Salicylate Hydroxylase, a Monooxygenase* Requiring Flavin Adenine Dinucleotide

I. PURIFICATION AND GENERAL PROPERTIES†

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Hydroxylation reactions play important roles in many biosynthetic and catabolic processes. The mechanism of enzymatic hydroxylation reactions, however, has not been fully understood, mainly because the enzymes that catalyze such reactions are particulate or unstable and the extensive purification has met with great difficulties. In a preliminary report (2), we described the isolation from a soil bacterium of salicylate hydroxylase which catalyzes the stoichiometric formation of catechol from salicylate and reduced pyridine nucleotide in the presence of flavin adenine dinucleotide as a specific cofactor.

This report deals with the purification of the enzyme and some of its properties. The accompanying paper (3) will describe the results of studies on the reaction mechanism, especially on the role of FAD and the results of experiments with 18O.

EXPERIMENTAL PROCEDURE

Chemicals - NAD, NADH, and NADPH were purchased from Calbiochem. FAD (approximately 85% purity) and FMN were gifts from Dr. Einosuke Ohmura of the Takeda Research Laboratory, and riboflavin was obtained from the Takeda Industries, Ltd. DEAE-cellulose was purchased from the Serva Entwicklungs labor and protamine sulfate from Nutritional Biochemicals. All other chemicals were from commercial sources.

Biological Materials - The organism used throughout this work was isolated from soil by enrichment culture with sodium salicylate as a sole source of carbon. This strain is an aerobic, gram-negative rod and was identified as a pseudomonad by Professor Tsunecaburo Fujino and Dr. Yoshihumi Takeda of the Research Institute for Microbial Diseases, Osaka University, Osaka. Glucose dehydrogenase was prepared from beef liver (4).

* A monooxygenase is defined as an enzyme that incorporates 1 atom of molecular oxygen per molecule of organic substrate (1). This term is preferred to the previously employed nomenclature such as hydroxylase or mixed function oxidase.

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1 In the preliminary communication (2) we erroneously described the organism as a gram-positive coccus. This was based on the observation of the organism cultured on the agar medium containing sodium salicylate as a sole carbon source.

Enzyme Assay - Since the rate of salicylate-dependent NADH oxidation was strictly proportional to that of catechol formation from salicylic acid (2), the amount of salicylic acid hydroxylated was estimated by the decrease of absorbance of NADH at 340 mμ. For the routine assay, the decrease of the absorbance at 340 mμ was followed with a Shimadzu spectrophotometer QR-50 in a cuvette with a 1-cm light path. Each cuvette contained 20 μmamoles of FAD, 200 μmamoles of potassium salicylate, 100 μmamoles of NADH, 60 μmules of Tris-HCl buffer, pH 8.0, and the enzyme in a total volume of 3.0 ml. Under the standard conditions, the reaction was linear with time and the rate was proportional to the amount of enzyme in the range of 0 to 0.15 optical density change per min. One unit of enzyme was defined as that amount which caused the oxidation of 1 μmole of NADH per min at 20°C, and the specific activity was defined as the number of enzyme units per mg of protein. Salicylic acid was determined by the method of Lowry et al. (5) with crystalline egg albumin as a protein standard, or calculated by the absorptions at 280 and 260 mμ from the equation of Kalckar (7). FAD was determined spectrophotometrically at 450 mμ with the molar extinction coefficient of 11.3 × 10².

RESULTS

Purification of Enzyme

Growth of Organisms - Three 5-liter Erlenmeyer flasks containing 1.5 liters of the medium previously described (2) were inoculated with an overnight culture of the microorganism from a peptone-bouillon agar slant. Incubation was carried out for 20 to 30 hours at 30°C in a reciprocating shaker. The contents of the flasks were then transferred to a jar fermenter containing 20 liters of the same medium. The cultures were aerated with an air compressor and stirred mechanically at 28°C for approximately 50 hours with occasional additions of sodium salicylate to maintain the concentration of the latter compound at about 0.1%. The cells were harvested in a continuous flow centrifuge. The paste of bacteria could be stored at −20°C without appreciable loss of activity at least for several months.

Isolation of Enzyme - All subsequent procedures were carried out at 0–5°C. Wet bacterial cells (100 g) were ground mechanically in a well chilled mortar with an equal weight of aluminum oxide (Wako W.800), and mixed with 400 ml of 0.033 m potassium phosphate buffer, pH 7.0. The resultant slurry was centrifuged at 13,000 × g for 10 min in order to separate the extract from
alumina and cell debris. The precipitate was washed with 300 ml of the same buffer by a similar centrifugation. The supernatant solutions were combined and centrifuged at 30,000 rpm for 30 min in the No. 30 rotor of a Spinco model L centrifuge to yield a clear and yellowish brown supernatant solution which is referred to as "crude extract." The protein concentration was adjusted to 5 mg per ml by the addition of the same buffer.

Treatement with Protamine Sulfate—Protamine sulfate solution (1%, 250 ml) was gradually added to 1000 ml of crude extract with mechanical stirring. After standing for more than 15 min the precipitate was removed by centrifugation at 13,000 × g for 10 min.

Ammonium Sulfate Fractionation—To 1000 ml of the protamine sulfate fraction 270 g of ammonium sulfate were added with mechanical stirring. After standing for more than 15 min the precipitate was collected by centrifugation, dissolved in a minimum volume of 0.033 M potassium phosphate buffer, pH 7.0, and dialyzed against the same buffer to remove ammonium sulfate. The enzyme fraction thus obtained will be referred to as "DEAE-cellulose fraction" or "purified enzyme." A typical purification procedure is summarized in Table I.

The purified enzyme preparation with a specific activity of 13.5 was obtained by only 10-fold purification starting from the supernatant solution of high speed centrifugation of the extracts. Apoenzyme was prepared from the DEAE-cellulose fraction by the method described previously (2).

Homogeneity of Purified Enzyme—The ultracentrifugation and the electrophoresis of the purified enzyme were carried out in collaboration with Dr. Kinji Kakuchi and Dr. Toshio Takagi, respectively, of the Institute for Protein Research, Osaka University, Osaka. The sedimentation pattern obtained in a Hitachi analytical ultracentrifuge showed a symmetrical peak under the conditions presented in Fig. 1. The electrophoresis of purified enzyme was carried out and only one moving boundary was present as shown in Fig. 2. The ultracentrifugation experiments provide 3.40 × 10⁻¹⁴ sec for D₄₀.₇₅. The diffusion constant D₄₀.₇₅ was found to be 5.80 × 10⁻₁⁰ cm² per sec in Veronal buffer, pH 8.0, and I/2 = 0.1, at 17°C. Assuming a partial specific volume of 0.75, these values correspond to a molecular weight of 57,200. Since the specific activity of the purified enzyme is 13.5 units per mg of protein, the molar activity of salicylate hydroxylase can be calculated to be 770.

<table>
<thead>
<tr>
<th>Table I</th>
</tr>
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<tbody>
<tr>
<td>Purification of salicylate hydroxylase</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein content</th>
<th>Specific activity</th>
<th>Total activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extracts</td>
<td>1000</td>
<td>4.83</td>
<td>1.48</td>
<td>7240</td>
<td>100</td>
</tr>
<tr>
<td>Protamine fraction</td>
<td>1125</td>
<td>3.43</td>
<td>1.84</td>
<td>7200</td>
<td>99</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fraction</td>
<td>100</td>
<td>10.4</td>
<td>4.06</td>
<td>4250</td>
<td>59</td>
</tr>
<tr>
<td>DEAE-cellulose fraction</td>
<td>5</td>
<td>12.5</td>
<td>13.5</td>
<td>845</td>
<td>12</td>
</tr>
</tbody>
</table>

Fig. 1. Ultracentrifugation pattern of the purified salicylate hydroxylase. The protein concentration was approximately 1% in sodium phosphate buffer, pH 7.0, and I/2 = 0.1. The progress of ultracentrifugation at 15°C is shown from right to left at 10-min intervals after reaching 57,600 rpm.

Fig. 2. Electrophoresis pattern of the purified salicylate hydroxylase. The protein concentration was approximately 0.9% in Veronal buffer, pH 8.0, and I/2 = 0.1. The pattern represents the ascending and descending boundary after 90 min at 17.3°C at 8 mA.
The stoichiometry of the reaction and the tentative identification of reaction products were reported previously (2).

Isolation of Reaction Product—A large scale incubation was carried out for the isolation and crystallization of the reaction product. The reaction mixture (30 ml) in a round flask contained 3.3 mmoles of sodium salicylate, 30 mmoles of NAD, 1.5 mmoles of FAD, 30 mmoles of glucose, 90 units of salicylate hydroxylase, 50,000 units of beef liver glucose dehydrogenase, and 3 mmoles of Tris-HCl buffer at pH 9.0. Salicylate hydroxylase was used as the ammonium sulfate fraction which was further treated with acid ammonium sulfate (2) and was free of pyrocatechase activity. After 3 hours of gentle rotation of the flask at 20°, when approximately half of the salicylate had disappeared judging from the estimation with the reagent of Trinder, 2 ml of 3.3 M HCl were added. The precipitate formed was removed by centrifugation and the supernatant solution was extracted three times with 60-ml portions of ether. The extracts were evaporated. The residue was recrystallized twice from toluene.

Identification of Reaction Product—Both the crystalline reaction product and the authentic catechol melted at 105°. The mixture of both also melted at this temperature. The ultraviolet spectrum of the reaction product was identical with that of catechol of the same concentration, and showed an absorption maximum at 277 mμ in the aqueous solution. The addition of purified pyrocatechase (8) caused the stoichiometric increase of absorbance at 260 mμ corresponding to cis,cis-muconic acid, and that of crystalline metapyrocatechase (9) resulted in the increase of absorbance at 375 mμ indicating the formation of α-hydroxymuconic semialdehyde. These results provide further evidence for the identification of the reaction product as catechol.

Fig. 3. FAD requirement of salicylate hydroxylase. The purified apo-enzyme (1.8 μg) was incubated with varying amounts of FAD (as indicated) in the standard assay system without NADH. After 10 min preincubination in an ice bath the reaction mixture was warmed to 20° and the reaction was started by the addition of NADH.

Isolation and Identification of Reaction Product

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Ascending paper chromatography was carried out on Toyo filter paper No. 51, and the spots of reaction product and authentic catechol were detected by spraying with 2% FeCl₃·6H₂O. Rₚ values for the reaction product and authentic catechol were identical: 0.98 with n-butyl alcohol-pyridine-water saturated with sodium chloride (1:1:2); 0.84 with chloroform-methanol-formic acid-water (1000:100:4:96); 0.84 with n-butyl alcohol-benzene-water (1.9:10); and 0.15 with benzene-formic acid-water (1000:2:98).

FAD Requirement

Kₘ for FAD—The apo-enzyme was completely inactive unless FAD was added. When a limited amount of FAD was added, about a 10-min preincubation at 0° was necessary to obtain the full activity. Fig. 3 shows the requirement of FAD for the salicylate hydroxylation as determined under such conditions. The concentration of FAD required for half-maximal velocity was estimated to be 0.07 μM. When the concentration of FAD was sufficiently high, preincubation appeared to be unnecessary. For example, in the standard assay system the apo-enzyme was activated almost instantaneously.

FAD Analysis—Since the enzyme-bound FAD gradually dissociated during the purification procedure, the equilibrium dialysis was adopted to determine the number of molecules of enzyme-bound FAD (Table II). Assuming the molar extinction of enzyme-bound FAD to be the same as that of free FAD, namely, 11,800, the number of molecules of FAD bound to 1 molecule of enzyme protein was calculated to be approximately 0.9 molecule. During the dialysis, the inactivation of enzyme was not observed to an appreciable extent.

Absorption Spectra of Enzyme—The absorption spectrum of DEAE-cellulose fraction in the visible region exhibited maxima at 450 and 370 mμ and minima at 400 and 320 mμ, respectively. The ratio of the absorbance at 375 and 450 mμ was 1.07 (Fig. 4). In Fig. 5 is shown the absorption spectrum of apo-enzyme with a peak at 280 mμ and a slight shoulder at 290 mμ.

General Properties

Stability—When the crude extracts (0.6 mg of protein per ml) were incubated at 37° in 0.033 M potassium phosphate or Tris-HCl buffer, the activity was most stable at pH 8. The purified DEAE-cellulose fraction in 0.02 M Tris-HCl buffer at pH 8.0 was kept in a test tube with 9 ml of the same buffer. FAD was added outside solution was frequently exchanged.

Table II

Equilibrium dialysis of salicylate hydroxylase and FAD

A cellophane membrane sac containing 1 ml of 20 μM solution of DEAE-cellulose fraction in 0.02 M Tris-HCl buffer at pH 8.0 was kept in a test tube with 9 ml of the same buffer. FAD was added inside and outside the membrane as depicted in the table. Dialysis was continued at 5° with stirring until the absorbance at 450 mμ outside the membrane stopped increasing. After about 45 hours of dialysis the absorbances at 450 mμ both inside and outside the membrane were measured.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>μM</td>
<td>μM</td>
<td>μM</td>
</tr>
<tr>
<td>Before dialysis</td>
<td>143</td>
<td>143</td>
<td>143</td>
<td>143</td>
</tr>
<tr>
<td>Inside</td>
<td>10.4</td>
<td>27.5</td>
<td>79.7</td>
<td>(0)*</td>
</tr>
<tr>
<td>Outside</td>
<td>0</td>
<td>19.3</td>
<td>85.5</td>
<td>(0)*</td>
</tr>
<tr>
<td>Difference</td>
<td>17.2</td>
<td>16.9</td>
<td>16.8</td>
<td>10.4</td>
</tr>
</tbody>
</table>

* The outside solution was frequently exchanged.

1 Purified pyrocatechase was kindly donated by Dr. Hiroshi Tanuieh of Kyoto University.

2 Crystalline metapyrocatechase was a gift from Dr. Mitsuhiro Nozaki of Kyoto University.
In the standard assay system where 0.7 mM potassium salicylate was estimated to be 1.9
following the time course curve of the decrease of absorbance at 340 nm with a Shimadzu recording spectrophotometer. The reaction rate was obtained from the slope of a tangential line that touches the curve and the 340 nm with a Shimadzu recording spectrophotometer. The protein concentration was 0.39 mg per ml.

apoenzyme was remarkably unstable above 25°. As shown in Fig. 6, when the apoenzyme was incubated at 38°, nearly complete inactivation occurred within 1/2 hour, whereas in the presence of 0.4 mM FAD more than 90% of the original activity remained even after a 2-hour incubation at 38°.

Effect of pH—The purified enzyme was most active between pH 7.5 and 8.0 (Fig. 7).

K<sub>v</sub> Values—The high affinity of salicylate hydroxylase for substrates made it difficult to determine K<sub>v</sub> values by the conventional method. Therefore the K<sub>v</sub> values were calculated by following the time course curve of the decrease of absorbance at 340 nm with a Shimadzu recording spectrophotometer. The reaction rate was obtained from the slope of a tangential line that touches the curve and the K<sub>v</sub> value was calculated from the concentration of remaining substrate at which the enzyme showed its half-maximal activity. When the substrate completely disappeared, the full restoration of enzyme activity was checked by the further addition of the substrate. K<sub>v</sub> for salicylate was estimated to be 1.9 μM when measured in the standard assay system containing 0.17 mM NADH and 0.03 mM potassium salicylate. K<sub>v</sub> for NADH was calculated to be 2.6 μM as determined in the standard assay system where 0.7 mM potassium salicylate and 0.08 mM NADH were present.

Substrate Specificity—As listed in Table III, NADH was required as a hydrogen donor of salicylate hydroxylase and NADPH was approximately 1% effective for the purified enzyme. Without salicylate NADH oxidase activity of the purified enzyme was almost negligible under the standard assay conditions.

Of a number of compounds tested in place of salicylic acid, the decrease of absorbance at 340 nm was observed at an appreciable rate with those listed in Table IV, whereas the following compounds were neither substrates nor inhibitors of the enzyme: benzoate, p-hydroxybenzoate, 2-hydroxy-3-naphthoate, α-nitro- benzoate, phthalate, salicylic acid methyl ester, sulfosalicylate, and thiosalicylate.

Judging from these results, the enzyme appears to be specific for the compounds having free carboxyl and hydroxyl groups in the α-position.

Effect of Inhibitors and Metals—The results of inhibition experiments are shown in Table V. Of the metal-chelating reagents tested, o-phenanthroline at 1 mM and 0.1 mM showed 59% and 13% inhibition, respectively. The preincubation of 0.5 mM o-phenanthroline with the purified enzyme in an ice bath for 1 hour resulted in 59% inhibition. However, upon dialysis...
against 0.02 except NADH. After 15 min the reaction mixture was warmed detected by spraying with diazotized sulfanilic acid followed by following solvent systems: n-amyl alcohol saturated with water; acid-water (125 : 72 : 3); n-butyl alcohol-glacial acetic acid-water (14 : 3 : 3); benzene-glacial acetic acid-water (4:1:5). The reaction products and authentic pyrogallol were detected by spraying with diazotized sulfanilic acid followed by the exposure to ammonia vapor. Pyrogallol was formed from the former compound could not be observed perhaps because of its instability. In either case, however, pyrogallol was never detected. These results are consistent with the presumption that the carbon atom of the benzene ring with a carboxyl group was decarboxylated and hydroxylated, assuming that one of the hydroxyl groups of catechol was derived from the original hydroxyl group of salicylic acid.

### Table V

<table>
<thead>
<tr>
<th>Compound added</th>
<th>Concentration</th>
<th>Activity</th>
<th>Zero time*</th>
<th>After 15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>m</td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>o-Phenanthroline</td>
<td>10^-4</td>
<td>87</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>α-α'-Dipyridyl</td>
<td>10^-4</td>
<td>41</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Ethylenediaminetetraacetate</td>
<td>10^-4</td>
<td>100</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td>Potassium cyanide</td>
<td>10^-4</td>
<td>95</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>Sodium azide</td>
<td>10^-4</td>
<td>102</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>p-Chloromercuribenzoate</td>
<td>10^-4</td>
<td>91</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>p-Chloromercuribenzoate‡</td>
<td>10^-4</td>
<td>91</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Cupric sulfate</td>
<td>10^-4</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Sodium molybdate</td>
<td>10^-4</td>
<td>79</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>10^-4</td>
<td>94</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>Ferrous sulfate + ascorbic acid</td>
<td>2 × 10^-3</td>
<td>97</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Ferrous sulfate + ascorbic acid</td>
<td>10^-4</td>
<td>97</td>
<td>96</td>
<td></td>
</tr>
</tbody>
</table>

* The reaction was started by the addition of the purified enzyme to the other components of standard assay system containing inhibitors or metals.
† The enzyme was preincubated in an ice bath with the inhibitors or metals and the other components of standard assay system except NADH. After 15 min the reaction mixture was warmed to 39° and the reaction was started by the addition of NADH.
‡ Apoenzyme in place of holoenzyme was preincubated without FAD.

Hydroxylation reactions are catalyzed by monooxygenases which incorporate 1 atom of molecular oxygen into a molecule of organic substrate. The other atom of oxygen is presumed to be reduced to water in the presence of a specific hydrogen donor. Various reducing agents such as NADH, NADPH, ascorbate, reduced FMN, reduced pteridine derivative, or the substrate itself, are reported to serve as the hydrogen donor (10). The mechanism of monooxygenase reactions which require reduced pyridine nucleotides as reducing agents has recently been under active investigation in several laboratories. Phenylation hydroxylase in mammalian liver has been reported to require the reduced pteridine derivative as a direct hydrogen donor in the hydroxylation of phenylalanine to tyrosine (11), while the lactonizing enzyme of 2,5-diketocamphane has been known to require FMN, through which NADH reduces the iron of the enzyme to the active ferrous state (12). In both of these cases, two separate enzymes are involved in the reaction. One catalyzes the reduction of pteridine derivative or FMN by reduced pyridine nucleotides, and the other is an oxygenase which requires the reduced pteridine derivative or FMN as a direct hydrogen donor.

Although the detailed discussion on the role of FAD in the hydroxylation of salicylate will be presented in the accompanying paper (8), salicylate appears to be hydroxylated in the presence of NADH by one FAD enzyme which was proved to be homogeneous upon electrophoresis and ultracentrifugation. Without salicylate NADH is not oxidized under the standard conditions; i.e. the oxidation of NADH is coupled with the hydroxylation of salicylate.

### Site of Hydroxylation of Salicylic Acid

Various metabolizable substrate analogues were incubated with purified salicylate hydroxylase and each reaction product was examined by paper chromatography. The reaction mixture, containing 5 μmoles of potassium phosphate buffer at pH 7.5, 1 μmole of substrate, 3 pmoles of NADH, 30 mmoles of FAD, and 1.5 units of purified enzyme in a total volume of 100 μl, was stopped by the addition of 10 μl of 1 N HCl. An aliquot, 11 μl, of the reaction mixture was spotted on Toyo filter paper No. 51. Ascending paper chromatography was carried out with the following solvent systems: n-aryl alcohol saturated with water; n-butyl alcohol-pyridine-water (14:3:3); benzene-glacial acetic acid-water (125:72:3); n-butyl alcohol-glacial acetic acid-water (4:1:5). The reaction products and authentic pyrogallol were detected by spraying with diazotized sulfanilic acid followed by the exposure to ammonia vapor. Pyrogallol was formed from either 2,3- or 2,6-dihydroxybenzoic acid. Hydroxyhydroxylase was not expected to appear after the incubation of 2,4- or 2,5-dihydroxybenzoic acid with the enzyme, but the formation of the former compound could not be observed perhaps because of its instability. In either case, however, pyrogallol was never detected.

**DISCUSSION**

Hydroxylation reactions are catalyzed by monooxygenases which incorporate 1 atom of molecular oxygen into a molecule of organic substrate. The other atom of oxygen is presumed to be reduced to water in the presence of a specific hydrogen donor. Various reducing agents such as NADH, NADPH, ascorbate, reduced FMN, reduced pteridine derivative, or the substrate itself, are reported to serve as the hydrogen donor (10). The mechanism of monooxygenase reactions which require reduced pyridine nucleotides as reducing agents has recently been under active investigation in several laboratories. Phenylation hydroxylase in mammalian liver has been reported to require the reduced pteridine derivative as a direct hydrogen donor in the hydroxylation of phenylalanine to tyrosine (11), while the lactonizing enzyme of 2,5-diketocamphane has been known to require FMN, through which NADH reduces the iron of the enzyme to the active ferrous state (12). In both of these cases, two separate enzymes are involved in the reaction. One catalyzes the reduction of pteridine derivative or FMN by reduced pyridine nucleotides, and the other is an oxygenase which requires the reduced pteridine derivative or FMN as a direct hydrogen donor.

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In addition to salicylate hydroxylase, other FAD-requiring monooxygenases have been reported recently: bacterial β-hydroxybenzoate hydroxylase (13) and acyl-CoA-desaturating enzyme from Mycobacterium (14).

The present report provides the first clear-cut evidence for the participation of FAD in an aromatic hydroxylation reaction. Although it may be premature to suggest that flavin might serve more generally as a cofactor in the hydroxylation of other aromatic compounds, it is tempting to postulate a more general role for flavins in the hydroxylation reactions since these have higher oxidation-reduction potentials and readily react with oxygen, and are known to participate in many biological oxidation reactions.

Participation of iron in addition to flavin has been reported in the enzymatic lactonization of 2,5-diketocamphane (12) and the enzymatic desaturation of acyl-CoA (14). Although the purified salicylate hydroxylase has not been analyzed for metals, the results of studies with inhibitors did not seem to provide definitive evidence for the participation of iron. It would seem that metal ions, if any, involved in salicylate hydroxylase must be firmly bound to the enzyme protein.
SUMMARY

1. Salicylate hydroxylase which catalyzes the hydroxylation of salicylate to catechol was purified from a pseudomonad and proved to be homogeneous upon ultracentrifugation and electrophoresis. The molecular weight was estimated to be approximately 57,200.

2. The reaction product was identified as catechol.

3. The enzyme was shown to require flavin adenine dinucleotide as a specific cofactor in the hydroxylation of salicylate. One mole of FAD was determined to be loosely bound to 1 mole of enzyme protein. FAD protected the inactivation of enzyme.

4. Reduced nicotinamide adenine dinucleotide was required as a hydrogen donor, but reduced nicotinamide adenine dinucleotide phosphate was practically inactive.

5. The oxidation of NADH could be observed in the presence of some of the derivatives of salicylic acid.

6. The inhibition of the enzyme by o-phenanthroline was observed, but the full activity was restored upon dialysis.

7. Available evidence indicates that the hydroxylation occurs at carbon atom 1 of the benzene ring concomitant with decarboxylation.

REFERENCES

Salicylate Hydroxylase, a Monooxygenase Requiring Flavin Adenine Dinucleotide: I. PURIFICATION AND GENERAL PROPERTIES
Shozo Yamamoto, Masayuki Katagiri, Hiroo Maeno and Osamu Hayaishi