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INTRAMOLECULAR DEUTERIUM ISOTOPE EFFECT AND ENANTIOTOPIC DIFFERENTIATION IN OXIDATIVE DEMETHYLATION OF CHIRAL [MONOMETHYL-D_3]METHOXYCHLOR IN RAT LIVER MICROSOMES

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Abstract—Intramolecular deuterium kinetic isotope effects on the O-demethylation of methoxychlor [2,2-bis-(4-methoxyphenyl)-1,1,1-trichloroethane] were measured in liver microsomes taken from rats treated with phenobarbital or β-naphthoflavone and from untreated rats. The substrates were (*R*)-, (*S*)- and racemic [monomethyl-d_3]methoxychlor, and the ratio of [d_3]- to [d_0]-mono-O-demethylated metabolites was measured by GC-MS selected-ion monitoring. The magnitude of the observed ratio of [d_3]- to [d_0]-metabolites in each microsomal preparation was largest on the reaction of the (*S*)-substrate, followed by racemic substrate, and then (*R*). Each value is a composite of the intramolecular kinetic isotope effect and enantiotopic differentiation during the reaction. Each intramolecular isotope effect value estimated from these values was smaller than the reported intrinsic value. A relatively slow intramolecular interchange of two methoxyl groups in the methoxychlor molecule in the enzyme–substrate complex was indicated during the reaction. There also was evidence of high enantiotopic differentiation.

Oxidative O-demethylation catalyzed by the cytochrome P-450 system is a common metabolic transformation of xenobiotics that carry a methoxyl moiety. Although this reaction proceeds through C–H bond oxygenation and forms an unstable hemiacetal intermediate which dissociates to an alcohol and formaldehyde, the detailed oxygenation mechanism has yet to be determined. Deuterium isotope effect studies are useful for obtaining detailed information on such mechanisms and, ideally, should use intrinsic isotope effect values, but often apparent intermolecular isotope effect values that are much suppressed are used rather than the full or almost full expression of the intrinsic isotope effect [1] because enzymatic reactions that have various slow steps not sensitive to isotopic substitution exist. Such slow steps might include enzyme-substrate association and dissociation, as well as product-release from the enzyme in the final stage of the reaction.

Northrop has proposed a method with which to obtain intrinsic isotope effect values. It is based on both the apparent deuterium and tritium isotope effect values [1] and is an excellent method with a sound theoretical background. But an inconvenient point is that usually we have to use a tritiated substrate that has a very high specific activity.

Another way to obtain intrinsic isotope effects is based on intramolecular isotope effect studies. Intramolecular competition of two or more reacting moieties should, and does, give a less suppressed isotope effect value [2–4] because of the faster interchange of the labeled and non-labeled reacting moieties in the ES complex† instead of the slower dissociation and association of an enzyme–substrate during intermolecular competition.

Several intramolecular deuterium isotope effect studies on cytochrome P-450-dependent C–H oxygenations and oxidative demethylation have been published [2–4] that report large isotope effect values of 10 to 14, which are believed to be close to the intrinsic isotope effect values. But, smaller intramolecular isotope effect values of 1.6 to 2.0 for the oxidative demethylation of the N-methyl group [5] also have been reported that, based on related experimental results [6], are assumed to be close to the intrinsic isotope effect.

We here describe an intramolecular deuterium isotope effect study of the oxidative demethylation of methoxychlor, a DDT analog and an important insecticide in rat liver microsomes. (*R*)-, (*S*)- and racemic [monomethyl-d_3]methoxychlor (hereafter, [d_3]methoxychlor) were the substrates used. The observed intramolecular values were compared with the intrinsic isotope effect values for the same reaction that we have reported elsewhere [7] using the method of Northrop [1] and the [d_6]- and [^{3}H]-substrates. Intramolecular isotope effect values for the two enantiomers also are compared.

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† Abbreviations: ES complex, enzyme–substrate complex; DDT, 2,2-bis-(4-chlorophenyl)-1,1,1-trichloroethane; methoxychlor, 2,2-bis-(4-methoxyphenyl)-1,1,1-trichloroethane; PB, phenobarbital; and NF, β-naphthoflavone.

MATERIALS AND METHODS

Compounds. Racemic, (*R*)- and (*S*)-[d_3]methoxychlor (Fig. 1) and [dimethyl-d_6]methoxychlor (hereafter, [d_6]methoxychlor) were prepared as reported

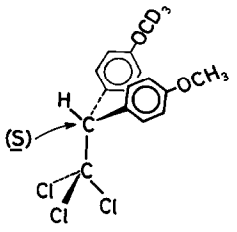


Fig. 1. Structure of (*S*)-[d_3]methoxychlor (one of the possible conformations).

elsewhere [8]. The degree of deuterium incorporation in each methyl group should be 99% or more, because we used [d_3]iodomethane with a 99 D-atom percent, or more. Our check of the degree of incorporation using the mass spectra of deuteriated substrates showed that the d_2-contamination (d_1 and d_0 also) in each [d_3]-substrate was less than 0.1%, evidence of 99.9%, or more, incorporation of deuterium in the [d_3]-substrates. Pentafluoropropionic anhydride was purchased from the Nakarai Chemical Co. Ltd. and distilled prior to use. All the other compounds used were purchased from the Wakenyaku Co. Ltd. and were of the highest purity available commercially.

Microsomes. Liver microsomes were obtained from male Wistar rats (190–230 g) treated with an inducer, or from untreated animals as reported previously [7]. Phenobarbital (PB) and β-naphthoflavone (NF) were the inducers used [7]. At least three separate preparations of liver microsomes from each of the untreated and treated rats were used to obtain the average value of the reaction rate. The cytochrome P-450 content of the microsomal suspension was determined by the method of Omura and Sato [9].

Metabolic conditions and assays. The reaction mixture (2 ml) consisted of liver microsomes containing 360–380 pmol cytochrome P-450, 1 μmol NADPH, the substrate (20 nmol in 10 μl ethanol) and 0.2 M potassium phosphate, pH 7.4. The reaction was started in a glass-stoppered tube by the addition of the substrate after the mixture had been incubated for 0.5 min at 37°. After subsequent incubations of 0.5 to 2.0 min at 37°, the mixture was rapidly cooled to 0°, and then shaken with 5 ml of hexane/ethyl acetate (4:1, v/v). Duplicate or triplicate runs were made for each reaction condition to work up at least two samples. We determined the amount of substrate and metabolites in 2-ml samples of the organic layer by HPLC as described elsewhere (Waters, column M&S Pack C/18, 20 cm, dioxane/methanol/water, 1:3:1, by vol.) [7]. A 2-ml sample of the organic layer was dried in a V-vial under a stream of nitrogen for the separate determination of the [d_0]- and [d_3]-metabolites. Pentafluoropropionic anhydride (80 μl) and distilled ethyl acetate (20 μl) that had been dried over calcium hydride were added. After being capped tightly, the vials were warmed in a heating block for 20 min at 60°, then cooled to room temperature, and their contents dried under a gentle stream of dry nitrogen. After ethyl acetate (20 μl) had been added to form a solution, 1- to 2-μl samples

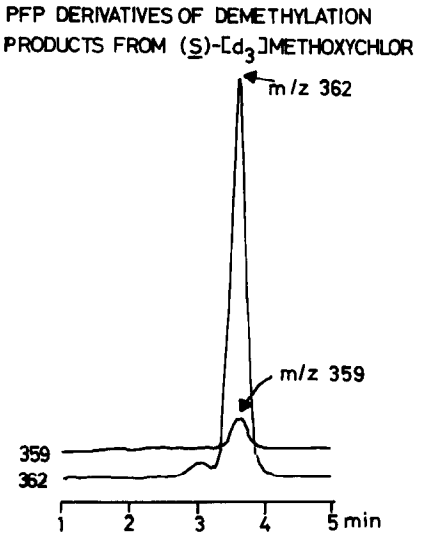


Fig. 2. Mass fragmentogram of the pentafluoropropionyl derivatives of metabolite mixtures. Mono-O-demethylated metabolites of [d_3]methoxychlor were pentafluoropropionylated (PFP); then an aliquot of the PFP derivative mixture was injected into the GC-MS. Ions at *m/z* 359 of [d_0]- and *m/z* 362 in the [d_3]-metabolite were monitored. An example from (*S*)-[d_3]methoxychlor is shown. In this particular analysis, the Shimadzu GCMS QP-1000 machine was used. GC column: 3% OV-3. 2-m, 285°; carrier: He 30 ml/min; electron energy: 70 eV.

were injected into the gas chromatography–mass spectrometer (GC–MS) for selected ion-monitoring analysis. Ions at *m/z* 359 and 362 that appeared as base peaks $[M\text{-}CCl_3]^+$ of the pentafluoropropionyl derivatives of the [d_0]- and [d_3]-metabolites [($C_{18}H_{12}O_3Cl_3F_5$)-(CCl_3) and ($C_{18}H_9D_3O_3Cl_3F_5$)-(CCl_3)] in the mass spectrum were monitored. A mass fragmentogram is shown in Fig. 2.

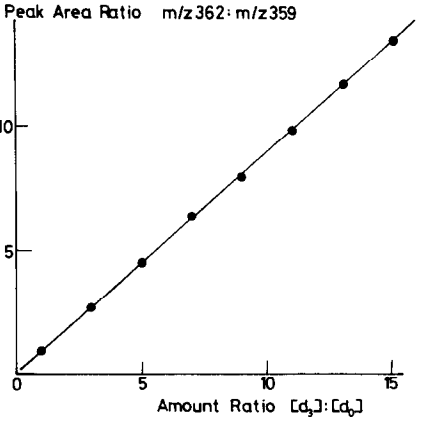


Fig. 3. A calibration curve for the determination of the ratio of the [d_3]- and [d_0]-metabolites. For each GC-MS assay, a series of a standard mixture was analyzed at least twice. The calibration curve was drawn from average values of the peak area ratios. Deviations of two determinations for the area ratios were less than 2%. Peak height ratio determinations gave a similar curve, differences from the area ratios being less than 1.5%.

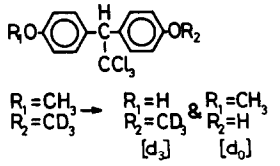


Fig. 4. Oxidative demethylation by microsomes of [d_3]methoxychlor that produces [d_3]- and [d_0]- mono-O-demethylated metabolites.

A linear standard curve (Fig. 3) was obtained by plotting the peak area ratio at m/z 359 and 362 against the ratio of the amount of a standard mixture of the pentafluoropropionyl derivatives of the [d_0]- and [d_3]-metabolites. The range of the ratio of [d_0]:[d_3] was from 1:1 to 1:15. From the curve and the measured peak area ratio of an unknown sample, we calculated the ratio of the [d_0]- and the [d_3]-metabolite.

Gas chromatography–mass spectrometry was done in a Shimadzu GCMS QP-1000 or GCMS-6020. The electron energy was 70 eV or 20 eV, and the gas chromatographic portion was equipped with a 3% OV-3, 2-m or a 3% OV-17, 1-m column. The column temperature was between 250° and 285°.

RESULTS

The brief reactions (see Materials and Methods) of [d_0]-, [d_6]-, (*R*)-[d_3]-, (*S*)-[d_3]- and the racemic [d_3]methoxychlor produced only mono-O-demethylated derivatives (Fig. 4). No di-O-demethylated compound was detected in the products of the short reactions. The demethylation rates of (*R*)-, (*S*)- and racemic [d_3]methoxychlor were calculated from the HPLC-determined amounts of 2-(4-methoxyphenyl)-2-(4-hydroxyphenyl)-1,1,1-trichloroethane ([d_0]-metabolite) and 2-(4-trideuteriomethoxyphenyl)-2-(4-hydroxyphenyl)-1,1,1-tricholorethane ([d_3]-metabolite). Results are shown in Table 1. The demethylation rates of (*S*)-[d_3]methoxychlor were almost identical with those of [d_0]methoxychlor for both drug-treated and untreated liver microsomes and rates for the (*R*)-[d_3]-enantiomer were close to the values found for [d_6]methoxychlor. This is evidence of a high degree of enantiotopic differentiation by the cytochrome P-450 system.

The observed degrees of regio-selectivity, which we have called "apparent intramolecular isotope effects", for the oxidative demethylation of methoxychlor by several preparations of rat liver microsomes are shown in Table 2. The magnitudes of the intrinsic isotope effects, obtained by the method of Northrop [1] from the apparent deuterium and tritium intermolecular isotope effect values on V_{max}/K_m and

Table 1. Demethylation rate of methoxychlor in rat liver microsomes

Substrate* (methoxychlor)	Demethylation rate† (mol/mol P-450/min)		
	Rat liver microsomes		
	Untreated	PB-treated	NF-treated
[d_0]	1.36 ± 0.05 (5)	0.51 ± 0.12 (3)	0.42 ± 0.01 (3)
[d_6]	0.47 ± 0.03 (5)	0.29 ± 0.05 (3)	0.15 ± 0.01 (3)
(*S*)-[d_3]	1.50 ± 0.10 (5)	0.52 ± 0.01 (3)	0.40 ± 0.00 (3)
(*R*)-[d_3]	0.57 ± 0.02 (5)	0.32 ± 0.01 (3)	0.20 ± 0.02 (3)
Racemic [d_3]	0.99 ± 0.03 (5)	0.39 ± 0.01 (3)	0.29 ± 0.01 (3)

* Initial concentration, 10 μM for each substrate.

† The demethylation rate is given as the average value ± SD. The number of independent microsomal preparations used for the reaction is in parentheses. Abbreviations: PB, phenobarbital; and NF, β-naphthoflavone.

Table 2. Apparent intramolecular deuterium isotope effects on the demethylation rate of methoxychlor by rat liver microsomes

Substrate* (methoxychlor)	[d_3]-Metabolite/[d_0]-Metabolite†		
	Rat liver microsomes		
	Untreated	PB-treated	NF-treated
(*S*)-[d_3]	13.63 ± 0.61 (5)	6.85 ± 0.08 (3)	6.20 ± 1.05 (3)
(*R*)-[d_3]	1.06 ± 0.10 (4)	0.71 ± 0.15 (3)	1.34 ± 0.10 (3)
Racemic [d_3]	4.71 ± 0.58 (5)	2.55 ± 0.00 (3)	4.27 ± 0.08 (3)

* Initial concentration, 10 μM for each substrate.

† The ratio of [d_3]-metabolite and [d_0]-metabolite was determined by selected ion monitoring of their pentafluoropropionyl derivative and is given as the average value ± SD. The number of independent microsomal preparations used for the reaction is in parentheses. Abbreviations: PB, phenobarbital; and NF, β-naphthoflavone.

reported elsewhere [7], are 15.2 (average of 17.0 and 13.4) for the O-demethylation of methoxychlor by microsomes from untreated rat livers and 19.2 for NF-treated rat liver. The intramolecular isotope effect value for each [d_3]-substrate was smaller than the reported intrinsic isotope effect value. The magnitude of the apparent intramolecular isotope effect on (*S*)-enantiomer demethylation was greater than on the (*R*)-enantiomer for each microsomal preparation, again a reflection of enantiotopic differentiation.

DISCUSSION

Values for the intramolecular deuterium isotope effect on the microsomal P-450-catalyzed oxygenation rate have been reported for several substrates: 11 ± 1 for [1,1-d_2]1,3-diphenylpropane [2], 11.5 ± 1 for [2,3,5,6-d_4]norbornane [3] and 10 for [methyl-d_3]4-methoxyanisole [4]. All the reported values are large and are believed to represent intrinsic isotope effect values [2]. Oxidative N-demethylation of some drugs catalyzed by the cytochrome P-450 system shows smaller intramolecular isotope effects (1.6 to 2.0 [5]), but these values also are close to the intrinsic isotope effect values based on other evidence [6].

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_5} EP \longrightarrow E + P$$

In enzyme-catalyzed reactions, as above, several slow steps that precede (or follow) the catalytic step may mask the intrinsic isotope effect values so that the observed values are much smaller than the intrinsic ones. For example, if the rate constant of the dissociation of the *ES* complex in a typical Michaelis–Menten type equilibrium step (k_2) is markedly small in comparison with the rate constant of the catalytic step (k_5), then the observed deuterium isotope effect on (V/K) [= (V_{max}/K_m)]

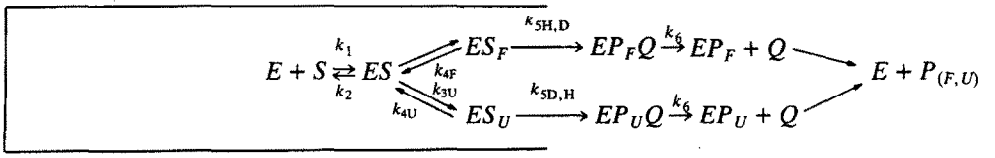
$$^{D}(V/K) = [^{D}k + (k_{5H}/k_2)]/[1 + (k_{5H}/k_2)]$$

will be much smaller than the intrinsic value. In this equation, $^{D}(V/K)$ is the observed deuterium isotope on (V/K), and ^{D}k is the intrinsic isotope effect value, (k_{5H})/(k_{5D}). (Equations and notations are according to Northrop [1].) If k_2 is much larger than k_{5H}, the observed isotope effect, $^{D}(V/K)$, will be very close to the ^{D}k value.

When two competing and reacting groups such as $-OCH_3$ and $-OCD_3$ are present in the same molecule, the interchange of groups in the *ES* complex (which corresponds to the equilibrium of $ES1 \rightleftharpoons ES2$ rather than $E + S \rightleftharpoons ES$) is expected to be more rapid than the intermolecular interchange of reacting molecules, making k_2 much larger than k_5 and $^{D}(V/K)$ very close to the value for ^{D}k.

In the examples of the intramolecular isotope effect studies [2–5] cited above, the reacting groups (methylene, methoxyl or methylamino) in the same molecule appear to undergo rapid interchange in the *ES* complex as expected. Our finding of a pair of chiral [d_3]methoxychlors suggests that the interchange of reacting groups in the substrate takes place slowly as follows.

The kinetic relations in the oxidative demethylation of methoxychlor in two pathways can be shown as



in which ES_F and ES_U are the enzyme–substrate complexes for the two possible conformers (favorable and unfavorable) that correspond to which of the two prochiral anisyl groups is interacting with the catalytic site. k_3 and k_4 control the two anisyl—actually two methoxyl—interchange at the catalytic site, and Q is the first product released after the step sensitive to the isotope effect of the methoxyl hydrogens (formaldehyde).

When we use (*R*)- and (*S*)-[d_3]methoxychlor in the reaction instead of unlabeled methoxychlor, the d_0-methoxyl group interacts with the catalytic site after which enzymatic demethylation in a favorable or unfavorable conformer of the enzyme–substrate complex takes place depending on the chirality of the molecule. We can predict from the apparent intramolecular isotope effect values that the d_0-methoxyl group will react in the favorable conformer in the enzyme-(*S*)-enantiomer complex (Fig. 5) and in the unfavorable conformer in the (*R*)-enantiomer complex.

In the above scheme, the ratio of the products formed from the (*S*)-enantiomer by the upper pathway through the favorable conformation of the *ES* complex versus the ratio for the lower pathway

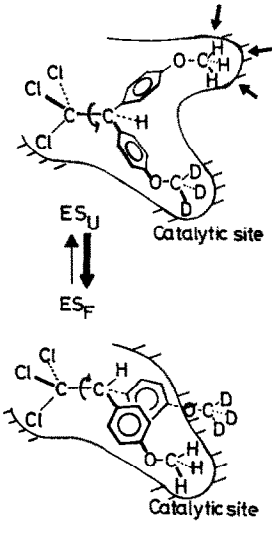


Fig. 5. Possible conformations of the enzyme–substrate complex. Favorable and unfavorable conformations of the *ES* complex containing (*S*)-[d_3]methoxychlor molecule are shown.

through the unfavorable conformation is expressed as

$$^{D}v_S = \frac{k_{5H}[ES_F]}{k_{5D}[ES_U]}$$

when the catalytic step is not reversible.*

At a steady-state concentration of ES_F and ES_U,

$$[ES_F] = \frac{k_{3F}[ES]}{k_{4F} + k_{5H}}$$

$$[ES_U] = \frac{k_{3U}[ES]}{k_{4U} + k_{5D}}$$

Thus,

$$^{D}v_S = \frac{k_{5H}k_{3F}[ES]/(k_{4F} + k_{5H})}{k_{5D}k_{3U}[ES]/(k_{4U} + k_{5D})} = \frac{k_{5H}k_{3F}(k_{4U} + k_{5D})}{k_{5D}k_{3U}(k_{4F} + k_{5H})}$$

and similarly for the (*R*)-[d_3]-enantiomer,

$$^{D}v_R = \frac{k_{5H}k_{3U}(k_{4F} + k_{5D})}{k_{5D}k_{3F}(k_{4U} + k_{5H})}$$

If we assume that the two reacting methoxyl groups are interchanged in the *ES* complex much faster than in the catalytic step, i.e. if k_{4F} (and k_{4U}) $\gg k_{5H}$ (and k_{5D}), then the two equations above can be written

$$^{D}v_S = \frac{k_{5H}k_{3F}k_{4U}}{k_{5D}k_{3U}k_{4F}}$$

$$^{D}v_R = \frac{k_{5H}k_{3U}k_{4F}}{k_{5D}k_{3F}k_{4U}}$$

and $\sqrt{^{D}v_S \times {}^{D}v_R}$ is equal to $(k_{5H})/(k_{5D})$. Substituting the observed $^{D}v_S$ and $^{D}v_R$ values into $\sqrt{^{D}v_S \times {}^{D}v_R}$ gives 3.8 for microsomes from untreated rats, a much smaller value than the 15.2 value reported for $(k_{5H})/(k_{5D})$. So we must abandon the assumption that the rate of interchange of the reacting groups in the *ES* complex would be much greater than the rate for the catalytic step. At least, k_{4F} or k_{4U} must not be much greater than k_{5H}.

We can calculate some relations between these rate constants from an intermolecular isotope effect study. As reported previously [7], the apparent intermolecular isotope effect obtained from the pair of the [d_0]- and [d_6]-methoxychlor in microsomes from untreated rats was about 2.5 for the overall rate v as well as V_{max} and V/K. assuming that each of these symmetrical substrates basically reacted through the favorable conformation of the *ES* complex, we obtain

$$v_H = \frac{k_{3F}k_{5H}k_6[ES]}{k_{4F} + k_{5H}}$$

and

$$v_D = \frac{k_{3F}k_{5D}k_6[ES]}{k_{4F} + k_{5D}}$$

Therefore,

$$\frac{v_H}{v_D} = 2.5 = \frac{k_{5H}(k_{4F} + k_{5D})}{k_{5D}(k_{4F} + k_{5H})}$$

From the relation $k_{5H} = k_{5D} \times 15.2$ (intrinsic isotope effect value) and the above equation, we obtain

$$(k_{5H})/(k_{4F}) = 8.47 \quad \text{and} \quad (k_{5D})/(k_{4F}) = 0.55.$$

Thus, k_{5H} is much larger than k_{4F}, and k_{5D} is approximately half the k_{4F} value. Therefore, intramolecular methoxyl interchange in the enzyme substrate complex is *not* a very fast process; it suppresses the intramolecular isotope effect to a value considerably lower than the intrinsic one.

For the drug-induced microsomes, we have an intrinsic isotope effect value for NF-induced microsomes: $(k_{5H})/(k_{5D}) = 19.2$ [7]. Taking into account this value as well as the experimental values $^{D}v_S = 6.20$ and $^{D}v_R = 1.34$ (Table 2), we conclude that methoxyl group interchange in the *ES* complex for NF-induced microsomes also is *not* a very fast process.

A probable cause of the slow interchange rate of methoxyl groups is the great distance between the groups separated by two benzene rings and one methine carbon, which differs from the structural characteristics reported for the substrates in the literature [2–5]. The relation between the distance that separates the two reacting groups in the molecule and their interchange rate in the *ES* complex is a topic to be investigated.

We have shown an example of an intramolecular isotope effect value that is considerably suppressed because of the slow interchange of two competing moieties in the substrate in the *ES* complex. We also have shown the usefulness of chirally deuteriated substrates in detecting enantiotopic differentiation in enzyme-catalyzed reactions.

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ADDENDUM

After the initial submission of this report (received October 23, 1986), a paper pertinent to our study was published by Sugiyama and Trager [10]. They studied prochiral selectivity and intramolecular isotope effects in the cytochrome P-450 catalyzed ω-hydroxylation of cumene using racemic, (*R*)- and (*S*)-[1,1,1-d_3]-substrates (and the [1-^{13}C]-substrate to study product stereoselectivity). Their discussion and conclusions are essentially the same as ours. Interestingly, from their data they posited a *small* degree of incomplete equilibrium of two prochiral methyl groups in the *ES* complex. For our substrates, the value $\sqrt{^{D}v_S \times {}^{D}v_R}$ was much smaller than the intrinsic isotope effect value, which suggests a *slow* interchange of the two prochiral anisyl groups and a *large* degree of incomplete equilibrium.

* When we used a [d_3]-substrate contaminated with $(a \times 100)$% of [d_0]-substrate, the observed $^{D}v_S$ should have been expressed as

$$k_{5H}[ES_F]/[(k_{5D} + ak_{5H})(ES_U)];$$

but, because the value "*a*" was less than 0.001 (as noted in Materials and Methods), we can neglect the term (ak_{5H}) without serious change in the following discussion.

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