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Yellow Pigments in the Wings of the Papilionid Butterflies. II. The Presence of a Pale Blue Fluorescent Substance Supposed to be Kynurenine in the Wings of the Zerynthiinae

By

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Ever since the pioneer work of Hopkins, it has been well known that the white and yellow pigments of the wings of the Pierid butterflies are pterins (Wigglesworth, 1950). The accumulation of pterin pigments in the wing scales is characteristic of that family (Ford, 1947). It is very interesting, however, that the yellow pigments of the closely related family Papilionidae are not pterins (Ford, 1947). Regarding the pigments of the wings of the Papilionidae, Ford (1941–1944) has examined the distribution and nature of the anthoxanthin and red pigments with reference to their bearing on systematics. According to his results, most of the yellow pigments of the Papilionidae, except *Parnassius* and most of *Graphium*, are not even anthoxanthin, and thus their nature remained unknown till recently. In relation to this problem, however, Umebachi and Nakamura (1954) found that a pale blue fluorescent substance which seemed to be kynurenine, a metabolite of tryptophan, was present in the wings of *Papilio xuthus*, *Papilio machaon*, *Papilio helenus*, *Papilio protenor* (male), and *Lühdorfia japonica*, all of which have yellow scales. Using these species of *Papilio*, Umebachi and Takahashi (1956) confirmed that kynurenine accumulated in the yellow scales of the wings. Moreover in the preceding paper of this series, Umebachi (1958) suggested that the yellow pigments of the wings of *P. xuthus* might contain kynurenine as a component. Since then, the author has investigated the nature of the yellow pigments and has also tried to determine the presence or absence of kynurenine in the wings of other Papilionid butterflies.

The present paper deals with the results obtained with the Zerynthiinae, one of the four subfamilies included in the Papilionidae. As a result of examining six among the eleven species of the four genera included in the Zerynthiinae, the presence of the pale blue fluorescent substance supposed to be kynurenine in the wings of *L. japonica* has been reconfirmed, and furthermore it has been found that the same substance is present also in the wings of all the other five species. All these species have pale yellow or yellow scales in the wings. It has been suggested that in the Zerynthiinae also, the yellow pigments have some relation with kynurenine (or some of its

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derivatives).

Materials and Methods

Materials. In the Zerynthiinae are included four genera, *Zerynthia* (with 3 known species), *Lühdorfia* (3 known species), *Bhutanitis* (4 known species), and *Sericinus* (1 known species). Among these species the following six were examined : *Zerynthia polyxena*, *Zerynthia rumina*, *Lühdorfia japonica*, *Lühdorfia puziloi*, *Bhutanitis lidderdalei*, and *Sericinus telamon*. All these butterflies have pale yellow or yellow pigments in the wings.

L. japonica was collected in Kanazawa. Both male and female butterflies were used. The butterflies of the other species were of commercial origin, and only the males were examined.

Extraction. The wings of the six species examined were treated by the following extracting method. The wings of four butterflies were cut out, placed in a beaker containing a little water, and heated on a boiling water bath. Kynurenine was very easily extracted by this procedure. The extract so obtained was centrifuged in order to remove the scales, and the supernatant fluid was placed in an evaporating dish and concentrated on a boiling water bath. The concentrate thus obtained will be denoted as "hot water extract" in this paper.

In the case of *L. japonica*, a larger quantity of the hot water extract using fifty butterflies of both sexes was also prepared for ultraviolet spectrophotometry. In addition, in order to determine in which part of the wings kynurenine was present, the yellow and the black scales were gathered separately and treated by the same extracting method.

Paper chromatography. The hot water extracts of the whole wings or the scales were subjected to one- or two-dimensional chromatography using Toyo filter paper No. 52. In one-dimensional chromatography, either 80 per cent methanol or the organic layer of *n*-butanol-acetic acid-water mixture, 4 : 1 : 5 (B. A.W.) was used as the solvent. In two-dimensional chromatography, 80 per cent methanol was used for the first dimension, and the B. A. W. solvent for the second dimension. After being developed and dried, the fluorescent substances on the filter paper were inspected under ultraviolet rays using a Mazda UV-Dl filter. The following color tests were then made on the filter paper:

(1) Ninhydrin reaction
(2) Reaction for aromatic amines by Tsuda's reagent
(3) Ehrlich's aldehyde reaction
(4) Ammoniacal silver nitrate reaction
(5) Ehrlich's diazo reaction

These color tests were made in the same manner as in the previous paper (Umebachi and Tsuchitani, 1955).

In *L. japonica*, the Rf values, fluorescence, and color tests of the pale blue fluorescent substance were compared with those of synthetic kynurenine.

Ultraviolet absorption. The ultraviolet absorption curve was taken only in *L. japonica*. The hot water extract of the wings of fifty butterflies was streaked a number of times along the starting lines 4.5 cm. from the margins of four sheets of filter paper 30×30 cm.. After the sheets were developed with 80 per cent methanol, dried, and inspected under ultraviolet rays, the areas of the sheets corresponding to the pale blue fluorescent substance were cut out, eluted with hot water, and the resuling eluate concentrated. This eluate was again streaked along the starting lines 4.5 cm. from the margins of four sheets of filter paper 30×30 cm.. After they were developed with the B. A. W. solvent, the areas of the sheets corresponding to the pale blue fluorescent substance were again cut out and eluted with 1/45 M phosphate buffer (pH 7.45), and its ultraviolet absorption was measured between 230 and 390 mμ with the Hitachi Photoelectric Spectrophotometer EPU-2. At the same time, an eluate of synthetic *dl*-kynurenine was prepared in the same manner, and the wave lengths of the absorption maxima were compared with those of the pale blue fluorescent substance from *L. japonica*.

Results

Paper chromatographic tests of the hot water extract of L. japonica. In the one-dimensional chromatogram of the hot water extract of the wings, a spot with pale blue fluorescence was found under ultraviolet illumination. The fluorescence and Rf values (0.35 in 80 per cent methanol; 0.43 in the B. A. W. solvent) of that spot agreed with those of *l*-kynurenine.**

The results of the color tests made for the pale blue fluorescent substance on the filter paper are shown in Table I. These also showed good agreement with the results

Table I. Color tests of the pale blue fluorescent substance of *L. japonica*

Reaction	
Ninhydrin	+ (red-purple)
Tsuda	+ (purple)
Ehrlich's aldehyde	+ (orange)
Ammoniacal silver nitrate	—
Ehrlich's diazo	—

** These Rf values are subject to variations with the developing conditions of chromatograms (for example, development chamber, filter paper etc.). Therefore, the identity of the Rf value of the pale blue fluorescent substance of butterflies with that of synthetic kynurenine was always confirmed by chromatographing both substances simultaneously in the same paper or by applying both substances in one spot and chromatographing. The Rf value of kynurenine in 80 per cent methanol has been reported to be 0.49 in the previous paper (Umebachi, 1958) and to be 0.35 in the present paper. This difference is due to the use of a different devepolment chamber. The Rf value of kynurenine is larger in the B. A. W. solvent than in 80 per cent methanol, irrespective of the developing condition.

obtained with synthetic kynurenine.

On the other hand, neither 3-hydroxykynurenine nor xanthurenic acid was detected in the chromatogram.

Ultraviolet absorption curve of the pale blue fluorescent substance of L. japonica. The ultraviolet absorption curve of the eluate of the pale blue fluorescent substance of the wings is shown in Fig. 1. The wave lengths of the absorption maxima were 275

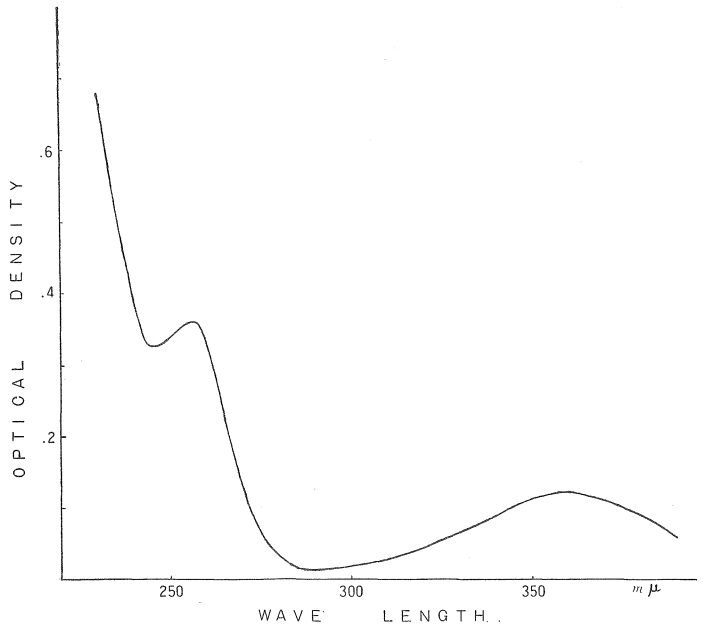

Fig. 1. Ultraviolet absorption curve of the eluate of the pale blue fluorescent substance from *L. japonica*.

and 360 mμ, which agreed with the data of the eluate of synthetic kynurenine.

The location of the pale blue fluorescent substance in the wings of L. japonica. The hot water extracts of the yellow and the black scales were separately examined by paper chromatography, and it was found that the pale blue fluorescent substance was present only in the yellow scales, but not in the black scales.

The presence of the pale blue fluorescent substance in the wings of the Zerynthiinae other than L. japonica. The hot water extracts of the wings of *Z. polyxena*, *Z. rumina*, *L. puziloi*, *B. lidderdalei*, and *S. telamon* were examined by one-dimensional chromatography. A substance with the same fluorescence and Rf value as the pale blue fluorescent substance of *L. japonica* was found in all these species. Like the substnace from *L. japonica*, the pale blue fluorescent substances from these five species also were found to be positive to the ninhydrin reaction (red-purple) and the Ehrlich's aldehyde reaction (orange).

Discussion

From the Rf values, fluorescence, color tests, and the wave lengths of absorption maxima, the pale blue fluorescent substance present in the wings of *L. japonica* seems to be kynurenine, although further experiments are necessary to confirm the identity. At least, it may be concluded that the substance is either kynurenine or one of its closely related derivatives (but not 3-hydroxykynurenine).

Up to the present, the presence or absence of kynurenine in the wings of many butterflies belonging to the Papilionidae has been examined by the author, and it has been found that some genera, but not all, have the pale blue fluorescent substance supposed to be kynurenine in the wings (unpublished data). There are in the Papilionidae four subfamilies, Zerynthiinae, Papilioninae, Parnassiinae, and Baroniinae. Among the genera of these subfamilies, *Papilio* accumulates kynurenine in the yellow scales as already reported. In the present paper, it has further been shown that the butterflies of four genera of the Zerynthiinae also have a substance supposed to be kynurenine in the wings. Now the facts, that, the wings of all these butterflies examined have the pale yellow or yellow pigments, and, that, in *L. japonica* the substance supposed to be kynurenine is present only in the yellow scales, may be interpreted to indicate that there exists some relation between the yellow pigments of the Zerynthiinae and kynurenine, like the yellow pigments of *P. xuthus*. It is very interesting that the yellow pigments of these genera of the Papilionidae, which is the closely related family of the Pieridae, are not pterins but seem to be kynurenine derivatives.

Ford (1941–1944) examined the presence or absence of anthoxanthins and the nature of red pigments in the wings of all genera belonging to the Papilionidae. According to his results, the wings of *Papilio*, *Zerynthia*, *Lühdorfia*, *Bhutanitis*, and *Sericinus* do not contain anthoxanthins. Therefore, *Papilio* and the Zerynthiinae are similar to each other regarding the presence or absence of kynurenine and anthoxanthins in the wings. So far as the author has examined up to the present, the species which have been reported to have anthoxanthins in the wings by Ford do not seem to accumulate kynurenine in the wings (unpublished data). On the other hand, as for red pigments reported by Ford, those of *Papilio* are of type B, while those of *Zerynthia*, *Lühdorfia*, *Bhutanitis*, and *Sericinus* are of type A. To summarize, with regard to the pigments of the wings, the characteristics of the Zerynthiinae are the following: (1) the pale blue fluorescent substance supposed to be kynurenine is present; (2) anthoxanthin is absent; (3) the red pigments are of type A. On the other hand, the characteristics of *Papilio* are the following: (1) kynurenine is present; (2) anthoxanthin is absent; (3) the red pigments are of type B. But even in *Papilio*, the species which have no yellow scales in the wings do not accumulate kynurenine in the wings. At any rate, it is very interesting that a highly evolved genus *Papilio* and the Zerynthiinae which has a number of primitive features both accumulate kynurenine in the wings.

The results of the present paper seem to indicate also that in the Papilionidae there

exists no correlation between the accumulation of kynurenine in the wings and the food plants (family) of the larvae. *P. xuthus*, *P. machaon*, *P. helenus*, *P. protenor* (male), and the Zerynthiinae all have the substance supposed to be kynurenine in the wings as already described, but their larvae feed on different plants. The larvae of *P. xuthus*, *P. helenus*, and *P. protenor* feed on the Rutaceae, those of *P. machaon* on the Umbelliferae and Rutaceae, and those of *Zerynthia*, *Lühdorfia*, and *Sericinus* on the Aristolochiaceae. Furthermore, *Chilasa agestor* and *Chilasa epycides*, which also have the pale blue fluorescent substance supposed to be kynurenine in the wings (unpublished data), are said to feed on the Lauraceae in their larval stage (Shirozu, 1955).

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Summary

(1) The pale blue fluorescent substance present in the wings of *L. japonica* reported in the previous paper by the author was shown to be identical with synthetic kynurenine with regard to its paper chromatographic behaviour, color tests, and wave lengths of absorption maxima of ultraviolet rays.

This substance was furthermore proved to accumulate in the yellow scales and to be absent in the black scales.

(2) Besides *L. japonica*, five species belonging to the Zerynthiinae were examined and were found to have the substance supposed to be kynurenine in their wings.

(3) It was suggested that there may exist a relation between kynurenine (or some of its derivatives) and the yellow pigments of the Zerynthiinae like the yellow pigments of *P. xuthus*.

Comparison was made between *Papilio* and the Zerynthiinae with regard to the pigments of the wings.

It was also described that no correlation seems to exist between the accumulation of kynurenine in the wings and the food plants (family) of their larvae.

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