Studies of a Flavoprotein, Salicylate Hydroxylase

I. PREPARATION, PROPERTIES, AND THE UNCOUPLING OF OXYGEN REDUCTION FROM HYDROXYLATION*

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SUMMARY

A flavoprotein, salicylate hydroxylase (salicylate, DPNH: oxygen oxidoreductase (1-hydroxylation, 1-decarboxylating)), has been induced and isolated from a soil bacterium grown on salicylate as sole carbon source. In contrast to another enzyme studied by Yamamoto, S., Katagiri, M., Maruno, H., and Hayashi, O. ((1965) J. Biol. Chem. 240, 3408) this enzyme is dimeric with two subunits and two FAD per 91,000 molecular weight (rather than one FAD per 57,000).

When benzoate is substituted for salicylate, DPNH is oxidized with the same Vmax as with salicylate but with higher Km for both benzoate and DPNH. With salicylate, the reaction products are catechol and H2O2; with benzoate, the benzoate is unchanged, but H2O2 is formed stoichiometrically with DPNH oxidized. Both salicylate and benzoate facilitate DPNII binding. Benzoate binds at the salicylate site, competitively inhibiting salicylate hydroxylation, and permitting DPNII binding and oxidation. But since benzoate, a "pseudosubstrate," cannot be hydroxylated, the oxygen utilized decomposes to H2O2, and oxygen reduction is considered as "uncoupled" from hydroxylation. A search for possible active intermediates in the reduction of O2 to H2O2 by the uncoupled reaction has failed to yield evidence for any oxygen radical species.

A partial "uncoupling" effect had been described (5, 8) and also

"...facilitates DPNII binding, but less effectively than does salicylate."

Salicylate hydroxylase is a flavoprotein which catalyzes the hydroxylation and simultaneous decarboxylation of salicylate to catechol. This enzyme, as isolated from a strain of Pseudomonas putida, has been described and extensively studied by Yamamoto et al. (1), Katagiri et al. (2, 3), and Takemori et al. (4, 5). Its mechanism of action has been studied by spectroscopic (3, 4), fluorometric (6), and stopped flow (5) techniques. The enzyme contains one FAD and one polypeptide chain per 57,200 molecular weight (1), and exhibits a strong specificity for substrates bearing hydroxyl and carboxyl substituents at ortho positions.

Recent publications from this laboratory (7-9) have described a salicylate hydroxylase, which has different physical and kinetic properties, from a different (and as yet unidentified) soil microorganism. Our enzyme consists of 2 moles of FAD and two subunits per 91,000 molecular weight, and has the distinctive property of catalyzing a benzoate-stimulated oxidation of DPNH with the same catalytic Vmax as salicylate hydroxylation, but without detectable alteration of the benzoate molecule (7, 8). The characteristics of this reaction suggest that, in the presence of benzoate, oxygen is activated or reduced in a "normal" fashion, but hydroxylation cannot proceed. The product of oxygen reduction in this case is H2O2. We have called benzoate a "pseudosubstrate" and have termed the benzoate effect "uncoupling of oxygen activation from hydroxylation" (7-9).

Since the publication of our original report (7) on the benzoate effect, two laboratories have reported analogous effects in other flavoprotein hydroxylases (10, 11). A partial "uncoupling" effect had been observed previously by Storm and Kaufman (12) with the pteridine-containing phenylalanine hydroxylase from rat liver. They found that structural changes in either substrate (4-fluoro derivative) or pteridine cofactor would affect the TPNII to tyrosine stoichiometry; where excess TPNH was utilized, H2O2 was also detected.

The present paper documents further some of our previous results, and describes additional experiments designed to examine more fully the effects of salicylate, benzoate, and other aromatic compounds. Stopped flow studies have been performed and will be reported in a subsequent paper (13). Such studies, combined with those reported in the present paper, have permitted us to propose a reaction mechanism (13) which accommodates "substrates," "pseudosubstrates," and compounds which display both modes of activity.
MATERIALS AND METHODS

DPNH, DPN\(^+\), and TPNH were purchased from Pabst Laboratories, made up fresh in distilled water, and checked for absorbance at both 340 and 260 nm before use. Only DPNH of better than 95% purity was used.

Sodium benzoate was Fisher U.S.P. grade, and sodium salicylate was Mallinckrodt analytical reagent grade. Other aromatic compounds were the purest commercially available. They were dissolved in pH 7.62 phosphate buffer, checked spectrophotometrically, and used fresh. Where necessary, aromatic compounds were precipitated from a solution of their salt with 6 M HCl, filtered, washed free of HCl, and redissolved in a 10% excess of NaHCO\(_3\). Easily oxidizable phenolic compounds, such as 2,5-dihydroxybenzoic acid, were made up in 95% ethanol and stored under a nitrogen atmosphere prior to use.

Guandine hydrochloride, used in molecular weight determinations, was the ultrahigh purity grade of Heico, Inc., Delaware Water Gap, Pa. 18327. Ammonium sulfate was Mann, enzyme grade. Protamine sulfate, from Mann Laboratories, was the U.S.P. grade Salmine. It was made up as a 1% suspension in water and titrated to pH 7.0 with KOH. The resultant mixture was quite turbid, but was stable in the cold. Hydrogen peroxide, purchased as the 30\(^\circ\) Mallinckrodt analytical reagent, was dissolved in pH 7.62 phosphate buffer, checked spectrophoto-

Crystalline beef liver catalase of 150,000 units per ml was obtained from Worthington. Yeast alcohol dehydrogenase and horse heart cytochrome c, type VI, were both from Sigma. Bovine superoxide dismutase was a gift from Dr. Irwin Fridovich (14).

Growth of Microorganism

The organism was maintained and subcultured in a liquid basal medium containing sodium salicylate as the sole carbon source. The medium, similar to that of Katagiri et al. (15), consisted of 0.2% sodium salicylate, 0.126% KNO\(_3\), 0.15% KH\(_2\)PO\(_4\), 0.05% KH\(_2\)PO\(_4\), 0.02% MgSO\(_4\)-7H\(_2\)O, 0.0067% CaCl\(_2\)-7H\(_2\)O, 0.3 mg per liter of riboflavin, and heavy metals. The riboflavin and heavy metals solutions were added as 1000 times concentrated solutions. The heavy metals formulation used was a modification of that of Ya-


data was monitored both by consumption of salicylate and by absorb-

ance, due to light scattering, measured at 600 nm. Special care was taken to harvest before the salicylate concentration had dropped below 0.03%; otherwise, enzyme yields would drop markedly.

The organisms were harvested in a Sharples, Lourdes, or De Laval continuous flow centrifuge, and when the volume of culture in the fermentor was reduced to about 1 liter, 40 liters of additional sterile medium were pumped in. This second lot of bacteria was grown up and harvested as previously described. The resultant cell paste from 80 liters of medium was washed three to four times with 0.033 \(\text{M potassium phosphate, pH 7.0, and frozen; about 300 g of bacteria were produced. The frozen cell paste was stable for months.}


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**Extraction and Purification of Enzyme**

The purification procedure was a modification of that of Yamamoto et al. (1). The progress of purification is summarized in Table I.

**Step 1. Sonication**—Frozen cell paste was thawed and homogenized with 1 volume of 0.033 M potassium phosphate buffer, pH 7.0, in a Brinkmann Polytron homogenizer for 2 min to break up clumps of bacteria. The suspension was then sonicated at full power in a beaker (in 500-ml batches) for 20 to 30 min, in 5-min bursts, with a Heat Systems-Ultrasonics, Inc., model W185D sonifier. Temperature was kept below 15° with an ice bath.

The sonicate was centrifuged for 1 hour at the highest speed (50,000 \(\times\) g) in the Sorvall RC2B centrifuge, SS 34 rotor. The dark brown supernatant solution was carefully decanted from the cell debris and a black, gelatinous precipitate.

**Step 2. Protamine Sulfate Treatment**—Protamine sulfate had been used by Hosokawa and Stanier (16) and Yamamoto et al. (1) to remove nucleic acid in the course of purification of their enzymes. We also used this technique but found that our salicylate hydroxylase was precipitable at higher protamine sulfate concentrations, permitting differential precipitation and subsequent elution of enzyme. The sonic supernatant was adjusted to a protein concentration of 5 mg per ml. Protamine sulfate was slowly added in the cold as a 1% suspension. One hundred fifty to 500 ml per liter of enzyme (higher amounts for higher protein concentration) were usually sufficient to sediment nucleic acid without precipitating enzyme. After this precipitate was removed by centrifugation (15 min at 15,000 rpm), additional protamine sulfate solution, in 150- to 200-ml increments per liter of original sonic supernatant was added, stirred for 2 to 3 hours, and recentrifuged. The supernatant was assayed between pro-

| Fraction                  | Volume | Protein concentration | Specific activity | Total activity | Yield  |
|---------------------------|--------|-----------------------|-------------------|----------------|--------|
| Sonic extract             | 1324   | 4.97                  | 0.734             | 4829           | 100    |
| Protamine eluate          | 576    | 0.895                 | 0.040             | 2813           | 58.3   |
| (NH\(_4\))\(_2\)SO\(_4\) fraction | 26.8   | 10.25                 | 5.72              | 2907           | 40.6   |
| DEAE-cellulose eluate     | 6.67   | 15.38                 | 10.57             | 1085           | 22.5   |

The purification procedure was a modification of that of Ya-

**TABLE I**

Purification of salicylate hydroxylase

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tamine additions, and the procedure was repeated until less than 10% of the original activity remained.

The enzyme-protamine sulfate precipitates were suspended in 25-ml portions of 0.033 M potassium phosphate buffer, pH 7.00, containing 0.05 M NaCl. One hour of stirring at 37° in 50-ml centrifuge tubes with ½ inch magnets was sufficient to clute most of the activity. The suspension was then centrifuged 15 min at 15,000 rpm and the yellow supernatant collected. Re-extraction of the protamine sulfate precipitate with the same buffer would increase yield without lowering specific activity.

Step 3. Ammonium Sulfate Fractionation—The combined eluates from the protamine sulfate step were fractionated between 47 and 65% ammonium sulfate saturation, using the formula of densities at 600 and 296 nm of the culture medium, are plotted as 5 ml of buffer, sonicated 1 min, and centrifuged at 27,000 X g, from the 65% fractionation was reconstituted quickly in 0.02 M K2HPO4 and dialyzed overnight against that solution.

Step 4. DEAE-cellulose Chromatography—The reconstituted ammonium sulfate fraction was applied to the top of a DEAE-cellulose column, 1.7 x 17 cm, previously equilibrated with 0.02 M K2HPO4. After washing the column with several hundred milliliters of K2HPO4, elution was begun with a linear gradient of 500 ml of 0.02 M K2HPO4 and 500 ml of 0.2 M K2HPO4. Fractions of 13 to 16 ml were collected. The peak of enzyme activity was eluted between 0.06 and 0.09 M phosphate concentration. The appearance of activity coincided with the appearance of protein and of 450-nm absorbance. The peak fractions were pooled, concentrated by a 45 to 65% ammonium sulfate fractionation, dialyzed against 0.02 M potassium phosphate, pH 7.0, and frozen. The yield of enzyme was about 20 to 25% of that in the original sonic extract.

In early preparations, an added step of hydroxyapatite chromatography (19) was included, and samples of enzyme purified through this step were used for molecular weight and electrophoresis experiments to be described. However, this procedure did not give appreciable additional purification, and subsequent preparations omitted this step.

**Enzyme Assays**

Protein concentration was determined by the microbiuret method (20) with bovine serum albumin as a standard. The albumin in turn was standardized by its extinction at 270 nm of 0.67 for 1 mg per ml (21). For procedures such as column chromatography, protein concentration was monitored from optical densities at 280 and 260 nm. The extinctions for free FAD, cited by Yagi (22) at 280, 260, and 450 nm, were assumed for bound FAD.

Enzyme assays were performed with a Gilford 2400 or Cary 14 recording spectrophotometer and a standard assay mixture of 1 mM EDTA, 135 μM sodium salicylate, 147 μM DPNH, and 0.02 M potassium phosphate buffer, pH 7.62, in a volume of 3 ml with a 1-cm path length cuvette. Addition of FAD (67 μM) or omission of EDTA had no effect on the activity. One unit of activity represented the oxidation of 1 μmole of DPNH per min measured at 340 nm and 27°. Occasional assays required cells of path lengths from 0.1 to 10 cm. These were calibrated before use with standardized solutions of DPNH. Assays at very high pyridine nucleotide concentrations (10 to 20 mM), and other procedures requiring measurement of oxygen uptake, were performed in a Gilson model KM Oxygraph equipped with a Clark oxygen electrode and thermostatted at 25°. Full scale deflection was set at standard oxygen tension at 25° which was assumed to be 240 μl. At high substrate concentrations, substrate absorption would sometimes interfere at 340 nm. In these cases spectrophotometric assays were performed at 360, 365, or 370 nm with the appropriate extinction coefficients for DPNH. It was sometimes advantageous to assay at 296 nm, the absorption maximum of salicylate. At this wave length a ΔA296 of -3400 was established for the salicylate to catechol reaction. This extinction, combined with the ΔA296 of -1300 for the reaction DPNH to DPNH+ gave an effective molar extinction coefficient of -4700 M⁻¹ cm⁻¹.

**Experimental Procedure and Results**

**Selection of Organism**—The salicylate hydroxylase described herein was isolated from an organism obtained from garden soil a few millimeters from a freshly creosoted telephone pole. The organism was selected by its ability to grow in medium containing 0.1% salicylate as sole carbon source. The culture was purified by selection of single colonies from plates, containing either 1% Difco yeast extract, or 0.1% sodium salicylate in minimal medium, with 2.5% Bacto-agar as a solid support. The colonies grown on salicylate-agar, like the liquid cultures, turned dark with growth. The organism, a gram-negative rod, has not been identified. It grows only aerobically, and will grow on dextrose or yeast extract broths. The sole nitrogen source used was KNO3 and, as expected, the bacterium has nitrate reductase activity.

The data of Fig. 1 indicate that salicylate hydroxylase is an
Fig. 2. Scans of polyacrylamide electrophoresis gels at three stages of purity. Protein plus bromthymol blue (BTB) to mark the front of the gel was applied to the top of the gels and electrophoresis performed by the method of Davis (23). Gels were stained with Amido Black T, destained with 7% acetic acid, and scanned at 615 nm by a Gilford 240 spectrophotometer equipped with a gel-scanning accessory. A scanning range of 0 to 2.5 A units was utilized for the hydroxylapatite eluate; otherwise a range of 0 to 0.5 was used. The $R_f$ values indicated were relative to the BTB marker.

inducible enzyme. Bacteria grown on 1% Difco yeast extract had no detectable salicylate-dependent DPNH oxidase activity. When these organisms, harvested without chilling, were washed and resuspended in salicylate minimal medium, salicylate hydroxylase activity appeared as depicted. Synthesis of enzyme by the cell preceded both the consumption of salicylate in the medium and growth of the organism. After an induction period of 9 hours, the specific activity of the enzyme reached a level of 0.27 unit per mg of protein. This compares with a level of 0.8 to 1.5 units per mg observed in crude sonic extracts of fermentor-grown bacteria.

Criteria of Purity—The eluate from hydroxylapatite column chromatography, as well as samples of protamine sulfate eluate and crude sonicate, were subjected to polyacrylamide gel electrophoresis by the method of Davis (23), and stained for protein with Amido Black T. The data are presented in Fig. 2. It can be seen that the enzyme peak is easily visible even in the crude supernatant. This observation is in accord with the data of Table I which show that pure enzyme is obtained after only 14- to 16-fold purification, i.e. that enzyme constitutes about 7% of the total protein in the sonic supernatant.

Electrophoresis of purified samples was also performed on cellulose acetate utilizing the Phoroslide Electrophoresis system of the Millipore Corp. Electrophoresis in 0.075 ionic strength barbiturate buffer, pH 8.6, for 30 to 45 min at 100 volts, followed by development with a 1.8% Ponceau S dye, 26.891, trichloroacetic acid, and 26.8% sulfosalicylic acid mixture indicated the presence of only one component for either DEAE-cellulose or hydroxylapatite eluates. The enzyme also migrated as one peak in the analytical ultracentrifuge; these experiments will be described shortly.

Characterization of Enzyme: Flavoprotein—The spectrum of the purified salicylate hydroxylase shown in Fig. 3 is that of a typical flavoprotein bearing no other chromophoric prosthetic groups. Addition of a small amount of sodium dithionite effected a reduction of the 450-nm peak to that of the fully reduced flavin. The fluorescence of solutions of enzyme and of free FAD, both having the same absorbance at 450 nm, was measured. At an excitation wave length of 462 nm, the fluorescence emission of free FAD at 520 nm was 13.3 times higher than that for the flavoprotein, indicating a quenching of flavin fluorescence upon binding to the protein. Suzuki et al. (6) had observed quenching of FAD fluorescence upon binding to the apoenzyme of their salicylate hydroxylase.

The supernatant from a boiled solution of the enzyme was chromatographed on paper by the method of Yagi (22). Solutions of free FAD, FMN, and enzyme supernatant were spotted in the dark onto Whatman No. 1 filter paper and developed with 5% Na$_2$HPO$_4$ as solvent. Samples of boiled enzyme supernatant were almost indistinguishable from samples of pure FAD, and were of significantly different $R_f$ values from those of FMN. There is no evidence for the presence of metals, nor is there...
evidence for catalytically significant reducible groups other than flavin. EDTA even at concentrations of 0.01 M does not inhibit. Zinc, copper, and iron sulfates, at concentrations of $1 \times 10^{-4}$ M, had no effect on activity, nor did potassium cyanide, sodium azide, or ascorbate. Yamamoto et al. (1) also reported the lack of effect of metals or metal-chelating agents, except o-phenanthroline, on the catalytic activity of his enzyme.

Physical Studies—Purified salicylate hydroxylase migrated as one homogeneous and symmetrical peak in the model E analytical ultracentrifuge, as shown in Fig. 4. With 0.1 M KCl as solvent, an $s_{20,w}$ of 5.91 x $10^{-1}$ sec was calculated. A plot of ln (radius from center of rotor to enzyme peak) versus time was linear over the 80-min period of measurement. A partial specific volume of 0.728 was determined by the method of Schachman (24) from an amino acid analysis of a 24-hour 6 N HCl hydrolysate. This $s_{20,w}$ of 5.91 compares with the value 3.4 obtained by Yamamoto et al. (1) for their enzyme.

Sedimentation equilibrium studies of native enzyme by the method of Yphantis (25) were performed at a protein concentration of 0.2 mg per ml in 0.1 M KCl, with a model E ultracentrifuge equipped with Rayleigh optics. Photographic plates were exposed after centrifuging at 23,141 rpm for 24 and 28 hours. A plot of the natural logarithm of the displacement of five fringes (averaged) versus the square of the fringe radius yielded a straight line, whose slope corresponded to a molecular weight of 91,000. This molecular weight compares with a value of 57,200 obtained by Yamamoto et al. (1) for their enzyme.

Sedimentation equilibrium studies of reduced protein by means of gel filtration. Salicylate hydroxylase (5 mg) was denatured and reduced in 6.0 M guanidine hydrochloride plus 0.1 M mercaptoethanol and carboxymethylated with a 10% excess of iodoacetamide at pH 8.5. The protein was dialyzed against 0.01 M acetic acid, lyophilized, and the entire reduction procedure repeated. The final product was taken up in 0.17 ml of 6 M guanidine-HCl, mixed with DNP-alanine and blue dextran as low and high molecular weight markers, and applied to the top of a 4% Agarose A-5M column. The column was eluted with 6.0 M guanidine-HCl by the method of Fish et al. (26), and fractions of approximately 0.9 ml were collected, weighed, and read for optical density at 280 nm. The elution curve indicated only one large symmetrical peak, whose position corresponded to a molecular weight of 43,000, in comparison with standard curves previously run on the same column (26). The symmetry and the presence of a single peak suggest the presence of only one type of subunit. Peptide mapping has not yet been performed.

The two peak tubes from the 6 M guanidine-HCl gel filtration column were diluted to an $A_{280}$ of 0.190 (about 0.2 mg per ml) with 6 M guanidine-HCl and analyzed by sedimentation equilibrium. The molecular weight was calculated as discussed previously, assuming a density of 1.141 g per cc for 6.0 M guanidine-HCl and a partial specific volume of 0.718 (0.01 cc per g less than that for native protein (27, 28)) for the denatured protein. The physical properties of purified salicylate hydroxylase are summarized in Table II and are consistent with a structure of two FAD and two subunits per mole. A minimum molecular weight per flavin of 48,700 was calculated from the peak fraction in the DEAE-cellulose eluate, measuring protein by microbiuret reaction (20) and using a millimolar extinction coefficient of 11.3 for FAD (22). This value is in reasonable agreement with the subunit molecular weight previously cited. Specific activity of various preparations was proportional to flavin content.

It is clear that the enzyme of this present study is physically different from the salicylate hydroxylase of Yamamoto et al. (1). Their enzyme is composed of one FAD and one subunit per 57,200 molecular weight. Our enzyme is a molecule of 91,000 molecular weight with two FAD and two subunits.

Characterization of Enzyme Activity—The requirements for salicylate hydroxylase activity are described in Table III. Salicylate hydroxylase was 76% as active with TPNH as with DPNH as with DPNH under those conditions. In the absence of salicylate, pyridine nucleotide oxidase activity was low: 2 to 4% of normal...
TABLE II

Physical properties of salicylate hydroxylase

Sedimentation equilibrium determinations were by the method of Yphantis (25) with a partial specific volume of 0.728 derived from amino acid analysis (24). Gel filtration was through a 4% Agarose A-5M column by the method of Fish, Mann, and Tanford (26). For further details see text. A molecular weight per flavin assumed an extinction coefficient of 11,300 at 450 nm and protein determination by the microbiuret method (20).

Sedimentation coefficient:
\[ \gamma_{s, o} = 5.91 \times 10^{-13} \sec \]

Molecular weight:
- Native (sedimentation equilibrium) \[ 91,000 \pm 3000 \]
- 6 M guanidine-HCl, reduced and alkylated \[ 46,000 \pm 3000 \]
- Gel filtration \[ 43,000 \pm 3000 \]
- Por flavin (from \( A_{450} \) and protein) \[ 48,700 \]

Structure:
- Two FAD and two subunits per mole

TABLE III

Requirements of salicylate hydroxylase reaction

The complete system below included in a 3-ml cuvette, 6.7 \( \mu \)M FAD, 167 \( \mu \)M pyridine nucleotide, 133 \( \mu \)M sodium salicylate, and 0.02 M potassium phosphate buffer, pH 8.00. Reactions were initiated by addition of enzyme and were followed at 340 nm (except where marked) in a Gilford 240 recording spectrophotometer.

| System            | Pyridine nucleotide | Activity units/ml |
|-------------------|---------------------|-------------------|
| Complete          | DPNH                | 146               |
| Complete          | TPNH                | 111               |
| Omit FAD          | DPNH                | 147               |
| Omit FAD          | TPNH                | 121               |
| Omit salicylate   | DPNH                | 3.58              |
| Omit salicylate   | TPNH                | 0.477             |
| Omit pyridine nucleotide* | DPNH | 0               |
| Omit enzyme       | DPNH                | 0                 |
| Use boiled enzyme | DPNH                | 0                 |

* Measured at 296 nm (see text).

An activity with DPNH and 0.4% with TPNH. The salicylate hydroxylase of Yamamoto et al. (1) also had low DPNH oxidase activity in the absence of substrate, but TPNH under their standard conditions was only 1.2% as effective as DPNH in catalysis of salicylate hydroxylation.

Attempts to determine the optimum pH for the DPNH- and TPNH-dependent salicylate hydroxylase activities were complicated by anion effects (see below) of the different buffers of overlapping pH ranges which were used. In phosphate buffer, which was not inhibitory, the pH optimum for DPNH-dependent salicylate hydroxylase activity was broad with only small differences between pH 7.0 and 8.5, and sharp drops in activity either below pH 6.0 or above pH 9.0. For TPNH-dependent salicylate hydroxylase, the pH optimum was shifted to pH 6.0 to 7.5.

Product Study—The data of Fig. 5 show the enzymatic conversion of salicylate to catechol, employing a coupled assay with salicylate hydroxylase and yeast alcohol dehydrogenase. Ethanol was added to 0.5 M concentration as a source of reducing power, and DPNH was added in only catalytic quantities. Change of the salicylate spectrum into that of catechol can be seen as the reaction progresses with time. Comparison with an independently run spectrum of catechol at the same concentration as the salicylate used indicated that 98% of the salicylate was converted to catechol. The existence of isosbestic points at 291 and 280 nm suggest that only two aromatic species were present during the reaction. Independent assays with separate enzymes showed that ethanol had no effect on salicylate hydroxylase activity at the levels used, and salicylate had no effect on alcohol dehydrogenase.

Stoichiometry—The product study in Fig. 5 indicated a 1:1 stoichiometry between salicylate consumed and catechol formed, and comparison of absorbance changes at 340 and 296 nm indicated a 1:1 ratio of salicylate to DPNH disappearance. The stoichiometry of oxygen consumption to DPNH utilization was measured in the oxygraph and compared with known systems of either washed mitochondrial suspensions from pig liver plus DPNH, or xanthine oxidase plus xanthine. With these as a calibration, for each mole of DPNH consumed in the presence of salicylate, between 0.94 and 0.97 mole of oxygen was utilized. These data indicate that salicylate hydroxylation proceeds with the expected stoichiometry.

\[
\text{Salicylate} + \text{DPNH} + \text{O}_2 + 2\text{H}^+ \rightarrow \text{catechol} + \text{DPN}^+ + \text{H}_2\text{O} + \text{CO}_2
\]
Values of Substrate and Pyridine Nucleotide—The initial rate of reaction at 340 nm with excess DPNH was measured as a function of salicylate concentration; the data so obtained are plotted in Fig. 6 by the method of Lineweaver and Burk (29). The $K_m$ so obtained was 2.7 $\mu M$.

Concentrations of salicylate in excess of 5 mM were found to be inhibitory. Concentrations of 0.2 $M$ inhibited 98%; 50% inhibition was achieved with 0.025 $M$ sodium salicylate. This inhibition is probably an anion effect, as will be described presently.

The data for the apparent $K_m$ values of pyridine nucleotides in the presence of high concentrations of salicylate, and at atmospheric $O_2$ tension, are presented in Fig. 7. It can be seen that, although the apparent $K_m$ for TPNH (99 $\mu M$) is approximately 6 times that for DPNH (17 $\mu M$), the $V_{max}$ values for the two nucleotides are virtually identical. As will be shown (Table IV), the $K_m$ values for DPNH and TPNH were highly dependent upon the presence of salicylate; without aromatic substrate, the DPNH apparent $K_m$ was 710 $\mu M$ and TPNH apparent $K_m$ was 20 $\mu M$. Thus salicylate markedly decreases the apparent $K_m$ of pyridine nucleotide. This phenomenon had also been observed with $P$. putida salicylate hydroxylase (2); with other bacterial flavoprotein hydroxylases, the inducer also appears to markedly decrease pyridine nucleotide $K_m$ (10, 11, 16, 30).

Inhibition by Monovalent Anions—The salicylate hydroxylase of Yamamoto et al. (1) was routinely assayed in Tris-hydrochloride at pH 8.0. Our salicylate hydroxylase showed diminished activity in Tris-HCl buffer as compared to phosphate, but Tris-sulfate was not inhibitory. It was established that the inhibiting factor was chloride, since NaCl would also inhibit catalysis; the curve of percentage inhibition versus molarity of $Cl^-$ was hyperbolic. The point of 50% inhibition was estimated graphically as 0.06 $M$ $Cl^-$. $K_i$ was calculated by Equation 2 of Friddy (31): $K_i = (V_0 - V_i)/V_i (I^-)$, where $V_0$ and $V_i$ are inhibited and uninhibited rates, respectively, and $K_i$ is the reciprocal of the anion concentration ($I^-$), required to cause 50% inhibition. Such a calculation gave $K_i$ values of 0.058 $M$ for KCI and 0.061 $M$ for NaCl. Such inhibition by monovalent anions has also been seen with the enzymes acetoacetic decarboxylase (31) and firefly luciferase (32). Sodium sulfate and potassium phosphate did not inhibit, even at 0.5 $M$ concentration.

Besides chloride, other monovalent anions inhibited salicylate hydroxylase activity. This inhibition was roughly related to anion size in a pattern following the Hofmeister Lyotropic series. The data are presented in Fig. 8. Fluoride, the smallest

![Excess Salicylate](image-url)

**Fig. 7.** Lineweaver-Burk plots of salicylate hydroxylase activity versus pyridine nucleotide concentration. Assay conditions were identical with those in Fig. 6 except that salicylate was 133 $\mu M$ and [DPNH] or [TPNH] were as indicated. The enzyme used was a different sample than that in Fig. 6, hence the different $V_{max}$ values. Both DPNH and TPNH $K_m$ values were determined at the same time.
50% Inhibition of Salicylate Hydroxylase Activity by Sodium Salicylate

The standard assay with a Gilford 240 recording spectrophotometer included in a 3-ml cuvette: 6.7 μM FAD, 133 μM sodium salicylate, 147 μM DPNH, 0.02 M potassium phosphate buffer, pH 7.62, 1.54 μg of purified salicylate hydroxylase, and the sodium salt at the concentrations indicated (except for KNOS). The "50% inhibition point" for fluoride was evaluated by use of Equation 2 of I. Fridovich (31).

Nature of Anionic Inhibition—Inhibition of salicylate hydroxylase by sodium chloride was shown to be competitive with respect to salicylate, as seen in Fig. 9. With other anions, the situation was more complex. With thiocyanate, the lines of a Lineweaver-Burk plot (1/v versus 1/[salicylate]) at different thiocyanate concentrations intersected somewhat to the left of the 1/v axis; with iodide the pattern diverged even more from that expected for competitive inhibition.

A small portion of the inhibition seen in the presence of excess NaBr and NaI (but not NaCl and NaSCN) could consistently be reversed by the addition of FAD. Thus, at 0.2 M NaI concentration, inhibition was 89% in the absence of FAD and 83% in the presence of 0.7 μM FAD; thus, at this NaI concentration, FAD provided a 55% stimulation of residual activity. These data suggest that I− and Br− may, in addition to other effects, cause some dissociation of FAD. Salicylate hydroxylase was inhibited by cyanide (KCN) and azide (NaN3) at concentrations 10 mM or higher, and by urea: 50% inhibition was observed in 2 M urea and 66% in 5 M urea. This inhibition has not yet been studied as a function of time. The previously cited inhibition by excess salicylate may be related to the anionic nature of this compound.

When enzyme previously incubated for short periods of time with varying concentrations of NaCl, NaSCN, or NaI was diluted into a cuvette and assayed, inhibition of activity was reversed by the dilution. In the presence of iodide and thiocyanate (but not chloride), prolonged (1 to 4 hours) incubations at 1 M concentrations caused 70 to 90% irreversible inactivation.

Effects of Mercurials—Enzyme previously incubated at 0°C in 1 mM p-chloromercuriphenylsulfonate was slowly inactivated; this inactivation was first order with a half-time of about 36 min. The presence of 100 μM salicylate slowed the rate of inactivation 5- to 6-fold, while 30 mM benzoate prolonged the half-time of inactivation 53% over that of a control lacking aromatic substrate.

Effects of Benzoate—In the course of studying the substrate or inhibitor specificity of salicylate hydroxylase with various organic compounds, it was found that addition of benzoate to a salicylate reaction would not inhibit, but rather appeared to stimulate (by about 10%) salicylate hydroxylation activity measured at 340 nm in the standard assay. In the absence of salicylate, benzoate caused the rapid disappearance of DPNH. A K_m for benzoate of 2.0 mM was determined, as shown in Fig. 10. This K_m is about 700 times that for salicylate. In this experiment, DPNH concentration was 147 μM.

Fig. 8. Monovalent anion inhibition of salicylate hydroxylase.

Fig. 9. Competitive inhibition of salicylate hydroxylase activity by NaCl. Reaction conditions were similar to those described in Fig. 8. Sodium chloride and salicylate concentrations were varied as indicated in the figure.

Fig. 10. Lineweaver-Burk plot of DPNH oxidase activity versus benzoate concentration. Reaction conditions were identical with those described in Fig. 6 except for the substitution of benzoate for salicylate at the concentrations described in the figure.
tion. Fifty percent inhibition was achieved with 0.2 mM benzoate, as compared to 0.025 mM salicylate.

Product Studies with Benzoate—Stimulation of DPNH oxidation by benzoate suggested that benzoate, like salicylate, was hydroxylated. An attempt to find a hydroxylated product of the benzoate reaction was made with the coupled assay described in Fig. 5, substituting 1 mM benzoate for the salicylate. No change in the benzoate spectrum could be seen after many hours, even when the concentrations of salicylate hydroxylase and alcohol dehydrogenase were increased 10-fold.

A large scale reaction was then attempted employing 1 mM concentrations of both benzoate and DPNH. Disappearance of DPNH (which ceased if the flask was not shaken) was followed by withdrawing aliquots of the mixture and assaying at 340 nm. When 98% of the DPNH had been oxidized, the reaction mixture was acidified with HCl, extracted repeatedly with ether, and the extracts were dried and evaporated. The residue reconstituted in ethanol had a spectrum identical with that of benzoic acid. A control reaction mixture omitting salicylate hydroxylase was run simultaneously. Either extraction of this control produced benzoic acid in the same amounts as the experimental in the incubation mixture. Benzoate thus appeared not to be changed in any way by the enzymatic reaction.

In an aerobic reaction mixture containing excess (300 μM) DPNH and 100 μM benzoate, all of the DPNH present was oxidized. The rate was much faster than in the absence of benzoate. In a similar experiment, salicylate only permitted rapid oxidation of about 100 μM DPNH. Thus, benzoate appeared to act catalytically whereas salicylate, as expected, served as a substrate.

The results of the three experiments described above indicated that, despite its effects on DPNH oxidation, benzoate was not itself hydroxylated. The hypothesis was therefore entertained that benzoate mimicked salicylate by binding at the salicylate site, facilitating DPNH binding, enzyme reduction, and reaction itself hydroxylated. The hypothesis was therefore entertained that benzoate mimicked salicylate by binding at the salicylate site, facilitating DPNH binding, enzyme reduction, and reaction itself hydroxylated. The hypothesis was therefore entertained that benzoate mimicked salicylate by binding at the salicylate site, facilitating DPNH binding, enzyme reduction, and reaction itself hydroxylated. The hypothesis was therefore entertained that benzoate mimicked salicylate by binding at the salicylate site, facilitating DPNH binding, enzyme reduction, and reaction itself hydroxylated. 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of oxygen activation from hydroxylation,” and have called benzoate a “pseudosubstrate” (7-9).

Identity of $V_{\text{max}}$ Values, Kinetic Data—The effects of benzoate and salicylate on apparent $K_m$ and $V_{\text{max}}$ values of pyridine nucleotides are summarized in Table IV. It can be seen that the $V_{\text{max}}$ for pyridine nucleotide disappearance is essentially identical with salicylate or benzoate, as well as TPNH or DPNH. The identity of $V_{\text{max}}$ found here suggests a common rate-limiting region for the reaction mechanism of the benzoate and salicylate reactions, despite the fact that salicylate is hydroxylated and benzoate is not. Stopped flow data, to be presented in the following paper (13), indicated that a combination of two steps rather than a single one was rate-limiting. The $V_{\text{max}}$ for DPNH disappearance in the absence of aromatic substrate was only 3.5% of the rate in the presence of benzoate or salicylate.

As previously stated, the apparent $K_m$ for DPNH is decreased 4.3- and 43-fold in the presence of benzoate and salicylate, respectively, as compared to this value in the absence of aromatic substrate. The same rough proportions are seen with TPNH, which in all cases has a $K_m$ about an order of magnitude higher than that for DPNH.

Search for Free Radical Oxygen Intermediates—A series of experiments were designed to see if oxygen intermediates could be detected in the benzoate-mediated DPNH oxidation reaction. Such intermediates could conceivably have been enzyme-bound in the presence of salicylate, but liberated in analogous reactions with benzoate. Both reducing and oxidizing radicals were sought, with methods utilizing both reduced and oxidized cytochrome c, superoxide dismutase, ethanol, mannitol, and the initiation of sulfite oxidation.

Superoxide (O$_2^-$) is known to reduce ferricytochrome c (14). If O$_2^-$ were produced in the benzoate reaction, or if it were an active intermediate in the hydroxylation of salicylate, then it might be detected by a superoxide dismutase-inhibitable reduction of cytochrome c in the former case, or in the latter, by superoxide dismutase inhibition of salicylate hydroxylation (14). All attempts to reduce cytochrome c by either salicylate or benzoate assay mixtures with salicylate hydroxylase failed, and superoxide dismutase had no effect upon salicylate- or benzoate-mediated DPNH oxidation. Thus if O$_2^-$ were an active intermediate in the hydroxylation reaction, it was tightly bound and inaccessible to the dismutase. No DPNH or TPNH cytochrome c oxidoreductase activity could be observed even at high levels of salicylate hydroxylase. Attempts were made to find oxidizing radicals, such as the hydroxyl radical (OH·) in assay mixtures. Oxidation of dithionite-reduced cytochrome c was used as a test. Enzyme-dependent oxidation of ferricytochrome c in the presence of DPNH and benzoate was seen but this was so slow and small in extent as to be uninterpretable. Mannitol, at 0.4 M concentration, did not interfere with the benzoate- or salicylate-mediated oxidation of DPNH, nor did ethanol at concentrations up to 1.5 M. We do not feel that the results of these attempts to detect oxidizing radicals are as yet interpretable.

Effect of Reaction Products—None of the reaction products, DPN$^+$, catechol, or (in the case of benzoate) H$_2$O$_2$, inhibited salicylate hydroxylation or benzoate-mediated DPNH oxidation. H$_2$O$_2$, even at 1 M concentration, could not substitute for DPNH in the enzymatic hydroxylation of salicylate.

Catalytic Activity of Salicylate Hydroxylase—The product of oxygen reduction in the benzoate-mediated DPNH oxidation was H$_2$O$_2$ (Fig. 12). The slow DPNH oxidase activity in the absence of substrate also produced H$_2$O$_2$, but in less than stoichiometric amounts. The lack of stoichiometry could be accounted for by a low catalatic activity of the purified enzyme, demonstrated in the oxygraph with 200 M H$_2$O$_2$. It has not been established whether the low catalatic activity is inherent, and of possible mechanistic significance, or due to a trace impurity.

Substrates and Pseudosubstrates—A variety of substituted benzoates, salicylates, and aromatic compounds were examined to ascertain enzyme specificity and determine which compounds are substrates like salicylate or pseudosubstrates like benzoate. These compounds were first tested under assay conditions identical with that of salicylate. The results are shown in Table V. Two salicylate analogues, p-amino- and p-hydroxysalicylate, exhibited reaction rates 163 and 196% of that with salicylate, respectively. Other analogues were less reactive. Besides benzoate, several compounds reported inactive with the salicylate hydroxylase of Yamamoto et al. (1) had some activity with this enzyme, i.e. p-hydroxybenzoate, o-nitrobenzoate, and m-hydroxybenzoate. But the activity of the latter two was low, and activity with p-hydroxybenzoate could have been artificial and due to trace contamination with salicylate; high concentrations of p-hydroxybenzoate caused a biphasic oxidation curve for DPNH, with a fast initial rate lasting a short time, followed by a slow rate. Phenol was neither a substrate nor a pseudosubstrate, but inhibited salicylate hydroxylation (50% at 0.05 M and 98% at 0.15 M). The enzyme of Yamamoto et al. (1) required both free hydroxyl and carboxyl groups to be a substrate. We observed the same general specificity. Thus neither methyl salicylate (COOH methyl ester) nor O-acetylsalicylate were substrates; apparent activity with the latter compound paralleled the rate of its spontaneous hydrolysis to salicylate.

Oxygraph Studies on Substrate Analogues—Oxygen uptake...
VI. The pH dependence of the percentage pseudosubstrate activity discussed above. The kinetic experiments performed on the salicylate analogues were indeed chemically altered and, presumably, hydroxylated. It was established by experiments similar to that described in Fig. 12. Most of the compounds listed in Table V with low relative activities (less than 10%) behaved as pseudosubstrates, that is, virtually all of the oxygen consumed was reduced to hydrogen peroxide. Besides benzoate, this category included o-nitrobenzoate, p-hydroxybenzoate, p-hydroxybenzoate, and salicylamide. For the substituted salicylates, DPNH to oxygen stoichiometry was intermediate between the substrate (salicylate) and pseudosubstrate (benzoate) modes. As shown in Table VI, with limiting concentrations of DPNH (200 µM), only part of the oxygen was reduced to water; the remainder was released upon addition of 1500 units of catalase was taken to represent one-half of that reduced to H₂O₂ during the course of the reaction.

The data of Table VI establish that salicylate and benzoate define the extremes in a continuum of types of reaction between the substrate and pseudosubstrate modes. The substituted salicylates, p-hydroxybenzoate, 2,5- and 2,6-dihydroxybenzoates, were at 1 mM concentration; p-amino- and 3-methyl salicylates and 2,3-dihydroxybenzoate were 400 µM; for salicylate and benzoate, see Table IV. The Vₘₐₓ values relative to salicylate (set at 100) were extrapolated to infinite DPNH concentration with the same large excess of aromatic substrate indicated already. The last column represents the percentage of oxygen consumed that is reduced enzymatically to H₂O₂ in the presence of the substrate indicated. These assays, performed in a Gilson oxygraph, were analogous to those in Fig. 12 except that 1 mM EDTA was substituted for FAD. In all cases, aromatic substrate was present at 400 µM concentration and DPNH was limiting at 200 µM. Reactions were initiated by the addition of 80 µg of salicylate hydroxylase; the oxygen released upon addition of 1500 units of catalase was taken to represent one-half of that reduced to H₂O₂ during the course of the reaction.

### Table V

**Activity of substrate analogues**

| Compound             | Relative activity % |
|----------------------|---------------------|
| Salicylate           | 100                 |
| 2,4-Dihydroxybenzoate| 106                 |
| p-Aminosalicylate    | 165                 |
| 2,5-Dihydroxybenzoate| 73.1                |
| 2,3-Dihydroxybenzoate| 63.8                |
| 2,6-Dihydroxybenzoate| 36.5                |
| p-Hydroxybenzoate    | 30.8                |
| o-Nitrobenzoate      | 8.1                 |
| m-Hydroxybenzoate    | 6.1                 |
| 3,4-Dihydroxybenzoate| 2.5                 |
| 3,5-Dihydroxybenzoate| 2.3                 |
| 3-Hydroxy-2-naphthoate| 2.3                |
| Methyl salicylate (ester) | 2.1              |
| 1-Hydroxy-2-naphthoate| 1.9                 |
| Sulfoxalicylate       | 1.1                 |

During reaction of substrate analogues, with and without catalase, was measured in a Gilson oxygraph in experiments similar to that described in Fig. 12. The pH dependence of the percentage pseudosubstrate activity and was ascertained as described in the legend for Table VI, by addition of catalase to the reaction mixture in the oxygraph (Fig. 12). These values were confirmed by experiments with catalase versus catalase-1% ethanol, both present initially. The difference in oxygen uptake between these sets of conditions was taken to represent half the H₂O₂ generated (33). This technique yielded figures in reasonable agreement with those in the last column of Table VI.

The percentage pseudosubstrate activity would vary by ±10% in different experiments and at pH 6.0 the figures were quantitatively substantially different from those shown in Table VI. The pH dependence of the percentage pseudosubstrate activity has not yet been fully investigated.

It was established by experiments similar to that described in Fig. 5, that the four hydroxysalicylates listed in Table VI were indeed chemically altered and, presumably, hydroxylated. Vₘₐₓ and Kₘ values for pseudosubstrates—The data in Table VI summarize the results obtained from Lineweaver-Burk plots of kinetic experiments performed on the salicylate analogues discussed above. The Kₘ values for DPNH as well as the Vₘₐₓ values were determined with large excesses of aromatic compound. Correlations between structure and kinetic behavior cannot be clearly drawn for most compounds; as will be shown in the subsequent paper, the structure of the compound specifically determines the rate of flavin reduction, and this step need not be rate-limiting in catalysis. No striking correlation can be extracted between structure and apparent Kₘ. The data of Table VI establish that salicylate and benzoate define the extremes in a continuum of types of reaction between the substrate and pseudosubstrate modes. The substituted salicylates, whose Kₘ values lie between that for salicylate and benzoate, can react in both substrate and pseudosubstrate modes. Salicylate is the inducer as well as the substrate of salicylate hydroxylase, and its Kₘ values both as a substrate and as an effector of DPNH binding are, as expected, the lowest observed. One other pseudosubstrate, o-nitrobenzoate, was found to behave like benzoate, with 100% pseudosubstrate activity in the oxygraph. Its Kₘ was identical with benzoate's 2 mM, but it had little effect upon the Kₘ for DPNH, which was close to that without aromatic substrate.
DISCUSSION

Salicylate hydroxylase has been induced, isolated, and purified from a soil organism grown on salicylate as sole carbon source. Like a similar enzyme of Yamamoto et al. (1), Katagiri et al. (2, 3), Takemori et al. (4, 5), Suzuki (6), and Katagiri and Take

mori (34), it contains FAD as the sole prosthetic group. The enzyme of the Japanese workers contained one FAD per 57,000 g; our salicylate hydroxylase is a dimer consisting of two FAD and two subunits with a molecular weight of 91,000.

Both enzymes are external monoxygenases, requiring reduced pyridine nucleotide as the external electron source. This requirement is met only by DPNH for Yamamoto's enzyme (1), but is satisfied either by DPNH or (at higher $K_m$ but with similar $V_{max}$), TPNH, with our enzyme. Both enzymes require, for active hydroxylation to occur, an aromatic substrate bearing free carboxyl groups ortho to a hydroxyl. This hydroxylation process fits into the mixed function oxidase (35, 36) or mono
oxynase (37) categories proposed by Mason and Hayashi, respectively, whereby a molecule of oxygen ($O_2$) is reduced, with 1 oxygen atom appearing in the hydroxylated product and the other oxygen atom appearing as water.

Perhaps the most striking difference between the enzyme described herein and that of the Japanese workers is the effect of benzoate, which was reported to be neither a substrate nor a competitive inhibitor (1-4) 

2 With the present enzyme, benzoate serves as a pseudosubstrate, causing the enzyme to assume a conformation favorable for the binding of DPNH and as will be shown (13), subsequent reduction of the flavin. However, since benzoate appears totally unsuitable as a hydroxylatable substrate, the oxygen which interacts with reduced flavin is released as $H_2O_2$. The enzyme is thus converted into a peroxide-producing DPNH oxidase. We have used the term “uncoupling of oxygen activation from hydroxylation” to describe this phenomenon (7). A partial uncoupling effect had previously been seen (12) with phenylalanine hydroxylase, a teridine-containing enzyme; the observations reported with salicylate hydroxylase represent stoichiometric rather than partial conversion of oxygen to $H_2O_2$ in the presence of a completely nonhydroxylatable compound. Since the publication of our original report (7), similar uncoupling phenomena have been described for other flavin hydroxylases: $p$-hydroxybenzoate hydroxylase (10) and orcinol hydroxylase (11, 39). Thus potential for diversion of oxygen from hydroxylation to peroxide formation may be a general phenomenon for flavin (and probably other types of) hydroxylases.

Salicylate, the inducer of the enzyme, behaves as a “perfect” substrate, with virtually no leakage of oxygen into $H_2O_2$. Benzoate, a compound completely unsuitable for hydroxylation, is the “perfect pseudosubstrate,” diverting all oxygen consumption into $H_2O_2$. Intermediate between these extremes are a series of compounds whose hydroxylation is “leaky,” yielding both hydroxylated product and peroxide, and utilizing more reduced pyridine nucleotide than in “perfectly efficient” hydroxylation. The subsequent paper in this series (13), which examines the reaction mechanism, permits a more detailed description of how a material may operate in the substrate, pseudosubstrate, or mixed mode.

It is becoming tempting to formulate certain generalizations which seem to hold for a small but perhaps representative number of bacterial flavoprotein hydroxylases. It appears that the compound on which the bacterium is grown induces an enzyme which (while maintaining a broad specificity) considers the inducing substrate as being uniquely perfect: i.e. it is hydroxylated without peroxide production, it is bound with the lowest $K_m$, and it also has the greatest effect in facilitating pyridine nucleotide binding and reduction of enzyme (1, 5, 7, 10, 11, 16, 30, 34). Other compounds, while capable of being bound to the enzyme and effecting the binding of pyridine nucleotide, are themselves bound at a higher $K_m$ than the inducing substrate, and are less effective in the facilitation of pyridine nucleotide binding. Such materials if hydroxylated at all, tend to be hydroxylated in a leaky manner. These hydroxylases, therefore, permit the employment of a novel criterion for the “perfectness” of an induced enzyme: the ratio of the frequencies with which the “natural” and the “unnatural” reactions occur.

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