Active Site Dynamics of Toluene Hydroxylation by Cytochrome P-450[1]

Robert P. Hanzlik* and Kah-Hiing John Ling

Department of Medicinal Chemistry, University of Kansas, Lawrence, Kansas 66045-2506

Received December 18, 1989

Rat liver cytochrome P-450 hydroxylates toluene to benzyl alcohol plus *o*-, *m*-, and *p*-cresol. Deuterated toluenes ($C_6D_5CH_3$, $C_6D_5CD_3$, $PhCD_nH_{3-n}$) were incubated under saturating conditions with liver microsomes from phenobarbital-pretreated rats, and product yields and ratios were measured. Stepwise deuteration of the methyl leads to stepwise decreases in the alcohol/cresol ratio without changing the cresol isomer ratios. Extensive deuterium retention in the benzyl alcohols from $PhCH_2D$ and $PhCHD_2$ suggests there is a large intrinsic isotope effect for benzylic hydroxylation. After replacement of the third benzylic H by D, the drop in the alcohol/cresol ratio was particularly acute, suggesting that metabolic switching from D to H within the methyl group was easier than switching from the methyl to the ring. Comparison of the alcohol/cresol ratio for $PhCH_3$ vs $PhCD_3$ indicated a net isotope effect of 6.9 for benzylic hydroxylation. From product yield data for $PhCH_3$ and $PhCD_3$, ^DV for benzyl alcohol formation is only 1.92, whereas ^DV for *total* product formation is 0.67 (i.e., inverse). These observations can be explained by postulating that product release is partly rate limiting in turnover when the product is benzyl alcohol but *not* when it is cresol; thus, deuterium-induced metabolic switching directs the substrate to a pathway (ring hydroxylation) that is intriniscally harder to enter but ultimately faster to traverse. Quantitative fitting of the data to a kinetic model of this situation supports these assumptions and indicates a net intrinsic kinetic isotope effect (k_H^{HH}/k_D^{DD}) of 7.4 for benzylic hydroxylation of toluene. From competitive incubations of $PhCH_3/PhCD_3$ mixtures $^D(V/K)$ isotope effects on benzyl alcohol formation and total product formation (3.6 and 1.23, respectively) are greatly reduced, implying strong commitment to catalysis. In contrast, $^D(V/K)$ for the alcohol/cresol ratio is 6.3, indicating that the majority of the intrinsic isotope effect is expressed through metabolic switching. Overall, these data are consistent with reversible formation of a complex between toluene and the *active oxygen form* of cytochrome P-450, which rearranges *internally* and reacts to form products faster than it dissociates back to release substrate; the benzylic position is more reactive than the aromatic ring, but release of benzyl alcohol is much slower than release of cresol (or its arene oxide precursor) and is partially rate limiting in turnover.

Of the various oxidation reactions catalyzed by cytochrome P-450 enzymes, benzylic hydroxylation is a particularly favorable process. Important examples include toluene,[2,3] the antioxidant BHT,[4] the analgesic alkaloid morphine,[5] and the carcinogen dimethylbenzanthracene.[6,7] For these otherwise quite diverse substrates it might logically be assumed that benzylic hydroxylation is favored over other possible reactions because of resonance stabilization of benzylic intermediates and the transition states leading to their formation (see Scheme I).

We have been keenly interested in testing this hypothesis through measurement of *secondary* kinetic deuterium isotope effects (DIEs) because such effects convey useful information about changes in hybridization geometry (e.g., sp^3 to sp^2; Scheme I) at the reaction center. We have focused on toluene as a simple prototype substrate for which benzylic hydroxylation is an important metabolic pathway.[2,8] Compared to primary DIEs, secondary DIEs are often small and thus more difficult to measure. Another significant challenge, experimentally, is to factor the *net* isotope effects observed with deuterated toluenes (e.g., $PhCD_nH_{3-n}$; Chart I) into their component primary and secondary effects. To this end we developed a model[8] that we thought could do this, but the model did not yield interpretable results when used to analyze data for hydroxylation of $PhCH_2D$ and $PhCHD_2$ by rat liver microsomal P-450. However, the model did appear to work well in three other situations, namely the free-radical chlorination and bromination of deuterated toluenes,[9] the P-450-catalyzed hydroxylation of *n*-octane,[10-13] and the (presumably P-450-mediated) fungal oxidation of deuterated toluenes.[14]

Scheme I. Abstraction-Recombination Mechanism for Cytochrome P-450

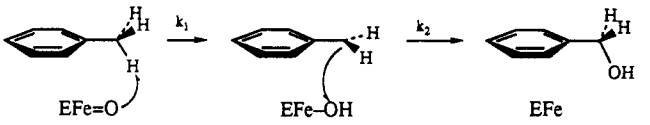
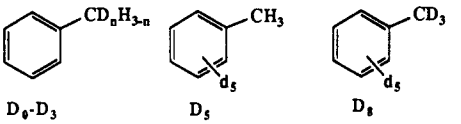


Chart I. Deuterated Substrates



In a further attempt to unravel and elucidate in detail the effects of deuteration on the mammalian P-450-catalyzed hydroxylation of toluene, we have now measured the effect of stepwise deuteration (viz. D_0–D_8, Chart I) on this process. With cytochrome P-450 from phenobarbital-induced rat liver microsomes the metabolites of toluene are benzyl alcohol and *p*-, *o*-, and *m*-cresol (ca. 65/15/15/5 for toluene-d_0).[2] As might have been expected, we find that

(1) Financial support for this study was provided in part by the National Institutes of Health (Grant GM-31910).

(2) Ling, K.-H. J.; Hanzlik, R. P. *Biochem. Biophys. Res. Commun.* **1989**, *160*, 844.
(3) Woiwode, W.; Wodarz, R.; Dupch, K.; Weichardt, H. *Arch. Toxicol.* **1979**, *43*, 93.
(4) Mizutani, T.; Yamamoto, K.; Tajima, K. *Toxicol. Appl. Pharmacol.* **1983**, *69*, 283.
(5) Krowech, G.; Caldera-Munoz, P. S.; Straub, K.; Castognoli, N., Jr.; Correia, M. A. *Chem. Biol. Interact.* **1986**, *58*, 29.
(6) Lamparczyk, H. S.; McKay, S.; Farmer, P. B.; Cary, P. D.; Grover, P. L.; Sims, P. *Xenobiotica* **1986**, *16*, 325.
(7) Myers, S. R.; Blake, J. W.; Flesher, J. W. *Biochem. Biophys. Res. Commun.* **1988**, *151*, 1441.
(8) Hanzlik, R. P.; Hogberg, L.; Moon, J. B.; Judson, C. M. *J. Am. Chem. Soc.* **1985**, *107*, 7164.
(9) Hanzlik, R. P.; Schaefer, A. R.; Moon, J. B.; Judson, C. M. *J. Am. Chem. Soc.* **1987**, *109*, 4926.
(10) Jones, J. P.; Korzekwa, K. R.; Rettie, A. E.; Trager, W. F. *J. Am. Chem. Soc.* **1986**, *108*, 7074.
(11) Jones, J. P.; Trager, W. F. *J. Am. Chem. Soc.* **1987**, *109*, 2171.
(12) Jones, J. P.; Korzekwa, K. R.; Rettie, A. E.; Trager, W. F. *J. Am. Chem. Soc.* **1988**, *110*, 2018.
(13) Jones, J. P.; Trager, W. F. *J. Am. Chem. Soc.* **1988**, *110*, 2018.
(14) Holland, H. L.; Brown, F. M.; Munoz, B.; Ninniss, R. W. *J. Chem. Soc., Perkin Trans. 2* **1988**, 1557.

Table I. Deuterium-Induced Metabolic Switching in Toluene Metabolism

substrate[a]	product[b] BA, mol %	2-MP, mol %	3- + 4-MP, mol %	total, nmol	BA/MP	2-MP/3- + 4-MP
D_0	69.0 ± 1.6	14.8 ± 0.8	16.2 ± 0.8	836 ± 80	2.22	0.91
D_1	63.2[c] ± 3.6	16.7 ± 2.6	20.1 ± 1.3	959 ± 177	1.71	0.83
D_2	51.3[d] ± 2.1	23.5 ± 1.5	25.2 ± 0.7	1138 ± 71	1.05	0.93
D_3	24.3 ± 1.9	34.7 ± 0.8	41.0 ± 2.2	1237 ± 85	0.32	0.85
D_5	67.9 ± 0.6	15.9 ± 1.1	16.2 ± 1.3	754 ± 153	2.11	0.98
D_8	19.6 ± 1.4	35.2 ± 0.5	45.2 ± 0.8	nd[e]	0.24	0.78

[a] Incubated in triplicate at saturating concentration (10.4 mM) with liver microsomes from phenobarbital-induced rats. [b] Key: BA, benzyl alcohol; 2-MP, *o*-cresol (2-methylphenol); 3- + 4-MP, *m*- plus *p*-cresol; BA/MP, alcohol/cresol ratio; 2-MP/3- + 4-MP, cresol isomer ratio. Data are the means ± SD for three experiments. [c] Contained 90.6 ± 0.6 mol % d_1. [d] Contained 74.2 ± 1.1 mol % d_2. [e] Not determined.

ring deuteration leads to no discernible isotope effect on the rates of benzyl alcohol or cresol formation. On the other hand, stepwise deuteration of the methyl group leads to stepwise decreases in the formation of benzyl alcohol *relative to cresols*. This effect is particularly dramatic with the introduction of the *third* deuterium onto the methyl group. Despite these effects the ratios of cresol isomers do not change with deuteration of the methyl group or aromatic ring. Quite unexpectedly, we observed that stepwise deuteration of the side chain leads to stepwise *increases* in the total yield of oxidation products (benzyl alcohol plus cresols), resulting in apparent *inverse* DIE on V_{max} of 0.67 for the net oxidation of toluene-d_3. To explain these effects, we proposed metabolic switching from benzyl alcohol to cresol formation leads to a net increase in overall turnover even though oxidative attack on the aromatic ring is intrinsically less favorable than benzylic hydroxylation (see above). In this paper we provide a complete kinetic analysis justifying this hypothesis. We also provide additional data on deuterium-induced metabolic switching (i.e., *intra*molecular isotope effects) in toluene metabolism and an analysis of competitive *inter*molecular isotope effects for toluene metabolism. The latter indicate that phenobarbital-induced rat liver microsomal P-450 also shows a substantial commitment to catalysis with toluene.

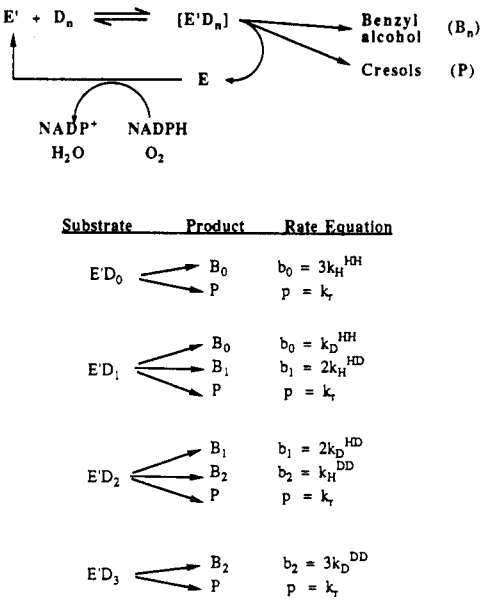
Experimental Procedures (Materials and Methods)

Deuterated toluenes D_1–D_3 were prepared as described previously[2] and contained 99.4, 98.9, and 98.7 mol % of the d_1, d_2, and d_3 species, respectively. Toluenes D_5 and D_8 were obtained from Merck Isotopes and were found to contain 98.3 and 97.8 mol % of the d_5 and d_8 species, respectively. Procedures for preparation of liver microsomes from phenobarbital-pretreated rats, incubation of substrates, and isolation and analysis of metabolites have also been described in detail.[2,8,15] Purified cytochrome P-450b (P450IIB1) and NADPH-cytochrome P-450 reductase were provided by Dr. Andrew Parkinson.

Results and Discussion

Deuterium-Induced Metabolic Switching. The relative yields of benzyl alcohol (B) and cresol isomers (P) from hydroxylation of toluene-d_0 (D_0) and five deuterated toluenes (D_1–D_8) are given in Table I. This predominance of benzyl alcohol over cresols is typical for toluene- and phenobarbital-induced rat liver cytochrome P-450b. Stepwise deuteration of the *methyl* group (viz. D_0–D_3) causes stepwise decreases in the B/P ratio (Table I). This change is most abrupt upon replacement of the last available methyl hydrogen by deuterium. Across this same D_0–D_3 series, however, the isomer ratios among the cresols remain essentially constant (i.e., o = p > m). In contrast to methyl deuteration, ring deuteration has only a slight

Scheme II. Intramolecular Metabolic Switching



effect on product ratios (viz. D_5 vs D_0 and D_8 vs D_3 in Table I).

The formation of multiple products from a single substrate in P-450-catalyzed reactions appears to be a consequence of the ability of some P-450 isozymes to bind some substrates in more than one orientation (i.e., to form several geometrically isomeric Michaelis complexes) such that several isomeric hydroxylation or other monooxygenation products are formed[10–13,16–20] (cf. Scheme II). If the Michaelis complex rearranges rapidly (either by intramolecular means or by dissociation–reassociation) relative to product formation, then disfavoring *one* mode of reaction by selective deuterium substitution should lead to changes in product *ratios* in accordance with (1) the magnitude of the intrinsic isotope effect on the susceptible reaction and (2) the relative importance of the various pathways with the undeuterated substrate. This phenomenon was first noted and dubbed "metabolic switching" by Horning and co-workers.[21] Its kinetic basis was later clarified by Trager and co-workers[10–13] who refer to the process as "isotopically-sensitive branching". The product ratio data in Table I conform well to the concept

(15) Hanzlik, R. P.; Hogberg, K.; Judson, C. M. *Biochemistry* **1984**, *23*, 3048.
(16) White, R. E.; McCarthy, M.-B.; Egeberg, K. D.; Sligar, S. G. *Arch. Biochem. Biophys.* **1984**, *228*, 493.
(17) Sligar, S. G.; Gleb, M. H.; Heimbrook, D. C. *Xenobiotica* **1984**, *14*, 63.
(18) Atkins, W. M.; Sligar, S. G. *J. Am. Chem. Soc.* **1987**, *109*, 3754.
(19) Atkins, W. M.; Sligar, S. G. *Biochemistry* **1988**, *27*, 1610.
(20) Sugiyama, K.; Trager, W. F. *Biochemistry* **1986**, *25*, 7336.
(21) Horning, M. G.; Haegele, K. D.; Sommer, K. R.; Nowlin, J.; Stafford, M.; Thenot, J. P. In *Proceedings of the Second International Conference on Stable Isotopes*; Klein, E. R., Klein, P. D., Eds.; NTIS: Springfield, VA, 1976; pp 41–54.

of isotopically sensitive branching. In particular, aromatic hydroxylation by P-450 enzymes typically shows no deuterium (or tritium) isotope effects on product formation rates.[22] Largely for this reason aromatic hydroxylation is thought to involve either arene oxide formation or an addition–rearrangement sequence with the initial addition step being rate limiting.[15] Such a step would be subject at most to a small *inverse* DIE.[23,24] Aliphatic (and benzylic) hydroxylations on the other hand are thought to proceed via an abstraction–recombination mechanism, with the former being rate limiting and potentially susceptible to a large intrinsic primary DIE.[25,26] Thus, methyl deuteration should significantly disfavor methyl hydroxylation relative to phenol formation, but ring deuteration should be almost inconsequential.

Before this simple view can be accepted, several alternatives must be considered. For example, with microsomes it is possible that the four metabolites of toluene do not all arise from a single isozyme of P-450. However, Waxman and Walsh[27] have shown using purified reconstituted systems that the major phenobarbital-induced isozyme of rat liver (i.e., P-450b) does indeed oxidize toluene to benzyl alcohol, *o*-cresol, and *m*/*p*-cresols. We have extended this work independently and have confirmed that purified rat liver cytochrome P-450b does indeed form benzyl alcohol and *all three* cresols from toluene. Another possibility is that Michaelis complex rearrangement might not occur by internal means but rather by a dissociation–reassociation mechanism. However, intermolecular competition isotope effect data presented below suggest there is significant commitment to catalysis with toluene, implying that the dissociation–reassociation process is slow relative to product formation. We therefore conclude that the Michaelis complex of toluene must be capable of rearranging *internally* at a rate in excess of the rate of product formation. We will return to this point later.

Intramolecular DIEs on Benzylic and Aromatic Hydroxylation. Deuterium analysis of the benzyl alcohol metabolite from D_1 showed it to contain 90.6 ± 0.6 mol % d_1, while that from D_2 contained 74.2 ± 1.1 mol % d_2 (Table I). These data are similar in magnitude but statistically more precise than we observed in a previous study.[8] If H or D abstraction had been purely statistical with no DIE involved, the expected values would have been 66.7 and 33.3 mol %, respectively. Assuming that rapid rotation of the phenyl–methyl bond in the Michaelis complex ensures statistical presentation of H or D for reaction, there is clearly a substantial DIE favoring loss of H over D during benzylic hydroxylation.

The deconvolution of the *net* DIEs into primary and secondary *component* DIEs has long been an objective of ours. Before we tackle this question again, it is interesting to combine the product ratio data and deuterium analysis data to deduce relative positional reactivities for the various deuterated toluenes as shown in Figure 1. Comparisons among the various possible sites of metabolism give a qualitative but clear impression of the effects of deuteration on metabolic switching within the methyl group as well as from the methyl to the ring. One can also

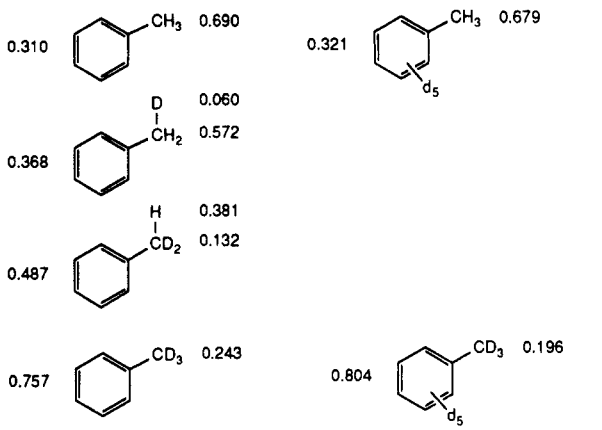


Figure 1. Relative positional reactivity of toluene and deuterated toluenes toward cytochrome P-450 catalyzed hydroxylation. For each molecule the data are expressed as fractional reactivities for the aromatic ring as a whole and for the benzylic hydrogen or deuterium atoms. For the relative total reactivities of the different molecules, see Table I.

Table II. Relative Rate Constants and Derived Isotope Effects for Microsomal Hydroxylation of Deuterated Toluenes

Rate Constants	
k_H^{HH} = 1.000 ± 0.056	k_D^{HH} = 0.217 ± 0.022
k_H^{HD} = 1.048 ± 0.084	k_D^{HD} = 0.183 ± 0.012
k_H^{DD} = 1.052 ± 0.057	k_D^{DD} = 0.144 ± 0.012
Isotope Effects	
P_1 = 4.59 ± 0.54	S_1 = 0.953 ± 0.094
P_2 = 5.72 ± 0.60	S_2 = 0.995 ± 0.097
P_3 = 7.30 ± 0.72	S_3 = 1.187 ± 0.146
	S_4 = 1.270 ± 0.134

observe the hint of a slight *inverse* isotope effect for ring deuteration; i.e., P/B ratios for D_0/D_5 and for D_3/D_8 are 0.966 ± 0.062 and 0.936 ± 0.030, respectively. While these effects are not statistically different from unity, they both tend to be inverse as expected.[23,24] The largest isotope effects clearly come from methyl deuteration, and comparing the $(CH_3/ring)/(CD_3/ring)$ ratios of reactivities for D_0 vs D_3 and for D_5 vs D_8 leads to CH_3/CD_3 isotope effects of 6.93 and 8.67, respectively, as discussed further below.

Primary and Secondary DIEs on Benzylic Hydroxylation of Toluene. Quantitative analysis of the data in Figure 1 begins with the kinetic models and equations presented in Scheme II, which are based on the assumption of a rapidly rearranging Michaelis complex that partitions into multiple pathways of product formation via irreversible initial steps. In this model all phenolic products are lumped into one kinetic term for simplicity. For example, the E–D_1 Michaelis complex may react via D abstraction, H abstraction, or ring attack to give benzyl alcohol-d_0 (B_0), benzyl alcohol-d_1 (B_1), or phenolic products (P) in mole fractions $b_0 + b_1 + p = 1.0$, respectively. These product mole fractions are proportional to the rate constants for the pathways into which the Michaelis complex partitions, i.e., D abstraction with two bystander hydrogens (k_D^{HH}), H abstraction with one bystander deuterium ($2k_H^{HD}$), or ring hydroxylation (k_r).

Relative reactivity values given in Figure 1 may be entered directly into the equations in Scheme II to determine four subsets of rate constants. Since the term k_r is common to each subset, and since *ring* hydroxylation is unlikely to be affected by *methyl* deuteration, the four subsets can be combined after each is normalized to its respective k_r value. Renormalizing the full set to $k_H^{HH} = 1.0$ then gives the relative rate constants tabulated in the upper half of

(22) Tomaszewski, J. E.; Jerina, D. M.; Daly, J. W. *Biochemistry* **1975**, *14*, 2024.
(23) Hanzlik, R. P.; Shearer, G. O. *Biochem. Pharmacol.* **1978**, *27*, 1441.
(24) Korzekwa, K. R.; Swinney, D. C.; Trager, W. F. *Biochemistry* **1989**, *28*, 9019.
(25) White, R. E.; Miller, J. P.; Favreau, L. V.; Bhattacharyya, A. *J. Am. Chem. Soc.* **1986**, *108*, 6024.
(26) Groves, J. T.; McClusky, G. A.; White, R. E.; Coon, M. J. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 154.
(27) Waxman, D. J.; Walsh, C. *J. Biol. Chem.* **1982**, *257*, 10446.

Table II. These six relative rate constants represent all possible benzylic H or D abstractions occurring within the D_0–D_3 series. In principle, they may be combined to define three primary and four secondary isotope effects as shown below.[9]

$$P_1 = k_H^{HH}/k_D^{HH} \qquad P_2 = k_H^{HD}/k_D^{HD} \qquad P_3 = k_H^{DD}/k_D^{DD}$$

$$S_1 = k_H^{HH}/k_H^{HD} \qquad S_2 = k_H^{HD}/k_H^{DD}$$

$$S_3 = k_D^{HH}/k_D^{HD} \qquad S_4 = k_D^{HD}/k_D^{DD}$$

The primary isotope effects involve H vs D abstraction (subscripts) in the presence of zero, one, or two bystander deuterium atoms (superscripts). The secondary isotope effects arise from one or two bystander deuterium atoms with either H or D as the transferring atom. According to the "rule of the geometric mean" (RGM), all P values should be equal and all S values should be equal.[28,29] Stating this another way, there should be no isotope effects on isotope effects. The free-radical chlorination of toluene is one reaction that appears to obey the RGM quite closely.[9]

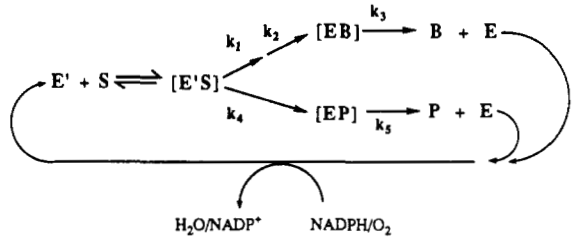
The corresponding P and S values for toluene hydroxylation are given in the lower half of Table II. Corresponding P and S values for hydroxylation of *n*-octane to 1-octanol by cytochrome P-450b are 7.6 and 1.11, respectively.[11,13] In the present case it is obvious that while P and S values for toluene hydroxylation are in this range, neither the primary effects nor the secondary effects are all equal to each other. There may be several reasons for this, the first and most likely being simple experimental variability in the measurement of product ratios and isotope ratios. The relative standard deviations (RSDs) on measured product ratios are generally 2–4% from one experiment to the next, while those on deuterium analyses are somewhat better (Table I). However, propagation of errors through several sequential calculations can easily result in RSDs as large as some secondary DIEs (viz. S values in Table II). Imperfect deuteration of the substrates is another potential source of error. For example, with D_2 there is less metabolic switching from benzylic to ring hydroxylation than with D_3, so the D_2 contaminant in the D_3 substrate will bias the alcohol/cresol ratio upward. However, in the present case the amounts of isotopic impurities were probably less than other sources of error and no corrections for this were made.

A further reason for the "apparent failure of the RGM" suggested by the nonequality of P values and S values in Table II may be that the rate of internal rearrangement of the Michaelis complex is not as fast, relative to product formation, as assumed in deriving the model (Scheme II). Thus, the normalization of data subsets to their respective k_r values prior to combining them (see above) may not accomplish as much of a correction as needed. This problem would also vary with the degree of methyl deuteration, further complicating any attempt to correct for it. Still another problem with Scheme II is that the associated equations ignore water formation (i.e., the *oxidase* activity of cytochrome P-450), which is another pathway to which the E–S Michaelis complex can branch.[17,18]

Another approach to testing the RGM for toluene hydroxylation requires only the measurement of intramolecular H/D discrimination in the benzyl alcohol product from D_1 and D_2.[9] For example, from D_1 the observed product ratio (b_1/b_0) is given by the ratio $k_H^{HD}/2k_D^{HH} = r_2$,

(28) Bigeleisen, J. *J. Chem. Phys.* **1955**, *23*, 2264.
(29) Amin, M.; Price, R. C.; Saunders, W. H., Jr. *J. Am. Chem. Soc.* **1988**, *110*, 4085.

Scheme III. Branching in Relation to Turnover of P-450



$k_1 = k_H$ or k_D S = toluene
B = benzyl alcohol P = cresols
E' = EFe=O E = EFe

while from D_2 the observed product ratio (b_2/b_1) is given by the ratio $k_H^{DD}/2k_D^{HD} = r_2$. The ratio of ratios r_1/r_2 thus equals $4S_2/S_3$ (and not simply 4 as deduced earlier[8] without the benefit of explicit definitions of S_1–S_4). From the product data summarized in Figure 1, $r_1 = 9.639 \pm 0.993$, $r_2 = 2.874 \pm 0.210$, and $r_1/r_2 = 4.437 \pm 0.639$; thus, $S_2/S_3 = 1.109 \pm 0.159$. Unfortunately, while the S_2/S_3 ratio appears to differ from unity in an interesting direction, this result is not statistically significant. To settle this issue for toluene hydroxylation by cytochrome P-450 will require data of greater precision than presently available.

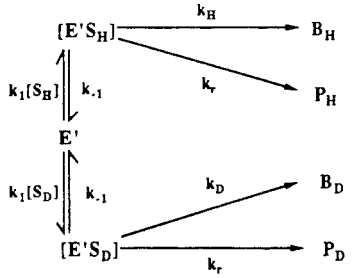
Isotope Effects on V_{max}. Table I also reports data on the absolute yields of oxidation products from toluenes D_0–D_5. Since substrate was initially present at saturating or near-saturating concentrations and total conversions were less than 2%, the total product yields are good estimates of V_{max}. The data in Table I indicate that ring deuteration leads to no significant isotope effects. As expected, however, methyl deuteration leads to decreased yields of benzyl alcohol with an apparant isotope effect $^DV = 1.92$ (i.e., $(0.69 \times 836)/(0.243 \times 1237)$). For comparison, the V_{max} isotope effect for toluene hydroxylation by rabbit liver cytochrome P-450-LM$_2$ is 2.68. Isotope effects on V_{max} report on the isotopic sensitivity of the step leading to the highest point on the overall reaction coordinate. Hence, if product release is not fast relative to the isotopically sensitive step (i.e., benzylic hydrogen abstraction), ^DV will be suppressed relative to the intrinsic isotope effect. We believe these low values for ^DV for benzylic hydroxylation of toluene reflect suppression of a large intrinsic isotope effect by a slow product release step; we will return to this point later.

Perhaps the most surprising aspect of the data in Table I is the fact that methyl deuteration actually leads to net *increases* in the *total* oxidation of toluene, such that $^DV = 0.67$ with D_3. The fact that ^DV with the rat liver enzyme is actually substantially *inverse* suggests that more than just simple kinetic suppression by product release is involved, as the limiting effect for total suppression should be unity, not less. We have previously attributed this to deuterium-induced metabolic switching to products (cresols) that are *released faster* than benzyl alcohol, thus enabling the enzyme to turnover faster,[2] and have now tested this hypothesis using the model depicted in Scheme III. Application of the steady-state approximation to this model (Appendix I) leads to eq 1, in which Σ_H is the sum

$$\frac{\Sigma_H}{\Sigma_D}\,\frac{k_D/k_4 + 1}{k_H/k_4 + 1} = \frac{1 + k_5/k_4 + (k_5/k_3)(k_D/k_4)}{1 + k_5/k_4 + (k_5/k_3)(k_H/k_4)} \quad (1)$$

of all products from D_0 and Σ_D is the sum of all products from D_3 (i.e., column 4 in Table I). From the observations in Table I we can calculate that $\Sigma_H/\Sigma_D = 0.675$, $k_H/k_4 = 2.33$, and $k_D/k_4 = 0.316$; thus, $k_H/k_D = 7.38$. By substi-

Scheme IV. Intermolecular Isotopic Competition



tuting these values into eq 1, one can derive the simpler expression in eq 2. The value of 7.38 for k_H/k_D compares

$$k_5/k_4 = 0.403(k_5/k_3) - 1 \quad (2)$$

very well to the values of 6.93 and 8.67 based solely on metabolic switching considerations (see above). The ratio k_5/k_3 is germane to our hypothesis that release of cresols (or perhaps their arene oxide precursors) is faster than release of benzyl alcohol. To evaluate this ratio, we first arbitrarily set $k_H = 1.0$; then, since $k_H/k_4 = 2.33$ (see above), $k_4 = 0.428$. If we allow k_5/k_4 to vary from 0.01 to 100, which would not seem unduly restrictive for the ratio of two successive steps in an enzymic reaction mechanism, one can calculate that k_3 varies from 0.0017 to 0.17, k_5 varies from 0.0043 to 43, and k_5/k_3 varies from 2.5 to 250. Thus, in this range of kinetic constraints, k_5 (cresol release) is always greater than k_3 (benzyl alcohol release) and k_3 is significantly rate limiting in the metabolism of toluene. Avoiding the benzyl alcohol pathway via deuterium-induced metabolic switching to ring hydroxylation thus leads to faster *overall* turnover of enzyme, net formation of more product from the *deuterated* toluene, and an *apparent* inverse isotope effect on V_{max}. This is because a rate-limiting step (k_3) occurs *after* an isotopically sensitive branch point (viz. Scheme III).

Competitive Intermolecular Isotope Effects on (V/K). When two isotopic forms of substrate compete for the same enzyme as in Scheme IV, only isotope effects on (V_{max}/K_m) may be observed. In the present study, mixtures of deuterated and nondeuterated toluenes were submitted to enzymic hydroxylation under conditions of high (saturating) concentrations of substrate and limited (<2%) conversion to products. Product ratios were measured by gas chromatography, and product deuterium content was measured by gas chromatography/mass spectrometry; the results are presented in Table III. From these data, *observed* isotope effects on (V/K) can readily be calculated on four parameters: benzyl alcohol formation, cresol formation, product ratio (benzyl alcohol/cresols), and product yield. Dividing these observed isotope effects by the isotope ratio in the starting substrate mixture gives the respective corrected isotope effects (Q_1–Q_4). Application of the steady-state approximation to Scheme IV (see Appendix II) leads to the following expressions for Q_1–Q_4

$$Q_1 = \frac{B_H}{B_D}\frac{[S_D]}{[S_H]} = \frac{k_H}{k_D}Z \quad (3)$$

$$Q_2 = \frac{P_H}{P_D}\frac{[S_D]}{[S_H]} = Z \quad (4)$$

$$Q_3 = \frac{B_H/P_H}{B_D/P_D}\frac{[S_D]}{[S_H]} = \frac{Q_1}{Q_2} = \frac{k_H}{k_D} = {}^Dk \quad (5)$$

$$Q_4 = \frac{\sum_H}{\sum_D}\frac{[S_D]}{[S_H]} = \frac{k_H + k_r}{k_D + k_r}Z \quad (6)$$

Table III. Intermolecular Competition Isotope Effects in Cytochrome P-450 Catalyzed Hydroxylation of Deuterated Toluenes

	product and isotopic composition, mole fraction			
species	toluene	BzOH	*o*-cresol	*p*-cresol
	Experiment 1 (d_0/d_3 Mixture)			
		39.6%	27.7%	32.3%
d_0	0.349	0.650 ± 0.005	0.248 ± 0.019	0.218 ± 0.002
d_1	0.000	0.007 ± 0.001	0.003 ± 0.001	0.009 ± 0.003
d_2	0.011	0.336 ± 0.005	0.010 ± 0.007	0.000
d_3	0.640	0.000	0.739 ± 0.026	0.773 ± 0.004
	Experiment 2 (d_0/d_5 Mixture)			
		68.4%	15.4%	16.2%
d_0	0.345	0.410 ± 0.009	0.408 ± 0.020	0.405 ± 0.012
d_3	0.000	0.001 ± 0.001	0.028 ± 0.009	0.005 ± 0.002
d_4	0.012	0.007 ± 0.001	0.565 ± 0.019	0.590 ± 0.014
d_5	0.643	0.582 ± 0.010	0.000	0.000
	Experiment 3 (d_0/d_8 Mixture)			
		35.9%	28.8%	36.2%
d_0	0.310	0.787 ± 0.002	0.295 ± 0.005	0.305 ± 0.012
d_6	0.000	0.008 ± 0.001	0.033 ± 0.001	0.000
d_7	0.015	0.205 ± 0.002	0.672 ± 0.004	0.695 ± 0.012
d_8	0.675	0.000	0.000	0.000

where $Z = (k_{-1} + k_D + k_r)/(k_{-1} + k_H + k_r)$.

Equations 3–6 are applicable to each of the three isotope competition experiments in Table III, but for simplicity only the D_0/D_3 competition will be analyzed in detail here. Evaluation of eq 5 using the appropriate data from Table III gives $k_H/k_D = 6.29$ for the combined effect of one primary and two secondary deuteriums. This is only slightly smaller than the CH_3/CD_3 isotope effects of 6.93 and 8.67 deduced from the product ratio data in Table I or the k_H/k_D isotope effect of 7.38 deduced from the product yield (i.e., V_{max}) data in Table I (see above). Considering the substantial differences in experimental formats between Tables I and III, i.e., intra- vs intermolecular, the agreement in CH_3/CD_3 ratios is very good.

Evaluation of eq 4 using data from Table III indicates that $Z = 0.56$. Since $k_H/k_r = 0.257/0.138 = 1.86$ (Table III, line 1) and $k_H/k_D = 6.29$ (from eq 5), evaluation of the expression for Z yields $k_{-1}/k_H = 0.682$ and $k_{-1}/k_D = 4.28$. Taking k_H to have a *relative* value of 1.0, the other rate constants in Scheme IV have values of $k_{-1} = 0.68$, $k_r = 0.53$, and $k_D = 0.159$. From this it is clear that dissociation of the E–S Michaelis complex is indeed rather slow compared to product formation and that there is substantial commitment to catalysis. For D_0 and D_3 the commitment factors are $(k_H + k_r)/k_{-1} = 2.25$ and $(k_D + k_r)/k_{-1} = 1.01$, respectively.

(V/K) isotope effects report on events occurring between free E and free S in solution and the first irreversible step *involving substrate* (i.e., excluding the irreversible O–O bond cleavage during oxygen activation by P-450 enzymes; see Harada et al.[30]). In cases where the first irreversible step involving substrate (e.g., H abstraction) is fast relative to dissociation of the E–S complex back to free enzyme and free substrate, the observed (V/K) isotope effects will be suppressed relative to the intrinsic isotope effect.[31] Thus, as demonstrated above for benzyl alcohol formation from D_3, the isotope effect on benzyl alcohol formation (Q_1) is only 3.6 while the intrinsic isotope effect (Q_3) is around 7. Much of the "unseen" portion of the intrinsic isotope

(30) Harada, N.; Miwa, G. T.; Walsh, J. S.; Lu, A. *J. Biol. Chem.* **1984**, *259*, 3005.

(31) Northrop, D. B. In *Isotope Effects on Enzyme-Catalyzed Reactions*; Cleland, W. W., O'Leary, M. H., Northrop, D. B., Eds.; University Park Press: Baltimore, MD, 1977; p 122.

effect is in fact expressed through metabolic switching to cresol formation (viz. Table I); consequently, there is almost no net isotope effect on total product formation (Q_4 = 1.23). There is also a large but "artifactual" inverse isotope effect on cresol formation (Q_2 = 0.56) arising purely from metabolic switching induced by methyl deuteration. Similar interplay of commitment to catalysis and metabolic switching has been reported by Jones and Trager[11,13] for the hydroxylation of deuterated *n*-octanes, by Miwa and co-workers[30] for the O-deethylation of 7-ethoxycoumarin, and by Atkins and Sligar[18,19] for hydroxylation of norbornanone and camphor derivatives. In this light it is especially interesting that, with rabbit liver P-450-LM_2, toluene is oxidized to benzyl alcohol *only*.[32] Since in this case $k_r = 0$, Q_2 and Q_3 are undefined and Q_1 and Q_4 simplify to the familiar Northrop formulation[30] of the effect of commitment on $^D(V/K)$, i.e.

$$^D(V/K) = \frac{k_H/k_D + C_f}{1 + C_f} \tag{7}$$

where C_f is the foreward commitment factor (k_H/k_{-1}). $^D(V/K)$ for oxidation of D_8 by rabbit liver P450-LM_2 is reported[32] to be 1.24 ± 0.28, which suggests very strong commitment to catalysis in this case. The failure of rabbit liver P-450-LM_2 to form cresols must indicate that the active site of this enzyme is unable to bind toluene in an orientation suitable for ring attack. In the absence of metabolic switching, the intrinsic isotope effect (presumably ≥6) is strongly suppressed.

Acknowledgment. We thank Dr. Andrew Parkinson for providing materials and technical assistance in connection with experiments involving reconstituted P-450 systems and Dr. Richard Schowen for stimulating discussions and helpful suggestions.

Appendix I

Scheme III represents the enzymic hydroxylation of a single substrate (S) to either of two products (B, P) through a branched pathway involving a Michaelis complex (E–S) that undergoes internal rearrangement rapidly with respect to the irreversible steps of product formation. Assuming saturating concentration of a nondeuterated substrate (S_H) and steady-state conditions, the model in Scheme III can be described by eqs 8–11, where e is total enzyme and Σ represents total products. Dividing eq 10 by $[ES_H]$ and substituting from eqs 8 and 9 leads to eq 12, with which one can write the expression for total product formation shown in eq 13. A similar treatment for the deuterated substrate leads to an equation like eq 13 but with the H subscripts replaced by D. Dividing eq 13 by its equivalent for the deuterated substrate and rearranging leads directly to eq 1 in the text.

$$k_H[ES_H] = k_3[EB_H] \tag{8}$$

$$k_4[ES_H] = k_5[EP_H] \tag{9}$$

$$[ES_H] + [EB_H] + [EP_H] = e \tag{10}$$

$$d\Sigma_H/dt = k_3[EB_H] + k_5[EP_H] \tag{11}$$

$$[ES_H] = (k_3k_5e)/(k_3k_5 + k_Hk_5 + k_4k_3) \tag{12}$$

$$d\Sigma_H/dt = (k_H + k_4)k_3k_5e/(k_3k_5 + k_Hk_5 + k_4k_3) \tag{13}$$

Appendix II

Scheme IV represents the competition between labeled and unlabeled substrate molecules for enzyme and the partitioning of each enzyme–substrate complex into two pathways (cf. Scheme III and Appendix I). Application of the steady-state approach leads to eq 14 and 15; dividing eq 14 by eq 15 then gives eq 16. Since the rates of formation of benzyl alcohols B_H and B_D are given by $k_H[ES_H]$ and $k_D[ES_D]$, respectively, we may use eq 16 to write eq 17 as an expression of the observed isotope ratio in the benzyl alcohol product. Rearrangement of eq 17 leads to the expression for the corrected isotope effect for benzyl alcohol formation Q_1 given in eq 3 in the text. Analogous derivations lead to expressions for the corrected isotope effects on phenolic products (Q_2), on product ratios (Q_3), and on total product yields (Q_4), as given by eqs 4–6 in the text.

$$d[ES_H]/dt = 0 = k_1[E][S_H] - [ES_H](k_{-1} + k_H + k_r) \tag{14}$$

$$d[ES_D]/dt = 0 = k_1[E][S_D] - [ES_D](k_{-1} + k_D + k_r) \tag{15}$$

$$\frac{[ES_H]}{[ES_D]} = \frac{[S_H](k_{-1} + k_D + k_r)}{[S_D](k_{-1} + k_H + k_r)} \tag{16}$$

$$\frac{B_H}{B_D} = \frac{k_H}{k_D}\frac{[S_H]}{[S_D]}\frac{k_{-1} + k_D + k_r}{k_{-1} + k_H + k_r} = \frac{k_H[S_H]}{k_D[S_D]}Z \tag{17}$$

(32) White, R. E.; McCarthy, M.-B. *Arch. Biochem. Biophys.* **1986**, *246*, 19.