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メタデータ	言語: English 出版者: 公開日: 2017-10-03 キーワード (Ja): キーワード (En): 作成者: Umebachi, Yoshishige, Nakamura, Akira, 梅鉢, 幸重 メールアドレス: 所属:
URL	https://doi.org/10.24517/00011534

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**Fluorescent Substances and Substances Positive to the Ehrlich's
Diazo Reagent in Mutants of *Drosophila melanogaster* as
revealed by Paper Chromatography ***

Yoshishige UMEBACHI and Akira NAKAMURA **

(Received 10. Jan. 1954)

It is generally recognized that the red and brown pigments are contained in the eye of *Drosophila melanogaster*. Though it has been suggested that the red pigment may be probably pterin (CHAN, HEYMANN & CLANCY, 1951), the process of its formation is yet unknown. On the other hand, it is known that the brown pigment is formed from tryptophan by way of kynurenine and 3-hydroxykynurenine (BUTENANDT, 1952). The continuation from 3-hydroxykynurenine to the brown pigment, however, is not yet known.

The authors have studied, by paper chromatography, the tryptophan metabolites and the fluorescent substances such as pterins in mutants of *Drosophila melanogaster*, and found a yellow fluorescent substance and a substance positive to the Ehrlich's diazo reagent, which is not 3-hydroxykynurenine. These two substances are supposed to have some relation to eye pigments. Paper chromatographical studies of fluorescent substances of *Drosophila melanogaster* have been already reported by HADORN & MITCHELL (1951) and BUZZATI-TRAVERSO (1953).

The authors wish to acknowledge their indebtedness to Professor H. KIKKAWA of Osaka University and to the Department of Biology, Faculty of Science, Tokyo Metropolitan University for kindly supplying several mutants of *Drosophila melanogaster*. Thanks are also due to Professor M. KUMANO and Assistant Professor K. MASHIKO for their encouragement and generous economical support. The authors are also indebted to Mrs. M. UMEBACHI for technical assistance. The authors also wish to express their gratitude to Dr. G. TOMITA of Tôhoku University for his kindness in reading and criticizing the manuscript.

Materials and Methods

The mutants of *Drosophila melanogaster* studied were as follows: white, cinnabar brown, vermilion, cinnabar, purple engrailed, sepia, brown, yellow, and ebony. The wild type was also studied for comparison.

The medium for growing the flies had the following composition: agar, 10g; sugar, 40g; malted rice, 100g; water, 720ml; K_2HPO_4 , 0.72g. After cooking and bottling, the

* This work was supported by a Grant in Aid for Miscellaneous Scientific Research from the Ministry of Education.

** Zoological Institute, Faculty of Science, Kanazawa University

medium was seeded with commercial yeast used for baking bread. All cultures were kept at $25 \pm 1^\circ\text{C}$.

Males and females were separately studied in all cases. The fly was separated, by decapitation, into the head and the posterior part (thorax and abdomen). Each part was mashed on filter paper with a glass rod. Ten samples were used to apply to a single spot. The filter paper was then allowed to dry in the air at room temperature (HADORN & MITCHELL, 1951) and developed. As for the head, extraction was also performed, without separating males and females. The heads of one hundred animals, including both sexes, were gathered, homogenized with cold 80 % ethanol, and centrifuged. The supernatant fluid was directly spotted on filter paper and developed.

For chromatographing, the one-dimensional procedure was used, with strips of filter paper (Toyo No. 2), 1.8×38 cm. The solvent used was the upper layer of a *n*-butanol-acetic acid-water (4:1:5) mixture. Development was made by the ascending method in a completely dark room. After developing and drying the chromatogram, the paper was inspected by ultraviolet rays, and the outline of the fluorescent spots was drawn with a pencil. The intensity of fluorescence of each spot was roughly estimated by the naked eye and recorded. After this, some papers were sprayed with the Ehrlich's diazo reagent and about 14% ammonia water; some papers were sprayed with a hydrochloric acid solution of *p*-dimethylaminobenzaldehyde and dried in warm air. The intensity of colour thus developed was roughly estimated by the naked eye and recorded.

Results

Substances positive to the Ehrlich's diazo reagent.

(1) The results obtained by the extracting method. The extracting method was performed only in the head, and two diazo-positive substances, spots 3 and 6, were found. The results are contained in table 1. Their R_f values are shown in table 2. As shown in table 1, spots 3 and 6 existed only in the wild type and certain mutants such as purple engrailed, sepia, brown, yellow, and ebony; it was not recognized in the mutants white, cinnabar brown, vermilion, and cinnabar. The colour for the Ehrlich's diazo reagent was rose-red in spot 3 and orange-red in spot 6. The colours of both spots appeared immediately after spraying the reagent; the colour of spot 3 then grew stronger gradually, whereas that of spot 6 faded gradually. Spot 6 had the same R_f value as 3-hydroxykynurenine in the white-2 mutant of *Bombyx mori*.

(2) The results obtained by the mashing method. The mashing method was performed both in the head and in the posterior part. Two diazo-positive substances, spots 3 and 6, also were found in both parts. The results obtained are shown in table 3. Their R_f values, which is smaller than those in the extracting method, are shown in table 2. As no sexual differences were seen in appearance of both spot 3 and spot 6, the results with both sexes are put together in table 3. As shown in table 3, both

Table 1. Summarized results of paper chromatographical studies of the head of mutants of *Drosophila melanogaster* (the results obtained by the extracting method)

Spot no.	Colour of fluorescence	Remarks	w	en,bw	v	en	wild	pr,en	se	bw	y	e
1	Yellow		-*	-	-	-	-	-	±	-	-	-
2**		Tryptophen	-	-	-	-	-	-	-	-	-	-
3†	unconfirmable	Diazo-positive substance	-	-	-	-	+	+	+	+	+	+
4	White blue	Kynurenine	-	-	-	?	-	?	?	-	?	?
5	Yellow		-	-	±	±	±	+	+++	?	±	+
6†		Diazo-positive substance	-	-	-	-	±	+	+	±	±	±
7	Blue		-	-	±	±	±	+	+	-	±	+
8	Yellowish green		-	-	±	±	±	±	?	-	±	+
9	Purple		-	-	±	±	?	±	?	-	±	±
10	Blue		-	-	-	-	-	-	±	-	-	-
11	Yellowish green		-	-	-	-	-	-	±	-	-	-
12	Orange	Red eye pigment	-	-	++	++	++	++	-	-	++	++
13	Orange	Red eye pigment	-	-	++	++	++	++	-	-	++	++
14	Orange	Red eye pigment	-	-	±	±	±	?	-	-	±	±
15	Red orange	Red eye pigment	-	-	+	+	+	+	-	-	+	+
16			-	-	-	-	±	±	±	±	±	±

* Intensity of colour of spots: +++, very strong; ++, strong; +, medium; ±, weak; ?, faint and unconfirmable; -, negative.

** Reaction to *p*-dimethylaminobenzaldehyde reagent.

† Reaction to the Ehrlich's diazo reagent.

spots also were recognized only in the wild type and those mutants such as purple engrailed, sepia, brown, yellow, and ebony. Spot 3 was much stronger in the head than in the posterior part, while spot 6 was stronger in the latter than in the former. The colours of both spots were respectively the same as those of spots 3 and 6 obtained by the extracting method.

Fluorescent substances.

(1) The results obtained by the extracting method. The extracting method was performed only in the head. The results obtained are contained in table 1. The Rf value of each spot is shown in table 2. Spot 1, the fluorescence of which was yellow, was present only in the mutant sepia of all the flies studied. Spot 4 (kynurenine) was not found in the head or even if a trace was found, it was extremely faint. The

Table 2. Rf values of spots obtained by paper chromatography of mutants of *Drosophila melanogaster*

Spot no.	Mashing method	Extracting method	Spot no.	Mashing method	Extracting method
1		0.57	9		0.26
2		0.51	10		0.18
3	0.48	0.51	11		0.15
4		0.47	12		0.17
5		0.41	13		0.14
6	0.37	0.41	14		0.10
7		0.38	15		0.07
8		0.34	16		0.03

Table 3. Summarized results of paper chromatographical studies of substances positive to the Ehrlich's diazo reagent in mutants of *Drosophila melanogaster* (the results obtained by the mashing method)

Spot no.	H e a d									
	w	cn,bw	v	cn	wild	pr,en	se	bw	y	e
3	-*	-	-	-	+	+	+	+	+	+
6	-	-	-	-	?	?	±	±	±	±
P o s t e r i o r p a r t										
w	cn,bw	v	cn	wild	pr,en	se	bw	y	e	
3	-	-	-	-	?	?	?	?	?	
6	-	-	-	-	+	+	±	+	+	?

* Intensity of colour of spots: +, medium; ±, weak; ?, faint and unconfirmable; - negative.

yellow fluorescence of spot 5 was extremely strong in the head of the mutant sepia. Spot 5 had the same Rf values as spot 6. The fluorescence of spot 7 was also strong in the mutant sepia. The fluorescence of spot 8 was generally weak. A purplish fluorescence in spot 9, which had the same Rf value as ichthyopterin, was very weak or unconfirmable in the head. Spot 10 and 11 were obtained only in the mutant seaia. Spots 12, 13, 14, and 15 were seen only in the head of the wild type and certain mutants: vermilion, cinnabar, purple engrailed, yellow, and ebony. The orange fluorescence of spots 12 and 13 was strong, but that of spot 14 was weak. The fluorescence of spot 15 was orange-red.

(2) The results obtained by the mashing method. The mashing method was

performed both in the head and in the posterior part. Although the spots of fluorescent substances produced "tailing" in this method, the clear results obtained were as follows: A spot with yellow fluorescence, which seemed to be the same substance as spot 1 in the extracting method (judging from the relative position on chromatogram, the "pattern" of chromatogram), was found only in the mutant sepia. This spot was more remarkable in the head than in the posterior part. Kynurenine was not present in the head or was extremely faint, if any, like that obtained by the extracting method. In the posterior part, this spot was recognized in all the mutants studied except the mutants vermilion and white, although its fluorescence was very weak. A spot with strong yellow fluorescence, which seemed to correspond to spot 5 in the extracting method (judging from the relative position on chromatogram), was present in the head of the mutant sepia. But this spot was very faint in the posterior part. This spot had also the same Rf value as the diazo-positive substance in spot 6. A spot with purplish fluorescence, which seemed to be the same substance as spot 9 in the extracting method (judging from the relative position on chromatogram), was found. Its fluorescence was very weak or unconfirmable in the head. This was also the case with the posterior part of the female, but the fluorescence of this spot was extremely strong in the posterior part of the male except for the mutants white, cinnabar brown, and brown

Table 4. Purple fluorescent substance (spot 9) in males and females of mutants of *Drosophila melanogaster* (the results obtained by the mashing method)

		<i>w</i>	<i>cn,bw</i>	<i>v</i>	<i>cn</i>	<i>wild</i>	<i>pr,en</i>	<i>se</i>	<i>bw</i>	<i>y</i>	<i>e</i>
Head	♂	—*	—	?	?	?	?	—	—	?	?
	♀	—	—	?	?	?	?	—	—	?	?
Posterior part	♂	?	?	+++	+++	+++	+++	+++	?	+++	+++
	♀	—	?	?	±	±	?	?	?	?	±

* Intensity of fluorescence of spots: +++, very strong; ±, weak; ?, faint and unconfirmable; —, negative.

(table 4). In this case, this spot made a stripe with strong purplish fluorescence almost from the origin of the chromatogram. In the heads of the wild type and of certain mutants such as vermilion, cinnabar, purple engrailed, yellow, and ebony, a spot with strong orange fluorescence and a spot with orange-red fluorescence were present. Although these spots also made a stripe almost from the origin of the chromatogram, the former seemed to correspond to spots 12, 13 and 14 in the extracting method, and the latter, to spot 15 judging from their fluorescence and the relative position on the chromatogram.

Other substances.

As shown in table 1, tryptophan (spot 2), which was examined by the *p*-dimethylaminobenzaldehyde reagent, was not found in the head in the extracting method. Tryptophan had the same Rf value as spot 3. As shown in table 1, spot 16, which had no fluorescence but was recognized by its dark colour when the papers were inspected by ultraviolet rays, was present in the wild type and the following mutants: purple engrailed, sepia, brown, yellow, and ebony. This spot was recognized also in the mashing method.

Discussion

Of the two diazo-positive substances, the chemical nature of spot 3 is not yet clear. However, as spot 3 was much stronger in the head than in the posterior part, and as it was not found in those mutants such as white, cinnabar brown, vermilion, and cinnabar (which have no brown pigment), this spot seems to have some relation to the formation of the brown eye pigment. But it is not yet clear whether spot 3 is a metabolite in the process of the synthesis of the brown pigment from 3-hydroxykynurenine, for spot 2 (tryptophan) and spot 4 (kynurenine), which are metabolites in the process of the formation of the brown pigment, were hardly recognizable in the head of any of the flies examined, quite irrespective of the presence or absence of the brown eye pigment. However, it is to be noticed that spot 3 was found only in the head of the wild type and of certain mutants such as purple engrailed, sepia, brown, yellow, and ebony, which have the brown pigment of spot 16.

As the diazo-positive substance of spot 3 is not 3-hydroxykynurenine in the Rf value, and as it may be said that Ehrlich's diazo reaction in the head of the flies depends mainly on spot 3, this reaction seems to be unable to be used for the examination of 3-hydroxykynurenine in the head, without separating the substance of spot 3 and 3-hydroxykynurenine.

The diazo-positive substance of spot 6 is supposed to be 3-hydroxykynurenine or, at least, to contain 3-hydroxykynurenine, for it had the same Rf value as 3-hydroxykynurenine in the white-2 mutant of *Bombyx mori*. Judging from only the fact that spot 6 had the same Rf value as spot 5 with yellow fluorescence, the substances of these two spots may be supposed to be the same. Namely, the fluorescent substance of spot 5 may be positive to the diazo reagent. But, as spot 5 with yellow fluorescence was negative to the Ehrlich's diazo reagent in the mutants vermilion and cinnabar, spot 5 and spot 6 seem to be two different substances. Thus, it may be taken that two different substances, the strong yellow fluorescent substance of spot 5 and the diazo-positive substance of spot 6 take the same position on the chromatogram. But, even if the yellow fluorescent substance of spot 5 is negative to the Ehrlich's diazo reagent, it is not clear whether the diazo-positive substance of spot 6 has yellow fluorescence or not. If the diazo-positive substance of spot 6 has yellow fluorescence, it should also be in the yellow fluorescence of spot 5. For example, 3-hydroxykynurenine is

positive to the Ehrlich's diazo reagent and has yellow fluorescence. As the diazo-positive substance of spot 6 seems to be 3-hydroxykynurenine (judging from its Rf value), spot 5 may be supposed to contain the fluorescence of 3-hydroxykynurenine. However, even if so, at least the extremely strong yellow fluorescence of spot 5 (in the head of the mutant sepia) seems to depend on other substance than 3-hydroxykynurenine. In the head of the mutant sepia, the yellow fluorescence of spot 5 was stronger and brighter than 3-hydroxykynurenine (which has a dull yellow fluorescence) and, to the naked eye, so similar to the colour of riboflavin that it was difficult to distinguish between them either by the colour of fluorescence or by visible colour, although these spots were different from each other in the Rf values. Moreover, 3-hydroxykynurenine reacted very strongly to the Ehrlich's diazo reagent even in cases where the fluorescence was weak. In the head of the mutant sepia, however, spot 5 reacted rather weakly to the Ehrlich's diazo reagent in spite of its very strong yellow fluorescence. The existence of a substance with strong yellow fluorescence in the mutant sepia has been already reported (HADORN & MITCHELL, 1951).

In *Drosophila melanogaster*, it was expected that fluorescence of kynurenine (spot 4) would be strong in the pupa of cinnabar, and this has been already reported (UMEBACHI & NAKAMURA, 1954). But it is interesting that kynurenine (spot 4) was scarcely recognized in the head of any of the mutant studied, including the mutant cinnabar. Although tryptophan (spot 2) also was not found in the head, this does not necessarily mean that the heads of the flies studied contain no free tryptophan at all. In addition to this case, the results also contain cases where, if there is any, its quantity is below the sensitivity to *p*-dimethylaminobenzaldehyde reagent. Also in the paper chromatographical studies of the butterfly, *Papilio xuthus*, kynurenine and tryptophan were not found in the head, although the diazo-positive substance of spot 3 was present. On the other hand, in the thorax and abdomen, however, the case seems quite contrary (unpublished data).

As the fluorescence of spot 9, which has been supposed to be ichthyopterin from its Rf value, was very strong in the posterior part of the male, this substance may be taken to have some bearings to the male sexual organs. Moreover, spot 9 is thought to have some relation with the red pigment formation (probably a metabolite in the process), for the spot was not found in the mutants white, cinnabar brown, and brown (table 4), which have no red eye pigment. The mutant sepia showed a strong fluorescence at spot 9 in the posterior part of the male, although it had no red eye pigment. If, in the mutant sepia, it is assumed that the process of the red pigment formation passes the substance with purplish fluorescence (spot 9) and attains finally to the substance with yellow fluorescence (spot 5), the above-mentioned facts may be understood.

As spots 12, 13, 14, and 15 were recognized only in the head of the wild type and certain mutants such as vermilion, cinnabar, purple engrailed, yellow, and ebony, these spots are thought to be some components of the red pigment. Although the spots that

have been reported in this paper in relation to the red eye pigment of *Drosophila melanogaster* are only four in number, one or more spots may be separated by extensive paper chromatography. Spot 16 is supposed to be the brown pigment, for this spot is recognized only in the head of the wild type and the following mutants: purple engrailed, sepia, brown, yellow, and ebony.

Summary

Of the wild type and several mutants of *Drosophila melanogaster*, substances positive to the Ehrlich's diazo reagent and fluorescent substances were revealed by paper chromatography. The mutants examined were as follows: white, cinnabar brown, vermilion, cinnabar, purple engrailed, sepia, brown, yellow, and ebony. Males and females of each mutant, and the head and the posterior part (thorax and abdomen) were separately examined. In the head of the flies, a diazo-positive substance, which is not 3-hydroxykynurenine, was found. The spot was recognized only in the wild type and certain mutants such as purple engrailed, sepia, brown, yellow, and ebony, but not in the mutants white, cinnabar brown, vermilion, and cinnabar. In the head of the mutant sepia, a spot with strong yellow fluorescence was found. A spot with strong purplish fluorescence, which is supposed to be ichthyopterin, was found in the posterior part of the male of the wild type and the following mutants: vermilion, cinnabar, purple engrailed, sepia, yellow, and ebony. These substances are supposed to have some relation to the eye pigment formation.

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