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# Participation of Cytochrome P450–2B and -2D Isozymes in the Demethylenation of Methylenedioxymethamphetamine Enantiomers by Rats

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## SUMMARY

The cytochrome P450 isozymes in rat liver microsomes that catalyze the demethylenation of methylenedioxymethamphetamine enantiomers to the corresponding dihydroxymethamphetamine were characterized. Dihydroxymethamphetamine formation in liver microsomes from male Sprague-Dawley rats exhibited multienzyme kinetics, with $K_m$ values in the micromolar/millimolar range. The stereoselectivity [(+)-isomer versus (−)-isomer] varied from 0.78 to 1.94 after pretreatment of the rats with phenobarbital, 3-methylcholanthrene, pregnenolone-16$\alpha$-carbonitrile, or pyrazole, suggesting that different isozymes participate in the reaction. The low-$K_m$ demethylenation was not induced by these compounds and was not inhibited by antibodies raised against CYP2C11. Liver microsomes from female Dark-Agouti rats, a strain genetically deficient in CYP2D1, exhibited demethylenation activities that were 9% of those in microsomes from male Sprague-Dawley rats. The low-$K_m$ demethylenation was also inhibited by CYP2D substrates such as sparteine, bufuralol, or desipramine and was almost completely inhibited by antibodies against P450 BTL, which belongs to the CYP2D family. The high-$K_m$ demethylenation activity was induced by phenobarbital and pregnenolone-16$\alpha$-carbonitrile and the activity in both untreated and phenobarbital-induced microsomes was suppressed by anti-CYP2B1 IgG. Experiments with IgG raised against cytochrome $b_5$ suggested that the hemoprotein contributed to the low-$K_m$ activity but not the high-$K_m$ activity. These results indicate that cytochrome P450 isozymes belonging to the CYP2D subfamily catalyze demethylenation with low $K_m$ values and that the reaction occurring with high $K_m$ values is likely to be mediated by members of the CYP2B family, but with the possible participation of other phenobarbital-inducible isoforms.

Systemic administration of MDMA to rats causes serotonergic neurotoxicity, manifested as a long term reduction in 5-HT levels and tryptophan hydroxylase activity (1–3). This neurotoxic effect is stereoselective; the (+)-isomer is more potent than the (−)-isomer, although both isomers deplete 5-HT acutely (3). *In vivo* studies have suggested that metabolites may be involved in this long term toxicity (4), and Gollamudi *et al.* (5) have reported that manipulation of P450 with inducers and inhibitors alters the extent to which MDMA elicits a short term 5-HT-depleting action (6). We (7) have examined the P450-mediated oxidation of MDMA by rat liver microsomes and described a metabolic pathway in which the methylenedioxy group is cleaved and the catechol metabolite is further oxidized to an *o*-quinone, which reacts readily with sulfhydryl compounds such as glutathione (7, 8). This is a major metabolic pathway at the low (7 $\mu$M) plasma concentrations found after pharmacological doses, accounting for >95% of the consumed substrate (9). The present report describes results of a study of the P450 isozymes that catalyze this demethylenation reaction. Kinetic studies revealed it to be a multienzyme process with $K_m$ values in the micromolar/millimolar range. The low-$K_m$ system was found to be a constitutive enzyme and the high-$K_m$ system a mixture of inducible isozymes.

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## Materials and Methods

**Chemicals.** (+)-MDMA, (−)-MDMA, and amphetamine were obtained from the Research Technology Branch of The National Institute

**ABBREVIATIONS:** MDMA, methylenedioxymethamphetamine; P450 or CYP, cytochrome P450; DHMA, dihydroxymethamphetamine; PB, phenobarbital; 3-MC, 3-methylcholanthrene; PCN, pregnenolone-16$\alpha$-carbonitrile; SD, Sprague-Dawley; DA, Dark-Agouti; SOD, superoxide dismutase; HPLC, high performance liquid chromatography; 5-HT, 5-hydroxytryptamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

on Drug Abuse (Rockville, MD). 3-MC, pyrazole, sparteine, and quinine were obtained from the Aldrich Chemical Co. Inc. (Milwaukee, WI). DHMA was synthesized according to the method of Smissman and Borchardt (10). PB was purchased from the Amend Drug & Chemical Co. (Irvington, NJ). PCN, desipramine, SOD, 7-pentoxyresorufin, resorufin, and HEPES were obtained from Sigma Chemical Co. (St. Louis, MO). Benzphetamine, bufuralol, and Emulgen 911 (913) were gifts from Upjohn Co. (Kalamazoo, MI), Hoffmann-La Roche (Nutley, NJ), and Kao Co. (Tokyo, Japan), respectively. Bio-Gel HTP and SM-2 were obtained from Bio-Rad Laboratories (Richmond, CA). DEAE-Sephacel, Sepharose 4B, Sephadex G-75, 2′,5′-ADP-Sepharose 4B, Protein A-Sepharose CL-4B, and Protein G-Sepharose 4 FF were obtained from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). Whatman DE-52 was obtained from Fisher Scientific Inc. (Pittsburgh, PA). The ω-aminooctyl-Sepharose 4B column was prepared by the method of Guengerich and Martin (11). All other chemicals used were of the highest grade available.

**Preparation of microsomes.** Male and female rats (strains, SD and DA; age, 2 months) were obtained from Bantin-Kingman (Fremont, CA). In the induction studies for P450, male SD rats were used and all inducers were injected intraperitoneally except for PCN, which was administered by lavage. PB (80 mg/kg) and pyrazole (200 mg/kg), dissolved in 0.9% NaCl solution, were given for 3 days. 3-MC (20 mg/kg) and PCN (100 mg/kg), dissolved in corn oil and water containing 2% Tween 80, respectively, were given for 4 days. After the final injection, animals were fasted for 24 hr before sacrifice. Livers were homogenized in 3 volumes of 1.15% KCl solution and the microsomes were isolated by procedures described earlier (12), except that the 105,000 × *g* pellets were resuspended in 100 mM sodium pyrophosphate buffer, pH 7.4, to remove contaminating hemoglobin (13). The microsomal pellets were stored at −70° before use.

**Purification of isozymes and antibody production.** CYP2B1 was purified from liver microsomes from PB-treated rats according to the method of Tanimoto *et al.* (14), with slight modifications. Briefly, fractions eluted from the ω-aminooctyl-Sepharose 4B column with 0.08% Emulgen 913 were applied to a DEAE-Sephacel column and isozyme 2B1 was eluted at room temperature with 10 mM potassium phosphate buffer, pH 7.7, containing 20% glycerol, 0.1 mM dithiothreital, 0.5% sodium cholate, and 0.2% Emulgen 911. Antibodies were raised in male New Zealand white rabbits. The purification of anti-CYP2C11 IgG fraction utilized Protein A-Sepharose CL-4B, according to the method of Nagata *et al.* (15). Anti-CYP2B1 IgG and anti-cytochrome $b_5$ IgG were purified by ammonium sulfate fractionation (20–50%), followed by Protein G-Sepharose 4 FF column chromatography. Preimmune and CYP2B1-immune IgG were further treated at 56° for 60 min, to minimize nonselective inhibition of the high-$K_m$ MDMA oxidation, following the procedure of Thomas *et al.* (16). Anti-P450 BTL IgG was obtained as reported previously (17). NADPH-P450 reductase was purified by Whatman DE-52 column chromatography and 2′,5′-ADP-Sepharose 4B affinity column chromatography according to the method of Yasukochi and Masters (18), except that 50 mM potassium phosphate buffer, pH 7.7, 20% glycerol, 0.1% sodium deoxycholate, 1 mM NADP, was used to elute the flavin enzyme from the affinity column. The specific activity of purified NADPH-P450 reductase was 54.8 units/mg of protein when cytochrome *c* reduction activity was assayed in 0.3 M potassium phosphate buffer, pH 7.7, containing 0.1 mM EDTA, according to the method of Phillips and Langdon (19). P450 concentration was determined by the method of Omura and Sato (20). Protein concentration was measured by the method of Bradford (21), with bovine serum albumin as standard.

**Enzyme assay.** A typical reaction mixture consisted of 100 mM HEPES buffer, pH 7.6, an NADPH-generating system (0.5 mM NADP, 8 mM glucose-6-phosphate, 5 mM $MgCl_2$, and 1 unit of glucose-6-phosphate dehydrogenase), microsomal preparation (1–2 mg of protein), 100 units of SOD, and substrate, in a final volume of 1.0 ml, unless otherwise noted. SOD was added to prevent further oxidation of the catechol metabolite to quinone (7, 8) and to minimize participation of hydroxyl radical-promoted demethylenation. Previous results have shown that incubation in the presence of 100 units of SOD allowed (9) demethylenation to proceed linearly with time for 5 min and with protein concentration up to 2 mg. In the immunoinhibition studies, liver microsomes were preincubated at 22°, with varying amounts of the IgG fraction purified from P450- and cytochrome $b_5$-immune or preimmune rabbit serum, for 10 min before initiation of the reaction by the addition of the NADPH-generating system, as described previously (22).

The reconstituted incubation mixture (1 ml) contained purified CYP2B1, NADPH-P450 reductase, dilauroylphosphatidylcholine, MDMA, and other components for the microsomal reaction described above. The reactions were initiated by addition of the NADPH-generating system.

DHMA production was assayed by HPLC with electrochemical detection, on a Biophase ODS column (4.6 × 250 mm; Bioanalytical Systems, Inc., West Lafayette, IN) with a glassy carbon working electrode (LC-4; Bioanalytical Systems) set at 0.7 V (versus a Ag/AgCl reference electrode). Separation was carried out with a mobile phase consisting of 0.1 M citrate buffer, pH 3.5, containing 1 mM octyl sodium sulfate/acetonitrile/methanol (8:1:1, by volume), at a flow rate of 0.75 ml/min. Benzphetamine *N*-demethylation activity was determined as formaldehyde produced, using the method of Nash (23).

Depentoxylation activity for 7-pentoxyresorufin was assayed by HPLC using a fluorometric detector set at 535 nm (excitation) and 580 nm (emission), according to the method of Yamazoe *et al.* (24). The incubation mixture contained 50 mM HEPES buffer, pH 7.6, 0.1 mM EDTA, microsomal preparation (1–2 mg of protein), the NADPH-generating system, and 0.01 mM 7-pentoxyresorufin and was incubated at 37° for 2 min. The reaction was terminated by addition of 0.5 ml of acetonitrile and 0.25 ml of *p*-acetanisidine (25 nmol) as internal standard. The reaction mixture was then centrifuged at 13,500 × *g* for 5 min and then a portion (50 μl) of the supernatant was injected into the HPLC system.

## Results

**Kinetics.** To investigate the properties of enzymes responsible for MDMA demethylenation (Fig. 1), the kinetics of catechol formation in the presence of hepatic microsomes from untreated male SD rats were determined over a substrate concentration range of 0.001–10 mM (Table 1). Lineweaver-Burk plots for the oxidation were nonlinear for both (+)- and (−)-isomers, indicating that at least two enzyme systems were present; nonlinear regression analysis of the kinetic data gave an excellent fit to a two-enzyme model with $K_m$ values differing by 3 orders of magnitude (Table 1). There was no significant difference between the $V_{max}/K_m$ ratios or the first-order rate constants for the two isomers. The kinetics of demethylenation in microsomes from PB-pretreated rats were also examined and induction was found to decrease the high $K_m$ values from 1.18 mM to 0.42 mM for the (+)-isomer and from 1.76 mM to 0.32 mM for the (−)-isomer (Table 1). There was a concomitant increase in $V_{max}$, so that the $V_{max}/K_m$ ratios for the two enantiomers increased by factors of 7.5–9 after PB pretreatment. The $K_m$ values corresponding to the low-$K_m$ system were unchanged after PB pretreatment but the maximal velocity value appeared to decrease.

**Characterization of isozymes.** The two $K_m$ systems were examined further by assessing changes in activity with inducers

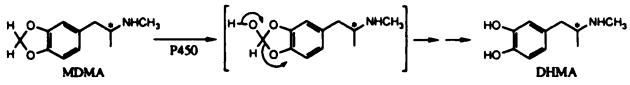


**Fig. 1.** Demethylenation of MDMA. *, Chiral carbon atom.

TABLE 1

**Kinetic values for stereoselective demethylenation of MDMA**

To determine $K_m$ and $V_{max}$ values for demethylenation of MDMA, substrate concentration ranges from 1 μM to 10 mM (28 data points) and from 50 μM to 2 mM (12 data points) were used with rat liver microsomes and the CYP2B1 reconstituted system, respectively. Reactions were carried out at 37° for 5 min, as described in Materials and Methods. The reconstituted system consisted of 0.1 nmol of purified CYP2B1, 0.2 units of NADPH-P450 reductase, and 30 μg/ml dilauroylphosphatidylcholine. Under these conditions, product formation was linear with respect to time and P450 concentration. The kinetic parameters were analyzed using a nonlinear regression program (BMDP), as reported previously (8) and shown ± asymptotic standard deviation.

| Microsomal preparation | Low-$K_m$ system | | | High-$K_m$ system | | |
|---|---|---|---|---|---|---|
| | $K_m$ | $V_{max}$ | $V_{max}/K_m$ | $K_m$ | $V_{max}$ | $V_{max}/K_m$ |
| | mM | nmol/min/nmol of P450 | | mM | nmol/min/nmol of P450 | |
| (+)-MDMA | | | | | | |
| Untreated | 0.003 ± 0.001 | 0.31 ± 0.01 | 115.0 | 1.18 ± 0.20 | 0.54 ± 0.03 | 0.46 |
| PB-treated | 0.002 ± 0.003 | 0.15 ± 0.06 | 78.95 | 0.42 ± 0.09 | 1.45 ± 0.07 | 3.45 |
| CYP2B1 | | | | 0.44 ± 0.02 | 1.11 ± 0.02 | 2.52 |
| (−)-MDMA | | | | | | |
| Untreated | 0.001 ± 0.000 | 0.19 ± 0.01 | 136.0 | 1.76 ± 0.15 | 0.91 ± 0.03 | 0.52 |
| PB-treated | 0.001 ± 0.003 | 0.08 ± 0.06 | 80.0 | 0.32 ± 0.06 | 1.51 ± 0.06 | 4.72 |
| CYP2B1 | | | | 0.45 ± 0.06 | 0.97 ± 0.06 | 2.15 |

and inhibitors of P450 isozymes. In induction studies, rats were pretreated with PB, 3-MC, PCN, and pyrazole, compounds that induce members of the CYP2B (25, 26), CYP1A (27), CYP3A (28), and CYP2E (29, 30) subfamilies, respectively. MDMA demethylenation activity in microsomes prepared from pretreated animals was compared with that in microsomes from untreated rats at low (0.01 mM) and high (10 mM) substrate concentrations (Table 2). These inducers increased the P450 content in the liver by factors of 1.78 (PB), 1.81 (3-MC), 1.33 (PCN), and 1.10 (pyrazole). Demethylenation activity at low substrate concentration (10 μM) decreased in all of these preparations, compared with untreated animals. However, at the 10 mM substrate concentration demethylenation activity for both enantiomers was induced by PB and PCN, whereas 3-MC and pyrazole enhanced the activities for only the (−)-enantiomer. Thus, the stereoselectivity, expressed as the ratio of demethylenation activities [(+)-isomer versus (−)-isomer], in microsomes from untreated and inducer-treated rats ranged from 0.78 to 1.94.

**Low-$K_m$ demethylenation.** Because the dominant P450 isozymes catalyzing demethylenation at low substrate concentration were not inducible, immunoinhibition experiments with antibodies against the constitutive isozymes CYP2C11 (31, 32) and CYP2D were conducted. Although the anti-CYP2C11 IgG effectively inhibited benzphetamine *N*-demethylation, a characteristic reaction for this isozyme (31), it had little effect on the low-$K_m$ demethylenation activity (Fig. 2). However, antibodies raised against P450 BTL, which is classified as a member of the CYP2D subfamily (33), suppressed the low-$K_m$ demethylenation activity by 93 ± 1% at a concentration of 1 mg of IgG/nmol of P450 (Table 3) and suppressed bunitrolol 4-hydroxylation and debrisoquine 4-hydroxylation activities by >95% under similar conditions (33).

Table 4 shows the effects of some substrates and inhibitors of the CYP2D isozyme on the low-$K_m$ oxidation. Bufuralol (34), sparteine (35), or desipramine (36) but not amphetamine (37) suppressed catechol formation in a concentration-dependent manner. Quinine, a selective inhibitor of CYP2D, also effectively suppressed the reaction at a concentration of 10 μM. To further assess CYP2D involvement, hepatic microsomes from female DA rats, a strain and gender deficient in CYP2D1 (38–40), were examined for their ability to catalyze demethylenation. The results, shown in Fig. 3, indicate that at low substrate concentrations female DA rats have very little activity, compared with male SD rats, but at higher concentrations the activities are comparable.

The requirement for cytochrome $b_5$ in the low-$K_m$ demethylenation was examined by addition to the microsomal incubation mixtures of antibodies raised against cytochrome $b_5$. The result showed a 31 ± 2% suppression of racemic MDMA demethylenation.

**High-$K_m$ demethylenation.** At high substrate concentration (10 mM) neither anti-CYP2C11 nor anti-cytochrome $b_5$ IgG had any effect on demethylenation (data not shown). When

TABLE 2

**Effects of inducers on the MDMA metabolism by rat liver microsomes**

Low- and high-$K_m$ demethylenation activities were determined at substrate concentrations of 0.01 and 10 mM. Animals were pretreated with PB (80 mg/kg), 3-MC (20 mg/kg), PCN (100 mg/kg), or pyrazole (200 mg/kg) under conditions described in Materials and Methods. MDMA was incubated with hepatic microsomal preparation (0.62–1.38 mg of protein), 100 units of SOD, the NADPH-generating system, and 100 mM HEPES buffer, pH 7.6, in a final volume of 1.0 ml. Production of DHMA from MDMA was determined by HPLC with electrochemical detection. The numbers in parentheses represent the percentage of values compared to untreated animals. Each value is the mean ± standard deviation of three determinations.

| Pretreatment | Low-$K_m$ system Activity | | | High-$K_m$ system Activity | | | Change in P450 content |
|---|---|---|---|---|---|---|---|
| | (+)-Isomer | (−)-Isomer | (+)/(−) | (+)-Isomer | (−)-Isomer | (+)/(−) | |
| | nmol/mg of protein/min | | | nmol/mg of protein/min | | | |
| None | 0.77 ± 0.05 (100%) | 0.43 ± 0.02 (100%) | 1.79 | 2.81 ± 0.24 (100%) | 2.29 ± 0.13 (100%) | 1.23 | 1.00 |
| PB | 0.56 ± 0.05 (73%) | 0.38 ± 0.06 (88%) | 1.47 | 6.79 ± 0.15 (241%) | 6.58 ± 0.05 (287%) | 1.03 | 1.78 |
| 3-MC | 0.37 ± 0.02 (48%) | 0.25 ± 0.01 (58%) | 1.48 | 3.43 ± 0.02 (122%) | 4.39 ± 0.50 (192%) | 0.78 | 1.81 |
| PCN | 0.60 ± 0.13 (78%) | 0.31 ± 0.01 (72%) | 1.94 | 3.94 ± 0.25 (140%) | 3.54 ± 0.12 (154%) | 1.11 | 1.33 |
| Pyrazole | 0.43 ± 0.04 (56%) | 0.28 ± 0.01 (65%) | 1.54 | 3.12 ± 0.24 (111%) | 3.21 ± 0.08 (140%) | 0.97 | 1.10 |

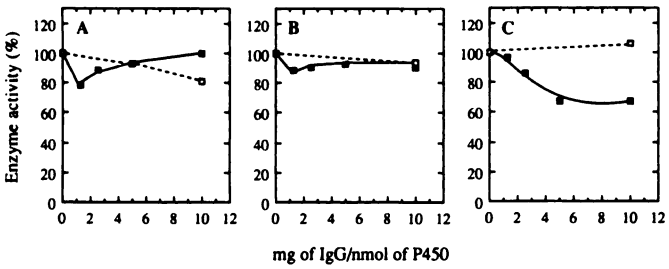


**Fig. 2.** Effect of anti-CYP2C11 IgG on the low-$K_m$ demethylenation of (+)-MDMA (A) and (−)-MDMA (B) and the *N*-demethylation of benzphetamine (C) by liver microsomes from untreated male SD rats. □, Preimmune IgG; ■, anti-CYP2C11 IgG. The reactions for low-$K_m$ demethylenation of MDMA were assayed with 0.01 mM substrate concentration, as described in Materials and Methods. The enzyme activities for (+)- and (−)-MDMA demethylenation in the absence of IgG were 0.30 and 0.21 nmol/min/nmol of P450, respectively. The rate of formaldehyde release from benzphetamine (1 mM) without IgG was 7.31 nmol/min/nmol of P450. Each *point* is the average of two determinations.

anti-CYP2B1 IgG was incubated with microsomes and 0.3 mM MDMA, however, demethylenation activity in PB-treated liver microsomes decreased by approximately one half [(+)-isomer, 55%; (−)-isomer, 62%) (Fig. 4). Under these conditions, the depentoxylation of 7-pentoxyresorufin, which is catalyzed mainly by CYP2B1 (41), was almost completely inhibited when 8 mg of antibody/nmol of P450 were added to the reaction mixture.

The ability of CYP2B1 to mediate MDMA demethylenation and the involvement of cytochrome $b_5$ was assessed in reconstitution experiments. The results showed that about 1.8 nmol of DHMA/min/nmol of CYP2B1 could be formed from 1 mM (±)-MDMA at a ratio of NADPH reductase to P450 of 4:1 and that demethylenation activity for both isomers was depressed at ratios of cytochrome $b_5$ to CYP2B1 of 2:1 or 1:1.

The effects of anti-CYP2D and anti-CYP2B IgG on DHMA formation by liver microsomes from untreated rats at different concentrations of MDMA are shown in Table 3. At a substrate concentration of 1 mM, only 29 ± 1% of the demethylenation activity was inhibited by anti-CYP2D IgG, whereas anti-CYP2B IgG inhibited activity by almost 50%. Together, anti-CYP2D and anti-CYP2B IgG inhibited DHMA formation by almost 80%. The immunoinhibition effects of anti-CYP2B and anti-CYP2D IgG both declined when the MDMA concentration was increased to 10 mM.

## Discussion

The multienzyme kinetics of P450-mediated demethylenation mean that at different tissue concentrations different isozymes catalyze the reaction. Because P450 isozymes have different regiochemistries and are regulated by different mechanisms, it is important to identify the isozymes involved to interpret results of *in vivo* studies.

The experiments with inducers have shown that the isozymes responsible for MDMA demethylenation at low substrate concentration are likely to be constitutive and not the inducible isozymes of the CYP1A, CYP2B, CYP2E, and CYP3A subfamilies. Polyclonal antibodies prepared against CYP2C11 that also cross-react with other members of CYP2C, such as CYP2C6, CYP2C7, CYP2C12, and CYP2C13 (42, 43), had no effect on the reaction, eliminating the possibility of participation of CYP2C subfamily members in the noninducible low-$K_m$ system. Next, the role of CYP2D was assessed. Liver microsomes from female DA rats, which are deficient in CYP2D1 (38–40), exhibited only about 9% of the demethylenation activity of male SD rats at low MDMA concentration (0.01 mM). In contrast, the activities of the two microsomal preparations at high substrate concentration (10 mM) were not substantially different. Additionally, inhibition experiments with CYP2D substrates such as bufuralol, sparteine, quinine, and desipramine (Table 3) and immunoinhibition experiments with anti-P450 BTL IgG, which cross-reacts with other P450 isozymes of the CYP2D family (33), also indicated that CYP2D1 or other members of this family are involved. In related studies, human CYP2D6, an orthologue of rat CYP2D1, expressed in yeast (*Saccharomyces cerevisiae*) (44) catalyzed demethylenation of MDMA with monophasic kinetics and with a $K_m$ value similar to the low $K_m$ value observed here (45). Overall, these lines of evidence indicate that isozymes belonging to the CYP2D subfamily catalyze the low-$K_m$ MDMA demethylenation. The kinetics exhibit little, if any, stereoselectivity.

The high-concentration reaction is more complex; treatment of the animals with PB and PCN induced demethylenation activity nonstereoselectively but 3-MC and pyrazole induced (−)-MDMA oxidation selectively. The fact that pretreatment with PB increased the $V_{max}/K_m$ value of the high-$K_m$ system by 7–9-fold suggested that the high-$K_m$ demethylenation may be catalyzed by PB-inducible isozyme(s) and/or a nonconstitutive isozyme that can be induced by PB. In rats, CYP2A1, CYP2B1, CYP2B2, CYP2C6, CYP2C7, CYP3A1, and CYP3A2 (46–50) are induced by PB. CYP2C6 and CYP2C7 are immunochemically similar to other CYP2C members, and the insensitivity of demethylenation to anti-CYP2C11 IgG would argue against their participation in this reaction. In addition, CYP2C7 and CYP2A1 are expressed at higher levels in females (49–51), but this sex-related difference was not observed in the reaction (Fig. 3). Of the 3A isozymes, CYP3A2 also seems unlikely to

TABLE 3

**Inhibition by anti-CYP2D and anti-CYP2B IgG of (±)-MDMA demethylenation by untreated rat liver microsomes**

Microsomal preparations were incubated with preimmune, anti-CYP2D, or anti-CYP2B IgG under the conditions described in Materials and Methods. Each value is the mean of two determinations. The numbers in parentheses represent the percent inhibition.

| Status | IgG amount | Demethylenation activity | | |
|---|---|---|---|---|
| | | 10 μM MDMA | 1 mM MDMA | 10 mM MDMA |
| | *mg of IgG/nmol of P450* | | *nmol/min/nmol of P450* | |
| Control | | 0.48 (0%) | 1.06 (0%) | 2.17 (0%) |
| Preimmune | 5 | 0.48 (0%) | 0.95 (10%) | 2.10 (3%) |
| Anti-CYP2D | 1 | 0.03 (93%) | 0.76 (29%) | 1.83 (16%) |
| Anti-CYP2B | 5 | | 0.55 (49%) | 1.36 (37%) |
| Anti-CYP2D + anti-CYP2B | 1 + 5 | | 0.24 (77%) | 1.03 (52%) |

TABLE 4

**Effects of CYP2D substrates or inhibitors on (+)- and (−)-MDMA demethylenation by untreated rat liver microsomes**

MDMA (0.01 mM) was incubated for 5 min with the microsomal preparation (1.41 mg of protein) in the presence of an NADPH-generating system. Under these conditions, the control activities of MDMA demethylenation were 0.55 ± 0.01 [(+)-isomer] and 0.36 ± 0.01 [(−)-isomer] nmol/min/mg of protein, respectively. Each value is the mean of two determinations.

| Addition | Concentration | Inhibition of demethylenation activity | |
|---|---|---|---|
| | | (+)-Isomer | (−)-Isomer |
| | μM | % | |
| Bufuralol | 1 | 13 | 6 |
| | 10 | 81 | 68 |
| Sparteine | 1 | 10 | 2 |
| | 10 | 33 | 23 |
| Quinine | 1 | 76 | 62 |
| | 10 | 96 | 92 |
| Desipramine | 1 | 40 | 18 |
| | 10 | 90 | 81 |
| Amphetamine | 1 | 15 | −13 |
| | 10 | 17 | −3 |

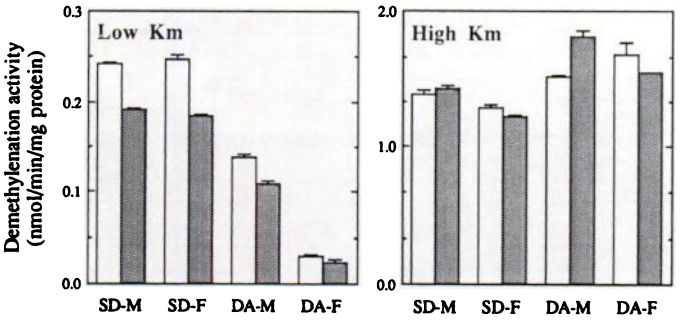


**Fig. 3.** Sex- and strain-related differences in MDMA demethylenation by rat liver microsomes. □, (+)-Isomer; ■, (−)-isomer. *SD-M*, SD male rats; *SD-F*, SD female rats; *DA-M*, DA male rats; *DA-F*, DA female rats. The low- and high-$K_m$ MDMA demethylenation reactions were determined at substrate concentrations of 0.01 mM and 10 mM, respectively. Incubations were performed under the conditions described in Materials and Methods. Each *data point* is the mean ± standard deviation of three determinations.

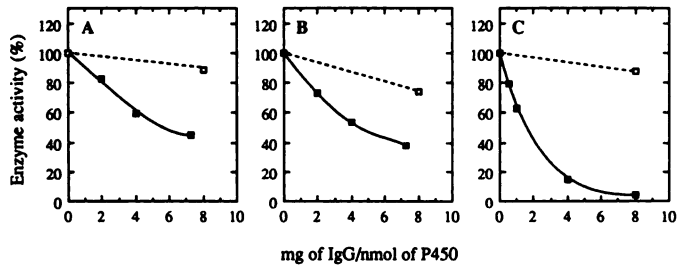


**Fig. 4.** Immunoinhibition by anti-CYP2B1 of the high-$K_m$ demethylenation of (+)-MDMA (A) and (−)-MDMA (B) and the depentylation of pentoxyresorufin (C) by liver microsomes from PB-treated male SD rats. □, Preimmune IgG; ■, anti-CYP2B1 IgG. Liver microsomes isolated from the PB-treated rats were incubated with preimmune IgG and varying amounts of anti-CYP2B1 IgG under the conditions described in Materials and Methods. The reactions were assayed at substrate concentrations of 0.3 mM. (+)- and (−)-MDMA demethylenation activities in the absence of IgG were 1.65 and 1.41 nmol/min/nmol of P450, respectively. Each *datapoint* is the average of two determinations.

TABLE 5

**Isozyme participation in MDMA demethylenation**

| Isozyme | Criterion | Effect on activity[a] | |
|---|---|---|---|
| | | 10 μM | 10 mM MDMA |
| 1A1/2 | 3-MC induction | − | + [(−)-Isomer] |
| | α-Naphthoflavone induction | − | ND |
| 2A1 | 3-MC induction | − | + [(−)-Isomer] |
| | PB induction | − | ++ |
| | PCN induction | − | + |
| | Female > male | − | − |
| 2B1/2 | PB induction | − | ++ |
| | PCN induction | − | + |
| | Anti-CYP2B IgG | − | ++ |
| | Reconstituted system | ND | + |
| 2C6 | PB induction | − | ++ |
| | Cross-reaction with anti-CYP2C11 | − | − |
| 2C7 | PB induction | − | ++ |
| | Female > male | − | − |
| | Cross-reaction with anti-CYP2C11 | − | − |
| 2C11 | Not inducible | + | − |
| | Male specific | − | − |
| | Anti-CYP2C11 IgG | − | − |
| 2D1 | Not inducible | + | − |
| | Female DA rat deficiency | +++ | − |
| | CYP2D substrate inhibition | +++ | + |
| | Anti-CYP2D IgG | +++ | + |
| | CYP2D6-expressing yeast microsomes | +++ | + |
| 2E1 | Pyrazole induction | − | ± [(−)-Isomer] |
| 3A1 | PB induction | − | ++ |
| | PCN induction | − | + |
| 3A2 | Male specific | − | − |
| | PB induction | − | ++ |
| | 3-MC repression | + | − |

[a] −, No effect; ±, marginal change; ND, not determined.

catalyze the high-$K_m$ reaction, for two reasons. Firstly, CYP3A2 mRNA decreases in female rats at puberty (CYP3A2 vanishes in 12 weeks) (52), and there was no dramatic sex difference in activity. Secondly, 3-MC administration, which decreases levels of CYP3A2 (52), did not suppress the high-$K_m$ demethylenation activity. CYP3A1 (another isozyme in the CYP3A family), although induced by both PB and PCN, is barely detectable in constitutive form and may participate only in pretreated microsomes.

CYP2B1 and CYP2B2 are the major PB-inducible isozymes, but only the CYP2B2 gene is constitutively expressed in rat liver. The levels of this isozyme appear to be low, approximately 5% of the total P450 content (26, 50, 51, 53). The ability of CYP2B1 to oxidize MDMA to DHMA was demonstrated in reconstitution experiments, with kinetic parameters that correspond to those of the high-$K_m$ system after PB induction (Table 1). The immunoinhibition by anti-CYP2B1 observed in untreated rat liver microsomes, however, suggests that CYP2B2, which could cross-react with this antibody, may be involved in MDMA demethylenation. The relatively strong immunoinhibition observed in microsomes from untreated animals may reflect higher turnover by CYP2B2, compared with CYP2B1. It is also possible that anti-CYP2B1 IgG is cross-reacting with another isozyme but it does not appear to be CYP2D, because the effects of anti-CYP2D and anti-CYP2B are additive (Table 3). Thus, although CYP2B2 may be involved in the reaction in microsomes from untreated rats, the data are not unambiguous. Table 5 summarizes the rat liver

isozymes considered in this analysis and the information used to assess their involvement.

The results of this study predict that demethylenation activity and its sensitivity to inducers and inhibitors would be dose dependent. Thus, at low concentrations (<100 μM) the constitutive CYP2D isozymes would be involved and demethylenation, which is the dominant reaction (9), would not be enhanced by typical P450 inducers. However, at higher concentrations (>100 μM) demethylenation would be enhanced by the added contributions of PB-inducible P450 isozymes, and at concentrations up to 1 mM isozymes other than CYP2D would contribute to about 70% of the overall activity. In studies of MDMA neurochemistry, Gollamudi *et al.* (5) reported that PB pretreatment only slightly increased the 5-HT-depleting actions of the drug. The marginal increment could reflect the participation of multiple isozymes in metabolism observed here. Although their analysis focused on *N*-demethylation (5), demethylenation could also be involved in the formation of active metabolites. Because catechol metabolites are unlikely to cross the blood-brain barrier, the significance of their formation in the liver might be questionable. However, isozymes belonging to CYP2D (54, 55) and CYP2B (56–59) subfamilies have been found in the brain, as has NADPH-P450 reductase (60–62). Thus, the catechol metabolite of MDMA can be formed in the central nervous system, and in *in vitro* experiments with brain microsomes we have observed the reaction (63). After 40 mg/kg doses, which cause substantial depletion of 5-HT, peak brain levels of MDMA were 200 nmol/g (64), which declined to 60 nmol/g over a 6-hr period. Assuming that these values reflect intracellular levels, peak cytoplasmic concentrations would be at least 200 μM. Thus, toxic doses of MDMA result in concentrations that encompass both high- and low-$K_m$ systems. Preliminary observations in this laboratory examining isozyme distribution in rat brain by immunohistochemistry revealed that CYP2D appears to be present in astrocytes, mainly Bergmann glia.[3] Additional studies are needed to determine whether the location of those isozymes responsible for MDMA demethylenation contributes to the central nervous system toxicity observed *in vivo*.

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