KYNURENINE FORMAMIDASE IN MUTANTS OF DROSOPHILA[1]

EDWARD GLASSMAN[2, 3]

Merganthaler Laboratory for Biology, The Johns Hopkins University, Baltimore, Maryland

Received January 30, 1956

MANY workers have shown that the brown eye pigment of *Drosophila melanogaster* is a derivative of tryptophan, and that gene mutations affecting the conversion of tryptophan to kynurenine (the v^+ substance) in Drosophila manifest themselves as deficiencies in this pigment (cf. EPHRUSSI 1942; GREEN 1949; KIKKAWA 1950a). Recent examination of the enzymes responsible for this conversion in mammalian liver has indicated that these reactions involve a peroxidation of tryptophan to N′-formylkynurenine (KNOX and MEHLER 1950), and the hydrolysis of the latter compound to kynurenine and formic acid (MEHLER and KNOX 1950). The enzyme for this hydrolysis, kynurenine formamidase (JAKOBY 1954), is apparently specific for formylated aromatic amines and is able to hydrolyze formylanthranilic acid, formylorthanilic acid, formylnitroaniline, and others, although at a much slower rate than it acts upon formylkynurenine. This enzyme is quite active in cell-free extracts of Drosophila, and some of its properties will be described below. Unfortunately it has not been possible to demonstrate the peroxidation of tryptophan to formylkynurenine in cell-free extracts (assayed by tryptophan disappearance) even though larvae, pupae, and adults have been examined in various ways.

Kynurenine formamidase was assayed in various strains in which the production of kynurenine is known to be altered. These included the vermilion mutants, in which the formation of kynurenine is blocked completely, and ruby, claret, and others, in which the amount is merely lessened (BEADLE and EPHRUSSI 1937). In order to determine the differences between strains, a variety of other strains in which kynurenine production is normal was also examined. The study was extended to include stocks of *D. virilis* because of a report by KIKKAWA and his associates (1950b) stating that the mutant cardinal of that species "seems to lack the enzyme which converts formylkynurenine into kynurenine". However, no details were presented by those authors.

The experiments of the present investigation demonstrated that kynurenine formamidase is present in all of the strains examined. This indicates that the reaction block in the pseudoallelic vermilion mutants, as well as the other strains, is in the conversion of tryptophan to N′-formylkynurenine.

[1] Supported in part by a contract No. At(30-1)-1472 from the Atomic Energy Commission.

[2] Adam T. Bruce Fellow. This material is taken in part from a dissertation presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[3] Present Address: Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California.

MATERIALS AND METHOD

Sources of chemicals

The N′-formyl-DL-kynurenine used in these investigations was the gift of DR. W. E. KNOX. The presence of kynurenine in the preparation was taken into account. Some of the formylanthranilic acid used was donated by DR. W. J. JAKOBY. An additional supply of this compound was prepared according to a method modified from VON MEYER and BELLMAN (1923). All other chemicals were of commercial origin.

Collection of flies

The wild type strain of *D. melanogaster* used in these investigations was an Oregon-R stock maintained in this laboratory. Most of the *D. melanogaster* strains used were obtained from the California Institute of Technology; the *D. virilis* strains, from the University of Texas.

Flies were raised at room temperature (except where noted otherwise) on a standard medium of cornmeal, dextrose, and agar, supplemented with 3.0% brewers' yeast. Collections of adults were usually made every day and then stored at −15°C until used. Animals frozen for over one year showed an enzymatic activity comparable to that of freshly frozen ones.

Enzyme preparation

The enzyme was prepared by grinding the frozen adult flies in a chilled mortar, and then homogenizing in a glass tissue-grinder with 10 times their weight of 0.1 *M* phosphate buffer, pH 7.3 to 7.6. The homogenate was centrifuged at 20,000 × g for 20 to 30 minutes, after which the cellular debris and the thick lipid layer were discarded. The supernatant solution, which contained about 5 mg of protein per ml, was used when the specific activity of the enzyme in the fly was determined. When the properties of the enzyme were to be examined, the homogenate (darkly colored with eye pigments) was further treated by dialyzing for two to three days against four or five changes of large volumes of distilled water. The eye pigments were removed, and about 80% of the proteins were precipitated, by this treatment; the kynurenine formamidase remained in solution with little loss in activity.

Enzyme assay

The assay of kynurenine formamidase is based on the absorption of kynurenine at 360 mμ. When ascertaining the properties of the enzyme the dialyzed preparation was assayed in a manner similar to that described by MEHLER and KNOX (1950). To a 1.0 ml cuvette were added 0.1–0.2 ml of the enzyme, 1.4 μ moles of N′-formylkynurenine and enough 0.1 *M* potassium phosphate buffer to bring the volume to 1.0 ml. A change in optical density of 0.440 at this wavelength indicates the formation of 0.1 μ moles of kynurenine per ml. A similar procedure was used for following the hydrolysis of formylanthranilic acid, except that 10 μ moles of this compound

were used and readings were taken at 330 mμ. A change in optical density of 0.150 indicates the formation of 0.1 μ moles of anthranilic acid per ml.

When stocks were examined for specific activity, however, a different procedure was employed. It was thought unwise to use dialyzed homogenates because of the possible presence of a diffusible inhibitor of the formamidase. Since the eye pigments present in the crude homogenate absorb in the ultraviolet, and because complete removal of particles by centrifugation was sometimes difficult, it was found to be more convenient to assay the crude homogenate in a test tube containing five times the amounts of materials used in the cuvette described above. The enzyme was added last, after the temperature had equilibrated to 30°C. Aliquots of 1.0 ml were taken 15 seconds after mixing and at 10 or 15 minute intervals thereafter, for 30 to 45 minutes. The reaction was stopped by deproteinizing these aliquots with 1.0 ml of 0.18 *N* sodium hydroxide and 1.0 ml of 5% zinc acetate (MEHLER and KNOX 1950). The amount of kynurenine produced was determined from the optical density of the resulting neutral protein-free solution at 360 mμ. The unit of enzyme activity, when assayed under these conditions, is defined as that amount of enzyme which will produce a change in optical density of 0.001 in five minutes. Specific activity is the number of enzyme units per mg of protein. Protein was determined by the method of LOWRY *et al.* (1951), using blood serum protein as a standard.

RESULTS

General properties of the enzyme

The general properties of this enzyme preparation were examined only sufficiently to ascertain that the reaction studied was the hydrolysis of N′-formylkynurenine. In all of these experiments enzymatic assays were carried out in the spectrophotometer cuvette.

The enzyme exhibits little change in activity when the pH varies from 7.0 to 7.6; there is a slight maximum at 7.3. Broad pH optima, such as this, have previously been reported for rat liver formamidase (pH 6 to 8, MEHLER and KNOX 1950) and Neurospora formamidase (pH 7.3 to 7.8, JAKOBY 1954).

The Km calculated by the method of LINEWEAVER and BURK (1934) is 3.1×10^{-4} *M*; the value reported for the Neurospora enzyme is 1.1×10^{-4} *M* (JAKOBY 1954).

The enzyme extracted from Drosophila appears to be more specific than the analogous enzymes obtained from rat liver or Neurospora, in that the Drosophila formamidase does not detectably hydrolyze formylanthranilic acid under the conditions described (fig. 2). However, formylanthranilic acid inhibits the hydrolysis of formylkynurenine by 60 percent at 10^{-2} *M* and by 15 percent at 10^{-3} *M*. Other reported substrates for formamidase have not been tried.

The enzyme is inhibited completely by 10^{-2} *M* sodium bisulfite, but not at all by 10^{-2} *M* sodium formate or 10^{-2} *M* thiourea. This last finding proved very useful when assaying the formamidase in larvae, since addition of thiourea inhibited the

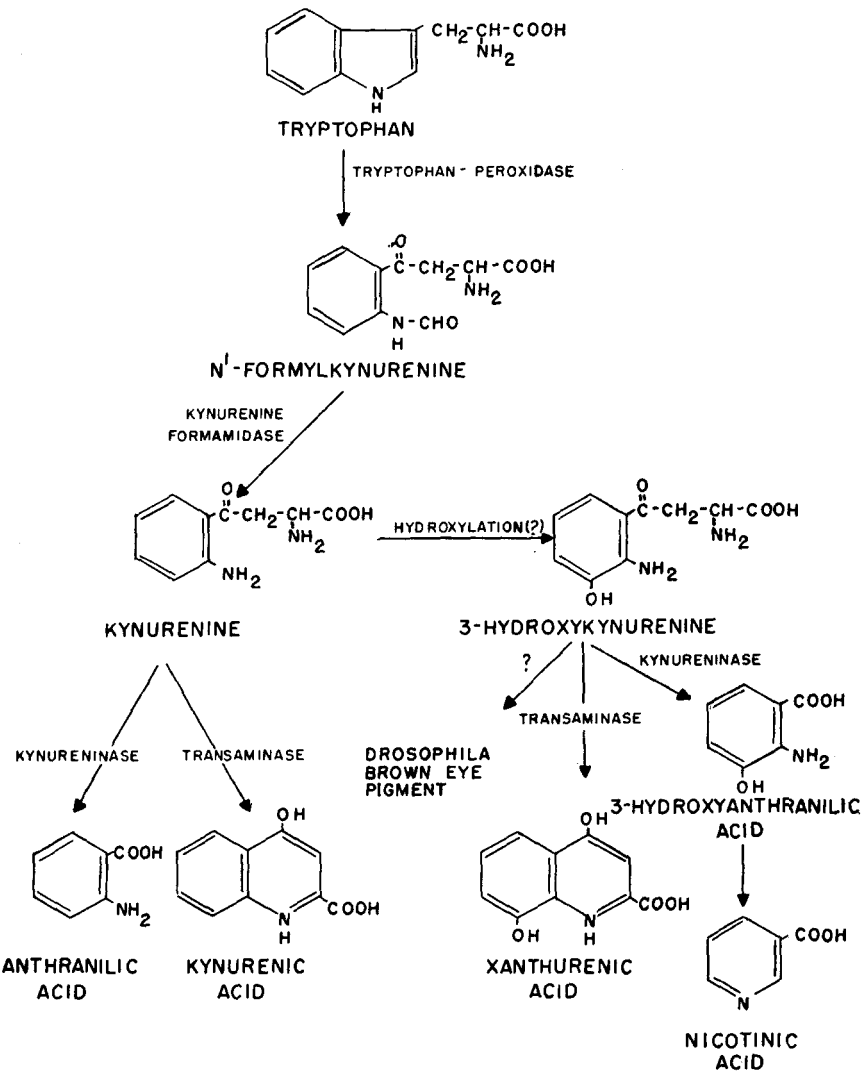

FIGURE 1.—Some aspects of the metabolism of tryptophan.

formation of substances (probably quinones), produced through the action of tyrosinase, which condense with kynurenine (GLASSMAN 1955).

The effect of age and temperature on the specific activity of kynurenine formamidase

In order to ascertain what effects the temperature at which the flies were raised, and the age of the fly after emergence, might have on the specific activity of the formamidase, flies were raised at 17–18°C, 22–23°C, 24–26°C, and 29–30°C, and collected at frequent intervals. Some flies were frozen immediately, while others were aged for 10 days at the temperature at which they were grown. Assays were per-

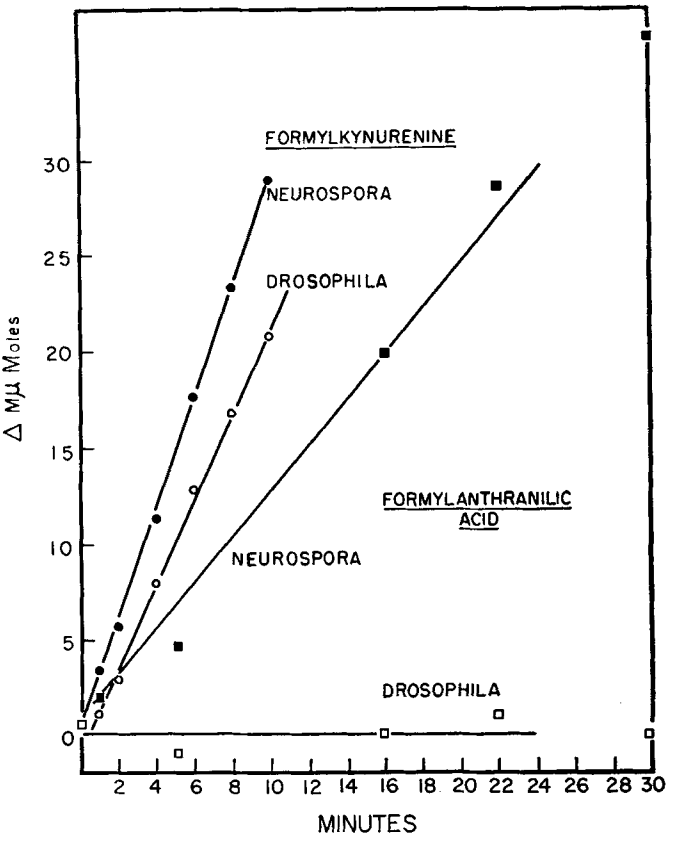

FIGURE 2.—Activities of kynurenine formamidase from Drosophila and Neurospora on N′-formylkynurenine and formylanthranilic acid. The Drosophila extract was prepared as described in the text. The Neurospora extract was prepared by homogenizing wild type mycelium with four times its weight of 0.1 *M* phosphate buffer, pH 7. The solution was centrifuged at 20,000 × *g* for 20 minutes and the supernatant was stored at −15°C until used. Assays were conducted in 1.0 ml volumes containing 0.2 ml of extract, 1.4 μ moles of N′-formylkynurenine or 10 μ moles of formylanthranilic acid, and 0.1 *M* buffer, pH 7.6. The data are presented as the change in millimicromoles of substrate against time; the two upper curves and the two lower curves are for the hydrolyses of N′-formylkynurenine and formylanthranilic acid, respectively.

TABLE 1

The effect of age of the adult flies and the temperature at which they were raised on the specific activity (number of enzyme units per mg of protein) of kynurenine formamidase

Age (days)	Specific activity			
	17–18°C	22–23°C	24–26°C.	29–30°C
0–1	24	22	23 26	24
	26	23	23 27	24
			24 27	24
			24 28	
			25	
9–10	25	25	28	23
	27	26		24
				27

formed as described above. The data are presented in table 1, where it can be seen that the specific activity of the enzyme is not detectably affected either by the temperature at which the flies are grown or by the age of the fly after emergence (up to 10 days). The specific activity of the wild type under the conditions stated is between 22 and 28 enzyme units per mg of protein.

Assay of mutant strains

The data for the specific activity of kynurenine formamidase in a number of mutant stocks of *D. melanogaster* and *D. virilis* are presented in figure 3. All stocks (within species) had similar levels of enzyme activity. Some differences exist, but the wide variation within strains makes it difficult to conclude that any stock differs significantly from the wild type. On the other hand, there does appear to be a significant difference between the two species. Whether the lower specific activity found in *D. virilis* is related to the larger size of the individuals or to some other factor is not known.

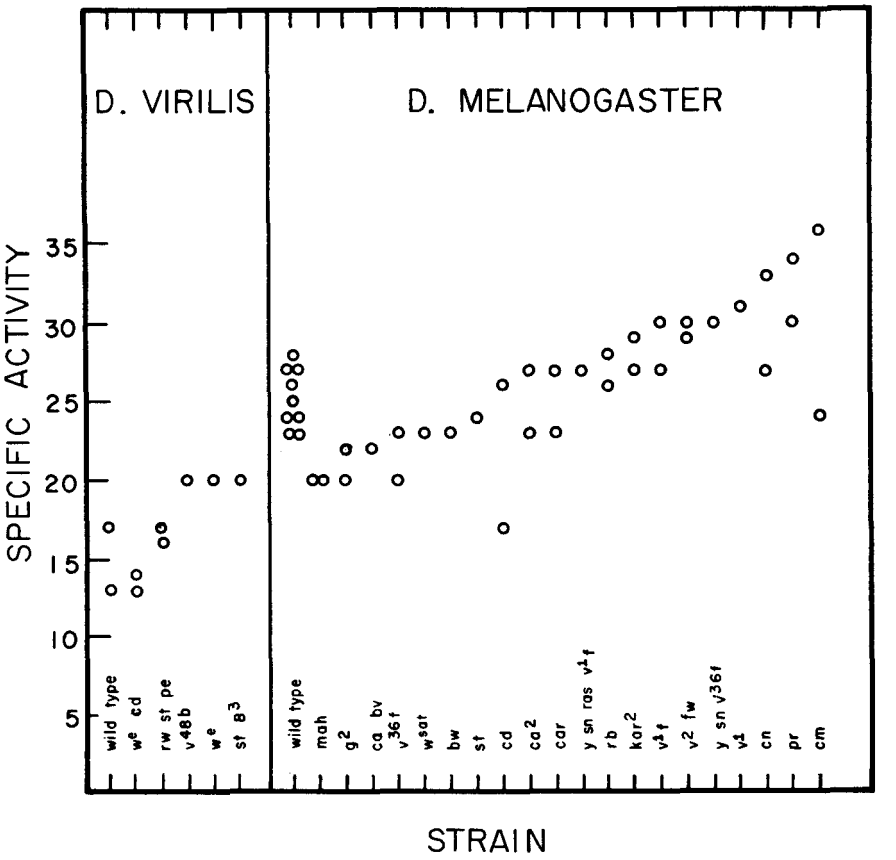

FIGURE 3.—The specific activity (number of enzyme units per mg of protein) of kynurenine formamidase in various strains of Drosophila.

DISCUSSION

In spite of the vast literature which implicates kynurenine and 3-hydroxykynurenine as precursors of the brown eye pigment in Drosophila, only few data are available which indicate that tryptophan is a precursor of these compounds in this organism. Not until relatively recently was it demonstrated that vermilion flies, which cannot form kynurenine, accumulate high levels of a compound which reacts positively in the colorimetric test of HORN and JONES for tryptophan, and which is presumably that compound (GREEN 1949). That this compound is a precursor is borne out by the fact that when the production of brown eye pigment is increased by a suppressor gene (*su*^{2}-*v*), the amount of this compound accumulated by vermilion flies is decreased. Further evidence that tryptophan is converted to kynurenine in Drosophila is provided by KIKKAWA (1950), who, by feeding tryptophan to cinnabar larvae, was able to demonstrate an increase in the amounts of kynurenine normally accumulated by this strain.

The demonstration that in Drosophila there is an enzyme capable of hydrolyzing N′-formylkynurenine to kynurenine and formic acid confirms these interpretations. However, tryptophan-peroxidase has not yet been demonstrated in cell-free extracts even though different methods of extraction of various stages have been tried. Similar attempts by M. M. GREEN, E. KNOX, and E. CASPARI (personal communication), working independently, have also proved fruitless. Whether this is because the concentration of the enzyme is too low to be detected, or because the enzyme is unstable, or because of some other factor which has not been recognized, is not known.

Kynurenine formamidase was present in all of the mutant stocks examined. Most of the strains were assumed to contain the enzyme because they produce sufficient kynurenine; they were used only to determine the variation to be expected from strain to strain. Vermilion strains, however, lack the ability to make kynurenine, while other strains, such as carmine, claret, and ruby, do not make quite as much as does the wild type (BEADLE and EPHRUSSI 1937). These latter stocks were examined in the hope that an absence of formamidase might turn out to be responsible for this condition. Since the enzyme was found to be present in all stocks, it may be concluded that the mutant genes block the reaction prior to the formation of formylkynurenine, i.e., in the tryptophan-peroxidase system. These data confirm the observations of GREEN (1952) who reported that the various vermilion pseudoallelic mutants could make brown eye pigment from formylkynurenine supplied in the medium. His data remained questionable, however, since in the acid medium used, formylkynurenine spontaneously hydrolyzes to formic acid and kynurenine, which is also active.

Formylanthranilic acid, which is hydrolyzed by kynurenine formamidase from rat liver or Neurospora, does not appear to be a substrate of the Drosophila formamidase; however, its presence does inhibit the hydrolysis of formylkynurenine. A similar situation has been recorded for kynureninase, which when extracted from Neurospora can convert formylkynurenine to formylanthranilic acid (JAKOBY and BONNER 1953), but when obtained from Pseudomonas is unable to do this (HAYAISHI

and STANIER 1952). A more detailed analysis of this point, supplemented with data on other reported substrates of the enzyme, would be of interest.

SUMMARY

1) Cell-free extracts of *Drosophila melanogaster* were examined for the enzymes that convert tryptophan to kynurenine. Only kynurenine formamidase was found. The tryptophan-peroxidase system could not be demonstrated in these extracts.

2) The Drosophila kynurenine formamidase has a broad pH optimum between 7.0 and 7.6. The Km was 3.1×10^{-4} *M*. The enzyme is inhibited completely by 10^{-2} *M* sodium bisulfite, but not by 10^{-2} *M* sodium formate or thiourea. It does not utilize formylanthranilic acid as a substrate, although it is inhibited by this compound. On the other hand, when extracted from rat liver or Neurospora, kynurenine formamidase does hydrolyze formylanthranilic acid.

3) The specific activity of the enzyme does not vary with the age of the adult, nor is it changed by raising the flies at various temperatures between 17°C and 30°C.

4) The specific activity of the enzyme was assayed in various mutant strains. In no strain was the activity significantly different from that of the wild type. Strains of *D. virilis* had lower specific activities than those of *D. melanogaster*.

ACKNOWLEDGMENTS

The author would like to express his gratitude to DR. BENTLEY GLASS, whose interest and counsel was invaluable throughout this investigation; and to DRS. W. D. MCELROY and H. K. MITCHELL for critically reading the manuscript. He is also indebted to ANN JUDITH TERRELL for her assistance, and to REBECCA K. RITTERHOFF and PAULINE LEE for the Drosophila stocks and food.

LITERATURE CITED

BEADLE, G. W., and B. EPHRUSSI, 1937 Development of eye colors in Drosophila: Diffusible substances and their interrelations. Genetics **22:** 76–86.

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

GLASSMAN, E., 1955 Ph.D. Thesis. The Johns Hopkins University, Baltimore, Maryland.

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.

HAYAISHI, O., and R. Y. STANIER, 1952 The kynureninase of *Pseudomonas fluorescens*. J. Biol. Chem. **195:** 735–740.

JAKOBY, W., 1954 Kynurenine formamidase from Neurospora. J. Biol. Chem. **207:** 657–663.

JAKOBY, W., and D. M. BONNER, 1953 Kynureninase from Neurospora: purification and properties. J. Biol. Chem. **205:** 699–707.

KIKKAWA, H., 1950a Tryptophan synthesis in insects. Science **111:** 495–496.

1950*b* Tryptophane metabolism in Drosophila. Drosophila Inf. Ser. **24:** 82–83.

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.

LINEWEAVER, H., and D. BURK, 1934 The determination of enzyme dissociation constants. J. Am. Chem. Soc. **56:** 658–666.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL, 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:** 265–275.

MEHLER, A. H., 1955 Metabolism of tryptophan. in *A Symposium on Amino Acid Metabolism.* pp. 882–908. Edited by W. D. MCELROY and B. GLASS. Baltimore: The Johns Hopkins Press.

MEHLER, A. H., and W. E. KNOX, 1950 The conversion of tryptophan to kynurenine in liver. II. The enzymatic hydrolysis of formylkynurenine. J. Biol. Chem. **187:** 431–438.

VON MEYER, E., and I. BELLMAN, 1886 Untersuchungen über Isatosäure und Abkömmlinge derselben. J. pract. Chem. **33:** 24.