STUDIES ON TRYPTOPHAN PYRROLASE IN DROSOPHILA MELANOGASTER

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DURING the course of an intensive study of eye pigment differentiation in *Drosophila melanogaster* using transplantation techniques, two diffusible precursors of the brown pigments were discovered (cf. Review by EPHRUSSI 1942). Originally called v^+ and cn^+ substances or hormones, these compounds were ultimately identified as kynurenine and 3-hydroxy-kynurenine, respectively. The metabolic pathway by which tryptophan is believed to be converted to ommochromes or brown pigment is shown in the following scheme:

$$\text{tryptophan} \xrightarrow{a} \text{formylkynurenine} \xrightarrow{b} \text{kynurenine}$$
$$\downarrow c$$
$$\text{hydroxykynurenine}$$
$$\downarrow d$$
$$\text{ommochrome}$$

Mutants of *Drosophila melanogaster* are known in which the single steps a, c and d are thought to be blocked. Of these, perhaps the most detailed genetic information is available for the vermilion (v) mutants in which reaction a appears to be blocked.

While tryptophan pyrrolase, the enzyme which catalyzes the conversion of tryptophan to formyl kynurenine, has been studied extensively in animal systems (KNOX and MEHLER 1950; TANAKA and KNOX 1959), until recently it could not be detected in *Drosophila melanogaster*. In 1960, BAGLIONI reported results of studies on the enzyme in various eye color mutants of *Drosophila melanogaster*. He demonstrated for the first time at the enzyme level that v mutants were partially blocked in the conversion of tryptophan to formylkynurenine; mutants believed to be blocked in later steps in the ommochrome pathway, such as cinnabar (*cn*) and scarlet (*st*) blocked at reaction c and d respectively, contained normal or increased amounts of tryptophan pyrrolase. It had already been reported that the enzyme could be detected in Ephestia and was missing in the *a* mutant, which is thought to be homologous with the *v* mutants of *Drosophila melanogaster* (EGELHAAF 1958).

In the present report, more sensitive assay procedures for assaying tryptophan pyrrolase will be described. Using these methods, the enzymes system has been studied in wild-type *Drosophila melanogaster* at various stages of development, as well as in several eye color mutants.

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MATERIALS AND METHODS

Flies were raised at 25° on a media of cornmeal, sugar, agar, propionic acid and dried brewers yeast.

Strains: *su-s* v^1;*bw*, *su-s* v^{36f};*bw*, and v^{36f} were obtained from DR. BUZZATI-TRAVERSO, Institute of Genetics, Pavia. Oregon R and *bw*, and a strain of v^1;*bw* was kindly supplied by DR. C. CLANCY, University of Oregon. The other strains including the Sevelin were from the collection of the Zoology Institute.

Enzyme assays, macro method: The method is a modification of the one used by BAGLIONI (1960). Flies were lightly etherized, weighed and homogenized in the cold with four volumes of 0.14 M KCl–0.0025 M KOH in an all glass homogenizer. Approximately 0.05–0.2 g of flies were homogenized at one time. After centrifugation at 0° at 16,000 × *g* for 15 minutes, the clear supernatant fluid was used for the enzyme assay. The reaction mixtures contained the following components (in micromoles): potassium phosphate buffer, pH 7.4, 100; ascorbate 4.0; L-tryptophan, 3.6; extract, 0.1 or 0.2 ml and water up to 1.0 ml. (Higher ascorbate concentrations than 4.0 μmoles/ml interfere in the BRATTON-MARSHALL test.) Incubations were carried out in open test tubes with shaking at 37° for about two hours. The reaction was stopped by the addition of 3.0 ml of 6.7 percent trichloroacetic acid and after centrifugation kynurenine was determined on 3.0 ml of the supernatant fluid by the BRATTON-MARSHALL (1939) procedure. The tubes were stored at 0° for 14 hours before determining the optical density at 560 mμ in a Beckman Model DU spectrophotometer. In each case a control tube in which tryptophan was omitted from the enzymatic incubation but added after trichloroacetic acid addition was carried through the whole procedure. Since tryptophan gives a slight color in the BRATTON-MARSHALL test, it is essential to add tryptophan prior to color determination. All reported values have been corrected for the color in the no tryptophan control tube.

It should be mentioned that assay of tryptophan pyrrolase by measuring kynurenine formation is valid only if kynurenine formamidase, the enzyme which converts formylkynurenine to kynurenine is not rate limiting. Since GLASSMAN (1956) has shown that the formamidase is present in all of the strains used in amounts which are far in excess of the levels of tryptophan pyrrolase noted here, the assay is probably a true reflection of tryptophan pyrrolase activity.

Enzyme assay, micro method: This procedure is based on the same principles as the assay which has been used for detecting the enzyme in Ephestia, where the enzymatically synthesized kynurenine is separated from interfering components in the reaction mixture by paper chromatography and then quantitatively determined by measuring the fluorescence (EGELHAAF 1958). The flies were homogenized in 1 ml conical glass centrifuge tubes, using .03–.04 ml of the KCl–KOH solution per fly. Between one and ten flies were homogenized at a time. The homogenate was centrifuged without transfer at approximately 5500 × *g* for 15 minutes. Exactly 0.03 ml of the supernatant fluid was added to a reaction mixture which was similar to the one described above, scaled down to a volume of 0.09–0.10 ml. The reaction was stopped by heating at 100° for 30 seconds and

centrifuged again for 15 minutes at 5500 × *g*. One third of the total supernatant fluid (i.e., 0.03 ml) was spotted on Whatman #1 paper and the paper was developed in *n*-propanol:one percent NH_3 (3:1) for 1-1/2–2 hours at 21–22°. Controls in which tryptophan was added only after the 30 second heating period were routinely included as in the macro assay. (Control experiments have also been performed in which tryptophan was added to a complete reaction mixture which was immediately heated for 30 seconds and then incubated for the regular period of two hours at 37°. No kynurenine formation was detected under these conditions.) A known amount of kynurenine was also spotted on the paper, added to a duplicate 0.03 ml sample of one of the reaction mixtures. This was necessary for the quantitation of the results since standard amounts of kynurenine cochromatographed with the reaction mixture gave consistently higher values than kynurenine chromatographed alone. This is probably due to a slow decomposition of the kynurenine during chromatography under these conditions, a decomposition which is partially prevented by some component in the reaction mixture. Finally, the fluorescence of the kynurenine spots was determined directly on paper by the method of HADORN and KÜHN (1953). With this micro assay, tryptophan pyrrolase activity could easily be detected in a single fly. For still greater sensitivity, a larger aliquot of the reaction mixture could be applied to the paper for chromatography.

The results of the micro assay are expressed as enzyme activity per fly while those of the macro assay are expressed as activity per g flies. If one assumes that a fly weighs approximately one mg the data from both assays can be compared by multiplying the micro assay figures by 1000.

RESULTS

General properties: Recent work on the mammalian tryptophan pyrrolase has clarified the role of H_2O_2 in the system; it has been shown that H_2O_2 stimulates the reaction by reducing the enzyme to the active form (TANAKA and KNOX 1959). In previous studies with the liver enzyme system, auto oxidizable dyes were added to generate H_2O_2 while in the more recent work, ascorbate was included for this purpose. When the effect of ascorbate was tested with extracts from flies, a twofold–threefold increase in kynurenine formation was observed, as shown in Figure 1. On further investigation, it was found that the ascorbate stimulation was markedly dependent on the age of the organisms used. In larvae and early pupae, little or no kynurenine formation could be detected in the absence of ascorbate. A clue to the lack of activity under these conditions was provided by the observation that after the two hour incubation at 37°, larvae and early pupae extracts turned black, presumably due to melanin formation. Since GLASSMAN (1957) has reported that kynurenine can disappear in larvae extracts of Drosophila through a nonenzymatic reaction with tyrosinase-produced quinones, it seemed likely that the absolute requirement for ascorbate shown by the larval tryptophan pyrrolase enzyme was due to a dual role of the ascorbate: (a) it served to generate H_2O_2 and thereby activate the enzyme, (b) it could nonenzymatically reduce the tyrosinase-produced quinones and thus prevent the

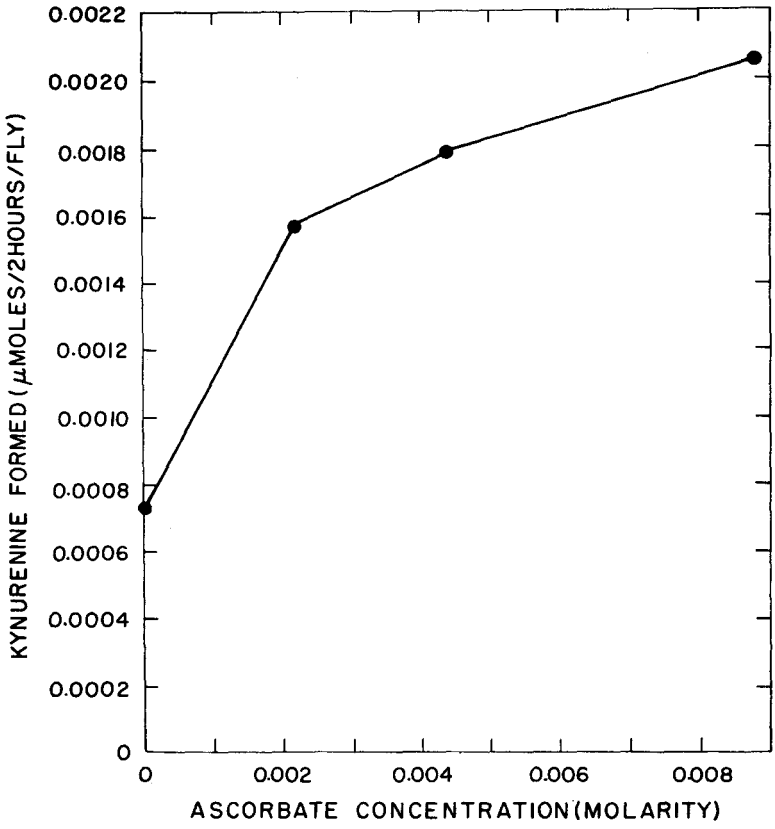

FIGURE 1.—The effect of ascorbate concentration on the rate of kynurenine formation. Micro assay using 11 day old flies.

loss of kynurenine. In support of these conclusions is the observation that larval extracts do not turn black when ascorbate is included in the reaction mixture. Furthermore, kynurenine can be quantitatively recovered from reaction mixtures containing larvae or pupae extracts only if ascorbate is present.

The effect of tryptophan concentration on the rate of kynurenine formation is shown in Figure 2. The K_m is approximately 1×10^{-3}M which is about two times higher than the value obtained in the mammalian enzyme (KNOX and MEHLER 1950). On a weight basis, the activity is six times greater than that reported by BAGLIONI (1960) and about one half the value in rat liver. Since in the latter comparison, enzyme activity in whole flies is being related to activity in liver, it seems likely that individual organs in *Drosophila melanogaster* may have a much higher specific activity than rat liver. The data in Figure 2 also serve to point up the fact that the enzyme activity values are minimal ones since in every case they represent tryptophan-dependent kynurenine formation, i.e., the activity due to the endogenous tryptophan in the crude extract has been subtracted. It can be seen, however, that the maximum rate is about ten times greater than the

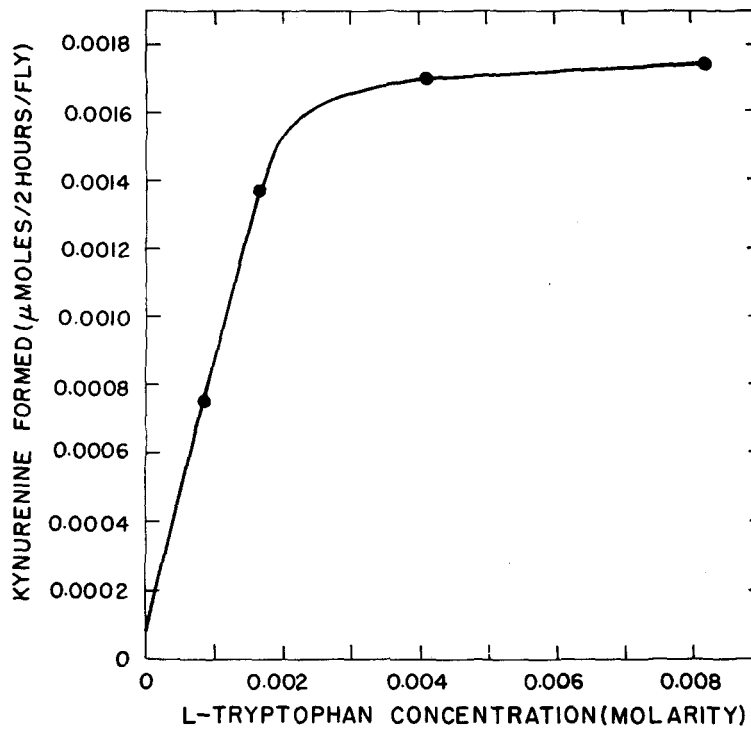

FIGURE 2.—The effect of L-tryptophan concentration on the rate of kynurenine formation. Micro assay using ten day old flies.

rate in the absence of added tryptophan (calculated from the ordinate intercept) and therefore the reported values are too low by a factor which probably does not exceed ten percent. In mutants such as *v*, where the endogenous tryptophan concentration may be three times higher than in wild-type strains (GREEN 1949), this factor could be greater. In spite of this theoretical possibility, with the assay methods used, no kynurenine formation could be detected when *v* extracts were incubated without added tryptophan.

Enzyme activity as a function of age: Figure 3 shows the levels of enzyme activity in the Sevelen strain at various stages of development. It can be seen that in the second instar larvae, the earliest stage tested, the activity is about one eighth the maximum adult level. (Since the activity is expressed on a constant weight basis, it is clear that a single second-instar larva has much less than one eighth the activity of a single fly.) The activity increases from the larvae to the pupae stage until it reaches a pupal maximum at six days and then declines.

Since brown pigment first appears in the eye about 50 hours after puparium formation, about 6–8 hours after v^+ substance formation begins in the eye (CLANCY 1940), this pupal peak in enzyme activity occurs around the same time as brown pigment formation begins.

Newly eclosed flies have about the same activity as late pupae. Within 24 hours

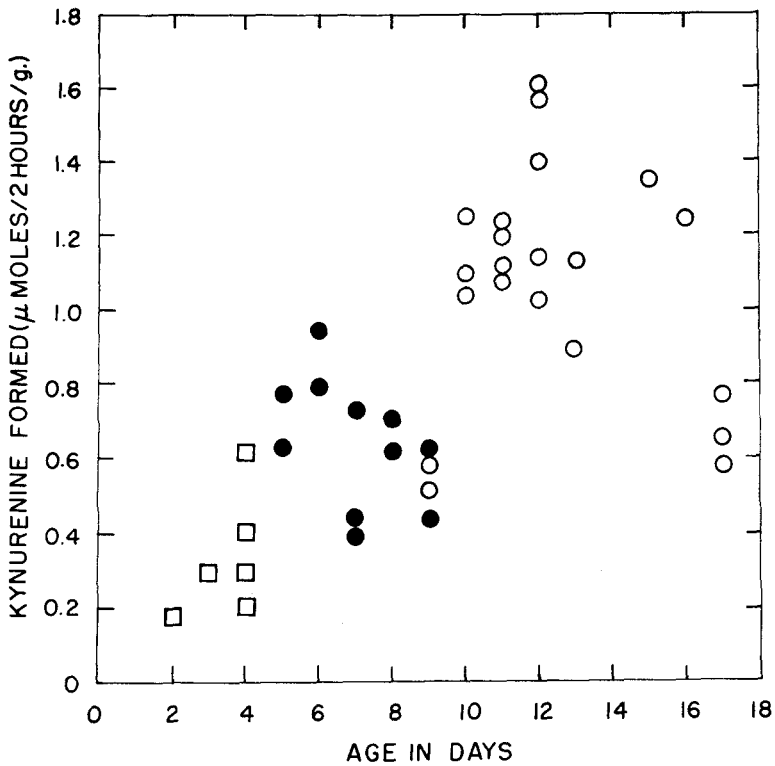

FIGURE 3.—Tryptophan pyrrolase activity as a function of age and developmental stage (macro assay). □, larvae; ●, pupae; ○, flies.

after eclosion, there is about a twofold increase in tryptophan pyrrolase activity which reaches a maximum at approximately 12 days, declining again in old flies.

The rise in enzyme activity after eclosion is somewhat surprising since most of the brown pigment has already been formed by this time. This second peak in activity may be a reflection of another metabolic role for the enzyme, independent of eye pigment formation. In relation to this rise after eclosion, it is of some interest that tryptophan pyrrolase in mammals is one of a group of enzymes which is almost completely absent from fetal liver, appearing only after birth (NEMETH and NACHMIAS 1958).

Enzyme in various strains: Table 1 summarizes the results of tryptophan pyrrolase determinations on flies of different strains. Oregon-R flies gave consistently lower results than the Sevelen strain. The marked difference in enzyme activity of the two different *bw* strains was also reproducible.

It can be seen that the *v* mutants have about 1–2 percent of the wild-type enzyme activities. This activity, while very low, is probably real since it has also been observed in the completely independent micro assay. The v^1 flies have somewhat more activity than the v^{36f} flies; indeed, the enzyme levels in v^{36f} flies are so low that they approach the sensitivity limits of the assays employed. The sup-

TABLE 1

Tryptophan pyrrolase activity in various strains of Drosophila melanogaster

Strain	Enzyme activity μmoles of kynurenine formed/2 hrs/g	
Sevelen	1.20	(1.04–1.62)
Oregon-R	0.92	(0.62–1.15)
v_1	0.02	
$v^1;bw$	0.02	(0.01–0.025)
v^{36f}	0.01	(0.005–0.015)
$su^2\text{-}s\ v^1;bw$	0.20	(0.08–0.23)
$su^2\text{-}s\ v^{36f};bw$	0.015	(0.007–0.020)
bw*	1.67	(1.43–1.93)
bw†	0.61	(0.45–0.79)

* Strain obtained at the Zoology Institute.
† Strain supplied by DR. C. CLANCY.
All assays carried out on flies between 11–12 days of age, using the macro assay. Figures in parentheses represent the range of values obtained in separate experiments.

pressor gene leads to about a tenfold increase in the activity in v^1 flies although this level is still only about 15 percent of the wild-type activity. On the other hand, the suppressor gene leads to only a slight and perhaps insignificant increase in enzyme activity in v^{36f} flies, confirming at the enzyme level the findings that v^1 is a suppressible and v^{36f} is a nonsuppressible mutant (GREEN 1952). Using a somewhat different assay, BAGLIONI (1960) found that v^1 flies had 10–15 percent of the wild-type activity instead of the much lower value reported here. It was also reported that $su^2\text{-}s\ v^1;bw$ flies had about twice the enzyme activity of the unsuppressed strains, in contrast to the tenfold increase due to the suppressor gene reported here. While the explanation for these discrepancies is not known with certainty[2], the values obtained here are consistent with older observations made on v mutants. Thus, it is known that v flies have little or no kynurenine (BEADLE, TATUM and CLANCY 1938) which suggests that the metabolic block in the mutant is almost complete. It would appear that the value reported here of 1–2 percent of wild-type activity in v^1 flies is more consistent with this idea than the value of 10–15 percent mentioned above.

It is also known that suppressors of vermilion lead to a dramatic increase in brown eye pigment in v^1 flies. Here again the reported tenfold increase in tryptophan pyrrolase in $su^2\text{-}s\ v^1;bw$ flies over the level found in the unsuppressed mutant would seem to be in better agreement with these observations than the previously reported twofold increase.

Enzyme in males and females: Because the v gene is sex-linked, it was of interest to see if a sex difference in the enzyme level could be detected. A comparison between tryptophan pyrrolase in males and females is shown in Table 2.

[2] In the previous study (BAGLIONI 1960), blanks in which tryptophan was omitted were used, but there is no indication that tryptophan was added after the enzymatic reaction had been stopped. Since tryptophan does give a slight color in the BRATTON-MARSHALL test, this omission could lead to falsely high values in the tryptophan-pyrrolase assay. The error could be quite significant when kynurenine formation is low, as is the case with extracts from v and $su^2\text{–}s\ v^1;bw$ flies.

When the enzyme activity is expressed per weight of flies, there is no difference between the sexes. Since females are heavier than males, this result indicates that the enzyme level may be somewhat higher in individual females than in males. Although females have two doses of the *v* gene and males only one, it is clear that this dosage is not quantitatively reflected in terms of enzyme levels.

Enzyme in v *heterozygotes*: A clearer picture of the effect of gene dosage on tryptophan pyrrolase levels was obtained by studying flies heterozygous for the *v* gene. To detect a maternal effect, possibly due to kynurenine supplied to the egg from +/+ mothers (GRAF 1957), both types of heterozygotes were prepared by the appropriate crosses and examined for tryptophan pyrrolase activity. The results shown in Table 3 demonstrate that the *v* heterozygotes contain less enzyme than the wild strain. It is also clear, however, that they contain significantly more than 50 percent of the wild strain enzyme, the range being 59–82 percent. Recently it was reported that the a^+/a heterozygote in Ephestia contains about 58 percent of the wild-type tryptophan pyrrolase (EGELHAAF and CASPARI 1960).

There is no indication of a maternal effect in the two different types of heterozygotes. Such an effect may only be a transitory one which could be detected by assaying for tryptophan pyrrolase activity at an earlier stage.

Enzyme localization: Some attempts have been made to localize the enzyme in various parts of the fly. While quantitative assays have not as yet been made on individual organs, the results shown in Table 4 indicate that the enzyme system is widely distributed. (The enzyme can be detected in malpighian tubes but quantitative data are not yet available.) In view of the role of this enzyme in eye pigment formation, the finding that 35–40 percent of the total activity is localized

TABLE 2

Tryptophan pyrrolase in males and females of Drosophila melanogaster *(Sevelen strain)*

Age (days)	Kynurenine formed μmoles/2 hours/g (macro assay)	
	Males	Females
9	0.54	0.51
10	1.07	1.11
13	0.89	0.92

TABLE 3

Tryptophan pyrrolase in vermilion heterozygotes

Assay method	Age	Kynurenine formed*		
			v/+	
	Days	+/+	*v*/*v* mother	+/+ mother
Macro	9	0.63	0.43	. . .
Macro	9	0.53	0.28	0.34
Micro	9	0.62	0.51	0.51
Micro	12	1.44	1.09	0.96

* For the macro assay, the figures represent μmoles kynurenine formed/2 hours/g. For the micro assay, the figures represent μmoles × 10^3 kynurenine formed/2 hours/fly.

TABLE 4

Tryptophan pyrrolase in different parts of Drosophila melanogaster *(Females)*

Tissue assayed	Enzyme activity μmoles kynurenine × 1000/2 hours/fly	
	10 day old flies	12 day old flies
Whole fly	1.56	1.89
Head	0.59	0.69
Thorax	0.59	0.47
Abdomen	0.26	0.45

in the head is of special interest. It had already been shown by transplantation and injection techniques that v^+ substance can be produced by the eye of Drosophila (CLANCY 1940).

DISCUSSION

The significance of the very low, but probably real, tryptophan pyrrolase activity in the v^1 mutant is difficult to evaluate at the present time. It is not known if it is due to a trace of the normal enzyme or to a slight catalytic activity of an altered protein produced by the mutant. The fact that the suppressor gene affects only the tryptophan pyrrolase activity of the v^1 mutant and apparently has no detectable effect on the enzyme activity of the v^+ allele (BAGLIONI 1960) may support the second possibility.

There is an impressive amount of evidence which indicates that the activity of many enzymes is far in excess of that required for maintaining the normal phenotype. Thus, in the majority of cases including the present one, heterozygotes in which the activity of a specific enzyme is decreased to almost half, have an essentially normal phenotype. Under special conditions, e.g., where the organism is subjected to a high concentration of the substrate of the affected enzyme, the heterozygote can usually be distinguished from the normal homozygote. Even more striking in this regard is the finding that in v^1 flies homozygous for the suppressor gene, an almost wild-type phenotype (i.e., eye color) can be maintained with only 15–20 percent of the normal amount of enzyme.

In the light of these considerations, it is possible that the low residual tryptophan pyrrolase activtiy in v^1 flies contributes significantly to their viability. It has been shown that under conditions of competition, the v gene has an effect on fitness of the flies, as well as on brown pigment synthesis (PARSON and GREEN 1959). It seems likely that an organism which is completely devoid of the enzyme would probably have an even lower fitness.

Two of the results obtained in the present study suggest a role for the tryptophan-pyrrolase enzyme system which is not directly related to brown pigment formation: (A) the finding of a large part of the enzyme in the abdomen plus thorax; and (B) the approximately 100 percent rise in enzyme activity after eclosion, after most of the brown pigment has already been synthesized. It is possible that this hypothetical second role for the enzyme, rather than the known one, is related to the decreased fitness of v flies.

SUMMARY

Tryptophan pyrrolase, the enzyme which, in the presence of an excess of kynurenine formamidase, converts tryptophan to kynurenine, has been followed at various developmental stages in wild-type *Drosophila melanogaster*. The activity can be detected in two day old larvae. It slowly rises to a peak in the middle of pupal life, followed by a second peak about two days after eclosion. Ascorbate, which stimulates the activity in the fly, is an obligatory requirement in larvae. One of the effects of ascorbate is to minimize the tyrosinase-mediated disappearance of kynurenine during the *in vitro* enzyme assay.

It has been shown that *v* flies have 1–2 percent of wild-type activity. Suppressor of vermilion leads to a tenfold increase in enzyme activity in v^1 mutants. By contrast, v^{36f} flies in the presence of the suppressor show only a negligible increase in activity. Vermilion heterozygotes have been shown to have less enzyme than wild type. While a significant percent of the total enzyme activity is present in the head, the enzyme has also been detected in thorax and abdomen.

The results have been correlated with some older observations on the production of v^+ substance.

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LITERATURE CITED

BAGLIONI, C., 1960 Genetic control of tryptophan pyrrolase in *Drosophila melanogaster* and *Drosophila virilis*. Heredity **15**: 87–96.

BEADLE, G. W., E. L. TATUM, and C. W. CLANCY, 1938 Food level in relation to rate of development and eye pigmentation in *Drosophila melanogaster*. Biol. Bull. **75**: 447–462.

BRATTON, A. C., and E. K. MARSHALL, 1939 A new coupling component for sulfanil-amide determination. J. Biol. Chem. **128**: 537–550.

CLANCY, E. B., 1940 Production of eye-color hormone by the eyes of *Drosophila melanogaster*. Biol. Bull. **78**: 217–225.

EGELHAAF, A., 1958 Nachweis eines gluabhängigen L-Tryptophanoxydase-Systems bei *Ephestia kühniella*. Z. Naturforsch. **13b**: 275–279.

EGELHAAF, A., and E. CASPARI, 1960 Über die Wirkungsweise und Genetische Kontrolle des Tryptophanoxydasesystems bei *Ephestia kühniella*. Z. Vererb. **91**: 373–379.

EPHRUSSI, B., 1942 Chemistry of "eye-color hormones" of Drosophila. Quart. Rev. Biol. **17**: 327–338.

GLASSMAN, E., 1956 Kynurenine formamidase in mutants of Drosophila. Genetics **41**: 566–574.

1957 Tyrosinase-produced quinones and the disappearance of kynurenine in larvae extracts of *Drosophila melanogaster*. Arch. Biochem. Biophys. **67**: 74–89.

GRAF, G. E., 1957 Biochemical predetermination in Drosophila. Experientia **13**: 1–4.

GREEN, M. M., 1949 A study of tryptophan in eye-color mutants of Drosophila. Genetics **34**: 564–572.

1952 Mutant isoalleles at the vermilion locus in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U. S. **38**: 300–305.

HADORN, E., and A. KÜHN, 1953 Chromatographische und fluorometrische Untersuchungen zur biochemischen Polypänie von Augenfarb-Genen bei *Ephestia kühniella*. Z. Naturforsch. **10b**: 582–589.

KNOX, W. E., and A. H. MEHLER, 1950 The conversion of tryptophan to kynurenine in liver. I. The coupled tryptophan peroxidase-oxidase system forming formylkynurenine. J. Biol. Chem. **187**: 419–430.

NEMETH, A. M., and V. T. NACHMIAS, 1958 Changes in tryptophan peroxidase activity in developing liver. Science **128**: 1085–1086.

PARSONS, P. A., and M. M. GREEN, 1959 Pleiotropy and competition at the vermilion locus in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U. S. **45**: 993–996.

TANAKA, T., and W. E. KNOX, 1959 The nature and mechanism of tryptophan pyrrolase (Peroxidase-Oxidase) reaction of Pseudomonas and rat liver. J. Biol. Chem. **234**: 1162–1170.