

- son, A., Eds.) Vol. III, p 187, Wiley, New York.
- Jencks, W. P. (1980) *Acc. Chem. Res.* 13, 161.
- Jenkins, W. T. (1961) *J. Biol. Chem.* 236, 1121.
- Jenkins, W. T., & Taylor, R. T. (1965) *J. Biol. Chem.* 240, 2907.
- Jenkins, W. T., & D'Ari, L. (1966) *J. Biol. Chem.* 241, 2845.
- Jenkins, W. T., & Harruff, R. C. (1979) *Arch. Biochem. Biophys.* 192, 421.
- Julin, D. A., Wiesinger, H., Toney, M. D., & Kirsch, J. F. (1989) *Biochemistry* (first of three papers in this issue).
- Klick, D. M., & Cook, P. F. (1983) *Biochemistry* 22, 375.
- Kirsch, J. F., Eichele, G., Ford, G. C., Vincent, M. G., Janssonius, J. N., Gehring, H., & Christen, P. (1984) *J. Mol. Biol.* 174, 497.
- Kokesh, F. C. (1976) *J. Org. Chem.* 41, 3593.
- Limbach, H. H., Hennig, J., Gerritzen, D., & Rumpel, H. (1982) *Faraday Discuss. Chem. Soc.* 74, 229.
- Maley, J. R., & Bruice, T. C. (1968) *J. Am. Chem. Soc.* 90, 2843.
- Martinez-Carrion, M., Riva, F., Turano, C., & Fasella, P. (1965) *Biochem. Biophys. Res. Commun.* 20, 206.
- McLeish, M. J., Julin, D. A., & Kirsch, J. F. (1989) *Biochemistry* (second of three papers in this issue).
- Meister, A. (1950) *J. Biol. Chem.* 184, 117.
- Menger, F. M. (1983) *Tetrahedron* 39, 1013.
- Michuda, C. M., & Martinez-Carrion, M. (1969) *J. Biol. Chem.* 244, 5920.
- More O'Ferrall, R. A. (1970) *J. Chem. Soc. B*, 785.
- Northrop, D. B. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) p 122, University Park Press, Baltimore, MD.
- Pascal, R. A., & Walsh, C. T. (1984) *Biochemistry* 23, 2745.
- Rendina, A. R., Hermes, J. D., & Cleland, W. W. (1984) *Biochemistry* 23, 6257.
- Rosenberg, S., & Kirsch, J. F. (1981) *Biochemistry* 20, 3189.
- Saier, M. H., & Jenkins, W. T. (1967) *J. Biol. Chem.* 242, 101.
- Segel, I. H. (1975) *Enzyme Kinetics*, Wiley, New York.
- Sonderegger, P., Gehring, H., & Christen, P. (1977) *J. Biol. Chem.* 252, 609.
- Swain, C. G., Stivers, E. C., Reuwer, J. F., Jr., & Schaad, L. J. (1958) *J. Am. Chem. Soc.* 80, 5885.
- Ulevitch, R. J., & Kallen, R. G. (1977) *Biochemistry* 16, 5350.
- Velick, S. F., & Vavra, J. (1962) *J. Biol. Chem.* 237, 2109.
- Walter, U., Luthe, H., Gerhart, F., & Soling, H.-D. (1975) *Eur. J. Biochem.* 59, 395.
- Westheimer, F. H. (1961) *Chem. Rev.* 61, 265.

## Inactivation of Dopamine $\beta$ -Hydroxylase by $\beta$ -Ethynyltyramine: Kinetic Characterization and Covalent Modification of an Active Site Peptide

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**ABSTRACT:**  $\beta$ -Ethynyltyramine has been shown to be a potent, mechanism-based inhibitor of dopamine  $\beta$ -hydroxylase (DBH). This is evidenced by pseudo-first-order, time-dependent inactivation of enzyme, a dependence of inactivation on the presence of ascorbate and oxygen cosubstrates, the ability of tyramine (substrate) and 1-(3,5-difluoro-4-hydroxybenzyl)imidazole-2-thione (competitive multisubstrate inhibitor) to protect against inactivation, and a high affinity of  $\beta$ -ethynyltyramine for enzyme. Inactivation of DBH by  $\beta$ -ethynyltyramine is accompanied by stoichiometric, covalent modification of the enzyme. Analysis of the tryptic map following inactivation by [<sup>3</sup>H]- $\beta$ -ethynyltyramine reveals that the radiolabel is associated with a single, 25 amino acid peptide. The sequence of the modified peptide is shown to be Cys-Thr-Gln-Leu-Ala-Leu-Pro-Ala-Ser-Gly-Ile-His-Ile-Phe-Ala-Ser-Gln-Leu-His\*-Thr-His-Leu-Thr-Gly-Arg, where His\* corresponds to a covalently modified histidine residue. In studies using the separated enantiomers of  $\beta$ -ethynyltyramine, we have found the *R* enantiomer to be a reversible, competitive inhibitor versus tyramine substrate with a *K*<sub>i</sub> of  $7.9 \pm 0.3 \mu\text{M}$ . The *S* enantiomer, while also being a competitive inhibitor (*K*<sub>i</sub> =  $33.9 \pm 1.4 \mu\text{M}$ ), is hydroxylated by DBH to give the expected  $\beta$ -ethynyltyramine product and also efficiently inactivates the enzyme [*k*<sub>inact</sub>(app) =  $0.18 \pm 0.02 \text{ min}^{-1}$ ; *K*<sub>i</sub>(app) =  $57 \pm 8 \mu\text{M}$ ]. The partition ratio for this process is very low and has been estimated to be about 2.5. This establishes an approximate value for *k*<sub>cat</sub> of  $0.45 \text{ min}^{-1}$  and reveals that (*S*)- $\beta$ -ethynyltyramine undergoes a slow turnover relative to that of tyramine (*k*<sub>cat</sub>  $\approx 50 \text{ s}^{-1}$ ), despite the nearly 100-fold higher affinity of the inactivator for enzyme.

**D**opamine  $\beta$ -hydroxylase (DBH;<sup>1</sup> EC 1.14.17.1) is a copper-containing mixed-function oxidase that catalyzes the hy-

droxylation of dopamine to norepinephrine (Scheme I; Levin et al., 1960; Skotland & Ljones, 1979; Rosenberg & Lovenberg, 1980; Villafranca, 1981; Ljones & Skotland, 1984). Despite its key physiological role in the biosynthesis of catecholamine neurotransmitters and its absolute stereochemical

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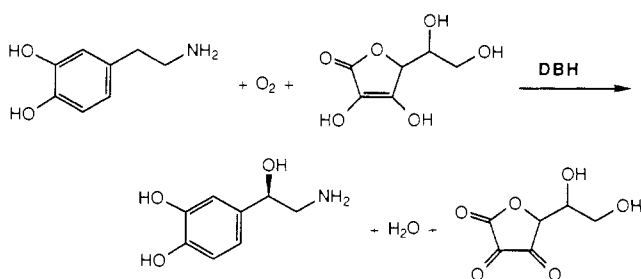
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<sup>1</sup> Abbreviations: DBH, dopamine  $\beta$ -hydroxylase; Eu[(+)-hfc]<sub>3</sub>, tris-[3-[(heptafluoropropyl)hydroxymethylene]-(+)-camphorato]europium (III); *R*<sub>T</sub>, retention time; TFA, trifluoroacetic acid.

Scheme I



specificity for removal of the *pro-R* benzylic hydrogen (Battersby et al., 1976), DBH displays a remarkable lack of specificity toward organic substrates. In addition to hydroxylating a number of substituted phenethylamines and phenylpropylamines (Creveling et al., 1962), DBH has been shown to catalyze sulfoxidation of phenyl thioethers (May & Phillips, 1980; May et al., 1981), epoxidation of styrenes, and N-dealkylation of methylamines (Padgett et al., 1985). In recent years, the indiscriminate nature of DBH has led to numerous reports of mechanism-based inhibitors; for an extensive review, see Fitzpatrick and Villafranca (1987). The therapeutic potential of most of these inhibitors is thought to be minimal since they exhibit  $K_i$ 's in the same range as the millimolar concentrations of catecholamine substrate found in chromaffin vesicles. However, the recent discovery that  $\beta$ -ethynyltyramine, a time-dependent inhibitor of DBH, has an affinity for the enzyme approximately 100-fold higher than that of tyramine substrate (Kruse et al., 1988) has raised the hope that sufficiently potent mechanism-based inhibitors can be developed. This paper presents the results of a detailed investigation of the inactivation of DBH by this compound with conventional steady-state kinetic and protein chemical methods. Evidence is provided demonstrating that this compound is an efficient and potent mechanism-based inhibitor of the enzyme and supporting the hypothesis that inactivation is by selective alkylation of a histidine residue in a single, major active site peptide.

## EXPERIMENTAL PROCEDURES

### Materials

The following were obtained from Aldrich: L-ascorbic acid, *N*-ethylmorpholine (4-ethylmorpholine), iodoacetic acid, sodium ascorbate, and trichloroacetic acid. Prior to use, the *N*-ethylmorpholine was refluxed for 1 h with excess ninhydrin and then distilled from KOH. Tyramine hydrochloride and D,L-octopamine were purchased from Sigma. Guanidine hydrochloride and trifluoroacetic acid (both Sequanal grade) were from Pierce. Crystalline catalase was the product of Boehringer Mannheim. 2-Mercaptoethanol was obtained from Bio-Rad. TPCK-treated trypsin was purchased from Cooper Biomedical, and the HPLC-grade solvents were supplied by J. T. Baker. 1-(3,5-Difluoro-4-hydroxybenzyl)imidazole-2-thione was prepared as described previously (Kruse et al., 1986). DBH was purified from bovine adrenal medulla according to the procedure of Scott et al. (1988). All other chemicals were commercial products of the highest purity available.

### Chemical Syntheses

**4-[(4-Methoxyphenyl)methoxy]benzaldehyde.** A solution of 4-hydroxybenzaldehyde (25.0 g, 0.205 mol) and 4-methoxybenzyl chloride (32.0 g, 0.205 mol) in DMF (150 mL) was stirred at ambient temperature under an argon atmosphere during the addition (30 min) of 50% sodium hydride-mineral

oil dispersion (9.9 g, 0.205 mol). The reaction mixture was stirred for 16 h at ambient temperature and poured into water (1 L). The crystalline product was filtered, dried, and recrystallized from ethanol to yield 38.1 g (77%) of product: mp 94–96 °C [lit. mp 98–99 °C (Taguchi et al., 1981)].

**2,2-Dimethyl-5-[[4-[(4-methoxyphenyl)methoxy]phenyl]methylene]-1,3-dioxane-4,6-dione (2).** A solution of Meldrum's acid (14.4 g, 0.1 mol) and 4-[(4-methoxyphenyl)methoxy]benzaldehyde (24.2 g, 0.1 mol) in toluene (100 mL) was treated with glacial acetic acid (2 mL) and piperidine (1 mL). The solution was stirred and heated at reflux until the removal of water with a Dean-Stark trap was complete. The reaction mixture was cooled, and the resulting solid orange product was filtered and washed with cold toluene to yield 24.9 g (68%) of **2**: mp 155–157 °C;  $R_f$  0.65 with 2:1 hexane-ethyl acetate as eluant. An analytical sample was recrystallized from toluene: mp 155–157 °C; IR (Nujol mull) 1740, 1713, 1545, 1180  $cm^{-1}$ ; NMR  $\delta$  1.77 (s, 6 H), 3.82 (s, 3 H), 5.08 (s, 2 H), 7.00 (d,  $A_2$  of  $A_2B_2$ ,  $J$  = 9 Hz, 2 H), 7.12 (d,  $A_2$  of  $A_2B_2$ ,  $J$  = 9 Hz, 2 H), 7.35 (d,  $B_2$  of  $A_2B_2$ ,  $J$  = 9 Hz, 2 H), 8.31 (d,  $B_2$  of  $A_2B_2$ ,  $J$  = 9 Hz, 2 H), 8.42 (s, 1 H); mass spectrum,  $m/e$  369 [(M + H)<sup>+</sup>], 121. Anal. Calcd for  $C_{21}H_{20}O_6$ : C, 68.47; H, 5.47. Found: C, 68.32; H, 5.66.

**2,2-Dimethyl-5-[[4-[(4-methoxyphenyl)methoxy]phenyl][(trimethylsilyl)ethynyl]methyl]-1,3-dioxane-4,6-dione (3).** A mixture of Mg turnings (1.4 g, 0.058 mol) and THF (75 mL) was stirred under argon while a solution of bromoethane (7.3 g, 0.067 mol) in THF (10 mL) was added. Iodomethane (0.05 mL) was added to initiate the reaction, and after the dissolution of the Mg turnings ceased (30–40 min), a solution of (trimethylsilyl)acetylene (6.34 g, 0.065 mol) in THF (20 mL) was added. The resulting solution was stirred at ambient temperature for 1 h. A solution of **2** (10.7 g, 0.029 mol) in THF (100 mL) was added, and the resulting solution was stirred until **2** had completely reacted as judged by TLC. The reaction mixture was poured into 1 N HCl, and the product was extracted with ethyl acetate. The organic extracts were washed with water, dried, and concentrated to yield 13.15 g (100%) of **3** which was used without further purification: mp 132–135 °C;  $R_f$  0.5 with 2:1 hexane-ethyl acetate as eluant. An analytical sample was recrystallized from ethanol: mp 130–132 °C; IR (Nujol mull) 2175, 1790, 1740, 1255, 1180, 845  $cm^{-1}$ ; NMR  $\delta$  0.20 (s, 9 H), 1.60 (s, 3 H), 1.70 (s, 3 H), 3.78 (m, 4 H total), 4.90 (d,  $J$  = 4 Hz, 1 H), 4.95 (s, 2 H), 6.90 (d,  $A_2$  of  $A_2B_2$ ,  $J$  = 9 Hz, 2 H), 6.93 (d,  $A_2$  of  $A_2B_2$ ,  $J$  = 9 Hz, 2 H), 7.40 (apparent t,  $2 \times B_2$  of  $A_2B_2$ ,  $J$  = 9 Hz, 4 H); mass spectrum,  $m/e$  484 [(M + NH<sub>4</sub>)<sup>+</sup>], 121. Anal. Calcd for  $C_{26}H_{30}O_6Si$ : C, 66.93; H, 6.48. Found: C, 66.54; H, 6.50.

**(*R,S*)- $\beta$ -Ethynyl-4-[(4-methoxyphenyl)methoxy]benzene-propanoic Acid (4).** A solution of **3** (14.2 g, 0.031 mol) in 3:1 pyridine-water (120 mL) was heated at 100 °C for 4 h and then cooled in ice. The cold solution was carefully acidified to pH 2 with 12 N HCl and extracted with ethyl acetate. The organic extracts were washed with water, dried, treated with activated carbon, and concentrated to yield 8.43 g (63%) of [[4-[(4-methoxyphenyl)methoxy]phenyl][(trimethylsilyl)ethynyl]methyl]propanoic acid intermediate. A solution of crude (trimethylsilyl)alkyne (8.43 g, 0.02 mol) and potassium fluoride (1.65 g, 0.029 mol) was heated at 50 °C for 90 min in DMF (50 mL). The reaction mixture was cooled, filtered, and washed with ether. The solid potassium salt in water (50 mL) was stirred and acidified to pH 2 with 12 N HCl, and the product was extracted with ethyl acetate. The organic extracts were washed with water, dried, and

concentrated to yield 5.44 g (70%) of **4**: mp 154–156 °C;  $R_f$  0.83 with 5:100:900 acetic acid–methanol–dichloromethane as eluant; IR (Nujol mull) 3300, 1710, 1610, 1180  $\text{cm}^{-1}$ ; NMR  $\delta$  2.40 (d,  $J = 3$  Hz, 1 H), 2.60–2.80 (m, AB of ABX, 2 H total), 3.80 (s, 3 H), 4.15 (d of t, X of ABX,  $J = 3$  and 7 Hz, 1 H), 4.95 (s, 2 H), 6.90 (d,  $2 \times A_2$  of  $A_2B_2$ ,  $J = 9$  Hz, 4 H), 7.45 (d,  $B_2$  of  $A_2B_2$ ,  $J = 9$  Hz, 2 H), 7.50 (d,  $B_2$  of  $A_2B_2$ ,  $J = 9$  Hz, 2 H).

#### Resolution of

#### $\beta$ -Ethynyl-4-[(4-methoxyphenyl)methoxy]benzene-propanoic Acid

(-)-(S)- $\beta$ -Ethynyl-4-[(4-methoxyphenyl)methoxy]benzene-propanoic Acid. To a solution of racemic **4** (12.48 g, 0.04 mol) in ethanol (500 mL) was added L-(+)-threo-2-amino-1-(4-nitrophenyl)-1,3-propanediol (8.82 g, 0.042 mol). The mixture was heated until a clear solution resulted. The solution was concentrated, and the residual diastereomeric salt (21.25 g) was dissolved with warming in 2-propanol (1 L). The solution was allowed to stand undisturbed at ambient temperature for 48 h. The precipitate (7.91 g) was collected and recrystallized twice from 2-propanol to yield 4.81 g (48%)<sup>2</sup> of the salt: mp 162–164 °C. Dowex 50-X2 (100 mesh) cation-exchange resin (8 g) was washed with methanol and added to a methanol (140 mL) solution of the resolved salt (4.0 g). The slurry was stirred for 15 min and then filtered. The filtrate was concentrated and the residue dried at ca. 0.5 Torr to constant weight to yield 2.3 g of solid: mp 144–146.5 °C;  $[\alpha]_D^{25} -19.4^\circ$  (c 1.5, DMF).

(+)-(R)- $\beta$ -Ethynyl-4-[(4-methoxyphenyl)methoxy]benzene-propanoic Acid. The combined mother liquors from the preceding preparation were concentrated, and the residue was dissolved in methanol (250 mL). Dowex 50-X2 (100 mesh) cation-exchange resin (30 g) was washed with methanol and then added. The slurry was stirred for 15 min and filtered. The filtrate was treated with activated carbon, filtered, and concentrated. The free carboxylic acid (6.54 g, 0.021 mol) was dissolved in ethanol (250 mL), and D-(-)-threo-2-amino-1-(4-nitrophenyl)-1,3-propanediol (4.68 g, 0.022 mol) was added. The mixture was heated on a steam bath until a clear solution resulted. The solution was concentrated, and the residue (10.7 g) was recrystallized twice from 2-propanol to yield 5.48 g (55%)<sup>2</sup> of the salt: mp 161–163 °C. The free carboxylic acid was obtained in the same manner as the (-)-enantiomer: mp 140–143 °C,  $[\alpha]_D^{25} +19.0^\circ$  (c 1.5, DMF).

(R,S)-[2-Ethynyl-2-[4-[(4-methoxyphenyl)methoxy]phenyl]ethyl]carbamic Acid (4-Methoxyphenyl)methyl Ester (**5**). A solution of **4** (17.36 g, 0.056 mol), diphenylphosphoryl azide (15.4 g, 0.056 mol), triethylamine (5.7 g, 0.056 mol), and 4-methoxybenzyl alcohol (9.0 g, 0.065 mol) in toluene (100 mL) was heated at 100 °C for 8 h under argon. The reaction mixture was cooled and concentrated under reduced pressure, and the residue was partitioned between ethyl acetate and water. The ethyl acetate layer was washed with water, dried, and concentrated under reduced pressure. The crude product was purified by flash chromatography with 3:1 hexane–ethyl acetate as eluant to yield 15.45 g (62%) of (R,S)-**5** contaminated with traces of 4-methoxybenzyl alcohol. An analytical sample was prepared by recrystallization from ethyl acetate–hexane: mp 112–114 °C;  $R_f$  0.57 with 2:1 hexane–ethyl acetate as eluant; IR (Nujol mull) 3310, 3300, 1698, 1250, 1180  $\text{cm}^{-1}$ ; NMR  $\delta$  2.27 (d,  $J = 2$  Hz, 1 H), 3.40–3.60

(m, AB of ABX, 2 H total), 3.81 (br s, 7 H total), 4.97 (s, 2 H), 5.05 (s, 2 H), 6.81–7.51 (m, 12 H total); mass spectrum,  $m/e$  463 [(M +  $\text{NH}_4$ )<sup>+</sup>], 282, 138, 121. Anal. Calcd for  $\text{C}_{27}\text{H}_{27}\text{NO}_5$ : C, 72.79; H, 6.11; N, 3.14. Found: C, 72.55; H, 6.11; N, 3.07.

(R)-[2-Ethynyl-2-[4-[(4-methoxyphenyl)methoxy]phenyl]ethyl]carbamic Acid (4-Methoxyphenyl)methyl Ester [(R)-**5**]. The reaction of (R)-**4** as in the preparation of (R,S)-**5** led to (R)-**5**: mp 118–120 °C;  $[\alpha]_D^{25} +25.4^\circ$  (c 1.5, DMF).

(S)-[2-Ethynyl-2-[4-[(4-methoxyphenyl)methoxy]phenyl]ethyl]carbamic Acid (4-Methoxyphenyl)methyl Ester [(S)-**5**]. The reaction of (S)-**4** as in the preparation of (R,S)-**5** led to (S)-**5**: mp 119–120.5 °C;  $[\alpha]_D^{25} -26.5^\circ$  (c 1.5, DMF).

(R,S)- $\beta$ -Ethynyl-4-hydroxybenzeneethanamine Hydrochloride (**1**). A solution of (R,S)-**5** (2.22 g, 5 mmol) in 1:1 ethyl acetate–ether (60 mL) was stirred during the addition of a saturated ethereal HCl solution (20 mL), and stirring was continued until the solution became cloudy. The solution was then allowed to stand at ambient temperature until crystallization was complete. The mixture was filtered, and the product was recrystallized from methanol–ethyl acetate: mp 160–163 °C;  $R_f$  0.81 with 75:23:2 ethyl acetate–methanol–14 N  $\text{NH}_4\text{OH}$  as eluant. An analytical sample of (R,S)-**1** was prepared by flash chromatography with 75:2 ethyl acetate–14 N  $\text{NH}_4\text{OH}$  as eluant followed by reconversion to the hydrochloride and recrystallization from methanol–acetonitrile: mp 171–173 °C; IR (Nujol mull) 3230, 1595, 1490, 832  $\text{cm}^{-1}$ ; NMR ( $[\text{H}_2\text{O}]$  DMSO- $\text{CDCl}_3$ )  $\delta$  2.73 (d,  $J = 3$  Hz, 1 H), 3.12 (d,  $J = 8$  Hz, 2 H), 4.20 (d of t,  $J = 2$  and 8 Hz, 1 H), 6.80 ( $A_2$  of  $A_2B_2$ ,  $J_{AB} = 9$  Hz, 2 H), 7.30 ( $B_2$  of  $A_2B_2$ ,  $J_{AB} = 9$  Hz, 2 H), 8.69 (br s, 1 H); mass spectrum,  $m/e$  162 [(M + H)<sup>+</sup>], 145, 132, 121. Anal. Calcd for  $\text{C}_{10}\text{H}_{12}\text{ClNO}$ : C, 60.72; H, 6.12; N, 7.09. Found: C, 60.74; H, 6.08; N, 7.17.

(R)- $\beta$ -Ethynyl-4-hydroxybenzeneethanamine Hydrochloride [(R)-**1**]. The deprotection of (R)-**5** as in the preparation of (R,S)-**1** led to (R)-**1**: mp 186–189 °C;  $[\alpha]_D^{25} +14.1^\circ$  (c 1.5, DMF).

(S)- $\beta$ -Ethynyl-4-hydroxybenzeneethanamine Hydrochloride [(S)-**1**]. The deprotection of (S)-**5** as in the preparation of (R,S)-**1** led to (S)-**1**: mp 193–195 °C;  $[\alpha]_D^{25} -17.1^\circ$  (c 1.5, DMF).

**Determination of the Absolute Configuration.** The absolute configuration of **1** was determined by chemical degradation of the intermediate (+)-**4** (Figure 1A) to (S)-(-)-ethylsuccinic acid as previously described (Kruse et al., 1988).

**Synthesis of [ $^3\text{H}$ ]- $\beta$ -Ethynyltyramine.** (R,S)-**1** (5 mg) was heated with approximately 10 Ci of high specific activity  $^3\text{H}_2\text{O}$  at 80 °C in trifluoroacetic acid to give 685 mCi of crude product. A portion of this (115 mCi) was purified by semipreparative HPLC on a Spherisorb ODS (10  $\mu\text{m}$ , 10  $\times$  250 mm) column with 0.1 M ammonium acetate, pH 4.2–15%  $\text{CH}_3\text{CN}$  as the mobile phase. The column was eluted at a flow rate of 4 mL/min and monitored at 254 nm. This was followed by preparative TLC on silica gel eluted with 90:10:1  $\text{CH}_2\text{Cl}_2$ – $\text{CH}_3\text{OH}$ – $\text{NH}_4\text{OH}$ . The appropriate band was scraped from the plate and eluted with 10:1  $\text{CHCl}_3$ – $\text{CH}_3\text{OH}$  to give 4.33 mCi of the final product. The specific activity of 40.6 Ci/mmol was calculated from the enrichment factor on the basis of ion intensities for the mass spectral fragmentation ion  $m/e$  132. It indicated the presence of three species: (1) unsubstituted, 35%; (2) disubstituted (ortho to the hydroxyl group), 55%; (3) trisubstituted (ortho disubstituted and acetylenic), 10%. The radiochemical purity was 96.8% on the basis of HPLC ( $\mu\text{Bondapak C}_{18}$ , 0.1 M ammonium acetate, pH 4.2–15%  $\text{CH}_3\text{CN}$ ) and silica-based TLC in two solvent

<sup>2</sup> The yield is based on the theoretical amount of the desired diastereomeric salt.

systems (90:10:1  $\text{CH}_2\text{Cl}_2$ - $\text{CH}_3\text{OH}$ - $\text{NH}_4\text{OH}$  and 5:1:1 ethyl acetate- $\text{CH}_3\text{OH}$ - $\text{NH}_4\text{OH}$ ).

#### Biochemical Methods

**Standard Assay Conditions for DBH.** The enzyme was incubated at 37 °C in the presence of 50 mM sodium acetate, pH 5.0, 1 mg/mL catalase, 10  $\mu\text{M}$   $\text{CuCl}_2$ , and enough 3.0 M NaCl to bring the ionic strength to 0.2. Substrates, inhibitors, and enzyme were varied as required for the specific experiments described below. The 1-mL reaction mixtures were agitated in a New Brunswick R-76 shaker/bath oscillating at 2 Hz to maintain equilibration with atmospheric oxygen. To measure the hydroxylation of tyramine, the reactions were first terminated by the addition of 0.2 mL of 3.0 M trichloroacetic acid, and the amount of octopamine produced was determined by a periodate cleavage procedure similar to that previously described (Nagatsu & Udenfriend, 1972). D,L-Octopamine was used as a standard.

**Steady-State Kinetic Studies.** Assays were performed as described above with various concentrations of substrates and inhibitors. The substrates tyramine, ascorbate, and oxygen were varied over the ranges of 0.68–10 mM, 0.17–1.0 mM, and 0.11–1.1 mM, respectively. Typically, six different concentrations of the varied substrate were used. Since only one substrate was varied in each experiment, the concentrations of the other, nonvaried substrates were fixed at 1 or 2 mM, 10 mM, and 0.24 mM for tyramine, ascorbate, and oxygen, respectively. The concentration of the inhibitor was usually varied from 0 to 3 times the inhibition constant ( $K_{is}$  or  $K_{ij}$ , whichever was appropriate), and approximately 0.4  $\mu\text{g}$  of DBH was used per sample. When the inhibitor was also an inactivator, the time of the incubation was kept short (10 min) in order to ensure the maintenance of steady-state conditions.

In experiments in which oxygen was the varied substrate, initial velocities were determined at 37 °C in 4-mL reaction mixtures in a Yellow Springs Instruments Model 53 oxygen monitor equipped with a custom-built offset amplifier (Sigmatronics, Inc., Bridgeport, PA). Prior to the addition of catalase and ascorbate, the reaction mixture was gassed for 3 min with the appropriate  $\text{O}_2/\text{N}_2$  mixture. Catalase (40  $\mu\text{L}$  of 20 mg/mL suspension) and sodium ascorbate (20  $\mu\text{L}$  of 2.0 M solution) were added, and the rate of autoxidation of ascorbate was measured for 4 min. Then, 10  $\mu\text{g}$  of DBH was added to initiate the enzymatic reaction.

**Inactivation Studies.** The inactivation of DBH was carried out at 37 °C in preincubation mixtures consisting of the standard incubation mixture (see above), 10 mM ascorbate, and appropriate concentrations of inactivator in a total volume of 0.25 mL. The reaction was initiated by the addition of 0.8–2  $\mu\text{g}$  of DBH. Beginning at approximately 30 s and every 5–10 min thereafter for periods up to 60 min, the remaining DBH activity was measured by removing 5- $\mu\text{L}$  samples and placing them into standard incubation mixtures containing 10 mM each of tyramine and ascorbate. After incubation of these for 1 h, the reaction was terminated with 3.0 M trichloroacetic acid, and the octopamine was measured as described above. The dependence of inactivation on ascorbate was determined by omitting it from the preincubation mixtures.

Experiments to show the oxygen dependence of inactivation in the presence and absence of ascorbate were similar to those described above except that the preincubation mixtures were placed into 0.6-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 argon and then stirred with positive pressure and continuous flow of gas throughout the experiment. The

preincubation mixtures were equilibrated under these conditions for 45 min prior to the addition of DBH. Aliquots (5  $\mu\text{L}$ ) were withdrawn and assayed at the specified time intervals as described above with a microliter syringe inserted through the Teflon/silicone septa.

Protection of DBH by tyramine substrate and the competitive inhibitor 1-(3,5-difluoro-4-hydroxybenzyl)imidazole-2-thione was demonstrated in the presence and absence of ascorbate with preincubation mixtures containing 1 or 50 mM tyramine or 0.5  $\mu\text{M}$  inhibitor. In order to account for the octopamine produced during the preincubation, additional aliquots were removed from the preincubation mixtures containing tyramine at the same time as those for the activity measurements and placed into previously quenched incubation mixtures. These were analyzed for octopamine as previously described.

**Demonstration of Irreversibility.** Approximately 40  $\mu\text{g}$  of DBH was inactivated by preincubation at 37 °C for 30 min in 5 mL of standard reaction mixture containing 10 mM ascorbate and 120  $\mu\text{M}$  (S)-1. The preincubation mixture containing the inactivated enzyme was divided into four 1-mL aliquots and dialyzed for 48 h at 4 °C vs  $2 \times 500$  mL of the following buffers: 50 mM sodium acetate, pH 4.5; 50 mM sodium acetate, pH 5.5, 5 mM tyramine; 50 mM sodium phosphate, pH 6.5; 50 mM Na-HEPPS, pH 8.5. All of the buffers also contained 0.15 M NaCl (to bring ionic strength near to 0.2) and 20  $\mu\text{g}/\text{mL}$  catalase. Two control samples were also preincubated and dialyzed similarly. The first contained the standard reaction mixture, ascorbate, and enzyme but no inactivator, and the second contained the reaction mixture, inactivator, and enzyme but no ascorbate. Activity measurements were made before and after dialysis.

**Hydroxylation of  $\beta$ -Ethynyltyramine by DBH.** Turnover of (R)- and (S)-1 into products was determined by incubating the separated enantiomers at a concentration of 1 mM with standard reaction mixture containing 10 mM ascorbate and 10 mg/mL DBH in a total volume of 100  $\mu\text{L}$ . Following incubation at 37 °C for 1 h, the samples were deproteinized by placing them into a Centricon microconcentrator (30 000 MW cutoff, Amicon Corp.) and centrifuging at 2000g for 20 min. The filtrate was then analyzed by reversed-phase HPLC on a Vydac 201 HS (4.6  $\times$  150 mm, 5  $\mu\text{m}$ ) column equipped with an RP-300 guard cartridge (7  $\mu\text{m}$ , Brownlee Labs). A 20-min, linear gradient from 0 to 9% aqueous  $\text{CH}_3\text{CN}$  containing 0.1% TFA was used at a flow rate of 1 mL/min. Alternatively, the samples were analyzed by HPLC-MS using a deactivated  $\text{C}_{18}$  column (LC-18-DB, 4.6  $\times$  150 mm, 5  $\mu\text{m}$ ; Supelco, Bellefonte, PA) and a mobile phase consisting of solvents A (0.1 M ammonium acetate, pH 5.3) and B ( $\text{C}-\text{H}_3\text{OH}$ ), with a 10-min hold at 2% B followed by a linear gradient to 50% B over 35 min at a flow rate of 1.1 mL/min. The HPLC-MS data were obtained on a triple quadrupole mass spectrometer (TSQ-45; Finnigan-MAT, San Jose, CA), with thermospray ionization. An electron filament was not used. Typical ion source conditions used a block temperature of 200 °C, a vaporizer temperature of 100–130 °C, and a repeller setting of  $\pm 30$  V. Spectra were recorded with either 1- or 2-s scans over the range 90–750 amu. Collision-assisted dissociation spectra were generated within the second quadrupole analyzer (Q2, operated in rf-only mode), with argon as a target gas at pressures of 110–130 mPa and energies ( $E_{lab}$ ) of  $\pm 20$  to  $\pm 25$  eV.

**Inactivation of DBH with [ $^3\text{H}$ ]- $\beta$ -Ethynyltyramine and Preparation of Radiolabeled Tryptic Peptides.** Approximately 5 mg of DBH was completely inactivated with (S)-1 to which

had been added tritiated (*R,S*)-**1**. It was assumed that 50% of the radiolabel in the undiluted [ $^3\text{H}$ ]-(*R,S*)-**1** resides in the *S* enantiomer, and thus the final specific activity of the diluted (*S*)-**1** in the preincubation mixture was calculated to be 43.7 Ci/mol. The preincubation mixture contained the standard reaction mixture, 10 mM sodium ascorbate, 200  $\mu\text{M}$  radio-labeled (*S*)-**1**, and enzyme in a total volume of 5 mL. The inactivation, isolation of DBH, S-carboxymethylation, and tryptic digestion were carried out as described previously (DeWolf et al., 1988). The tryptic peptides were fractionated by the two-step reversed-phase HPLC procedure of DeWolf et al. (1988) except that the  $\text{C}_4$  and phenyl columns were run at 40 and 25  $^\circ\text{C}$ , respectively.

**Peptide Sequencing and Liquid Scintillation Counting of the PTH-Amino Acids.** The collected peaks of radioactivity were evaporated under a stream of nitrogen to a final volume of 150  $\mu\text{L}$ . Aliquots consisting of one-tenth or one-fifth of the sample were applied to the sequencer (Applied Biosystems 477A, Cheshire, U.K.) to check for purity and N-terminal sequence. After peptide identification, the remaining sample was sequenced, and the radioactivity of the collected PTH fractions was measured to locate the site of modification. The PTH fractions, in 120  $\mu\text{L}$  of 20%  $\text{CH}_3\text{CN}$ , were added to Poly Q minivials (Beckman Instruments) containing 5 mL of Pico-Fluor 15 scintillant (Parkard Instrument Co.). The samples were counted for 10 min each in a Beckman LS 580L using the tritium counting program with automatic quench correction.

**Mass Spectroscopy of the Native Peptide.** The FAB-MS analysis of unmodified DBH peptides was carried out on a VG Analytical ZAB high-field instrument at  $V_{\text{acc}} = 8 \text{ kV}$  for 3000 mass range. The spectra were generated from an M-Scan FAB gun with xenon gas operating at 10 kV and a beam strength of 15  $\mu\text{A}$ .

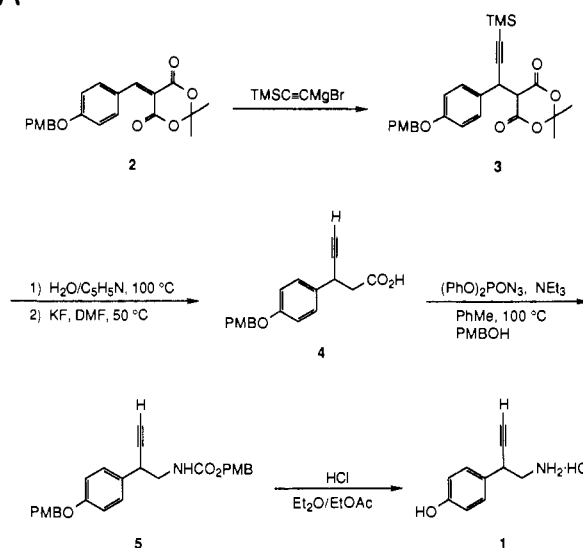
**Analysis of Data.** Multiple-parameter nonlinear regression analysis of the steady-state inhibition data was accomplished by using the COMP, NONCOMP, and UNCOMP programs of Cleland (1979). The  $k_{\text{obsd}}$  values were determined from linear regression fits of log (percent activity remaining) vs time. The values of  $k_{\text{inact}}(\text{app})$  and  $K_{\text{I}}(\text{app})$  were determined by nonlinear regression analysis of the inactivation data to

$$V = V_0 e^{-k_{\text{inact}}(\text{app})t/[K_{\text{I}}(\text{app})+I]} \quad (1)$$

## RESULTS

**Synthesis of Racemic  $\beta$ -Ethynyltyramine and Its Enantiomers.** The chemical synthesis and resolution of (*R,S*)-**1** was carried out as depicted in Figure 1A. Thus, conjugate addition of alkynylmagnesium bromide to **2** occurred in quantitative yield. The success of this reaction was critically dependent upon the use of the highly activated Meldrum's acid derivative of **2**. Other simple diester analogues of **2** (such as the corresponding benzal malonate) failed to react, a result we attribute to the low nucleophilicity of the acetylenic anion and resonance deactivation by the electron-releasing 4-alkoxy group. Hydrolysis of **3** and removal of the silicon protecting group under standard conditions yielded **4**. Modified Curtius reaction of **4** yielded carbamate **5**. Subsequent deprotection with anhydrous hydrogen chloride provided (*R,S*)-**1**. Synthesis of the two optical antipodes of **1** was accomplished by resolving the acid intermediate **4** via the crystalline diastereomeric salts produced with either L-(+)- or D-(-)-*threo*-2-amino-1-(4-nitrophenyl)-1,3-propanediol. The choice of this resolving agent was based on the formation of nicely crystalline diastereomeric salts of substantially different solubilities as well as the commercial availability of both optical antipodes of the

## A



## B

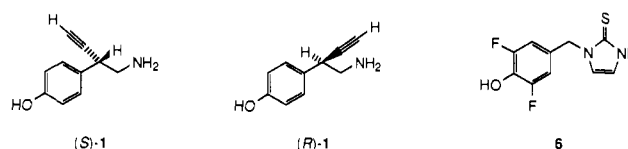


FIGURE 1: (A) Synthetic scheme for the synthesis of compound **1**; (B) structures of the DBH inhibitors.

Table I: Observed Inhibition Patterns for  $\beta$ -Ethynyltyramine and Its Separated Enantiomers<sup>a</sup>

compd	varied substrate		
	tyramine	oxygen	ascorbate
( <i>R,S</i> )- <b>1</b>	C 13.8 $\pm$ 0.8	N $K_{\text{is}} = 26.1 \pm 3.2$ $K_{\text{ii}} = 31.1 \pm 2.2$	U 33.4 $\pm$ 0.8
( <i>R</i> )- <b>1</b>	C 7.9 $\pm$ 0.3	N $K_{\text{is}} = 9.9 \pm 1.7$ $K_{\text{ii}} = 22.8 \pm 3.2$	U 20.8 $\pm$ 0.5
( <i>S</i> )- <b>1</b>	C 33.9 $\pm$ 1.4	N $K_{\text{is}} = 139 \pm 22$ $K_{\text{ii}} = 137 \pm 11$	U 112 $\pm$ 3

<sup>a</sup> C, competitive; N, noncompetitive; U, uncompetitive. Kinetic constants are reported in  $\mu\text{M}$  units with standard errors. Slope inhibition constants ( $K_{\text{is}}$ 's) are reported for competitive patterns and intercept inhibition constants ( $K_{\text{ii}}$ 's) for uncompetitive. Experimental conditions and the method for data analysis are given under Experimental Procedures.

resolving agent. The optical purity of the resolved acids was established by 90-MHz NMR studies using the diastereomeric complex formed between the chiral chemical shift reagent  $\text{Eu}[(+)\text{hfc}]_3$  and the methyl ester of **4** in either  $\text{CDCl}_3$  or [ $^2\text{H}_6$ ]benzene solution. This technique showed the presence of as little as 2–3% of enantiomeric **4**. The absolute configuration of (*R*)- and (*S*)-**4** was established by chemical degradation of the (*R*)-**4** isomer to (*S*)-(-)-ethylsuccinic acid of known absolute configuration (Kruse et al., 1988). Subsequent conversion of (*R*)- and (*S*)-**4** led to the enantiomerically pure isomers of **1** (Figure 1B). The difference in reported optical rotations for the enantiomers is a result of variable water content of these very hygroscopic salts.

**Steady-State Kinetic Studies.** Inhibition studies were carried out with (*R,S*)-, (*R*)-, and (*S*)-**1**. The results as given in Table I show that each compound is competitive vs tyramine, noncompetitive vs oxygen, and uncompetitive vs as-

Table II:  $k_{\text{obsd}}$  Values for the Inactivation by (*R*)- and (*S*)-1 under Various Experimental Conditions

expt	$k_{\text{obsd}}$ (min <sup>-1</sup> )
enantiomeric specificity	
control (no inactivator)	0.0021 ± 0.0004
1 mM ( <i>R</i> )-1	0.0028 ± 0.0004
1 mM ( <i>S</i> )-1	0.200 ± 0.003
ascorbate and oxygen dependence <sup>a</sup>	
control (no inactivator)	0.0042 ± 0.0015
+ascorbate, +oxygen	0.132 ± 0.002
+ascorbate, -oxygen	0.0091 ± 0.0008
-ascorbate, +oxygen	0.054 ± 0.002
-ascorbate, -oxygen	0.0073 ± 0.0019
protection experiments	
+ascorbate	
control (no inactivator)	0.0017 ± 0.0004
120 μM ( <i>S</i> )-1	0.125 ± 0.001
+50 mM tyramine	0.0122 ± 0.0004
0.5 μM inhibitor <sup>b</sup>	0.0027 ± 0.0003
-ascorbate	
control (no inactivator)	0.0007 ± 0.0001
120 μM ( <i>S</i> )-1	0.0197 ± 0.0008
+1 mM tyramine	0.0035 ± 0.0004
+0.5 μM inhibitor <sup>b</sup>	0.0012 ± 0.0002

<sup>a</sup> The concentration of (*S*)-1 was 120 μM. The preincubation mixtures containing ascorbate and/or oxygen had these substrates fixed at 10 mM and 0.24 mM, respectively. The control sample contained both substrates. <sup>b</sup> The specific compound used was the competitive multisubstrate inhibitor 1-(3,5-difluoro-4-hydroxybenzyl)imidazole-2-thione, which has a  $K_{\text{is}}$  of 5.7 nM vs tyramine (Kruse et al., 1986).

corbate. These inhibition patterns are consistent with both the expectation that these compounds should mimic tyramine in their binding to DBH and the proposed kinetic mechanism for the enzyme (Ahn & Klinman, 1983; Kruse et al., 1986). The composite kinetic constants given for the racemate can be seen to be the result of having a mixture of inhibitors (*R*)- and (*S*)-1 with different affinities for the enzyme (Cleland et al., 1973). In this case, the *R* enantiomer binds approximately 4-fold tighter than the *S* enantiomer with respect to tyramine substrate. However, each has a substantially higher affinity for DBH than does tyramine, with  $K_{\text{i}}$  values ranging from 40- to 170-fold lower than the  $K_{\text{m}}(\text{app})^3$  of 1.4 mM for tyramine. Interestingly, the *R* enantiomer which is neither a substrate nor an inactivator (see below) is the more potent inhibitor.

**Preliminary Inactivation Studies.** Initial studies had shown that racemic  $\beta$ -ethynyltyramine is a time-dependent inactivator of DBH. Therefore, preincubations were carried out with 1 mM (*R*)- or (*S*)-1 to determine the stereochemical specificity of this inactivation. As can be seen from a comparison of the  $k_{\text{obsd}}$  values in Table II, only the *S* enantiomer was effective in causing inactivation. The fact that this enantiomer has the benzylic hydrogen in the proper configuration for replacement with a hydroxyl group by the enzyme implies that inactivation results from an abortive, normal catalytic event. This requires that the inactivation be dependent upon both oxygen and ascorbate cosubstrates. The data presented in Table II show that inactivation is completely dependent upon the presence of oxygen. However, while ascorbate is required for optimal rates of inactivation, there is an ascorbate-independent component that results in a rate of inactivation in the absence of the reductant equal to 40% of that obtained in its presence and greater than 10-fold over that of the control sample which contained no inactivator. Thus the inactivation observed in samples containing no ascorbate is both real and significant.

<sup>3</sup> The kinetic constants reported here,  $K_{\text{m}}(\text{app})$ ,  $k_{\text{inact}}(\text{app})$ , and  $K_{\text{I}}(\text{app})$ , are properly designated as *apparent* kinetic constants since they were determined at a subsaturating concentration (0.24 mM) of the nonvaried substrate oxygen.

Table III: Kinetic Constants for the Inactivation of DBH by (*R,S*)- and (*S*)-1<sup>a</sup>

inhibitor	$k_{\text{inact}}(\text{app})$ (min <sup>-1</sup> )	$K_{\text{I}}(\text{app})$ (μM) <sup>b</sup>	$k_{\text{inact}}/K_{\text{I}}$ (M <sup>-1</sup> min <sup>-1</sup> )
( <i>R,S</i> )-1	0.023 ± 0.001	15.7 ± 2.3	1500
( <i>S</i> )-1			
+ascorbate	0.184 ± 0.015	57.0 ± 7.9	3200
-ascorbate	0.088 ± 0.004	208 ± 26	420

<sup>a</sup> These reported values with standard errors are the result of a computer fit of the raw data to eq 1. <sup>b</sup>  $K_{\text{I}}$ , the concentration of inhibitor that gives a half-maximal rate of inactivation. Since this constant is a complex ratio of multiple microscopic rate constants, it does not necessarily represent a true dissociation constant.

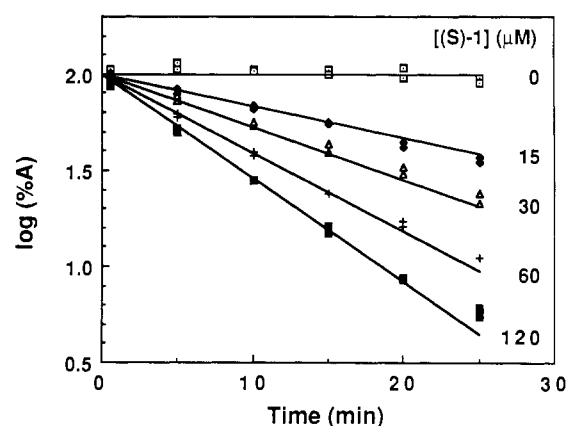


FIGURE 2: Inactivation of DBH by (*S*)-1. The preincubation was carried out in the presence of ascorbate and the indicated concentrations of (*S*)-1 as described under Experimental Procedures. A plot of the raw data of percent activity remaining vs time is shown along with the result of a computer fit of the data to eq 1 (lines).

Like its ascorbate-dependent counterpart, it also requires oxygen (Table II).

Experiments were then conducted to determine whether the substrate tyramine or the potent multisubstrate inhibitor 1-(3,5-difluoro-4-hydroxybenzyl)imidazole-2-thione (**6**; Figure 1B) could protect DBH from inactivation. Tyramine was included during the preincubation at a concentration of 50 mM, which is 35 times its  $K_{\text{m}}(\text{app})$ . The concentration of the competitive inhibitor was 0.5 μM or about 100 times its  $K_{\text{is}}$  vs tyramine. As shown in Table II, both compounds afforded virtually complete protection from the (*S*)-1-induced inactivation. In order to further characterize the unexpected ascorbate-independent inactivation, the effect of these compounds was also tested in preincubation mixtures *lacking* ascorbate. Both were effective in protecting the enzyme, but surprisingly, it was discovered that *in the absence of ascorbate cofactor* tyramine at the relatively low concentration of 1 mM completely prevented inactivation (Table II) even though the concentration of inactivator was approximately twice its  $K_{\text{I}}(\text{app})$  (Table III). These results suggested that the protective effect of tyramine is not due to simple competition with the inactivator at the active site and that a second mechanism is also most likely involved (see Discussion).

**Determination of the Kinetic Constants for Inactivation.** The apparent kinetic constants for the inactivation at 0.24 mM oxygen were determined for (*R,S*)- and (*S*)- $\beta$ -ethynyltyramine in experiments where the rate of inactivation was measured in preincubation mixtures containing various concentrations of the inactivator. The results from a typical experiment are shown in Figure 2 for (*S*)-1 in which the lines represent the result of a nonlinear regression fit of the raw data to eq 1. These results indicate a pseudo-first-order loss of enzymatic activity that is dependent upon the concentration of the in-

hibitor and displays saturation kinetics. Similar results were obtained for (*R,S*)-1 and for (*S*)-1 in the absence of ascorbate. A replot of the slopes of the individual lines is not shown here because eq 1 allows all of the data at different concentrations of inhibitor to be fit simultaneously. The kinetic constants that result from such a fit are presented in Table III. The  $k_{\text{inact}}(\text{app})$  for (*S*)-1 is  $0.184 \pm 0.015 \text{ min}^{-1}$ , indicating a rapid rate of inactivation that is 8-fold faster than that obtained for (*R,S*)-1. Thus the inactivation by (*S*)-1 in the racemate is largely masked due to the protective effect of the *R* enantiomer which is a potent, reversible inhibitor. As suggested in the earlier experiments at a single, fixed concentration of inhibitor (Table II), the  $k_{\text{inact}}(\text{app})$  for the ascorbate-independent inactivation is approximately half of the rate in its presence. The  $K_{\text{I}}(\text{app})$ 's for (*R,S*)- and (*S*)-1 are close to their  $K_{\text{is}}$  values vs tyramine, indicating that the commitment to catalysis of these compounds is probably low relative to the dissociation rate and that the  $K_{\text{I}}(\text{app})$ 's approximate true dissociation constants.

**Demonstration of Irreversibility.** DBH that had been completely inactivated by (*S*)-1 in the presence of ascorbate was subjected to prolonged dialysis (48 h) at pH 4.5, 6.5, and 8.5 and at pH 5.5 in the presence of 5 mM tyramine. Control samples containing active DBH were also included in the dialysis. Except at pH 4.5 where 95% of the control activity was lost upon dialysis, 80% or more of the starting activity of the controls was retained. Under these conditions, no recovery of activity was observed in any of the inactivated samples.

**Turnover of  $\beta$ -Ethynyltyramine by DBH.** Incubation mixtures were set up to measure whether  $\beta$ -ethynyltyramine is hydroxylated by DBH. In these studies the concentration of  $\beta$ -ethynyltyramine was fixed at 1 mM, and the concentration of enzyme was increased approximately 1000-fold over the normal concentration in the previous inactivation studies to 10 mg/mL ( $1.43 \times 10^{-4} \text{ M}$ ). Following incubation for 1 h at 37 °C and deproteinization, the incubation mixtures were analyzed by reversed-phase HPLC. Figure 3A depicts an elution profile following incubation of DBH with (*S*)-1 and ascorbate. Apart from the peaks corresponding to ascorbate, its oxidative products, and buffer salts which elute near the solvent front, the chromatogram consists primarily of four distinct peaks when monitored at 275 nm. One of the peaks (labeled  $\beta$ -Ethynyltyramine,  $R_{\text{T}} = 18.0 \text{ min}$ ) corresponds to unreacted (*S*)-1 and exhibits a  $\lambda_{\text{max}}$  at 271 nm. Comparison of the area of this peak to that of an unreacted control reaction mixture (not shown) indicates that approximately 50% of the starting material has been converted to products. Since the DBH was completely inactivated during the incubation, this allowed the estimation of a partition ratio of 2.5. Since the partition ratio reflects the ratio  $k_{\text{cat}}/k_{\text{inact}}$ , the calculated estimate of  $k_{\text{cat}}$  for (*S*)-1 is  $0.45 \text{ min}^{-1}$ . Thus, despite its relatively high affinity, (*S*)-1 undergoes turnover much more slowly than does tyramine.

The remaining three peaks in Figure 3A represent products formed during hydroxylation and/or inactivation. The first of these (labeled  $\beta$ -Ethynyltyramine,  $R_{\text{T}} = 6.7 \text{ min}$ ) is the major product produced and has a  $\lambda_{\text{max}}$  of 269 nm. Support for a structural assignment as the benzylic hydroxylated product of (*S*)-1 comes from a comparison of the  $R_{\text{T}}$  for octopamine relative to the  $R_{\text{T}}$  for tyramine and the observation that there is a similar hypsochromic shift in the  $\lambda_{\text{max}}$  of octopamine relative to tyramine (272 vs 274 nm). Additional support for the proposed structure was obtained when the reaction mixture was subjected to HPLC-MS using positive-ion thermospray ionization. The background-subtracted

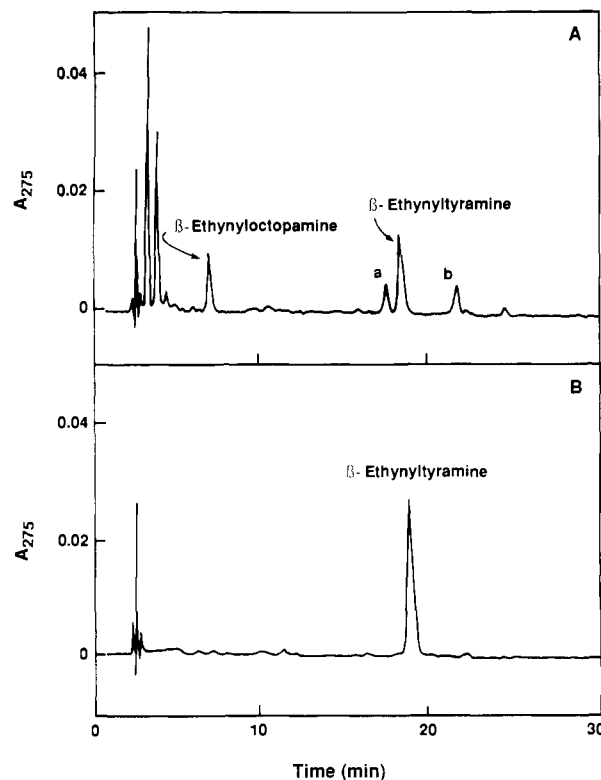


FIGURE 3: Reversed-phase HPLC profile of the products produced from the turnover of (*S*)-1 in the presence (panel A) and absence (panel B) of ascorbate. The concentration of (*S*)-1 was 1 mM, and that of DBH was 10 mg/mL (0.14 mM subunits). The conditions for the incubation and HPLC are as described under Experimental Procedures.

mass spectrum (not shown) gave  $m/e$  178 corresponding to  $(M + H)^+$ , along with an intense signal at  $m/e$  160. The latter fragment results from the loss of water from the proposed parent compound and is an expected mode of fragmentation on the basis of the structure of the hydroxylated compound and the fact that a similar mass spectral pattern is obtained with octopamine.

The remaining products (peaks a and b, Figure 3A) at 17.2 and 21.5 min could not be identified and are presumed to be degradation products produced during turnover. These compounds exhibited  $\lambda_{\text{max}}$ 's of 261 and 262 nm, respectively, but did not give detectable  $m/e$  signals when subjected to HPLC-MS analysis. This coupled with the fact that they are produced in small amounts prevented their isolation and further structural analysis.

The HPLC elution profile of a reaction mixture containing 1 mM (*S*)-1 but no ascorbate is shown in Figure 3B. No products were observed under these conditions, and in contrast to the previously reported experiments run in the absence of ascorbate, no inactivation of the enzyme occurred during the 1-h incubation. Since the difference between these turnover studies and the normal inactivation experiments is the 1000-fold difference in enzyme concentration, this result provides an important clue to understanding the ascorbate-independent inactivation (see Discussion). In a related experiment (not shown), no products were produced upon incubation of this high concentration of DBH with the *R* enantiomer, consistent with its behavior as a reversible, competitive inhibitor.

**Inactivation by [ $^3\text{H}$ ]-(*S*)- $\beta$ -Ethynyltyramine and Analysis of the Tryptic Digest.** DBH was completely inactivated in the presence of ascorbate and (*S*)-1 containing a small amount of [ $^3\text{H}$ ]-(*R,S*)-1. The final specific activity of the inactivator, accounting for the fact that 50% of the radiolabel was the



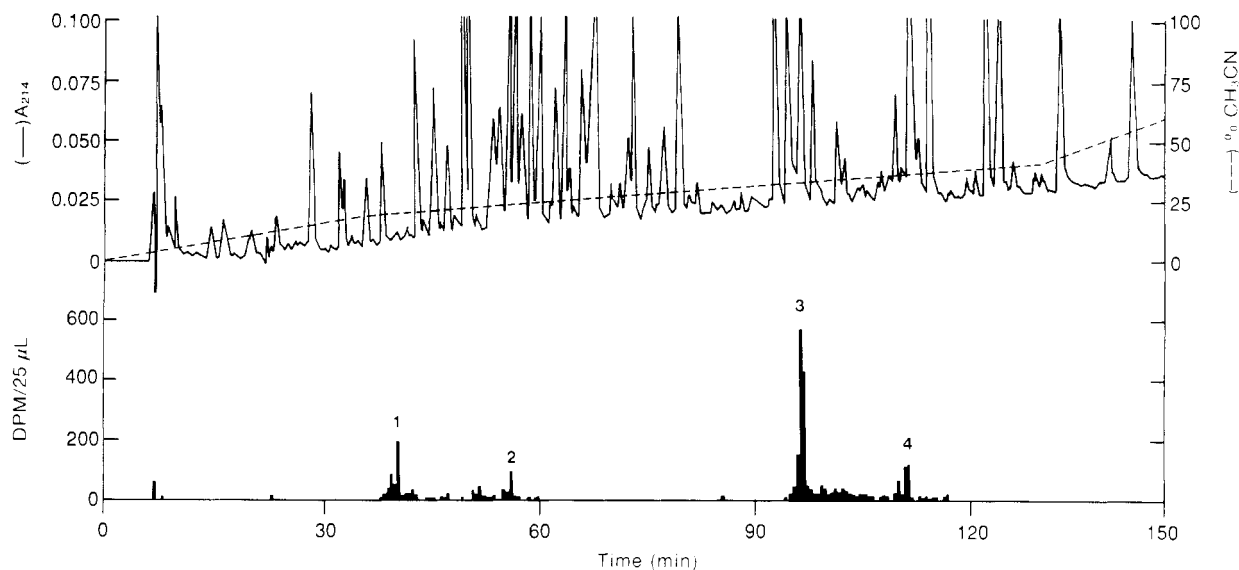


FIGURE 4: (Top) Tryptic map of  $[^3\text{H}]$ -(*S*)-1-inactivated DBH. The acidified tryptic digest was applied to a Vydac Protein  $\text{C}_4$  column and eluted as described under Experimental Procedures. The absorbance was monitored at 214 nm. (Bottom) Histogram showing the distribution of radioactivity during elution of the  $\text{C}_4$  column. A portion of each of the collected fractions was analyzed for radioactive content. The large peak at approximately 100 min was isolated and further characterized.

inactive *R* isomer, was  $43.7 \mu\text{Ci}/\mu\text{mol}$ . Analysis of the DBH and the radioactivity recovered following gel filtration and dialysis gave a stoichiometry of incorporation of 1.14 per subunit,<sup>4</sup> supporting the hypothesis that inactivation is accompanied by a specific, covalent modification of the active site of DBH. The enzyme was then subjected to reduction, carboxymethylation, and tryptic digestion as previously reported (DeWolf et al., 1988). The reversed-phase HPLC map of this digest is shown in Figure 4 (top), along with its radioactive profile (bottom) which shows four peaks of radioactivity. Purification of the major peak (3) by reversed-phase HPLC on a phenyl column followed by automated Edman degradation gave the following partial sequence:

Cys-Thr-Gln-Leu-Ala<sup>5</sup>-Leu-Pro-Ala-Ser-Gly<sup>10</sup>-Ile-His-Ile-Phe-Ala<sup>15</sup>-Ser-Gln-Leu-xxx-Thr<sup>20</sup>-His-Leu-Thr-Gly-Arg<sup>25</sup>

The "hole" at residue 19 was shown by liquid scintillation counting of the resulting PTH-amino acids to be the one that is covalently modified (Figure 5). Sequence analysis of the corresponding native peptide by Edman chemistry and confirmation by FAB-MS exact mass determination enabled the assignment of residue 19 as a histidine. Unfortunately, the low quantities (5–20 pmol) of PTH-amino acids resulting from this study prevented further structural analysis of the covalent adduct. Peak 4 (Figure 4, bottom) gave the same N-terminal residues as peak 3 (eight cycles) showing it to have arisen from the same tryptic cleavage, but low amounts of this peptide ( $\approx 10$  pmol) precluded further characterization. Preliminary partial sequence data<sup>5</sup> indicate that peak 4 could have arisen from incomplete tryptic digestion and may contain additional C-terminal residues. The combined radioactivity in peaks 3 and 4 accounts for 85% of the total recovered from the  $\text{C}_4$  column.

#### DISCUSSION

(*S*)- $\beta$ -Ethynyltyramine has been shown to be a potent and rapid time-dependent inactivator of DBH. It also fulfills the criteria set forth recently for a mechanism-based inhibitor

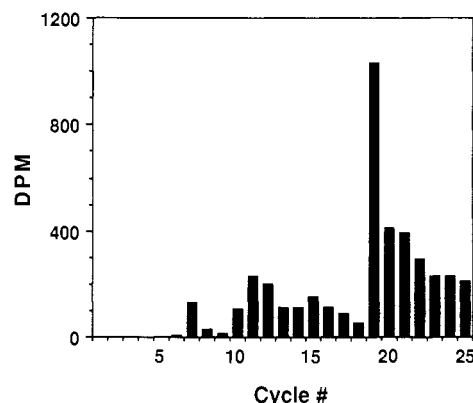


FIGURE 5: Histogram showing the radioactivity released during each cycle of the Edman degradation of the radiolabeled peptide. The scintillation counting of the PTHs was accomplished as described under Experimental Procedures. The spike of radioactivity at cycle 19 corresponds to the site of modification by (*S*)-1.

(Silverman, 1988). Thus, the loss of enzyme activity is pseudo first order, is proportional to low concentrations of inhibitor, and exhibits saturation kinetics. Inactivation is prevented in the presence of substrate and competitive inhibitor, and activity does not return upon dialysis or gel filtration. A 1:1 stoichiometry of incorporation of radiolabel into DBH is obtained. The dependence of inactivation on the presence of ascorbate and oxygen argues for the conversion of the inhibitor to a reactive intermediate that alkylates the enzyme as a result of catalytic activation during turnover to hydroxylated product. The high enantiomeric specificity of the inactivation and the fact that the active enantiomer has the benzylic hydrogen in the proper configuration to be a substrate also argue strongly for an enzyme-mediated activation step. Finally, there is no detectable lag time for inactivation, indicating that the reactive species is generated from the starting material and not the hydroxylated (or other) product.

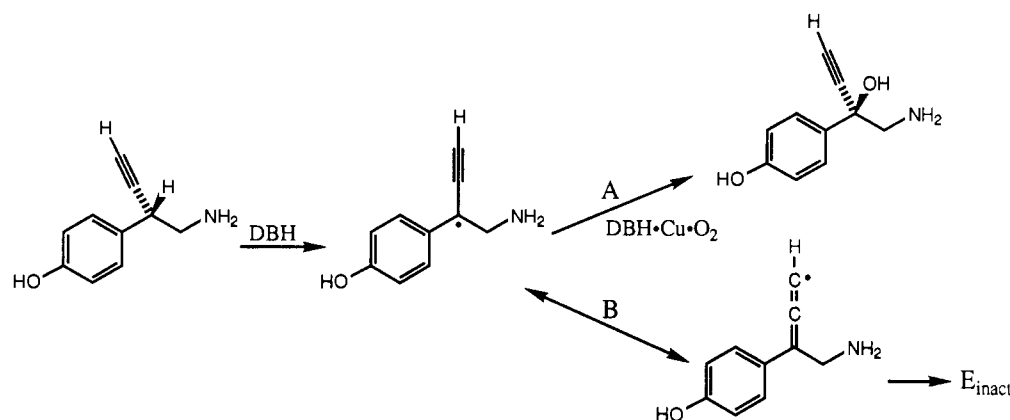
A discrepancy has been observed with respect to the ascorbate dependence of the inactivation such that, at a low concentration of DBH ( $0.14 \mu\text{M}$ ) with no ascorbate, inactivation proceeds at a rate of about half the rate with ascorbate while at high DBH ( $0.14 \text{ mM}$ ) no inactivation is observed. This apparent paradox can be understood if one postulates the

<sup>4</sup> This assumes an average subunit molecular weight of 70 000.

<sup>5</sup> C. Southan, unpublished observations.



Scheme II



presence of minute amounts of a one-electron reductant in the preincubation mixtures containing no ascorbate. The identity of this reducing agent is unknown, but we believe it may be a small amount of free ferrous ion introduced into the preincubation mixture with the catalase. Alternative one-electron reducing agents such as  $\text{Fe}(\text{CN})_6^{4-}$  have been shown to be efficient electron donors for DBH (Ljones & Flatmark, 1974; Rosenberg et al., 1980). Unfortunately, a direct test of this hypothesis was impossible, since DBH assays can be conducted neither in the absence of catalase nor in the presence of an appropriate metal chelator. The ascorbate-independent inactivation is at least in part the consequence of having a low partition ratio. The calculated value of 2.5 is among the lowest yet reported. With such a low partition ratio, only a slight stoichiometric excess of a contaminating reductant will produce complete inactivation. Thus less than 1  $\mu\text{M}$  reducing equivalent is all that would be required to inactivate DBH at a concentration of 0.14  $\mu\text{M}$  in the absence of ascorbate. At 0.14 mM DBH however, that same 1  $\mu\text{M}$  reducing equivalent would be capable of inactivating only about 1% of the enzyme and would, therefore, go undetected. While a high concentration of tyramine was shown to protect against inactivation in the presence of ascorbate, only a relatively low concentration was required in its absence. This is most likely the result of rapid exhaustion of the contaminating reductant brought about by the hydroxylation of the substrate. The conclusion, therefore, is that the inactivation by (S)-**1** is indeed completely dependent upon ascorbate (or other reductant) and that the rate observed at low enzyme concentrations in its absence is the result of an artifact of the preincubation conditions and the very low partition ratio for this compound. It also suggests why this phenomenon may have gone previously undetected.

The inactivation of DBH with (S)-**1** results in the stoichiometric incorporation of inhibitor into the enzyme. The modification has been localized to residue 19 of a single, major, 25 amino acid tryptic peptide, and on the basis of sequence data from the native protein, the modified amino acid is a histidine. The low partition ratio and the low calculated  $k_{\text{cat}}$  for hydroxylation of (S)-**1** indicates that this compound is turned over very slowly by DBH, but once catalysis is initiated, it results in a very efficient inactivation. This, coupled with the high degree of specificity of the covalent modification, provides a compelling argument that this peptide constitutes part of the active site.

The modified peptide shares a high degree of homology (96%) with residues 380–404 of the recently cloned human DBH (Lamoureux et al., 1987). Recognizing this and the fact that it is relatively rich in histidine residues (3 out of 25), it is tempting to hypothesize that the modified residue and thus the peptide also represent a portion of the copper binding site.

This proposal comes as the result of several observations. First, the His-Thr-His sequence in this peptide (residues 398–400 of the human enzyme) is homologous to the His-X-His sequences that have been shown by X-ray diffraction to ligate the copper atoms of superoxide dismutase (Richardson et al., 1975; Richardson, 1977; Tainer et al., 1983) and hemocyanin (Gaykema et al., 1984). Second, spectroscopic studies (EPR and EXAFS) involving DBH have provided evidence in support of coordination of the copper atoms by imidazole-like ligands (Peisach & Blumberg, 1974; Hasnain et al., 1984; McCracken et al., 1988; Scott et al., 1988). Third, direct evidence for a close spatial relationship between the catalytic site and at least one of the copper atoms was recently obtained as the result of EXAFS experiments (Scott et al., 1988) with the reduced ( $\text{Cu}^+$ ) form of DBH and the multisubstrate inhibitor **6** previously shown to be competitive vs both tyramine and oxygen (Kruse et al., 1986). From the EXAFS it was demonstrated that direct coordination of the sulfur atom of the imidazole-2-thionyl moiety apparently results upon binding of the inhibitor to the enzyme. Thus all of the available evidence supports our hypothesis that the modified peptide constitutes a portion of a copper binding site that is spatially close to or part of the catalytic site.

The covalently modified peptide reported here is different from the one that is alkylated by *p*-cresol (DeWolf et al., 1988). This is not unexpected, since we have hypothesized this peptide to be part of the "phenethylamine binding site". In this case, a tyrosine was the modified residue, and this suggested the participation of that amino acid in the binding of substrate to the enzyme via a  $\pi$ - $\pi$  interaction of the phenyl rings. Modification of a different peptide by (S)- $\beta$ -ethynyltyramine may be the result of structural differences in the reactive intermediates for the two compounds or differences in steric requirements for the two inactivators. For *p*-cresol, the evidence supported a simple 4-hydroxybenzyl radical intermediate although limited quantities of the isolated adducts prevented definitive structural identification. In the present case, the frustratingly small amounts of the modified PTH-amino acid that can be prepared also preclude structural analysis of the covalent adduct, and without this information, any mechanistic conclusions must remain highly speculative. Nevertheless, if abstraction of the benzylic hydrogen occurs to form a radical intermediate as is believed to occur during the normal activation of substrate (Miller & Klinman, 1985), then delocalization of this radical away from the benzylic position to the energetically favored allenic radical form (Scheme II, pathway B) will not only cause shunting away from product formation (pathway A) but may make other areas of the active site (i.e., the binding site for copper) accessible to alkylation.

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## REFERENCES

- Ahn, N., & Klinman, J. P. (1983) *Biochemistry* 22, 3096-3106.
- Battersby, A. R., Sheldrake, P. W., Staunton, J., & Williams, D. C. (1976) *J. Chem. Soc., Perkin Trans. 1*, 1056-1062.
- Cleland, W. W. (1979) *Methods Enzymol.* 63, 103-138.
- Cleland, W. W., Gross, M., & Folk, J. E. (1973) *J. Biol. Chem.* 248, 6541-6542.
- Creveling, C. R., Daly, J. W., Witkop, B., & Udenfreund, S. (1962) *Biochim. Biophys. Acta* 64, 125-134.
- DeWolf, W. E., Jr., Carr, S. A., Varrichio, A., Goodhart, P. J., Mentzer, M. A., Roberts, G. D., Southan, C., Dolle, R. E., & Kruse, L. I. (1988) *Biochemistry* 27, 9093-9101.
- Fitzpatrick, P. F., & Villafranca, J. J. (1987) *Arch. Biochem. Biophys.* 257, 231-250.
- Gaykema, W. P. J., Hol, W. G. J., Vereijken, J. M., Soeter, N. M., Bak, H. J., & Beintema, J. J. (1984) *Nature* 309, 23-29.
- Goodhart, P. J., DeWolf, W. E., Jr., & Kruse, L. I. (1987) *Biochemistry* 26, 2576-2583.
- Hasnain, S. S., Diakun, G. P., Knowles, P. F., Binsted, N., Garner, C. D., & Blackburn, N. J. (1984) *Biochem. J.* 221, 545-548.
- Kruse, L. I., DeWolf, W. E., Jr., Chambers, P. A., & Goodhart, P. J. (1986) *Biochemistry* 25, 7271-7278.
- Kruse, L. I., Kaiser, C., DeWolf, W. E., Jr., Chambers, P. A., Goodhart, P. J., Ezekiel, M., & Ohlstein, E. H. (1988) *J. Med. Chem.* 31, 704-706.
- Lamouroux, A., Vigny, A., Faucon Biguet, N., Darmon, M. C., Franck, R., Henry, J.-P., & Mallet, J. (1987) *EMBO J.* 6, 3931-3937.
- Levin, E. Y., Levenberg, B., & Kaufman, S. (1960) *J. Biol. Chem.* 235, 2080-2086.
- Ljones, T., & Flatmark, T. (1974) *FEBS Lett.* 49, 49-52.
- Ljones, T., & Skotland, T. (1984) in *Copper Proteins and Copper Enzymes* (Lontie, R., Ed.) Vol. 2, pp 131-157, CRC Press, Boca Raton, FL.
- May, S. W., & Phillips, R. S. (1980) *J. Am. Chem. Soc.* 102, 5981-5983.
- May, S. W., Phillips, R. S., Mueller, P. W., & Herman, H. H. (1981) *J. Biol. Chem.* 256, 8470-8475.
- McCracken, J., Desai, P. R., Papadopoulos, N. J., Villafranca, J. J., & Peisach, J. (1988) *Biochemistry* 27, 4133-4137.
- Miller, S. M., & Klinman, J. P. (1985) *Biochemistry* 24, 2114-2127.
- Nagatsu, T., & Udenfriend, S. (1972) *Clin. Chem.* 18, 980-983.
- Padgett, S. R., Wimalasena, K., Herman, H., Sirmanne, S. R., & May, S. W. (1985) *Biochemistry* 24, 5826-5839.
- Peisach, J., & Blumberg, W. E. (1974) *Arch. Biochem. Biophys.* 165, 691-708.
- Richardson, D. C. (1977) in *Superoxide and Superoxide Dismutases* (Michelson, A. M., McCord, J. M., & Fridovich, I., Eds.) pp 217-224, Academic Press, London.
- Richardson, J. S., Thomas, K. A., Rubin, B. H., & Richardson, D. C. (1975) *Proc. Natl. Acad. Sci. U.S.A.* 72, 1349-1353.
- Rosenberg, R. C., & Lovenberg, W. (1980) in *Essays in Neurochemistry and Neuropharmacology* (Youdin, M. B. H., Sharman, D. F., Lovenberg, W., & Lagnado, J. R., Eds.) Vol. 4, pp 163-208, Wiley, Chichester.
- Rosenberg, R. C., Gimble, J. M., & Lovenberg, W. (1980) *Biochim. Biophys. Acta* 613, 62-72.
- Scott, R. A., Sullivan, R. J., DeWolf, W. E., Jr., Dolle, R. E., & Kruse, L. I. (1988) *Biochemistry* 27, 5411-5417.
- Silverman, R. B. (1988) *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology*, Vol. 1, pp 9-12, CRC Press, Boca Raton, FL.
- Skotland, T., & Ljones, T. (1979) *Inorg. Perspect. Biol. Med.* 2, 151-180.
- Taguchi, H., Yosioka, I., Yamasaki, K., & Kim, I. H. (1981) *Chem. Pharm. Bull.* 29, 55-62.
- Tainer, J. A., Getzoff, E. D., Richardson, J. S., & Richardson, D. C. (1983) *Nature* 306, 284-287.
- Villafranca, J. J. (1981) in *Copper Proteins* (Spiro, T. G., Ed.) pp 263-289, Wiley, New York.