DEVELOPMENTAL AND GENETIC STUDIES ON KYNURENINE HYDROXYLASE FROM DROSOPHILA MELANOGASTER

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ABSTRACT

The level of kynurenine hydroxylase was measured throughout the development of wild type and the eye color mutants v, en, st, ltd, cd, kar, w, ca, bri and p of Drosophila melanogaster. In all cases except en a bimodal distribution of enzyme activity during development was observed. Activity is initially detectable in second instar. A maximum is reached in early third instar. Activity declines prior to puparium formation. Shortly after pupation, activity rises dramatically to reach a maximum about five times the peak larval level. Maximum activity persists for a short time, and then falls sharply prior to emergence. No activity is detectable in en, en, or en. In pupae which have zero, one, two or three doses of the en allele, activity is proportional to the number of the + alleles. This provides further evidence that the en locus contains the structural gene for kynurenine hydroxylase. Kynurenine hydroxylase is a useful gene product for studying the events of imaginal disc differentiation.

The imaginal discs of Drosophila are potentially useful for investigating the genetic and molecular events involved in cellular differentiation. Primarily through the efforts of HADORN and his co-workers, it has been shown that cells in imaginal discs are rather rigidly determined as to their developmental fate. Only rarely is the state of determination altered. During pupation the imaginal discs will realize their developmental potential in the course of differentiation. An experimental approach aimed at understanding the genetic and molecular bases for these phenomena is to elucidate the mechanisms by which a specific gene product, characteristic of cells derived from a specific imaginal disc, is regulated during the course of the differentiation of these cells. A problem to date has been the selection of a gene product which is both conceptually useful and technically approachable.

It would be useful therefore to characterize an enzyme system which appears during the differentiation of a specific imaginal disc and thereby could be considered an appropriate molecular indicator for the processes of cellular differentiation. For technical reasons, it would be most useful if these differentiating cells were the sole, or at least major, site where this enzyme was localized. Finally, if studies on the genetic regulation of the enzyme’s appearance are to be pursued, the map position of the structural gene for the enzyme should be identi-

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fied. With these objectives in mind we have studied the developmental history and tissue localization of kynurenine hydroxylase. We have also studied the relation between kynurenine hydroxylase activity and the number of doses of the cn+ (2.57.5) allele. The results of these studies are reported in this paper.

This enzyme catalyzes the conversion of kynurenine to 3-hydroxykynurenine (3-OH-K). It is the third enzyme in the pathway which converts tryptophan to xanthommatin, the brown eye pigment. The enzyme from Drosophila has previously been described by Ghosh and Forrest (1967). These authors reported that enzyme activity reaches its highest level midway through pupation. They also reported its absence from the eye color mutant cinnabar (cn) and low activity in the eye color mutant white (w).

**MATERIALS AND METHODS**

Wild-type *Drosophila melanogaster*, strain Oregon-R, and the mutants white (w), white apricot (w1), scarlet (st), pink-peach (pP), cinnabar (cn), claret (ca), bright (bri), cardinal (cd), karmoisin (kar), and scute, zeste, echinus, cut6(sc, z, ec, cut6) were obtained from the stock center at California Institute of Technology. The mutants white-honey (w1), lightoid (lid), vermilion (p86F), cn34K and cn5/Y;Z were obtained from the stock center at Bowling Green University.

Animals were maintained at 25° on standard corn meal-sugar-yeast extract media (Lewis 1960), except that all food contained 0.1% methyl-1-p-hydroxybenzoate (Eastman Chem) as the mold inhibitor. Mass culture and synchronization of animals was according to the procedure of Mitchell and Mitchell (1964). Eggs were collected onto plastic trays containing food and a thick suspension of live bakers yeast for a period of 2–3 hours, depending on the size of the breeding population. Larval ages are expressed as hours from the midpoint of this collection period. Animals were collected five days after oviposition; those that would float were discarded. The remaining animals were floated at four-hour intervals and the pupae obtained at these floats were allowed to develop until the desired stage. When F1 progeny were desired, a breeding cage was set up using appropriate males and virgin females collected from half-pint milk bottles at eight-hour intervals.

Three counted samples from each batch of animals were weighed. The mean animal weight in each batch was used to calculate the number of animals contained in each assay. Enzyme activities are expressed on per-animal basis.

Extracts were prepared by grinding animals in four volumes of 0.36M sucrose, 5 mM KCN, 1 mM 2-mercaptoethanol, 50 mM Tris-HCl, pH 8.1 in a motor driven glass-teflon homogenizer. Kynurenine hydroxylase activity was measured using modifications of the procedures of Ghosh and Forrest (1967). Each assay contained 0.36M sucrose, 5 mM KCN, 1 mM 2-mercaptoethanol, 1.33 mM NADPH (Sigma), 3 mM L-kynurenine sulfate (Sigma) and 50 mM Tris-HCl, pH 8.1. The final volume of the assay was 1.5 ml when 3-OH-K was to be measured spectrophotometrically and 0.4 ml when 3-OH-K was to be measured by radioactivity determination. Incubation was conducted in a shaking water bath at 37° in open straight-sided vials. Controls were identical reaction mixtures that had kynurenine added at the end of the reaction time.

Colorimetric determination of 3-OH-K was by the nitric acid technique of Inagami (1954). The reaction was stopped by the addition of 1.5 ml cold 10% trichloroacetic acid (TCA). The contents of the vials were removed and centrifuged in a clinical table-top centrifuge for 10 min. The supernatant was removed by pouring through a fine mesh nylon cloth to remove any floating lipoidal material. 0.25 cc of a fresh 1% (w/v) solution of NaNO2 was placed in the bottom of a standard spectrophotometric cuvette. 2 cc of the 5% TCA extract containing 3-OH-K was then added. The contents were rapidly mixed, the cuvette housing was covered and the mixture was allowed to stabilize for 3–5 minutes. The absorbance of the light-sensitive diazoxoide product was then measured at 410 nm in a Gilford model 240 spectrophotometer. In our experience 410 is a
somewhat more satisfactory wavelength than 400. There is a slight sacrifice in sensitivity but an increase in specificity. Controls were identical tubes which had kynurenine added at the end of the incubation. Control values were subtracted from the experimental values and the difference in absorbance compared to an appropriate standard curve obtained using commercial 3-OH-K (Sigma) to determine the amount of 3-OH-K produced. We have checked for the stability of 3-OH-K under these assay conditions and have found it is not degraded during incubation.

A more sensitive assay for kynurenine hydroxylase was developed using radioactive kynurenine as substrate followed by chromatographic isolation of 3-OH-K. H3 kynurenine, generally labeled, was obtained from New England Nuclear at a specific activity of 877 mC/mmole. Each 0.4 ml assay contained 8.0 µC of H3 kynurenine. The conditions of incubation were as described above. The reactions were stopped by the addition of 0.05 ml of 1 N perchloric acid. The mixture was neutralized with 0.05 ml of 1 N KOH. 0.5 ml of a solution 2 mM with respect to 3-OH-K was then added. The mixture was centrifuged to remove insoluble material. 10 µl aliquots of the resulting supernatant were spotted on Whatman No. 1 paper for chromatography. Descending chromatography was run for 4 to 5 hours using methanol, butanol, benzene, water (2:1:1:1) freshly adjusted to 1% NH4OH. In this system the Rf of kynurenine is 0.55 and of 3-OH-K 0.32. After chromatography the spots were located by their fluorescence using a hand U. V. lamp. The 3-OH-K spots were marked, cut out, placed in scintillation vials and eluted with 1 ml of H2O. 10 ml of Bray's solution (Instabray, Yorktown Research) were added to each vial. Counting was performed using a Beckman LS-255 liquid scintillation counter. The recovery of material through this procedure was determined using known amounts of H3 kynurenine and is essentially complete.

RESULTS

Optimum conditions for kynurenine hydroxylase activity: The optimum conditions for assaying kynurenine hydroxylase from Drosophila have not been previously reported. The assay as described is linear for at least two hours. Activity has a single pH optimum at 8.1. 50% of maximal activities are found at pH 7.4 and 8.7. Maximal activities are observed between 1.0 to 3.0 mM kynurenine and between 0.8 mM to 2.0 mM NADPH. NADH shows a relative activity of about 10%. Kynurenine hydroxylase is sensitive to metal ions only when added in fairly high concentrations. Monovalent ions, Na, Li, and K at 0.1 M result in nearly 50% inhibition. Divalent cations Mn++, Mg++, and Fe++ give partial inhibition in concentrations greater than 0.02 M. Activity is relatively resistant to chelating agents. Ethylenediaminetetraacetate at 0.01 M results in 20% inhibition. Diethylthiocarbamate at the same concentration results in 70% inhibition. More extensive analysis of the biochemical properties of kynurenine hydroxylase will be reported elsewhere.

Development Profile of Kynurenine Hydroxylase: The levels of kynurenine hydroxylase found during development are shown in Figure 1. Activity becomes detectable during second instar and increases in the third instar. In late third instar larvae, the activity levels off and falls prior to puparium formation. Activity begins to rise again shortly after puparium formation and reaches a maximum at about 70 hours after puparium formation. Maximum activity persists for a short period before dropping sharply as the time for emergence approaches. We have detected low levels of kynurenine hydroxylase in adults up to four days after emergence.

The developmental profiles of kynurenine hydroxylase in several eye color mutants which are known to have defects which primarily affect ommochome
Figure 1.—Kynurenine hydroxylase activities found during developmental stages in wild-type animals, strain Oregon-R. Each point represents an average of duplicate enzyme assays. Growth is at 25°. On the abscissa is hours from oviposition for larval ages and hours from pupariation formation for pupal and adult ages.

Figure 2.—Kynurenine hydroxylase activities found during developmental stages of the eye color mutants vermilion (v36f), scarlet (st), lightoid (ltd) and karmoisin (kar). Each point represents an average of duplicate enzyme assays.
metabolism are shown in Figures 2 and 3. The mutant v^{agg} does not synthesize kynurenine but shows normal regulation of kynurenine hydroxylase activity. Therefore it appears that the presence of substrate has no effects on the control of kynurenine hydroxylase. The mutant cd has a developmental profile that is generally similar to wild type except that the duration of maximal activity is slightly longer. This difference is small and its relation to the cd phenotype is not clear. However it has been reproduced in several independent experiments. The mutants st, ltd and kar, whose biochemical bases have not yet been established, have kynurenine hydroxylase profiles equivalent to wild type. It appears unlikely that the phenotypic basis of these mutants directly involves kynurenine hydroxylase.

We have also examined several mutants which have major defects in both pteridine and ommochrome metabolism. The analysis of the white alleles w, w^{a} and w^{h} is presented in Figure 3. Their patterns of kynurenine hydroxylase activity are indistinguishable from one another and from wild type. In addition to the data on the above mutants we have analyzed, bri, p^{a} and ca. Their developmental profiles of kynurenine hydroxylase are also indistinguishable from wild type.

Relationship between dosage of cn^{+} alleles and kynurenine hydroxylase activity: Table 1 shows the levels of kynurenine hydroxylase found in pupae containing zero, one, two or three doses of the cn^{+} allele. Group A animals are standard wild type, strain Oregon-R.

One dose of the cn^{+} allele is found in the animals of groups B, C, D, E and F.
The kynurenine hydroxylase levels between these groups does not differ significantly. Group B are F₁ progeny obtained by crossing + males with cn females. Progeny from a reciprocal cross are group C. Groups D and E are F₁ progeny obtained by crossing mutant females with + males. Group F are males from the stock cn3/T(Y;2)C. Their sibling females are group H. This stock carries a translocation of chromosome 2, which includes a cn⁺ allele, on the Y chromosome. Males of this stock have wild-type eyes while females have cinnabar eyes. Males from this stock, crossed to females carrying any other cn allele, yield a line in which all males are wild type and females are mutant. There is no difficulty with respect to viability or male sterility of stocks derived from the original cn¹/T(Y;2)C males. Sex can be distinguished quite easily by observing eye color in intact pupae. The eye of males contain ommochromes and are brown. The females do not contain these pigments and appear colorless prior to pteridine appearance. It is noteworthy that one-dose group F animals are no different than other one-dose animals even though these have one wild-type allele in the presence of two mutant alleles, whereas other heterozygotes have one wild-type allele in the presence of only one mutant allele. In addition to group H, cn²/cn² individuals of mixed sex have no detectable activity. No activity is present in cn/cn (GHOSH and FORREST 1967) or cn²/cn² (Figure 3). There is no complementation between different mutant cn alleles, as judged by the eye color phenotype of the respective F₁ progeny.

Group G individuals with three doses of cn⁺ were obtained by introducing the Y chromosome carrying the cn⁺ allele from the cn²/T(Y;2)C stock into Oregon-R. Females of the resultant line have one dose of cn⁺ on each second chromosome. Males have an additional dose of cn⁺ on the Y. The genetic identity of this line was established by crossing males to cn/cn females. No cinnabar females were obtained in the F₁. The segregation ratio of females with respect to eye color showed the expected mendelian ratio of 3:1 in the F₂. All males in successive generations had wild-type eyes. The above line has been maintained in stock cul-
ture for over six months. At each transfer it has been examined for cn segregants, and none have been found.

Since only males contained three doses of cn+, a method for selecting male pupae was necessary. The sex-limited eye color mutant zeste was introduced into this stock. Three-dose cn+ males were crossed to females that were sc z ec ct'. The resulting stock is then sc z ec ct', T(Y;B)C. Males of this stock at 74 to 76 hours after puparium formation have brown, slightly reddish eyes. Females have eyes that appear to be colorless when viewed through the pupal case. When the case is opened the eyes of these females are seen to be pale yellowish with a slight pink hue, i.e. like the standard zeste females.

The animals in group G have approximately 1.5 times the kynurenine hydroxylase activity of Group A and close to 3 times that found in one-dose pupae. Controls for effect of the zeste mutation on kynurenine hydroxylase activity have indicated no significant difference between male and female zeste pupae. Furthermore, their activity is not significantly different from that of Oregon-R pupae of mixed sex.

*Tissue localization of kynurenine hydroxylase: Since kynurenine hydroxylase functions in the production of eye pigments we hypothesized that this enzyme might be localized in the developing eye. Wild-type pupae, 72-76 hours after puparium formation, were opened and the anterior section of the head was removed. Head pieces from 25 to 50 animals were combined and assayed for kynurenine hydroxylase. The portions of the animals remaining after the removal of the head (posterior section) were also combined and assayed. Other animals were dissected and the heads and posterior sections were recombined prior to homogenization and assay. These are referred to as sham-operated. The results of these experiments are shown in Table 2. All of the activity in intact or sham-operated animals can be accounted for in the head fraction. The posterior section has essentially no detectable activity. The dissected heads were obtained by cutting down on the dorsal surface from a point immediately behind the pigmented hemisphere of the eye. This dissection resulted in a piece of tissue which contained the eye, the epidermis between and below the eyes, the developing antennal region the optic stalk, a few fragments of the brain and some loose internal tissue. In order to determine if the activity found in the head was in the eye or

<table>
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<th>Samples</th>
<th>Activity*</th>
<th>S.E.M.†</th>
<th>N</th>
</tr>
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<tr>
<td>Intact</td>
<td>4,049</td>
<td>150.9</td>
<td>14</td>
</tr>
<tr>
<td>Head</td>
<td>3,802</td>
<td>298.3</td>
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<td>85</td>
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<tr>
<td>Sham operated</td>
<td>3,669</td>
<td>71.2</td>
<td>6</td>
</tr>
<tr>
<td>Eyes</td>
<td>2,801</td>
<td>383.3</td>
<td>8</td>
</tr>
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* Activity = c.p.m. 3-hydroxykynurenine/animal/hour.
† S.E.M. = standard error of the mean.
‡ Animals were assayed 72-76 hours after puparium formation.
the surrounding tissue a more extensive dissection was accomplished. Heads were obtained as before and these were cleaned of as much tissue as possible, leaving intact the pigmented eye hemispheres. The resulting tissue contained the pigmented layer of the eyes and fragments of attached epidermis. In some of the dissections the two eyes of one animal were intentionally left attached to one another by the epidermal and antennal region between them. This facilitated transfer of the eye tissue. Comparison of the "eyes" and "head" samples (Table 2) by a t-test or by Wilcoxon's rank test (Snedecor 1956) does not reveal statistically significant differences ($P > 0.05$) between them. We therefore conclude that the great majority of kynurenine hydroxylase is found in the developing eye. A simple comparison of the mean values for "head" and "eyes" in Table 2 might suggest that not all of the head hydroxylase is found in the eye. The radioassay is subject to a high degree of variation. This is the basis for lack of statistical significance between the samples. We would only argue that most of the kynurenine hydroxylase is found in the eye. However, some activity loss might reasonably be expected to occur during the period (about 1½-2 hours) from the initiation of dissection until enough sufficiently clean, intact eyes are obtained for reliable activity measurements. For this reason we believe that the level of kynurenine hydroxylase found in the eye (Table 1) represents a minimum value.

DISCUSSION

The data we have presented concerning the developmental profile of pupal kynurenine hydroxylase are generally comparable to those reported by Ghosh and Forrest (1967), with the exception that these authors could not detect enzyme activity in larvae. The only confirmed function for this enzyme in Drosophila is in the production of ommochrome pigments. These are normally confined to the eye. The tissue localization and the timing of enzyme appearance coincident with pigment synthesis in pupae are consistent with this function. The existence of kynurenine hydroxylase in larvae may indicate an additional role, perhaps in some aspect of tryptophan catabolism. However, whatever this function may be, it is not essential since $cn$ mutants lack detectable activity in larvae as well as pupae.

The developmental profiles of kynurenine hydroxylase in the eye color mutants we have analyzed do not differ significantly from wild type. Small differences may exist with respect to the regulation of the larval enzyme. However, the relatively low level of activity found in larvae do not at present allow for distinguishing small differences.

The mutant ($w$) was reported by Ghosh and Forrest (1967) to be deficient in kynurenine hydroxylase activity. This observation was used as the basis for a model concerning the biochemical basis of the white-eyed phenotype. We were unable to confirm these observations for $w$, $w^d$, or $w^a$. We cannot positively account for the failure of Ghosh and Forrest to detect normal activity in $w$. One possible reason is that these authors report measurements on $w$ at only one developmental stage. This is the time when maximal activity is found in wild type.
GHOSH and Forrest time their animals from oviposition. We have observed that ommochrome-deficient mutants take about six hours longer to reach pupation than does strain Oregon-R. Since kynurenine hydroxylase is regulated quite sharply during development, any fluctuations in culture conditions which affect developmental rate or any increase in asynchrony can easily lead to lowering the apparent activity. PHILLIPS, FORREST and KULKARNI (1973) have claimed that the mutant st has significantly lower than wild-type levels of kynurenine hydroxylase. We have also been unable to confirm these observations.

The data we presented show that the level of kynurenine hydroxylase is proportional to the number of doses of cn+ alleles. This is circumstantial but strong evidence that the cn locus contains the structural gene for kynurenine hydroxylase. In several other cases in Drosophila, enzyme activity has been shown to be proportional to the number of structural genes. In these cases independent observations have shown that an altered protein is produced by mutations at the locus under study. GLASSMAN, KARAM and KELLER (1962) and GRELL (1962) have shown that xanthine dehydrogenase (XDH) activity is proportional to the number of ry+ alleles. YEN and GLASSMAN (1965) subsequently mapped electrophoretic variants of XDH to the ry locus. TOBLER, BOWMAN and SIMMONS (1971) showed that tryptophan pyrrolase activity is proportional to the number of v+ alleles. TARTOF (1969) and BAILLIE and CHOVNICK (1971) have shown that a mutation at the v locus, vk, produces an altered form of the tryptophan pyrrolase molecule. O'BRIEN and MACINTYRE showed that flies heterozygous for mutations at the aGpdh locus, which cause an absence of α-glycerophosphate dehydrogenase (αGPDH), and the wild-type allele of this locus have intermediate levels of αGPDH activity.

It is difficult to account for the observed relation between kynurenine hydroxylase and doses of cn+ with any other hypothesis except that this locus contains the structural gene for this enzyme. However, knowledge of the nature of genetic regulatory elements in Drosophila is not sufficient to allow prediction of the effects of mutations at such sites on enzyme activities. The only direct way of showing that a given mutation is a structural gene mutation is to demonstrate that a protein having an altered primary structure is produced by the mutant locus. In some systems, e.g. tryptophan pyrrolase (TARTOF 1969), it has been possible to demonstrate an altered protein by means of super-additive effects in heterozygotes of v mutants and wild type. In cn mutants we have examined, there is no evidence of super-additive effects in heterozygotes. Heterozygotes have one-half the level of activity found in wild type. These cn mutants apparently produce an extremely altered product or no product at all.

The data on the tissue localization of pupal kynurenine hydroxylase are consistent with the results of BEADLE and EPURUSSI (1936) on eye disc transplants. These authors showed that wild-type disc transplanted in to a cn host would develop into an eye with wild-type pigmentation. This result implies that the developing eye has the enzymatic capability of producing 3-hydroxykynurenine. The hypothesis that all or most of the kynurenine hydroxylase is located in the eye might, at first glance, appear to be in conflict with the non-autonomy of cn
discs transplanted to wild-type hosts. However, Phillips, Simmons and Bowman (1970) and Donnelly and Sullivan (unpublished observation) have measured substantial levels of 3-hydroxykynurenine in larvae and early pupae. This is before the rise in enzyme activity occurs in pupae. This 3-hydroxykynurenine is the result of larval enzyme function and may well be available to an implanted disc. Therefore an implanted crz disc may be getting its 3-hydroxykynurenine from the hemolymph and may well be independent of the developing host eye. The possibility also exists that the eye may, in addition to using 3-hydroxykynurenine for pigment synthesis, release excess to the hemolymph (Ephrussi 1942).

The results presented here indicate that kynurenine hydroxylase has several properties which will make it a useful model system for studying the biochemical and genetic events of imaginal disc differentiation. It is primarily, if not exclusively, found in tissue which develops from a specific disc. The rise in its activity begins fairly soon after pupation, near the onset of differentiation. Since it is probable that the cn locus contains the structural gene for kynurenine hydroxylase and mutations at this locus produce a visible alteration in phenotype, extensive genetic characterization of this enzyme can be accomplished.

It is possible to depict one aspect of eye disc differentiation as the initiation and maintenance of activity at the crz locus in response to the stimuli (hormonal?) present at pupation or shortly thereafter. Presumably the state of determination of the disc as presumptive eye tissue involves this locus, either directly or indirectly, as well. The principal advantage of this enzyme is that the progress of a specific disc's differentiation can be monitored by measuring the levels of kynurenine hydroxylase activity in whole animals. Presently no other enzyme systems offer the unique potential of kynurenine hydroxylase for studying genetic and biochemical aspects of imaginal disc differentiation. The ultimate utility of this enzyme will depend on demonstrating that the appearance of kynurenine hydroxylase in the developing eye is due to de novo synthesis in the cells derived from the eye disc. Also, more extensive analysis of enzyme levels from puparium formation through the pupal molt are needed in order to determine more precisely the timing of the increase in activity.

LITERATURE CITED


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