Metabolism of the Nigrostriatal Toxin 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine by Liver Homogenate Fractions

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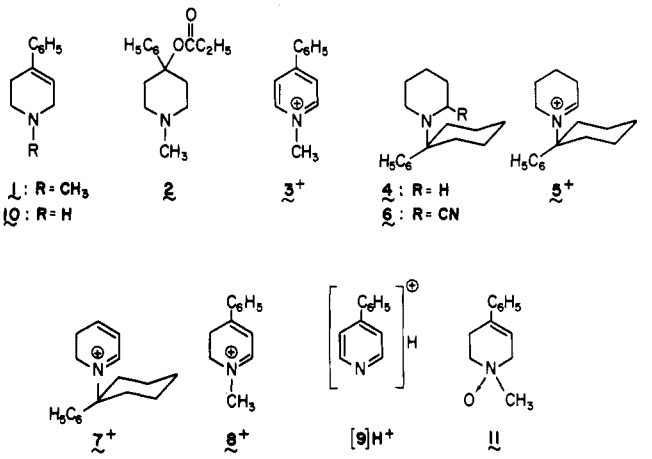
The metabolic fate of the nigrostriatal toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been examined in rat and rabbit liver mitochondrial and rabbit liver microsomal preparations. The mitochondrial preparations rapidly oxidized MPTP, in a pargyline-sensitive reaction, to a polar material that was shown to contain the 1-methyl-4-phenylpyridinium species as the principal product. NADPH-supplemented microsomal preparations converted MPTP to two principal products: 4-phenyl-1,2,3,6-tetrahydropyridine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine *N*-oxide. Carbon monoxide and SKF 525A selectively inhibited the oxidation of MPTP to the nor compound, indicating that this N-demethylation reaction is cytochrome P-450 catalyzed. Attempts to trap possible unstable iminium metabolites of MPTP in microsomal incubation mixtures with sodium cyanide led to the isolation of a monocyano adduct that proved to be the *N*-cyanomethyl derivative. Thus, hepatic mitochondrial and microsomal enzyme systems catalyze the oxidation of MPTP by different pathways, the former leading to the generation of species that may possess neurotoxic properties.

The piperideine derivative 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, 1), a thermal breakdown product of the abused narcotic analgetic agent 1-methyl-4-phenyl-4-(propionyloxy)-1,2,3,6-tetrahydropyridine (2), has been reported to cause a clinical syndrome indistinguishable from idiopathic Parkinson's disease in man[1-3] and to destroy selectively the nigrostriatal system in monkeys.[4,5] We recently presented evidence suggesting that MPTP is a substrate for brain monoamine oxidase (MAO) since brain mitochondrial preparations catalyze the oxidation of the parent drug in a pargyline-sensitive reaction to form, eventually, the 1-methyl-4-phenylpyridinium species MPP^+ (3^+).[6] MPP^+ is the same four-electron oxidation product of MPTP that had been isolated from the substantia nigrae of monkeys displaying an MPTP-induced parkinsonian-like syndrome.[7,8] Since pretreatment of monkeys[9] and mice[10] with MAO inhibitors also blocks the nigrostriatal toxicity of MPTP, this oxidative conversion becomes the first well-documented biotoxification metabolic pathway that is mediated by brain enzymes.

As part of our continuing efforts to characterize biotransformation reactions that may contribute to the toxicological properties of tertiary amines,[11-13] we have extended our metabolic studies on MPTP to liver mitochondrial and microsomal preparations. This undertaking was stimulated in part by the results we have obtained with the psychotomimetic piperidine derivative phencyclidine (4, PCP).[14,15] PCP undergoes cytochrome P-450 catalyzed α-carbon oxidation to the iminium species 5^+, which can be trapped in situ with sodium cyanide as the corresponding α-cyano amine 6. This transformation also appears to be a classical bioactivation pathway that leads to reactive intermediates that bind covalently to biomacromolecules and destroy cytochrome P-450. Preliminary evidence suggests that the iminium ion itself must undergo further oxidation in an NADPH-dependent process to generate the chemically reactive intermediate ultimately responsible for the bioalkylation reactions.[15] One possible product of such an oxidation reaction would be the dihydropyridinium species 7^+. An analogous α-carbon oxidation at the C-6 allylic carbon atom of MPTP would lead to the dihydropyridinium species 8^+. Compound 8^+ is structurally similar to the proposed PCP metabolite and is a possible intermediate in the metabolic conversion of MPTP to MPP^+ by brain mitochondrial enzymes.[6]



(1) Davis, G. C.; Williams, A. C.; Markey, S. P.; Ebert, M. H.; Caine, E. D.; Reichert, C. M.; Kopin, I. J. *Psychiat. Res.* **1979**, *1*, 249.
(2) Langston, J. W.; Ballard, P.; Tetrud, J. W.; Irwin, I. *Science* **1983**, *219*, 979.
(3) Wright, J. M.; Wall, R. A.; Perry, T. L.; Paty, D. W. *N. Engl. J. Med.* **1984**, *310*, 325.
(4) Burns, R. S.; Chieuh, C. C.; Markey, S. P.; Ebert, M. H. E.; Jacobowitz, D. M.; Kopin, I. J. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 4546.
(5) Langston, J. W.; Forno, L. S.; Rebert, C. S.; Irwin, I. *Brain Res.* **1984**, *292*, 390.
(6) Chiba, K.; Trevor, A. J.; Castagnoli, N., Jr. *Biochem. Biophys. Res. Commun.* **1984**, *120*, 574.
(7) Langston, J. W.; Irwin, I.; Langston, E. B.; Forno, L. S. *Neurosci. Lett.* **1984**, *48*, 87.
(8) Markey, S. P.; Johannessen, J. N.; Chieuh, C. C.; Burns, R. S.; Herkenhan, M. A. *Nature (London)* **1984**, *311*, 464.
(9) Langston, J. W.; Irwin, I.; Langston, E. B. *Science* **1984**, *225*, 1480.
(10) Heikkila, R. E.; Manzino, L.; Cabbat, F. S.; Duvoisin, R. C. *Nature (London)* **1984**, *311*, 467.
(11) Ho, B.; Castagnoli, N., Jr. *J. Med. Chem.* **1980**, *23*, 133.
(12) Zeigler, R.; Ho, B.; Castagnoli, N., Jr. *J. Med. Chem.* **1981**, *24*, 1133.
(13) Ward, D.; Kalir, A.; Trevor, A.; Adams, J.; Baillie, T.; Castagnoli, N., Jr. *J. Med. Chem.* **1982**, *25*, 491.
(14) Ward, D. P.; Trevor, A. J.; Adams, J. D.; Baillie, T. A.; Castagnoli, N., Jr. *Drug Metab. Dispos.* **1982**, *10*, 690.
(15) Hoag, M. K. P.; Trevor, A. J.; Asscher, Y.; Weissman, J.; Castagnoli, N., Jr. *Drug Metab. Dispos.* **1984**, *12*, 371.

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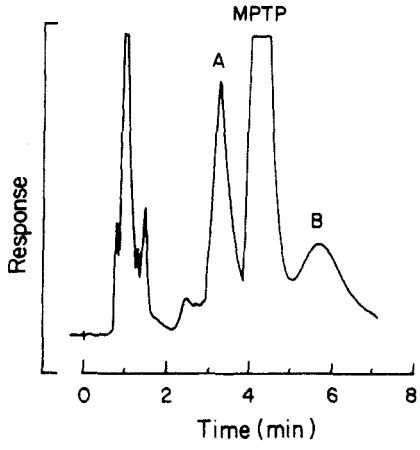


Figure 1. An HPLC tracing of an acetonitrile extract obtained from a rabbit liver microsomal incubation mixture of MPTP. The peak marked A contained the nor compound **10**; the peak marked B contained the *N*-oxide **11**.

Results and Discussion

Comparison of the HPLC tracing of rat and rabbit hepatic mitochondrial incubation mixture extracts with the corresponding tracing obtained with a rat brain mitochondrial incubation mixture[6] indicated that, as with the brain enzymes, MPTP is converted by hepatic mitochondrial enzymes to MPP^+. The direct insertion probe CI mass spectrum of the polar fraction isolated from both preparations displayed a prominent ion at m/z 156, which we have assigned to the protonated 4-phenylpyridine species $[\mathbf{9}]H^+$ and which is generated by pyrolytic N-demethylation of synthetic MPP^+I^-.[6] Comparison of the ^1H NMR spectrum of the liver mitochondrial generated product with that of synthetic MPP^+I^- confirmed the structure assignment. Although the rate of metabolism of MPTP by rat and rabbit liver mitochondrial enzymes is somewhat faster [1.31 nmol/mg of protein (rat) and 1.09 nmol/mg of protein (rabbit)] than the corresponding rate observed with rat brain mitochondrial enzymes (0.62 nmol/mg of protein), the basic features of the two processes appear to be similar. As with the brain enzyme system, the metabolism of MPTP by liver mitochondrial enzymes was completely inhibited by pargyline (10^{-6} M), an observation that is consistent with the catalytic role of MAO in this transformation.

An HPLC system modified after that developed to analyze extracts of mitochondrial incubation mixtures allowed us to monitor the metabolic rate (9.35 nmol/mg of protein) and profile (Figure 1) of MPTP in rabbit liver microsomal preparations. Two principal metabolite containing HPLC peaks (labeled A and B) were observed in the tracing. When the incubations were performed under a nitrogen atmosphere, only trace amounts of peak A were present. In the absence of NADPH, no evidence of metabolite formation could be detected. Use of a $CO–O_2$ (90:10) atmosphere led to a major reduction in the conversion of MPTP to the metabolite present in peak A and a partial reduction in the conversion to the metabolite present in peak B. Coincubation of MPTP with SKF 525A also led to the selective inhibition of the formation of the metabolite present in peak A (Table I).

Analysis of the HPLC effluent with a diode array detector established that the two metabolites displayed the same chromophore as MPTP (λ_{max} 243 nm). Preparative HPLC provided adequate quantities of the metabolites present in peaks A and B to characterize their structures as 4-phenyl-1,2,3,6-tetrahydropyridine (**10**) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine *N*-oxide (**11**),

Table I. Formation of Metabolites Corresponding to HPLC Peaks A and B by Rabbit Liver Microsomal Preparations under Varying Incubation Conditions

	metabolite formation	
conditions[a]	nmol of **10** mL^{-1} $(2\ h)^{-1}$ (%)	nmol of **11** mL^{-1} $(2\ h)^{-1}$ (%)
+ NADPH	130 (26)	190 (38)
− NADPH	0 (0)	0 (0)
N_2	16 (3)	0 (0)
$CO–O_2$ (90:10)	53 (11)	160 (33)
SKF525A	70 (15)	170 (34)

[a] See Experimental Section for detailed description of incubation conditions.

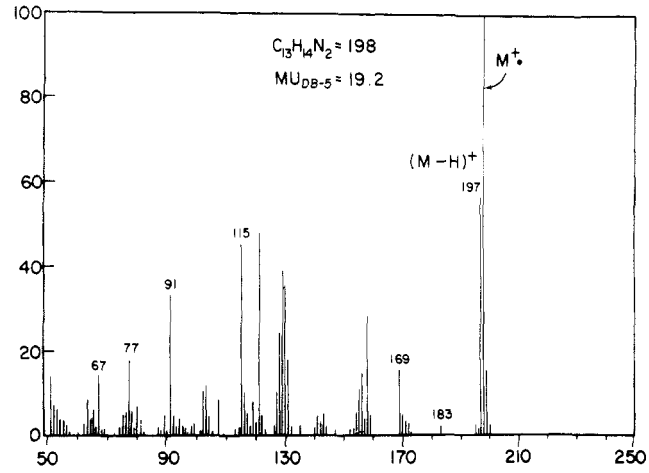


Figure 2. The GC–EIMS spectrum of the monocyano adduct isolated from a rabbit liver microsomal incubation mixture of MPTP and sodium cyanide.

respectively. The HPLC retention times as well as the CI and, for compound **10**, the GC–EI mass spectral characteristics of the metabolites were essentially identical with those of commercially available **10** and a synthetic standard of **11** that had been obtained by treatment of MPTP with hydrogen peroxide. ^1H NMR assignments of the spectrum for the *N*-oxide metabolite were confirmed by addition of an authentic standard that led to the enhancement of all assigned signals. Since the formation of the nor compound **10** was selectively inhibited by CO and SKF 525A, we assume that this biotransformation pathway is cytochrome P-450 catalyzed. The marginal effect of these agents on the formation of **11** suggests that N-oxidation of MPTP may be catalyzed, at least in part, by the NADPH-dependent amine oxidase first reported by Poulsen and Ziegler.[16]

In an attempt to trap possible iminium metabolites, we examined the metabolism of MPTP by microsomal preparations in the presence of sodium cyanide. We observed the NADPH-dependent formation of an organic soluble product that was radiolabeled with $Na^{14}CN$. This material was collected and analyzed by direct insertion probe CI, EI, and GC–EI mass spectrometry. All of the resulting mass spectral data confirmed that this product was a monocyano adduct of MPTP. The GC–EI mass spectrum (Figure 2) displayed a parent ion and fragment ions consistent with 6-cyano-1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (**12**) or 4-cyano-1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (**13**), the two possible cyano adducts that would result from the 1,2- and 1,4-addition of cyanide ion, respectively, to the dihydropyridinium intermediate 8^+. The presence of a fragment ion at m/z 183 $((M-15)^+)$,

(16) Poulsen, L. L.; Ziegler, D. M. *J. Biol. Chem.* **1979**, *254*, 6449.

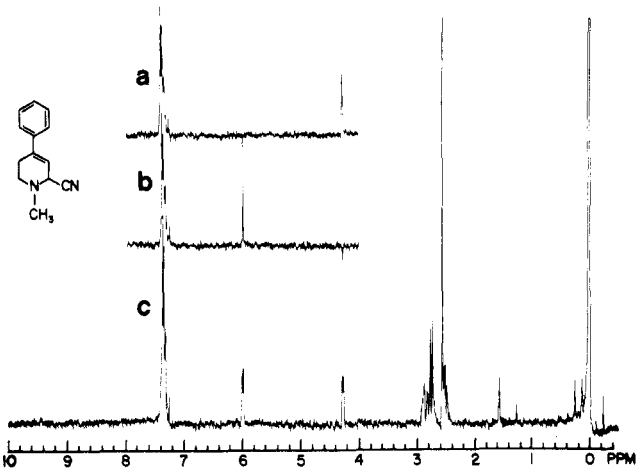
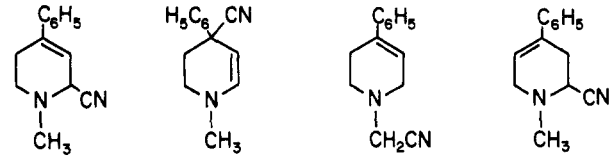


Figure 3. The ^1H NMR spectrum of the synthetic 6-cyano derivative of MPTP taken at 240 MHz. The two scans shown above the main spectrum display the results of the decoupling experiments.

assumed to be due to loss of a CH_3 fragment from the molecular ion, led us to rule out the *N*-cyanomethyl isomer **14** as a possible structure of this product.



In order to characterize further the structure of this cyano adduct, we undertook the synthesis of **12** (and/or **13**) by a pathway modified after that reported by Portoghese et al.[17] Thus, MPTP was converted first to the corresponding *N*-oxide **11**, which was obtained as its crystalline monohydrate. Treatment of **11** with trifluoroacetic anhydride in dichloromethane led to the formation of an intermediate species that was observed by UV spectroscopy (λ_{max} 359 nm) and that was assumed to be the dihydropyridinium species **8+**. The reaction of this intermediate at pH 4 with potassium cyanide led to the formation of a cyano adduct that was obtained in crystalline form and that had the same melting point as that reported for **12**.[17] The ^1H NMR spectrum of this product (Figure 3) confirmed this structure assignment. In particular, the spectrum displayed one olefinic proton signal at δ 6.0 and a second low field signal at δ 4.22, which can be assigned to the C-6 methine proton. The absence of two olefinic proton signals ruled out the 4-cyano isomer **13**. Furthermore, a homonuclear decoupling experiment established that the two low-field protons are coupled. This observation eliminated another possible isomeric structure, 2-cyano-1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (**15**), an important conclusion because of the recent finding by Bonin et al. that 6-cyano compounds similar to **12** tend to undergo double-bond rearrangement to the corresponding 2-cyano isomers.[18]

The direct insertion probe CI mass spectrum of synthetic **12** was similar to, but not identical with, that of the metabolically derived adduct. Its GC–EI mass spectral behavior, however, was completely different. The total ion

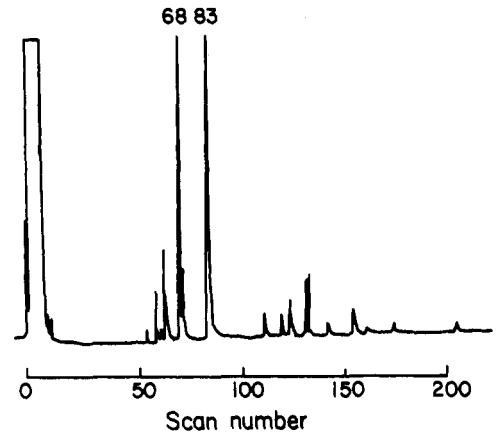


Figure 4. The total ion chromatogram of synthetic 6-cyano-1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

current chromatogram of **12** (Figure 4) established that this α-cyano amine undergoes thermal decomposition to a variety of products. Scans of the two principal products (scans 68 and 83) were found to be identical with the corresponding GC–EI mass spectra of 4-phenylpyridine (**9**) and MPTP, respectively. Compound **9** presumably arises from pyrolytic- N-demethylation of the pyridinium species MPP^+ (3^+). The conversion of **12** to MPTP and 3^+ represents a formal disproportionation reaction, a reaction that recently was observed for a 2,3-dihydropyridinium system.[19] It seems reasonable to suggest, therefore, that the present reaction proceeds via initial loss of cyanide to form the reactive dihydropyridinium species 8^+, which presumably participates in the redox chemistry.

Although the 4-cyano isomer **13** remained a possible candidate, a more likely structure for the unknown cyano product was the *N*-cyanomethyl isomer **14**. This compound was readily synthesized by reaction of 4-phenyl-1,2,3,6-tetrahydropyridine (**10**) with formaldehyde and sodium cyanide. The GC–EI mass spectral characteristics of synthetic **14** were identical with the metabolism derived product (including the previously misassigned fragment ion at *m/z* 183). Thus, the cytochrome P-450 catalyzed carbon oxidation of MPTP occurs primarily (if not exclusively) at the *N*-methyl substituent. This is somewhat surprising in view of the many well-documented cases of ring α-carbon oxidation of *N*-alkyl-substituted pyrrolidine and piperidine compounds.[20]

These results have shown that the in vitro oxidative metabolism of MPTP is mediated by enzymes present in both mitochondrial and microsomal fractions of liver tissues. As with rodent brain mitochondrial preparations,[6] the incubation of MPTP with liver mitochondrial preparations results in its four-electron oxidation to the pyridinium species 3^+, the formation of which presumably proceeds via the dihydropyridinium intermediate 8^+. This biotransformation is sensitive to pargyline and thus appears to be catalyzed by MAO. Since the in vivo[9,10] and in vitro[21] neurotoxicity of MPTP also is blocked by MAO inhibitors, the metabolism of MPTP by mitochondrial enzymes is a classical bioactivation pathway leading to reactive intermediates, the exact nature of which remains to be characterized. In contrast, liver microsomal enzymes catalyze the N-demethylation and N-oxidation of MPTP.

(17) Grouts, W. C.; Essawi, M.; Portoghese, P. S. *Synth. Commun.* **1980**, *10*, 495.
(18) Bonin, M.; Romesco, J. R.; Grierson, D. S.; Havsson, H. P. *J. Org. Chem.* **1984**, *49*, 2392.
(19) Sundberg, R. J.; Grierson, D. S.; Nusson, H. P. *J. Org. Chem.* **1984**, *49*, 2400.
(20) Low, L. K.; Castagnoli, N., Jr. In "The Basis of Medicinal Chemistry", 4th ed., Wolff, M. E.; Wiley: New York, 1980; Part 1, p 157.
(21) Mytilineou, C.; Cohen, G. *Science* **1984**, *225*, 529.

The N-demethylation of MPTP appears to be cytochrome P-450 catalyzed and may be classified as a detoxification process since the resulting secondary amine **10** is devoid of neurotoxic effects.[22] The more detailed analysis of the metabolism of MPTP by mitochondrial enzymes and the characterization of the enzymatic pathway and biological importance of the *N*-oxide **11** are presently under study.

Experimental Section

All synthetic reactions were carried out under a nitrogen atmosphere. Proton NMR spectra were recorded on a custom-built 240-MHz FT instrument and were recorded in $CDCl_3$. Chemical shifts are reported in parts per million (ppm) relative to Me_4Si as an internal standard; s = singlet, d = doublet, t = triplet, m = multiplet, br = broad. Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Low-resolution CI mass spectra were obtained on a modified AEI MS-902 instrument using isobutane (0.7 torr) as reagent gas. Low-resolution probe and GC–EI mass spectra were obtained on a VG 70-70H mass spectrometer equipped with a VG Model 2035 data system or a Finnegan TSQ Model 45 B equipped with a 30-m DB 5 capillary column. HPLC separations were performed on a Beckman Model 330 instrument employing a precolumn (4.6 mm × 5 cm) and analytical column (4.6 mm × 25 cm) packed with 5-μm Altex Ultrasphere-ODS reverse-phase packing material and an HP 3390A integrator. In general, the effluent was monitored at 254 nm. Electronic absorption spectra (205–600 nm) of the HPLC effluent were obtained with the aid of an on-line HP Model 1040A diode array detector. Microanalyses were performed by the microanalytical laboratory, University of California, Berkeley, CA.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine *N*-Oxide (11). A solution of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (**1**; 1.75 g, 10 mmol) and 2 mL of 30% H_2O_2 in dichloromethane (5 mL) and ethanol (5 mL) was heated under reflux at 60 °C for 20 h at which time 10% Pd/C was added. The resulting mixture was stirred an additional 3 h at 60 °C and then was filtered through Celite. Azeotropic distillation in vacuo three times with 4 volumes of anhydrous ethanol and three times with 4 volumes of anhydrous benzene gave a light-yellow solid mass, which upon crystallization from benzene yielded 0.84 g (4.6 mmol, 46%) of pure **11** as its monohydrate: mp 110.0 °C; UV (CH_3OH) λ_{max} 244 nm (ε 13 500); CIMS 190 (MH^+, 20), 174 (100), 172 (67), 156 (22); ^1H NMR ($CDCl_3$) δ 2.45 [m, 2 H, C(3)-H_2], 3.1 [t, 2 H, C(2)-H_2], 3.5 (q, 2 H, C(6)-H_2], 6.1 [m, 1 H, C(5)-H], 7.3 (m, 5 H, Ar H). Anal. ($C_{12}H_{15}NO$-H_2O) C, H, N.

6-Cyano-1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (12). To a solution of the above *N*-oxide (0.57 g, 3 mmol) in dichloromethane (32 mL) maintained at 0 °C was added trifluoroacetic anhydride (750 μL, 5 mmol). The disappearance of starting material was followed by silica gel TLC [CH_2Cl_2–CH_3OH (4:1)]. After stirring for 1 h, the reaction mixture was allowed to warm to room temperature and 15 min later was concentrated on a rotary evaporator in vacuo to yield a reddish oil [UV (CH_2Cl_2) λ_{max} 359 nm]. To the residue in 24 mL of dichloromethane was added an aqueous solution of potassium cyanide (0.32 g, 5.2 mmol in 16 mL of water). The reaction mixture was stirred vigorously and the pH was adjusted quickly to 4. After an additional 15 min, 10% aqueous sodium carbonate was added to adjust the pH to 10 and the organic layer was separated. The remaining aqueous phase was extracted with dichloromethane (3 × 10 mL), and the combined organic extracts were washed with sodium chloride saturated water, dried over anhydrous sodium sulfate, and filtered through a short silica gel bed to remove a polar impurity. Recrystallization from ethanol–water yielded 0.4 g (2.0 mmol, 67%) of pure product: mp 66 °C (lit.[18] mp 66 °C); ^1H NMR ($CDCl_3$) δ 2.55 (s, 3 H, CH_3), 2.5–2.9 [m, 4 H, C(2)-H_2 and C(3)-H_2], 4.22 [m, 1 H, C(6)-H], 6.0 [d, 1 H, C(5)-H)], 7.2 (m, 5 H, Ar H); UV (CH_3OH) 245 nm (ε 11 900); probe EIMS 198 (M^+, 8), 171 (30), 170 (100), 155 (10), 154 (9), 128 (10), 127 (9), 115 (9); CIMS 199 (MH^+, 20), 198 (22), 172 (100), 171 (18), 170 (16). Anal. ($C_{13}H_{14}N_2$) C, H, N. Attempted analysis of this product by GC–EIMS led to extensive pyrolytic decomposition (see Figure 4) accompanied by the formation of two major products, the first of which provided a spectrum identical with that of 4-phenylpyridine [155 (M^+, 100), 154 (25)] and the second of which provided a spectrum identical with that of MPTP [173 (M^+, 100), 172 (75), 144 (20), 129 (50), 115 (50), 96 (75)].

1-(Cyanomethyl)-4-phenyl-1,2,3,6-tetrahydropyridine (14). A solution of 4-phenyl-1,2,3,6-tetrahydropyridine (1.60 g, 10 mmol) in 10 mL of methanol was treated with a mixture of sodium cyanide (1.5 g, 30 mmol) and formaldehyde (1.5 g, 50 mmol) in 15 mL of 1 M sodium acetate buffer (pH 5.6) at room temperature with vigorous stirring for 1 h. After addition of an additional 25 mL of water, the pH was adjusted to 10 with potassium carbonate and the resulting mixture was extracted with dichloromethane (3 × 15 mL). The combined organic layers were washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness on a rotary evaporator. The residue was recrystallized from ethanol–water to yield 1.56 g (7.8 mmol, 78%) of pure product: mp 92 °C; ^1H NMR ($CDCl_3$) δ 2.6 [m, 2 H, C(3)-H_2], 2.8 [m, 2 H, C(2)-H_2], 3.3 [m, 2 H, C(6)-H_2], 3.65 (s, 2 H, CH_2CN), 6.0 [m, 1 H, C(5)-H], 7.3 (m, 5 H, Ar H); probe EIMS 198 (M^+, 100) 197 (56), 183 (3), 169 (16), 158 (30), 129 (41), 121 (47), 115 (45), 91 (34); CIMS 199 (MH^+, 22), 198 (20), 172 (100), 156 (17); GC–EIMS (see Figure 2). Anal. ($C_{13}H_{14}N_2$) C, H, N.

Preparation of Liver Fractions. New Zealand white male rabbits and Wister male rats (150–200 g) were used for the preparation of liver fractions. For microsomal studies using phenobarbital-treated animals, rabbits received 0.1% (w/v) sodium phenobarbital, which was administered in the drinking water (pH adjusted to 6.9) for 6 days prior to sacrifice. All animals were sacrificed by CO_2 asphyxiation. All buffers were purged with nitrogen. The livers were first perfused in situ with 250 mL of an ice-cold solution of 0.25 M sucrose and 0.05 M Tris hydrochloride buffer (pH 7.4) and then were homogenized in 3 volumes of the same buffer. The homogenate was centrifuged at 700*g* for 10 min and the resulting supernatant was centrifuged at 10000*g* for 20 min. The pellet was resuspended in 3 volumes of buffer and resedimented at 10000*g* for 20 min. This crude mitochondrial fraction (P2) was stored at −70 °C in 0.1 M potassium phosphate buffer, pH 7.4, at a final concentration of 10–20 mg of protein/mL. The 10000*g* supernatant fraction was centrifuged at 100000*g* for 75 min. The resulting pellet in a solution of 0.15 M KCl and 0.02 M potassium phosphate buffer (pH 7.4) was recentrifuged at 100000*g* for 75 min. This microsomal pellet (P3) was stored in the above buffer at −70 °C at a final concentration of 40–60 mg of protein/mL. Protein concentrations were measured by the method of Lowry et al.[23]

Metabolic Incubations. For liver mitochondrial incubation studies, MPTP (0.1 mM) was incubated with P2 fractions (2 mg of protein/mL) in 1 mL of 100 mM potassium phosphate buffer (pH 7.4) at 37 °C for 30 min. Incubations were terminated by the addition of 2 volumes of ice-cold acetonitrile. The resulting mixture were centrifuged to remove protein and the supernatant fractions subjected to HPLC. MAO inhibition studies were carried out with P2 fractions that had been preincubated in phosphate buffer for 5 min with 10^{-6} M pargyline prior to the addition of MPTP. The microsomal incubation studies were conducted with the P3 fractions (2–3 mg of protein/mL) in 100 mM Hepes buffer (pH 7.6) containing an NADPH-generating system (0.5 mM $NADP^+$, 8 mM glucose 6-phosphate, 1 unit/mL glucose-6-phosphate dehydrogenase, 4 mM magnesium chloride) and 0.5–1.0 mM MPTP. Rate studies were conducted for 30 min with 1-mL incubation mixtures consisting of 0.25 mg of microsomal protein/mL and 0.1 mM MPTP. In most experiments, incubations were carried out in air at 37 °C for time periods of 1–2 h. In some cases, buffers were saturated with either CO–O_2 (90:10) or 100% N_2 and the incubations were carried out in sealed vials under these gases. In cyanide trapping experiments, sodium cyanide (0.5 mM) or ^{14}C-labeled sodium cyanide (1 mCi/mmol) was added to the incubation mixtures. Experiments with SKF 525A involved preincubation of the P3 fractions with the inhibitor (5×10^{-4} M) for 5 min prior to the addition of MPTP. Microsomal incubations

(22) Heikkila, R. E.; Hess, A.; Duvoisin, R. C. *Science* **1984**, *224*, 1451.

(23) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.

were terminated by the addition of 2 volumes of ice-cold acetonitrile followed by centrifugation. Preparative-scale incubations were run for 2 h with 1 mM MPTP and mitochondrial or microsomal incubations preparations containing 3–5 mg of protein/mL and were terminated by the addition of 2 volumes of cold acetonitrile.

HPLC Separations. The acetonitrile fractions from the mitochondrial incubation experiments were chromatographed with acetonitrile/100 mM acetic acid (85:15, v/v) treated with triethylamine to pH 5.6. Metabolite isolation was accomplished as described previously.[6] The aqueous acetonitrile phases obtained in the microsomal incubation experiments were chromatographed with acetonitrile/100 mM acetic acid (65:35, v/v) treated with triethylamine to pH 5.6. Isolation of the microsomal metabolites was achieved by 35 400-μL injections. The effluents contained in the 3–4 and 5–6-min windows were collected, and the solvent was removed under vacuum followed by a stream of nitrogen at 40 °C. The CI mass spectrum of the fraction isolated from the mitochondrial incubation mixture was obtained at a source temperature of 130 °C and displayed a single intense ion at m/z 156. The ^{1}H NMR spectrum displayed all of the signals characteristic of the 1-methyl-4-phenylpyridinium species.[6] The two principal fractions separated by HPLC of the microsomal incubation mixture extract (see Figure 1) also were analyzed by mass spectrometry. Fraction A (4-phenyl-1,2,3,6-tetrahydropyridine, **10**) displayed a strong CIMS ion at m/z 160 (MH^+) and the following GC–EI mass spectrum: 159 (M^+, 100), 158 (70), 156 (98), 130 (75), 129 (68), 128 (68), 115 (80), 102 (13), 91 (19), which was essentially identical with the corresponding spectrum of authentic **10**. Fraction B (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine *N*-oxide, **11**) displayed a CI mass spectrum similar to that of the synthetic *N*-oxide and the following ^{1}H NMR signals: δ 3.55 (s, NCH_3), 6.0 [br s, C(5)-H], 7.4 (m, Ar H). All signals were enhanced by the addition of authentic **11**. Biological contaminants prevented the unambiguous assignments of the remaining signals. The CI, EI, and GC–EI mass spectra of the cyano adduct isolated from microsomal incubation mixtures of MPTP and sodium cyanide were identical with the corresponding spectra reported above for synthetic 1-(cyanomethyl)-4-phenyl-1,2,3,6-tetrahydropyridine (**14**).

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Psoralenamines. 3.[1] Synthesis, Pharmacological Behavior, and DNA Binding of 5-(Aminomethyl)-8-methoxy-, 5-[[(3-Aminopropyl)oxy]methyl]-, and 8-[(3-Aminopropyl)oxy]psoralen Derivatives

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A series of derivatives of 5-(aminomethyl)-8-methoxypsoralens, 8-[(3-aminopropyl)oxy]psoralens, and 5-[[[3-(trimethylammonio)propyl]oxy]methyl]-8-methoxypsoralen has been synthesized and their potential as PUVA reagents examined. While the DNA association constants of selected psoralens were found to be 10^5–10^6 L mol^{-1}, corresponding to efficient binding, flow linear dichroism studies indicated that only the 8-substituted psoralens bind to DNA by intercalation. Furthermore, the ability to photoinduce interstrand cross-links in calf thymus DNA, in vitro, was as efficient as that of 8-methoxypsoralen for the 8-substituted psoralens, which were up to 25 times as efficient as the 5-substituted psoralens. Four of the psoralens studied were radiolabeled and used to study photobinding to DNA. Analogously to the cross-binding results, the 8-substituted psoralens were more efficiently photobound than the 5-substituted, while only slight differences were found in the photobinding–cross-linking ratio. The photoreactivity of the aminopsoralens toward cyclohexene and 2′-deoxythymidine was enhanced compared to that of 8-methoxypsoralen, the effect being most pronounced when the amino group is close to the furocoumarin ring system. Most of the new compounds were less photocytotoxic than 8-methoxypsoralen to NHIK 3025 cells, in vitro, and they caused less light-induced DNA interstrand cross-linking, in situ, in these cells. A clear correlation between the photocytotoxicity and the DNA cross-linking ability of the psoralens was observed. Several of the derivatives showed more pronounced effects in the light-dependent skin thickening (inflammatory) test on mice than 8-methoxypsoralen. No correlation between DNA cross-linking capacity, in vitro, and skin phototoxicity was found for this series of psoralens.

Psoralen derivatives are currently used as photochemotherapeutical drugs in the PUVA (psoralen-ultraviolet A) therapy of dermatological disorders like psoriasis, vitiligo, and alopecia, as well as mycosis fungoides (a malignant cutaneous lymphoma).[3-6] Such compounds are also useful as tools for studying the structure of nucleic acids in molecular biology.[7-9]

The pharmacological mechanism of action of these drugs has not yet been established. However, psoralens photoreact with nucleic acids, in particular DNA, forming both mono- and diadducts (interstrand cross-links). These

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(2) Hansen, J. B.; Buchardt, O. *Tetrahedron Lett.* **1981**, *22*, 1847.
(3) Scott, B. R.; Pathak, M. A.; Mohn, G. R. *Mutat. Res.* **1976**, *39*, 29.
(4) Anderson, T. F.; Voorhees, J. J. *Annu. Rev. Pharmacol. Toxicol.* **1980**, *20*, 235.
(5) Parsons, B. J. *Photochem. Photobiol.* **1980**, *32*, 813.
(6) Cahn, J.; Forlot, P.; Grupper, C.; Meybeck, A.; Urbach, F., eds. "Psoralens in Cosmetics and Dermatology"; Pergamon Press: New York, 1981.

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