The Conversion of Shikimic Acid to Anthranilic Acid by Extracts of Neurospora crassa

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The role of shikimic acid as a common intermediate in the synthesis of phenylalanine, tyrosine, tryptophan, and \( p \) amino benzoic acid has been recognized for some time (1-3). The enzymes which catalyze the synthesis of shikimic acid from intermediates of carbohydrate metabolism have been observed in extracts of bacteria (4-6) as well as Neurospora crassa (7). On the other hand, the reactions leading from shikimic acid to specific intermediates in the synthesis of the various aromatic amino acids have been poorly understood. Only recently, studies with extracts from bacterial mutants (8-13) have implicated the sequence of reactions presented in Fig. 1. From the bacterial studies, it appears that shikimic acid is converted by three enzymatic reactions to chorismic acid, which serves as a direct precursor of the various specific pathways of aromatic biosynthesis.

Genetic and nutritional studies with \( N. \) crassa indicate that the first enzyme specifically involved in tryptophan biosynthesis catalyzes the formation of anthranilic acid (14). As an initial approach toward the investigation of this enzyme in \( N. \) crassa, attempts were made to show the conversion of shikimic acid to anthranilic acid. Under the conditions employed in the bacterial studies, crude extracts of \( N. \) crassa did not catalyze this conversion. The studies presented here were undertaken to define the conditions necessary for the synthesis of anthranilic acid from shikimic acid in \( N. \) crassa and to investigate the reactions involved.

**EXPERIMENTAL PROCEDURE**

*Escherichia coli* K12 was grown on minimal medium (15) plus 0.5% glucose at 37° with forced aeration for 24 hours. The cells were harvested in a Sharples centrifuge, suspended in 4 volumes of 0.02 M potassium phosphate buffer, pH 6.8, and treated for 60 seconds in a Branson sonifier. The crude extract was prepared by centrifuging the broken suspension at 20,000 \( \times \) g for 20 minutes.

The strain of \( N. \) crassa used in these studies, \( \text{td} 48R \) (16), is an indole-utilizing \( \text{td} \) mutant; i.e. it cannot convert indole glycerol phosphate to tryptophan but can convert indole to tryptophan. This organism was grown on minimal medium (17) plus 20 \( \mu \)g of indole per ml at 30° with forced aeration. The cultures were incubated for 12 hours after the exhaustion of indole (approximately 48 hours). The mycelia were harvested by filtration on cheesecloth, lyophilized, powdered in a Wiley mill, and stored in a deep freeze. The lyophilized powder was stirred for 20 minutes with 15 volumes of 0.02 M Tris buffer, pH 8.0, in an ice bath. The crude extract was prepared by centrifuging this suspension at 13,000 \( \times \) g for 20 minutes to remove cell debris. For assaying anthranilic acid synthesis, it was necessary to pass the crude extract over a Sephadex G-25 column to remove small molecules which interfered with assay procedures.

The crude extract of \( N. \) crassa was fractionated by the following procedures. The extract was first treated with protamine sulfate (18). The protamine sulfate supernatant solution was adjusted to 40% saturation with solid ammonium sulfate, stirred for 10 minutes in an ice bath, and centrifuged for 10 minutes at 10,000 \( \times \) g. The precipitate was dissolved in 0.02 M Tris buffer, pH 8.0. By the same procedure, 40 to 50% and 50 to 60% saturation fractions were prepared. Each of the above fractions was passed over a Sephadex G-25 column for use in assays or for further fractionation.

The 40 to 50% fraction was adjusted to pH 6.0 with 1 M acetic acid and added to the top of a brushtite column, prepared according to Burns (19), at 2.0 mg of protein per ml of brushtite. The column was eluted with potassium phosphate buffer as follows: 1 column volume at pH 6.0; 1 column volume, pH 6.5; and 2 column volumes at pH 7.0. Fractions active in the conversion of chorismic acid to anthranilic acid (see Table VII) were eluted in the last buffer. The most active fractions were combined and reassayed as indicated.

Anthranilic acid formation was assayed by two procedures. (a) A 0.4-ml sample of incubation mixture was acidified with 0.1 ml of 1 N HCl and extracted with 5.0 ml of ethyl acetate. Anthranilic acid in the ethyl acetate layer was determined in the Aminco-Bowman spectrophotofluorometer. Determinations were carried out at both zero time and the end of the incubation. (b) Alternatively, anthranilic acid formation was determined directly in the incubation mixture in the spectrophotofluorometer. In each case, authentic anthranilic acid was used as a standard under the conditions of the assay. The range in either determination is from 0.01 to 10 \( \mu \)g of anthranilic acid per ml of incubation mixture.

Chorismic acid was prepared by the procedure of Gibson (13) from the growth filtrate of \( \text{Aerobacter aerogenes} \) 62-1.

Protein was determined according to the Lowry procedure (20).

**RESULTS**

The general conditions utilized to show the conversion of shikimic acid to anthranilic acid with bacterial extracts (9, 12) were used in an attempt to demonstrate a similar conversion with extracts from \( N. \) crassa. When a crude extract from \( N. \) crassa was incubated with shikimic acid, Mg\(^{2+}\), ATP, P-enolpyruvate,
Conversion of Shikimic Acid to Anthranilic Acid

**Fig. 1.** Pathway of aromatic biosynthesis in bacteria. PEP, phosphoenolpyruvate

**TABLE I**

Formation of anthranilic acid from shikimic acid by crude extracts from *N. crassa* and *E. coli*

<table>
<thead>
<tr>
<th>Extract</th>
<th>Anthranilic acid formed* μmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude <em>N. crassa</em> (0.8 mg of protein)</td>
<td>0.000</td>
</tr>
<tr>
<td>Crude <em>E. coli</em> (2.0 mg of protein)</td>
<td>0.051</td>
</tr>
<tr>
<td>Crude <em>N. crassa</em> (0.8 mg of protein) and crude <em>E. coli</em> (2.0 mg of protein)</td>
<td>0.010</td>
</tr>
</tbody>
</table>

* Conditions: Tris buffer, pH 8.2, 50 μmoles; MgCl₂, 5 μmoles; shikimic acid, 2 μmoles; ATP, 1 μmole; DPNH, 0.75 μmole; L-glutamine, 10 μmoles; phosphoenolpyruvate, 1 μmole; extract as indicated; 1.0 ml final volume; incubated for 30 minutes at 37°. Anthranilic acid was determined after extraction into ethyl acetate (see "Experimental Procedure").

**TABLE II**

Fractionation of inhibitor present in crude extract of *N. crassa*

<table>
<thead>
<tr>
<th>Fraction of <em>N. crassa</em> extract added*</th>
<th>Protein in fraction mg</th>
<th>Anthranilic acid formed† μmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.051</td>
<td></td>
</tr>
<tr>
<td>Protamine sulfate supernatant</td>
<td>0.4</td>
<td>0.014</td>
</tr>
<tr>
<td>0-40% ammonium sulfate</td>
<td>2.0</td>
<td>0.084</td>
</tr>
<tr>
<td>40-50% ammonium sulfate</td>
<td>3.1</td>
<td>0.111</td>
</tr>
<tr>
<td>50-60% ammonium sulfate</td>
<td>2.9</td>
<td>0.000</td>
</tr>
</tbody>
</table>

* For fractionation procedure, see "Experimental Procedure."
† Conditions as in Table I.

Glutamine, and DPNH, no anthranilic acid synthesis was detected (Table I). Variations in substrate concentrations and conditions of extract preparation were without effect in attempts to observe anthranilic synthesis from shikimic acid. On the other hand, crude extracts from *E. coli* K₁² formed anthranilic acid from shikimic acid under the conditions employed with the *N. crassa* extract. The addition of the crude extract from *N. crassa* inhibited the accumulation of anthranilic acid about 80%. This result suggested that the failure to obtain the conversion of shikimic acid to anthranilic acid with crude extracts of *N. crassa* might be due to the presence of an inhibitor.

When the crude extract was fractionated by protamine sulfate treatment and ammonium sulfate precipitation, the apparent inhibitor was selectively precipitated between 50 and 60% saturation with ammonium sulfate (Table II). On the other hand, the fractions precipitated between 0 and 50% saturation with ammonium sulfate caused a significant increase in anthranilic accumulation by the crude extract of *E. coli*. The most active fraction, the fraction precipitated between 40 and 50% saturation with ammonium sulfate, converted shikimic acid to anthranilic acid in the absence of the bacterial extract, but at a rate far less than expected from the magnitude of stimulation of the bacterial extract by the fraction. These results suggested that the bacterial extract was supplying some required factor to the extract from *N. crassa* which was missing from the substrate additions.

The data in Table III show that a boiled extract of *E. coli* also stimulated the conversion of shikimic acid to anthranilic acid by *N. crassa* extracts. A crude extract from *E. coli*, which had been boiled for 3 minutes, cooled, and centrifuged for 5 minutes at 20,000 × g, was completely inactive by itself. However, the formation of anthranilic acid by the extract from *N. crassa* was stimulated about 3-fold by the boiled extract. On the possibility that the boiled extract was supplying some sources of reducing
TABLE III
Effect of various additions on anthranilic acid formation by extracts from *N. crassa*

<table>
<thead>
<tr>
<th>Additions*</th>
<th>Anthranilic acid formed† (μmole/hr/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. crassa</em> extract</td>
<td>0.05</td>
</tr>
<tr>
<td><em>N. crassa</em> extract + boiled extract (E. coli)</td>
<td>0.18</td>
</tr>
<tr>
<td><em>N. crassa</em> extract + 0.01% yeast extract</td>
<td>0.01</td>
</tr>
<tr>
<td><em>N. crassa</em> extract + 0.5 μmole of glutathione</td>
<td>0.01</td>
</tr>
<tr>
<td><em>N. crassa</em> extract + 0.67 μmole of TPNH</td>
<td>0.91</td>
</tr>
<tr>
<td>Boiled extract (E. coli)</td>
<td>0.00</td>
</tr>
</tbody>
</table>

* N. crassa extract used was 1.1 mg of protein of a 40 to 50% ammonium sulfate fraction (see “Experimental Procedure”). For boiled extract (E. coli), see text.
† Conditions: Tris buffer, pH 8.2, 50 μmoles; MgCl₂, 5 μmoles; shikimic acid, 2 μmoles; ATP, 1 μmole; DPNH, 0.75 μmole; L-glutamine, 10 μmoles; phosphoenolpyruvate, 1 μmole; 1 ml final volume; incubated for 30 minutes at 37°; anthranilic acid added after extraction into ethyl acetate (see “Experimental Procedure”).

Table III shows the effect of various additions on anthranilic acid formation by extracts from *N. crassa*. The presence of DPNH was not required for the extract in ether and subsequently converted to anthranilic acid by the same ture, an intermediate accumulated which could be extracted into ATP, P-enolpyruvate, L-glutamine, and TPNH, in addition to the following properties (13): (a) the intermediate, in 0.01 Tris buffer, pH 8.0, exhibited an absorption spectrum similar to that of chorismic acid, with an absorption maximum at 270 μm; (b) the intermediate preparation exhibited a single component which reacted with the p-nitroaniline spray reagent (21) after paper chromatography in either of the two solvent systems utilized by Gibson (13). This component migrated with a rate identical with that of chorismic acid in each solvent system; (c) when the intermediate preparation was acidified and heated in a boiling water bath for 5 minutes, a new compound was formed which reacted readily with the p-nitroaniline spray reagent (21) and which migrated at a rate identical with that of p-hydroxybenzoic acid on paper in a benzene-acetic acid-water solvent system (21).

The accumulation of this intermediate varied quantitatively from a narrow range of extract concentration as indicated in Table V. The amount of anthranilic acid formed was dependent on the presence of Mg++, yeast extract, glutathione, and TPNH must be added to obtain the conversion of shikimic acid to anthranilic acid in extracts from *N. crassa* was dependent on the presence of Mg++, yeast extract, glutathione, and TPNH were tested for their ability to replace the boiled extract. TPNH was more stimulatory than the boiled extract. The presence of DPNH was not required for the TPNH effect, and the boiled extract did not produce a further stimulation.

Therefore, in contrast to the bacterial system studied, to obtain the conversion of shikimic acid to anthranilic acid in extracts from *N. crassa*, an inhibitor must be removed by selective ammonium sulfate precipitation and TPNH must be added as the source of reduced pyridine nucleotide rather than DPNH. Under these conditions, the formation of anthranilic acid by extracts from *N. crassa* was dependent on the presence of Mg++, ATP, P-enolpyruvate, L-glutamine, and TPNH, in addition to shikimic acid (Table IV).

When L-glutamine was eliminated from the incubation mixture, an intermediate accumulated which could be extracted into ether and subsequently converted to anthranilic acid by the same extract in the presence of L-glutamine (Table V). The amount of anthranilic acid formed was used to determine quantitatively the amount of intermediate accumulated by this procedure. The accumulation of this intermediate varied quantitatively from preparation to preparation but was invariably confined to a narrow range of extract concentration as indicated in Table V. All the components present in the protocol of Table V were required for the accumulation of the intermediate, while only L-glutamine and Mg++ were required for its subsequent conversion to anthranilic acid.

The intermediate was accumulated on a large scale by the procedure outlined in Table V and found to be identical with chorismic acid (see “Experimental Procedure”) on the basis of the following properties (13): (a) the intermediate, in 0.01 M Tris buffer, pH 8.0, exhibited an absorption spectrum identical with that of chorismic acid, with an absorption maximum at 270 μm; (b) the intermediate preparation exhibited a single component which reacted with the p-nitroaniline spray reagent (21) after paper chromatography in either of the two solvent systems utilized by Gibson (13). This component migrated with a rate identical with that of chorismic acid in each solvent system; (c) when the intermediate preparation was acidified and heated
The $K_m$ determined for these data is $0.23 \times 10^{-5}$ M. Therefore, $1.5 \times 10^{-5}$ M chorismic acid was routinely used in the assay procedure outlined in Fig. 2. Under these conditions, an extract from N. crassa, which had been precipitated with ammonium sulfate, exhibited absolute requirements for Mg$^{2+}$ and L-glutamine, in addition to chorismic acid, for anthranilic acid synthesis (Table VI). NH$_4^+$ partially replaced glutamine in crude extracts but not in the more purified preparation described below. Low concentrations of L-tryptophan inhibited the reaction (Table VI), and this inhibition was competitively reversed by chorismic acid (Fig. 3).

The conversion of chorismic acid to anthranilic acid would appear to be a complex reaction which might well involve more than one enzyme. However, the activity has been purified 83-fold by protamine sulfate treatment, ammonium sulfate precipitation, and chromatography on a brushite column (Table VII). The activity was eluted as a single peak from the brushite column, and only the four most active fractions were combined for the data in Table VII. Other fractions, from the two edges of the activity peak were assayed individually and in mixtures to test for the possible involvement of two components. In each case, the activities were strictly additive, indicating that only a single active component was being eluted from the column.

**DISCUSSION**

The removal of the inhibitory factor present in crude extracts of N. crassa has made it possible to study the conversion of shikimic acid to anthranilic acid in extracts from N. crassa. The inhibitor has not been studied further, but would appear to be a macromolecular component, since passage over Sephadex G-25 failed to remove it from the excluded protein fractions, and since it is precipitable by a limited range of ammonium sulfate concentration.

For the conversion of shikimic acid to anthranilic acid, extracts from N. crassa exhibit requirements identical with those of the bacterial systems studied except that DPNH must be replaced by TPNH. The requirement for reduced pyridine nucleotide is
of considerable interest since the reaction in which it is required for the bacterial system does not involve a net transfer of electrons.

The accumulation of chorismic acid from shikimic acid when glutamine is omitted from the incubation mixture provides further evidence that the sequence of reactions involved in \textit{N. crassa} is identical with the sequence observed in the bacterial system. The accumulation of this compound by extracts of wild type \textit{N. crassa} is contrary to the findings with the bacterial systems. In that case, a mutant blocked in each of the pathways utilizing chorismic acid was required to demonstrate chorismic acid accumulation (12). The difference between the bacterial and fungal wild type extracts in accumulating chorismic acid is presumably a reflection of a difference in the relative levels of enzymes involved in the formation and utilization of chorismic acid in these preparations.

The conversion of chorismic acid to anthranilic acid would appear to be the first enzymatic step specific for tryptophan biosynthesis. In spite of the apparent complexity of this reaction, the activity has not been resolved into more than one component over an 83-fold purification range. Furthermore, the reaction is completely inhibited by low concentrations of \textit{l-tryptophan}, and this inhibition is competitively reversed by chorismic acid. By analogy to observations on feedback inhibition in other biosynthetic pathways (22), these findings suggest that conversion of chorismic acid to anthranilic acid is specifically involved in tryptophan biosynthesis. Finally, the activity is absent from all \textit{tryp-2} mutants of \textit{N. crassa} which have been examined.\footnote{1 J. A. DeMoss and J. Wegman, unpublished results.} This auxotrophic class is known to be blocked prior to anthranilic acid in the tryptophan pathway.

On the basis of the above observations, it is tentatively concluded that the conversion of chorismic acid to anthranilic acid in the presence of glutamine and Mg\textsuperscript{2+} is catalyzed by a single enzyme, anthranilate synthetase.

**SUMMARY**

Extracts of \textit{Neurospora crassa} catalyze the conversion of shikimic acid to anthranilic acid after an inhibitor, present in crude extract, has been removed by partial purification. In addition to shikimic acid, Mg\textsuperscript{2+}, ATP, phosphoenolpyruvate, triphosphopyridine nucleotide, and \textit{l}-glutamine are required for this conversion. When \textit{l}-glutamine is omitted from the reaction mixture, an ether-extractable intermediate accumulates which is converted to anthranilic acid in the presence of Mg\textsuperscript{2+}, \textit{l}-glutamine, and an extract of \textit{N. crassa}. On the basis of several properties, the intermediate is identical with chorismic acid. The conversion of the intermediate to anthranilic acid is inhibited competitively by low concentrations of \textit{l}-tryptophan. This enzyme activity has been purified 83-fold from crude extracts with a 30% recovery of activity. It is proposed that a single enzyme, anthranilate synthetase, catalyzes the conversion of chorismic acid to anthranilic acid in \textit{N. crassa}.

**Acknowledgment**—I am indebted to Mrs. Edith Edlin for her excellent technical assistance.

**REFERENCES**

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