

A SIMPLE, RAPID METHOD FOR THE MULTIPLE ASSAY OF TRYPTOPHAN PYRROLASE IN *DROSOPHILA*^{1, 2}

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ABSTRACT

This report describes the development of a simple, rapid method for the kinetic assay of tryptophan pyrrolase in *Drosophila*. By means of this method, one can readily make simultaneous measurements of tryptophan pyrrolase activity in as many as 36 samples.

INTRODUCTION

Tryptophan pyrrolase is essential in *Drosophila* for the formation of eye pigments. Vermilion eye mutants, for example, have low levels of tryptophan pyrrolase activity (Baglioni, 1960; Kaufman, 1962; Marzluf, 1965). Although an assay for tryptophan pyrrolase in *Drosophila* has been reported (Kaufman, 1962), it involves a modification of a diazotization procedure (Bratton and Marshall, 1939) whereby kynurenine is measured after the reaction has been stopped with acid. Because this is cumbersome and time-consuming, we decided to develop a method that was both simpler and more rapid. We also investigated the components of the system with the intention of developing an optimal reaction mixture. The result of these studies was the development of a simple, rapid method for the multiple assay of tryptophan pyrrolase in *Drosophila*. This assay, as well as the one previously reported, is based on the following principle. Tryptophan pyrrolase converts L-tryptophan to N-formyl kynurenine. The latter is subsequently converted to kynurenine by a second enzyme, kynurenine formamidase. Because kynurenine formamidase occurs in *Drosophila* in great excess (Glassman, 1956), the amount of kynurenine formed can be used as an index of tryptophan pyrrolase activity.

METHODS

Wild-type *Drosophila* larvae were homogenized in 3 volumes of a 0.14 M KCl-0.0025 N KOH medium. The resulting homogenate was centrifuged at 15,000 x g for 15 minutes. The supernatant was decanted and used as the source of the enzyme in the reaction mixture.

The reaction mixture contained, in final concentrations: 7 micromoles/ml of L-tryptophan; 0.00125 mg/ml of hematin; 10 micromoles/ml of sodium ascorbate; and 110 micromoles/ml of sodium phosphate buffer, pH 7.0. The reaction mixture also contained 1.0 ml of the 15,000 x g supernatant and water. The final volume was adjusted to 4.53 ml with water so that the values of optical density at 365 m μ would be equal to the concentration of kynurenine in micromoles/ml. The solutions of tryptophan, hematin, and ascorbate were prepared fresh weekly, adjusted to a pH of 7.0, and stored at 5°C.

The reaction mixture was incubated at 37°C in open 10 ml DeLong culture flasks on a Dubnoff shaker. The use of a Dubnoff shaker with racks made possible the simultaneous incubation of up to 36 flasks. At intervals of 30 minutes, the mixture in each flask was decanted into a quartz cuvette, and the amount of kynurenine present was determined by absorbancy measurements at 365 m μ against water. The mixture was then decanted back into the same flask, and the flask was returned to the incubator. Because the entire process required less

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than one minute, the absorbancy of the contents of 30 flasks could be measured every 30 minutes. By incubating for a period of three hours, it was thus possible to obtain six 30-minute determinations for each of the 30 flasks. The reaction was no longer linear after three hours, so the measurements were discontinued at that time. Because all reaction mixtures were measured in the same cuvette, no window corrections were necessary. The values obtained for "no tryptophan" blanks were subtracted from the experimental values to determine the amount of kynurenine formed. Tryptophan pyrrolase activity was expressed as micromoles of kynurenine formed per hour per milliliter of the 15,000 x g supernatant.

RESULTS

From figure 1 it is evident that kynurenine has an absorption maximum at 365 m μ . It is also evident that L-tryptophan does not absorb light at this wavelength, even in concentrations 100 times as great.

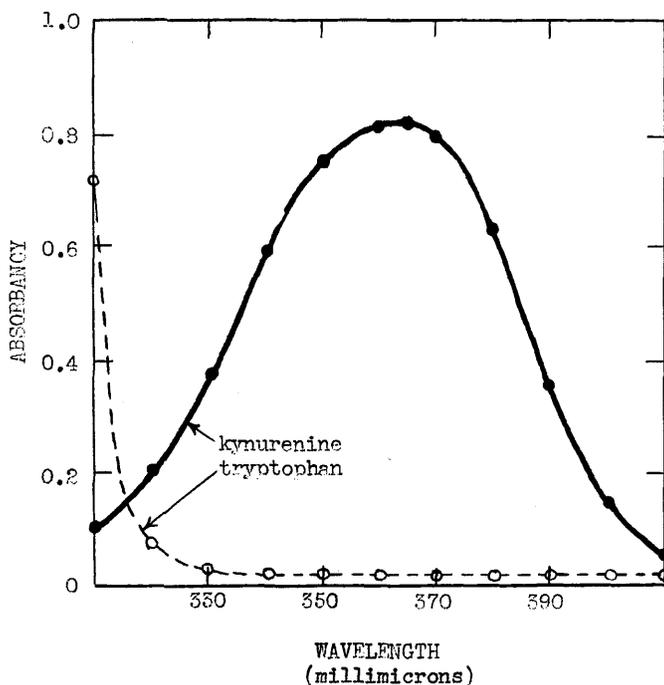


FIGURE 1. The Absorption Spectra of DL-kynurenine and L-tryptophan. The absorption of solutions of DL-kynurenine (0.3 micromoles/ml) and L-tryptophan (30.0 micromoles/ml) was measured at various wavelengths from 310 to 410 m μ .

Hematin has been used in the assay of tryptophan pyrrolase in rat liver (Freigelson and Greengard, 1961). We found its use to be advantageous, but not essential, in the assay of tryptophan pyrrolase in *Drosophila*. The results of studies made to determine the optimal concentrations of L-tryptophan and hematin in the assay system are illustrated in figures 2 and 3. An optimal concentration was considered to be one high enough to achieve zero order kinetics, yet low enough to cause no absorption at 365 m μ . As is evident, the velocity of the reaction reached a plateau when the L-tryptophan concentration was about 7 micromoles/ml and when the hematin concentration was about 0.00125 mg/ml. With L-tryptophan no absorption occurred at any of the concentrations measured.

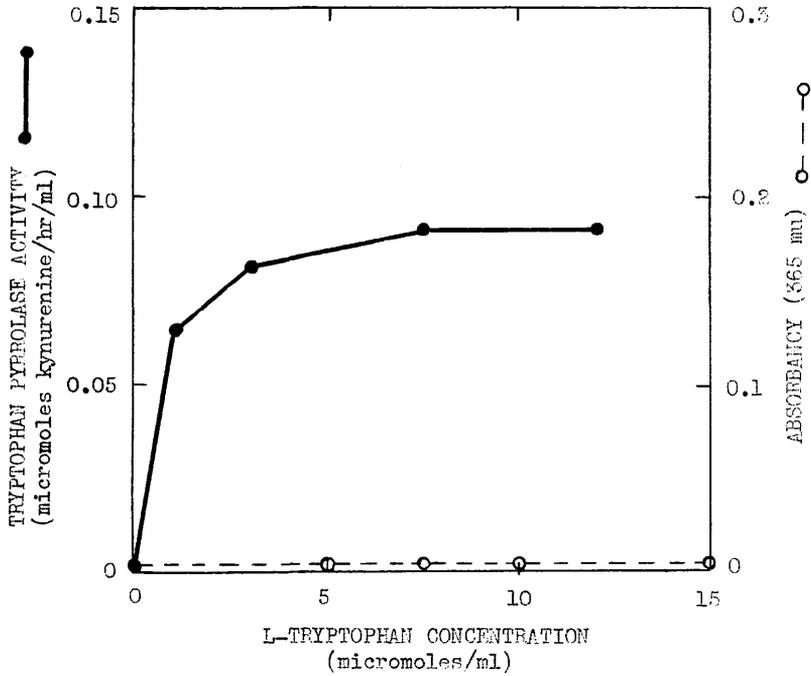


FIGURE 2. The Effect of the Concentration of L-tryptophan on Tryptophan Pyrrolase Activity and on Absorption at 365 mμ. Assays were performed on the 15,000 x g supernatant as described in the text.

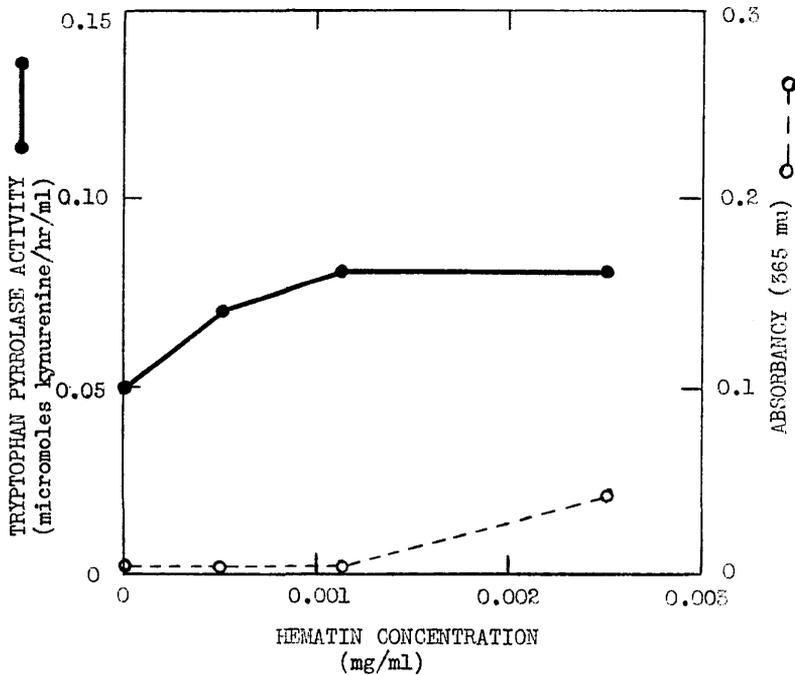


FIGURE 3. The Effect of the Concentration of Hematin on Tryptophan Pyrrolase Activity and on Absorption at 365 mμ. Assays were performed on the 15,000 x g supernatant as described in the text.

With hematin, however, absorption occurred at a concentration above 0.00125 mg/ml. This meant that L-tryptophan could be used in the assay system at any concentration above 7 micromoles/ml, while it was necessary to use hematin at a concentration of 0.00125 mg/ml.

Sodium ascorbate is necessary to prevent the disappearance of kynurenine. It acts by reducing the tyrosinase-produced quinones in the larval homogenate, and thus prevents their non-enzymatic condensation with kynurenine (Glassman, 1957). Ascorbate is necessary, therefore, for the net formation of kynurenine. In fact, in some experiments, performed without ascorbate, the amount of kynurenine actually decreased with time. Although ascorbate seemed to produce a continual increase in tryptophan pyrrolase, it began to absorb light at a concentration of between 10 and 15 micromoles/ml (fig. 4). Ascorbate, therefore, was

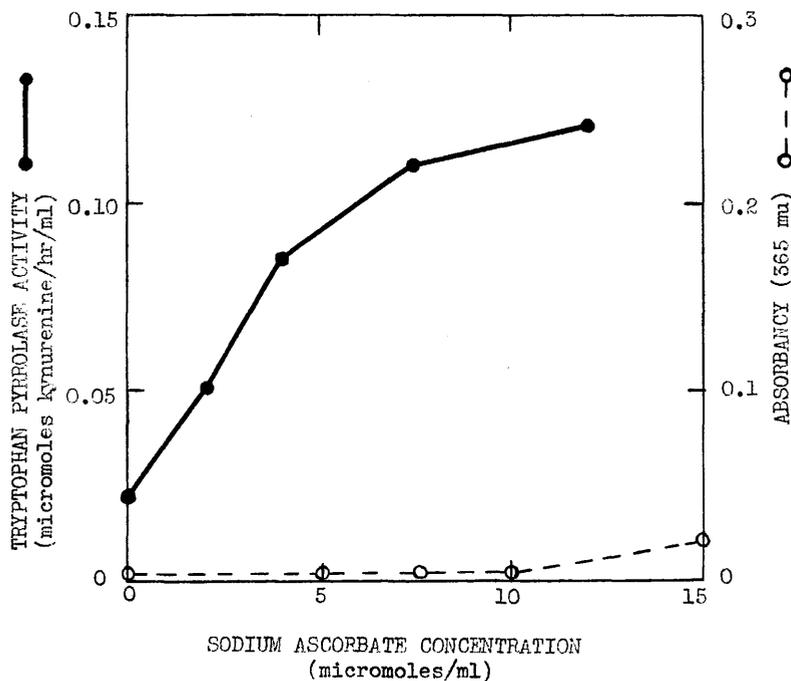


FIGURE 4. The Effect of the Concentration of Sodium Ascorbate on Tryptophan Pyrrolase Activity and on Absorption at 365 $m\mu$. Assays were performed on the 15,000 x g supernatant as described in the text.

added to the assay system at a concentration of 10 micromoles/ml.

The assay system was linear within certain time limits (fig. 5). Measurements made at less than 30 minutes were very irregular, in some instances showing an initial drop. After 180 minutes, sedimentation sometimes occurred. Determinations were made, therefore, between 30 and 180 minutes only.

The validity of the assay system is evident from the fact that the addition of increasing amount of the 15,000 x g supernatant resulted in corresponding increases in the amount of tryptophan pyrrolase activity measureable (fig. 6).

DISCUSSION

The first step in our effort to develop a simple, rapid assay for tryptophan pyrrolase in *Drosophila* was to work out a method whereby kynurenine could be

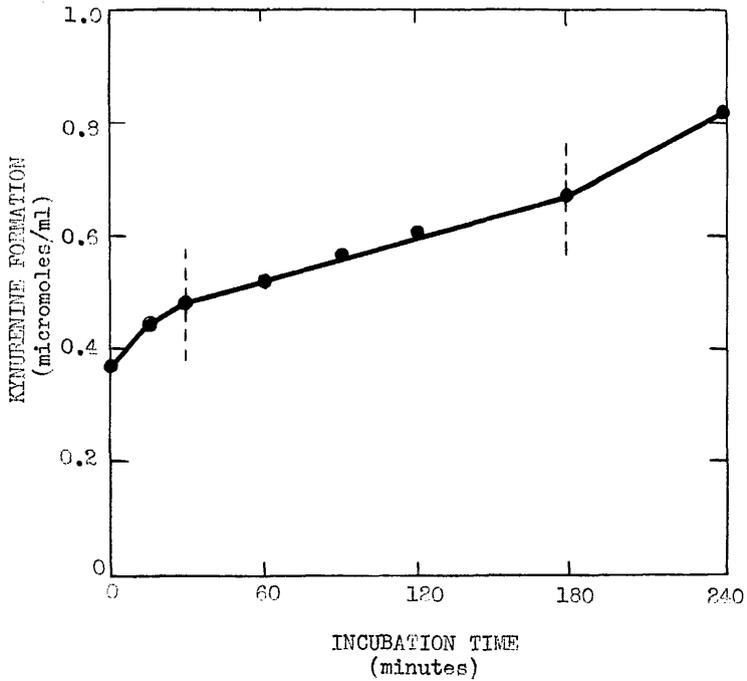


FIGURE 5. The Linearity of the Assay System. Aliquots of the 15,000 x g supernatant were incubated at 37° as described in the text. The amount of kynurenine present in each system was determined, at various time intervals, by absorbancy measurements at 365 m μ . The dotted lines indicate the time limits within which the reaction was linear.

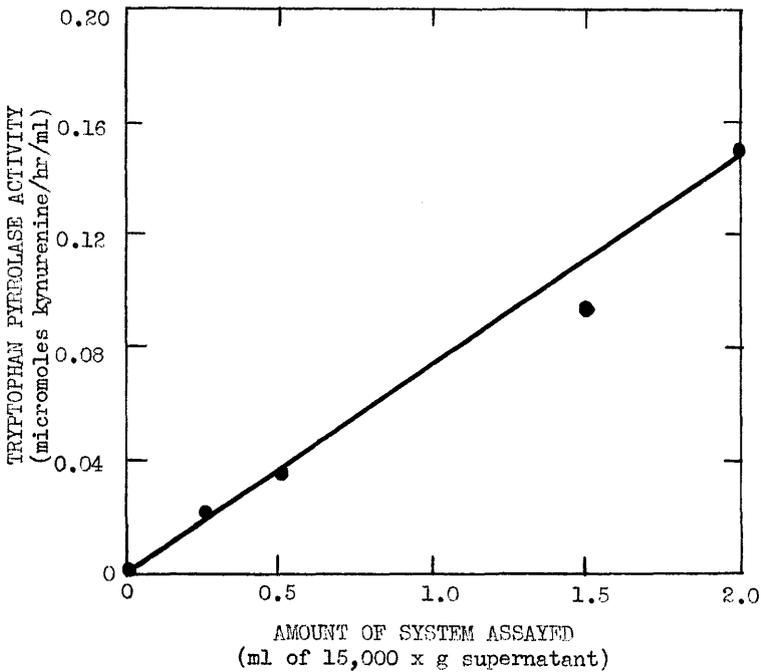


FIGURE 6. The Validity of the Assay System. Aliquots of the 15,000 x g supernatant were assayed as described in the text.

measured as it formed, rather than after the reaction had been stopped. A kinetic assay for tryptophan pyrrolase in rat liver had been reported by Greengard and Feigelson (1962). Their method, however, involved the use of a Beckman DU spectrophotometer equipped with thermal spacers maintained at 37°C and a Gilford continuous-recording attachment. This limited the method to the assay of only three reaction mixtures and a reaction blank. With a Dubnoff shaker, however, we were able to incubate and assay as many as 36 samples simultaneously. Because the reaction was linear for three hours, and because we could make an absorbancy measurement every minute, we could obtain six 30-minute determinations for each of 30 reaction mixtures (or a total of 180 readings in three hours). Other combinations are, of course, possible. For example, one could also obtain twelve 15-minute determinations for each of 15 reaction mixtures, and so on.

By decanting the contents of the incubation flasks into and out of the cuvette for the absorbancy measurements (rather than removing aliquots at various time intervals), it was possible to assay a smaller amount of material. Also, by making all measurements in a single cuvette, no window corrections were necessary.

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