Characteristics of Catechol Oxygenase from Brevibacterium fuscum

By HACHIRO NAKAGAWA, HIDEO INOUE and YOSHIRO TAKEDA*

(From the Department of Physiological Chemistry, Medical School, and the Department of Biochemistry, Dental School, Osaka University, Osaka)

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It has been reported by Suda, Hayashi and Oda (1, 2) and by Stanier (3) that anthranilate and benzoate could be metabolized through catechol in bacteria. An enzyme, which catalyzes the further degradation of catechol to cis,cis-muconic acid, was obtained in a cell-free state and partially purified from anthranilate-adapted cells of a pseudomonad (3—8). This enzyme was named pyrocatechase originally (2) and is now classified as catechol oxygenase [EC 1.99.2.2].

Further investigations on the characteristics of pyrocatechase revealed that this enzyme requires ferrous ion as the sole cofactor (9—11) and that the two atoms of atmospheric oxygen are directly incorporated into the double bond between the two hydroxyl groups during the reaction (12).

On the other hand, it has been shown that various microorganisms could form pyrocatechase inducibly when grown on one of the metabolic precursors of catechol such as phenol (3, 7, 13, 14), benzoate (3, 7, 10, 14), anthranilate (2, 5) or tryptophan (2, 5, 6) and several reports on the purification and properties of pyrocatechases from different sources have appeared recently (10, 12). However the differences between these pyrocatechases have not yet been established. For example, it is not known whether the enzymes derived from different microorganisms show the same pattern of substrate specificity.

Recently we have isolated phenol hydroxylase from Brevibacterium fuscum (15). This enzyme hydroxylates phenol to form catechol in the presence of NADPH. The preparation was still contaminated with highly active pyrocatechase and therefore the reaction proceeded beyond the cleavage of the benzene ring of phenol. In addition, the preparation catalyzes the oxidation of various phenol derivatives besides phenol, thus leading to the rupture of the benzene ring of these substrates. These facts suggest that the pyrocatechase present in this preparation has broader substrate specificity than those previously reported for other pyrocatechases (10, 12). This stimulated us to study the characteristics of our enzyme and to compare it with pyrocatechases from other bacterial sources. To avoid confusion our enzyme will be tentatively designated as Brevibacterium pyrocatechase.

The present communication describes the purification and characteristics of Brevibacterium pyrocatechase and the identification of the reaction products formed from various substrates.

EXPERIMENTAL PROCEDURE

Biological Materials—Brevibacterium fuscum, which was used throughout the present experiments unless otherwise noted, was isolated from soil and was capable of growing in a synthetic medium containing phenol as the sole carbon source.

This bacterium was subcultured for 16 hours at 30°C with vigorous shaking in the following medium (in grams): phenol 1 (or sodium benzoate 3), (NH₄)₂SO₄ 0.5, KH₂PO₄ 2, Na₂HPO₄·7H₂O 3, NaCl 2.5, MgCl₂·7·H₂O 0.2, dried yeast extract (Oriental Yeast Co.) 0.5 and 1 liter of tap water. The pH was adjusted to 7.2 with KOH. After subculture, the cells were transferred to a jar-fermenter containing 20 liters of fresh medium of the same composition and grown with vigorous aeration. After 16 hours, the cells were harvested by centrifugation and stored at —20°C until needed.

* Address requests for reprints to the Department of Biochemistry, Dental School, Osaka University, Osaka, Japan.
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Pseudomonas fluorescens A-12, Pseudomonas aeruginosa B-23 and Micrococcus ureas Et were obtained by cultivation on a medium similar to the above, except that the carbon and nitrogen sources were replaced by 3 g. per liter of anthranilic acid. Crystalline catalase [EC 1.11.1.6, H$_2$O$_2$: H$_2$O, oxidoreductase] from beef liver was kindly supplied by Dr. K. Okunuki of this University.

Chemical Materials—3-Methylcatechol was prepared from 2-vanillin (16). 4-Methylcatechol was synthesized from vanillin by a similar method. 3- and 4-nitrocatechols were obtained by nitration of catechol (17). 3- and 4-aminocatechols were synthesized from the corresponding nitrocatechols by reduction with tin and hydrochloric acid. 2-Hydroxyhexanone was synthesized from cyclohexanone (18, 19), acetoin from diacetyl (20) and $\alpha$-hydroxymuconic acid from the diethyl esters of crotonic and oxalic acids (21). $\alpha$-Pyrone 6-carboxylic acid was kindly provided by Dr. S. Senoh of the Institute of Food Chemistry, Osaka and trans-1,2-hexane diol by Sumitomo Chemical Co. Other chemical compounds were purchased commercially.

Assay Methods—The activity of pyrocatechase was followed by measuring the oxygen consumption in a conventional Warburg apparatus at 30°C. The reaction mixture contained (in /moles): substrate 10, conventional Warburg apparatus at 30°C. The reaction system contained (in /moles): substrate 10, ferrous sulfate 1, Tris buffer (pH 7.5) 120, substrate at various concentration and the purified enzyme (6.83 g. of protein) in a total volume of 3 ml. After 10 minute equilibration, the reaction was started by tipping the substrate into the main compartment from the side arm. One unit is defined as the amount of enzyme catalyzing the uptake of 1/1 of oxygen per minute.

The determination of Km's with various substrates was carried out in a Beckman model DU spectrophotometer by following the increase in optical density at 260 mp for catechol, 3-methylcatechol and 4-methylcatechol, and at 300 mp for pyrogallol. The increase in optical density represents the formation of the corresponding muconic acid from each substrate.

The reaction system contained (in /moles): glutathione 6, Tris buffer (pH 7.5) 120, substrate at various concentration and the purified enzyme (6.83 g. of protein) in a total volume of 3 ml. A difficulty in these experiments was that the purified enzyme showed very low activity without the addition of ferrous ion to the reaction mixture and that addition of ferrous ion caused the formation of a colored catechol complex, thus interfering with the spectrophotometric analyses. To overcome this difficulty, the enzyme preparation was dialyzed against 5 x 10$^{-4}$ M ferrous sulfate for 1 hour and then against distilled water for 2 hours. After this treatment the enzyme retained fairly high activity without the addition of ferrous ion; approximately two-thirds of that obtained on addition of ferrous ion.

Catechols were determined by the method of Arnow (22). Protein was measured by the method of Lowry et al. (23). Ultracentrifugal analysis of the enzyme was carried out with a Spinco model E ultracentrifuge by Dr. K. Fukai of the Research Institute for Microbial Diseases of this University.

RESULTS AND DISCUSSION

Purification of Brevibacterium Pyrocatechase

Step I, Crude Extract—Ten grams (wet weight) of cells were suspended in 90 ml. of 0.05 M Tris buffer, pH 7.5, and disrupted by sonic vibration in a 10-kc oscillator for 15 minutes with the aid of 2 g. of alumina. The cellular debris was removed by centrifugation at 24,500 x g for 20 minutes. A crude extract was also prepared by aqueous extraction of acetone-dried cells.

All subsequent operations were carried out in the cold and centrifugation was at 13,200 x g unless otherwise specified.

Step II, Protamine Treatment—One-tenth volume of 2 per cent protamine sulfate solution, pH 6.7, was added dropwise to the crude extract, previously adjusted to pH 6.7 with acetate buffer, pH 4.0. The mixture was allowed to stand for 20 minutes with stirring. It was then centrifuged and the precipitate discarded.

Step III, First Ammonium Sulfate Fractionation—The protamine supernatant was adjusted to pH 7.5 with KOH and diluted with 0.02 M Tris buffer, pH 7.5, so as to give 15 mg. of protein per ml. Powdered ammonium sulfate was added to the supernatant to 50 per cent saturation, and the pH was adjusted to 7.5 with 10 per cent ammonium hydroxide solution. After 30 minutes' stirring, the solution was centrifuged, and additional ammonium sulfate was added to the supernatant to 75 per cent saturation. The precipitate thus formed was dissolved in a half volume of 0.05 M Tris buffer, pH 7.5.

Step IV, Calcium Phosphate Gel Adsorption—Step III enzyme was dialyzed against approximately 50 volumes of distilled water for 16
hours, and the outer water was changed twice during the dialysis period. Dialyzed enzyme was adjusted to pH 6.7 with 0.2 M acetate buffer, pH 4.0, and then mixed with calcium phosphate gel (1.5 mg. of gel per mg. of protein). After 15 minutes' stirring, the mixture was centrifuged and the supernatant was discarded. The precipitate was washed once with ice-cold distilled water and then eluted twice with half the original volume of 0.05 M potassium phosphate buffer, pH 7.2.

**Step V, C1 Alumina Gel Treatment**—The eluate was dialyzed against 50 volumes of distilled water for 3 hours with two changes of water and the dialyzed enzyme was adjusted to pH 6.7 with 0.2 M acetate buffer, pH 4.0. This fraction was applied to a column of C1 alumina gel (equivalent to approximately half the amount of protein) containing an appropriate amount of cellulose powder (Whatman No. 1, equivalent to 5 to 7-fold the weight of protein). The column was washed with 100 ml. of 0.005 M potassium phosphate buffer, pH 7.2, and then eluted with the same buffer at a concentration of 0.02 M.

**Step VI, Second Ammonium Sulfate Fractionation**—The eluate was fractionated with saturated ammonium sulfate solution at pH 7.2. The fraction precipitating between 62 and 68 per cent saturation was dissolved in a small volume of 0.005 M potassium phosphate buffer, pH 7.2, and dialyzed against the same buffer for 2 hours.

**Step VII, DEAE-Cellulose Column Chromatography**—Step VI enzyme was passed through a DEAE-cellulose column containing 1 g. of DEAE-cellulose per 200 mg. of protein, which had been equilibrated with 0.02 M potassium phosphate buffer, pH 7.2. Elution was effected by a continuous gradient of potassium chloride in 0.005 M potassium phosphate buffer at pH 7.5, with initial concentrations of 0.1 M in the mixing chamber (150 ml.) and M in the reservoir (150 ml.). The flow rate was maintained at 1 ml. per minute, and the eluate was collected in 4 ml. fractions. Active fractions were pooled, concentrated by lyophilization, and dialyzed overnight against 0.05 M KCl with two changes of the external solution. By this purification procedure, the specific activity of the enzyme was increased to approximately 40-fold that of the crude extract (Table I). The purified enzyme was completely free of muconate cycloisomerase [EC 5.5.1.1, 4-carboxy-...
methyl-4-hydroxyisocrotonolactone lyase (de-cyclizing) and had an absorbancy ratio at 280 m/ to 260 m/ of 1.71.

Properties of Purified Brevibacterium Pyrocatechase

Ultracentrifugal Analysis — The ultracentrifugal pattern of the purified enzyme exhibited only one sharp and symmetrical boundary, indicating the homogeneity of the preparation (Fig. 1). The sedimentation constant was found to be 4.36 Svedberg units and the diffusion coefficient 5.47 x 10^-7 cm^2 per second. Assuming the partial specific volume to be 0.75, the molecular weight of this pyrocatechase was calculated as about 78,000. This value is similar to those of pyrocatechases from other bacterial strains (10, 12).

Substrate Specificity — Table II shows a comparison of the substrate specificities of Brevibacterium pyrocatechase and pyrocatechases from other bacterial strains. The most striking difference between them is in the oxidation of 3-methylcatechol and pyrogallol. Brevibacterium pyrocatechase oxidized these catechols at nearly the same rate as catechol, while other pyrocatechases do not attack these substrates or, if at all, only slightly. On the other hand, none of the pyrocatechases tested are active with 3- and 4-aminocatechols, 3- and 4-nitrocatechols, guaiacol, protocatechuic acid, 2, 3, 4-trihydroxybenzoic acid, 3, 4-dihydroxyphenylacetic acid, 3, 4-dihydroxyphenyl-ethylamine, trans-1, 2-hexane diol, 2-hydroxyhexanone or acetoin.

It should be mentioned that Brevibacterium pyrocatechase, derived from cells grown on a different precursor of catechol, such as benzoate, shows exactly the same substrate specificity as when grown on phenol. This means that the inducibly formed enzyme in Brevibacterium fuscum is identical regardless of the carbon source used.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Enzyme source</th>
<th>Substrate</th>
<th>3-methyl-&lt;br&gt;catechol</th>
<th>4-methyl-&lt;br&gt;catechol</th>
<th>Pyrogallol</th>
<th>( \mu l ) of oxygen uptake/10 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Brevibacterium fuscum grown on phenol</td>
<td>Catechol: 35.8(100)</td>
<td>39.6(111)</td>
<td>35.0(97.8)</td>
<td>35.8(100)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Brevibacterium fuscum grown on benzoate</td>
<td>Catechol: 55.5(100)</td>
<td>65.8(121)</td>
<td>57.5(103)</td>
<td>4.2(7.7)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Pseudomonas fluorescens A3-12</td>
<td>Catechol: 53.5(100)</td>
<td>6.9(12.9)</td>
<td>16.6(35)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Pseudomonas aeruginosa B-23</td>
<td>Catechol: 58.6(100)</td>
<td>4.0(6.8)</td>
<td>30.4(51.8)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Micrococcus urea Et</td>
<td>Catechol: 45.4(100)</td>
<td>2.9(6.4)</td>
<td>21.9(48.9)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

The reaction system contained 5 \( \mu moles \) of substrate, 1 \( \mu mole \) of ferrous sulfate, 5 \( \mu moles \) of ascorbate, 100 \( \mu moles \) of Tris buffer, pH 7.5, and sonic extract (320 \( \mu g. \) of protein in Expt. 1, 590 \( \mu g. \) in Expt. 2, 765 \( \mu g. \) in Expt. 3, 940 \( \mu g. \) in Expt. 4, and 1060 \( \mu g. \) in Expt. 5) in a total volume of 2 ml. The figures in parentheses indicate the percentage of the activity with catechol in each preparation.
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TABLE III

Effect of Ferrous Ion and Reducing Agents on Catechol Oxidation by Brevisbacterium Pyrocacthase

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Ferrous ion concentration</th>
<th>Reducing agents</th>
<th>Activity</th>
<th>Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X \times 10^{-7}M</td>
<td></td>
<td>µl of O₂ uptake/10 min.</td>
<td>%</td>
</tr>
<tr>
<td>I</td>
<td>—</td>
<td>—</td>
<td>14.5</td>
<td>+5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Glutathione</td>
<td>15.3</td>
<td>+5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Glutathione</td>
<td>15.3</td>
<td>+5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Glutathione</td>
<td>49.6</td>
<td>+342</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Glutathione</td>
<td>11.6</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>5</td>
<td>Glutathione</td>
<td>43.2</td>
<td>+370</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>L-Cysteine</td>
<td>52.1</td>
<td>+450</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Ascorbic acid</td>
<td>36.2</td>
<td>+520</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>38.1</td>
<td>+330</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>45.5</td>
<td>+390</td>
</tr>
</tbody>
</table>

Step VII enzyme was used (10.7 µg of protein per vessel).

Cofactor Requirements—As shown in Table III, the purified enzyme shows maximal activity only in the presence of both ferrous ion and reducing agents. In this high ferrous ion-dependence, it differs from other pyrocatechases reported previously, which require no ferrous ion (12), or require it only after special treatments such as exhaustive dialysis against α-phenanthroline (10). Other metallic ions tested, such as Cu⁺, Cu²⁺, Mn⁴⁺, Mg²⁺, Ni²⁺, Zn²⁺, Ca²⁺ and Cd²⁺, did not affect the reaction rate.

In the case of pyrogallol oxidation, a considerable activation by ferrous ion and reducing agents is observed even in the crude extract, though in the crude extract the combination of these two agents has no effect in the oxidation of other catechols. As shown in Fig. 2, the rate of oxidation of pyrogallol decreases with time in the presence of either ferrous ion or reducing agents, whereas in the presence of both agents the reaction is strikingly enhanced and proceeds linearly during the experimental period. The decrease in the rate of oxidation is not due to either the formation of hydrogen peroxide by autooxidation of pyrogallol or to the accumulation of the oxidation product of pyrogallol, α-hydroxymuconic acid, because the addition of catalase and α-hydroxymuconic acid to the reaction mixture completely prevented its autooxidation.

Fig. 2. The effect of ferrous ion and reducing agent on pyrogallol oxidation. The reaction mixture contained 10 µmoles pyrogallol (or catechol in curve H), 2 µmoles ferrous sulfate, 5 µmoles ascorbate, 100 µmoles Tris buffer (pH 7.5) and enzyme (crude extract, 2.43 µg of protein) in a total volume of 2 ml. All incubations were carried out at 30°C under air. Curve A, complete system; curve B, ferrous ion omitted; curve C, ascorbate omitted; curve D, both ferrous ion and ascorbate omitted; curve E, both ferrous ion and ascorbate omitted and 10 minutes after ferrous ion added (indicated by arrow); curves F and G, autooxidation of pyrogallol (without enzyme) in the presence or absence of ferrous ion, respectively; curve H, catechol instead of pyrogallol as substrate.
acid (1 x 10^{-4} M) does not affect the reaction rate. In addition, this phenomenon cannot be ascribed to the inactivation of the enzyme during incubation, because the addition of ferrous ion when the rate has decreased, reactivates the enzyme (Fig. 2, curve E). Therefore it is probable that benzoquinone formed by autooxidation of pyrogallol strongly inhibits the reaction by oxidizing ferrous ion, an essential component of the enzyme, to ferric ion and that the added reducing agent reduces the benzoquinone or the ferric ion, or both, thus releasing the inhibition.

Other Properties—The pH activity curve of Brevibacterium pyrocatechase is given in Fig. 3. The optimal pH range is wide, extending from 6.8 to 9.0.

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**Fig. 3.** Activity at various pH values. Step VI enzyme (18.6 μg. of protein) was used. A, Tris buffer; B, potassium phosphate buffer (100 μmoles per vessel). The activities are expressed as μl. of oxygen uptake per 10 minutes. For other conditions see text.

Brevibacterium pyrocatechase is not affected by SH-inhibitors, such as p-chloromercuribenzoate or mercuric chloride, which strongly inhibit other pyrocatechases.

Brevibacterium pyrocatechase is strikingly different in stability from other pyrocatechases. The purified enzyme can be kept for at least one week at pH 5.5—9.5 in a refrigerator without loss of activity. Furthermore the purified enzyme can be lyophilized and the lyophilized preparation can be stored for over one month at room temperature in the presence or absence of ferrous ion without loss of activity.

**Isolation and Identification of Reaction Products**

As mentioned before, Brevibacterium pyrocatechase differs in substrate specificity from other known pyrocatechases. Therefore it is necessary to check whether the mode of action of this enzyme is similar to that of other pyrocatechases. Experiments were conducted to isolate and identify the reaction products of catechol and its derivatives.

The reaction system contained: ferrous sulfate 20 μmoles, ascorbate 100 μmoles, Tris buffer (pH 7.5) 2 mmoles, substrate 200 mg., enzyme (step VII enzyme, 3 mg. of protein) in a total volume of 50 mL. Incubations were continued at 30°C until the color reaction of catechols with ferric chloride had almost disappeared.

After incubation, the mixture was deproteinized by the addition of perchloric acid at a final concentration of 3 per cent and centrifuged. The precipitate was washed twice with a small volume of 90 per cent ethanol and the combined supernatant was continuously extracted with ether. The ether extract was evaporated to dryness under reduced pressure and the residue was dissolved in a small volume of 10 per cent sodium bicarbonate solution, and then treated with charcoal. Crystals were formed on standing the solution in a refrigerator after acidifying with HCl and were collected by filtration and recrystallized from 60 per cent ethanol. Thus, 120 mg., 95 mg., and 85 mg. of crystalline reaction products were obtained with catechol and 3- and 4-methylcatechols respectively as substrates.

The characteristics of the oxidation product from catechol are as follows: Absorption spectrum, λ_{max}^{7.0} 258 (log ε=4.15); melting point, 183°C (uncorrected). These figures accord well with those of authentic cis,cis-muconic acid (5, 24—26). Therefore it is concluded that the reaction mechanism of Brevibacterium pyrocatechase is the same as that reported previously for other pyrocatechases,
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involving attack on the double bond between the two hydroxyl groups of catechol, and differs from that of \( m \)-pyrocatechase (27, 28).

The absorption spectrum of the reaction product from 3-methylcatechol is given in Fig. 4-I. The absorption maxima and molar extinction coefficients at various pH's are as follows: \( \lambda_{\text{max}}^{\text{HCl}} \) 271, \( \lambda_{\text{max}}^{\text{HCl}} \) 266 (log \( \varepsilon \) = 4.17), \( \lambda_{\text{max}}^{\text{KOH}} \) 266 (log \( \varepsilon \) = 4.17). However, immediately after completion of the reaction, the absorption maximum of the reaction mixture is at \( 262 \text{ m\( \mu \)} \) at pH 7.0. This shift of absorption maximum may be explained by assuming that isomerization of the product occurs during its isolation. The crystalline product from 3-methylcatechol melts at 279°C.

Calculated for \( \text{C}_{11} \text{H}_{10} \text{O}_{8} \): C 53.84%, H 5.16%

Found: C 54.03%, H 5.26%

The chemical analysis of the product accords well with that of methylmuconic acid. From these results and by analogy with catechol oxidation, it is probable that the actual reaction product from 3-methylcatechol is \( \text{cis}, \text{cis} \)-\( \alpha \)-methylmuconic acid.

![Graph](image1.png)

**Fig. 4.** The absorption spectra of the oxidation products of 3-methylcatechol (I) and 4-methyl catechol (II). The concentration of the product was in each case \( 2.5 \times 10^{-2} \text{ M} \). Curve A, in \( 0.02 \text{ M} \) potassium phosphate buffer (pH 7.0); curve B, in \( \text{N KOH} \); curve C, after standing for 30 minutes in \( \text{N HCl} \); curve D, after standing for 60 minutes in \( \text{N HCl} \).

The absorption spectra of the oxidation product from 4-methyl catechol are as follows: \( \lambda_{\text{max}}^{\text{HCl}} \) 267, \( \lambda_{\text{max}}^{\text{HCl}} \) 263 (log \( \varepsilon \) = 4.16), \( \lambda_{\text{max}}^{\text{KOH}} \) 263 (log \( \varepsilon \) = 4.16) (Fig. 4-II). The melting point of the product is 235°C (uncorrected). The chemical analysis of this compound (Found: C 53.74%, H 5.05%) is consistent with that of methylmuconic acid. For the same reason as in the case of 3-methyl catechol, it is likely that the actual oxidation product from 4-methyl catechol is \( \text{cis}, \text{cis} \)-\( \beta \)-methylmuconic acid.

It should be noted that the absorption maxima of the oxidation products from 3- and 4-methyl catechols, \( \text{cis}, \text{cis} \) and \( \text{cis}, \text{trans} \)-methylmuconic acids, decrease with time in \( \text{N HCl} \) and this makes it impossible to calculate their molar extinction coefficients at an acidic pH. In addition, the rate of decrease in absorbancy is greater with the latter than with the former. These phenomena may be explained by assuming that the formation of muconolactone from the corresponding muconic acid occurs on acidification. These suggest that the geometric structure of the muconic acids formed enzymatically is the \( \text{cis}, \text{cis} \) or \( \text{cis}, \text{trans} \)-form, because \( \text{trans}, \text{trans} \)-muconic acid fails to form a lactone (24).

The oxidation product of pyrogallol was obtained in almost the same way, except that the incubation time was prolonged to 5 hours and crude extract was used instead of purified enzyme, because the reaction rate with the crude preparation decreased less with time during pyrogallol oxidation. Twenty three mg. of the crystalline product were obtained.

![Graph](image2.png)

**Fig. 5.** The absorption spectra of the oxidation product of pyrogallol. The concentration of the product was \( 2.5 \times 10^{-4} \text{ M} \). Curve A, immediately after dissolving in \( \text{N HCl} \); curve B, after standing for 60 minutes in \( \text{N HCl} \); curve C, in \( \text{N NaOH} \).
TABLE IV
Identification of Oxidation Product of Pyrogallol

<table>
<thead>
<tr>
<th>Reaction product</th>
<th>α-Hydroxymuconic acid</th>
<th>α-Pyron-6-carboxylic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Chemical analysis</td>
<td>Found:</td>
<td>Calculated (C₇H₇O₄):</td>
</tr>
<tr>
<td></td>
<td>C 45.72%</td>
<td>C 45.58%</td>
</tr>
<tr>
<td></td>
<td>H 4.06%</td>
<td>H 3.83%</td>
</tr>
<tr>
<td>2. λ&lt;sub&gt;max&lt;/sub&gt;&lt;sup&gt;296&lt;/sup&gt;</td>
<td>296 μμ</td>
<td>296 μμ</td>
</tr>
<tr>
<td>3. Ferric chloride reaction</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4. R&lt;sub&gt;f&lt;/sub&gt; values on paper chromatograms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solvent A</td>
<td>0.80</td>
<td>0.79</td>
</tr>
<tr>
<td>Solvent B</td>
<td>0.41</td>
<td>0.41</td>
</tr>
<tr>
<td>Solvent C</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>


from 200 mg of the substrate.

As in the identification of the oxidation product of pyrogallol, attention must be paid to two possible compounds, α-hydroxymuconic acid and α-pyron-6-carboxylic acid. The absorption spectrum of the product is λ<sub>max</sub><sup>310</sup> 329 (log ε = 4.16) (Fig. 5). It completely coincides with that of authentic α-hydroxymuconic acid at an alkaline pH, but at acidic or neutral pH values results are variable, because the spectrum of the compound gradually changes with time. The latter phenomenon is also true for authentic α-hydroxymuconic acid. Furthermore, other properties of the product, such as its paper chromatographic patterns, reaction with ferric chloride and chemical analysis, are consistent with those of α-hydroxymuconic acid, but not with those of α-pyron-6-carboxylic acid (Table IV). From these results, it is concluded that the oxidation product from pyrogallol is α-hydroxymuconic acid rather than α-pyron-6-carboxylic acid, although the steric configuration of the product has not yet been determined.

TABLE V
Stoichiometry

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Substrate added</th>
<th>Substrate disappeared</th>
<th>Oxygen consumed</th>
<th>Muconic acid formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmole</td>
<td>μmole</td>
<td>μmole</td>
<td>μmole</td>
</tr>
<tr>
<td>Catechol</td>
<td>10</td>
<td>10</td>
<td>10.62</td>
<td>9.31</td>
</tr>
<tr>
<td>3-Methylocatechol</td>
<td>10</td>
<td>9.98</td>
<td>10.18</td>
<td>8.71</td>
</tr>
<tr>
<td>4-Methylocatechol</td>
<td>10</td>
<td>9.90</td>
<td>9.92</td>
<td>6.46</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>5</td>
<td>4.87</td>
<td>4.67</td>
<td>4.07</td>
</tr>
</tbody>
</table>

The reaction system contained: substrate, 1 μmole FeSO₄, 5 μmole glutathione, 100 μmole Tris buffer (pH 7.5), and the enzyme (step VII enzyme, 41 μg. of protein) in a total volume of 2 ml. After oxygen uptake had ceased, the reaction mixture was transferred to volumetric flasks and then diluted to 200 ml with 0.05 M potassium phosphate buffer, pH 7.0. The muconic acids formed were determined from their extinction coefficients at their absorption maxima around 260 μμ at pH 7.0. For pyrogallol, the enzyme was replaced by the protamine supernatant (4.86 mg. of protein per vessel). After oxygen consumption had ceased, the reaction mixture was deproteinized with perchloric acid at a final concentration of 3 per cent, and the precipitate was washed twice with a small amount of 3% perchloric acid. The α-hydroxymuconic acid in the combined supernatant was determined in N KOH after neutralization and dilution with N KOH.
The formation of α-hydroxymuconic acid from catechol through the action of m-pyrocatechase has been reported recently (29). In this case, however, α-hydroxymuconic acid is not formed primarily from catechol, but secondarily through α-hydroxymuconic semialdehyde, the primary product of the oxidation. Therefore the mechanism of formation of this acid is different in the two reactions.

**Stoichiometry**

Table V shows that various catechols are quantitatively converted to the corresponding muconic acids by *Brevibacterium pyrocatechase*, as calculated from the molar extinction coefficients at their absorption maxima. However, the formation of α- and β-methylmuconates, and especially of the latter, are considerably lower than the expected values. This may be due to the instability of these acids on acid treatment, such as deproteinization with perchloric acid. A similar consideration may apply to α-hydroxymuconic acid, the oxidation product of pyrogallol.

**Determination of Km Values**

It is difficult to determine the Km value for catechol exactly even by spectrophotometric analysis, because of the high affinity of catechol for the enzyme. The reaction velocity is maximal even at a concentration of catechol as low as 2×10⁻⁴ M. The same is true for pyrogallol.

The Km values for 3- and 4-methylcatechols are calculated as 2.25×10⁻⁴ M and 1.67×10⁻⁴ M respectively, according to the formula of Michaelis and Menten (3).

**SUMMARY**

A pyrocatechase was isolated from a strain of *Brevibacterium fuscum* and purified about 40-fold. This enzyme was homogeneous by ultracentrifugation. Its molecular weight was estimated as 78,000.

*Brevisibacterium* pyrocatechase has unique substrate specificity. Thus it catalyzes the oxidative cleavage of the benzene ring of various catechols including 3- and 4-methylcatechols and pyrogallol unlike the pyrocatechases from other bacterial species.

The purified enzyme requires both ferrous ion and a reducing agent for maximal activity.

The reaction products from catechol, 3- and 4-methylcatechols and pyrogallol were identified as cis, cis-muconic acid, α- and β-methylmuconic acids and α-hydroxymuconic acid, respectively.

It was also demonstrated that the substrate specificity of the enzyme obtained from *Brevibacterium fuscum* was the same on whatever metabolic precursors of catechol the bacteria were grown.

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**REFERENCES**


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