Intramolecular Isotope Effects for Benzylic Hydroxylation of Isomeric Xylenes and 4,4′-Dimethylbiphenyl by Cytochrome P450: Relationship between Distance of Methyl Groups and Masking of the Intrinsic Isotope Effect†

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ABSTRACT: Intramolecular isotope effects associated with the benzylic hydroxylation of a series of selectively deuterated isomeric xylenes and 4,4′-dimethylbiphenyl as catalyzed by various rat liver microsomal preparations and CYP2B1 were determined. Substrate analogs in which each methyl group contained either one (d_2 substrates) or two (d_4 substrates) deuterium atoms were used to determine the intrinsic isotope effect for the reaction. Specific values of the individual primary (*P*) and secondary isotope effects (*S*) were determined. *P* ranged from a low of 5.32 ± 0.48 to a high of 7.57 ± 0.42 depending upon the specific cytochrome P450 preparation used for catalysis. *S* had an average value of 1.03. The d_3 substrates allowed exploration of the effect of distance on the magnitude of the observed isotope effect. The results indicate that the distance of 6.62 Å that separates the carbon atoms of the para methyl groups of *p*-xylene is insufficient to suppress (mask) the intrinsic isotope effect for benzylic hydroxylation by all of the enzyme preparations examined. Conversely, a distance of 11.05 Å, the minimal separation between the carbon atoms of the para methyl groups of *p,p′*-dimethylbiphenyl, is large enough to almost completely mask the intrinsic isotope effect for benzylic hydroxylation by the same set of enzymes.

The enzymatic behaviors of the isoforms that comprise the cytochrome P450 (P450)[1] superfamily are unusual. Most enzymes tend to show high substrate selectivity and to effect catalysis by stabilizing the transition state. However, the P450s operate very differently. Individual isoforms not only are able to oxidize a broad range of structurally diverse organic compounds but they effect catalysis by first activating molecular oxygen to generate the active oxidant, which is believed to be an iron heme bound atomic oxygen atom, termed an oxene (Ortiz de Montellano, 1996). Binding "*per se*" would seem to have little to do with the mechanism, or energetics of the oxidative event, but does determine the orientation of the substrate within the active site of the enzyme. An iron oxene, by itself, is reactive enough to oxidize virtually any organic species it contacts, including hydrocarbons. Thus, the mechanism of P450 oxidations can be expected to mirror the reactivity of oxene while the stereochemistry of substrate transformation can be expected to reflect the architecture of the active site. All of the P450s are believed to have the same reactive oxene species (Jones et al., 1990; Kharki et al., 1995). Thus, each P450 would be expected to catalyze the same type of reaction, e.g. alkane hydroxylation, by essentially the same chemical mechanism.

The intrinsic isotope effect, k_H/k_D, i.e. the isotope effect associated exclusively with the bond-breaking step of a reaction, is the single most important piece of information that needs to be determined in any deuterium isotope effect experiment intended to probe mechanism. It directly characterizes the transition state of the bond-breaking event (Melander & Saunders, 1980) which is the molecular descriptor of mechanism. Unfortunately, using traditional methods of measurement, the observation of a k_H/k_D for an enzymatically mediated reaction is rare. This is because of the multiplicity of steps involved in an enzymatically catalyzed reaction, which may limit the overall rate of product formation. These steps include substrate binding and debinding, bond cleavage, bond formation, product release, etc. The net effect is to decrease (mask) the magnitude of the isotope effect that is actually observed, $(k_H/k_D)_{obs}$, relative to the magnitude of the required quantity, the intrinsic isotope effect, k_H/k_D (Northrop, 1975) . Our current understanding of the relationship between $(k_H/k_D)_{obs}$ and k_H/k_D in biochemically mediated reactions rests on the seminal works of Northrop (1975, 1978, 1981) and Cleland (1982).

An effective strategy for obtaining k_H/k_D values for P450 catalyzed oxidations, introduced by Hjelmeland et al. (1977) and expanded upon by others (Miwa et al., 1980; Gelb et al., 1982, Lindsay Smith et al., 1984; Hanzlik et al., 1985; Jones et al., 1986), is to use experiments of intramolecular design. In such experiments, a substrate is chosen that by symmetry has two chemically equivalent, catalytically susceptible, intramolecular sites. One of the sites is then deuterated. The enzyme is thereby provided the option of oxidizing either a protio site or an electronically equivalent deutero site within the same molecule. The kinetic model for an intramolecular deuterium isotope effect experiment, as proposed by Miwa et al. (1980), is shown in Scheme 1.

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[1] Abbreviations: P450, cytochrome P450; PB, phenobarbital; 3MC, 3-methylcholanthrene; THF, tetrahydrofuran; MTBSTFA, *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide; GC/MS, gas chromatography/mass spectrometry; *P*, primary isotope effect; *S*, secondary isotope effect; k_H/k_{Dobs}, observed intramolecular deuterium isotope; k_H/k_D, intrinsic isotope effect.

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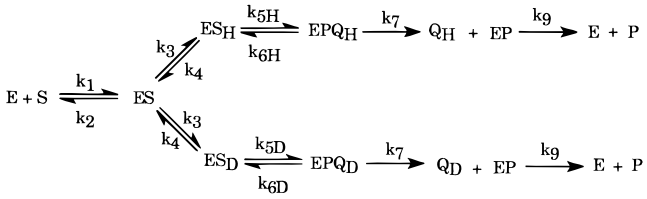
Scheme 1



In the model ES symbolizes an undefined initial complexation between enzyme and substrate which fractionates to the catalytically competent enzyme−substrate complexes, ES_H and ES_D, that define substrate orientation for cleavage of either a C−H or C−D bond, respectively. The rate constants k_3 and k_4 control interchange between the protio and deutero catalytic sites of the substrate at the active site, while Q_H and Q_D are the first isotopically sensitive products released. Kinetically, the relationship between the observed isotope effect and the intrinsic isotope effect is given by eq 1 (Miwa et al., 1980)

$$(k_H/k_D)_{obs} = {}^D\upsilon = k_H/k_D + C_{if} + C_r{}^DKeq/(1 + C_{if} + Cr) \quad (1)$$

where C_{if} is defined as the forward commitment to catalysis and is equal to k_{5H}/k_4, C_r is the reverse commitment to catalysis and is equal to k_{6H}/k_7, and ${}^DKeq$ is the equilibrium isotope effect. If transfer of the activated oxygen atom from enzyme to substrate is irreversible, as is generally assumed for cytochrome P450 reactions, k_6 approaches zero and eq 1 simplifies to

$$(k_H/k_D)_{obs} = {}^D\upsilon = k_H/k_D + C_{if}/(1+ C_{if}) \quad (2)$$

Equation 2 states that if the rate of exchange (equilibration) between protio and deutero catalytic sites is rapid relative to bond breaking, i.e. C_{if} approaches zero, $(k_H/k_D)_{obs}$ will equal k_H/k_D. A substrate that is most likely to have a rate of protio and deutero exchange that is sufficiently fast to meet this condition (C_{if} approaches zero) would contain a methyl group(s) that is either mono- or dideuterated. Having both isotopically distinct (protio and deutero) catalytically sensitive sites within a methyl group means that interchange, with respect to the active site of the enzyme, depends solely on the rate of methyl group rotation, a process that is known to be very fast (Gough et al., 1985). Thus, using a substrate that contains a partially deuterated methyl group(s) would provide one of the best means possible of experimentally estimating the intrinsic deuterium isotope effect associated with the P450-catalyzed hydroxylation of a primary carbon-hydrogen bond of a normal alkane.[2] In contrast, in a substrate that contains deuterium in only one of the two symmetrically disposed methyl groups, the magnitude of C_{if} would depend upon the rate of interchange between methyl groups and the catalytically susceptible orientation of the methyl group with the active site of the enzyme.

Equation 3 also expresses the fundamental relationship between $(k_H/k_D)_{obs}$ and k_H/k_D (Miwa et al., 1980) but in terms of the relative concentrations of ES_H and ES_D.

$$(k_H/k_D)_{obs} = {}^D\upsilon = (k_H/k_D)(ES_H/ES_D) \quad (3)$$

The power of eq 3 is that it highlights the importance of maintaining equal concentrations of the isotopically distinct but catalytically competent enzyme−substrate complexes (ES_H/ $ES_D = 1$) through the course of reaction, if k_H/k_D is to be observed.

A parameter that could modulate the relative concentrations of ES_H and ES_D is the intrasubstrate distance between equivalent but isotopically distinct catalytic sites. The physical closeness of hydrogens and deuteriums bonded to the same carbon atom, coupled to the rapidity of methyl group rotation, ensures that the magnitude of an observed isotope effect associated with hydroxylation of this group will reflect the magnitude of the intrisic isotope effect for the reaction. In contrast, as the distance between the sites containing protium and deuterium atoms increases, the slower the rate of interchange (equilibration) is likely to be.

To test this hypotheses, several selectively deuterated *o*- and *p*-xylenes and 4,4′-dimethylbiphenyl were targeted for study. These particular compounds were chosen because they provided a group of substrates in which the intramolecular distances between equivalent protio and deutero methyl groups were different, but fixed and known. In addition, the lack of ionic, polar, or hydrogen bonding sites within these substrates allowed the focus to center on distance effects and precluded complications that would be expected to arise from decreased substrate motion through specific binding of the substrate to the enzyme.

MATERIALS AND METHODS

Chemicals. Lithium aluminum deuteride and lithium aluminum hydride were obtained from Fluka Chemical Co. (Buchs, Switzerland). Diazald, silica gel, sodium borohydride, sodium borodeuteride, chromium trioxide, *o*-methyltoluate, *p*-methyltoluate, dimethylphthalate, terephthalic acid, phthalic dicarboxaldehyde, terephthalaldehyde, 4,4′-dimethylbiphenyl, and pentane were obtained from Aldrich Chemical Co. (Milwaukee, WI). All other organic solvents were purchased from J. T. Baker Inc. (Phillipsburg, NJ) and were of analytical grade. Phenobarbital sodium was purchased from Spectrum Chemical Manufacturing Co. (New Brunswick, NJ). *N*-Methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) was purchased from Pierce Chemical Co. (Rockford, IL), and *p*-toluenesulfonyl chloride was obtained from Eastman Kodak Co. (Rochester, NY). Biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Synthesis of Substrates. (A) o-Xylene-α-$^2H_3$. *o*-Methyltoluate (5.2 g, 0.036 mol) in 20 mL of anhydrous diethyl ether was added dropwise to lithium aluminum deuteride (1.6 g, 0.04 mol) suspended in 50 mL of anhydrous diethyl ether. The reaction was stirred at room temperature for 10 h under a nitrogen atmosphere after which 1 mL of water, 2 mL of 15% sodium hydroxide, and 2 mL of water were added cautiously. The solution was filtered and extracted with 2 × 75 mL of pentane. The pentane layer was dried over

[2] Because it is impossible for a methyl group to contain an equal number of deuteriums and hydrogens, the experimentally determined isotope effect would not be directly equal to k_H/k_D, the intrinsic isotope effect. The monodeuteromethyl substrate has twice as many hydrogens as deuteriums and thus would equal $2k_H/k_D$. The deuteromethyl substrate has twice as many deuteriums as hydrogens and thus is equal to $k_H/2k_D$. Dividing the former isotope effect by 2 and multiplying the latter by 2 statistically corrects the observed value and provides k_H/k_D. The k_H/k_{Dobs} values reported in Table 1 are corrected for the number of hydrogens and deuteriums present in each methyl group.

sodium sulfate and evaporated to yield *o*-methylbenzyl alcohol (4.1 g, 0.033 mol, 92% yield). The alcohol was then reacted with *p*-toluenesulfonyl chloride (6.6 g, 0.035 mol) in 150 mL of dichloromethane containing 6 mL of triethylamine. After 12 h of stirring, the reaction mixture was washed with 2 × 150 mL of water and then 2 × 150 mL of saturated sodium bicarbonate. The organic layer was dried over sodium sulfate and evaporated to yield crude *o*-methylbenzyl tosylate. The residue was dissolved in 20 mL of anhydrous diethyl ether and added dropwise to lithium aluminum deuteride (1.2 g, 0.03 mol) suspended in diethyl ether. The reaction was stirred at room temperature for 10 h under nitrogen after which time was added 1 mL of water, 2 mL of 15% sodium hydroxide, and 2 mL of water. The reaction was extracted with 2 × 75 mL of pentane. The pentane layer was dried over sodium sulfate and evaporated to yield crude *o*-xylene. The *o*-xylene was purified by silica gel column chromatography (60 Å, 230−240 mesh, 15 g) with pentane as solvent. The resulting *o*-xylene (0.01 mol) was >99% pure by GC and by MS had a deuterium content of 98.48 atom % deuterium-d_3, 1.45 atom % deuterium-d_2, and 0.07 atom % deuterium-d_1.

(B) p-Xylene-α-$^2H_3$. This compound was prepared according to the procedure for *o*-xylene-α-$^2H_3$ except *p*-methyltoluate was used as starting material. The resulting *p*-xylene was >99% pure by GC and by MS had a deuterium content of 98.20 atom % deuterium-d_3, 1.59 atom % deuterium-d_2, and negligible d_1 or d_0.

(C) o-Xylene-α-$^2H_2$-α′-$^2H_2$. Dimethylphthalate (2.76 g, 0.015 mol) was dissolved in 15 mL of ether and added dropwise to lithium aluminum deuteride (1.2 g, 0.03 mol) suspended in 40 mL of ether. The reaction was stirred overnight and then terminated by the addition of 0.6 mL of water, 1.2 mL of 15% sodium hydroxide, and 1.2 mL of water. The resulting mixture was filtered and extracted with 2 × 30 mL of ether. The ether layer was dried over sodium sulfate and evaporated to yield 1.93 g (0.014 mol) of the dialcohol as a clear oil. The product alcohol was stirred with 25 mL of 48% hydrobromic acid for 30 min. The resulting orange solid was recrystallized from methanol to yield 1.58 g (0.006 mol) of the greenish-white dibromo product. The dibromo compound was suspended in 20 mL of dimethyl sulfoxide, added to sodium borohydride (0.5 g, 0.012 mol) dissolved in 20 mL of dimethyl sulfoxide, and stirred for 2 h before 50 mL of pentane was added. Water (10 mL) was then added cautiously to the reaction and the reaction mixture extracted with 2 × 50 mL of water. The pentane layer was evaporated and the resulting oil partitioned between pentane and water. The pentane layer was dried over sodium sulfate and evaporated to yield *o*-xylene. The *o*-xylene was purified by neutral alumina column chromatography (15 g) with pentane as solvent. The resulting *o*-xylene (0.002 mol) was >97% pure by GC and by MS had a deuterium content of 97.71 atom % deuterium-d_4, 1.64 atom % deuterium-d_3, and 0.65 atom % deuterium-d_2.

(D) p-Xylene-α-$^2H_2$-α′-$^2H_2$. This compound was prepared according to the procedure used for *o*-xylene-α-$^2H_2$-α′-$^2H_2$ except terephthalic acid was used as starting material. The resulting *p*-xylene was >98% pure by GC and by MS had a deuterium content of 97.90 atom % deuterium-d_4, and 1.92 atom % deuterium-d_3, and 0.22 atom % deuterium-d_2.

(E) o-Xylene-α-$^2H_1$-α′-$^2H_1$. Phthalic dicarboxaldehyde (5.36 g, 0.04 mol) was suspended in methanol-*d* (30 mL), and sodium borodeuteride (2.1 g, 0.05 mol) dissolved in 10 mL of methanol-*d* was added dropwise with stirring. The reaction was terminated by the addition of ether (25 mL) followed by 5 mL of water. The reaction mixture was extracted with 2 × 50 mL of ether and the organic layer dried over sodium sulfate and evaporated. The resulting dialcohol (4.14 g, 0.03 mol) was brominated in 40 mL of 48% HBr and the dibromo compound reduced as described in the synthesis of *o*-xylene-α-$^2H_2$-α′-$^2H_2$. The resulting *o*-xylene (0.01 mol) was >97% pure by GC and by MS had a deuterium content of 96.98 atom % deuterium-d_2, 2.97 atom % deuterium-d_1, and 0.05 atom % deuterium-d_0.

(F) p-Xylene-α-$^2H_1$-α′-$^2H_1$. This compound was prepared according to the procedure used for *o*-xylene-α-$^2H_1$-α′-$^2H_1$ except that terephthalaldehyde was used as starting material. The resulting *p*-xylene was >98% pure by GC and by MS had a deuterium content of 95.84 atom % deuterium-d_2, 3.84 atom % deuterium-d_1, and 0.07 atom % deuterium-d_0.

(G) 4′-Methylbiphenyl-4-carboxylic Acid. To 2.3 g (0.013 mol) of 4,4′-dimethylbiphenyl in 100 mL of glacial acetic acid was slowly added 4.58 g (0.046 mol) of chromium trioxide with stirring (Carnelley, 1877). The reaction mixture was slowly heated and maintained at 90 °C for 1 h. The reaction was then cooled and poured into 500 mL of ice-cold water. The green solution was filtered and the precipitate washed with cold water till the washings were colorless. The precipitate was dissolved in a minimum volume of 30% ammonia, filtered, and then reprecipitated by acidifying with concentrated HCl. The precipitate was filtered and oven-dried at 120 °C to yield 0.4 g (0.002 mol) of 4′-methylbiphenyl-4-carboxylic acid.

(H) Dimethylbiphenyl-4,4′-dicarboxylate. This compound was synthesized from 4,4′-dimethylbiphenyl (2.3 g, 0.013 mol) according to the method outlined for the monocarboxylic acid except that 14.0 g (0.14 mol) of chromium trioxide was used and the reaction heated at 90 °C for 3 h. After workup as described above, the dicarboxylic acid was treated with excess diazomethane in ether for 8 h at room temperature to yield 0.81 g (0.003 mol) of the dimethyl ester.

(I) 4-$^2H_3$,4′-Dimethylbiphenyl. To lithium aluminum deuteride (210 mg, 0.005 mol) suspended in 20 mL of dry tetrahydrofuran was slowly added 4′-methylbiphenyl-4-carboxylic acid (500 mg, 0.0024 mol) suspended in 20 mL of dry tetrahydrofuran. The reaction was stirred for 16 h at room temperature under a nitrogen atmosphere. The reaction was terminated with 1 mL of water, 2 mL of 15% NaOH, and 2 mL of water. The mixture was filtered, dried over sodium sulfate, and evaporated to yield the crude alcohol. The alcohol was purified by silica gel (12 g) column chromatography with chloroform/methanol (9:1) as eluant. The alcohol (310 mg, 0.0016 mol) was dissolved in 50 mL of dichloromethane and reacted with *p*-toluenesulfonyl chloride (380 mg, 0.0020 mol) and triethylamine (1 mL) for 48 h. The mixture was washed with 2 × 75 mL water and 2 × 75 mL saturated sodium bicarbonate. The organic layer was dried over sodium sulfate and evaporated to yield the tosylated product. The product was purified by silica gel (5 g) column chromatography with hexane as eluant. The tosylate (65 mg, 0.2 mmol) was reduced with lithium aluminum deuteride (0.1 g, 0.003 mol) to yield 4-$^2H_3$,4′-dimethylbiphenyl. This compound was purified by silica gel (25 g) column chromatography with hexane as eluant. The resulting dimethylbiphenyl was >99% pure by GC and by

Table 1: Statistically Corrected Values of $(k_H/k_D)_{obs}$ for Microsomal or Purified CYP2B1-Catalyzed Methyl Group Hydroxylation of *o*- and *p*-Xylenes and 4,4′-Dimethylbiphenyl in Which Both Methyl Groups of Each Substrate Are either Mono- or Dideuterated

	isotope effect[a] with substrate					
enzyme source	*o*-xylene-α-$^2H_1$-α′-$^2H_1$	*p*-xylene-α-$^2H_1$-α′-$^2H_1$	4-$^2H_1$,4′-$^2H_1$-dimethylbiphenyl	*o*-xylene-α-$^2H_2$-α′-$^2H_2$	*p*-xylene-α-$^2H_2$-α′-$^2H_2$	4-$^2H_2$,4′-$^2H_2$-dimethylbiphenyl
PB induced microsomes	5.27 ± 0.14 (3)[b]	5.29 ± 0.10 (3)	8.32 ± 1.02 (3)	6.37 ± 0.19 (3)	6.35 ± 0.11 (5)	7.31 ± 0.16 (3)
3-MC induced microsomes	5.00 ± 0.16 (3)	4.57 ± 0.08 (3)	5.77 ± 0.46 (3)	5.54 ± 0.02 (3)	5.33 ± 0.15 (4)	5.72 ± 0.14 (3)
control microsomes	5.75 ± 0.15 (3)	5.30 ± 0.04 (3)	5.84 ± 0.12 (3)	6.38 ± 0.08 (3)	5.76 ± 0.13 (5)	6.07 ± 0.03 (3)
purified CYP2B1	5.81 ± 0.06 (5)	6.06 ± 0.02 (3)	7.54 ± 0.35 (3)	7.38 ± 0.11 (5)	6.63 ± 0.13 (5)	6.68 (2)

[a] Numerical value of the isotope effect plus or minus the standard deviation. [b] Number in parentheses is the number of determinations.

MS had a deuterium content of 94.42 atom % deuterium-d_3, 4.46 atom % deuterium d_2, 0.389 atom % deuterium-d_1, and 0.297 atom % deuterium-d_0.

(J) 4-$^2H_2$,4′-$^2H_2$-Dimethylbiphenyl. This compound was prepared according to the procedure described for the preparation of *o*-xylene-α-$^2H_2$-α′-$^2H_2$ except the methyl ester of biphenyl-4,4′-dicarboxylic acid was used as starting material. The resulting 4-$^2H_2$,4′-$^2H_2$-dimethylbiphenyl was >98% pure by GC and by MS had a deuterium content of 96.63 atom % deuterium-d_4, 1.48 atom % deuterium-d_3, and 1.47 atom % deuterium-d_2.

(K) 4-$^2H_1$,4′-$^2H_1$-Dimethylbiphenyl. This compound was prepared according to the procedure described for the preparation of *o*-xylene-α-$^2H_1$-α′-$^2H_1$ except the methyl ester of biphenyl-4,4′-dicarboxylic acid was used as starting material. In addition, the first reduction was done with lithium aluminum hydride but, after bromination, the final reduction was done with sodium borodeuteride. The resulting 4-$^2H_1$,4′-$^2H_1$-dimethylbiphenyl was >98% pure by GC and by MS had a deuterium content of 89.50 atom % deuterium-d_2, 9.79 atom % deuterium-d_1, and 0.366 atom % deuterium-d_0.

Preparation of Microsomes and Enzyme Purification. Microsomes were prepared from induced (phenobarbital and 3-methylcholanthrene) male Sprague-Dawley rats (200−250 g) as described elsewhere (Porter et al., 1977). CYP2B1 was purified according to the procedure of Sugiyama et al. (1989). Rabbit cytochrome P450 reductase was purified according to the method of Ardies et al. (1987).

Incubation Conditions. Microsomal incubations were run for 30 min at 27 °C and contained 7−8 nmol of P450 as determined by the method of Omura and Sato (1964), 12.3 mmol (1.0 mg) of NADPH, and substrate (xylene, 4.08 mM; or 4,4′-dimethylbiphenyl, 0.67 mM) in a total volume of 2 mL of Tris buffer (0.2 M, pH 8.2 at 25 °C). The incubations were terminated by the addition of 5 mL of pentane and stored at −78 °C until analysis.

The incubations with purified CYP2B1 were run for 30 min and contained 0.5 nmol of CYP2B1, 0.5 nmol of rabbit cytochrome P450 reductase, 20 mg of L-(α-dilauroylphosphatidylcholine), 0.3 mmol of NADPH, and substrate (xylene, 4.08 mM; or 4,4′-dimethylbiphenyl, 0.67 mM) in a final volume of 2 mL of Tris buffer (0.2 M, pH 8.2 at 25 °C). The incubations were terminated and stored as described above. For analysis the incubations were extracted with 2 × 5 mL of pentane. The pentane layers were pooled, dried over sodium sulfate, and concentrated under a stream of nitrogen gas to 30 μL. The samples were derivatized with 150 mL of 60% MTBSTFA in acetonitrile at 65 °C for 3 h and analyzed by GC/MS.

Aromatic Hydroxylation. The ratio of benzylic to aromatic hydroxylation was determined in phenobarbital-induced microsomes as follows. Incubations were terminated by rapid freezing in an acetone/dry ice bath and spiked with an internal standard solution (3,5-dimethylphenol for *o*-xylene incubations and 2,3-dimethylphenol for *p*-xylene incubations). The samples were processed for GC analysis as described above except hexane/ether (8:2) was used for extraction. Standard curve samples, with each concentration in triplicate, were processed along with the incubations for each of the possible metabolites. Metabolites were quantified by determination of the GC peak area ratios of the metabolite to the internal standard.

GC analysis for the determination of the ratio of benzylic to aromatic hydroxylation was conducted utilizing the same column and chromatographic conditions as used for the xylenes except that detection was accomplished with a flame ionization detector and the data acquisition was achieved with an HP 3396A integrator.

GC/MS Analysis. GC/MS analysis was performed on a VG7070 mass spectrometer, interfaced to a HP-5710A GC fitted with a 15 m J&W DB-5 fused silica capillary column, and operated in the selected ion monitoring mode. The derivatized xylenes were cold trapped at 40 °C, and the temperature was raised linearly at 15 °C/min to 135 °C followed by isothermal elution for 5 min. For the derivatized biphenyls an initial temperature of 100 °C was used for 1 min, followed by a linear ramp at 15 °C/min to 250 °C, and completed by isothermal elution for 5 min. The $[M - 57]^+$ ion of the silyl derivative was monitored for the various xylene isomer metabolites and 4,4′-dimethylbiphenyl metabolites. Mass spectral conditions were as follows; 50 ms dwell time, −70 eV ionizing voltage, and 200−205 °C source temperature. Deuterium incorporation in each substrate was determined using the same GC parameters as those used for each of the respective metabolites or by bleeding the compound through the reference inlet. The mass spectral conditions were the same as those used for the metabolites except that the ionizing voltage was −12.5 eV. The measured ion intensity of each ion was corrected for the natural isotopic abundance of ^2H, ^{13}C, ^{14}C, ^{18}O, and ^{29}Si (Korzekwa et al., 1990).

Calculation of Isotope Effects. The calculations and corrections used for the determination of observed isotope effects were as described previously (Jones et al., 1986).

RESULTS

Determination of k_H/k_D. The $(k_H/k_D)_{obs}$ values for the *o*- and *p*-xylenes and 4,4′-dimethylbiphenyl in which each of the methyl groups contained either one (d_2 substrates) or two (d_4 substrates) deuterium atoms were determined and are listed in Table 1. The statistically corrected[2] values of $(k_H/k_D)_{obs}$ over the four different enzyme preparations and the

Table 2: Values of $(k_H/k_D)_{obs}$ for the Microsomal or CYP2B1-Catalyzed Methyl Group Hydroxylation of Trideuteromethyl-*o*- and *p*-Xylenes and 4,4′-Dimethylbiphenyl

	isotope effect[a] with substrate		
enzyme source	*o*-xylene-α-$^2H_3$	*p*-xylene-α-$^2H_3$	4-$^2H_3$,4′-dimethylbiphenyl
PB-induced microsomes	7.44 ± 0.18 (6)[b]	6.04 ± 0.76 (6)	1.70 ± 0.05 (3)
3-MC-induced microsomes	6.48 ± 0.17 (6)	5.30 ± 0.39 (3)	1.66 ± 0.04 (3)
control microsomes	7.41 + 0.30 (6)	5.75 ± 0.61 (3)	1.67 ± 0.05 (3)
purified CYP2B1	6.66 ± 0.26 (7)	7.73 ± 0.30 (3)	2.09 ± 0.14 (3)

[a] Numerical value of the isotope effect plus or minus the standard deviation. [b] Number in parentheses is the number of determinations.

three different d_2 substrates were found to range from a low of 4.57 ± 0.08 for hydroxylation of *p*-xylene-α-$^2H_1$-α′-$^2H_1$ with microsomes from 3-methylcholanthrene-pretreated rats to a high of 8.32 ± 1.02 for hydroxylation of dimethylbiphenyl-4-$^2H_1$-4′-$^2H_1$ with microsomes from phenobarbital-pretreated rats (Table 1). The value of $(k_H/k_D)_{obs}$ over the four different enzyme preparations and the three different d_4 substrates was much tighter, ranging from a low of 5.33 ± 0.15 for hydroxylation of *p*-xylene-α-$^2H_2$-α′-$^2H_2$ with microsomes from 3-methylcholanthrene-pretreated rats to a high of 7.38 ± 0.11 for hydroxylation of *o*-xylene-α-$^2H_2$-α′-$^2H_2$ from purified CYP2B1 (Table 1). The reason for the narrower range of $(k_H/k_D)_{obs}$ for the d_4 substrates relative to the d_2 substrates is not apparent, but a possible explanation is the inherently greater accuracy of the mass spectral measurement of the d_4/d_3 product ratio over that of the d_2/d_1 product ratio because of the smaller difference in individual peak area measurements (d_4 versus d_3 compared to d_2 versus d_1).

Determination of $(k_H/k_D)_{obs}$ for the Trideuteromethyl Substrates. Having defined the range of k_H/k_D to be expected for the system the influence of distance could now be probed using the set of substrates in which one of the two possible methyl groups in each individual member was trideuterated (Table 2). A cursory glance of the values in Table 2 indicates that as expected the differences in $(k_H/k_D)_{obs}$ are in general much greater than those seen in Table 1. The $(k_H/k_D)_{obs}$ values for 4,4′-dimethylbiphenyl-α-$^2H_3$ range from 1.66 ± 0.04 to 2.09 ± 0.14, while those for *o*- and *p*-xylene-α-$^2H_3$ range from 6.48 ± 0.17 to 7.44 ± 0.18 and from 5.30 ± 0.39 to 7.73 ± 0.30, respectively.

Determination of Branching to Aromatic Hydroxylation. If the rate of methyl group hydroxylation is sufficiently retarded by deuteration, the rate of ring hydroxylation would increase provided it is an alternate product-forming pathway available to the enzyme. Branching of this kind would tend to unmask the isotope effect because it would provide an alternate mechanism for equalizing [ESH] and [ESD] (Jones et al., 1986; Korzekwa et al., 1989). As a result any masking of k_H/k_D that would occur because of an increased distance between protio and deutero intramolecular sites would be countered by the effects of product branching. Thus, the extent of branching for each of the substrates needed to be determined. The results are reported in Table 3. The relative levels of benzylic and aromatic hydroxylation in the case of *o*- and *p*-xylene indicated that benzylic hydroxylation accounts for 92% and 97% of the total metabolism, respectively, (Table 2). Introduction of deuterium, as in the case of *p*-xylene-α-$^2H_3$, decreased the extent of benzylic hydroxylation to about 94%. No evidence for the formation of a ring hydroxylated metabolite, e.g. in the form of an unidentified peak, could be found in the GC/FID or GC/MS chromatograms of the product mixture obtained upon incubation of 4-trideuteromethyl-4′-methylbiphenyl with any of the enzyme preparations.

Table 3: Ratio of Benzylic to Aromatic Hydroxylation for *o*- and *p*-Xylene and *p*-Xylene-α-$^2H_3$ in Phenobarbital-Induced Microsomes

	metabolite (site of hydroxylation)		
substrate	benzylic (mol %)	aromatic (mol %)	ratio Bz/Ar
o-xylene	92.00	7.99	11.51
p-xylene	97.06	2.94	33.01
p-xylene-α-$^2H_3$	94.80	5.43	17.45

Determination of Individual Values of Primary (P) and Secondary (S) Isotope Effects. The values of experimentally observed intramolecular isotope effects, $(k_H/k_D)_{obs}$, for the hydroxylation of mono- or dideuteromethyl groups will contain contributions from the secondary isotope effects associated with the reaction. When $(k_H/k_D)_{obs}$ is equal to k_H/k_D, as is assumed for these substrates, *P* and *S* are both fully expressed. In addition, because of the minimal motion necessary for equilibration to be achieved between methyl groups, the values of $(k_H/k_D)_{obs}$ for *o*-xylene-α-$^2H_3$ are also assumed to be fully expressed. With these assumptions and the individual values of $(k_H/k_D)_{obs}$ for corresponding d_2, d_4, and d_3 substrates it is possible to calculate individual values for *P* and *S* as we have done previously (Jones & Trager, 1987) using the method of Hanzlik et al. (1985). The values of *P* and *S* calculated in this way are presented in Table 4. The entries for d_2- and d_4-*p*-xylene were calculated using $(k_H/k_D)_{obs}$ for *p*-xylene-α-$^2H_3$ by assuming that it represents a fully expressed value of PS^2. Similarly, the entries for d_2- and d_4-4,4′-dimethylbiphenyl were calculated using $(k_H/k_D)_{obs}$ for *p*-xylene-α-$^2H_3$ by assuming that it also represents a close estimate of a fully expressed value of PS^2 for dimethylbiphenyl-α-$^2H_3$. The justification for both assumptions will be presented under Discussion.

DISCUSSION

The purpose of this study was to test the hypothesis that, in an intramolecular isotope effect experiment, the extent of k_H/k_D masking is distance dependent. To test this hypothesis, it was imperative that k_H/k_D be known for the reaction being investigated. To minimize the variables that could affect the magnitude of $(k_H/k_D)_{obs}$ and to simplify interpretation, it was also necessary to use substrates that lacked polar and hydrogen bonding sites but had well-defined distances between methyl groups. A set of substrates that meet these requirements are the *o*- and *p*-xylenes and 4,4′-dimethylbiphenyl (Figure 1). They not only lack polar and hydrogen bonding sites, but within a substrate, the distance between methyl groups is fixed by the phenyl ring(s). Molecular mechanics calculations indicate that the shortest distances

Table 4: Individual *P* and *S* Isotope Effects Determined from the $(k_H/k_D)_{obs}$ Values for the Microsomal or CYP2B1-Catalyzed Hydroxylation of *o*-Xylene-α-$^2H_1$-α′-$^2H_1$, *o*-Xylene-α-$^2H_2$-α′-$^2H_2$, *p*-Xylene-α-$^2H_1$-α′-$^2H_1$, *p*-Xylene-α-$^2H_2$-α′-$^2H_2$, 4,4′-Dimethylbiphenyl-α-$^2H_1$-α′-$^2H_1$, and 4,4′-Dimethylbiphenyl-α-$^2H_2$-α′-$^2H_2$

	primary (*P*) and secondary (*S*) isotope effect[a] with substrate											
	o-xylene-α-$^2H_1$-α′-$^2H_1$		*o*-xylene-α-$^2H_2$-α′-$^2H_2$		*p*-xylene-α-$^2H_1$-α′-$^2H_1$		*p*-xylene-α-$^2H_2$-α′-$^2H_2$		dimethylbiphenyl-α-$^2H_1$-α′-$^2H_1$		dimethylbiphenyl-α-$^2H_2$-α′-$^2H_2$	
enzyme source	*P*	*S*	*P*	*S*	*P*	*S*	*P*	*S*	*P*	*S*	*P*	*S*
PB-induced microsomes	5.91 ± 0.20	1.12 ± 0.01	6.71 ± 0.24	1.05 ± 0.01	5.53 ± 0.83	1.04 ± 0.04	6.25 ± 0.95	0.99 ± 0.04	7.47 ± 1.29	0.90 ± 0.05	6.87 ± 1.04	0.94 ± 0.04
3-MC-induced microsomes	5.46 ± 0.28	1.09 ± 0.02	5.83± 0.18	1.05 ± 0.01	4.81 ± 0.43	1.05 ± 0.03	5.32 ± 0.48	1.00 ± 0.03	5.61 ± 0.56	0.97 ± 0.04	5.57 ± 0.50	0.98 ± 0.03
control microsomes	6.25 ± 0.32	1.09 ± 0.02	6.71 ± 0.33	1.05 ± 0.01	5.45 ± 0.69	1.03 ± 0.04	5.76 ± 0.74	1.00 ± 0.04	5.81 ± 0.75	1.00 ± 0.04	5.97 ± 0.76	0.98 ± 0.04
purified CYP2B1	6.08 ± 0.29	1.05 ± 0.01	7.20 ± 0.35	0.97 ± 0.01	6.57 ± 0.31	1.03 ± 0.01	6.99 ± 0.34	1.05 ± 0.02	7.57 ± 0.42	1.01 ± 0.02	7.01 ± 0.33	1.05 ± 0.01

[a] Numerical value of the isotope effect plus or minus the standard deviation.

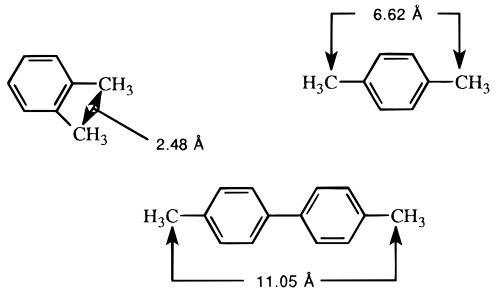


FIGURE 1: Intramolecular distances between methyl group carbon atoms for *o*- and *p*-xylene and 4,4′-dimethylbiphenyl calculated using Spartan Molecular Mechanics software.

between the carbon atoms[3] of the two methyl groups are as follows: *o*-xylene, 2.48 Å; *p*-xylene, 6.62 Å; and 4,4′-dimethylbiphenyl, 11.05 Å (Figure 1).

For k_H/k_D to be determinable, the rate of equilibration between chemically equivalent but isotopically distinct oxidizable groups within the substrate must be rapid relative to bond breaking. Therefore, substrates chosen for study must meet this requirement. Substrates which in general meet this requirement are those that contain a partially deuterated methyl group as the target for hydroxylation. The rapidity of methyl group rotation ensures that the active iron−oxygen species samples the statistical distribution of catalytically competent orientations of hydrogen and deuterium at all times during the course of reaction (Gough et al., 1985) and that $(k_H/k_D)_{obs}$ for these substrates is close to k_H/k_D. The good estimates of k_H/k_D provided by the d_2 and d_4 substrates serve as the necessary standards that make the analysis of distance-induced masking effects possible. Thus, the bis monodeuteromethyl (d_2) and bis dideuteromethyl (d_4) analogs of each of the selected substrates were included in the present study.

The $(k_H/k_D)_{obs}$ values for all the d_2 and d_4 substrates with the different enzyme preparations are listed in Table 1. The results overall suggest that while there is some variation, k_H/k_D is reasonably constant over the range of conditions employed, particularly under the same set of conditions, i.e. the same enzyme preparation and the same extent of deuterium incorporation. In theory, because the rule of the geometric mean states that isotope effects associated with a reaction center are independent and multiplicative, identical $(k_H/k_D)_{obs}$ values should be obtained for the d_2 and d_4 substrates after statistical correction when they are subjected to identical reaction conditions and protocols. In the present study we have found that the statistically corrected values of $(k_H/k_D)_{obs}$ from the d_4 substrates are 10−20% larger than the statistically corrected values of $(k_H/k_D)_{obs}$ from the d_2 substrates. The reason(s) for the discrepancy is (are) not known. As a result, the most valid comparisons of $(k_H/k_D)_{obs}$ that can be made between the different substrates are ones that are restricted to substrates having identical deuterium substitution or ones that are obtained once the individual values of *P* and *S* are known.

[3] The carbon atoms of the methyl groups are taken as the frame of reference for defining the distance between methyl groups since it is fixed, unlike the distances between inter methyl group hydrogens, which are conformationally variable. The distance of closest approach that can be achieved between two hydrogens that are bonded to different methyl groups is approximately 1.4 Å for *o*-xylene, 6.7 Å for *p*-xylene, and 11.1 Å for 4,4′-dimethylbiphenyl.

Having established k_H/k_D for each substrate under each experimental condition, the influence of distance could now be probed using the set of substrates in which one of the two possible methyl groups in each individual substrate was trideuterated (d_3 substrates) Table 2. Comparison of the data presented in Tables 1 and 2 for each of the compounds showed the most dramatic differences in $(k_H/k_D)_{obs}$ with the deuterated analogs of 4,4′-dimethylbiphenyl. The values of $(k_H/k_D)_{obs}$ for the d_2 and d_4 analogs of 4,4′-dimethylbiphenyl range from 5.72 ± 0.14 to 8.32 ± 1.02 (Table 1), while those for the d_3 analogs range from only 1.66 ± 0.07 to 2.09 ± 0.14, (Table 2). These results suggest that at a distance of 11.05 Å (Figure 1) distance-induced masking of the intrinsic isotope effect is almost total. In contrast, much smaller differences are seen between the values of $(k_H/k_D)_{obs}$ for the deuterated analogs of both *o*- and *p*-xylene (Tables 1 and 2), thus providing little indication of significant distance-induced masking effects with these substrates. However, before the possibility of a distance-induced masking effect could be removed from consideration for these substrates, an additional parameter, that of isotopically sensitive branching, needed to be assessed.

If an alternate product can be formed from the substrate, the intrinsic isotope effect can be unmasked (Jones et al., 1986). For the *o*- and *p*-xylene substrates (Figure 1), aromatic hydroxylation can serve as an alternate product-forming pathway. Since aromatic hydroxylation could counter any masking of k_H/k_D that would occur as a result of an increased distance between protio and deutero sites, the actual value of $(k_H/k_D)_{obs}$ would be a summation of the effects of branching and distance on k_H/k_D. Thus, the relatively narrow range of $(k_H/k_D)_{obs}$ values determined for the deuterated analogs of the *o*- and *p*-xylene substrates could mean either (1) the distance between methyl groups is not sufficient to slow the rate of interchange to a level at which masking becomes evident or (2) the distance is sufficient to cause masking but is negated by isotopically sensitive branching to an alternate product.

To distinguish between these two possibilities, the relative levels of benzylic and aromatic hydroxylation were determined for the three substrates (Table 3). As indicated under Results, no evidence for ring hydroxylation of 4,4′-dimethylbiphenyl could be found and only minor conversion to ring hydroxylated products occurred with *o*- and *p*-xylene (8% and 3%, respectively). Thus, the large difference seen in the values of $(k_H/k_D)_{obs}$ for the d_3 4,4′-dimethylbiphenyls, versus those for d_2 and d_4 4,4′-dimethylbiphenyls, can be attributed to distance-induced masking effects (Table 3). In contrast, the similarity of $(k_H/k_D)_{obs}$ values for the d_2, d_3, and d_4 *p*-xylenes coupled to a lack of a significant branched pathway (5%, Table 3) suggests that these values are fully expressed and correspond to values of k_H/k_D. The 6.62 Å separation between the *p*-xylene methyl group carbons (Figure 1) is not sufficient to slow methyl group/active site interchange to a level that allows masking to be apparent. Since the 6.62 Å methyl group separation in *p*-xylene does not induce masking, we assume the 2.48 Å separation between the methyl carbons of *o*-xylene would also not induce masking. Thus, all of the $(k_H/k_D)_{obs}$ values for the d_2, d_3, and d_4 *o*-xylenes can also be considered as being fully expressed intrinsic isotope effects.

The analysis above indicates that for each individual substrate and set of experimental conditions, $(k_H/k_D)_{obs}$ is a good approximation of k_H/k_D for all of the isotopic variants of *o*- and *p*-xylene studied in this investigation. However, in general, the $(k_H/k_D)_{obs}$ values for the d_3 *o*- and *p*-xylene substrates tend to be larger than the corresponding values for the d_2 and d_4 *o*- and *p*-xylene substrates (Tables 1 and 2). Since the latter compounds are substrates for which the isotope effects are believed to be fully expressed because of the rapidity of methyl group rotation, it would seem to be impossible for these compounds to have $(k_H/k_D)_{obs}$ values that are lower than those for the corresponding d_3 analogs. The results can be understood, however, once the contribution of secondary isotope effects is taken into account. The value of the observed isotope effect for any of the d_3 substrates is a composite of a primary isotope effect times the square of the secondary isotope effect, i.e. PS^2, while the value of the observed isotope effect for any of the d_2 or d_4 substrates is a composite of a primary isotope effect divided by a secondary isotope effect, i.e. P/S.

The calculation of individual values of P and S requires that the observed isotope effect, $(k_H/k_D)_{obs}$, equals the intrinsic isotope effect, k_H/k_D. While this appears to be the case for *o*- and *p*-xylene-α-$^2H_3$, it is not for dimethylbiphenyl-α-$^2H_3$. To be able to determine P and S for dimethylbiphenyl, a surrogate was needed that expresses what k_H/k_D would be, if it could be measured. Because of the similarity in structures, and because $(k_H/k_D)_{obs}$ fully expresses k_H/k_D, *p*-xylene-α-$^2H_3$ was chosen as the surrogate for dimethylbiphenyl-α-$^2H_3$. The calculated values for P and S (Hanzlik et al., 1985) are presented in Table 4.

The reasonably constant values of P over all the substrates and enzyme preparations provides further support for the notion that the intrinsic isotope effect, k_H/k_D, for a given oxidative reaction is likely to be substantially independent of the specific P450 catalyzing the reaction (Bush & Trager, 1982; Gelb et al., 1982; Hanzlik et al., 1984; White et al., 1984; Jones et al., 1990). The magnitude of k_H/k_D, 5−7, was found to be significantly less than that determined for the terminal methyl group hydroxylation of *n*-octane, a reaction that is known to proceed with a symmetrical transition state (Jones & Trager, 1987). A probable reason for the lower intrinsic isotope effects for these substrates compared to that for *n*-octane is their ability to resonance stabilize the transition state. Classical resonance stabilization is impossible for aliphatic oxidation. This means that k_H/k_D for benzylic hydroxylation is inherently smaller than k_H/k_D for the methyl hydroxylation of *n*-octane and its value would appear on the ascending slope of the bell-shaped curve defining the relationship between the magnitude of k_H/k_D and reaction coordinate symmetry (Melander & Saunders, 1980).

In conclusion, we have demonstrated that the distance between symmetrically disposed but isotopically distinct metabolic sites must be considered in the design of an intramolecular isotope effect experiment. If the distance is large enough, equilibration between the two sites will not be reached and the magnitude of the observed isotope effect will be less than the intrinsic isotope effect. As stated earlier, the intrinsic isotope effect must be determined if deductions about transition state structure are to be made and if meaningful mechanistic interpretations are to be reached. However, masking effects are not without value. They offer a methodology to gain valuable insight into the active site constraints of the enzyme. The observation that $(k_H/k_D)_{obs}$ for *p*-xylene-α-$^2H_3$ is essentially equivalent to k_H/k_D for all

enzymes preparations suggests that the minimal distance of 6.62 Å between carbon atoms of the two para methyl groups is not sufficiently large, nor are the active sites of the enzymes studied restrictive enough, to slow the rate of methyl group equilibration to a level at which masking begins to appear. In contrast, the 11.05 Å between the carbon atoms of the two para methyl groups of *p*,*p*′-dimethylbiphenyl is sufficiently large and/or the active sites of the enzymes involved are restrictive enough to slow the rate of methyl group equilibration to a level at which masking is significant. Thus, differences of 6.62 and 11.05 Å between isotopically sensitive sites would appear to be sufficient to convert a fully expressed intrinsic isotope effect to one that is almost completely masked.

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