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Yellow Pigments in the Wings of the Papilionid Butterflies I. The Relation between Kynurenine and the Yellow Pigments of Papilio xuthus

By

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Among the pigments of the wings of butterflies, the white and yellow pigments of the Pierid butterflies, such as *Pieris rapae*, *Colias hyale*, *Eurema hecabe*, and *Gonepterix mahaguru* are definitely known to be pterins (Wigglesworth, 1950 ; Umebachi, 1954 ; Yagi and Saitoh, 1955), and the anthoxanthin and the red pigments in the Pieridae, Papilionidae, and other families have also been studied by Ford (1941–1944), with reference to their bearing on systematics. Little work, however, has been done on the others. So the author examined the pigments of many kinds of Japanese butterflies, including the Pieridae, Papilionidae, Satyridae, Nymphalidae, and Hesperiidae, by means of paper chromatography, and found to his surprise that kynurenine, a metabolite of tryptophan, accumulated in the yellow scales of the wings of the Papilionid butterflies (Umebachi and Nakamura, 1954 ; Umebachi and Takahashi, 1956). As kynurenine is, however, a colorless substance, not a yellow pigment, the author tried to separate the yellow pigments of the butterflies of this family by means of paper chromatography, and to study the relation between each of them and kynurenine. For this purpose, *Papilio xuthus*, which can be got in large numbers in Japan, was chosen as the subject. It was found that in the yellow scales of this species there existed three yellow pigments, two of which decomposed easily into kynurenine and other substances. This result, which seemed to suggest that these two pigments contain some tryptophan metabolite (kynurenine or its precursor) as a component, led the author to see whether the C^{14} of tryptophan-C^{14} injected into butterflies is incorporated into the yellow pigments, which was shown by experiments to be actually the case. The present paper deals with these experiments on the relation between kynurenine and the yellow pigments.

Materials and Methods

The materials used were the scales in the upper side of the wings of the males of *Papilio xuthus* except in the case of butterflies injected with tryptophan-C^{14}, in which case the females also were used.

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As the yellow pigments are soluble in distilled water and 80 per cent ethanol, and insoluble in ether, petroleum ether, chloroform, and carbon tetrachloride at room temperature, the following extraction method was adopted. The yellow scales of the wings were gathered, and the yellow pigments were extracted with 80 per cent ethanol after being throughly washed with petroleum ether and ether. The extraction was repeated till the scales became completely colorless. In this procedure, the extraction was speedily finished by using hot ethanol. The extract so obtained will be denoted as "ethanol extract" in this paper. For comparison, the black scales also were treated in the same manner and examined.

The ethanol extract was subjected to one- or two-dimensional paper chromatography, which was carried out by the ascending technique using Toyo No. 52 filter paper. In one-dimensional chromatography, the ethanol extract was streaked a number of times along the starting line 5 cm. from the margin of a sheet of filter paper 30×30 cm. or 30×15 cm., and developed with 80 per cent methanol. In two-dimensional chromatography, the ethanol extract was spotted 5 cm. from the lower corner of a sheet 30×30 cm., and developed with 80 per cent methanol for the first dimension and with an upper layer of *n*-butanol-acetic acid-water mixture, 4 : 1 : 5 (B. A. W.) for the second dimension. After being developed and dried, the chromatogram was first observed in daylight, and the yellow substances were marked with a pencil. Next, the fluorescence on the chromatogram was inspected under ultraviolet rays using a Mazda UV-Dl filter. After that, one of the following color tests was made on the filter paper :

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

These color tests were made in the same way as in a previous paper (Umebachi and Tsuchitani, 1955) except the ferric chloride reaction, in which the filter paper was heated a little after being sprayed with 2 per cent ferric chloride dissolved in 10 per cent ethanol.

In one-dimensional chromatography, an elution experiment was made. After the filter paper had been developed, dried, and inspected under ultraviolet rays, the area of the paper corresponding to the yellow pigment was cut out and eluted with 80 per cent ethanol. The eluate was concentrated using a boiling water bath, and examined again by one-dimensional chromatography using 80 per cent methanol.

In order to ascertain whether tryptophan metabolites are incorporated into the yellow pigments, the ethanol extract obtained from butterflies injected with tryptophan-C^{14} was examined. For this purpose, *dl*-tryptophan-2-C^{14} (200 μc per m

mol) which was obtained from the Daiichi Pure Chemicals Co., Ltd. was used. Thirty prepupae were taken, and 0.02 to 0.03 ml. of a saturated aqueous solution of tryptophan-C^{14} was injected into each of them. After emergence of the butterflies, the wings were cut out and subjected to the following procedure. (1) In the first place, in order to see whether the C^{14} of tryptophan-C^{14} is incorporated only into the yellow scales or also into the black scales, the radioautographs of the wings of three arbitrarily selected butterflies (1 male, 2 females) were taken as follows. The wings were dried and brought into contact with the X-ray film (Fuji). The time of exposure was a week. (2) The ethanol extract of the yellow scales of the remaining butterflies was subjected to one- and two-dimensional chromatography. After being devoloped and dried, the paper was cut into small sections, and the radioactivity of each section was measured with a gas flow counter (Nuclear Chicago U. S. A., Model 47). In the case of the one-dimensional chromatogram, the radioautograph also was taken in contact with the X-ray film (Fuji).

Results

Paper chromatograms of ethanol extracts

The one-dimensional chromatogram of the extract of the yellow scales and the description of each spot are shown in Figure 1 and Table I respectively. This chromatogram clearly indicates that in the yellow scales there exist three yellow pigments which can be distinctly separated from kynurenine. These three yellow pigments will be called yellow pigments I, II, and III, respectively, in the present paper.

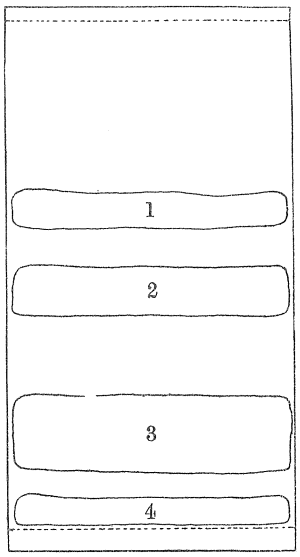


Figure 1. One-dimensional chromatogram of the ethanol extract of the yellow scales. The solvent : 80 per cent methanol.

The two-dimensional chromatogram of the extract of the yellow scales is shown in Figure 2. In this case also, kynurenine and yellow pigments I, II, and III were clearly separated, although yellow pigment III (spot 5 in Fig. 2) seemed to be decomposed in the second dimension. For comparison, a two-dimensional chromatogram of the extract of the black scales is given in Figure 3, which shows that the black scales have no yellow pigment nor

Table I. Rf value, fluorescence, and color test of the spots in Fig. 1.

Spot No.	Substance	Rf	Color	Fluorescence	Ninhydrin
1	Yellow pigment I	0.64	yellow		
2	Kynurenine	0.49	colorless	white-blue	red-purple
3	Yellow pigment II	0.22	yellow	yellow	yellow-brown
4	Yellow pigment III	0.04	yellow	yellow	yellow-brown

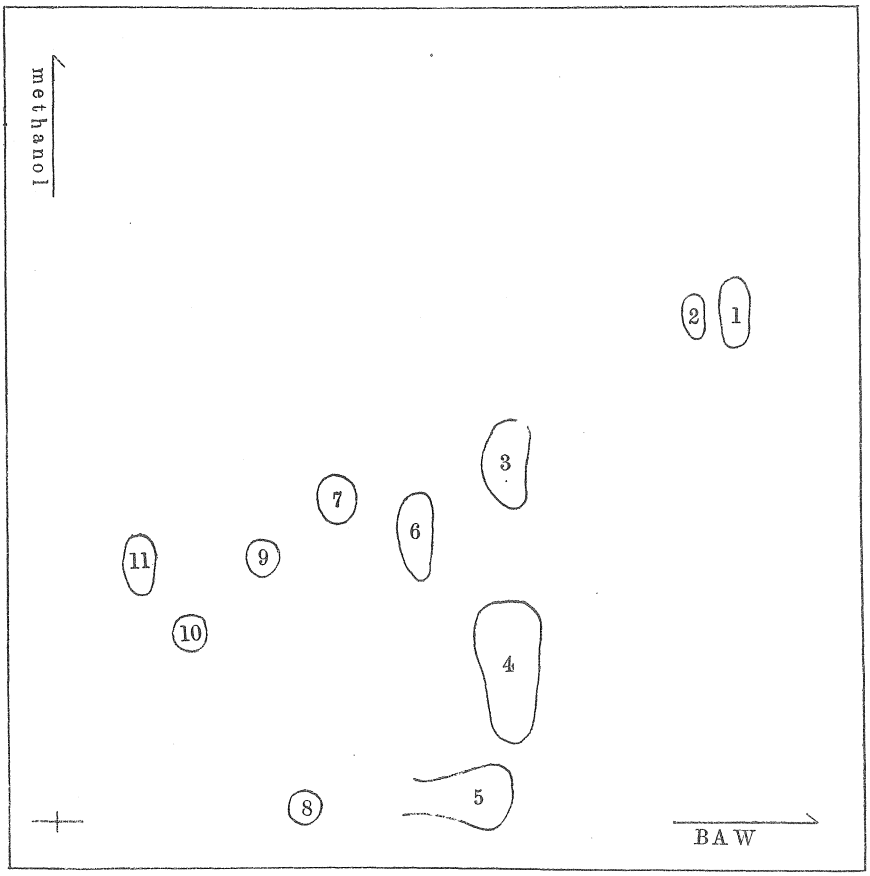


Figure 2. Two-dimensional chromatogram of the ethanol extract of the yellow scales.

Table II. Fluorescence and color tests of the spots in Fig. 2. Y, yellow ; P, purple ; W, white ; B, blue ; R, red ; Or, orange ; Gr, green ; Br, brown.

Spot No.	Substance	Color	Fluorescence	Color tests						
				Ninhydrin	$AgNO_3$	Diazo	Tsuda	Aldehyde	Millon	$FeCl_3$
1	Yellow pigment I	Y	—	—	—	—	—	—	—	—
2		—	—	RP(?)	+	—	—	—	—	+
3	Kynurenine	—	WB	RP	—	—	P	Or	—	—
4	Yellow pigment II	Y	Y	YBr	+	R	—	Or	+	PB
5	Yellow pigment III	Y	W(?)	?	?	—	—	?	—	—
6		Pale P	—	RP(?)	+	R	—	—	?	dark Gr
7	Uric acid	—	—	—	+	—	—	—	—	—
8		—	—	—	+	?	—	—	—	?
9		—	—	—	+	?	—	—	—	—
10		—	—	RP	—	—	—	—	—	—
11		—	—	RP	—	—	—	—	—	—

kynurenine at all. The descriptions of the spots in Figures 2 and 3 are given in Tables II and III respectively. As spot 7 of Figure 2 and spot 2 of Figure 3 both corresponded to uric acid in position and in color tests, they were identified as uric acid. Spot 1 in Figure 3, which was not present in the yellow scales, appeared to be isoxanthopterin, judging from its position and fluorescence. In the chromatogram

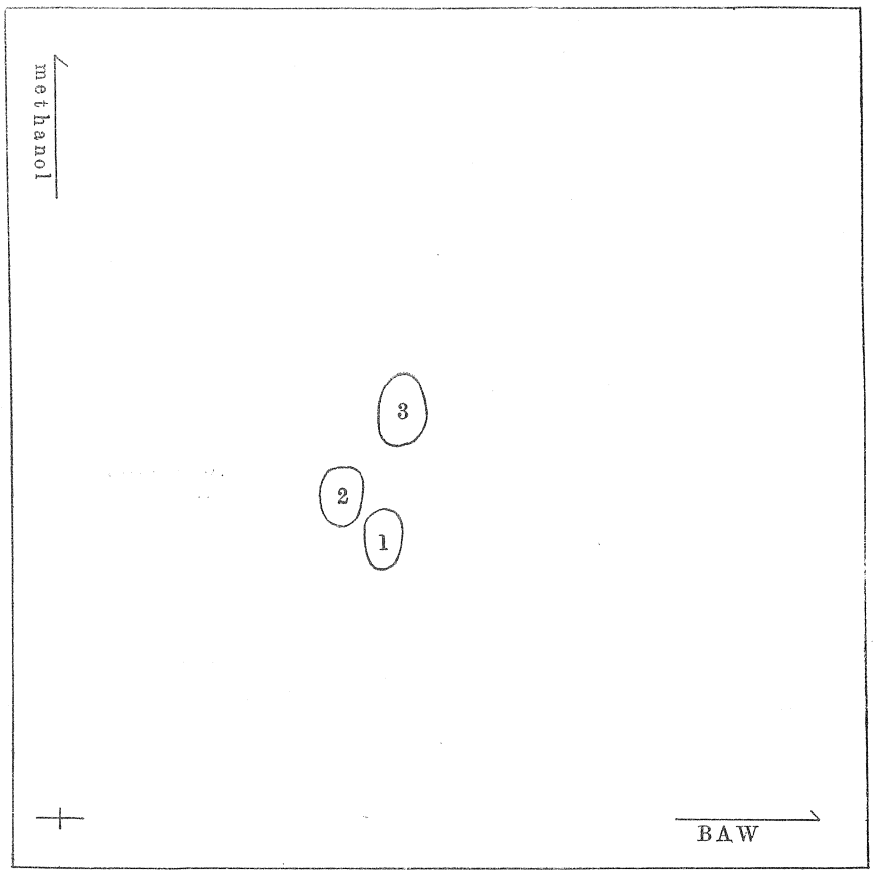


Figure 3. Two-dimensional chromatogram of the ethanol extract of the black scales.

Table III. Fluorescence and color tests of the spots in Fig. 3.

Spot No.	Substance	Color	Fluorescence	Color tests					
				Ninhydrin	$AgNO_3$	Diazo	Tsuda	Aldehyde	Millon
1	Isoxanthopterin(?)	—	Purple	—	—	—	—	—	—
2	Uric acid	—	—	—	+	—	—	—	—
3		—	—	—	+	—	—	—	—

of the yellow scales as well as that of the black scales, free tyrosine, tryptophan, 3-hydroxykynurenine, and xanthurenic acid were not recognized. Spot 6 in Figure 2

was similar to 3-hydroxykynurenine in color tests, but the former was pale violet in daylight and did not emit yellow fluorescence under ultraviolet rays. Besides, as the positions on the chromatogram of these two substances were a little different from each other although they overlapped, it was concluded that spot 6 was not 3-hydroxykynurenine.

Paper chromatograms of the concentrated eluates of the yellow pigments

The concentrated eluates of yellow pigments I, II, and III respectively were prepared and again subjected to one-dimensional chromatography. The chromatograms so obtained and the description of each spot are given in Figure 4 and Table IV respectively.

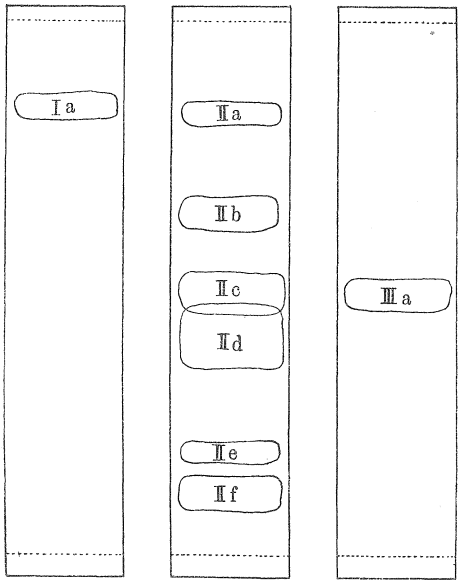


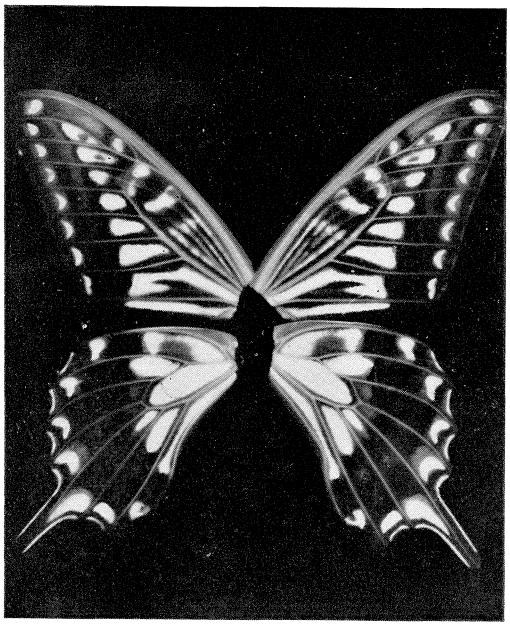
Figure 4. One-dimensional chromatogram of the concentrated eluates of yellow pigments I, II, and III (from left to right). The solvent: 80 per cent methanol.

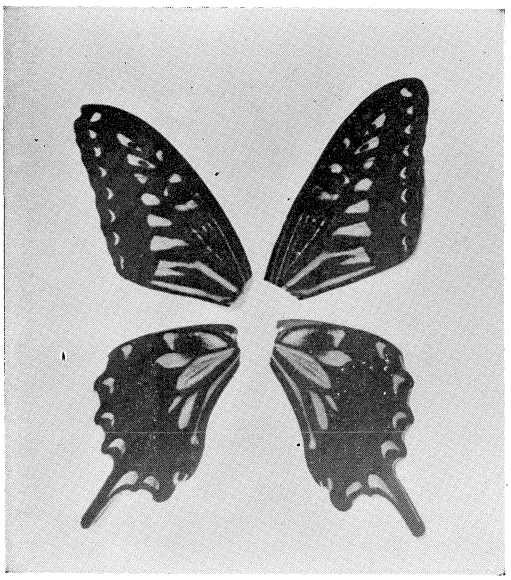
The concentrated eluate of yellow pigment I produced a yellow substance (spot Ia in Fig. 4), the Rf value of which was larger than that of yellow pigment I. In this case kynurenine was not detected.

Table IV. Fluorescence and color tests of the spots in Fig. 4. Y, yellow; W, white; B, blue; P, purple; Or, orange; Ro, rose; R, red.

Spot No.	Substance	Color	Fluorescence	Color tests			
				Ninhydrin	$AgNO_3$	Aldehyde	Tsuda
Ia		Y	?	—	—		?
IIa		Y	?	—	—	RoOr	?
IIb		—	?	—	+	—	P
IIc	Kynurenine	—	WB	RP	—	Or	—
IId		—	—	—	+	—	—
IIe		—	—	—	+	—	—
IIf		Y	Y	±	+	—	?
IIIa	Kynurenine	—	WB	RP	—	Or	P

In the concentrated eluates of yellow pigments II and III, there was found unexpectedly a whitish blue fluorescent substance (spots II c and III a in Fig. 4), which was presumed to be kynurenine from the Rf value, fluorescence, and color tests. This was especially conspicuous in the case of yellow pigment II. In addition, in this case, two yellow substances (spots II a and II f in Fig. 4) and substances showing positive ammoniacal silver nitrate reaction (spots II b, d, and e) were recognized. Of them, a yellow substance of spot II f seemed to be the un-decomposed





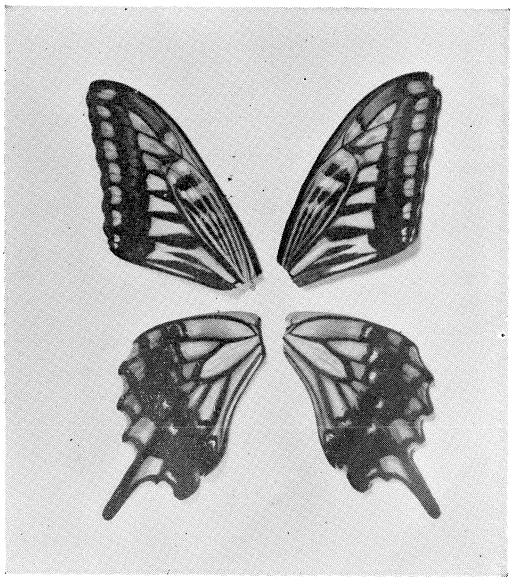


Figure 5. Radioautograph of the wings of the butterfly injected with tryptophan-C^{14}. Above is the radioautograph of the upper side in the male. Below are the ordinary photographs of the upper and under sides (left to right) of the same butterfly as in the upper picture.

residue of yellow pigment II. On the other hand, in the concentrated eluate of yellow pigment III, no clear yellow substance was recognized. Some substances showing positive ammoniacal silver nitrate reaction seemed to be present in some cases.

Examination in the butterflies injected with tryptophan-C^{14}

The radioautograph of the wings of the butterflies injected with tryptophan-C^{14} is shown in Figure 5 with the ordinary photographs which are given for comparison. This clearly indicates that the C^{14} of tryptophan-C^{14} accumulates in the yellow scales but not in the black scales.

The result of the radioactivity measurement of the one-dimensional chromatogram of the yellow scale extract is illustrated in Figure 6, which indicates that the C^{14} of

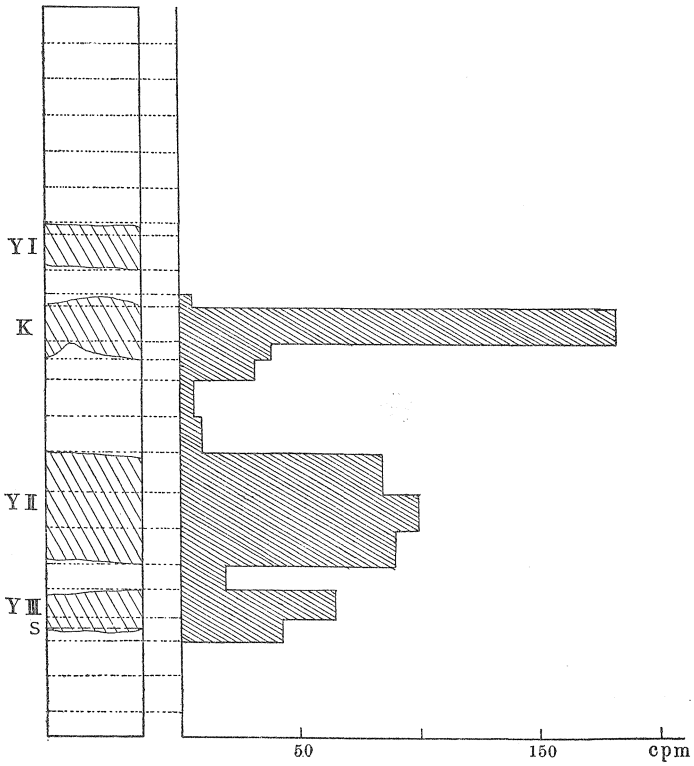


Figure 6. The result of radioactivity measurement of the one-dimensional chromatogram of the yellow scale extract of butterflies injected with tryptophan-C^{14}. K, kynurenine; Y I, II, and III, yellow pigments I, II, and III; S, starting line.

tryptophan-C^{14} is incorporated into both kynurenine and yellow pigments II and III but not into yellow pigment I. The radioautograph of the one-dimensional chromatogram also gave the same result. The result of the radioactivity measurement of the two-dimensional chromatogram is shown in Figure 7. In this case also, the C^{14} of tryptophan-C^{14} can be recognized in kynurenine and yellow pigments II and III, especially in the first two. In Figure 7, section A, which was the position of tryptophan, had radioactivity. Though section B also had radioactivity, the substance in this section was not identified.

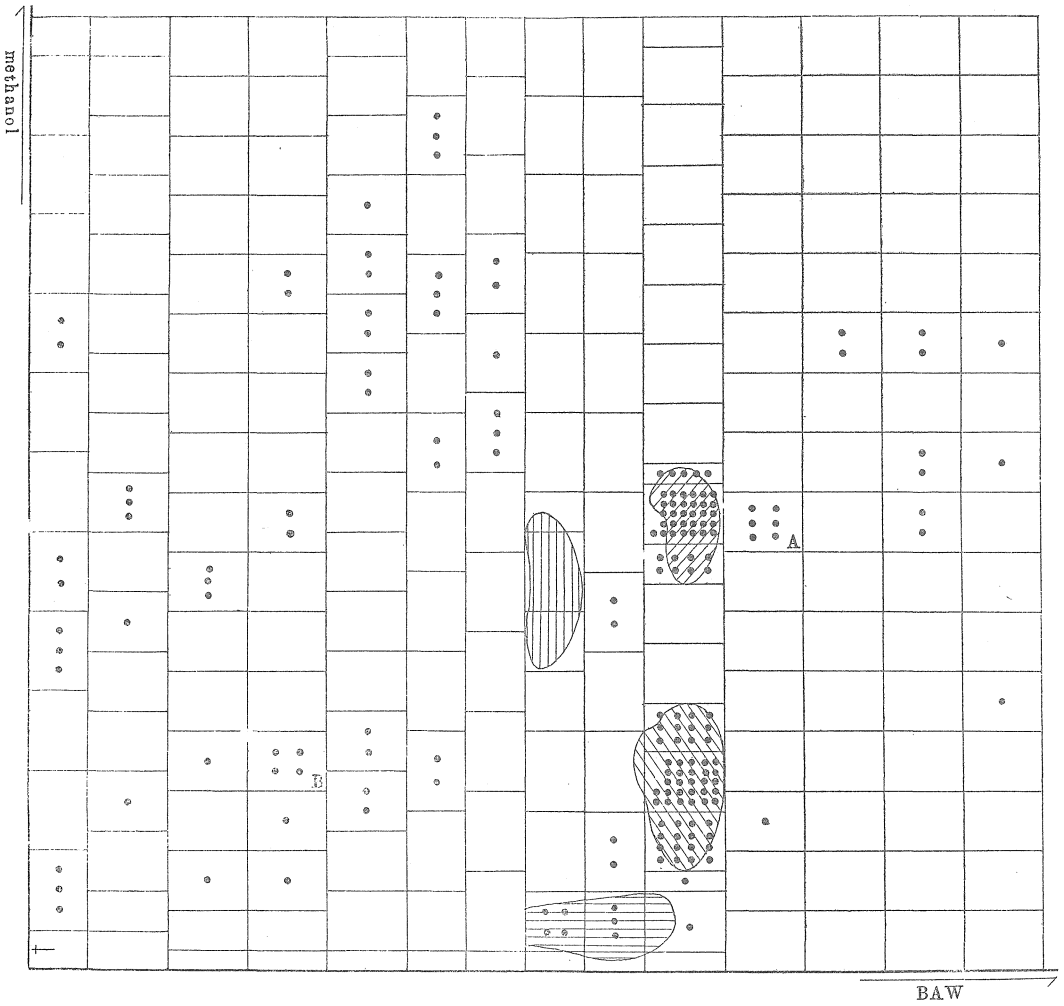


Figure 7. The result of radioactivity measurement of the two-dimensional chromatogram of the yellow scale extract of butterflies injected with tryptophan-C^{14}. One dot represents 2 counts per minute. ▨, kynurenine; ▨, yellow pigment II; ▨, yellow pigment III; ▨, spot 6 in Fig. 2.

Discussion

The preceding report that kynurenine accumulates in the yellow scales of the wings of the Papilionid butterflies (Umebachi and Takahashi, 1956) has been again confirmed here by the chromatogram of the yellow scale extract of the butterflies injected with tryptophan-C^{14}. Moreover, the absence of kynurenine in the black scales has also been verified again by the radioautograph of the wings.

In the present paper, three yellow pigments were separated in the one-dimensional chromatogram, while in the preceding papers (Umebachi and Nakamura, 1954; Umebachi and Takahashi, 1956) no yellow pigment was detected although kynurenine was found. This difference may be ascribed to the fact that in the preceding papers,

the quantity of the sample spotted on the filter paper was smaller than in the present paper. For detection of kynurenine alone on the chromatogram, the extract from one fore-wing and one hind-wing of one butterfly was sufficient as the sample to be spotted, while in order to detect the yellow pigments besides kynurenine, a large quantity of the extract (that is to say, the extract from many butterflies) was necessary. In addition, the solvent B. A. W. which was used for one-dimensional chromatography in the preceding papers seems to be less suitable for separating the yellow pigments than 80 per cent methanol.

Although the structures of the three yellow pigments have not yet been made clear, the relation between them and kynurenine can be, to some extent, guessed from the present experiments. Among the three, yellow pigment I seems not to have any relation to kynurenine, for in the tryptophan-C^{14} injection experiment, the C^{14} was not incorporated into it. But since the tryptophan injected was tryptophan-2-C^{14}, there remains the possibility that the part (benzen ring) other than the alanine side chain of kynurenine may be incorporated into yellow pigment I.

As the concentrated eluate of yellow pigment II produced a large quantity of kynurenine and as the C^{14} of tryptophan-C^{14} injected was incorporated into yellow pigment II in large quantities, this yellow pigment is presumed to have some tryptophan metabolite (probably kynurenine or its precursor) as a component. Here it is of interest that the concentrated eluate of yellow pigment II produced a yellow substance and some substances showing positive ammoniacal silver nitrate reaction besides kynurenine and the un-decomposed yellow pigment II (spot II f in Fig. 4). This fact suggests the possibility that yellow pigment II may be a combination of some tryptophan metabolite (probably kynurenine or its precursor) and some other substance (for example, yellow substance of spot II a and substances of spot II b, d, and e which give positive ammoniacal silver nitrate reaction).

Yellow pigment III also seems to have some tryptophan metabolite (probably kynurenine or its precursor) as a component, for the concentrated eluate of yellow pigment III produced kynurenine, although in a smaller quantity than that of yellow pigment II, and the C^{14} was incorporated into yellow pigment III. But it should be noted that in the concentrated eluate of yellow pigment III, no clear yellow substance is recognized in contrast to the concentrated eluates of yellow pigments I and II. This point has been left for further study.

Yellow pigments II and III seem to be different from any other insect pigment reported up to the present in that they easily produce kynurenine. It is well known through the biochemical genetics of *Ephestia*, *Drosophila*, and *Bombyx* that kynurenine is a precursor of ommochromes (Butenandt, 1952; Kikkawa, 1953). Today ommochromes are understood to be the pigments derived from 3-hydroxykynurenine (Oxydations-und Abwandlungsprodukte des Oxykynurenins) (Butenandt, 1955), and most of these pigments are dark in color and not yellowish. Up to the present, xanthommatin, which is a condensate of two molecules of 3-hydroxykynurenine has been reported

as a yellowish brown ommochrome by Butenandt (1955). Now it is not certain whether yellow pigments II and III of *P. xuthus* belong to the ommochrome group or not, but it should be noted that these two pigments produce kynurenine easily and that the extracts of the yellow scales contain a large quantity of kynurenine but no 3-hydroxykynurenine nor xanthurenic acid. Judging from the two-dimensional chromatogram of the yellow scale extract of the butterflies injected with tryptophan-C^{14}, the amount of 3-hydroxykynurenine present must be only a trace if it is present at all. The interpretation of such a metabolic pattern and the identification of the yellow substances of spot Ia and II a will be a key to make clear the nature of the yellow pigments in the yellow scales of *P. xuthus*. Further experiments are being carried out.

Among the Papilionidae, *Parnassius glacialis*, *Lühdorfia japonica*, *Papilio xuthus*, *Papilio machaon*, *Papilio helenus*, *Papilio protenor*, *Papilio bianor*, and *Graphium sarpedon* were examined up to the present in regard to the presence of kynurenine in the wings, and *L. japonica*, *P. xuthus*, *P. machaon*, *P. helenus*, and *P. protenor* were found to accumulate kynurenine in their yellow scales (Umebachi and Nakamura, 1954). In the present paper, only *P. xuthus* was used as a representative in studying the relation between kynurenine and the yellow pigments. But the yellow pigments of *L. japonica*, *P. helenus*, and *P. protenor* seem to be similar to those of *P. xuthus* judging from their solubility and color. The deep yellow pigments of *P. machaon* are different from these yellow pigments, although its yellow scales contain kynurenine. Most of the yellow pigments of this species are insoluble in water and ethanol. Some of them are insoluble even in 80 per cent formic acid (unpublished data). On the other hand, *P. glacialis* has very little kynurenine in the wings, and *G. sarpedon* has none at all. Accordingly the accumulation of kynurenine seems to be characteristic of the yellow scales of the wings of *Papilio* and *Lühdorfia*. Hereupon, Ford's papers (1941—1944), which say that *Graphium* and *Parnassius* have anthoxanthin, but *Papilio* and *Lühdorfia* have not, are of much interest. Moreover, according to Ford (1944 a, b) most of *Papilio* have type B red pigment, while most of *Graphium*, *Parnassius*, and *Lühdorfia* have type A red pigment. In other families than the Papilionidae, it is already well known that the white and yellow wing scales of the Pierid butterflies except the Dismorphiinae have large quantities of pterins, and this is characteristic of this family. These facts will have an interesting bearing on the metabolism and distribution of the pigments in all the families of butterflies and on the systematics of butterflies.

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Summary

(1) The ethanol extract of the yellow scales of the wings of *P. xuthus* was examined by means of paper chromatography, and was found to contain three yellow pigments (I, II, and III) besides kynurenine.

(2) Yellow pigments II and III were proved to decompose easily and to produce a whitish blue fluorescent substance which was presumed to be kynurenine, but yellow pigment I did not produce kynurenine in the same way.

(3) In the butterflies which emerged from the prepupae injected with tryptophan-C^{14}, the C^{14} was incorporated into yellow pigment II and III, but not into yellow pigment I.

(4) From these results, it was suggested that yellow pigments II and III have some tryptophan metabolite (probably kynurenine or its precursor) as a component.

(5) The relation between these yellow pigments and ommochromes, and also the bearing of these yellow pigments on the systematics of butterflies were discussed.

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