Cytochrome P450-Catalyzed Hydroxylation of Hydrocarbons: Kinetic Deuterium Isotope Effects for the Hydroxylation of an Ultrafast Radical Clock[†,‡]

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ABSTRACT: The ultrafast radical clock probe *trans*-1-methyl-2-phenylcyclopropane (**1**CH_3) and its mono-, di-, and trideuteriomethyl analogues were oxidized by phenobarbital-induced rat liver microsomal enzymes. This cytochrome P450-catalyzed hydroxylation of **1**CH_3 gave three products: the alcohol *trans*-(2-phenylcyclopropyl)methanol (**2**), the rearranged alcohol 1-phenylbut-3-en-1-ol (**3**), and the phenol *trans*-2-(*p*-hydroxyphenyl)-1-methylcyclopropane (**4**). The identification of both the unrearranged and rearranged products of oxidation, **2** and **3**, is consistent with the formation of a radical intermediate via a hydrogen atom abstraction from the methyl group by the catalytically active iron–oxo center. Hydroxylation of three deuteriomethyl forms of **1**CH_3 produced the analogous deuterated products, although in different amounts of each. Perdeuteration of the methyl group (**1**CD_3) disfavored oxidation at the methyl group and caused an increase in the oxidation of the phenyl ring (metabolic switching). By comparing the amounts of alcohols and phenol formed from the individual, noncompetitive oxidation of **1**CH_3 and **1**CD_3, the overall (i.e., combined primary and secondary) deuterium kinetic isotope effect (DKIE) was found to be 12.5. *Intra*molecular DKIEs for **1**CHD_2 and **1**CH_2D were 2.9 and 13.2, respectively. From these results, the primary and secondary DKIEs were calculated to be 7.87 and 1.26, respectively, values that indicate that there is extensive C—H bond stretching in the transition state for the rate-controlling step in P450-catalyzed hydroxylation of **1**CH_3. Microsomal incubations performed with optically enriched samples of (*R,R*)-**1**CH_3 and (*S,S*)-**1**CH_3 and with pseudoracemates composed of equal amounts of an unlabeled enantiomer and a d_3-labeled enantiomer showed that there was a low selectivity for the oxidation of the (*R,R*) enantiomer and only a small difference in the regioselectivity between the two enantiomers for oxidation of the methyl groups vs the aromatic ring.

It is now widely accepted that during the cytochrome P450-catalyzed hydroxylation of alkanes, an iron–oxo species (Fe^{V}=O) abstracts a hydrogen atom from the substrate to generate an intermediate carbon-centered radical (Akhtar & Wright, 1991; Groves, 1985; Ortiz de Montellano, 1987, 1989; White, 1991). The intermediate radical then combines with an iron-bound hydroxyl radical to form an alcohol in a process termed "oxygen-rebound" (Groves, 1985; Groves *et al.*, 1978). Our laboratories have recently reported the calibration of a series of methylcyclopropane radical clocks (Bowry *et al.*, 1989, 1990; Griller & Ingold, 1980; Newcomb *et al.*, 1991, 1992; Newcomb & Manek, 1990) and their use in the investigation of the hydroxylation of alkanes by microsomal cytochrome P450 from rat livers (Atkinson & Ingold, 1993; Bowry & Ingold, 1991). It was evident from the later work (Atkinson & Ingold, 1993) that when methylcyclopropanes were oxidized by P450, only those substrates that yielded intermediate cyclopropylcarbinyl radicals that underwent ring opening with rate constants $k_r \gtrsim 2 \times 10^9$ s^{-1} gave both the unrearranged cyclopropylmethanols and the rearranged, ring-opened alkenols. This is illustrated for the radical clock substrate *trans*-1-methyl-2-phenylcyclopropane (**1**CH_3) in Figure 1. The rearrangement of **1**CH_2· to form **3**· (see Scheme 1) is very fast ($k_r \sim 4 \times 10^{11}$ s^{-1}) and hence is highly competitive with oxygen-rebound; for this reason the rearranged alcohol, **3**, could easily be detected. However, for those substrates yielding cyclopropylcarbinyl radicals for which $k_r \lesssim 2 \times 10^9$ s^{-1}, oxygen-rebound occurs so much more rapidly than the radical rearrangement that cyclopropylmethanols are formed exclusively (Atkinson & Ingold, 1993).

For cyclopropylcarbinyl radicals that undergo rapid ring opening at known rates, the molar ratio of the unrearranged product alcohol to rearranged product alcohol (e.g., **2**/**3**) formed by oxidation of the parent methylcyclopropane allows the rate constant for oxygen-rebound, k_{OH}, to be calculated. The magnitude of k_{OH} would appear to be substrate dependent (Atkinson & Ingold, 1993). Thus, the hydrocarbons hexamethylcyclopropane and 1,1,2,2-tetramethylcyclopropane, each of which forms a primary alkyl radical after hydrogen abstraction, both gave a calculated value for k_{OH} of $(2.4 \pm 0.1) \times 10^{11}$ s^{-1}, whereas the fused bicyclic system, bicyclo[2.1.0]pentane, which forms a secondary alkyl radical after hydrogen abstraction, gave $k_{OH} = 1.4 \times 10^{10}$ s^{-1}. Intriguingly, the ultrafast radical clocks, **1**CH_3, and 2,2-diphenyl-1-methylcyclopropane, generated the remarkable values for k_{OH} of 1.5×10^{12} and 7×10^{12} s^{-1}, respectively. These values for k_{OH} are getting very close to the maximum possible value of

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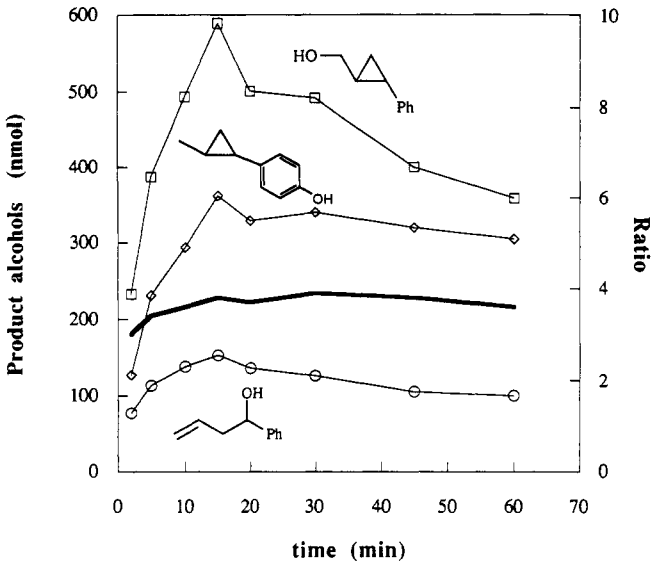


FIGURE 1: Hydroxylation product profiles for the rat liver microsomal oxidation of *trans*-1-methyl-2-phenylcyclopropane (**1**CH_3). The bold line is the ratio of the two product alcohols: *trans*-(2-phenylcyclopropyl)methanol (**2**) and the rearranged alcohol, 1-phenylbut-3-en-1-ol (**3**).

ca. 10^{13} s^{-1} (which is dictated by oxygen-rebound occurring on the first vibration of the $Fe^{IV}OH\cdots R^{\cdot}$ couple). Atkinson and Ingold (1993) have therefore suggested that the high rate constants found for oxygen-rebound with these two substrates are artifacts that arise because both of these ultrafast radical clocks may have run slow when bound within the pocket of the enzyme's active site. That is, the rate constants for ring opening of these phenyl-substituted cyclopropylcarbinyl radicals may have been reduced below those measured in homogeneous solution, because spatial restrictions in the enzyme's active site may not have allowed the phenyl group(s) to become optimally oriented so as to provide their full potential resonance stabilization to the new radical center, which is developing as the cyclopropane ring opens. As a consequence, the true values of k_{OH} for **1**CH_3 and 2,2-diphenyl-1-methylcyclopropane could be lower by up to 3 orders of magnitude than the values given here, which were calculated by assuming that the radicals' ring openings occurred with the same rate constants in the enzyme's active site pocket as in homogeneous solution (Atkinson & Ingold, 1993). However, regardless of the values of k_{OH}, it is important to note that the oxidations appear to proceed through radical intermediates, since both the unrearranged and rearranged product alcohols were formed.

The results for the P450-catalyzed oxidation of **1**CH_3 stand in sharp contrast to those of the oxidation of **1**CH_3 by the non-heme monooxygenase from *Pseudomonas oleovorans* (Fu *et al.*, 1991), where only the rearranged alcohol 1-phenylbut-3-en-1-ol (**3**) was obtained. The variety of behavior that can be observed when **1**CH_3 is oxidized by monooxygenases is further illustrated by the fact that the methane monooxygenase (MMO) from *Methylococcus capsulatus* (Bath) gave an equal amount of the unrearranged alcohol *trans*-(2-phenylcyclopropyl)methanol (**2**) and the phenol **4** (formed by oxidation of the aromatic ring) but no rearranged alcohol **3**, whereas the MMO from *Methylosinus trichosporium* OB3b gave mostly unrearranged alcohol **2** and a small amount (3–5%) of the rearranged material **3** (Liu *et al.*, 1993).

From the foregoing, it would appear that there may be significant mechanistic differences between hydroxylations catalyzed by bacterial monooxygenases and those catalyzed by cytochrome P450. We report herein a study on the oxidation of mono-, di-, and trideuteriomethyl analogues of **1**CH_3, which was undertaken in the hope of gaining a more detailed appreciation of some of the mechanistic subtleties involved in the P450-catalyzed oxidation of **1**CH_3. Our results have allowed us to calculate the magnitudes of the primary and secondary deuterium kinetic isotope effects (DKIEs) for attack of the iron–oxo center on the methyl group of **1**CH_3. The oxidation of the optically enriched antipodes of **1**CH_3 and the regioselectivity thereof also have been examined.

EXPERIMENTAL PROCEDURES

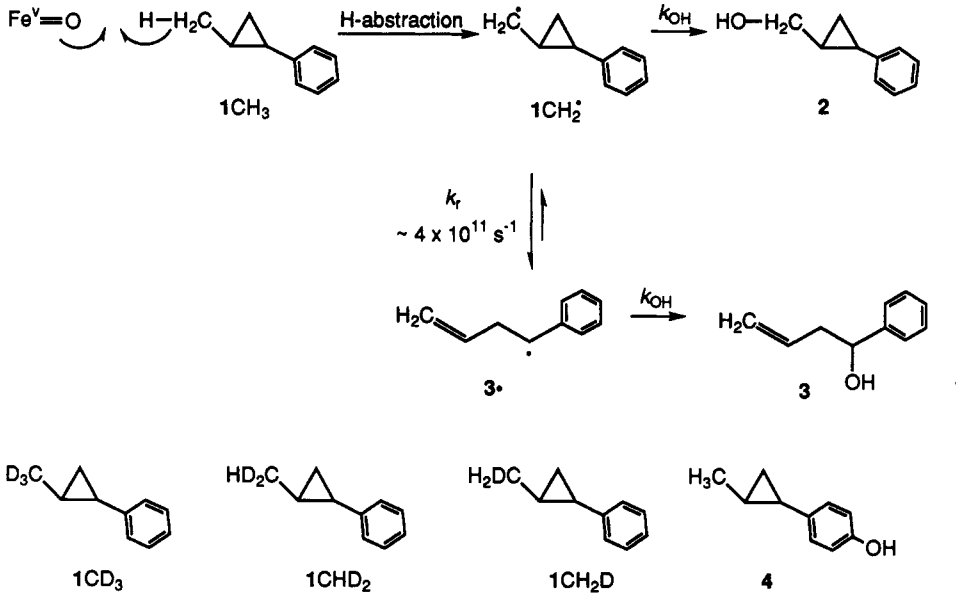
Instrumentation. Product analyses at NRCC were performed on a FID-equipped HP5890 Series II gas chromatograph with a cross-linked methyl silicon column (HP-1, 10 m × 0.22 mm i.d.). The carrier gas was helium, and the temperature program was 90 °C for 4 min and then 15 °C/min to 220 °C for the oxidation products of *trans*-1-methyl-2-phenylcyclopropane (**1**CH_3). Mass spectral analyses were done using a Hewlett-Packard HP5890 instrument and a cross-linked methyl silicon column (HP-1, 12 m × 0.2 mm i.d.) interfaced with an HP 5970 (EI, 70 eV) mass selective detector. Selected ion mass spectra (SIMS) were recorded spanning the expected molecular weights of the protiated and deuterated products. The dwell time on each peak was 30 ms, which allowed the accumulation of about 15 spectra for each ion over a single chromatographic peak.

Analyses at WSU were accomplished on FID-equipped Varian 3400 chromatographs and on an HP5890 chromatograph interfaced to an HP5971 mass selective detector. A variety of columns were employed; superior resolutions were obtained on bonded phase Carbowax columns (15 m × 0.53 mm i.d. capillary column for FID quantitation and 15 m × 0.25 mm i.d. capillary column for MS analysis).

Syntheses. The synthesis of *trans*-1-methyl-2-phenylcyclopropane (**1**CH_3) was described previously (Fu *et al.*, 1991), as were the syntheses of the mono-, di-, and trideuteriomethyl-substituted analogues and the phenol, *trans*-1-methyl-2-(4-hydroxyphenyl)cyclopropane (**4**) (Liu *et al.*, 1993).

Optically enriched samples of the probe substrate were obtained by resolution of *trans*-2-phenylcyclopropanecarboxylic acid (Aldrich Chemical). The resolved acids were converted into the probe substrates by methods previously described (Liu *et al.*, 1993). In a typical resolution, methyl *trans*-2-phenylcyclopropanecarboxylate (10.0 g, 56.8 mmol) was added to a solution containing 5.0 g of crude P-30 lipase (Amano International Enzyme Co., from *Pseudomonas cepacia*, formerly classified as *Pseudomonas fluorescens*) (Xie, 1991) in 200 mL of 0.1 M aqueous potassium phosphate buffer (pH 7.5), and the mixture was stirred at room temperature. The progress of the enzymatic hydrolysis was monitored by removing a 1-mL aliquot from the mixture, adding this to 1 mL of a stock solution of ca. 5% benzophenone in toluene, and analyzing the resulting organic phase by GC to determine the ester to benzophenone ratio. At ca. 40% conversion of the ester (2 days), the reaction mixture was treated with 200 mL of CH_2Cl_2 and 10 mL of 3 N aqueous HCl solution. The mixture was filtered through a pad of Celite, the phases were separated, and the aqueous phase was extracted twice with CH_2Cl_2. The combined organic phases were extracted twice with aqueous 10% NaOH solution, washed with saturated aqueous NaCl solution, and dried over $MgSO_4$. Filtration and concentration of this solution gave 4.4 g (25.0 mmol) of ester. The basic extracts were acidified with HCl and extracted

Scheme 1



with CH_2Cl_2, and the resulting organic solution was washed with saturated aqueous NaCl solution and dried over $MgSO_4$. Filtration and concentration of this solution gave 4.2 g (25.9 mmol) of the acid as a viscous oil. The isolated acid had $[\alpha]^{25}_D$ = +294° (c = 1.95, $CHCl_3$), indicating 77% optical purity (op) (*S,S*) acid (Berson *et al.*, 1976). The recovered ester was saponified with KOH in MeOH, and the recovered acid had $[\alpha]^{25}_D$ = −276° (c = 2.20, $CHCl_3$) or 72% op (*R,R*) acid (Berson *et al.*, 1976). Further resolution of the ca. 75% op acids, both (+) and (−), was achieved by recrystallization of the dehydroabietylamine salts by the method of Berson *et al.*, (1976) ultimately to yield acids of 94–97% op. The acids used for the syntheses of (*S,S*)-**1**CH_3, (*R,R*)-**1**CH_3, (*S,S*)-**1**CD_3, and (*R,R*)-**1**CD_3 had $[\alpha]^{25}_D$, (c, $CHCl_3$), and op, respectively, as follows: +358.6° (2.04) 94.1%; −363.6° (2.03) 95.4%; +361.8° (2.00) 95.0%; −368.8° (2.03) 96.8%.

Pseudoracemic mixtures of *trans*-1-methyl-2-phenylcyclopropane were prepared by mixing equal amounts of a **1**CD_3 enantiomer and a **1**CH_3 enantiomer.

Microsomal Oxidations. At the NRCC, microsomes were prepared from phenobarbital-treated rats (80 mg/kg body weight) following the general methods described earlier (Ortiz de Montellano *et al.*, 1981). The microsomes were stored at −80 °C in small aliquots to avoid multiple freeze/thaw cycles. The concentration of P450 in the microsomes was determined following the method of Estabrook *et al.* (1972). As prepared, the microsomes represented 1.2–1.5 g of liver/mL of final suspension and generally contained 20–30 nmol of P450/mL. Experimental details for substrate incubations with microsomes and extraction of products have been described previously (Atkinson & Ingold, 1993). Briefly, for a typical experiment in which four time points (2, 5, 10, and 15 min) and a blank were necessary, 1.20 mL of microsomes was diluted with 10.05 mL of buffer (50 mM sodium phosphate, 1 mM desferrioxamine, and 0.075 M KCl, pH 7.4) in a 20-mL scintillation vial and chilled in ice. Ten microliters of the neat alkane was added, and the mixture was allowed to equilibrate for 10–20 min with occasional swirling by hand. The vial was then placed in a thermostatted water bath at 37 °C and equilibrated for 2 min. The reaction was initiated by the addition of a solution of 10 mg of NADPH in 0.75 mL of buffer. At the moment of initiation, the concentration of NADPH was 1.2 mM, the P450 was 2.5–3.0 nmol/mL (a 10-fold dilution of stock microsomes), and the substrate was approximately 5 mM, in a total volume of 12 mL. At each time point, 2 mL of the reaction mixture was removed, cyclooctanol (400 nmol) was added as an internal standard, and the mixture was extracted with 3 × 2 mL of CH_2Cl_2.

For oxidations conducted at WSU, microsomes were prepared from the livers of inbred Fischer 344 male rats (8–10 weeks old), which were fed rodent lab chow and water *ad libitum*. These rats were pretreated with phenobarbital (0.1% in drinking water). The microsomes were suspended in 100 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA and 20% glycerol. They were stored at −70 °C. Reaction mixtures of 2.0 mL total volume in 5-mL round-bottomed flasks were prepared containing 5 nmol of microsomal cytochrome P450 and ca. 2 mg (15 μmol) of substrate **1**CH_3 in 50 mM potassium phosphate buffer, pH 7.4, containing 1 mM desferrioxamine and 0.075 M KCl. The reaction mixtures were preincubated at 37 °C in a shaking water bath, and the reaction was initiated by the addition of 110 μL of 22 mM NADPH solution in buffer (the final concentration of NADPH was 1.2 mM). The reaction mixtures were incubated at 37 °C for varying lengths of time and terminated by placing the reaction flasks on ice and adding 50 μL of 0.5 M aqueous sodium dodecyl sulfate solution. The reaction mixture was stirred via a vortex stirrer for 0.5 min and then mixed with 2.0 mL of CH_2Cl_2. The mixture was transferred to a test tube, stirred with a vortex stirrer for 1 min, and centrifuged at 2000 rpm for 10 min on a clinical centrifuge. The organic layer was removed, and the extraction procedure was repeated twice. The combined organic extracts were dried over $MgSO_4$, and a standard was added (100 μL of a solution containing 20 mg of hexadecane in 10 mL of EtOH). The mixtures were concentrated under a gentle stream of nitrogen and analyzed by GC.

Intermolecular Competitive Incubations. For the determination of an intermolecular competitive isotope effect where both labeled and unlabeled substrates were present in the same incubation, approximately equal volumes of **1**CH_3 and **1**CD_3 were added to a small tube using a positive displacement microsyringe. An equal volume of ethanol (20 μL) was added, and the contents of the tube were thoroughly mixed by vortexing. A small sample of this mixture was examined by GC/MS to determine the exact **1**CH_3/**1**CD_3 ratio. The

remaining portion of the solution was added to the diluted microsomes in the usual manner. On completion of the oxidations and extractions, the ratios of deuterated and nondeuterated products were corrected for the relative abundance of the reactants in the initial mixture.

Noncompetitive Assay (Metabolic Switching). For the noncompetitive experiments, microsomes were diluted and then divided into two equal portions. Equal volumes of **1**CH_3 and **1**CD_3 substrates (10 μL) were added to each portion using the microsyringe. All other manipulations were as described.

Determination of Observed Isotope Effects. Observed isotope effects were obtained by comparing the molecular ion intensities of the appropriately deuterated *trans*-(2-phenylcyclopropyl)methanols. The nondeuterated material has molecular mass 148.20 Da, the monodeuterated 149.21 Da, and the dideuterated 150.22 Da. The ion intensities were also corrected for the isotopic abundance of ^{13}C in (M − 1) peaks.

RESULTS AND DISCUSSION

We will analyze the deuterium kinetic isotope effect (DKIE) found for the microsomal P450-catalyzed oxidation of **1**CH_3 and its deuterated derivatives using the original, simple formalism developed by Hanzlik *et al.* (1985). Implicit in this simple formalism, although not in Hanzlik's later, more complete formalism (Hanzlik & Ling, 1990, 1993), is the reasonable assumption that primary and secondary isotope effects contribute independent of one another. For a freely rotating methyl or partially deuterated methyl group, a simple primary DKIE is represented by $P = k_H^{HH}/k_D^{HH}$, a simple α-secondary DKIE is represented by $S = k_H^{HH}/k_H^{HD}$, where the subscript to each rate constant, k, identifies the atom (H or D) that is removed from the methyl group, and the two superscripts identify the two atoms that remain attached to the carbon atom. For the monodeuterated compound **1**CH_2D, the product ratio, r_1, is related to the statistically corrected ratios of the specific rate constants for the bond cleavages indicated by the dashes associated with eq 1:

$$r_1 = \frac{\textbf{2}\text{-}d_1 + \textbf{3}\text{-}d_1}{\textbf{2}\text{-}d_0 + \textbf{3}\text{-}d_0} = \frac{2k_H^{HD}}{k_D^{HH}} = \frac{2(\textbf{1}\text{CHD–H})}{(\textbf{1}\text{CHH–D})} \quad (1)$$

Similarly, for the dideuterated compound, **1**CHD_2, we obtain:

$$r_2 = \frac{\textbf{2}\text{-}d_2 + \textbf{3}\text{-}d_2}{\textbf{2}\text{-}d_1 + \textbf{3}\text{-}d_1} = \frac{k_H^{DD}}{2k_D^{HD}} = \frac{(\textbf{1}\text{CDD–H})}{2(\textbf{1}\text{CHD–D})} \quad (2)$$

In these equations, **2**-d_0, **2**-d_1, and **2**-d_2 refer to the yields of undeuterated and mono- and dideuterated unrearranged alcohols, respectively, and **3**-d_0, **3**-d_1, and **3**-d_2 refer to the yields of the corresponding rearranged alcohols. Some simple algebraic manipulations of these two equations (Hanzlik *et al.*, 1985) lead to eqs 3 and 4:

$$r_1 = \frac{2P}{S} \quad (3)$$

$$r_2 = \frac{P}{2S} \quad (4)$$

The observed *intra*molecular DKIEs for the oxidation of **1**CH_2D and **1**CHD_2 were

$$r_1 = 13.2 \pm 0.2 \quad \text{i.e., } P = 6.6S \quad (5)$$

$$r_2 = 2.9 \pm 0.4 \quad \text{i.e., } P = 5.8S \quad (6)$$

two results that are in rather gratifying numerical agreement.

In order to determine the absolute magnitudes of the primary and secondary DKIEs (rather than their ratios, cf. eqs 5 and 6), a different kind of *intra*molecular competition is required. One technique is to use a tritiated substrate and analyze the tritium content in the oxidation products (Northrop, 1982), followed by application of the normal relationship between deuterium and tritium isotope effects (Swain *et al.*, 1958). This technique has been employed successfully to estimate primary DKIEs for various P450-catalyzed oxidations (Guengerich *et al.*, 1988; Miwa *et al.*, 1984). However, it should be noted that the tritium method will yield true, primary DKIEs for only two classes of substrates. In the first class are those substrates that contain two or more equivalent hydrogen atoms (a small portion of which are monotritiated) at the reaction site. In the second class are those substrates that, although they contain only a single hydrogen atom (partially tritiated) at the reaction site, also exhibit isotopically sensitive branching or metabolic switching of the reaction (Atkins & Sligar, 1987; Guengerich *et al.*, 1988; Harada *et al.*, 1984; Jones *et al.*, 1986; Jones & Trager, 1987; Korzekwa *et al.*, 1989). The phenomenon of metabolic switching is dependent (generally) upon there being two (or more) oxidizable sites, not necessarily equivalent, within the molecule under study. If the P450-catalyzed oxidation at one site involves significant C—H bond stretching in the transition state, then tritiation or, for that matter, deuteration (particularly perdeuteration, when this is possible) at this site will decrease the rate at which this site is oxidized. Since the substrate cannot escape from the enzyme once the enzyme has been activated (Harada *et al.*, 1984), i.e., it cannot escape from the P450 Fe^V=O intermediate, the rate of oxidation at the other site(s) in the substrate must increase. Indeed, the very observation of an *inter*molecular DKIE of significant magnitude (i.e., $k_H/k_D \gtrsim 2$) in a P450-catalyzed oxidation implies that there must be another metabolite (Gillette & Korzekwa, 1991). This usually arises from oxidation at another site within the substrate, but it can also arise from a reduction of oxygen to hydrogen peroxide or water.

For the P450-catalyzed oxidation of **1**CH_3, we were fortunate in having an isotopically sensitive metabolic switch. That is, in addition to the two alcohols, **2** and **3** (which were identified by mass spectrometry and cochromatography with authentic standards), there was a third product of the oxidation (see Figure 1). This third product was again identified by mass spectrometry (M^+, *m/z* 148) and cochromatography with an authentic standard sample of *trans*-1-methyl-2-(4-hydroxyphenyl)cyclopropane (**4**). The formation of **4** provides the internal calibration needed to derive the *true* primary and secondary DKIEs for the reaction under study, since it allows the yields of alcohols produced in matched P450-catalyzed oxidations of **1**CH_3 and **1**CD_3 to be normalized (with the reasonable assumption that the rate at which the aromatic ring is hydroxylated is unlikely to be influenced by perdeuteration of the methyl group). Thus, we have

$$r_3 = \frac{[(\textbf{2}\text{-}d_0 + \textbf{3}\text{-}d_0)/\textbf{4}\text{-}d_0]}{[(\textbf{2}\text{-}d_2 + \textbf{3}\text{-}d_2)/\textbf{4}\text{-}d_3]} = \frac{k_H^{HH}}{k_D^{DD}} = \frac{(\textbf{1}\text{CHH–H})}{(\textbf{1}\text{CDD–D})} \quad (7)$$

and algebraic manipulation yields:

$$r_3 = PS^2 \quad (8)$$

Table 1: Yields of Hydroxylated Products (nmol) from the Matched Microsomal Enzyme-Catalyzed Oxidations of Racemic *trans*-1-Methyl-2-phenylcyclopropane ($1CH_3$) and Racemic *trans*-1-(Trideuteriomethyl)-2-phenylcyclopropane ($1CD_3$)[a]

time (min)	2	3	4	total product (nmol)	(2 + 3)/4
			Racemic $1CH_3$		
2	136.9 (2.7)	50.2 (1)	86.6 (1.7)	274	2.16
5	197.2 (3.0)	65.8 (1)	135.2 (2.0)	398	1.95
10	314.4 (3.6)	86.9 (1)	216.4 (2.5)	618	1.85
15	300.9 (3.7)	82.0 (1)	225.5 (2.7)	608	1.70
av[b]	**(3.3)**	**(1)**	**(2.2)**		**1.92**

time (min)	2-d_2	3-d_2	4-d_3	total product (nmol)	(2-d_2 + 3-d_2)/ 4-d_3
			Racemic $1CD_3$		
2	23.5 (2.4)	9.6 (1)	221.2 (23.0)	254	0.150
5	42.6 (3.4)	12.7 (1)	342.5 (27.0)	398	0.161
10	62.1 (3.5)	18.0 (1)	499.3 (27.7)	580	0.160
15	60.4 (3.3)	18.1 (1)	535.4 (29.6)	614	0.147
av[b]	**(3.2)**	**(1)**	**(26.8)**		**0.154**

[a] Relative product yields, 2/3, 4/3, 2-d_2/3-d_2, and 4-d_3/3-d_2, are given in parentheses. [b] The foldface numbers are the averages of the ratios of the products.

The products obtained during matched P450-catalyzed oxidation of $1CH_3$ and $1CD_3$ are given in Table 1. There would appear to be a slight tendency for the 2/3, 4/3, 2-d_2/3-d_2, and 4-d_3/3-d_2 ratios to increase with the length of the incubation period. This suggests that there may be some small *relative* loss of the ring-opened alcohols, 3 and 3-d_2, over 15 min. However, these trends are not large, and in view of the difficulties involved in these experimental measurements and the associated uncertainties we have decided to calculate the true primary and secondary DKIEs using averaged data (rather than trying to extrapolate to zero time). Thus, from Table 1 and eq 8, we have

$$(r_3)_{av} = PS^2 = 1.92/0.154 = 12.5 \quad (9)$$

and taking the mean of eqs 5 and 6, i.e.,

$$P = 6.2S$$

we obtain $P = 7.87$ and $S = 1.26$.

The primary and secondary DKIEs found in this work for the microsomal P450-catalyzed oxidations of the methyl group in $1CH_3$ are very similar to those reported for the oxidation of methyl groups (labeled with 0–3 deuterium atoms and using various P450 isozymes) in the following substrates: *n*-octane, $P = 7.61$–9.18, $S = 1.09$–1.14 (Jones & Trager, 1987); $P = 7.69$–9.18, $S = 1.13$–1.25 (Jones *et al.*, 1990); toluene, $P = 4.59$–7.30, $S = 0.95$–1.27 (Hanzlik & Ling, 1990); *o*-xylene, $P = 5.25$–7.20, $S = 1.15$–1.44, *p*-xylene, $P = 5.58$–7.87, $S = 0.87$–1.19 (Hanzlik & Ling, 1993). Perhaps this similarity is not too remarkable, but it does imply that the fact that the $1CH_2$· radical can rearrange whereas the *n*-octyl radical and the three benzylic radicals cannot does not significantly affect the degree of C—H bond stretching or the degree of carbon-centered radical formation in the transition states for these two reactions. This provides some justification for the use of the radical clock technique (Griller & Ingold, 1980) to probe P450-catalyzed alkane oxidation mechanisms and kinetics.

In earlier work, the P450-catalyzed oxidation of $1CH_3$ gave a ratio of unrearranged to rearranged alcohols (2/3) of 3.8/1 for microsomal P450 and 3.0/1 for reconstituted P450 2B4 (Atkinson & Ingold, 1993). These values are agreeably bridged by the averaged ratios of 3.3/1 found in the present work for $1CH_3$ and 3.2/1 found for $1CD_3$ in separate, but matched, noncompetitive incubations (see Table 1). This illustrates the intuitively obvious fact that the chemistry that occurs *after* radical formation does not depend significantly on whether the radical is centered on a CH_2· or a CD_2· moiety. Furthermore, it illustrates that the diminished yields of 2 and 3, which were formed from $1CD_3$ relative to $1CH_3$, and metabolic switching had no effect on the chemistry of the radical clock itself. Thus, the data obtained with $1CH_3$ and $1CD_3$ are wholly consistent with the intermediate formation of the carbon-centered radical, $1CH_2$·, followed by a fast oxygen-rebound step.

It can also be seen from Table 1 that the total yields of products 2, 3, and 4 were the same at each time point for the matched oxidations of $1CH_3$ and $1CD_3$ (within experimental error). This means that the metabolic shift induced by deuterium was a shift from the alcohols 2 and 3 to only the phenol 4. That is, catalytic oxidation proceeds with the same degree of efficiency for $1CH_3$ and $1CD_3$, which suggests that there are no undetected products. (The pseudoracemate oxidation discussed below confirms that the labeled and unlabeled substrates were oxidized to similar extents.) These results are consistent with the expectation (mentioned above) that once the substrate is bound to the enzyme and the iron has been activated, the substrate cannot leave the active site until reaction has occurred (Harada *et al.*, 1984). That is, for the P450-catalyzed oxidation of $1CH_3$, the commitment to catalysis (Northrop, 1982) must be close to 100%.

All of our results are consistent with the rapid-equilibrium kinetic model, which has been developed to interpret DKIEs obtained by the analysis of metabolic switching in P450-catalyzed oxidations (Gillette & Korzekwa, 1991). In this model, the substrate first binds reversibly with the enzyme, and once it is in the active site, it exists in a rapid equilibrium between several orientations with respect to the catalytic iron center. However, once the iron has been irreversibly activated (formation of Fe^{V}=O), only the orientational equilibrium remains, and the substrate cannot leave the active site until deactivation has occurred (formation of Fe^{III}, usually with the concomitant formation of a hydroxylated organic compound).

The experiments described thus far utilized *racemic* $1CH_3$. In order to discover whether microsomal P450 discriminated between the two enantiomers, (*R*,*R*)-$1CH_3$ in 72% (86/14) and (*S*,*S*)-$1CH_3$ in 77% (88.5/11.5) optical purity were studied at the NRCC. In a matched pair of experiments, each enantiomerically enriched mixture was subjected to P450-catalyzed oxidation following the usual procedures. The total yields of products formed from the two mixtures after 1, 2, 5, and 10 min of reaction were essentially equal within experimental error (e.g., 435 and 406 nmol at 10 min for the (*R*,*R*) and (*S*,*S*) enriched mixtures, respectively). This means that phenobarbital-induced rat liver microsomal P450 has little or no enantioselectivity in its oxidation of $1CH_3$. However, the enzyme did exhibit a slightly different regioselectivity in these two oxidations. For the (*R*,*R*)-$1CH_3$ enriched sample, the averaged 2/3/4 ratios were 4.2/1/2.7 so that the (2 + 3)/4 ratio was 1.94, whereas for the (*S*,*S*)-$1CH_3$ enriched sample the corresponding averaged ratios were 3.7/1/4.4 and 1.09. Thus, oxidation of the aromatic ring is favored relative to oxidation at the methyl group for the (*S*,*S*)-$1CH_3$ enriched sample. This enantiomerically induced example of metabolic switching is much less dramatic than the switching observed between (racemic) $1CH_3$ and (racemic) $1CD_3$. The difference in the regioselectivities between the

Table 2: Yields of Hydroxylated Products (nmol) from the Microsomal Enzyme-Catalyzed Oxidations of Racemic $1CH_3$ and Two Pseudoracemate Mixtures of a $1CD_3$ Enantiomer and a $1CH_3$ Enantiomer[a]

	group[b]	time (min)	2	3	4	total product (nmol)	(2 + 3)/4
racemic							
$1CH_3$	1[c]	5	137 (4.3)	32 (1)	101 (3.2)	270	1.67
		20	249 (4.9)	51 (1)	167 (3.3)	467	1.80
		40	270 (4.0)	68 (1)	194 (2.9)	532	1.74
		60	249 (5.0)	50 (1)	170 (3.4)	469	1.76
		av[d]	**(4.6)**	**(1)**	**(3.2)**		**1.74**
	2[e]	5	158 (4.5)	35 (1)	91 (2.6)	284	2.12
		20	281 (5.6)	50 (1)	130 (2.6)	461	2.55
	3[e]	30	101 (3.7)	27 (1)	86 (3.2)	214	1.49
	4[e]	5	91 (3.5)	26 (1)	76 (2.9)	193	1.54
		15	173 (4.1)	42 (1)	127 (3.0)	342	1.69
		30	224 (3.7)	60 (1)	149 (2.5)	433	1.91
		av[d]	**(4.2)**	**(1)**	**(2.8)**		**1.88**
pseudoracemate[f]							
(*S*,*S*)-$1CH_3$	5[e]	15	70 (1.6)	44 (1)	53 (1.2)	167 (177)[g]	2.15
(*R*,*R*)-$1CD_3$		15	30 (3.0)	10 (1)	109 (10.9)	149	0.37
(*S*,*S*)-$1CH_3$		30	68 (1.5)	45 (1)	49 (1.1)	162 (172)[g]	2.31
(*R*,*R*)-$1CD_3$		30	28 (2.5)	11 (1)	104 (9.5)	143	0.38
(*S*,*S*)-$1CH_3$		av[d]	**(1.6)**	**(1)**	**(1.2)**		**2.23**
(*R*,*R*)-$1CD_3$		av[d]	**(2.8)**	**(1)**	**(10.2)**		**0.38**
pseudoracemate[h]							
(*S*,*S*)-$1CD_3$	6[e]	15	16 (2.3)	7 (1)	125 (18)	148 (154)[g]	0.18
(*R*,*R*)-$1CH_3$		15	146 (2.0)	74 (1)	64 (0.86)	284	3.44
(*S*,*S*)-$1CD_3$		30	21 (3.0)	7 (1)	168 (24)	196 (204)[g]	0.17
(*R*,*R*)-$1CH_3$		30	184 (2.0)	90 (1)	85 (0.94)	359	3.22
(*S*,*S*)-$1CD_3$		av[d]	**(2.6)**	**(1)**	**(21)**		**0.18**
(*R*,*R*)-$1CH_3$		av[d]	**(2.0)**	**(1)**	**(0.9)**		**3.33**

[a] Relative yields, **2**/**3** and **4**/**3**, are given in parentheses. [b] Group numbers indicate sets of experiments conducted simultaneously. [c] NRCC aliquot method used. [d] The foldface numbers are the averages of the ratios of the products. [e] Batch isolation method used. [f] The mixture consisted of (*S*,*S*)-$1CH_3$ (94% ee) and (*R*,*R*)-$1CD_3$ (96% ee) in a 0.94/1.00 ratio. [g] The total yields for the (*S*,*S*) compounds have been normalized (values in parentheses) according to the initial ratios of substrate enantiomers. [h] The mixture consisted of (*S*,*S*)-$1CD_3$ (96% ee) and (*R*,*R*)-$1CH_3$ (95% ee) in a 0.96/1.00 ratio.

two enantiomeric mixtures is due to the switching of only ca. 14% of the total reaction from one site to the other, whereas for the $1CH_3/1CD_3$ pair, ca. 53% of the total reaction was switched from site to site.

Because the samples of (*R*,*R*)-$1CH_3$ and (*S*,*S*)-$1CH_3$ used to generate the foregoing results were of relatively low enantiomeric purity, and because of the low product yields (a total product yield of 400 nmol corresponds to the oxidation of only ca. 0.6% of the $1CH_3$ added to the system), there was obviously a possibility that only one of the two enantiomers was actually being oxidized by the P450. It was therefore decided to conduct experiments at WSU with pseudoracemic mixtures composed of approximately equal quantities of a $1CH_3$ enantiomer and the opposite $1CD_3$ enantiomer. The enantiomers used to prepare the pseudoracemic mixtures were of 94–96% ee. The results of these microsomal oxidations are given in Table 2, together with the results of oxidation studies at WSU on racemic $1CH_3$. In these experiments, a total product yield of 400 nmol corresponds to the oxidation of ca. 2.8% of the initial substrate. The product isolation procedure for one of the oxidations of racemic $1CH_3$ was the aliquot method used earlier at the NRCC, whereas the batch method of analysis was used for the remaining reactions (see Experimental Procedures section).

Comparison of the results in Table 2, which were obtained with *racemic* $1CH_3$ by the aliquot method (first entry, group 1) and by the batch method, reveals no significant differences between the two procedures. That is, the total product yields at a given time are rather similar. Furthermore, the averaged relative product yields and the averaged regioselectivities are quite similar. However, comparison of these Table 2 results within themselves and with those presented for *racemic* $1CH_3$ in Table 1 indicates that the total product yields at a given time depend on the particular experiment, i.e., presumably on the catalytic activity of the microsomes that were employed. It is clear from the data in Table 2 that the oxidations are generally complete within ca. 15 min and that thereafter the products are slowly lost (see also Figure 1). It should also be noted that the averaged **2**/**3**/**4** product ratios are not always identical for *racemic* $1CH_3$, viz., 3.3/1/2.2 from Table 1 and 4.6/1/3.2 and 4.2/1/2.8 from Table 2. This serves as a reminder that our results should not be over interpreted; these are difficult experiments and the measurement errors are larger than we would like. In contrast, the **(2 + 3)**/**4** regioselectivity ratios for *racemic* $1CH_3$ are remarkably similar, viz., 1.92 from Table 1 and 1.74 and 1.88 from Table 2.

For the pseudoracemate oxidations, the total yields of products **2**–**4** were determined by GC. The reaction mixtures were then treated with acetic anhydride and pyridine to convert the alcohols to acetates, which displayed larger relative M^+ peaks in their mass spectra than did the alcohols. The isotopic compositions of the products were determined by GC/MS by comparing the M^+ and $(M + 2)^+$ peaks for the acetates from **2** and **3** and the M^+ and $(M + 3)^+$ peaks for the acetate from **4**. By design, the enzyme activity must be the same for each enantiomer in a given experiment, but the two pseudoracemate oxidations were not matched in terms of the enzyme lot.

The results of the pseudoracemate oxidations in Table 2 indicate that the two enantiomers were oxidized at nearly the same rates with a small selectivity for oxidation of the (*R*,*R*) enantiomer and a small isotope effect, resulting in a reduced rate of oxidation of the $1CD_3$ enantiomers. The **2**/**3** ratios for the two enantiomers were comparable. For the $1CH_3$ substrates, both enantiomers were regioselectively oxidized at methyl, with the oxidation of the (*R*,*R*) enantiomer being slightly more selective in favoring this group, a result that is in agreement with the results obtained with the less enantiomerically pure samples employed initially (*vide supra*).

Comparison of the results *between* the two pseudoracemate oxidations (which are not matched experiments) suggests an apparent DKIE for the (*S,S*) enantiomer of 12 and an apparent DKIE for the (*R,R*) enantiomer of 9, values that are in reasonable agreement with the racemate value of 12.5 found in the matched oxidation experiments (see Table 1 and eq 9).

Conclusions. The large magnitude of the primary DKIE (7.87) indicates extensive C—H bond stretching in the transition state for hydroxylation of the methyl group in **1**CH_3. Similarly, the large magnitude of the secondary DKIE (1.26) indicates that the conversion of an sp^3-hybridized carbon in the reactant (CH_3) to the sp^2-hybridized carbon in the product ($^{\cdot}CH_2$) is well advanced in the transition state. These two DKIEs are fully consistent with the formation of a carbon-centered radical as a discrete intermediate in this P450-catalyzed hydrocarbon oxidation. The formation of a discrete carbon-centered radical is also indicated by the detection of the unrearranged (ring-closed) *and* rearranged (ring-opened) alcohols, **2** and **3**, and is further emphasized by the fact that, in matched experiments, the **2**/**3** ratio is equal within experimental error for **1**CH_3 and **1**CD_3, despite the large differences between these two substrates in the fraction of the overall reaction, which involves initial attack at the methyl group. Thus, our results provide further supporting evidence for Groves' oxygen-rebound mechanism for alkane hydroxylation by P450 (Groves, 1985; Groves *et al.*, 1978).

As a substrate, **1**CH_3 must be fairly mobile within the enzyme's active site since both the methyl group and the phenyl group are accessible to the catalytically active Fe^V=O species. This implies a fairly *large* and *loose* enzyme pocket. In contrast, Ortiz de Montellano and Stearns (1987) have shown that, in the P450-catalyzed oxidation of bicyclo[2.1.0]pentane, not only does hydrogen abstraction occur from the more congested *endo* face of the molecule but also oxygen rebound (with $k_{OH} = 1.4 \times 10^{10}$ s^{-1}; Atkinson & Ingold, 1993) occurs with retention of stereochemistry. This implies that the bicyclo[2.1.0]pent-2-yl radical is not very mobile in the P450 active site despite its small size relative to **1**CH_3, i.e., it implies a *small* and *tight* pocket. The most plausible explanation for these apparently divergent conclusions is that substrate rotation is fast relative to its rate of reaction with the iron–oxo species, but that rotation of the carbon-centered radical derived from the substrate is slow relative to the rate of oxygen rebound. The large magnitudes of the primary and secondary DKIEs for hydrogen atom abstraction from **1**CH_3 are *only* consistent with a relatively slow reaction of this substrate with Fe^V=O (rate constant probably $<10^6$ s^{-1}), whereas the lifetimes of the carbon-centered radicals derived from the various radical clocks employed range from a maximum of ca. 10^{-10} s for bicyclo[2.1.0]pent-2-yl to even shorter times ($<10^{-11}$ s) for all the other clocks.

Atkinson and Ingold (1993) suggested that the **1**$CH_2^{\cdot}$ radical clock rearrangement probably ran slow in the enzyme because steric constraints enforced an unfavorable orientation for overlap of the phenyl group's π electrons with the developing radical center (see introduction), and hence they attributed the dramatic variation in the *apparent* values of k_{OH} calculated using methylcyclopropane probes to the failure of the ultrafast, phenyl-substituted cyclopropylcarbinyl radicals to keep to time. The problem with these suggestions is that the postulated enzymatically induced retardation of the rate of ring opening of **1**$CH_2^{\cdot}$ must occur to a roughly similar extent with both enantiomers of this radical clock (see the **2**/**3** ratios for (*R,R*)- and (*S,S*)-**1**CH_3 given in Table 2). Although it is not easy to accept that the degree of twisting of the phenyl group from its optimum orientation for stabilization of the incipient benzylic radical should be so similar for the two enantiomeric **1**$CH_2^{\cdot}$ radicals, there would appear to be only two alternatives: first, that there is fairly substantial error in the measured rate constant for ring opening of **1**$CH_2^{\cdot}$, and second, that the generally accepted oxygen-rebound mechanism for P450-catalyzed hydroxylation of alkanes does not apply to **1**CH_3. Since neither of these alternatives is attractive, additional experiments are planned with some new cyclopropylcarbinyl ultrafast clocks having an accelerating substituent that is conformationally locked with respect to the cyclopropane ring.

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