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Effect of Tryptophan Metabolites on Fluorescent Granules in the Malpighian Tubules of Eye Color Mutants of *Drosophila melanogaster*

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ABSTRACT—Fluorescent granules that are stained with Nile blue sulfate are present in larval Malpighian tubules in the wild type strain of *Drosophila melanogaster*, *Oregon-R*. These granules emit a weak blue fluorescence and most of them are about 2 μm or more in diameter. The ommochrome precursor 3-hydroxy-kynurenine (3-HK) is actively transferred into the tubules of *Oregon-R*. Changes in the fluorescent granules in the Malpighian tubules on administration of ommochrome precursors were investigated in eye color mutants of *Drosophila*. The fluorescent granules in the tubules of the nonautonomous mutants *v;bw* and *cn bw* emit a strong blue fluorescence and most of them are about 1 μm or less in diameter. When *v;bw* and *cn bw* larvae were cultured on medium supplemented with kynurenine or 3-HK, respectively, the fluorescence intensity of their granules decreased, and their size increased. These additions resulted in almost equal accumulation of 3-HK to that in *Oregon-R*. On the other hand, no 3-HK accumulated in the tubules of larvae of the autonomous mutants *bw;st*, *ltd bw* and *w*, which lack the fluorescent granules. These findings indicate that the fluorescent granules are an important intracellular site for uptake or storage of ommochrome precursors in larval Malpighian tubules of *Drosophila*.

INTRODUCTION

The organ specificity and the pathway of pigment biosynthesis in *Drosophila melanogaster* have been studied extensively for fifty years, but little is yet known about the mechanism of formation of pigment-containing granules (Phillips and Forrest, 1980; Summers *et al.*, 1982). The wild type eye color of *D. melanogaster* is due to two types of pigments, brown ommochrome derived from tryptophan and red pteridines synthesized from guanosine triphosphate (Phillips and Forrest, 1980; Summers *et al.*, 1982). Most of *Drosophila* eye color mutants are known to be deficient in Malpighian tubule pigment. The wild type alleles of *v*, *cn*, *st*, *ltd* and *w* encode products that are required for the brown pigmentation. Two of them, *v* and *cn* are nonautonomous mutants and encode the enzymes tryptophan oxygenase (Baglioni, 1959, 1960; Kaufman, 1962; Baillie and Chovinick, 1971) and kynurenine-3-hydroxylase (Ghosh and Forrest, 1967; Sullivan *et al.*, 1973), respectively. The roles of the autonomous mutant genes *st*, *ltd* and *w* have not been clarified.

Malpighian tubules in *D. melanogaster* are known to be sources of two precursors of brown pigment, kynurenine and 3-hydroxy-kynurenine (3-HK) (Beadle, 1937a,b; Wessing and Danell, 1961; Wessing and Eichelberg, 1968, 1972; Sullivan and Sullivan, 1975) and kynurenine-3-hydroxylase activity can be detected only in larval tissue (Sullivan *et al.*, 1973; Sullivan and Sullivan, 1975). During the larval stage, tryptophan metabolites are taken up by the tubules and the stores of pigment precursors are released into the haemolymph at the time of pupation (Beadle, 1937a,b). Transport of the pigment precursors is severely impaired in *st*, *ltd* and *w* (Beadle, 1937a,b; Beadle and Ephrussi, 1937; Howells and Ryall, 1975; Sullivan and Sullivan, 1975; Sullivan *et al.*, 1979, 1980; Sullivan, 1984). The *st* and *w* gene products are thought to be structural components of pigment granules or proteins involved in the transport of pigment precursors across to cell membrane (O'Hare *et al.*, 1984; Tearle *et al.*, 1989; Tearle, 1991). A well characterized system such as *Drosophila* eye color is suitable for studies on the cytological consequences of gene actions at a cellular level. Since the cytoplasmic granules in the Malpighian tubules differ significantly in wild type and ommochrome-deficient mutants (Yagi, 1969), we investigated the mechanism of brown pigmentation by analysing the effects of tryptophan metabolites on the fluorescent granules in tubules of eye color mutants, and the storage of 3-HK.

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MATERIALS AND METHODS

Drosophila strains and culture

The strains of *Drosophila melanogaster* used were as follows: the wild-type *Oregon-R* and eye color mutant strains, *vermilion brown* (*v;bw*), *cinnabar brown* (*cn bw*), *brown scarlet* (*bw;st*), *lightoid brown* (*ltd bw*) and *white* (*w*). Double mutant strains were obtained by crossing these single mutants. All the strains used in this study are listed by Lindsley and Zimm (1992). Animals were maintained at 25°C on standard corn meal glucose-yeast medium.

Administration of tryptophan and its metabolites

The larvae of eye color mutants were transferred 72–84 hr after egg laying from standard medium to medium supplemented with 3 mg of L-tryptophan, DL-kynurenine or 3-hydroxy-DL-kynurenine (3-HK) per 5 ml of glucose-agar medium at 25°C. In supplementation experiments, crowding was avoided by placing 10–20 individuals in each bottle.

Measurements of sizes and fluorescence intensities of granules in Malpighian tubules

After an appropriate feeding period, animals were dissected in the Ringer's solution described by Ephrussi and Beadle (1936). The Malpighian tubules were freed from the gut and were transferred a hole in a glass slide and vitally stained with 0.05% Nile blue sulfate in Ringer's solution, pH 6.8. The number of granules per cell was counted and the sizes of the granules in the proximal portions of anterior and posterior strands of the Malpighian tubules were measured under a microscope using an ocular micrometer with a 40×objective and a 10×ocular lens. The diameters of the granules were determined as more than about 2 μm and less than about 1 μm.

The Malpighian tubules of living larvae were dissected in Ringer's solution and the fluorescence of the granules in the tubules was examined directly under a microscope. A Nikon microfluorometer with a comparative photometric device was used to determine the fluorescence intensity of the granules. The fluorescence intensity of the granules is a measure of the relative value of the two posterior and two anterior strands of the tubules. Excitation light from a 200-watt high-pressure mercury lamp (Toshiba) was filtered through a UV-glass filter so that only the 365 nm region was transmitted. The fluorescence was measured with a 40×objective, a fluorescence filter (Wratten 2B), and a 10×ocular lens, focusing the objective sharply on the granules. A modified microspectrofluorometer attached to a microspectrophotometer (Zeiss, UMSP-1) was used to record the emission spectra from the granules within a spot of 20 μm diameter on excitation of 365 nm from a 450-watt xenon lamp (Osram). Fluorescence emission was measured with a 100×objective using a filter for cut off below 360 nm.

Determination of 3-HK

Individual Malpighian tubules or whole bodies were homogenized with 200 μl of 0.5 M perchloric acid / 50 μM sodium hydrogen sulfite in an ice cold microhomogenizer (Tokai M-100) set at scale 10 for 2 min. The homogenates were centrifuged at 15,000 g for 10 min at 4°C, and the supernatants were used for determination of the amount of 3-HK. 3-HK was determined by high-performance liquid chromatography (HPLC) with electrochemical detection (ECD). A Yanaco HPLC apparatus (model L-5000) equipped with a column (φ4.6×250 mm) packed with Yanaco ODS-T and a Yanaco ECD detector (model VMD-101 A) were used. 3-HK was eluted at 25°C from the column with a degassed solution of 0.1 M sodium dihydrogen phosphate containing 10 mM EDTA at a flow rate of 1.5 ml/min and then determined at a voltage of 0.5 V (Ag vs AgCl).

Chemicals

L-tryptophan, DL-kynurenine and 3-hydroxykynurenine were purchased from Sigma Chemical Co., USA. Nile blue sulfate was from Schmid & Co., Chroma-Geselshaft, Germany. Other chemicals were of analytical grade.

RESULTS

Cytological observations

Figure 1 shows the appearance of excised Malpighian tubules of early 3rd instar larvae (84 hr) of the wild-type *Or-R* and the eye color mutants *cn bw* and *w* observed by light (A, B and C) and fluorescence (D, E and F) microscopes. Intact cells of Malpighian tubules of *Or-R* and *cn bw* contained numerous fluorescent granules, usually of a spherical form which emitted blue fluorescence on excitation at 365 nm and were stained with Nile blue sulfate. These granules appeared in the tubules of first instar larvae within 24 hr after hatching. During later larval and pupal stages, the stainability with Nile blue of the granules decreased, as reported previously (Yagi, 1969). The granules were also vitally stained with other basic dyes such as neutral red and Janus green. The wild-type strain *Or-R* contained fluorescent granules with a maximum diameter of 2–3 μm that showed weak blue fluorescence with an emission maximum of around 480 nm (Figs. 1A, D and 2A). In contrast, the fluorescent granules of the nonautonomous mutants *v;bw* and *cn bw* were about 1 μm in diameter and emitted a strong blue fluorescence (Fig. 1B and E). The peak of the emission spectrum of *v;bw* was at around 440 nm and that of *cn bw* at around 470 nm (Fig. 2 B and C). The autonomous eye color mutants *bw;st, ltd bw* and *w* were devoid of fluorescent granules (Fig. 1C and F). For accurate determination of the number of granules, the procedure using Nile blue sulfate by light microscopy was more suitable than that using fluorescence microscopy. Vital staining with Nile blue sulfate was employed to examine the number and size of granules. In general, granules in the proximal part of the tubules were somewhat larger than those in the distal part. In this study we examined the granules in only the proximal part of the tubules.

As shown in Tables 1 and 2, the strains examined were classified into the following three groups according to the fluorescence intensity and size of the granules in early 3rd instar larvae (84 hr).

Group 1: [*Or-R*]. The fluorescent granules in the Malpighian tubules emitted weak fluorescence and were mostly about 2 μm or more in diameter (Figs. 1A, D and 2A).

Group 2: [*v;bw, cn bw*]. The fluorescent granules in the Malpighian tubules emitted strong blue fluorescence and were mostly about 1 μm or less in diameter (Figs. 1B, E and 2B, C).

Group 3: [*bw;st, ltd bw, w*]. Fluorescent granules were not present or were difficult to detect (Fig. 1C and F).

Changes of fluorescent granules induced by ommochrome precursors

We attempted to modify the formation of the fluorescent granules in the Malpighian tubules in the eye color mutant strains by adding ommochrome precursors. Feeding of DL-kynurenine (400 μg/ml) to *v;bw* and 3-hydroxy-DL-kynurenine (400 mg/ml) to *cn bw* caused the formation of eye pigment which could not be distinguished in color from that of the wild-type (Schwabl and Linzen, 1972). The mutant *bw* lacked red pigments and produced white eyes in combination with the mutants *v, cn, st* and *ltd*. To monitor the brown eye pigmentation by ommochrome precursors, we used the double mutants *v;bw, cn bw, bw;st* and *ltd bw* in this study.

The fluorescence intensity of the granules decreased when *v;bw* larvae were given kynurenine and when *cn bw*

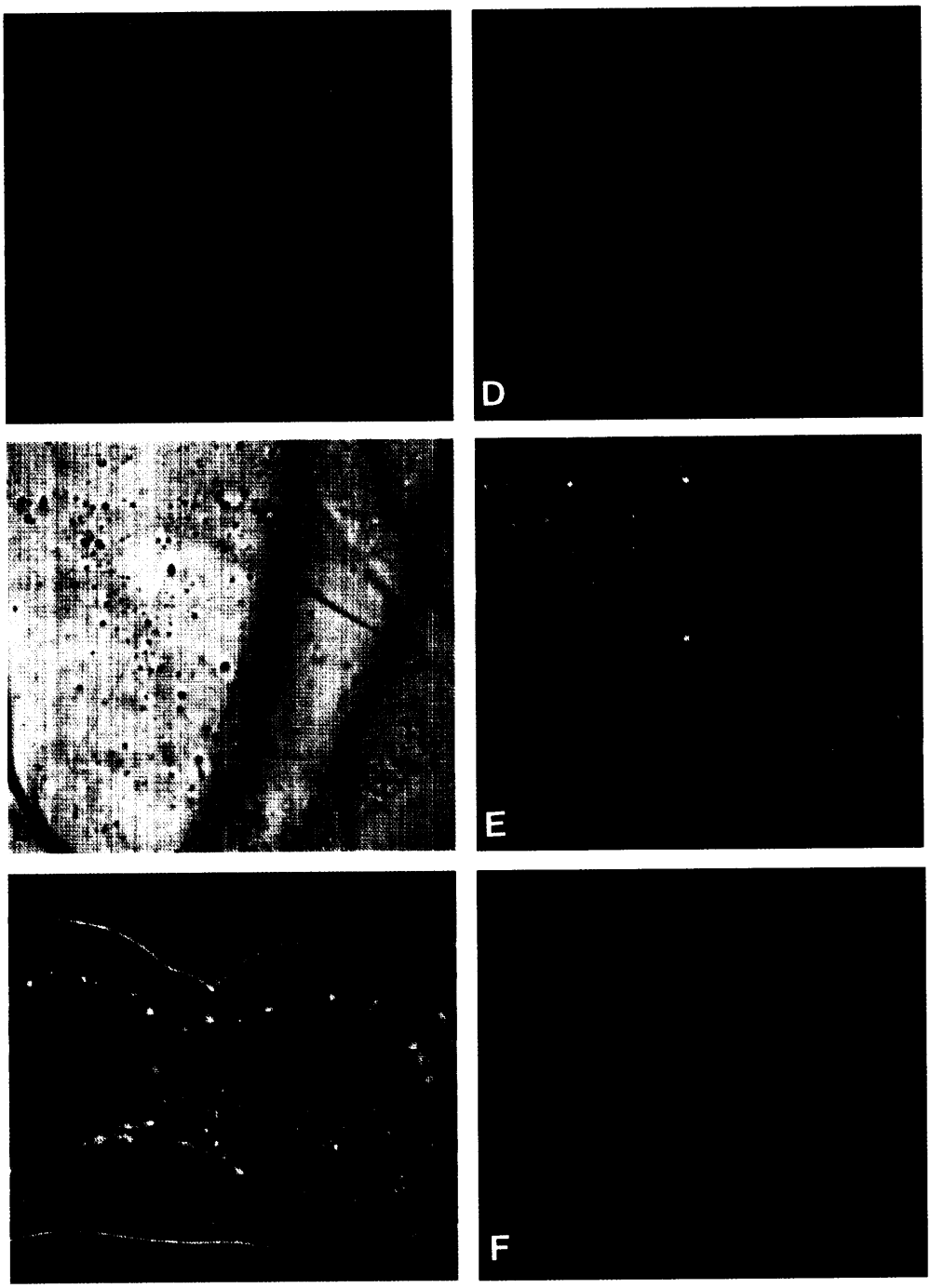

Fig. 1. Nile blue staining (left) and fluorescence (right) of Malpighian tubules in early 3rd instar (84 hr) larvae of wild type *Or-R* (A, D) and the eye color mutants *cn bw* (B, E) and *w* (C, F). Bar, 5 μm.

larvae were given 3-HK (Fig. 3A and B). These decreases continued from about 3 hr after giving the compounds, reaching lower levels. No fluorescence changes were observed when *v;bw* larvae and *cn bw* larvae were given tryptophan. The size of the granules began to increase within 7 hr after administration of kynurenine to *v;bw* larvae and 3-HK to *cn bw* larvae (Tables 3 and 4), shifting towards the level of the wild-type and reaching on maximum after about one day (23–29 hr). No size alteration was detected in *v;bw* or *cn bw* larvae even one day (28–34 hr) after feeding tryptophan. The number of the granules per cell increased slightly in *v;bw* one day after feeding kynurenine, but did not change in *cn bw* after feeding 3-HK.

The above observations indicated that pigment precursors are probably incorporated into the fluorescent granules. As the tubule cells in the *bw;st, ltd bw* and *w* strains were devoid of the granules, they failed to take up or deposit the precursors and there should have been no cytological changes after feeding tryptophan metabolites. To examine these possibilities, we administered the larvae of the autonomous mutant strains

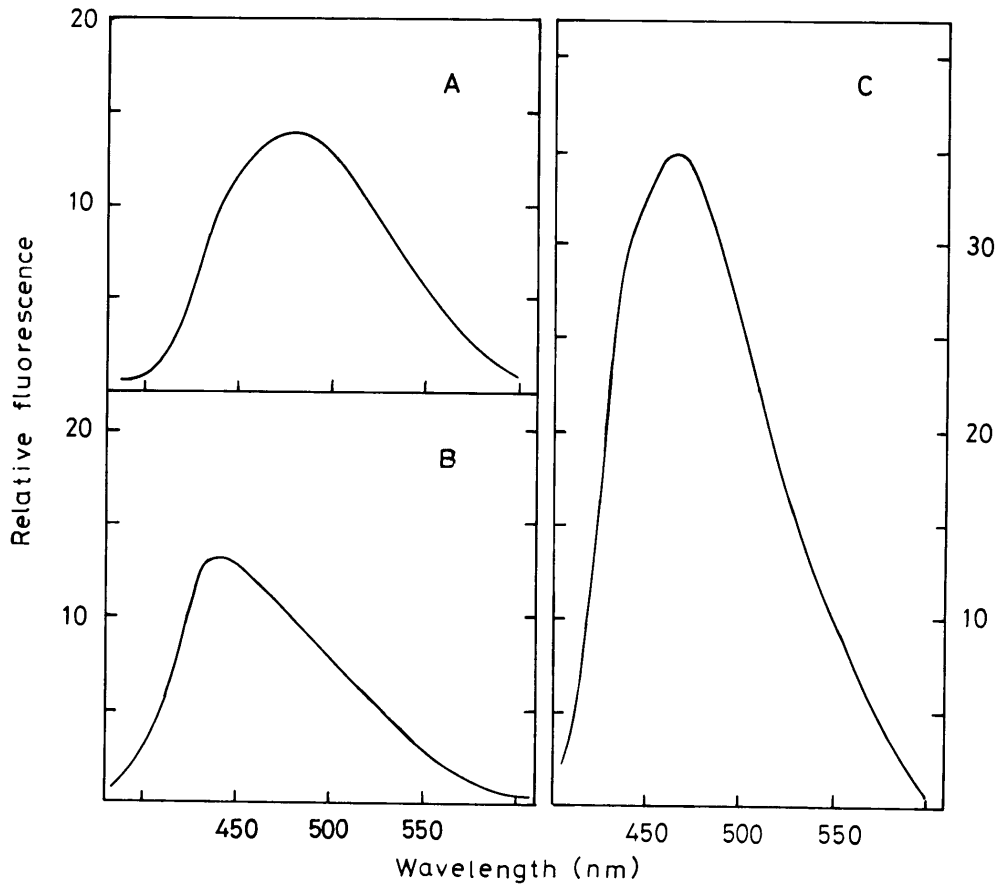

Fig. 2. Fluorescence spectra of granules in Malpighian tubules in early 3rd instar (84 hr) larvae of wild type *Or-R* (A) and eye color mutants *v;bw* (B) and *cn bw* (C). All fluorescence intensities are means for three to six determinations.

Table 1. Fluorescence intensities of granules in Malpighian tubules of early 3rd instar (84 hr) larvae of the wild type and eye color mutants

Strain	Number of individuals examined	Fluorescence intensity in arbitrary units (Mean ± SE)
Or-R	10	11.9 ± 0.6
v;bw	9	16.9 ± 0.4
cn bw	7	19.5 ± 0.5

Table 2. Relative sizes and numbers of granules per cell in Malpighian tubules of early 3rd instar (84 hr) larvae of wild type and eye color mutants

Strain	Number of individuals examined	Average number of granules per cell (Mean ± SE with percentage in parenthesis)	
		Large*	Small**
Or-R	8	42.0 ± 9.7(63.9)	23.7 ± 7.9(36.1)
v;bw	8	0	30.1 ± 4.8(100)
cn bw	13	11.0 ± 4.1(20.3)	43.2 ± 8.8(79.7)

*: Granules of about 2 μm or more in diameter.
**: Granules of about 1 μm or less in diameter.

bw;st, ltd bw and *w* with tryptophan, kynurenine and 3-HK, respectively. No fluorescent granules appeared in the Malpighian tubules after feeding the larvae with these compounds for one day or two days (19–51 hr).

Accumulation of 3-HK

Third instar larvae (72–90 hr) were isolated and dissected in Ringer's solution. The contents of 3-HK per individual of the wild-type, *Or-R* and the mutant strains *v;bw*, *cn bw*, *bw;st*, *ltd bw* and *w* were then determined by HPLC analysis. In *Or-R*, 3-HK was actively transferred into, and accumulated in the tubules, whereas it was not in *v;bw*, *cn bw*, *bw;st* , *ltd bw* or *w* (Table 5). The 3-HK contents of the tubules were approximately equal to that of *Or-R* after administration of kynurenine to *v;bw* or 3-HK to *cn bw* for 24 hr, whereas administration of tryptophan to *v;bw* and *cn bw* larvae did not result in accumulation of 3-HK in the tubules. In the nonautonomous mutant strains *v;bw* and *cn bw*, pigment precursors might also have been deposited in the granules resulting in fluorescence changes and increase in size of the granules. On the other hand, when larvae of the mutant strains *bw;st, ltd bw* and *w*, which lack the fluorescent granules, were given the tryptophan metabolites tryptophan, kynurenine and 3-HK, no accumulation of 3-HK

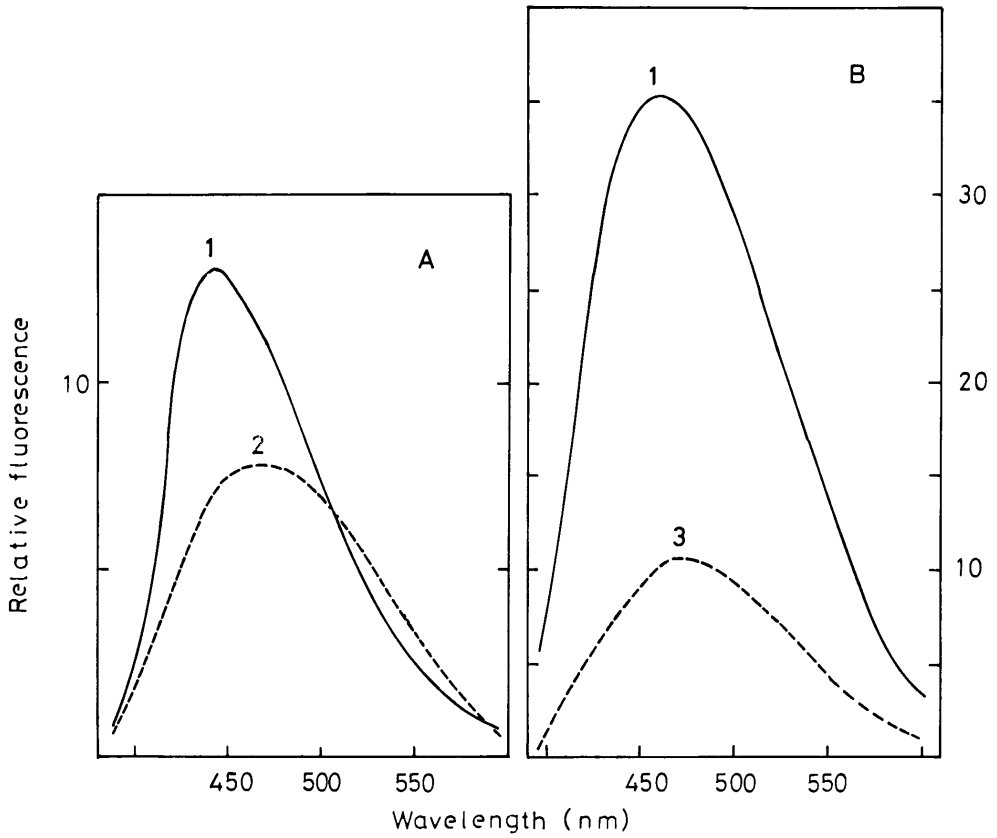

Fig. 3. Effects of tryptophan metabolites on the relative fluorescence of granules in the Malpighian tubules of eye color mutants *v;bw* (A) and *cn bw* (B). *v;bw* and *cn bw* larvae were cultured for 18 to 46 hr in the presence of tryptophan (1), kynurenine (2), or 3-hydroxy-kynurenine (3). All fluorescence intensities are means for three to six determinations.

Table 3. Effects of dietary administrations of tryptophan and kynurenine on the relative sizes and numbers of granules in mutant strain *v;bw*

Addition to diet	Duration of treatment (hr)	Number of individuals examined	Average number of granules per cell (Mean ± SE with percentage in parenthesis)	
			Large*	Small**
trp***				
	4–4.5	9	0.1± 0.1 (0.3)	34.2± 7.5 (99.7)
	8	3	0 (0.0)	29.2±18.1 (100)
	28–34	8	0.1± 0.3 (0.2)	40.6±11.6 (99.8)
kyn****				
	2.5–3	12	1.1± 1.0 (2.7)	39.8±12.9 (97.3)
	5–5.5	6	7.9± 6.3 (16.7)	39.5±11.7 (83.3)
	6–7	6	12.0±10.2 (22.6)	41.2±13.5 (77.4)
	27–28	7	30.8±19.7 (50.2)	30.6±17.7 (49.8)

*, **: For abbreviations, see Table 2
Diets supplemented with 3 mg of tryptophan (***) and kynurenine (****), respectively, in 5 ml of culture medium.

Table 4. Effects of dietary administrations of tryptophan and 3-hydroxykynurenine on the relative sizes and numbers of granules in mutant strain *cn bw*

Addition to diet	Duration of treatment (hr)	Number of individuals examined	Average number of granules per cell (Mean ± SE with percentage in parenthesis)	
			Large*	Small**
trp***				
	4–4.5	9	3.6± 1.7 (7.2)	46.4± 5.4 (92.8)
	8	7	6.3± 2.5 (10.3)	54.9±21.2 (89.7)
	29–34	9	4.0± 3.1 (7.1)	52.3±18.4 (92.9)
3-HK****				
	3	10	5.6± 3.5 (9.9)	51.1± 8.5 (90.1)
	5	7	12.1± 4.8 (19.1)	51.3±13.0 (80.9)
	6–7	13	17.6± 6.8 (30.5)	40.2±12.2 (69.5)
	9	4	26.3± 5.0 (48.1)	28.4±12.2 (51.9)
	23–29	9	47.8±11.4 (64.0)	26.9±13.6 (36.0)

*, **: For abbreviations, see Table 2
Diets supplemented with 3 mg of tryptophan (***) and 3-HK (****), respectively, in 5 ml of culture medium.

Table 5. Contents of 3-hydroxykynurenine in the body and Malpighian tubules of wild type and eye color mutants

Strain	3-HK pmol/individual (Mean ± SE)		
	WB	MT	MT/WB(%)
Or-R	909±68	930±5.3	102
v;bw	41± 4	21±3	51
cn bw	ND	ND	0
bw;st	ND	ND	0
ltd bw	ND	ND	0
w	ND	ND	0

Values are means of separate five experiments.
ND; not detectable. WB; whole body. MT; Malpighian tubules.

was observed in the tubules. These results support the hypothesis that the components in the fluorescent granules of the tubules were involved in the uptake or storage of pigment precursors.

DISCUSSION

The mechanism of the metabolic conversion of 3-hydroxy-kynurenine to xanthommatin is still unknown. Cytoplasmic granules have been considered to be important for ensuring the final enzyme activity of the pathway and for binding 3-HK in particles within pigment cells. The *wa* mutant of the Mediterranean meal moth, *Ephestia kühniella* (Caspari and Gottlieb, 1975) and the *cream* and *pearl* mutants of the honey bee, *Apis melifera* (Dustmann, 1968, 1975) appear to lack the capacity to bind 3-HK to granules in eye pigment cells. The formation of granular 3-HK in these cells is suggested to be a necessary prerequisite for the enzymatic conversion of the intermediate to xanthommatin, which probably also takes place in association with pigment granules. The initial phase of granular 3-HK is not observed in the eye pigment cells of any mutants of *D. melanogaster*. Ommochrome pigment in the primary and secondary pigment cells of ommatidia, is believed to develop as a vesicular secretion by way of the Golgi apparatus (Shoup, 1966). In the mutants *v* and *cn* of *Drosophila*, which contain neither ommochrome eye pigment nor "empty" pigment granules, feeding of kynurenine or 3-HK causes the formation of wild-type pigment granules (Schwabl and Linzen, 1972). However, the reduction in the amounts of brown pigments in *w*, *st* and *ltd* mutants is due to a transport deficiency of ommochrome precursors (Sullivan and Sullivan, 1975). In addition, Sullivan *et al.* (1980) suggests that Malpighian tubules have a transport system that enables entry of tryptophan into a cellular pool and that the specific membrane transport systems result in the compartmentalization of cellular pools.

The cytoplasmic granules in the Malpighian tubules are different among wild-type and eye color mutants, and in the latter, *w* and *st* are absent in these granules (Yagi, 1969). The result indicates that the cellular site of transport system or storage of ommochrome precursors might be involved in the cytoplasmic granules. In the present study, attention was focused on the mechanism of uptake and storage of precursors in relation to the granules in larval Malpighian tubules.

When *v;bw* and *cn bw* larvae were cultured in the medium supplemented with kynurenine or 3-HK, respectively, the accumulation of the compounds in the Malpighian tubules was always accompanied by a decrease in the fluorescence intensity of the granules (data not shown). Only then the fluorescent granules begin to increase in size (Tables 3 and 4). These observations suggest that the increase in the size of the fluorescent granules could be caused by the molecular interaction of fluorescent components with the incorporated tryptophan metabolites in the granules but could not be caused by the accumulation of 3-HK. In addition, our micro-spectrophotometric studies show that the maximum emission

of the fluorescent granules at early 3rd instar larvae of wild-type, *v;bw* and *cn bw* was around 480, 440 and 470 nm, respectively, but that the net fluorescence intensity of wild-type was much weaker in the granules that in *v;bw* and *cn bw* (Table 1 and Fig. 2).

When the Malpighian tubules of *Or-R* were treated with 1% OsO_4, a black color develops in the fluorescent granules after incubation for 30 min. This staining is due to the reaction of OsO_4 with 3-HK (Wessing and Eichelberg, 1968). Treatment with OsO_4 stained the small fluorescent granules of *v;bw* and *cn bw* yellow. The small granules of *bw;st, ltd bw* and *w* were also stained pale yellow by OsO_4 treatment (Yagi, unpublished data). The fluorescent materials in the Malpighian tubules can be separated by TLC and HPLC to yield the three major components pteridines, riboflavin and tryptophan metabolites, and they present the specific pattern in the pool levels of the components by these mutations (Wessing and Eichelberg, 1968; Yagi *et al.*, 1991). Both pteridines and riboflavin are known to form complexes with tryptophan or similar compounds (Fujimori, 1959). This is probably the cause of the differences in emission spectra of the granules in the Malpighian tubule cells among the wild-type, *v;bw* and *cn bw*. We have confirmed this with an experiment in which *v;bw* and *cn bw* strains were cultured in the medium with kynurenine and 3-HK (Fig. 3). The fluorescence emission pattern of these larvae were very close to that of the fluorescent granules in the wild-type tubule cells because the quenching process tended to decrease fluoresence. In view of our findings, we confirm that the presence of fluorescent materials, pteridines and riboflavin in the granules is related to the incorporation and storage of tryptophan metabolites and precursors of xanthommatin in Malpighian tubule cells.

3-HK is subjected to known detoxication mechanism and the compound has a protective role against peroxidation of membrane because of high reactivity with active oxygen (Linzen, 1974; Wadano *et al.*, 1993). Meanwhile, xanthine oxidase and dehydrogenase activities of the Malpighian tubule of wild-type flies are demonstrable cytochemically in bodies like those containing catalase (Beard and Holtzman, 1987). The oxidase activity is critical to the degradation of purines in many organisms; the dehydrogenase function is central to the formation of pteridine eye color pigments in *Drosophila*. The *rosy* mutation, with a deletion for xanthine dehydrogenase, appears to affect the morphological characteristic, and excretory materials of the tubules (Hadorn, 1952). Sullivan *et al.* (1979) proposed that the connection between the ommochrome and pteridine biosynthetic pathways resides in the sharing of a common system for precursor transport. Our suggestion regarding the changes in the fluorescent granules resulting in the interelation between 3-HK and pteridine or riboflavin is supported, albeit indirectly, by the available data. Thus, it is likely that an interaction between precursors of xanthommatin and fluorescent materials in the cytoplasmic granules is responsible for the increase in size of granules in the Malpighian tubules.

An issue to be settled is the identity of the fluorescent compound components of the granules in the Malpighian tubules which may be involved in the brown pigmentation mechanism, and the storage of 3-HK. We are hopeful that understanding of the nature of the compound for these incorporation in the granules, and understanding of the storage systems will enable the testing of hypotheses regarding the role of fluorescent granules in pigmentation.

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