A New Anthranilic Acid Hydroxylase from *Aspergillus niger*

PURIFICATION AND PROPERTIES

(Received for publication, October 11, 1968)

N. S. Sreeleela, P. V. SubbaRao, R. Premkumar, and C. S. Vaidyanathan

From The Department of Biochemistry, Indian Institute of Science, Bangalore 12, India

**SUMMARY**

A new anthranilic acid hydroxylase which catalyzes the conversion of anthranilic acid to 2,3-dihydroxybenzoic acid, has been partially purified from crude extracts of the mycelial felts of *Aspergillus niger*, grown in the presence of anthranilic acid. Neither 3-hydroxyanthranilic acid nor salicylic acid is an intermediate in this conversion.

The enzyme is stabilized by glutathione and requires substrate amounts of NADPH. The optimum pH for anthranilic acid hydroxylase activity is around 8.2. The $K_m$ values for anthranilic acid and NADPH at pH 8.2 and 30° are 0.15 and 0.16 mM, respectively. The partially purified enzyme is highly specific for anthranilic acid and does not act on related substances such as 3-hydroxyanthranilic acid, salicylic acid, m-hydroxybenzoic acid, methylanilinic acid, and ethylanthranilic acid. Cupric, mercuric, and cadmium ions have a pronounced inhibitory effect on enzyme activity. Metal-chelating agents like o-phenanthroline, $\alpha,\alpha'$-dipyridyl, salicyldialdoxime, and diethyl dithiocarbamate also inhibit the reaction. Sulfhydryl reagents such as $\beta$-hydroxymercaptobenzozoate and $N$-ethylmaleimide irreversibly inhibit anthranilic acid hydroxylase activity.

Crude extracts of the mycelium have the ability to hydroxylate anthranilic acid to 3-hydroxyanthranilic acid in addition to 2,3-dihydroxybenzoic acid. This activity, however, is lost on purification.

The conversion of anthranilic acid to catechol by a partially purified anthranilic acid hydroxylase was reported from *Pseudomonas* (1, 2). This enzyme, which shows a requirement for NADH or NADPH and molecular oxygen, does not seem to involve any detectable intermediate in the conversion (1-3). Nair and Vaidyanathan (4) demonstrated a second type of anthranilic acid hydroxylase from *Tecoma stans*, which catalyzes a simple hydroxylation of anthranilic acid to 3-hydroxy-

*Council of Scientific and Industrial Research Scientists' Pool Officer. Present address, Department of Biology and Botany, University of British Columbia, Vancouver 8, Canada.

Anthraniilic acid in the presence of NADPH and tetrahydrofolic acid. An NADPH-dependent hydroxylase localized in rat liver microsomes converts anthranilic acid to 5-hydroxyanthranilic acid (5). In a preliminary communication we have reported the conversion of anthranilic acid to 2,3-dihydroxybenzoic acid by a soluble enzyme from *Aspergillus niger* (6). The present paper is concerned with the partial purification and properties of this enzyme, which catalyzes a dioxygenase type of reaction analogous to that reported by Kobayashi et al. (7).

**EXPERIMENTAL PROCEDURE**

*Materials*

Anthranilic acid purchased from Eastman Kodak Company, Rochester, was recrystallized from water. 2,3-Dihydroxybenzoic acid was obtained from K and K Laboratories, Inc., California, and recrystallized from hot water. Protanil sulfate was obtained from Mann. Hydroxylapatite (Bio-gel, HTP) and alumina Cy gel were purchased from Calbiochem. NAD, NADP, NADH, NADPH, FMN, FAD, folic acid, glucose 6-phosphate dehydrogenase, and DEAE-cellulose were from Sigma. Glucose 6-phosphate (barium salt) was purchased from Reanal Chemical Company, Budapest, Hungary. Sephadex G-25 was from Pharmacia, Uppsala. Tetrahydrofolic acid was prepared by catalytic hydrogenation of folic acid with platinum oxide as catalyst (8). Other chemicals were of analytical reagent grade available commercially.

*Organism*—*Aspergillus niger* UBC 814 was obtained from Dr. R. J. Bandoni, Department of Botany, University of British Columbia.

*Media and Conditions of Cultivation*—A. niger was grown for 48 to 60 hours at 30° in 1-liter flasks on a synthetic medium (9) supplemented with 0.1% anthranilic acid. Stock cultures were maintained on the same medium supplemented with 1.5% agar. The mycelia were harvested before the advent of sporulation and washed thoroughly with distilled water. The mycelia was used fresh or stored at $-20^\circ$ until required.

*Methods*

Assay of Anthranilic Acid Hydroxylase—Anthranilic acid hydroxylase activity was measured by incubating for 20 min at 30° a reaction mixture (1 ml) containing Tris-HCl buffer, pH 8.2
(30 μmoles), anthranilic acid (0.2 μmole), NADP (0.2 μmole), glucose 6-phosphate (0.25 μmole), glucose 6-phosphate dehydrogenase (10 milliunits), and 0.5 ml of anthranilic acid hydroxylase (4 to 8 milliunits). The components of the NADPH-regenerating system (NADP, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase) were incubated with the buffer for 5 min prior to the addition of anthranilic acid hydroxylase and substrate. The reaction was stopped by the addition of 0.2 ml of 0.5 N HCl and the mixture was extracted with 2.5 ml of peroxide-free ether. One-milliliter aliquots were taken to dryness and the amount of 2,3-dihydroxybenzoic acid was determined colorimetrically by the method described by Nair and Vaidyanathan for the estimation of catechol substances (10). To follow the disappearance of anthranilic acid, 0.5-ml aliquots from the ether layer were used for the colorimetric determination (11).

Oxygen Consumption—Oxygen consumption was measured by using Gilson Oxygraph, model K.

Ammonia Formation—Ammonia liberated during the course of the reaction was determined by nesslerization by using the Conway microdiffusion method (12).

Determination of Protein Concentration—The protein concentrations of various fractions obtained in the course of purification were determined with Folin-Phenol reagent (13) with bovine serum albumin as the standard.

RESULTS

Stabilization of Anthranilic Acid Hydroxylase

The mycelium can be stored at −20° for 1 month without appreciable loss in activity. The enzyme activity is rapidly lost during purification. Thiol compounds have a profound protective action, GSH being the most effective.

Purification of Anthranilic Acid Hydroxylase

All operations were performed at 0–4°.

Preparation of Crude Extract—The mycelium (30 g) was macerated in a chilled mortar for 15 min with an equal weight of glass powder and extracted with 90 ml of 0.025 M sodium phosphate buffer (pH 7) containing 0.001 M GSH. The extract was passed through a cheesecloth and centrifuged at 12,000 × g for 20 min. The supernatant solution containing about 5 to 6 ml of protein per ml was used for purification.

Treatment with Protamine Sulfate—A 2% solution of protamine sulfate in 0.025 M sodium phosphate buffer, pH 7 (9 ml) was added to the crude extract (81 ml) with gentle stirring. After 15 min, the mixture was centrifuged for 10 min at 12,000 × g and the precipitate was discarded.

DEAE-cellulose Treatment—DEAE-cellulose was washed according to the procedure described by Peterson and Sober (14) and suspended in 0.025 M sodium phosphate buffer (pH 7). An aliquot of the suspension was filtered through a Buchner funnel. To the protamine sulfate extract (90 ml) were added 6 g of the washed DEAE-cellulose cake and the mixture was stirred for 30 min. The suspension was filtered through a Buchner funnel and the filtrate was collected.

Alumina Cy Treatment—The enzyme solution obtained after DEAE-cellulose treatment (70 ml) was treated with alumina Cy gel (solids obtained from 70 ml of a preparation containing 10 mg per ml) and stirred for 15 min. The gel was separated by centrifugation and washed with 70 ml of 0.025 M sodium phosphate buffer, pH 7, containing 0.001 M GSH. The enzyme was finally eluted from the gel twice with 70 ml (35 ml each time) of a solution containing sodium phosphate buffer (0.06 M, pH 8) and GSH (0.001 M).

Hydroxylapatite Treatment—The enzyme preparation from the previous step (50 ml) was treated with hydroxylapatite (500 mg). After stirring for 15 min, the suspension was centrifuged and the precipitate was discarded. The clear supernatant was used as the antranthilic acid hydroxylase.

The balance sheet for the purification of anthranilic acid hyrdrxylase is shown in Table I. Crude extracts of the mycelium exhibited very low anthranilic acid hydroxylase activity. These preparations were found to contain a potent NADPH oxidase. Even in the presence of a sufficient excess of the NADPH-regenerating system (NADP, 1 μmole; glucose 6-phosphate, 1.25 μmoles; and glucose 6-phosphate dehydrogenase, 50 milliunits), there was no further increase in the enzyme activity in crude extracts. These results seem to suggest that there are endogenous inhibitors other than NADPH oxidase in crude preparations. The enzyme activity, however, increases rapidly after treatment with DEAE-cellulose, giving over-all recoveries as high as 850%. Preparations obtained after DEAE-treatment were completely free from NADPH oxidase activity.

Attempts to purify the enzyme extensively have been limited by its extremely instability. The enzyme was inactivated by treating with ammonium sulfate or acetone, or by positive adsorption on DEAE-cellulose or carboxymethyl cellulose. Filtration through Sephadex G-25 or dialysis against 0.025 M sodium phosphate buffer, pH 7, containing GSH (1 mM) irreversibly inactivated the enzyme. There was 50% inactivation when partially purified enzyme solutions were kept at −20°.

Identification of Reaction Product

A large scale incubation mixture (400 ml) consisting of Tris-HCl buffer (30 μmoles), anthranilic acid (0.2 μmole), NADP (0.2 μmole), glucose 6-phosphate (0.25 μmole), glucose 6-phosphate dehydrogenase (10 units), and partially purified anthranilic acid hydroxylase (2 units) was incubated at 30°. After 2 hours, the pH of the reaction mixture was adjusted to 2 with 1 N HCl and it was extracted twice with equal volumes of peroxide-free ether. The organic layer was taken to dryness at room temperature after shaking with anhydrous sodium sulfate. The residue was dissolved in ethyl acetate and chromatographed on Whatman No. 3 filter paper with 2% formic acid as a solvent. The fluorescent band corresponding to 2,3-dihydroxybenzoic acid was detected.
acid was eluted with hot 95% ethanol. The substance was finally crystallized from hot water.

The enzymic product was identified as 2,3-dihydroxybenzoic acid by comparing its properties with those of an authentic sample. The $R_f$ values on paper chromatograms in benzene-acetic acid-water (10:7:3) and benzene-ethyl methyl ketone-formic acid-water (900:100:2:98) were 0.52 and 0.38, respectively. Color reactions with phenolic reagents like $p$-nitroaniline, diazotized sulfanilic acid, and ferric chloride (15, 16) were identical with those of synthetic, 2,3-dihydroxybenzoic acid. The ultraviolet spectrum of the isolated compound which showed absorption maxima at 248 and 316 μm in ethanol was indistinguishable from that of 2,3-dihydroxybenzoic acid.

Decarboxylation of Anthranilic Acid Hydroxylase

Reaction Product to Catechol

Incubation of the enzymatically isolated product from anthranilic acid with purified 2,3-dihydroxybenzoic acid carboxy-lyase from A. niger, resulted in its decarboxylation to catechol (17). The identity of the decarboxylated product was established by comparing its properties with those of authentic catechol. The decarboxylation product, like authentic catechol, gave a melting point of 108°. It was indistinguishable from catechol when subjected to paper chromatography in water-ethyl methyl ketone-diethylamine (77:92:1) and formic acid-water (2:98) as solvents, and the $R_f$ values were 0.88 and 0.73, respectively.

Properties of Partially Purified Anthranilic Acid Hydroxylase

Effect of pH on Enzyme Activity—Fig. 1 shows the effect of pH on anthranilic acid hydroxylase activity in the range 7.2 to 10. The maximum activity was observed around pH 8.2. For the pH range between 5 and 8, activity determinations were made in buffer consisting of sodium citrate (30 μmole) and disodium phosphate (60 μmole) and it was found that there was a steady increase in activity when the pH was raised from 5 to 8. It was also observed that the activities were invariably higher when citrate-phosphate buffer was used. For example, at pH 7.5 the activity in citrate-phosphate buffer was about 25% higher than the corresponding value in Tris-HCl buffer. The enzyme was only about 26% active at pH 5 when compared to the activity at the optimum pH.

Stoichiometry—The stoichiometry of the reaction catalyzed by the purified anthranilic acid hydroxylase from A. niger is shown in Table II. For the formation of 1 mole of 2,3 dihydroxybenzoic acid from anthranilic acid there was a consumption of 2 atoms of oxygen and oxidation of 1 mole of NADPH. From the data it is evident that the over-all reaction catalyzed by the enzyme is

$$\text{COOH} + \text{NADPH} + \text{H}^+ + \text{O}_2 \rightarrow \text{COOH} \text{OH} + \text{NADP}^+ + \text{NH}_3$$

![Fig. 1. pH activity curve for the conversion of anthranilic acid to 2,3-dihydroxybenzoic acid. A reaction mixture (1 ml, total volume) consisting of enzyme, 8 milliunits; anthranilic acid, 0.2 μmole; NADP, 0.2 μmole; glucose 6-phosphate, 0.25 μmole, and glucose 6-phosphate dehydrogenase, 10 milliunits, was incubated with 30 μmole of Tris-HCl (pH 7.2 to 9) or sodium carbonate-sodium bicarbonate buffer (pH 9 to 10). After incubation for 20 min at 30°, the enzyme activity was assayed as described under "Experimental Procedure."](image)

Substrate Specificity for Pyridine Nucleotides—The enzyme showed an absolute requirement for NADPH. There was approximately 2% activity when NADP was replaced by NADP. FMN, FAD, or tetrahydrofolic acid had no effect on enzyme activity. The reaction rate increased with an increase in the concentration of NADPH, the optimum concentration being 0.2 mM (Table III). The absorption spectrum of the purified enzyme in 0.06 M sodium phosphate buffer (pH 7) was that of a simple protein and did not show any indication for the presence of a flavin prosthetic group.

Substrate Specificity—Anthranilic acid is the only substrate known to be hydroxylated by the A. niger enzyme. No activity was detected with 3-hydroxyanthranilic acid, benzoic acid, salicylic acid, m-hydroxybenzoic acid, p-hydroxybenzoic acid, p-aminobenzoic acid, m-aminobenzoic acid, methylanthranilate, and ethylanthranilate.

Substrate Affinities—A study of the effect of different concentrations of NADPH and of anthranilic acid on the rate of 2,3-dihydroxybenzoic acid formation in the presence of partially purified anthranilic acid hydroxylase gave typical Lineweaver-Burk plots. The $K_m$ values for anthranilic acid and NADPH at pH 5.2 were 0.15 and 0.16 mM, respectively.

Effect of Metal Ions—Anthranilic acid hydroxylase is not activated by any divalent cation. Mercuric, cadmium, and
cupric ions inhibited the enzyme activity to the extent of 100, 75, and 50%, respectively, at a final concentration of 0.5 mm.

**Effect of Inhibitors**—Table IV shows the effect of various inhibitors on the reaction. Even though the enzyme activity was not enhanced by any metal ion, partial inhibition by agents forming complexes with metals like α,α'-dipyridyl, salicylaldoxime, diethyl dithiocarbamate, and 8-hydroxyquinoline seems to suggest that a tightly bound metal ion, probably Fe²⁺, is required for enzyme activity. The inhibition, however, was found to be irreversible as neither Fe²⁺ nor any other metal ion could restore the activity.

A might be expected from the protective effect of GSH, the enzyme is sensitive to inhibition of thiol groups (Table IV), even though an excess of GSH is present.

Inhibitors like Atabrine and aminopterin had no effect on anthranilic acid hydroxylase activity.

**Effect of Structural Analogues**—Structurally related compounds like salicylic acid, methylanthranilate, and ethylanthranilate had no effect on the enzyme activity. 3 Hydroxyanthranilic acid and m-hydroxybenzoic acid inhibited the formation of 2,3-dihydroxybenzoic acid to the extent of 30%.

**Formation of 3-Hydroxyanthranilic acid in Crude Extracts**

A reaction mixture consisting of Tris-HCl buffer, pH 8.2 (300 μmole), anthranilic acid (2 μmole), NADP (2 μmole), glucose

![Table II](https://example.com/table2.png)

**Table II**

Stoichiometry of reaction catalyzed by anthranilic acid hydroxylase from A. niger

In Experiment 1, each reaction mixture (2 ml) contained Tris-HCl buffer, pH 8.2, 0 μmole; anthranilic acid, 0.4 μmole; NADP, 0.4 μmole; glucose 6-phosphate, 0.5 μmole; glucose 6-phosphate dehydrogenase, 20 milliunits, and anthranilic acid hydroxylase from Step V, 9 milliunits. The reaction mixtures were incubated at 30° for various time intervals. The consumption of oxygen, anthranilic acid disappearance, and formation of 2,3 dihydroxybenzoic acid and ammonia were estimated as described under "Experimental Procedure," and expressed as millimicromoles per ml of standard reaction mixture. To determine the amount of NADPH oxidized, the reaction was allowed to proceed until all the anthranilic acid was utilized. The 2,3-dihydroxybenzoic acid formed was converted quantitatively to catechol by the inclusion in the reaction mixture of o-pyrocatecholic acid carboxyl-lyase (IV) and the amount of NADPH remaining was calculated from the measured absorbance at 340 mμ. The reaction mixture in this case (Experiment 2) contained, in a final volume of 2 ml, sodium phosphate buffer, pH 7, 60 μmole; anthranilic acid, 0.2 μmole; NADPH, 0.4 μmole; anthranilic acid hydroxylase from Step V, 10 milliunits, and o-pyrocatecholic acid carboxyl-lyase, 20 milliunits, and it was incubated for 60 min at 30°. The composition of the blank was the same as described above but anthranilic acid was added at the end of the incubation period. The following molar extinction coefficients in 0.1 m sodium phosphate buffer, pH 7, were used for the calculation of the amounts of anthranilic acid and NADPH: anthranilic acid, ε₂₅₀ = 2750 and ε₂₈₀ = 900.1; NADPH, ε₂₈₀ = 6220. The amount of catechol formed was estimated as follows: the reaction mixture (1 ml) after acidification with 0.2 ml of 0.5 N HCl, was extracted with 2.5 ml of peroxide-free ether. A 1-ml aliquot of ether layer was taken to dryness and the residue was dissolved in 1 ml of 0.125 N HCl. The absorbance at 278 mμ was determined. By reference to the standard graph, the amount of catechol present was determined.

![Table III](https://example.com/table3.png)

**Table III**

Effect of different concentrations of NADPH on reaction

The standard assay conditions were used except for the replacement of regenerating system (NADP, 0.2 μmole, glucose 6-phosphate, 0.25 μmole; and glucose 6-phosphate dehydrogenase, 10 milliunits) with the indicated concentration of NADPH. The amount of anthranilic acid hydroxylase added to the reaction mixtures was 8 milliunits.

<table>
<thead>
<tr>
<th>NADPH concentration</th>
<th>2,3-Dihydroxybenzoic acid formed</th>
<th>mμmole</th>
<th>mμmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>91</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>160</td>
<td>117</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>130</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regenerating system</td>
<td>131</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Table IV](https://example.com/table4.png)

**Table IV**

Effect of different inhibitors on anthranilic acid hydroxylase activity

The substrate-free standard reaction system was first incubated for 10 min with the test compound and the reaction was started by the addition of anthranilic acid. 2,3-Dihydroxybenzoic acid was determined colorimetrically as described under "Experimental Procedure."

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mμc</td>
<td>%</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>1.0</td>
<td>37</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>0.5</td>
<td>42</td>
</tr>
<tr>
<td>o-Phenanthrolin</td>
<td>0.5</td>
<td>46</td>
</tr>
<tr>
<td>α-Phenanthrolin</td>
<td>1.0</td>
<td>60</td>
</tr>
<tr>
<td>α,α'- Dipropyridyl</td>
<td>1.0</td>
<td>40</td>
</tr>
<tr>
<td>Salicylaldoxime</td>
<td>0.5</td>
<td>42</td>
</tr>
<tr>
<td>Salicylaldoxime</td>
<td>1.0</td>
<td>38</td>
</tr>
<tr>
<td>p-Hydroxymercuribenzoate</td>
<td>0.5</td>
<td>96</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>0.5</td>
<td>96</td>
</tr>
<tr>
<td>Atabrine</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>Aminopterin</td>
<td>1.0</td>
<td>0</td>
</tr>
</tbody>
</table>
6-phosphate (2.5 μmoles), glucose 6-phosphate dehydrogenase (2 units), and 5 ml of crude cell-free extract was incubated for 30 min at 30°. The reaction was arrested by acidifying to pH 2 with 1 N HCl and the precipitate was removed by centrifugation. The supernatant solution was extracted thrice with equal volume of peroxide-free ether. The ether extract was taken to dryness in a vacuum and the residue was dissolved in 0.5 ml of ethyl acetate. Suitable aliquots were spotted on Whatman No. 1 filter paper and subjected to two-dimensional chromatography (16). In addition to 2,3-dihydroxybenzoic acid and anthranilic acid, the chromatograms contained an additional spot corresponding to 3-hydroxyanthranilic acid. The identity of this compound was established by its characteristic fluorescence and color reactions (15). Partially purified preparations, however, did not convert anthranilic acid to 3-hydroxyanthranilic acid, and 2,3-dihydroxybenzoic acid was invariably detected as the only product of anthranilic acid hydroxylation in such preparations. The second hydroxylation activity, i.e., hydroxylation of anthranilic acid to 3-hydroxyanthranilic acid, which is confined to crude preparations, was lost during purification and attempts to purify this enzyme were unsuccessful.

**DISCUSSION**

Results presented in this paper show that partially purified preparations from *A. niger* catalyze the stoichiometric conversion of anthranilic acid to 2,3-dihydroxybenzoic acid. Terui, Enatsa, and Tobata (19) identified 3-hydroxyanthranilic acid, 2,3-dihydroxybenzoic acid, and catechol as metabolites of anthranilic acid in the culture filtrates of *A. niger*. Radioactive studies by Subba Rao et al. (20) with tryptophan-¹⁴C also showed that the label from tryptophan is incorporated into anthranilic acid, 3-hydroxyanthranilic acid, 2,3-dihydroxybenzoic acid, and catechol. These results, together with the ability of tryptophan, kynurenine, anthranilic acid, 3-hydroxyanthranilic acid, and 2,3-dihydroxybenzoic acid to induce o-pyrocatechuic acid carboxylase led these workers to postulate tentatively that 3-hydroxyanthranilic acid and 2,3-dihydroxybenzoic acids are intermediates in the conversion of anthranilic acid to catechol.

Present studies, however, have established that 3-hydroxyanthranilic acid, even though it is a metabolite of tryptophan or anthranilic acid, is not an isolatable intermediate in the conversion of anthranilic acid to 2,3-dihydroxybenzoic acid. The enzyme is highly specific for anthranilic acid and failed to convert either 3-hydroxyanthranilic acid or salicylic acid to 2,3-dihydroxybenzoic acid. The studies in vivo of Tyler et al. (21) and Groeger et al. (22) on the metabolism of tryptophan by *Claniceps paspali* are also consistent with such a direct conversion.

The ability of crude extracts of *A. niger* to convert anthranilic acid to 3-hydroxyanthranilic acid suggests the occurrence of another anthranilic acid hydroxylase similar to that reported by Nair and Vaidyanathan from *Tecoma stanee* (4). 3-Hydroxyanthranilic acid, even though it is not converted to 2,3-dihydroxybenzoic acid, may serve as an intermediate in the biosynthesis of NADH (23).

Four different mechanisms are described in literature for the formation of catechol substances in biological systems, namely (a) by two successive single hydroxylations, (b) by further hydroxylation of a mono-oxygenated compound, (c) by abstraction of hydrogen from dihydrodiol compounds, and (d) by simultaneous introduction of 2 atoms of oxygen into the aromatic molecule followed by reduction (24).

Tanizaki et al. (2) obtained a partially purified anthranilic acid hydroxylase from *Pseudomonas* which is the only example of the fourth type of double hydroxylation reaction. This enzyme which catalyzes the stoichiometric formation of 1 mole each of catechol, carbon dioxide, and ammonia from 1 mole of anthranilic acid consumes 1 mole each of oxygen and NADH (or NADPH). By using O₂ and H₂O₂, it has been established that both the atoms of oxygen in catechol originate from molecular oxygen (7). Kobayashi (7) reported that the *Pseudomonas* enzyme is activated by ferrous ions. On the basis of these studies, Hayashi (24) suggested a reaction mechanism, implicating a role for Fe³⁺. It is presumed that the activation of oxygen may involve the formation of perferryl ion and the oxygen atoms may add to the double bond between C-1 and C-2 of anthranilic acid. The unstable cyclic peroxide thus formed, might then undergo deamination, decarboxylation, and reductive cleavage in a concerted manner leading to the formation of catechol. In analogy with the mechanism of Hayashi for the conversion of anthranilic acid to catechol, a similar one may hold good for the conversion of anthranilic acid to 2,3 dihydroxybenzoic acid. In contrast to *Pseudomonas*, the *A. niger* enzyme hydrolylates anthranilic acid in positions C-2 and C-3 instead of in positions C-1 and C-2 of the aromatic ring. Further, decarboxylation is not part of the *A. niger* anthranilic acid hydroxylase activity. Although there is no direct evidence for the participation of Fe³⁺ in the conversion of anthranilic acid to 2,3-dihydroxybenzoic acid, the inhibition by metal-chelating agents does not rule out the possible involvement of a tightly bound metal ion in the reaction.

**Acknowledgments**—We wish to thank Dr. G. H. N. Towers, Department of Botany, University of British Columbia, for his interest in this investigation. The generous gift of NADPH by Sigma is gratefully acknowledged.

**REFERENCES**