RE-EVALUATION OF A SYSTEM FOR THE IN VITRO SYNTHESIS OF TRYPTOPHAN PYRROLASE

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Morrison and Frajola (1964) reported the in vitro synthesis of tryptophan pyrrolase in a protein synthesizing system isolated from Drosophila melanogaster. Since tryptophan pyrrolase activity is known to be controlled by the vermilion locus of Drosophila (Baglioni, 1960; Kaufman, 1962) this system promised to be useful in analyzing the action of the mutant suppressor of vermilion. In attempting to use this system we added three controls to ensure the validity of our results. These controls, however, have led to a re-evaluation of the system and are the subjects of this report.

In Drosophila, the oxidation of tryptophan to N-formylkynurenine is catalyzed by the enzyme tryptophan pyrrolase. N-formylkynurenine is converted to kynurenine in a reaction mediated by kynurenine formamidase (Glassman, 1956). Thus, in the presence of excess kynurenine formamidase, the formation of kynurenine serves as a measure of tryptophan pyrrolase activity.

Morrison and Frajola incubated various RNA extracts in a protein synthesizing system (PSS). Aliquots of this PSS were then added to an incubation mixture known to allow the formation of kynurenine from tryptophan when tryptophan pyrrolase is present. Thus the formation of kynurenine would re-

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flect the in vitro synthesis of tryptophan pyrrolase. To detect the formation of kynurenine, Morrison and Frajola measured the increase in OD at λ = 365 mμ, the absorption maximum of kynurenine. According to their report, an increase in $OD_{365\ m\mu}$ in the incubation mixture was observed when a cell-free PSS isolated from a tryptophan pyrrolase deficient mutant (vermilion) and a wild type Drosophila extract thought to contain tryptophan pyrrolase messenger RNA were used. This increase was apparently dependent on the energy generating system in the PSS and was sensitive to the addition of chloramphenicol, puromycin, ribonuclease, and "sometimes" deoxyribonuclease.

A possible source of error in this method of determining tryptophan pyrrolase activity lies in the use of ascorbate as a reducing agent in the enzyme incubation system. Marzluf (1965) has shown that a tryptophan dependent reaction which intereferes with the assay of kynurenine occurs in this system, and that this reaction is eliminated by replacing ascorbate with 2-mercaptoethanol. Thus, as one control, we used the following incubation system modified from Marzluf (1965). 0.4 ml aliquots of the enzyme preparation were incubated at 37°C in a 1.0 ml total volume which also contained tryptophan, 3.6 μm; 2-mercaptoethanol, 100.0 μm; and potassium phosphate buffer, pH 7.4, 0.75 μm.

As an additional method for determining kynurenine we utilized the highly sensitive Bratton-Marshall test (Bratton and Marshall, 1939). We determined that the components of the PSS, which are present in the assay samples, do not adversely affect the Bratton-Marshall test. This second control, modified from Kaufman (1962), was as follows. 1.0 ml samples of the incubation mixture were placed in tubes containing 3.0 ml trichloroacetic acid (6.7%) after which the precipitated protein was removed by filtration. To 0.5 ml of the filtrate were added 0.2 ml sodium nitrite (0.2%), 0.2 ml ammonium sulfamate (0.5%), and 0.2 ml N-(naphthyl)ethylenediamine dihydrochloride (0.1%) in that order, allowing three minutes between each addition to permit completion of the reactions. The mixture was

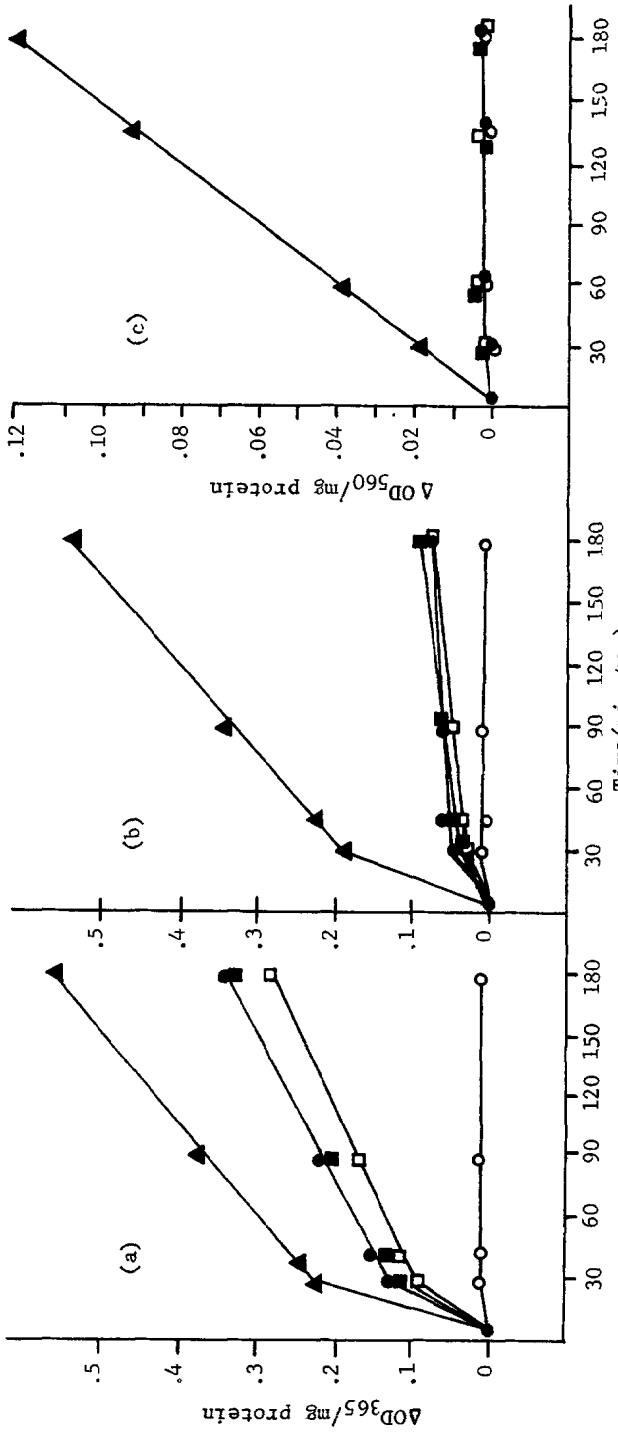

Figure 1. Increases in OD with variations in the PSS, enzyme incubation, and method of assay. (a) ascorbate incubation-direct absorption assay; (b) ascorbate incubation-Bratton-Marshall assay; (c) 2-mercaptoethanol incubation-Bratton-Marshall assay. See text for details.

	RNA ADDED TO PSS	TRYPTOPHAN PRESENT IN INCUBATION SYSTEM
(●-●)	Canton-S	yes
(○-○)	Canton-S	no
(■-■)	v^{36f}	yes
(□-□)	no RNA added	yes

(▲-▲) crude tryptophan pyrrolase preparations from Canton-S run in each experiment as a control on the incubation and assay systems.

then allowed to stand for 18-24 hours in the dark, after which the $OD_{560\ m\mu}$ was determined. All steps were carried out at 0-5°C.

As the final control, the response of the PSS to an RNA extract from a nonleaky, tryptophan pyrrolase deficient stock (v^{36f}) was tested.

The methods used for RNA extraction, *in vitro* protein synthesis, ascorbate enzyme incubation, and direct absorption assay were those specified by Morrison and Frajola.

If, in fact, tryptophan pyrrolase is synthesized in the *in vitro* system used by Morrison and Frajola, then its enzymatic activity should also be

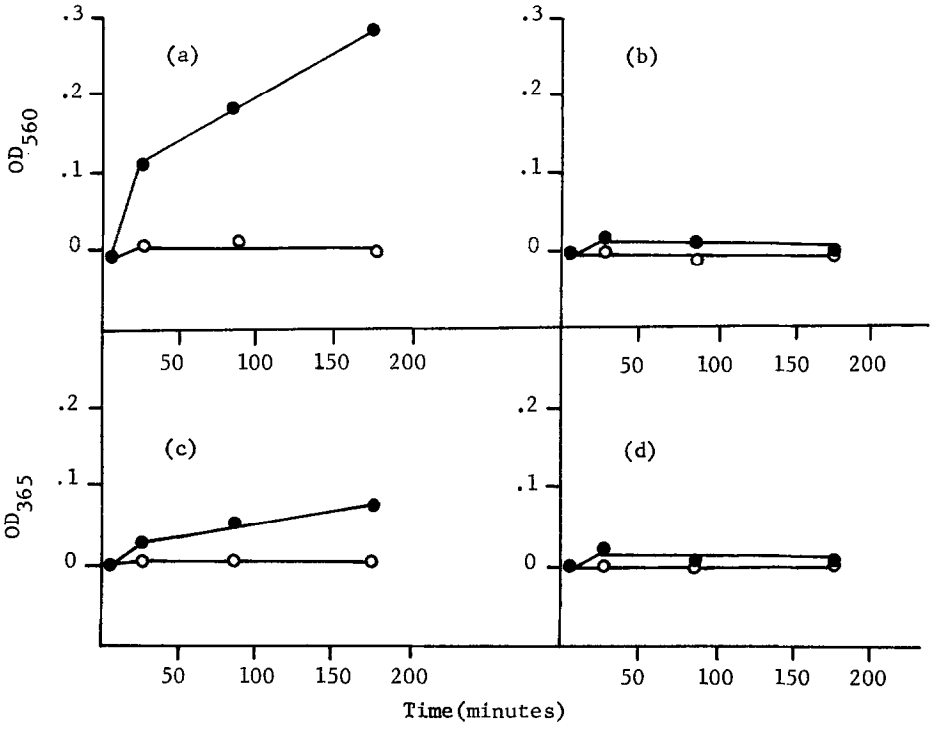

Figure 2. Increase in OD occurring in the enzyme incubation systems in the absence of enzyme. Distilled H_2O was added in place of the enzyme preparation.
(a) ascorbate incubation system assayed by direct absorption
(b) 2-mercaptoethanol incubation system assayed by direct absorption
(c) ascorbate incubation system assayed by Bratton-Marshall test
(d) 2-mercaptoethanol incubation system assayed by Bratton-Marshall test
(●-●) tryptophan present; (o-o) tryptophan absent

amenable to assay by the 2-mercaptoethanol-Bratton-Marshall system. Fig 1 shows the results of applying various methods for determining the *in vitro* synthesis of tryptophan pyrrolase in response to various RNA extracts. Note that any semblance of activity observed through the ascorbate-direct absorption system used by Morrison and Frajola (Fig 1a) is not evident in the 2-mercaptoethanol-Bratton-Marshall system (Fig 1c). The apparent substrate dependence of the "activity" observed when the ascorbate-direct absorption system is used (Fig 1a, 1b) is shown in Fig 2 to be the result of the tryptophan dependent, nonenzymatic reaction occurring in the ascorbate incubation system.

Finally, the PSS showed an identical response to RNA extracts from Canton-S (a wild type strain) and $\underline{v}^{36f}$ (Fig 1a, 1b, 1c). In addition, neither the Canton-S nor the $\underline{v}^{36f}$ RNA extracts could elicit a positive response in the PSS as determined by the 2-mercaptoethanol-Bratton-Marshall system.

The controls, therefore, demonstrated that the system reported by Morrison and Frajola (1964) does not support the *in vitro* synthesis of tryptophan pyrrolase.

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