0026-895X/92/040695-08$03.00/0

MOLECULAR PHARMACOLOGY, 42:695–702

Regiochemical Differences in Cytochrome P450 Isozymes Responsible for the Oxidation of Methylenedioxyphenyl Groups by Rabbit Liver

YOSHITO KUMAGAI, LENA Y. LIN, RICHARD M. PHILPOT, HIDEYUKI YAMADA, KAZUTA OGURI, HIDETOSHI YOSHIMURA,[1] and ARTHUR K. CHO

Department of Pharmacology, UCLA School of Medicine, Center for the Health Sciences, Los Angeles, California 90024 (Y.K., L.Y.L., A.K.C.), National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709 (R.M.P.), and Faculty of Pharmaceutical Sciences, Kyushu University 62, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan (H.Ya., K.O., H.Yo.)

Received April 20, 1992; Accepted July 20, 1992

SUMMARY

The cytochrome P450 isozymes catalyzing the oxidation of the methylenedioxyphenyl compounds methylenedioxybenzene (MDB) and methylenedioxyamphetamine (MDA) have been investigated in rabbit liver preparations. The aromatic ring in MDB undergoes both demethylenation to catechol and aromatic hydroxylation to sesamol, whereas that in MDA undergoes only demethylenation to dihydroxyamphetamine. Formation of catechol and sesamol from MDB in microsomal incubation mixtures was enhanced about 5- and 3-fold, respectively, by pretreatment of the rabbits with phenobarbital, which induced CYP2B4 and CYP4B1. The cytochrome P450 isozyme responsible for aromatic hydroxylation of MDB was induced by β-naphthoflavone and was inhibited by α-naphthoflavone. Microsomal demethylenation of MDA was minimally sensitive to pretreatment of the rabbits with phenobarbital, β-naphthoflavone, pyrazole, or rifampicin. However, MDA competitively inhibited the *N*-demethylation of erythromycin. Antibodies against CYP2B4, but not those against CYP4B1, caused a marked inhibition of the demethylenation and aromatic hydroxylation of MDB. Antibodies against CYP2C3 did not inhibit the demethylenation of MDA, nor did substrates or inhibitors of the CYP2D family except for bufuralol. MDB and MDA were both capable of forming metabolic intermediate complexes, and the rate of complex formation was accelerated by phenobarbital induction. Reconstitution experiments with CYP2B4 suggested that phenobarbital-inducible complex formation from MDA was not due to the carbene pathway involving the methylenedioxy group but was due to oxidation of the amino group. These results indicate that CYP2B4 oxidizes different regions of methylenedioxyphenyl compounds depending on their structure. MDB undergoes oxidation at the methylenedioxy group (major) and the benzene ring (minor). MDA is oxidized at the alkylamino side chain at the nitrogen and α-carbon. The results suggested that one or more constitutive isoforms (probably unknown) of cytochrome P450 present in rabbit liver microsomes are primarily responsible for MDA demethylenation but that CYP3A6 contributes slightly.

The methylenedioxyphenyl (1,3-benzodioxole) group is present in numerous biologically active organic compounds. The best known example is a class of insecticide synergist, most commonly exemplified by piperonyl butoxide (1). The synergistic activity of these compounds is based on their ability to inhibit cytochrome P450 by acting as a competitive substrate or by forming a so-called MI complex (2). The MI complex is the result of hydroxylation of the methylenedioxy carbon and subsequent conversion to a carbene, which forms a complex with the heme of cytochrome P450 (3). A second group of methylenedioxyphenyl compounds that has received attention recently are the methylenedioxyamphetamines, with MDA as the prototype. One of the reasons for the interest in these compounds is their neurotoxicity, expressed as a loss of serotonin neuronal activity (4, 5). This neurotoxicity is thought to be based on metabolic transformation, but details of the pathway and the identity of the actual toxin are not known. These biochemical interactions and the metabolic transformations involved have generated considerable interest in the biochemistry of the methylenedioxy function, which, in addition to conversion to a carbene, can also undergo scission to formic acid and a catechol (6, 7).

This laboratory has been examining the metabolic transformations of methylenedioxy compounds, focusing on the conversion of this function to the catechol (8, 9) and, more recently,

This research was supported by United States Public Health Service Grant DA 04206 and National Science Foundation Grant EET-8807418.
[1] Present address: Nakamura Gakuen College, Fukuoka 814–01, Japan.

ABBREVIATIONS: MI complex, metabolic intermediate complex; MDB, methylenedioxybenzene; MDA, methylenedioxyamphetamine; DHA, dihydroxyamphetamine; PB, phenobarbital; β-NF, β-naphthoflavone; α-NF, α-naphthoflavone; SOD, superoxide dismutase; PNP, *p*-nitrophenol; HPLC, high performance liquid chromatography; ECD, electrochemical detection; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

TABLE 1

Effects of inducers on oxidation of typical substrates by rabbit liver microsomes

Substrate (acetanilide, 4 mM; benzphetamine, 1 mM; PNP, 0.1 mM; erythromycin, 0.4 mM) was incubated with microsomal preparations in the presence of an NADPH-generating system (acetanilide, benzphetamine, and erythromycin) or 1 mM NADPH (PNP) for 10 min (acetanilide, benzphetamine, and erythromycin) or 3 min (PNP). The reaction mixture for PNP hydroxylation contained 1 mM ascorbate to prevent oxidation of the *p*-nitrocatechol formed (24). Each assay was performed as described in Materials and Methods, and the value shown is the mean of four determinations. The numbers in parentheses represent the percentage of control activity.

Pretreatment[a]	Enzyme activity			
	Acetanilide hydroxylation	Benzphetamine *N*-demethylation	PNP hydroxylation	Erythromycin *N*-demethylation
	nmol/min/mg of protein			
None	2.83 ± 0.38 (100)	14.49 ± 0.39 (100)	2.03 ± 0.29 (100)	1.05 ± 0.02 (100)
PB	4.62 ± 0.52 (163)	29.87 ± 1.84 (201)	4.00 ± 0.22 (197)	3.64 ± 0.07 (347)
β-NF	4.74 ± 0.51 (167)	16.44 ± 0.32 (113)	3.16 ± 0.45 (156)	0.70 ± 0.05 (66)
Pyrazole	5.24 ± 1.25 (185)	15.42 ± 1.17 (106)	8.65 ± 0.40 (426)	2.12 ± 0.06 (202)
Rifampicin	2.53 ± 0.44 (89)	13.89 ± 0.74 (96)	2.11 ± 0.54 (104)	5.97 ± 0.13 (569)

[a] The specific induction of CYP1A2 by β-NF (43), of CYP2B4 by PB (45), and of CYP2E1 (59) and CYP3A6 (40) by rifampicin (48) was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis or immunoquantitation.

TABLE 2

Effects of inducers on oxidation of MDB and MDA by rabbit liver microsomes

MDB or MDA (1 mM) was incubated with hepatic microsome preparation (0.33–0.61 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. Reactions were carried out at 37° for 2 min (MDB) or 5 min (MDA). Production of catechol and sesamol from MDB and of DHA from MDA was determined by HPLC-ECD, as described in Materials and Methods. Each value is the mean of three determinations. The numbers in parentheses represent the percentage of control activity.

Pretreatment	Product formation		
	Catechol	Sesamol	DHA
	nmol/min/mg of protein		
None	8.21 ± 0.11 (100)	0.80 ± 0.01 (100)	1.30 ± 0.01 (100)
PB	43.35 ± 1.95 (528)	2.32 ± 0.13 (290)	1.93 ± 0.10 (148)
β-NF	7.77 ± 0.30 (95)	1.52 ± 0.06 (190)	1.07 ± 0.02 (82)
Pyrazole	9.65 ± 0.37 (118)	0.52 ± 0.04 (65)	1.49 ± 0.08 (115)
Rifampicin	8.32 ± 0.46 (101)	0.54 ± 0.10 (68)	1.74 ± 0.05 (134)

hydroxylation of the aromatic ring (10). Because these reactions have been implicated in the biological actions of 1,3-benzodioxole compounds, the cytochrome P450 isozyme(s) involved are of considerable interest. This report describes results of a study of the regiochemistry of cytochrome P450 isozymes obtained from rabbit liver as they oxidize the unsubstituted MDB and the arylalkylamine MDA. The results show that demethylenation of the two compounds is mediated by different isozymes and that MI complex formation is based on oxidation of different sites of the molecules.

Materials and Methods

Chemicals. MDB, acetanilide, PNP, sesamol, 4-acetamidophenol, *p*-nitrocatechol, α-NF, β-NF, pyrazole, sparteine, quinine, and quinidine were obtained from the Aldrich Chemical Co. Inc. (Milwaukee, WI). A small amount of catechol contained in the MDB was removed as described previously (11). MDA was obtained from the Research Technology Branch of The National Institute on Drug Abuse (Rockville, MD). DHA was obtained from Merck Sharp & Dohme Laboratories (West Point, PA). PB was purchased from the Amend Drug & Chemical Co. (Irvington, NJ). Catechol, erythromycin, rifampicin, SOD, and HEPES were obtained from the Sigma Chemical Co. (St. Louis, MO). Bufuralol was a gift from Hoffmann-La Roche (Nutley, NJ). All other chemicals used were of the highest grade available.

Animal treatment and preparation of microsomes. Male New Zealand white rabbits (2.0–2.5 kg) were used and fasted 24 hr before sacrifice. For inducing individual cytochrome P450 isozymes, PB (60 mg/kg, 3 days), β-NF (80 mg/kg, 2 days), rifampicin (50 mg/kg, 4 days), or pyrazole (200 mg/kg, 1 day) was given to animals by intraperitoneal injection. PB, rifampicin, and pyrazole were dissolved in 0.9% NaCl, and β-NF was dissolved in corn oil. Liver and lung microsomes were prepared by the method established earlier (12), except that 105,000 × *g* pellets were resuspended in 100 mM pyrophosphate buffer (pH 7.4) to remove contaminating hemoglobin (13). The resulting pellets were stored at −80° before use.

Antibody production. Rabbit liver cytochromes P450 are referred to according to the nomenclature of Nebert *et al.* (14). CYP2B4, CYP4B1, and CYP2C3 were purified from untreated rabbit lung (CYP2B4 and CYP4B1) and liver (CYP2C3), as described elsewhere (15–17). It should be noted that Gasser *et al.* (18) have demonstrated the presence of multiple forms of CYP2B4 in rabbit tissue. In the studies conducted here, the CYP2B4 was isolated from a large number of animals and, therefore, utilized a mixture of isoforms. Antibodies against CYP2B4 and CYP4B1 were raised in yellow goats and those against CYP2C3 were raised in White Leghorn chickens. Each IgG fraction was isolated from the serum obtained according to previously described methods (17, 19, 20). The final preparation of IgG fractions purified from CYP2B4-, CYP4B1-, and CYP2C3-immune sera inhibited benzphetamine *N*-demethylation, 2-aminofluorene hydroxylation, and amphetamine deamination, respectively.

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 preparations, and substrate, in a final volume of 1.0 ml, unless otherwise noted. Catalytic activities of MDB or MDA demethylenation and MDB aromatic hydroxylation were assayed as described previously (9, 10), except that 100 units of SOD instead of 1 mM ascorbate were added to the incubation mixture in order both to prevent further oxidation of catechol metabolite to quinone (8, 9) and to minimize participation of hydroxyl radical-promoted demethylenation of methylenedioxyphenyl compound (11). Metabolites produced from MDB or MDA were determined by HPLC-ECD, using a Biophase ODS column (4.6 × 250 mm; Bioanalytical

Systems, Inc., West Lafayette, IN) and 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. Detection was performed with a glassy carbon working electrode (LC-4; Bioanalytical Systems, Inc.) set at 0.7 V (versus a Ag/AgCl reference electrode). Hydroxylation of acetanilide to 4-acetamidophenol (acetaminophen) was determined by the method of Johnson and Muller-Eberhard (21), modified as follows. After addition of perchloric acid (final concentration of 2.5%) to the incubation mixture containing acetanilide (4 mM), microsomal suspension (0.7–1 mg of protein), and NADPH-generating system, 2-acetamidophenol was added as internal standard and the mixture was centrifuged at 13,500 × *g* for 5 min. No 2-acetamidophenol formation was detected with hepatic microsomes from rabbits, as reported previously by Koop *et al.* (22). The supernatant (1.0 ml) was extracted with 10 ml of ethyl acetate, the organic layer was evaporated to dryness under nitrogen, and the residue was dissolved in 0.2 ml of methanol. An aliquot (20 μl) of the methanol solution was filtered and then injected into an HPLC system equipped with a Microsorb C_{18} column (4.6 × 250 mm; Rainin Instrument Co., Inc., Woburn, MA). The mobile phase was 5% acetic acid/acetonitrile (4:1, v/v), used at a flow rate of 1 ml/min; the products were detected at 245 nm. Under these conditions, the retention times of 4-acetamidophenol, 2-acetamidophenol, and acetanilide were 3.7, 6.1, and 9.5 min, respectively. *N*-Demethylation activities with benzphetamine and erythromycin were determined as the formation of formaldehyde, by the method of Nash (23). PNP hydroxylation was assayed according to the method of Koop (24). The reaction mixture containing 0.1 mM PNP and microsomal preparation (~0.2 mg of protein) was incubated at 37° for 3 min, and the reaction was terminated by addition of 0.2 ml of 15% perchloric acid. After centrifugation, supernatants of incubated and nonincubated controls were transferred to sample and reference cuvettes, respectively. 4-Nitrocatechol formation was determined by absorption at 510 nm after addition of 0.2 ml of 5 N NaOH to each cuvette (extinction coefficient = 15.4 mM^{-1} cm^{-1}). Cytochrome P450 content for liver and lung microsomes was determined by the methods of Omura and Sato (25) and Matsubara *et al.* (26), respectively. Protein concentration was measured by the method of Bradford (27), using the Bio-Rad protein assay kit and bovine serum albumin as standard.

MI complex formation. A mixture of microsomal preparation (1.2–1.6 mg of protein), 1 mM MDB or MDA, and 0.1 M HEPES buffer (pH 7.6) was equally divided into two cuvettes (0.8 ml each), and the base line between 390 and 500 nm was recorded. Then, potassium phosphate buffer (pH 7.4, 0.05 M, 0.2 ml) was added to the reference cuvette and the NADPH-generating system (0.2 ml) was added to the sample cuvette. The absorbance change between 455 and 490 nm due to complex formation was measured according to the method of Lindeke *et al.* (28). When a reconstituted system with purified isozyme was used as the enzyme source, incubations were carried out in the following manner. A reaction mixture consisting of CYP2B4 (0.2 nmol), NADPH-cytochrome P450 reductase (1.0 unit), and dilauroylphosphatidylcholine (30 μg) was added to reference and sample cuvettes, maintained for 5 min at 37°, and then incubated as described above, except that 0.2 mM NADPH was used instead of the NADPH-generating system.

CYP2B4 that was purified from liver microsomes of PB-treated rabbits by polyethylene glycol fractionation (8–12%), followed by DEAE-cellulose, hydroxylapatite, and CM-Sepharose column chromatography, according to the methods of Coon *et al.* (29), had a specific content of 18.9 nmol/mg of protein. NADPH-cytochrome P450 reductase was purified by DEAE-Sephadex A-25 column chromatography and 2′,5′-ADP-agarose affinity column chromatography, as described previously (30). Its specific activity was 64.9 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 (31).

Results

Induction of MDB and MDA oxidation activity by xenobiotic treatment. In order to investigate whether the cytochrome P450 isozymes responsible for the oxidation of MDB and MDA were constitutive or inducible, the effects of inducers were checked and typical reactions, such as acetanilide hydroxylation for CYP1A2 (32), benzphetamine *N*-demethylation for CYP2B4 (33), PNP hydroxylation for CYP2E1 (34), and erythromycin *N*-demethylation for CYP3A6 (35, 36), were determined as controls. Pretreatment of rabbits with PB, β-NF, rifampicin, and pyrazole enhanced the specific content of cytochrome P450 by 2.1, 1.6, 1.4, and 1.3 times, respectively, compared with a cytochrome P450 content of 2.0 nmol/mg of protein in microsomes from untreated livers. The increases in enzyme activities after β-NF, PB, pyrazole, and rifampicin treatment are shown in Table 1. Surprisingly, the induction of acetanilide hydroxylase by pyrazole was greater than that by β-NF.

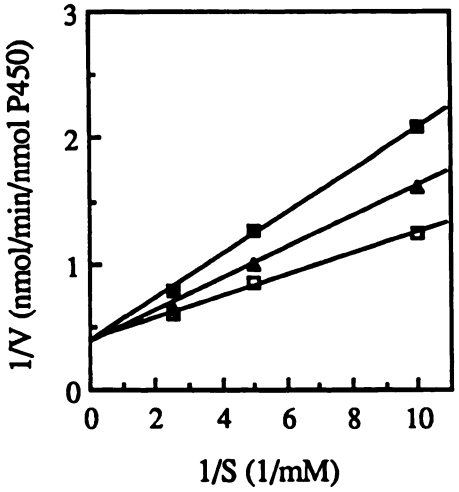

Fig. 1. Lineweaver-Burk plot of the inhibition of erythromycin *N*-demethylation by MDA. Erythromycin was incubated with liver microsomes from rifampicin-treated rabbits in the presence of 0.01 mM (□), 1 mM (▲), or 10 mM (■) MDA, under the conditions described in Materials and Methods. Formaldehyde formed from erythromycin was determined by the Nash method. Addition of MDA at any of these concentrations did not interfere with the assay. Each *point* is the average of two determinations.

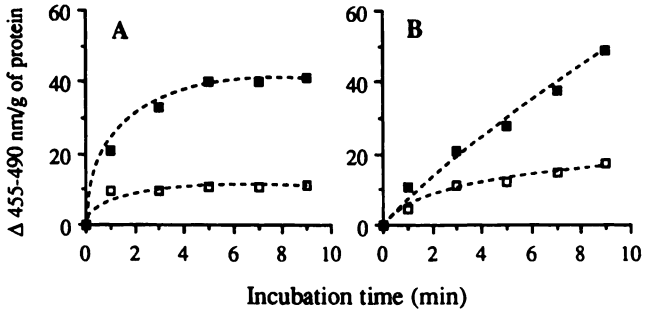

Fig. 2. Effect of PB induction on MI complex formation from MDB (A) and MDA (B), using untreated liver microsomes (□) and PB-treated liver microsomes (■). The reaction mixtures contained 100 mM HEPES buffer (pH 7.6), 1 mM substrate, hepatic microsomes from untreated or PB-treated rabbits, and the NADPH-generating system, in a final volume of 1.0 ml. The incubations were initiated by addition of NADPH-generating system to the sample cuvette and were carried out at 37°. The production of MI complex was determined as described in Materials and Methods. Each *point* is the mean of three determinations.

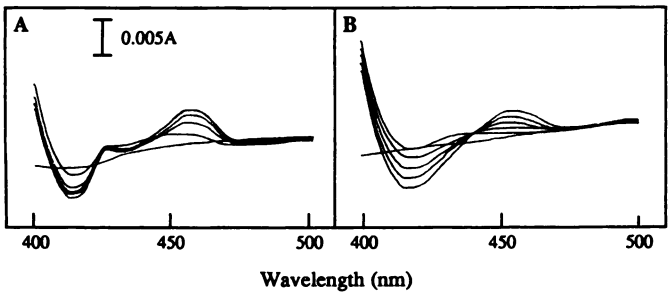

Fig. 3. MI complexes generated from methylenedioxyphenyl compounds in a reconstituted system with CYP2B4. A, MDB; B, MDA. The reaction mixture contained 0.2 nmol of CYP2B4, 1 unit of cytochrome P450 reductase, 30 μg of dilauroylphosphatidylcholine, 0.2 mM NADPH, and 100 mM HEPES (pH 7.6), in a final volume of 1.0 ml. After the components (except for NADPH) were mixed, a base line was obtained and the reactions were then initiated by the addition of NADPH (dissolved in 0.1 M potassium phosphate, pH 7.4) to the sample cuvette and buffer to the reference cuvette. The difference spectra shown were recorded at 0, 1, 3, 5, 7, and 9 min.

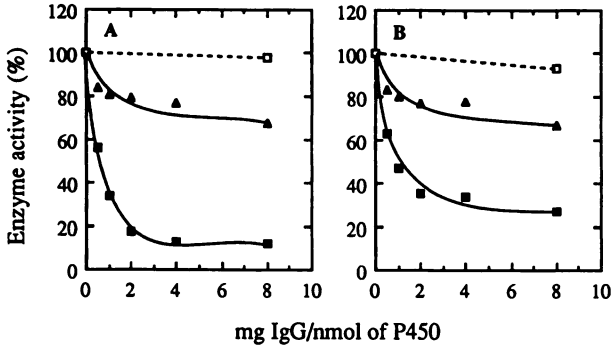

Fig. 4. Inhibition by anti-CYP2B4 and anti-CYP4B1 of demethylenation (A) and aromatic hydroxylation (B) of MDB by liver microsomes of PB-treated rabbits. □, Preimmune IgG; ■, anti-CYP2B4; ▲, anti-CYP4B1. Liver microsomes from PB-treated rabbits were incubated at 22° for 10 min with varying amounts of an IgG fraction purified from CYP2B4- or CYP4B1-immune or preimmune goat serum before the reaction was initiated by the addition of the NADPH-generating system. Incubations were carried out at 37°. The enzyme activities of catechol and sesamol production in the absence of IgG were 9.46 ± 0.25 and 0.57 ± 0.02 nmol/min/nmol of cytochrome P450, respectively (duplicates). Each *point* is the average of two determinations.

Table 2 shows the effects of these inducers on catechol and sesamol formation from MDB and DHA formation from MDA. The ring-hydroxylated product of MDB, sesamol, has been identified by gas chromatography-mass spectrometry (10). MDB demethylenation was increased 5.3-fold after PB treatment and ring hydroxylation activity was increased 2.9-fold after PB and 1.9-fold after β-NF treatment. In contrast, MDA demethylenation activity was induced slightly by PB and rifampicin and decreased in preparations from β-NF-pretreated animals. Because the demethylenation of MDA exhibited low (3.8 μM) and high (141 μM) K_m values (9), experiments were conducted at substrate concentrations of 10 μM and 1 mM. Although only the data for 1 mM are shown (Table 1), results from inducer pretreatment were similar at both concentrations. MDA competitively inhibited erythromycin oxidation by liver microsomes from rabbits pretreated with rifampicin (Fig. 1). When these data were analyzed by nonlinear regression to fit a competitive inhibition model, a K_i value of 14 ± 2 mM was obtained for MDA.

MI complex formation during oxidation of MDB and MDA. NADPH-dependent MI complex formation from MDB and MDA was compared in untreated and PB-treated liver microsomes. MDB exhibited a type I binding spectrum, whereas MDA showed type IIa binding characteristics. The addition of an NADPH-generating system to the incubation mixtures containing untreated microsomes and either compound gave an MI complex, or a type III spectrum with peaks at 424 and 452–456 nm. Because the former peak also reflects reduction of cytochrome b_5 (37), the increase in the peak at 455 nm was used to determine MI complex formation. As shown in Fig. 2, PB-treated enzyme preparations exhibited greater MI complex formation than did untreated controls, with both compounds. With PB-treated microsomes, the production of MI complex from MDB reached a plateau at about 5 min, whereas that from MDA oxidation was linear up to 10 min. The increase in 455-nm complex formation from MDA after PB pretreatment did not parallel the change in demethylenation activity (see Table 2). Fig. 3 shows the complex formation from MDB and MDA in a reconstituted system with CYP2B4, a major PB-inducible isozyme (38). The rates of MI complex formation from MDA and MDB at 5 min were 0.011 and 0.02 $\Delta A_{455-490}$/nmol of cytochrome P450. At 10 min, the complex formed from MDA was 70% greater than that formed from MDB. In addition, the type III binding spectrum for MDB exhibited absorbance maxima at 426 and 455–457 nm, whereas that for MDA showed an absorbance maximum at 453 nm only.

Inhibition by antibodies of MDB and MDA oxidation. Because PB induces not only CYP2B4 but also CYP4B1 (39), experiments on immunoinhibition of MDB metabolism with anti-CYP2B4 and anti-CYP4B1 IgG were performed. As shown in Fig. 4, demethylenation and aromatic hydroxylation of MDB by liver microsomes from PB-treated rabbits were inhibited by addition of anti-CYP2B4 IgG, in a concentration-dependent manner. The inhibitory effect of the IgG on MDB metabolism reached a plateau at 4 mg/nmol of cytochrome P450. Under these conditions, anti-CYP2B4 IgG suppressed 90% of catechol formation and about 70% of sesamol formation. In contrast, addition of anti-CYP4B1 IgG to reaction mixtures of microsomes from PB-treated liver and untreated lung caused only a slight suppression of both activities. Lung microsomes, in which CYP2B4 and CYP4B1 are expressed as major forms in untreated rabbit (40), were also incubated with MDB in the presence of the antibodies, to confirm the role of CYP2B4 in the oxidation (Fig. 5). As expected, MDB demethylenation and aromatic hydroxylation activities in untreated lung microsomes were greater than those in PB-treated liver microsomes, when expressed as product formed per minute per nanomole of cytochrome P450 (see legends to Figs. 4 and 5). Anti-CYP2B4 IgG suppressed lung microsome-promoted demethylenation and aromatic hydroxylation of MDB to the same degree (about 90%), whereas anti-CYP4B1 IgG had a minimal (20% inhibition) effect. The enzyme activities for MDA demethylenation by liver and lung microsomes from untreated rabbits were 0.66 and 0.69 nmol/min/nmol of cytochrome P450, respectively. Addition of antibodies against CYP2B4 and CYP4B1 (4 mg/nmol of cytochrome P450) to the incubation mixtures caused a slight reduction of PB-treated liver-mediated (19 and 28%) and untreated lung-mediated (9 and 29%) DHA formation.

Effects of α-NF, a selective inhibitor for the CYP1A family (41, 42), on MDB and acetanilide metabolism were examined because aromatic hydroxylation of MDB was also induced by

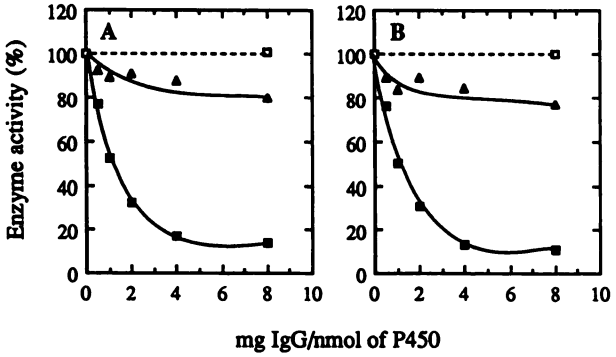

Fig. 5. Inhibition by anti-CYP2B4 and anti-CYP4B1 of demethylenation (A) and aromatic hydroxylation (B) of MDB by lung microsomes from untreated rabbits. □, Preimmune IgG; ■, anti-CYP2B4; ▲, anti-CYP4B1. Lung microsomes isolated from untreated rabbits were incubated with varying amounts of an IgG fraction purified from CYP2B4- or CYP4B1-immune or preimmune goat serum, under the conditions described in Fig. 3. The enzyme activities of catechol and sesamol production in the absence of IgG were 22.63 ± 0.25 and 1.19 ± 0.01 nmol/min/nmol of cytochrome P450, respectively (two experiments). Each *point* is the average of two determinations.

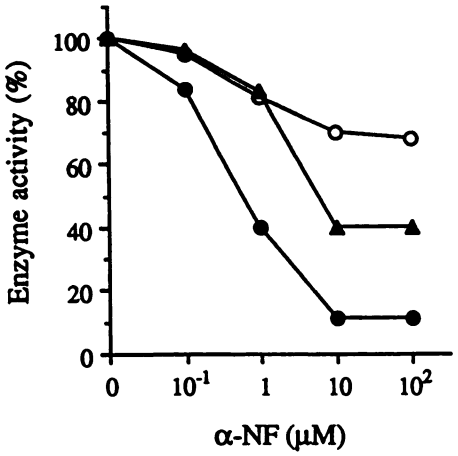

Fig. 6. Inhibition by α-NF of MDB and acetanilide oxidation by hepatic microsomes from rabbits. MDB (1 mM) and acetanilide (4 mM) were incubated with β-NF-treated liver microsomes (0.96 mg of protein) under the conditions described in Materials and Methods. α-NF was dissolved in acetonitrile. The rates of catechol (○), sesamol (●), and 4-acetamidophenol (▲) formation in the presence of 1% acetonitrile (without the inhibitor) were 7.61 ± 0.23, 1.14 ± 0.04, and 3.48 ± 0.47 nmol/min/mg of protein, respectively. The addition of acetonitrile had little if any effect on these activities under these conditions. Each *point* is the average of two determinations.

β-NF, which induces CYP1A isozymes (43, 44). α-NF (10 μM) inhibited production of sesamol from MDB by 89%, that of 4-acetamidophenol from acetanilide by 60%, and that of catechol from MDB by only 30% (Fig. 6).

Because MDA demethylenation activity was not induced, participation of CYP2C3, a major constitutive isozyme (22, 33), in the reaction was examined by use of antibody against CYP2C3 (Fig. 7). Although deamination of amphetamine (17) was markedly inhibited by antibody against CYP2C3, the IgG fraction exhibited no inhibitory effect on the MDA demethylenation.

Finally, although members of the CYP2D family have not been found in rabbit liver, the effects of substrates or inhibitors for CYP2D isozymes on DHA formation were examined (Table 3). Bufuralol (10 μM), a typical substrate for CYP2D isozymes

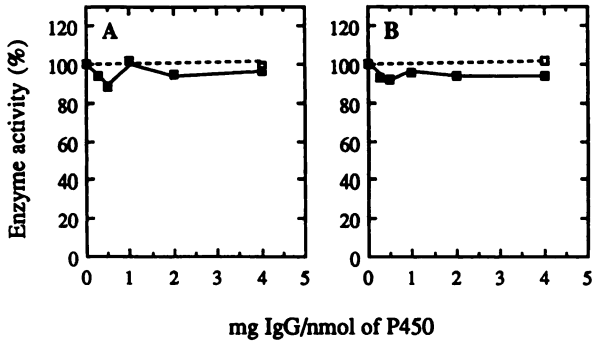

Fig. 7. Effect of antibody to CYP2C3 on the demethylenation of MDA by liver microsomes from untreated rabbits. A, MDA; 10 μM; B, 1 mM MDA. □, Preimmune IgG; ■, anti-CYP2C3 IgG. Liver microsomes isolated from untreated rabbits were incubated with varying amounts of an IgG fraction purified from preimmune or CYP2C3-immune chicken serum, under the conditions described in Fig. 3. The enzyme activities of DHA formation in the absence of IgG were 0.28 ± 0.01 and 0.44 ± 0.01 nmol/min/nmol of cytochrome P450 for 10 μM and 1 mM, respectively. Each *point* is the average of two determinations.

TABLE 3

Participation of cytochrome P450 2D family in the demethylenation of MDA by liver microsomes from untreated rabbits

MDA (10 μM or 1 mM) was incubated with liver microsomes from untreated rabbits as described for Table 2. Under these conditions, the catalytic activities of MDA demethylenation were 0.33 ± 0.02 and 0.58 ± 0.11 nmol/min/nmol of cytochrome P450 for 10 μM and 1 mM, respectively. Each value is the average of two determinations.

Addition	Concentration	Demethylenation activity	
		10 μM MDA	1 mM MDA
	μM	% of control	
Bufuralol	1	100 ± 4	
	10	47 ± 1	100 ± 0
	100		85 ± 1
Sparteine	1	106 ± 1	
	10	104 ± 1	104 ± 1
	100		101 ± 1
Quinine	1	103 ± 0	
	10	94 ± 0	92 ± 1
	100		100 ± 0
Quinidine	1	105 ± 2	
	10	94 ± 0	101 ± 1
	100		102 ± 8

(45), inhibited the demethylenation activity by about 50%, but sparteine had no effect. Quinine and quinidine, potent inhibitors for CYP2D1 (46) and CYP2D6 (47), had no effect on DHA formation.

Discussion

Previous work showed that cleavage of the methylenedioxy group of MDB and MDA to yield catechol and DHA, respectively, was the predominant reaction catalyzed by cytochrome P450 for both compounds. However, the isozyme catalyzing demethylenation was different for the two substrates (9). The object of the present study was to identify the cytochrome P450 isozyme(s) involved, using inducers, polyclonal antibodies, and selective inhibitors as probes. A second reaction, aromatic hydroxylation, was observed for MDB but not for MDA. However, ring hydroxylation of *N*-methyl-MDA has been observed (10), indicating that a minor change appears to have a major effect on the isozyme selectivity. These apparent differences in regiochemistry were the basis for the present study.

TABLE 4
Isozymes responsible for oxidation of MDB and MDA

Isozyme	Criterion	MDB demethylenation	MDB hydroxylation	MDA demethylenation
1A1,2	α-NF inhibition	±[a]	++	ND[b]
	β-NF induction	−	+	−
2B4	PB induction	++	+	±[c]
	Phencyclidine inhibition[d]	++	ND	−
	Anti-CYP2B4	++	++	−
	Reconstituted system[d,e]	++	+	−
2C3	Anti-CYP2C3	ND	ND	−
2D	Spartein, etc., inhibition	ND	ND	−
2E1	Pyrazole induction	−	−	−
3A6	Rifampicin induction	−	−	±
	Erythromycin inhibition	−	−	+
4B1	Anti-CYP4B1	±	±	±

[a] −, no effect.
[b] ND, not determined.
[c] ±, marginal change.
[d,e] These data were taken from Refs. 9 and 10, respectively.

The cytochrome P450 isozyme responsible for the demethylenation of MDB was induced by PB, but not by β-NF, pyrazole, or rifampicin, suggesting that CYP2B4 or CYP4B1 participates in the reaction. The exclusive participation of CYP2B4 in the reaction was demonstrated by the ability of anti-CYP2B4 IgG to suppress MDB demethylenation by 90% in either PB-treated liver or untreated lung microsomes, whereas anti-CYP4B1 IgG hardly affected it.

Aromatic hydroxylation of MDB was induced by PB and β-NF. Antibody experiments with PB-induced microsomes showed that CYP2B4 but not CYP4B1 effects ring hydroxylation of MDB. The possibility of β-NF-inducible isozyme participation was confirmed by the effects of α-NF, which selectively inhibits CYP1A isozymes. The presence of residual (~30%) hydroxylation activity in PB-treated liver microsomes and the complete suppression (>90%) in untreated lung microsomes after addition of anti-CYP2B4 were probably associated with differences in the contents of CYP1A isozymes between liver and lung (37, 44). From these results it is concluded that most of the MDB demethylenation is mediated by CYP2B4, not CYP4B1, and that aromatic hydroxylation is catalyzed by CYP2B4 and CYPIA1 (2) (see Table 4).

MDA demethylenation activity was only slightly increased by PB or rifampicin and was decreased by β-NF pretreatment, whereas MDB demethylenation was increased 5-fold by PB (Table 2). This marginal increase and the inability of antibodies against CYP2B4 and CYP4B1 to suppress MDA demethylenation excluded participation of PB-inducible isozymes in the reaction. Although inhibition of erythromycin *N*-demethylation by MDA was competitive (Fig. 1), the low rate of induction by rifampicin, a selective inducer for CYP3A6 (48), suggested that a constitutive isozyme other than CYP3A6 plays an important role in the MDA demethylenation. For this reason, suppression by antibody against CYP2C3, a predominant constitutive form in rabbit liver (22, 33), was evaluated. However, the antibody had no inhibitory effect on DHA formation. Substrates or inhibitors of CYP2D, with the exception of bufuralol, were also shown to be without effect. CYP2C5, reported by Johnson and Schwab (49), may participate in MDA demethylenation, but it is unlikely because the content of CYP2C5 varies widely among liver microsomes prepared from individual New Zealand white rabbits (50, 51). We found that MDA demethylenation activity was uniform among a large number of rabbits, thereby eliminating the possibility of participation of CYP2C5.[2] Thus, the major isozyme catalyzing MDA

[2] Y. Kumagai and A. K. Cho, unpublished observations.

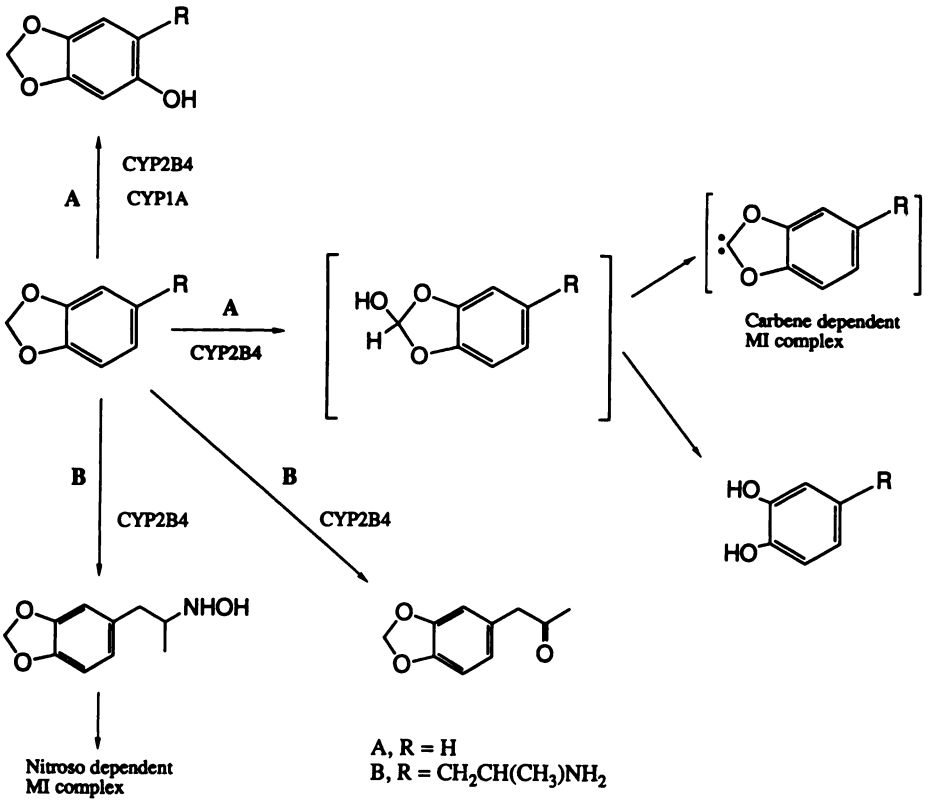

Fig. 8. Cytochrome P450 isozyme-promoted oxidation of and MI complex formation from MDB (A) and MDA (B).

demethylenation in rabbit liver seems to be an unidentified constitutive form(s) other than CYP1A1, CYP1A2, CYP2B4, CYP2C3, CYP2C5, CYP2E1, CYP3A6, and CYP4B1 (Table 4). It should be noted that the demethylenation of MDA was also observed in lung microsomes, and the specific activity (nanomoles of product formed per minute per nanomole of cytochrome P450) from lung was almost the same as that from liver of untreated animals.

The MI complex generated during NADPH-dependent oxidation of methylenedioxyphenyl compounds by cytochrome P450 has been examined extensively (52). Complex formation is thought to require formation of a carbene by hydroxylation of the methylene carbon and dehydration (1–3). The hydroxylated intermediate can also undergo hydrolysis to yield the catechol metabolite (53), so catechol and carbene share a common initial oxygenation step (6). In the case of MDB, a good correlation between the rates of catechol and MI complex formation was obtained from induction studies with PB, and reconstitution experiments showed that CYP2B4 forms an MI complex with MDB. In the case of MDA, however, complex formation and demethylenation activity did not correlate after PB pretreatment. In spite of the fact that the MDA demethylenation activity of CYP2B4 was about 1% of that for MDB (9), the formation of CYP2B4-mediated MI complex from MDA was >50% of that from MDB. CYP2B4 catalyzes the *N*-hydroxylation of MDA (7), and complex formation from *N*-hydroxy-MDA is greater than that from MDA in both PB-treated liver microsomes and reconstituted preparations with CYP2B4.[2] These data suggest that the complex produced from MDA is based on the nitroso derivative generated by oxidation of the side chain amino group, as reported previously with amphetamine analogues (28). Thus, CYP2B4 appears to oxidize different regions of methylenedioxyphenyl compounds; MDB undergoes oxidation on the benzene ring to produce catechol, sesamol, and carbene-promoted MI complex. MDA mainly undergoes oxidation of the alkylamine group, i.e., *N*-oxidation and deamination (7). Based on these findings, a scheme for the relationship between regiochemistry and MI complex formation by CYP2B4 is shown in Fig. 8. It should be pointed out that methylenedioxyphenyl compounds with side chains are not necessarily poor substrates for CYP2B4 in the demethylenation reaction. Compounds such as safrole (4-allyl-1,2-methylenedioxybenzene) and isosafrole (4-propenyl-1,2-methylenedioxybenzene) were effectively oxidized by the isozyme and produced MI complexes and carbon monoxide (54). We have also found that CYP2B4 is capable of demethylenating methylenedioxyphenyl acetone, whereas *N*-hydroxy-MDA undergoes little catechol formation.[2] Thus, it appears that the presence of an ionizable group on the side chain strongly influences the substrate specificity for the demethylenation by CYP2B4.

The present findings demonstrate the participation of different cytochrome P450 isozymes in the cleavage of the methylenedioxy group on MDB and MDA. Because the regulation and extrahepatic localization of these isozymes differ, the identification of the isozyme catalyzing specific reactions is vital to our understanding of metabolism-based pharmacological and toxicological events. In this study, the ultimate products, catechol and MI complex, are involved in cell toxicity (55, 56) and inhibition of drug metabolism (57, 58).

References

1. Hodgson, E., and R. M. Philpot. Interaction of methylenedioxyphenyl(1,3-benzodioxole) compounds with enzymes and their effects on mammals. *Drug Metab. Rev.* **3**:231–301 (1974).
2. Mansuy, D., J.-P. Battioni, J.-C. Chottard, and V. Ullrich. Preparation of a porphyrin-iron-carbene model for the cytochrome P 450 complexes obtained upon metabolic oxidation of the insecticide synergists of the 1,3-benzodioxole series. *J. Am. Chem. Soc.* **101**:3971–3973 (1979).
3. Wilkinson, C. F., M. Murray, and C. B. Marcus. Interactions of methylenedioxyphenyl compounds with cytochrome P450 and effects on microsomal oxidation. *Rev. Biochem. Toxicol.* **6**:27–63 (1984).
4. Ricaurte, G., G. Bryan, L. Strauss, L. Seiden, and C. Schuster. Hallucinogenic amphetamine selection destroys brain serotonin nerve terminals. *Science (Washington D. C.)* **229**:986–988 (1985).
5. Battaglia, G., S. Y. Yeh, E. O'Hearn, M. E. Molliver, M. J. Kuhar, and E. B. De Siuza. 3,4-Methylenedioxymethamphetamine and 3,4-methylenedioxyamphetamine destroy serotonin terminals in rat brain: quantification of neurodegeneration by measurement of [^{3}H]paroxetine-labeled serotonin uptake sites. *J. Pharmacol. Exp. Ther.* **242**:911–916 (1987).
6. Anders, M. W., J. M. Sunram, and C. F. Wilkinson. Mechanism of metabolism of 1,3-benzodioxoles to carbon monoxide. *Biochem. Pharmacol.* **33**:577–580 (1984).
7. Fukuto, J. M., Y. Kumagai, and A. K. Cho. Determination of the mechanism of demethylenation of (methylenedioxy)phenyl compounds by cytochrome P450 using deuterium isotope effects. *J. Med. Chem.* **34**:2871–2876 (1991).
8. Hiramatsu, M., Y. Kumagai, S. E. Unger, and A. K. Cho. Metabolism of methylenedioxymethamphetamine (MDMA):formation of dihydroxymethamphetamine and a quinone identified as its glutathione adduct. *J. Pharmacol. Exp. Ther.* **254**:521–527 (1990).
9. Kumagai, Y., K. A. Wickham, D. A. Schmitz, and A. K. Cho. Metabolism of methylenedioxyphenyl compounds by rabbit liver preparations: participation of different cytochrome P450 isozymes in the demethylenation reaction. *Biochem. Pharmacol.* **42**:1061–1067 (1991).
10. Kumagai, Y., D. A. Schmitz, and A. K. Cho. Aromatic hydroxylation of methylenedioxybenzene (MDB) and methylenedioxymethamphetamine (MDMA) by rabbit cytochrome P450. *Xenobiotica* **22**:395–403 (1992).
11. Kumagai, Y., L. Y. Lin, D. A. Schmitz, and A. K. Cho. Hydroxyl radical mediated demethylenation of (methylenedioxy)phenyl compounds. *Chem. Res. Toxicol.* **4**:330–334 (1991).
12. Florence, V. M., E. W. DiStefano, C. Y. Sum, and A. K. Cho. The metabolism of *R*-(−)-amphetamine by rabbit liver microsomes: initial products. *Drug Metab. Dispos.* **10**:312–315 (1982).
13. Van Der Hoeven, T. A., and M. J. Coon. Preparation and properties of partially purified cytochrome P450 and reduced nicotinamide adenine dinucleotide phosphate-cytochrome P450 reductase from rabbit liver microsomes. *J. Biol. Chem.* **249**:6302–6320 (1974).
14. Nebert, D. W., D. R. Nelson, M. J. Coon, R. W. Estabrook, R. Feyereisen, Y. Fujii-Kuriyama, F. J. Gonzalez, F. P. Guengerich, I. C. Gunsalus, E. F. Johnson, J. C. Loper, R. Sato, M. R. Waterman, and D. J. Waxman. The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature. *DNA Cell Biol.* **10**:1–14 (1991).
15. Wolf, C. R., S. R. Slaughter, J. P. Marciniszyn, and R. M. Philpot. Purification and structural comparison of pulmonary and hepatic cytochrome P450 from rabbits. *Biochim. Biophys. Acta* **624**:409–419 (1980).
16. Slaughter, S. R., C. R. Wolf, J. P. Marciniszyn, and R. M. Philpot. The rabbit pulmonary monooxygenase system: partial structural characterization of the cytochrome P450 components and comparison to the hepatic cytochrome P450. *J. Biol. Chem.* **256**:2499–2503 (1981).
17. Yamada, H., S. Honda, K. Oguri, and H. Yoshimura. A rabbit liver constitutive form of cytochrome P450 responsible for amphetamine deamination. *Arch. Biochem. Biophys.* **273**:26–33 (1989).
18. Gasser, R., M. Negishi, and R. M. Philpot. Primary structures of multiple forms of cytochrome P-450 isozyme 2 derived from rabbit pulmonary and hepatic cDNA. *Mol. Pharmacol.* **32**:22–30 (1987).
19. Serabjit-Singh, C. J., C. R. Wolf, and R. M. Philpot. The rabbit pulmonary monooxygenase system: immunochemical and biochemical characterization of enzyme components. *J. Biol. Chem.* **254**:9901–9907 (1979).
20. Vanderslice, R. R., B. A. Domin, G. T. Carver, and R. M. Philpot. Species-dependent expression and induction of homologues of rabbit cytochrome P450 isozyme 5 in liver and lung. *Mol. Pharmacol.* **31**:320–325 (1987).
21. Johnson, E. F., and U. Muller-Eberhard. Purification of the major cytochrome P450 of liver microsomes from rabbits treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *Biochem. Biophys. Res. Commun.* **76**:652–659 (1977).
22. Koop, D. R., A. V. Persson, and M. J. Coon. Properties of electrophoretically homogenous constitutive forms of liver microsomal cytochrome P450. *J. Biol. Chem.* **256**:10704–10711 (1981).
23. Nash, T. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.* **55**:416–421 (1953).
24. Koop, D. R. Inhibition of ethanol-inducible cytochrome P450IIE1 by 3-amino-1,2,4-triazole. *Chem. Res. Toxicol.* **3**:377–383 (1990).
25. Omura, T., and R. Sato. The carbon monoxide-binding pigment of liver microsomes. *J. Biol. Chem.* **239**:2370–2378 (1964).
26. Matsubara, T., M. Koike, A. Touchi, Y. Tochino, and K. Sugeno. Quantitative

determination of cytochrome P450 in rat liver homogenate. *Anal. Biochem.* **75**:596–603 (1976).
27. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
28. Lindeke, B., U. Paulsen, and E. Anderson. Cytochrome P455 complex formation in the metabolism of phenylalkyamines. IV. Spectral evidences for metabolic conversion of methamphetamine to *N*-hydroxyamphetamine. *Biochem. Pharmacol.* **28**:3629–3635 (1979).
29. Coon, M. J., T. A. Van Der Hoeven, S. B. Dahl, and D. A. Haugen. Two forms of liver microsomal cytochrome P450, P450 LM_2 and P450 LM_4 (rabbit liver). *Methods Enzymol.* **52**:109–117 (1978).
30. Duncan, J. D., and A. K. Cho. *N*-Oxidation of phentermine to *N*-hydroxyphentermine by a reconstituted cytochrome P450 oxidase system from rabbit liver. *J. Biol. Chem.* **22**:235–238 (1982).
31. Phillips, A. H., and R. G. Langdon. Hepatic triphosphopyridine nucleotide-cytochrome *c* reductase isolation, characterization, and kinetic studies. *J. Biol. Chem.* **237**:2652–2660 (1962).
32. Norman, R. L., E. F. Johnson, and U. Muller-Eberhard. Identification of the major cytochrome P450 form transplacentally induced in neonatal rabbits by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J. Biol. Chem.* **253**:8640–8647 (1978).
33. Johnson, E. Isolation and characterization of a constitutive form of rabbit liver microsomal cytochrome P450. *J. Biol. Chem.* **255**:304–309 (1980).
34. Koop, D. Hydroxylation of *p*-nitrophenol by rabbit ethanol-inducible cytochrome P450 isozyme 3a. *Mol. Pharmacol.* **29**:399–404 (1986).
35. Bonfils, C., C. Dalet, I. Dalet-Beluche, and P. Maurel. Cytochrome P450 isozyme LM_{3b} from rabbit liver microsomes: induction by triacetyloleandomycin purification and characterization. *J. Med. Chem.* **258**:5358–5362 (1983).
36. Watkins, P. B., S. A. Wrighton, P. Maurel, E. G. Schuetz, G. Mendez-Picon, G. A. Parker, and P. S. Guzelian. Identification of an inducible form of cytochrome P450 in human liver. *Proc. Natl. Acad. Sci. USA* **82**:6310–6314 (1985).
37. Werringloer, J., and R. W. Estabrook. Evidence for an inhibitory product cytochrome P450 complex generated during benzphetamine metabolism by liver microsomes. *Life Sci.* **13**:1319–1330 (1973).
38. Domin, B. A., C. J. Serabjit-Singh, and R. M. Philpot. Quantitation of rabbit cytochrome P450, form 2, in microsomal preparations bound directly to nitrocellulose paper using a modified peroxidase-immunostaining procedure. *Anal. Biochem.* **136**:390–396 (1984).
39. Robertson, I. G. C., C. Serabjit-Singh, J. E. Croft, and R. M. Philpot. The relationship between increases in the hepatic content of cytochrome P450, form 5, and in the metabolism of aromatic amines to mutagenic products following treatment of rabbits with phenobarbital. *Mol. Pharmacol.* **24**:156–162 (1983).
40. Wolf, C. R., M. W. Szutowski, L. M. Ball, and R. M. Philpot. The rabbit pulmonary monooxygenase system: characteristics and activities of two forms of pulmonary cytochrome P450. *Chem.-Biol. Int.* **21**:29–43 (1978).
41. Johnson, E. F., G. E. Schwab, and U. Muller-Eberhard. Multiple forms of cytochrome P450: catalytic differences exhibited by two homogeneous forms of rabbit cytochrome P450. *Mol. Pharmacol.* **15**:708–718 (1979).
42. Domin, B. A., and R. M. Philpot. The effect of substrate on the expression of activity catalyzed by cytochrome P450: metabolism mediated by rabbit isozyme 6 in pulmonary microsomal and reconstituted monooxygenase systems. *Arch. Biochem. Biophys.* **246**:128–142 (1986).
43. Haugen, D. A., and M. J. Coon. Properties of electrophoretically homogeneous phenobarbital-inducible and β-naphthoflavone-inducible forms of liver microsomal cytochrome P450. *J. Biol. Chem.* **251**:7929–7939 (1976).
44. Chiang, J. Y. L., A. G. DiLella, and A. W. Steggles. Effects of inducers and aging on rabbit liver microsomal drug metabolizing enzymes. *Mol. Pharmacol.* **23**:244–251 (1983).
45. Gut, J., T. Catin, P. Dayer, T. Kronbach, U. Zanger, and U. A. Meyer. Debrisoquine/sparteine-type polymorphism of drug oxidation: purification and characterization of two functionally different human liver cytochrome P450 isozymes involved in impaired hydroxylation of the prototype substrate bufuralol. *J. Biol. Chem.* **261**:11734–11743 (1986).
46. Kobayashi, S., S. Murray, D. Watson, D. Sesardic, D. S. Davies, and A. R. Boobis. The specificity of inhibition of debrisoquine 4-hydroxylase activity by quinidine and quinine in the rat is the inverse of that in man. *Biochem. Pharmacol.* **38**:2795–2799 (1989).
47. Otton, S. V., T. Inaba, and W. Kalow. Competitive inhibition of spartein oxidation in human liver by β-adrenoceptor antagonists and other cardiovascular drugs. *Life Sci.* **34**:73–80 (1984).
48. Lange, R., C. Balny, and P. Maurel. Inductive and repressive effects of rifampicin on rabbit liver microsomal cytochrome P450. *Biochem. Pharmacol.* **33**:2771–2776 (1984).
49. Johnson, E. F., and G. E. Schwab. Constitutive forms of rabbit-liver microsomal cytochrome P450: enzymatic diversity, polymorphism and allosteric regulation. *Xenobiotica* **14**:3–18 (1984).
50. Dieter, H. H., U. Muller-Eberhard, and E. F. Johnson. Identification of rabbit microsomal cytochrome P450 isozyme, form 1, as a hepatic progesterone 21-hydroxylase. *Biochem. Biophys. Res. Commun.* **105**:515–520 (1952).
51. Dieter, H. H., U. Muller-Eberhard, and E. Johnson. Rabbit hepatic progesterone 21-hydroxylase exhibits a bimodal distribution activity. *Science* (Washington D. C.) **217**:741–743 (1982).
52. Franklin, M. R. The enzymic formation of a methylenedioxyphenyl derivative exhibiting an isocyanide-like spectrum with reduced cytochrome P450 in hepatic microsomes. *Xenobiotica* **1**:581–591 (1971).
53. Casida, J. E., J. L. Engel, E. G. Essac, F. X. Kamienski, and S. Kuwatsuka. Methylene-C^{14}-dioxyphenyl compounds: metabolism in relation to their synergistic action. *Science* (Washington D. C.) **153**:1130–1133 (1966).
54. Delaforge, M., D. R. Koop, and M. J. Coon. Role of isosafrole as complexing agent and inducer of $P450_{LM4}$ in rabbit liver microsomes. *Biochem. Biophys. Res. Commun.* **108**:59–65 (1982).
55. Benford, D. J., and J. W. Bridges. The cytotoxicity of benzodioxoles and related compounds to isolated rat hepatocytes. *Hum. Toxicol.* **2**:563–564 (1983).
56. Patel, N., Y. Kumagai, S. E. Unger, J. M. Fukuto, and A. K. Cho. Transformation of dopamine and α-methyldopamine by NG108–15 cells: formation of thiol adducts. *Chem. Res. Toxicol.* **4**:421–426 (1991).
57. Marcus, C. B., M. Murray, and C. F. Wilkinson. Spectral and inhibitory interactions of methylenedioxyphenyl and related compounds with purified isozymes of cytochrome P450. *Xenobiotica* **15**:351–362 (1985).
58. Murray, M., L. Zaluzny, and G. C. Farrel. Selective reactivation of steroid hydroxylases following dissociation of the isosafrole metabolite complex with rat hepatic cytochrome P450. *Arch. Biochem. Biophys.* **251**:471–478 (1986).
59. Koop, D. R., B. L. Crump, G. D. Nordblom, and M. J. Coon. Immunochemical evidence for induction of the alcohol-oxidizing cytochrome P450 of rabbit liver microsomes by diverse agents: ethanol, imidazole, trichloroethylene, acetone, pyrazole, and isoniazid. *Proc. Natl. Acad. Sci. USA* **82**:4065–4069 (1985).

Send reprint requests to: Arthur K. Cho, Ph.D., University of California, Los Angeles, Department of Pharmacology, UCLA School of Medicine, Center for the Health Sciences, Los Angeles, CA 90024–1735.