The Science Reports of the Kanazawa University, Vol. VII, No. 2, pp. 139—150, August, 1961.

Yellow Pigments in the Wings of the Papilionid Butterflies. V. Some Chemical Properties of the Yellow Pigments of *Papilio xuthus*

By

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(Received September 30, 1960)

In previous papers (Umebachi and Takahashi, 1956 ; Umebachi, 1958), it was reported that (1) kynurenine is accumulated in the yellow scales of wings of *Papilio xuthus*, (2) in the yellow pigments of this species, three components (Y-I, Y-II, Y-III) are present, (3) Y-II and-III undergo a change easily and produce kynurenine, and (4) the C^{14} of tryptophan-C^{14} injected into the pupa is incorporated into Y-II and-III. Since then, some chemical properties of these yellow pigments have been examined in further detail. It has been proved that (1) the natural yellow pigments of *P. xuthus* are of only two kinds : Y-II and-III, (2) Y-I is not the natural pigment of this species but is either a degradation-product or a changed substance produced from Y-II and-III, (3) both Y-II and-III are phenolic compounds, (4) aqueous solutions of Y-II and-III produce kynurenine and some phenolic compound easily by being heated, and (5) Y-II and-III produce kynurenine by being left exposed to the air also. Moreover, it has been confirmed that Y-II and-III do not produce xanthurenic acid by alkali hydrolysis, and do not produce 3-hydroxykynurenine but a large quantity of kynurenine by acid hydrolysis. From these results, it has been supposed that Y-II and-III are pigments derived from tryptophan but do not belong to the ommochrome group reported up to now. The present paper deals with these chemical properties of the yellow pigments of *P. xuthus*.

Material and Methods

Material. —— The yellow scales of the wings of *Papilio xuthus* from Japan and Formosa were used as materials. Only the male was used, for the male can be obtained more easily than the female.

Extraction of the yellow pigments. —— The yellow pigments of *P. xuthus* are insoluble in petroleum ether, ether, benzene, acetone, ethyl acetate, and chloroform, and soluble in water and 80 per cent ethanol. As these yellow pigments are labile to heat and exposure to the air, the extraction had to be made speedily with as little exposure to heat

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or air as possible. The yellow scales were gathered in a centrifuge tube with petroleum ether. After removing petroleum ether by centrifugation, the scales were washed with ether. Ether was removed and evaporated completely, and then the yellow pigments were extracted with 80 per cent ethanol. When the scales were not fresh, the yellow pigments could not be thoroughly extracted with 80 per cent ethanol at room temperature, and so the scales were warmed on a water bath for as short a duration as possible to prevent decomposition of the pigment. The ethanol extract so obtained was evaporated to dryness under reduced pressure. The residue will be denoted as the crude sample of yellow pigments in the present paper.

Paper chromatography. —— Both one- and two-dimensional chromatographies were carried out using Toyo No. 50 filter paper. The solvents for development were (1) 80 per cent methanol, (2) a methanol-benzene-butanol-water mixture, 2 : 1 : 1 : 1 (MBBW), and (3) an upper layer of *n*-butanol-acetic acid-water mixture, 4 : 1 : 5 (BAW). After development, the paper was dried either in the air at room temperature or in the warm air, and the fluorescence on the chromatogram was inspected under ultraviolet light (Mazda, UV–Dl filter). And then, the following color tests were made :

(1) Ninhydrin reaction
(2) Reaction for aromatic amines by Tsuda's reagent (Umebachi and Tsuchitani, 1955)
(3) Ehrlich's aldehyde reaction
(4) Dragendorff's reaction (Kariyone et al., 1953)
(5) Millon's reaction
(6) Ehrlich's diazo reaction
(7) Ferric chloride reaction
(8) Phosphomolybdic acid reaction (Riley, 1950)
(9) α-Nitroso-β-naphthol reaction (Correale and Cortese, 1953)
(10) Ammoniacal silver nitrate reaction
(11) Diphenyl carbazid reaction (Blazsek, 1958)
(12) Lithium aluminium hydride reaction (Hayashi, 1954)
(13) Benzidine reaction
(14) Aniline hydrogen phthalate reaction (Cramer, 1954)
(15) Resorcin reaction (Momose, 1954)

Among these tests, the tests (5), (6), (7), (8), and (10) are for phenols, the test (9) for mono-phenol (for example tyrosine), the test (11) for purine and pyrimidine, the test (12) for flavonoid, and the tests (10), (13), (14), and (15) for sugars. As to kynurenine and uric acid, their positions on the chromatogram were confirmed using synthetic kynurenine and uric acid respectively.

Preparation of the crude sample of Y–II. —— The crude sample of yellow pigments was dissolved in a little water and applied as a streak across the filter paper sheet. After being developed with 80 per cent methanol one-dimensionally, the paper was inspected under ultraviolet light. Then, the strip of Y – II was cut out excluding uric acid and

eluted with 80 per cent ethanol. The eluate was evaporated to dryness under reduced pressure. The residue will be denoted as the crude sample of Y－II in the present paper.

Measurement of ultraviolet absorption spectrum. —— The ultraviolet absorption spectra of Y－II and a substance named SN－1 were measured. Each substance was eluted from the chromatogram with 80 per cent ethanol, and evaporated to dryness under reduced pressure. The residue was again dissolved in 80 per cent ethanol and centrifuged. The ultraviolet absorption of the supernatant fluid was measured between 230 and 400 mμ using a Hitachi EPU－2 spectrophotometer.

Spot test. —— In order to examine the chemical properties of SN－1, the following spot tests were made according to the methods of Feigl (1956) :

(1) a test for phenol by natrium nitrite
(2) a test for phenol by 5-nitroso-8-hydroxy-quinoline
(3) a test for phenol by chloroform and hydrazine sulfate
(4) a test for phenol by sodium cobaltinitrite
(5) a test for aromatic primary amine by 1 (4-pyridyl)-pyridinium chloride hydrochloride

The tests (1) and (2) are positive to phenol which has not *p*-substituent, and the test (3) is positive to phenol which has not *o*-substituent. And so, the test (3) is negative for catechol and positive for resorcinol. On the contrary, the test (4) is positive for both catechol and resorcinol.

Radioactivity measurement of decomposition-products obtained from Y－II labeled with the C^{14} from tryptophan-C^{14}. —— It has been already reported that the C^{14} of tryptophan-C^{14} injected into the butterfly is incorporated into Y－II and－III. In the present paper, Y－II which incorporated the C^{14} of tryptophan-C^{14} was decomposed to kynurenine and a phenolic compound (SN－1) by being heated, and it was seen whether the C^{14} was incorporated into SN－1 or not. The crude sample of Y－II prepared from the butterflies which had been injected with tryptophan-2-C^{14} in the third paper of this series was dissolved into a little water, heated in a boiling water bath, and applied to a chromatographic paper. After being developed with the BAW solvent, the paper was dried, inspected under ultraviolet light, and subjected to the ammoniacal silver nitrate test. Then, the paper was cut into small sections, and the radioactivity of each section was measured in the same manner as in the first paper of this series.

Hydrolysis. —— The crude sample of yellow pigments was hydrolysed with 1 N NaOH in a boiling water bath for four hours. After that, the solution was acidified with HCl to pH 3, and shaken repeatedly with *n*-butanol saturated with water. The upper phase was evaporated to dryness under reduced pressure, then dissolved in 80 per cent ethanol, and subjected to one- or two-dimensional chromatography. The crude sample of yellow pigments was also hydrolysed with 6 N HCl in a boiling water bath for ten hours. After that, the hydrolysate was freed from HCl under reduced pressure, and evaporated to dryness. The residue was dissolved in 80 per cent ethanol and examined by two-dimensional chromatography. Moreover, hydrolysis of the crude sample of Y－II was carried out as follows.

The crude sample of Y-II was dissolved in a little 80 per cent ethanol, removed from insoluble substances by centrifugation, and again brought to dryness under reduced pressure. The residue was hydrolysed with alkali or acid in the same way as mentioned above and examined by two-dimensional chromatography. As the solvents for two-dimensional chromatography after hydrolysis, 80 per cent methanol was used for the first direction, and the BAW solvent for the second direction. In all the cases of hydrolysis, after development, xanthurenic acid and 3-hydroxykynurenine were examined by Ehrlich's diazo reaction.

Results

The paper chromatographic separation and color tests of the yellow pigments. —— The crude sample of yellow pigments was dissolved in a small amount of 80 per cent ethanol or water, and subjected to two-dimensional chromatography after removing insoluble substances. When applied to the paper, the sample was dried in the air at room temperature and not heated. As solvents, 80 per cent methanol was used for the first direction, and the MBBW solvent for the second direction. Although the best solvent for the yellow pigments of *P. xuthus* has not been sought out yet, 80 per cent methanol was comparatively good, and the MBBW solvent was secondarily available. Although the BAW solvent also was sometimes used, this solvent was not so good, for it decomposes the yellow pigments to some extent. The two-dimensional chromatogram so obtained is shown in Figure 1, and the color tests of each spot are gived in Table 1. It will be seen from Figure 1 that the crude sample of yellow pigments contains four substances of spots K, U, Y-II, and Y-III. Y-I reported in the previous paper (Umebachi, 1958) was not found in the present experiment. So, Y-I does not seem to be the natural pigment of *P. xuthus*. Spots K and U corresponded respectively to synthetic kynurenine and uric acid in their positions and color tests.* Y-II and -III corresponded to Y-II and -III of the previous paper respectively. Table 1 shows

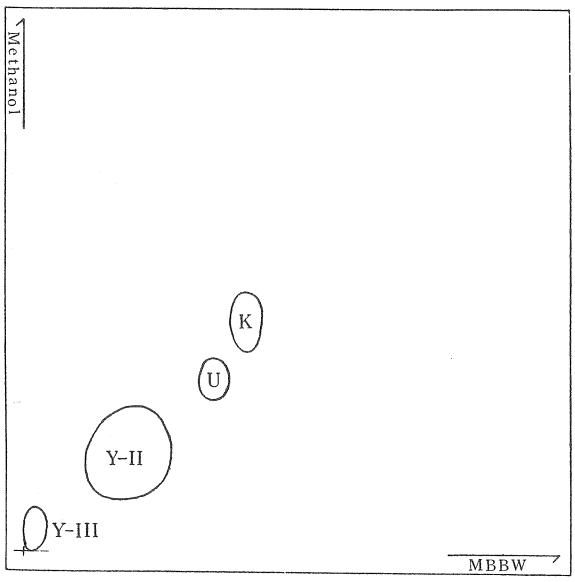

Figure 1. Paper chromatogram of the crude sample of yellow pigments.

* These two spots have been proved to correspond to synthetic kynurenine and uric acid respectively in their ultraviolet absorption spectra also (unpublished data).

Table 1. Color tests of the spots in Fig. 1.

	Spot			
	K	U	Y-II	Y-III
Color	Colorless	Colorless	Pale yellow	Pale yellow
Fluorescence	Pale blue	None	White-yellow	White-yellow
Ninhydrin	Red-purple	Negative	Yellowish brown*	Yellowish brown*
Tsuda	Purple	Negative	Pale purple after several hrs.	Pale purple after several hrs.
Aldehyde	Orange	Negative	Orange	Orange
Dragendorff	Negative	Negative	Negative	Negative
Millon	Negative	Negative	Yellowish brown	Yellowish brown
Diazo	Negative	Negative	Red	Red
$FeCl_3$	Negative	Negative	Dark brown after heating	Dark green
Phospho-molybdic	Negative	Blue**	Blue**	Blue**
$AgNO_3$	Negative	Gray	Brown	Brown
Benzidine	Negative	Negative	Negative	Negative
Aniline hydrogen phthalate	Negative	Negative	Negative	Negative
Resorcin	Negative	Negative	Negative	Negative
Identity	Kynurenine	Uric acid		

* In some cases, a reddish brown color appeared.
** The blue color appeared after the paper was exposed to ammonia.

clearly that both Y-II and -III are phenolic compounds. Morever, it seems that Y-II and -III contain no sugar component. Y-II and -III reacted to Ehrlich's aldehyde reagent in the same manner as kynurenine, and gave a yellowish brown color with ninhydrin, although in some cases they gave a reddish brown color. For Tsuda's reagent, both yellow pigments were negative for a while after spraying the reagent, but gave a slight purple color after several hours. Moreover, Y-II and -III were confirmed to be neither 3-hydroxykynurenine nor 5-hydroxykynurenine by comparison of the positions in the chromatogram. Although Y-II and -III are similar to each other in their color tests, they can be distinguished easily by their different reactions to ferric chloride.

Ultraviolet absorption spectrum of Y-II. —— The crude sample of Y-II was dissolved in a little 80 per cent ethanol, and its ultraviolet absorption spectrum was measured. The result is given in Figure 2. The absorption maxima were 261-264, 282-283, and 383-385 mμ.

Treatment by heat of an aqueous solution of Y-II. —— The crude sample of Y-*II* was dissolved in a little water and centrifuged. The supernatant fluid was heated in a boiling water bath for twenty minutes and chromatographed with the BAW solvent or 80 per cent methanol one-dimensionally. The chromatograms so obtained are shown in Figure 3, and the color tests for each spot are given in Table 2. Spot K is kynurenin and was

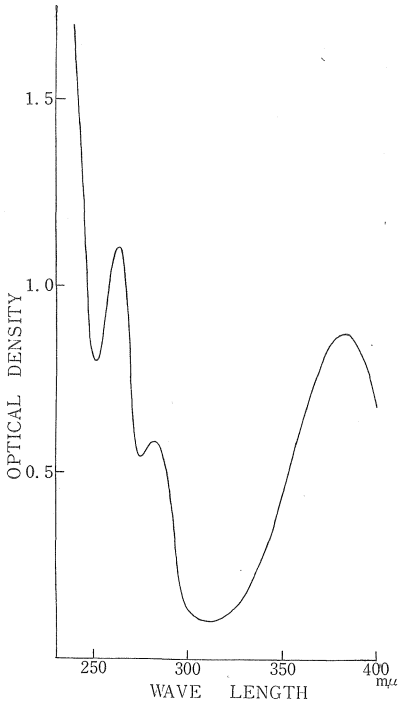

Figure 2. Ultraviolet absorption spectrum of Y-II in 80 percent ethanol.

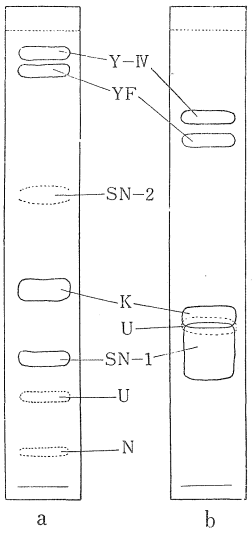

Figure 3. Chromatograms obtained after heating the aqueous solution of the crude sample of Y-II. Solvent : (a), BAW ; (b), 80 per cent methanol.

present in large quantities. Spots Y-IV and YF are probably degradation-products of kynurenine, for these two spots were produced from synthetic kynurenine treated in the same manner also. Spot Y-IV seems to correspond to the spot IIa of Figure 4 in the first paper of this series. Spot U seems to be uric acid, for the former has the same Rf values as the latter for both the BAW solvent and 80 per cent methanol. As spot U was found only in a small quantity, it is conceivable that Y-II fraction may have been contaminated with uric acid. Spot SN-1 turned pale purple when left exposed to the air. From Table 2, it is clear that SN-1 is a phenolic compound, but not tyrosine, purine derivative, flavonoid, and sugar. It was also evident from the fluorescence and the position on the chromatogram that SN-1 is neither 3-hydroxykynurenine nor 5-hydroxykynurenine. Moreover, spot SN-1 was confirmed not to be dopa by comparison with synthetic dopa. Spot SN-1 corresponds to spot 6 of Figure 2 and spot IId of Figure 4 of the first paper of this series. Probably an aqueous solution of Y-II seems to decompose by being heated, and to produce kynurenine and the phenolic compound SN-1. In Figures 2 and 4 of the first paper of this series, the sample was extracted (or eluted) and concentrated by heating, and so a part of Y-II must have been decomposed. Besides spots K, U, Y-IV, YF, and SN-1, spots SN-2 and N also were found in some cases, although absent in some cases. Spot SN-2 was positive to the ammoniacal silver nitrate test although in very weak,

Table 2. Color tests of the spots in Fig. 3.

	Spot				
	Y-IV	YF	K	U	SN-1
Color	Yellow	Colorless	Colorless	Colorless	Colorless*
Fluorescence	None	Yellow	Strong pale blue	None	None
Ninhydrin	Negative	Negative	Red-purple	Negative	Slightly brownish purple or ?
Tsuda	Negative or ?	Negative	Purple	Negative	Negative
Aldehyde	Rose	Negative	Orange	Negative	Negative
$AgNO_3$	Slightly brown or ?	Negative	Negative	Grayish brown	Dark brown
Millon	Negative	Negative	Negative	Negative	Brownish yellow
Phospho-molybdic	Negative	Negative	Negative	Blue**	Blue**
$FeCl_3$	Negative	Negative	Negative	Negative	Dark green
Diazo	Negative	Negative	Negative	Negative	Orange-red
α-Nitroso-β-naphthol	Negative	Negative	Negative	Negative	Negative
Aniline hydrogen phthalate	Negative	Negative	Negative	Negative	Negative
Diphenyl carbazid	Negative	Negative	Negative	?	Negative
$LiAlH_4$	Negative	Negative	Negative	Negative	Negative
Identity			Kynurenine	Uric acid	

* SN-1 turned pale purple when left exposed to the air.
** The blue color appeared after the paper was exposed to ammonia.

and seemed to correspond to spot IIb of Figure 4 of the first paper of this series. Spot N was positive to ninhydrin reaction. The eluate of Y-III has not yet examined in so detail as in Y-II, but it has been proved that an aqueous solution of Y-III also produces both kynurenine and a phenolic compound by being heated.

Spot tests of SN-1. —— An aqueous solution of the crude sample of Y-II was decomposed by heating in the same manner as mentioned above and chromatographed with the BAW solvent one-dimensionally. After developing, the strip of spot SN-1 was cut out, eluted with 80 per cent ethanol, evaporated to dryness under reduced pressure, and subjected to spot tests. The results obtained are given in Table 3. The table shows clearly that SN-1 is a phenolic compound and is not an aromatic primary amine. It is conceivable that SN-1 may be *o*-

Table 3. Spot tests of SN-1.

Test	
$NaNO_2$	+ (yellow)
5-Nitroso-8-hydroxyquinoline	+ (pale purple)
Hydrazine sulfate	−
Sodium cobaltinitrite	+ (weak brown)
1 (4-Pyridyl)-pyridinium chloride hydrochloride	−

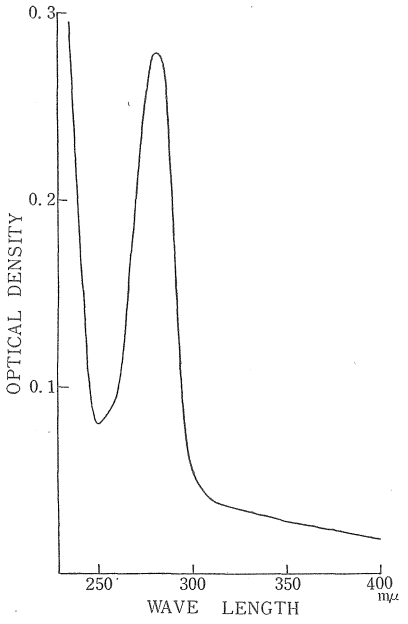

Figure 4. Ultraviolet absorption spectrum of SN-1 in 80 per cent ethanol.

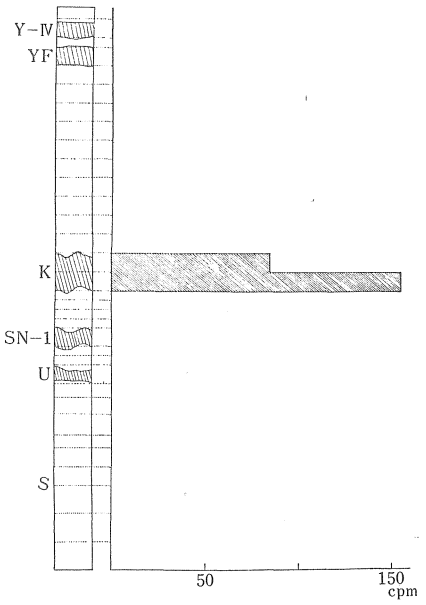

Figure 5. The result of radioactivity measurement of the one-dimensional chromatogram of the decomposition-products of Y-II obtained from the butterflies injected with tryptophan-C^{14}. *S*, starting line.

phenol, for the negative result was obtained in the spot test using chloroform and hydrazine sulfate.

Ultraviolet absorption spectrum of SN-1. —— In the same manner as mentioned above, SN-1 was eluted from the chromatogram, brought to dryness under reduced pressure, and again dissolved in 80 per cent ethanol. The ultraviolet absorption spectrum of the solution was measured. The result obtained is shown in Figure 4. The absorption maximum was 281 – 282 mμ. This spectrum also suggests that SN-1 is a phenolic compound.

Radioactivity measurement of SN-1 obtained from Y – II labeled with the C^{14} from tryptophan-C^{14}. —— The result of radioactivity measurement of the decomposition-products of the crude sample of Y – II labeled with the C^{14} from tryptophan-C^{14} is shown in Figure 5. It will be seen from the figure that the C^{14} of tryptophan-2-C^{14} was incorporated into kynurenine but not into spot SN-1.

Hydrolysis of the yellow pigments. —— The alkali and acid hydrolysates of the crude sample of yellow pigments were chromatographed and examined with Ehrlich's diazo reagent. It has been confirmed that the yellow pigments including Y – II and – III do not produce xanthurenic acid by alkali hydrolysis, and do not produce 3-hydroxykynurenine but a large quantity of kynurenine by acid hydrolysis. The same results have been obtained also in the alkali and acid hydrolyses of the crude sample of Y – II.

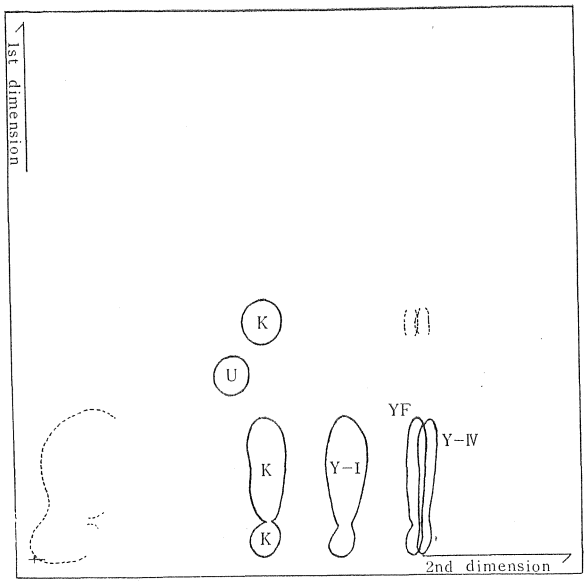

Figure 6. Two-dimensional chromatogram of the yellow pigments heated after one-dimensional development.

Treatment by heat of the chromatogram of Y-II and-III. —— After the crude sample of yellow pigments was applied to the paper for two-dimensional chromatography and developed with 80 per cent methanol in the first direction, the paper was dried, put in an electric drying apparatus, and kept at 80-100°C for about two hours. And then the paper was developed again with 80 per cent methanol in the second direction. The chromatogram thus obtained is shown in Figure 6, and the color tests of each spot are given in Table 4. It will be seen from Figure 6 that the chromatogram of Y-II and-III produces not only kynurenine but also Y-I when heated. This Y-I seems to correspond to Y-I described in the previous paper. Moreover, Y-II and-III produced Y-IV and YF also. In some cases, kynurenine also produced the two spots, although in small quantity. It is of interest that the heating of an aqueous solution of Y-II and the

Table 4. Color tests of the spots in Fig. 6.

	Spot			
	K	Y-I	YF	Y-IV
Color	Colorless	Yellow	Colorless	Yellow
Fluorescence	Pale blue	None	Yellow	None
Ninhydrin	Red-purple	Negative	Negative	Negative
Tsuda	Purple	Negative	Negative	Pale purple*
Aldehyde	Orange	Rose-orange after heating**	Negative	Immediate rose-orange
Diazo	Negative	Negative	Negative	Negative
$FeCl_3$	Negative	Negative	Negative	Negative
Phosphomolybdic	Negative	Slightly blue	Negative	?
$AgNO_3$	Negative	Very slightly yellowish brown	Negative	Negative
Millon	Negative	?	Negative	Negative
Benzidine	Negative	Negative	Negative	Negative

* The pale purple color appeared immediately after spraying the reagent and disappeared after a while.

** Slightly.

heating of the chromatogram of Y－II gave the different results. That is to say, in the former case, Y－I is not produced.

Exposure of Y－II and－III to the air. —— The crude sample of yellow pigments was chromatographed with 80 per cent methanol in the first direction in the same manner as in the treatment by heat of the chromatogram. After development, the paper was left exposed to the air in a dark room for periods varying from one day to two months, and then was developed again with 80 per cent methanol in the second direction. The chromatogram so obtained is shown in Figure 7. Spot K corresponds to kynurenine. Figure 7 shows clearly that Y－II and－III produce kynurenine easily by exposure to the air. From Y－II, a small quantity of kynurenine arises with as short an exposure as one day. After about a month, a large quantity of kynurenine was proved to arise from both Y－II and－III.

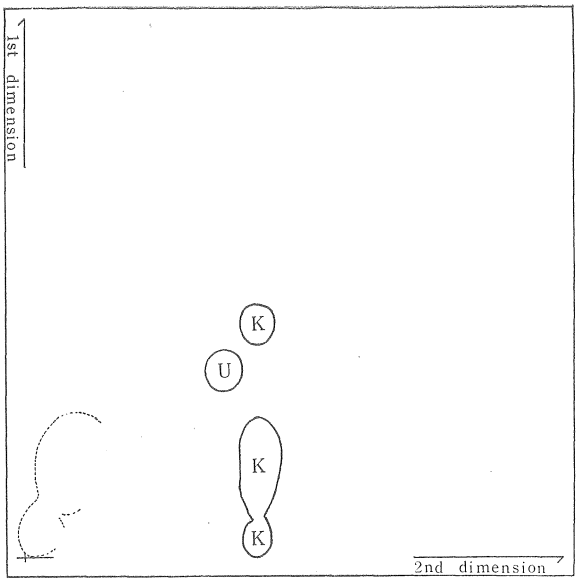

Figure 7. Two-dimensional chromatogram of the yellow pigments exposed to the air after one-dimensional development.

Discussion

It was already reported in the previous paper (Umebachi, 1958) that kynurenine is accumulated in the yellow scales of *P. xuthus*, and that the yellow pigments have a close relation to kynurenine. At that time, the two-dimensional chromatogram of the yellow pigments was already illustrated, but in the previous experiments, the extraction was performed under heating, and the application of the extract to the filter paper was made in hot air. Moreover, the BAW solvent was used for development. Accordingly, as some parts of the yellow pigments seem to have undergone a change, a part of the results has been corrected in the present paper. It has been presumed from the results of the present paper that Y－I described in the previous paper is either a degradation-product or a changed substance from Y－II and－III. Accordingly, the natural yellow pigments of *P. xuthus* seem to be of only two kinds : Y－II and－III. The occurrence of Y－I in the experiment of the previous paper seems to have resulted from applying the sample to the filter paper in hot air.

It is evident from the injection experiment of tryptophan-C^{14} reported in the previous paper that Y－II and－III are the pigments derived from tryptophan. Then, on what

relation are Y－II and－III with ommochrome which is the most commonly known pigment derived from tryptophan? As for ommochrome, a detailed investigation of Butenandt (1957, 1959) has been reported. When the chemical properties of Y－II and－III (that is to say, (1) they are phenolic compounds, (2) they produce kynurenine and some phenolic compound, (3) they do not produce xanthurenic acid by alkali hydrolysis, and (4) they do not produce 3-hydroxykynurenine but kynurenine by acid hydrolysis), are compared with the chemical properties of ommochrome reported up to the present, Y－II and－III do not seem to belong to the ommochrome group. The experimental results so far obtained for Y－II and－III strongly suggest that Y－II and－III may be a combination of kynurenine and some other substance (probably a phenolic compound). Although the phenolic compound (SN-1) obtained by heating Y－II is not dopa, it is of interest whether SN-1 is derived from dopa or not. More detailed discussion of Y－II and－III will be reported in the seventh paper of this series.

Finally, as such yellow pigments related to kynurenine as Y－II and－III are found only in the Papilionidae so far as the author has examined up to the present, the names "Papiliochrome－II and－III" are here proposed respectively for Y－II and－III. Although it is difficult to determine the structure of Papiliochrome－II and－III as they are both labile substances and as the number of the butterflies available is not so large, further experiments are being carried out. The author thinks that such pigments related to kynurenine as Y－II and－III may be called "kynurenine-pigment".

The author wishes to express his gratitude to Professor K. Makino of the Tokyo Jikeikai School of Medicine for a generous gift of synthetic 5-hydroxykynurenine. Thanks are also due to Miss Y. Muroki and Mr. Y. Aburano for their practical assistance in carrying out the experiments.

Summary

(1) The yellow pigments of wings of *P. xuthus* were extracted with as little exposure to heat or air as possible and subjected to two-dimensional chromatography. Since Y－I described in the previous paper was not found, the natural yellow pigments of *P. xuthus* have been presumed to be of only two kinds: Y－II and－III, and both of them have been shown to be phenolic compounds and not to contain sugars. Moreover, Y－II and－III have been confirmed to be neither 3-hydroxykynurenine nor 5-hydroxykynurenine.

(2) The ultraviolet absorption spectrum of Y－II was determined. It has three absorption maxima 261－264, 282－283, and 383－385 mμ.

(3) The aqueous solution of Y－II was decomposed by heat and examined by paper chromatography. Kynurenine and a substance (SN-1) positive to ammoniacal silver nitrate were found. The latter has been presumed to be a phenolic compound from the color tests, spot tests, and ultraviolet absorption spectrum. It has been proved that the C^{14} of tryptophan-2-C^{14} is not incorporated into SN-1 and that SN-1 is neither 3-hydroxykynurenine nor 5-hydroxykynurenine. Moreover, SN-1 has been confirmed not to be dopa.

(4) The crude sample of yellow pigments including Y-II and-III did not produce xanthurenic acid by alkali hydrolysis and did not produce 3-hydroxykynurenine but a large quantity of kynurenine by acid hydrolysis. The same results were obtained also by using the crude sample of Y-II.

(5) The chromatogram of Y-II and-III produced Y-I easily by being heated. So, Y-I has been presumed to be either a degradation-product or a changed substance produced from Y-II and-III.

(6) Both Y-II and-III produced kynurenine easily by exposure to the air also.

(7) It has been supposed that although the yellow pigments of *P. xuthus* are derived from tryptophan, they do not belong to the ommochrome group reported up to now. Y-II and-III have been named "Papiliochrome-II and-III" respectively.

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