Determination of the Mechanism of Demethylenation of (Methylenedioxy)phenyl Compounds by Cytochrome P450 Using Deuterium Isotope Effects

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The mechanism of demethylenation of (methylenedioxy)benzene (MDB), (methylenedioxy)amphetamine (MDA), and (methylenedioxy)methamphetamine (MDMA) by purified rabbit liver cytochrome P450IIB4 has been investigated by using deuterium isotope effects. A comparison of the magnitude and direction of the observed kinetic isotope effects indicates that the three compounds are demethylenated by different mechanisms. The different mechanisms of demethylenation have been proposed on the basis of comparisons of the observed biochemical isotope effects with the isotope effects from purely chemical systems.

Introduction

The (methylenedioxy)phenyl group is found in several classes of pharmacologically active compounds. Biochemically, the function can be oxidized by cytochrome P450 to an intermediate that binds to the heme and inhibits the monooxygenase.[1] This feature is exploited by insecticide synergists to enhance the toxicity of insecticides by inhibiting their degradation.[2] The drugs of abuse, (methylenedioxy)amphetamine (MDA) and (methylenedioxy)methamphetamine (MDMA), also contain this functionality and are of current interest because of their neurotoxicity.[3-5] In order to study the possible effect of the rate of metabolic demethylenation on the neurotoxicity of MDA and MDMA, we prepared deuterium-substituted derivatives (substituted in the methylenedioxy group) with the intent of utilizing isotope-dependent differences in metabolism of the methylenedioxy group in differentiating potential pathways of potential neurotoxicity. However, initial investigations on the in vitro metabolism of the deuterium- and hydrogen-substituted compounds revealed significant and unusual isotope effects, both in magnitude and direction, as a function of substrate and enzyme source. Therefore, before the effect of the demethylenation rate on neurotoxicity can be assessed, the basis of these differing isotope effects must be addressed.

This paper describes results of a study investigating the basis for these differences. MDA, MDMA, the unsubstituted analogue, (methylenedioxy)benzene, MDB, and their corresponding deuterated analogues (structures shown in Figure 1) were compared as substrates for cytochrome P450 in microsomal and reconstituted preparations of isozyme IIB4. To assess potential reaction pathways, isotope effects in model chemical reactions representing possible steps in the metabolic demethylenation pathway were determined for comparison. The results show that changes in structure can cause significant mechanistic differences in the cytochrome P450 mediated oxidation of the methylenedioxy group.

Experimental Section

Materials. MDB obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI) contained small amounts of catechol, which were removed by washing with 1 N NaOH. MDA and MDMA were obtained from the Research Technology Branch of The National Institute on Drug Abuse (Rockville, MD). (Methylenedioxy)-phenylacetone was synthesized according to the procedure of Shulgin and Jacob.[6] *N*-Hydroxy-MDA was synthesized by a method described previously.[7] Deuterium-substituted MDB, MDA, and MDMA were synthesized from the corresponding catechols according to the method of Clark et al.[8] Dihydroxyamphetamine (DHA, α-methyldopamine) was donated from Merck Sharp and Dohme Laboratories (West Point, PA). Dihydroxymethamphetamine (DHMA) was synthesized according to the method of Smissman and Borchardt.[9] Catechol, NADPH, NADP, scopoletin, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, horseradish peroxidase, HEPES, hydrogen peroxide, cytochrome *c*, and dilauroylphosphatidylcholine were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of the highest grade available.

Preparation of Microsomal Cytochrome P450. Control rabbit liver and lung microsomes were prepared according to the general method of Hiramatsu et al.[5] Phenobarbital-induced microsomes were prepared according to the method of Florence et al.[10] General incubation conditions for all three microsomal preparations have been described previously.[5]

Purification of Cytochrome P450IIB4 and NADPH–Cytochrome P450 Reductase. Isozyme IIB4 was purified from liver microsomes of phenobarbital-treated male New Zealand rabbits according to the method of Coon and co-workers,[11] the specific content was 16.5 nmol/mg protein. The concentration of cytochrome P450 was determined by the method of Omura and Sato.[12]

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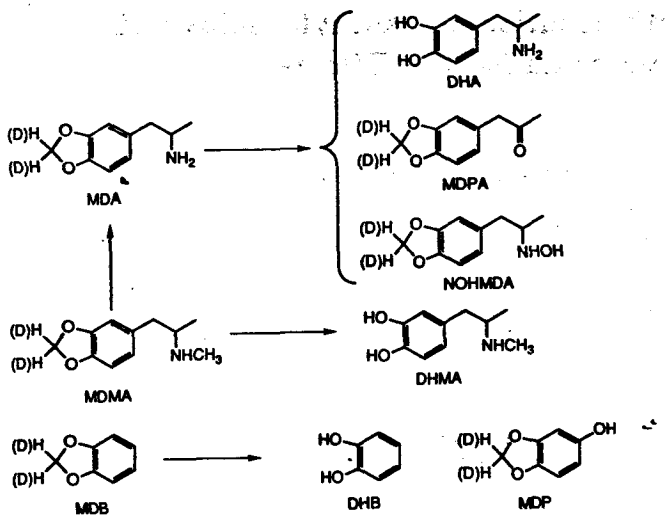

Figure 1. Structures of the (methylenedioxy)phenyl compounds and their metabolites. (Methylenedioxy)amphetamine, MDA; (methylenedioxy)methamphetamine, MDMA; (methylenedioxy)benzene, MDB; dihydroxyamphetamine, DHA; (methylenedioxy)phenylacetone, MDPA; *N*-hydroxy(methylenedioxy)-amphetamine, NOHMDA; dihydroxymethamphetamine, DHMA; dihydroxybenzene, DHB; (methylenedioxy)phenol, MDP.

NADPH–cytochrome P450 reductase was purified from liver microsomes of phenobarbital-treated rabbits as described previously.[13] The specific activity for the purified preparation was 64.9 units/mg protein when cytochrome *c* reductase activity was measured in 0.3 M potassium phosphate buffer, pH 7.7, containing 0.1 mM EDTA, according to the method of Yasukochi and Masters.[14] One unit of NADPH–cytochrome P450 reductase activity was expressed as 1 μmol of cytochrome *c* reduced/min at 25 °C under the above conditions. Each preparation of isozyme IIB4 and NADPH–cytochrome P450 reductase showed a single band on SDS–polyacrylamide gel electrophoresis. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as standard.

Enzyme Assay. The reconstituted incubation mixture contained purified cytochrome P450IIB4 (0.2 nmol), 1.5 units of NADPH–cytochrome P450 reductase, 30 μg of dilauroylphosphatidylcholine, 1 mM substrate, 1 mM ascorbate (for preventing further oxidation of catechol metabolite formed), and 0.2 mM NADPH, in a total volume of 1.0 mL, unless otherwise noted. The reactions were initiated by addition of the NADPH and terminated by addition of 7.5% perchloric acid (final concentration, 2.5%). Incubations were performed at 37 °C for 2 min (MDB) and 5 min (MDA and MDMA). Under these conditions, the demethylenation activities for MDB, MDA, and MDMA were linear with regard to time and P450 concentration. The quenched reaction mixtures were centrifuged at 13500*g* for 5 min, and a portion (10 μL) of the supernatant was analyzed by high-performance liquid chromatography with electrochemical detection (HPLC-ECD, conditions described later). The stoichiometry of NADPH consumption, catechol (and other metabolite) formation, and hydrogen peroxide production was determined. To monitor NADPH consumption, the incubation components were the same as those described above except that 0.2 mM NADPH was used instead of the NADPH-generating system. NADPH consumption was measured by the decrease in absorbance at 340 nm, using an extinction coefficient of 6.26 mM^{-1} cm^{-1}, employing a UVIKON 810 spectrophotometer (Kontron Instruments, Hayward, CA) at 37 °C. The hydrogen peroxide generated during oxidation of MDB, MDA, and MDMA was determined by the fluorometric assay of Hildebrandt and Roots[15] with the following modifications: To stop the reaction, 0.5 mL of 6% trichloroacetic acid was added and the mixture centrifuged. The supernatant (1.0 mL) was mixed with 0.06 mL of 2 M triethanolamine buffer, pH 10.3, containing 0.15 M KOH and 25 mM scopoletin. After preincubation for 2 min, a second reaction was initiated by addition of 0.1 mL of horseradish peroxidase (40 units/mL). The incubations were carried out at 25 °C for 2 min. The reaction was terminated by the addition of 2.84 mL of 0.15 M tetraborate buffer, pH 10, and then each sample (50 mL) was quickly diluted 100 times with the tetraborate buffer. The diluted samples were measured at 380 nm (excitation) and 460 nm (emission) on an Aminco-Bowman spectrophotofluorometer (American Instrument Co. Inc., Silver Spring, MD). For the quantitation of non-catechol metabolites, standard curves were generated with [$^2H_2$]MDMA as the internal standard for MDA, and 1-phenyl-2-butanone was the internal standard for *N*-hydroxy-MDA and (methylenedioxy)phenylacetone. After centrifugation, the supernatants were extracted and treated according to the method described previously[16] and were assayed by gas chromatography–mass spectroscopy (GC-MS).

Chemical Demethylenation of MDB, MDA, and MDMA by Hydroxyl Radical. Hydroxyl radical mediated cleavage of the methylenedioxy function of MDB, MDA, and MDMA were examined by using a hydroxyl radical generating system containing ascorbate.[17] The reaction mixture consisted of MDB (2 mM), MDA or MDMA (1 mM), 10 μM ferric chloride, 20 μM EDTA, and 30 mM potassium phosphate buffer, pH 7.4, in a final volume of 1.0 mL. The reaction was initiated by the addition of ascorbate (final concentration of 1 mM). Incubations were carried out at 37 °C for 5 min and terminated by addition of 0.5 mL of 7.5% perchloric acid containing 30 mM thiourea (the ascorbic acid system mediated reaction could not be completely stopped by perchloric or trichloroacetic acid only). The reaction mixtures were then assayed for products as described in the enzyme assay.

HPLC. Catechols obtained from the oxidation of MDB, MDA, or MDMA were separated on a Biophase ODS column (4.6 × 250 mm, particle size 5 μm, Bioanalytical Systems, Inc.) using a mobile phase consisting of 0.1 M citrate buffer, pH 3.5, containing 1 mM sodium octyl sulfate/acetonitrile/methanol (8:1:1, by volume) at a flow rate of 0.8 mL/min. The compounds were detected with an electrochemical detector equipped with a glassy carbon working electrode (LC-4, Bioanalytical system, Inc.) set at +0.7 V (vs Ag/AgCl reference electrode). Under these conditions, retention times of catechol, dihydroxyamphetamine, and dihydroxymethamphetamine were 9.8, 10.0, and 11.6 min, respectively.

GC–MS. A Hewlett-Packard 5971A GC-MS system was used. The GC was equipped with an HP fused silica capillary column (12.5 m × 0.2 mm i.d.) with cross-linked methylsilicone operating with a temperature program from 70 to 195 °C at a rate of 25 °C/min. Under these conditions, the retention times of MDA, *N*-hydroxy-MDA, and (methylenedioxy)phenylacetone were 5.50, 5.20, and 4.78 min, respectively.

Determination of the Isotope Effect for the Hydrolysis of Phenyl Formate. Phenyl formate was synthesized according to the method of Van Es and Stevens.[18] The deuterium-sub-

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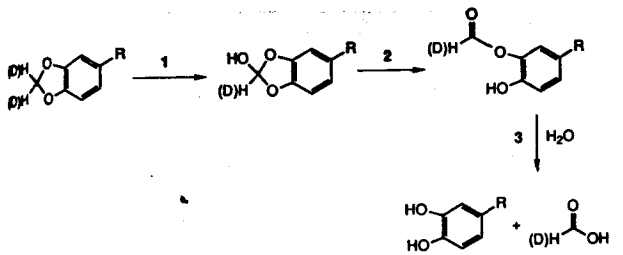

Figure 2. Proposed pathway for the metabolic demethylenation of (methylenedioxy)phenyl compounds.

Table I. Stoichiometry of MDB, MDA, and MDMA Oxidation with Reconstituted Rabbit Liver Cytochrome P4502B4[a]

species measured	(methylenedioxy)phenyl substrate (nmol/min) MDB	MDA	MDMA
NADPH consumption	−8.76	−1.6	−1.41
catechol formation	+8.47	+0.13	+0.20
other metabolites	0	+0.24[b]	+0.47[c]
H_2O_2 formation[d]	−2.02	+0.53	+1.44

[a] These numbers were determined with the cytochrome as the rate-limiting component in the presence of a saturating level of NADPH-cytochrome P450 reductase. In the absence of substrate, the rates of NADPH consumption and H_2O_2 production were 4.98 and 3.90 nmol/min, respectively. Each value is the mean of two to four determinations. Incubations were carried out under the conditions described in the Experimental Section. [b] Besides the catechol metabolite, production of (methylenedioxy)phenylacetone (0.15 nmol/min) and *N*-hydroxy-MDA (0.09 nmol/min) was determined by GC–MS. [c] Besides catechol metabolite, production of (methylenedioxy)phenylacetone (0.09 nmol/min), *N*-hydroxy-MDA (0.02 nmol/min), and MDA (0.36 nmol/min) was determined by GC–MS. [d] Hydrogen peroxide determinations were performed in the absence of ascorbate in separate experiments. Therefore, H_2O_2 levels presented here cannot be stoichiometrically compared to other values in the table.

stituted analogue (deuterium substituted on the carbonyl) was synthesized by the same method except that deuterated formic acid (MSD Isotopes, 95% in D_2O, 99.6 atom % D) was used. The isotopic purity of the deuterio phenyl formate was determined to be >98% by ^1H NMR. A 1 mM solution of the substrate was made up in 0.1 M HEPES buffer, pH 7.6, and the increase in phenol concentration was monitored at 274 nm at 37 °C. The experiment was repeated three times each for the protio and deuterio substrates, and the averages of the pseudo-first-order rate constants were determined. The rate constant for protio phenyl formate was 0.00186 ± 0.00004 s^{-1}, and the rate constant for deuterio phenyl formate was 0.00212 ± 0.00008 s^{-1}.

Results

The demethylenation of (methylenedioxy)phenyl compounds generates the corresponding catechol and formic acid,[19] presumably according to the pathway shown in Figure 2. For MDB, demethylenation was the major reaction. A small quantity of the ring-hydroxylated product 3,4-(methylenedioxy)phenol was formed with liver microsomes and the purified cytochrome P450IIB4 isozyme (Kumagai et al., unpublished observation). The presence of the 2-aminopropyl side chain on the amphetamine analogues results in additional oxidation products, the nature of which were established by comparison with authentic compounds. The products generated in microsomal and reconstituted preparations are shown in Figure

Table II. Isotope Effects for Demethylenation of (Methylenedioxy)phenyl Compounds[a]

enzyme source	demethylenation activity MDB	MDA (nmol min^{-1} mg^{-1} protein)	MDMA
1. With Saline-Treated Liver Microsomes			
D0 substrate	3.60 ± 0.14	0.62 ± 0.02	1.29 ± 0.02
DD2 substrate	3.92 ± 0.10	0.65 ± 0.01	1.22 ± 0.01
(k_H/k_D)	(0.92*)[b]	(0.95**)[c]	(1.06**)
2. With Phenobarbital-Treated Liver Microsomes			
D0 substrate	17.49 ± 0.41	0.94 ± 0.02	1.17 ± 0.06
DD2 substrate	21.97 ± 0.30	0.92 ± 0.02	1.33 ± 0.08
(k_H/k_D)	(0.80**)	(1.02)	(0.88*)
3. With Saline-Treated Lung Microsomes			
D0 substrate	4.92 ± 0.36	0.12 ± 0.02	0.18 ± 0.04
DD2 substrate	5.35 ± 0.85	0.06 ± 0.02	0.15 ± 0.04
(k_H/k_D)	(0.92)	(2.00**)	(1.20**)
4. With Cytochrome P450 IIB4[d]			
D0 substrate	30.63 ± 0.95	0.32 ± 0.01	0.55 ± 0.03
DD2 substrate	34.45 ± 1.52	0.10 ± 0.01	0.48 ± 0.05
(k_H/k_D)	(0.89*)	(3.20**)	(1.15*)

[a] Each substrate (1 mM) was incubated under the conditions described in the Experimental Section. Each value is the mean ±SD of four determinations. [b] (*) $P < 0.05$. [c] (**) $P < 0.01$. [d] Activities were expressed as nmol min^{-1} $nmol^{-1}$ of P450.

1. For the amphetamine compounds, N-hydroxylation, deamination, and N-demethylation of MDMA were observed and the nature and quantity of each compound were established. The quantities of each product formed under the incubation conditions for purified cytochrome P450IIB4 are indicated in Table I (see footnotes).

The nature of the observed variation in isotope effects (k_H/k_D) on catechol formation by various enzyme sources is shown in Table II. Microsomes from untreated and phenobarbital-pretreated liver and lungs from rabbits were compared for their activity toward the three substrates. The absolute rates and ratios of k_H/k_D were different for all three substrates. The rate of MDB demethylenation was more rapid than the corresponding rate for either MDA or MDMA and was much greater in liver from rabbits pretreated with phenobarbital. The reconstituted preparation had the highest activity for MDB and the lowest for both amphetamine analogues.

In an effort to clarify these variations in the isotope effect, the demethylenation reaction was studied in further detail with purified and reconstituted cytochrome P450 isozyme IIB4, the major phenobarbital-inducible isozyme,[20] and cytochrome P450 reductase. As shown in Table II, in purified, reconstituted systems MDB was the best overall substrate (most rapid turnover) of the three compounds. Its rate of demethylenation was 50–100 times faster than that of the corresponding MDA or MDMA compounds. The increase in specific activity with phenobarbital induction and in lung microsome preparations and the high specific activity in the reconstituted system indicate that MDB is demethylenated for the most part by cytochrome P450IIB4. The rates of the MDA and MDMA compounds were much lower, and the low specific activities in the reconstituted preparation indicate a dominant participation by other, constitutive isozymes. In the purified cytochrome P450IIB4 preparation, deuterium

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for hydrogen substitution on the methylenedioxy group resulted in an increased rate of catechol formation for MDB ($k_H/k_D = 0.89$) and a decreased rate for both MDA and MDMA. MDA experienced the greatest rate decrease ($k_H/k_D = 3.2$), while MDMA was demethylenated only slightly slower with deuterium substitution ($k_H/k_D = 1.15$).

The efficiencies (efficiency of NADPH utilization) of the three compounds as substrates for cytochrome P450IIB4 were also determined (Table I). The rates of catechol formation, NADPH consumption, and hydrogen peroxide production were measured as a function of substrate. MDB was found to be the most efficient substrate (Table II) in that the amount of catechol formed was almost equal to the amount of NADPH consumed and no hydrogen peroxide was produced. In fact, MDB reduced the basal formation of hydrogen peroxide (discussed later). MDA and MDMA were relatively inefficient substrates; the concentrations of catechols and other products were low, and the rate of NADPH consumption was far greater than the rate of product formation. Hydrogen peroxide concentration in the reaction mixtures was greater in the presence of MDA and MDMA than in their absence, indicating that they were uncouplers of cytochrome P450, generating H_2O_2 by a NADPH-dependent process. MDMA was the most potent uncoupler of the three substrates, producing hydrogen peroxide at almost 3 times the rate of MDA.

Since hydrogen peroxide is formed as a result of inefficient MDA and MDMA metabolism, the possibility exists that hydrogen peroxide (or rather hydroxyl radical generated from hydrogen peroxide by Fenton reactions) may be at least partially responsible for some of the demethylenation of these two substrates. Therefore, a chemical system for the generation of hydrogen peroxide under Fenton conditions was utilized to obtain reference isotope effects for hydroxyl radical mediated demethylenation. In enzymatic systems, this active oxygen species could be generated through uncoupling of cytochrome P450 to hydrogen peroxide, which could be then converted to hydroxyl radical by Fe^{II} present in trace concentrations or by cytochrome P450 itself.[21] The deuterium isotope effect for the demethylenation of MDB, MDA, and MDMA by hydroxyl radical (produced from the reduction of hydrogen peroxide by Fe^{II}; see Experimental Section) was found to be between 1.1 and 1.2 for all three substrates (data not shown). Thus, oxidative demethylenation by hydroxyl radical exhibits a small and positive deuterium isotope effect.

The proposed reaction pathway for demethylenation shown in Figure 2 indicates that the reaction is a multistep process. In order to assess the deuterium isotope effect for the hydrolysis of the formate ester (Figure 2, step 3), another chemical model was used. The hydrolysis of phenyl formate was examined. Both protio and deuterio phenyl formate (substituted on the carbonyl carbon) were hydrolyzed in 0.1 M HEPES buffer, pH 7.6, at 37 °C. The relative rates of hydrolysis were determined by monitoring the formation of phenol at 274 nm. The kinetic isotope effect (k_H/k_D) for this reaction was found to be 0.88 (Figure 3).

It should be noted that the isotope effects reported for the enzymatic reactions are the observed and not the intrinsic effects on V_{max} only. In other words, they represent possible combinations of effects in any rate-determining

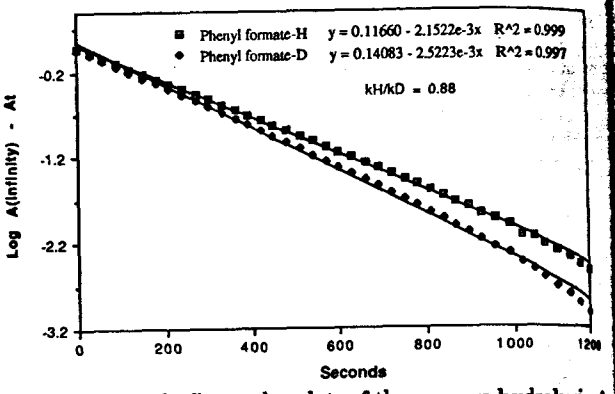

Figure 3. Pseudo-first-order plots of the aqueous hydrolysis of phenyl formate and phenyl [^{2}H]formate. Hydrolysis performed in 0.1 M HEPES buffer, pH 7.6 at 37 °C.

or partially rate determining step. Due to experimental difficulties associated with working with these substrates (inhibitory complex formation, solubility, etc.), intrinsic isotope effects were not obtainable.

Discussion

The results of this study indicate that the demethylenation of MDB, MDA, and MDMA by cytochrome P450 can occur by processes with different rate-limiting steps that can be distinguished on the basis of the direction and magnitude of deuterium isotope effects in purified systems. Deuterium for hydrogen substitution has been used many times previously as a mechanistic probe for a variety of reactions catalyzed by cytochrome P450. For example, kinetic deuterium isotope effects have been used to study the mechanism of cytochrome P450 catalyzed N-dealkylation[22-24] and O-dealkylation.[25] Deuterium isotope effects have led to an improved understanding of arene[26] and benzylic[27] oxidations performed by cytochrome P450. The magnitude of the deuterium isotope effect has also been used to distinguish between possible oxidative mechanisms for dihydropyridines.[28] Also, in a study on the bioactivation of (methylenedioxy)phenyl compounds utilized as insecticide synergists, deuterium substitution dramatically lowered the effectiveness of the compound.[29]

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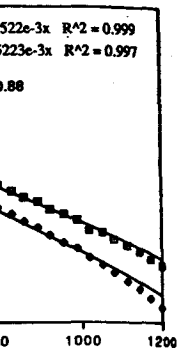

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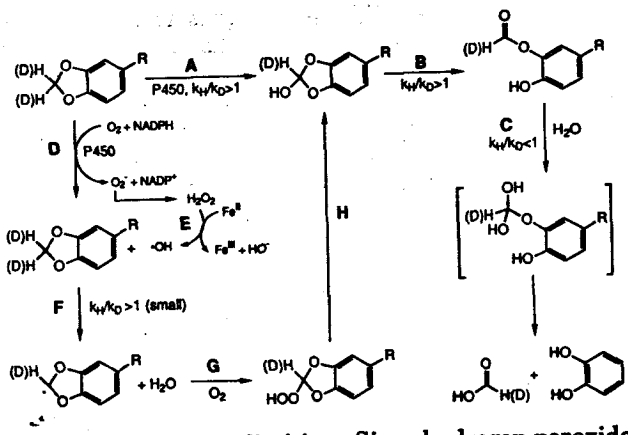
Presumably this effect was due to decreased reactivity with cytochrome P450. The results presented in this paper indicate that the magnitude and direction of an observed deuterium kinetic isotope effect can be useful information for distinguishing between differing oxidative mechanisms catalyzed by cytochrome P450.

The demethylenation of (methylenedioxy)phenyl compounds is thought to occur by a three-step process involving initial oxidation of the methylene carbon, followed by a rearrangement and hydrolysis step (Figure 2). It would be predicted that step 1 would exhibit a normal primary deuterium isotope effect. That is, deuterium for hydrogen substitution would decrease the rate of carbon–hydrogen bond cleavage ($k_H/k_D > 1$). Step 2 does not involve the breaking of a carbon–hydrogen bond but should still exhibit a normal, but small, secondary isotope effect since there is a hybridization change of sp^3 to sp^2.[30] However, in step 3, deuterium substitution should actually increase the rate of formate ester hydrolysis due to the hybridization change from sp^2 to sp^3 in the transition state. Thus, step 3 would be expected to exhibit an inverse isotope effect ($k_H/k_D < 1$). The magnitude of this effect was determined by measuring the aqueous hydrolysis of a model compound, phenyl formate, and found to be 0.88. This confirms the predicted inverse isotope effect and is in almost exact agreement with the isotope effect for the cytochrome P450 mediated demethylenation of MDB ($k_H/k_D = 0.89$). Other workers have also observed inverse isotope effects for the acid- and base-catalyzed hydrolysis of alkyl formates.[31] Since the observed deuterium isotope effect, k_H/k_D, for the demethylenation of MDB by cytochrome P450IIB4 is inverse and similar to what is observed for the hydrolysis of phenyl formate, step 3 must be the predominant, rate-determining step for the overall reaction sequence.

MDB was also found to be the most efficient substrate in that it not only is rapidly demethylenated but, on the basis of NADPH consumption and hydrogen peroxide concentration, did not uncouple cytochrome P450. In fact, addition of MDB to the reaction mixture reduces the basal production of hydrogen peroxide by cytochrome P450IIB4. Cytochrome P450 is capable of producing hydrogen peroxide in the absence of substrate by catalyzing the direct reduction of molecular oxygen by NADPH,[32] and a good substrate will inhibit this process. These data suggest that the rate of carbon oxidation is rapid and not rate limiting for a good substrate for cytochrome P450IIB4 such as MDB, and that the overall rate of catechol formation is at least partially limited by the rate of hydrolysis of the aryl formate intermediate.

MDA, on the other hand, is a relatively poor substrate for cytochrome P450IIB4. It is only slowly oxidized by the purified enzyme system and appears to slightly uncouple the enzyme to generate hydrogen peroxide. The deuterium isotope effect for the demethylenation of MDA (k_H/k_D), as measured by catechol formation, was determined to be 3.2, a significant normal primary isotope effect. In the case of MDA, the initial oxidation step, which involves carbon-hydrogen bond cleavage (step 1 in Figure 2), must be at least partially rate limiting. Since hydrogen peroxide is also produced from the apparent uncoupling of the enzyme by MDA, some of the oxidative demethylenation of MDA could be a result of hydroxyl radical generation from the reaction of hydrogen peroxide and the reduced form of the iron protein (Fenton-type processes). The contribution of the hydroxyl radical mediated oxidative pathway to the overall oxidation of MDA is likely to be minimal, however, since the isotope effect for the demethylenation of MDB, MDA, and MDMA by hydroxyl radical alone was found to be much smaller than that observed in the enzymatic system (approximately 1.2 compared to 3.2). Therefore it appears that MDA is demethylenated by a mechanism in which the cytochrome P450IIB4 catalyzed oxidation of the methylene group is at least partially rate determining.

Scheme I. Proposed Mechanism for the Demethylenation of MDA, MDMA, and MDB by a Cytochrome P450



MDMA, like MDA, is also a poor substrate for cytochrome P450IIB4. It is converted to the corresponding catechol only slowly and is the most potent uncoupler of the enzyme of the three substrates tested. It produces hydrogen peroxide at almost 3 times the rate of MDA (Table I). The deuterium isotope effect, k_H/k_D, for the demethylenation of MDMA, as measured by catechol formation, was found to be 1.15. This effect is significantly smaller than that for MDA and is similar to that found for the hydroxy radical mediated demethylenation of methylenedioxy compounds. However, it is impossible to determine, from this data, whether the observed isotope effect is a result of a hydroxyl radical mediated process or simply a combination of the isotope effects from several steps in the demethylenation process. Though hydroxyl radical mediated oxidations catalyzed by cytochrome P450 have been previously reported (see, for example, Perrson et al.[33]), we are currently unable to distinguish between this and other possible mechanisms.

It should be mentioned that the cytochrome P450 catalyzed demethylenation of MDB, MDA, and MDMA was also performed in the presence of catalase in order to assess the importance of hydrogen peroxide in the respective oxidations (data not shown). Catalase addition had no effect on the rate of MDB oxidation and had only a small inhibitory effect (approximately 7%) on the rate of MDA

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demethylenation. Catalase did inhibit the rate of oxidation of MDMA more than either MDB and MDA to the extent of only about 10%. This result argues against the participation of hydroxyl radical mediated processes for MDMA hydroxylation. Interpretation of these results must be approached with some caution, however, since it is possible that the hydrogen peroxide responsible for demethylenation of MDMA is generated directly at the active site of the enzyme (where the uncoupler MDMA resides) and performs its chemistry without ever leaving the enzyme and, therefore, cannot be efficiently trapped by catalase.

The general processes possibly involved in the demethylenation of the three substrates MDB, MDA, and MDMA are summarized in Scheme I. MDB, a good substrate for cytochrome P450IIB4, is rapidly oxidized to the hydroxylated acetal (step A), which then rapidly rearranges to the formate ester (step B). The formate ester is then hydrolyzed, in a partially rate determining step, to the catechol and formic acid (step C). MDA is also demethylenated by the same pathway. However, since it is a poorer substrate for cytochrome P450IIB4, the initial hydroxylation (step A) becomes significantly rate determining as evidenced by the relatively large and positive primary observed isotope effect of 3.2. MDMA is also a poor substrate for P450IIB4. The small positive isotope effect may reflect a combination of opposing isotope effects (i.e., steps A, B, and C) or possibly be due to a significant contribution by hydroxyl radical as an oxidant. MDMA is capable of generating significant levels of hydrogen peroxide. The hydrogen peroxide thus formed may be converted to hydroxyl radical by the iron heme protein, step E, and possibly participate in the overall oxidation, step F, by abstracting a hydrogen (deuterium) atom. This step would occur with a small positive isotope effect. The radical intermediate can then react with molecular oxygen and eventually decompose to give the hydroxylated acetal species, steps G and H. The corresponding catechol is then formed by the previously described pathway. The degree of participation by hydroxyl radical to the overall demethylenation process is unknown, and though any further speculation is unwise, it must be considered as a mechanistic possibility since hydrogen peroxide is generated in the incubation mixtures. It should be emphasized that these pathways would be available to all the substrates and that the observed isotope effects may only reflect a change in the contribution of a single step to the overall rate of catechol formation.

The initial results, shown in Table II, can now be accounted for, in part, by the role of the isozyme IIB4 in the oxidation of each substrate. MDB is a highly efficient substrate primarily for the IIB4 isozyme in all enzyme preparations, and its rate of demethylenation reflects the levels of this isozyme in the different microsomal preparations, i.e., very high in lung and phenobarbital liver microsomes.[34] The inverse isotope effect observed in all enzyme preparations for MDB is also consistent with this hypothesis. MDA and MDMA, on the other hand, are poor substrates for the IIB4 isozyme in microsomal preparations, and the contributions of other constitutive isozymes in their demethylenation is more significant. The varied isotope effects for MDA and MDMA as a function of the enzyme source probably reflect the sum of contributions from a variety of isozymes and cannot be easily explained or dissected. In the purified cytochrome P450IIB4 system, however, it is clear that both MDA and MDMA are demethylenated by mechanisms that exhibit different isotope effects. The contribution of the IIB4 isozyme isotope effects for MDA and MDMA to the effects observed in other microsomal preparations is likely to be small since neither is a particularly good substrate for cytochrome P450IIB4. In order to properly evaluate the origin of the various MDA and MDMA isotope effects, studies with other purified isozymes (constitutive enzymes, for example) are needed. The results presented herein, however, indicate that the use of isotope effects is a powerful tool in the dissection and determination of the relevant metabolic pathways of the kind described in this study. Both the magnitude and direction of the effect can be used to discern mechanistic distinctions which may otherwise be inaccessible.

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Registry No. MDA, 4764-17-4; MDMA, 42542-10-9; MDB, 274-09-9; DHA, 555-64-6; MDPA, 4676-39-5; NOHMDA, 74696-47-8; DHMA, 15398-87-5; DHB, 120-80-9; MDP, 69393-72-2; cytochrome P450, 9035-51-2; NADPH cytochrome P450 reductase, 9039-06-9.