Materials

The red scales from the underside of the hind wings of *Pachliopta aristolochiae* and the reddish brown scales from the upperside of the anal eye spot of *Papilio demoleus* were used. These scales were scraped and stored. The butterflies were all obtained through the Ōkura Biological Institute, and only male butterflies were used.

Extraction of the red pigment of P. aristolochiae

The red pigment is soluble in 70% ethanol. The red scales were treated with 70% ethanol successively at room temperature, 40°, 50°, 60°, 70°, and 80°C. The suspension was centrifuged each time, and the supernatants were combined. The red pigment solution thus obtained is called the crude extract in the present paper. The remaining scales were still red. This shows that although a large portion of the red pigment can be extracted by the above treatment, some of the pigment remains insoluble in the scales. In the present paper, the above crude extract was used as the starting material.

Extraction of the reddish brown pigment of P. demoleus

The reddish brown scales were treated first with 70% ethanol at room temperature, five times. The combined extract was colorless or very slightly brownish and will be referred to as the EtOH fraction in the present paper. Next, the scales were treated with 4% HCl-methanol in the cold, five times. The combined extract was slightly brownish and will be called the HCl-MeOH fraction. If the extraction with 4% HCl-methanol is done at 30°C, the extract is bluish brown. Next, the scales were

* In the previous paper (Umebachi and Aburano, 1978), the generic name *Menelaides* was used. According to Professor T. Shirozu, the Kyushu University, however, *Pachliopta* is better. So, the latter generic name is used throughout the present paper.

washed with 99.5% ethanol and ethyl ether, and dried. Then the scales were treated with 1 N NaOH at room temperature, five or six times. The pigment was extracted by this treatment. The combined extract was brown and named the NaOH fraction.

Thin-layer chromatography

Pre-coated cellulose thin-layer sheet (Merck No. 5552, 20×20 cm) was used. For two-dimensional chromatography, the first solvent was 70% methanol (MeOH) and the second, a mixture of *n*-butanol-glacial acetic acid-water (12 : 3 : 5) (BAW). For one-dimensional chromatography, the solvent was either 70% MeOH or BAW. After development, the chromatogram was inspected under ultraviolet light. Then, either the ninhydrin test or the phosphomolybdic acid-NH_3 test was made. The latter test is for phenolic substances (Umebachi and Yoshida, 1970).

Paper chromatography

One-dimensional chromatography was carried out on Toyo 51A filter paper with 70% MeOH.

Biogel column

Biogel P-30 was used for purifying the type A red pigment. A 1.1×20 cm column was prepared and washed only with water. After the sample was applied on the column, the red pigment was eluted with water.

Dowex 50 W column

A 1.1 × 14 cm column of Dowex 50W × 4 (H^+) was used for purifying the type B reddish brown pigment. After the sample was applied, the column was washed with water. Then, the pigment was eluted with 2 N ammonia water. The pigment fraction and the amino acid fraction were eluted separately. The former fraction moved faster than the latter.

Centriflow and collodion bag

In order to see whether or not the red pigment of *P. aristolochiae* in the crude extract is present as a protein-bound molecule, the Centriflow (Amicon, CF-25) and collodion bag (Sartorius Membrane Filter Co., No. 12) were used.

The crude extract of the red pigment was put in the Centriflow cone and centrifuged at about 1,000*g*. Proteins with molecular weights greater than about 25,000 do not go out through the Centriflow.

The crude extract was also placed in the collodion bag and dialyzed against water. Proteins with molecular weights smaller than about 12,400 pass through the membrane.

Salting-out by ammonium sulfate

In order to see whether or not the red pigment of *P. aristolochiae* in the crude extract is in a protein-bound state, a salting-out with ammonium sulfate was tried, too. To the pigment solution, a saturated ammonium sulfate solution was added to 50 per cent saturation. After the solution was kept in the cold overnight, the precipitate was separated by centrifugation. Then, to the supernatant, crystals of ammonium sulfate was added to 100 per cent saturation. The same technique was also used for the purification of the pigment.

Hydrolysis

The sample to be hydrolyzed was refluxed in 6 N or 1 N HCl in a boiling water bath for 5 hr. The

hydrolysate was evaporated to dryness in a rotary evaporator at 60°C or in a vacuum desiccator at room temperature.

Atomic absorption spectrophotometry

The presence or absence of iron in the type A red pigment was checked with a Shimazu AA-640 atomic absorption spectrophotometer.

Results

Chemical properties of the crude extract of the red pigment from P. aristolochiae

(1) *Color change by acid and alkali* After the crude extract of the red pigment was evaporated to dryness in a rotary evaporator at 40°C, the residue was dissolved in water. The solution thus obtained was of course red.

When, to an aliquot of the above red solution, the same volume of 0.4 N HCI was added, the solution quickly turned yellow. The absorption spectrum is given in Fig. 1, which shows a peak at about 275 nm and a shoulder between 410 and 440 nm.

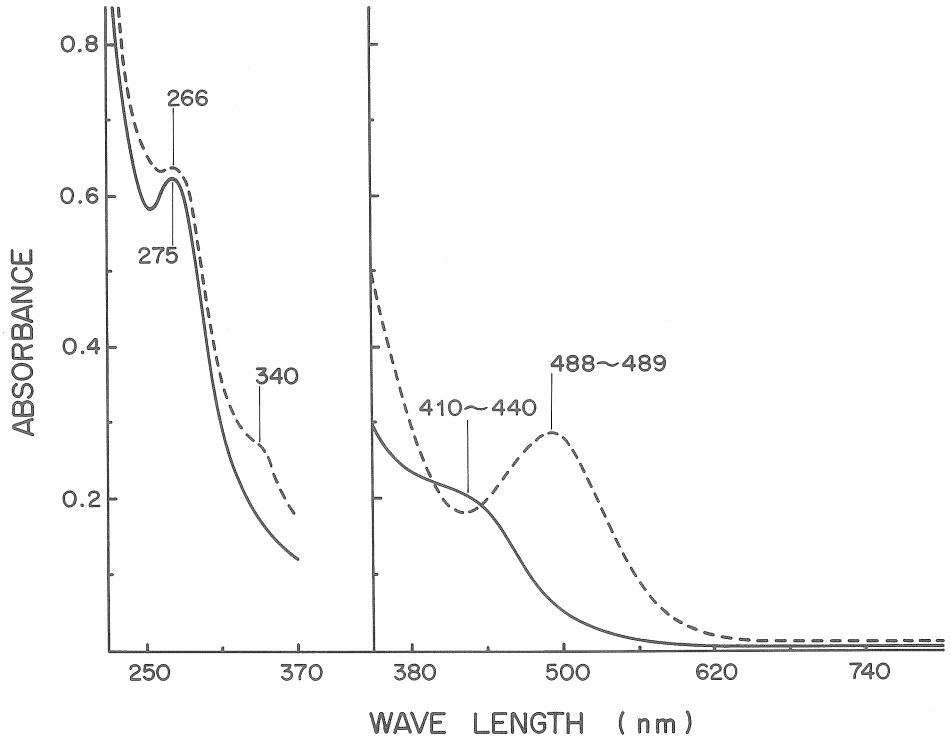

Fig. 1. Absorption spectra of the crude extract of the red pigment from *P. aristolochiae*. Solid line, in 0.2 N HCI ; broken line, in 0.2 N $NaHCO_3$.

When the same volume of 0.4 N $NaHCO_3$ was added to another aliquot of the red pigment solution, the color remained red. The absorption spectrum showed peaks at 266 and 488–489 nm and a shoulder at about 340 nm (Fig. 1).

Next, several drops of saturated $NaHCO_3$ solution was added to the above yellow solution in 0.2 N HCI. The color turned red back again.

To the above red solution in 0.2 N $NaHCO_3$, two drops of conc. HCI was added. The color immediately turned yellow.

(2) *Thin-layer chromatography* When the crude extract was one-dimensionally chromatographed with 70% methanol, two red bands (A_2 and A_4) were always found (Fig. 2). In some cases, two more red bands (A_1 and A_3) were also found, though the A_1 was uncertain. These red pigments were all negative to the ninhydrin reaction. Moreover, no ninhydrin-positive substance was found on the chromatogram. So, free amino acids were absent or, if any present, an undetectable amount. The phosphomolybdic acid-NH_3 test was positive only near the starting line. The red pigments A_1, A_2, A_3, and A_4 were negative to the test.

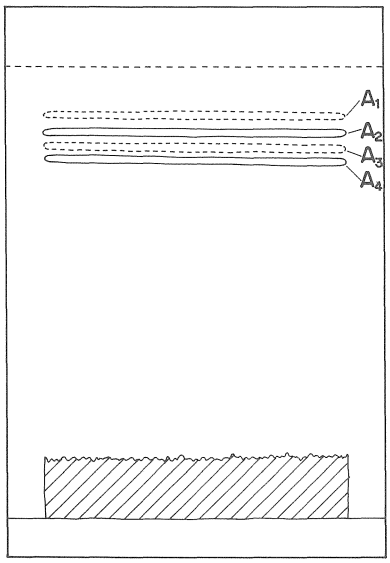

Fig. 2. One-dimensional thin-layer chromatogram of the crude extract of the red pigment from *P. aristolochiae*. Solvent, 70% MeOH. Slant lines show the area positive to the phoshomolybdic acid-NH_3 test.

(3) *Hydrolysis* The crude extract was hydrolyzed in 1 N HCI, and the hydrolysate was evaporated to dryness, dissolved in water, and submitted to two-dimensional thin-layer chromatography. The ninhydrin test showed the presence of at least six amino acids including glycine, aspartic acid, glutamic acid, and α-alanine. Threonine was also present, though it was faint. Interestingly, a considerable quantity of β-alanine was present. Kynurenine was not found. The phosphomolybdic acid-NH_3 test showed at least three positive substances. As the solvent BAW is not suited for the red pigment of this type, no red pigment was not found on the chromatogram.

The area of the red pigment A_2 or A_4 in Fig. 2 was scraped and treated with water.

After centrifugation, the supernatant was hydrolyzed in 1 N HCI. Two-dimensional chromatogram of the hydrolysate showed at least six amino acids including glycine, aspartic acid, glutamic acid, and α-alanine. A considerable quantity of β-alanine was found but kynurenine was not present. These results suggest that the red pigment is extracted as a protein-bound molecule from the red scales with 70% ethanol.

(4) *The absence of iron* In order to see whether the red pigment contains iron or not, the crude extract was submitted to atomic absorption spectrophotometry. As the result, the absorption by iron was negligible. It was sure that the red pigment is not an iron-containing pigment.

(5) *Evidence for protein-bound pigment* In order to confirm that the pigment in the crude extract is present as a protein-bound molecule, the crude extract was submitted to the following three treatments: (a) the Centriflow, (b) collodion bag, and (c) salting-out by ammonium sulfate.

When the crude extract of pigment was placed in the Centriflow cone and centrifuged, only a trace amount of the red pigment went out through the cone. A large portion of the pigment remained in the cone.

When the crude extract was placed in the collodion bag and dialyzed, the red pigment did not pass through the membrane. A large portion of the pigment was adsorbed in the membrane.

A large portion of the red pigment precipitated in 50% saturation of ammonium sulfate, and a little of the pigment precipitated in 100% saturation of the salt. Furthermore, some pigment remained soluble in the 100% saturated salt solution.

All these results and the presence of amino acids in the hydrolysate indicate that a large portion of the red pigment in the crude extract is present as a protein-bound molecule.

Purification of the protein-bound red pigment of P. aristolochiae

The crude extract of the pigment was evaporated to dryness in a rotary evaporator at 40°C. The residue was dissolved in water. Some of the red pigment remained insoluble. After centrifugation, the supernatant was brought to 50% saturation of ammonium sulfate. After being kept in the cold overnight, the red precipitate was separated by centrifugation. As mentioned above, some of the red pigment remained soluble. The red precipitate was dissolved in water, applied on the Biogel P-30 column, and eluted with water. The effluents of the red pigment were collected and evaporated to dryness under reduced pressure. In some cases, the residue thus obained was further dissolved in water and chromatographed one-dimensionally with 70% methanol on filter paper. The area of the red pigment was cut out and extracted with water. The red extract was again applied on the Biogel P-30 column and eluted with water. The red effluents were collected and evaporated to dryness.

Hydrolysis of the purified protein-bound red pigment of P. aristolochiae

The protein-bound red pigment purified by the above procedure was hydrolyzed in 1 N HCl. The hydrolysate was evaporated to dryness under reduced pressure, dissolved in water, and submitted to two-dimensional thin-layer chromatography. The ninhydrin reaction showed distinct spots of β-alanine and glycine. In addition, α-alanine, glutamic acid, and aspartic acied were found. Threonine might be also present, though it was uncertain. Kynurenine was not found.

Chemical properties of the extracts from the reddish brown scales of P. demoleus

As mentioned in the section of methods, the reddish brown scales were treated first with 70% ethanol. Two-dimensional chromatogram of the EtOH fraction showed small quantities of β-alanine and kynurenine. Two distinct and two faint spots positive to the phosphomolybdic acid-NH_3 test were present. The hydrolysate of this fraction was also submitted to two-dimensional chromatography, and at least seven amino acids including glycine, aspartic acid, glutamic acid, α-alanine, β-alanine, and kynurenine were found. One distinct spot positive to the phosphomolybdic acid-NH_3 test was present.

Next, the scales were treated with 4% HCl-methanol. Two-dimensional chromatogram of the HCl-MeOH fraction showed at least five amino acids including glycine, β-alanine, tyrosine and kynurenine. An unidentified ninhydrin-positive substance was also present. To the phosphomolybdic acid-NH_3 test, there were several faint or uncertain spots. The hydrolysate of this fraction showed at least ten amino acids including glycine, aspartic acid, glutamic acid, α-alanine, β-alanine, tyrosine, isoleucine, leucine, and kynurenine. The above-mentioned unidentified ninhydrin-postitive substance was also present.

Purification of the reddish brown pigment of P. demoleus

After the scales were washed with 70% ethanol and then with 4% HCl-methanol as mentioned above, the brown pigment was extracted with 1 N NaOH. A large portion of the reddish brown pigment was extracted by this treatment. The remaining scales was slightly yellowish.

The NaOH fraction thus obtained was submitted to the Dowex 50W column. The amino acid fraction and the pigment fraction were obtained by the elution with ammonia water. Two-dimensional chromatogram of the amino acid fraction showed the presence of at least ten ninhydrin-positive substances including glycine, aspartic acid, glutamic acid, α-alanine, β-alanine, tyrosine, phenylalanine, leucine and kynurenine. Probably, threonine was also present. Besides, an unidentified ninhydrin-positive spot was present.

The pigment fraction was evaporated to dryness and washed with water. The residue was dissolved in 2 N ammonia water and submitted to two-dimensional thin-layer chromatogram. The pigment did not move from the original point in both 70% MeOH and BAW. Free amino acids were not found at all. Substance positive to

the phosphomolybdic acid-NH_3 test was not found, either. Thus, at least thin-layer chromatographically, the pigment fraction was pure, though it was a protein-bound pigment as described later. The color was dark brown or chocolate.

Hydrolysis of the reddish brown pigment of P. demoleus

The dark brown pigment purified by the above-mentioned procedure was hydrolyzed in 1 N or 6 N HCI. After the hydrolysate was centrifuged, the supernatant was evaporated to dryness in a rotary evaporator at 60°C. The residue was dissolved in water and submitted to two-dimensional chromatography. At least ten ninhydrin-positive substances including glycine, aspartic acid, glutamic acid, α-alanine, tyrosine, phenyalanine, valine, leucine, and kynurenine were found. Interestingly, a considerable quantity of β-alanine was present. No substance positive to the phosphomolybdic acid-NH_3 test was found. These results show that the reddish brown pigment is extracted as a protein-bound molecule from the scales.

After the above hydrolysis, some pigment remained insoluble. After centrifuging the hydrolysate, the precipitate was washed with water, 99.5% ethanol, and ethyl ether, and dried. The dried pigment was dissolved in 1 N NaOH, and absorpuion spectrum was taken. As seen in Fig. 3, no absorption peak was found. The absorption progressively increases from 800 to 223 nm.

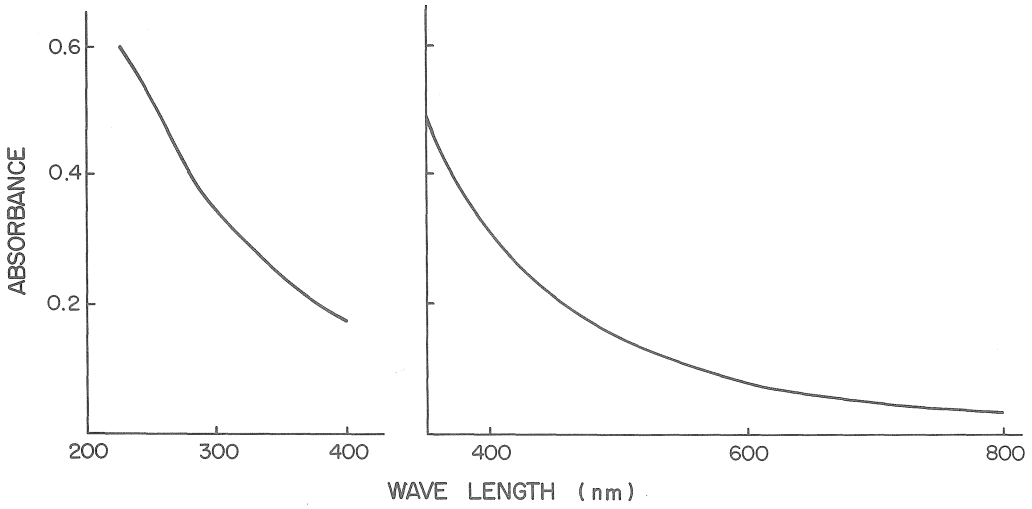

Fig. 3. Absorption spectrum of the brown pigment from *P. demoleus*. Solvent, 1 N NaOH.

Discussion

Ford (1942) investigated red pigments of the Lepidoptera and divided them into five types (A, B, C, D, and E). Afterward, in 1944, he added the sixth type. In the Papilioni-

dae, the types A and B are found. The type A red pigment is widely distributed in the Lepidoptera. Among the Papilionidae, this type occurs in almost all genera except *Papilio, Chilasa,* and *Battus*. On the other hand, the type B red pigment occurs in *Papilio, Chilasa,* and *Battus*. Exactly speaking, the pigment of this type is not red but reddish brown or brown. According to Ford (1944a, b), the red pigment of *P. aristolochiae* is of type A, and the reddish brown pigment of *P. demoleus* is of type B.

Ford (1942) reported that the type A red pigment shows a quick and remarkable color change by acid and alkali. The results of the present paper has spectrophotometrically confirmed this property.

From the chemical properties of the red pigment in the crude extract, there is no doubt that the pigment of *P. aristolochiae* is extracted as a protein-bound molecule from the scales. And it is certain that the red pigment is not an iron-containing pigment. It is interesting that the hydrolysate of the protein-bound red pigment showed the presence of β-alanine. Whether the β-alanine is attached to the protein molecule or to the red pigment itself has remained unsettled. In comparison with the reddish brown pigment of *P. demoleus,* it is important that the type A red pigment does not contain kynurenine.

In the reddish brown scales of *P. demoleus*, it is interesting that the EtOH and HCl-MeOH fractions contatin β-alanine and kynurenine. Furthermore, the hydrolysate of the purified reddish brown pigment showed β-alanine and kynurenine, too. The presence of several amino acids in the hydrolysate indicates that the reddish brown pigment of type B is extracted as a protein-bound molecule from the scales and that the purified reddish brown pigment is a protein-bound molecule. Whether the β-alanine and kynurenine are attached to the protein moiety or to the pigment itself has remained unsettled. Next, as to the color of the pigment, exactly speaking, the purified reddish brown pigment is not reddish brown but dark brown or chocolate. In 1 N NaOH, it is brown. From the solubility properties, the presence of kynurenine in the hydrolysate (absence of 3-hydroxykynurenine), and the absorption spectrum (Fig. 3), there is no doubt that the pigment of this type does not belong to ommochrome.

Umebachi (1975a, b) and Rembold et al. (1978) reported that the pale yellow pigments (Papiliochrome) of the wings of the genus *Papilio* readily decompose to kynurenine and N-(β-alanyl) noradrenaline. The N-(β-alanyl) compound releases β-alanine on a mild hydrolysis (Umebachi and Yamashita, 1977). Bodnaryk and Levenbook (1969) reported that the β-alanine which is in the carbonyl side of peptide bond and is present as a N-terminal is readily released on a mild hydrolysis.

Now, it is very interesting that the reddish brown pigment (type B) which occurs in the genus *Papilio* is related to both β-alanine and kynurenine. On the other hand, the type A red pigment is related to β-alanine but not to kynurenine. Further experiments on the type A red pigment itself and the type B reddish brown pigment itself are now being carried out.

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