Synthesis of Several 2-Substituted 3-(p-Hydroxyphenyl)-1-propanes and Their Characterization As Mechanism-based Inhibitors of Dopamine \( \beta \)-Hydroxylase*

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Three substrate analogs of dopamine \( \beta \)-hydroxylase, viz. 2-X-3-(p-hydroxyphenyl)-1-propanes (where \( X = Br, Cl, H \)), have been synthesized, and all behave as substrates requiring \( O_2 \) and ascorbate for the enzyme-catalyzed hydroxylation reaction. The products have been characterized by mass spectrometry as the respective 2-X-3-hydroxy-3-(p-hydroxyphenyl)-1-propanes. The relative \( k_{\text{cat}} \) values for these compounds at pH 5.5, 0.25 mM \( O_2 \) are 49 min\(^{-1} \) (2-H), 8.6 min\(^{-1} \) (2-Cl), and 7.0 min\(^{-1} \) (2-Br). All three compounds have the characteristics of mechanism-based inhibitors of dopamine \( \beta \)-hydroxylase since incubation of enzyme with these compounds under turnover conditions leads to a time-dependent loss of activity. The \( k_{\text{cat}} \) values at pH 5.5, 0.25 mM \( O_2 \) are 0.08, 0.20, and 0.51 min\(^{-1} \), respectively, for the 2-Br-, 2-Cl-, and 2-H-substituted analogs. No reactivation was observed after oxidative dialysis of enzyme inactivated by 2-Br-3-(p-hydroxyphenyl)-1-propane, suggesting irreversible inactivation of dopamine \( \beta \)-hydroxylase.

Dopamine \( \beta \)-hydroxylase (EC 1.14.17.1) is a copper-containing monoxygenase involved in the biosynthesis of noradrenaline (Keuffman and Friedman, 1965; Skotland and Ljones, 1979; Rosenberg and Lovenberg, 1980), a neurotransmitter whose physiological level is critical for the functioning of the central nervous system (Molinoff and Axelrod, 1971). Thus, dopamine \( \beta \)-hydroxylase is an interesting target for the development of mechanism-based inactivators (Rando, 1977; Abeles and Maycock, 1976; Walsh, 1977, 1982). In the presence of molecular oxygen and ascorbate as electron donor, dopamine \( \beta \)-hydroxylase catalyzes in vitro the conversion of several substituted \( \beta \)-phenylethylamines to phenethanolamines. Recent investigations have focused on a number of structural analogs of dopamine which, in addition to being substrates, behave as mechanism-based or suicide inhibitors of this enzyme (Baldoni and Villafranca, 1980; Colombo et al., 1984a; Klinman and Krueger, 1982; May et al., 1983). These analogs can provide information pertinent to the bond-making and breaking steps in the enzymatic reaction, can serve as useful tools to study structure-function relationships in catalysis, and also help to identify amino acid residues at the active site of the enzyme.

Previous work from our laboratory showed that \( p \)-hydroxybenzyl cyanide specifically inactivates dopamine \( \beta \)-hydroxylase (Baldoni and Villafranca, 1980). Recent experiments demonstrate that several ring-substituted benzyl cyanides also inactivate dopamine \( \beta \)-hydroxylase in a mechanism-based fashion (Colombo et al., 1984a). The proposed mechanism involves a tight binding complex and/or covalent adduct formation as a result of activation of an enzyme-bound species produced during catalysis (Colombo et al., 1984b). Our present report deals with several substrate analogs that contain a potential allene or allylic radical functionality as the latent reactive group which could be unraveled during catalysis. This electrophilic moiety could react with an amino acid residue at the active site resulting in alkylation. Data in this paper show that 3-(p-hydroxyphenyl)-1-propene (V) and the corresponding 2-bromo and 2-chloro congeners are substrates of dopamine \( \beta \)-hydroxylase (Equation 1) and have the characteristics of mechanism-based inhibitors of this enzyme. We report here the synthesis of these compounds and their kinetic data as substrates.

\[
\begin{align*}
&\text{HO-CH}_2-C=CH_2 \\
&\text{X} \\
&\text{I. X.$\cdot$ Br} \\
&\text{II. X.$\cdot$ Br} \\
&\text{III. X.$\cdot$ Cl} \\
&\text{IV. X.$\cdot$ Cl} \\
&\text{V. X.$\cdot$ H} \\
&\text{VI. X.$\cdot$ H}
\end{align*}
\]

EXPERIMENTAL PROCEDURES AND RESULTS*

Preparation of Various 3-(p-Hydroxyphenyl)-1-propanes—To extend our investigations of the mechanism of the hydroxynase*

1 The abbreviations used are: I, III, and V, 2-X-3-(p-hydroxyphenyl)-1-propanes with \( X = Br, Cl, H \), respectively; II, IV, and VI, 2-X-3-hydroxy-3-(p-hydroxyphenyl)-1-propanes with \( X = Br, Cl, H \), respectively; VII, 2-Br-3-(p-methoxyphenyl)-1-propene; VIII, 2-Cl-3-(p-methoxyphenyl)-1-propene; DMF, N,N-dimethylformamide; MES, 2-(N-morpholino)ethanesulfonic acid; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); HPLC, high performance liquid chromatography; THF, tetrahydrofuran.

2 Portions of this paper (including "Experimental Procedures," part of "Results," Fig. 1, and Scheme 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-3334, cite the authors, and include a check or money order for $3.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
ylation reaction catalyzed by dopamine β-hydroxylase, we have synthesized propenyl substrate analogs that also have the potential of being mechanism-based inhibitors of this enzyme. The preparation and characterization of compounds I, III, and V are given in Miniprint.

Product Identification—If dopamine β-hydroxylase catalyzes the benzyl hydroxylation of I, the expected product would be II. Initially, two separate experiments were performed at pH 5.5 in the presence of O2, ascorbate, and I. One reaction mixture contained dopamine β-hydroxylase and the other did not. These separate reaction mixtures were incubated with 100% O2 for 30 min, extracted with ether, concentrated, and subjected to mass spectrometric analysis (Fig. 1). The mass spectra obtained from both experiments show that I is indeed converted to II only in the presence of the enzyme. Similar results were obtained with 2-Cl-3-(p-hydroxyphenyl)-1-propene (III) and 3-(p-hydroxyphenyl)-1-propene (V), i.e., III is converted to IV, and V is converted to VI.

Steady State Kinetics with 2-Br-3-(p-hydroxyphenyl)-1-propene—In initial velocity experiments at pH 5.0 and 37 °C, I is a linear competitive inhibitor versus tyramine with a Ki value of 4.9 mM (Fig. 2).

When I was incubated with O2, ascorbate, and enzyme, a small rate of oxygen uptake was detected employing the oxygraph assay, indicating that I is a very poor substrate for dopamine β-hydroxylase. The low sensitivity of this assay did not permit reliable evaluation of kinetic constants. Thus, to quantify the amount of product formed and to determine the steady state kinetic parameters for the dopamine β-hydroxylase-catalyzed hydroxylation of I, an HPLC method was developed. A typical HPLC analysis of a reaction mixture, as described in Miniprint, showed three elution peaks. The peaks with retention times of 6.6 and 2.6 min corresponded, respectively, to the substrate I and product II. If the enzyme was omitted from the assay mixture, no peak at 2.6 min was observed. The third peak with a retention time of 2 min contained the remaining components of the assay mixture.

Kinetic data obtained under identical conditions (25 °C, pH 5.5, 100% O2) for the dopamine β-hydroxylase-catalyzed oxidation of compound I and of tyramine showed that both I and tyramine have similar Km values (5.9 and 4.3 mM, respectively). However, I is processed at a rate which is only 1.1% that of tyramine (Vmax = 0.19 and 17.3 μmol of O2/min/mg, respectively). It should be kept in mind that the actual values of Vmax depend on the magnitude of the Km for oxygen. The data above were obtained at a single oxygen concentration (1.21 mM). The Km and kcat values for compounds I, III, and V were obtained at 0.25 mM O2 under identical conditions for a direct comparison and are presented in Table I.

Inhibition of Dopamine β-Hydroxylase by Compounds I, III, and V—Experiments were conducted to evaluate whether all three 3-(p-hydroxyphenyl)-1-propenes were mechanism-based inhibitors of dopamine β-hydroxylase. This was the case as established by several criteria. 1) Inactivation was first order and displayed saturation kinetics (Fig. 3 for compound I). The value for kcat/Km, the rate of inactivation at infinite inhibitor concentration, was evaluated from a plot of 1/kcat versus 1/inhibitor (Fig. 3, inset) to be 0.09 min⁻¹. The value for the dissociation constant of the enzyme inhibitor complex calculated from this plot is 4.9 mM. The inactivation of dopamine β-hydroxylase by compounds III and V also followed first order kinetics. The kcat/Km values for all

| X | Km (mM) | kcat (min⁻¹) | Vmax (μmol O2/min/mg) | kcat/Km (min⁻¹) |
|---|---------|--------------|-----------------------|-----------------|
| H | 0.97 ± 0.33 | 49 ± 7.4 | 1.3 ± 0.2 | 0.04 ± 0.04 |
| Cl | 4.0 ± 1.7 | 8.6 ± 1.5 | 3.4 ± 0.6 | 0.20 ± 0.01 |
| Br | 0.8 ± 0.2 | 7.0 ± 1.4 | 2.9 ± 0.4 | 0.08 ± 0.00 |

*Method A used the ratio of kcat and kcat given in this table, while Method B used the HPLC analysis given under “Experimental Procedures,” i.e., the total micromoles of product formed was divided by the total micromoles of enzyme used in the experiment.

![Fig. 2. Lineweaver-Burk plot of the effect of I on the reaction catalyzed by dopamine β-hydroxylase. The assays were performed at 37 °C and pH 5.0 in 200 mM sodium acetate buffer containing 13.8% DMF, 10 mM fumarate, 20 mM ascorbate, 0.22 mM O2, and 60 μg/ml of catalase. Tyramine was varied from 1.66 to 10 mM. The concentration of I is given adjacent to each line. The reaction was started by the addition of enzyme after the nonenzymatic rate of ascorbate oxidation had been monitored for at least 5 min.](image1)

![Fig. 3. Time course of the inactivation of dopamine β-hydroxylase with I. Dopamine β-hydroxylase (0.2 mg/ml, 8 Ctl/tetramer) was incubated at room temperature with 0.25 mM O2 in 200 mM sodium acetate buffer, pH 6.0, containing 13.8% DMF, 20 mM fumarate, 156 μg/ml of catalase, and 4.5 mM I in the absence of ascorbate (C, control) or with 38 mM ascorbate and I at the indicated concentrations. Inset, double reciprocal plot of kcat versus concentration of I.](image2)
three compounds were compared at 0.25 mM substrate analogs inactivate dopamine \( \beta \)-hydroxylase in a manner consistent with the following model:

\[
E + S \rightarrow E \cdot S \rightarrow E \cdot P \rightarrow E + P + P
\]

2) Inactivation required the presence of the other two substrates, i.e. oxygen and ascorbate (Table II, incubation conditions 1–5 for compound I and incubation conditions 6 and 7 for compound V). 3) Tyramine protects against inactivation of dopamine \( \beta \)-hydroxylase by I, III, or V (not shown). The results in Table II clearly show that inactivation is strictly dependent on catalysis.

Inspection of the data in Table II (incubation conditions 4 and 5) also shows that the observed rate of inactivation by I increased about 4-fold when the concentration of oxygen was increased from 21 to 100%. This indicates that, as was the case with the benzyl cyanide analogs (Colombo et al., 1984a), the value for the \( K_m \) for \( O_2 \) is higher when compound I is the substrate than when tyramine is the substrate. Because inactivation follows saturation kinetics, i.e. a reversible complex is formed prior to the first order inactivation step, the true value of the \( K_m \) for \( O_2 \) with I was determined from incubation experiments. Fig. 4 shows that at saturating ascorbate, a plot of \( 1/k_{\text{obs}} \) versus \( 1/[I] \) at three different levels of \( O_2 \) gives a set of straight lines intersecting to the left of the vertical axis and above the horizontal axis. This pattern is similar to one expected for a sequential mechanism in initial velocity experiments. The \( K_m \) for \( O_2 \) and the maximal rate of inactivation were 2.7 mM and 0.77 min\(^{-1}\), respectively, as calculated from the intercept replot (Fig. 4, inset).

**Partition between Inactivation and Turnover**—The kinetic constants for inactivation by all three compounds are given in Table I, and the steady-state kinetic parameters for these compounds were determined under the same conditions as were used for inactivation, allowing partition coefficients \( (k_{\text{obs}}/k_{\text{inact}}) \) to be calculated. The previous kinetic model accounts for this partitioning. The partition coefficients were also determined directly by measuring the amount of product formed after complete inactivation of the enzyme using HPLC to analyze for the product (Table I, Method B). These numbers are given in Table I and agree quite well with those calculated from the individually determined rate constants (Table I, Method A). For these calculations, it was assumed that there is one active site/dopamine \( \beta \)-hydroxylase monomer.

**Irreversibility of the Inhibition of Dopamine \( \beta \)-Hydroxylase by Compound I**—Dopamine \( \beta \)-hydroxylase inactivated in the presence of \( O_2 \), ascorbate, and 4.5 mM compound I did not regain significant activity when dialyzed against a 500-fold excess of buffer (four changes) for 48 h (Table III). These results indicate that compound I causes a time-dependent, first order irreversible inactivation of the enzyme.

**DISCUSSION**

The data presented in this paper establish that the newly synthesized compounds I, III, and V are alternate substrates for dopamine \( \beta \)-hydroxylase and are also inhibitors of the enzyme during catalysis (suicide substrates).

In agreement with the known substrate specificity of dopamine \( \beta \)-hydroxylase for phenethylamines (Creveling et al., 1962a, 1962b), benzyl cyanides (Baldoni and Vilafranca, 1980; Colombo et al., 1984a), and \( \beta \)-substituted (pro-3 hydrogen) phenethylamines (Klinman and Krueger, 1982), analogs I, III, and V are hydroxylated at the benzylic carbon to give the corresponding carbinols (Equation 1). The products have been identified by mass spectrometry. Furthermore, the mass spectra (Fig. 1) clearly show that the oxidation of I is exclusively enzyme-catalyzed. Other substrate analogs, viz. 1-

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**TABLE I**

| Incubation conditions | \( k_{\text{obs}} \) (min\(^{-1}\)) |
|-----------------------|----------------------------------|
| 1 Compound I + 21% \( O_2 \) | 2.1 |
| 2 Ascorbate + 21% \( O_2 \) | 1.7 |
| 3 Compound I + ascorbate + 100% \( N_2 \) | 1.8 |
| 4 Compound I + ascorbate + 21% \( O_2 \) | 4.6 |
| 5 Compound I + ascorbate + 100% \( O_2 \) | 170 |
| 6 Compound V + ascorbate + 100% \( CO \) | <1 |
| 7 Compound V + ascorbate + 21% \( O_2 \) | 107 |

* \( \beta \)-hydroxylase (0.12 mg/ml, 11.4 mg/100 ml of catalase) was incubated at room temperature at the indicated oxygen saturation in 110 mM MES, pH 5.5, containing 14% DMF, 156 \( \mu \)g/ml of catalase, and 38 mM ascorbate. The concentration of I was varied from 0.9 to 5.1 mM. At intervals, aliquots were removed and assayed for dopamine \( \beta \)-hydroxylase activity in the 37 °C standard assay at pH 5.5 as indicated under "Experimental Procedures." At each concentration of I, the value of \( k_{\text{obs}} \) was obtained from semilog plots of residual enzyme activity versus time.

**TABLE II**

| Incubation conditions \( k_{\text{obs}} \) (min\(^{-1}\)) |
|-----------------------|----------------|
| 1 Compound I + 21% \( O_2 \) | 2.1 |
| 2 Ascorbate + 21% \( O_2 \) | 1.7 |
| 3 Compound I + ascorbate + 100% \( N_2 \) | 1.8 |
| 4 Compound I + ascorbate + 21% \( O_2 \) | 4.6 |
| 5 Compound I + ascorbate + 100% \( O_2 \) | 170 |
| 6 Compound V + ascorbate + 100% \( CO \) | <1 |
| 7 Compound V + ascorbate + 21% \( O_2 \) | 107 |

* Dopamine \( \beta \)-hydroxylase (250 \( \mu \)g/ml) was incubated at room temperature in acetate/MES (0.1 M) buffer, pH 5.0, containing 20 mM fumarate, 14% DMF, and 156 \( \mu \)g/ml of catalase. As indicated in the left column, the incubation solutions were equilibrated with 21% \( O_2 \), 100% \( O_2 \), 100% \( N_2 \) or 100% \( CO \). When present, the concentration of ascorbate was 38 mM and the concentrations of the analogs were 4.5 mM (compound I) and 0.33 mM (compound V). The value for \( k_{\text{obs}} \) was determined from semilog plots of activity versus time.
Mechanism-based Inhibitors of Dopamine \( \beta \)-Hydroxylase

In initial velocity studies at pH 5.0 (Fig. 2), I was found to be a competitive inhibitor versus tyramine. When assayed under identical conditions (100% \( O_2 \)), the 2-Br-substituted analog was hydroxylated at a rate much slower than tyramine (relative \( V_n < 1.1% \)). The true \( V_n \) for I is probably higher inasmuch as the true \( K_m \) for \( O_2 \) estimated from inactivation experiments is 2.7 mM (Fig. 4). In this respect, compound I behaves similarly to \( p \)-hydroxybenzyl cyanide (Colombo et al., 1984a) in that the \( K_m \) value for \( O_2 \) is 2.8 mM with this compound.

In incubation experiments, the interaction of 3-(\( p \)-hydroxyphenyl)-1-propene and of the 2-Cl- and 2-Br-substituted compounds with dopamine \( \beta \)-hydroxylase had the characteristics of a mechanism-based inhibitor (Rando, 1974; Abeles and Maycock, 1976; Walsh, 1982). The rate of inactivation by these compounds was strictly dependent on catalysis (Table I). The \( V/K \) values are, respectively, 50.5, 2.2, and 3.9 \( \text{min}^{-1} \text{mM}^{-1} \) for compounds V, III, and I. In initial velocity studies at pH 5.0 (Fig. 2), I was found to be a competitive inhibitor versus tyramine. When assayed under identical conditions (100% \( O_2 \)), the 2-Br-substituted analog was hydroxylated at a rate much slower than tyramine (relative \( V_n < 1.1% \)). The true \( V_n \) for I is probably higher inasmuch as the true \( K_m \) for \( O_2 \) estimated from inactivation experiments is 2.7 mM (Fig. 4). In this respect, compound I behaves similarly to \( p \)-hydroxybenzyl cyanide (Colombo et al., 1984a) in that the \( K_m \) value for \( O_2 \) is 2.8 mM with this compound.

In incubation experiments, the interaction of 3-(\( p \)-hydroxyphenyl)-1-propene and of the 2-Cl- and 2-Br-substituted compounds with dopamine \( \beta \)-hydroxylase had the characteristics of a mechanism-based inhibitor (Rando, 1974; Abeles and Maycock, 1976; Walsh, 1982). The rate of inactivation by these compounds was strictly dependent on catalysis (Table I) and followed saturation kinetics (Fig. 3). For each analog, the time-dependent loss of activity was first order for several half-lives, indicating that the processing of compounds I, III, and V is accompanied by irreversible enzyme inactivation. Also, the partition ratio for these compounds ranged from ~40 to ~100 (Table I). Last, no recovery of enzyme activity occurred after prolonged dialysis of dopamine \( \beta \)-hydroxylase inactivated by incubation with I under turnover conditions (Table III).

The requirement for all the substrates (ascorbate and \( O_2 \)) indicates that dopamine \( \beta \)-hydroxylase catalyzes the first step or steps in the oxidation of compounds I, III, or V prior to inactivation. An inactivation mechanism involving an activated intermediate should account for the fact that compound V, the unsubstituted analog, is also a suicide substrate of the enzyme. This observation rules out a mechanism in which an electrophilic allene is generated prior to product release from the enzyme-product complex by the abstraction of the benzylic proton followed by halogen (Br\(^-\) or Cl\(^-\)) elimination.

Several other mechanisms for the inactivation reaction can be postulated. Kinetic data for the dopamine \( \beta \)-hydroxylase-catalyzed hydroxylation of ring-substituted phenethylamines and benzylic cyanides analyzed as a function of the \( \sigma \) substituent constant using the Hammett equation suggest a radical-cation intermediate in the enzyme-catalyzed hydroxylation reaction.\(^3\) This mechanism is also reasonable for the benzylic hydroxylation of compounds I, III, and V. Thus, following initial enzyme reduction, binding of substrate and \( O_2 \) occurs as demonstrated by the kinetic results of Miller and Klinman (1983). Recent data from our laboratory demonstrate that maximum activity occurs with a stoichiometry of two copper ions at the active site (Ash et al., 1984). With \( O_2 \) binding between two \( Cu^{2+} \) ions, removal of one electron from bound substrate would yield a cation radical which could ultimately rearrange to an enzyme-bound intermediate that could partition between formation of product and inactivation. The inactivation may result from attack by an active site base, resulting in formation of a covalent enzyme adduct. The mechanism of inactivation is still unknown, and experiments designed to further explore the inactivation reaction and the enzyme residues modified during the inactivation reaction are underway in this laboratory.

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\(^3\) B. Rajashekhar and J. J. Villafranca, unpublished results.
Mechanism-based Inhibitors of Dopamine β-Hydroxylase

Mechanism-based Inhibitors of Dopamine β-Hydroxylase: Structure-Activity Relationships and Their Characterization. Mechanism-based inhibitors of dopamine β-hydroxylase (DBH) have been developed as potential therapeutic agents for the treatment of neurodegenerative diseases such as Parkinson's disease. DBH is a key enzyme in the biosynthesis of norepinephrine and epinephrine, and its inhibition can lead to the accumulation of dopamine and other potential therapeutic effects.

Experimental Procedures

Preparation of 4-Chloro-3,4-dihydroxyphenylalanine (DOPA) and 3,4-Dihydroxyphenylalanine (DOPA): DOPA and DOPA were prepared by oxidation of L-DOPA with performic acid.

Synthesis of 4-Chloro-3,4-dihydroxyphenylalanine (DOPA): A solution of 0.1 M 4-chloro-3,4-dihydroxyphenylalanine (DOPA) in 7 M HCl was prepared and added to the reaction mixture. The reaction mixture was heated to 90 °C for 24 h and then cooled to room temperature.

Results of 4-Chloro-3,4-dihydroxyphenylalanine (DOPA): A solution of 0.1 M 4-chloro-3,4-dihydroxyphenylalanine (DOPA) in 7 M HCl was prepared and added to the reaction mixture. The reaction mixture was heated to 90 °C for 24 h and then cooled to room temperature.

Discussion: The results show that the synthesis of 4-chloro-3,4-dihydroxyphenylalanine (DOPA) was successful, and the product was characterized by spectroscopic methods such as UV and NMR. The results also indicate that the inhibition of DBH by 4-chloro-3,4-dihydroxyphenylalanine (DOPA) is more potent than that of DOPA, suggesting its potential use as a therapeutic agent.

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Summary: The synthesis and characterization of 4-chloro-3,4-dihydroxyphenylalanine (DOPA) as a mechanism-based inhibitor of dopamine β-hydroxylase (DBH) is described. The results indicate that DOPA is a more potent inhibitor than DOPA, suggesting its potential use as a therapeutic agent for the treatment of neurodegenerative diseases.

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Appendix: Materials and Methods

Materials: All reagents and solvents were purchased from commercial suppliers and were used without further purification. The substrates for the inhibition experiments were prepared according to the procedures described above.

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5. J. Med. Chem. 1996, 39, 2148-2150.
Mechanism-based Inhibitors of Dopamine β-Hydroxylase

Substrate Activity of L-Dopa (hydroxylase)-L-dopamine. For each substrate concentration, the progress of the reaction was monitored using the ELC method (see above) following the formation of product as a function of time. In initial experiments with 1.0 ml aliquots were removed from the incubation reaction at 0-15 min intervals over a 90 min period and mixed with 20 µl of a 2:1 mixture of TFA buffer, and the product formed was quantified by ELC. The data (i.e., rates of product vs time) were computer fitted to the hyperbolic form of the Michaelis-Menten equation (Cotman, 1947) to determine initial rates. The values for kcat and K were then obtained using a Lineweaver-Burk plot. The partition coefficient for a given substrate was being determined, the reaction was followed until an further linear to the product formation was seen. The concentration of product at this point was then divided by the enzyme concentration to give the partition coefficient.

In order experiments a different method was used (see Table II). Twenty µl aliquots were withdrawn from the reaction mixture at 30-60 second intervals and mixed with 20-40 µl TFA buffer. The reaction was followed until the initial rate began to drop off due to enzyme inactivation, typically 2-4 minutes. The amount of product present at a given time was then determined by injecting an aliquot into the ELC, monitoring it at 200 nm. All of the products had elution times of 2.6-3.5 min under the conditions used: for A and D, 20-40 µl acetate-acetonitrile, 60:40, and for A, 70:30. Initial rates determined in this manner were then computer fitted to the hyperbolic form of the Michaelis-Menten equation (Cotman, 1947).

Inhibition Studies with L-Dopa. Initial studies indicated that both of the dopamine-β-hydroxylase catalyzed hydroxylase, of tyrosine (Figure 1) were performed using the continuous galvanographic system. Inhibition of dopamine-β-hydroxylase with L-Dopa prior to assay was performed as indicated in the legend of each table or figure. All incubation solutions contained 250 mM HEPES to ensure solubility of L-Dopa, and K were performed at 30-32°C. Two controls were readily run: 1) enzyme with substrate and no substrate using 10-20 µl aliquots were withdrawn to determine the residual enzyme activity in the 9 ml assay at pH 1.7 and 37°C.

RESULTS
Identification of Product of the Reaction of Dopamine-β-hydroxylase with L-Dopa and K. Dopamine-β-hydroxylase was incubated with L-Dopa according to the method given in Experimental Procedures and some assay was analyzed by mass spectrometry. Figure 1 above the mass spectrum for L-Dopa and for the reagents isolated from a solution of L-Dopa incubated with enzyme and the other substrate. Pools at m/z 128 (M+H) and m/z 200 (M+2H) represent the molecular ions, and the fragmentation peaks at m/z 149 (M-H) and m/z 127 (tryptic ion) clearly indicate that the product of the enzyme catalyzed hydroxylation of L-Dopa is dopamine-β-hydroxylase. The signals at m/z 128, 149 and 127 were not present in the control experiment. Conclusively, the oxygen atom transferred to L-Dopa exclusively from an enzyme catalyzed reaction. The fragmentation pattern for the product is similar to that described for L-Dopa. Some loss of mass at m/z 168 and 170, and for K at m/z 166 and 158. The molecular ions were at m/z 154 for K and at m/z 132. The fragmentation patterns for L-Dopa and K were identical to that of L-Dopa and K.