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Prochiral Selectivity and Intramolecular Isotope Effects in the Cytochrome P-450 Catalyzed ω-Hydroxylation of Cumene†

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ABSTRACT: A kinetic model is presented from which steady-state equations are derived that describe the intramolecular competition for the enzymatically mediated hydroxylation of two like groupings of a prochiral substrate. The observed isotope effect in such a system if one of the groupings is isotopically labeled is shown to be a function of three parameters: (a) the equilibrium constant for the catalytically sensitive orientations of the two prochiral groupings at the active site, (b) the intrinsic isotope effect associated with the bond-breaking step, and (c) the relative rates of bond breaking vs. enzyme–substrate dissociation. The expected isotope effects associated with the ω-hydroxylation of racemic, (*R*)-, and (*S*)-2-phenylpropane-*1,1,1*-d_3 and the product stereoselectivity associated with the ω-hydroxylation of (*R*)- and (*S*)-[1-^{13}C]-2-phenylpropane were determined with microsomal preparations (cytochrome P-450) from untreated and phenobarbital- and β-naphthoflavone-pretreated male Sprague-Dawley rats. The data from these experiments allow the observed isotope effect to be evaluated in terms of its component parts, i.e., expected isotope effects, product stereoselectivity, and equilibrium constant. These data further suggest that (a) the intramolecular isotope effect is consistent with a hydrogen abstraction recombination mechanism and is largely dependent upon the chemical nature of the porphyrin–Fe–oxene complex but independent of specific apoprotein structure, (b) product stereoselectivity is primarily dependent upon apoprotein structure, and (c) product stereoselectivity is a good measure of the equilibrium constant and both parameters are dependent upon the chirality of the active site.

One of the major approaches to the elucidation of reaction mechanisms is via the measurement of isotope effects. The critical value to be determined in such experiments is the intrinsic isotope effect, i.e., the isotope effect associated exclusively with the bond-breaking step. The magnitude of the intrinsic isotope effect can then be directly related to transition-state structure, which describes the mechanism of the reaction in detail. The successful application of isotope effects to enzymatically mediated systems, however, has proven to be difficult because of the complexity of the multistep reaction sequences that describe many of these processes. In general, an observed intermolecular isotope effect provides a measure of the overall rate limitations for a given reaction and includes such factors as product release and substrate binding in addition to other steps that may lower (mask) the observed magnitude of the intrinsic isotope effect associated with the actual bond-breaking step (Northrop, 1975).

The problems associated with these complicated schemes and the attenuation or "masking" of an intrinsic isotope effect have been minimized by workers in the field through the application of intramolecular isotope effect experiments (Miwa et al., 1980; Gelb et al., 1982; Hjelmeland et al., 1977). In such an experiment a molecule that has two positions that are equivalent in all respects except for isotopic substitution is used as substrate, and the observed isotope effect reflects the intramolecular competition between the two otherwise equivalent sites. In most cases intramolecular isotope effects more nearly approximate the intrinsic isotope effect since they are primarily dependent upon the product-determining step rather than other rate-limiting steps (Lindsay Smith et al., 1984; Miwa et al., 1980).

A parameter that, at least theoretically, could have a large effect on the observed magnitude of an intramolecular isotope effect is stereoselectivity. If a substrate is chiral, the relationship between the two possible enantiomeric substrate–enzyme complexes must be diastereomeric. Thus, the equilibrium constant for their formation may be different and/or

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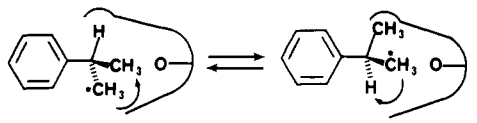

FIGURE 1: Two distinct catalytically sensitive orientations of cumene at the active site formed by a 120° rotation. The *pro-R* methyl group is identified by an asterisk.

the activation energies for subsequent reaction may be different. Even if a substrate is prochiral the two orientations of the substrate to the enzyme in the enzyme–substrate complex, formed by a 120° rotation of the two like groupings, will be inherently different (Figure 1). If one had a means of distinguishing the two like groupings they might give rise to different observed isotope effects for the same reaction.

A study of the cytochrome P-450 catalyzed ω-hydroxylation of the hydrocarbon cumene (2-phenylpropane) was therefore undertaken to determine (1) if the intramolecular isotope effect associated with the reaction is consistent with a hydrogen abstraction–recombination mechanism as has been established for the hydroxylation of norbornane (Groves et al., 1978), (2) how stereoselectivity might perturb the observed intramolecular isotope effect associated with a prochiral substrate, and (3) if a single mechanism exists for the specific cytochrome P-450 isozymes that may be involved in catalyzing this reaction. To answer these questions, optically pure cumene was synthesized such that it contained either deuterium (d_3) or carbon-13 in a specific methyl group. The isotopically labeled compounds of known absolute configuration were then incubated with various microsomal preparations of cytochrome P-450, and products were analyzed by a sensitive capillary GC/mass spectrometry assay.

Materials and Methods

Materials. All chemical starting materials and solvents were of reagent quality and purchased from readily available commercial sources.

Analytical Methods. Mass spectra were obtained on either a Hewlett-Packard 5985 GCMS or a VG 70-70H mass spectrometer with a VG 2000 data system, both operating in the EI mode at 70 eV. Gas chromatography was performed with a Hewlett-Packard 5840A gas chromatograph equipped with an FID detector and a Hewlett-Packard fused-silica capillary column. Optical rotations were obtained on a Jasco-DIR-4 digital polarimeter.

Synthesis of Racemic 2-Phenylpropane-1,1,1-d_3. Dimethyl 2-(1-phenyl-2,2,2-trideuterioethyl)malonate was obtained from the copper-catalyzed addition of trideuteriomethylmagnesium iodide (99.5 atom % excess) to dimethyl benzylidenemalonate (Allen & Spangler, 1955) according to the method of Bergson et al. (1977). The product was hydrolyzed in base and then decarboxylated according to the method of Bergson et al. (1977), to yield 3-phenyl-4,4,4-trideuteriobutyric acid. Reaction of the acid with iodine and lead tetracetate followed by reduction with lithium aluminum hydride gave the title compound (Streitweiser & Stang, 1965).

(R)- and (S)-2-Phenylpropane-1,1,1-d_3. (+)-(S)-3-Phenyl-4,4,4-trideuteriobutyric acid was obtained upon liberation after the fractional recrystallization of its salt (10× ethanol–water) formed from the reaction of racemic acid with (−)-1-phenylethylamine (Bergson et al., 1977): specific rotation, $[\alpha]^{26}_D$, +52.4°; enantiomeric purity, 96.3%. (−)-(R)-3-Phenyl-4,4,4-trideuteriobutyric acid was obtained upon liberation after the fractional recrystallization of its salt (10× ethanol–water) formed from the reaction of racemic acid with (+)-1-phenylethylamine: specific rotation, $[\alpha]^{26}_D$, −53.4°; enantiomeric purity, 97.3%. Enantiomeric purities were calculated from optical purities (c = 0.5, benzene) as determined by comparison with the published values for (−)-(S)-phenylbutyric acid (Cram, 1952). The method of Streitweiser and Stang (1965), as described above, was then used to obtain (R)-2-phenylpropane-1,1,1-d_3 from the S acid and (S)-2-phenylpropane-1,1,1-d_3 from the R acid.

Synthesis of Racemic, (R)-, and (S)-[1-^{13}C]-2-Phenylpropane. An exactly analogous sequence of reactions was utilized to obtain the title compounds, as those described above, except [^{13}C]methylmagnesium iodide (99 atom % excess) was used in place of trideuteriomethyl iodide in the initial condensation reaction: Enantiomeric purity (R)-[1-^{13}C]-2-phenylpropane, 92.2%; (S)-[1-^{13}C]-2-phenylpropane, 89.6%.

Preparation of Liver Microsomes. Rat microsomes were prepared from either uninduced or phenobarbital (Pb)- or β-naphthoflavone (BNF)-induced male Sprague-Dawley rats (200 g) as previously reported (Porter et al., 1981). Protein concentrations were determined according to the modified Lowry method using bovine serum albumin as a standard (Omura et al., 1964).

Cumene Hydroxylation. The incubation system (2.0 mL) consisted of microsomes ca. 4 mg of protein, 8.5 μmol of D-glucose-6-phosphate, 4 units of glucose-6-phosphate dehydrogenase, 4.0 μmol of $MgCl_2$, and 200 μmol of Tris buffer, pH 7.9, at a temperature of 38 °C. The reactions were initiated by the addition of 1 mmol of substrate. They were incubated aerobically at 38 °C for 20 min and then terminated by chilling (ice bath). Metabolites were extracted 3 times with 3 mL of *n*-pentane, combined, and dried over sodium sulfate. The *n*-pentane extracts were transferred to concentration tubes, and 3 μL of *N*-methyl(trimethylsilyl)trifluoracetamide was added to each. The sample volumes were then reduced to 50–100 μL by evaporation of the solvent under a stream of dry nitrogen.

Metabolite Analysis. Gas chromatography–mass spectrometry analysis was performed on a VG 70-70H mass spectrometer operating in the selected ion mode while interfaced to a HP 5710 GC. A Hewlett-Packard fused-silica capillary column (cross-linked 5% phenylmethylsilicone, film thickness: 0.33 μm, 25 m × 0.20 mm i.d.) was used. Conditions for GC analysis were carrier-gas, helium; head pressure, 10 psi; injection temperature, 250 °C; initial oven temperature, 80 °C for 2.5 min; rate, 6 °C/min up to 120 °C for 3 min and then 30 °C/min up to 250 °C; final temperature, 250 °C for 3 min. The retention time for the Me_4Si derivatives of 2-phenylpropanol was 11.5 min. Mass spectrometry conditions were electron impact; ionization energy, 70 eV; source temperature, 200 °C; filament current, 200 μA; accelerating potential, 4 kV; dwell time 5–10 ms.

Product Stereoselectivity. The electron impact mass spectrum of the trimethylsilyl derivative of 2-phenylpropanol is shown in Figure 2. Three major fragmentation processes characterize the spectrum: loss of methyl from the molecular ion to generate the ion at m/z 193, cleavage of the α–β bond which effectively divides the molecule into two, a fragment ion at m/z 105 bearing the phenyl half of the molecule, a fragment ion at m/z 103 bearing the trimethylsilyl half of the molecule, and finally loss of the trimethylsilyl group to yield the base peak ion at m/z 73. On the basis of these fragmentation processes the intense ion at m/z 103 would give rise to corresponding ions at m/z 104 and 103 in the spectrum of the trimethylsilyl derivative of [1-^{13}C]-2-phenylpropanol and 2-phenyl[3-^{13}C]propanol, respectively (Figure 3). Hence, the product ratio of [1-^{13}C]-2-phenylpropanol to 2-phenyl[3-

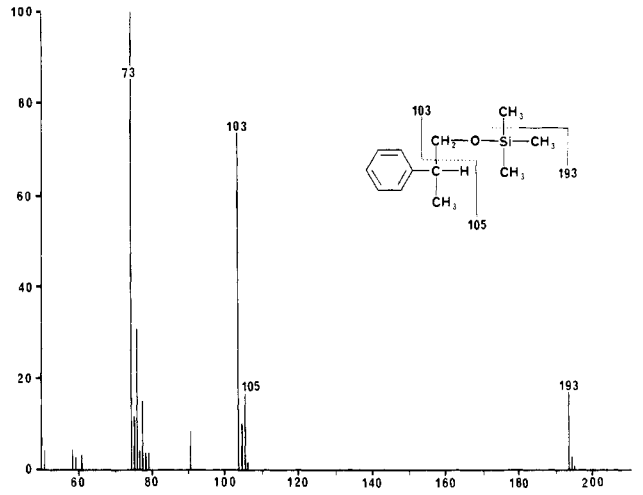

FIGURE 2: Electron impact mass spectrum of 2-phenylpropanol trimethylsilyl ether.

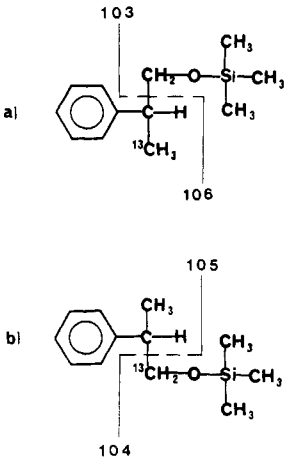

FIGURE 3: (a) Diagnostic fragmentation giving an ion at m/z 103 in the mass spectrum of 2-phenyl-[3-^{13}C]-propanol. (b) Diagnostic fragmentation giving an ion at m/z 104 in the mass spectrum of [1-^{13}C]-2-phenylpropanol.

^{13}C]propanol is readily determined by the relative abundance of ions at m/z 104 vs. m/z 103.

If product stereoselectivity $^{R/S}P_S$, is defined as the ratio of the amount of product obtained from ω-hydroxylation of the *pro-R* methyl group to that of the *pro-S* methyl group at saturating conditions, it is given by the ratio of the amount [1-^{13}C]-2-phenylpropanol to 2-phenyl[3-^{13}C]propanol from the (*R*)-[1-^{13}C]-2-phenylpropane substrate at any time t. Experimentally $^{R/S}P_S$ could simply be determined by measuring the ratio of the ion intensities at m/z 104 to m/z 103, provided the substrate contains 100% ^{13}C exclusively in the *pro-R* methyl group. Since, in practice, it is impossible to obtain a substrate that is 100% isotopically and enantiomerically pure, the measured ion ratios must be corrected to account for any departure from these ideal values in addition to ion intensity from natural isotopic abundance and background.

If the substrate is considered to be composed of α% of (*S*)-[1-^{13}C]-2-phenylpropane, β% of (*R*)-[1-^{13}C]-2-phenylpropane, and γ% of nonlabeled 2-phenylpropane, then an equation relating measured ion ratios, e.g., ^{13}C/^{12}C, of the product to product stereoselectivity can be derived from Figure 4.

Inspection of Figure 4 reveals that the ratios of ions at m/z 104 (^{13}C) to m/z 103 (^{12}C) in the products is given by

$$\frac{^{13}C}{^{12}C} = \frac{b + c}{a + d + e + f} = \frac{F_{1-x}\alpha + F_x\beta}{F_x\alpha + F_{1-x}\beta + (F_x + F_{1-x})\gamma} \quad (1)$$

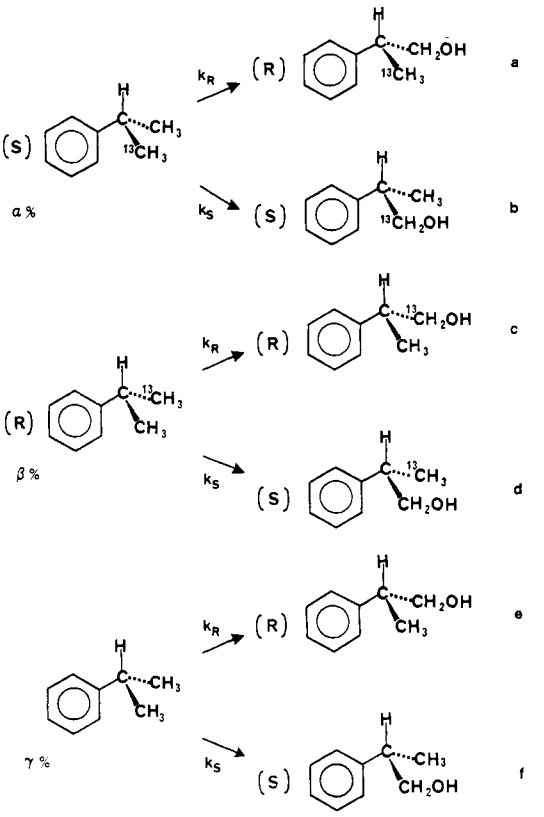

FIGURE 4: ω-Hydroxylation of cumene; k_R is the rate constant for hydroxylation of the *pro-R* methyl group and k_S is the rate constant for hydroxylation of the *pro-S* methyl group.

where F_x is the fraction of product formed from ω-hydroxylation of the *pro-R* methyl group and F_{1-x} is the fraction of product formed from ω-hydroxylation of the *pro-S* methyl group. Since $^{R/S}P_S$ is defined as F_x/F_{1-x}, dividing top and bottom by F_{1-x} gives

$$\frac{^{13}C}{^{12}C} = \frac{\alpha + {}^{R/S}P_S\beta}{{}^{R/S}P_S\alpha + \beta + ({}^{R/S}P_S + 1)\gamma} \quad (2)$$

In order to calculate $^{R/S}P_S$, eq 2, which is nonlinear, was converted to linear form, eq 3, by substituting $(1 - \alpha - \gamma)$ for β, and adding 1 to each side of the equation, and taking reciprocals of both sides.

$$\frac{1}{(^{13}C/^{12}C) + 1} = \frac{1 + \gamma\,{}^{R/S}P_S}{{}^{R/S}P_S + 1} + \frac{{}^{R/S}P_S - 1}{{}^{R/S}P_S + 1}\alpha \quad (3)$$

Linear regression analysis of $1/[(^{13}C/^{12}C) + 1]$ vs. α provided an estimation of the slope $({}^{R/S}P_S - 1/({}^{R/S}P_S + 1))$ from which $^{R/S}P_S$ was calculated.

Observed Intramolecular Isotope Effects. One of the major fragmentation processes found in the mass spectrum of the trimethylsilyl derivative of 2-phenylpropanol is loss of one of the methyl groups from the trimethylsilyl grouping present in the molecular ion to generate an ion at m/z 193, Figure 2. Hence the observed intramolecular isotope effect associated with the ω-hydroxylation of racemic, (*R*)-, and (*S*)-2-phenylpropane-*1,1,1-d*$_3$ should be equal to the ratio of the ions at m/z 196–195, once the substrate is corrected for the 1% contamination by species containing eithe d_0, d_1, or d_2.

Under saturating conditions, the product ratio of d_3 to d_2 for a substrate that is less than 100% optically pure can be derived from the scheme shown in Figure 5, where α is the precent of the *R* enantiomer in the 2-phenylpropane-*1,1,1-d*$_3$ substrate and $^{R/S}K_{eq}$ is the equilibrium constant for the catalytically susceptible orientation of the *pro-R* methyl group

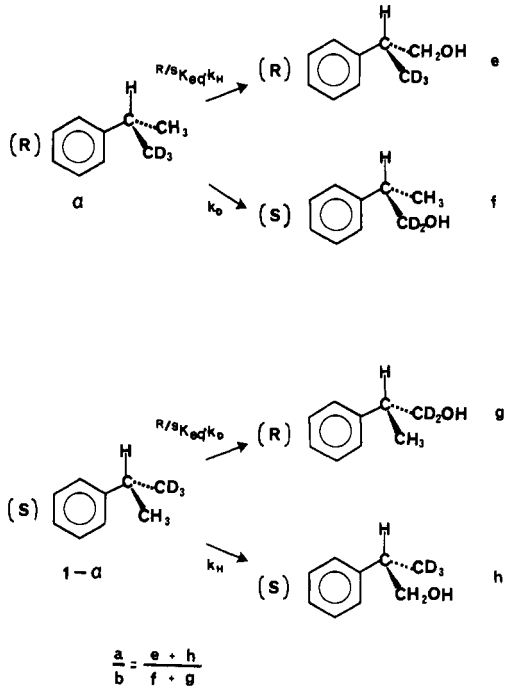

FIGURE 5: Product profile for the ω-hydroxylation of 2-phenylpropane-*1,1,1-d*$_3$ of less than 100% optical purity. $^{R/S}K_{eq}$ is the equilibrium constant for the catalytically susceptible conformation of the *pro-R* vs. the *pro-S* methyl group at the active site, k_H is the rate constant for ω-hydroxylation of a carbon–hydrogen bond, and k_D is the rate constant for ω-hydroxylation of a carbon–deuterium bond.

vs. the *pro-S* methyl at the active site of the enzyme (see the development of the concept of $^{R/S}K_{eq}$ under Theory). Thus the ratio of the velocity for ω-hydroxylation of the methyl group vs. the trideuteriomethyl group, $^{D}V_{obsd}$, is

$$^{D}V_{obsd} = \frac{d_3}{d_2} = \frac{e+h}{f+g} = \frac{^{R/S}K_{eq}\alpha k_H + (1-\alpha)k_H}{k_D\alpha + (1-\alpha)^{R/S}K_{eq}k_D} = \frac{k_H}{k_D}\frac{^{R/S}K_{eq}\alpha + (1-\alpha)}{\alpha + (1-\alpha)^{R/S}K_{eq}} \quad (4)$$

Equation 4 can be converted to linear form by adding 1 to each side of the equation and taking reciprocals of both sides

$$\frac{1}{^{D}V_{obsd}/^{D}V_{exp} + 1} = \frac{1 - ^{R/S}K_{eq}}{1 + ^{R/S}K_{eq}} + \frac{1 - ^{R/S}K_{eq}}{1 + ^{R/S}K_{eq}}\alpha \quad (5)$$

The slope $(1 - ^{R/S}K_{eq})/(1 + ^{R/S}K_{eq})$ was estimated by linear regression analysis of the data points obtained from the (*R*)-2-phenylpropane-*1,1,1-d*$_3$ (95.3% optically pure), (*S*)-2-phenylpropane-*1,1,1-d*$_3$ (96.8% optically pure), and *rac*-2-phenylpropane-*1,1,1-d*$_3$ substrates. $^{R/S}K_{eq}$ was calculated from the slope.

THEORY

If one of the two like groupings in a prochiral molecule undergoes a covalent change, an asymmetric center and a single enantiomer is generated. Such a change will only be stereospecific if the prochiral center is transformed by a chiral reactant, e.g., the active site of an enzyme. In this context the ω-hydroxylation of cumene by cytochrome P-450 could involve stereoselective (one prochiral methyl group preferred to some degree over the other) oxidation to generate optically active β-(methylphenyl)ethanol. Just how the active site of the enzyme might distinguish between the two methyl groups is illustrated in Figure 1. If we assume that the *pro-R* and

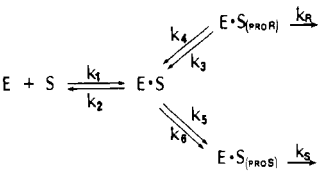

FIGURE 6: Kinetic model for the interaction between a prochiral substrate and an enzyme to generate enantiomeric products. E·S is any enzyme–substrate complex that has a spatial orientation that is not susceptible to catalysis, E·$S_{pro\text{-}R}$ is the enzyme–substrate complex that has the necessary spatial orientation for reaction to occur at the *pro-R* grouping, and E·$S_{pro\text{-}S}$ is the enzyme–substrate complex that has the necessary spatial orientation for reaction to occur at the *pro-S* grouping.

pro-S methyl groups are indeed distinguishable, then we can model the reaction by the scheme shown in Figure 6 and write (see Appendix)

$$^{R/S}P_S = ^{R/S}K_{eq}\frac{(k_R/k_S) + (k_R/k_6)}{1 + (k_R/k_4)} \quad (7)$$

where $^{R/S}P_S$, or product stereoselectivity, is defined as the ratio of product formed from the *pro-R* methyl group relative to product formed from the *pro-S* methyl group and where $^{R/S}K_{eq}$ is the equilibrium constant for the catalytically susceptible orientation of the *pro-R* vs. *pro-S* methyl group at the active site of the enzyme.

If eq 7 is evaluated under various conditions, it is found that when $k_4, k_6 \gg k_R$, i.e., the rate of dissociation of the enzyme substrate complex is much faster than the rate of bond breaking

$$^{R/S}P_S = ^{R/S}V_{obsd} = \frac{k_R}{k_S}{}^{R/S}K_{eq} \quad (8)$$

and if in addition $k_R = k_S$, i.e., the energy of activation as reflected by the rate constants for the hydroxylation is independent of stereochemistry

$$^{R/S}P_S = ^{R/S}V_{obsd} = ^{R/S}K_{eq} \quad (9)$$

Hence, if there is rapid equilibrium at the active site and no differences in activation energy for hydroxylation of the *pro-R* or *pro-S* methyl groups, product stereoselectivity should be a good measure of the equilibrium constant, $^{R/S}K_{eq}$.

If we initially label one of the methyl groups with deuterium in order to distinguish it from the other, the ratio of products obtained will reflect not only a stereoselective component as discussed above but also an isotope effect due to the inclusion of deuterium. Thus, if we label the *pro-S* methyl group with deuterium we may write eq 13 as per eq 7, provided there are no binding effects due to deuterium

$$^{D}V_{obsd,R} = ^{R/S}K_{eq}\frac{(k_{H,R}/k_{D,S}) + (k_{H,R}/k_6)}{1 + (k_{H,R}/k_4)} \quad (13)$$

where $^{D}V_{obsd,R}$ is the ratio of velocities for hydroxylation of an *R* methyl group relative to the *S* trideuteriomethyl group, $k_{H,R}$ is the rate constant for hydroxylation of the *R* methyl group and $k_{D,S}$ is the rate constant for hydroxylation of the *S* trideuteriomethyl group.

Similarly, if we label the *pro-R* methyl group with deuterium we may write

$$^{D}V_{obsd,S} = \frac{1}{^{R/S}K_{eq}}\frac{(k_{H,S}/k_{D,R}) + (k_{H,S}/k_4)}{1 + (k_{H,S}/k_6)} \quad (14)$$

Both eq 13 and 14 describe the isotope effect including any effects due to stereoselectivity that will exist for hydroxylation between methyl groups i.e., $CH_3(R)$ vs. $CD_3(S)$ or $CH_3(S)$ vs. $CD_3(R)$.

Table I: Stereochemical Distribution of ^{13}C in Racemic, *R*, and *S* ^{13}C-Enriched 2-Phenylpropane Substrates

substrate	α^a	β^b	γ^c
R	7.1	91.4	1.5
S	88.9	9.6	1.5
racemic[d]	50.7	47.8	1.5

[a] Percent of (*S*)-[1-^{13}C]-2-phenylpropane. [b] Percent of (*R*)-[1-^{13}C]-2-phenylpropane. [c] Percent of nonlabeled 2-phenylpropane. [d] An approximate racemic mixture generated by mixing "equal" quantities of (*R*)- and (*S*)-[1-^{13}C]-2-phenylpropane.

$^{D}V_{exp,rac}$, the value of the isotope effect expected to be observed for the racemic mixture, can be calculated from

$$(^{D}V_{obsd,R}\,^{D}V_{obsd,S})^{1/2} = {}^{D}V_{exp,rac} \quad (24)$$

provided that the model in Figure 6 applies to the combination of the two enantiomers, there is rapid equilibration at the active site, and the activation energy for hydroxylation is independent of stereochemistry (see Appendix). Comparison of the calculated values of $^{D}V_{exp,rac}$ with experimentally determined values of $^{D}V_{obsd,rac}$ should provide an estimation of how good the assumptions are.

If the rate of enzyme substrate dissociation is rapid relative to bond breaking and if the activation energy for hydroxylation of a methyl group is independent of stereochemistry, combination of eq 13 and 14 leads to an expression for the equilibrium constant, $^{R/S}K_{eq}$.

$$\left(\frac{^{D}V_{obsd,R}}{^{D}V_{obsd,S}}\right)^{1/2} = {}^{R/S}K_{eq} \quad (27)$$

Equations 13 and 14 provide a measure of the isotope effect associated with the breaking of a carbon–hydrogen bond on one methyl group vs. the breaking of a carbon–deuterium bond on the opposite methyl group, e.g., $k_{H,R}/k_{D,S}$. The value for the isotope effect at a specific methyl group $k_{H,R}/k_{D,R}$ or $k_{H,S}/k_{D,S}$ can be derived by combining the concepts of P_S from eq 7 and $^{D}V_{obsd}$ from eq 13 and 14 assuming rapid equilibration of the enzyme substrate complex relative to bond breaking; thus

$$\frac{^{D}V_{obsd,R}}{^{R/S}P_S} = \frac{k_{H,R}}{k_{D,S}}\frac{k_S}{k_R} = {}^{D}V_{exp,pro\text{-}R} \quad (31)$$

and

$$^{D}V_{obsd,S}\,^{R/S}P_S = \frac{k_{H,S}}{k_{D,R}}\frac{k_R}{k_S} = {}^{D}V_{exp,pro\text{-}S} \quad (32)$$

Where $^{D}V_{exp,pro\text{-}R}$ and $^{D}V_{exp,pro\text{-}S}$ are defined as the expected isotope effect for a given methyl group (*pro-R* or *pro-S*) calculated from the experimentally determined observed isotope effect and the experimentally determined product stereoselectivity for the reaction.

Further if the activation energy for hydroxylation of a methyl group is independent of stereochemistry, we can equate eq 31 and 32, i.e.

$$\frac{^{D}V_{obsd,R}}{^{R/S}P_S} = {}^{D}V_{obsd,S}\,^{R/S}P_S = {}^{D}V_{exp,pro\text{-}R} = {}^{D}V_{exp,pro\text{-}S} = \frac{k_H}{k_D} = {}^{D}V_{intrinsic,pro\text{-}R\ or\ pro\text{-}S} \quad (33)$$

Results

Product Stereoselectivity. The values of α, β, and γ experimentally determined for the (*S*)-[1-^{13}C]-2-phenylpropane, the (*R*)-[1-^{13}C]-2-phenylpropane and the racemic [1-^{13}C]-2-phenylpropane substrates are given in Table I. The measured $^{13}C/^{12}C$ ion ratios in the 2-phenylpropanol product obtained from the various substrates and the various microsomal preparations are presented in Table II. These values are corrected for contributions from natural isotopic abundance (^{13}C, ^{2}H, ^{18}O, and ^{29}Si) and background. The values calculated for the product stereoselectivities, $^{R/S}P_S$, associated with the ω-hydroxylation of cumene by various rat liver microsomal preparations were 2.23 ± 0.27, uninduced, 1.65 ± 0.21, phenobarbital pretreated, and 0.89 ± 0.02, β-naphthoflavone pretreated. The standard deviations are based on 27 data points from two separate experiments. It is readily apparent from the results that the hydroxylation reaction is stereoselective and that the degree of stereoselectivity depends upon the specific composition of the isozymes comprising the cytochrome P-450 content in each microsomal preparation.

Table II: Ratio of Ions at *m/z* 104 vs. *m/z* 103 (Corrected for Background and Natural Isotopic Abundance) in ^{13}C-Enriched 2-Phenylpropanol Obtained from the Hydroxylation of Racemic, (*R*)-, and (*S*)-[1-^{13}C]-2-Phenylpropane by Microsomes from Normal and Phenobarbital- and β-Naphthoflavone-Pretreated Rats

	ion ratios			
substrates	normal	Pb	BNF	n^a
R	1.953 (0.064)[b]	1.496 (0.071)	0.834 (0.026)	9
S	0.445 (0.002)	0.559 (0.005)	1.028 (0.030)	9
racemate	0.950 (0.003)	0.946 (0.006)	0.966 (0.032)	9

[a] Total number of determinations from two separate experiments. [b] Number in parentheses is plus or minus the standard deviation.

Table III: Observed Intramolecular Isotope Effect Associated with the ω-Hydroxylation of Racemic, (*R*)-, and (*S*)-2-Phenylpropane-*1,1,1-d*$_3$ by Hepatic Microsomes from Phenobarbital- and β-Naphthoflavone-Pretreated and Normal Male Sprague-Dawley Rats

		treatment			
substrate	parameter	normal	Pb	BNF	n^a
racemic	$^{D}V_{obsd,rac}$	8.21 (0.40)[b]	8.68 (0.22)	7.54 (0.20)	7
R	$^{D}V_{obsd,R}$	15.57 (0.63)	11.00 (0.32)	7.21 (0.23)	7
S	$^{D}V_{obsd,S}$	4.24 (0.27)	5.71 (0.31)	8.12 (0.62)	7

[a] Total number of determinations from two separate experiments. [b] Number in parentheses is plus or minus the standard deviation.

Intramolecular Isotope Effects. The observed intramolecular isotope effects, $^{D}V_{obsd,R}$, $^{D}V_{obsd,S}$, and $^{D}V_{obsd,rac}$, for the ω-hydroxylation of the *R* and *S* enantiomers of the trideuteriomethyl analogue of cumene after incubation with microsomal preparations from normal rats and those pretreated with Pb and BNF are given in Table III. As can be seen, the magnitude of the observed intramolecular isotope effect for the individual enantiomers is highly dependent upon both the stereochemistry of the substrate and the specific microsomal preparation utilized for the reaction. Values of approximately 15 and 4 are observed for the *R* and *S* enantiomers with normal microsomes, 11 and 6 with microsomes from Pb-pretreated animals, and 7 and 8 with microsomes from BNF-pretreated animals. With racemic substrate the observed intramolecular isotope effect was approximately 8 for all treatments. From these values the expected intramolecular isotope effect $^{D}V_{exp,rac}$ and the equilibrium constant $^{R/S}K_{eq}$ can readily be calculated. The results of the calculations are given in Table IV. The value of $^{D}V_{exp,rac}$ is approximately 8 for all microsomal preparations while that of $^{R/S}K_{eq}$ varies from 0.94 (BNF) to 1.99 (normal). The isotope effect associated with the hydroxylation of a specific methyl, i.e., *pro-R* or *pro-S*, can also be calculated. Their values, $^{D}V_{exp,pro\text{-}S}$ or $^{D}V_{exp,pro\text{-}R}$, are given in Table V. Although the values are not statistically different at the 95% confidence level, a trend does seem to exist. $^{D}V_{exp,pro\text{-}R}$ for microsomes from BNF-pretreated rats appears to be smaller than $^{D}V_{exp,pro\text{-}R}$ for microsomes from

Table IV: Expected Intramolecular Isotope Effect, $^{D}V_{exp,rac}$, and Equilibrium Constant, $^{R/S}K_{eq}$, Associated with the ω-Hydroxylation of 2-Phenylpropane-*1,1,1-d*$_3$ by Hepatic Microsomes from Phenobarbital- and β-Naphthoflavone-Pretreated and Normal Male Sprague-Dawley Rats

	treatment			
parameter	normal	Pb	PNF	n^a
$^{D}V_{exp,rac}$	8.13 (0.31)[b]	7.92 (0.24)	7.63 (0.31)	7
$^{R/S}K_{eq}$	1.99 (0.02)	1.41 (0.02)	0.94 (0.01)	21

[a] Total number of determinations (7) or data points (21) from two separate experiments. [b] Numbers in parentheses are plus or minus the standard deviation.

Table V: Calculated Intramolecular Isotope Effects Associated with the ω-Hydroxylation of the *Pro-R* and *Pro-S* Methyl Groups of Cumene by Hepatic Microsomes from Phenobarbital- and β-Naphthoflavone-Pretreated and Normal Male Sprague-Dawley Rats

	treatment		
parameter	normal	Pb	BNF
$^{D}V_{exp,proR}$	9.50 (1.19)[a]	9.46 (1.19)	7.29 (1.10)
$^{D}V_{exp,proS}$	6.96 (1.16)	6.63 (1.16)	8.02 (1.06)

[a] Number in parentheses is plus or minus the standard deviation.

Pb-pretreated and normal animals. In contrast, $^{D}V_{exp,pro\text{-}S}$ for microsomes from BNF-pretreated rats appears to be larger than $^{D}V_{exp,pro\text{-}S}$ for microsomes from Pb-pretreated and normal animals.

Discussion

The observation of product stereoselectivity in the transformation of a prochiral substrate is a direct reflection of asymmetry in the structure of the active site. As such, prochiral substrates offer potentially powerful probes for mapping the catalytic site of a given enzyme.

The degree of product stereoselectivity is a function of the interplay of three parameters as can be seen from eq 7. The first, the equilibrium expressed by $k_3k_6/(k_5k_4)$ relates to the ground-state conformational preference for the oxidation of one methyl group over the other in relation to the orientation of the oxygen atom being transferred. The second, k_R/k_S relates to possible differences in activation energy (hence transition states) for transfer of the oxygen atom to either methyl group once in the proper orientation. The third, k_R/k_6, k_R/k_4 takes into account the relative rates of forward reaction, k_R, to the rates k_6 and k_4 of specific E·S complex dissociation.

The experimentally determined values of $^{R/S}P_S$ vary considerably as a function of the specific microsomal preparation used to catalyze the oxidation. Since structurally distinct cytochrome P-450s are known to have overlapping reactivities (Lu & West, 1980) it is likely that more than one isozyme in any given microsomal preparation catalyzes the ω-hydroxylation of cumene. This would suggest that the product stereoselectivities that are obtained represent a composite value that primarily reflects the stereoselective preference of the major P-450 isozyme catalyzing that specific reaction. A comparison of the results from normal microsomes to those obtained after BNF induction implies that either the major enzyme catalyzing the reaction in normal microsomes is not present in BNF microsomes and those enzymes present have a low degree of stereoselectivity or if the major enzyme from normal BNF is present in microsomes the latter must contain a second major isozyme that catalyzes the reaction but with opposite stereoselectivity.

As seen from Table III, the observed isotope effects for the deuterated cumenes vary significantly as a function of both substrate stereochemistry and enzyme preparation. For example, the large difference in the observed isotope effect $^{D}V_{obsd,R} \cong 15$ for the (*S*)-trideuteriomethylcumene vs. that of $^{D}V_{obsd,S} \cong 4$ for the (*R*)-trideuteriomethylcumene from normal microsomes is on first inspection surprising since the nature of the reaction, hydroxylation of a terminal methyl group, is fundamentally the same in the two substrates. However, inspection of eq 13 and 14 reveals that the observed isotope effect is not only a function of the intrinsic isotope effect for the bond-breaking step but also dependent upon rates of processes that govern the relative orientations of the substrate at the active site and the relative rate of bond breaking vs. dissociation. In contrast to the variation in the observed isotope effects for the individual enantiomers, $^{D}V_{obsd,rac}$ is reasonably constant at an approximate value of 8 for all microsomal preparations.

$^{D}V_{exp,rac}$ may be calculated for the reaction from eq 24 and the values are given in Table IV. As can be seen from Tables III and IV, corresponding values of $^{D}V_{obsd,rac}$ and $^{D}V_{exp,rac}$ are statistically indistinguishable at the 95% confidence level. The overall similarity in values suggests that not only is the scheme presented in Figure 6 a reasonable model for the system but that eq 24 applies and therefore $k_6, k_4 \gg k_{H,R}, k_{H,S}$ and $k_{H,R} = k_{H,S}$. The magnitudes of $^{D}V_{obsd,rac}$ and $^{D}V_{exp,rac}$ are consistent with the hydrogen abstraction–recombination mechanism for the cytochrome P-450 catalyzed hydroxylation of norbornane proposed by Groves et al. (1978). In addition, the relative constancy over the various microsomal preparations suggests that the mechanism is largely independent of the specific isozyme catalyzing the reaction and therefore is dependent primarily upon the chemical nature of the porphyrin–Fe–O complex rather than the apoprotein portion of the enzyme.

This conclusion can be further tested by comparing the computed values for $^{D}V_{exp,pro\text{-}R}$ and $^{D}V_{exp,pro\text{-}S}$ from eq 31 and 32 presented in Table V. The degree of similarity of the values obtained irrespective of stereochemistry or enzyme preparation strongly suggests that the basic assumption of rapid equilibration at the active site is largely correct while the degree of their nonidentity probably reflects a small degree of incomplete equilibration and some relatively minor differences in transition state, i.e., $k_R \cong k_S$.

If the isotope effects between methyl groups are assumed to be equal, $^{R/S}K_{eq}$ can be calculated from eq 27, and the values are given in Table IV. Not unexpectedly, they are found to vary considerably as a function of enzyme preparation. The dependency of $^{R/S}K_{eq}$ on the specific microsomal preparation is, in each case, clearly a reflection of the averaged chiralities of the active sites of the isozymes catalyzing product formation. Similar results, independence of the basic mechanism but dependence of stereoselectivity on the specific isozyme(s) catalyzing the reaction, have recently been demonstrated for the aromatic hydroxylation of warfarin (Bush & Trager, 1985).

Product stereoselectivity, $^{R/S}P_S$, is an experimentally determinable quantity that has incorporated into its value both ground-state conformational and transition-state differences that might exist between the *pro-R* and *pro-S* methyl groups of cumene in its interaction with the catalytic site of the enzyme, eq 7. In contrast, $^{R/S}K_{eq}$ is a quantity that can be calculated from an independent data set, eq 27, provided the following assumptions are true: (1) there is rapid equilibration of the two methyl groups at the active site relative to bond breaking, (2) there is no significant deuterium binding isotope effect, and (3) the kinetic isotope effects for the two enantiomerically deuterated substrates are equal. Moreover, given

these assumptions, ${}^{R/S}P_S = {}^{R/S}K_{eq}$ according to eq 9.

Comparison of the averaged ${}^{R/S}P_S$ values to the corresponding averaged ${}^{R/S}K_{eq}$ values provides an assessment of the validity of the assumptions invoked in order to calculate ${}^{R/S}K_{eq}$. Clearly the results would seem to imply that to a first approximation product stereoselectivity is a good measure of the equilibrium constant and, as such, is a highly sensitive parameter that directly reflects topographical properties of the active site.

In summary the magnitude of the intramolecular isotope effect associated with the ω-hydroxylation of cumene is consistent with a hydrogen atom abstraction–recombination mechanism involving a reasonably symmetrical transition state irrespective of the specific cytochrome P-450 isozyme catalyzing the reaction. Product stereoselectivity is primarily a function of isozyme structure and the chirality of the active site and provides a good estimation of the equilibrium constant governing the catalytically susceptible orientations of the prochiral methyl groups. It would thus appear reasonable to conclude that determination of the K_{eq}, ${}^{D}V_{exp,rac}$ and P_s parameters for various purified forms of the enzyme using topologically well-defined series of prochiral substrates would provide considerable insight into the architecture of the active sites of this biologically important family of enzymes. In addition such an approach potentially offers a valuable tool for the identification of various purified cytochrome P-450s from different tissues and different laboratories.

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Appendix

If we assume steady-state conditions and irreversible product formation for the reaction scheme shown in Figure 6, we may write

$$[E\cdot S]_{pro\text{-}R} = \frac{k_3[E\cdot S]}{k_4 + k_R}, \qquad [E\cdot S]_{pro\text{-}S} = \frac{k_5[E\cdot S]}{k_6 + k_S}$$

and

$${}^{R}V_{obsd} = k_R[E\cdot S_{pro\text{-}R}], \; {}^{S}V_{obsd} = k_S[E\cdot S_{pro\text{-}S}]$$

where ${}^{R}V_{obsd}$ is the velocity of hydroxylation at the *pro-R* methyl group and ${}^{S}V_{obsd}$ is the velocity of hydroxylation at the *pro-S* methyl group. Thus the ratio of the velocity of the reaction at the *pro-R* methyl vs. the *pro-S* methyl, ${}^{R/S}V_{obsd}$, becomes

$${}^{R/S}V_{obsd} = \frac{k_R}{k_S}\frac{k_3(k_6 + k_S)}{k_5(k_4 + k_R)} = {}^{R/S}P_S \tag{6}$$

where ${}^{R/S}P_S$, or product stereoselectivity, is defined as the ratio of product formed from the *pro-R* methyl group relative to product formed from the *pro-S* methyl group.

Although eq 6 is a general solution for the model depicted in Figure 6, a more useful form of the equation can be obtained as follows; dividing both numerator and denominator by k_6 gives

$${}^{R/S}P_S = \frac{k_R}{k_S}\frac{k_3}{k_5}\frac{1 + (k_S/k_6)}{(k_4/k_6) + (k_R/k_6)}$$

isolating k_4/k_6 in the denominator gives

$${}^{R/S}P_S = \frac{k_3}{k_5}\frac{(k_R/k_S) + (k_R/k_6)}{(k_4/k_6)(1 + (k_R/k_4))} = \frac{k_3}{k_5}\frac{k_6}{k_4}\frac{(k_R/k_S) + (k_R/k_6)}{1 + (k_R/k_4)} = {}^{R/S}K_{eq}\frac{(k_R/k_S) + (k_R/k_6)}{1 + (k_R/k_4)} \tag{7}$$

where the ratio $(k_3k_6)/(k_5k_4)$ expresses the equilibrium constant ${}^{R/S}K_{eq}$ for the *pro-R* vs. *pro-S* methyl group at the active site. When limits are examined, it is found that when k_4, $k_6 \gg k_R$

$${}^{R/S}P_S = {}^{R/S}V_{obsd} = \frac{k_R}{k_S}{}^{R/S}K_{eq} \tag{8}$$

and if in addition $k_R = k_S$

$${}^{R/S}P_S = {}^{R/S}V_{obsd} = {}^{R/S}K_{eq} \tag{9}$$

Finally, when k_4, $k_6 \ll k_R$

$${}^{R/S}P_S = {}^{R/S}V_{obsd} = k_3/k_5 \tag{10}$$

If we initially label one of the methyl groups with deuterium, e.g., *pro-S*, in order to distinguish it from the other, eq 6 can simply be rewritten as eq 11, provided there are no binding effects due to deuterium

$${}^{D}V_{obsd,R} = \frac{k_{H,R}}{k_{D,S}}\frac{k_3(k_6 + k_{D,S})}{k_5(k_4 + k_{H,R})} \tag{11}$$

where ${}^{D}V_{obsd,R}$ is the ratio of velocities for hydroxylation of an *R* methyl group relative to the *S* trideuteriomethyl group, $k_{H,R}$ is the rate constant for hydroxylation of the *R* methyl group, and $k_{D,S}$ is the rate constant for hydroxylation of the *S* trideuteriomethyl group.

Similarly, if we label the *pro-R* methyl group with deuterium we may write

$${}^{D}V_{obsd,S} = \frac{k_{H,S}}{k_{D,R}}\frac{k_5(k_4 + k_{D,R})}{k_3(k_6 + k_{H,S})} \tag{12}$$

If eq 11 and 12 are rearranged in the same manner as eq 6 was rearranged to obtain eq 7 we obtain eq 13 and 14, respectively.

$${}^{D}V_{obsd,R} = {}^{R/S}K_{eq}\frac{(k_{H,R}/k_{D,S}) + (k_{H,R}/k_6)}{1 + (k_{H,R}/k_4)} \tag{13}$$

$${}^{D}V_{obsd,S} = \frac{1}{{}^{R/S}K_{eq}}\frac{(k_{H,S}/k_{D,R}) + (k_{H,S}/k_4)}{1 + (k_{H,S}/k_6)} \tag{14}$$

When k_6, $k_4 \gg k_{H,R}$, $k_{H,S}$

$${}^{D}V_{obsd,R} = {}^{R/S}K_{eq}\frac{k_{H,R}}{k_{D,S}} \tag{15}$$

and

$${}^{D}V_{obsd,S} = \frac{1}{{}^{R/S}K_{eq}}\frac{k_{H,S}}{k_{D,R}} \tag{16}$$

When k_6, $k_4 \ll k_{H,S}$, $k_{H,R}$

$${}^{D}V_{obsd,R} = k_3/k_5 \tag{17}$$

and

$${}^{D}V_{obsd,S} = k_5/k_3 \tag{18}$$

Multiplying eq 13 by eq 14 and taking the square root, we obtain

$$({}^{D}V_{obsd,R}{}^{D}V_{obsd,S})^{1/2} = \left[\frac{(k_{H,R}/k_{D,S}) + (k_{H,R}/k_6)}{1 + (k_{H,R}/k_4)}\frac{(k_{H,S}/k_{D,R}) + (k_{H,S}/k_4)}{1 + (k_{H,S}/k_6)}\right]^{1/2} \tag{19}$$

When $k_6, k_4 \gg k_{H,R}, k_{H,S}$

$$({}^{D}V_{obsd,R}{}^{D}V_{obsd,S})^{1/2} = \left(\frac{k_{H,R}}{k_{D,S}}\frac{k_{H,S}}{k_{D,R}}\right)^{1/2} \quad (20)$$

and when $k_{H,R} = k_{H,S}$

$$({}^{D}V_{obsd,R}{}^{D}V_{obsd,S})^{1/2} = \frac{k_H}{k_D} = {}^{D}V_{intrinsic} \quad (21)$$

when $k_6, k_4 \ll k_{H,R}$

$${}^{D}V_{exp} = ({}^{D}V_{obsd,R}{}^{D}V_{obsd,S})^{1/2} \rightarrow 1 \quad (22)$$

and no isotope effect is observed. If there is rapid equilibration of the two enantiomers at the active site, the isotope expected for a racemic mixture may be defined as

$${}^{D}V_{exp,rac} = \frac{k_{H,R}f_R(E{\cdot}S) + k_{H,S}f_S(E{\cdot}S)}{k_{D,R}f_R(E{\cdot}S) + k_{D,S}f_S(E{\cdot}S)} \quad (23)$$

where $f_R(E{\cdot}S)$ is the fraction of enzyme–substrate complex oriented for hydroxylation to occur at the R methyl group and $f_S(E{\cdot}S)$ is the fraction of enzyme–substrate complex oriented for hydroxylation to occur at the S methyl group.

If $k_{H,R} = k_{H,S}$ and $k_{D,R} = k_{D,S}$, i.e., the isotope effect is independent of stereochemistry, then

$${}^{D}V_{exp,rac} = \frac{f_R + f_S}{f_R + f_S}\frac{k_H}{k_D} = \frac{k_H}{k_D} = {}^{D}V_{intrinsic} = ({}^{D}V_{obsd,R}{}^{D}V_{obsd,S})^{1/2} \quad (24)$$

Dividing eq 13 by eq 14 and taking the square root, we obtain

$$\left(\frac{{}^{D}V_{obsd,R}}{{}^{D}V_{obsd,S}}\right)^{1/2} = {}^{R/S}K_{eq}\left[\frac{(k_{H,R}/k_{D,S}) + (k_{H,R}/k_6)}{1 + (k_{H,R}/k_4)}\,\frac{1 + (k_{H,S}/k_6)}{(k_{H,S}/k_{D,R}) + (k_{H,S}/k_4)}\right]^{1/2} \quad (25)$$

when $k_6, k_4 \gg k_{H,R}, k_{H,S}$

$$\left(\frac{{}^{D}V_{obsd,R}}{{}^{D}V_{obsd,S}}\right)^{1/2} = {}^{R/S}K_{eq}\left(\frac{k_{H,R}}{k_{D,S}}\frac{k_{D,R}}{k_{H,S}}\right)^{1/2} \quad (26)$$

further if $k_{H,R} = k_{H,S}$

$$\left(\frac{{}^{D}V_{obsd,R}}{{}^{D}V_{obsd,S}}\right)^{1/2} = {}^{R/S}K_{eq} \quad (27)$$

When $k_6, k_4 \ll k_{H,R}, k_{H,S}$, then

$$\left(\frac{{}^{D}V_{obsd,R}}{{}^{D}V_{obsd,S}}\right)^{1/2} = \frac{k_3}{k_5} \quad (28)$$

Combining the concepts of P_S and ${}^{D}V_{obsd}$ by dividing eq 13 by eq 7, we obtain

$$\frac{{}^{D}V_{obsd,R}}{{}^{R/S}P_S} = \frac{(k_{H,R}/k_{D,S}) + (k_{H,R}/k_6)}{1 + (k_{H,R}/k_4)}\,\frac{1 + (k_R/k_4)}{(k_R/k_S) + (k_R/k_6)} \quad (29)$$

Since ${}^{R/S}P_S = 1/{}^{S/R}P_S$, an analogous derivation for the opposite enantiomer leads to

$${}^{D}V_{obsd,S}{}^{R/S}P_S = \frac{(k_{H,S}/k_{D,R}) + (k_{H,S}/k_4)}{1 + (k_{H,S}/k_6)}\,\frac{(k_R/k_S) + (k_R/k_6)}{1 + (k_R/k_4)} \quad (30)$$

when $k_6, k_4 \gg k_{H,R}, k_R, k_{H,S}$, eq 29 becomes

$$\frac{{}^{D}V_{obsd,R}}{{}^{R/S}P_S} = \frac{k_{H,R}}{k_{D,S}}\frac{k_S}{k_R} = {}^{D}V_{exp,pro\text{-}R} \quad (31)$$

and eq 30 becomes

$${}^{D}V_{obsd,S}{}^{R/S}P_S = \frac{k_{H,S}}{k_{D,R}}\frac{k_R}{k_S} = {}^{D}V_{exp,pro\text{-}S} \quad (32)$$

Where ${}^{D}V_{exp,pro\text{-}R}$ and ${}^{D}V_{exp,pro\text{-}S}$ are defined as the apparent isotope effect for a given methyl group (*pro-R* or *pro-S*) calculated from the experimentally determined observed isotope effect and the experimentally determined product stereoselectivity for the reaction.

Further if the activation energy for hydroxylation of a methyl group is independent of stereochemistry

$$\frac{{}^{D}V_{obsd,R}}{{}^{R/S}P_S} = {}^{D}V_{obsd,S}{}^{R/S}P_S = {}^{D}V_{exp,pro\text{-}R} = {}^{D}V_{exp,pro\text{-}S} = \frac{k_H}{k_D} = {}^{D}V_{intrinsic,pro\text{-}R\ or\ pro\text{-}S} \quad (33)$$

Registry No. (±)-$CD_3CHPhMe$, 104713-89-5; (*R*)-$CD_3CHPhMe$, 10059-20-8; (*S*)-$CD_3CHPhMe$, 104713-90-8; (*R*)-$^{13}CH_3CHPhMe$, 104642-03-7; (*S*)-$^{13}CH_3CHPhMe$, 104642-04-8; D_2, 7782-39-0; ^{13}C, 14762-74-4; cytochrome P-450, 9035-51-2; monooxygenase, 9038-14-6; cumene, 98-82-8.

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