Beta-alanine and Dopamine in the Reddish Brown Scales of Papilio Butterflies

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Beta-alanine and Dopamine in the Reddish Brown Scales of *Papilio* Butterflies

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Abstract (1) Reddish brown scales of the anal eye spot in the hind-wings of *P. demoleus* and *P. machaon* have been examined for β-alanine and dopamine.

(2) The scales were fractionated into 70% ethanol-soluble fraction, 4% HCl-methanol -soluble fraction, and the residual scales, and the β-alanine content of each fraction was determined. Most of the β-alanine present in the scales has been found in the residual scales. On acid hydrolysis of the residual scales, the β-alanine has been rather rapidly released, and the hydrolysate has contained a large amount of β-alanine.

(3) The protein-bound brown pigment (HCl-ppt fraction), which was extracted with 1 N NaOH and precipitated by being acidified with HCl, has contained a large amount of β-alanine. In most or at least some of the β-alanine, the NH_2-group has been proved to be free.

(4) ^{14}C-Labelled β-alanine and ^{14}C-dopamine, which were injected at prepupal or pupal stage, have been incorporated in the highest degree into the residual scales. And the ^{14}C has been confirmed to be present in the HCl-ppt fraction.

(5) All these results indicate that the pigment of the reddish brown scales contains β-alanine and dopamine.

Introduction

Wing-pigments of *Papilio* butterflies are neither pteridine pigments nor ommochromes but belong to the pigment group, Papiliochrome (Umebachi, 1980). Among Papiliochromes, the pale yellow pigments have been investigated in most detail, and the main pale yellow pigment, Papiliochrome II has been proved to be N^{ar}−[α−(3−aminopropionylaminomethyl)−3, 4−dihydroxybenzyl]−L−kynurenine (Rembold and Umebachi, 1983). The pigment readily decomposes to L-kynurenine and N-β-alanylnoradrenaline on being heated (Umebachi and Yamashita, 1976, 1977 ; Rembold et al., 1978). The deep yellow and reddish brown pigments have also been studied to some extent and named Ppapiliochrome M and R, respectively (Umebachi, 1977a, 1978, 1979).

Chemical properties of the reddish brown pigment reported in the previous papers

(Umebachi, 1978, 1979, 1983) are as follows : (1) The pigment contains kynurenine and β-alanine. (2) The pigment is extracted as a protein-bound form with 1 N NaOH from the reddish brown scales and precipitated by HCl. But, during this procedure, most of the kynurenine is lost. (3) The solubility of the pigment is similar to that of melanin but the pigment is not melanin. (4) The protein-bound pigment does not contain copper. And (5) the absorption spectrum of the protein-bound pigment does not have any peak and the absorbance progressively increases from 800 to 230nm.

The present paper deals with more detailed investigations of β-alanine in the reddish brown scales and moreover shows that the pigment contains also dopamine.

Materials and Methods

Materials

Reddish brown scales of the anal eye spot in the hind-wings of *Papilio demoleus* and *Papilio machaon* were used.

For the incorporation-experiments of ^{14}C-labelled compounds, young larvae of *P. machaon* were collected in the field and raised in the laboratory. Pupal stage was 10 to 13 days.

Extraction and fractionation

Scales were first extracted with 70% ethanol at room temperature and 40°C several times, and the combined extract is called 70% EtOH fraction. The residue was then extracted with 4% HCl-methanol at room temperature five times. The combined extract is named 4% HCl-MeOH fraction, and the residue is called residual scales, which were still dark brown.

The residual scales were further treated with 1 N NaOH at room temperature eight times. Most of the dark brown pigment was extracted by this extraction. The combined extract is referred to below as 1 N NaOH-soluble fraction. The final residue was brownish yellow.

When the 1 N NaOH-soluble fraction was acidified with conc. HCl and kept in the cold, the dark brown pigment precipitated. After centrifugation, the supernatant was almost colorless. The precipitate is called HCl-ppt fraction.

Hydrolyses

The above-mentioned fractions (70% EtOH-soluble, 4% HCl-MeOH-soluble, residual scales, and HCl -ppt) as well as the original scales before fractionation were hydrolyzed in 6 N HCl at 100°C for 24 hr. In some cases, the hydrolysate was diluted to 1 N HCl with water and applied to the Dowex 50W×4 column (1×13cm). After being washed with water, amino acids were eluted with 2 N ammonia water and lyophilyzed. In some other cases, the hydrolysate was evaporated to dryness in a rotary evaporator at 60° C, and the residue was kept over NaOH in a vacuum desiccator. The β-alanine content of the residue thus obtained was determined.

In some cases, the time of hydrolysis and the concentration of HCl were changed.

Determination of β-alanine

This was performed by the DNP-method (Sanger and Thompson, 1953). The sample (the above -mentioned hydrolysate) was dissolved in 0.1ml of 1% trimethylamine, and 0.2ml of 5% FDNB (1-fluoro -2, 4-dinitrobenzene) in ethanol was added. After standing for 2hr in the dark, the solution was submitted

to two-dimensional thin-layer chromatography with silica gel sheet (Merck, No. 5553). The solvent for the first direction was the organic layer of toluene, chloroethanol, pyridine, and 0.8 N ammonia water mixture (5 : 3 : 1.5 : 3) and for the second direction, a mixture of chroloform, benzylalcohol, and acetic acid (70 : 30 : 3). After development, absorbance of the yellow spot of DNP-β-alanine on the chromatogram was measured with a Shimazu 920 TLC scanner, which could perform a zig-zag scan over a spot and integrate the values of absorbance.

Amino acid analyses

The residual scales and HCl-ppt fraction from *P. demoleus* were hydrolyzed as mentioned above, and amino acids of the hydrolysates were analyzed with a Nihon-Denshi 200A amino acid analyzer.

Dinitrophenylation of the HCl-ppt fraction

The HCl-ppt fraction from *P. demoleus* was dissolved in 1 N NaOH and applied to the Dowex 50W × 4 column (1 × 13cm). The column was washed with water, and then the brown pigment was eluted with 2 N ammonia water (Umebachi, 1978, 1979). The pigment was eluted ahead of amino acid fraction. The dark brown effluent was collected and lyophilyzed. The residue, which was the protein-bound dark brown pigment, was washed with water, dissolved in 1 N NaOH, and precipitated with conc. HCl. The dark brown precipitate was further washed with water and dissolved in 1ml of 5% $NaHCO_3$, and 2ml of 5% FDNB in ethanol was added. After standing for 3hr in the dark, the solution was acidified with three drops of conc. HCl and washed with ethyl ether. After being further washed with water, acetone, and ethyl ether, the precipitate was hydrolyzed in 6 N HCl at 105°C for 15hr in a sealed tube. The hydrolysate was diluted to 1 N HCl with water and extracted with ethyl ether three times. The combined ether layer was evaporated to dryness, dissolved in acetone, and submitted to two-dimensional thin-layer chromatography in the same way as used in the determination of β-alanine.

Incorporation of ^{14}C-labelled β-alanine and dopamine

The labelled compounds used were β-alanine [1-^{14}C] (New England Nuclear; specific activity, 54.7μCi per m mol) and dopamine [side chain-1-^{14}C] (Amersham; specific activity, 56μCi per m mol). Experiments were all performed with *P. machaon*. The labelled compounds were used as a water solution, respectively, and 0.02 to 0.06ml (0.6 to 2μCi) of it was injected at the prepupal stage or 5—7days after pupation.

After emergence of butterfly, autoradiograph of the wings was taken with a Sakura Medical X—ray film, type A. The time of exposure was 14 to 21 days. Then, the reddish brown scales were scraped and treated with 70% ethanol and 4% HCl-methanol successively as mentioned above. The 70% EtOH fraction and 4% HCl - MeOH fraction were evaporated to dryness in counting vials, respectively. The residual scales were also transferred to a counting vial. To the dry sample in the counting vial thus obtained, 0.05ml of water and 1ml of the solubilizer (NCS of Amersham) were added. After the sample was warmed at 50°C for several hours, 0.03ml of glacial acetic acid and 8ml of scintillation cocktail (ACS II of Amersham) were added. After the counting vial containing the sample was kept in the refrigerator for 2 to 6days, dpm of ^{14}C was measured with a Beckman LS—9000 liquid scintillation counter.

In some cases, the radioactive reddish brown scales were washed with 70% ethanol repeatedly and then were hydrolyzed in 6 N HCl at 100°C for 24 hr. The hydrolysate was filtered through Centriflow CF—25 (Amicon), and the filtrate was evaporated to dryness in a rotary evaporator at 60°C. After being kept over NaOH in a vacuum desiccator, the residue was dissolved in 70% ethanol and submitted to one-dimensional thin-layer chromatography on a cellulose sheet (Merck, No. 5552) with a mixture of *n*

-butanol-acetic acid-water (12 : 3 : 5). Radioactivity of the chromatogram was measured with a Berthold radioactive chromatogram scanner. The ninhydrin reaction was also carried out.

In some other cases, the radioactive reddish brown scales were treated with 70% ethanol, 4% HCl -methanol, and 1 N NaOH successively as mentioned above. The HCl-ppt fraction was prepared from the 1 N NaOH-soluble fraction, and its radioactivity was measured with the above-mentioned liquid scintillation counter.

Results

Incorporation of ^{14}C-β-alanine

An autoradiograph of the wings from the butterfly (*P. machaon*) which was injected with ^{14}C-β-alanine at the pupal stage is given in Fig. la, which shows that the ^{14}C was incorporated into not only the deep yellow scales but also the reddish brown scales of the anal eye spot. The butterfly which was injected at the prepupal stage gave also substantially the same autoradiograph.

The radioactive reddish brown scales were scraped and hydrolyzed in 6 N HCl, and the hydrolysate was submitted to one-dimensional thin-layer chromatography. The result of radioactivity scanning on the chromatogram is given in Fig. 2, which shows that the scales incorporated ^{14}C-β-alanine and that radioactive substance other than ^{14}C-β-alanine is absent or, if any present, it is only a little. Of course, many ninhydrin-positive cold

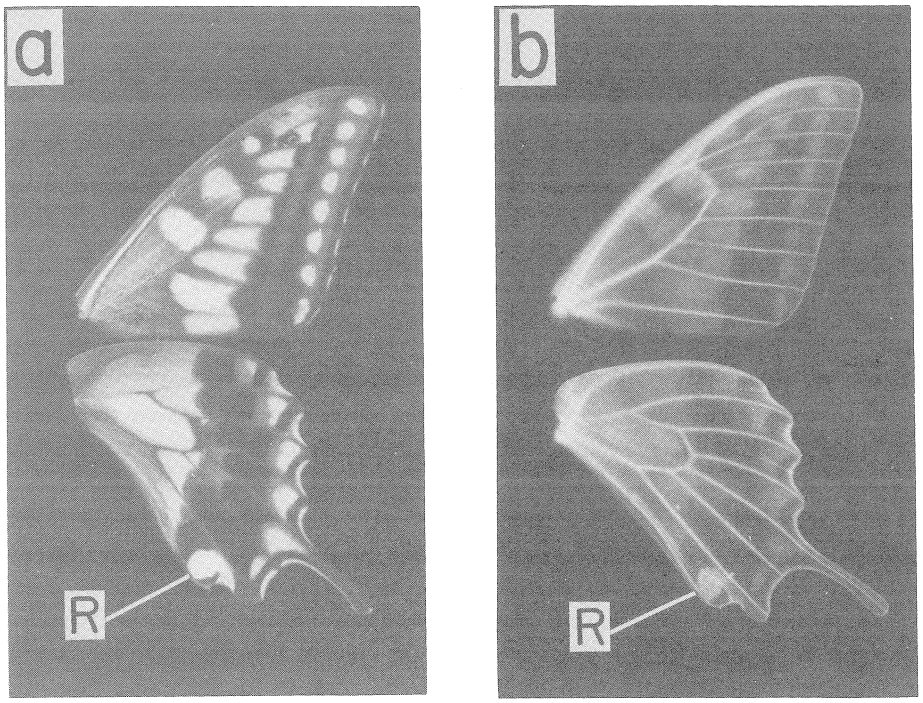

Fig. 1. Autoradiographs of the wings of *P. machaon* which was injected with (a) ^{14}C-β-alanine or (b) ^{14}C-dopamine at the pupal stage. R, reddish brown part.

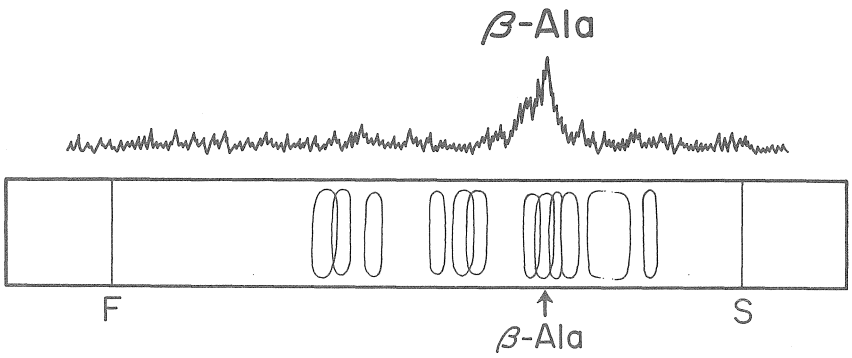

Fig. 2. Radioactivity scanning of the thin-layer chromatogram obtained with the hydrolysate of ^{14}C-β-alanine-incorporated reddish brown scales (*P. machaon*). The chromatogram shows ninhydrin-positive substances. S, starting line ; F, the front of solvent.

Table 1. Distribution of radioactivity in the ^{14}C-β-alanine-incorporated reddish brown scales (*P. machaon*) (Percentage of the total radioactivity)

Fraction	70% EtOH	4% HCl-MeOH	Residual scales
Radioactivity dpm, %	3.2	14.5	82.3

substances including ordinary amino acids were present.

From the radioactive reddish brown scales, 70% EtOH fraction, 4% HCl-MeOH fraction, and residual scales were prepared, and radioactivity of each fraction was measured. The results are given in Table 1, which shows that most of the ^{14}C is present in the residual scales.

Moreover, the radioactive residual scales were extracted with 1 N NaOH, and the HCl-ppt fraction prepared from the 1 N NaOH-soluble fraction was confirmed to be radioactive.

Table 2. Beta-alanine contents in the reddish brown scales of *Papilio* (μg per mg dry weight of scales)

Species	*demoleus*	*machaon*
β-Alanine	73.6±3.8* (3)**	61.9 (1)

* Mean±S.E.

** The number of determination is shown in parenthesis.

Beta-alanine contents of scales

The reddish brown scales of *P. demoleus* and *P. machaon* were hydrolyzed in 6 N HCl at 100°C for 24hr, and the β-alanine contents were determined. The results are given in Table 2, which shows that the reddish brown scales contain about 60—75μg β-alanine per

mg dry weight of scales.

Distribution of β-alanine in the scales

The 70% EtOH fraction, 4% HCl-MeOH fraction, and residual scales from the reddish brown scales were hydrolyzed in 6 N HCl, and their β-alanine contents were

Table 3. Distribution of β-alanine in the reddish brown scales (Percentage of the total β-alanine)

Species	*demoleus*	*machaon*
Fraction	%	%
70% EtOH	3.9	1.7
4% HCl-MeOH	9.9	5.7
Residual scales	86.2	92.5

determined. The results are given in Table 3, in which the quantity of β-alanine in each fraction is given as percentage of the total β-alanine content. The table shows that about 90 per cent of the total β-alanine is insoluble in 70% ethanol and 4% HCl-methanol and remains in the residual scales.

Release of β-alanine by acid hydrolysis

The residual scales of *P. demoleus* were hydrolyzed in 6 N HCl at 100°C for 2.5, 5.5, 11. 5, and 24hr, and β-alanine of the hydrolysate was determined. As seen in Fig. 3, β-alanine is rather rapidly released on acid hydrolysis, though it takes 24hr to release the total β-alanine.

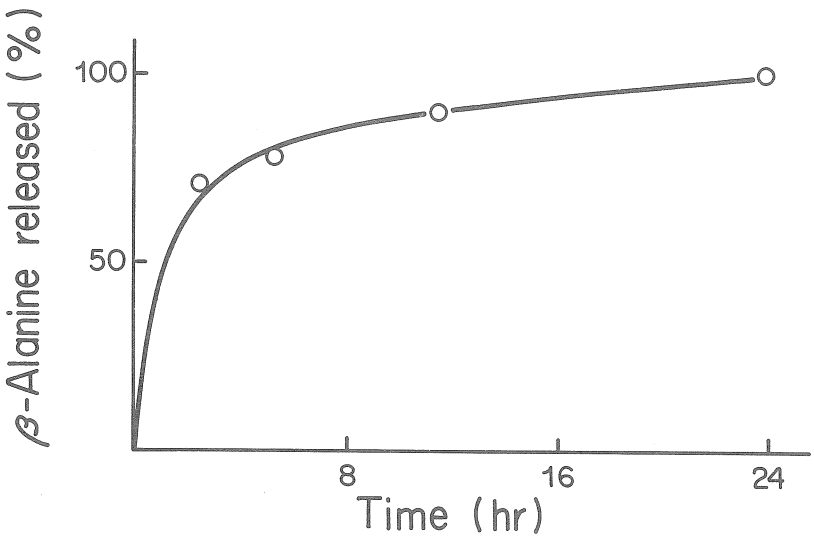

Fig. 3. Release of β-alanine from the residual scales of *P. demoleus* by acid hydrolysis (6 N HCl, 100°C).

Table 4. Molar ratios of amino acids in the hydrolysates of the residual scales and HCl-ppt fraction from *P. demoleus*

Amino acid	Experimental No. 1	2	3
	Residual* scales	Residual** scales	HCl-ppt** fraction
Met††	3.3†	3.7	27.6
Asp	31.8	40.7	36.6
Thr	16.4	26.2	23.0
Ser	31.5	41.9	31.1
Glu	57.6	42.8	88.0
Pro	57.1	55.1	44.4
Gly	100.0	100.0	100.0
Ala	102.1	94.7	72.0
Val	58.7	111.0	84.4
Ile	33.1	32.4	37.7
Leu	47.0	46.3	53.0
Tyr	32.0	41.0	23.0
Phe	8.9	9.5	19.5
β-Ala	285.0	197.8	498.1
Kyn	4.9	12.3	Trace
Lys	13.0	15.5	20.2
His	23.9	27.5	64.6
Arg	—	21.7	—

* The hydrolysate was desalted through the Dowex 50 W column.
** The hydrolysate was evaporated to dryness in a rotary evaporator.
† Molar ratios are given as the value for 100 of glycine.
†† This includes methionine and methionine sulfoxide.

Amino acid analyses of the residual scales and HCl- ppt fraction

The residual scales of *P. demoleus* were hydrolyzed in 6 N HCl at 100°C for 24hr, and the amino acid composition was determined with the amino acid analyzer. The results are given in Experimental Nos. 1 and 2 of Table 4, in which the molar ratios of amino acids are given as the values for 100 of glycine. The Expermental Nos. 1 and 2 show that the residual scales contain much more β-alanine than other amino acids.

Moreover, the HCl-ppt fraction from *P. demoleus* was hydrolyzed in the same way as in the residual scales, and the amino acid composition was determined. The result is given in Experimental No. 3 of Table 4, which shows that the HCl- ppt fraction contains also a great quantity of β-alanine.

Dinitrophenylation of the HCl- ppt fraction

The HCl- ppt fraction from *P. demoleus* was partially purified, dinitrophenylated, hydrolyzed, and submitted to thin-layer chromatography as desciribed in the section of methods. The chromatogram clearly showed the presence of DNP-β-alanine, the yellow spot of which was distinct and predominant. Although DNP-α-alanine and DNP-glycine were also found, their spots were slight.

Incorporation of ^{14}C-dopamine

An autoradiograph of the wings from the butterfly (*P. machaon*), which was injected with ^{14}C-dopamine at the pupal stage, is given in Fig. 1b, which shows that the ^{14}C was incorporated into not only the deep yellow scales but also the reddish brown scales of the anal eye spot.

Table 5. Distribution of radioactivity in the ^{14}C-dopamine-incorporated reddish brown scales (*P. machaon*) (Percentage of the total radioactivity)

Fraction	70% EtOH	4% HCl-MeOH	Residual scales
Radioactivity dpm, %	7.6±1.0* (3)**	11.3±1.2 (3)	80.1±1.5 (3)

* Mean±S.E.

** The number of determination is shown in parenthesis.

From the radioactive reddish brown scales, the 70% EtOH fraction, 4% HCl-MeOH fraction, and residual scales were prepared, and the radioactivity of each fraction was measured. The results are given in Table 5, which shows that most of the ^{14}C is present in the residual scales.

The radioactive residual scales were extracted with 1 N NaOH, and the HCl-ppt fraction prepared from the 1 N NaOH-soluble fraction was confirmed to be radioactive.

Discussion

From the results of the previous papers (Umebachi, 1978, 1979, 1983) and the present paper, there is no doubt that the reddish brown pigment of *Papilio* is not ommochrome but the pigment which is related to kynurenine, β-alanine, and dopamine. For this reason, the pigment has been named Papiliochrome R. Ford (1944a, b) has divided red pigments of the Papilionidae into two types A and B. The pigment of type A is widely distributed in Lepidoptera, while the pigment of type B is found only in the Papilionidae, especially in *Papilio*, *Chilasa*, and *Battus*. Papiliochrome R of the present paper corresponds to the red pigment of type B. Exactly speaking, the color of the pigment is not red but reddish brown.

The present paper indicates that the HCl-ppt fraction contains a great quantity of β-alanine, that the β-alanine is readily released on acid hydrolysis, that the NH_2-group of most or at least some of the β-alanine is free, and that the ^{14}C-β-alanine incorporated into the pigment can be recovered as such. The present paper furthermore shows that dopamine is also incorporated into the pigment, but the property of the dopamine has remained unsettled. The elucidation of the structure of Papiliochrome R is rather difficult, since the pigment is extracted as a protein-bound form with 1 N NaOH and since most of the kynurenine present in the pigment is lost by the extraction with 1 N NaOH. But, judging from thc fact that Papiliochrome II is composed of kynurenine and N-β-alanyldopamine, there is the possibility that Papiliochrome R is a kind of polymer of N-β-alanyldopamine, with which some kynurenine combines and that such pigments may be bonded to the scale protein.

The reddish brown pigments of *P. machaon* and *P. demoleus* seem to be substancially the same. But the reddish brown scales of *P. machaon* contain small quantities of pale yellow pigments (Papiliochrome II and III) in addition to Papiliochrome R. On the other hand, the reddish brown scales of *P. demoleus* hardly contain thc pale yellow pigments (Umebachi, 1977b). Therefore the reddish brown scales of *P. machaon* is more brownish than those of *P. demoleus*.

Figs. la and lb show that both ^{14}C-β-alanine and ^{14}C-dopamine are incorporated into not only the reddish brown scales but also the deep yellow scales. In fact, the deep yellow pigment is also related to kynurenine, β-alanine, and dopamine. Chemical properties of the pigment will be reported elsewhere.

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