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 traces 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 benzenglycol (5,6-dihydroxy-cyclohexanecarboxylic acid) 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-1-14C 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 Hayaishi 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 (specific activity, 518) (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 x 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° with vigorous aeration in a medium containing 0.1% benzoic acid, 0.15% K2HPO4, 0.05% KH2PO4, 0.2% NH4Cl, 0.1% Difeo 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 x g for 10 minutes. To 10 ml of the supernatant solution, 1 ml of well aged alumina Cγ gel (19 mg of dry matter per ml) was added. After stirring for 10 minutes, the precipitate was removed by centrifugation at 20,000 x 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, will be 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 formanidase 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 X 10⁴ 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, 2% cross-linked, 1 cm2 X 8.5 cm. The sample was washed in with 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).

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 X 10⁶ 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 0.60).

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 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 0.1 M of metaphyrocatechase purified to the step of the first ammonium sulfate fraction, and 0.09 M of anthranilic acid. Incubation was carried out at 30°. O-O denotes the radioactivity of each fraction. The numbers indicate time in minutes after the reaction was started.

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 metaphyrocatechase purified to the step of the first ammonium sulfate fraction, and 0.09 ml of anthranilic acid. 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°. O-O, anthranilic acid; O-O, catechol.
(0.1 to 5.0 n) 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 identification of the material and measurement of radioactivity. The specific activity was calculated to be 240,000 c.p.m. per μmole on the basis of absorbance at 310 μm at pH 7.0 (ε = 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), Rf 0.94, and propanol-water (80:20), Rf 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. β-Ketoacidipic acid was detected by its color reaction with nitroprusside (24) and determined quantitatively by CO₂ evolution after decarboxylation with 4-aminooantipyrine in acid 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 FeCl₃.

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 efficiency difference between the gas flow counter and the scintillation counter by measuring benzoic acid-1-14C 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 μm 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 μmole of anthranilic acid per minute under these conditions. An optical density change of 0.06 per minute at 310 μm 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 (10⁻⁵ M) and HgCl₂ (10⁻⁴ M), respectively. Inhibition by p-chloromercuribenzoate was partially reversed by the addition of either 2-mercaptothanol, cysteine, or GSH at 10⁻³ M to yield 46, 45, and 68% of the original activity, respectively.

The optimal pH was found to be 7.5 when examined in Tris-maleate 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 μm due to the disappearance of substrate was observed and was accompanied by an increase in absorbance at 275 μm 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 μm, corresponding to that of α-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-2-14C. The reaction mixture, containing Tris buffer, pH 9.0, 25 μmoles; glucose, 25 μmoles; GSH, 20 μmoles; NADPH, 1.0 μmole; catechol, 20 μmoles; anthranilic acid-2-14C, 0.36 μmole (86,000 c.p.m.); and 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 33 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 vacuum at 15°, and the residue was dissolved in 1.0 ml of water. A total of 3.1 μmoles of catechol as judged by ultraviolet absorption was recovered (8,800 μmoles of catechol was detected by its color reaction with nitroprusside (24).

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

Metapyrocatechase is known to convert catechol to α-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 α-hydroxymuconic semialdehyde from catechol by metapyrocatechase was demonstrated spectrophotometrically in the reaction mixture, which was similar to that described in the text. The amount of catechol was calculated by using an ε₁₀₀ value of 2900.
This radioactive material gave an $R_f$ value identical with that of authentic catechol upon paper chromatography with the following solvent systems: m-cresol-acetic acid-water (50:2:48), $R_f$ 0.68; isopropyl alcohol-28% ammonia-water (20:1:1), $R_f$ 0.88; and 20% KCl, $R_f$ 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 oxygen consumption of 1 mole each of NADH (or NADPH) and oxygen.

### Site of Hydroxylation

The supernatant fraction obtained from cells of *Pseudomonas* No. 23 adapted to tryptophan could not oxidize salicylic acid and o-anisidophenol, but protocatechuate acid and 2,3-dihydroxybenzoic acid were oxidized by this system (Table III). These results suggest that one of the latter two compounds may serve as an intermediate in the conversion of anthranilic acid to catechol. However, the possibility that 2,3-dihydroxybenzoic acid is an intermediate appeared unlikely, since the rate of oxygen consumption with this substrate was 1,097. 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.

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same reaction mixture (see the text) after the same treatment. 

2,4-dinitrophenylhydrazone of levulinic acid isolated from the reaction mixture by the treatment described above and that of the hydrazone formed was extracted from the acidic solution with 2,4-dinitrophenylhydrazine at 80°. After 30 minutes, the activity in the (\(\gamma\) carboxyl carbon of \(\beta\)-ketoadipic acid (Table IV). Subsequent to decarboxylation, levulinic acid was isolated as the 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>Protocatechueic acid</td>
<td>28.6</td>
</tr>
<tr>
<td>trans-Benzene-glycol</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 \(\beta\)-ketoadipic acid formed from anthranilic acid-2-\(^{14}\)C**

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 aminoantipyrine. 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-aminoantipyrine 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-dinitrophenyhydrazone 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 (\beta)-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 (\beta)-ketoadipic acid.</td>
<td>48%</td>
</tr>
</tbody>
</table>

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

**Trapping Experiments with Benzene-glycol**—Since it is unlikely that o-aminophenol, salicylic acid, 2,3-dihydroxybenzoic acid, and protocatechuic acid are intermediates in the conversion of anthranilic acid to catechol, benzeneglycol (15) was considered as a possible intermediate since it has been shown that catechol could be formed from benzeneglycol 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 benzeneglycol 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 benzeneglycol dehydrogenase activity, as determined by the assay method of Ayengar et al. (23). 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 benzeneglycol 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; paper chromatography on Whatman No. 3MM paper with butanol-water-28% ammonia (4:1:0.5). A yellow band having an *Rf* value (0.71) identical with that of an authentic sample of 2,4-dinitrophenyhydrazone 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 \(\beta\)-ketoadipic acid was essentially equal to that of the levulinic acid moiety, indicating that the radioactive carbon of anthranilic acid-2-\(^{14}\)C 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 \(\beta\)-ketoadipic acid from anthranilic acid-2-\(^{14}\)C by a *Pseudomonas* species (29, 30).
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 radioactive \( \beta \)-ketoacidic acid formed from anthranilic acid-2-\( ^{14} \)C and that the radioactivity of labeled benzene glycol would be diluted with anthranilic acid and benzene glycol added as carriers. The radioactivity of \( \beta \)-ketoacidic acid was determined from the radioactivity of CO\(_2\) evolved from its \( \alpha \) carbonyl carbon (see the text).

<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 ( \beta )-ketoacidic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>benzene glycol</td>
<td>c.p.m./( \mu )mole</td>
<td>c.p.m./( \mu )mole</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>25</td>
</tr>
</tbody>
</table>

* The amount of the isolated carrier was 0.0 \( \mu \)moles.

There are several examples of biological hydroxylations which are catalyzed by hydroxylases, the so-called mixed function oxygenases (32, 33). The characteristic stoichiometry of the reaction catalyzed by this group of enzymes is well established. In this reaction, 1 mole each of oxygen and NADH are consumed with the result that 1 atom of molecular oxygen is incorporated into the hydroxyl group of the product and the other is converted to water (34-36). Recently, another type of enzymatic hydroxylating resulting in the simultaneous hydroxylation at 2 adjacent carbon atoms of the aromatic ring has been recognized (13, 37, 38). The mechanism of this new type of hydroxylation has been elucidated in principle with kynurenic acid hydroxylase (13, 14). Kynurenic acid (Compound I, Equation 1) is converted to 7,8-dihydroxykynurenic acid 7,8-diol (III) with the consumption of 1 mole each of oxygen and NADH (or NADPH). The latter compound undergoes dehydrogenation by an NAD-linked dehydrogenase to form 7,8-dihydroxykynurenic acid (IV). Kynurenic acid 7,8-oxide (II) has been proposed as an intermediate in this sequence of reactions.
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 \( \beta \)-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 1 carbon of benzoic acid into carbon atoms 1 and 2 of catechol, the following reaction sequence (Equations 4 to 7) may be postulated.

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

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 benzene glycol dehydrogenase. The dehydrogenase activity found in extracts prepared from \( P. \text{fluorescens} \) A 312 cells was the same whether or not the conversion of 1 mole of anthranilic acid to 1 mole of catechol (Equation 2).

There is an apparent difference in the over-all stoichiometry of the reaction sequences involving the conversion of kynurenic acid to 7,8-dihydroxykynurenic acid and the conversion of anthranilic acid to catechol. Only 1 mole of oxygen is consumed in the former case, while 1 mole of oxygen as well as 1 mole of NADH are consumed in the latter, since the consumption of NADH in the initial reaction is balanced by the formation of NADH in the conversion of kynurenic acid to 7,8-dihydroxykynurenic acid (Equation 1).

In view of the observations that benzene glycol is not an intermediate and that carbon 2 of anthranilic acid is converted to carbon atoms 1 and 2 of catechol, together with the stoichiometry described above, a reaction sequence (Equation 3) in which only the initial reaction is enzymatic and the others are spontaneous may be postulated as a probable mechanism of the formation of catechol from anthranilic acid.

\[
\begin{align*}
\text{COOH} + \text{NADH} & \rightarrow \\
\text{COOH} & \quad \text{(3)} \\
\end{align*}
\]

The meaning of theoretically minimum specific activity of benzene glycol is the same as described in Table V.

**Table VI**

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

\( ^4 \) H. Takeda, M. Mori, K. Ueda, and H. Taniuchi, to be published.
Summary

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

2. The role of catechol as an intermediate in the degradation of anthranilic acid by a \textit{Pseudomonas} species (ATCC 11250) has been described.

3. A partially purified enzyme preparation from \textit{Pseudomonas} species 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-7a-⁴C and extracts of \textit{Pseudomonas} species adapted to anthranilic acid.

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 \textit{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 sequences postulated for a new type of kynurenic acid hydroxylase nor benzoic acid hydroxylase (Equation 4) is strict as proposed by Ichihara et al. (42).

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References


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