# Mechanism-Based Inactivation of Dopamine β-Hydroxylase by *p*-Cresol and Related Alkylphenols

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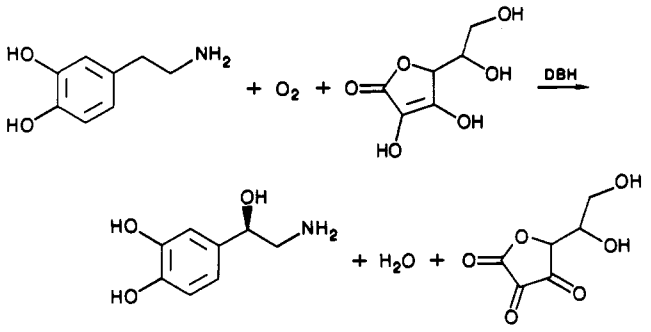
ABSTRACT: The mechanism-based inhibition of dopamine β-hydroxylase (DBH; EC 1.14.17.1) by *p*-cresol (4-methylphenol) and other simple structural analogues of dopamine, which lack a basic side-chain nitrogen, is reported. *p*-Cresol binds DBH by a mechanism that is kinetically indistinguishable from normal dopamine substrate binding [DeWolf, W. E., Jr., & Kruse, L. I. (1985) *Biochemistry 24*, 3379]. Under conditions (pH 6.6) of random oxygen and phenethylamine substrate addition [Ahn, N., & Klinman, J. P. (1983) *Biochemistry 22*, 3096] *p*-cresol adds randomly, whereas at pH 4.5 or in the presence of fumarate "activator" addition of *p*-cresol precedes oxygen binding as is observed with phenethylamine substrate. *p*-Cresol is shown to be a rapid ($k_{inact}$ = 2.0 min$^{-1}$, pH 5.0) mechanism-based inactivator of DBH. This inactivation exhibits pseudo-first-order kinetics, is irreversible, is prevented by tyramine substrate or competitive inhibitor, and is dependent upon oxygen and ascorbic acid cosubstrates. Inhibition occurs with partial covalent incorporation of *p*-cresol into DBH. A plot of −log $k_{inact}$ vs. pH shows maximal inactivation occurs at pH 5.0 with dependence upon enzymatic groups with apparent p$K$ values of 4.51 ± 0.06 and 5.12 ± 0.06. *p*-Cresol and related alkylphenols, unlike other mechanism-based inhibitors of DBH, lack a latent electrophile. These inhibitors are postulated to covalently modify DBH by a direct insertion of an aberrant substrate-derived benzylic radical into an active site residue.

Dopamine β-hydroxylase (DBH;[1] EC 1.14.17.1) is a copper-containing, glycosylated mixed-function oxidase that, under physiological conditions, catalyzes the benzylic hydroxylation of dopamine to norepinephrine (Skotland & Ljones, 1979; Levin et al., 1960; Rosenberg & Lovenberg, 1980; Villafranca, 1981; Ljones & Skotland, 1984):




The stereochemical course of this oxidation proceeds with retention of configuration at the benzylic carbon with absolute specificity for the *pro-R* hydrogen (Battersby et al., 1976). In contrast to this stereochemical specificity, DBH shows surprisingly little discrimination toward organic substrates and will even catalyze sulfoxidation at sulfur (May & Phillips, 1980), epoxidation of olefins, and N-dealkylations of methylamines (Padgette et al., 1985), in addition to hydroxylating a number of other simple substrate analogues. The relative lack of specificity shown by DBH for organic substrates has already been exploited in the design of β-substituted phenethylamines as mechanism-based inhibitors (Klinman & Krueger, 1982; Mangold & Klinman, 1984; Padgette et al., 1985). In addition, in an approach reminiscent of those successfully applied to P-450 enzymes, alternate substrates bearing cyano (Colombo et al., 1984), ethenyl (Rajashekhar et al., 1984), or ethynyl (Colombo & Villafranca, 1984) substituents and lacking the basic dopamine nitrogen exhibit the properties of mechanism-based inhibitors. Implicit in these studies and in consequent mechanistic arguments has been the assumption that irreversible inhibition derives from a covalent alkylation of the active site by a radical derived from the pendent functional group present in the alternate substrate (Fitzpatrick & Villafranca, 1985), i.e. that cyano, ethynyl, or ethenyl groups are required for inactivation. Here we demonstrate this not to be the case.

In the course of steady-state inhibition studies directed toward the kinetic characterization of multisubstrate inhibitors of DBH, *p*-cresol was employed as a dopamine substrate analogue (DeWolf & Kruse, 1985; Kruse et al., 1986b). We report here that *p*-cresol, in addition to being a competitive inhibitor, is also an alternate substrate for DBH which forms a ternary enzyme–oxygen–*p*-cresol complex in a fashion that is kinetically indistinguishable from that formed with phenethylamine substrates. In addition, we report experiments which establish that *p*-cresol is one of the most effective $k_{cat}$ inactivators yet described for DBH and, more importantly, that this mode of inactivation occurs with a molecule that *lacks* a latent electrophile. Consistent with these observations, a new mechanistic alternative for the inactivation of DBH is suggested to occur via the insertion into an active site residue of a benzylic radical derived from alternative substrate. The mechanism-based inactivation of DBH by *p*-cresol, a molecule that lacks a latent electrophilic group, cautions against the assumption that inactivation of DBH by alternate substrates

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[1] Abbreviations: DBH, dopamine β-hydroxylase; HPLC, high-performance liquid chromatography; HEPPS, *N*-(2-hydroxyethyl)-piperazine-*N*′-3-propanesulfonic acid; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry.

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bearing latent electrophilic groups necessarily involves these groups.

## Experimental Procedures

*Materials.* Tyramine hydrochloride, L-ascorbic acid, and DL-octopamine were purchased from Sigma. Crystalline catalase (specific activity 65 000 units/mg as claimed by supplier) was obtained from Boehringer. AG 50W-X8 ion-exchange resin was obtained from Bio-Rad. Aquasol-2 was purchased from Du Pont, and Ready-Solv MP was from Beckman. *p*-Cresol, *m*-cresol, phenol, 4-hydroxybenzyl alcohol, 3-hydroxybenzyl alcohol, 4-hydroxybenzaldehyde, 3-hydroxybenzaldehyde, 4-methylcatechol, and 4-ethylphenol were all obtained from Aldrich. *m*-Cresol and 3-hydroxybenzaldehyde were used without further purification. *p*-Cresol was purified by distillation. Phenol was recrystallized from petroleum ether. 4-Hydroxybenzyl alcohol was purified by silica gel flash chromatography (Still et al., 1978) using 1:1 methylene chloride–ethyl acetate as eluant followed by trituration with ether–hexane. 3-Hydroxybenzyl alcohol was recrystallized twice from methylene chloride containing a small amount of ethyl acetate. 4-Hydroxybenzaldehyde was purified by two recrystallizations from ether. 4-Methylcatechol was recrystallized from carbon tetrachloride following silica gel flash chromatography using 5% methanol in methylene chloride as eluant. 4-Ethylphenol was recrystallized 3 times from hexane. The multisubstrate inhibitor 1-(3,5-difluoro-4-hydroxybenzyl)imidazole-2-thione was synthesized as recently reported (Kruse et al., 1986a). All other chemicals were commercial products of the highest purity available. Bovine dopamine β-hydroxylase was isolated from either whole adrenal medulla or partially purified chromaffin vesicles according to our improved procedure.[2] Enzyme from whole medulla (specific activity 22–31 units/mg in the octopamine assay at 37 °C and pH 5.0 with tyramine substrate) was used routinely in the present study except for the [$^3$H]-*p*-cresol labeling experiments. These were conducted with DBH of higher specific activity (42 units/mg in the octopamine assay) which had been isolated from partially purified chromaffin vesicles.

*Synthesis of [$^3$H]-p-Cresol.* High specific activity tritium-labeled *p*-cresol was prepared by reductive tritiation of 3-bromo-4-methylphenol (Baddeley & Plant, 1943) over 10% palladium on carbon in ethanol with carrier-free $^3H_2$. Purification of crude [$^3$H]-*p*-cresol was accomplished by flash chromatography on silica gel with methylene chloride as eluant followed by HPLC on a 10 × 250 mm Lichrosorb Diol (10-μm) column with 0.1% trifluoroacetic acid in methylene chloride as the mobile phase. A radiochemical purity of 99.89% for [$^3$H]-*p*-cresol was established on an analytical Lichrosorb column. The specific activity of the sample prepared in this way was 15 Ci/mmol with the $^3$H incorporated specifically at the 3-position as evidenced by $^3$H and $^1$H NMR.

*General Methods.* The activity of DBH was determined by measuring the production of octopamine from tyramine substrate by using a periodate cleavage procedure similar to that reported (Nagatsu & Udenfriend, 1972) or by following the uptake of oxygen substrate with a Yellow Springs Instruments Model 53 oxygen electrode. With the exception of $V_{max}$(app) both assays led to identical results. Specific assay conditions are described below for each experiment. Unless specified otherwise, all reaction mixtures contained 50 mM buffer at the desired pH (sodium acetate for pH 5.5 and below and sodium phosphate above pH 5.5), 10 mM ascorbic acid, 1 mg/mL catalase, $CuCl_2$ (10 μM at pH 5.5 and lower, 5 μM above pH 5.5), and enough NaCl to bring the ionic strength to 0.2. Other additions are specified in the methods for particular experiments. Except where noted, all incubations were performed at 37 °C in a New Brunswick R-76 shaker/bath oscillating at 120 Hz. Oxygen electrode experiments were also performed at 37 °C. Scintillation counting was performed by using a Beckman LS-5801 liquid scintillation spectrometer and a standard tritium program with quench correction. The scintillation samples contained 10 mL of Aquasol-2.

*Steady-State Inhibition Studies.* Initial velocity kinetic studies of several of the reported inactivators were carried out in standard incubation mixtures containing various concentrations of tyramine and inactivator. When fumarate was present, this was included at a concentration of 10 mM. To ensure minimal inactivation of the enzyme during the octopamine method of assay, incubation times were kept to 5 min for *p*- and *m*-cresol and 20 min for the slower inactivators. When oxygen was the varied substrate, incubations were carried out in the oxygen electrode, but velocities were based on the assay (Nagatsu & Udenfriend, 1972) for octopamine produced. This allowed a facile correction for oxygen consumption due to *p*-cresol turnover. When the oxygen electrode assay was used, shorter (30 s–2 min) incubation times were used.

*HPLC Identification of Hydroxylation Products.* Standard incubation mixtures at pH 6.5 that contained 10 mM *p*-cresol in a total volume of 2.0 mL were used for product analysis of *p*-cresol turnover. The incubation was carried out with or without 0.25 mg of DBH. After 60 min, the incubation mixtures were quenched into acetonitrile, filtered, and injected onto a Beckman Ultrasphere ODS C-18 reverse-phase column equilibrated with 10% acetonitrile in water and running at a flow rate of 1.0 mL/min. A gradient of 10–70% acetonitrile was used to elute the products, which were detected by UV absorbance at 275 nm. Identity of the products was confirmed by comparing the retention times with those of known standards and by GC/mass spectral analysis of the collected HPLC fractions. This analysis was performed on a Finnigan–MAT quadrupole mass spectrometer operating in both the electron impact and chemical ionization modes using methane as the reagent gas. For gas chromatography, a fused silica capillary DB-17 column (15 m) was used with a programmed temperature gradient from 70 to 250 °C changing at 10 °C/min. The molecular weight scanning range was 50–500.

*Inactivation Experiments.* Inactivation of DBH was carried out by using a preincubation mixture consisting of the standard incubation mixture without tyramine and an appropriate concentration of inactivator in a total volume of 0.25 mL. The inactivation was initiated by the addition of 0.016–0.065 mg of DBH. Beginning at approximately 20 s and every 5 or 10 min thereafter for periods up to 60 min, aliquots (5 μL) were withdrawn and added to 1.0-mL samples of the standard incubation mixture containing 10 mM tyramine. These were incubated for an appropriate time and then quenched by the addition of 0.2 mL of 3.0 M trichloroacetic acid. Octopamine production was determined as described above. Ascorbic acid dependence was demonstrated by omitting it from the preincubation mixtures.

The inactivation of DBH at pH 5.0 was shown to be oxygen-dependent by procedures similar to those described above with the following exceptions. Preincubation mixtures (0.4

[2] W. E. DeWolf, Jr., P. J. Goodhart, and L. I. Kruse, unpublished results.

mL) were placed in 1-mL Reacti-Vials (Pierce) fitted with spin vanes and set in the heating block of a Reacti-Therm stirrer/heater (Pierce). Each vial was partially evacuated and flushed repeatedly with either argon or the appropriate oxygen–nitrogen mixture and then stirred with positive pressure and continuous flow of gas. The inactivation was started by the addition of 0.032 mg of DBH. Aliquots (15 μL) were removed at 20 s and every 1 min thereafter for a period of 5 min and assayed as described above.

Protection of DBH by tyramine substrate was demonstrated at pH 5.0 in preincubation mixtures identical with those described above with the following exceptions. The volume of the preincubation mixture was 1.0 mL. Tyramine was included in the preincubation at concentrations ranging from 2 to 48 mM. In order to minimize turnover of substrate during the preincubation, the concentration of DBH was lowered 10-fold to 0.0065 mg/mL. The size of the aliquots assayed for remaining DBH activity was increased to 25 μL, and appropriate blanks were run to correct for the octopamine produced during the preincubation.

Protection by the competitive inhibitor 1-(3,5-difluoro-4-hydroxybenzyl)imidazole-2-thione was investigated at pH 5.0 by using the standard conditions described above in preincubation mixtures containing inhibitor at concentrations ranging from 25 to 500 nM.

To demonstrate that catalase was not inactivated under conditions of DBH inactivation, preincubation mixtures (pH 5.0) containing the highest concentration of inactivator used for a particular $k_{inact}$ experiment were incubated without DBH. Aliquots (10 μL) were withdrawn at 0, 25, and 60 min and diluted into 3.0 mL of 0.5 M potassium phosphate, pH 7.0. Then, 20 μL aliquots of diluted preincubation mixture were assayed for catalase activity by continuous monitoring at 240 nm according to the assay described by Bergmeyer (1955).

*Demonstration of Irreversibility.* DBH that had been partially inactivated with *p*-cresol was divided into three 1-mL aliquots, and each was dialyzed for 48 h at 4 °C against 4 × 1 L of 0.1 M buffer at different pHs. The buffers used were sodium acetate, pH 4.5, sodium phosphate, pH 6.5, and HEPPS sodium salt, pH 8.5, each containing 20 μg/mL catalase and NaCl to bring the ionic strength to 0.2. In a separate experiment, inactivated enzyme was dialyzed for 48 h at 4 °C against 4 × 1 L of 0.2 M sodium acetate, pH 5.5, which contained 5 mM tyramine. Activity was measured before and after dialysis and compared with noninactivated DBH controls which were dialyzed under identical conditions. Additional evidence for irreversibility was obtained by assaying fully inactivated DBH before and after HPLC size-exclusion chromatography on a Bio-Rad TSK-250 column (7.5 × 300 mm) equilibrated with 5 mM potassium phosphate, pH 6.5, containing 0.2 M KCl and running at a flow rate of 0.5 mL/min.

*Inactivation with [$^3H$]-p-Cresol.* Test tubes containing DBH (0.2 mg/mL, 2.68 μM), $CuCl_2$ (10 μM), and catalase (1 mg/mL), with or without *p*-cresol (10 mM of either 18 or 36 μCi/μmol) and with or without ascorbate (10 mM) in 0.2 M pH 5.5 acetate buffer (1.25-mL total sample volume), were incubated at 37 °C for 3 h. Enzyme activity was determined immediately after the inactivation had been initiated by the addition of ascorbate and after 2.5 h by diluting 5 μL of the inactivation sample into 1-mL standard assays. After 3 h the samples were stored at 4 °C until 250 μL of each was injected onto a Bio-Rad 7.5 × 300 mm TSK-250 size-exclusion HPLC column run (0.5 mL/min) with 0.2 M KCl in 5 mM $KH_2PO_4$, pH 6.5 buffer. Fractions (0.5 mL) were collected, and 200- or 400-μL portions were counted in 10 mL of Aquasol-2 in a Beckman LS-5801 scintillation counter. The concentration of DBH in each fraction was determined by integrating the $A_{280}$ absorbance relative to the $A_{280}$ absorbance of reference samples of DBH. Results were calculated in the form of mole ratio *p*-cresol: DBH subunit, with a DBH subunit molecular weight of 70 000.

*Mass Action Experiments.* DBH peak fractions (0.5 or 1.0 mL) from the TSK-250 HPLC runs were incubated with 50 or 100 μL, respectively, of 100 mM *p*-cresol solution (final *p*-cresol concentration of 9.1 mM) for 72 h in a reciprocating shaker bath at 37 °C. Samples were stored at 4 °C, and 250-μL aliquots were reinjected onto the TSK-250 column as previously described. Mole ratios of *p*-cresol to DBH were determined as before.

In order to ensure that the inactivated DBH did not bind to either the sample vessels or the size-exclusion column, an $A_{280}$ recovery control was performed with approximately 40 μg of DBH inactivated with unlabeled *p*-cresol. The absorbance yield was measured on a similar sample of native DBH and compared to the inactivated DBH recovered following fractionation on the TSK-250 column.

*Data Analysis.* Multiple-parameter nonlinear regression analysis of the steady-state inhibition data was accomplished by using the COMP and NONCOMP programs of Cleland (1979). The $k_{obsd}$ values were determined from linear regression fits of log (percent activity) vs. time. The values for $k_{inact}$ and $K_I$ were determined by fitting replots of $1/k_{obsd}$ vs. 1/[inactivator] to either the HYPER or HYPERL programs of Cleland (1979). The pH data from the inactivation studies were analyzed by using BELL (Cleland, 1979). A value for the true $k_{inact}$ was achieved by fitting the $k_{obsd}$ values obtained at pH 5.0 with different concentrations of *p*-cresol and oxygen to the EQORD program (Cleland, 1979).

## RESULTS

*Steady-State Kinetics.* *p*-Cresol is an excellent competitive inhibitor vs. tyramine substrate at both pH 4.5 ($K_{is}$ = 0.21 mM, Figure 1A) and pH 6.6 [$K_{is}$ = 5.5 mM, tyramine $K_m$(app) = 5.7 mM, data not shown], the pH extremes employed in the present study. At high pH the random addition of oxygen and phenethylamine substrates has been demonstrated (Klinman et al., 1980; Miller & Klinman, 1983; Ahn & Klinman, 1983) whereas at lower pH or in the presence of fumarate the kinetic mechanism becomes predominantly ordered with phenethylamine substrate binding before oxygen. Steady-state experiments with *p*-cresol support this change in kinetic mechanism and underscore the similarity in binding of this inhibitor and normal phenethylamine substrates to DBH. At pH 6.6 (random conditions) *p*-cresol is a noncompetitive inhibitor with oxygen substrate (Figure 1B), but at pH 4.5 (Figure 1C) or in the presence of fumarate activator (data not shown) induced substrate inhibition is observed. This is consistent with an ordered kinetic mechanism where *p*-cresol now binds prior to oxygen (Cleland, 1977). Unfortunately, a more detailed study of this induced substrate inhibition is limited by the solubility of oxygen substrate at 37 °C and 760-torr pressure.

*p-Cresol Turnover Studies.* Incubation of *p*-cresol with DBH in the presence of ascorbate and oxygen cosubstrates leads to product formation as demonstrated by HPLC analysis of the quenched incubation mixtures (Figure 2). HPLC of the incubation mixtures on a C-18 reverse-phase column demonstrates the production of 4-hydroxybenzyl alcohol and 4-hydroxybenzaldehyde under standard assay conditions. The HPLC retention times of products, as compared with those

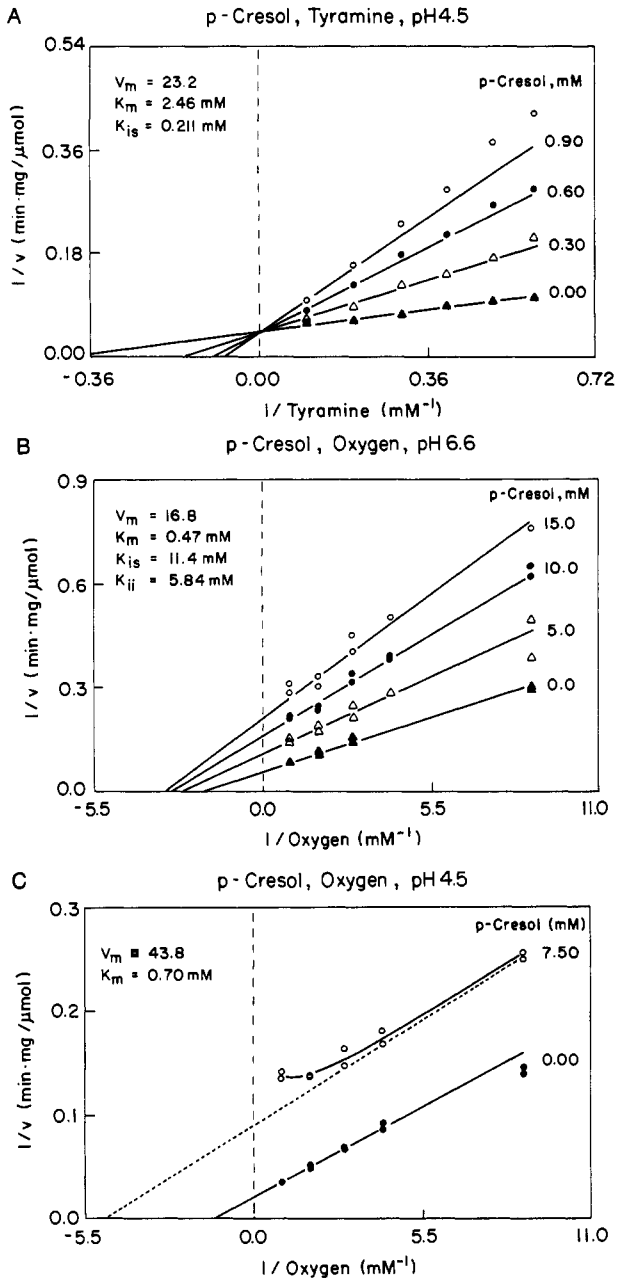



FIGURE 1: Lineweaver–Burk steady-state kinetic patterns for *p*-cresol. Oxygen substrate concentrations were varied in an oxygen electrode. In order to separate the true rate of tyramine turnover from the total rate of oxygen consumption due to combined turnover of both tyramine and *p*-cresol, velocities were based on octopamine production as described under Experimental Procedures. (A) Competitive binding of *p*-cresol vs. tyramine substrate under ordered conditions at pH 4.5. (B) Noncompetitive binding of *p*-cresol vs. oxygen substrate under random conditions at pH 6.6. (C) *p*-Cresol-induced inhibition by oxygen substrate under ordered (tyramine binding precedes oxygen) conditions at pH 4.5.

for known standards, and GC/MS analysis of collected HPLC fractions allowed an unambiguous assignment of chemical structure.

Kinetic constants for the turnover of *p*-cresol were determined by oxygen uptake experiments under initial velocity conditions where HPLC product analysis confirmed that further oxidation of the small amounts of 4-hydroxybenzyl alcohol product did not occur. These experiments show *p*-cresol to be a good alternate substrate for DBH (Table I). The $K_m$(app) for *p*-cresol is comparable to the $K_m$(app) for tyramine substrate under these conditions while $V_{max}$(app) is substantially reduced. Unlike tyramine substrate, *p*-cresol

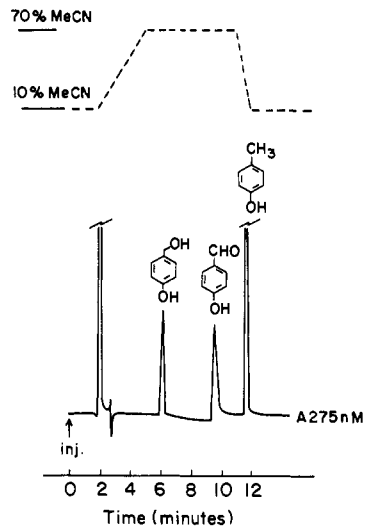



FIGURE 2: Reverse-phase C-18 HPLC product analysis of *p*-cresol turnover by DBH. A 10–70% gradient of acetonitrile in water was used to elute products, the identities of which were established by comparison to known standards by the procedures reported in the text.

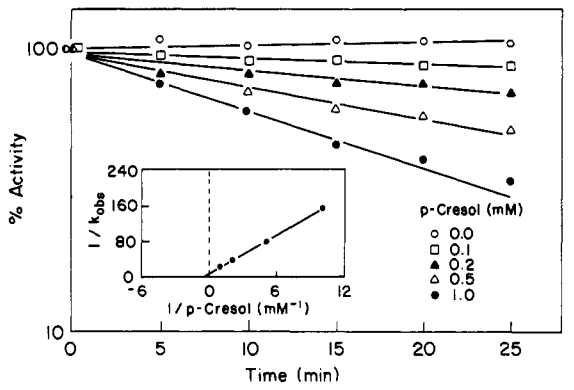



FIGURE 3: Semilog plot of percent activity remaining vs. time for the pseudo-first-order, irreversible inhibition of DBH by *p*-cresol at pH 5.0. Inset: Replot of $1/k_{inact}$(obsd) vs. 1/[*p*-cresol] demonstrates saturation.

Table I: Kinetic Constants for Tyramine and *p*-Cresol[a]

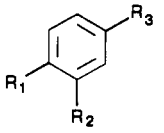
| | $V_{max}$(app)[b] | | $K_m$(app)[c] | |
|---|---|---|---|---|
| compd | 0.24 mM $O_2$ | 1.14 mM $O_2$ | 0.24 mM $O_2$ | 1.14 mM $O_2$ |
| tyramine | 59.2 ± 2.6 | 82.3 ± 3.8 | 1.4 ± 0.2 | 1.3 ± 0.2 |
| *p*-cresol | 6.9 ± 0.3 | 17.0 ± 0.7 | 2.4 ± 0.2 | 1.6 ± 0.2 |

[a] Determined by oxygen uptake experiments at pH 5.0 in the standard assay. [b] Units of μmol/(min·mg of DBH). [c] Units of mM.

shows an appreciable increase in $V_{max}$(app) at pH 5.0 as oxygen concentration is increased from 0.24 to 1.14 mM.

*Irreversible Inactivation by p-Cresol.* Incubation of DBH with *p*-cresol under the standard assay conditions leads to a rapid, pseudo-first-order, time-dependent loss of catalytic activity for more than one half-life (Figure 3). A replot of the inactivation data shows saturation by *p*-cresol and a rapid inactivation, with $k_{inact}$(app) = 0.3 ± 0.06 $min^{-1}$. The inactivation event is dependent upon the presence of both ascorbate and oxygen substrates. Protection against inactivation is afforded by either tyramine substrate or the multisubstrate inhibitor 1-(3,5-difluoro-4-hydroxybenzyl)imidazole-2-thione (DeWolf & Kruse, 1985; Kruse et al., 1986b). Sufficient bovine catalase was included in all inactivation experiments to protect DBH from irreversible $H_2O_2$-mediated inactivation. Additionally, in appropriate control experiments the catalytic activity of catalase was assayed and was found to be unaltered

Chart I: Substituted Hydroxyphenyl Inhibitors of DBH



| no. | compound | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|---|
| **1** | *p*-cresol | OH | H | $CH_3$ |
| **2** | *m*-cresol | H | OH | $CH_3$ |
| **3** | 4-methylcatechol | OH | OH | $CH_3$ |
| **4** | 4-ethylphenol | OH | H | $CH_2CH_3$ |
| **5** | 3-hydroxybenzyl alcohol | H | OH | $CH_2OH$ |
| **6** | phenol | OH | H | H |

by the inactivation conditions. This excludes the possibility of *p*-cresol-mediated inactivation of catalase resulting in buildup of $H_2O_2$ and inactivation of DBH by this autoxidation product of ascorbate. Lastly, the inactivation of DBH by *p*-cresol is completely irreversible as evidenced by a failure to reactivate upon prolonged dialysis (48 h) at pH 4.5, 6.5, or 8.5 with frequent changes of buffer. Dialysis at pH 5.5 in the presence of tyramine substrate also fails to reactivate. Further evidence for irreversibility of the inactivation event is found in the failure of analytical size-exclusion HPLC to reactivate *p*-cresol-inactivated DBH (vide infra).

*pH Dependence of Inactivation.* The inactivation of DBH by *p*-cresol is pH-dependent, as shown by a plot of −log $k_{inact}$(app) vs. pH. This experiment demonstrates a maximum rate of inactivation at pH 5.0, and the bell-shaped curve yields two apparent p$K$ values for inactivation as 4.51 ± 0.06 and 5.12 ± 0.06, one of which is reminiscent of a value 5.39 ± 0.25 determined for normal catalysis with dopamine (Ahn & Klinman, 1983).

*Oxygen Dependence, True $k_{inact}$, and Partition Ratios for p-Cresol Inactivation.* The dependence of $V_{max}$(app) for *p*-cresol turnover (Table I) upon oxygen concentration led to the expectation that the true $k_{inact}$ for *p*-cresol would be substantially larger than the $k_{inact}$(app) at 0.24 mM oxygen. An increase in oxygen concentration to 1.14 mM led to an increase in $k_{inact}$(app) to 0.51 min$^{-1}$. The use of several oxygen and *p*-cresol concentrations allowed the calculation of a true $k_{inact}$ of 2.0 ± 0.6 min$^{-1}$ at pH 5.0 and 37 °C from the equation that describes equilibrium ordered binding:

$$k_{inact} = k_{inact}(\text{app})(1 + K_{O_2}/[O_2] + K_{i,p\text{-cresol}}K_{O_2}/[p\text{-cresol}][O_2])$$

Partition ratios were calculated from values for *p*-cresol $V_{max}$(app) (Table I) and *p*-cresol $k_{inact}$(app) at an oxygen concentration of 0.24 mM, assuming a subunit molecular weight of 70000.[3] Partition ratios were also calculated from the mole ratio of [$^3$H]-4-hydroxybenzyl alcohol product to the DBH subunit present under conditions that produce complete inactivation. The partition ratio estimated from the kinetic constants at 0.24 mM oxygen (1600 ± 330:1)[4] corresponds well to that calculated from 4-hydroxybenzyl alcohol product formation (1300:1). These partition ratios establish *p*-cresol as a suicide inactivator comparable in effectiveness to others previously reported (Mangold & Klinman, 1984; Padgette et al., 1985; Fitzpatrick & Villafranca, 1985).

*Other Inactivators of DBH.* The irreversible inactivation of DBH reported here is not restricted to the simple substrate analogue *p*-cresol. Virtually all dopamine or tyramine substrate analogues that lack the basic side-chain nitrogen show time-dependent, pseudo-first-order inactivation of DBH which is consistent with a mechanism-based inactivation (Chart I, Table II). The insolubility of added 4-hydroxybenzyl alcohol in the standard assay has precluded study of this compound as a potential inactivator. However, it is worth noting that under prolonged turnover conditions the 4-hydroxybenzyl alcohol produced in situ undergoes further hydroxylation to the aldehyde, presumably via the *gem*-diol. The $k_{inact}$(app) rates observed for compounds **2**–**5** (Table II) demonstrate some of these, like *p*-cresol, to be among the most effective yet reported for DBH. In contrast, phenol (**6**), which is a competitive inhibitor vs. tyramine (data not shown), is not a time-dependent inactivator of DBH.

Table II: Kinetic Constants for Competitive and Irreversible Inhibition of DBH by Compounds **1**–**6**

| compd no. | $K_{is}$ (mM)[a] | $K_I$ (mM)[b] | $k_{inact}$(app) (min$^{-1}$)[c] |
|---|---|---|---|
| **1** | 0.18 ± 0.01 | 2.0 ± 0.5 | 0.30 ± 0.06 |
| **2** | 2.6 ± 0.2 | 12.8 ± 7.6 | 0.30 ± 0.06 |
| **3** | 3.0 ± 0.4 | 1.5 ± 0.4 | 0.033 ± 0.004 |
| **4** | | 1.1 ± 0.3 | 0.11 ± 0.02 |
| **5** | | 10.4 ± 1.2 | 0.026 ± 0.001 |
| **6** | 1.7 ± 0.1 | | 0 |

[a] This value is the apparent slope inhibition constant vs. tyramine substrate at pH 4.5. [b] This $K_I$ value results from a replot of $1/k_{obsd}$ vs. 1/[*p*-cresol] and is therefore a complex ratio of multiple microscopic rate constants that does not represent a true dissociation constant. [c] These experiments were performed at a single oxygen concentration, 21%, and therefore these inhibition rates are not true $k_{inact}$ values.

Table III: Stoichiometry of [$^3$H]-*p*-Cresol Labeling of DBH

| experimental conditions | % DBH activity remaining | initial label[a] | after mass action[a] |
|---|---|---|---|
| +ascorbate, +[$^3$H]-*p*-cresol | ~0 | 0.95 ± 0.12 ($N$ = 5)[b] | 0.56 ± 0.13 ($N$ = 6) |
| −ascorbate, +[$^3$H]-*p*-cresol | >90 | 0.39 ± 0.05 ($N$ = 3) | 0.11 ± 0.03 ($N$ = 6) |
| +ascorbate, −[$^3$H]-*p*-cresol | >95 | | |

[a] Molar ratio *p*-cresol:DBH subunit; subunit molecular weight of 70000. [b] $N$ refers to the number of experiments.

Covalent Labeling of DBH by [$^3$H]-*p*-Cresol. Because of the efficient mechanism-based inactivation shown by **1**–**5**, the most effective inhibitor, *p*-cresol, was chosen for active site titration by radiochemical labeling. An efficient radiochemical synthesis of high specific activity [$^3$H]-*p*-cresol was accomplished with complete incorporation of $^3$H at the ring position meta to the phenolic hydroxyl group, as evidenced by $^1$H and $^3$H NMR analysis. This meta regiochemistry of labeling was essential to ensure against potential nonenzymatic exchange of label which is known to occur at the ortho position of phenols.

Incubation of DBH with [$^3$H]-*p*-cresol under conditions that produce total inactivation, followed by HPLC of the incubation mixture on an analytical TSK-250 HPLC size-exclusion column, led to clean separation of DBH from both catalase and small molecules (Figure 4). Gel electrophoresis of the HPLC-separated proteins demonstrated an efficient resolution between DBH and catalase (Figure 5).

Control experiments established the quantitative recovery of either native or inactivated DBH from this HPLC separation technique. Appropriate protein-containing HPLC fractions were collected, and $^3$H content was established by scintillation counting. The 4-hydroxybenzyl alcohol fraction was also collected and counted for partition ratio calculations.

[3] This molecular weight is the average of the subunit molecular weights reported for DBH.

[4] Standard errors were derived for the expression $W = ax/y$, where $a$ is a constant, $x$ represents *p*-cresol $V_{max}$(app), $y$ represents $k_{inact}$(app), and the resulting standard error in $W$ ($S_W$) is propagated from the standard errors in $x$ and $y$: $S_W^2 = (a^2/y^2)S_x^2 + (a^2x^2/y^4)S_y^2$.

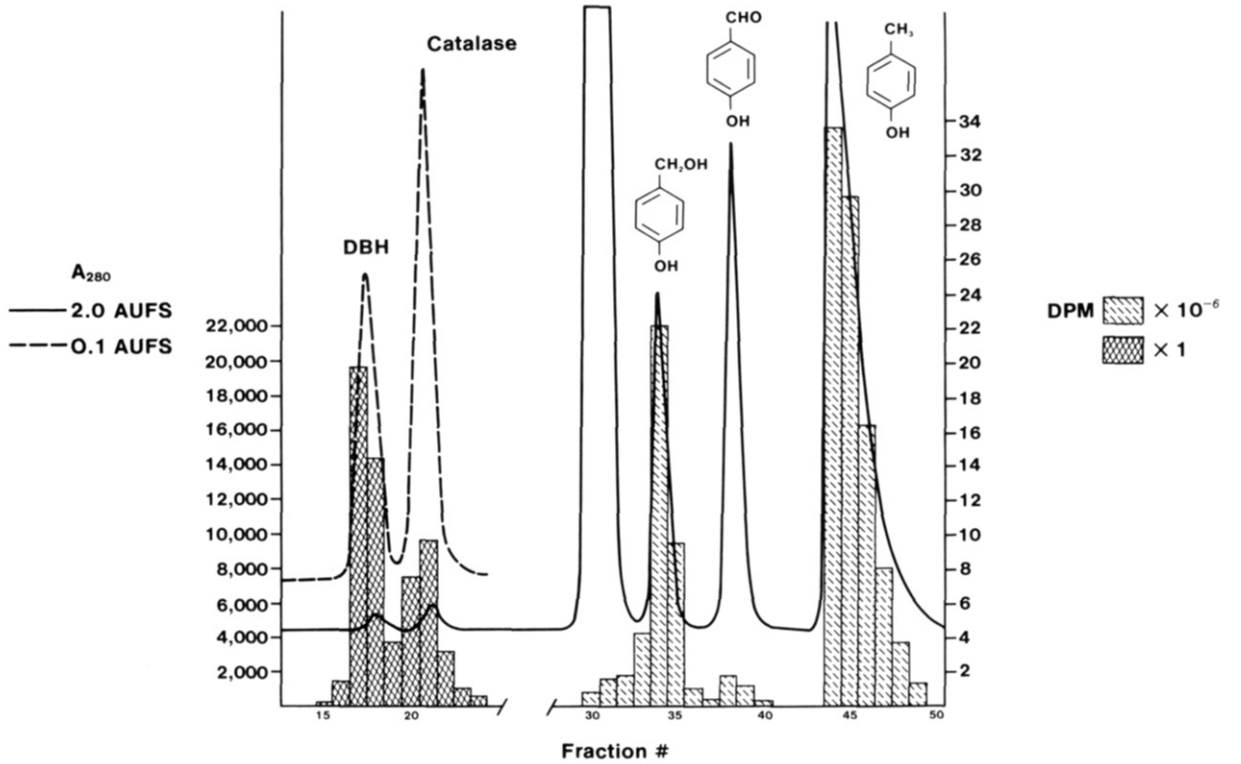



FIGURE 4: Analytical size-exclusion HPLC of incubation mixture from [$^3$H]-*p*-cresol inactivation of DBH. A Bio-Rad TSK-250 7.5 × 300 mm column was run at 0.5 mL/min with pH 6.5 phosphate buffer containing 0.2 M KCl.

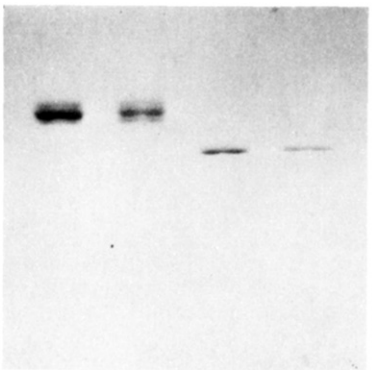



FIGURE 5: Laemmli gel (1.5-mm slab gel, 10% polyacrylamide) of pooled protein fractions from HPLC separation of incubation mixture (Figure 4). Coomassie brilliant blue stain was used to visualize protein. Lanes 1 and 2, fractions 17 and 18 (Figure 4); lanes 3 and 4, fractions 20 and 21 (Figure 4).

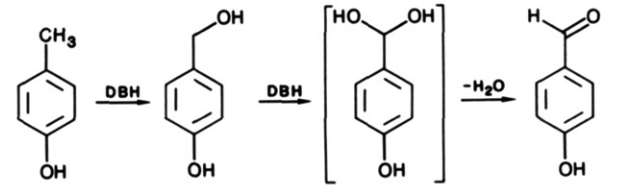
Additional control experiments were performed by omitting ascorbate cofactor or *p*-cresol.

The mass action experiments demonstrated that a fraction of the $^3$H label associated with the completely inactivated DBH is tightly but reversibly bound and that this binding is not dependent upon catalysis (Table III). A comparable amount of $^3$H label also binds to DBH in the absence of ascorbate cofactor, and this binding does not affect catalytic activity of enzyme. While the elution of DBH from the size-exclusion HPLC failed to dissociate completely the noncovalent [$^3$H]-*p*-cresol label, this was accomplished by equilibration with an excess of unlabeled *p*-cresol.

## Discussion

*p*-Cresol was initially chosen for study as a simple tyramine substrate analogue in competitive inhibition studies (DeWolf & Kruse, 1985; Kruse et al., 1986b). These studies, together with additional data presented here (Tables I and II), demonstrate an affinity of *p*-cresol for DBH which is comparable to that of tyramine substrate, suggesting the basic side-chain nitrogen of substrate is unimportant for recognition and binding by enzyme. Phenol, a simple phenethylamine analogue that even lacks an alkylamino side chain, still shows appreciable affinity for enzyme (Table II) although no evidence for turnover or inactivation has been observed with this compound.

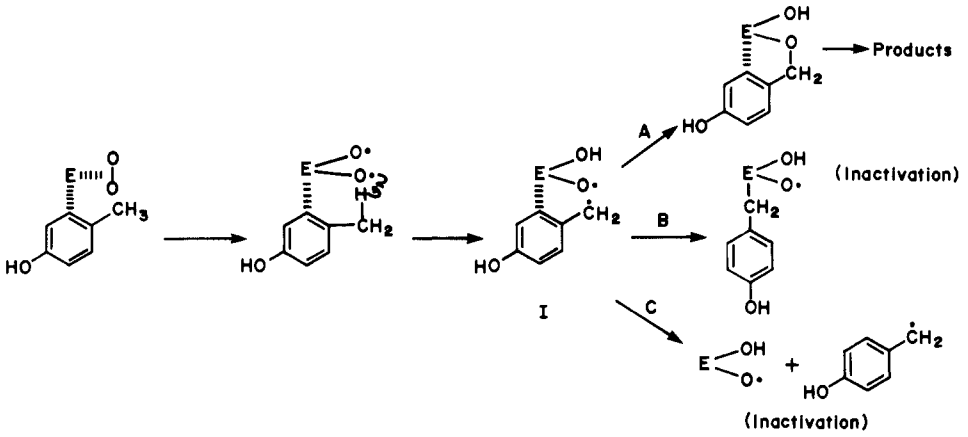
*p*-Cresol is a reasonably good substrate as shown by the data in Table I. Product analysis shows that an initial hydroxylation of *p*-cresol to 4-hydroxybenzyl alcohol occurs and that under conditions of prolonged turnover this is followed by an "overoxidation" of product to 4-hydroxybenzaldehyde. This is reminiscent of the oxidation of the unnatural *S* enantiomer of octopamine to the corresponding α-aminoacetophenone (May et al., 1981; Klinman & Krueger, 1982):




An appreciable increase in $V_{max}$(app) and a decrease in $K_m$-(app) occur for *p*-cresol upon increasing oxygen substrate concentration from 0.24 to 1.14 mM. This suggests a higher dissociation constant $K_D$ for oxygen from the *p*-cresol ternary complex relative to that from the tyramine ternary complex. The magnitude of the oxygen $K_D$ for DBH has been shown to vary depending upon structural variations in phenethylamine substrate (Miller & Klinman, 1985). A similar effect apparently occurs with *p*-cresol.

Isotope effect studies at high pH have demonstrated a random kinetic mechanism for phenethylamine and oxygen addition to form ternary complex whereas at low pH or in the presence of fumarate "activator" the random mechanism converts to a predominantly ordered addition of tyramine prior to oxygen (Ahn & Klinman, 1983). A corresponding change in kinetic order for *p*-cresol is consistent with the induced

Scheme I




inhibition for oxygen substrate observed at low pH (Figure 1C) or in the presence of fumarate activator (data not shown) although this could conceivably arise from a substantially tighter binding of *p*-cresol to ternary complex relative to its binding in the binary complex.

The irreversible inactivation of DBH by *p*-cresol exhibits all the necessary characteristics of a mechanism-based event. Oxygen and ascorbic acid cosubstrates are required, the inactivation event demonstrates a pH dependence which is similar to that for normal catalysis, and saturation kinetics are observed (Table II, Figure 3). Tyramine substrate and the multisubstrate inhibitor 1-(3,5-difluoro-4-hydroxybenzyl)-imidazole-2-thione both protect against inactivation. As is expected from the dependence of *p*-cresol turnover upon oxygen concentration, the rate of inactivation by *p*-cresol is dependent upon oxygen concentration with a true $k_{inact}$ of 2.0 $min^{-1}$. This rate of inactivation establishes *p*-cresol as one of the most effective mechanism-based inhibitors reported for DBH. Partition ratios of ~1300:1 based upon product formation and ~1600:1 based upon kinetic constants are reasonably consistent with the range of partition ratios reported for other classes of mechanism-based DBH inhibitors (Padgette et al., 1975; Fitzpatrick & Villafranca, 1985).

The data in Table II allow the identification of the minimal structural requirements for mechanism-based inhibition of DBH by alternative substrates. Phenol (**6**), which lacks an oxidizable benzylic $sp^3$-hybridized C–H bond, is inactive as a mechanism-based inhibitor in spite of a reasonable affinity for enzyme as a competitive inhibitor of tyramine. 4-Hydroxybenzaldehyde, a compound with an $sp^2$-hybridized benzylic C–H bond, also shows no time-dependent inactivation even at concentrations of 30 mM (data not shown). Placement of ring hydroxyls, or the substitution and oxidation state of the benzylic position, appears unimportant, so long as an oxidizable $sp^3$-hybridized benzylic C–H bond is present and the molecule lacks the basic side-chain nitrogen found in normal phenethylamine substrates.

Mechanism-based inactivation of DBH occurs with covalent labeling of enzyme by *p*-cresol although the labeling event is substoichiometric and is complicated by a high-affinity, reversible binding of *p*-cresol to enzyme (Table III) which is independent of catalysis. Under conditions where *p*-cresol completely inactivates enzyme, no significant loss of activity occurs with either *p*-cresol or ascorbate alone despite the reversible incorporation of 0.39 mol of *p*-cresol (Table III) when this is present. This noncovalent incorporation of *p*-cresol accounts for a large fraction of the total label associated with *p*-cresol-inactivated DBH and is readily reversed by mass action effects with unlabeled *p*-cresol. The substoichiometric labeling of completely inactivated DBH could be explained in two ways. Enzyme in which only a fraction of the subunits were catalytically active could account for the incorporation of only 0.56 mol equiv of *p*-cresol to subunit in fully inactivated enzyme. However, the excellent specific activity of the DBH used in these experiments (Table I) would argue against this possibility. A more satisfying explanation for the substoichiometric labeling by *p*-cresol could be found in a divergence of catalysis into competing mechanistic pathways that lead to turnover or inactivation (Scheme I). A benzylic radical intermediate has been demonstrated for catalysis with tyramine substrates (Miller & Klinman, 1985) and has been implicated in the turnover of alternate substrate inhibitors (Fitzpatrick & Villafranca, 1985). In the case of *p*-cresol the intermediate diradical I (Scheme I) will frequently recombine with release of 4-hydroxybenzyl alcohol product and regeneration of free enzyme (path A). An occasional dislocation of the hydroxybenzyl radical could lead to insertion into an active site residue (path B) and inactivation which results in covalent incorporation of *p*-cresol.[5] Alternatively, a dissociation of the benzylic radical from the active site (path C) could result in autoxidation and inactivation of enzyme without covalent incorporation of *p*-cresol (DeWolf et al., 1986). This autoxidation could involve hydroxylation of a copper ligand. Dehydration of 4-hydroxybenzyl alcohol product to an electrophilic quinone methide could also lead to inactivation. However, inactivation of DBH by *m*-cresol (**2**), a compound incapable of forming a quinone methide, argues against this mechanistic possibility. Peptide sequencing efforts are presently under way to determine the chemical mechanism of the inactivation event and the structure(s) of covalently modified residues.

In summary, an efficient mechanism-based inactivation of DBH has been demonstrated for simple tyramine substrate analogues that lack a latent electrophile and that do not possess the basic nitrogen present in phenethylamines. The inactivation by one inhibitor, *p*-cresol, has been characterized in detail and has been shown by labeling experiments to result from a partial covalent modification of enzyme. These and the other results presented here support mechanism-based inactivation of DBH as a generic property of simple alternate substrates that lack a side-chain nitrogen.

In light of the facile inactivation of DBH by these substrate analogues that lack a latent electrophile, a demonstrable involvement of latent electrophilic groups present in other

[5] Since the submission of this paper, we have determined that a major portion of label is incorporated into a >24 amino acid hydrophobic tryptic peptide (W. E. DeWolf, Jr., unpublished results).

mechanism-based inhibitors of DBH must be accepted as a minimum criterion before elaborate mechanistic conclusions can be drawn from inactivation results.

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