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Secondary Isotope Effects and Structure–Reactivity Correlations in the Dopamine β-Monooxygenase Reaction: Evidence for a Chemical Mechanism†

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ABSTRACT: The chemical mechanism of hydroxylation, catalyzed by dopamine β-monooxygenase, has been explored with a combination of secondary kinetic isotope effects and structure–reactivity correlations. Measurement of primary and secondary isotope effects on V_{max}/K_m under conditions where the intrinsic primary hydrogen isotope effect is known allows calculation of the corresponding intrinsic secondary isotope effect. By this method we have obtained an α-deuterium isotope effect, $^{D}k_{\alpha} = 1.19 \pm 0.06$, with dopamine as substrate. The β-deuterium isotope effect is indistinguishable from one. The large magnitude of $^{D}k_{\alpha}$, together with our previous determination of a near maximal primary deuterium isotope effect of 9.4–11, clearly indicates the occurrence of a stepwise process for C–H bond cleavage and C–O bond formation and hence the presence of a substrate-derived intermediate. To probe the nature of this intermediate, a structure–reactivity study was performed by using a series of para-substituted phenylethylamines. Deuterium isotope effects on V_{max} and V_{max}/K_m parameters were determined for all of the substrates, allowing calculation of the rate constants for C–H bond cleavage and product dissociation and dissociation constants for amine and O_2 loss from the enzyme–substrate ternary complex. Multiple regression analysis yielded an electronic effect of $\rho = -1.5$ for the C–H bond cleavage step, eliminating the possibility of a carbanion intermediate. A negative ρ value is consistent with formation of either a radical or a carbocation; however, a significantly better correlation is obtained with σ_p rather than σ_p^+, implying formation of a radical intermediate via a polarized transition state. Additional effects determined from the regression analyses include steric effects on rate constants for substrate hydroxylation and product release and on $K_{D\,amine}$, consistent with a sterically restricted binding site, and a positive electronic effect of $\rho = 1.4$ on product dissociation, ascribed to a loss of product from an enzyme-bound Cu(II)–alkoxide complex. These results lead us to propose a mechanism in which O–O homolysis [from a putative Cu(II)–OOH species] and C–H homolysis (from substrate) occur in a concerted fashion, circumventing the formation of a discrete, high energy oxygen species such as hydroxyl radical. The substrate and peroxide-derived radical intermediates thus formed undergo a recombination, kinetically limited by displacement of an intervening water molecule, to give the postulated Cu(II)–alkoxide product complex.

Dopamine β-monooxygenase, a copper-containing enzyme, catalyzes the hydroxylation of dopamine to norepinephrine concomitant with the reductive cleavage of dioxygen. Despite its key role in neurotransmitter biosynthesis, the enzyme displays an unexpected lack of substrate specificity, catalyzing the hydroxylation of a variety of both 2- and aryl-substituted phenylethylamines (Kaufman & Friedman, 1965; May et al., 1981; Klinman & Krueger, 1982) and the benzylic oxidation of such groups as sulfides (May et al., 1981), olefins (May et al., 1983; Colombo et al., 1984), and aldehydes (Bossard & Klinman, 1985). As summarized in eq 1, an additional two electrons and two protons are required to complete the reduction; ascorbic acid is believed to be the physiologic reductant (Terland & Flatmark, 1975), but other one- and

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two-electron donors work as well (Rosenberg & Lovenberg, 1980; Mangold & Klinman, 1984).

$$ArCH_2CH_2NH_3^+ + O_2 \xrightarrow{2e^-,\ 2H^+} ArCHOHCH_2NH_3^+ + H_2O \quad (1)$$

Recent studies have shown that two coppers per subunit are required for full expression of dopamine β-monooxygenase activity, indicating that both reducing equivalents required by the reaction stoichiometry can be stored within a single subunit (Klinman et al., 1984; Ash et al., 1984). Additional evidence demonstrating the mandatory presence of a proton prior to substrate activation (Ahn & Klinman, 1983) and an absence of magnetic coupling between Cu(II) sites when enzyme containing two coppers per subunit is examined by ESR (D. E. Edmondson et al., unpublished data) has led to the suggestion that a single catalytic site copper undergoes a direct interaction with oxygen to generate Cu(II)–OOH as an intermediate (Klinman et al., 1984). Further properties of the enzyme have been reviewed (Skotland & Ljones, 1979; Rosenberg & Lovenberg, 1980; Villafranca, 1981).

Despite extensive investigations of dopamine β-monooxygenase, fundamental questions regarding the mechanism of oxygen and substrate activation remain unanswered. In an earlier study, the intrinsic primary deuterium isotope effect in the dopamine β-monooxygenase catalyzed hydroxylation of dopamine was determined to be 9.4–11. Since the large magnitude of this isotope effect appeared inconsistent with a failure to observe re-formation of dopamine from enzyme-bound product, carbon–hydrogen bond cleavage was proposed to be uncoupled from the oxygen insertion step (Miller & Klinman, 1983). In the present investigation, intrinsic α- and β-secondary deuterium isotope effects have been determined. As demonstrated herein, the large magnitude of the α-deuterium effect, $^Dk_\alpha = 1.19 \pm 0.06$, provides strong support for a two-step mechanism in which C–H bond cleavage precedes C–O bond formation to generate a substrate-derived intermediate.

Structure–reactivity correlations can provide considerable insight into the nature of chemical intermediates in enzyme-catalyzed reactions, provided that substituent-sensitive step(s) can be isolated and examined independently. In dopamine β-monooxygenase, the major rate-limiting step with dopamine as substrate occurs after the C–H bond cleavage step (Ahn & Klinman, 1983); however, microscopic rate constants can be calculated for the substrate hydroxylation and product desorption step, in the event that observed isotope effects on V_{max} are different from unity and the intrinsic isotope effect (Miller & Klinman, 1982). As shown in the present investigation, the magnitude of deuterium isotope effects for a series of eight ring-substituted phenylethylamines permits an evaluation of electronic effects on individual steps in the overall reaction. Although May and co-workers (1981) have reported a study of structure–reactivity correlations in the dopamine β-monooxygenase reaction, these authors failed to consider either changes in the K_m for oxygen with changing substrate or the possibility of multiple rate-limiting steps contributing to V_{max}, precluding a quantitative evaluation of substituent effects on the chemical step.

Overall, the data presented in this paper support a mechanism in which substrate undergoes homolytic carbon–hydrogen bond cleavage to generate a transient radical intermediate. The recombination of this intermediate with an oxygen-derived radical is proposed to be kinetically limited by displacement of an intervening water molecule and to lead to the formation of an inner-sphere complex between Cu(II) and product. Additionally, a chemical mechanism for dioxygen activation, which will accommodate the observed properties of substituted phenylethylamine hydroxylation, has been postulated.

Experimental Procedures

Materials. All chemicals were reagent grade unless otherwise specified. Disodium fumarate, dopamine hydrochloride, norepinephrine hydrochloride, and 2-mercaptoethanol were from Sigma. Catalase was from Boehringer. Ascorbic acid was from BDH Chemicals, Ltd. All substituted phenylacetonitriles and *p*-(trifluoromethyl)benzyl alcohol were from Aldrich. Lithium aluminum [^{3}H]hydride (LAH)[1] (180.5 mCi/mmol), tritiated water (10.5 mCi/mmol), and Liquifluor PPO–POPOP–toluene concentrate were from New England Nuclear. 2-(3,4-Dihydroxyphenyl)[1-^{14}C]ethylamine hydrochloride (56 mCi/mmol) was from Amersham. Ag 50W-X12 ion-exchange resin (100–200 mesh) was from Bio-Rad.

General Methods. Radioactivity was determined on a Beckman LS-8000 liquid scintillation spectrometer with a toluene-diluted Liquifluor/absolute ethanol cocktail; for ^{3}H/^{14}C ratios, channels were set with 33.1% ^{14}C spillover into the tritium window and <0.1% ^{3}H spillover into the ^{14}C window. A Radiometer type PHM 26 pH meter was used for pH readings. Absorbance measurements were made on a Cary 118B UV–visible spectrometer, fluorescence assays on a Perkin-Elmer MPF-44A fluorescence spectrometer, and oxygen uptake assays on a Yellow Springs Instrument polarographic oxygen electrode, Model 53. HPLC was performed on a Beckman Model 332 gradient liquid chromatographic system equipped with a Model 155 variable wavelength detector using a Water's C_{18} analytical reverse-phase column with a mobile phase of 0.1% acetic acid (by volume). Under conditions of a flow rate of ca. 1.5 mL/min, norepinephrine eluted with a retention time of 4 min, relative to dopamine (7 min). Melting points were determined with a Buchi (capillary) apparatus and are uncorrected. NMR spectra were obtained on a Varian EM-390 (90 MHz) and were recorded in $CDCl_3$ with tetramethylsilane as reference at 0 ppm or in D_2O with the DOH peak as reference at 4.65 ppm. Chemical shifts are expressed in parts per million; the couplings and widths at half-height ($w_{1/2}$) for multiplets are expressed in hertz. Apparent couplings which are actually part of a more complex pattern are denoted as the apparent (in quotes) followed by the actual (e.g., "d"A_2B_2). Elemental analyses were performed by the Analytical Laboratory, College of Chemistry, University of California, Berkeley, CA. Dopamine β-monooxygenase was prepared from bovine chromaffin granules as previously described (Klinman & Krueger, 1982). Enzyme activity was assayed by measuring the rate of oxygen uptake or norepinephrine production as previously described (Klinman et al., 1980).

1-(3,4-Dimethoxyphenyl)[1-^{3}H]acetonitrile. A mixture of the nitrile (179.2 mg, 1.01 mmol), TOH (1.21 mL, 1.05×10^7 cpm/μmol), and K_2CO_3 (1.24 mg, 9μmol) was heated at 70 °C with magnetic stirring in a flask fitted with a wired-on serum stopper. After 4 h, the mixture was cooled, and crystallization of the nitrile was induced by momentary immersion of the flask in a −70 °C bath. The TOH was pipetted away for purification, and the nitrile was washed once with water

[1] Abbreviations: PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyloxazol-2-yl)benzene; ^{3}H or T, tritium; ^{2}H or D, deuterium; LAH, lithium aluminum hydride; UV, ultraviolet; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; BDE, bond dissociation energy; DM, dopamine.

and then dissolved in $CHCl_3$. The solution was dried with $MgSO_4$, filtered into a clean flask, and evaporated under N_2 flow for 24 h. Crystallization of the nitrile was induced by immersion in an ice bath, and the remaining solvent was removed under vacuum with warming of the flask in a water bath at 45 °C for 1 h. Recovery was 160.4 mg (0.905 mmol) with a specific activity of 9.2×10^6 cpm/μmol (relative to a maximum possible incorporation of 2.1×10^7 cpm/μmol).

2-(3,4-Dimethoxyphenyl)[2-^{3}H]ethylamine Hydrochloride. The foregoing nitrile was reduced essentially by the method of Nystrom (1955) as described for 2-(4-methylphenyl)-ethylamine hydrochloride with the following exceptions: (1) the reaction was run on a millimole scale; (2) the final acidified ether was evaporated under vacuum, and the residue was transferred in MeOH to a 10-mL flask followed by further evaporation of solvent to an oily residue. Upon addition of acetone, the amine hydrochloride crystallized from solution and was filtered. The yield was 122.5 mg (0.563 mmol, 62.2%) with an estimated specific activity of at least 8×10^6 cpm/μmol.

2-(3,4-Dihydroxyphenyl)[2-^{3}H]ethylamine Hydrochloride ([2-^{3}H]Dopamine Hydrochloride). A mixture of 2-(3,4-dimethoxyphenyl)[2-^{3}H]ethylamine hydrochloride (122.5 mg, 0.563 mmol) and freshly distilled constant-boiling HBr (2 mL) was refluxed at 115 °C under N_2 with stirring for 10 h. After the mixture was cooled, the HBr was evaporated under vacuum. The residue was dissolved in 0.5 mL of H_2O and applied to a previously H_2O-equilibrated Ag 50W-X12 ion-exchange column (6 cm height in a Pasteur pipet). The column was washed with H_2O (10 mL) until the eluent was neutral (pH paper) and then with 2 N HCl to elute the amine as the hydrochloride. Fractions (2.3 mL) were collected and monitored by absorbance readings at 280 nm and by radioactivity. After combination of the amine fractions, the water was evaporated under vacuum and the crystalline product dissolved in 0.01 N HCl (4 mL). The final solution was 118 mM with a specific activity of 8.4×10^6 cpm/μmol. The material was stored frozen at −20 °C in 0.1-mL aliquots. The purity was established by two means: (1) when a 50-nmol aliquot was subjected to HPLC, a single peak was observed at 280 nm corresponding to commercial dopamine retention time, and tritium counts were found only in fractions corresponding to the peak; (2) when an aliquot was completely converted to norepinephrine with dopamine β-monooxygenase, 48.1% of the total tritium was released to water, which is within experimental error of the expected 50% release. Therefore, this material was used without further purification.

2-(3,4-Dimethoxyphenyl)[1-^{3}H]ethylamine Hydrochloride. A mixture of [^{3}H]LAH (5.3 mg, 0.14 mmol, 180.5 mCi/mmol) and cold LAH (15.8 mg, 0.416 mmol) was placed in a three-neck flask. Ether (1 mL, dried over activated 4-Å sieves) was added by cannula followed immediately by addition of an ethereal solution of $AlCl_3$ (75.2 mg, 0.564 mmol in 2 mL) by cannula. After 1 min, addition of an ethereal solution of (3,4-dimethoxyphenyl)acetonitrile (169.3 mg, 0.955 mmol in 9 mL) was begun. Addition was performed dropwise (ca. 10–12 drops at once) with TLC monitoring until all of the nitrile had reacted, at which point more was added. At 4.5 mL, some nitrile still remained by TLC 20 min after the last addition; at this point, a solution of cold LAH/$AlCl_3$ (0.25 mmol each in 2 mL of ether) was added. An additional 2 mL of the nitrile solution (for a total of 6.5 mL) was added dropwise until the nitrile persisted by TLC. The reaction was quenched and worked up as above. Addition of acetone to the oily residue afforded crystalline 2-(3,4-dimethoxyphenyl)[1-^{3}H]ethylamine hydrochloride (52.5 mg, 0.241 mmol, 35% based on addition of 72% of the nitrile solution; specific activity 1.1×10^7 cpm/μmol).

2-(3,4-Dihydroxyphenyl)[1-^{3}H]ethylamine Hydrochloride ([1-^{3}H]Dopamine Hydrochloride). The [1-^{3}H]dopamine hydrochloride was prepared by deprotection of the 2-(3,4-dimethoxyphenyl)[1-^{3}H]ethylamine with HBr and purification by ion-exchange chromatography as described above. After evaporation of solvent, the 2-(3,4-dihydroxyphenyl)[1-^{3}H]-ethylamine hydrochloride was dissolved in 0.01 N HCl (2 mL) yielding a final solution of 95.9 mM with a specific activity of 1.4×10^7 cpm/μmol which was stored at −20 °C in 0.10-mL aliquots. Reverse-phase HPLC showed the presence of a well-separated contaminant by both absorbance at 280 nm and tritium counts. Therefore, a sufficient aliquot was purified by HPLC under the same conditions just before use in isotope effect experiments.

Tritium Isotope Effects. Primary tritium isotope effects were determined as previously described (Klinman et al., 1980). Secondary isotope effects were measured by using mixtures of [1-^{14}C]dopamine and either [1-^{3}H]- or [2-^{3}H]-dopamine as substrate, from a comparison of the ^{3}H/^{14}C ratios in product at complete vs. partial conversion. The [1-^{14}C]-dopamine was purified by HPLC before mixing with the tritiated dopamine. Both primary and secondary isotope effects were determined with the same enzyme preparation in identical reaction mixtures containing 100 mM potassium phosphate, pH 6.07, 10 mM sodium ascorbate, 40 μg/mL catalase, 1 μM $CuCl_2$, 1 mM dopamine hydrochloride (see Tables I and II for ^{3}H/^{14}C ratios), and 10 μg/mL dopamine β-monooxygenase at 35 °C and air saturation. Reactions were initiated by addition of dopamine β-monooxygenase (20 μL/mL) after a 2-min incubation at 35 °C. At appropriate time points 0.17 mL of the reaction was quenched with 0.10 mL of 16.7 mM disodium fumarate in 0.1 N $HClO_4$ and kept on ice. Zero points were taken by incubating 0.60 mL of reaction mix minus enzyme at 35 °C for the entire length of the reactions and then quenching with 0.36 mL of 16.7 mM disodium fumarate in 0.1 N $HClO_4$. Complete conversion was obtained in separate reactions containing 100 mM potassium phosphate, pH 6.07, 10 mM sodium ascorbate, 10 mM disodium fumarate, 40 μg/mL catalase, 1 μM $CuCl_2$, 0.1 mM dopamine hydrochloride (see Tables I and II for ^{3}H/^{14}C ratios), and 25 μg/mL dopamine β-monooxygenase at 35 °C and air saturation. For all points, a 30–50-μL aliquot of the quenched reaction mixture was set aside for assay of norepinephrine by fluorescence; the remaining volume was filtered through an Amicon YMT membrane in an Amicon MPS-1 micropartition system by centrifugation in a Sorvall SS-34 rotor at 3000 rpm for 10 min. The filtrates were frozen at −20 °C and removed individually for HPLC purification of norepinephrine. Purification of each reaction sample required 4 × 50-μL injections. As a result of a time-dependent increase in base-line counts, zero points were obtained for every two to three time points. Infinity points and their zero points were purified in alternating single 50-μL injections. Following the recovery of product from the HPLC, solvent was removed by evaporation under vacuum, and the residue redissolved and evaporated 2× with 0.01 N HCl. Finally, each sample was dissolved in 0.2 mL of 0.01 N HCl and the ^{3}H and ^{14}C content determined.

Synthesis of Para-Substituted Phenylethylamine Hydrochlorides. All of the phenylethylamines were synthesized by reduction of the appropriately substituted phenylacetonitriles essentially according to Nystrom (1955). With the exception of the [*p*-(trifluoromethyl)phenyl]acetonitrile, which was

prepared from *p*-(trifluoromethyl)benzyl alcohol [supplementary materials (see paragraph at end of paper regarding supplementary material)], substituted phenylacetonitriles were obtained commercially. Yields ranged from 64% to 90% depending on the amine. A typical procedure is given below for 2-(4-methylphenyl)ethylamine hydrochloride.

Dideuterated phenylacetonitriles were obtained by base-catalyzed exchange of the benzylic protons with D_2O. Typically 5–10 g of the nitrile, 10–20 mL of D_2O, and 0.5 g of K_2CO_3 were heated at 70–80 °C with vigorous stirring in a flask equipped with a drying tube. Exchange was monitored by ^1H NMR (90 mHz, $CDCl_3$) and usually required three to five changes of D_2O and base over a period of 12–48 h. When deuteration was complete by NMR, the mixture was cooled, and the nitrile was extraced with ether. After the mixture was dried over $MgSO_4$ and filtered, the ethereal nitrile was reduced under conditions identical with reduction of protonated nitrile.

2-(4-Methylphenyl)ethylamine Hydrochloride. LAH (2.64 g, 69.4 mmol) was dissolved in ether (100 mL) under N_2 in a dry 1-L three-neck flask equipped with a mechanical stirrer, dropping funnel, and condenser. An ethereal solution of $AlCl_3$ (9.25 g, 69.4 mmol in 130 mL) was run in quickly with stirring. After 5 min an ethereal solution of (*p*-methylphenyl)acetonitrile (7.0 g, 53 mmol in 200 mL) was added dropwise over 15 min and the reaction stirred vigorously for 1 h at 25 °C. While the solution was being cooled in an ice bath, the excess LAH was quenched by cautious dropwise addition of water (50 mL) followed by acidification with 6 N H_2SO_4 (50 mL). An additional 100 mL of water was added to dissolve fully aluminum salts. After separation, the aqueous layer was washed once with ether (110 mL), taken to pH ≥12 (pH paper) with KOH pellets, and extracted with ether (4 × 200 mL). The ether was dried over $MgSO_4$ and filtered; HCl gas was bubbled through it to precipitate the amine hydrochloride. After filtration, the product was recrystallized from methanol/ether in 64% yield: mp 217–218 °C [lit. mp 217–218 °C; Speer & Hill, 1937]; ^1H NMR (D_2O) δ 2.23 (s, 3 H), 2.76 (m, 2 H, $w_{1/2}$ = 10.5), 3.2 (m, 2 H, $w_{1/2}$ = 12), 7.15 (s, 4 H). Anal. Calcd for $C_9H_{14}ClN$: C, 63.0; H, 8.2; N, 8.2. Found: C, 62.9; H, 8.1; N, 8.1.

The physical properties for each purified protonated and deuterated amine used in these studies are available as supplementary material.

Steady-State Kinetics. Initial velocities were measured at varied oxygen and para-substituted phenylethylamine concentrations by the rate of oxygen consumption at 35 °C and pH 6.2. Reaction mixtures contained 50 mM potassium phosphate, 10 mM sodium ascorbate, 10 mM disodium fumarate, 40 μg/mL catalase, and 1 μM $CuCl_2$. A total ionic strength of 0.15 M (adjusted with KCl) was maintained throughout with the following exception: (1) at concentrations of 100 mM for *p*-methoxy and *p*-bromo substrates, μ = 0.18 M; (2) the entire experiment with 2-[4-(trifluoromethyl)-phenyl]ethylamine was conducted at an ionic strength of 0.21 M due to a high K_m for substrate. Individual reaction mixtures were made from stock solutions within 15 min of rate measurements with addition of ascorbate, catalase, and $CuCl_2$ immediately prior to equilibration with oxygen. Oxygen concentrations were varied by stirring reaction mixtures for 3.25 min in atmospheres obtained by using premixed O_2–N_2 standards (Matheson) alone and/or mixed with pure oxygen or nitrogen (Ohio Medical). Stock solutions of phenylethylamine substrates were obtained by weighing ca. 1-g samples (to 0.05 mg) and dissolving these up to known volumes in water. Velocities for both unlabeled and dideuterated substrate were obtained in a single experiment using the same stocks of the other reaction components to minimize fluctuations in experimental conditions.

Kinetic constants, V_{max} and V_{max}/K_m, were obtained from initial velocities and substrate concentrations as fit to the expression

$$v/E_T = V_{max}S/(K_m + S) \quad (2)$$

by using the program HYPER as previously described (Ahn & Klinman, 1983). Since velocity is divided by E_T, the units for V_{max} and V_{max}/K_m are s^{-1} and M^{-1} s^{-1}, respectively. Deuterium isotope effects for each parameter equal the ratio of parameter for unlabeled over that for dideuterated substrate. Microscopic rate constants, k_5 and k_7, were calculated for the mechanism in eq 3. For a minimal mechanism involving a single pre-

$$\begin{array}{l} E\cdot DM \underset{k_4}{\overset{+O_2}{\rightleftharpoons}} E\cdot DM\cdot O_2 \xrightarrow{k_5} E\cdot P \xrightarrow{k_7} E + P \\ E\cdot O_2 \underset{+DM}{\overset{k_4}{\rightleftharpoons}} \end{array} \quad (3)$$

catalytic complex, the constants k_5 and k_7 are described by eq 4 and 5 (Miller & Klinman, 1982; Ahn & Klinman, 1983)

$$k_5 = \frac{V_H(^Dk - 1)}{^DV - 1} \quad (4)$$

$$k_7 = \frac{V_H(^Dk - 1)}{^Dk - ^DV} \quad (5)$$

where V_H is the rate constant for protonated amine, ^Dk is the intrinsic deuterium isotope effect, and ^DV is the observed isotope effect on V_{max}. The effect of an additional precatalytic step is addressed in the Appendix. Dissociation constants from enzyme ternary complex for oxygen and amine substrates were calculated from eq 6 (Klinman & Matthews, 1985) where K_D

$$K_D = \frac{K_m[^D(V/K) - 1]}{^DV - 1} \quad (6)$$

is the substrate dissociation constant, K_m is the Michaelis constant for protonated amine, and ^DV and $^D(V/K)$ are observed isotope effects on V_{max} and V_{max}/K_m.

Results

Secondary Isotope Effects. Both α- and β-secondary isotope effects on V_{max}/K_{DM} were determined by using a double-label method with either [2-^{3}H]- or [1-^{3}H]dopamine, respectively, and [1-^{14}C]dopamine as the tracer for unlabeled substrate. In this method the isotope effects were directly determined from the $^3H/^{14}C$ ratios of purified product at complete (∞) vs. partial (t) conversion.[2]

$$^T(V/K) = \frac{(^3H/^{14}C)_\infty}{(^3H/^{14}C)_t} \quad (7)$$

Results of the α experiments are summarized in Table I and of the β experiments in Table II. As can be seen from the

[2] It should be noted that although fairly large percent conversions were used in these experiments, calculations of $^T(V/K)$ according to eq 7 rather than the full expression

$$^T(V/K) = \frac{\ln(1-f)}{\ln(1-f)[(^3H/^{14}C)_t/(^3H/^{14}C)_\infty]}$$

(where f equals the fractional conversion) leads to uncertainties significantly lower than the overall reproducibility. For example, at 12.3% conversion and a ratio of ratios of 1.100, the $^T(V/K)$ calculated from the full expression is 1.107, whereas experimental error is ±2%.

Table I: Measurement of $^{T}(V/K)_{\alpha}$[a]

% conversion	$^{3}H/^{14}C$ in norepinephrine	$^{T}(V/K)_{\alpha}$
	Experiment 1[b]	
∞[c]	4.328	
2.7	3.987	1.086
2.5	3.940	1.098
1.9	4.039	1.072
4.4	3.953	1.095
4.2	3.961	1.093
3.2	3.895	1.111
4.9	3.873	1.117
5.0	3.839	1.127
4.2	3.873	1.117
	Experiment 2[d]	
∞[c]	4.144	
5.8	3.725	1.112
7.9	3.758	1.103
12.3	3.718	1.115
		1.104 ± 0.016 (av)

[a] Details of these experiments are described under Experimental Procedures. [b] The initial $^{3}H/^{14}C$ ratio of dopamine was 8.641. Three separate reactions were run, consisting of three time points each. Time points were analyzed in the order listed. [c] Four infinity points were taken at 1, 2, 3, and 4 h and their $^{3}H/^{14}C$ ratios averaged. All points were at complete conversion as assayed by fluorescence. [d] The initial $^{3}H/^{14}C$ ratio of dopamine was 8.231.

Table II: Measurement of $^{T}(V/K)_{\beta}$[a]

% conversion	$^{3}H/^{14}C$ of norepinephrine	$^{T}(V/K)_{\beta}$
	Experiment 1[b]	
∞[c]	10.705	
1.5	10.771	0.994
1.5	10.367	1.033
1.2	11.040	0.970
2.9	10.946	0.978
2.6	10.754	0.995
2.3	10.819	0.989
4.7	11.013	0.972
4.0	10.632	1.007
3.4	10.819	0.989
	Experiment 2[d]	
∞[c]	10.683	
2.8	10.704	0.998
2.6	10.481	1.019
5.3	10.645	1.004
4.7	10.790	0.990
6.5	10.799	0.989
6.9	10.445	1.023
		0.997 ± 0.018 (av)

[a] Details of these experiments are described under Experimental Procedures. [b] The initial $^{3}H/^{14}C$ ratio of dopamine was 10.750. Three separate reactions were run, consisting of three time points each. Time points were analyzed in the order listed. [c] Infinity points represent the average $^{3}H/^{14}C$ ratio of four time points taken at 1, 2, 3, and 4 h. All of these points were at complete conversion, as assayed by fluorescence. [d] The initial $^{3}H/^{14}C$ ratio of dopamine was 10.750. Two reactions of three points each were run. Time points were analyzed in the order listed.

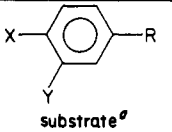
data, the method gives a high degree of reproducibility, yielding a value of 1.106 ± 0.016 for $^{T}(V/K)_{\alpha}$ (see footnote 3) and a value within experimental error of one for $^{T}(V/K)_{\beta}$.

As Northrop (1975) has discussed, the interpretation of any isotope effect on a kinetic parameter such as V_{max}/K_m is limited by the kinetic complexity of the reaction being studied. Typically the kinetic parameters are dependent on more than one microscopic rate constant, and the resultant observable isotope effect is diminished from the true or intrinsic isotope effect on the isotopically sensitive step. Once again, it is the intrinsic isotope effect which must be determined. For dopamine β-monooxygenase, the general expression for a tritium isotope effect can be written as

$$^{T}(V/K)_{x} = \frac{^{T}k_{x} + c}{1 + c} \qquad (8)$$

where $^{T}k_{x}$ is the intrinsic isotope effect ($x = 1°, \alpha, \beta$) and c is a complex term containing several rate constants and the oxygen concentration if it is less than saturating (Klinman et al., 1980). Since the primary intrinsic hydrogen isotope effect has been determined (Miller & Klinman, 1983), c can be obtained from the primary isotope effect, $^{T}(V/K)_{1°}$, and combined with $^{T}(V/K)_{2°}$ (measured under identical reaction conditions) to calculate $^{T}k_{2°}$.[4] Conversion to the intrinsic deuterium isotope effect is then accomplished by using the Swain relationship (1955). Under conditions of the experiments in Table I, $^{T}k_{1°} = 31.4 \pm 7.9$ (Miller & Klinman, 1983) and $^{T}(V/K)_{1°}$ was measured to be 12.10 ± 0.29, leading to a value for $^{D}k_{\alpha}$ of 1.19 ± 0.06.[5]

Table III: Comparison of V_{max} and $^{D}(V)$ for Substituted Phenylethylamines



substrate[a]	V_{max} (s^{-1})	$^{D}(V)$
Y, X = OH[b]	12.7 ± 0.3	1.19 ± 0.05
Y = H; X = OH	13.3 ± 0.5	1.18 ± 0.05
	17.8 ± 0.5	1.43 ± 0.04
Y = H; X = H	18.9 ± 0.7	1.40 ± 0.07
	16.3 ± 0.1	1.34 ± 0.04
	20.2 ± 0.8	1.38 ± 0.10
Y = H; X = CH_3[c]	24.4 ± 0.8	2.74 ± 0.12
Y = H; X = OCH_3	26.6 ± 0.8	4.44 ± 0.53
Y = H; X = F	49.8 ± 2.5	3.85 ± 0.22
Y = H; X = Cl	40.8 ± 1.9	6.62 ± 0.39
Y = H; X = Br	15.4 ± 0.3	8.08 ± 0.16
Y = H; X = CF_3[d]	1.9 ± 0.2	≥11.2

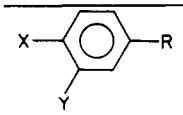
[a] R = $CH_2CH_2NH_3Cl$. [b] Data from Ahn & Klinman (1983), plus fumarate, pH 6.0, μ = 0.14 M; similar data were obtained at pH 6.2. [c] Kinetics for the dideuterated substrate were obtained at a later time due to extremely noisy traces from the oxygen electrode in the first set of data. One oxygen concentration was repeated for the unlabeled substrate, which looked identical with the first set of data. [d] $^{D}(V)$ was not actually determined (see text) but $^{D}(V/K_{amine}) = 18.5$, and therefore, $^{D}(V)$ was assumed to be greater than or equal to $^{D}(k)_{1°}{}^{D}(k)_{\alpha}$.

[3] Our observed value for $^{T}(V/K)_{\alpha}$ is significantly smaller than a previously reported secondary deuterium isotope effect (Bachan et al., 1974). We attribute this difference to changes in experimental conditions, as well as possible contamination of [2(S)-^{2}H]dopamine by the 2(R) enantiomer in the earlier study.

[4] By use of this method, the error equation for $^{T}k_{2°}$ is

$$\frac{\sigma(^{T}k_{2°})}{^{T}k_{2°}} = \left\{ \left[\frac{\sigma(^{T}(V/K)_{2°})}{^{T}(V/K)_{2°} - \left[\frac{^{T}k_{1°} - ^{T}(V/K)_{1°}}{^{T}k_{1°} - 1} \right]} \right]^{2} + \left[\frac{\sigma(^{T}k_{1°})}{[^{T}(V/K)_{1°} - 1]\left[\frac{^{T}(V/K)_{2°}}{^{T}(V/K)_{2°} - 1} + \frac{^{T}k_{1°} - ^{T}(V/K)_{1°}}{^{T}(V/K)_{1°} - 1} \right]} \right]^{2} + \left[\frac{\sigma[^{T}(V/K)_{1°}][1 - ^{T}k_{1°}]}{[^{T}(V/K)_{1°} - 1]^{2}\left[\frac{^{T}(V/K)_{2°}}{^{T}(V/K)_{2°} - 1} + \frac{^{T}k_{1°} - ^{T}(V/K)_{1°}}{^{T}(V/K)_{1°} - 1} \right]} \right]^{2} \right\}^{1/2}$$

Table IV: Comparison of V/K and $^{D}(V/K)$ for Amine and Oxygen Substrates



substrate[a]	V_{max}/K_{amine} ($\times 10^3$ M^{-1} s^{-1})	$^{D}(V/K_{amine})$	V_{max}/K_{O_2} ($\times 10^4$ M^{-1} s^{-1})	$^{D}(V/K_{O_2})$
X, Y = OH[b]	26.0 ± 3.2	1.37 ± 0.25	25.5 ± 2.1	3.88 ± 0.38
Y = H; X = OH	51.2 ± 1.2	1.0 ± 0.1	33.4 ± 4.9	1.65 ± 0.26
	58.8 ± 3.5	1.6 ± 0.12	32.8 ± 1.8	1.73 ± 0.10
Y = H; X = H	35.3 ± 2.8	7.82 ± 0.7	15.3 ± 0.8	4.9 ± 0.3
	20.7 ± 1.2	9.45 ± 0.56	23.6 ± 0.6	7.9 ± 0.3
	46 ± 17	3.1 ± 1.9	27.5 ± 3.8	8.5 ± 1.2
Y = H; X = CH_3[c]	6.59 ± 0.12	9.98 ± 0.46	4.69 ± 0.15	3.07 ± 0.19
Y = H; X = OCH_3	0.98 ± 0.13	8.75 ± 0.22	3.52 ± 0.27	5.75 ± 0.50
Y = H; X = F	5.15 ± 0.36	1.06 ± 0.16	12.6 ± 0.6	3.13 ± 0.16
Y = H; X = Cl	4.38 ± 0.29	6.50 ± 0.68	4.60 ± 0.08	4.40 ± 0.11
Y = H; X = Br	5.37 ± 0.13	12.6 ± 0.7	3.15 ± 0.08	6.06 ± 0.17
Y = H; X = CF_3	(6.7 ± 0.1)	18.5 ± 1.4[d]	0.52 ± 0.04	≥11.2[e]

[a] R = $CH_2CH_2NH_3Cl$. [b] Data from Ahn & Klinman (1983). [c] H and D kinetics determined at separate times but with the same enzyme stock. [d] Due to the extremely high K_{amine}, $^{D}(V/K_{amine})$ was determined at a single concentration (130 mM). $^{D}(V/K_{amine})$ was taken as the ratio of intercept values from $1/v$ vs $1/[O_2]$ plots for the H and D amine. [e] Not determined, but assumed to be greater than or equal to $^{D}(k)_1 \cdot {}^{D}(k)_\alpha$.

Kinetics of Para-Substituted Phenylethylamines. By use of a series of para-substituted phenylethylamines, an investigation of the substituent effect on C–H bond cleavage was undertaken. Several investigators have pointed out [e.g., see Klinman (1972), Northrop (1975), and Cleland (1975)] that examination of substituent or isotope effects on kinetic parameters is ambiguous unless it is certain that the parameter is limited by a single microscopic rate constant; to avoid this limitation, microscopic rate constants must be isolated and examined independently. It has been demonstrated (Miller & Klinman, 1982) that if the intrinsic isotope effect has been measured, parameter isotope effects different from unity and the intrinsic value allow the calculation of microscopic rate constants for a minimal kinetic mechanism. Since $^{D}k_{1\circ}$ has been measured for dopamine β-monooxygenase, the substituent effect was pursued by obtaining deuterium isotope effects on the kinetic parameters V_{max} and V_{max}/K_m through a kinetic analysis of both unlabeled and dideuterated phenylethylamines; the results are summarized in Tables III and IV. Previous studies have shown that, in the presence of saturating reductant (ascorbate), the minimal kinetic mechanism is one of random binding of substrates (Klinman et al., 1980); therefore, complete kinetic analyses of the parameters $V_{max}/K_{O_2\,H,D}$, $V_{max}/K_{amine\,H,D}$, and $V_{max\,H,D}$ have been obtained from initial velocities by variation of both oxygen and amine substrates with subsequent extrapolation to infinite amine or oxygen, respectively, for V_{max}/K_m parameters and to both infinite oxygen and amine for V_{max} (cf. Experimental Procedures).

With the primary focus of this study being the effect of substituent on C–H cleavage (k_5 in eq 3), the greatest concern is with the results for V_{max} shown in Table III. It can be seen from the repetitive measurements listed for *p*-tyramine and phenylethylamine that the method affords reasonably reproducible values for both V_{max} and $^{D}(V)$. Focusing on the values for $^{D}(V)$ across the series, a wide variation is observed, which indicates a change in the relative rate limitation of V_{max} from k_7 for "good" substrates with low isotope effects to k_5 for "poor" substrates with high isotope effects. With this observation, it becomes obvious that any attempts to correlate the substituent effects directly with V_{max} would yield faulty conclusions. One assumption to be noted in Table III is the value of $^{D}(V)$ for *p*-CF_3. Rates for the dideuterated substrate were extremely low and difficult to obtain; therefore, only $^{D}(V/K_{amine})$ was obtained as described in Table IV. Because this value was $\geq {}^{D}k$ and the V_{max} was significantly lower than for any other amine, it was assumed that $^{D}(V) \geq {}^{D}k$ for dopamine and that k_5 is completely rate limiting.

Turning to the results in Table IV, it is noted that the reproducibility of both V_{max}/K_m and $^{D}(V/K)$ values is somewhat less than that of V_{max}. This result can be attributed, in part, to the fact that V_{max}/K_m and $^{D}(V/K)$ measurements depend on the concentration of unlabeled and dideuterated substrates. Concentrations of the stock substrate solutions should be accurate ±5%, which will account for some lack of reproducibility. Additionally, in the third experiment with phenylethylamine, where $^{D}(V/K_{amine}) = 3.1$, all of the concentrations of substrate were above the K_m, resulting in relatively poor determination of V/K_{amine}. In any case, the values presented are judged to be good estimates and as shown below permit a description of the effect of substituent on calculated constants.

Comparison of the V_{max} to V_{max}/K_m values yields the K_m values for substrate and oxygen. Special attention should be given to the resultant magnitudes of $K_{m\,O_2}$ across the series of substrates. For *p*-tyramine and phenylethylamine, the $K_{m\,O_2}$ is in the range 0.05–0.08 mM which is approximately the same as for dopamine. However, for all of the other amines, $K_{m\,O_2}$ is greater than the 0.21 mM oxygen concentration obtained from air saturation at 35 °C, indicating the importance of varying oxygen in kinetic studies aimed at comparing V_{max} values for a range of substrates.

Calculation of Rate and Dissociation Constants. Rate constants for C–H bond cleavage, k_5, and product dissociation, k_7, and dissociation constants for amine and oxygen from enzyme–ternary complex have been calculated as described under Experimental Procedures for the minimal mechanism in eq 3 and are summarized in Table V. Since the deuterium isotope effects were measured by using dideuterated substrates, ^{D}k in eq 4 and 5 reflects both the primary and secondary intrinsic isotope effects and as such will be equal to the product of $^{D}k_{1\circ}$ $^{D}k_\alpha$.[6] Under the conditions employed herein, $^{D}k_{1\circ}$ =

[5] It should be noted that these results were obtained in the absence of the anion activator fumarate. Ideally, $^{D}k_\alpha$ also would have been determined in the presence of fumarate; however, since fumarate significantly lowers the observed kinetic isotope effects (Ahn & Klinman, 1983; Miller & Klinman, 1983), the experimental error was expected to dominate $^{T}(V/K)_\alpha$. Furthermore, in the case of the intrinsic primary isotope effect, addition of fumarate caused only a minor decrease in $^{D}k_{1\circ}$ (Miller & Klinman, 1983) and insignificant change in the resulting value of the rate constant for the C–H bond cleavage step (Ahn & Klinman, 1983). If this rate constant is essentially unaltered by fumarate, then the transition state for C–H bond cleavage and hence $^{D}k_\alpha$ are also expected to be unchanged.

9.4 ± 1.3 (Miller & Klinman, 1983), and from above $^{D}k_{\alpha}$ = 1.19 ± 0.06, giving a final value of ^{D}k = 11.2 ± 1.6; this was used in eq 4 and 5 to calculate k_5 and k_7.

Two underlying assumptions in calculating the rate and dissociation constants are that (1) the kinetic mechanism and (2) the intrinsic isotope effects are the same throughout the series. Since all of the substrates, with the possible exception of *p*-tyramine and (*p*-fluorophenyl)ethylamine, exhibit isotope effects greater than one for both $^{D}(V/K_{amine})$ and $^{D}(V/K_{O_2})$, it is clear that both substrates must dissociate from enzyme-ternary complex. For *p*-tyramine and *p*-fluorophenylethylamine, $^{D}(V/K_{amine})$ is within experimental error of 1 (1.3 ± 0.3 for *p*-OH), indicating a very low dissociation of amine from enzyme–ternary complex reminiscent of dopamine under similar conditions. $K_{D\,amine}$ values calculated for these two substrates are much less reliable and possibly should be dropped from the correlation analysis (see below). Evidence that V_{max} depends on two steps for each substrate (with the exception of the *p*-trifluoromethyl substituent) derives from the observation that $^{D}(V)$ is generally smaller than one or both of the isotope effects on the V_{max}/K_m parameters. Since all parameter isotope effects for a substrate reflect the same intrinsic isotope effect, a value of $^{D}(V)$ smaller than $^{D}(V/K)$ indicates the presence of a kinetically significant step subsequent to C–H bond cleavage. Although the *p*-fluoro and *p*-chloro substituents do not meet this criterion, they exhibit $^{D}(V)$ values less than ^{D}k for dopamine, consistent with a partially rate-limiting postcatalytic step.

In considering the magnitude of ^{D}k across the series, it is again of interest to examine the values of the parameter isotope effects. Large isotope effects near the value of ^{D}k for dopamine are observed with both electron-donating and electron-withdrawing substituents, suggesting that ^{D}k is at least as large as 11.2 for the whole series. For [*p*-(trifluoromethyl)-phenyl]ethylamine the only measured isotope effect, $^{D}(V/K_{amine})$, is greater than ^{D}k = 11.2 for dopamine. Given the experimental difficulties in determining kinetic parameters for this substrate, it is difficult to be certain that its intrinsic isotope effect exceeds the value of 11.2; in any case, it was assumed that all the parameter isotope effects are maximally expressed, such that k_5 was set equal to V_{max} and k_7 assigned a minimal value that would allow the full expression of ^{D}k in $^{D}(V)$. Analogous to [*p*-(trifluoromethyl)phenyl]ethylamine, (*p*-bromophenyl)ethylamine also shows an isotope effect on V_{max}/K_{amine} somewhat greater than ^{D}k. The appearance of observed isotope effects larger than ^{D}k for dopamine introduces the possibility of an increase in intrinsic isotope effects for electron-withdrawing substituents and, hence, some uncertainty in the calculated values for k_5 and k_7. In general, underestimation of ^{D}k leads to an underestimation of k_5 and an overestimation of k_7. Importantly, errors in calculation of k_5 and k_7 will be smallest when the observed isotope effect on V_{max} is significantly different from unity or the intrinsic isotope effect, respectively. As can be seen from Table IV, the trend in observed values for $^{D}(V)$ parallels a possible trend in ^{D}k, such that errors in the calculation of k_5 and k_7 are not expected to be very great. For example, if ^{D}k for *p*-Br is actually 15, k_5 and k_7 would both be ca. 30 s^{-1}; if ^{D}k for *p*-Cl is 13, k_5 = 87 s^{-1} and k_7 = 77 s^{-1}. Compared to the overall range of values listed in Table V, these changes in k_5 and k_7 are fairly insignificant. Thus, variations in ^{D}k may be expected to produce a slight reduction in the magnitudes of the observed correlations described in the next section, but qualitatively the effects should remain the same.

It should be noted that the calculated constants of Table V are defined by the minimal mechanism of eq 3. In consideration of the chemistry in this reaction, an expansion of the mechanism to include an equilibrium reduction of oxygen in the enzyme–substrate ternary complex prior to C–H bond cleavage may be necessary (Klinman et al., 1984). As detailed in the Appendix, inclusion of this step slightly alters the definitions of the calculated constants but is not expected to influence the regression analyses described below.

Multiple Regression Analysis. It is well established that substituent effects in biological systems can reflect a combination of electronic, steric, and hydrophobic contributions. Thus, multiple regression analyses were performed to evaluate which effects were most significant for each set of calculated constants in Table V; the method employed the general expression

$$\log k,K = \sigma\rho + \pi b + Sc + d \qquad (9)$$

where k and K represent the rate and dissociation constants, σ, π, and S are the electronic, hydrophobic, and steric substituent constants, and ρ, b, and c are the contributions of each factor to a given rate or equilibrium process. The major constants employed in this study include the electronic substituent constants σ_p, σ_p^+, and σ_p^0, and the hydrophobic substituent constant, π (Hansch & Leo, 1979).[7] Choice of the correct steric constant can be ambiguous and will depend on the nature of the substrate binding pocket. For example, a dependence on E_s (Taft, 1952), which is highly correlated to the van der Waals radius of the first atom of the substituent (Charton, 1969), implies that branched substituents can assume conformations where the bulk of the substituent does not interact with the active site. Alternatively, a more constrained binding pocket is expected to correlate with substituent constants reflecting steric bulk. In this regard, Hansch and co-workers (1964) have proposed the potential use of Bondi's calculated van der Waals volumes, V_w (Bondi, 1964). Thus, in the present study, both E_s and V_w values[7] were examined, since the "branched" substituents *p*-OH and *p*-OCH_3 were expected to correlate well with only one type of steric parameter.

Multiple linear regression analyses were performed on variations of eq 9 with weighted values for log k by using the SAS General Linear Models Procedure.[8] Correlations with

[6] Assuming the motions of the two hydrogens attached to carbon are uncoupled, the isotope effects are orthogonal. As considered in the discussion of the magnitudes of $^{D}k_{1^\circ}$ and $^{D}k_{\alpha}$, we do not believe the motions of the benzylic hydrogens are appreciably coupled in the dopamine β-monooxygenase reaction.

[7] With regard to electronic substituent constants, σ_p is based on the ionization of benzoic acids, σ_p^+, the S_N1 solvolysis reaction of (dimethylphenyl)methyl chlorides and σ_p^0, the ionization of phenylacetic acids. The hydrophobicity substituent constant, π, derives from octanol–water partition studies. For steric substituent constants, E_s results from studies of the acid-catalyzed hydrolysis of α-substituted esters, and V_w represents calculated van der Waals volumes. With the exception of V_w, each of the substituent constants has been defined and tabulated in Hansch & Leo (1979). van der Waals volumes are from Bondi (1964) but have been scaled by 0.1 to obtain values of comparable magnitude to E_s. In addition, V_w has been calculated for the *p*-CH_3O, *p*-CF_3, and *p*-H substituents as described in Table VI.

[8] Statistical analysis system is available from SAS Institute Inc., Cary, NC 27511. For least-squares analyses on experimental data having unequal experimental uncertainties, each data point is weighted by the reciprocal of its uncertainty (error) squared (Bevington, 1969). In the present study, the experimental data points are values of k ± error; but the correlation function is linear in log k. Thus, the weighting factors were derived from the limiting values of log (k ± error), specifically as the reciprocal of the square of the average deviation of log k_{limit} from log k where $k_{limit} = k \pm$ error (K was treated identically).

Table V: Calculated Constants[a]

X =	k_5 (s^{-1})	k_7 (s^{-1})	$K_{D\ amine}$ (mM)[b]	$K_{D\ O_2}$ (mM)[b]
OH[c]	590 ± 170	16 ± 2	(0.5 ± 0.1)[d]	0.12 ± 0.03
H[c]	500 ± 71	19 ± 2	14 ± 5.3	1.3 ± 0.1
CH_3	140 ± 24	29 ± 2	19 ± 1.4	0.62 ± 0.06
OCH_3	79 ± 15	40 ± 7	61 ± 0.8	1.0 ± 0.1
F	180 ± 29	69 ± 7	(0.2 ± 0.1)[d]	0.30 ± 0.02
Cl	74 ± 12	91 ± 21	9.1 ± 1.0	0.54 ± 0.03
Br	22 ± 3.5	50 ± 18	(4.1 ± 0.7)[e]	0.35 ± 0.01
CF_3	1.9 ± 0.3	(≥200 ± 70)[f]	(300 ± 57)[e]	(0.36 ± 0.07)[g]

[a] Corresponding values for dopamine are k_5 = 680 ± 210, k_7 = 13 ± 0.3, $K_{D\ amine}$ = 0.96 ± 0.64, and $K_{D\ O_2}$ = 0.76 ± 0.21. [b] Dissociation constants from ternary enzyme–substrate complex are equal to $k_{4\ amine}/k_{3\ amine}$ and $k_{4\ O_2}/k_{3\ O_2}$, respectively (eq 3). [c] The values for these substrates represent averages of repetitive determinations (see Table III). [d] $^D(V/K_{amine})$ is very close to 1.0; therefore, dissociation constants can only be estimated from eq 6 in text. [e] $^D(V/K_{amine})$ > 11.2; therefore, the expression for V_{max}/K_{amine} was simplified to $V_{max}/K_{amine} = k_5/K_{D amine}$, and $K_{D\ amine}$ was calculated from V_{max}/K_{amine} and k_5. The corresponding error function is $\sigma(K_{D\ amine})/K_{D\ amine} = [(\sigma(k_5)/k_5)^2 + (\sigma(V/K_{amine})/V/K_{amine})^2]^{1/2}$. [f] Estimated as the minimum value consistant with the full expression of k_5 in V_{max}, i.e., $k_7/k_5 \geq 100$. [g] Estimated as in footnote e assuming a maximal isotope effect on $^D(V/K_{O_2})$.

one and two parameters in the form of eq 9 were examined in each case; however, with only eight experimental observations, the full three-parameter correlation of eq 9 would not be statistically valid and therefore is not included. The best one- and two-parameter equations for each constant with n = 8 observations are summarized below together with the following statistical data: r is the correlation coefficient; F relates the variance of the null hypothesis to the variance of each correlation; p is the probability that a random set of data would yield a higher value of F; for two-parameter equations, $F_x = F_{1,5}$ is the test for the significance of adding the indicated variable to the previous one-parameter equation and is interpreted by the corresponding p value, which is the probability that the coefficient of the added variable is zero (Bevington, 1969).

rate of C–H bond cleavage

$$\log k_5 = [0.958\ (\pm 0.177)]E_s + 2.75\ (\pm 0.20) \quad (10)$$
$$r = 0.911 \qquad F_{1,6} = 29.31 \qquad p = 0.0016$$

$$\log k_5 = [-1.03\ (\pm 0.24)]V_w + 3.08\ (\pm 0.31) \quad (11)$$
$$r = 0.865 \qquad F_{1,6} = 17.80 \qquad p = 0.0056$$

$$\log k_5 = [-0.810\ (\pm 0.096)]V_w - [1.48\ (\pm 0.23)]\sigma_p + 2.93\ (\pm 0.12) \quad (12)$$
$$r = 0.986 \qquad F_{2,5} = 86.57 \qquad p = 0.0001$$
$$F_{x(\sigma_p)} = 39.91 \qquad p_x = 0.0015$$

rate of product dissociation

$$\log k_7 = [1.13\ (\pm 0.35)]\sigma_p^0 + 1.55\ (\pm 0.05) \quad (13)$$
$$r = 0.798 \qquad F_{1,6} = 10.55 \qquad p = 0.018$$

$$\log k_7 = [1.41\ (\pm 0.24)]\sigma_p^0 + [0.239\ (\pm 0.075)]V_w + 1.30\ (\pm 0.08) \quad (14)$$
$$r = 0.938 \qquad F_{2,5} = 18.19 \qquad p = 0.005$$
$$F_{x(V_w)} = 10.0 \qquad p_x = 0.025$$

amine dissociation

$$\log K_{D\ amine} = [1.56\ (\pm 0.42)]V_w - 0.929\ (\pm 0.627) \quad (15)$$
$$r = 0.834 \qquad F_{1,6} = 13.66 \qquad p = 0.010$$

For the C–H bond cleavage step the most significant single parameter correlations were obtained with the rescaled Taft steric parameter E_s (Unger & Hansch, 1976) and, to a slightly lesser extent, with a van der Waals volume parameter V_w (Bondi, 1964). Alghough the fit is slightly better with E_s, the coefficient (c) is nearly identical for the two equations (with opposite signs because E_s values are negative), indicating that both parameters predict a similar steric dependence. Additional single-parameter correlations with σ_p and π were both significant at the 97% confidence level, suggesting that one or both of these parameters may also be important. Examination of two-parameter correlations yielded eq 12 as the most significant overall fit to the data with 97.2% (r^2) of the variance explained. All other two-parameter combinations, including $E_s + \sigma_p$, gave poorer correlations than the single parameter eq 10 and 11.[9] It also should be mentioned that correlations attempted with σ_p^+ gave much poorer fits than those with σ_p. Figure 1 shows the adjusted and unadjusted correlations of k_5 to σ_p.

For the product dissociation step, k_7, the best single-parameter correlation was obtained with σ_p^0, eq 13, and this was significantly improved statistically by inclusion of the steric parameter V_w, eq 14; i.e., there is only a 2.5% chance that the coefficient (c) of V_w is actually zero. Inclusion of E_s instead of V_w gave a poorer fit, and all correlations using σ_p instead of σ_p^0 were much less significant. Figure 2 shows both adjusted and unadjusted correlations of k_7 to σ_p^0. In light of the dominance of V_{max} for p-bromo- and especially [p-(trifluoromethyl)phenyl]ethylamines by k_5, the deviation of these two substrates from the adjusted correlation is not unexpected, and arises from uncertainty in calculated values for k_7.[10]

As indicated in eq 15, V_w was the only parameter to give a significant correlation for $K_{D\ amine}$. In this case, the correlation with E_s had an F value of only 0.14 (p = 0.724) as compared to 13.66 (p = 0.010) with V_w. All two-parameter equations gave worse fits than eq 15. In the case of $K_{D\ O_2}$, no one- or two-parameter equations yielded any significant correlations. The best appeared with σ_p having an F value of 2.72 (p = 0.150) and r = 0.558. With a correlation coefficient and an F value this low, the fit is hardly better than chance.

The lack of any significant variation in $K_{D\ O_2}$ provides an important conclusion concerning the expanded mechanism mentioned above. As summarized in the Appendix, the addition of an equilibrium step for reduction of oxygen yields expressions for the apparent K_D values which contain the true K_D and the equilibrium constant for reduction (K_{eq}). With the absence of an effect of substituent on $K_{D\ O_2}$, we can conclude that there is no substituent effect on K_{eq}. Hence, the correlations observed on both $K_{D\ amine}$ and k_5, which also

[9] An important question in regression analysis is the extent of parameter collinearity among the substituents investigated. In the present study, V_w shows very little collinearity with either σ or π, whereas σ_p and π indicate some collinearity, where r^2 = 0.66. This is undoubtedly the reason that both σ_p and π gave identical one-parameter correlations with log k_5. Despite this problem, in the two-parameter analysis of log k_5, inclusion of π with V_w reduced the overall fit, whereas σ_p showed the dramatically improved fit of eq 12, indicating the importance of the electronic effect as opposed to the hydrophobic effect.

[10] Elimination of k_7 for p-trifluoromethylphenylethylamine led to a very small change in eq 14, yielding log k_7 = [1.59 (±0.28)]σ_p^0 + [0.28 (±0.08)]V_w + 1.26 (±0.09).

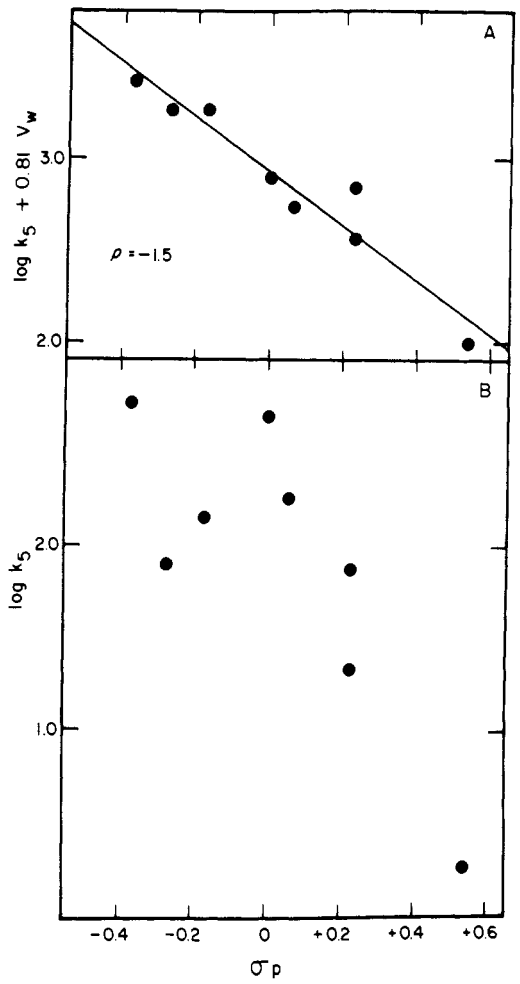


FIGURE 1: Correlation of k_5 with the electronic substituent constant σ_p. (Panel A) Corrected for the contribution of a steric component to catalysis; (panel B) uncorrected.

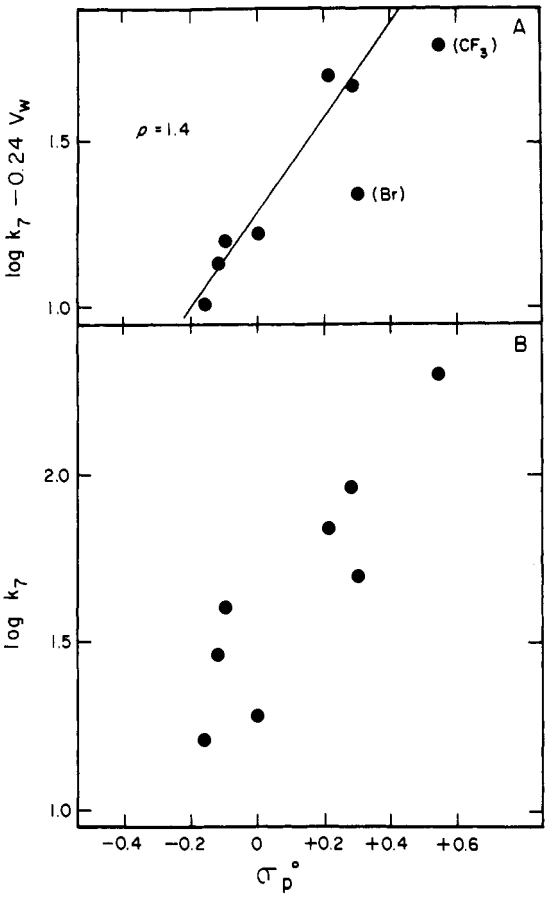


FIGURE 2: Correlation of k_7 with the electronic substituent constant σ_p^0. (Panel A) Corrected for the contribution of a steric component; (panel B) uncorrected.

contain K_{eq} in the expanded mechanism, can be ascribed directly to amine binding and C–H bond cleavage, respectively.

DISCUSSION

Secondary Isotope Effects. In the dopamine β-monooxygenase reaction, the large magnitude of the intrinsic primary hydrogen isotope effect, together with the irreversibility of the C–H bond cleavage step, has led to the postulate of a stepwise mechanism in which C–H bond cleavage precedes C–O bond formation. Independent of the mode of C–H bond activation (heterolytic vs. homolytic), a stepwise mechanism predicts measurable bond rehybridization at the benzylic carbon in the transition state, leading to a kinetic α-deuterium isotope effect greater than the equilibrium effect for the production of norepinephrine from dopamine [estimated as ${}^{D}K_{\alpha} \simeq 0.84$ from fractionation factors tabulated by Cleland (1982)]. Typical kinetic isotope effects for solvolysis reactions range from 1.10 to 1.25 (Melander & Saunders, 1980), and although very few studies have been reported for radical reactions, Seltzer and co-workers (1961, 1963, 1965, 1967) report values of 1.13–1.16 in the decomposition of azo compounds to radical intermediates. From the very substantial kinetic α-secondary isotope effect reported herein for the dopamine β-monooxygenase catalyzed hydroxylation of dopamine, ${}^{D}k_{\alpha} = 1.19 \pm 0.06$, we exclude a concerted oxygen insertion mechanism, confirming our previous conclusion of a stepwise mechanism to generate a substrate-derived intermediate. Since a large secondary isotope effect could arise from formation of either a carbon radical or carbocation, and possibly also with a carbanion because of charge delocalization into the ring, the chemical nature of the intermediate cannot be inferred from these data. However, as discussed below, combination of the isotope effect with substituent effect data does provide a complete picture of the nature of the intermediate formed from hydrogen abstraction.

Interpretation of α-secondary hydrogen isotope effects in terms of transition state structure has undergone significant change in the last several years. Although it is widely accepted that these effects reflect rehybridization changes, kinetic α-secondary isotope effects have been found to exceed equilibrium effects in several systems involving reaction at sp^3 hybridized methylene carbon centers to generate sp^2 hybridized products [cf. Cook et al. (1981)]. These unexpectedly large kinetic isotope effects have been attributed to coupled motion between the primary and secondary positions in the transition state. Modeling of transition states involving such coupling indicates relatively small primary hydrogen isotope effects; i.e., the increase in the α-secondary effect is at the expense of the primary isotope effect. Husky & Schowen (1983) have shown that only with inclusion of a significant tunnel correction can *both* observed primary (${}^{D}k_{1^\circ} = 4.7$) and observed secondary (${}^{D}k_{2^\circ} = 1.25$) hydrogen isotope effects in NAD^+-dependent reactions be effectively modeled.

Regarding dopamine β-monooxygenase, the very large magnitude of the intrinsic primary hydrogen isotope effect, ${}^{D}k_{1^\circ} = 9.4$–11 (Miller & Klinman, 1983) implies an excessively large tunnel correction in the event of a coupled motion between the benzylic hydrogens in the transition state. In the absence of evidence for such a high degree of tunneling, we conclude that changes in the stretching and bending frequencies of the α-hydrogen dominate the expression of ${}^{D}k_{\alpha}$.

This statement implies a fairly linear relationship between the magnitude of $^{D}k_{\alpha}$ and the bond order between the α-carbon and transferred hydrogen in the transition state (cf. Saunders, 1984). Thus, in principle, the symmetry of the transition state can be deduced from the magnitude of the $^{D}k_{\alpha}$ in relationship to $^{D}K_{\alpha}$ for formation of the enzyme-bound intermediate. However, as noted, the C–H bond cleavage step in the dopamine β-monooxygenase reaction is irreversible, precluding an experimental determination of $^{D}K_{\alpha}$ (Miller & Klinman, 1983). In the absence of experimental data for $^{D}K_{\alpha}$, two approaches can be taken for reactions involving the conversion of an sp^3 to an sp^2 carbon center. The first is to use the aldehydic C–H bond as a model for an sp^2 intermediate, which led Streitwieser and co-workers (1958) to predict a maximum $^{D}K_{\alpha} = 1.4$. Alternatively, fractionation factors for a variety of C–H bonds have been tabulated and can be used to estimate secondary equilibrium isotope effects. For example, for the conversion of a saturated to an unsaturated carbon center, e.g., the dehydration of malate to fumarate, fractionation factors indicate $^{D}K_{\alpha} = 1.13$ (Cleland, 1982). Comparison of the measured α-deuterium isotope effect in the dopamine β-monooxygenase to this range for $^{D}K_{\alpha}$ values suggests a transition state in which C–H bond cleavage has proceeded to at least 50%. A fairly symmetrical transition state was previously proposed, from the magnitude of $^{D}k_{1^\circ}$ in relation to literature correlations of primary hydrogen isotope effects with the thermodynamic properties of these hydrogen transfer reactions.[11]

β-Secondary isotope effects are generally believed to arise from hyperconjugative interaction of a C–H bond with the p orbital of an adjacent incipient radical or carbocation. The magnitude of such isotope effects depends on several factors: (1) the extent of orbital overlap and therefore the molecular geometry; (2) the degree of electron deficiency in the reacting bond at the transition state; (3) the degree to which additional stabilization of the transition state occurs such as by resonance interaction with an adjacent π system. Reported values of $^{D}k_{\beta}$ for radical-forming reactions are usually on the order of 1.02 but may be as large as 1.08 where no stabilization of the radical is present (Tsolis et al., 1976). For carbocation-forming reactions, the values typically run about 5–15% (Melander & Saunders, 1980) but cluster at the low end for solvolyses of benzylic compounds; for example, with 1-phenylethyl chlorides, $^{D}k_{\beta}$ (for β-d_3) was 1.11 for the p-OMe compound and 1.22 for the unsubstituted compound corresponding to 1.035 and 1.069 per deuterium, respectively (Shiner et al., 1968). In the present study, $^{D}(V/K)_{\beta}$ is within experimental error (2%) of one, precluding the determination of $^{D}k_{\beta}$. On the basis of the factors listed above, the apparent lack of a β-secondary isotope effect may be due to geometrical constraints on the binding of substrate; or, alternatively, $^{D}k_{\beta}$ may be very small, 1.02–1.04, due to the benzylic nature of the incipient intermediate, such that $^{D}(V/K)_{\beta}$ would be unobservable (1.01–1.015) under the experimental conditions. In light of the multiple explanations, no definitive conclusion can be made from these data regarding the nature of the transition state.

Structure–Reactivity Correlations. The electronic and steric contributions obtained by multiple linear regression analysis for the calculated constants in Table V are summarized in Table VI; no hydrophobic contributions were observed. As indicated in Table V, $K_{D\,O_2}$ exhibits very little variance with substituent, and no statistically significant one- or two-parameter correlations were obtained for $K_{D\,O_2}$. As indicated in Table VI, the variance in $K_{D\,amine}$ is best explained by the change in volume of the substituent, where substrates with larger substituents bind loosely. This simple correlation with volume implies a reasonably constrained binding pocket, although both tyramine and dopamine [and possibly (*p*-fluorophenyl)ethylamine] bind more tightly than the rest of the series, implying an additional interaction(s) with the natural substrate.

Table VI: Electronic and Steric[a] Contributions to Calculated Constants

process	calcd constant	$\log k,K = \sigma_p,\sigma_p^0\rho + V_w c + d$	
		ρ	c
(1) C–H bond cleavage	k_5[b]	−1.5 ± 0.2[c]	−0.81 ± 0.10[c]
(2) product dissociation	k_7[b]	1.4 ± 0.2[d]	0.24 ± 0.08[d]
(3) substrate dissociation	$K_{D\,amine}$[b]	0	1.6 ± 0.4[e]

[a] Van der Waals volumes, V_w, are from Bondi (1964) except for the following which were calculated from group increments with the indicated assumptions: (1) For p-CH_3O, $V_w = V_w(CH_3) + V_w(-O-)$; the two assumptions in this calculation are that the cited methyl group volume which is correct for attachment to a carbon atom is also correct for attachment to oxygen and that a poly(phenyl) ether oxygen has a volume similar to that of a phenylalkyl ether. (2) For p-CF_3, the volume was calculated from the volumes for a tetrahedral carbon and for fluorine atoms in perfluorinated alkanes with no assumptions necessary. (3) For p-H, V_w is calculated by subtracting the volume of an aromatic carbon atom when it is attached to an alkyl group, V_w(aromatic C–R), from the total volume of an aromatic C–H group, V_w(aromatic C–H). [b] Table V. [c] Equation 12 in text; uses σ_p. [d] Equation 14 in text; uses σ_p^0. [e] Equation 15 in text.

Examination of Table VI shows that the C–H bond cleavage step, k_5, exhibits a significant dependence on both electronic and steric parameters. The negative value for ρ indicates some development of positive charge at the benzylic carbon in the transition state and as such rules out a proton abstraction mechanism. Although some development of positive charge is indicated, formation of a carbocation is not implied since σ_p gives a significantly better fit than σ_p^+. By contrast, several ρ values of similar sign and magnitude have been reported for radical processes [e.g., see Walling et al., (1963)]. Typically, negative ρ values (usually correlated to σ_p) ranging from −0.5 to −1.6 have been observed for hydrogen atom abstraction from para-substituted toluenes by radical species which are more electronegative than carbon. For example, Russell & Williamson (1964) report ρ = −0.8 with chlorine atoms and ρ = −1.4 with bromine atoms, and Pryor and co-workers (1980) report ρ = −1.6 with iodine atoms. In light of these results, we conclude that our value of ρ = −1.5 for k_5 is indicative of a net hydrogen atom abstraction from the amine substrate producing a radical intermediate which rapidly collapses to product.

The steric dependence of k_5 can be understood in the context of the correlation of $K_{D\,amine}$ with V_w, which implies that substrates with larger para substituents do not bind as well to enzyme, presumably because the favorable energy of the normal binding interaction is insufficient to overcome the increased van der Waals repulsions of the substituent. Although tight binding interactions with substrate in the ground state are generally viewed as detrimental to catalysis, they are expected to lower the energy barrier for catalysis if they serve to pre-position a substrate for the transition state. This appears to be the case for dopamine β-monooxygenase where we propose that smaller substrates (with a lower K_D) are properly

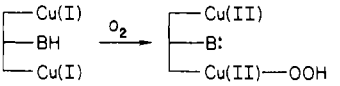
[11] In a recent study, Saunders (1984) has concluded coupled motion from a comparison of his observed kinetic α-secondary isotope effect, $^{D}k_{\alpha} = 1.21$, to a calculated equilibrium isotope effect, $^{D}K_{\alpha} = 1.12$. It is of interest that modeling of his reaction coordinate, in which both coupled motion and tunneling have been included, indicates ca. 40–50% bond cleavage when $^{D}k_{\alpha}$ is maximal and equal to his observed value.

positioned for C–H cleavage, whereas larger substrates (with a higher K_D) are not.

It should be mentioned that May and co-workers (1981) have reported substituent effects for dopamine β-monooxygenase giving rise to $\rho = -0.4$ for hydroxylation and $\rho = -3.6$ for sulfoxidation. Examination of their work, however, shows two major problems with the analysis. First, their kinetic constants were determined at a single oxygen concentration (atmospheric), making distinction between V_{max} and V_{max}/K_m difficult. Second, their analysis assumed that the measured parameter is limited to a single step. Comparison of the $^D(V)$ values in Table III with the intrinsic isotope effect of 11.2 ± 1.6 clearly shows that another step is kinetically important in V_{max} and that the extent of rate limitation by the C–H bond cleavage step varies with substrate. Thus, even comparison of true V_{max} values would not give a correct correlation equation. In light of these problems, we conclude that their reported ρ values are complex and cannot be attributed to the chemical step.

A very interesting correlation can be discerned for the product dissociation step, k_7, from Table VI. The major contributor to k_7 is an electronic effect described by σ_p^0, a normalized constant obtained from and used for correlations where the reaction center is insulated from direct resonance with the aromatic ring. [Standard reactions used to define σ_p^0 include the measurement of pK_a's of phenylacetic acids and of rates of alkaline hydrolysis of phenylacetates (Taft et al., 1958, 1959; Yukawa et al., 1966).] The sign and magnitude of ρ indicates a very large development of negative charge, presumably on the product benzylic oxygen, at the transition state for k_7. For comparison, Yukawa and coworkers (1972) have reported a ρ value of 0.98 for the alkaline hydrolysis of substituted benzyl benzoates in 70% aqueous acetone. This result strengthens the proposal by Ahn & Klinman (1983) that the enzyme–product complex is actually an enzyme–Cu(II)–OR complex above the pK of an active site residue (pK_4 = 5.2–5.4). We conclude that dissociation of the alkoxide from Cu(II) is either the slow step controlling k_7 or possibly an equilibrium process followed by a rate-limiting protonation and/or loss of product from enzyme. The small steric contribution to k_7 is attributed to a poorer fit of large substrates in the binding pocket with a consequently less stable geometry in the Cu(II)–OR complex and a faster dissociation rate.

Chemical Mechanism. As presented above, both the primary and α-secondary hydrogen isotope effects are consistent with a nearly isoenergetic abstraction of a hydrogen atom from substrate to generate a radical intermediate which then proceeds downhill to product. While these data provide an excellent description of the bond-breaking process at carbon, they provide no insight into the activation of dioxygen except that the energetics of the two processes must agree. Additional observations which bear more directly on oxygen activation include the following: (1) maximal enzymatic activity is observed with a *total* stoichiometry of two coppers per monomer (Klinman et al., 1894; Ash et al., 1984); (2) no other transition metal can replace the second copper for activity, and further, the tritium isotope effect for dopamine hydroxylation is independent of copper occupancy, indicating an essential role for copper in the catalytic mechanism (Klinman et al., 1984); (3) no evidence of spin coupling between copper centers is obtained with enzyme containing two coppers per subunit by EPR spectroscopy (Edmondson et al., unpublished results); (4) enzyme requires a protonated base for catalysis attributed to general acid catalysis of oxygen reduction (Ahn & Klinman, 1983). A mechanism consistent with these findings involves a two-electron reduction of oxygen to a protonated peroxy species prior to substrate hydroxylation.



(16)

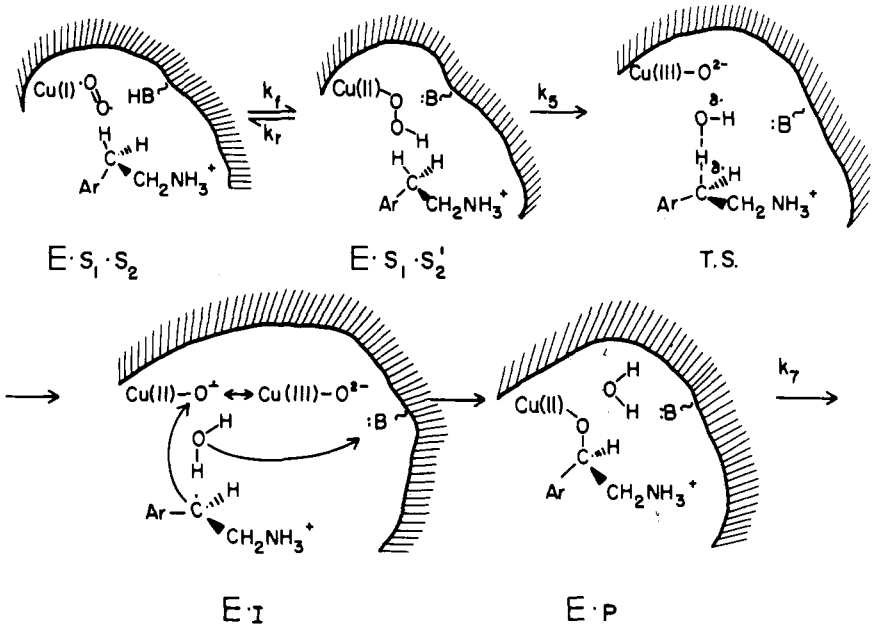
The formation of ECu(II)–OOH could occur by sequential 1e transfers from Cu(I) to O_2, giving rise to Cu(II)–O_2^{-}· as an intermediate; however, this seems unlikely considering the high reduction potential for enzyme copper (Ljones et al., 1978; Walker et al., 1977) coupled to the highly unfavorable 1e reduction potential for O_2 (Sawyer & Nanni, 1981). As illustrated in eq 16, activation of dioxygen is written as a simultaneous two-electron, one-proton-transfer process. The structure and energy level of the transition state for this process will depend upon the binding site for the second copper, but in any case it cannot be either rate limiting or irreversible since large V_{max}/K_m isotope effects, especially $^D(V/K_{O_2})$, have been observed.

At this point, the question arises as to how a Cu(II)–OOH with no unpaired electron density on oxygen could abstract a hydrogen atom from substrate. By analogy to cytochrome P-450, further activation via O–O bond cleavage may occur prior to substrate activation. As one possible mechanism, a heterolytic cleavage would give rise to hydroxide ion, $^-$OH, and a copper–oxene species [Cu(II)→O ↔ Cu(IV)←O^{2-} ↔ Cu(III)–O^-·] similar to the ferryl species postulated for P-450 (Coon & White, 1980). Such a mechanism implies that the metal ion is nucleophilic, i.e., that it has an electron-rich environment which allows it to achieve an *effective* valence state two oxidation equivalents above normal in the activated intermediate. In the case of P-450, the porphyrin and thiolate ligands can readily supply electrons to the iron, making the ferryl intermediate feasible; with dopamine β-monooxygenase the ligands to copper remain to be clarified, but almost certainly are nothing like a porphyrin. In fact, comparison of the reduction potential of 310–370 mV for the enzyme–copper (Walker et al., 1977; Ljones et al., 1978) to that of 160 mV for free Cu(II) → Cu(I) (Weast, 1971) suggests an enzyme environment which stabilizes reduced copper. This observation greatly diminishes the feasibility of a discrete Cu(IV)–O^{2-} intermediate in dopamine β-monooxygenase.

As a result of experiments with peroxyphenylacetic acid, White et al. (1980) have suggested that cytochrome P-450 hydroxylates organic substrates via a homolytic O–O bond cleavage process. Such a bond cleavage could, in principle, occur in the dopamine β-monooxygenase reaction, yielding hydroxyl radical, ·OH, and a Cu(II)–O^-· ↔ Cu(III)–O^{2-} species as intermediates. Viewed strictly as a homolysis, this process would require ca. 51 kcal/mol, employing H_2O_2 → 2HO· (Weast, 1971) as a model. Although catalysis could be afforded by an effective 1e oxidation of Cu(II) → Cu(III) coupled to a 1e reduction of the peroxide [cf. Hamilton (1974)], the $\Delta G°$ for the activation of oxygen would still be estimated at ≃20 kcal/mol, probably nearer 30 kcal/mol.[12]

[12] The reduction potential for Cu(III) + e^- → Cu(II) is ≥ +0.8 V (Kust, 1979) unless the ligand environment is very electron rich [cf. Margerum et al. (1975)]. In light of the positive potential for Cu(II)/Cu(I) at the dopamine β-monooxygenase active site relative to aqueous copper, the Cu(III)/Cu(II) potential may be equal to or greater than +1 V. The 1e reduction potential for H_2O_2 + e^- → HO^- + ·OH is estimated from Sawyer & Nanni (1981) to be between +0.3 (H_2O_2 + H^+ + e^- → H_2O + ·OH; pH 7) and −0.25 V (HO_2^- + H_2O + e^- → 2HO^- + ·OH; pH 14). Taking the largest overall estimate for $\Delta\epsilon°$, the change in free energy may be on the order of 30 kcal/mol.

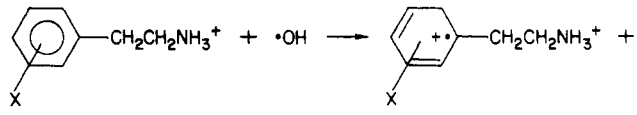
Scheme I: Postulated Chemical Mechanism for the Conversion of Dopamine to Norepinephrine at the Active Site of Dopamine β-Monooxygenase[a]



[a] Since the distance between coppers in the $E \cdot S_1 \cdot S_2$ complex is not yet known, the second copper has been omitted.

By comparison, the energy of activation for C–H bond cleavage with dopamine as substrate, which will include any endothermic oxygen activation step, is only 14.2 kcal/mol. Therefore, complete homolysis to form a discrete hydroxyl radical is energetically inconsistent with our data unless the enzyme is capable of considerable stabilization of such an intermediate. Importantly, even if such stabilization does occur, *C–H bond cleavage is unlikely to be effected by a simple hydrogen atom transfer to either an ·OH or Cu(II)–O·⁻ species*, since this process is expected to be highly exothermic, e.g., by 34 kcal/mol in the case of ·OH [calculated from the BDE_{HO-H} = 119 kcal/mol and BDE_{ArCH_2-H} = 85 kcal/mol (Weast, 1971)].[13] On the basis of the Hammond postulate (1955), such an exoenergetic process predicts a highly unsymmetrical transition state with very little C–H bond cleavage, which is incompatible with the isotope effect data presented in this paper.

Although our findings for dopamine β-monooxygenase appear to rule out a hydrogen atom abstraction from substrate to an activated form of oxygen such as ·OH, the question arises whether alternate routes exist for substrate activation. Specifically, could a substrate-derived radical arise via a two-step activation process to generate first a radical cation followed by proton loss? An attractive feature of this mechanism is that radical cation formation could, in principle, lower the pK_a of the benzylic C–H bonds to the region of an active site residue. In such an instance, proton activation could occur via an isoenergetic process, consistent with the magnitudes of $^{D}k_{1^\circ}$ and $^{D}k_{\alpha}$. However, the oxidation of a benzene ring is a very high energy process on the order of 1–2 V (Bard, 1976). Although the presence of two ring hydroxyl groups would be expected to facilitate a one-electron oxidation of substrate, the linear free energy plot in Figure 1 indicates that radical cation formation would have to be fast relative to C–H bond cleavage for substrates containing electron-withdrawing substituents as well. Even if the enzyme were capable of greatly stabilizing a radical cation, such that its energy of formation were consistent with the overall energy of C–H bond activation, 14.2–17.8 kcal/mol, it would still be necessary to postulate a reactive species capable of a one-electron oxidation of substrate. In principle, a homolytic cleavage of Cu(II)–OOH could generate enzyme species, e.g., Cu(II)–O⁻· or ·OH, capable of catalyzing radical cation formation.



OH^- (17)

However, the subsequent abstraction of a proton from substrate must then be catalyzed by an active site residue of a lower pK_a than hydroxide ion, since if the substrate-derived proton were transferred directly to OH^-, the net reaction would be equivalent to a hydrogen atom transfer from substrate to an activated form of oxygen; as discussed, the available data are inconsistent with such a process.

We therefore postulate a concerted mechanism for the oxidation of substrate, catalyzed by dopamine β-monooxygenase (Scheme I). As depicted, generation of a partial radical character at oxygen, via partial O–O homolysis with substrate properly juxtaposed, could initiate C–H homolysis leading to a transition state where both the O–O and C–H bonds are significantly broken and the O–H bond is significantly formed. Consistent with the large primary hydrogen isotope effect, an in-line geometry would be favored for this process in order to maximize the overlap of the incipient radical on oxygen with the C–H σ orbital. In this concerted mechanism, the highly exoenergetic hydrogen atom transfer between the benzylic carbon of substrate and a hydroxyl radical is directly coupled to the highly endoenergetic O–O homolysis of the Cu(II)–O-OH, such that the excess energy released in O–H bond formation drives the O–O homolysis to completion. By necessity, the extent of C–H homolysis/O–H formation lags behind the O–O homolysis, as no O–H interaction can occur until some radical character is generated. The extent of C–H/H–O bond homolysis/formation at the transition state can be estimated from the data presented herein: The large primary isotope

[13] The highly exothermic nature of monooxygenase-type reactions was pointed out several years ago by Hamilton (1974).

effect and α-secondary isotope effect of 1.19 suggest that the force constants of the C–H and H–O pseudobonds are approximately equal and that C–H bond cleavage has proceeded to at least 50%. If we assume the transition state is reached when the hydrogen is ca. 50% transferred, 42.5 kcal/mol is needed to partially break the C–H bond, and 59.5 kcal/mol is released in partially forming the O–H bond, yielding an excess energy of 17 kcal/mol which can be used to drive the O–O homolysis to completion. Combination of this excess energy with the energy of activation for $k_5 \geq 14.2$ kcal/mol gives a total energy of ≥ 31 kcal/mol for the O–O homolysis process itself; this value is similar to the estimate obtained by using the Cu(III) → Cu(II) and $H_2O_2 \rightarrow HO^- + \cdot OH$ reduction potentials as models.[12] The overall change in free energy for the formation of E·I from E·S_1·S_2′ (Scheme I) is estimated as −4 kcal/mol from $\Delta G° = 30$ kcal/mol for the O–O homolysis and $\Delta G° = -34$ kcal/mol for the hydrogen atom transfer. This value is consistent with the concept of a nearly isoenergetic C–H bond cleavage step as suggested by the magnitude of the observed isotope effects, and is well within the range of observed $\Delta G°$ values where maximal primary isotope effects, are obtained in correlations of k_H/k_D with ΔpK_a or ΔH_{BDE}.

Briefly, four key features of the enzymic catalysis can be discerned in this mechanism. First, a proton is provided for oxygen reduction without which the reaction could not proceed. Second, in the binding of substrate and oxygen, the C–H and O–O bonds are linearly juxtaposed such that no additional entropy need be overcome to attain maximal orbital overlap at the transition state. Third, the active site copper provides a catalyst for homolysis of the O–O bond, lowering the energy for complete homolysis of H_2O_2 from 51 to ca. 30 kcal/mol. Fourth, and perhaps most importantly, two highly energetic processes—one endoenergetic and one exoenergetic—are coupled in a concerted reaction such that one directly drives the other to completion at a significantly lowered energy level.

To complete the catalytic cycle, the radical intermediate rapidly decomposes in a very exoenergetic process to H_2O and the Cu(II)–alkoxide complex, which then undergoes a slow dissociation to yield free enzyme and products. For simplicity, the conversion of E·I to E·P has been written as a single step, although it must involve the diffusion away of water prior to recombination of the radicals. Diffusion of water may be accelerated by a hydrogen bonding to the base which originally donated a proton to oxygen in Cu(II)–OOH formation. In any case, as described in our previous work (Miller & Klinman, 1983), the activation barrier for this conversion must be very small. The proposal of a Cu(II)–alkoxide complex for E·P has evolved from the effect of pH on product release (Ahn & Klinman, 1983) and the large rate enhancement observed on k_7 with electron-withdrawing substituents (Table VI). The regression analysis also showed a very small rate enhancement on k_7 with increased steric bulk. As discussed above, this appears to be due to impairment of active site binding interactions with poorly fitting larger substituents. The smaller magnitude of the steric effect on k_7, relative to either to $K_{D\,amine}$ or k_5, is consistent with a geometric change from an outer-sphere to an inner-sphere complex in the conversion of the enzyme-bound substrate to product complexes (Scheme I).

ACKNOWLEDGMENTS

We thank Dr. Chris Murray for helpful discussions on the analysis of secondary isotope effects.

APPENDIX

Expanded Mechanism and Implications. Inclusion of an equilibrium step for reduction of bound oxygen prior to the C–H bond cleavage step introduces new terms into the kinetic expressions and the isotope effect expressions. Since k_5, k_7, and K_D are all calculated from the expressions for a simpler mechanism, it is important to evaluate how the new terms will affect the analyses presented herein. The expanded mechanism (showing only one binary complex for simplicity) is shown in eq A1.

$$\mathrm{E{\cdot}S_1} \underset{k_4}{\overset{k_3\,S_2}{\rightleftharpoons}} \mathrm{E{\cdot}S_1{\cdot}S_2} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \mathrm{E{\cdot}S_1{\cdot}S_2'} \xrightarrow{k_5} \mathrm{E{\cdot}P} \xrightarrow{k_7} \quad \text{(A1)}$$

In this expanded mechanism, the C–H bond cleavage step corresponds to conversion of E·S_1·S_2′ to E·P. The expressions for V_{max}, V_{max}/K_m, $^D(V)$, and $^D(V/K)$ are shown in eq A2–A5.

$$V_{max} = \frac{k_f k_5 k_7}{k_5(k_f + k_7) + k_7(k_f + k_r)} \quad \text{(A2)}$$

$$\frac{V_{max}}{K_m} = \frac{k_3 k_f k_5}{k_4 k_r + k_5(k_4 + k_f)} \quad \text{(A3)}$$

$$^D(V) = \frac{^Dk_5 + [k_5(1/k_7 + 1/k_f)]/(1 + k_r/k_f)}{1 + [k_5(1/k_7 + 1/k_f)]/(1 + k_r/k_f)} \quad \text{(A4)}$$

$$^D(V/K) = \frac{^Dk_5 + (k_5/k_r)(1 + k_f/k_4)}{1 + (k_5/k_r)(1 + k_f/k_4)} \quad \text{(A5)}$$

Rearrangement of these equations leads to

$$k_{5\,calcd} = \frac{k_5}{1 + k_r/k_f} \quad \text{(A6)}$$

$$k_{7\,calcd} = \frac{k_5}{k_5/k_7 + k_5/k_f} \quad \text{(A7)}$$

$$K_{D\,calcd} = \frac{K_D}{1 + k_f/k_r} \quad \text{(A8)}$$

As discussed by Palcic & Klinman (1983), the impact of k_r/k_f is expected to be small, with the exception of a mechanism in which both E·S_1·S_2 and E·S_1·S_2′ are significantly populated in the ground state. For the mechanism described by dopamine β-monooxygenase, the conversion of E·S_1·S_2 to E·S_1·S_2′ is attributed to a two-electron reduction of oxygen to Cu(II)–OOH. This equilibrium is unlikely to affect k_7 since the large magnitude of $^D(V/K_{amine})$ for dopamine reported by Ahn & Klinman (1983) implies that $k_f > k_5$ and that $k_{7\,calcd} \simeq k_7$. It is important to note that the same equilibrium constant contributes to the calculated expressions for both k_5 and K_D. As shown in Table V in the text, K_D for oxygen is independent of substrate structure. Thus, while the calculated values for k_5 may be modified to some extent by the $1 + k_r/k_f$ term, this term is expected to be invariant with changing substrate structure.

SUPPLEMENTARY MATERIAL AVAILABLE

Syntheses, melting points, NMR spectral data, and elemental analyses of the compounds discussed in this paper (4 pages). Ordering information is given on any current masthead page.

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