

# ENZYMATIC STUDIES ON THE HYDROXYLATION OF KYNURENINE IN *DROSOPHILA MELANOGASTER*<sup>1</sup>

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THE classic pathway for brown pigment synthesis in *Drosophila melanogaster*, as determined by the accumulation of intermediates, involves the sequence, tryptophan → formylkynurenine → kynurenine → 3-hydroxykynurenine → brown pigments (for review see ZIEGLER 1961). Two of the enzymes in this pathway, tryptophan pyrrolase (BAGLIONI 1959, 1960; KAUFMAN 1962; MARZLUF 1965a, b) and kynurenine formamidase (GLASSMAN 1956) have been described, and the absence of tryptophan pyrrolase in the vermilion mutant of *Drosophila* has been documented.

However, the enzyme catalyzing the conversion of kynurenine into hydroxykynurenine has never been described in *Drosophila*, although it has been shown to be present in a mammalian system (DE CASTRO, PRICE and BROWN 1956), and some of its characteristics, as studied in this system, are known (SAITO, HAYAISHI, and ROTHBERG 1957). In this paper the existence of this enzyme in *Drosophila*, its virtual absence in the mutant cinnabar, and its very greatly reduced activity in the mutant white, are demonstrated. On the other hand, tryptophan pyrrolase activity in extracts of the cinnabar and white mutants is the same as in extracts of wild type.

## MATERIALS AND METHODS

Wild type Oregon-R and the white mutant of *Drosophila melanogaster* were from stocks maintained in our laboratories. The cinnabar mutant was obtained from the California Institute of Technology. Flies were raised at a temperature of 23° in large populations according to the method of MITCHELL and MITCHELL (1964). Eggs were collected over 4 to 5 hours in plastic bread boxes spread with standard cornmeal agar medium. Samples of larvae and pupae, at different stages of development, were harvested for experiments following the method of MITCHELL and MITCHELL.

*Preparation, partial purification and assay of kynurenine hydroxylase in wild-type.* (A) *Crude homogenate:* To each gram of chilled material (larvae, pupae or adults) were added 1.4 μmoles NADPH<sup>3</sup>, 2.0 units glucose-6-phosphate dehydrogenase (Sigma) (KORNBERG 1950), 130 μmoles glucose-6-phosphate, 1.2 μmoles potassium cyanide, and 260 μmoles phosphate buffer (pH 7.5). Total volume of fluid added was 1.4 ml. A glass-Teflon tissue grinder fitted into a mechanical

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<sup>3</sup> The following abbreviations are used throughout the paper: NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADP, nicotinamide adenine dinucleotide phosphate; NADH, reduced nicotinamide adenine dinucleotide; G-6-P, glucose-6-phosphate; G-6-PD, glucose-6-phosphate dehydrogenase.

press was used to homogenize each sample. With pupae and adult flies, homogenates were next squeezed through cheese cloth to remove pupal cases and other coarse particles.

Protein concentrations of such extracts, estimated by the method of Lowry *et al.* (1951), varied from 60 to 65 mg/ml. This crude homogenate (1 ml) was treated with a solution of DL-kynurenine sulfate (0.2 ml; 5  $\mu$ m); the whole was shaken 2 hr in an open 50 ml test tube at 37°C. The reaction was stopped by adding trichloroacetic acid (TCA; 40%; 0.25 ml/ml). A similarly incubated mixture without DL-kynurenine was used as a control. In this case, DL-kynurenine was added after the TCA. 3-Hydroxykynurenine was assayed in both samples by the method given below, and the net synthesis was calculated by difference.

(B) *Partial purification of kynurenine hydroxylase*: Crude homogenates of larvae, pupae, or adult flies were prepared as before using 2 ml/g tissue of cold sucrose solution (0.5 M), but with no other additions. An aliquot was assayed, as described below for kynurenine hydroxylase activity. The crude homogenate was then centrifuged at 1000  $\times g$  for 10 min, using a Model RC-2 Sorvall refrigerated centrifuge. The supernatant was recovered using a syringe, without disturbing the fat layer at the top and the cell debris at the bottom. An aliquot of this supernatant was saved for assay, and the remainder was centrifuged 1½ hr at 37,000  $\times g$ . The supernatant and pellet thus obtained were likewise assayed for kynurenine hydroxylase activity.

The fractions described above were shaken 2 hr in an open 50 ml test tube at 37°C with the following components: NADPH, 1.08  $\mu$ moles; glucose-6-phosphate, 100  $\mu$ moles; glucose-6-phosphate dehydrogenase, 0.2 unit; DL-kynurenine sulfate, 8  $\mu$ moles; phosphate buffer (pH 7.5), 200  $\mu$ moles; potassium cyanide, 10  $\mu$ moles; protein 16 to 20 mg; total volume, 2 ml. A control was run, as before, with no DL-kynurenine added until the mixture was deproteinized by addition of TCA (40%; 0.25 ml/ml). After centrifugation, aliquots of the various supernatants were assayed for 3-hydroxykynurenine and net synthesis was obtained by difference between the experimental and the appropriate control. *Estimation of 3-hydroxykynurenine*: 3-Hydroxykynurenine, an  $\alpha$ -aminophenol, was estimated by the nitrous acid technique of INAGAMI (1954). On treatment with nitrous acid, 3-hydroxykynurenine forms a diazo-oxide which has appreciable absorption at 390–400 m $\mu$ . Kynurenine does not interfere with the reaction. Thus the net increase in absorbancy at 400 m $\mu$ , after incubation and treatment with nitrous acid, gives a measure of enzymatically produced 3-hydroxykynurenine. Endogenous 3-hydroxykynurenine was assayed by the same method in homogenized, deproteinized, tissue samples. *Preparation and assay of tryptophan pyrrolase*: Preparation and assay of this enzyme were carried out using the method of KAUFMAN (1962) as modified by MARZLUF (1965). Estimations of endogenous diazotizable amine were made using the Bratton-Marshall procedure, as modified by BAGLIONI (1959), on extracts without incubation.

## RESULTS

Kynurenine hydroxylase activity was investigated at various stages of development of the insect. Figure 1 shows activities obtained from crude extracts. There is a pronounced peak in enzyme activity about 10¼ days from the time of egg collection. The majority of pupae in such samples were in the middle of brown pigment synthesis (i.e., eyes were light brown). At later stages of development, there is a continuous decline of kynurenine hydroxylase activity. Thus newly emerged flies have activity, but it is of a low order of magnitude. This may well account for the absence of information concerning this enzyme in *Drosophila*.

Substrate saturation of the enzyme was achieved with 0.004 M DL-kynurenine (Figure 2). A requirement for NADPH was shown, although NADH was also slightly effective. The amount of 3-hydroxykynurenine formed was proportional to the amount of protein in the reaction mixture up to 6–8 mg protein/ml. After

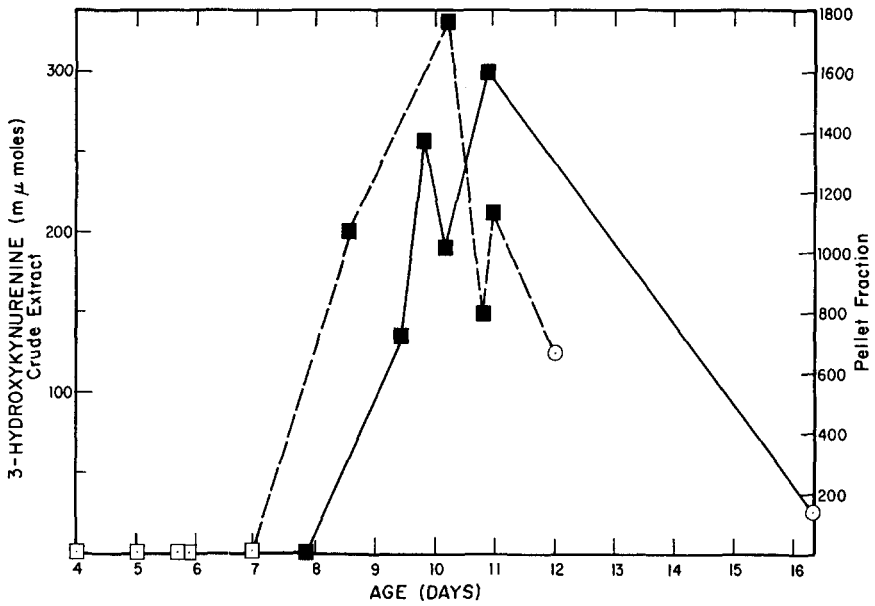


FIGURE 1.—Kynurenine hydroxylase activity in Oregon-R. □, larvae; ■, pupae; ○, adult flies. ---, activity in crude extracts per gram tissue after incubation for 2 hr at 37°; —, activity in pellet fractions/100 mg protein under the same incubation conditions.

a lag of about 20 min, 3-hydroxykynurenine production was linear for about 70 min and then continued at a slower rate for at least a further 30 min.

Using homogenates of pupae with maximum hydroxylase activity, attempts were made to purify the enzyme by differential centrifugation. The results are shown in Table 2. It can be seen that the highest activity was obtained with the pellet from high-speed centrifugation (mitochondria?).

Similar pellets from different developmental stages were then prepared and

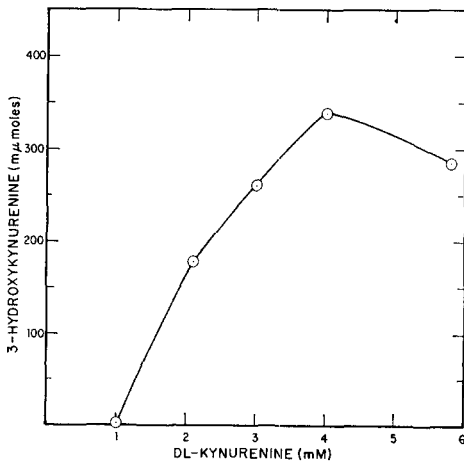


FIGURE 2.—Activity per gram tissue of kynurenine hydroxylase in crude pupal extracts (10 days after egg laying) as a function of DL-kynurenine concentration.

TABLE 1

*Effect of pyridine nucleotides on kynurenine hydroxylase in pupal homogenates*

	Additions	mμmoles 3-hydroxykynurenine per g pupae per 2 hr
Experiment I	none	25
	NADP, G-6-P, G-6-PD	212
Experiment II	none	39
	NADP, G-6-P, G-6-PD	204
	NADPH	304
	NADH	54

Crude pupal homogenates were prepared 8 to 8½ days from egg laying. Quantities of reagents were as given in METHODS. NADPH was added at a concentration of 2.4 μmoles/ml, and NADH at 2.7 μmoles/ml.

TABLE 2

*Kynurenine hydroxylase activity of fractions from centrifugation of wild-type (Oregon-R) pupae (ca. 11 days from egg collection)*

Fraction	mμmoles of 3-hydroxykynurenine*	Protein mg-ml	Specific activity†
Crude extract	103	39	2.6
Cell free extract (1000 × g-10 min)	58	21	2.7
37,000 × g-1½ hr supernatant	33	20	1.6
37,000 × g-1½ hr pellet	328	21	15.9

\* mμmoles formed per ml after 2 hr at 37°.

† mμmoles formed per mg protein after 2 hr at 37°.

assayed. The results (Figure 1) confirm those obtained with crude homogenates. There is a peak of activity at 10½ days after egg laying, and, thereafter, a pronounced drop in activity. No activity was found in larvae, at least as early as we have tested them. It should be pointed out, however, that there was very little "pellet fraction" from larvae. As with crude homogenates, the pellet fractions require NADPH, or a system generating NADPH for activity (mμmoles 3-hydroxykynurenine formed 100 mg pellet protein per 2 hours at 37° in the absence of NADPH generating system, 60; in the presence of NADPH generating system, 1015).

Enzyme activity in "pellet" fractions was only slightly affected (13 to 16% loss in activity) by freezing (-20°) and thawing if the pellet was suspended in 0.5 M sucrose at a protein concentration of 20 to 24 mg/ml.

Cinnabar and white mutants were assayed for kynurenine hydroxylase activity in crude homogenates and in "pellet" fractions at the developmental stage (pupae) when the wild type shows maximum activity. The results are shown in Table 3. It can be seen that cinnabar has no hydroxylase activity (the very small activity shown by crude homogenates is of doubtful significance), and white has very greatly reduced activity.

Endogenous 3-hydroxykynurenine—as assayed by the nitrous acid technique

TABLE 3

*Comparative activities of kynurenine hydroxylase in pupae of wild-type Oregon-R, white and cinnabar*

	Crude homogenate*		Range	Pellet†	
	Range	Average		Range	Average
Oregon-R	304-308	327 (4)	1020-1600	1260 (4)	
white	74-91	83 (2)	470-660	540 (4)	
cinnabar	0-18	10 (3)	0	0	

\* Activities are expressed as  $\mu$ moles 3-hydroxykynurenine formed per g tissue after 2 hr incubation at 37°C.

† Activities are expressed as  $\mu$ moles of 3-hydroxykynurenine formed per 100 mg "pellet" protein after 2 hr incubation at 37°C.

Numbers in parentheses indicate the number of experiments performed.

—was also assayed in wild type and in the cinnabar and white mutants. The results are shown in Figure 3. There is a considerable spread of values for 3-hydroxykynurenine. In general, however, cinnabar contains very little "diazo-oxide positive" material, as does white. Since no further identification of the "diazo-oxide" positive material was made, it cannot be ruled out that the results obtained in this assay are due to other *o*-aminophenols.

Tryptophan pyrrolase activity in wild type and in white and cinnabar, is recorded in Table 4. At all stages of development, these activities are essentially similar. Thus, white is no different from wild type in its capacity to oxidize tryptophan, at least as measured by this assay system. Table 5 lists the endogenous amounts of diazotizable amine at various points in the life cycle for wild type and the same two mutants. It is not unreasonable to suppose that "diazotizable amine" can be equated with kynurenine, and, as would be expected on this

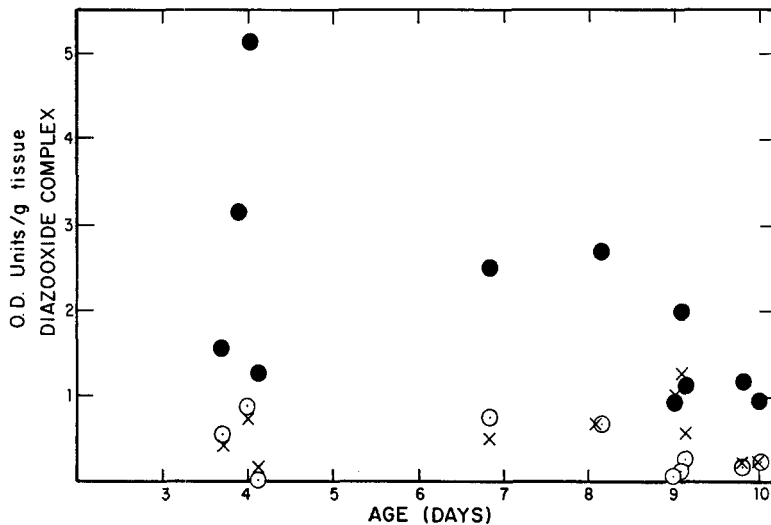


FIGURE 3.—Endogenous "diazo-oxide forming" material ( $\equiv$  3-hydroxykynurenine) at various stages of development in wild type, ●; white, ×; and cinnabar, ○.

TABLE 4

*Endogenous diazotizable amines (kynurenine?) in extracts of wild type and mutants\**

	Age		
	5½ days (larvae)	9 days (pupae)	13 days (adults)
Oregon-R	0.5 (2)	1.2 (4)	1.0
white	0.5 (2)	1.6 (3)	0.6 (2)
cinnabar	0.8 (2)	2.7 (5)	1.5 (2)

\* Amounts expressed in optical density units per gram tissue. Numbers in parentheses give the number of determinations.

basis, the level in cinnabar is considerably higher than in white or in wild type. On the other hand, the white mutant has essentially the same content as the wild type.

## DISCUSSION

The results described above demonstrate for the first time the existence of the enzyme, kynurenine hydroxylase, in *Drosophila melanogaster*. This completes—at least with regard to known steps—the enzymatic basis for the biosynthesis of the brown pigments. Furthermore, the cinnabar mutant has little or no enzymatic activity, as would be expected on the basis of the classical chemical work on eye-color mutants.

Another point of considerable interest is the fact that there is a peak of enzyme activity during the pupal stage of development. This peak of activity coincides with the onset of brown pigment formation, as again might be expected. Enzyme activity in the adult fly, on the other hand, is quite low. This suggests that the synthesis of brown pigments is not continuous, if indeed it occurs at all in adult flies. The low level of activity may also be the reason why this enzyme has not been described previously in *Drosophila*, since many enzymatic studies are conducted using 1 to 3-day old adults. Furthermore, the overall amount of 3-hydroxy-kynurenine formed enzymatically, even at maximum activity, is very small compared with other systems, being about 1/10 of that obtained using equivalent amounts of pig liver mitochondria.

TABLE 5

*Tryptophan pyrrolase activity in wild type and mutants\**

	Age					
	5½ days (larvae)		9 days (pupae)		13 days (adult flies)	
	Range	Average	Range	Average	Range	Average
Oregon-R	5.5-9	7.2 (3)	4.0-7.0	5.4 (6)	3.8-6.0	5.1 (3)
white	5.5-7.0	6.2 (3)	6.3-8.5	7.0 (6)	4.5-6.0	5.0 (3)
cinnabar	7.5-8.5	8.0 (3)	4.8-8.8	6.7 (6)	2.5-4.8	3.9 (3)

\* Specific activities expressed in optical density units per gram tissue. Incubation for 2 hr at 37°. Figures in parentheses give number of determinations.

XANTHURENIC ACID ⊕ 3-HYDROXYKYNURENINE ⊕	XANTHURENIC ACID ⊖ 3-HYDROXYKYNURENINE ⊕	XANTHURENIC ACID ⊖ 3-HYDROXYKYNURENINE ⊖
$w^{Bwx} \begin{matrix} \oplus \\ \square \end{matrix}$	$w^{bl} \begin{matrix} \oplus \\ \oplus \end{matrix}$ $w^{cf} \begin{matrix} \oplus \\ \square \end{matrix}$ $w^{sat} \begin{matrix} \oplus \\ \oplus \end{matrix}$	$w^{bf} \begin{matrix} \ominus \\ \oplus \end{matrix}$
	$w^{bf2} \begin{matrix} \ominus \\ \square \end{matrix}$	$w^a \begin{matrix} \ominus \\ \oplus \end{matrix}$ $w^{a2} \begin{matrix} \ominus \\ \square \end{matrix}$ $w^{co} \begin{matrix} \circ \\ \oplus \end{matrix}$
		$w \begin{matrix} \ominus \\ \square \end{matrix}$
		$sp-w \begin{matrix} \oplus \\ \square \end{matrix}$
		$w^h \begin{matrix} \ominus \\ \oplus \end{matrix}$ $w^i \begin{matrix} \ominus \\ \square \end{matrix}$ $w^t \begin{matrix} \circ \\ \square \end{matrix}$ $w^e \begin{matrix} \oplus \\ \oplus \end{matrix}$
		<p style="text-align: center;">No Action on Zeste</p> <p style="text-align: center;">Suppressors of Zeste</p>

FIGURE 4.—Biochemical division of the white locus. Xanthurenic acid detected, after chromatography (*n*-propanol-1% ammonia), by spraying with N,2,6-trichloroquinoneimine (“DCC”). Results for 3-hydroxykynurenine are those of WESSING (1961). An empty box or circle means that no determination has been made on that mutant. Genetic ordering of the white mutants is after JUDD (1964).

Some years ago (FORREST 1963), it was shown that mutants at the white locus could be divided into three distinct groups on the basis of their content of 3-hydroxykynurenine and xanthurenic acid (Figure 4). Thus the first group had both of these compounds, albeit in smaller amount than the wild type; the second group had 3-hydroxykynurenine but not xanthurenic acid; and the third group had neither compound. The white mutant belonged to this latter class. These three subdivisions correspond accurately to the genetic subdivisions of the locus.

This work then led to the theory that this group of mutants had reduced capacity to synthesize 3-hydroxykynurenine and that this inability was due to a reduction in the amount of, or absence of, a pteridine cofactor which, as a substrate, was also involved in red pigment formation. The basis for assuming that kynurenine hydroxylase had a pteridine cofactor was the formal analogy (NADPH requirement, O<sub>2</sub> requirement) between the enzymatic conversion of kynurenine into 3-hydroxykynurenine, and the conversion of phenylalanine into tyrosine—a reaction which is known to be catalyzed by an enzyme having a reduced pteridine as cofactor.

The work described in this paper was in part an attempt to substantiate the theory, according to which the white mutant would have no kynurenine hydroxylase activity, owing to the absence of an essential pteridine cofactor. In fact the kynurenine hydroxylase activity of the white mutant is lower than that of wild

type, but is not absent. However, it should be pointed out that, as is the case in the mammalian system, this enzyme is apparently associated with the mitochondria, and we have not as yet been able to obtain a soluble preparation. (There are two reasons for this: the low activity mentioned above, and the logistic difficulty of obtaining enough material to work with.) Thus problems of cofactor removal, and addition of exogenous cofactor, are compounded because of the mitochondrial nature of the enzyme. Possibly for this reason, for example, we have not been able to increase kynurenine hydroxylase activity from the white mutant, by the addition of reduced or aromatic pteridines, or by any other means. This may well be because such a cofactor is very tightly bound and activity cannot be affected by exogenously added materials. The low enzyme activity of white is then assumed to be due to a deficiency, but not a complete absence of cofactor. In experiments to be reported elsewhere, we have demonstrated that compounds of the biopterin type inhibit the first enzyme in the biosynthetic pathway leading to brown pigment formation, i.e., tryptophan pyrrolase, although, as reported above, this enzyme has the same activity in vermilion and white as in wild type, at least, using the *in vitro* assay. However, if the lesion resulting in the white-eye phenotype was in, for example, the production of a redox enzyme affecting pteridine metabolism, a low production of reduced pteridine cofactor for kynurenine hydroxylase, and an accumulation of the corresponding aromatic compound capable of inhibiting tryptophan pyrrolase, could lead to the virtual cessation of brown pigment production. In addition, since it is very probable that red pigment synthesis occurs through reduced pteridine intermediates, this too would be blocked. It has to be borne in mind that this state of affairs need only exist for a very limited period of time, i.e., around the time when kynurenine hydroxylase activity is at its peak, to produce the observed phenotypic effect. Thus, even if enough cofactor was finally produced as a result of inefficient or indirect enzyme activity, the phenotype would not be affected, since the appropriate enzyme system would no longer be functional. This theory predicts then that there will be a difference, between wild type and white, in the pattern of pteridine accumulation during pupal development. This prediction will be tested in future work.

#### SUMMARY

Kynurenine hydroxylase activity has been detected in wild-type *Drosophila melanogaster* for the first time. The cinnabar mutant, as expected, lacks this activity, and the white mutant has about one half the wild-type activity. During the life cycle of the fly, there is a peak of enzyme activity at about the time the brown pigments are being laid down. As in the mammalian system, the enzyme seems to be associated with the "mitochondrial fraction." A theory as to the nature of the biochemical lesion in white mutants is proposed, involving the supposition that kynurenine hydroxylase has a pteridine cofactor.



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