Dopamine β-Hydroxylase: Activity and Inhibition in the Presence of β-Substituted Phenethylamines†

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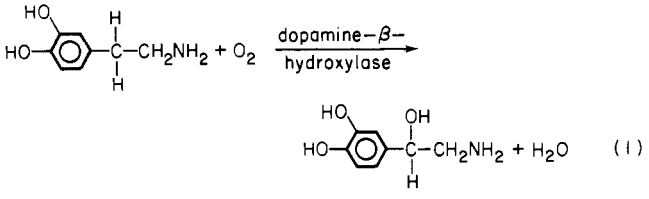
ABSTRACT: Functionalization of the β carbon of phenethylamines leads to a new class of substrates and inhibitors of dopamine β-hydroxylase. The rate of O_2 uptake in the presence of a series of β-hydroxyphenethylamines approaches that observed with dopamine (in the limit of infinite amine concentration, 0.2 mM O_2). Uncoupling of O_2 reduction from amine hydroxylation has been ruled out from observed stoichiometries of $K_4Fe(CN)_6$ oxidation to O_2 consumption in the presence of catalase and peroxidase. Characterization of products indicates formation of α-aminoacetophenone from both β-chloro- and β-hydroxyphenethylamine. Normal monooxygenase activity is supported by our failure to observe net incorporation of $H_2{}^{18}O$ into product derived from the dopamine β-hydroxylase catalyzed conversion of β-chlorophenethylamine to α-aminoacetophenone. Analysis of tritium release from [2-^{3}H]dopamine, following prolonged incubation in the presence of dopamine β-hydroxylase, rules out any "over" hydroxylation of product (*R*)-norepinephrine, establishing (*S*)-norepinephrine, and by analogy the *S* enantiomer of β-substituted phenethylamines, as substrate. Preincubation of dopamine β-hydroxylase with β-chlorophenethylamine under turnover conditions (K_m = 5.1 mM, k_{cat} = 1.1 s^{-1}) leads to a time-dependent loss of enzyme activity (K_i = 4.4 mM and k_i = 0.94 × 10^{-4} s^{-1}) consistent with a mechanism-based inactivation once in every 12 000 turnovers. Failure to recover enzymatic activity by extensive dialysis, subsequent to preincubation with β-chlorophenethylamine, may indicate covalent modification of enzyme concomitant with inactivation. Whereas phenethylamine, β-hydroxyphenethylamine derivatives, and α-aminoacetophenone do not inactivate enzyme under turnover conditions (+10 mM ascorbate), α-aminoacetophenone leads to efficient inactivation in the absence of reductant. These results suggest alternate enzymatic forms, E·Cu^{2+} vs. E·Cu^{1+}, differentially susceptible to inactivation by α-aminoacetophenone. The unique ability of β-chlorophenethylamine to inactivate dopamine β-hydroxylase may indicate an enzyme-catalyzed hydrogen activation at the α carbon of product, leading to the generation of the enzyme-bound enol of aminoacetophenone. Alternatively, loss of HCl from the highly unstable chlorohydrin derived from β-chlorophenethylamine is suggested to produce enzyme-bound α-aminoacetophenone in equilibrium with its enol as the dopamine β-hydroxylase inactivating species.

The properties of dopamine β-hydroxylase are of considerable interest in light of the important role played by this enzyme in the synthesis of the hormone/neurotransmitter norepinephrine, the unusual packaging of this enzyme within either chromaffin vesicles in the adrenal gland or synaptic vesicles in the adrenergic nervous system (together with the release of dopamine β-hydroxylase into cerebrospinal fluid and serum concomitant with nervous system activity), and the unresolved roles of the soluble vs. membrane-bound forms of dopamine β-hydroxylase in catecholamine biosynthesis in vivo (cf. review by Winkler, 1976; Nagatsu, 1977; Skotland & Ljones, 1979).

As illustrated in eq 1, dopamine β-hydroxylase is a mono-



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oxygenase catalyzing replacement of the *pro-R* hydrogen at the β carbon of dopamine by a hydroxyl group to form norepinephrine (Kaufman et al., 1962; Levin et al., 1960; Goldstein et al., 1965; Taylor, 1974). The enzymatic reaction also requires the presence of an exogenous electron donor (e.g., ascorbate), which has been demonstrated to reduce enzyme-bound Cu^{2+} to Cu^{1+} (Freidman & Kaufman, 1966; Blumberg et al., 1965; Walker et al., 1977). Although the intermediacy of reduced copper is well established, the precise order in which reducing equivalents are added to enzyme under turnover conditions is unclear (Ljones et al., 1978; Skotland et al., 1978). Recent measurements of primary tritium isotope effects in the dopamine β-hydroxylase reaction reveal a random kinetic mechanism for the formation of enzyme ternary complexes (Klinman et al., 1980), in contrast to experiments on dioxygenases, which indicate that addition of substrate is a prerequisite for both oxygen binding and its subsequent activation (Fujisawa et al., 1971, 1972). In the absence of information regarding the nature of enzyme-bound intermediates, hydrogen abstraction from the β carbon of dopamine can be postulated to occur either directly to an activated form of oxygen or to be mediated by an enzyme-bound functional group. Studies by May & Phillips (1980), indicating the stereospecific conversion of phenyl 2-aminoethyl sulfide to phenyl 2-aminoethyl sulfoxide can be rationalized in terms of the proximity of the sulfur atom of bound substrate to an activated form of oxygen. In contrast, Baldoni & Villafranca (1980) have demonstrated that *p*-hydroxybenzyl cyanide behaves as a suicide reagent toward dopamine β-hydroxylase; these authors suggest an enzyme-catalyzed proton abstraction from bound product (*p*-hydroxymandelonitrile) to generate the chemically reactive ketenimine.

In this paper, we demonstrate that functionalization of the *pro-S* hydrogen at the β carbon of phenethylamine with

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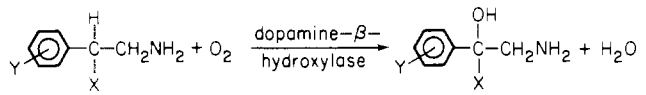
Table I: Kinetic Parameters for the Hydroxylation of β-Substituted Phenethylamines, Catalyzed by Dopamine β-Hydroxylase[a]

name	structure	k_{cat} (s^{-1})[b]	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})
dopamine	$3,4\text{-}(OH)_2C_6H_3CH_2CH_2NH_2$	13.0	1.0	1.3×10^4
(*RS*)-octopamine	$4\text{-}OHC_6H_4CHOHCH_2NH_2$	14.0	8.7	1.5×10^3
(*RS*)-norepinephrine	$3,4\text{-}(OH)_2C_6H_3CHOHCH_2NH_2$	8.3	7.6	1.1×10^3
(*RS*)-β-hydroxyphenethylamine	$C_6H_5CHOHCH_2NH_2$	4.2	24	1.8×10^2
(*RS*)-β-chlorophenethylamine	$C_6H_5CHClCH_2NH_2$	1.1	5.1	2.2×10^2
(*RS*)-synephrine	$4\text{-}OHC_6H_4CHOHCH_2NHCH_3$	2.5	53	49
(*R*)-norepinephrine	$3,4\text{-}(OH)_2C_6H_3CHOHCH_2NH_2$	0.90	42	21

[a] Standard assay conditions were 100 mM potassium phosphate, pH 6, 8 mM KCl, 10 mM sodium fumarate, 10 mM ascorbic acid, 10 μg/mL catalase, 1 μM $CuCl_2$, 0.2 mM O_2, and 2–20 mM amine, 35 °C. [b] Calculated from V_{max}, V_{max}/K_m, and a subunit molecular weight of 70 000.

heteroatoms leads to a new class of substrates and inhibitors of dopamine-β-hydroxylase.



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In the case of X = OH, the rate of oxygen uptake in the presence of dopamine β-hydroxylase approaches (and may exceed) rates observed with dopamine. Similar results have been reported recently by May et al. (1981) for the oxidation of octopamine (X, Y = OH) by dopamine β-hydroxylase. Replacement of X = OH by the more efficient leaving group X = Cl leads to a slow inactivation of enzyme concomitant with turnover. As discussed, the observed inhibition may indicate an enzyme-catalyzed hydrogen activation at the α carbon of product, leading to the generation of the enzyme-bound enol of α-aminoacetophenone. Alternatively, data are presented which suggest a loss of HCl from the highly unstable chlorohydrin derived from β-chlorophenethylamine to produce enzyme-bound α-aminoacetophenone as the dopamine β-hydroxylase inactivating species.

Materials and Methods

All chemicals were reagent grade unless otherwise noted. Dopamine, norepinephrine, synephrine, octopamine, ascorbic acid, sodium fumarate, catalase, and peroxidase were from Sigma. β-Hydroxyphenethylamine was purchased from ICN. β-Chlorophenethylamine was prepared from β-hydroxyphenethylamine according to the method of Barnett et al. (1944). [2-^{3}H]Dopamine (specific activity 5–10 Ci/mmol) and [^{3}H]$NaBH_4$ (specific activity 222 mCi/mmol) were from New England Nuclear. [2-^{3}H]Dopamine was purified as previously described (Klinman et al., 1980). $H_2{}^{18}O$, 53.4% oxygen-18 enriched, was from Alfa. Silica gel thin-layer chromatography plates were from Merck, DE-52 and CM-52 ion-exchange resins from Whatman, and Sephadex G-25 and Con-A Sepharose from Pharmacia. Methyl mannoside was purchased from Calbiochem. Radioactivity was determined on a Beckman LS 8000 scintillation spectrometer, absorbance spectroscopic determinations on a Cary 118B, fluorescence assays on a Perkin-Elmer MPF-48A fluorometer, and oxygen uptake assays on a Yellow Springs Instrument polarographic oxygen electrode. High-pressure liquid chromatography was conducted with a Beckman Model 332 gradient liquid chromatographic system, equipped with a Model 155 variable wavelength detector. Mass spectral analyses were obtained on an A.E.I. Model 12 mass spectrometer.

Dopamine β-hydroxylase activity was assayed by the rate of O_2 consumption employing a polarographic electrodes as previously described (Klinman et al., 1980). Where appropriate, norepinephrine levels were assayed by the production of fluorescent product according to a modification (Klinman et al., 1980) of the method of Von Euler & Floding (1955). Conversion of $K_4Fe(CN)_6$ to $K_3Fe(CN)_6$ was monitored at 420 nm ($\Delta\epsilon$ = 1 mM^{-1} cm^{-1}; Ljones & Flatmark, 1974). Extinction coefficients for β-hydroxyphenethylamine hydrochloride, β-chlorophenethylamine hydrochloride, and α-aminoacetophenone hydrochloride were determined in water at 22 °C.

The preparation of dopamine β-hydroxylase differed from a previously published procedure (Klinman et al., 1980) in that a Con A–Sepharose column chromatography step preceded DE-52 chromatography. In a typical preparation, dialyzed soluble enzyme derived from ~12 lb of adrenal glands is added to a Con A–Sepharose column (2 × 15.0 cm). The column is washed with 50 mM potassium phosphate, pH 6.0, containing 0.2 M NaCl until an absorbance of 0.05 is observed at 280 nm. The enzyme is then eluted with 200 mL of a linear gradient: starting buffer 50 mM potassium phosphate, 0.2 M NaCl; final buffer 50 mM potassium phosphate, 0.2 M NaCl containing 10% (w/v) methyl mannoside. Dopamine β-hydroxylase fractions eluting in the region of the gradient containing 3–7% methyl mannoside are pooled, concentrated to a volume of ~4 mL, and desalted by chromatography on a Sephadex G-25 column (1.5 × 20 cm). Protein is further purified by chromatography on a DE-52 column (1.5 × 20 cm) equilibrated with 5 mM potassium phosphate. Enzyme is eluted with a three-chamber gradient, with chambers one and two containing 200 mL of 5 mM potassium phosphate, pH 6.6, and chamber three containing 200 mL of 5 mM potassium phosphate and 0.7 M NaCl. In general, two peaks of dopamine β-hydroxylase activity are observed, a minor peak at 198–258 mL and a major peak at 264–319 mL. These pools are concentrated to a final concentration of 0.5–2.0 mg/mL (specific activity = 5.6 and 6.5 units/mg for the minor and major pools, respectively). Enzyme is stored in plastic tubes at −70 °C in 0.1–0.2-mL aliquots.

Results

Substrate Activity of β-Substituted Phenethylamines. Initial studies of enzyme activity with β-substituted phenethylamines focused on the properties of β-chloro- and β-hydroxyphenethylamine. High concentrations of these substrates (10 mM) lead to an enzyme-dependent rate of oxygen consumption which is 20% of that observed in the presence of 1 mM dopamine under standard assay conditions. Examination of the effect of amine concentration indicated a larger k_{cat} and K_m for β-hydroxyphenethylamine than for β-chlorophenethylamine (Figure 1A and Table I).

Although β-chloro- and β-hydroxyphenethylamine were pure as judged by NMR and melting point determination, it was important to establish that enzyme activity arises from the β-substituted phenethylamine rather than a trace contaminant. In addition, the possibility existed that oxygen uptake was due to an (partial) uncoupling of oxygen reduction from substrate

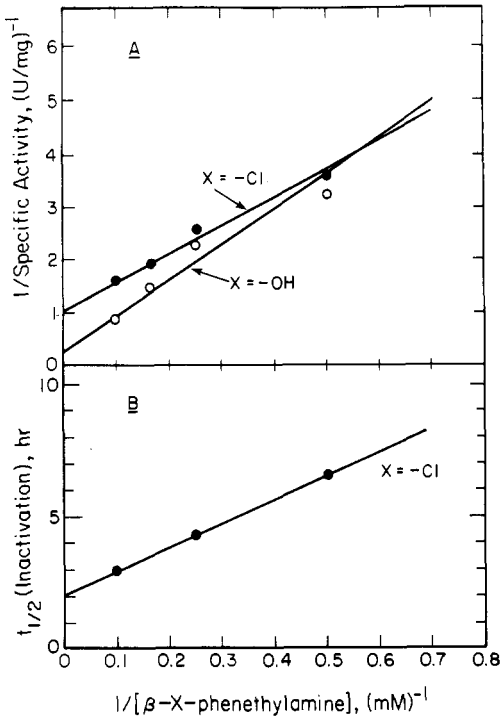


FIGURE 1: Reciprocal plots of enzymatic activity (A) or half-time for enzyme inactivation (B) as a function of β-X-phenethylamine concentration. Enzymatic assays were determined as described in Table I. Half-times for inactivation are derived from the plots of activity vs. time in Figure 4B.

hydroxylation. Initially, we examined the stoichiometry of O_2 uptake under conditions of limiting β-hydroxyphenethylamine (0.1 mM vs. 0.2 mM O_2). The very slow enzymatic reaction under these conditions, relative to base-line changes in control samples, precluded following the reaction to completion; however, the observation of a consumption of oxygen ≥26% of the starting β-hydroxyphenethylamine effectively ruled out the presence of a trace contaminant as the source of O_2 uptake. Uncoupling of oxygen reduction from substrate hydroxylation was examined by comparison of $K_4Fe(CN)_6$ oxidation to oxygen uptake for the dopamine β-hydroxylase catalyzed oxidation of β-chloro- and β-hydroxyphenethylamines in the presence of either catalase or peroxidase. In the event that β-substituted phenethylamines were acting as effectors (rather than substrates) for a two-electron reduction of O_2 to H_2O_2, the stoichiometry of Fe^{3+} production/O_2 consumption would be expected to deviate from a normal value of 2.0 (Ljones et al., 1976; May & Phillips, 1980): in the presence of catalase, any H_2O_2 produced would be disproportionated to O_2 and H_2O, leading to values for Fe^{3+} production/O_2 consumption = 3.0; in the presence of peroxidase, product H_2O_2 would be further reduced to H_2O by $K_4Fe(CN)_6$, leading to values for Fe^{3+} production/O_2 consumption = 4.0. As summarized in Table II, Fe^{3+} production/O_2 consumption = 2.0 ± 0.1, indicating little or no production of H_2O_2 concomitant with O_2 uptake.

Subsequent studies focused on characterization of products derived from β-substituted substrates. In the case of β-chloro- and β-hydroxyphenethylamines, hydroxylation is expected to lead to either the chlorohydrin or hydrate of α-aminoacetophenone; elimination of HCl or water produces the unstable product α-aminoacetophenone,[1] which can be trapped by

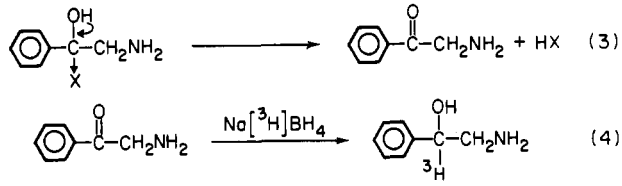
[1] Above pH 7.0, α-aminoacetophenone undergoes spontaneous dimerization and oxidation to 2,5-diphenylpyrazine (Vinot & Pinson, 1968).

Table II: Stoichiometry of $K_4Fe(CN)_6$ Oxidation to Oxygen Consumption for the Reaction of β-Substituted Phenethylamines With Dopamine β-Hydroxylase[a]

substrate	μmol of Fe^{3+} produced min^{-1} mg^{-1}	μmol of O_2 consumed min^{-1} mg^{-1}	Fe^{3+} production/O_2 consumption
β-hydroxyphenethylamine +catalase	0.74	0.38	1.9
β-chlorophenethylamine +catalase	0.36	0.18	2.0
+peroxidase	0.32	0.15	2.1

[a] Enzyme assays contained 100 mM potassium phosphate, pH 6, 10 mM sodium fumarate, 1 μM $CuCl_2$, 250 μM $K_4Fe(CN)_6$, 10 mM amine, and either 40 μg/mL peroxidase or 10 μg/mL catalase in a final volume of 1-3 mL. The reaction was initiated by the addition of 20-50 μL of dopamine β-hydroxylase, 0.5 mg/mL. Assays of initial rates for the production of Fe^{3+} and consumption of O_2 were carried out at 35 °C, as described under Materials and Methods.

$NaBH_4$ reduction regenerating β-hydroxyphenethylamine (eq 3 and 4).



The production of α-aminoacetophenone was monitored by production of tritiated β-hydroxyphenethylamine with [^{3}H]-$NaBH_4$. In initial studies, $K_4Fe(CN)_6$ was added as enzyme reductant, since under these conditions α-aminoacetophenone would be the only component of the reaction mixture undergoing incorporation of tritium upon reduction with [^{3}H]Na-BH_4. However, oxygen uptake in the presence of β-chlorophenethylamine and $K_4Fe(CN)_6$ was observed to proceed to a very small extent, corresponding to ~2% conversion to product. Ascorbate enhances both the rate and percent conversion in a similar manner for phenethylamine and β-chlorophenethylamine, and the characterization of reaction products was carried out in the presence of ascorbate, necessitating chromatographic separation of tritiated products (β-hydroxy[β-^{3}H]phenethylamine and [^{3}H]ascorbate, formed by reduction of α-aminoacetophenone and dehydroascorbate, respectively). Figure 2 illustrates ion-exchange chromatography of reaction products derived from the incubation of β-chlorophenethylamine either in the presence or in the absence of high concentrations of dopamine β-hydroxylase. The addition of enzyme leads to a unique peak of radioactivity eluting in fractions 8–19. This material was pooled, concentrated, and rechromatographed on thin-layer chromatographic plates under conditions of solvent leading to R_f values of 0.51 and 0.10 for β-hydroxyphenethylamine. As indicated in Figure 3, radioactivity cochromatographs with authentic β-hydroxyphenethylamine under conditions of both acidic (butanol–water–acetic acid) and basic (chloroform–methanol–ammonium hydroxide) mixed solvents.

Quantitation of α-aminoacetophenone formation from total tritium incorporated into β-hydroxyphenethylamine is difficult, due to an anticipated less than 100% recovery of β-hydroxy-[β-^{3}H]phenethylamine from ion-exchange chromatography and an unknown tritium isotope effect in the reduction of

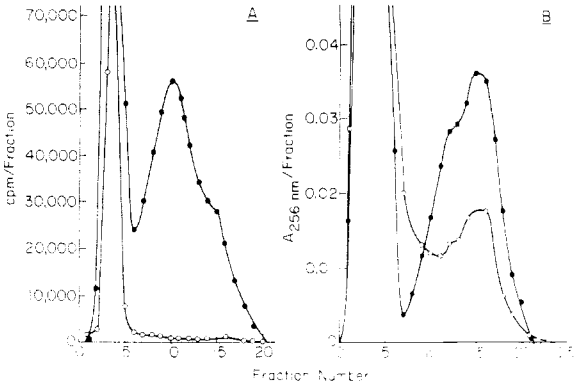


FIGURE 2: Elution of radioactivity (A) and absorbance (B) from ion-exchange chromatography of reaction products derived from incubation of β-chlorophenethylamine with dopamine β-hydroxylase, according to eq 2–4 in text. The reaction mixture contained 100 mM potassium phosphate, pH 6.0, 17 mM KCl, 10 mM sodium fumarate, 10 mM ascorbate, 1 μM $CuCl_2$, 100 μg of catalase, and 1 mM β-chlorophenethylamine. The reaction was initiated with the addition of either 100 μg of dopamine β-hydroxylase or water for a final volume of 1.0 mL and incubated at 35 °C for 2 h. At the end of the incubation time, 0.4 mL of 1 M K_2HPO_4 was added to bring the pH to 7.5, followed by the addition of 10 μL of 0.1 M [^{3}H]$NaBH_4$ (specific activity 1.3×10^7 cpm/equiv). After 15 min, the reaction mixture was quenched with 1 M H_3PO_4 and stirred for 15 min to facilitate loss of tritium gas derived from the breakdown of [^{3}H]$NaBH_4$. The resulting mixture was lyophilized to dryness and redissolved in 1 mL of H_2O. Cation-exchange chromatography was carried out on CM-52 columns (0.8 × 16 cm) equilibrated in 50 mM sodium acetate, pH 5.0. After addition of the sample, the column was washed with 50 mM sodium acetate until the absorbance at 256 nm was at the base line, followed by the addition of 1 M acetic acid. The column flow rate was 18 mL/h, and 2-mL fractions were collected. Absorbance of the eluant was monitored at 256 nm; for the measurement of radioactivity, 200 μL of each fraction was counted. In the case of the dopamine β-hydroxylase (●) and control experiments (○) a total of 3.2×10^6 (representing 89% of the initial counts) and 0.6×10^6 cpm (representing 86% of the initial counts), respectively, were recovered from the column.

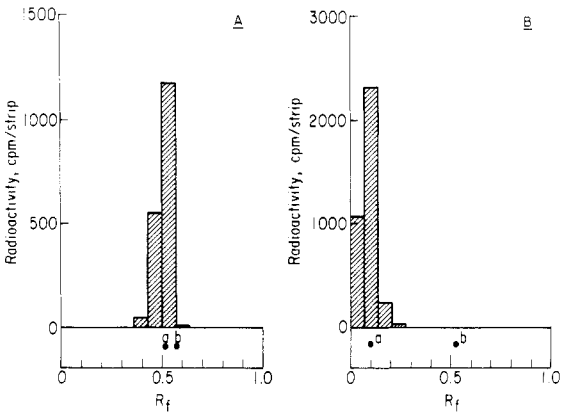


FIGURE 3: Thin-layer chromatography of radiolabeled amine derived from reaction of β-chlorophenethylamine with dopamine β-hydroxylase. (A) Histogram of thin-layer chromatography of a mixture of 10 μL of 1 mM β-hydroxyphenethylamine and 10 μL of tritiated product derived from fraction 8–12 in Figure 2. The thin-layer chromatographic plate was developed with butanol–water–acetic acid (4:1:2). Amines were located with ninhydrin spray. Radioactivity was determined by dividing the region of the plate between the origin and solvent front into 14 equivalent steps, which were scraped from the plate into scintillation vials and counted. Approximately 1950 cpm were recovered from the plate for a yield of 77%. Under the conditions of this experiment, β-hydroxyphenethylamine (a) and β-chlorophenethylamine (b) were characterized by R_f values of 0.51 and 0.57, respectively. (B) Histogram of thin-layer chromatography of a mixture of 10 μL of 1 mM β-hydroxyphenethylamine and 20 μL of tritiated product derived from fractions 8–12 in Figure 2. The thin-layer chromatographic plate was developed with chloroform–methanol–ammonium hydroxide (84:15:1). Amine were located with ninhydrin spray. Radioactivity was determined by dividing the region of the plate between the origin and solvent front into 14 equivalent strips, which were scrapped from the plate into scintillation vials and counted. Approximately 3620 cpm were recovered from the plate for a yield of 72%. Under the conditions of this experiment, β-hydroxyphenethylamine (a) and β-chlorophenethylamine (b) were characterized by R_f values of 0.10 and 0.52, respectively.

aminoacetophenone by [^{3}H]$NaBH_4$. In addition, Figure 2 indicates an increase in absorbance due to amines retained by the CM-52 resin; whereas the extinction coefficients for β-chloro- and β-hydroxyphenethylamine hydrochloride are similar, (ϵ_{256} = 0.24 and 0.19 mM^{-1} cm^{-1}, respectively), α-aminoacetophenone HCl is characterized by a 50-fold higher extinction coefficient (ϵ_{256} = 10 mM^{-1} cm^{-1}), suggesting either incomplete reduction of α-aminoacetophenone or its dimerization and oxidation to 2,5-diphenylpyrazine (ϵ_{256} = 15 mM^{-1} cm^{-1} for 2,5-diphenyl-3,6-diethylpyrazine; Vinot & Pinson, 1968). Despite these limitations, a comparison of oxygen uptake to tritium fixed in product under experimental conditions similar to those in Figure 2 allows us to establish a *minimum* value for the ratio of oxygen uptake (0.33 μmol) to β-hydroxy[β-^{3}H]phenethylamine formation (0.16 μmole) of 0.48.

Although not shown, [^{3}H]$NaBH_4$ trapping of reaction products from the oxidation of β-hydroxyphenethylamine yields β-hydroxy[β-^{3}H]phenethylamine in a manner strictly analogous to our observations with β-chlorophenethylamine. The generality of hydroxylation of β-substituted phenethylamines was tested with the series of analogues summarized in Table I. The data in Table I indicate a wide range of enzymatic activities with β-hydroxyphenethylamine derivatives.

The direct formation of either the chlorohydrin or *gem*-diol of α-aminoacetophenone by hydroxylation of β-chloro- and β-hydroxyphenethylamine, respectively, was presumed to occur via a classical monooxygenase pathway involving incorporation of O_2 into the product. Possible incorporation of solvent into product was examined by turnover of β-chlorophenethylamine in $H_2{}^{18}O$, 53.4% enriched in oxygen-18. Product α-aminoacetophenone was reduced by $NaBH_4$ to β-hydroxyphenethylamine, followed by mass spectral analysis as a pentafluoropropionyl derivative. The fragmentation patterns of this derivative occur at m/e 225, 253, and 266 (Willner et al., 1974). In the event that every mole of product involved the incorporation of solvent, the ratio of $(m + 2)/m$ would be 1.15; inspection of Table III indicates an $(m + 2)/m = 0.23 \pm 0.06$ for β-hydroxyphenethylamine derived from the enzymatic oxidation of β-chlorophenethylamine. The ultimate product of the enzymatic reaction, α-aminoacetophenone, is expected to undergo an exchange with solvent; in addition, substrate β-chlorophenethylamine might undergo limited hydrolysis under the conditions of the enzymatic reaction. Hence, a control reaction was run in the absence of enzyme, containing β-chlorophenethylamine and α-aminoacetophenone in the ratio of 80:20 (corresponding to the observed 20% conversion of β-chlorophenethylamine to α-aminoacetophenone in the presence of dopamine β-hydroxylase). As summarized in Table III, β-hydroxyphenethylamine isolated from the control was characterized by an $(m + 2)/m = 0.20 \pm 0.05$, within experimental error of the ratio observed for product derived from the enzymatic oxidation of β-chlorophenethylamine. These results support a classical monooxygenase pathway for the hydroxylation of β-substituted phenethylamines. In addition, the similarity of $(m + 2)/m$ ratios for control and experimental incubations rules out any significant catalysis by dopamine β-hydroxylase of an exchange reaction of α-aminoacetophenone with solvent.

Table III: Assay for Incorporation of $H_2{}^{18}O$ into β-Hydroxyphenethylamine Derived from Enzymatic Oxidation of β-Chlorophenethylamine

source of β-hydroxyphenethylamine	m/e (%)								
	225	227	227/225	253	255	255/253	266	268	268/266
standard α-aminoacetophenone[a]	34.6			73.4			60.6		
$H_2{}^{16}O$	9.05	(1.5)	(0.11)	16.1			17.7		
$H_2{}^{18}O$	23.5	3.95	0.17	43.4	11.1	0.26	36.0	6.52	0.18
β-chlorophenethylamine[b]									
$H_2{}^{16}O$	50.1			100			90.5		
$H_2{}^{18}O$	21.7	4.54	0.20	31.6	9.36	0.30	37.0	7.30	0.20

[a] Incubation mixtures contained 20 mM potassium phosphate, pH 6, 10 mM sodium fumarate, 1 μM $CuCl_2$, 50 μg/mL catalase, 10 mM ascorbate, 0.8 mM β-chlorophenethylamine, and 0.2 mM α-aminoacetophenone in a final volume of 1 mL of $H_2{}^{18}O$ 53.4% enriched in oxygen-18. The reaction was incubated for 20 min at 35 °C and frozen, and $H_2{}^{18}O$ was removed by bulb to bulb distribution in vacuo. The residue was dissolved in 0.24 mL of H_2O, followed by the addition of 10 μL of 1.25 M $NaBH_4$ in 0.01 N NaOH. Reduction was allowed to proceed for 15 min, followed by chilling in an ice-water bath and centirfugation through a centriflo cone to remove protein. The sample was injected onto an Beckman ultrasphere high-pressure liquid chromatographic column, 4.6 × 25 cm. Amines were eluted with a solvent comprised of methanol–5% acetic acid containing 5 μM sodium dodecyl sulfate and 5 μM EDTA (30:70). The flow rate was 1 mL/min. Retention times were 11.2 and 28.2 min for β-hydroxy- and β-chlorophenethylamines, respectively. β-Hydroxyphenethylamine was pooled, taken to dryness, and derivatized by treatment with 100 μL of pentafluoropropionic anhydride in 20 μL of ethyl acetate. Samples were heated at 70 °C for 45 min, and the solvent was removed. Prior to mass spectral analysis, the residue was dissolved in *n*-heptane. The major fragmentation patterns for derivatized β-hydroxyphenethylamine occur at *m/e* values of 225, 253, and 266 (Willner et al., 1974). [b] Reactions mixtures were similar to footnote *a*, with the exception that α-aminoacetophenone was omitted and the final concentration of β-chlorophenethylamine was 1 mM in a final volume of 1 mL. The reaction was initiated by the addition of 50 μL of dopamine β-hydroxylase, 1 mg/mL. Incubations and isolation of product β-hydroxyphenethylamine were identical with (*a*) above. The yield of β-hydroxyphenethylamine from high-pressure liquid chromatography corresponded to an approximately 20% conversion of β-chlorophenethylamine to product.

With the exception of norepinephrine, all of the amines tested were a *RS* mixture. The question of whether one or both enantiomers are substrates for dopamine β-hydroxylase was addressed in two ways: first, by comparison of the rate of oxidation of (*R*)- to that of (*RS*)-norepinephrine and, second, by the time course for tritium release from [2-^{3}H]-dopamine. Inspection of k_{cat} and k_{cat}/K_m values for (*R*)- vs. (*RS*)-norepinephrine indicates preferential oxidation of the *S* isomer. Although a low level of enzyme activity is observed with (*R*)-norepinephrine, this material was prepared by chemical synthesis, introducing the possibility of contamination by the *S* isomer. Evidence that (*R*)-norepinephrine is not turned over is presented in Table IV; long term incubation of [2-^{3}H]dopamine leads to the production of 1 equiv of norepinephrie and release of ~50% of the total tritium to water. Since further oxidation of product (*R*)-norepinephrine would have both increased the level of tritium release and reduced the level of norepinephrine production, the results in Table IV establish (*S*)-norepinephrine, and by analogy the *S* enantiomer of β-substituted phenethylamines, as substrates.

Table IV: Stoichiometry of Norepinephrine and ^{3}HOH Production from [2-^{3}H]Dopamine Catalyzed by Dopamine β-Hydroxylase[a]

time (h)	μmol of dopamine	μmol of norepinephrine	cpm (×10^6) residue	cpm (×10^6) volatile	volatile (%)
0	0.36		2.6	0.013	0.05
2.5		0.35	1.1	1.2	52
3.0		0.35	1.1	1.2	52

[a] [2-^{3}H]Dopamine was incubated with 20 μg of dopamine β-hydroxylase under conditions of 50 mM potassium acetate, pH 5.8, 10 mM ascorbate, 10 mM sodium fumarate, 10 μg of catalase, and 0.36 mM dopamine in a volume of 0.5 mL, 35 °C. At the indicated time, 0.1 mL of the reaction mixture was removed to 0.1 mL of 0.1 N $HClO_4$. The initial concentration of dopamine was calculated from absorbance at 280 nm. The concentration of norepinephrine in quenched reaction mixtures was determined by fluorescence. Water was removed by bulb to bulb distillation in vacuo to determine volatile tritium.

Inhibition of Dopamine β-Hydroxylase by β-Chlorophenethylamine. Previous work from this laboratory has indicated that dopamine β-hydroxylase undergoes oxidative inactivation at variable rates, which depend on the presence of anion effector (which increases the rate of inactivation) and substrates (which decrease the rate of inactivation) (Klinman, 1979). In contrast to a stabilization of enzyme by β-chlorophenethylamine noted earlier, inclusion of ascorbate in incubation mixtures leads to a β-chlorophenethylamine-dependent loss of enzymatic activity (Figure 4B). Control experiments involving the preincubation of enzyme in the presence of 2–10 mM phenethylamine lead to no detectable loss of enzyme activity over a 5-h time period (Figure 4A). A plot of the half-time for enzyme inactivation as a function of the reciprocal of β-chlorophenethylamine concentration, illustrated in Figure 1B, indicates a limiting value for $t_{1/2}$ of 2.1 h and a K_i of 4.4 mM. The latter parameter is almost identical with the K_m = 5.1 mM summarized in Table I for β-chlorophenethylamine turnover. Preincubation of enzyme with 10 mM of octopamine, β-hydroxyphenethylamine, and synephrine indicated little or no loss of enzyme activity relative to controls. The failure to observe enzyme inactivation with β-hydroxyphenethylamine and synephrine could be due in part to the high K_m values for these substrates (Table I). However, octopamine and β-chlorophenethylamine are characterized by similar K_m values; as summarized in Figure 4C, enzyme was stable up to 5 h in the presence of 10 mM octopamine, in contrast to a loss of 50% enzyme activity with 10 mM β-chlorophenethylamine.

As summarized in eq 2–4, both β-chloro- and β-hydroxyphenethylamine produce the same product; hence, α-aminoacetophenone was considered unlikely to be the enzyme inhibitor. Addition of up to 10 mM α-aminoacetophenone to assay mixtures containing 0.5–5.0 mM dopamine indicated little effect on enzyme activity. Similarly, preincubation of dopamine β-hydroxylase with α-aminoacetophenone in the presence of 10 mM ascorbate led to no loss of enzyme activity (Figure 5A). By contrast, preincubation of enzyme with α-aminoacetophenone in the absence of reductant led to a time-dependent inactivation under conditions of enzyme stability in the presence of β-chlorophenylamine (Figure 5B). Although not shown, loss of activity was observed to be a first-order process up to 97% inactivation in the presence of 10 mM α-aminoacetophenone. A replot of half-times for inactivation vs. the reciprocal of α-aminoacetophenone con-

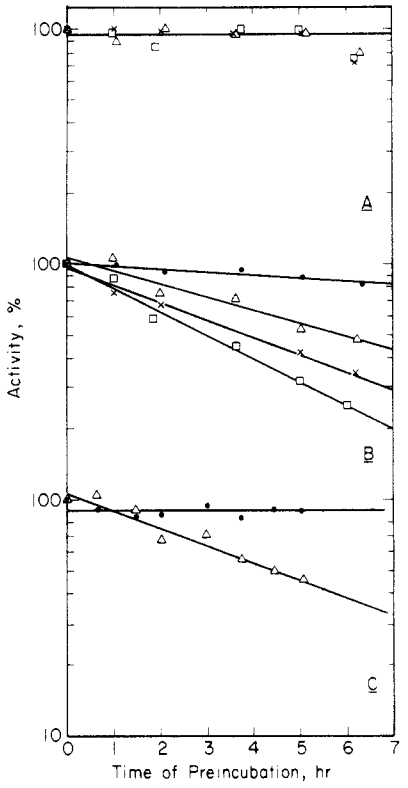


FIGURE 4: Time course for dopamine β-hydroxylase activity following preincubation in the presence of phenethylamine (A), β-chlorophenethylamine (B), and octopamine vs. β-chlorophenethylamine (C). Enzyme was preincubated under conditions of 100 mM potassium phosphate, pH 6, 10 mM sodium fumarate, 10 mM ascorbate, 1 μM $CuCl_2$ and 100 μg/mL catalase and 60 μg/mL dopamine β-hydroxylase, 35 °C. Phenethylamine concentrations in (A) were 2 mM (×), 4 mM (△), and 10 mM (□); β-chlorophenethylamine concentrations in (B) were 0 (●), 2 (△), 4 (×), and 10 mM (□); both octopamine (●) and β-chlorophene thylamine (△) were 10 mM in (C). At the indicated times, 10 μL of the preincubation mixture was diluted into 290 μL of assay mix containing at a final concentration 100 mM potassium phosphate, pH 6, 10 mM sodium fumarate, 17 mM KCl, 1 mM dopamine, and 10 μg/mL catalase. Following incubation for 3 min at 35 °C, the reaction was quenched by the addition of 300 μL of 0.1 N $HClO_4$. Product norepinephrine was assayed by flurescence as described under Materials and Methods.

Table V: Irreversibility of Dopamine β-Hydroxylase Inactivation by β-Chlorophenethylamine

treatment	sp act. (units/mg)[a]	
	no addition	+10 mM β-chlorophenethylamine
initial	5.81	5.49
preincubation for 9.5 h[b]	5.14	1.42
dialysis for 16 h[c]	3.36	0.81

[a] Determined under conditions of the standard assay described in Table I. [b] Preincubation mixtures contained 100 mM potassium phosphate, pH 6, 10 mM sodium fumarate, 1 μM $CuCl_2$, 100 μg/mL catalase, 10 mM ascorbate, and 60 μg/mL dopamine β-hydroxylase; either water or 10 mM β-chlorophenethylamine was added for a final volume of 0.8 mL, 35 °C. [c] Dialysis against a 500-fold volume excess of 20 mM potassium phosphate, pH 6, and 1 μM $CuCl_2$, 4 °C. Fresh dialysate was added after 6 h.

centration (inset in Figure 5) suggests extremely weak binding of α-aminoacetophenone ($K_i \approx 50$ mM) together with rapid inactivation in the limit of infinite concentration of ketone ($t_{1/2} \approx 0.2$ h).

The reversibility of enzyme inhibition of β-chlorophenethylamine following prolonged incubation was examined (Table V). As summarized, a 9.5-h preincubation of enzyme

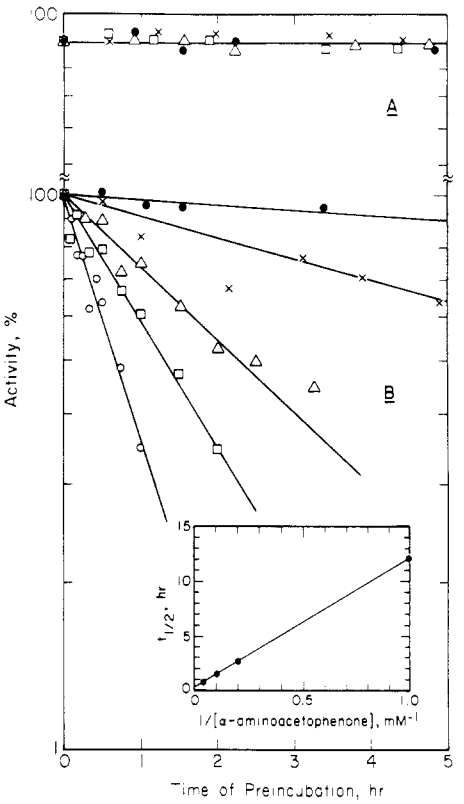


FIGURE 5: Time course for dopamine β-hydroxylase activity following preincubation in the presence of α-aminoacetophenone ± ascorbate. (A) +Ascorbate: Preincubations contained 100 mM potassium phosphate, pH 6, 10 mM sodium fumarate, 1 μM $CuCl_2$, 10 mM ascorbate, 100 μg/mL catalase, and 60 μL/mL dopamine β-hydroxylase and aminoacetophenone at 0 (●), 0.5 (△), 1.0 (×), and 5.0 mM (□) and were carried out at 35 °C. At the indicated times, 20 μL of the preincubation mixture was diluted into 220 μL of assay mix containing at a final concentration 100 mM potassium phosphate, pH 6.0, 10 mM sodium fumarate, 17 mM KCl, 1 mM dopamine, and 10 μg/mL catalase. Following incubation for 3 min at 35 °C, the reaction was quenched by the addition of 240 μL of 0.1 N $HClO_4$. Product norepinephrine was assayed by fluorescence as described under Materials and Methods. (B) −Ascorbate: Preincubations contained 100 mM potassium phosphate, pH 6, 10 mM sodium fumarate, 5 μM $CuCl_2$, 50 μg/mL catalase, and 30 μg/mL dopamine β-hydroxylase in the presence of 10 mM β-chlorophenethylamine (●) and 1 (×), 5 (△), 10 (□), and 20 mM (○) α-aminoacetophenone. The concentration of $CuCl_2$ and catalase were increased and decreased, respectively, in the absence of ascorbate in order to maintain the same stoichiometry of $CuCl_2$–catalase–dopamine β-hydroxylase as was employed in turnover studies. Assays for enzymatic activity were carried out as described above in (A), with the exception that 30 μL of the preincubation mixture was diluted into 150 μL of assay mix.

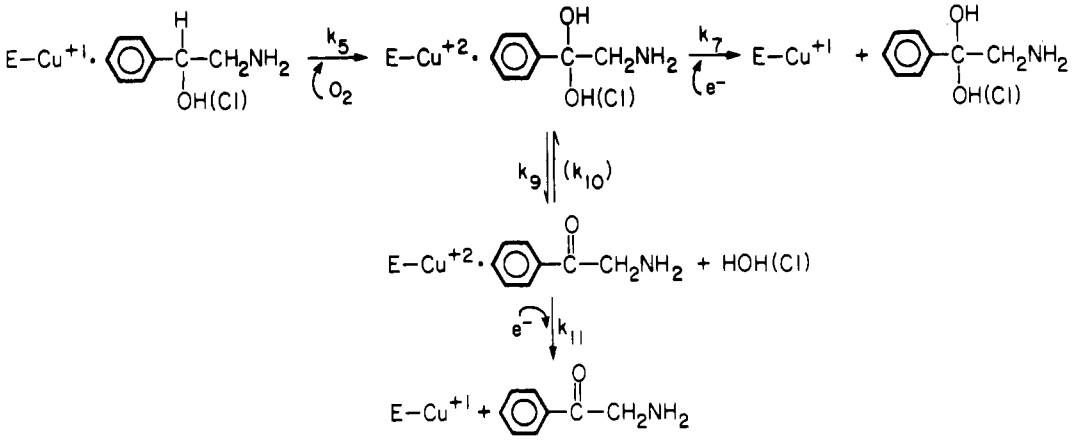
in the presence of β-chlorophenethylamine led to a 74% loss of enzyme activity, relative to a 12% loss of activity for a control carried out in the absence of inhibitor. Subsequent dialysis led to further loss of activity comparable to the control rather than any regain of activity, and we conclude that the observed loss of activity with β-chlorophenethylamine is irreversible.

Discussion

Recent evidence for an unusually broad substrate specificity for dopamine β-hydroxylase has been accumulating: specifically, the production of benzaldehyde from benzyl cyanide (Baldoni & Villafranca, 1980) and phenyl 2-aminoethyl sulfoxide from phenyl 2-aminoethyl sulfide (May & Phillips, 1980).

In this paper, we demonstrate that dopamine β-hydroxylase is an excellent catalyst for the hydroxylation of a series of β-X-phenethylamines where X = OH and Cl. These obser-

Scheme I: Kinetic Mechanism for the Hydroxylation of β-Substituted Phenethylamines[a]



[a] As illustrated, bound product has the potential to partition between product release, k_7, and elimination of HX to produce the thermodynamically stable ketone, k_9.

vations suggested a possible "over" hydroxylation of dopamine producing both the dihydroxy derivative of α-aminoacetophenone and (*R*)-norepinephrine. However, comparison of enzyme activity with (*R*)-norepinephrine to an *RS* mixture indicated the preferential hydroxylation of the *S* enantiomer (Table I). In addition, failure to observe a greater than 50% release of tritium from randomly labeled [2-^{3}H]dopamine (Table IV) rules out any significant activity of dopamine β-hydroxylase with (*R*)-norepinephrine, establishing (*S*)-norepinephrine and, by analogy, *S*-β-substituted phenethylamines as substrate for dopamine-β-hydroxylase. The occurrence of hydroxylation via a classical monooxygenase pathway is supported by three lines of evidence: (1) our failure to detect uncoupling of O_2 reduction from substrate hydroxylation (Table II), (ii) the absence of any net exchange of $H_2{}^{18}O$ into product derived from the enzyme-catalyzed oxidation of β-chlorophenethylamine (Table III), and (iii) characterization of product formed from β-chlorophenethylamine as α-aminoacetophenone (Figures 2 and 3).

In a recent study, reported subsequent to completion of this work, May et al. (1981) have characterized the interaction of octopamine with dopamine β-hydroxylase. These authors observed an enrichment of (*R*)-octopamine from the enzymatic oxidation of an *RS* mixture, indicating preferential oxidation of (*S*)-octopamine. Isolation of product from a preparative scale incubation of (*S*)-octopamine with enzyme led to identification of product as *p*-hydroxy-α-aminoacetophenone. Steady-state kinetic parameters obtained with (*S*)-octopamine were k_{cat} = 33 s^{-1}, $k_{cat}/K_m = 2.4 \times 10^3$ M^{-1} s^{-1}, and K_m = 14 mM at pH 5.0. Comparable kinetic values for the hydroxylation of (*S*)-octopamine can be estimated as k_{cat} = 56 s^{-1}, $k_{cat}/K_m = 12.4 \times 10^3$ s^{-1}, and K_m = 4.2 mM at pH 6.0.[2]

The kinetic constants summarized in Table I represent a single oxygen concentration (0.2 mM); thus, depending on the magnitude of the K_m for oxygen, the reported values reflect either k_{cat} or k_{cat}/K_{O_2}. Preliminary kinetic studies with β-hydroxyphenethylamine indicate very large values of K_m for both β-hydroxyphenethylamine, K_m = 32 mM in the limit of infinite oxygen, and oxygen, K_m = 1.2 mM in the limit of infinite amine; K_m values for dopamine hydroxylation obtained under comparable conditions are considerably smaller: K_m (dopamine) = 0.87 mMand K_m (O_2) = 0.049 mM. It therefore appears likely that true k_{cat} values (obtained under conditions of oxygen saturation) for the more active β-substituted phenethylamines in Table I will exceed the value of k_{cat} for dopamine.

In the case of dopamine, kinetic isotope effect measurements with [2-^{3}H]dopamine lead to variable values for $(k_{cat}/K_m)_H/(k_{cat}/K_m)_T$ dependent on oxygen concentration, diagnostic of a random order of dopamine and oxygen addition to enzyme (Klinman et al., 1980); examination of primary deuterium isotope with [2-^{2}H$_2$]dopamine under the experimental conditions of these studies indicates a value for $(k_{cat})_H/(k_{cat})_D$ close to one, consistent with a largely rate-limiting dissociation of norepinephrine (N. Ahn and J. P. Klinman, unpublished results).[3] To the extent that β-substituted phenethylamines behave in an analogous manner, enzyme-bound product will have the option to partition between product loss, k_7 in Scheme I, vs. elimination of HX to form the thermodynamically favored ketone, k_9 in Scheme I. The latter reaction is discussed below in the context of enzyme inactivation by β-chlorophenethylamine.

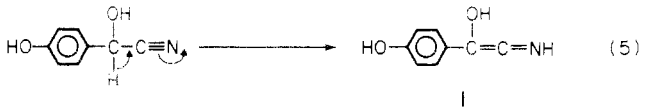
Of the β-substituted phenethylamines investigated (Table I), only β-chlorophenethylamine produces a time-dependent loss of dopamine β-hydroxylase activity. In contrast to a stabilization of enzyme activity following preincubation with β-chlorophenethylamine in the absence of reductant (Figure 5B), the addition of 10 mM ascorbate leads to the observed enzyme inactivation (Figure 4B,C). From the dependence of inactivation on turnover conditions, together with the similarity of *K* values derived from enzyme inactivation, K_i = 4.4 mM, vs. substrate hydrozylation, K_m = 5.1 mM (Figure 1), enzyme inactivation is concluded to occur in the course of the catalytic conversion of β-chlorophenethylamine to α-aminoacetophenone. The failure to recover enzyme activity following exhaustive dialysis of β-chlorophenethylamine-inhibited dopamine β-hydroxylase (Table V) is consistent with inactivation arising from covalent modification of enzyme. A comparison of the rate of enzyme inhibition ($k_i = 0.94 \times 10^{-4}$ s^{-1}) to

[2] These are from the data in Table I and a molar molecular weight of 280 000. Although May et al. (1981) did not state the molecular weight used for calculation of k_{cat} and k_{cat}/K_m, comparison of reported constants to enzyme specific activity indicates a value of 280 000, as opposed to the subunit molecular weight of 70 000 used in Table I. We have assumed that (*R*)-octopamine does not interact with enzyme, consistent with our failure to detect product inhibition by (*R*)-norepinephrine up to a concentration of 10 mM.

[3] In a previous study, Bachan et al. (1974) observed a deuterium isotope effect of 2.0 for [2(*R*)-^{2}H]phenethylamine hydroxylation in the presence of fumarate at a single concentration of O_2 (air). Depending on the extent of enzyme saturation by oxygen, their reported isotope effect may reflect either k_{cat}/K_M or k_{cat}.

turnover (k_{cat} = 1.1 s^{-1}) indicates that inactivation is a very slow event, occurring only once in every 12000 turnovers.

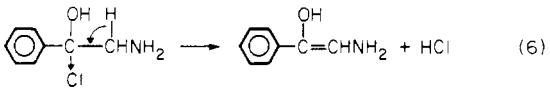
Many of the properties of enzyme inhibition by β-chlorophenethylamine are similar to those reported by Baldoni & Villafranca (1980) for enzyme inhibition by β-hydroxybenzyl cyanide. In the latter case, the authors have demonstrated inhibition every 8000 turnovers with a rate constant of 8.3 × 10^{-4} s^{-1}. In addition, the cyanohydrin derived from *p*-hydroxybenzaldehyde and HCN has been shown to inactivate dopamine β-hydroxylase rapidly in the absence of reductant. These observations led Baldoni and Villafranca to suggest a mechanism involving proton abstraction from *p*-hydroxymandelonitrile to generate a reactive ketenimine:



(5)

I

A similar mechanism for dopamine β-hydroxylase inhibition by β-chlorophenethylamine would involve an α,β elimination of HX from bound product to generate the enol of α-aminoacetophenone (**2** in eq 6). Analogous to the similarity in



(6)

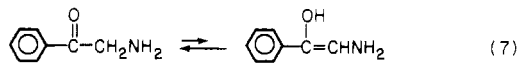
2

kinetic properties for enzyme inhibition by β-chlorophenethylamine and *p*-hydroxybenzyl cyanide, structures **1** (eq 5) and **2** (eq 6) are quite similar. It should be noted, however, that formation of **2** according to eq 6 requires activation of hydrogen at the α rather than β carbon of product; the ability of dopamine β-hydroxylase to catalyze hydrogen activation at the more inert, nonbenzylic position of phenethylamine derivatives has not yet been demonstrated. Furthermore, eq 6 fails to take into account the properties of α-aminoacetophenone inhibition described in this study.

A potentially key finding in the current study is the failure to observe enzyme inhibition by α-aminoacetophenone in the presence of ascorbate (Figure 5A) together with a time- and concentration-dependent inhibition by α-aminoacetophenone in the absence of reductant (Figure 5B). The opposing effects of α-aminoacetophenone in the presence and absence of reductant strongly suggest that enzyme-bound copper must be in a +2 valence state for enzyme to undergo inactivation by α-aminoacetophenone. The observation of a nonzero intercept in plots of $t_{1/2}$ (inactivation) vs. the reciprocal of α-aminoacetophenone concentration (inset in Figure 5B) supports the formation of a complex between α-aminoacetophenone and E–Cu^{2+} in the course of enzyme inactivation, although the apparent very weak binding of α-aminoacetophenone precludes a precise determination of either K_i (≈50 mM) or $t_{1/2}$ (≈0.2 h). The absence of enzyme inactivation by α-aminoacetophenone in the presence of ascorbate may simply be a consequence of inefficient binding at the enzyme active site, consistent with our failure to observe significant inhibition of initial rates for dopamine hydroxylation in the presence of up to 10 mM α-aminoacetophenone. Although mechanistic details were not reported, previous authors have noted the inhibitory effect of the α-aminoacetophenone analogues of adrenaline and norepinephrine (adrenalone and arterenone, respectively) toward dopamine-β-hydroxylase both in vitro and in vivo (Goldstein et al., 1962; Goldstein, 1966).

As illustrated in Scheme I, the initial product derived from hydroxylation of β-X-phenethylamines has the potential to partition between product release, k_7, and ketone formation, k_9. It is generally believed that enzyme-bound copper cycles between Cu^{1+} in E·S complexes and Cu^{2+} in E·P complexes, and this feature of the dopamine β-hydroxylase mechanism has been incorporated into Scheme I. Inspection of Scheme I indicates that elimination of HX from enzyme-bound product would generate the enzyme form proposed to undergo rapid inactivation, E·Cu^{2+}·$C_6H_5C(=O)CH_2NH_2$. Thus the unique ability of β-chlorophenethylamine among the β-substituted phenethylamines examined to cause enzyme inhibition may derive from the significantly lower stability of the chlorohydrin than *gem*-diol derivative of α-aminoacetophenone. The inefficiency of enzyme inactivation by β-chlorophenethylamine ($t_{1/2}$ = 2.1 h) compared to α-aminoacetophenone ($t_{1/2}$ ≈ 0.2 h) suggests (i) that even in the case of the chlorohydrin of α-aminoacetophenone, the rate of formation of α-aminoacetophenone from bound product does not compete effectively with product release ($k_7 > k_9$) and/or (ii) that bound α-aminoacetophenone undergoes a rapid dissociation relative to enzyme inactivation ($k_{11} > k_i$).

The chemical mechanism whereby α-aminoacetophenone irreversibly inactivates dopamine β-hydroxylase remains unclear. Although nucleophilic addition of an active site residue to the ketone carbonyl would generate a covalent adduct, such adducts are unstable in solution and would require stabilization at the enzyme active site. A route other than eq 6 for production of **2** is the direct enolization of α-aminoacetophenone.



(7)

2

The position of equilibrium for eq 7 is expected to lie very much in favor of ketone (K_{eq} = 2 × 10^{-7} for acetophenone; Guthrie, 1979). It is conceivable that dopamine β-hydroxylase perturbs the position of this equilibrium for bound α-aminoacetophenone. Alternatively, the observed rate of inactivation of dopamine β-hydroxylase by α-aminoacetophenone, $k_i \simeq 10^{-3}$ s^{-1}, might be the consequence of a very rapid inactivation process, $k_i' \geq 5 \times 10^3$ s^{-1}, by the minor enol species, ≤2 × 10^{-7} E_T. Analogous to ketone, however, direct nucleophilic attack by an active-site residue at either the α or β carbon of **2** is not expected to lead to a stable covalent adduct. Oxidation of **2** coupled to the production of E·Cu^{1+} from E·Cu^{2+} could, in principle, generate a radical cation capable of irreversible binding to dopamine β-hydroxylase. It is anticipated that ongoing studies focused on the demonstration and characterization of putative covalent adducts will provide insight into the nature of the chemical intermediate(s) leading to enzyme inhibition.

The generality of β-substituted phenethylamines as substrates will require further investigation to determine the range of steric and electronic alterations in X which can be tolerated without loss of enzyme activity. Nonetheless, it is of interest to speculate on the significance of a very broad substrate specificity to dopamine β-hydroxylase activity in vivo. As a consequence of compartmentation of enzyme to either chromaffin or synaptic vesicles, intravesicular enzyme specificity is expected to reside in the capacity of phenethylamine derivatives to be transported into storage vesicles as either passive diffusion or an ATP-dependent active transport process (Kirshner, 1962; Taugner & Hasselbach, 1968; Phillips, 1974). By contrast, the specificity of circulating dopamine β-hydroxylase, released from synaptic vesicles concomitant with sympathetic nervous system activity, can be expected to more closely resemble the in vitro properties of dopamine β-hydroxylase; in the event that circulating dopamine β-hydroxylase

retains monooxygenase activity in vivo, its function could be distinct from the well-characterized role of intravesicular dopamine β-hydroxylase in norepinephrine biosynthesis.

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Solution Structure of 5-Keto-D-fructose: Relevance to the Specificity of Hexose Kinases†

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ABSTRACT: 5-Keto-D-fructose (5KF) is isolated from cultures of *Gluconobacter cerinus* growing on D-fructose as the sole carbon source. 5KF is a substrate for hexokinase, fructokinase, and several polyol dehydrogenases. ^1H and ^{13}C nuclear magnetic resonance studies show that 5KF exists in different forms in anhydrous dimethyl-d_6 sulfoxide and D_2O. In dimethyl-d_6 sulfoxide, 5KF exists as a spirane dimer with linked furanose and pyranose rings, similar to the structure reported for crystalline 5KF [Hassen, L., Hordvik, A., & Hove, R. (1976) *J. Chem. Soc., Chem. Commun.*, 572]. In D_2O, 5KF exists predominantly (>95%) in a β-pyranose form with the 5-keto group hydrated to form a *gem*-diol. ^{13}C–^1H coupling patterns, ^{13}C relaxation measurements, and ^{13}C deuterium-induced differential isotope shifts confirm this structure of 5KF. The phosphorylation of 5KF by fructokinase can be accounted for by an approximately 2% proportion of the β-furanose form in solution at 25 °C. Both the β-pyranose and β-furanose forms of 5KF are proposed to be substrates for yeast hexokinase.

5-Keto-D-fructose (5KF,[1] D-*threo*-2,5-hexodiulose) is produced by several strains of *Acetobacter* growing on D-fructose as the sole carbon source (Terada et al., 1960; Avigad & Englard, 1965; Ameyama et al., 1981). Specific NADPH-linked dehydrogenases have been isolated and purified from *Gluconobacter cerinus* (Avigad et al., 1966) and yeast (Englard et al., 1972) which reduce 5KF and 5KF-1-P to D-

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[1] Abbreviations: 5KF, 5-keto-D-fructose; 5KF-1-P, 5-keto-D-fructose 1-phosphate; Me_2SO-d_6, dimethyl-d_6 sulfoxide; NMR, nuclear magnetic resonance; DDS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; Me_4Si, tetramethylsilane.

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