Comparison between Yellow Pigments of the Wings of Papilio machaon and Those of P. xuthus

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Comparison between Yellow Pigments of the Wings of *Papilio machaon* and Those of *P. xuthus*

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Abstract (1) The yellow scales of wings of *P. machaon* and *P. xuthus* were treated with 70 % ethanol and then with 4 % HCl-methanol. Each extract was, without any treatment or after hydrolysis in 1 N HCl, submitted to two-dimensional thin-layer chromatography. The remaining scales were also hydrolyzed and chromatographed.

(2) In both species, the 70 % ethanol extract contains kynurenine and Papiliochrome IIa, IIb, IIIa, and IIIb. But the main pigments of *P. machaon* are the brownish yellow pigments which are extracted with 4 % HCl-methanol and have been tentatively named the yellow pigments M_1 and M_2. As in Papiliochrome II, the M_1 and M_2 have also been presumed to be related to kynurenine, β-alanine, and an *o*-diphenolic substance. This indicates that the deep yellow pigments of *P. machaon* may also belong to the Papiliochrome group.

(3) In both species, the remaining scales have also proved to contain β-alanine.

Introduction

As already reported, the yellow pigments of wings of the papilionid butterfly, *Papilio xuthus,* are neither pterin nor ommochrome but the pigments which are related to both kynurenine and DOPAmine, and were named Papiliochrome (Umebachi, 1958, 1961, 1962; Umebachi and Yoshida, 1970). In this species, three kinds of Papiliochrome (II, III, and V) were found with paper chromatography. Among them, Papiliochrome II is the main pigment of this species and has been investigated in detail. The yellow pigment readily decomposes to kynurenine and the DOPAmine derivative, SN-1, by being heated (Umebachi and Yoshida, 1970; Umebachi, 1975a). Interestingly, the SN-1 contains β-alanine and has been presumed to be a N-(β-alanyl) DOPAmine derivative (Umebachi, 1975a and b; Umebachi and Yamashita, 1976, 1977). Thus, Papiliochrome belongs to a new group of insect pigments and is also interesting from the standpoint of the chemistry of cuticle.

On the other hand, no such detailed investigation has been made on the yellow

pigments of other *Papilio* species, though it was reported that ^{14}C-labeled tryptophan is incorporated into the yellow scales of wings of *P. machaon, P. protenor,* and *P. helenus* (Umebachi, 1959).

The present paper deals with the yellow pigments of the wings of *P. machaon.* The yellow scales were treated with 70 % ethanol and then with 4 % HCl-methanol. Both extracts were, without any treatment or after hydrolysis, chromatographed on thin-layer sheet. The scales remaining after extraction were also hydrolyzed and chromatographed. For the purpose of comparison, experiments of the same kind were also made with *P. xuthus.* It has proved that, in *P. machaon* also, there are Papiliochrome II and III. But the main pigments of this species are the brownish yellow pigments which can be extracted with HCl-methanol. These brownish yellow pigments have also been presumed to be related to kynurenine, β-alanine, and an *o*-diphenolic substance.

Materials and Methods

Materials

Male adults of *P. machaon* and *P. xuthus* were used. Some of them were raised from the larval stage in the laboratory, and others were obtained through the Okura Biological Institute. The yellow scales of wings were scraped and stored.

Extraction

As the starting material, the yellow scales were always used. The extraction of yellow pigments were carried out with 70 % ethanol and 4 % HCl-methanol as shown in Fig. 1. The remaining scales were named ghost scales.

Hydrolysis

Each fraction of Fig. 1 was, without any treatment or after hydrolysis, submitted to thin-layer chromatography. The hydrolysis was carried out under reflux in 1 N HCl at 100°C for 2.5 hr. After the hydrolyzate was evaporated to dryness under reduced pressure, the residue was dissolved in water and chromatographed. The ghost scales were also hydrolyzed in 1 N HCl at 100°C for 5 hr and chromatographed in the same way.

Thin-layer chromatography

Cellulose thin-layer sheet (Merck, No. 5552; 20×20 cm) was used. For two-dimensional chromatography, the first solvent was 70 % methanol, and the second, a *n*-butanol-glacial acetic acid-water mixture (120 : 30 : 50) (BAW). For one-dimensional chromatography, the solvent was either 70 % methanol (MeOH) or BAW. After development, the chromatogram was inspected under ultraviolet light. Then, one of the ninhydrin, phosphomolybdic acid-ammonia, and sodium molybdate tests (Umebachi and

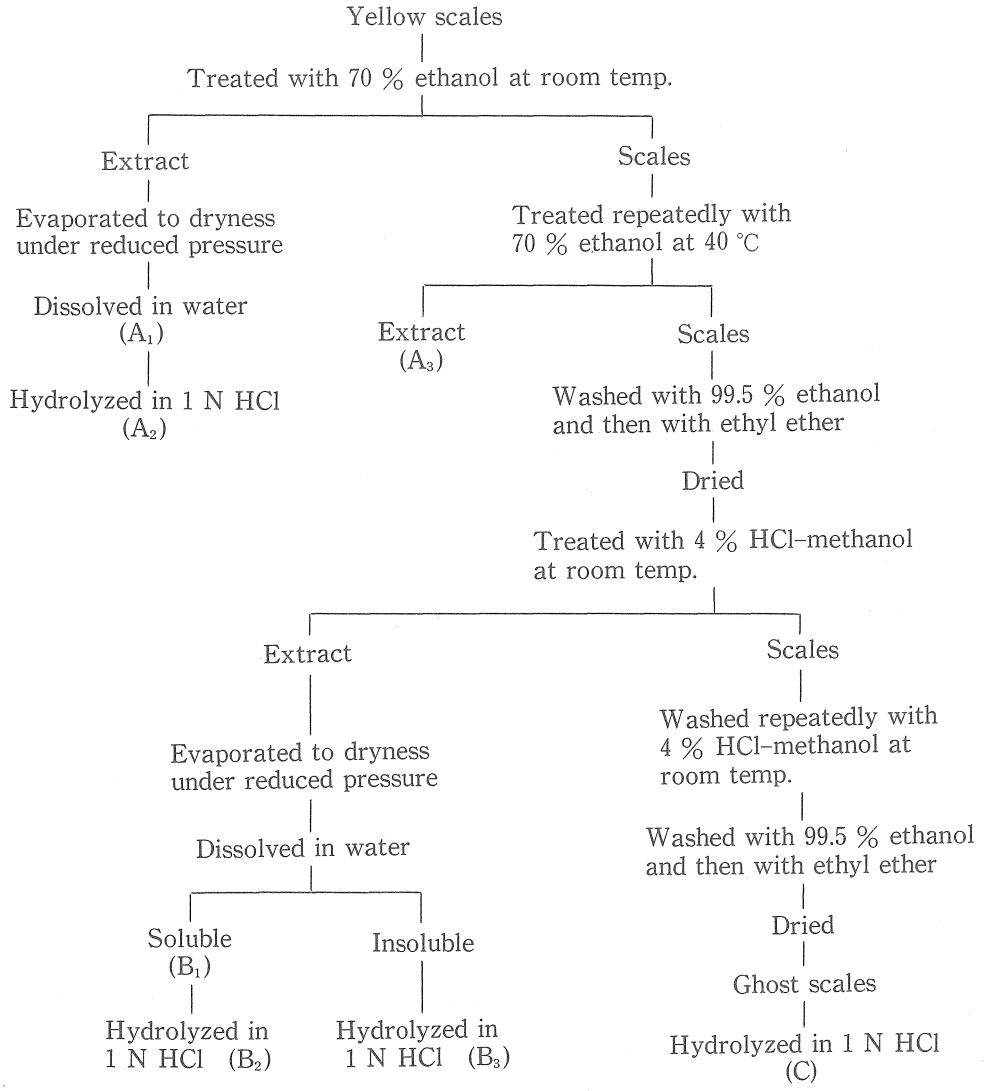

Fig. 1. Extraction of yellow pigments from the yellow scales.

Yoshida, 1970) was performed. The last two tests are respectively for phenolic substances and for *o*-diphenolic substances.

DEAE-Cellulose column

The DEAE-Cellulose powder was washed with 0.2 N HCl and water successively and packed to the 1×5 cm column. This column was used only for the deep yellow pigments of *P. machaon*. A large portion of the pigments were washed down with water.

Purification of SN-1

Some of the hydrolyzates in the fraction A_2 were compared with those of the purified SN-1. The purification of SN-1 was performed in essentially the same way as described in the previous paper (Umebachi and Yamashita, 1977).

Results

Chromatographies of the fractions A_1, A_2, and A_3

The 70 % ethanol extracts (Fraction A_1) of the yellow scales of *P. machaon* and *P. xuthus* were brownish yellow and light yellow, respectively. First, the fraction A_1 was, without any treatment, submitted to two-dimensional thin-layer chromatography. The chromatograms obtained are given in Figs. 2a and c. Fig. 2c corresponds to Fig. 1 of the previous paper (Umebachi and Yoshida, 1970). As the fraction A_1 is a crude extract, it is natural that it contains a few or several substances other than yellow pigments. Spots U and P-1 correspond respectively to the spots U and P of the previous paper.

Figs. 2a and c clearly show that, in both species, free kynurenine and Papiliochrome IIa, IIb, IIIa, and IIIb are present. But, in *P. machaon,* Papiliochrome V was absent or, if any present, in a small quantity. Instead of that, the extract A_1 of this species contained small quantities of brownish yellow pigments which remained near the origin. Generally speaking, a large portion of the light yellow pigments of *P. xuthus* are extracted with 70 % ethanol. On the other hand, in *P. machaon,* the yellow pigments which can be extracted with 70 % ethanol are only a part, and a large portion of the deep yellow pigments which are characteristic of this species remain in the scales.

The extract A_1 was evaporated to dryness under reduced pressure, and the residue was dissolved in 1 N HCl and hydrolyzed at 100°C for 2.5 hr. After the hydrolyzate was evaporated to dryness under reduced pressure, the residue was dissolved in water (Fraction A_2) and submitted to two-dimensional chromatography. The chromatograms are given in Figs. 2b and d, which show that, in both species, the yellow pigments disappeared and that β-alanine and some phenolic substances appeared. Although kynurenine was already present in free form before hydrolysis as seen in Figs. 2a and c, much more kynurenine was found after the hydrolysis. On the other hand, β-alanine and some phenolic substances were found only after hydrolysis. Of the phenolic substances, at least spot P-2 seems to be o-diphenolic, because it was positive to the sodium molybdate test. The spot P-2 corresponded to the DOPAmine derivative, SN-1a, which had been reported as a constituent of Papiliochrome in the previous papers (Umebachi, 1975a ; Umebachi and Yamashita, 1976). Because the SN-1a obtained from the purified SN-1 showed the same chromatographic behavior as the spot P-2. Spots x and y were probably the secondary products which came from SN-1a, because these spots were also found in the chromatogram of the hydrolyzate of purified SN-1.

In Figs. 2b and d, glycine, aspartic acid, glutamic acid, α-alanine, and leucine are found, though they were only in small quantities. This is not surprising, for the fraction

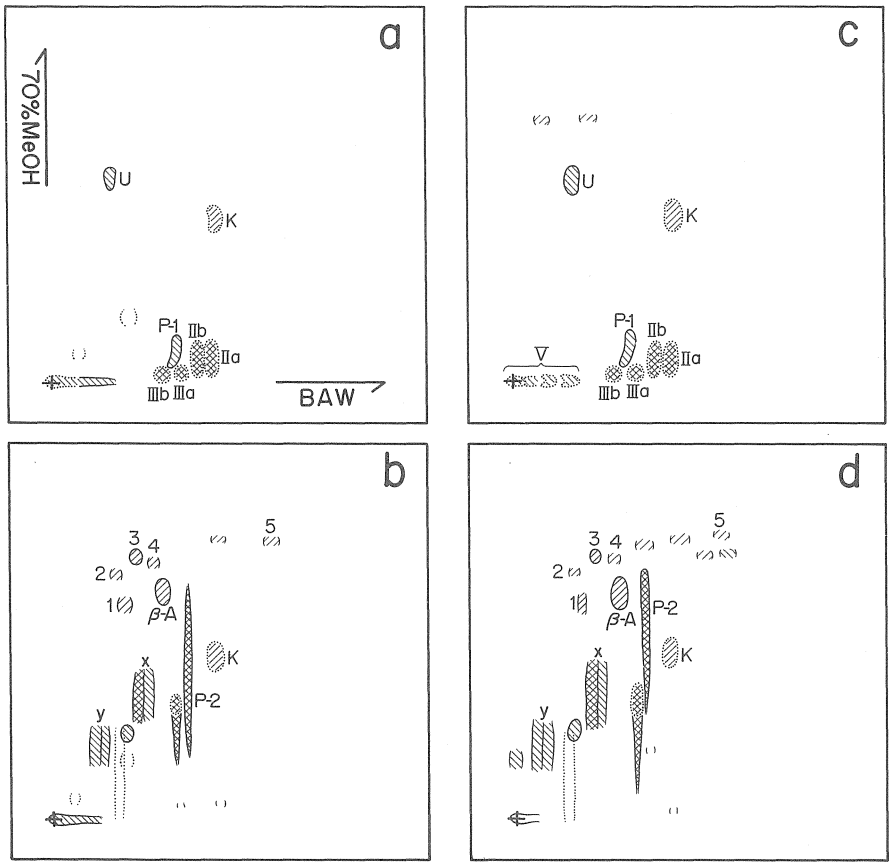

Fig. 2. Thin-layer chromatograms of the 70 % ethanol extract of yellow scales (Fraction A_1) and its hydrolyzate (Fraction A_2).

a and b, Fractions A_1 and A_2 of *P. machaon,* respectively; c and d, Fractions A_1 and A_2 of *P. xuthus,* respectively.

Dotted circle, fluorescent substances; ▨, ninhydrin-positive substances; ▧, substances positive to the phosphomolybdic acid–ammonia test. These symbols are common to all the figures.

Spot K, kynurenine; IIa, IIb, IIIa, IIIb, and V, Papiliochrome; β-A, β-alanine; P-2, x, and y, phenolic substances.

Spots 1-5 are glycine, aspartic acid, glutamic acid, α-alanine, and leucine, respectively.

Spots U and P-1 correspond to the spots U and P of the previous paper (Umebachi and Yoshida, 1970).

A_1 is a crude extract and may contain a small quantity of protein.

After the extract A_1 was obtained at room temperature, the scales were repeatedly treated with 70 % ethanol at 40°C. After the extract was evaporated to dryness under reduced pressure, the residue was dissolved in water (Fraction A_3) and chromatographed. In *P. xuthus,* the extract contained Papiliochrome IIa, IIb, IIIa, IIIb, and V. In *P. machaon,* on the other hand, the extract contained, in addition to Papiliochrome IIa, IIb, IIIa, and IIIb, much more brownish yellow pigments which remained near the origin

than in the extract A_1. These brownish yellow pigments are identical with the M_1 and M_2 described later.

Furthermore, in both species, the extraction with 70% ethanol at 40°C was repeated until the extract became completely colorless. Finally, the scales were washed with 99.5 % ethanol and ethyl ether, and dried.

Chromatographies of the fractions B_1, B_2, and B_3

The above-mentioned dried scales were treated with 4 % HCl-methanol at room temperature. The extract, deep yellow in *P. machaon* and slightly yellow in *P. xuthus,* was evaporated to dryness under reduced pressure. The residue was dissolved in water (Fraction B_1) and two-dimensionally chromatographed on thin-layer sheet. The chromatograms are given in Figs. 3a and c, which show that, in both species, kynurenine

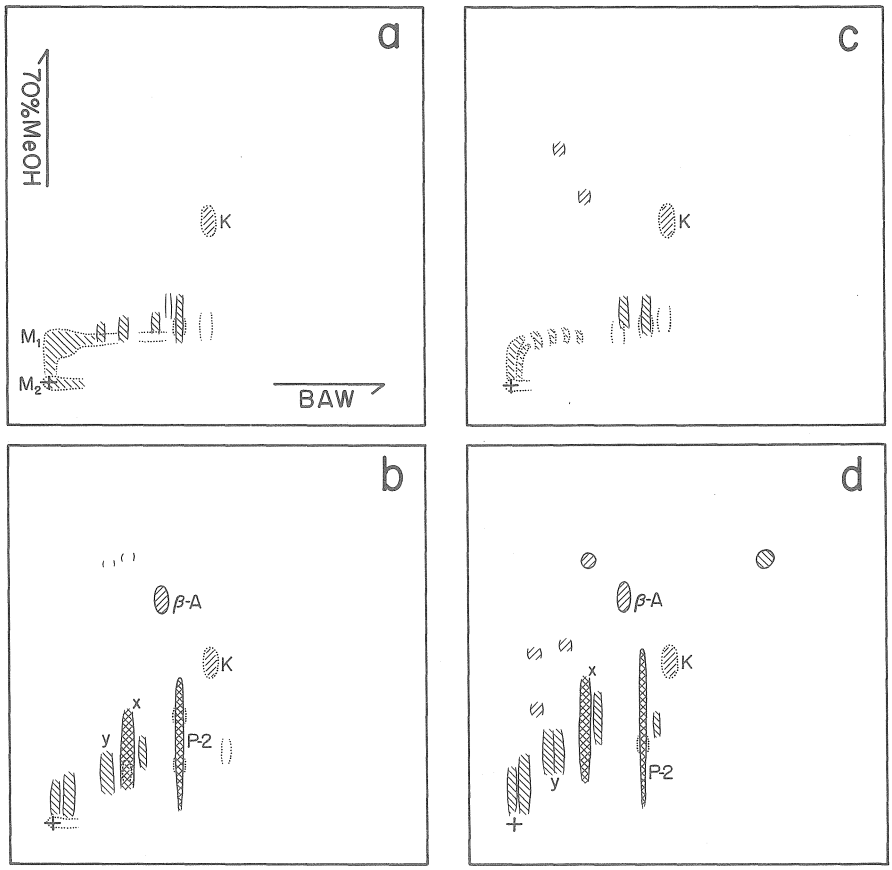

Fig. 3. Thin-layer chromatograms of the 4% HCl-methanol extract of yellow scales (Fraction B_1) and its hydrolyzate (Fraction B_2).

a and b, Fractions B_1 and B_2 of *P. machaon,* respectively; c and d, Fractions B_1 and B_2 of *P. xuthus,* respectively.

Spots K, β-A, P-2, x, and y are the same as in Fig. 2. Spots M_1 and M_2, brownish yellow pigments of *P. machaon.*

is again found and that Papiliochrome IIa, IIb, IIIa, and IIIb are absent or, if any present, only a trace. In *P. xuthus,* some phenolic substances were found near the origin. In *P. machaon,* on the other hand, two kinds of brownish yellow pigments were found near the origin. These two pigments were tentatively named the yellow pigments M_1 and M_2 respectively. Although the thin-layer chromatography used in the present paper was not suited for these two yellow pigments, it was sure that a large quantity of these yellow pigments are present in *P. machaon* and that these are the main yellow pigments which are responsible for the deep yellow of this species. A part of them were already found in the extract A_3. In *P. xuthus,* the yellow pigments M_1 and M_2 were absent or, if any present, only in small quantities.

After the extract B_1 was evaporated to dryness under reduced pressure, the residue was dissolved in 1 N HCl and hydrolyzed at 100°C for 2.5 hr. The hydrolyzate was again evaporated to dryness under reduced pressure, and the residue was dissolved in water (Fraction B_2) and two-dimensionally chromatographed. The chromatograms are given in Figs. 3b and d, which show that the yellow pigments M_1 and M_2 in Fig. 3a of *P. machaon* and the phenolic substances near the origin of Fig. 3c of *P. xuthus* disappeared and that, in both species, β-alanine and some phenolic substances appeared. In addition, kynurenine was present in both species. A part of the kynurenine was already present before hydrolysis as seen in Figs. 3a and c. The phenolic substance P-2 seems to be *o*-diphenolic, because it was positive to the sodium molybdate test.

In *P. machaon,* when the above-mentioned 4 % HCl-methanol extract (Fraction B_1) was evaporated to dryness and then dissolved in water, a part (dark brown) remained insoluble as a residue. On the other hand, in *P. xuthus,* there was no such residue. The residue of *P. machaon* was repeatedly washed with water and finally dissolved in 1 N HCl. The brown pigment of this fraction was referred to as M_3 and was hydrolyzed at 100°C for 2.5 hr. The hydrolyzate was again evaporated to dryness under reduced pressure, dissolved in water (Fraction B_3), and two-dimensionally chromatographed. The chromatogram showed the presence of kynurenine, β-alanine, and some phenolic substances including the spot P-2.

Hydrolysis of the yellow pigments M_1 and M_2

The fraction B_1 of *P. machaon* was applied on the DEAE-Cellulose column and washed with water. Although a small quantity of yellow substance was absorbed on the top of the column, a large portion of the yellow pigments were washed down with water. The yellow fractions including the yellow pigments M_1 and M_2 were collected and evaporated to dryness under reduced pressure. The residue was dissolved in water and applied as a streak on a cellulose thin-layer sheet. After being developed with 70 % methanol, the chromatogram was inspected under ultraviolet light. The pigments M_1 and M_2 were recognized by their brownish yellow color and yellow fluorescence. The areas of the M_1 and M_2 were separately scraped and extracted with the mixture of formic acid, methanol, and conc. HCl (80 : 15 : 0.5) (FMN). After the extracts of M_1

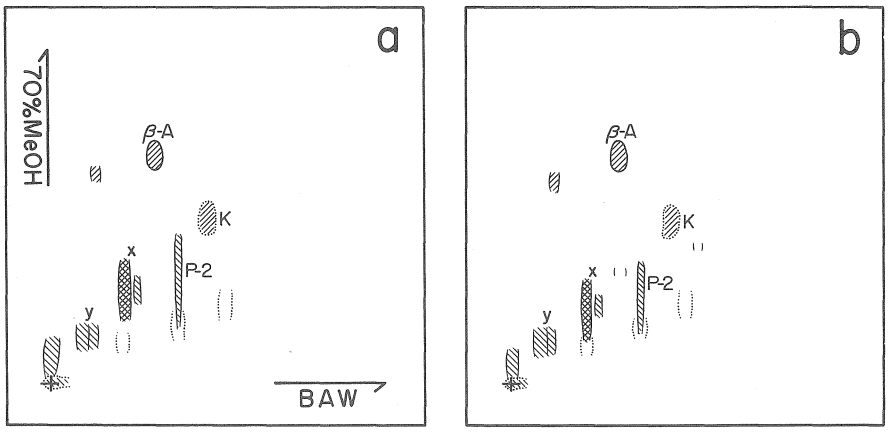

Fig. 4. Thin-layer chromatograms of the hydrolyzates of the brownish yellow pigments M_1 and M_2 of *P. machaon*.
a, M_1 ; b, M_2. Spots K, β-A, P-2, x, and y are the same as in Fig. 2.

and M_2 were respectively evaporated to dryness under reduced pressure, the residues were dissolved in water and, respectively, applied again as a streak on the cellulose thin-layer sheet. After being developed with BAW, the area of the M_1 or M_2 was scraped and extracted with the above FMN. The extract was evaporated to dryness under reduced pressure, and the residue was dissolved in 1 N HCl and hydrolyzed at 100 °C for 2.5 hr. After the hydrolyzate was evaporated to dryness under reduced pressure, the residue was dissolved in water and two-dimensionally chromatographed. The chromatograms obtained for M_1 and M_2 are respectively given in Figs. 4a and b, which show the presence of kynurenine, β-alanine, and the phenolic substance P-2.

Hydrolysis of ghost scales

After the extract B_1 was obtained, the remaining scales were further treated repeatedly with 4 % HCl-methanol at room temperature until the supernatant became colorless. After that, the scales was washed with 99.5 % ethanol and ethyl ether, and dried.

The ghost scales thus obtained were pale brown in *P. machaon* and almost white (but not pure white) in *P. xuthus*. These ghost scales were hydrolyzed in 1 N HCl at 100 °C for 5 hr. After the hydrolyzate was evaporated to dryness under reduced pressure, the residue was dissolved in water (Fraction C) and two-dimensionally chromatographed. The chromatograms are shown in Figs. 5a and b. In both species, at least fourteen ninhydrin-positive substances were found. Among them, glycine, aspartic acid, glutamic acid, α-alanine, tyrosine, and leucine gave a distinct spot, pespectively. In addition, interestingly, a clear blue spot of β-alanine was found. All other spots including phenylalanine, valine, and threonine were faint. Probably serine was also present, though it partly overlapped glycine. In *P. machaon,* kynurenine was faintly

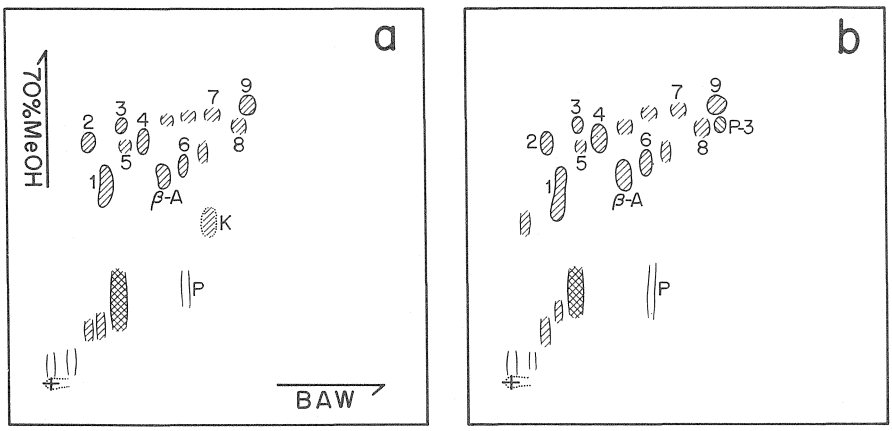

Fig. 5. Thin-layer chromatograms of the hydrolyzate of ghost scales.
a, *P. machaon*; b, *P. xuthus*.
Spots 1–9 are respectively glycine, aspartic acid, glutamic acid, α-alanine, threonine, tyrosine, valine, phenylalanine, and leucine.
Spots K and β-A are the same as in Fig. 2. Spot P–3 is an *o*-diphenolic substance.

found, but absent in *P. xuthus*.

As seen in Figs. 5a and b, the hydrolyzates contain a few substances positive to the phosphomolybdic acid - NH_3 test. Among them, the spot P–3 seems to be *o*-diphenolic, because it was positive to the sodium molybdate test. This spot was especially distinct in *P. xuthus*. Spot P might correspond to the spot P–2 of Figs. 2, 3, and 4, though it could not be confirmed because of its slight amount.

Discussion

In the previous paper (Umebachi and Yoshida, 1970), Papiliochrome IIa, IIb, IIIa, IIIb, and V were reported as the yellow pigments of *P. xuthus*. Among these pigments, Papiliochrome II, the main yellow pigment of this species, has been investigated in detail (Umebachi and Yoshida, 1970; Umebachi, 1975a; Umebachi and Yamashita, 1976, 1977). It has been proved that the yellow pigment readily decomposes to kynurenine and the DOPAmine derivative, SN-1, by being heated and that the SN-1 is cleaved to yield β-alanine and the DOPAmine derivative, SN-1a, by a mild hydrolysis (1 N HCl, 100 °C 2 hr). From these and some other results including dinitrophenylation and i. r. spectrum, it has been presumed that the SN-1 is a N-(β-alanyl)DOPAmine derivative. The possibility was suggested that the yellow pigment might be a kind of molecular complex of kynurenine and the N-(β-alanyl)DOPAmine derivative (Umebachi, 1975a; Umebachi and Yamashita, 1976, 1977). And the IIa and IIb are probably optically isomeric with each other.

From the previous and present papers, it is sure that a large portion of the yellow pigments of *P. xuthus* can be extracted with water or 70 % ethanol. Although the

extract B_1 with 4 % HCl-methanol also contains kynurenine and releases β-alanine and phenolic substances on hydrolysis, the amount of the substances in this fraction is, in this species, rather small. And the ghost scales are almost white.

On the other hand, although the 70 % ethanol extract (Fractions A_1 and A_3) of *P. machaon* also contains Papiliochrome IIa, IIb, IIIa, and IIIb, the characteristic of this species is that the scales after the repeated extraction with 70 % ethanol are still deep yellow. And the main yellow pigments of this species are the brownish yellow or brown pigments (M_1, M_2, and M_3) which are extracted with 4 % HCl-methanol. Although the M_1 and M_2 after extraction are soluble in water, most of them are extracted from scales not with water or 70 % ethanol but with 4 % HCl-methanol. These brownish yellow pigments also release kynurenine, β-alanine, and the *o*-diphenolic substance, P-2, on hydrolysis. Interestingly enough, the spots P-2 of Figs. 2b, 2d, 3b, 3d, 4a, and 4b are probably identical with each other. The conclusion of the present paper is that the brownish yellow pigments of *P. machaon* may also be formed from kynurenine, β-alanine, and an *o*-diphenolic substance, in other words, are based on the same principle as in the case of Papiliochrome II. Therefore it is probable that the brownish yellow pigments of *P. machaon* belong to the group of Papiliochrome.

Here I would like to define Papiliochrome as the pigments which are formed from both kynurenine and a DOPAmine derivative. The investigations made until now indicate that the latter is a N-(β-alanyl)DOPAmine derivative. There is the possibility that Papiliochrome II and III and the brownish yellow or brown pigments M_1, M_2, and M_3 all contain the N-(β-alanyl)DOPAmine derivative as their constituent, because, as mentioned above, the spots P-2 of Figs. 2, 3, and 4 of the present paper seem to be identical with each other.

In both *P. xuthus* and *P. machaon,* the ghost scales of yellow scales release β-alanine on hydrolysis. This is interesting as it has been reported that β-alanine is present in the hardened cuticle of some insects (Bodnaryk, 1971a ; Bodnaryk and Levenbook, 1969 ; Karlson et al., 1969 ; Gilby and McKellar, 1970 ; Hackman and Goldberg, 1971 ; Srivastava, 1971) and that the cuticle of black mutants of some insects lacks β-alanine (Seki, 1962 ; Fukushi and Seki, 1965 ; Fukushi, 1967 ; Jacobs and Brubaker, 1963 ; Jacobs, 1966, 1968). Furthermore it is interesting that the two-dimensional chromatographic patterns of the hydrolyzate of ghost scales are much the same in the two exmined species (Figs. 5a and b).

Finally, it is important that whenever β-alanine is found in the hydrolyzates (Fractions A_2, B_2, B_3, and C ; the hydrolyzates of M_1 and M_2), it is released by a mild hydrolysis, that is, 1 N HCl, 100°C, 2.5-5 hr. The fact might be taken as indicating that, in all the fractions, β-alanine is present in the same way of bonding. In the above-mentioned SN-1, the β-alanine has been presumed to be joined, through a peptide bond, to the amino group of the side chain of DOPAmine derivative (Umebachi and Yamashita, 1977). It has been reported that the β-alanine present as a N-terminal of

peptide is split by a very mild hydrolysis (Bodnaryk, 1971b).

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