FUNCTION OF PYRIDOXAL PHOSPHATE: RESOLUTION AND PURIFICATION OF THE TRYPтопHANASE ENZYME OF ESCHERICHIA COLI

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Since Hopkins and Cole (1) demonstrated the formation of indole from tryptophan by Bacterium coli, numerous investigators have attempted to find the mechanism of this reaction (2-5). At least three mechanisms have been suggested. Woods (3) postulated an oxidative degradation of tryptophan to yield indole, carbon dioxide, water, and ammonia, 5 atoms of oxygen being used in the process. Baker and Happold (6) suggested a primary fission into indole and alanine, followed by the oxidation of alanine. Krebs et al. (7) proposed that the mechanism involved a preliminary oxidation of the indole ring, followed by oxidation of the side chain to yield o-aminophenylacetdehyde, which condensed to indole spontaneously. The data, however, did not substantiate this view and Krebs was led to state that, while Escherichia coli would form indole from o-amino-β-phenyl-ethanol, via the analogous aldehyde, the mechanism of tryptophanase action very probably did not involve this compound as an intermediate.

Woods (3) and Baker and Happold (6) studied a series of possible oxidative intermediates between tryptophan and indole, and concluded that an unaltered alanine side chain was necessary for tryptophanase action. More recently, Dawson (8) found that mepacrine (atabrine) inhibits tryptophanase, and Dawes, Dawson, and Happold (9) have been able to recover alanine concurrently with indole formation in the presence of mepacrine. They have thus strengthened Baker and Happold's postulate of primary fission to indole and alanine.

In the present study, Escherichia coli cells with a very active tryptophanase system have been obtained by growing the culture with aeration. The cells have been vacuum- or acetone-dried to yield cell preparations which contain most of the activity present in the living cells. The enzyme is stable in these preparations and may be obtained in a cell-free state by autolysis. The resolution and purification of the enzyme have been accomplished by precipitation of the cell-free extracts with ammonium sulfate and by calcium phosphate adsorption. Pyridoxal phosphate will reactivate the resolved enzyme, thereby adding tryptophanase to the group of vitamin B₆ enzymes.

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The products of the action of these purified preparations, as well as of the dried cell preparations, are indole, pyruvate, and ammonia, in an equimolar ratio.

The purified tryptophanase enzyme does not deaminate alanine or serine; thus neither of these is an intermediate in the tryptophanase reaction.

Methods

Culture—The Crookes strain of Escherichia coli from the departmental collection was used. With this culture a very active tryptophanase enzyme was obtained by growth in a medium composed of 1 per cent tryptone, 1 per cent yeast extract, 0.5 per cent K$_2$HPO$_4$, and 0.1 per cent glucose. The medium was dispensed in 200 ml. amounts in 500 ml. Erlenmeyer flasks, inoculated, incubated 4 to 6 hours at 30°, then placed in a mechanical shaker (approximately a hundred 3 inch strokes per minute) and incubated for an additional 18 to 20 hours. The cells were harvested with a Sharples centrifuge. The cell paste from 6 liters of medium was washed with 250 ml. of water, centrifuged, resuspended in 15 to 20 ml. of distilled water, and dried in vacuo over drierite to yield about 10 gm. of dried cells. Acetone-dried cells were prepared by pipetting the washed cell suspensions into 10 volumes of ice-cold acetone. The cells were collected on a Büchner funnel and washed with ether. Both the vacuum-dried and acetone-dried preparations contained two-thirds or more of the tryptophanase activity present in the living cells, the enzyme being stable in the dried state.

Tryptophanase Determination—Tryptophanase activity was determined by measuring indole formation. The usual assay was performed in 2 ml. volume containing the following: 0.2 ml. of 1 M phosphate buffer, pH 8.3; 20 γ of barium pyridoxal phosphate; 50 to 500 γ of the cell preparations described above; 2 mg. of L-tryptophan. The enzyme, buffer, and co-enzyme were incubated at 37° in a volume of 1.8 ml. for 10 minutes. The substrate (0.2 ml.) was added, the reaction allowed to proceed 10 minutes, and then stopped with 0.2 ml. of 100 per cent trichloroacetic acid. The indole was extracted by shaking with 2 ml. of toluene, and a portion of the toluene layer was removed for analysis.

Analytical Methods—Indole was determined by Erhlich's method, modified as follows: From 0.2 to 1 ml. of toluene layer, depending on the level of indole expected, was pipetted into a colorimeter tube and 1 ml. of 5 per cent p-dimethylaminobenzaldehyde in ethyl alcohol was added. The tubes were filled to the 10 ml. line with an acid alcohol solution (1 liter of ethyl alcohol plus 50 ml. of concentrated sulfuric acid), allowed to stand 10 minutes, and read in an Evelyn colorimeter, with the No. 540
filter. With this method, indole can be determined over a range of 1 to 15 γ; the method may be used to 20 γ without great deflection from linearity.

Pyruvate—For most determinations, the direct method of Friedemann and Haugen (10) with 2,4-dinitrophenylhydrazine was used. The identity of the pyruvate was established by the toluene extraction method, the quantitative data agreeing with the direct method.

Ammonia—Ammonia was determined by nesslerization after distillation from a Pregl still (11). The reaction mixture was deproteinized with trichloroacetic acid, neutralized to nearly pH 7, and pipetted into the still. 2 ml. of a borate buffer (12) were added and the sample steam-distilled for 5 minutes, about 6 ml. of distillate being collected. The distillate was nesslerized by adding 2 ml. of Johnson’s reagent (13) and 1 ml. of 6 N alkali, and then diluted to 10 ml. After 10 minutes the color was read in the Evelyn colorimeter with the No. 490 filter.

Results

With the dried cell preparations, it was found that the quantity of indole formed was proportional to the cell concentration only at the lower levels (Fig. 1). The lack of proportionality of indole production at the higher cell concentrations was later shown to be due to the inhibitory effect of indole, as previously described by Fildes (2). The enzyme could be assayed reasonably well, however, over a range of cell concentrations from 50 to 300 γ; equivalent to 1.5 to 12 γ of indole formed in a 10 minute incubation period. As shown in Fig. 2, the rate of indole production by a given cell concentration also decreased with time. Inasmuch as the cell preparations were sufficiently active to give accurate analytical values within a 10 minute period, there was no need for a longer incubation time.

The dried preparations did not show an oxygen uptake with tryptophan, and so no effort was made to run the experiments anaerobically. An attempt to determine the products of tryptophanase action showed that pyruvate was formed in approximately equimolar ratio to the indole formed. The preparations contained serine and alanine deaminases, which also formed pyruvate; thus one of these might be an intermediate in the tryptophanase reaction. The alanine would be considered as a possible intermediate in view of the work of Dawes, Dawson, and Happold (9); and serine might be considered in view of the formation of tryptophan from serine and indole by the Neurospora enzyme (14, 15). In order to determine whether either of these was an intermediate in the reaction, conditions were sought in which the tryptophanase was active whereas the deaminases were not.

In view of the function of pyridoxal phosphate as the coenzyme of tryptophan synthesis with the Neurospora enzyme (15), the possibility of
its function in the tryptophanase reaction was obvious. Attempts to demonstrate the action of pyridoxal phosphate with the dried preparations resulted in approximately 50 per cent stimulation of the rate of indole formation. Thus, even in the dried preparations, the enzyme system which forms indole from tryptophan was partially resolved and could be activated by pyridoxal phosphate as the coenzyme.

Fig. 1. Tryptophanase activity of dried cell preparation. 2 ml. reaction volume containing 0.2 ml. of 1 M phosphate buffer, pH 8.3; 20 γ of pyridoxal phosphate (barium salt); dried cells as indicated; water to 1.8 ml.; let stand 10 minutes at 37°; add 0.2 ml. (2 mg.) of L-tryptophan; incubate 10 minutes at 37°.

Fig. 2. Tryptophanase activity and incubation time. Conditions are the same as in Fig. 1.

**Enzyme Purification and Resolution**

10 gm. of an acetone-dried preparation were evenly suspended in 500 ml. of water in a Florence flask and placed in a cold room overnight and allowed to freeze. The next morning the flask was removed from the cold room and placed in a 37° water bath, where thawing and autolysis were allowed to proceed for 2 hours. The cell débris was removed by centrifugation and the supernatant treated at room temperature, with an equal quantity of saturated ammonium sulfate neutralized to a pH of about 8.5. Upon standing in the refrigerator for a short time, the precipitate flocculated and was removed by centrifugation. The supernatant was saturated with solid ammonium sulfate and 7 ml. of 0.01 M sodium cyanide
added (final concentration 0.0001 M). The solution was allowed to stand in the refrigerator until the precipitate flocculated, and was then centrifuged. The precipitate was suspended in 270 ml. of water and the insoluble matter removed by centrifugation. The supernatant solution was again treated with ammonium sulfate to 55 per cent saturation and the small quantity of precipitate which formed was discarded. The enzyme was then precipitated by adding ammonium sulfate to 68 per cent saturation and the precipitate removed by centrifugation. This precipitate, which contained about 10 per cent of the enzyme originally present in the cells, was suspended in 50 ml. of water. Pilot experiments were performed to determine the concentration of calcium phosphate gel necessary just to adsorb the enzyme, and the indicated amount was added with mixing and allowed to stand 10 minutes. The phosphate gel was collected by centrifugation and washed with five 250 ml. portions of distilled water, after which the enzyme was eluted from the gel with 50 ml. of 1 M phosphate buffer, pH 6.0. The enzyme at this stage of purification was completely resolved and free from serine and alanine deaminases (see Table I).

Table I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pyruvate formed</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 mg. cell preparation</td>
<td>0.05 ml. cell-free enzyme</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>18.3</td>
<td>42.5†</td>
</tr>
<tr>
<td>without coenzyme</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>DL-Alanine</td>
<td>1.0</td>
<td>0.3</td>
</tr>
<tr>
<td>DL-Serine</td>
<td>8.1</td>
<td>0</td>
</tr>
<tr>
<td>L-Serine*</td>
<td>5.0</td>
<td>0.1</td>
</tr>
</tbody>
</table>

* We wish to thank Dr. J. S. Fruton for a supply of L-serine.
† 50 γ of indole formed; without coenzyme no indole formed.

The characteristics of Tryptophanase

The influence of tryptophan concentration on the reaction rate is shown in Fig. 3. With the cell-free enzyme, the half maximum rate is obtained with 35 γ of tryptophan per ml. (Michaelis constant (16), $K = 2.5 \times 10^{-5}$ mole per liter). With the dried cells, a somewhat higher substrate concentration is required, the rate of indole formation dropping sharply below 200 γ of tryptophan per ml.
The coenzyme saturation curve for tryptophanase is shown in Fig. 4, the half saturation concentration of barium pyridoxal phosphate being 0.9 \( \gamma \) per ml. (Michaelis constant, \( K = 2.1 \times 10^{-6} \) mole per liter). Thus the dissociation constant of the pyridoxal phosphate-tryptophanase complex approximates that of the glutamic-aspartic transaminase, \( K = 1.5 \times 10^{-6} \), but higher than that of the tyrosine decarboxylase, \( K = 1.5 \times 10^{-8} \) mole per liter. Both the tryptophanase and the transaminase reactions are run at neutral reaction, but the tyrosine decarboxylase is run at an acid pH.

2 Gunsalus, I. C., and Umbreit, W. W., unpublished data.
Fig. 5. Influence of products upon tryptophanase activity. Conditions are the same as in Fig. 1. 0.03 ml. of purified enzyme; indole pyruvate and ammonia, added in the quantities indicated, before the enzyme is added.

**Table II**

*Inhibition of Tryptophanase by NaCN*

Conditions are the same as in Fig. 1; 0.03 ml. of purified enzyme; cyanide added after enzyme and coenzyme.

<table>
<thead>
<tr>
<th>Concentration of cyanide</th>
<th>Indole formed</th>
<th>Per cent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>γ</td>
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<tr>
<td>$10^{-2}$</td>
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<td>100</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>1.0</td>
<td>95</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>5.2</td>
<td>70</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>11.5</td>
<td>35</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>17.5</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>17.5</td>
<td></td>
</tr>
</tbody>
</table>

**Table III**

*Products of Tryptophanase Reaction*

Conditions: 7 ml., reaction volume; 0.7 ml. of 1 M phosphate buffer, pH 8.3; 70 γ of pyridoxal phosphate (barium salt); 0.3 ml. of purified enzyme. Water to 6.3 ml.; let stand 10 minutes, 37°; 0.7 ml. (7 mg.) of L-tryptophan added and incubated 10 minutes.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Micromoles of products formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Indole</td>
</tr>
<tr>
<td>2057</td>
<td>0.92</td>
</tr>
<tr>
<td>2101</td>
<td>1.37</td>
</tr>
<tr>
<td>2102</td>
<td>1.44</td>
</tr>
<tr>
<td>2103</td>
<td>1.02</td>
</tr>
<tr>
<td>2103a</td>
<td>0.82</td>
</tr>
</tbody>
</table>

Molar ratio (average) 1.00 1.05 1.05
Tryptophanase is sensitive to indole accumulation, as indicated by the cell preparations, Figs. 1 and 2, and as shown with the cell-free enzyme, Fig. 5. The presence of pyruvate plus ammonia has a slight influence.

The enzyme is sensitive to cyanide (Table II). This sensitivity, however, may not indicate an iron catalyst but a reaction between cyanide and the free carbonyl group of pyridoxal phosphate. A similar sensitivity of pyridoxal phosphate-containing enzymes to cyanide is mentioned by Blaschko (17) and by Gale (18) for the animal and bacterial decarboxylases respectively.

**Products of Tryptophanase Reaction**

The tryptophanase reaction, as catalyzed by the dried cell preparations and by the partially purified enzyme, can be expressed by the following equation: tryptophan → indole + pyruvic acid + ammonia. Analyses of the products formed in several experiments with the purified enzyme are shown in Table III.

**DISCUSSION**

From the data presented, it is apparent, that the tryptophanase system of *Escherichia coli* is not the reversal of the indole and serine condensation which leads to tryptophan with the *Neurospora* enzyme. With purified tryptophanase the products are indole, pyruvate, and ammonia. Serine and alanine do not yield pyruvate, and are not, as such, intermediates in the reaction. The intermediates postulated by various investigators (3, 5, 7), with the possible exception of amino acrylic acid (12), do not appear to be involved.

**SUMMARY**

Tryptophanase has been obtained in a cell-free state from *Escherichia coli* and has been partially purified.

The enzyme has been resolved and shown to require pyridoxal phosphate as the coenzyme.

The enzyme preparation catalyzes the breakdown of tryptophan according to the following reaction: tryptophan → indole + pyruvic acid + ammonia. No oxidation occurs in the process, nor does alanine or serine occur as an intermediate.

**BIBLIOGRAPHY**

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