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Contribution of *N*-Oxygenation to the Metabolism of MPTP (1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine) by Various Liver Preparations

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SUMMARY

Liver microsomes from uninduced mice and rats catalyze NADPH- and oxygen-dependent *N*-oxygenation of the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). The *N*-oxide is the principal product and accounts for 95–96% of the total MPTP metabolized by microsomes. Demethylation of MPTP is detectable but the rate of nor-MPTP formation was never more than 4–6% of the rate of *N*-oxygenation. Studies on the biochemical mechanisms for *N*-oxygenation of MPTP suggest that this reaction is catalyzed exclusively by the flavin-containing monooxygenase. This conclusion is based on the effects of selective cytochrome P-450 inhibitors, positive effectors, and alternate substrates for the flavin-containing monooxygenase as well as on studies with the purified hog liver enzyme. MPTP is an excellent substrate for this monooxygenase with a K_m of 30–33 μM. Limited studies with human liver whole homogenates suggest that *N*-oxygenation is also a major route for the metabolism of MPTP in man and the rate of *N*-oxide formation is approximately equal to the rate of mitochondrial monoamine oxidase-dependent MPDP$^+$ (1-methyl-4-phenyl-2,3-dihydropyridinium species) production.

MPTP, a thermal decomposition product of a "street narcotic," is a highly selective neurotoxin that is responsible for a parkinsonism-like syndrome in humans (1–3) and that, upon administration to monkeys (4, 5), leads to degeneration of the substantia nigra. MPTP appears to require metabolic activation for expression of its neurotoxic effects. The metabolism of MPTP has been examined in rat and rabbit liver mitochondrial and in rabbit liver microsomal preparations (6). The mitochondrial preparations rapidly oxidize MPTP to MPP$^+$ via the intermediate MPDP$^+$ (6). Apparently, MAO is the enzyme primarily responsible since rat brain, beef liver, and human placenta MAOs efficiently metabolize MPTP to MPDP$^+$ (7–9). Microsomal preparations supplemented with NADPH convert MPTP to the nor compound, 4-phenyl-1,2,3,6-tetrahydropyridine and MPTP *N*-oxide (6). Mouse liver homogenates also produce some lactam and pyridone metabolites arising from initial α-oxidation of MPTP (10).

The neurotoxic effects of MPTP are dependent on its biotransformation since: (a) pretreatment of animals sensitive to MPTP neurotoxicity with MAO type B inhibitors blocks nigrostriatal destruction (11, 12), (b) 2 mol of the MAO oxidation product, MPDP$^+$, can disproportionate to 1 mol of MPTP and 1 mol of MPP$^+$ (7), and (c) MPP$^+$ accumulation may be the basis for the selective toxicity of MPTP (13–15). Regardless of the mechanism, oxidative biotransformation undoubtedly plays a role in MPTP-mediated neurotoxicity. In addition to cytochrome P-450 and MAO, the flavin-containing monooxygenase (EC 1.14.13.8) must also contribute to the oxidative metabolism of this neurotoxin. MPTP is a tertiary amine similar in structure to other *N*-methylpiperidine substrates for this enzyme. Weissman *et al.* (6) have shown that MPTP is oxidized, at least in part, to MPTP *N*-oxide by liver microsomes from rabbits pretreated with phenobarbital, although the biochemical basis for the formation of the *N*-oxide was not clearly defined.

This report describes studies on the biotransformation of MPTP by liver microsomes and by the purified flavin-containing monooxygenase from hog liver. MPTP is efficiently *N*-oxygenated by this monooxygenase and, in contrast to previous reports by other workers (6, 10), MPTP *N*-oxide is demonstrated to be the major metabolite formed in reactions catalyzed by liver microsomes.

Materials and Methods

Reagents. MPTP was obtained from Aldrich Chemical Co.; its *N*-oxide (MPTP *N*-oxide) was synthesized and isolated by the method described by Weissman *et al.* (6). [^{3}H]-MPTP (10 Ci/mmole) was

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ABBREVIATIONS: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP$^+$, 1-methyl-4-phenylpyridinium ion; MAO, monoamine oxidase; MPTP *N*-oxide, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine *N*-oxide; HPLC, high pressure liquid chromatography.

purchased from New England Nuclear and diluted with MPTP to afford working stocks of 5 mCi/mmol. Pargyline as well as all compounds of the NADPH generating system were obtained from Sigma Chemical Co. Deprenyl and clorgyline were gifts of Jim Salach, Veterans Administration Hospital, (San Francisco, CA); the other MAO inhibitors were generous gifts from the companies indicated: amitriptyline, Merck Sharp and Dohme; imipramine, Geigy Pharmaceuticals.

Liver preparations. Microsomal fractions were isolated by the method described earlier (16, 17) from homogenates of liver from male Sprague-Dawley rats (200–225 g) and male C57 Black mice (10–25 g). The microsomes were washed and resuspended into 0.05 M potassium phosphate, pH 7.4, containing 1 mM ethylenediaminetetraacetate. To minimize inactivation of the flavin-containing monooxygenase, all steps were carried out as quickly as possible at 4°. The flavin-containing monooxygenase was purified from hog liver by minor modifications of the published procedure (16). Human liver biopsy samples were provided by Dr. L. Ferrell, Department of Pathology, University of California, San Francisco School of Medicine. Samples obtained from uncompromised portions of the liver were rapidly frozen on dry ice and stored at −78°. Immediately before metabolic measurements the tissue was rapidly thawed and homogenized in 0.05 M phosphate, pH 7.4.

Metabolic incubations and product analyses. The basic incubation medium contained 50 mM potassium phosphate, pH 7.4, 0.5 mM $NADP^+$, 2.0 mM glucose-6-phosphate, 1 IU of glucose-6-phosphate dehydrogenase, and 2–4 mg/ml of microsomes or whole homogenate protein. After a brief temperature equilibration at 37°, the reaction was initiated by the addition of MPTP and the incubation was continued with constant shaking to maintain adequate oxygen. At timed intervals the reaction was quenched and analyzed for products by the procedures given below.

In assays initiated by the addition of [^{3}H]MPTP, the reaction was quenched by adding an equal volume of cold methanol containing 0.2 mg of MPTP and MPTP *N*-oxide. After thorough mixing, insoluble material was separated by brief centrifugation. A 50-μl aliquot of the supernatant was applied to the loading zone of a Whatman LK5DF thin layer chromatography plate and air dried for about 5 min. The plate was developed in chloroform/methanol (9:1). The bands (R_f) corresponding to the *N*-oxide (0.16) and MPTP (0.49) were scraped into scintillation vials for counting. The conversion of MPTP to MPTP *N*-oxide was essentially quantitative and greater than 98% of the radioactivity applied to the thin layer chromatography plate was recovered.

The profile of metabolites was also determined by HPLC analysis of CH_3CN extracts of the reaction mixture essentially by the procedure described by Weissman *et al.* (6). The reaction was quenched by adding 2 volumes of cold acetonitrile, and metabolic products in the resulting extract were separated and quantitated by HPLC (Beckman model 330 employing a UV detector at 254 nm fitted with a precolumn and 5-μliter Altex ultrasphere-ODS reverse phase analytical column). The mobile phase consisted of acetonitrile:100 mM acetic acid (86:14, v/v) adjusted to pH 5.6 with triethylamine. This system efficiently separates MPTP, nor-MPTP, and MPTP *N*-oxide which have retention volumes of 8.0, 9.0 and 14.4 ml, respectively. The recovery of radioactivity as judged by HPLC was > 94%, and 96% of this material was MPTP or MPTP *N*-oxide.

The major metabolite formed in reactions catalyzed by rat liver microsomes and the purified hog liver flavin-containing monooxygenase was also subjected to mass spectral analysis. The metabolite isolated by HPLC was extracted into chloroform. The combined extracts from several runs were dried over anhydrous sodium sulfate and then taken to dryness *in vacuo*. The samples were azeotroped with toluene to remove the last traces of solvent and water, and the residue was dissolved in acetonitrile for mass spectral analysis.

Chemical ionization mass spectra were taken on a modified Kratos AEI MS 9025 at 8 kV using isobutane (about 1 torr). The sample (about 10 μg) in acetonitrile was coated on the ceramic probe tip and inserted into the instrument—initially above the source. The filament was energized, the background spectrum recorded, and the probe tip lowered into the source which was then heated at 10°/min. At a source temperature of 120°, synthetic MPTP *N*-oxide gave prominent ions at *m/z* (relative abundance) 190 (MH^+, 27), 174 (100), 172 (63), 160 (29), and 156 (17).

Other analytical methods. Kinetic constants for the oxidation of MPTP and other substrates for the purified flavin-containing monooxygenase were calculated from substrate-dependent oxygen uptake at 37°, pH 7.4, with variable substrate by the procedure described earlier (18). The basic assay medium was essentially the same as that used for microsomal and homogenate activity measurements except that phosphate was increased to 100 mM. Concentration of enzyme as well as programs used to calculate kinetic constants were identical to those listed earlier (18).

The concentration of protein was determined by the method of Lowry *et al.* (19).

Results

Preliminary studies showed that rat and mouse liver microsomes supplemented with NADPH catalyze rapid oxidation of MPTP to the *N*-oxide. Although some nor-MPTP could be detected, its rate of formation at pH 7.4 in three separate determinations did not exceed 0.07 ± 0.008 and 0.04 ± 0.005 nmol/min/mg of protein for rat and mouse liver microsomes, respectively. The rate of MPTP *N*-oxygenation was always 20–25 times faster under the same conditions. The formation of the *N*-oxide was also a linear function of protein concentration (1–4 mg/ml) and with incubation time for at least 7 min. Authentic synthetic MPTP *N*-oxide added to the complete reaction medium was recovered quantitatively after 10 min incubation. In addition, neither MPTP *N*-oxide nor $MPDP^+$ serves to inhibit the flavin-containing monooxygenase as judged by thiobenzamide *S*-oxidation (17).

n-Octylamine-stimulated microsomes catalyzed the formation of the *N*-oxide more than 2-fold (Table 1), and in the presence of this primary alkylamine the *N*-oxide was the only metabolite detected in acetonitrile extracts analyzed by HPLC. The chemical ionization mass spectra of this product gave prominent ions at *m/z* (relative abundance) 190 (MH^+, 17) 174 (100), 172 (64), and 160 (9), which is virtually identical with the spectrum of authentic MPTP *N*-oxide (cf. Materials and Methods).

These results suggest that *N*-oxygenation of MPTP is cata-

TABLE 1
Effects of inhibitors on MPTP *N*-oxide formation by microsomes from rat and mouse liver

Incubation conditions	MPTP *N*-oxide formation	
	Rat liver	Mouse liver
	nmol/min/mg protein	
Complete system[a]	4.32 ± 0.39	2.47 ± 0.19
Omit *n*-octylamine[b]	1.60 ± 0.16	1.00 ± 0.09
Omit NADPH	0.08 ± 0.02	0.0
+ Thiobenzamide (1 mM)	0.47 ± 0.06	0.06 ± 0.03
+ *N*-Methylmercaptoimidazole (2 mM)	0.56 ± 0.10	0.04 ± 0.05
+ Aminobenzotriazole (0.5 mM)[c]	3.8 ± 0.61	2.68 ± 0.13

[a] The complete system contained 50 mM phosphate, pH 7.4, the NADPH generating system, 100 μM [^{3}H]MPTP, 4.5 mM *n*-octylamine, and 1.6–2.1 mg microsomal protein in a final volume of 0.35 ml. MPTP *N*-oxide was determined by the radiometric procedure unless otherwise indicated. The results are averages of three determinations (±SD) using pooled microsomes from five mice or six rats.

[b] Incubations were performed in the absence of *n*-octylamine. Metabolites were quantitated by the HPLC procedure: desmethyl MPTP accounted for 0.07 ± 0.008 and 0.04 ± 0.005 nmol/min/mg of protein from rat and mouse liver microsomes, respectively.

[c] Inhibitor was preincubated with microsomes containing NADPH for 10 min.

lyzed largely by the flavin-containing monooxygenase since *n*-octylamine is a good inhibitor of cytochrome P-450 (20) and a known positive effector for the flavin-containing monooxygenase (21). The molecular basis for formation of the *N*-oxide was further examined by measuring the effects of various compounds listed in Table 1. Aminobenzotriazole, a potent mechanism-based inhibitor of cytochrome P-450 (22) did not significantly inhibit *N*-oxygenation of MPTP by either rat or mouse liver microsomes. This indicates that the *n*-octylamine-stimulated *N*-oxygenation was not due to some unusual cytochrome P-450 isozyme. Conversely, thiobenzamide (23) and methimazole (21), two well documented specific alternate substrates for the flavin-containing monooxygenase, markedly decreased the rate of MPTP *N*-oxygenation.

Kinetic constants for the *N*-oxygenation of MPTP catalyzed by rat liver microsomes were calculated from the rate of *N*-oxide formation at variable substrate concentrations by the radiometric procedure described under Materials and Methods. The K_m and V_{max} values obtained from double reciprocal plots of velocity versus substrate are 45 μM and 4.8 nmol/min/mg of protein, respectively.

As shown by the kinetic constants listed in Table 2, MPTP is also an excellent substrate for the purified hog liver flavin-containing monooxygenase. The concentration required for half-maximal activity is only slightly lower than that required for rat liver microsomes and compares favorably with that of the tricyclic phenothiazines and the other better amine substrates for this enzyme (21). For comparison, the kinetic constants for oxidation of several MAO inhibitors catalyzed by the purified monooxygenase are also included in Table 2. The turnover at infinite MPTP concentration is essentially the same as that for all other known substrates for the flavin-containing monooxygenase. This is consistent with earlier studies on mechanism (18) which predict that at infinite concentrations all substrates for this enzyme are oxygenated at the same velocity.

The only product detected in acetonitrile extracts of reactions catalyzed by the purified monooxygenase eluted with retention volume identical with that of authentic *N*-oxide upon separation by HPLC. The mass spectrum of this metabolite gave prominent ions at *m/z* (relative abundance) 190 (MH^+, 23), 174 (100), 172 (27), and 153 (18). This spectrum is virtually identical to that of the synthetic MPTP *N*-oxide and clearly shows that the flavin-containing monooxygenase catalyzes *N*-oxygenation of MPTP.

The metabolism of MPTP was also examined in whole homogenates of two different human liver biopsy samples supplemented with NADPH. As shown in Table 3, MPTP is metabolized in equal amounts to MPTP *N*-oxide and $MPDP^+$ and, to a lesser extent, to nor-MPTP. Limited size of the liver samples available precluded an extensive analysis of the biochemical basis for the formation of the different metabolites. However, the rate of MPTP *N*-oxidation is in the same range as that described for the *N*-oxidation of dimethylaniline by human liver homogenates (24). These studies, although limited, show that MPTP *N*-oxide is a major metabolite of this neurotoxin in reactions catalyzed by human liver.

TABLE 2
Kinetic constants for *N*-oxygenation of MPTP and other tertiary amines catalyzed by the purified flavin-containing monooxygenase
Kinetic constants were calculated from initial velocity measurements by the methods described earlier (18) at pH 7.4, 37° with variable substrate in the presence of 4.5 mM *n*-octylamine.

Substrate	K_m	V_{max}
	μM	nmol/min/mg protein
MPTP	32	730
Amitriptyline	98	740
Imipramine	22	730
Pargyline	65	750
Deprenyl	49	750
Clorgyline	2	730

TABLE 3
Metabolism of [^{3}H]MPTP by human liver homogenates
Whole liver tissue was quickly thawed, chopped, and homogenized. Protein (2.93 mg female, 6.25 mg male) was added to a temperature-equilibrated solution containing the NADPH generating system and 1 mM [^{3}H]MPTP in 50 mM phosphate, pH 7.4. Triplicate incubations were performed for 5 min, aerobically, with shaking and were quenched with cold CH_3CN. The resultant mixtures were centrifuged and decanted, and the supernatant fractions were analyzed by HPLC. The work-up and analysis of the samples (as described in Materials and Methods) were carried out as quickly as possible because of the instability of $MPDP^+$. Radiolabeled metabolites cochromatographed with authentic synthetic standards.

Sample	Metabolite		
	MPTP *N*-oxide	$MPDP^+$	nor-MPTP
	nmol/min/mg of protein		
J.C. (female)	1.7	1.9	0.11
J.W. (male)	0.5	0.4	0.06

Discussion

The kinetic constants (Table 2) demonstate that MPTP is an excellent substrate for the purified hog liver monooxygenase. The concentration of this toxin required to half-saturate the enzyme is in the micromolar range and the turnover at infinite MPTP is essentially equal to that of all other substrates for this enzyme (21). Like other tertiary amines, MPTP is converted quantitatively to the *N*-oxide. The low K_m coupled with the high concentration of the flavin-containing monooxygenase in liver tissue (25) indicates that the *N*-oxide would be a major metabolite in the liver.

This conclusion is consistent with the oxidation of MPTP catalyzed by rat and mouse liver microsomes (Table 1). In preparations from uninduced animals the *N*-oxide is formed at a rate more than 20 times that of desmethyl MPTP—the only other metabolite detected in the relatively short incubation times used in these studies. The rate of MPTP demethylation with microsomes from phenobarbital-pretreated animals is considerably faster (6, 10) than we found in the same preparations from normal mice or rats. While phenobarbital pretreatment increased microsomal cytochrome P-450 and decreases the concentration of the flavin-containing monooxygenase, the large differences in the ratio of nor-MPTP to MPTP N-oxide found in this and in previous reports (6) cannot be due only to changes in the concentration of these monooxygenases in the intact liver. Other factors are undoubtedly involved. Although they were not identified, the extreme thermal lability of the flavin-containing monooxygenase may be largely responsible for the apparent differences. This microsomal enzyme is rapidly inactivated upon incubation in the absence of NADPH and substantial portions are also frequently destroyed during the routine preparation of microsomes (21). The measurements described in this report were made under conditions that preserve maximal activity of this enzyme.

The oxidative metabolism of MPTP by human liver homog-

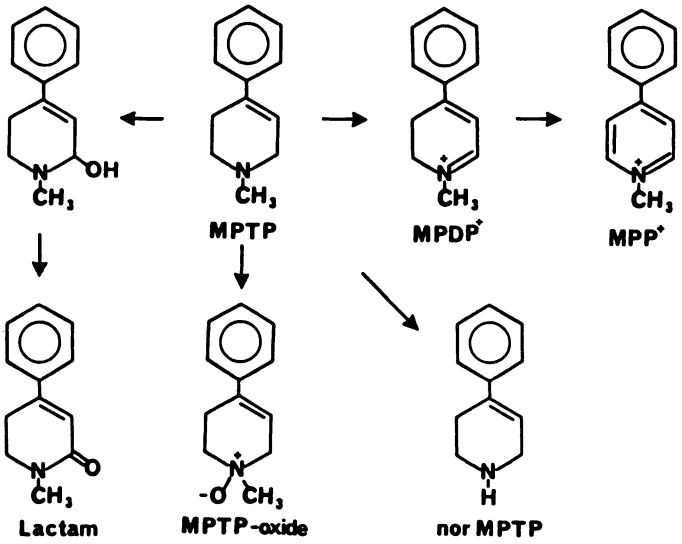

Fig. 1. Known metabolites of MPTP formed *in vitro* by liver preparations. The biochemical bases for biotransformation listed are given in the text.

enate (Table 3) yields three detectable products. MPTP *N*-oxide and $MPDP^+$ are formed at approximately equal rates but, as observed with rat and mouse liver microsomes, nor-MPTP is produced at less than 6% of the rate of MPTP *N*-oxide. These results suggest that, in the normal human, cytochrome *P*-450-dependent monooxygenases play a minor role in the metabolism of MPTP.

Known metabolites of MPTP formed *in vitro* by liver tissue are summarized in Fig. 1. The mitochondrial MAO-catalyzed oxidation of MPTP to $MPDP^+$ and MPP^+ (6–9) appears to be largely responsible for the bioactivation of this neurotoxin. However, at the present time it is not clear whether MPP^+ is formed enzymically or by disproportionation of $MPDP^+$ (7), and the nature of the molecular events leading to destruction of the substantia nigra in susceptible animals is also not fully defined.

In contrast, the metabolites produced by microsomal enzymes may represent routes for detoxication of MPTP. Of the three listed in Fig. 1 (α-hydroxy MPTP, nor-MPTP, and MPTP *N*-oxide), this report shows that in hepatic preparations from normal animals the latter is the major metabolite and *N*-oxygenation may be the major route for detoxication of MPTP. However, this interpretation is not completely consistent with the observation that MAO inhibitors prevent neurotoxicity of MPTP (11, 12). Without exception, these inhibitors are also alternate substrates for the flavin-containing monooxygenase (Table 2) and, *in vivo*, they, like other alternate substrates (i.e., methimazole, thiobenzamide; Table 1), should act as competitive inhibitors for the flavin-containing monooxygenase-catalyzed *N*-oxygenation of MPTP. To what extent MAO inhibitors decrease formation of the *N*-oxide in whole animals is not known, and further studies will be required to determine whether this is, in fact, a route for detoxication of MPTP, or whether metabolism of MPTP *N*-oxide in tissues other than the liver can produce more reactive intermediates. In agreement with our studies, Weli and Lindeke (26) have shown that pargyline is converted mainly to pargyline *N*-oxide in normal rat liver microsome preparations. By employing rat liver microsomes from phenobarbital-pretreated animals, a significant decrease in pargyline *N*-oxide formation was observed by these workers (26). Although the studies were not extensive, Weli and Lindeke (26) suggested that *N*-oxide formation was mediated by the flavin-containing monooxygenase.

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