

Metabolism of Methylenedioxymethamphetamine: Formation of Dihydroxymethamphetamine and a Quinone Identified as Its Glutathione Adduct

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ABSTRACT

The *in vitro* conversion of (+)-3,4-methylenedioxymethamphetamine and (–)-3,4-methylenedioxymethamphetamine to the corresponding catecholamine, 3,4-dihydroxymethamphetamine (N-methyl- α -methyldopamine), by rat liver microsomes was examined. Metabolite formation was monitored after short-term incubations using high-performance liquid chromatography-electrochemical detection to determine concentrations of the catecholamine. The formation of N-methyl- α -methyldopamine exhibited enantioselectivity and levels were significantly higher after incubation of the (+)-isomer. The reaction appears to be cytochrome P-450 dependent as it was sensitive to SKF 525A and carbon monoxide. The catecholamine was unstable and was metabolized rapidly to a compound capable of forming an adduct with glutathione (GSH) and other thiol compounds. This second

oxidation did not appear to be cytochrome P-450-dependent but required NADPH and microsomal protein. Catecholamine oxidation was inhibited by superoxide dismutase and by reducing agents. The same catecholamine oxidation product, characterized as the GSH adduct, could be generated by a xanthine-xanthine oxidase mixture and by tyrosinase. Mass spectral data showed that it was a 1:1 amine GSH adduct. These results indicate that MDMA is oxidized by cytochrome P-450 to the catechol and the catecholamine oxidized by superoxide to a quinone to which GSH or other thiol functions add. The formation of this quinone and its thiol adducts may account for some of the irreversible actions of this compound on serotonergic neurons.

MDMA is selectively toxic to serotonergic neurons after s.c. administration to rats. Short-term, reversible reductions in brain concentrations of 5-HT and 5-hydroxyindoleacetic acid are followed by long-term, toxic actions on serotonergic neurons (Schmidt, 1987). The long-term neurotoxicity is characterized by loss of tryptophan hydroxylase (Stone *et al.*, 1987), impairment of 5-HT uptake (Battaglia *et al.*, 1987; Commins *et al.*, 1987) and irreversible cytopathological damage of serotonergic neurons (O'Hearn *et al.*, 1988). Dopaminergic neurons are also involved in the toxicity as it is blocked by pretreatment with dopamine receptor antagonists (Johnson *et al.*, 1988a,b; Stone *et al.*, 1988). This interaction with dopamine neurons also exhibits stereoselectivity as *in vivo* microdialysis studies have shown that only (+)-MDMA causes dopamine release in the striatum (Hiramatsu and Cho, 1990). In pharmacokinetic studies of MDMA, the formation of MDA, which also has neurotoxic actions (Ricaurte *et al.*, 1985), exhibited a marked enan-

tioselectivity and levels of this metabolite were higher after dosage with (+)-MDMA (Hiramatsu *et al.*, 1989). These pharmacodynamic and pharmacokinetic differences in the enantiomers of MDMA raise the possibility that some of the actions of this compound could involve MDA as well. Furthermore, because direct injection of MDMA into rat brain does not produce 5-HT neurotoxicity, this action is thought to be mediated by metabolites (Schmidt and Taylor, 1988). Therefore, the characterization of MDMA metabolism is essential to an understanding of its pharmacology and toxicology.

In preliminary studies we have demonstrated that the catecholamine, N-Me- α -MeDA, is the major metabolite of MDMA in rat liver microsomal incubations (Hiramatsu *et al.*, 1989). As this compound has also been reported to form in brain tissue (Lim and Foltz, 1988), its formation and properties are of toxicological and pharmacological interest. This paper describes the biochemical characteristics and enantiomeric selectivities of the catecholamine metabolites of MDMA and its N-demethylated analog, MDA.

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ABBREVIATIONS: MDMA, 3,4-methylenedioxymethamphetamine; 5-HT, serotonin; N-Me- α -MeDA, N-methyl- α -dopamine; MDA, 3,4-methylenedioxymethamphetamine; α -MeDA, α -methyldopamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 1,4-DABCO, 1,4-diazabicyclo(2,2,2)octane; HPLC-EC, high-performance liquid chromatography-electrochemical detection; FAB, fast atom bombardment; MS, mass spectrum.

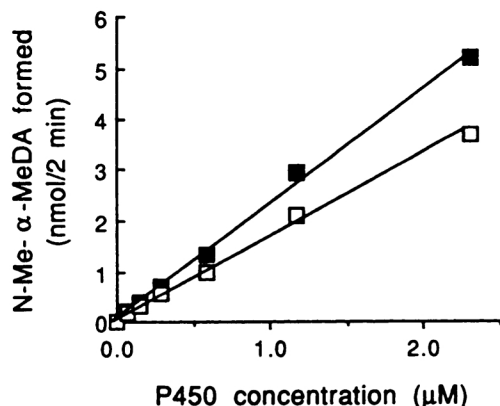


Fig. 1. Plot of catechol formed at 2 min vs. cytochrome P-450 concentration. ■, (+)-MDMA; □, (-)-MDMA.

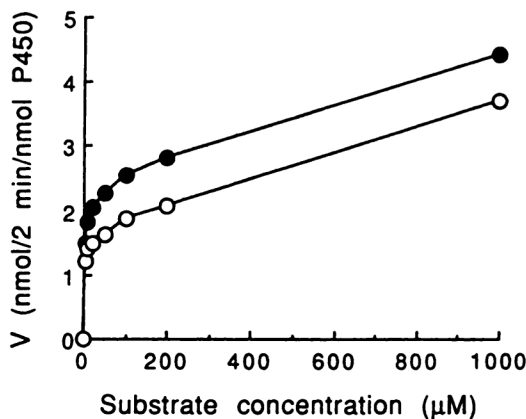


Fig. 2. Kinetic plots of the conversion of (+)- or (-)-MDMA to N-Me-α-MeDA by rat liver microsomes. ●, (+)-MDMA; ○, (-)-MDMA. Substrate is the initial concentration (micromolar). Each data is the mean ± S.E. of four determinations.

TABLE 1

Enzymatic parameters for catechol formation

The substrates were incubated with rat liver microsomes as described under "Materials and Methods." The data were analyzed by nonlinear regression procedures for their fit to a single- and two-enzyme model using the *F* test for evaluation. The results showed the two-enzyme model to be significant at a *P* value of .01. Units for K_m are micromolar, V_{max} , nanomoles per nanomole of cytochrome P-450 × 2 min⁻¹.

	(+)-MDMA	(-)-MDMA
K_{m1}	3.1 ± 1.1	1.6 ± 0.7
K_{m2}	1757 ± 2195	2010 ± 1521
V_{max1}	1.75 ± 0.16	1.22 ± 0.08*
V_{max2}	5.11 ± 3.76	5.51 ± 2.62

* *P* < .01 vs. (+)-MDMA.

Materials and Methods

Animals. Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 300 to 350 g were used. The rats were housed under a 12-12 hr light-dark cycle and the room was maintained between 22-24°C. Rat chow and water were available *ad libitum*. Food was removed the night before microsome preparation.

Chemicals and drugs. (+)- or (-)-MDMA and (+)- or (-)-MDA were obtained from the Research Technology Branch of the National Institute on Drug Abuse (Rockville, MD). N-Me-α-MeDA was synthesized in our laboratory by procedures described by Chavdarian *et al.* (1978). α-MeDA was obtained from Merck Sharp and Dohme Laboratories (West Point, PA).

NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, HEPES, GSH, 1,4-DABCO, catalase, xanthine, xanthine oxidase, ty-

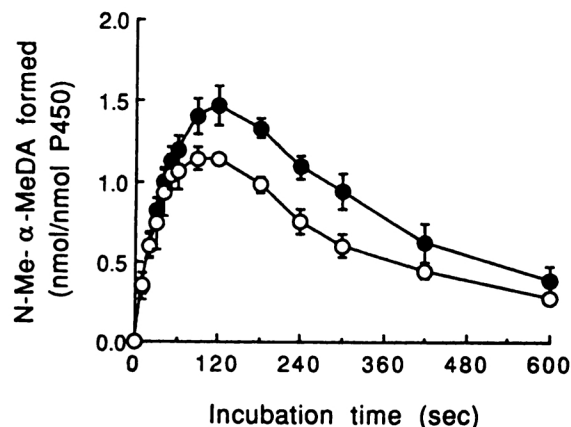


Fig. 3. Time course of N-Me-α-MeDA formation by rat liver microsomes. ●, (+)-MDMA; ○, (-)-MDMA. (+)-MDMA or (-)-MDMA (20 μM) was incubated at 37°C for 10 to 600 sec with rat liver microsomes as described under "Materials and Methods." Each data is the mean ± S.E. of four determinations.

TABLE 2

N-Me-α-MeDA formed (nanomoles per nanomoles of cytochrome P-450)

Reaction Mixture	N-Me- α -MeDA formed P-450		<i>n</i>
	(+)-MDMA	(-)-MDMA	
	nmol/nmol of cytochrome		
Complete (air atmosphere)	1.54 $\pm$ 0.14 [†]	1.17 $\pm$ 0.09	16
Boiled enzyme	0**	0**	2
-NADPH	0**	0**	2
+CO-O ₂ (9/1) atmosphere	0.45 $\pm$ 0.01**	0.43 $\pm$ 0.01**	2
+SKF 525A			
0.3125 μ M	0.48 $\pm$ 0.04**	0.43 $\pm$ 0.02**	3
1.25 μ M	0.12 $\pm$ 0.03**	0.10 $\pm$ 0.03**	4
5 μ M	0**	0**	4
20 μ M	0**	0**	4
+Methimazole			
0.25 mM	1.59 $\pm$ 0.32	1.19 $\pm$ 0.16	4
0.5 mM	1.56 $\pm$ 0.33	1.17 $\pm$ 0.19	4
1 mM	1.48 $\pm$ 0.35	1.15 $\pm$ 0.23	4

** *P* < .01 vs. complete; [†] *P* < .01 vs. (-)-MDMA group.

TABLE 3

Comparison of the demethylation of (+)- and (-)-MDMA by rat liver microsomes in the presence of NADPH and hydrogen peroxide

(+)- and (-)-MDMA (20 μM) were incubated with microsomes for 2 min under conditions described under "Materials and Methods." Each value is the mean ± S.D. of two to four determinations.

System	N-Me-α-MeDA formed P-450	
	(+)-MDMA	(-)-MDMA
nmol/nmol of cytochrome		
Microsomes + NADPH	1.76 ± 0.06 (100%)	1.27 ± 0.10 (100%)
Microsomes + H ₂ O ₂ (10 mM)	0.86 ± 0.23 (49%)	0.79 ± 0.14 (62%)
Horseradish peroxidase (0.15 μg) + H ₂ O ₂ (10 mM)	0.25 nmol*	

* (+)-MDMA (0.5 mM) was incubated with horseradish peroxidase for 20 min.

rosinase and superoxide dismutase were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade and obtained from commercial sources.

Enzyme preparation. The rats were decapitated and the livers removed, cleaned and weighed. Portions of liver (10 g) were homogenized in 30 ml of 1.15% KCl using a Potter-Elvehjem homogenizer and Teflon pestle (Florence *et al.*, 1982). The homogenates were centrifuged at 9000 × *g* for 20 min in a refrigerated centrifuge. The supernatants were recentrifuged at 100,000 × *g* for 60 min. The resulting pellets were

TABLE 4

Catechol catabolism in rat liver microsomes

N-Me- α -MeDA and α -MeDA (2 μ M) was incubated at 37°C for 2 min with rat liver microsomes as described under "Materials and Methods." Each value is the mean $\pm$ S.D. of number (*n*) of determinations. Values are expressed as percentage of the starting catecholamine levels (control, incubation time 0 min) after the 2-min incubation. N.D., not determined.

Reaction Mixture	% of Initial concentration		<i>n</i>
	N-Me- α -MeDA	α -MeDA	
Control			
incubation time 0 min	100.0	100.0	38
incubation time 2 min	46.9 $\pm$ 3.6*	57.6 $\pm$ 2.6*	7
-NADPH	93.2 $\pm$ 5.0**, †	93.8 $\pm$ 4.6**, †	4
Boiled microsomes	103.2 $\pm$ 3.7**	98.6 $\pm$ 5.4**	4
+100% N ₂ gas atmosphere	64.8 $\pm$ 1.5**, ‡	75.8 $\pm$ 2.7**, ‡	4
+CO-O ₂ (9/1) atmosphere	89.7 $\pm$ 2.3**, ‡	95.5 $\pm$ 0.8**, †	3
+SKF 525A, 20 μ M	53.3 $\pm$ 3.6*, ‡	63.1 $\pm$ 4.5*, ‡	4
+Methimazole, 1 mM	50.1 $\pm$ 0.6*, ‡	N.D.	
+Ascorbic acid, 1 mM	98.0 $\pm$ 4.8**	97.8 $\pm$ 4.3**	6
+EDTA, 20 mM	77.7 $\pm$ 7.3**, ‡	82.9 $\pm$ 6.3**, ‡	6

* $P < .05$; ** $P < .01$ vs. control (incubation time 2 min); † $P < .05$; ‡ $P < .01$ vs. control (incubation time 0 min).

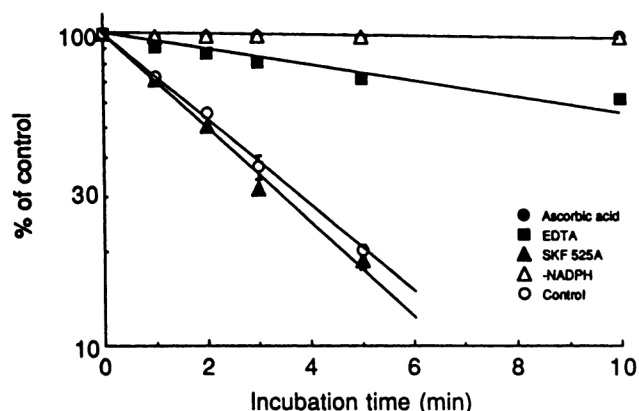


Fig. 4. Time course of N-Me- α -MeDA catabolism in rat liver microsomes. N-Me- α -MeDA (2 μ M) was incubated at 37°C for 2 min in the presence of ascorbic acid (1 mM), EDTA (20 mM) or SKF 525A (20 μ M) and in the absence of an NADPH generating system. Values are expressed as percentage of the starting catechol levels after the 2- to 10-min incubation. Each data is the mean $\pm$ S.E. of four determinations.

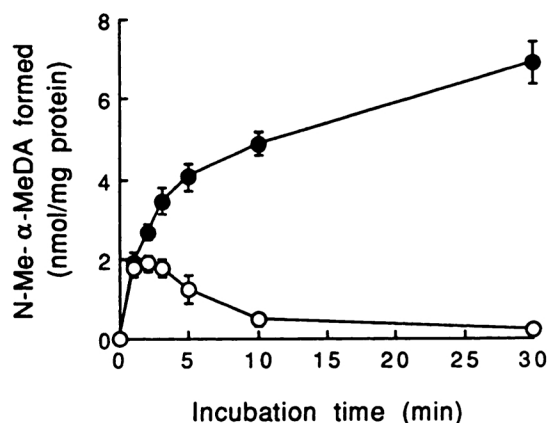


Fig. 5. Time course of N-Me- α -MeDA formation from (+)-MDMA in the presence of ascorbic acid. ●, (+)-MDMA in the presence of ascorbic acid (1 mM); ○, (+)-MDMA in the absence of ascorbic acid. (+)-MDMA (20 μ M) was incubated at 37°C for 1 to 30 min with rat liver microsomes as described under "Materials and Methods." Each data is the mean $\pm$ S.E. of four determinations.

TABLE 5

Effects of active oxygen scavenging agents on the stability of N-Me- α -MeDA generated from (+)-MDMA

(+)-MDMA (20 μ M) was incubated with microsomes for 5 min under conditions described under "Materials and Methods." Each value is the mean $\pm$ S.D. of four determinations. SOD, superoxide dismutase.

Reaction Mixture	N-Me- α -MeDA Formed nmol/5 min/mg protein
Complete	1.03 $\pm$ 0.12
+SOD (50 U)	3.89 $\pm$ 0.21
+Catalase (100 U)	0.92 $\pm$ 0.09
+SOD + catalase	3.90 $\pm$ 0.14
+1,4-DABCO (1 mM)	1.00 $\pm$ 0.11
+Benzoic acid (1 mM)	1.05 $\pm$ 0.15

TABLE 6

Oxidation of N-Me- α -MeDA by microsomes and by xanthine oxidase

N-Me- α -MeDA (2 μ M) was incubated with the microsomes or xanthine oxidase for 5 min and assay was described under "Materials and Methods." Each value is the mean $\pm$ S.D. of four determinations. SOD, superoxide dismutase.

Conditions	N-Me- α -MeDA oxidized nmol/ml
Microsomes + NADPH	1.70 $\pm$ 0.08
Microsomes + SOD (50 U)	0.2 $\pm$ 0.11
Xanthine (0.5 mM)-xanthine oxidase (0.1 U)	2.00
Xanthine-xanthine oxidase + SOD (50 U)	0.52 $\pm$ 0.06

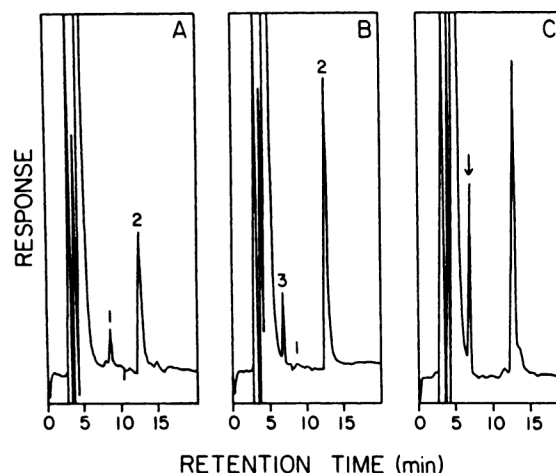


Fig. 6. HPLC tracing of the microsomal incubation mixture of MDMA and an NADPH generating system. A, in the absence of GSH; B, in the presence of GSH; C, B + authentic GSH adduct. The peaks are 1) unknown; 2) N-Me- α -MeDA; and 3) new product. MDMA (20 μ M) was incubated with GSH (1 mM) for 5 min and assayed as described under "Materials and Methods."

washed with the same volume of fresh 1.15% KCl and recentrifuged at 100,000 $\times$ *g* for 30 min. The pellets were stored at -80°C.

In vitro incubation. The standard incubation mixture contained 150 μ mol of HEPES buffer, pH 7.4, 1.0 μ mol of NADP, 11.4 μ mol of glucose-6-phosphate, 4.5 μ mol of MgCl₂ and 2.5 U of glucose-6-phosphate dehydrogenase in a volume of 0.95 ml and 0.05 ml of (+)- or (-)-MDMA or (+)- or (-)-MDA (20 μ M final concentration) and 0.5 ml of a suspension of rat liver microsomes (1-2 nmol/ml of cytochrome P-450) in a final volume of 1.5 ml. The reactions were carried out at 37°C and started by adding microsomal suspension. The reactions were stopped by adding 0.3 ml of 12% HClO₄ and immersing the incubation tubes in an ice bath. After centrifugation (13,500 $\times$ *g*, 5 min), the supernatant (10 μ l) was analyzed for catecholamine.

Catecholamine analysis by HPLC-EC. The metabolites were separated by reverse phase chromatography using Biophase ODS 5 μ m

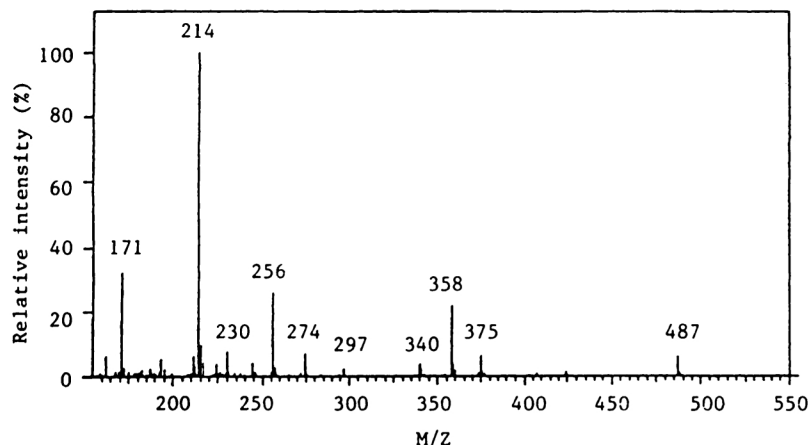


Fig. 7. HPLC-MS of GSH adduct formed from the action of tyrosinase on N-Me- α -MeDA. The product was subjected to HPLC separation and the MS of the peak at 8.4 min was determined by thermospray analysis.

(4.6 $\times$ 250 mm, Bioanalytical Systems, Inc, W. Lafayette, IN) and 0.1 M citrate buffer, pH 5.5, containing 7.5% methanol, 50 μ M EDTA and 65 μ M octyl sodium sulfate at a flow rate of 0.9 ml/min. The GSH adduct of the N-Me- α -MeDA oxidation product was monitored with a HPLC solvent mixture consisting of citrate buffer (0.1 M, pH 3.5 containing 1 mM octyl sodium sulfate), acetonitrile and methanol in a ratio of 8:8:1 at a flow rate of 0.7 ml/min. Electrochemical measurements were made using a glassy carbon working electrode (LC-4, Bioanalytical Systems, Inc.) set at +0.7 V (*vs.* Ag/AgCl reference electrode). In the analysis, the samples were injected directly on to the HPLC column without pretreatment using a refrigerated autoinjector. Samples were stable for at least 24 hr under these conditions. Signals were recorded with a Hewlett Packard 3390A recording integrator and the peak height of each compound was compared with that of standard samples. Retention times for α -MeDA and N-Me- α -MeDA were 13.0 and 15.5 min, respectively.

Data analysis. Kinetic parameters were calculated using the BMDP iterative nonlinear regression program AR (Dixon, 1988). The requirement for a two enzyme model for the substrate *vs.* concentration data was significant at F 0.99. The statistical significance was determined with Student's t test. The difference between two comparable sets of results was considered significant when $P < .05$.

Synthesis of N-Me- α -MeDA-GSH adduct. N-Me- α -MeDA-GSH adduct was synthesized according to the procedure of Ito and Prota (1977). A mixture of the N-Me- α -MeDA (2 mM), GSH (10 mM), tyrosinase (20,000 U) in a 20 ml volume of potassium-phosphate buffer (0.50 mM, pH 7.4) was incubated at 37°C. The reaction was quenched after 10 min by addition of 4 ml of 15% HClO₄. Under these conditions, most of the N-Me- α -MeDA was converted into its GSH adduct. After centrifugation, the supernatant was adjusted to neutrality with 5 M NaOH and mixed with 20 ml of 100 mM potassium-phosphate buffer, pH 7.4. The solution was poured onto Sep-Pak C₁₈ cartridges (Waters Associates, Milford, MA) which had been pretreated with 5 ml of methanol, 5 ml of water and 3 ml of 100 mM potassium-phosphate buffer, pH 7.4. The cartridges were washed with 1 ml of water and the adduct eluted with 3 ml of 1% acetic acid-methanol (1:1, v/v). The eluate was lyophilized overnight and the resulting powder was analyzed as authentic N-Me- α -MeDA-GSH adduct by means of mass spectrometry. In the FAB and thermospray LC/MS spectra the protonated molecule was seen at m/z 487, indicating a molecular weight of 486 for the monoadduct.

Mass spectra were obtained using a Finnigan TSQ-70 mass spectrometer (Finnigan MAT, San Jose, CA). FAB mass spectra were recorded from a water and glycerol matrix using 8 keV Xe fast neutrals generated from a FAB ion source (Ion Tech, Middlesex, England). Thermospray mass spectra were recorded after separation on an Adsorbosphere HS C₁₈ 5 μ m 4.6 $\times$ 250 mm column (Alltech Associates, Deerfield, IL) using 95:5 1% acetic acid-acetonitrile at a flow rate of 1.0 ml/min. The UV response at 254 nm was monitored (ABI, Ramsey, NJ) simultaneously with the mass spectrum. Source and vaporizer

temperatures of 260 and 80°C, respectively, were found to give best conditions. Daughter ion spectra were obtained by mass-selecting the protonated molecule in the first quadrupole, dissociating it at 20 eV collisions with Ar in the second quadrupole, and scanning the fragments in the third quadrupole.

Results

N-Me- α -MeDA formation from (+)- and (–)-MDMA in incubates of rat liver microsomes was proportional to the concentration of cytochrome P-450 over a range of 0.07 to 2.3 μ M (fig. 1). The apparent kinetics of the reaction were determined at different concentrations of MDMA (fig. 2) in a 2-min incubation. At this time about 5% of the substrate is consumed and the catecholamine accounts for essentially all of it. An Eadie-Hofstee plot of the data was nonlinear suggesting more than one enzyme was involved. The effect of substrate concentration on observed rate was consistent with the action of two enzymes whose kinetic parameters are shown in table 1. As the higher K_m value was in the millimolar range and not likely to be of pharmacological significance, subsequent experiments investigating the biochemical properties of the reaction were conducted at 20 μ M concentrations. This concentration was also more compatible with plasma concentrations observed after pharmacological doses (5–19 μ M after a dose of 10 mg/kg; Hiramatsu and Cho, 1989).

The time course of N-Me- α -MeDA formation (fig. 3) was unusual for a sequence of reactions involving saturating concentrations of substrate in that N-Me- α -MeDA levels reached a maximum at about 2 min then declined rapidly. This time course was noted for both enantiomers although absolute levels of catecholamines were greater from (+)-MDMA. The catechol metabolite of MDA, α -MeDA, was not detected when MDMA was incubated although it was present in separate incubations of MDA. A similar time course for α -MeDA formation was obtained when MDA (final concentration, 20 μ M) was incubated under the same incubation conditions (data not shown).

The nature of the demethylenation was characterized in incubations conducted under the conditions shown in table 2. Thus, conversion of MDMA to N-Me- α -MeDA did not occur with boiled microsomes or in the absence of an NADPH generating system. Catechol formation was inhibited by an atmosphere of CO-O₂ (9:1). The demethylenation of MDMA was inhibited by SKF 525A at low concentrations, but not by methimazole, a competitive inhibitor of the flavin containing monooxygenase. These results indicated that the conversion of MDMA to N-Me- α -MeDA is mediated by the cytochrome P-

450 monooxygenase system and is consistent with prior studies of the demethylenation of many other methylenedioxyphenyl compounds including MDA (Marquardt and DiStefano, 1974; DiStefano, 1978a). The demethylenation is not limited to cytochrome P-450 action in its normal cycle, however, and could be effected in a peroxidatic pathway in the presence of hydrogen peroxide (table 3). The reaction also occurs with horseradish peroxidase and hydrogen peroxide.

The nature of the subsequent reaction was examined with racemic N-Me- α -MeDA. At concentrations of 2 μ M, about 50% of the catechol was consumed in a 2-min incubation by a system that required intact microsomes and an NADPH generating system (table 4). Although the reaction was not sensitive to SKF 525A, it was inhibited when the air was replaced by a carbon monoxide-oxygen mixture. Ascorbic acid (1 mM) and EDTA (20 mM) also inhibited the metabolism of N-Me- α -MeDA (fig. 4) with α -MeDA as the catechol, results obtained were similar to those described above (data not shown). Ascorbate also blocked the further metabolism of N-Me- α -MeDA so that levels of the catecholamine increased progressively with time (fig. 5). Addition of ascorbate to the reaction mixture after the reaction did not reverse the oxidation, indicating that ascorbate was preventing the initial oxidation. Because these reactions appear to be oxygen-dependent, the involvement of different oxygen species in the reaction sequence was examined with MDMA and microsomes (table 5). Levels of N-Me- α -MeDA were increased almost 4-fold by the addition of superoxide dismutase, indicating that superoxide anion was involved in the second step of MDMA metabolism. Catalase, 1,4-DABCO and benzoic acid had no effect on the reaction sequence indicating that hydrogen peroxide, singlet oxygen or hydroxyl radicals were not involved (table 5). When incubated with microsomes, over 80% of N-Me- α -MeDA (2 μ M) was metabolized in 5 min (fig. 4; table 6). When incubated with xanthine and xanthine oxidase (Nelson *et al.*, 1976; Monks *et al.*, 1984) the compound was oxidized completely. Addition of superoxide dismutase prevented the reaction (table 6).

A likely product of superoxide-mediated oxidations of catechols are the corresponding *o*-quinones (Nelson *et al.*, 1978). Inasmuch as semiquinones and quinones react readily with thiol functions (Doherty and Cohen, 1984; Monks *et al.*, 1984) the possible reaction of the oxidation product with GSH was examined. As shown in figure 6, A and B, addition of GSH to the incubation mixture resulted in a new electrochemically active product from MDMA whose formation was dependent on GSH, microsomes and the NADPH generating system. Similar results were obtained when N-Me- α -MeDA was used as the substrate. Addition to thiols was not limited to GSH as other thiol containing compounds such as cysteine or N-acetylcysteine gave new HPLC peaks under equivalent conditions (data not shown). The adduct was demonstrated to coelute GSH adduct prepared from N-Me- α -MeDA and tyrosinase in two HPLC systems; one of which is shown in figure 6. The tyrosinase-GSH product was identified by analysis of its mass and NMR spectra as the GSH adduct of the *o*-quinone.

The positive FAB mass spectrum showed the protonated and sodiated molecule of the mono adduct at m/z 487 and 509, respectively, and no evidence of the bis adduct at m/z 792. The daughter spectrum of the protonated molecule showed fragments at m/z 214 and 358, ascribed to the loss of GSH with retention of the thiol group and loss of $C_8H_7NO_3$ from the glutamic acid portion, respectively. The thermospray mass

spectrum of the compound after HPLC (retention time, 8.4 min.) showed similar features (fig. 7) to the FAB mass spectrum. Although no ammoniated molecule was observed, the ammoniated fragment analogous to the m/z 358 ion was seen at m/z 375. Loss of H_2O , CO and NH_3 was responsible for the m/z 424 fragment. Additional ions not observed in the FAB MS or MS/MS spectra at m/z 274 and 256 are assigned as the addition of acetic acid and its dehydrated form to the m/z 214 base peak, likely adducts produced from thermospray ionization in 1% acetic acid. These results indicate that one equivalent of the thiol has added to the catechol or its oxidation product.

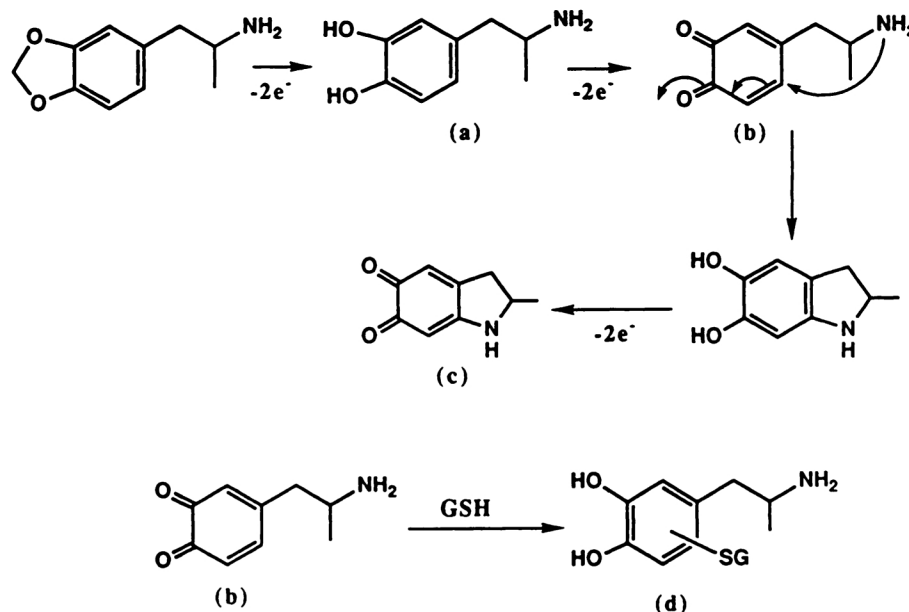
Discussion

MDMA is thought to exert its neurotoxic actions through metabolism (Schmidt and Taylor, 1988) to an as yet, unidentified compound. Because virtually all of the consumed substrate at early times is accountable by catecholamine derived product, demethylenation is a major route of its metabolism by rat liver *in vitro* (Hiramatsu *et al.*, 1989). This reaction must also occur in the brain inasmuch as this highly polar compound is found in the brain after peripheral administration of MDMA (Lim and Foltz, 1988). The catecholamine is formed rapidly and further transformed to a compound capable of reacting with GSH. Demethylenation occurs by a cytochrome P-450 pathway and the metabolism of the catechol is a superoxide-mediated reaction. Cytochrome P-450 appears to be involved in this second reaction, however, as it is reduced in the presence of carbon monoxide. These observations can be accommodated by a reaction sequence in which the cytochrome P-450 system is uncoupled to generate superoxide and is analogous to a reaction observed with the oxidation of a hydroxylamine by superoxide generated by uncoupling of cytochrome P-450 (Duncan *et al.*, 1985). These results and those reported earlier (Brady *et al.*, 1986), however, differ from those reported by Marquardt and DiStefano (1978a). These workers reported that MDA was metabolized by cytochrome P-450 to α -MeDA and stated that α -MeDA was not metabolized further. This discrepancy may be explained by the difference in assay procedure used for the catecholamine products. Marquardt and DiStefano (1974) used a flurometric procedure in which the catechol was oxidized to fluorescent product and the total fluorescence measured. This reaction was the basis of the highly popular fluorescent assay of catecholamines used before the development of HPLC/EC systems.

Catecholethylamine derivatives can be oxidized to quinones that can then ring close to fluorescent indole derivatives (Udenfriend, 1962). This product (scheme 1c) would form from both the catechol (scheme 1a) and its initial oxidation product, quinone (scheme 1b).

The assay used by Marquardt and DiStefano (1974) measured scheme 1a and scheme 1b together whereas the HPLC procedure used here readily distinguishes them. The report of Marquardt and DiStefano (1974) does suggest, however, that the subsequent reaction of the catecholamine is to the quinone amine which is capable of forming GSH adducts (Monk *et al.*, 1984) such as scheme 1d.

In related studies of the catechol amino acid, α -methyldopa, Dybing *et al.* (1976) have reported that covalent binding occurred after incubation with rat liver microsomes in the presence of an NADPH generating system. These and other experimental observations were interpreted to indicate that α -meth-



Scheme 1.

yldopa was being oxidized by cytochrome P-450 generated superoxide anion to a reactive semiquinone and/or quinone. The results of the present study are analogous in that the disappearance of catechols from the incubation required NADPH, and was inhibited by ascorbic acid and that a compound capable of forming adducts with thiols was formed from MDMA by a superoxide dismutase sensitive pathway. The formation of covalent bonds with GSH is indicative of an electrophilic center and a potential pathway for toxicological lesions, so that the oxidation products of the catecholamine could be involved in the toxicology of MDMA.

The catecholamine metabolites of MDMA and MDA are highly polar compounds and unlikely to penetrate the blood brain barrier. For the reaction sequence described above to occur therefore, the initial demethylation must take place in the brain and the report of Lim and Foltz (1988) indicates that it does. The brain has low concentrations of cytochrome P-450 that could mediate the reaction (Sasame *et al.*, 1977), but does have other heme proteins that could effect the reaction by a peroxide-mediated mechanism. The experiments with microsomes and with horseradish peroxidase using hydrogen peroxide as the oxidant indicate that alternate pathways for the initial step of quinone formation exist.

Pharmacokinetic studies of MDMA (Cho *et al.*, 1989) showed that the formation of MDA exhibited a marked enantioselectivity and levels of this metabolite were higher after dosage with (+)-MDMA. Furthermore, 5 hr after (+)-MDMA dosage, plasma levels of MDA exceed those of the parent compound. Because MDA is also a potent 5-HT neurotoxin, its participation in the neurotoxicity of MDMA must be considered.

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