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METABOLISM OF METHYLENEDIOXYPHENYL COMPOUNDS BY RABBIT LIVER PREPARATIONS

PARTICIPATION OF DIFFERENT CYTOCHROME P450 ISOZYMES IN THE DEMETHYLENATION REACTION

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Abstract—The cytochrome P450-mediated oxidative demethylenation of the benzo-1,3-dioxoles (methylenedioxyphenyl compounds, MDPs), methylenedioxybenzene (MDB), methylenedioxyamphetamine (MDA), and methylenedioxymethamphetamine (MDMA), by rabbit liver microsomes and cytochrome P450IIB4 (*CYP2B4*) was examined. Material balance studies indicated that demethylenation to catechol derivatives is a major metabolic pathway for MDB, MDA and MDMA. The reactions required NADPH and were inhibited by CO/O_2 (4:1, v/v). Biphasic double-reciprocal plots of MDMA, MDA and MDB oxidation suggested participation of more than one isozyme of cytochrome P450 in the reaction. Phenobarbital (PB) induction was selective in that the V_{max} values for MDB were increased but not those for MDA and MDMA. Exposure of liver microsomes from PB-pretreated animals to phencyclidine (PCP) markedly suppressed MDB oxidation but had little effect on MDA and MDMA demethylenation. Reconstitution experiments with *CYP2B4* demonstrated that MDB is a good substrate for the isozyme; but the relative demethylenation activities for MDA and MDMA were 1 and 2% of that for MDB. These results indicate that the PB-inducible isozymes such as *CYP2B4* appear to play an important role in MDB demethylenation, whereas MDA and MDMA oxidation is mediated mainly by constitutive isozymes.

Methylenedioxyamphetamine (MDA§) and its *N*-methyl derivative, methylenedioxymethamphetamine (MDMA), are hallucinogens that affect serotonergic (5HT) neurotransmitter systems. These compounds also cause a neurotoxicity evidenced by a long-term depletion of brain 5HT [1] that is associated with histologically demonstrated lesions [2]. This long-term depletion appears to be metabolism dependent and, for this reason, the metabolism of these compounds has come under scrutiny [3–5]. Recently, we demonstrated that MDMA is demethylenated to dihydroxymethamphetamine (DHMA) by cytochrome P450 and the DHMA is oxidized by superoxide in rat liver preparations to a semiquinone or quinone species, which reacts easily with nucleophiles such as glutathione [6]. Since demethylenation is an important first step in the generation of a potentially toxic species, the scope and structural requirements of the reaction are of general interest. The present report describes results of an investigation of the demethylenation reactions of three MDPs, methylenedioxybenzene (MDB), MDA, and MDMA, mediated by rabbit liver microsomes and by P450IIB4 (*CYP2B4*) purified from liver microsomes of phenobarbital (PB)-treated rabbits.

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§ Abbreviations: MDA, methylenedioxyamphetamine; MDB, methylenedioxybenzene; MDMA, methylenedioxymethamphetamine; MDPA, methylenedioxyphenylacetone; PB, phenobarbital; DHA, dihydroxyamphetamine; PCP, phencyclidine; DHMA, dihydroxymethamphetamine; and HPLC–ECD, high pressure liquid chromatography–electrochemical detection.

MATERIALS AND METHODS

Chemicals. MDB and formaldehyde were obtained from the Aldrich Chemical Co. Inc. (Milwaukee, WI). The MDB contained small amounts of catechol which was removed by washing with 1 N NaOH. MDA·HCl and MDMA·HCl were obtained from the Research Technology Branch of The National Institute on Drug Abuse (Rockville, MD). Dihydroxyamphetamine (DHA) was obtained from Merck Sharp & Dohme Laboratories (West Point, PA). Methylenedioxyphenylacetone (MDPA) was prepared according to the procedure of Shulgin and Jacob [7]. *N*-Hydroxy MDA was synthesized by a method described previously [4]. Benzphetamine was a gift from the Upjohn Co. (Kalamazoo, MI). DHMA was synthesized according to the method of Smissman and Borchardt [8]. Phencyclidine hydrochloride (PCP·HCl) was synthesized by the method of Kalir *et al.* [9] using piperidine as the starting amine. Catechol, HEPES, NADP, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, cytochrome *c* and dilauroylphosphatidylcholine were obtained from the Sigma Chemical Co. (St. Louis, MO). Phenobarbital was purchased from the Amend Drug & Chemical Co. (Irvington, NJ). All other chemicals used were of the highest grade.

Preparation of microsomes and their incubation conditions. Liver microsomes from male New Zealand white rabbits (2.5 to 3.0 kg) were prepared by procedures published previously [10] and were stored at −80° before use. For studies on PB induction, rabbits were injected with sodium PB (60 mg/kg, i.p.) every day for 3 days. Control animals were injected with the same volume of 0.9% saline. To obtain the PCP-pretreated microsomes, liver microsomes from PB-pretreated rabbits were incubated with PCP (1 mM) in the presence of an NADPH-generating system at 37° for 90 min under the conditions reported previously [11]. The PCP-exposed microsomes were obtained according to the method of Hoag *et al.* [12]. The activity of these microsomes was always compared with microsomes preincubated in the absence of PCP. Protein concentrations were determined by the Bio-Rad Protein assay (Bio-Rad Laboratories, Richmond, CA) with bovine γ-globulin (Cohn Fraction II) as standard. The incubation mixture contained substrate (1 mM), an NADPH-generating system consisting of 0.5 mM NADP, 8 mM glucose-6-phosphate, 5 mM $MgCl_2$ and 1 unit of glucose-6-phosphate dehydrogenase, microsomal preparation (0.5 to 0.9 mg of protein), 1 mM ascorbate and 0.1 M HEPES, pH 7.6, in a final volume of 1.0 mL, unless otherwise noted. After preincubation for 5 min, the reactions were initiated by addition of the enzyme solution and terminated by adding 0.5 mL of 7.5% perchloric acid. Incubations were carried out for 5 min at 37°. The quenched reaction mixture was centrifuged at 13,500 *g* for 5 min, and a portion of the supernatant (10 μL) was analyzed by high pressure liquid chromatography with electrochemical detection (HPLC-ECD). Benzphetamine (1 mM) was incubated under the same conditions, and the formaldehyde produced was determined by the method of Nash [13]. In the material balance studies of MDPs, the microsomal incubation mixtures (3 mL) consisted of the same components described above with a substrate concentration of 5 μM and a protein concentration of 4.5 nmol of cytochrome P450. The reactions were stopped by addition of 1.5 mL of 7.5% perchloric acid containing internal standard ($^2H_2$-MDMA was used for the analysis of MDB, MDA, and MDMA, and 1-phenyl-2-butanone was used for *N*-hydroxy-MDA and MDPA). After centrifugation, the supernatant was divided into two portions. One sample was used for the assay of catechol metabolites using HPLC-ECD. The other sample was processed [14] for the assay of MDB, MDA, MDMA and their metabolites by gas chromatography–mass spectrometry (GC–MS). For analysis of hydrogen peroxide production, the untreated microsomal preparations were incubated with and without 50 μM MDPs in the presence of the NADPH-generating system and 0.2 mM sodium azide. The supernatants (0.2 mL) obtained from the quenched reaction mixtures were assayed for H_2O_2 by the $FE(SCN)_3$ assay of Hildebrandt and Roots [15].

Enzyme assay with purified P450. P450 *CYP2B4* was purified from liver microsomes of PB-treated rabbit according to the method of Coon and co-workers [16, 17]. Cytochrome P450 concentration was determined by the method of Omura and Sato [18] using an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$. The lower apparent specific activity may be the result of the Bio-Rad protein assay with γ-globulin as standard (with bovine serum albumin as the standard, the specific content of *CY2B4* and the specific activity of NADPH–cytochrome P450 reductase were 16.5 nmol/mg and 64.9 units/mg, respectively). The specific content of the final preparation was 8.0 nmol/mg protein. NADPH–cytochrome P450 reductase was purified from hepatic microsomes of PB-treated rabbits by DEAE-Sephadex A-25 column chromatography and 2′,5′-ADP-agarose affinity column chromatography as described previously [19]. Cytochrome P450 reductase activity was determined by measuring cytochrome *c* reduction at 550 nm using an extinction coefficient of 21 $mM^{-1}\,cm^{-1}$ according to the method of Yasukochi and Masters [20]. The final preparation had a specific activity of 31 units/mg protein when cytochrome *c* reductase activity was assayed in 0.3 M potassium phosphate buffer, pH 7.7, 0.1 mM EDTA. One unit of NADPH–cytochrome *c* reductase activity is defined as the amount of enzyme catalyzing the reduction of cytochrome *c* at an initial rate of 1 μmol/min at 25°. Each preparation of *CYP2B4* and NADPH–cytochrome P450 reductase gave a single major band, upon sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. The incubation mixture used 0.1 nmol *CYP2B4* for MDB and 0.2 nmol for MDA and MDMA together with cytochrome P450 reductase at levels of 0.8 units. These proteins were mixed with 30 μg of dilauroylphosphatidylcholine, 1 mM substrate, 1 mM ascorbate, the NADPH-generating system, and 0.1 M HEPES, pH 7.6, in a final volume of 1.0 mL. The reaction was initiated by the addition of the NADPH-generating system and was carried out at 37° for 2 min for MDB and 5 min for MDA and MDMA. The reactions were terminated by the same method described in the microsomal experiments.

HPLC. HPLC separation of the reaction components utilized a Biophase ODS column (4.6 × 250 mm, particle size 5 μM, bioanalytical System, Inc., West Lafayette, IN) and 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 vol.) at a flow rate of 0.7 mL/min. Metabolites 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, the retention times of catechol, DHA and DHMA were 9.8, 10.0 and 11.6 min, respectively.

GC–MS. A Hewlett-Packard 5971A GC–MS system was employed in the selected ion monitoring mode with the internal standards. The GC was equipped with an HP fused silica capillary column (12.5 × 0.2 mm i.d.) with cross-linked methylsilicone operated with a temperature program from 70 to 195° at a rate of 25°/min. Under these conditions, the retention times of MDB, MDA, MDMA, *N*-hydroxy MDA and MDPA were 2.07, 5.59, 6.20, 5.20 and 4.78 min, respectively. The quantitation of

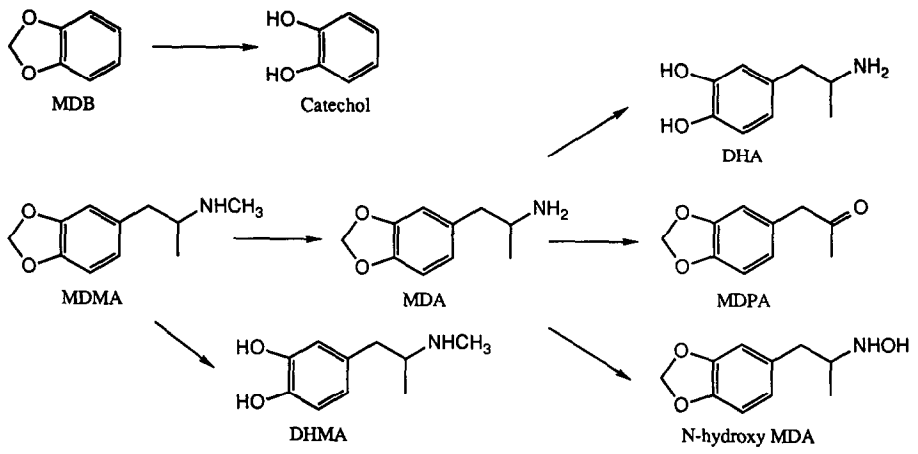

Fig. 1. Proposed biotransformation of methylenedioxybenzene (MDB), methylenedioxyamphetamine (MDA) and methylenedioxymethamphetamine (MDMA) in rabbit liver.

Table 1. Biotransformation of MDPs in rabbit liver microsomes

	Metabolite produced (nmol)						
Substrate	Catechol	DHA	DHMA	MDA	MDPA	*N*-Hydroxy MDA	Total
MDB	8.43	—	—	—	—	—	8.43
MDA	—	5.91	—	—	0.34	6.12	12.37
MDMA	—	1.03	5.31	2.29	0.24	3.84	12.71

Substrate (15 nmol) was incubated with the microsomal preparation (4.5 nmol of P450) for 5 min under the conditions described in Materials and Methods. Levels of catechol, DHA and DHMA were measured by HPLC–ECD and those of MDB, MDA, MDMA, MDPA and *N*-hydroxy MDA were determined by GC–MS. During the reaction period, the amounts of the substrates consumed were 8.67 (MDB), 11.95 (MDA) and 13.65 nmol (MDMA), respectively. Each value is the mean of two determinations.

these MDPs was performed by previously published procedures [14].

RESULTS

Characterization and identification of metabolites. The formation of metabolites was dependent on microsomes, substrates and NADPH; products were identified by comparison with authentic compounds. The metabolites generated when substrate (15 nmol) was incubated with rabbit microsomes containing 4.5 nmol of cytochrome P450 for 5 min are shown in Fig. 1. Results of material balance studies for each substrate (Table 1) show that under the incubation conditions, demethylenation of the amphetamine derivatives accounted for almost 50% of consumed substrate. The material balance under these conditions was essentially complete although a small quantity of an unidentified electrochemically active product was present in MDMA incubates. The catechols were identified by co-chromatography using HPLC–ECD, whereas *N*-hydroxy MDA and MDPA were identified by GC–MS. Demethylenation is thus a major metabolic pathway for the three MDPs, and the nature of this particular reaction was examined in more detail.

Properties of demethylenation. Various compounds were added to the incubation mixture to characterize the enzymes catalyzing demethylenation and revealed differences in susceptibility (Table 2). Hydrogen peroxide (5 mM) could replace NADPH in the reaction, but while the rate for MDA was similar to that with NADPH, the rate for MDB in hydrogen peroxide-supported oxidation was much slower. The sensitivity of the reaction to SKF-525A also varied with substrate and MDB demethylenation was least sensitive. Replacement of the incubation atmosphere with CO/O_2 (4:1) suppressed all demethylenation reactions, consistent with involvement of the cytochrome P450 monooxygenase. Ascorbic acid and superoxide dismutase (SOD), which convert superoxide to hydrogen peroxide [21, 22], increased the levels of catechols from MDA and MDMA, but not from MDB, suggesting that the catechols were reacting further with superoxide. Previous studies with rat liver microsomes showed that DHMA formed from MDMA is oxidized rapidly by superoxide [6]. Superoxide, which dismutates to

Table 2. Effects of P450 inhibitors and active oxygen scavenging agents on the demethylenation of MDB, MDA and MDMA by rabbit liver microsomes

Addition	Demethylenation (% of control)		
	MDB	MDA	MDMA
None	100	100	100
Hydrogen peroxide* (5 mM)	9 ± 2	92 ± 10	55 ± 13
SKF-525A (1 μM)	108	89 ± 5	82 ± 5
SKF-525A (10 μM)	105	48 ± 2	39 ± 3
SKF-525A (100 μM)	78 ± 3	23 ± 1	0
CO/O_2 (4:1, v/v)	51 ± 1	57 ± 6	64 ± 2
Ascorbic acid (1 mM)	107 ± 2	126 ± 9	139 ± 8
SOD (100 units)	115 ± 4	122 ± 6	135 ± 6

All substrates (50 μM) were incubated with rabbit liver microsomes (1.75 nmol of P450) in the presence of an NADPH-generating system for 5 min. Under these conditions, the amounts of each demethylenated product formed from MDB, MDA and MDMA were 7.39 ± 0.31, 3.35 ± 0.12 and 3.20 ± 0.3 nmol, respectively. Each value is the mean ± SD of four determinations.

* Incubation was carried out in the absence of the NADPH-generating system.

Table 3. Effects of MDB, MDA and MDMA on the NADPH-dependent hydrogen peroxide production by rabbit liver microsomes

Substrate	Hydrogen peroxide formed (nmol)
None	26.08 ± 2.18 (100)
MDB	29.40 ± 1.07 (113)
MDA	33.75 ± 0.55* (129)
MDMA	40.69 ± 0.82† (156)

Liver microsomes from untreated rabbits (1.92 nmol of P450) were incubated with an NADPH-generating system in the absence and presence of MDPs (50 μM) for 5 min. Each value is the mean ± SD of four determinations. The values in parentheses represent the percentage of the hydrogen peroxide generated in the absence of substrate.

* $P < 0.01$ vs none.

† $P < 0.01$ vs MDA.

hydrogen peroxide, can be generated by microsomes through uncoupling of cytochrome P450 [23]. Consistent with this notion, each substrate caused an increase in hydrogen peroxide by an NADPH-dependent process with the amphetamines being the most effective (Table 3). Ascorbate at 1 mM was shown to block superoxide-mediated oxidation of MDA and MDMA so that subsequent experiments were carried out in its presence [6].

Under these conditions, the rates of the oxidation of these methylenedioxyphenyl compounds were linear with time for at least 5 min and linearly dependent on microsomal protein concentration up to 2 mg. To determine kinetic parameters for demethylenation of MDB, MDA and MDMA, substrate concentration ranges of 2 to 1000 μM were employed. Figure 2 shows the Lineweaver–Burk plots of MDB, MDA and MDMA demethylenation by liver microsomes of saline- and PB-treated rabbits. The double-reciprocal plots were biphasic in all cases (Fig. 2), suggesting that more than one cytochrome P450 isozyme participates in the reaction. The data were then analyzed by a non-linear regression program (BMDP) to generate kinetic parameters. The parameters, which were found to be significant at $a = 0.01$ (F statistic, [24]) for all substrates, are shown in Table 4. The K_m and V_{max} values for MDB were greater than those for either MDA or MDMA. PB induction resulted in a marked increase in both low K_m and V_{max} values for MDB but had little effect on the values for MDA or MDMA.

Isozyme specificity. Osawa and Coon [25] have reported recently that pretreatment of rabbit liver microsomes with PCP causes selective inactivation of *CYP2B4* without affecting other forms such as isozymes *CYP1A1*, *CYP1A2*, *CYP2C3* and *CYP2E1*. Therefore, PCP pretreatment of microsomes from PB-treated animals was considered a convenient method for removal of *CYP2B4*. Exposure of PCP to PB-treated microsomes under conditions described in Materials and Methods decreased P450 content to 55% of control. The activities of benzphetamine N-demethylation and MDB demethylenation were reduced by 55 and 64%, respectively (Fig. 3), in the PCP-treated microsomes, whereas MDA and MDMA demethylenation were only minimally altered.

The selectivity toward MDB by isozyme *CYP2B4* was demonstrated by direct experiments. The results of oxidative cleavage of the methylenedioxyphenyl compounds by reconstituted isozyme *CYP2B4* are shown in Table 5. When the dilauroylphosphatidylcholine concentration (30 μg/mL) was fixed, the maximal rate of MDP metabolism was obtained at reductase/*CYP2B4* ratios of about 8 for MDB and 3 for MDA and MDMA. The activity was reduced by omission of the phospholipid. MDB oxidation was linear up to 0.2 nmol of the isozyme *CYP2B4* and for at least 2 min, whereas metabolite formation from MDA and MDMA was almost linear at isozyme *CYP2B4* concentrations of up to 0.4 nmol and for 10 min. The results of the comparison show

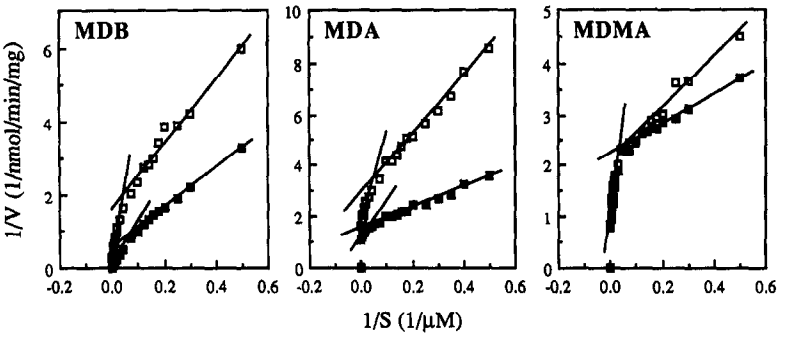

Fig. 2. Double-reciprocal analysis of demethylenation activities for MDB, MDA and MDMA in livers from saline- and PB-treated rabbits. Key: (□) saline-treated microsomes; and (■) with PB-treated microsomes. Incubations were carried out under the conditions described in Materials and Methods. Each point is the mean of two determinations.

Table 4. K_m and V_{max} values for demethylenation of MDB, MDA and MDMA in rabbit liver microsomes

		K_m (μM)		V_{max} (nmol/min/mg protein)	
Substrate	Treatment	K_1	K_2	V_1	V_2
MDB	Saline	21.65 ± 3.70	3408.02 ± 2136.88	1.19 ± 0.11	10.64 ± 4.82
	PB	182.89 ± 15.92	9000	17.16 ± 1.12	29.26 ± 8.06
MDA	Saline	3.76 ± 0.36	141.12 ± 19.03	0.32 ± 0.02	0.34 ± 0.01
	PB	2.86 ± 0.14	840.30 ± 281.15	0.67 ± 0.01	0.50 ± 0.07
MDMA	Saline	1.79 ± 0.42	234.40 ± 26.65	0.40 ± 0.02	1.07 ± 0.03
	PB	1.65 ± 0.27	419.35 ± 56.46	0.45 ± 0.02	1.00 ± 0.04

Each value is the mean of ±SD of 42–48 data points.

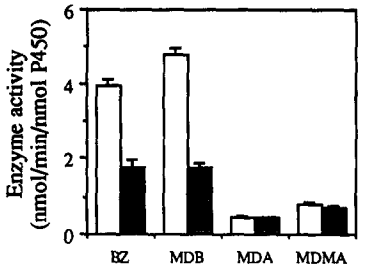

Fig. 3. Effects of PCP pretreatment on benzphetamine (BZ) N-demethylation and MDP demethylenation activities in PB-treated microsomes. Key: (□) control; and (▨) PCP pretreatment. Incubations were carried out under the conditions described in Materials and Methods. Each value is the mean ± SD of three to four determinations.

Table 5. Demethylenation activities toward MDB, MDA and MDMA by the reconstituted system

Compound	Activity (nmol/min/nmol of P450)	Relative rate (%)
MDB	30.63	100
MDA	0.32	1
MDMA	0.55	2

Substrate (1 mM) was incubated with a reconstituted mixture of isozyme *CYP2B4* under the conditions described in Materials and Methods.

that MDB was demethylenated effectively by isozyme *CYP2B4* but MDA and MDMA were not.

DISCUSSION

Compounds containing the methylenedioxy group have been investigated for some time because of their ability to form inhibitory complexes with cytochrome P450 [26, 27]. This complex is thought to involve a carbene generated by the action of cytochrome P450 on the methylene carbon [28]. However, the complexation does not appear to be a high efficiency process, and other reactions, including demethylenation to formic acid and catechol, occur [29–31]. The amphetamines also form inhibitory complexes with cytochrome P450 by an N-oxidation reaction [32] so that the methylenedioxy amphetamines could inhibit cytochrome P450 function by two distinct metabolism-dependent pathways. Under the short incubation times of the present study, however, neither of these pathways of inhibition affected substrate oxidation.

Demethylenation of MDPs by cytochrome P450 is a major reaction by rabbit liver microsomes.

However, the cytochrome P450 isozyme responsible differs with substrate structure. The neutral and monofunctional MDB appears to be demethylenated efficiently by *CYP2B4*.

The differential sensitivity of MDB, MDA and MDMA oxidation to SKF-525A and nonlinear double-reciprocal plots suggested the participation of different P450 isozymes. The kinetics studies show that isozymes with K_m values in the micromolar range effected the reaction in microsomes from untreated rabbits. Upon PB induction, there was a substantial increase in MDB demethylenation activity whereas that for the amphetamines was changed only marginally. At the higher concentrations (1 mM), MDB demethylenation was mediated mostly by isozyme *CYP2B4*. MDA and MDMA oxidation appeared to be catalyzed mainly by constitutive P450 isozymes. This possibility was demonstrated in two ways. One was to use PCP-pretreated microsomes as Osawa and Coon [25] had reported that PCP selectively inactivates *CYP2B4*. Exposure of microsomes from PB-pretreated animals to PCP affected only MDB oxidation. The second approach was to use isozyme *CYP2B4* in a reconstitution experiment. The activity of isozyme *CYP2B4* in MDB demethylenation was 100 times that of MDA and 50 times that of MDMA. MDB, with a turnover of 31 mol/min/nmol of P450, is comparable to benzphetamine (turnover of 56 nmol/min/nmol of P450 [17]) as a substrate for this isozyme. The isozymes responsible for demethylenating MDA and MDMA are likely to be the constitutive forms, but the higher levels of H_2O_2 suggest that the reaction also elicits a higher degree of uncoupling.

These results show that a metabolic reaction leading to a reactive intermediate capable of binding tissue nucleophiles [6] involves different isozymes for different substrates. This same reaction has been demonstrated in brain homogenates [3] but little is known of the pathway involved. Since P450 isozymes have been found to be distributed unequally within the brain [33], it is possible that demethylenation occurs in different areas to result in the selective CNS toxicity of MDA and MDMA.

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