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DRUG METABOLISM & DISPOSITION

THE USE OF DEUTERIUM ISOTOPE EFFECTS TO PROBE THE ACTIVE SITE PROPERTIES, MECHANISM OF CYTOCHROME P450-CATALYZED REACTIONS, AND MECHANISMS OF METABOLICALLY DEPENDENT TOXICITY

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ABSTRACT:

Critical elements from studies that have led to our current understanding of the factors that cause the observed primary deuterium isotope effect, (k_H/k_D)obs, of most enzymatically mediated reactions to be much smaller than the "true" or intrinsic primary deuterium isotope effect, k_H/k_D, for the reaction are presented. This new understanding has provided a unique and powerful tool for probing the catalytic and active site properties of enzymes, particularly the cytochromes P450 (P450). Examples are presented that illustrate how the technique has been used to determine k_H/k_D, and properties such as the catalytic nature of the reactive oxenoid intermediate, prochiral selectivity, the chemical and enzymatic mechanisms of cytochrome P450-catalyzed reactions, and the relative active site size of different P450 isoforms. Examples are also presented of how deuterium isotope effects have been used to probe mechanisms of the formation of reactive metabolites that can cause toxic effects.

Historically, the determination of deuterium isotope effects has been a powerful tool to help unravel the intricacies of carbon-hydrogen bond cleavage and define the mechanism of specific chemical reactions. The intrinsic primary isotope effect, k_H/k_D, for a reaction is the magnitude of the isotope effect on the rate constant for the bond-breaking step (C–H versus C–D) of the reaction and is related to the symmetry of the transition state for that step. The larger the isotope effect, the more symmetrical the transition state, with the theoretical limit being 9 at 37°C in the absence of tunneling effects (Bell, 1974). The chemical events leading to the transition state and subsequent formation of products are the descriptors of a chemical mechanism. It is the relationship of k_H/k_D to transition state that provides mechanistic insight. Thus, k_H/k_D is the quantity that needs to be known, and for homogenous chemical reactions, the experimentally observed deuterium isotope effect, (k_H/k_D)obs, where (k_H/k_D)obs is defined as the ratio of a kinetic parameter such as V_{max} or V_{max}/K_m obtained from a nondeuterated substrate to a deuterated substrate, is identical to k_H/k_D.

This is generally not true of enzymatically mediated reactions and is the reason for the much less successful application of deuterium isotope effects to such reactions until the system was better understood. The experimentally observed isotope effect, (k_H/k_D)obs, was invariably found to be much smaller than k_H/k_D, the intrinsic isotope effect for that reaction, thereby obscuring both meaning and mechanism. Even more perplexing was the observation that (k_H/k_D)obs for the same enzymatic reaction could vary with different experimental designs. For example, (k_H/k_D)obs determined at saturating substrate concentrations could differ from values determined at decreasing substrate concentrations. The disparity between k_H/k_D and (k_H/k_D)obs is a consequence of the multistep nature (substrate binding, debinding, product release, etc.) of the catalytic cycle of enzymatically mediated reactions. If deuterium isotope effects are to be successfully applied to such reactions, then the relationship between k_H/k_D and (k_H/k_D)obs must be known, since experiment can only yield (k_H/k_D)obs. Fortunately, the relationship between k_H/k_D and (k_H/k_D)obs has been largely clarified (Northrop, 1975, 1978, 1981a; Cleland, 1982); and how partially rate-limiting steps exclusive of the bond-breaking step can mask k_H/k_D by lowering the magnitude of (k_H/k_D)obs is now clearly understood.

Even the simplest of enzymatic reactions is composed of three components (Northrop, 1981b). The first involves substrate combining with enzyme to form an enzyme-substrate complex. Rate constants k_{12} and k_{21} govern the process. The second component is the catalytic component that transforms the enzyme-substrate complex into an enzyme-product complex with rate constant k_{23} (Reaction 1). In this simplest of cases the catalytic step is considered to be irreversible. The third component is the product release step that entails dissociation of the complex to free enzyme and product with rate constant k_{31}. Equations 1 to 3 define the kinetic expressions for the maximum velocity, V, the Michaelis constant, K, and the ratio of the two, V/K (Northrop, 1975, 1981b). In analyzing the kinetic

$$V = \frac{k_{23}k_{31}Et}{k_{23} + k_{31}} \quad (1)$$

$$K = \frac{k_{31}(k_{12} + k_{23})}{k_{12}(k_{23} + k_{31})} \quad (2)$$

$$V/K = \frac{k_{12}k_{23}Et}{k_{21} + k_{23}} \quad (3)$$

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parameters, Northrop (1975) demonstrates that the expression of deuterium isotope effects on V and V/K should be the most revealing and important as they depend on the fewest variables and on rate constants for different parts of the kinetic scheme. Since V is determined at saturating concentrations, the binding component is eliminated and its value reflects the catalytic and product release components. V/K, which is reflective of the kinetics at low substrate concentration, is dependent on all rate constants up to and including the first irreversible step. In this case it is also the catalytic step. Isotope effects on V and V/K for this example are described by eqs. 4 and 5.

$$^{D}V = V_H/V_D = \frac{k_{23H}/k_{23D} + k_{23H}/k_{31}}{1 + k_{23H}/k_{31}} \quad (4)$$

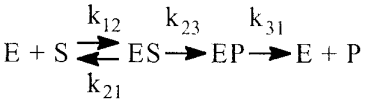
$$^{D}(V/K) = (V/K)_H/(V/K)_D = \frac{k_{23H}/k_{23D} + k_{23H}/k_{21}}{1 + k_{23H}/k_{21}} \quad (5)$$

^{D}V and $^{D}(V/K)$ are short-form terms introduced by Northrop (1975) for V_H/V_D and $(V/K)_H/(V/K)_D$, respectively. If eqs. 4 and 5 are rewritten in general form as eqs. 6 and 7, as proposed by Northrop (1975), the factors

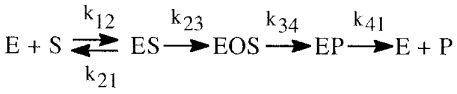
$$^{D}V = \frac{^{D}k + R}{1 + R} \quad (6)$$

$$^{D}(V/K) = \frac{^{D}k + C}{1 + C} \quad (7)$$

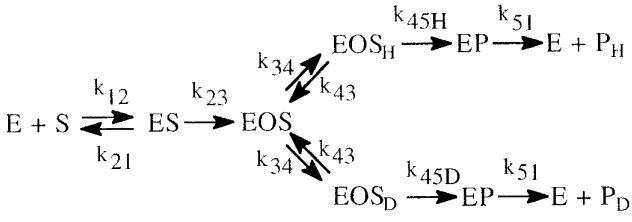
that can lead to the observed isotope effect, ^{D}V or $^{D}(V/K)$, having a much lower value than the intrinsic isotope effect, ^{D}k (Northrop's nomenclature), become clear. In the equation for the isotope effect on V, R, termed the "ratio of catalysis," is a measure of rate of the catalytic step relative to the rate of the other forward steps contributing to maximal velocity. In the present example, the other forward step would be the product release step. The value of ^{D}V [the value of (k_H/k_D)obs for V_{max} conditions] will only be close to ^{D}k (intrinsic isotope effect, k_H/k_D) when product release is fast relative to the catalytic step. In the equation for the isotope effect on V/K, C, termed



Reaction 1.



Reaction 2.



Reaction 3.

"commitment to catalysis," is a measure of the enzyme-substrate complex tendency to proceed through the first irreversible step versus reversing to free enzyme and substrate. The value of $^{D}(V/K)$ [the value of (k_H/k_D)obs for V/K conditions] will only be close to ^{D}k if the first irreversible step is also the catalytic step and the reverse step to free enzyme and substrate is fast relative to the catalytic step. What makes Northrop's presentation of isotope effects in this way so powerful is that steady-state equations for much more complex enzyme mechanisms can all be reduced to this form. R and C simply become more complex expressions of a collection of rate constants. In more complicated schemes involving a greater number of reversible steps, an additional grouping of rate constants, C_r, termed the "commitment to reverse catalysis," can be factored from both ^{D}V and $^{D}(V/K)$ experiments. C_r is a measure of the substrates tendency to return to E + S. In such schemes ^{D}V experiments now contain both R and C_r, while $^{D}(V/K)$ experiments will contain C_r and C_f, where C_f is a collection of rate constants termed the "commitment to forward catalysis" that measures the substrate's tendency to move forward to E + P. Despite the greater complexity, the general forms of the equations still indicate that modification of (k_H/k_D)obs relative to k_H/k_D can be readily understood in the interplay of a collection of specific rate constants.

The simplest formulation for a cytochrome P450 (P450[1])-catalyzed reaction involves one extra step over the general scheme presented above. This is the single irreversible step prior to substrate transformation in which the enzyme-substrate complex, ES, is irreversibly transformed into the substrate-bound active oxygenating perferryl oxene enzymatic species, EOS, which then proceeds to the enzyme-product complex, EP, with the rate constant k_{34} (the isotopically sensitive step) (Korzekwa et al., 1989) (Reaction 2). The kinetics of the system have been described (Northrop, 1978, 1981; Cleland, 1982) and the following equations for the isotope effect derived. The term $k_{34H}(1/k_{23} + 1/k_{41})$ in

$$V_H/V_D = ^{D}V = \frac{k_{34H}/k_{34D} + k_{34H}(1/k_{23} + 1/k_{41})}{1 + k_{34H}(1/k_{23} + 1/k_{41})} \quad (8)$$

$$(V/K)_H/(V/K)_D = ^{D}V/K = 1 \quad (9)$$

eq. 8 is termed the V ratio and is the factor that will increasingly mask the intrinsic isotope effect, k_{34H}/k_{34D}; the larger k_{34} is relative to k_{23} and k_{41}. So if product formation is faster than product release, the formation of the perferryl oxene species, the magnitude of the intrinsic isotope effect, will be suppressed; i.e., (k_H/k_D)obs will be smaller than k_H/k_D. Thus, the faster product formation is, the greater the suppression of k_H/k_D. Equation 9 indicates that for V/K conditions [$(^{D}V/K)$], no isotope effect should be observed, i.e., k_H/k_Dobs must equal 1. This is because the first irreversible step in the scheme, conversion of ES to EOS, is not isotopically sensitive. Once EOS is formed, it is committed to continue on to product irrespective of whether or not substrate contains deuterium. Under V/K conditions, when an irreversible step precedes the isotopically sensitive step, the decreased rate constant, k_{34D}, generated by deuterium substitution, will be compensated for by an equal and opposite increase in the concentration of EOS_D, i.e., $(EOS_H)k_{34H} = (EOS_D)k_{34D}$. Thus, for cytochrome P450 reactions, deuterium isotope effects on V/K should in general never be observed (Korzekwa et al., 1989). Isotope effects can only be

[1] Abbreviations used are: P450, cytochrome P450; NDMA, *N*-nitrosodimethylamine; EDB, ethylene dibromide; GSH, glutathione; Tris-BP, tris(2,3-dibromopropyl)phosphate; DBCP, 1,2-dibromo-3-chloropropane; NMF, *N*-methylformamide; NDPS, *N*-(3,5-dichlorophenyl)succinimide; 3-MC, 3-methylcholanthrene; BHT, butylated hydroxytoluene; VPA, valproic acid; 3MI, 3-methylindole.

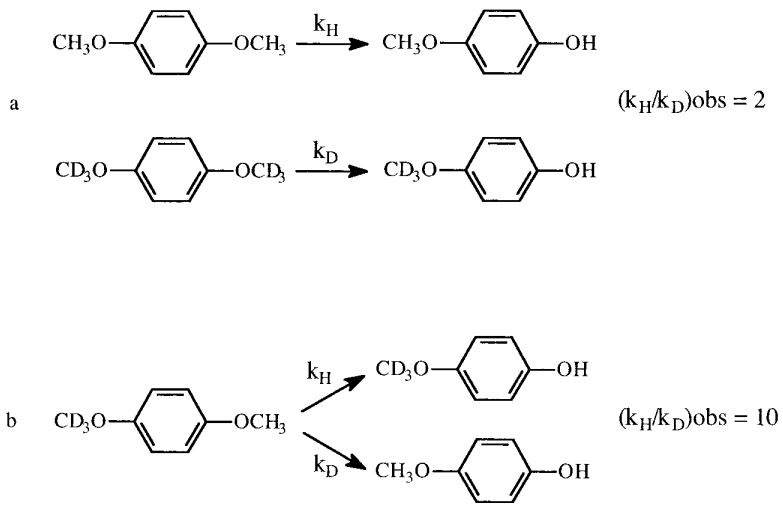
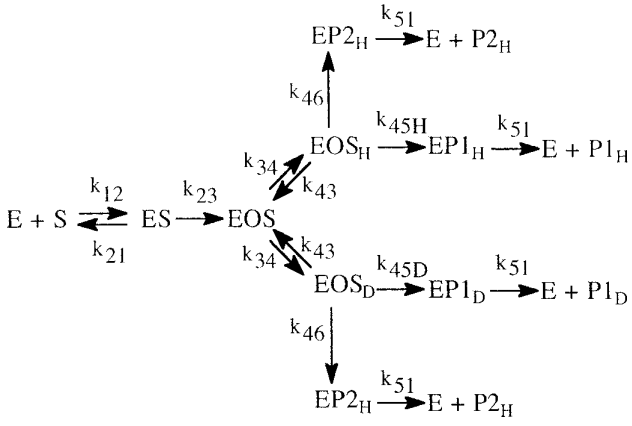


FIG. 1. *The $(k_H/k_D)obs$ value for the O-demethylation of p-methoxyanisole determined from an experiment of intermolecular design (a) and intramolecular design (b).*

observed if some mechanism or some special experimental condition exists that diminishes the increase in EOS_D that occurs in response to the decreased value of k_{34D} relative to k_{34H}. Fortunately, there are both mechanisms and experimental conditions that will tend to equalize (EOS_H) and (EOS_D) and thereby unmask the intrinsic isotope effect, i.e., cause $(k_H/k_D)obs$ to approach k_H/k_D. One mechanism that can achieve this goal is the presence of a branched reaction pathway from the substrate-bound active oxygenating perferryl oxene species, EOS_D, to a nonisotopically sensitive alternate product and free enzyme. A second and similar mechanism is reduction of the perferryl oxene EOS_D to free substrate, free enzyme, and water (Atkins and Sligar, 1987, 1988; Korzekwa et al., 1989). A special experimental condition that can achieve the same end is an isotope effect experiment of symmetrical intramolecular design (Hjelmeland et al., 1977; Miwa et al., 1980; Gelb et al., 1982; Lindsay Smith et al., 1984).

Symmetrical Intramolecular Design

In an isotope effect experiment of intramolecular design, a substrate is chosen that is susceptible to enzymatic attack at either of two symmetrically equivalent sites (Hjelmeland et al., 1977; Miwa et al., 1980; Gelb et al., 1982; Lindsay Smith et al., 1984). One site contains deuterium and the other retains its normal complement of hydrogen. The enzyme then has the choice of attacking a deuterated or an equivalent protio site within the same molecule. It can be viewed as a special case of branching and can be modeled as shown. The observed isotope effect, $(k_H/k_D)obs$, measured as the ratio of product resulting from attack at the protio site versus product resulting from attack at the deutero site, P_H/P_D, reflects the intramolecular competition between these two sites, i.e., $P_H/P_D = k_H[EOS_H]/k_D[EOS_D]$ (Reaction 3). Kinetically, the experimental conditions correspond to a V/K isotope effect (Korzekwa et al., 1989). Thus, $(k_H/k_D)obs$ is independent of all kinetic steps following branching of EOS and can be described by eq. 10. The equation reveals that the faster the rate of reorientation of the substrate is in the active site of the enzyme, k_{43}, relative to the



REACTION 4.

$$P_H/P_D = (k_H/k_D)_{obs} = \frac{k_{45H}/k_{45D} + k_{45H}/k_{43}}{1 + k_{45H}/k_{43}} \quad (10)$$

commitment to catalysis (catalytic step), k_{45H}, i.e., $k_{43} > k_{45H}$, the more the concentrations of EOS_D and EOS_H will equalize and the closer $(k_H/k_D)obs$ will be to k_{45H}/k_{45D}. Conversely, the higher the commitment to catalysis relative to the rate of reorientation, the more the intrinsic isotope effect will be suppressed. An early, if the not first, example of the power of an intramolecular isotope effect experiment to unmask the intrinsic isotope effect is provided by the work of Foster et al. (1974). These investigators measured the deuterium isotope effect associated with the oxidative *O*-demethylation of *p*-methoxyanisole using two different experimental designs. The first was the traditional experiment of intermolecular design in which the rate of demethylation of the hydrogen-containing substrate was measured and compared with the rate of demethylation of the deuterium-containing substrate determined in a separate experiment (Fig. 1a). The observed isotope, $(k_H/k_D)obs$, was found to be 2. The second experiment was of intramolecular design in which the hydrogens of one of the *O*-methyl groups were replaced with deuterium. Incubation of the substrate with a microsomal preparation of cytochrome P450 followed by measurement of the *p*-methoxyphenol to *p*-trideuteromethoxyphenol product ratio gave a $(k_H/k_D)obs$ of 10 (Fig. 1b). The observed products, *p*-methoxyphenol and formaldehyde, are consistent with at least two distinct mechanisms that might be operative. The first would be an insertion mechanism in which an oxygen atom inserts between a carbon-hydrogen bond of one of the methoxy groups to generate a hemiacetal intermediate. The hemiacetal is then hydrolyzed to generate the observed products. This mechanism should be accompanied

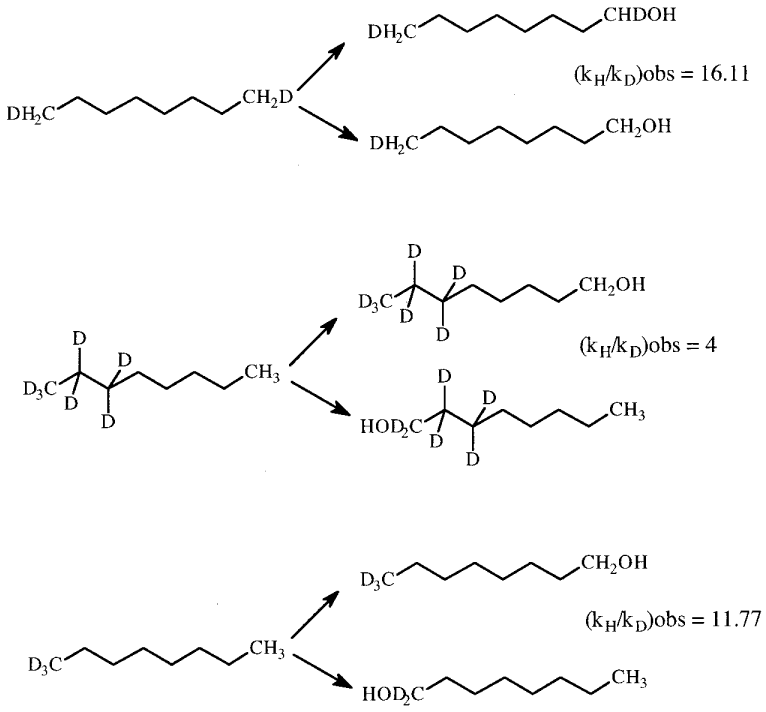


FIG. 2. *The (k_H/k_D)obs values for the formation of n-octanol from n-octane-1,8-$^2H_2$, n-octane-1-$^2H_3$, and n-octane-1,2,3-$^2H_7$.*

by a primary deuterium isotope effect of low magnitude, approximately 2, since the transition state for oxygen insertion would necessarily be nonlinear (Shea et al., 1983). The second mechanism would involve initial hydrogen atom abstraction from the methyl group to form methylene and hydroxy radicals followed by recombination to generate the intermediate hemiacetal. This is the mechanism first postulated by Groves et al. (1978) to account for the cytochrome P450-catalyzed hydroxylation of norbornane, a mechanism that is now generally accepted as the mechanism for all cytochrome P450-catalyzed hydroxylations of aliphatic carbon-hydrogen bonds. Since direct hydrogen abstraction would, if possible, involve a linear transition state, the magnitude of the primary deuterium isotope effect could approach the theoretical value of approximately 9, depending upon the symmetry of the transition state. The (k_H/k_D)obs of 2 given by the intermolecular isotope effect experiment is supportive of a mechanism involving oxygen insertion. In contrast, the (k_H/k_D)obs of 10 from the intramolecular isotope effect experiment is only consistent with the abstraction recombination mechanism. Since (k_H/k_D)obs from the intramolecular isotope effect experiment is much closer to k_H/k_D than is (k_H/k_D)obs from the intermolecular experiment, the choice between possible mechanisms is clear. Indeed the

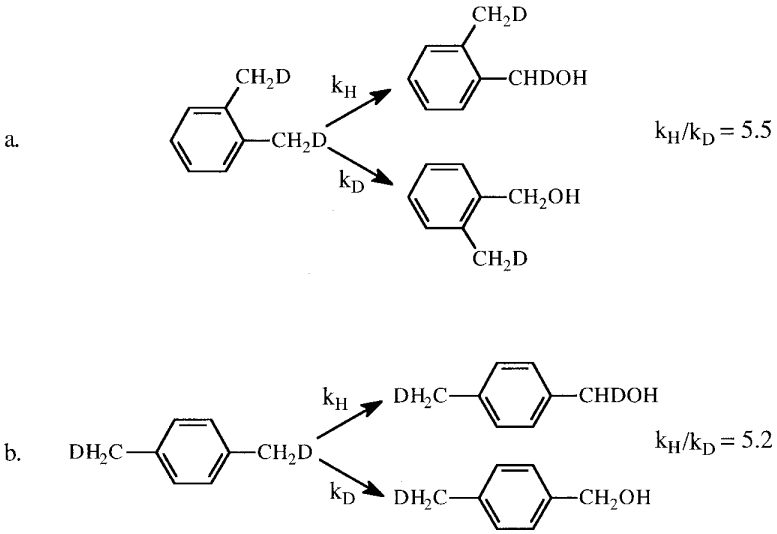


FIG. 3. *The intrinsic isotope effect, k_H/k_D, for the cytochrome P450-catalyzed hydroxylation of (1,2)-dideuteromethyl-o-xylene (a) and (1,2*)-dideuteromethyl-p-xylene (b).*

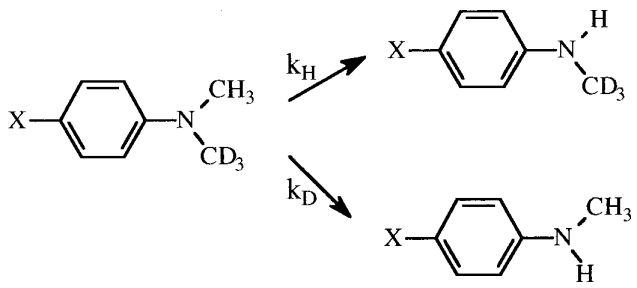


FIG. 4. *The cytochrome P450-catalyzed N*-demethylation of various (X = H, Cl, CN, and NO_2) p-substituted N,N-dimethylaniline analogs.

(k_H/k_D)obs of 10, which is a composite value for one primary and two secondary isotope effects, i.e., PS^2 (Hanzlik et al., 1985) is still large enough to suggest that the transition state is close to symmetrical and that the primary isotope effect is not too far from its maximum value.

Although the ratio of the concentration of $[EOS_H]$ to the concentration of $[EOS_D]$ must equal 1 for (k_H/k_D)obs to be equal to k_H/k_D, rapid equilibration of equivalent but isotopically distinct intramolecular oxidation sites is not the only means by which this condition can be met. As indicated above, the presence of a branched reaction pathway from the substrate-bound active oxygenating perferryl oxene species, EOS_D, to a nonisotopically sensitive alternate product and free enzyme or reduction of the perferryl oxene EOS_D to free substrate, free enzyme, and water can also equalize $[EOS_H]$ and $[EOS_D]$ and lead to (k_H/k_D)obs being equal to k_H/k_D. A model for a branched reaction pathway in competition with the isotopically sensitive step follows (Harada et al., 1984; Jones et al., 1986). In the model, substrate can reversibly reorient in the active site of the enzyme with rate constants k_{34} and k_{43} (Reaction 4). This allows it to present either the deutero site, EOS_D, or the protio site, EOS_H, for catalysis. EOS_H and EOS_D then go on to form products. P1 is formed from EOS_H and EOS_D with rate constants, k_{45H} or k_{45D}, respectively, whereas a single rate constant, k_{46}, characterizes the formation of P2 from EOS_H and EOS_D. This is because P2 arises from oxidative attack at a molecular site remote from the site of deuterium substitution. The observed isotope effect for the model is given by eq. 11. Equation 11 reveals that the rates of substrate reorientation in the active site, k_{43}, and formation of P2, k_{46}, relative to the rate of formation of isotopically sensitive P1 are the factors that govern the magnitude of (k_H/k_D)obs and define the degree of masking of k_{45H}/k_{45D}. The larger k_{43} and/or k_{46} are relative to k_{45H}, the closer (k_H/k_D)obs will be to k_{45H}/k_{45D}.

$$(k_H/k_D)_{obs} = \frac{k_{45H}/k_{45D} + k_{45H}/(k_{43} + k_{46})}{1 + k_{45H}/(k_{43} + k_{46})} \quad (11)$$

The values of (k_H/k_D)obs found for cytochrome CYP2B1-catalyzed ω-hydroxylation of the dideuterated analog of *n*-octane, *n*-octane-1,8-$^2H_2$, the heptadeuterated analog, *n*-octane-1,2,3-$^2H_7$, and the trideuterated analog, *n*-octane-1-$^2H_3$, of 16.11, 4.0, and 11.77, respectively, nicely illustrate the effects of k_{43} and k_{46} (Fig. 2). Before comparing values, it is first necessary to determine k_H/k_D for the reaction. Of the three substrates, the ω-hydroxylation of *n*-octane-1,8-$^2H_2$ can best be expected to provide k_H/k_D. Both terminal methyl groups are isotopically equivalent, and it can be assumed that the inherent rate of rotation of a methyl group is a much faster process than the rate of cleavage of a carbon-hydrogen bond via hydrogen atom abstraction. Thus, the enzyme always has the choice of oxidizing a carbon-hydrogen or carbon-deuterium bond irrespective of which methyl group is oriented for catalysis. The value of (k_H/k_D)obs = 16.11 for this substrate corresponds to an intrinsic primary isotope effect of 9.18 once it has been corrected for the contribution of secondary isotope effects and the fact that the methyl group contains two hydrogens but only one deuterium (Jones and Trager, 1987). As indicated above, an intrinsic primary isotope effect of 9 is the theoretical limit for cleavage of a carbon-hydrogen bond in the absence of tunneling effects (Bell, 1974). This provides compelling evidence that the cytochrome CYP2B1-catalyzed ω-hydroxylation of *n*-octane involves a highly symmetrical transition state and is consistent with the abstraction-recombination mechanism for aliphatic hydroxylation (Groves et al., 1978). In the case of *n*-octane-1,2,3-$^2H_7$, the enzyme has the choice of oxidizing either a carbon-hydrogen bond or a carbon-deuterium bond, depending on which terminal methyl group of the substrate is properly oriented for catalysis. According to eq. 11, if (k_H/k_D)obs is to be close to k_H/k_D, the rate of methyl group interchange, k_{43}, must be much faster than the rate of bond breaking, k_{45H}. The (k_H/k_D)obs of 4 indicates that this is clearly not true for this substrate and suggests that the distance between terminal methyl groups is large enough so that the rate of interchange is slow enough to prevent the concentrations of $[EOS_H]$ and $[EOS_D]$ from equalizing. For the case of *n*-octane-1-$^2H_3$, the enzyme also has the choice of oxidizing either a carbon-hydrogen bond or a carbon-deuterium bond, depending on which terminal methyl group of the substrate is properly oriented for catalysis. What is different about this substrate relative to *n*-octane-1,2,3-$^2H_7$ is that it also has the choice of a branched reaction pathway, (ω-1)-hydroxylation, that is blocked by deuteration in the case of *n*-octane-1,2,3-$^2H_7$.

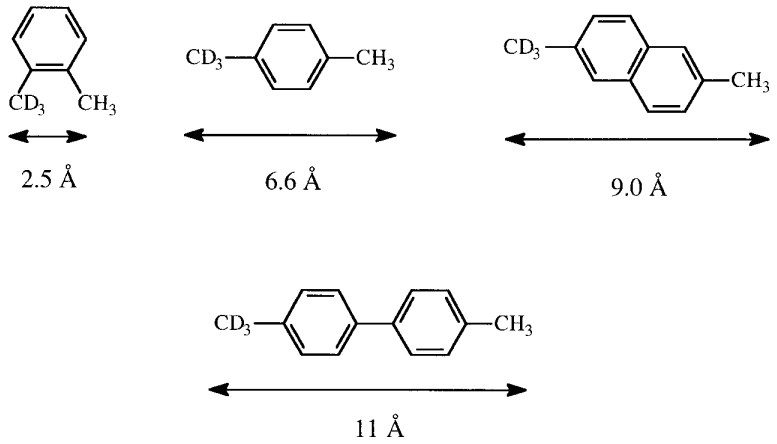


FIG. 5. *The fixed intramolecular distance between methyl and trideuteromethyl groups of o-xylene-α-$^2H_3$, p-xylene-α-$^2H_3$, 2-$^2H_3$-6-dimethylnaphthalene, and 4-$^2H_3$-4′-dimethylbiphenyl.*

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