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# Hydroxylation of *o*-Halogenophenol and *o*-Nitrophenol by Salicylate Hydroxylase[1]

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**Salicylate hydroxylase [EC 1.14.13.1] from *Pseudomonas putida* catalyzed the formation of catechol from substrate analogues such as *o*-nitro-, *o*-amino-, *o*-iodo-, *o*-bromo-, and *o*-chloro-phenol by removing the ortho-substituted groups. They are converted into nitrite, ammonia, and halide ions, respectively. Kinetic parameters of these reactions were determined by spectrophotometric and polarographic methods. Hydroxylation of *o*-nitro- or *o*-iodophenol proceeds with the unusual stoichiometry of 2 : 1 : 1 for consumed NADH, $O_2$-uptake, and catechol formed. Other ortho-substituted phenols examined also gave the same results. Like salicylate, these substrates perturb the absorption spectrum of salicylate hydroxylase in the visible region, indicating the formation of enzyme·substrate complexes. Titration experiments with ortho-substituted phenols gave the dissociation constants of the complexes. The complexes were quantitatively reduced with NADH or dithionite without detectable formation of the intermediates. The fact that one atom of $^{18}O_2$ was incorporated into the produced catechol in hydroxylation of *o*-nitrophenol indicates that the reaction is of monooxygenase nature. It is concluded that salicylate hydroxylase cleaves the C-N and C-X bonds of ortho-substituted phenols.**

Salicylate hydroxylase [salicylate, NADH:oxygen oxidoreductase (1-hydroxylating, decarboxylating), EC 1.14.13.1] from *Pseudomonas putida*, S-1, catalyzes the decarboxylative hydroxylation of salicylate (*1-3*). It is a flavin-dependent monooxygenase containing 1 mol of FAD per mol enzyme with a molecular weight of 54,000, and has also been purified from other Pseudomonads (*4-6*). The enzyme is a unique monooxygenase for catalyzing hydroxylation with decarboxylation of salicylate or deformylation of salicylaldehyde (*7*). It has a relatively narrow specificity for the substrates such as phenol compounds with a carboxyl or carbonyl substituent in the ortho position (*1, 7*). When salicylate was the substrate, the nascent decarboxylation product was proved to be not a carbonate ion but carbon dioxide (*7*). The evidence suggested that hydroxylation of salicylate is carried out by attack of a cationic species of activated oxygen on the carbon atom bound on the carbonyl group. Therefore, we have particularly focussed our study on the enzyme reactions for *o*-phenolic compounds substituted with a nucleophilic or electrophilic group.

In this paper, we describe the hydroxylation of *o*-nitro-, *o*-halogeno-, and *o*-amino-phenol by the salicylate hydroxylase. The enzyme catalyzes oxygenation of these ortho-substituted phenols with an unusual stoichiometry. The reaction might proceed by the oxene mechanism proposed by Entsch *et al.* (*8*). A part of this work has been reported previously (*9*).

## MATERIALS AND METHODS

*Materials*—*o*-Aminophenol, *o*-chlorophenol, *o*-iodophenol, *o*-nitrophenol, salicylaldehyde, and FAD were purchased from Wako Pure Chemicals. NADH was obtained from Kyowa Hakko. Salicylic acid was from Nacalai Tesque, L-epinephrine bitartrate from Sigma, and *o*-bromophenol from Tokyo Kasei. *o*-Nitrophenol was recrystallized from toluene, and *o*-aminophenol was purified by sublimation (*10*). $H_2{}^{18}O$ (43.467 atom%) was obtained from Yeda, and $^{18}O_2$ was prepared by electrolysis (*11*).

Salicylate hydroxylase was purified from *Pseudomonas putida*, S-1 (*12*). Partially purified nitrate reductase was prepared from *E. coli* (*13*). Superoxide dismutase was purified from bovine erythrocytes (*14*), and metapyrocatechase was from *Pseudomonas putida*, T-2 (*15*).

All other reagents were of reagent grade and available from commercial sources.

*Enzyme Activities*—Salicylate hydroxylase was assayed generally by the reported procedures (*1*). The NADH-oxidation activity of salicylate hydroxylase was determined from decrease of the absorbance at 340 nm. The reaction mixture (1 ml) contained the enzyme, 7 $\mu$M FAD, substrates, NADH, and 30 mM phosphate buffer. The concentrations of substrate and NADH, and the pH of the buffer used are described in the legend of the figures for each experiment.

Oxygen uptake was measured by the polarographic method with a Clark-type electrode mounted in a 3.5-ml temperature-controlled reaction vessel (Yellow Spring Instrument). The reaction mixture (3.5 ml) contained 30 mM phosphate buffer (pH 7.0), 7 $\mu$M FAD, 200 $\mu$M

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NADH, and the varied concentrations of substrates and salicylate hydroxylase. After the reaction, the amount of hydrogen peroxide produced was estimated from that of the oxygen generated by the addition of 0.2 $\mu$M catalase.

The coupling ratio (%) of hydroxylation is defined as the ratio of the moles of catechol formed to a half of the NADH oxidized. In the case of salicylate, the value is the ratio of formed catechol to oxidized NADH (*16*).

*Anaerobic Experiments*—Anaerobic spectral observations of the enzyme reaction were carried out in a 3-ml Thunberg-type cuvette with a vaccine rubber stopper. The enzyme solution (40 $\mu$M) was alternately evacuated and purged with oxygen-free nitrogen three times. An oxygen-free solution of *o*-iodophenol was injected into the cuvette after the second cycle of purging of nitrogen to avoid denaturation of the enzyme. The enzyme was reduced by injection of NADH or dithionite with a gas-tight microsyringe.

*$^{18}O_2$ Incorporation Experiments*—The experiments were carried out in Thunberg-type tubes by the previously reported procedures (*17*). The reaction mixture for *o*-nitrophenol hydroxylation contained 7 $\mu$M FAD, 20 mM *o*-nitrophenol, 3.8 $\mu$M salicylate hydroxylase, 1 mM $NAD^+$, 1 M glucose, 7 $\mu$g catalase, and 200 mM phosphate buffer (pH 7.5) in the main tube (1.95 ml) and 2.4 units of glucose dehydrogenase in 0.05 ml of the same buffer in the side arm. For salicylate hydroxylation, 40 mM salicylate and 100 mM Tris-HCl buffer (pH 9.0) were used. The gas phase was exchanged with a mixture of $^{18}O_2$ (25.1 atom % excess) and nitrogen in a 1 : 4 ratio. For the control experiments, air was used. Reactions with *o*-nitrophenol were carried out at 25°C for 2 1/2 h and for 3 h with salicylate. After incubation, 0.05 ml of 4 N HCl was added; phenol compounds were extracted three times with 2 ml diethylether, and the extract was concentrated under a nitrogen flow. Mass-spectra of the samples were measured with a Nihon Denshi JMS-D100 mass spectrophotometer with a JGD20-KP-gas chromatographic inlet mounted with a column (2 mm×1m, glass) packed with Gas-chrom Q coated with Silicone OV-1 (2%). Helium was used as the carrier gas. The column temperature was 96°C for nitrophenol and catechol, and 140°C for salicylate and catechol; ion source temperature, 190°C; electron energy, 22 eV; accelerating voltage, 3 kV; and ionizing current, 300 $\mu$A. Each analysis represents an average of at least six independent measurements for a given sample. The signal intensities of both the molecular ion, $M^+$, and $(M+2)^+$ in each mass spectrum were used to calculate the incorporation of $^{18}O_2$ into each compound.

*Chromatographic Methods*—TLC was used for identification of catechol. Samples in ether extract were spotted on a silica gel sheet, Eastman Chromagram Sheet, No. 6060 (Eastman Kodak), and developed with the following solvent mixtures: methylethylketone-$H_2O$-diethylamine (921 : 77 : 2, v/v/v), benzene-dioxane-acetic acid (90 : 25 : 4, v/v/v); benzene-ethanol-acetic acid (48 : 8 : 4, v/v/v), or benzene-ethanol (95 : 4, v/v). Catechol was detected by irradiation with a UV lamp and by reaction with phenol reagent ($FeCl_3$ solution).

Gas chromatography was carried out with a Nihon Denshi model JGC-20 KEP gas chromatograph. $N_2$ was used as the carrier gas. The column (4 mm×2 m) was packed with Gas-chrom Q (80-100 mesh) coated with Silicone OV-1 (2%). The retention time for catechol was 1 min at 140°C column temperature.

*Other Methods*—For determination of catechol, metapyrocatechase was added to the reaction mixtures and the amount of $\alpha$-hydroxymuconic semialdehyde formed was measured spectrophotometrically at 375 nm using the molecular coefficient $3.3\times10^4$ $M^{-1}\cdot cm^{-1}$ (*18*). Nitrite was determined with Griess-Ilosvey reagent (*13*), and nitrate with the same reagent after its reduction to nitrite with nitrate reductase (*13*). Ammonia was determined by a micro diffusion method on a Conway dish and nesslerization (*19*). Hydroxylamine was by the method of Novak *et al.* (*20*). Iodide was determined by the method of Iwasaki *et al.* (*21*). Protein was determined by a modified microbiuret method (*22*) with crystalline bovine serum albumin as the standard.

## RESULTS AND DISCUSSION

*Hydroxylation of o-Nitro- and o-Halogeno-Phenols*—Previous work on salicylate hydroxylase has suggested that *o*-phenols having an electron-attractive, substituted group are also oxygenated to catechol by the enzyme (*9*). We examined the enzymic oxidation of NADH and the consumption of oxygen depending on the presence of *o*-phenols such as *o*-nitro-, *o*-halogeno-, and *o*-amino-phenols and found that like salicylate and salicylaldehyde, the enzyme oxidizes these phenols, except *o*-fluorophenol. Catechol formation from these compounds was confirmed by chromatographic methods and by the metapyrocatechase method (*18*). *o*-Fluorophenol was not converted to catechol, though NADH oxidation was observed. Other substrate analogs such as salicylalcohol, cresol, phenol, and guaiacol were not oxidized to catechol at pH 7-8. Meta- and para-substituted halogenophenols had little effect on NADH oxidation at pH 8.0.

Table I shows the kinetic parameters of salicylate hydroxylase for those phenols, which were determined from the initial rate of NADH oxidation at the pH-optimum for each substrate. The results indicate that *o*-nitrophenol and *o*-iodophenol are relatively good substrates for the enzyme. The coupling ratios, determined from catechol formation and NADH oxidation or from molecular oxygen uptake, are slightly lower than that for salicylate. With *o*-chlorophenol and *o*-aminophenol, the coupling ratios are very low and most of the NADH consumed was used for formation of hydrogen peroxide and not for substrate hydroxylation. The data in Table I suggest that the apparent $K_m$ values for *o*-phenols correlate to p*K*a values for the hydroxyl groups, and these groups participate in the binding of phenol substrates to the active site of the enzyme. On the other hand, considering the apparent $V_{max}$ and coupling ratios, the velocity of hydroxylation correlates with the induction effect of the substituted group, because the effects of these substituents on the benzene ring are in the following order: $NO_2$, I, Br, Cl, and $NH_2$. The results support a mechanism of anionic elimination of the substituent group following the attack of a hypothetical $OH^+$ or oxene, generated from the peroxyflavin of the enzyme, on the carbon atom in the ortho-position of phenolic carbon (*8*).

The NADH oxidation activity of the enzyme in the presence of 1 mM *o*-iodophenol and the molar ratio of

TABLE I. **Steady-state kinetic parameters of phenols substituted at the ortho-position with salicylate hydroxylase.** The rate of NADH oxidation was spectrophotometrically measured at 20°C, and the concentrations of substrate phenol or NADH were varied. The kinetic parameters were obtained by double reciprocal methods. The assay mixture (1 ml) contained 30 mM phosphate buffer (pH 8.0), 7 $\mu$M FAD, 0.35 mM NADH, 0.4 $\mu$M salicylate hydroxylase (0.08 $\mu$M for the salicylate reaction), and substrate phenol (0.5 mM salicylate, 0.5 mM *o*-nitrophenol, 1 mM *o*-bromophenol, 1 mM *o*-chlorophenol, 1 mM *o*-fluorophenol, 1 mM *o*-aminophenol, or 1.5 mM *o*-iodophenol).

| Substrate | p*K*a[a] | $K_m'$ ($\mu$M) for | | | $V_{max}'$ ($min^{-1}$)[c] | Coupling ratio (%)[e] | |
|---|---|---|---|---|---|---|---|
| | | Substrate[b] | NADH[c] | Oxygen[d] | | from catechol formed | from oxygen consumed[f] |
| *o*-Nitrophenol (pH 7.2) | 7.23 | 34 | 76 | 130 | 320 | 72 | 75 |
| *o*-Iodophenol (pH 7.0) | 8.51 | 290 | 83 | 98 | 280 | 63 | 50 |
| *o*-Iodophenol | | 76 | 240 | 220 | 160 | 81 | 73 |
| *o*-Bromophenol | 8.42 | 190 | 370 | — | 110 | 55 | 33 |
| *o*-Chlorophenol | 8.48 | 210 | 390 | — | 96 | 12 | 15 |
| *o*-Fluorophenol | 8.81 | 550 | 400 | — | 98 | 0 | 0 |
| *o*-Aminophenol | 9.71 | 2,000 | 170 | — | 28 | 3.8 | 8.8 |
| Salicylate (pH 7.0) | 2.97 | 1.6 | 3.9 | 100 | 830 | 98 | 83 |

[a] p*K*a value for phenolic hydroxyl group of substrate (*23*). [b] Concentration of substrate phenol was varied from 0.1 to 2,000 $\mu$M. [c] NADH concentration was varied from 0.5 to 500 $\mu$M. [d] $K_m'$ for oxygen was estimated at 25°C by the polarographic method. The assay system contained 30 mM phosphate buffer (pH 8.0), 7 $\mu$M FAD, 200 $\mu$M NADH, 0.86 $\mu$M salicylate hydroxylase (0.1 $\mu$M for the salicylate reaction), and substrate (1 mM salicylate, 1 mM iodophenol, or 1.5 mM for the other phenols). [e] The definition of the coupling ratio is stated in "MATERIALS AND METHODS." [f] The values were estimated from the data obtained by the polarographic assay method using the equation $1-X/Y$, where $Y$ is the total $O_2$-uptake and $X$ is the hydrogen peroxide estimated by the addition of catalase.

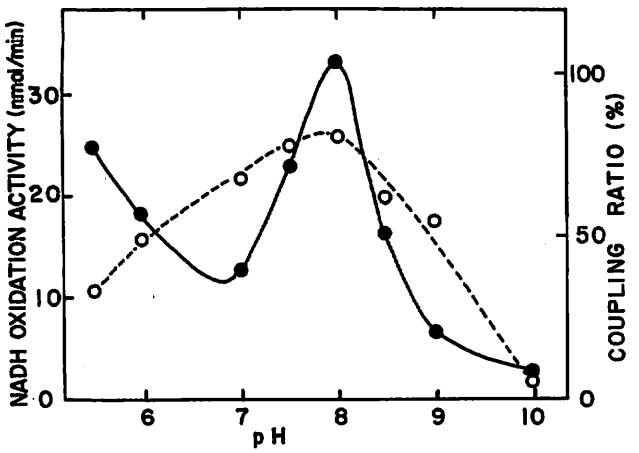



Fig. 1. **Effect of pH on *o*-iodophenol hydroxylation with salicylate hydroxylase.** The incubation mixture (1 ml) contained 7 $\mu$M FAD, 1 mM *o*-iodophenol, 100 $\mu$M NADH, and 0.4 $\mu$M salicylate hydroxylase in 30 mM buffer solutions. The buffer systems employed were sodium acetate (pH 5.5 and 6.0), potassium phosphate (pH 6.5-8.5), and glycine-NaOH (pH 9.0-10.0). Reaction was initiated by addition of NADH, and the initial velocities of its oxidation were measured (●). After complete oxidation of the added NADH, aliquots of the reaction mixture were taken for determination of the amount of catechol formed. The molar ratio of catechol formed per half of the NADH oxidized (the coupling ratio) was plotted (○).

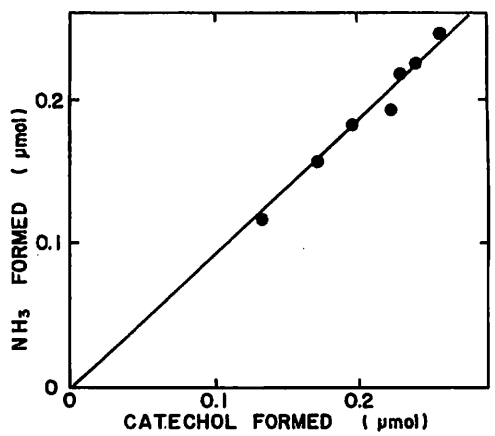



Fig. 2. **Hydroxylation of *o*-aminophenol by salicylate hydroxylase.** The reaction mixture (1 ml) contained 7 $\mu$M FAD, 4-34 mM *o*-aminophenol, 1.2 mM NADH, 0.94 $\mu$M salicylate hydroxylase, and 30 mM phosphate buffer (pH 7.6). The reaction was started by addition of NADH at 20°C. After complete oxidation of NADH, an aliquot of 0.4 ml of the incubation mixtures was used to determine catechol, and the other portion was taken for ammonia determination. Catechol and ammonia were determined as described under "MATERIALS AND METHODS."

catechol formed to NADH oxidized (the coupling ratio) were plotted against pH (Fig. 1). Maximum catechol formation was observed at pH 8.0 and the curve is different from that of the NADH oxidation activity. The maximum NADH oxidation was determined to be at pH 8.0 with *o*-bromophenol, 8.2 with *o*-chlorophenol, 7.2 with *o*-nitrophenol, and 8.0 with *o*-aminophenol. *o*-Aminophenol is a poor substrate (Table I), and its deaminative hydroxylation produced an equimolar amount of ammonia and catechol at pH 7.6 (Fig. 2). Hydroxylamine was not detected in the incubated medium. This indicates that the deamination product formed by cleavage of the C-N bond is not $NH_2^+$ species.

*Stoichiometry of o-Nitrophenol Hydroxylation*—Salicylate hydroxylase catalyzes hydroxylation of *o*-nitrophenol and produces catechol and nitrite. Nitrate was not detected in the incubation mixture by the nitrate-reductase method (*13*), nitrite being the only denitrification product. The stoichiometry of this reaction was determined as shown in Table II. The reaction products and $O_2$-consumption were determined after complete oxidation of the added NADH. The results indicate that the stoichiometry is 2 : 1.08 : 0.87 : 0.90 for NADH oxidized, *o*-nitrophenol consumed, catechol formed, and nitrite formed (Expt. A). By the polarographic method, similar values were obtained, namely 2 : 0.92 : 0.83 : 0.98 for NADH oxidized, oxygen consumed, catechol formed, and nitrite formed (Expt. B). The obtained value is quite different from that of hydroxylation for salicylate (*3*) and allows us to write the following

TABLE II. **Stoichiometry of *o*-nitrophenol hydroxylation.** Experiment (A) was carried out by spectrophotometric methods at 20°C. The reaction mixture (2 ml) contained NADH, 1 mM *o*-nitrophenol, 7 $\mu$M FAD, 0.47 $\mu$M salicylate hydroxylase, and 30 mM phosphate buffer (pH 7.0). Experiment (B) was carried out by polarographic methods. The reaction mixture (3.5 ml) contained NADH, 7 $\mu$M FAD, 1 mM *o*-nitrophenol, 0.47 $\mu$M salicylate hydroxylase, and 30 mM phosphate buffer (pH 7.0). After complete oxidation of the added NADH, catechol and nitrite were determined by the methods described in "MATERIALS AND METHODS." *The enzyme was heated at 100°C for 3 min.

| Experiments | | NADH added | Nitrophenol disappeared | Oxygen consumed | Catechol formed | Nitrite formed |
|---|---|---|---|---|---|---|
| | | | (nmol) | | | |
| (A) | | | | | | |
| I | Complete | 362 | 186 | — | 146 | 164 |
| II | Complete | 212 | 114 | — | 94 | 90 |
| III | −NADH | 0 | 0 | — | 0 | 1.6 |
| IV | −*o*-Nitrophenol | 362 | — | 0 | 0 | 1.0 |
| V | −Enzyme, +Boiled enzyme* | 362 | 0 | — | 0 | 1.0 |
| (B) | | | | | | |
| VI | Complete | 721 | — | 378 | 266 | 322 |
| VII | Complete | 308 | — | 151 | 118 | 138 |
| VIII | −NADH | 0 | — | 33 | 0 | 19 |
| IX | −*o*-Nitrophenol | 634 | — | 420 | 1.8 | 16 |
| X | −Enzyme, +Boiled enzyme* | 634 | — | 49 | 0 | 16 |

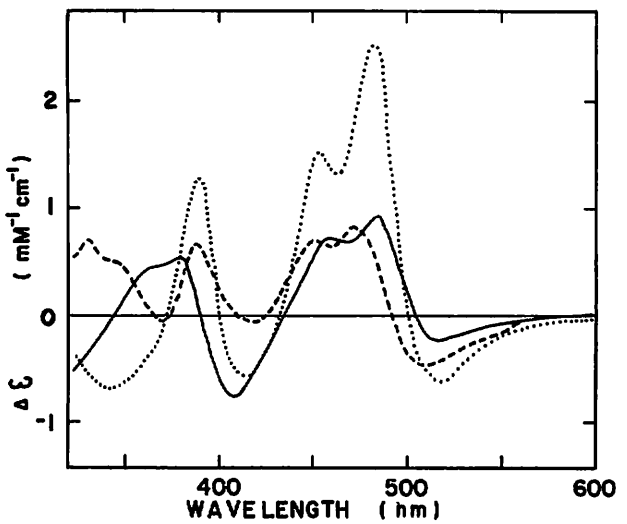
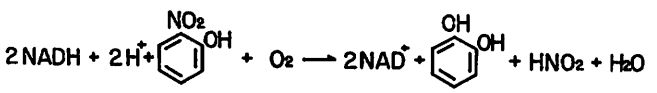



Fig. 3. **Spectral perturbation of salicylate hydroxylase by substrate phenol.** Difference spectra of the enzyme mixed with salicylate (·····), *o*-nitrophenol (- - -), or *o*-iodophenol (——) *versus* the enzyme were taken using a reference and a sample cell (1 ml), which contained 40 $\mu$M salicylate hydroxylase and 30 mM phosphate buffer (pH 7.0, 7.2, or 8.0, respectively). Substrate solution (0 to 201 $\mu$mol) was added to the sample cell and the same volume of water was added to the reference cell, and the difference spectra were recorded. Volume corrections were carried out for each spectrum. Each spectrum was expressed as the millimolar extinction coefficient of the difference with respect to the enzyme, which was obtained by extrapolation of the spectral change to infinite substrate concentration.

equation for hydroxylation of *o*-nitrophenol:




When *o*-iodophenol was the substrate, the oxidation products were identified as catechol and iodide, and a similar stoichiometry was also observed; *i.e.* NADH oxidized, oxygen consumed, catechol formed, and iodide formed = 2 : 1.08 : 0.81 : 0.84. These values include about 19% uncoupling of the hydroxylation from the oxidation of NADH. *o*-Bromophenol and *o*-chlorophenol were also oxidized by the enzyme with similar stoichiometries (data not shown).

TABLE III. **$^{18}O_2$ Incorporation into the reaction product in the salicylate hydroxylase reaction.** The data were calculated by subtracting the values of the relative intensities of the molecular ion peak, $M^+$ and $(M+2)^+$, in the air-experiment from those of the $^{18}O_2$-experiment. The data represent mean value ± SD for six determinations.

| Exp. | Substrate | Atom % excess of $^{18}O$ in medium | Atom % excess of $^{18}O$ in catechol | $^{18}O$ enrichment |
|---|---|---|---|---|
| I | Salicylate | 25.1 | 25.9±0.1 | 103±1 |
| II | *o*-Nitrophenol | 25.1 | 22.9±1.0 | 91±5 |

*Formation of Enzyme·Substrate Complex*—Addition of *o*-iodophenol or *o*-nitrophenol to a solution of salicylate hydroxylase induced spectral perturbation by formation of a binary complex between the enzyme and phenol (Fig. 3). The degree of perturbation is lower than that induced with salicylate. Titration of the enzyme with *o*-iodophenol yielded an apparent dissociation constant of the enzyme·substrate complex of 240 $\mu$M in 30 mM phosphate buffer at pH 7.0, and 130 $\mu$M at pH 8.0. When the concentration of phosphate buffer, pH 8.0, was increased to 400 mM, the dissociation constant changed to 210 $\mu$M. The value for salicylate in 30 mM phosphate buffer, pH 7.0, is 3.5 $\mu$M (*12*). Similar perturbations were also observed by addition of *o*-bromophenol, *o*-aminophenol, or phenol to the enzyme. The facts indicate that binding of substrate phenol induces some changes of hydrophobicity and conformation of the flavin environment of the enzyme.

*Reduction of Enzyme·Substrate Complex*—When the complex of enzyme and *o*-iodophenol was titrated anaerobically with NADH, the enzyme-bound FAD was converted quantitatively into the fully reduced form without detectable formation of any intermediate, such as semiquinone or charge transfer complex (Fig. 4). Further addition of NADH did not change the spectrum. Dithionite titration of the enzyme·substrate complex gave also the same result. The reduced complex was reoxidized by air with concomitant production of catechol and iodide in a ratio of 0.45 mol product per mol enzyme. The ratio suggests that the reaction proceeds on oxidation of a half amount of the reduced enzyme·substrate complex and then rapid reduction of the nascent product with the other half of the complex. In a catalytic turnover reaction, the second

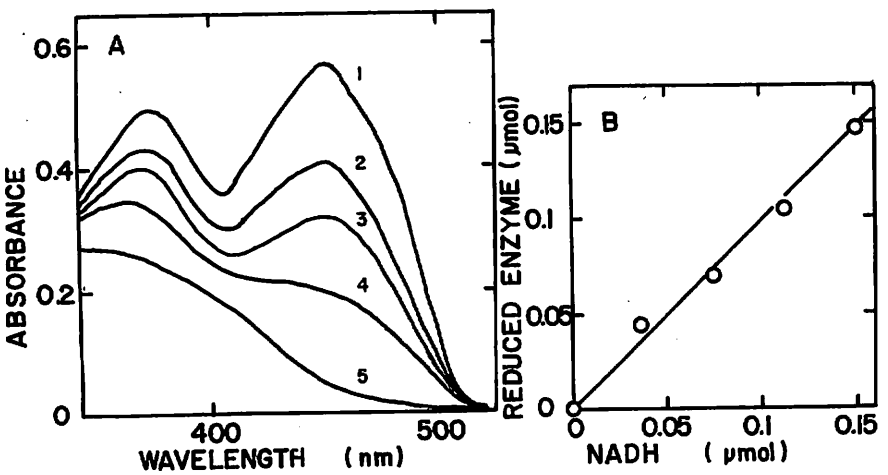



Fig. 4. **Reduction of the salicylate hydroxylase·*o*-iodophenol complex with NADH.** A: A Thunberg-type cuvette (3 ml) containing 50 $\mu$M salicylate hydroxylase and 1 mM *o*-iodophenol in 30 mM phosphate buffer (pH 8.0) was used. The titrations with 5 mM NADH were made at 20°C. The molar ratios of NADH to the enzyme were 0 (1), 1/4 (2), 2/4 (3), 3/4 (4), and 1 (5). B: Plots of the data from A against the amounts of added NADH.

NADH molecule might play the same role for the reduction.

*$^{18}O_2$ Incorporation and an Attempt to Detect $O_2^-$ Intermediate*—To clarify what the oxidant of the second NADH molecule is, incorporation of molecular oxygen and its intermediates were studied. *o*-Nitrophenol hydroxylation by the salicylate hydroxylase was carried out under an atmosphere of $^{18}O_2/N_2$ (1 : 4), and the produced catechol was analyzed by mass-spectrometry (Table III). The results exhibit the incorporation of one atom of $^{18}O_2$ into the hydroxyl group of the produced catechol and ruled out the possibility of incorporation of oxygen from water. Exchange of oxygen atoms between that of the substrate phenol and the atmosphere was not observed by analysis of recovered *o*-nitrophenol and salicylate. Like hydroxylation of salicylate, the reaction of *o*-nitrophenol is also monooxygenation with the unusual stoichiometry.

Epinephrine was oxidized in the oxidation of *o*-nitrophenol or *o*-iodophenol by salicylate hydroxylase. Attempts were made to detect superoxide anion in the reaction. Superoxide dismutase did not affect the oxidation of epinephrine at pH 7.0, but was inhibitory at pH 9.0. Half inhibition was obtained with 4 units of superoxide dismutase. This enzyme (100 units), however, did not inhibit the formation of catechol and nitrite in the *o*-nitrophenol hydroxylation by 85 or 850 nM hydroxylase at pH 9.0. Neither mannitol (40 mM) nor sodium azide (0.2 mM) inhibited *o*-iodophenol hydroxylation. These results suggest that no free oxygen radical participates in *o*-nitro- or *o*-iodo-phenol hydroxylation, though inhibition of the epinephrine oxidation by superoxide dismutase is not yet clear.

The present study demonstrates that salicylate hydroxylase catalyzes hydroxylation and dehalogenation of *o*-halogenophenols and also denitrification of *o*-nitrophenol with an unusual stoichiometry. Enzymatic dehalogenations of halogenated compounds have been reported on hydroxylation of 4-fluorophenylalanine by pteridine-containing phenylalanine hydroxylase (*24*), $\beta$-elimination of $Cl^-$ from $\beta$-chloroalanine by D-amino acid oxidase (*25*), $\beta$-chlorolactate by lactate oxygenase (*26*), hydroxylation of fluoroderivatives of *p*-hydroxybenzoate by *p*-hydroxybenzoate hydroxylase (*27*), and defluorination of fluoro-L-tyrosine by mushroom tyrosinase (*28*). 2-Nitropropane dioxygenase was reported to catalyze denitrification of 2-nitropropane (*29, 30*). In these studies, the unusual stoichiometry described in this paper was found with phenylalanine hydroxylase (*24*) and *p*-hydroxybenzoate hydroxylase (*27*). It is of interest to compare the mechanisms of these reactions with the present results and to identify the oxidant involved with the second NADH molecule.

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