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METAPYROCATECHASE

III. SUBSTRATE SPECIFICITY AND MODE OF RING FISSION

MITSUHIRO NOZAKI, SHIGEKO KOTANI, KATSUHIKO ONO* AND SIRO SENOH[a]

Department of Medical Chemistry, Kyoto University Faculty of Medicine, Kyoto (Japan) and [a]Laboratory of Organic Chemistry, The Institute of Food Chemistry, Osaka (Japan)

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SUMMARY

Metapyrocatechase (catechol:oxygen 2,3-oxidoreductase, EC 1.13.1.2) is a dioxygenase which catalyzes the conversion of catechol to α-hydroxymuconic ε-semialdehyde with the insertion of two atoms of O_2 into the bond adjacent to a hydroxyl group. The crystalline enzyme preparation is also found to catalyze oxygenative cleavage of the benzene ring of some 4- or 3-substituted catechol derivatives. Among them, 4-methylcatechol (homocatechol), is found to be oxygenated by the enzyme at the same initial rate as that for catechol. The reaction product is identified as α-hydroxy-δ-methylmuconic ε-semialdehyde. Likewise, the ring fission product of protocatechuic acid by the action of metapyrocatechase is not α-hydroxy-γ-carboxymuconic ε-semialdehyde but α-hydroxy-δ-carboxymuconic ε-semialdehyde which is nonenzymatically decarboxylated to form α-hydroxymuconic ε-semialdehyde. 3-Methylcatechol appears to be cleaved by the enzyme at the bond between carbon atom bearing a hydroxyl group and the adjacent carbon carrying a methyl group.

In the light of these findings, the mode of ring fission of 4- or 3-substituted catechol derivatives by metapyrocatechase is discussed.

INTRODUCTION

Among a number of *o*-dihydroxyphenyl compounds which are cleaved by the action of individual dioxygenases, three modes of ring fission by microbial enzymes have so far been clearly demonstrated: (A) oxygenative cleavage of the bond between carbon atoms bearing the hydroxyl groups of an *o*-dihydroxyphenyl compound (intradiol cleavage**, A in Fig. 1); (B) cleavage of the bond between, the carbon atoms of 2 and 3 position (extradiol cleavage, proximal**, B in Fig. 1); and (C) that of 4 and 5 (extradiol cleavage, distal**, C in Fig. 1).

* Present address: Laboratory of Viral Oncology, Aichi Cancer Center Research Institute, Nagoya.

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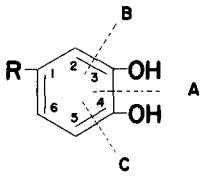


Fig. 1. Three modes of ring fission of *o*-dihydroxyphenyl compounds by dioxygenases.

Pyrocatechase which catalyzes the conversion of catechol to *cis,cis*-muconic acid[1] is a typical example of Type A. Protocatechuic acid is cleaved by the action of protocatechuate 3,4-dioxygenase[2] and 4,5-dioxygenase[3] to form *cis,cis*-β-carboxymuconic acid and α-hydroxy-γ-carboxymuconic ε-semialdehyde, respectively. The former is an example of Type A, and the latter of Type C.

Recently, ADACHI *et al.*[4] reported that 3,4-dihydroxyphenylacetate 2,3-oxygenase from a pseudomonad catalyzes the ring fission between the carbon atoms of position 2 and 3 of 3,4-dihydroxyphenylacetate to form α-hydroxy-δ-carboxymethylmuconic ε-semialdehyde (Type B).

Metapyrocatechase (catechol: oxygen 2,3-oxidoreductase, EC 1.13.1.2) catalyzes the cleavage of the bond of catechol between the carbon atom bearing a hydroxyl group and an adjacent carbon atom carrying hydrogen, to form α-hydroxymuconic ε-semialdehyde.

In previous papers of this series, we have reported the purification and crystallization procedure of metapyrocatechase[5] and some of its chemical nature and reaction mechanism[6]. In this paper, we wish to present some lines of evidence to indicate that substituted catechol derivatives are exclusively cleaved at proximal site (Type B) by the action of metapyrocatechase.

EXPERIMENTAL

Materials

Crystalline metapyrocatechase with a specific activity of 116 μmoles/min per mg of protein was prepared as previously described[5] from *Pseudomonas arvilla*, grown with benzoate as a major carbon source. α-Hydroxymuconic semialdehyde dehydrogenase was prepared from cat liver by the method of ICHIYAMA *et al.*[7]. Catechol was a product of E. Merck AG, Darmstadt, Germany. 4-Methylcatechol (homocatechol) and protocatechuic acid was purchased from Tokyo Kasei Co. and recrystallized from benzene. Lactate dehydrogenase, catalase and picolinic acid were products of Sigma.

4-Methylpicolinic acid and 5-methylpicolinic acid were synthesized following the method of NAKAJIMA[8] from 2,4-dimethylpyridine and 2,5-dimethylpyridine, respectively. 2,4-Quinolinic acid and 2,5-quinolinic acid were synthesized by oxidation with $KMnO_4$ from 4-methylpicolinic acid and 5-methylpicolinic acid, respectively.

α-Hydroxymuconic acid and γ- and δ-methyl-substituted α-hydroxymuconic acid were synthesized by condensation of dimethyl oxalate with corresponding β-methyl acrylate, β,β-dimethyl acrylate and α,β-dimethylacrylate, respectively, and followed by hydrolysis[9,10].

Methods

Enzyme assay and protein determination. The activity of metapyrocatechase with various substrates was assayed polarographically at 25° by measuring O_2 consumption[5]. The reaction mixture contained in a final volume of 2.2 ml: 110 μmoles of potassium phosphate buffer, pH 7.5, 10 μmoles of the substrate and the enzyme. The enzyme used was in a range between 1 and 1000 μg to give a measurable rate of O_2 consumption for each substrate.

Protein concentration was determined by the method of LOWRY *et al.*[11], using crystalline bovine serum albumin as a reference protein.

Identification of reaction products as pyridine derivatives. The conversion of the reaction products to their pyridine derivatives was achieved by the method of

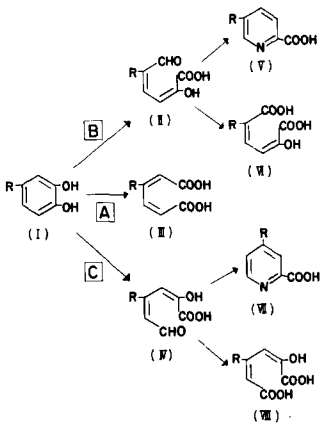


Fig. 2. Predictable mode of ring fission and reaction products of 4-substituted catechol derivatives.

ADACHI *et al.*[4]. After enzymic oxidation was complete, 2 vol. of glacial acetic acid saturated with ammonium acetate were added to the solution. After 24 h, an aliquot of the reaction mixture was taken for spectral analysis with a Cary spectrophotometer Model 15 or a Shimadzu multipurpose spectrophotometer MPS-50.

Principle for the determination of cleavage site of 4- or 3-substituted catechol derivatives. As shown in Fig. 2, intradiol cleavage of 4-substituted catechol derivatives (I) gives a product, β-substituted muconic acid derivatives (III), whereas two different products are expected to be formed by extradiol cleavage: namely, α-hydroxy-δ-substituted muconic ε-semialdehyde derivatives (II) by proximal cleavage and α-hydroxy-γ-substituted muconic ε-semialdehyde derivatives (IV) by distal cleavage. These α-hydroxymuconic ε-semialdehyde derivatives can be converted either to picolinic acid derivatives (V, VII) in the presence of NH_4^+ or to muconic acid derivatives (VI, VIII) by the action of α-aminomuconic ε-semialdehyde dehydrogenase in the presence of NAD^+.

Likewise, extradiol ring fission products of 3-substituted catechol by proximal and distal cleavage can be converted to 6- and 3-substituted picolinic acids, respectively. By examining ring fission products, it is possible to determine the specificity of cleavage site of the substituted catechol derivatives under the action of metapyrocatechase.

TABLE I

SUBSTRATE SPECIFICITY OF METAPYROCATECHASE

The activity of the enzyme was estimated as described under EXPERIMENTAL.

Compound	*Relative activity (%)*
Catechol	100
4-Methylcatechol	100
3-Methylcatechol	62
4-Chlorocatechol	51
Pyrogallol	32.8
Protocatechualdehyde	21
Protocatechuic acid	0.15
3,5-Dichlorocatechol	0.17
Dopamine	0.014

RESULTS

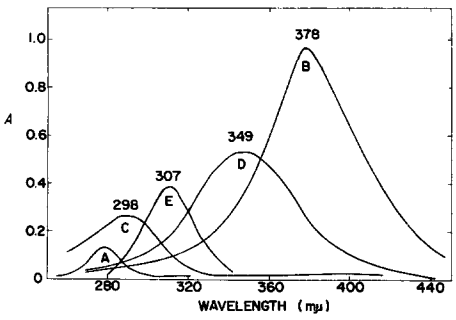
Substrate specificity of metapyrocatechase

The substrates studied and their relative activities are summarized in Table I. Concentrations of all substrates used were 3.3 mM and enzyme concentration was varied over a 1000-fold range. Among the *o*-dihydroxyphenyl compounds tested, 4-methylcatechol was oxygenated at almost the same initial rate as that of the physiological substrate, catechol. When catechol analogues were used as substrate, the reaction rate decreased with time in a manner different from simple first order kinetics. Therefore, the initial reaction rate was determined in a range in which the rate was reasonably proportional to the reaction time, usually within 10 sec. The following compounds were either not oxygenated or oxygenated at rates less than 0.01% of that for catechol: 3,4-dihydroxyphenylalanine, 3,4-dihydroxyphenylacetic acid, *o*-aminophenol, 3-hydroxyanthranilic acid, *o*-phenylenediamine, 4,5-dichlorocatechol, *p*-hydroquinone, resorcinol, phenol, salicylic acid, gentisic acid, *p*-hydroxybenzoic acid, homogentisic acid and guaiacol.

Spectrophotometric identification of the reaction product formed from 4-methylcatechol by metapyrocatechase

As shown in Fig. 3, 4-methylcatechol (A) was converted, using metapyrocatechase, to a product having an absorption maximum at 378 mμ (B). When α-aminomuconic ε-semialdehyde dehydrogenase which is equally active against α-hydroxymuconic ε-semialdehyde was added to the solution of the product, the peak at 378 mμ decreased with the simultaneous appearance of a new peak at 298 mμ (C) indicating the formation of a muconic acid derivative. This product showed absorption maxima at 349 (D) and 307 mμ (E) in strongly alkaline and acidic solution, respectively. Spectral characteristics of the muconic acid derivative thus formed were far from those of α-hydroxy-γ-methylmuconic acid (λ_{max}: 269 mμ at pH 1.4; 258 mμ at pH 7.1; 258 mμ at pH 12.5)* and resembled those of α-hydroxy-δ-methylmuconic acid (λ_{max}: 307 mμ at pH 1.4; 298 mμ at pH 7.2; 349 mμ at pH 12.4)*.

* These values were obtained experimentally with authentic samples.



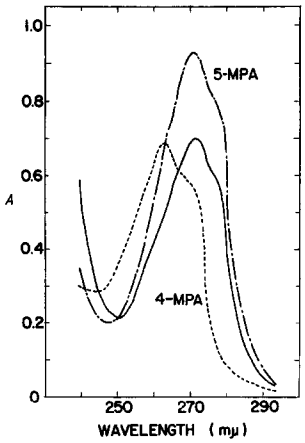


Fig. 3. Absorption spectra of reaction products from 4-methylcatechol. The reaction mixture contained in a 1-cm light-path quartz cuvette: 200 μmoles Tris–acetate buffer, pH 7.5; 0.1 μmole 4-methylcatechol; 0.1 μmole NAD^+; 5 μmoles pyruvate and 0.05 ml lactic dehydrogenase in a total volume of 2.7 ml. Reference cuvette contained all the above components except 4-methylcatechol. To both cuvettes, 10 μl of metapyrocatechase were added (about 50 μg). After completion of the reaction, 0.4 ml of α-aminomuconic ε-semialdehyde dehydrogenase was added. When the reaction was completed, the pH of the reaction mixture was brought to 13 with the dropwise addition of 5 M KOH and then to 1.0 with 10 M HCl. Incubation was at 24°. Curves A and B represent difference spectra of the reaction mixture before and after the addition of metapyrocatechase, respectively, and Curve C, after addition of the dehydrogenase. Curves D and E show the spectra of the final product at pH 13 and 1, respectively.

Fig. 4. Absorption spectra of picolinic acid derivative derived from ring fission product of 4-methylcatechol and reference compounds. The reaction mixture contained in a final volume of 3.0 ml: 150 μmoles potassium phosphate buffer (pH 7.5); 30 μmoles 4-methylcatechol; and 20 μg catalase. To the reaction mixture, using the magnetic stirrer, 10-μl volumes of metapyrocatechase solution (14 μg) were added stepwise until absorbance at 378 mμ, which was measured with an aliquot of the reaction mixture, reached a maximum value. The reaction product thus obtained was converted to picolinic acid derivatives as described under EXPERIMENTAL. All the spectra were measured in 1.0 M HCl with a 1-cm light-path quartz cuvette. Solid line represents the absorption spectrum of the final product, and dotted lines, those of authentic samples as indicated: 5-MPA, 5-methylpicolinic acid; 4-MPA, 4-methylpicolinic acid.

Absorption spectra of the pyridine derivative, produced from the product of 4-methylcatechol in the presence of NH_4^+, are shown in Fig. 4, together with those of authentic samples. The pyridine derivative of the 4-methyl-catechol product showed an absorption spectrum identical with that of an authentic sample of 5-methylpicolinic acid in 1.0 M HCl and in alkaline solution. This spectrum was distinguishable from that of 4-methylpicolinic acid. Identity of the product was also confirmed by paper chromatography with a solvent system of butanol–acetic acid–water (4:1:2, by vol.). R_F value of the product was 0.59, which was distinct from that of 4-methylpicolinic acid (0.52) and identical to that of 5-methylpicolinic acid. The product cochromatographed with 5-methylpicolinic acid but not with 4-methylpicolinic acid.

Stoichiometry of the reaction with 4-methylcatechol

Stoichiometry between 4-methylcatechol oxidized and O_2 consumed was determined polarographically with a limited amount of substrate. One mole of O_2

was found to be consumed per mole of 4-methylcatechol added. Stoichiometry between 4-methylcatechol consumed and product formed was also established with the experiment described in the legend for Fig. 4. 5-Methylpicolinic acid formed from 30 μmoles of homocatechol was calculated to be 28.17 μmoles and 30.15 μmoles when determined in 1.0 M HCl (λ_{max}: 272 mμ; $\varepsilon = 9350\ M^{-1} \cdot cm^{-1}$)* and in 1.0 M NaOH ($\lambda_{max}$: 270 m$\mu$; $\varepsilon = 5450\ M^{-1} \cdot cm^{-1}$)*, respectively. No 4-methylpicolinic acid, which was expected to be formed from 4-methylcatechol by its distal cleavage, was detected.

Identification of reaction product of 3-methylcatechol

Using metapyrocatechase 3-methylcatechol gave a product having an absorption maximum at 390 mμ. The product was not oxidized to its muconic acid derivative by α-aminomuconic ε-semialdehyde dehydrogenase in the presence of NAD^+. Upon addition of NH_4^+ to the product, it was converted to a pyridine derivative, having an absorption maximum at 275 mμ with a shoulder at 280 mμ in 1.0 M HCl and a maximum at 270 mμ with a shoulder at 278 mμ in 1.0 M NaOH. The methylpicolinic acid was further converted to a quinolinic acid derivative by oxidation with $KMnO_4$.

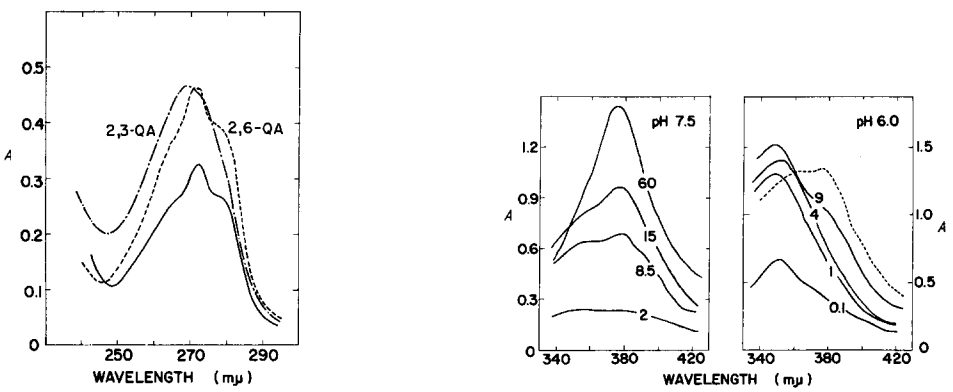


Fig. 5. Absorption spectra of quinolinic acid derivative derived from ring fission product of 3-methylcatechol and reference compounds. The reaction mixture contained in a final volume of 200 ml: 0.05 M potassium phosphate buffer (pH 7.5); 5 mM 3-methylcatechol; 400 μg catalase; and 1 drop of octanol. To the reaction mixture, stirred with magnetic stirrer, 10-μl volumes of metapyrocatechase solution (140 μg) were added stepwise until absorbance at 390 mμ reached a maximum value. 400 ml of glacial acetic acid saturated with ammonium acetate were then added to the reaction mixture. After standing overnight, the picolinic acid derivative formed was further purified on a Dowex 50 column and converted to a quinolinic acid derivative by $KMnO_4$ oxidation according to the method of ADACHI *et al.*[4]. Solid line represents the absorption spectrum of the final product and dotted lines those of authentic samples as indicated: 2,3-QA, 2,3-quinolinic acid; 2,6-QA, 2,6-quinolinic acid.

Fig. 6. Absorption spectra of reaction mixture during the oxygenation of protocatechuic acid. The reaction mixture contained in a final volume of 6.0 ml: 300 μmoles potassium phosphate buffer (pH 7.5 or 6.0 as indicated); 0.4 μmole protocatechuic acid; and about 700 μg metapyrocatechase. The reactions were carried out with a special cuvette (see the legend for Fig. 7) and started by the addition of protocatechuic acid at 24°. Numbers in the figure represent the reaction time in min. Dotted curve in right figure shows the spectrum measured immediately after the addition of 0.1 ml of 1.0 M NaOH to the reaction mixture at 12 min. The pH was about 8.0.

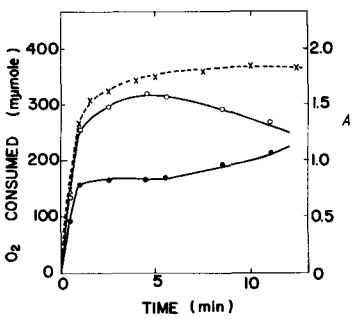
* These values were obtained experimentally with authentic samples.

The quinolinic acid showed an absorption peak at 272 mμ and shoulders at 265 and 278 mμ and was indistinguishable from that of an authentic sample of 2,6-quinolinic acid (Fig. 5).

Identification of the reaction product of protocatechuic acid

The reaction product following the action of metapyrocatechase on protocatechuic acid showed two peaks, one at 350 mμ and the other at 375 mμ, the former peak appearing earlier. The relative rates of increase in absorbance were influenced by pH, the formation of the peak at 375 mμ being markedly retarded at lower pH values (Fig. 6). A plot of pH *vs.* rate of O_2 uptake revealed a sharp peak with an optimum at pH 6.0. The rate at pH 7.5 was about one-tenth the maximum rate.

When O_2 uptake and the appearance of each peak were measured simultaneously at pH 6.0, the rate of increase in absorbance at 350 mμ was almost parallel



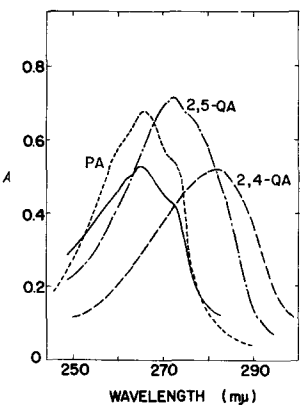


Fig. 7. Rates of O_2 uptake and increase in absorbance during the oxygenation reaction of protocatechuic acid. O_2 uptake and appearance of absorbance at 350 and 375 mμ were simultaneously measured by a special cuvette of 1-cm light path, to which was connected a rotating O_2 electrode to measure O_2 uptake polarographically. The reaction was carried out at pH 6.0 with the same reaction system as described for Fig. 6. O_2 consumed, ×——×; $A_{350\ m\mu}$, ○—○; $A_{375\ m\mu}$, ●—●.

Fig. 8. Absorption spectra of pyridine derivative derived from ring fission product of protocatechuic acid and reference compounds. The reaction mixture contained in a final volume of 3.0 ml: 150 μmoles potassium phosphate buffer (pH 7.0); 10 μmoles protocatechuic acid; about 200 μg catalase; and a total 560 μg metapyrocatechase, which were added stepwise. The reaction was carried out at 30° with Warburg manometer vessels. When O_2 uptake ceased, the product formed was converted to a picolinic acid derivative as described under EXPERIMENTAL. Solid line represent the absorption spectrum of the final reaction product in 0.1 M HCl and dotted lines those of authentic samples as indicated. PA, picolinic acid; 2,5-QA, 2,5-quinolinic acid; 2,4-QA, 2,4-quinolinic acid.

to that of the O_2 uptake in the initial stage of the reaction. In the later stages of the reaction, the peak at 375 mμ developed slowly with concurrent decrease in the peak at 350 mμ without consumption of O_2 (Fig. 7). These results suggest that the compound having a peak at 350 mμ is a primary oxygenated product of protocatechuic acid and is converted to the other at 375 mμ by a secondary reaction.

When NH_4^+ was added to the product solution formed in a range of pH from

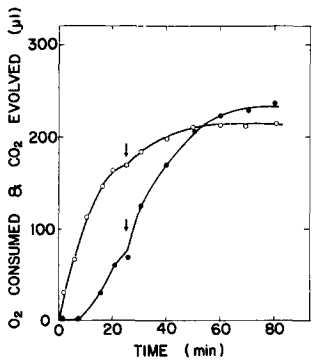


Fig. 9. Time-course of O_2 uptake and CO_2 evolution during the reaction with protocatechuic acid. The reaction was carried out at 30° in Warburg manometer vessels with 2 sidearms. The reaction mixture contained: 150 μmoles potassium phosphate buffer (pH 7.0); 10 μmoles protocatechuic acid; and 200 μg catalase in a main chamber; and 280 μg of metapyrocatechase each in 2 sidearms. In one vessel 0.2 ml of 20% KOH was placed in the center well, while in an other it was not. Total volume was 3.0 ml. The reaction was started by the addition of the enzyme from one sidearm and the enzyme in the other sidearm was tipped in at the time indicated by the arrow. CO_2 evolved was determined from the difference between the reaction system without KOH and the one with KOH. ○—○, O_2 consumed, ●—●, CO_2 evolved.

7.5 to 6.0, the product was converted to a pyridine derivative, having an absorption peak at 265 mμ with two shoulders at 260 and 272 mμ. The spectrum of this pyridine compound was indistinguishable from that of picolinic acid (Fig. 8).

Stoichiometry of the reaction

In order to explore the possibility that the primary product having an absorption peak at 350 mμ is decarboxylated probably nonenzymatically to form α-hydroxymuconic ε-semialdehyde, CO_2 evolution during the reaction was measured with a Warburg manometer. As shown in Fig. 9, O_2 uptake occurred right after the reaction started whereas the rate of CO_2 evolution showed a lag period at the beginning of

TABLE II

STOICHIOMETRY OF THE REACTION WITH PROTOCATECHUIC ACID

The stoichiometry of the reaction was determined from the experiment described in the legend for Fig. 9. After incubation for 80 min, the reaction product formed was converted to picolinic acid as described under EXPERIMENTAL. The amount of picolinic acid thus formed was determined spectrophotometrically using the molar extinction coefficient described in the text. O_2 consumed and CO_2 evolved were determined as described in the legend for Fig. 9. Numbers in the table are expressed in μmoles.

Protocatechuic acid added	*O_2 consumed*	*CO_2 evolved*	*Picolinic acid formed*
10	7.9	9.3	7.9* 9.8**

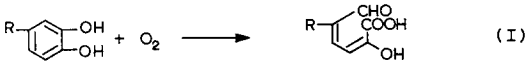
* The value determined in acidic pH.
** The value determined in alkaline pH.

the reaction. CO_2 evolution was much faster at higher pH and depressed at low pH. The stoichiometry between O_2 consumed, CO_2 evolved and the product formed, measured as picolinic acid (λ_{max}: 265 mμ; $\varepsilon = 8100\ M^{-1}\cdot cm^{-1}$ in 1.0 M HCl, $\varepsilon = 3700\ M^{-1}\cdot cm^{-1}$ in 1.0 M NaOH)*, at the end of the reaction was established to be approximately 1:1:1 (Table II).

DISCUSSION

Several dioxygenases, including metapyrocatechase, have recently been obtained in crystalline forms[5,12–14], and the availability of a large quantity of pure enzyme has facilitated the precise determination of their substrate specificity. Although action of dioxygenases has been believed to be highly specific, metapyrocatechase showed rather wide substrate specificity. The enzyme acted on various *o*-dihydroxyphenyl compounds, and all the compounds serving as substrates could also act as competitive inhibitors for catechol oxygenation, suggesting that these substrate analogues combine with the enzyme at the same site as that for physiological substrate. However, since none of *m*- or *p*-dihydroxyphenyl compounds, monohydroxyphenyl compounds or guaiacol served as substrates for the enzyme, *o*-dihydroxyphenyl structure seemed to be an essential requirement.

SENOH *et al.*[15] reported that 3,4-dihydroxyphenylacetate 2,3-oxygenase cleaves the C–C bond between the 2 and 3 position of 3,4-dihydroxyphenylacetate and has fairly broad substrate specificity for the 4-substituted catechol derivatives. The data presented in this paper revealed that metapyrocatechase also cleaves the C–C bond between the 2 and 3 position of 4-substituted catechol derivatives to form α-hydroxy-δ-substituted muconic ε-semialdehyde (Eqn. I). Recently, 3- and 4-methylcatechol were reported to be metabolized *via* ring fission products formed with extradiol, proximal cleavage by *Pseudomonas desmolyticum*[16] and by crude extracts of a fluorescent Pseudomonas[17].



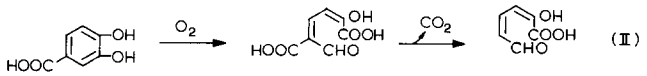
(I)

The reaction product of 4-methylcatechol by the action of metapyrocatechase was converted to either α-hydroxy-δ-methylmuconic acid in the presence of α-amino-muconic ε-semialdehyde dehydrogenase and NAD^+, or 5-methylpicolinic acid in the presence of NH_4^+. Both compounds were expected to be formed from the ring fission product of 4-methylcatechol by proximal cleavage (see Fig. 2). Neither α-hydroxy-γ-muconic acid nor 4-methylpicolinic acid, which were expected to be formed by the distal cleavage, were detected during the reaction. These results, together with the stoichiometry presented in RESULTS, indicate that 4-methylcatechol was exclusively cleaved at the proximal site to form α-hydroxy-δ-methylmuconic ε-semialdehyde which is consistent with previous reports[17,18].

The reaction of metapyrocatechase with protocatechuic acid was more complex in that during the reaction a sequence of peaks of ultraviolet absorption appeared, first at 350 mμ, and second at 375 mμ. These peaks were different from those of the

* These values were obtained experimentally with authentic samples.

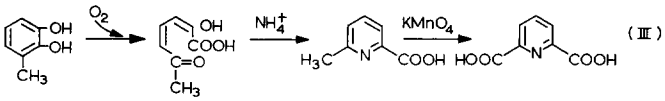
reported ring fission products of protocatechuic acid[2,19]. It is plausible to assume that the primary oxygenated product is α-hydroxy-δ-carboxymuconic ε-semialdehyde. With simultaneous decarboxylation the compound was then converted to the secondary compound having an absorption at 375 mμ and was identified as α-hydroxymuconic ε-semialdehyde (Eqn. II).



(II)

An attempt at positive identification of the intermediate was made by carrying out a reaction at pH 6.0 and stopping it before the development of the second peak at 375 mμ by the addition of glacial acetic acid saturated with ammonium acetate. However, all the product formed was identified as picolinic acid, probably due to the nonenzymic decarboxylation of the product during the ring formation.

Since the authentic samples of the methylpicolinic acids expected to be formed from the ring fission product of 3-methylcatechol were not available, the methylpicolinic acid formed was further converted to a quinolinic acid derivative by oxidation with $KMnO_4$. The acid formed was identified as 2,6-quinolinic acid. Although the stoichiometry of the reaction was not established, quinolinic acid derivatives other than 2,6-quinolinic acid were not detected, suggesting that 3-methylcatechol was exclusively cleaved at the bond between the carbon atom bearing an hydroxyl group and the adjacent carbon-carrying methyl group (Eqn. III). This result is consistent with a previous report that the ring fission product of 3-methylcatechol by *Pseudomonas desmolyticum* was isolated and then identified as 2-hydroxy-6-oxo-*trans*-4,*trans*-haptadienoic acid[16].



(III)

It should be noted that metapyrocatechase was easily inactivated during catalysis especially when substrate analogues were used as substrates. This was probably due to the removal of the functional iron in the enzyme as proposed for the similar inactivation of protocatechuate 4,5-dioxygenase[20]. The detailed kinetic analyses of this inactivation are now being undertaken in our laboratory.

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