Enzymatic Formation of Catechol from Anthranilic Acid*

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Anthranilic acid is known to be formed from kynurenine in animals and microorganisms (3–6) and to be degraded by cell-free extracts of a Pseudomonas species adapted to L-tryptophan (7). Catechol has been postulated as an intermediate in the catabolism of anthranilic acid on the basis of experiments utilizing sequential enzyme induction (8). Several groups of investigators have recently succeeded in partially purifying and characterizing the enzyme responsible for the oxidation of anthranilic acid (9–12), but because of the instability of the enzyme and the complex nature of the reaction, properties of the enzyme and the reaction mechanism still remain obscure. Furthermore, the role of catechol in the catabolism of anthranilic acid has not been established, since these enzyme preparations invariably contained significant levels of pyrocatechase. Direct evidence for the formation of catechol from anthranilic acid is necessary to provide a firmer basis for further studies on the mechanism of anthranilic acid oxidation. In the work presented, catechol has been identified as an intermediate in the degradation of anthranilic acid by a Pseudomonas species. In addition, the mechanism of this transformation is investigated in connection with the enzymatic hydroxylation of the aromatic ring at 2 adjacent carbon atoms (13, 14).

EXPERIMENTAL PROCEDURE

Reagents—The trans and cis forms of benzene glycol (5,6-dihydroxy-1,2-cyclohexadiene-1,2) were synthesized according to the method of Nakajima et al. (15, 16). The 2,4-dinitrophenylhydrazone of levulinic acid was synthesized by the conventional method (17). DL-Tryptophan was a gift from Takeda Chemical Industries, Ltd. DL-Tryptophan-7α-14C was kindly furnished by Dr. M. Rothstein. Benzoic acid-1,4C was purchased from the New England Nuclear Corporation. β-Ketoadipic acid was obtained from the Sigma Chemical Company. All other chemicals were commercial products.

Biological Materials—The ammonium sulfate fraction of kynureninase was prepared from cells of Pseudomonas fluorescens No. 23 (ATCC 11250) according to the method of Hayashi and Stanier (6). The enzyme was dialyzed against a large volume of water overnight before use. The crude extract obtained from P. fluorescens No. 6 (ATCC 11299B) was dialyzed against a large volume of 0.02 M potassium phosphate buffer, pH 7.5, for 3 hours and was used for the enzymatic preparation of L-kynurenine (18). Metapyrocatechase was prepared by the method of Kojima, Itada, and Hayaishi (19). Glucose dehydrogenase was purified according to the method of Straub (20). Rat liver (8.8 g) was homogenized in 45 ml of 1.15% KCl in a Potter-Elvehjem homogenizer and filtered through gauze. The filtrate was centrifuged at 105,000 × g for 30 minutes. The supernatant solution obtained is referred to as the liver supernatant fraction. Cell-free extracts capable of degrading benzoic acid were obtained from P. fluorescens A 312 (21), which was grown for about 16 hours at 25°C with vigorous aeration in a medium containing 0.1% benzoic acid, 0.15% KH₂PO₄, 0.05% KH₂PO₄, 0.2% NH₄Cl, 0.1% Difco yeast extract, and 0.02% MgSO₄·7H₂O. The preparation of the cell-free extracts and the fractionation by high speed centrifugation were carried out according to the method described previously (22). Benzeneglycol dehydrogenase was purified from rabbit liver according to the method of Ayengar et al. (23).

Purification of Anthranilic Acid Hydroxylase—P. fluorescens No. 23 (ATCC 11250) was used as the source of enzyme. Culture methods and preparation of cell-free extracts and their supernatant fractions from high speed centrifugation were the same as those described previously (22). The supernatant fraction (22 mg of protein per ml) was stirred with 0.1 volume of 2% proteamine sulfate solution, pH 7.5, and the precipitate was removed by centrifugation at 20,000 × g for 10 minutes. To 10 ml of the supernatant solution, 1 ml of well aged alumina C or gel (19 mg of dry matter per ml) was added. After stirring for 10 minutes, the precipitate was removed by centrifugation at 20,000 × g for 10 minutes. The supernatant solution was fractionated by the addition of a neutral saturated ammonium sulfate solution prepared by the method described previously (14). The precipitate obtained between 50 and 60% saturation was dissolved in 10 ml of 0.02 M Tris buffer, pH 7.5, and reprecipitated by adjusting the ammonium sulfate concentration to 60% saturation. The precipitate was collected by centrifugation and redissolved in 10 ml of the same buffer. This solution, containing 3.4 mg of protein per ml, was referred to as the anthranilic acid hydroxylase fraction. Fractions were classified according to their ammonium sulfate fractionation.

Anthranilic acid hydroxylase was located exclusively in the fraction between 50 and 60% ammonium sulfate saturation, with...
a rather poor yield (30%). The addition of other ammonium sulfate fractions, including those between 0 and 30%, 30 and 40%, 40 and 50%, and 60 and 70% saturation, did not further stimulate the hydroxylase activity.

Preparation of Anthranilic Acid-2-14C—The enzymatic synthesis of anthranilic acid-2-14C is based upon conversion of L-tryptophan to L-kynurenine by tryptophan pyrrolase and formanilidase of Pseudomonas cells (18), followed by hydrolysis of L-kynurenine to anthranilic acid by the action of kynureninase (6).

A reaction mixture containing DL-tryptophan-7α-14C, 4.2 μmoles (4.16 × 10^4 c.p.m.); L-tryptophan, 8 μmoles; potassium phosphate buffer, pH 7.9, 70 μmoles; and dialyzed enzyme (2.3 mg of protein) obtained from P. fluorescens No. 6, in a total volume of 2.0 ml was incubated at 35° for 80 minutes with shaking. The reaction was stopped by the addition of 0.3 ml of 20% HCl followed by gradient elution with HCl.

The supernatant solution and washings were combined and brought to a total volume of 10 ml with water. The solution was acidified with 30 ml of 0.1 N HCl and applied to a Dowex 50-H+ column, 200 to 400 mesh, 8% cross-linked, 1 cm² X 8.5 cm. The sample was washed in with 15 ml of water, and eluted with 30 ml of 1 N HCl and 120 ml of 5 N HCl were successively mixed with the content of the mixing chamber, and the column was eluted finally with 50 ml of 12 N HCl. Fractions of 4 ml each were collected at a rate of 50 ml per hour. The fractions were read for absorbance at 240 to 400 nm and their aliquots were tested for radioactivity (Fig. 1).

The fractions containing L-kynurenine were combined and dried in a vacuum at 35–40°. The dried material was dissolved in 2 ml of water and neutralized with 0.1 N KOH. An aliquot of the labeled kynurenine was assayed spectrophotometrically and the radioactivity was determined. The total radioactivity of the L-kynurenine isolated was 1.81 × 10^6 c.p.m. (87% of the theoretical yield). The specific activity was 220,000 c.p.m. per μmole on the basis of the absorbance of kynurenine at 360 nm at pH 7.0 (18). The purity of the radioactive material was tested by paper chromatographic analysis. Kynurenine was the only radioactive compound detected by paper chromatography with the following solvent systems: methanol-butanol-water-acetic acid (2:1:1:1:0.05) with ascending technique (RF 0.57), and butanol-acetic acid-water (4:1:5) with descending technique (RF 0.80).

The L-kynurenine-2-14C thus obtained was used in the preparation of anthranilic acid-2-14C. The reaction mixture, containing L-kynurenine-2-14C, 4.0 μmoles (880,000 c.p.m.); Tris buffer, pH 8.5, 150 μmoles; and kynureninase, 1.0 ml (2 mg of protein), in a total volume of 3.0 ml, was incubated for 12 minutes at 30°. The reaction mixture was deproteinized with perchloric acid (final concentration, 2%), neutralized, and brought to a total volume of 10 ml with water. The solution was passed through a Dowex 1-formate column (200 to 400 mesh, 2% cross-linked, 1 cm² X 5 cm), washed with 15 ml of water, and eluted with 30 ml of 1 N formic acid. The formic acid eluate was concentrated in a vacuum at 35–40° to about 3 ml. This solution, containing 500,000 c.p.m. of radioactivity, was added to a Dowex 50-H+ column, 200 to 400 mesh, 8% cross-linked, 1 cm² X 10.5 cm. The column was eluted with an HCl concentration gradien.

Fig. 1 (left). Ion exchange separation of various products formed from DL-tryptophan-7α-14C by the action of extracts of P. fluorescens No. 6. Tryptophan (○), kynurenine (●), kynurenin acid (△), and anthranilic acid (Δ) were identified by their ultraviolet absorption spectra and determined by comparing absorbance with those of authentic materials dissolved in water. The solution was acidified with 30 ml of 0.1 N HCl and applied to a Dowex 50-H+ column, 200 to 400 mesh, 8% cross-linked, 1 cm² X 8.5 cm. The sample was washed in with 30 ml of 0.1 N HCl followed by gradient elution with HCl. The mixing chamber was filled with 300 ml of 0.1 N HCl and closed with a stopper to keep the volume of liquid in the chamber constant throughout the elution. Then 300 ml of 5 N HCl and 120 ml of 8 N HCl were successively mixed with the content of the mixing chamber, and the column was eluted finally with 50 ml of 12 N HCl. Fractions of 4 ml each were collected at a rate of 50 ml per hour. The fractions were read for absorbance at 240 to 400 nm and their aliquots were tested for radioactivity (Fig. 1).

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Fig. 2 (center). Spectrophotometric evidence for the formation of catechol from anthranilic acid. The reaction mixture contained, in a total volume of 1.0 ml, 100 mM Tris buffer, pH 7.9, 100 mM glucose, 0.2 mM NADPH, 0.2 mM anthranilic acid, 300 units of glucose dehydrogenase, 0.1 ml of metapyrocatechase purified to the step of the first ammonium sulfate fraction, and 0.08 ml of anthranilic acid hydroxylase fraction. A reference cuvette contained all the components except for the omission of anthranilic acid. The reaction was initiated by addition of the hydroxylase fraction. The numbers indicate time in minutes after the reaction was started.

Fig. 3 (right). Manometric experiments with the supernatant fraction of Pseudomonas No. 23. The assay system contained, in a volume of 2.0 ml, 2 μmoles of substrate and 1.8 ml of the supernatant fraction. Both the assay system with catechol and its reference system contained 2 μmoles of GSH. The rates of endogenous oxygen uptake was less than half of that with anthranilic acid. Incubation was carried out at 30°. ●—●, anthranilic acid; ○—○, catechol.
(0.1 to 5.0 x) by a method similar to that described above. Ten fractions of 5 ml each were collected per hour. Each fraction was tested for ultraviolet absorption and radioactivity. No radioactive or ultraviolet-absorbing material other than anthranilic acid was observed. The fractions containing anthranilic acid (tubes 47 through 53) were combined and evaporated to dryness in a vacuum at 35-40°. The dried material was dissolved in water and neutralized with 0.1 N NaOH, and the volume was adjusted to 5 ml. An aliquot was used for the spectrophotometric measurement of the material and measurement of radioactivity. The specific activity was calculated to be 240,000 c.p.m. per pmole on the basis of absorbance at 310 m\(\mu\) at pH 7.0 \((\epsilon_{310} = 2,890)\). Paper chromatography by the descending technique gave further evidence of the identity and purity of the material. The following solvent systems were employed: butanol-acetic acid-water (4:1:1), \(R_f\) 0.94, and propanol-water (80:20), \(R_f\) 0.97.

**Analytical Methods**—A Shimadzu recording spectrophotometer and a Beckman DU spectrophotometer were used for spectrophotometric examinations. Manometric experiments were performed in a conventional Warburg apparatus. \(\beta\)-Ketoadipic acid was detected by its color reaction with ninhydrin (24) and determined quantitatively by CO\(_2\) evolution after decarboxylation with 4-aminooxycinnamic acid in acidic solution as catalyst (25). Protein was determined spectrophotometrically (26). Paper chromatographic analyses were done on Whatman No. 1 filter paper by the descending technique at 23° unless otherwise specified. Fluorescent materials on paper were located with the aid of a Mineralight apparatus (2537 A), and catechol was detected by spraying with a 0.1% aqueous solution of FeCl\(_3\).

Radioactivity was determined with samples of infinite thinness in a Nuclear-Chicago Geiger-Müller gas flow counter equipped with a Micromil thin window; the radioactive areas on paper chromatograms were determined with an Actigraph paper strip counter. A Packard Tri-Carb scintillation counter was used to check the radioactivity in trapping experiments with benzene-glycol, by employing scintillator solution made up of toluene containing 1.5% 2,5-diphenyloxazole and 0.005% p-bis-2'- (5'-phenyloxazolyl)benzene. Corrections were made for the difference in efficiency between the gas flow counter and the scintillation counter by measuring benzoic acid-1\(^{14}\)C in both counters. No quenching was observed with any of the samples. All values are expressed in terms of gas flow counting.

**Assay of Anthranilic Acid Hydroxylase**—The standard reaction mixture contained 100 mm Tris buffer, pH 7.5, 0.1 mm anthranilic acid, 0.2 mm NADH, and enzyme solution in a total volume of 1.0 ml. The reference cuvette contained all the components except the substrate. The activity was assayed by measuring the initial rate of the decrease in absorbance at 310 m\(\mu\) at 23°. All enzymatic reactions were carried out at 23° unless otherwise specified. One unit of enzyme was defined as that amount which causes the disappearance of 1 pmole of anthranilic acid per minute under these conditions. An optical density change of 0.06 per minute at 310 m\(\mu\) corresponded to 10 units of enzyme activity (see Table II, Experiment 2).

**RESULTS**

**Properties of Anthranilic Acid Hydroxylase**—The requirement by anthranilic acid hydroxylase for NADH or NADPH as a co-factor is shown in Table I. NAD or NADP did not replace NADH or NADPH. Further addition of NAD, boiled extracts, or a liver supernatant fraction did not result in stimulation of hydroxylase activity, in contrast to the manometric data of Hosokawa, Nakagawa, and Takeda (12). Ferrous ion had no effect on the stimulation of the reaction by this enzyme preparation.

The enzyme was inhibited to the extent of 85 and 76% by the addition of \(p\) chloromercuribenzoate \((10^{-4} \text{m})\) and \(MgCl_2\) \((10^{-4} \text{m})\), respectively. Inhibition by \(p\)-chloromercuribenzoate was partially reversed by the addition of either 2-mercapto- ethanol, cysteine, or GSH at \(10^{-3} \text{m}\) to yield 46, 45, and 68% of the original activity, respectively.

The optimal pH was found to be 7.5 when examined in Trismaleate buffer (0.1 m). At pH 7.2, 78%, and at pH 8.2, 82%, of the maximal activity was observed.

**Direct Evidence for Formation of Catechol from Anthranilic Acid**—Since the partially purified anthranilic acid hydroxylase preparation still contained a considerable amount of pyrocatechase, the accumulation of a stoichiometric quantity of catechol could not be demonstrated. However, the accumulation of some catechol during the initial stage of the reaction was observed spectrophotometrically. When anthranilic acid was incubated with the anthranilic acid hydroxylase together with an NADPH-generating system, the gradual decrease in absorbance at 310 m\(\mu\) due to the disappearance of substrate was observed and was accompanied by an increase in absorbance at 275 m\(\mu\) during the initial 6 minutes, suggesting the accumulation of catechol. The formation of catechol was confirmed by adding a partially purified preparation of metapyrocatechase to the reaction mixture described above. The appearance of an absorption spectrum with a peak at 375 m\(\mu\), corresponding to that of \(\alpha\)-hydroxy-muconic semialdehyde, indicated the formation of catechol (Fig. 2).

Further evidence that catechol is an intermediate during the degradation of anthranilic acid was obtained from the following experiments with anthranilic acid-\(2-^{14}\)C. The reaction mixture, containing Tris buffer, pH 9.0, 25 \(\mu\)moles; glucose, 25 \(\mu\)moles; GSH, 20 \(\mu\)moles; NADPH, 1.0 \(\mu\)mole; catechol, 20 \(\mu\)moles; anthranilic acid \(-2^{14}\)C, 0.36 \(\mu\)mole (86,000 c.p.m.); glucose dehydrogenase, 900 units; and the 50 to 60% ammonium sulfate fraction, 1.2 ml, in a total volume of 2.4 ml, was incubated with shaking at 30° for 35 minutes. The reaction was stopped by the addition of 0.3 ml of 1 N HCl to the reaction mixture in the cold. The precipitate was removed by centrifugation, and the supernatant solution was neutralized with 1 N NaOH and bromothymol blue as an indicator. The neutral solution was extracted three times with 6-ml aliquots of ether, the ether layers were combined and evaporated to dryness in a vacuum at 15°, and the residue was dissolved in 1.0 ml of water. A total of 3.1 \(\mu\)moles of catechol, as judged by ultraviolet absorption was recovered (8,800 c.p.m.).

S. Kobayashi and S. Kuno found the stimulation effect of ferrous ion with more purified anthranilic acid hydroxylase. Details will be published elsewhere.

1 Metapyrocatechase is known to convert catechol to \(\alpha\)-hydroxymuconic semialdehyde. The metapyrocatechase preparation used was found to be inactive toward anthranilic acid per se. In the presence of anthranilic acid hydroxylase preparations, the formation of \(\alpha\)-hydroxymuconic semialdehyde from catechol by metapyrocatechase was demonstrable spectrophotometrically in the reaction mixture, which was similar to that described in the text.

4 The amount of catechol was calculated by using an \(\epsilon_{375}\) value of 2300.
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This radioactive material gave an RF value identical with that of authentic catechol upon paper chromatography with the following solvent systems: m-cresol-acetic acid-water (50:2:48), RF 0.68; isopropyl alcohol-28% ammonia-water (20:1:1), RF 0.85; and 20% KCl, RF 0.73. Further evidence for the identity of this material was obtained from the constant specific activity obtained after repeated recrystallization with added authentic catechol from hot toluene.

Stoichiometric Formation of Catechol from Anthranilic Acid—The data presented in Table II and Fig. 3 indicate that 1 mole of anthranilic acid is converted to 1 mole of catechol with the consumption of 1 mole each of NADH (or NADPH) and oxygen. Although the stoichiometry of NADH (or NADPH) consumed to consumption of 1 mole each of NADH (or NADPH) and oxygen.

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The following molar extinction coefficients at 375 nm in 0.1 M Tris buffer, pH 7.5, for 3 hours at 4°C. The amounts of additions were as follows: glucose dehydrogenase, 300 units; boiled extracts,* 0.1 ml; liver supernatant fraction, 0.1 ml, ferrous ammonium sulfate, 0.1 mmole; and glucose, 100 mmole.

**Table I**

<table>
<thead>
<tr>
<th>Experiment and additions</th>
<th>Anthranilic acid hydroxylase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. None</td>
<td>0</td>
</tr>
<tr>
<td>2. NAD, 2 × 10^{-4} M</td>
<td>0</td>
</tr>
<tr>
<td>3. NADP, 2 × 10^{-4} M</td>
<td>0</td>
</tr>
<tr>
<td>4. NADH, 2 × 10^{-4} M</td>
<td>5.3</td>
</tr>
<tr>
<td>5. NADH, 2 × 10^{-4} M Fe^{2+}</td>
<td>5.3</td>
</tr>
<tr>
<td>6. NADPH, 2 × 10^{-4} M</td>
<td>4.9</td>
</tr>
<tr>
<td>7. NADPH, 2 × 10^{-4} M; NAD, 2 × 10^{-4} M</td>
<td>4.3</td>
</tr>
<tr>
<td>8. NADH, 10^{-4} M; glucose; glucose dehydrogenase</td>
<td>4.5</td>
</tr>
<tr>
<td>9. NADPH, 10^{-4} M; glucose; glucose dehydrogenase</td>
<td>3.5</td>
</tr>
<tr>
<td>10. NADH, 10^{-4} M; glucose; glucose dehydrogenase, boiled extracts*</td>
<td>3.2</td>
</tr>
<tr>
<td>11. NADPH, 10^{-4} M; glucose; glucose dehydrogenase; liver supernatant fraction</td>
<td>3.5</td>
</tr>
</tbody>
</table>

* The cell-free extracts of Pseudomonas No. 23 were heated at 60°C for 5 minutes. After centrifugation, the supernatant solution was used.

**Table II**

Stoichiometry of formation of catechol from anthranilic acid

In Experiment 1, the assay system contained, in a volume of 2.8 ml, Tris buffer, pH 7.5, 300 mmole; NADPH, 0.4 mmole; anthranilic acid, 0.3 mmole; metapyrocatechase purified to the step of the second ammonium sulfate fraction (19), 40.4 units in Experiment 1a, and 22.2* units in Experiment 1b; and anthranilic acid hydroxylase purified to the step of the C-2 gel treatment (see the text), 0.2 ml, containing 0.45* unit of pyrocatechase, which was determined according to the method of Hayaishi et al. (27). The amount of α-hydroxymuconic semialdehyde formed were determined by measuring absorbance at 375 nm (19) at 30-second intervals after the reaction had been started by adding anthranilic acid hydroxylase. The disappearance of anthranilic acid was assayed by measuring the decrease in absorbance at 310 nm with the reaction mixture as described above, from which metapyrocatechase was omitted because the formation of α-hydroxymuconic semialdehyde made it difficult to measure accurately the changes in absorbance at 310 nm. The assumption was made that the presence of the metapyrocatechase preparation did not affect the rate of the disappearance of anthranilic acid. The amount of catechol formed was calculated from the amount of α-hydroxymuconic semialdehyde formed. It was assumed that the amount of each product of pyrocatechase and metapyrocatechase was proportional to each enzyme activity in the reaction mixture, because both enzymes showed identical K, values for catechol (19, 27). The following molar extinction coefficients at 310 nm in 0.1 M Tris buffer, pH 7.5, were used for calculation: α-hydroxymuconic semialdehyde, 32,000 (19), and NADPH, 1.580. In Experiment 2, the reaction mixture contained, in a volume of 1.0 ml, Tris buffer, pH 7.5, 100 mmole; NADPH, 0.1 mmole; anthranilic acid, 0.1 mmole, and 0.05 ml of the dialyzed fraction (see Table I) between 50 and 60% ammonium sulfate saturation. Spectral changes of the reaction mixture were recorded at 300 to 400 nm at 2-minute intervals after the reaction had been started by adding the enzyme. Decreases in absorbance at 310 and 340 nm for the initial 10 minutes were used to determine the stoichiometry.

The following molar extinction coefficients in 0.1 M Tris buffer, pH 7.5, were used for the calculation: anthranilic acid, ε_{410} = 2,900 and ε_{440} = 1,000; NADPH, ε_{410} = 3,100 and ε_{440} = 6,220.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time</th>
<th>Anthranilic acid consumed</th>
<th>Catechol formed</th>
<th>NADPH consumed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>1 min</td>
<td>17 mmoles</td>
<td>20 mmoles</td>
<td>20 mmoles</td>
</tr>
<tr>
<td>1b</td>
<td>1 min</td>
<td>17 mmoles</td>
<td>18 mmoles</td>
<td>18 mmoles</td>
</tr>
<tr>
<td>2</td>
<td>10 min</td>
<td>20 mmoles</td>
<td>26 mmoles</td>
<td>26 mmoles</td>
</tr>
</tbody>
</table>

* The unit of metapyrocatechase is expressed as 0.01 μmole per minute (19), and that of pyrocatechase, as 1 μmole per minute (27).

† The calculation was based on ε_{410} = 6,220 at pH 7.0 (28).

Positions 1 and 2 of catechol, as shown by the following experiment.

A reaction mixture in a volume of 2.1 ml, containing Tris buffer, pH 8.0, 100 mmole; GSH, 5 mmole; NADH, 2 mmole; glucose, 100 mmole; anthranilic acid, 2 mmole; anthranilic acid-2-14C, 0.22 mmole (53,000 c.p.m.); glucose dehydrogenase, 300 units; and 1.1 ml of the cell-free extract, was incubated at 30°C with constant shaking. After 30 minutes, the mixture was cooled in an ice bath, and 8 mmole of neutralized β-ketoadipic acid dissolved in 0.2 ml were added to the incubation mixture. Aliquots were immediately removed for determination of radio-
TABLE III

Oxidation of various compounds by supernatant fraction

The assay system contained, in a volume of 2.0 ml, 2 μmoles of substrate and 1.5 ml of the enzyme (the supernatant fraction). Incubation was carried out at 30°. The initial rates of oxygen consumption are presented.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Oxygen uptake μl/10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7.4</td>
</tr>
<tr>
<td>Anthranilic acid</td>
<td>27.0</td>
</tr>
<tr>
<td>Catechol</td>
<td>30.9</td>
</tr>
<tr>
<td>2,3-Dihydroxybenzoic acid</td>
<td>10.3</td>
</tr>
<tr>
<td>Protocatechluic acid</td>
<td>28.6</td>
</tr>
<tr>
<td>trans-Benzenglycol</td>
<td>19.7</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>7.4</td>
</tr>
<tr>
<td>o-Aminophenol</td>
<td>7.0</td>
</tr>
</tbody>
</table>

TABLE IV

Distribution of radioactivity in β-ketoadipic acid formed from anthranilic acid-2-14C

The main compartment of a Warburg flask contained, in a volume of 2.0 ml, acetate buffer, pH 4.2, 200 μmoles; and a 0.4-ml aliquot of the reaction mixture (see the text). The center well contained 0.2 ml of 20% KOH, and the side arm contained 0.2 ml of 1.4 M aminooantipyrine. After the solution in the side arm had been poured into the contents of the main compartment, the flask was incubated at 30° for 20 minutes with reciprocal shaking, after which the vessel was opened. To the alkali solution in the center well, 0.2 ml of water was added and mixed. Two 0.05-ml aliquots of the diluted alkali solution were used for the determination of radioactivity. A control experiment was carried out with a flask containing the same components as those described above except for the replacement of 4-aminooantipyrine with water. In the latter case no radioactivity was found in the alkali solution. Data present the radioactivity of CO₂ evolved from 0.8 ml of the reaction mixture by the treatment described above and that of the 2,4-dinitrophenylhydrazone of levulinic acid isolated from the same reaction mixture (see the text) after the same treatment.

<table>
<thead>
<tr>
<th>Material assayed</th>
<th>Radioactivity c.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aliquot of reaction mixture used*</td>
<td>20,000</td>
</tr>
<tr>
<td>C-1 of β-ketoadipic acid</td>
<td>4,640</td>
</tr>
<tr>
<td>Levulinic acid 2,4-dinitrophenylhydrazone</td>
<td>5,000</td>
</tr>
<tr>
<td>Yield of radioactivity in β-ketoadipic acid</td>
<td>48%</td>
</tr>
</tbody>
</table>

* Almost all of the radioactivity other than that of β-ketoadipic acid was found in unreacted anthranilic acid upon paper chromatography.

activity in the α carboxyl carbon of β-ketoadipic acid (Table IV). Subsequent to decarboxylation, levulinic acid was isolated as the hydrazone derivative by the following method. An aliquot (0.8 ml) of the incubation mixture was treated as described in Table IV and centrifuged to remove the precipitate. The supernatant solution was poured into 5 ml of 3 N HCl containing 0.1% 2,4-dinitrophenylhydrazine at 80°. After 30 minutes, the hydrazone formed was extracted from the acidic solution with several 5-ml aliquots of ethyl acetate. The extracts were combined and evaporated to dryness in a vacuum. The residue was dissolved in a small volume of ethyl acetate and subjected to paper chromatography on Whatman No. 3MM paper with butanol-water-28% ammonia (4:1:0.5). A yellow band having an Rf value (0.7) identical with that of an authentic sample of 2,4-dinitrophenylhydrazine of levulinic acid was cut out and eluted with ethyl acetate. The eluate was evaporated to dryness in a vacuum, and the residue was redissolved in 1.0 ml of ethyl acetate. An aliquot was rechromatographed as described above. The hydrazone was shown to be chromatographically pure and to coincide with the only radioactive area on the chromatogram. As shown in Table IV, the radioactivity of carbon 1 of β-keto acid was essentially equal to that of the levulinic acid moiety, indicating that the radioactive carbon of anthranilic acid-2-14C was distributed equally in the carbon atoms 1 and 2 of catechol. These results are consistent with the interpretation that the hydroxylation occurs either at carbon atoms 1 or 2 or at carbon atoms 2 and 3 of anthranilic acid. Scheme 1 illustrates the formation of β-keto acid from anthranilic acid-2-14C by a Pseudomonas species (29, 30).

Trapping Experiments with Benzenglycol—Since it is unlikely that o-aminophenol, salicylic acid, 2,3-dihydroxybenzoic acid, and protocatechluic acid are intermediates in the conversion of anthranilic acid to catechol, benzenglycol (15) was considered as a possible intermediate since it has been shown that catechol could be formed from benzenglycol by an NADP-linked dehydrogenase of rabbit liver described by Ayengar et al. (23). A similar enzyme has also been found in a Pseudomonas species (14), and more recently this enzyme was found to be rather ubiquitous (31). However, that benzenglycol is an intermediate in the formation of catechol from anthranilic acid was ruled out in part by the following observations.

Cell-free extracts prepared from tryptophan-adapted and unadapted cells contained essentially the same level of benzenglycol dehydrogenase activity, as determined by the assay method of Ayengar et al. (28). The total dehydrogenase activity contained in the crude extracts was located in the fractions between 0 and 30% ammonium sulfate saturation (65%) and in the fractions between 30 and 40% saturation (35%), with none detectable in either the 40 to 50% or 50 to 60% fractions. As indicated above, the fraction between 50 and 60% ammonium sulfate saturation catalyzes the conversion of anthranilic acid to catechol.

An additional series of experiments was conducted in an effort to rule out benzenglycol as an intermediate in a more conclusive manner.

A reaction mixture, in a volume of 8.0 ml, contained potassium phosphate buffer, pH 7.5, 50 μmoles; anthranilic acid, 2 μmoles;
The reaction mixture and details of procedures are described in the text. The theoretically minimum specific activity of isolated benzene glycol refers to the value expected on the assumption that the amount of radioactive benzene glycol formed from anthranilic acid would be at least equal to the amount of radio-active β-ketoacidic acid formed from anthranilic acid-2-14C and that the radioactivity of labeled benzene glycol would be diluted with anthranilic acid and benzene glycol added as carriers. The radioactivity of β-ketoacidic acid was determined from the radioactivity of CO₂ evolved from its α-carboxyl carbon (see the text).

TABLE V

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Specific activity of anthranilic acid</th>
<th>Specific activity of isolated benzene glycol</th>
<th>Recovery of radioactivity in β-ketoacidic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>benzene glycol</td>
<td>c.p.m./μmole</td>
<td>c.p.m./μmole</td>
<td>%</td>
</tr>
<tr>
<td>trans</td>
<td>30,000</td>
<td>241</td>
<td>18</td>
</tr>
<tr>
<td>cis*</td>
<td>24,000</td>
<td>326</td>
<td>0</td>
</tr>
</tbody>
</table>

* The amount of the isolated carrier was 0.0 μmole.

Anthranilic acid-2-14C, 0.29 μmole (70,000 c.p.m.); trans-benzeneglycol, 50 μmole; GSH, 15 μmole; and the supernatant fraction, 6.0 ml. Incubation was carried out at 30° with constant shaking. After 42 minutes, the reaction was stopped by heating at 60–65° for 10 minutes. The heated reaction mixture was centrifuged, and the precipitate was washed four times with 2-ml portions of water. The supernatant solution and washings were combined to make a total volume of 15.7 ml, and a 1-ml aliquot was used to determine the radioactivity of carbon atom 1 of β-ketoacidic acid as described in Table IV. It was found that a total of 13,000 c.p.m. of the radioactivity was incorporated into the β-ketoacidic acid. The solution was then passed through a column of Dowex 1 resin (2% cross-linked, 200 to 400 mesh, formate form, 1 cm² X 5 cm), and the column was eluted with 15 ml of water. The eluates were combined and evaporated to dryness in a vacuum at 30°. The residue was extracted three times with 2-ml aliquots of ether. The ether extracts were combined and evaporated to dryness in a vacuum at 15° and redissolved in 2.0 ml of water. Aliquots were analyzed for ultra-violet absorption and radioactivity. The absorption spectrum was found to be identical with that of benzene glycol. The identity was confirmed by its activity with benzene glycol dehydrogenase. The amount of benzene glycol recovered was calculated to be 7.8 μmole as judged by absorbance at 260 μμ in water solution (Table V).

A second reaction mixture, containing Tris buffer, pH 7.9, 100 μmole; anthranilic acid, 2.23 μmole; anthranilic acid-2-14C, 0.23 μmole (56,000 c.p.m.); cis-benzeneglycol, 41 μmole; and the supernatant fraction, 1.0 ml, in a volume of 2.0 ml, was incubated and treated by a method essentially identical with that described above.

The data presented in Table V, and the additional fact that the fraction (50 to 60% ammonium sulfate saturation) responsible for the conversion of anthranilic acid to catechol was free of benzeneglycol dehydrogenase, indicate that neither trans- nor cis-benzeneglycol is an intermediate in the conversion.
The reaction mixture, containing, in a volume of 2.0 ml, Tris buffer, pH 7.5, 100 μmoles; benzoic acid, 2 μmoles; benzoic acid-1-\(^{14}C\), 0.76 μmole (200,000 c.p.m.); trans-benzeneglycol, 40 μmoles; and the supernatant fraction of Pseudomonas A 312 cells (9 mg of protein), was incubated at 30° for 30 minutes with reciprocal shaking. Another reaction mixture contained, in a volume of 2.0 ml, the same components as described above except that trans-benzeneglycol was replaced by 40 μmoles of cis benzeneglycol and incubated by the same method. After the incubation, benzeneglycol was isolated from each reaction mixture and its quantity and radioactivity were determined by the same method as described in the text with anthranilic acid. The amount of the isolated carrier after reaction was calculated to be 23.2 μmoles in the former case and 20.5 μmoles in the latter. The radioactivity incorporated into β-ketoadipic acid (41) was also measured by methods essentially identical with those described for anthranilic acid-2-\(^{14}C\). The meaning of theoretically minimum specific activity of benzeneglycol is the same as described in Table V.

### Table VI

<table>
<thead>
<tr>
<th>Carrier benzeneglycol</th>
<th>Specific activity of benzoic acid</th>
<th>Specific activity of isolated carrier</th>
<th>Recovery of radioactivity in β-ketoadipic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c.p.m./μmole</td>
<td>c.p.m./μmole</td>
<td>%</td>
</tr>
<tr>
<td>trans</td>
<td>74,500</td>
<td>2,160</td>
<td>45</td>
</tr>
<tr>
<td>cis</td>
<td>74,500</td>
<td>58</td>
<td>1.2</td>
</tr>
</tbody>
</table>

In connection with these reaction mechanisms, the conversion of benzoic acid to catechol (39, 40) is also of interest and may be helpful in understanding the mechanism of action of anthranilic acid hydroxylase. If benzoic acid is also converted to catechol by a reaction sequence involving epoxidation, it is still possible that benzeneglycol is an intermediate in the conversion. Furthermore, the requirement of NADPH in the oxidation of benzoic acid to β-ketoadipic acid by cell-free extracts of a Pseudomonas species has been shown manometrically by Tanaka and Wada (38). Takeda et al.\(^4\) were able to show, by using benzoic acid-carboxyl-\(^{14}C\) and partially purified enzymes from a soil bacterium adapted to benzoic acid, that one fraction of the enzymes accumulated an unidentified intermediate in the presence of both NADPH (or NADH) and oxygen, which was decarboxylated by the action of another fraction of the enzymes. These facts suggest that benzoic acid is oxidized by an enzyme similar to a hydroxylase in its requirement for NADPH, and that the system contains an NADPH-regenerating mechanism, since the balance in the conversion of benzoic acid to catechol is similar in overall stoichiometry to that in the conversion of kynurenic acid, but differs from that involving anthranilic acid. The data presented in Table VI, however, indicate that neither trans- nor cis-benzeneglycol is a dissociable intermediate in the conversion of benzoic acid to catechol. From these considerations and the experiments of Sleeper (41), which show the incorporation of carbon 1 of benzoic acid into carbon atoms 1 and 2 of catechol, the following reaction sequence (Equations 4 to 7) may be postulated.

\[
\begin{align*}
\text{COOH} + \text{O}_2 + \text{NADPH} + \text{H}^+ &\rightarrow (4) \\
\text{COOH} + \text{NADP}^+ + \text{H}_2\text{O} &\rightarrow (5) \\
\text{COOH} + \text{H}_2\text{O} &\rightarrow (6) \\
\text{COOH} - \text{NAD}^+ &\rightarrow (7)
\end{align*}
\]

The reactions in Equation 7 are postulated to be the same as those involved in the conversion of anthranilic acid and those suggested by Sleeper (41). Since the reaction in Equation 6 might be enzymatic, it would be important to determine whether the enzyme concerned is identical with benzeneglycol dehydrogenase. The dehydrogenase activity found in extracts prepared from P. fluorescens A 312 cells was the same whether or

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\(^4\) H. Takeda, M. Mori, K. Ueda, and H. Taniuchi, to be published.
not the bacteria were adapted to benzoic acid. Ichihara et al. (42) have reported the interesting observation that extracts prepared from Pseudomonas species adapted to anthranilic acid oxidize benzoic acid at a lower rate than anthranilic acid, while those adapted to benzoic acid oxidize anthranilic acid at a lower rate than benzoic acid. These findings can be explained in terms of the reaction sequences presented above for anthranilic acid and benzoic acid, if it is assumed that the reaction shown in Equation 6 is catalyzed by the constitutive benzeneglycol dehydrogenase as well as that the specificity of neither anthranilic acid hydroxylase nor benzoic acid hydroxylase (Equation 4) is strict as proposed by Ichihara et al. (42).

SUMMARY

1. The enzymatic synthesis of anthranilic acid-2-14C from tryptophan-7a-14C has been described.

2. The role of catechol as an intermediate in the degradation of anthranilic acid by a Pseudomonas species (ATCC 11250) adapted to tryptophan has been demonstrated by enzymatic and radioisotope techniques.

3. A partially purified enzyme preparation from Pseudomonas species cells catalyzes the formation of catechol from anthranilic acid with the consumption of equimolar amounts of reduced nicotinamide adenine dinucleotide (or reduced nicotinamide adenine dinucleotide phosphate) and oxygen. This enzyme is referred to as anthranilic acid hydroxylase.

4. The results of experiments with anthranilic acid-2-14C show that carbon 2 of anthranilic acid is one of the carbon atoms hydroxylated and that neither trans- nor cis-benzeneglycol is a dissociable intermediate in this process. Benzeneglycol dehydrogenase was present to the same extent in extracts of tryptophan-adapted as well as nonadapted cells.

5. Attempts to show that either trans- or cis-benzeneglycol is an intermediate in the degradation of benzoic acid 14C with extracts of another strain of a Pseudomonas species were unsuccessful.

6. The mechanism of the formation of catechol from anthranilic acid or benzoic acid is discussed in connection with the reaction sequence postulated for a new type of kynurenic acid hydroxylation.

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Enzymatic Formation of Catechol from Anthranilic Acid
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