Title

Biotransformation and neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its two-electron oxidation product, the1-methyl-4-phenyl 1-2,3,-dihydropyridinium (MPDP+) species

Permalink

https://escholarship.org/uc/item/9533g8ct

Author

Wu, Ellen Yung-hua

Publication Date

1989

Peer reviewed|Thesis/dissertation

Biotransformation and Neurotoxicity of 1-Methyl-4-phenyl-
1,2,3,6-tetrahydropyridine (MPTP) and its Two-electron
Oxidation Product, the 1-Methyl-4-phenyl-2,3-dihydro-
pyridinium (MPDP+) Species

by

Ellen Yung-hua Wu

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of
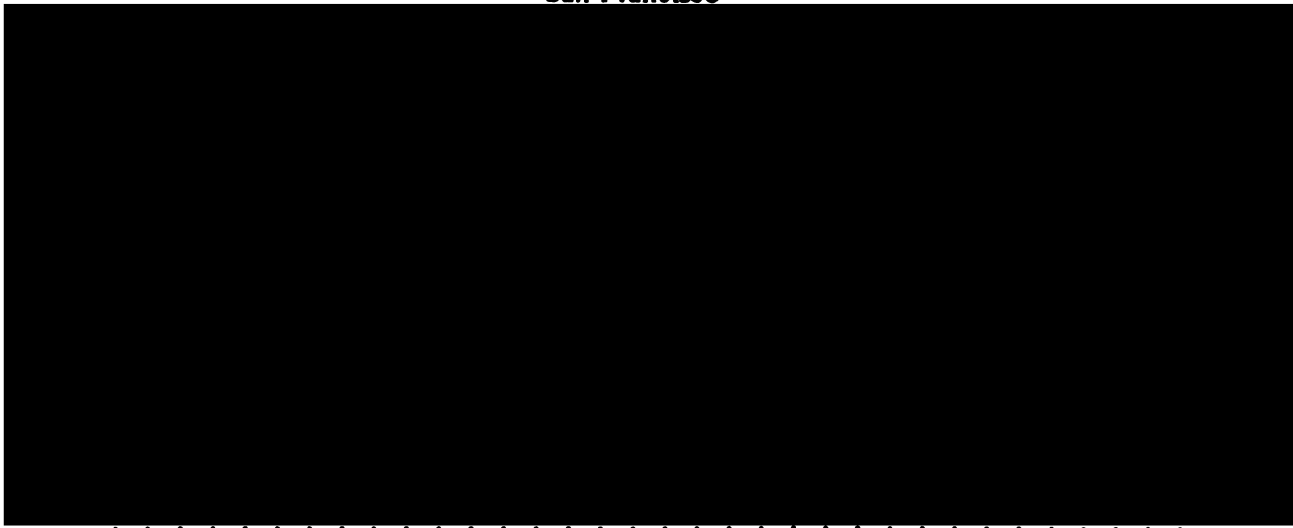
DOCTOR OF PHILOSOPHY

in

Pharmacology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

. . . .
Date   **University Librarian**

JAN 2 1987

Degree Conferred: . . . .

To my parents, Sinmin and Betty,
for their love, encouragement, and
unending faith in me.

ACKNOWLEDGMENTS

Biotransformation and Neurotoxicity of 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its Two-electron Oxidation Product, the 1-Methyl-4-phenyl-2,3-dihydropyridinium (MPDP+) Species

Ellen Y. Wu

ABSTRACT

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) specifically destroys the nigrostriatal tract in humans and certain laboratory animals; the neurotoxin depletes striatal dopamine and produces a parkinsonian syndrome. The neurotoxic action of MPTP is dependent upon its monoamine oxidase B (MAO B) catalyzed biotransformation to the 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP+) species, which undergoes further transformation to the 1-methyl-4-phenylpyridinium (MPP+) species. The mechanism of cellular toxicity induced by the metabolites, however, has not been elucidated. MPP+, though much studied, is quite stable and has not been shown to be further transformed. MPDP+, on the other hand, is a potentially reactive intermediate and its role in MPTP neurotoxicity is not well-understood. In this dissertation, the potential contribution of MPDP+ to the mechanism of MPTP neurotoxicity was examined by the investigation of the *in vitro* and *in vivo* metabolic behavior of MPTP and MPDP+, and the possible role of factors other than MAO in determining the fate of these species.

Neuromelanin, the black pigment found in the brains of species susceptible to MPTP neurotoxicity, was found to enhance the rate of oxidation of MPDP+ to MPP+. Melanin may also act as a reservoir to "trap" MPP+ and prolong its exposure to susceptible brain neurons. Diethyldithiocarbamate (DDC), a compound previously reported to increase the neurotoxic effect of MPTP in mice, was found to potentiate the formation of MPDP+ from the MAO B catalyzed biotransformation of MPTP. Brain dopamine levels were found to be

significantly lower in animals which had been pretreated with DDC before MPTP administration.

Access of the toxic metabolites to the susceptible dopaminergic neurons is expected to be critical for MPTP-induced neurotoxicity. Therefore, the ability of the first metabolite, MPDP+, to act as a substrate for the dopamine uptake system was assessed. Since MPDP+ itself is unstable, the stable 3,4-dihydro-2-methyl-9-H-indeno[2,1-C]pyridinium (DMIP+) species was used. DMIP+, however, was found to be a poor inhibitor of [^{3}H] DA and of [^{3}H] MPP+ uptake.

Incubations of MPTP with mouse brain preparations resulted in the expected MAO B catalyzed formation of MPDP+ and MPP+. NADPH-supplemented mouse liver microsomes converted MPTP to MPTP N-oxide and 4-phenyl-1,2,3,6-tetrahydropyridine (PTP), products of the flavin monooxygenase and the cytochrome P-450 systems, respectively. At high initial concentrations of MPTP, NADPH-dependent pargyline-insensitive mouse liver microsomal enzymes also catalyzed the oxidation of MPTP to MPDP+. Liver aldehyde oxidase catalyzed the oxidation of MPDP+ to the corresponding lactam, the 1-methyl-4-phenyl-5,6-dihydro-2-pyridone, which in turn may be oxidized to the 1-methyl-4-phenyl-2-pyridone by a NADPH-dependent microsomal enzyme system. In the isolated perfused rat liver MPTP is converted to MPDP+, MPP+, the N-oxide and PTP, and MPDP+ to MPP+. Results from the *in vivo* metabolism of MPTP suggest that the relatively longer retention of MPDP+ and MPP+ in the brain compared to the liver may explain why the brain is more susceptible to the neurotoxic action of MPTP.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES AND SCHEMES

Scheme   Page

1

2

3

4

5

6

7

8

9

10

11

12

13

14

15

16

17

18

19

20

21

22

23

xv

24

25

26

27

28

29

30

31

xvi

INTRODUCTION

Parkinson's Disease

Parkinson's disease affects one in a thousand of the general population and one in two hundred of those over the age of 50 years. Most of the major clinical features of Parkinson's disease were described by James Parkinson in 1817. The classic clinical triad includes resting tremor, bradykinesia, and rigidity. There is gradual loss of motor functions involving gait, speed of movement, and coordination. A fundamental biological characteristic of this progressive neurodegenerative disorder is a damaged nigrostriatal dopamine system, where the loss of the pigmented cell bodies in the pars compacta of the substantia nigra with axons ascending to terminate in the corpus striatum (Ungerstedt, 1971) is accompanied by a large decrease in the striatal dopamine content (Hornykiewicz, 1966; Marsden, 1982). The noradrenergic neurons in the locus coeruleus are also lesioned. Although this degeneration is known to be central to the motor abnormalities associated with Parkinson's disease, its etiology remains unclear. The number of nigrostriatal neurons steadily decline with age at the rate of about 10 percent per decade (Riederer and Woketich, 1976). Clinical symptoms of parkinsonism begin to appear when the striatal dopamine levels decline to about 20 percent of normal. The primary role of the loss of dopamine-containing neurons in Parkinson's disease is demonstrated by the success of a wide variety of synthetic dopamine agonists to reverse the motor deficits. However, despite advances in the treatment of Parkinson's disease, there are still problems. For instance, after some years of drug treatment, the effectiveness of α-amino acid L-dopa (**1**), the chemical precursor of dopamine (DA, **2**), often starts to wane and many patients experience a variety of other side effects including the appearance of dyskinesias after each dose and abrupt swings in mobility (Marsden *et al.*, 1982).
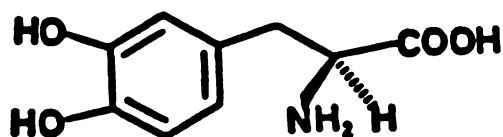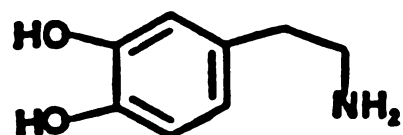
1   **2**

At the present time Parkinson's disease is an affliction whose pathology and biochemistry are well-understood, but whose cause is unknown. In addition, while the treatment of the disease provides substantial benefit at first, the reasons for the loss of drug effect and for the complications accompanying long-term therapy remain uncertain.

Discovery of MPTP

A recent series of chance occurrences has provided the potential for a considerable advance in our understanding of Parkinson's disease. In the quest to synthesize 1-methyl-4-phenylpropionoxy-piperidine (MPPP, **4**), a potent analog of the narcotic pethidine (meperidine, **3**), "designer drug" chemists inadvertantly formed a by-product of the synthesis. At low pH or at elevated temperature, MPPP readily forms 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, **5**). The first reported case by Davis *et al.* (1979) of a young male graduate student who developed persistent parkinsonism following self-administration of the product from an attempt to synthesize MPPP was

2

overlooked until 1983. In that year Langston *et al.* (1983) reported four further cases of young drug addicts developing a parkinsonian state which responded to L-dopa therapy. Again, these subjects were attempting to self-administer MPPP. The material injected by the addicts, however, was found to be contaminated with MPTP.

3

4

5
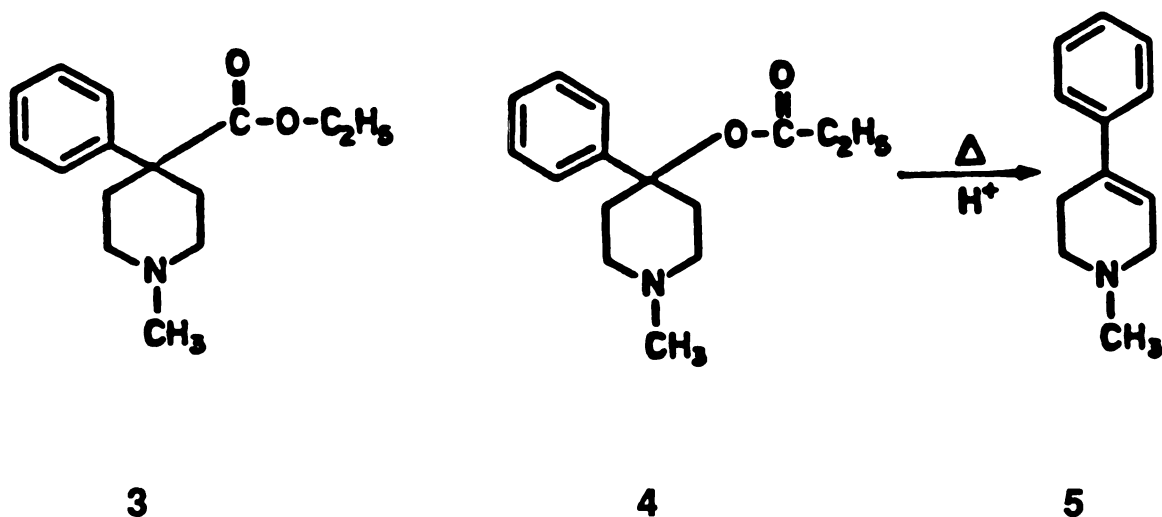
The patient initially described by Davis *et al.* (1979) subsequently died of drug overdosage. On examination of the brain neuronal loss and gliosis limited to the zona compacta of the substantia nigra was discovered, whereas the locus coeruleus was spared. Furthermore, a darkly staining eosinophilic body similar to the Lewy bodies characteristic of Parkinson's disease (Forno, 1982) was discovered in the substantia nigra. Pathologically, it appeared that MPTP had indeed induced a parkinsonian state due to the selective destruction of the nigrostriatal tract.

Direct proof of the neurotoxicity of MPTP came from the systematic administration of the isolated compound to primates (Burns *et al.*, 1983; Langston *et al.*, 1984a). The neurological symptoms were typical of those seen in human parkinsonism and the histological changes in the midbrain on autopsy were strikingly similar to those in idiopathic Parkinson's disease. There

is, however, much variability in species susceptibility to the neurotoxic effects of MPTP. Low doses of MPTP cause relatively specific damage to the dopaminergic neurons in the nigrostriatal pathway of primates and are also effective in dogs (Parisi and Burns, 1986). Larger doses are required to affect mice (Hallman *et al.*, 1985; Heikkila *et al.*, 1985a). In mice, however, though the initial extent and specificity of neuronal damage appear to parallel that seen in primates, over a long period (six months) there is a recovery of biochemical and histological measures, presumably due to the recovery of damaged cells (Chiueh *et al.*, 1986). Other rodents (rats, guinea pigs) appear almost invulnerable to the systemically administered toxin (Chiueh *et al.*, 1984). It has been postulated that the occurrence of neuromelanin (the dark pigment which is increasingly accumulated in dopaminergic nigrostriatal neurons with advancing age) in primates and dogs but not in rodents might be related to the vulnerability of certain species to MPTP toxicity (Burns *et al.*, 1984).

Metabolism of MPTP

Consideration of the chemical structure of MPTP suggests that, as a neutral molecule, it may be lipophilic and might readily pass the blood-brain barrier. However, with no highly reactive group present, MPTP's neurotoxic activity is unexpected--unless it is metabolized to a more reactive intermediate(s) which may interact with critical biological macromolecules. If a compound covalently binds to one or more of the subunits of a macromolecule, unless there is a specific enzyme to reverse this bonding, it may interfere with the function of that molecule; hence, this type of bonding may inactivate enzymes, stimulate peroxidation leading to decomposition of cellular lipids, derange structural proteins, and alter DNA which may cause mutations in genetic material (Langston *et al.*, 1987a). While the cell has ongoing repair mechanisms which can cope with a certain level of damage and some level of inactivated macromolecules can be compensated for by new synthesis, the capacity for repair and restoration can be exceeded, and cell death ensues.

Most drugs and other chemicals that are not normally present in the body are metabolized by a variety of enzymes to more polar compounds which can then be excreted via the kidneys. Some drugs, such as the "prodrug" phenacetin, are biotransformed to therapeutically active compounds, such as

acetaminophen (Hinson, 1980). The same enzymes, however, may also catalyze the formation of reactive, toxic metabolites from other drugs. For example, when administered at very high doses, acetaminophen is metabolized into a hepatotoxin. Whether the biotransformation of a compound produces a detoxification product or a potentially toxic metabolite is determined by factors such as structural features of the drug and its metabolites and the dose of the drug. Factors which influence the ability of a toxin to attack and destroy specific cells include the relative rates of activation and detoxification of the toxin, the effeciency of the diffusion or active transport of the compound or its metabolite to the target site(s), the critical "importance" of the target site(s) to the survival of the cell/organism, and the ability of the cell to repair or replace damaged organelles or enzymes.

Although drug metabolism is primarily a hepatic event, it occurs to some extent in other organs such as the lung (Bend *et al.*, 1973), skin (Alvares *et al.*, 1973), kidney (Orrenius *et al.*, 1973), placenta (Juchau *et al.*, 1975), intestine (Born *et al.*, 1983), and brain (Paul *et al.*, 1977; Marietta *et al.*, 1979). However, the bioactivation of a foreign compound via brain enzyme systems, resulting in the generation of neurotoxic metabolites, appears to be a rare event.

Initial metabolism studies on MPTP were guided by the expectation that, like other cyclic tertiary amines, MPTP would be bioactivated by the cytochrome P-450 system. For instance, the psychotomimetic piperidine derivative phencyclidine (PCP, 6) undergoes cytochrome P-450 catalyzed α-carbon oxidation to a reactive iminium species (7) (Fig. 1), a pathway which leads to the formation of drug-protein covalent adducts (Ward *et al.*, 1982). This iminium species can be trapped *in situ* with sodium cyanide as the corresponding α-cyano amine (8).
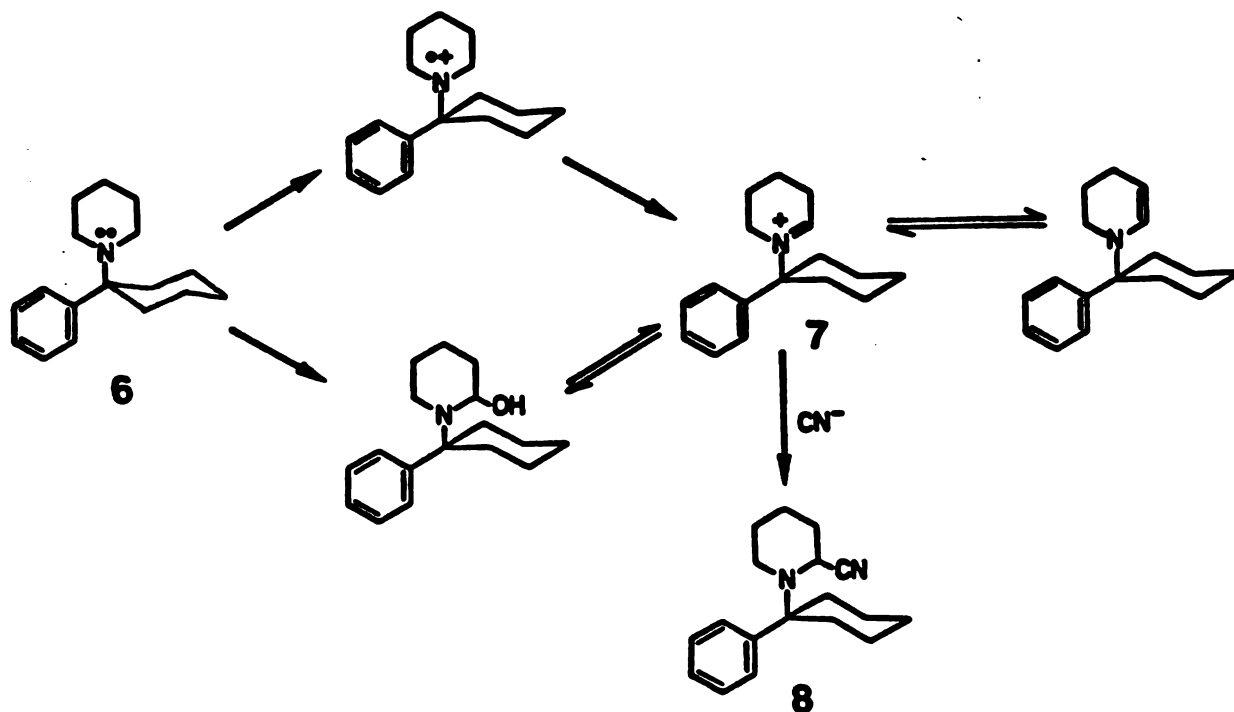
Fig. 1. Pathway of metabolic formation of iminium ion metabolite of PCP.

However, mammalian brain tissues exhibit only low enzymic activities associated with cytochrome P-450 catalyzed reactions (Marietta *et al.*, 1979) but have high MAO activity (Demarest *et al.*, 1980). It was shown by Chiba *et al.* (1984) and Castagnoli *et al.* (1985) that though α-carbon oxidation at the C-6 allylic carbon atom of MPTP does occur, brain mitochondrial and not brain microsomal preparations catalyze the oxidation of MPTP to the four-electron oxidation product, the 1-methyl-4-phenylpyridinium species (MPP+, **10**) via the initial two-electron product, the 1-methyl-4-phenyl-2,3-dihydropyridinium species (MPDP+, **9**), and the process is blocked by selective inhibition of the B form of monoamine oxidase (MAO). Using pure enzymes, Salach *et al.* (1984) reported the rapid oxidation of MPTP by both the A and B enzymes. Oxidation of MPTP is particularly rapid for MAO B--fourteen-fold higher than that by the A form--and is 40 percent of the rate of benzylamine, the best substrate known. There is a powerful product inhibition of the A form by MPDP+ and MPP+, with K_i

values for competitive inhibition being 2.4 and 3 μM, respectively (Singer *et al.*, 1986). These products are much less inhibitory for the B form of the enzyme (K_i values are 200 and 300 μM, respectively). MPTP and MPDP+ are also time-dependent, mechanism based inhibitors of both forms of MAO. However, dissociation of the product far outstrips covalent adduct formation with the enzyme so that essentially complete oxidation of MPTP occurs.

Several laboratories have demonstrated that deprenyl and pargyline, selective inhibitors of MAO B, but not clorgyline (a MAO A selective inhibitor) individually prevented the neurotoxicity of MPTP in mice and primates (Heikkila *et al.*, 1984a; Langston *et al.*, 1984b). Of the two types of MAO it appears that only the B form plays a significant role in the bioactivation of MPTP.

MAO-B

MPTP	**MPDP+**	**MPP+**
5	**9**	**10**
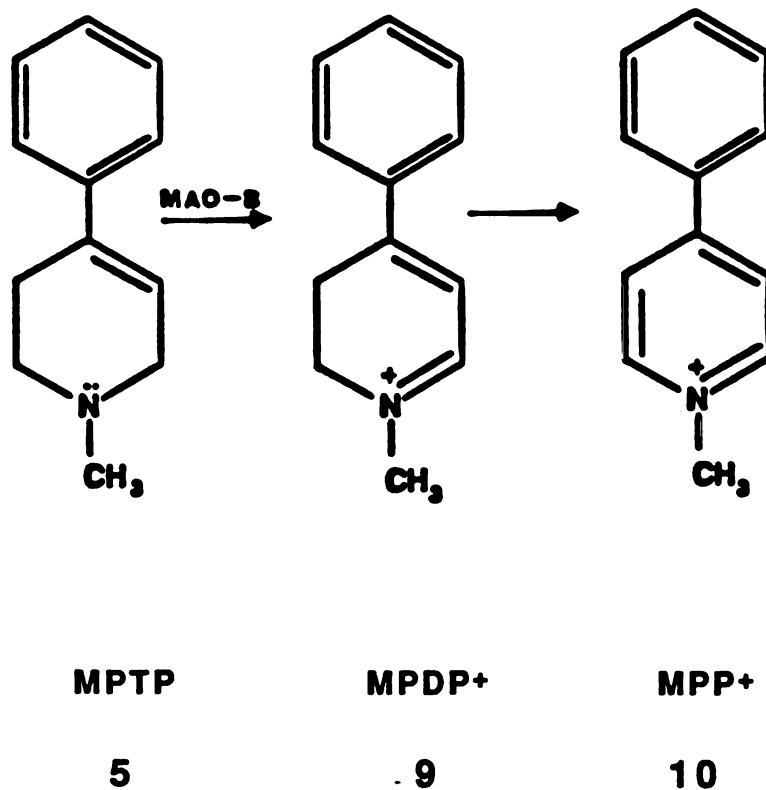
Although there had been general agreement that the neurotoxic actions of MPTP are initiated by its two-electron oxidation via MAO B to form MPDP+, relatively little else was known about other enzyme systems which may contribute to the biological fate of this cyclic tertiary allylamine. Rodent liver microsomes do not convert low levels of MPTP to MPDP+ (Weissman *et al.*, 1985; Cashman and Ziegler, 1986). Under these conditions the NADPH-dependent microsomal metabolism of MPTP leads to the cytochrome P-450 catalyzed product, 4-phenyl-1,2,3,6-tetrahydropyridine (PTP, **11**) and the flavin monooxygenase (FMO) catalyzed product, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine N-oxide (MPTP N-oxide, **12**) .

11   **12**
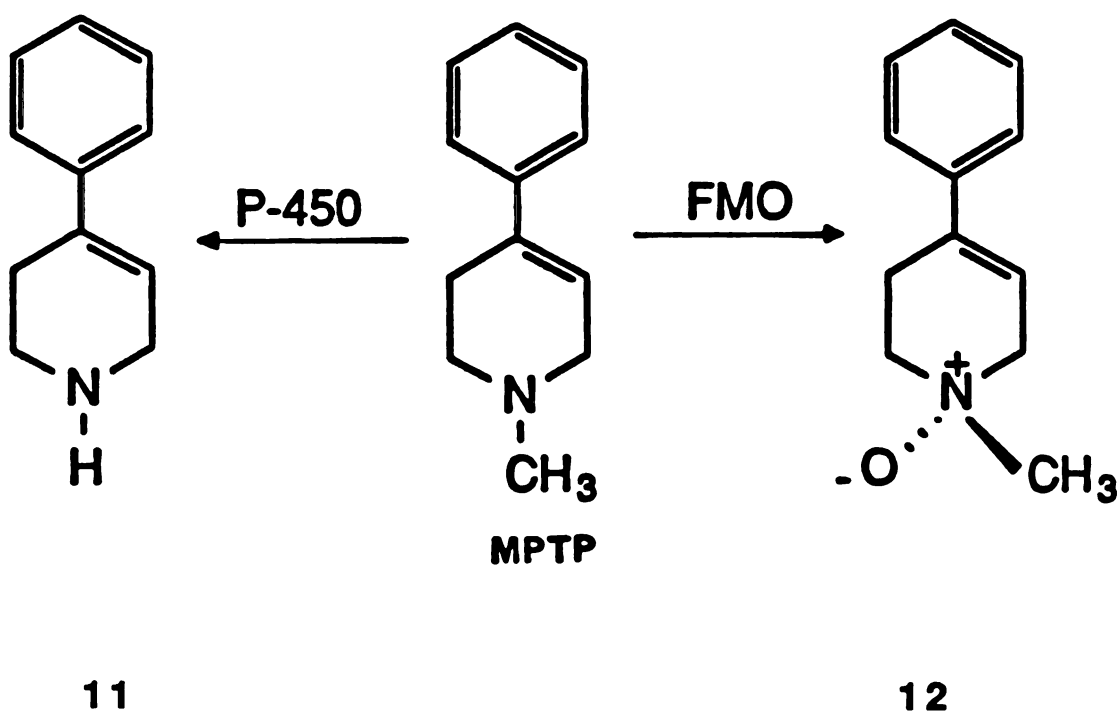
While the mechanism of the MAO B catalyzed oxidation of MPTP to form MPDP+ may be well-understood, there still exists uncertainty concerning the mechanisms involved in the further oxidation of this intermediate to generate the MPP+ pyridinium species, the four-electron oxidation product. Results from previous studies have shown that both MAO A and B oxidize MPDP+ to MPP+ (Salach *et al.*, 1984; Singer *et al.*, 1985) although estimation of the catalytic rate is complicated by the slow turnover of the substrate, its mechanism-based inactivation of MAO and the tendency for MPDP+ to undergo spontaneous air oxidation and disproportionation (Peterson *et al.*, 1985). Although MPDP+ has been shown to undergo disproportionation to form MPTP and MPP+ *in vitro*, this mechanism is unlikely to occur at a significant rate *in vivo* because it requires high concentrations of the compound and is favored by alkaline pH. It is also possible that the oxidation of MPDP+ may be catalyzed by constituents, either enzymic or nonenzymic, endogenous to brain cells. Potentially, then, the metabolic route for the formation of the pyridinium ion might involve a number of toxic reactive intermediates. For a better understanding of its metabolic profile, a recent quantitative study, which will be discussed later in detail, was made on the fate of MPTP and MPDP+ with a variety of mouse brain and liver subcellular fraction preparations (Wu *et al.*, 1988).

Neurotoxicity of MPTP

Whatever the mechanism(s) involved in MPTP's metabolism, it appears that much of the systemically administered neurotoxin selectively accumulates as MPP+ in specific regions of the primate CNS, particularly in the vulnerable nigrostriatal cells (Irwin and Langston, 1985; Markey *et al.*, 1984). It has been shown that intracerebral injection of MPP+ causes dopamine depletion and certain behavioral effects similar to parkinsonism (Heikkila *et al.*, 1985b; Bradbury *et al.*, 1986). MPP+ also appears to be toxic to isolated cells (Mytilineou *et al.*, 1985; DiMonte *et al.* 1986a). Attention, therefore, has been concentrated on MPP+ as the metabolite responsible for the toxicity of MPTP. However, the role of the dihydropyridinium intermediate, the MPDP+ species, or of a possible labile and reactive compound arising from it in the development of the neurotoxicity should not be ruled out. Not only is the dihydropyridinium product itself a potentially reactive iminium species but its conversion to MPP+,

which may involve possible other reactive intermediates, is not well-understood. Moreover, the involvement of MPDP+ in alternative metabolic routes is not well-delineated. Furthermore, while MPP+ is selective for dopaminergic neurons, it is by no means specific. Bradbury *et al.* (1985) have shown that the infusion of MPP+ into the medial raphe nucleus of rats produces a 33 percent decrease in striatal serotonin as compared to a 53 percent loss of striatal dopamine following infusion into the ventral tegmental nucleus. Furthermore, if MPP+ intraneuronally trapped is the principal toxic agent it is necessary to explain its selective cell toxicity. Its presence per se does not ensure neurotoxicity, as evidenced by the lack of toxicity in the adrenal of both rodents and primates where it is highly accumulated, and by its high concentrations in several unaffected brain regions, namely the hypothalamus and the nucleus accumbens (Markey *et al.*, 1984; Johannessen *et al.*, 1985; Irwin and Langston, 1985).

Athough the dopamine-containing cells of the nigrostriatal system contain little MAO B, this enzyme form is localized in nearby brain glial cells and serotonergic neurons (Westlund *et al.*, 1985), which do not appear to be as sensitive to the potentially toxic metabolites they generate from MPTP as are the dopaminergic neurons. The dihydropyridinium metabolite can exist as the uncharged free base (13) and thus can diffuse across the glial or serotonergic membrane into the extracellular space, where it may undergo disproportionation or oxidation to form MPP+. Presumably, extracellular MPP+ (or MPDP+) must then enter the dopamine neurons to destroy the cells.
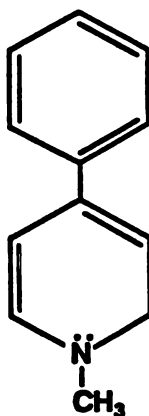
13

Synaptosomal preparations of dopaminergic striatal nerve endings have been shown to accumulate MPP+ via the dopamine uptake system with similar values of K_m and V_{max} as found for dopamine itself, and in a manner prevented by dopamine uptake inhibitors such as mazindol (Javitch *et al.*, 1985; Chiba *et al.*, 1985a). That this uptake process for MPP+ has relevance to the expression of the neurotoxicity of MPTP is suggested by the protective effects of dopamine uptake-blocking drugs on the striatal dopamine-depleting effects of the neurotoxin in rodents *in vivo* (Melamed *et al.*, 1985; Sundstrom and Jonsson, 1985). Using primates, Schultz *et al.* (1986) reported that inhibition of uptake appears to be effective in blocking MPTP-induced nigrostriatal damage but only if the inhibitor (nomifensine) is given for prolonged intervals after the administration of MPTP . However, in spite of repeated attempts, Langston and colleagues have not been able to duplicate these results in the primate (Langston, personal communication).

In its apparent dependence on a high-affinity uptake system for neurotoxicity, MPTP is similar to another neurotoxin, 6-hydroxydopamine (6-OHDA, **14**). In 1968, Tranzer and Thoenen (1968) reported the discovery that 6-OHDA selectively destroys catecholaminergic systems. The compound does not cross the blood-brain barrier, but once administered intraventricularly, intracisternally, or directly into the brain parenchyma, 6-OHDA selectively destroys central dopaminergic and noradrenergic neurons (Ungerstedt,1968; Bloom, 1971; Breese and Traylor, 1970). This destruction has been documented both neurochemically and morphologically. Although exogenous 6-OHDA has never been postulated to be a cause of Parkinson's disease (due to its inability to cross the blood-brain barrier to reach target brain sites and its profound destruction of peripheral noradrenergic neurons, a destruction not seen in Parkinson's disease) it has been used extensively to study this disorder. 6-Hydroxydopamine selectively destroys the two most prominently affected cell groups in the brains of patients suffering from Parkinson's disease--the dopaminergic neurons in the substantia nigra and the noradrenergic neurons in the locus coeruleus.

The mechanism of action of 6-OHDA has never been fully elucidated. However, its selectivity for and its attainment of a critical toxic concentration in dopaminergic and noradrenergic neurons is believed to be due to its accumulation by the high-affinity uptake systems. Indeed, compounds which interfere with catecholamine uptake protect against the neurotoxic action of 6-

OHDA (Thoenen and Tranzer, 1973; Kostrzewa and Jacobowitz, 1974). Though the exact series of molecular events by which 6-OHDA produces cell death remains incompletely understood, the two most commonly offered explanations are the production of free radicals and covalent binding of quinone oxidation products to biomacromolecules (Heikkila and Cohen, 1971; Saner and Thoenen, 1971; Graham *et al.*, 1978).
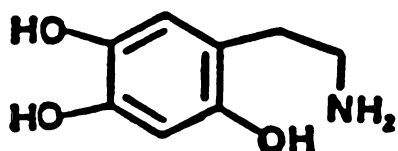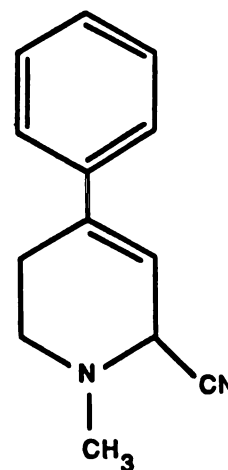
14   15

Parallels to 6-OHDA neurotoxicity may be found, perhaps, in the mechanism of neurotoxicity of MPTP. It is possible that a reactive intermediate, such as MPDP+, might covalently bond with one or more enzyme systems-- particularly in light of the work by Chiba *et al.* (1985b) demonstrating that MPDP+ binds covalently with cyanide at the 2-position of the nitrogen-containing ring when the two are coincubated (15). Furthermore, MPDP+ has been shown to interact with the pigment contained in nigral neurons, neuromelanin--a process which may contribute to nigral cell damage (Wu *et al.*, 1986). Moreover, once MPDP+ or MPP+ enters the nigral neuron, interactions with neuromelanin allow MPP+ to bind to the pigment (D'Amato *et al.*, 1986). It is possible that this pigment in binding MPP+ may facilitate its accumulation and

retention in the nigrostriatal dopaminergic neurons to reach a concentration that is critical for the exertion of cytotoxic effects. Neuromelanin may also act as a reservoir where MPP+ may be stored and then gradually released, thus causing more severe neuronal damage in the substantia nigra (D'Amato *et al.*, 1987).

The finding by Corsini *et al.* (1985) that diethyldithiocarbamate (DDC), a superoxide dismutase inhibitor, exacerbates MPTP-induced neurotoxicity appears to support some type of free radical generation in MPTP neurotoxicity . Recently, however, Irwin *et al.* (1987a) found that DDC enhances the rate of oxidation of MPTP *in vitro* and that DDC pretreatment increases concentrations of MPP+ in the brain *in vivo* for a longer period of time. These results suggest that DDC enhances MPTP-induced neurotoxicity by increasing brain exposure to either MPP+ or an intermediate(s) which results in MPP+.

Recent interest has also concentrated on the effects of MPTP and its metabolites on mitochondrial function. Nicklas *et al.* (1986) demonstrated that MPP+ is an inhibitor of NADH pyruvate and glutamate (but not succinate) oxidation in rat liver and brain mitochondria preparations. This block of oxidative phosphorylation appears to occur in the same region where barbiturates, rotenone, and piercidin A inhibit respiration--a block of NADH dehydrogenase between substrate and coenzyme Q (Fig. 2). The neurotoxicity of MPTP, then, might result from depletion of ATP production and NADH reoxidation leading to cell death. *In vivo* brain dialysis techniques have provided further evidence for this mechanism. Since the storage of dopamine is ATP-dependent, the massive release of dopamine observed when rat striata are perfused with 1 mM MPP+ can be explained by ATP depletion (Rollema *et al.*, 1986). Consistent with the inhibition of aerobic glycolysis, MPP+ and MPTP were also shown to result in lactate accumulation in slices from mouse neostriatum, with the effects of MPTP (but not those of MPP+) being prevented by the administration of the MAO B inhibitor pargyline (Nicklas *et al.*, 1986). *In vivo* dialysis with an on-line lactate assay has confirmed that MPP+ (10 mM, 1 min) induces a four-fold increase over basal levels in lactate output from rat striatum (Rollema *et al.*, 1988a). Furthermore, the time courses of the MPP+-induced efflux of dopamine and efflux of lactate are similar which suggests that both effects are consequences of inhibition of aerobic glycolysis by MPP+ (Rollema *et al.*, 1988b).

NADH
+
H⁺

nH⁺ ↑

MPP⁺

nH⁺ ↑

nH⁺ ↑

| NADH DEHYDROGENASE (4 Fe-S clusters) | ⇨ | Q | ⇨ | CYTOCHROMES b,c₁ | ⇨ | CYT. c | ⇨ | CYTOCHROME OXIDASE |

NAD⁺

⇧

SUCCINATE
DEHYDROGENASE
(3 Fe-S clusters)

↑

SUCCINATE

$2H^+ + \frac{1}{2}O_2$ H_2O
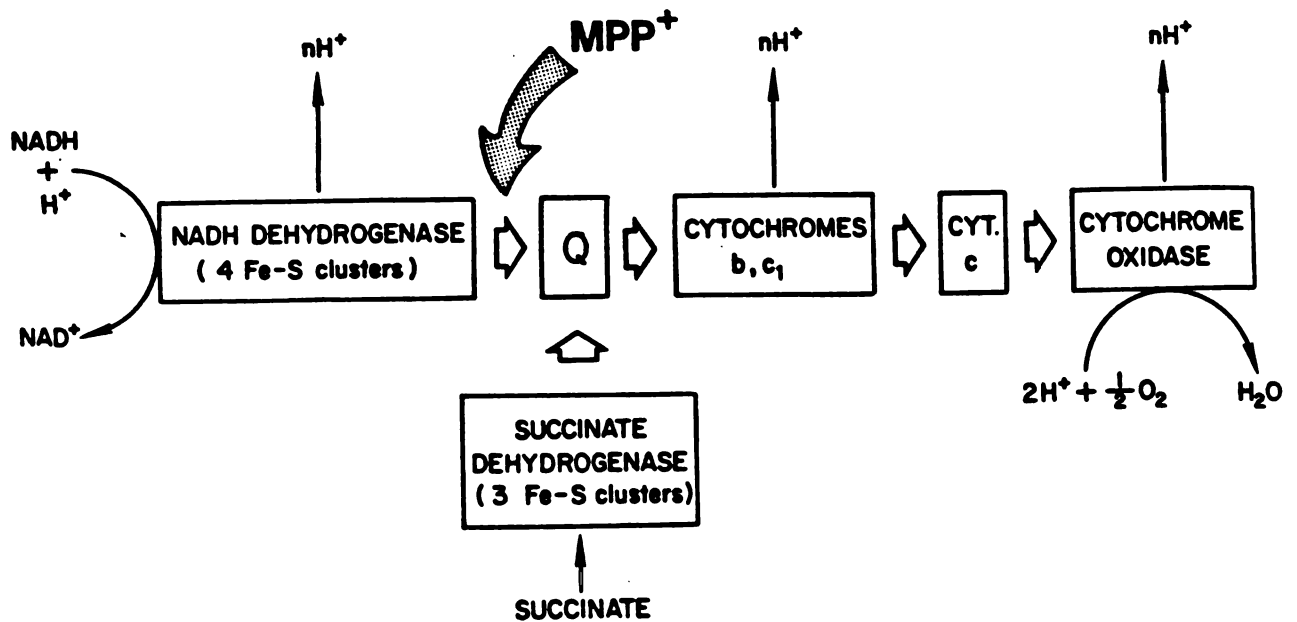
Fig. 2. Inhibition site of MPP+ in the mitochondrial respiratory chain.

The inhibition of NADH oxidation has been found to be much greater in intact rather than disrupted mitochondria due to an energy-dependent transport system that is able to concentrate MPP+ within mitochondria (Ramsay *et al.*, 1986; Ramsay and Singer, 1986). The uptake process appears to be dependent on the electrochemical gradient of the mitochondrial membrane, since valinomycin and K+, which abolish the gradient, abolish MPP+ uptake. So far, only positively charged compounds structurally close to MPP+ have been found to react with this mitochondrial uptake carrier. In the intact mitochondria, MPTP and MPDP+ are much less inhibitory than MPP+. Mitochondrial uptake of MPP+ is not inhibited by dopamine uptake inhibitors (*e.g.*, mazindol) but is blocked by respiratory decouplers such as dinitrophenol.

14

Calcium ions also interfere with the uptake of MPP+ by isolated rat brain mitochondria (Frei and Richter, 1986); they may compete for the same electrochemical gradient. On the other hand, excess calcium release has been implicated as a cause of cell death (Frei and Richter, 1986). Although neither 6-OHDA nor MPP+ separately have much effect on the rate of release of calcium sequestered in mitochondria, together they greatly increase the rate of calcium efflux. Therefore, it was suggested that the toxic effects of MPTP might be mediated through some mechanism of calcium release from mitochondria.

Since mitochondria from liver and brain appear to be similar in their abilities to concentrate MPP+ and in their sensitivities to this compound as an inhibitor of NADH oxidation or as a promoter (in the presence of 6-OHDA) of calcium efflux, it is assumed that the selective toxicity of MPP+ arises from the specific ability of dopaminergic neurons to concentrate it. However, the existence of alternative pathways for the oxidation of MPTP in liver (Baker *et al.*, 1984; Wu *et al.*, 1988) may also limit its peripheral toxicity.

This brief overview of some of the work which has been done since the relatively recent discovery of MPTP clearly establishes that the neurotoxic actions of this tertiary allylamine are initiated by its biotransformation via the enzyme MAO B. Although much evidence points to the four-electron oxidation product, the stable MPP+ species, as the ultimate neurotoxin, the mechanism of its toxicity has not been determined. Of additional interest and importance is the investigation into the possible role of the two-electron oxidation product, the potentially reactive MPDP+ species, in the mechanism of MPTP neurotoxicity. The inherent chemical instability of the dihydropyridinium makes it difficult to study and this is likely the reason little attention has been focused on MPDP+ in the past. However, it is precisely its chemical reactivity which makes MPDP+ a potential candidate in interactions with biomacromolecules and thus possibly play a role in producing neurotoxicity. In fact, MPDP+ has been shown to be more toxic than MPTP or MPP+ in isolated hepatocytes (DiMonte *et al.*, 1987). Moreover, although much is known about the MAO B catalyzed formation of MPDP+ from MPTP, little is known about the step from MPDP+ to MPP+ or from MPDP+ to other possible products. Since much is still unknown about the dihydropyridinium, the objects of study in this dissertation involve the investigation of the *in vitro* and *in vivo* behavior of this potentially reactive species. The interaction of MPDP+ with neuromelanin, a specifically localized brain pigment thought to play a role in MPTP neurotoxicity, has been assessed.

15

Because access to dopaminergic neurons is crucial for the onset of neurotoxicity, the ability of MPDP+ (via a stable MPDP+ analog) to act as a substrate for the dopamine uptake system is also assessed. For a better understanding of their biodisposition, metabolic profile, and neurotoxicity, studies on MPTP and MPDP+ using subcellular tissue fractions, the isolated perfused liver, and purified MAO B as well as the intact animal (the mouse and the rat) are discussed in detail.

INTERACTIONS OF THE 1-METHYL-4-PHENYL-2,3-DIHYDROPYRIDINIUM SPECIES WITH SYNTHETIC DOPAMINE-MELANIN

Introduction

Neuromelanin, the black pigment derived from the autoxidation and polymerization of catecholamines, is deposited within the cytoplasm of certain catecholamine-containing brain neuronal cell bodies (Marsden, 1969). The biological significance of neuromelanin remains obscure; however, the pigment is present in the substantia nigra of the midbrain and the locus coeruleus of the pons (Van Woert and Ambani, 1974), precisely those brain regions which degenerate in idiopathic Parkinson's disease (Farley et al., 1977). A general correlation also appears to exist between the density of neuromelanin in the nigral cells of various species and the susceptibility of the species to the toxic effects of MPTP (Langston et al., 1984a; Parisi and Burns, 1986; Schneider et al., 1986). It has been shown that MPTP-elicited destruction of dopamine and norepinephrine nerve terminals is followed by their regeneration unless the cell bodies are also destroyed (Ricaurte et al., 1985a). In mice (in which no neuromelanin is detected), depletion of striatal dopamine can be produced by high doses of MPTP, but neuronal cell bodies are not readily destroyed (Hallman et al., 1985). Primates have a high content of neuromelanin in the substantia nigra and show a dramatic loss of nigral neurons following low doses of MPTP. In addition, the amount of neuromelanin in the primate substantia nigra increases with age, paralleling the increase in neurotoxicity of MPTP to aged monkeys (Burns et al., 1984). The brains of rats, rabbits, and guinea pigs, species which are resistant to the toxicity of MPTP (Chiueh et al., 1984), are reported to contain no neuromelanin (Barden and Levine, 1983). Given all these facts, it appears that the selective destruction of dopamine cell bodies by MPTP may involve neuromelanin.

Previous studies established that the toxicity of MPTP is dependent upon its MAO B catalyzed oxidative transformation to the unstable charged $MPDP^+$ species which undergoes further oxidation to the MPP^+ species (Chiba et al., 1984; Chiba et al., 1985b). The observation that MPP^+ localizes in the

substantia nigra of monkeys treated with MPTP (Irwin and Langston, 1985; Markey *et al.*, 1984) suggests that MPP+ may bind to neuromelanin, a process which may contribute to nigral cell damage. Lyden *et al.*, 1983; Lyden *et al.* (1985) have demonstrated the binding of MPTP to synthetic melanin, but only low affinity interactions were observed. D'Amato *et al.* (1986) have described the high affinity binding of MPP+ to this pigment. In an effort to characterize further the possible contributions which neuromelanin may make in the mediation of the nigrostriatal toxicity of MPTP, we (Wu *et al.*, 1986) examined the interactions of MPDP+ with human neuromelanin and synthetic dopamine-melanin (D-M), a polymeric pigment that is structurally similar to naturally occuring neuromelanin.

Results

Using MPTP and MPP+, we conducted binding studies similar to those by Lyden *et al.* (1983) and D'Amato *et al.* (1986). The UV-visible spectra of 50 μM MPTP (λ_{max} 244 nm, ε 12,000 M-1 cm-1) and 50 μM MPP+ (λ_{max} 295 nm, ε 18,000 M-1 cm-1) were monitored before and after the addition of D-M (100 μg polymer/mL). Upon incubation with D-M at 37º C in phosphate buffer (0.1 M, pH 7.4), the amounts of MPTP and MPP+, as determined spectrally, were decreased by about 20 percent (data not shown). However, the bound material does not undergo irreversible chemical change since upon acidification of the incubation mixture with hydrochloric acid to pH 2 the starting compounds are recovered quantitatively. (See Experimental section for details). These binding studies were extended to characterize D-M interactions with MPDP+. Although stable when stored at low pH (4 or less) this dihydropyridinium compound at concentrations greater than about 1 mM undergoes disproportionation to yield equimolar amounts of MPTP and MPP+ (Chiba *et al.*, 1984) and self-condensation to yield a hexahydroisoquinoline (Leung *et al.*, in press). At concentrations below 100 μM, the rates of such bimolecular reactions at pH 7.4 and 37º C are insignificant. In such dilute solutions, however, MPDP+ undergoes slow but stoichiometric autoxidation to MPP+ with a half life of about 6 h. Under these conditions the addition of trace metals, which are reported to catalyze the oxidation of catecholamines and other readily oxidizable compounds (Donaldson *et al.*, 1981), was found to have no effect on the rate of

18

autoxidation of MPDP+. Moreover, in contrast to a report that ferrous ion catalyzes the oxidation of MPTP to MPDP+ (Poirier and Barbeau, 1985), in our hands, a pH 7.4 solution of MPTP (50 to 100 μM) was stable in the presence of ferrous sulfate (50 to 500 μM).

The possibility that MPDP+ may bind to neuromelanin was examined with the synthetic polymer under conditions similar to those used to demonstrate the binding of MPTP and MPP+. Under these conditions, however, MPDP+ proved to be extremely unstable. UV spectral analysis showed a time-dependent decrease in the chromophore corresponding to MPDP+ (λ_{max} 345 nm) and a concomitant increase in a new chromophore with maximum absorbance at 290 nm (Fig. 3). A semilog plot of the 345 nm absorbance (which is directly proportional to MPDP+ concentration) against time gave a straight line (Fig. 4) indicating that the reaction was a pseudo first-order process. Under these conditions (50 μM MPDP+ in the presence of 100 μg/mL D-M in pH 7.4 at 37° C) the half life of MPDP+ was 8.9 min and the yield of MPP+ was quantitative (Fig. 5). Extraction of a typical post-incubation mixture with chloroform provided a product which displayed HPLC and diode array UV as well as 240 MHz ^{1}H NMR spectral characteristics identical to those of authentic MPP+. When neuromelanin isolated from human nigral tissue replaced the D-M, the half life of MPDP+ was 90 min--still considerably shorter than the 360 min half life observed in the absence of pigment.
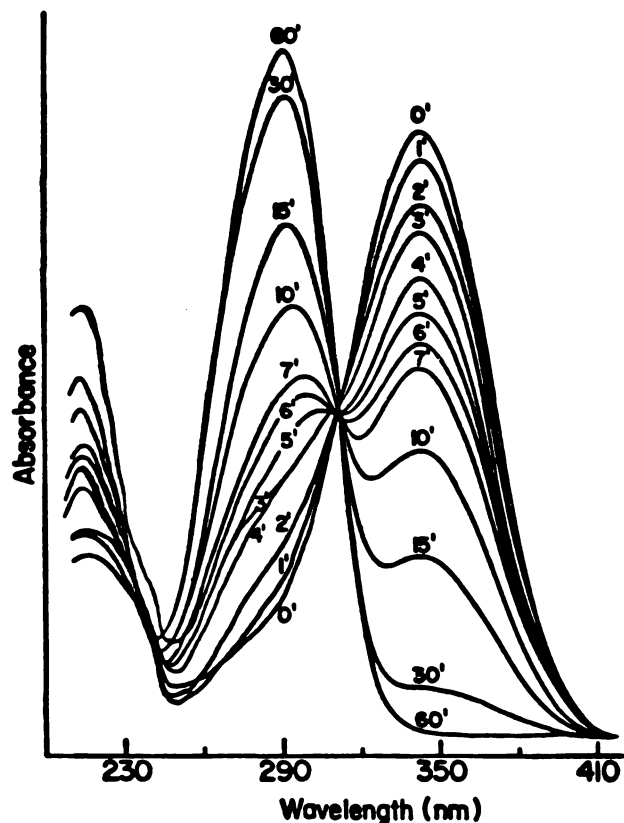
Fig. 3. Serial scans of 50 μM MPDP+ following incubation in 0.1 M sodium phosphate buffer (pH 7.4) at 37° C in the presence of synthetic dopamine-melanin (100 μg/mL).
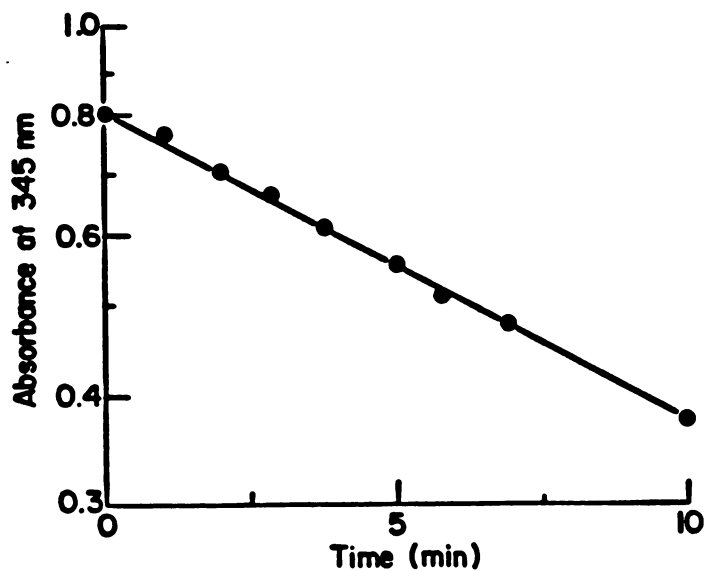
Fig. 4. Semilog plot of absorbance at 345 nm (corresponding to MPDP+) vs time of the scans (between 0 and 10 min) shown in Fig. 3.

20
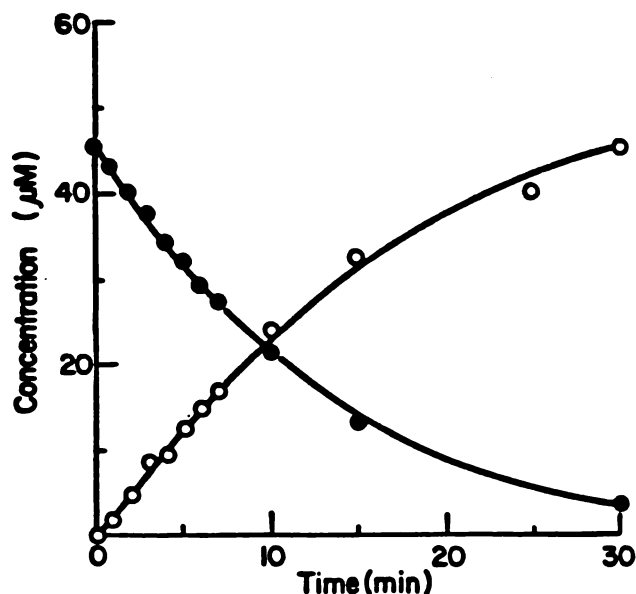
Fig. 5. Plot of concentrations of MPDP+ (closed circles) and MPP+ (open circles) vs time of the scans (between 0 and 30 min) shown in Fig. 3.

The interaction of MPDP+ with D-M was examined under a variety of conditions. The possibility that dioxygen is the species which oxidizes MPDP+ seems unlikely since replacing air with 100 percent oxygen had only a slight rate enhancing effect (half life of 6.9 min) while anaerobic conditions (nitrogen atmosphere and preincubations for up to 180 min in the presence of D-glucose-6-phosphate, glucose oxidase, and catalase) increased the half life to 17.0 min. The use of dopamine polymers prepared in the presence of various transition metals (see Experimental section) to mimic more closely natural neuromelanin which is reported to contain such metals (Marsden, 1969) or the addition of these transition metals to the D-M incubation mixtures also was without effect on the rate of MPDP+ oxidation.

21

Discussion

The mechanism by which D-M enhances the rate of oxidation of MPDP+ to MPP+ is unclear. Since anaerobic conditions have only a modest effect on the reaction rate, it seems likely that the D-M itself is the oxidizing species, accepting electrons directly from MPDP+ or rather, from the dihydropyridinium free base **13**. This type of reaction has been observed previously with synthetic DOPA melanin (33 μg/mL) which was shown to oxidize NADH (128 μM) at pH 7.2 and 23° C at a rate of 30 pmol/min·μg polymer (Menon *et al.*, 1977). This value is similar to the rate of 28 pmol/min·μg D-M we have observed with 50 μM MPDP+ and 100 μg/mL D-M (calculated from half life of 8.9 min in incubation volume of 15 mL).

It has been suggested that neuromelanin is a waste product of catecholamine metabolism and is derived from the oxidation of dopamine (an established neurotoxin itself), norepinephrine, and related compounds to semiquinone radicals and other quinone species which polymerize to form the pigment (Graham, 1978) (Fig. 6). A variety of studies suggests that melanins are free radical bearing polymers containing both catechol and *o*-benzoquinone moieties which are capable of participating in redox reactions (Sealy *et al.*, 1980). Melanins also may act as radical traps in which case these pigments may protect cells exposed to carbon radicals (Slawinska *et al.*, 1983) as well as toxic oxygen radicals (Goodchild *et al.*, 1981). A possible role for neuromelanin, then, would be to protect the intricate biochemical mechanisms which operate in dopamine containing neuronal cell bodies by scavenging such free radical species--a proposal first made by Marsden (1969). Consistent with this proposal is the reported accumulation of radioactive dopamine on melanin (Lindquist, 1973) and the observation that chlorpromazine [which is known to bind essentially irreversibly to neuromelanin (Lindquist, 1973)] may be responsible for the degeneration of nigrostriatal cells observed at autopsy of patients suffering from drug-induced tardive dyskinesias (movement disorders) (Christensen *et al.*, 1970).
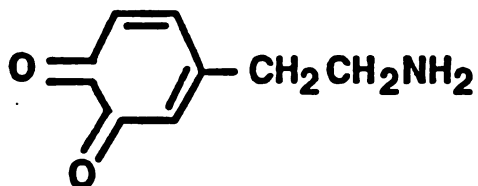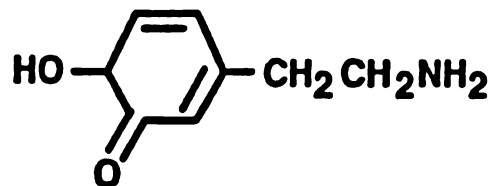
DA quinone   **DA semiquinone**
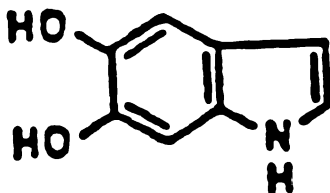
5,6-dihydroxyindole   **5,6-Indolequinone**
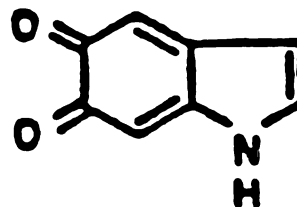
Fig. 6. Possible quinone species in dopamine-melanin.

If MPDP+ were to have access to the pigmented cells of the substantia nigra, redox interactions with the neuromelanin would lead to the deposition of MPP+ on the polymer which could compromise the ability of the neuromelanin to scavenge toxic free radical species. The localization of MPP+ in nigral cell bodies following administration of MPTP to monkeys is consistent with this proposal. At 24 h after radiolabeled MPTP administration Markey *et al.* (1984)

reported high concentrations of radioactivity in the monkey locus coeruleus, thalamus, and corpus striatum, and low density labeling in the cerebral cortex, cerebellum, and substantia nigra. However, between 1 and 72 h after monkeys receive MPTP, MPP+ concentrations in the substantia nigra almost doubled, while they fell ten-fold in the cerebellum and declined by 50 percent in the corpus striatum (Irwin and Langston, 1985). MPP+ which is bound intracellularly to neuromelanin may form a depot for gradual release and subsequent damage to the neurons. The neurotoxicity of other substances such as chlorpromazine, chloroquine (Larson and Tjalve, 1979), and manganese (which produces a parkinsonian syndrome characterized by tremor, bradykinesia, postural difficulties, dystonia, and psychic disturbances) (Cotzias *et al.*, 1964), may also depend on their high affinity for melanin. The toxin-binding capacity of neuromelanin in the substantia nigra could account for a component of the selective loss of nigral neurons in idiopathic Parkinson's disease. In normal humans there is an age-related selective loss of dopamine neurons which may also be caused by the accumulation of toxic substances on nigral neuromelanin.

THE EFFECT OF DIETHYLDITHIOCARBAMATE (DDC) ON THE MAO B CATALYZED FORMATION OF THE 1-METHYL-4-PHENYL-2,3-DIHYDROPYRIDINIUM (MPDP+) SPECIES FROM 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE (MPTP)

Introduction

Diethyldithiocarbamate (DDC) (16) has been reported to increase the neurotoxic effects of MPTP in mice (Corsini *et al.*, 1985). Whereas two MPTP (30 mg/kg i.p.) injections to untreated mice induced about a 70 percent decrease of neostriatal dopamine (DA) compared to control, in DDC (400 mg/kg i.p.) pretreated mice the same dose of MPTP decreased DA by over 95 percent. DDC treatment alone had no effect on DA levels. Concomitant decreases of striatal DOPAC and HVA (products of dopamine metabolism) but not of 5-HT (serotonin) were observed in the mice treated with both DDC and MPTP, suggesting that the drug treatments selectively affect the striatal dopaminergic system. Because DDC inhibits copper containing enzymes, including superoxide dismutase in the brain as well as in various other tissues of mice (Heikkila *et al.*, 1976), it was suggested that these findings supported a free radical mediated mechanism of MPTP toxicity (Corsini *et al.*, 1985) involving the redox cycling of MPP+ in a manner similar to paraquat.
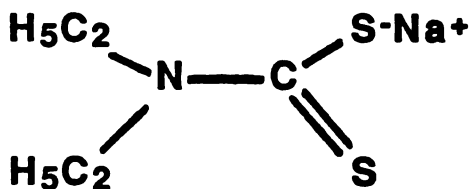
16
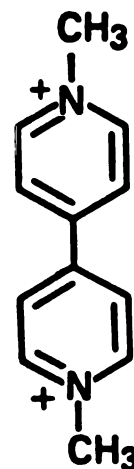
17

The similarity in structure of MPP+ and paraquat (17) suggests that the mechanisms of their toxicity might be similar. Bus *et al.* (1974) proposed that the toxic effects of paraquat were mediated by redox cycling in the presence of oxygen to generate free radicals. After depletion of protective antioxidants (*e.g.*, glutathione) superoxide anion (O_2), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\cdot OH$) are formed. These three radical species have been referred to as the "toxic triad" because they are all potentially damaging to biological systems. The superoxide anion has a deleterious effect on enzymes, DNA, cell membranes, virus particles, prokaryotic and eukaryotic cells in culture and animal tissues which have been exposed to them (Fridovich, 1979). In the cell the presence of enzymes such as superoxide dismutase, catalase, and glutathione peroxidase normally protect against these effects. When free radical formation is accelerated, however, the protective mechanism may be inadequate. The subcellular effects of peroxide and the hydroxide radical probably involve the peroxidation of polyunsaturated lipids in membranes (McBrien and Slater, 1982). Free radical chain reactions are then promoted throughout the entire membrane. In addition to this primary damage, the attack on membranes may result in even greater secondary damage. In the mitochondria, the family of enzymes which generate and utilize active oxygen species are positioned carefully in a chain-like physical arrangement on the outer mitochondrial membrane. For instance, MAO produces one molecule of hydrogen peroxide every time a molecule of substrate (including MPTP) is oxidized. If the structure of the membrane is disrupted, then not only is the enzymatic process disrupted and the cell deprived of energy but, because systems are no longer contained, there may be an uncontrolled release of radicals which can attack the membrane further and precipitate a chain reaction (Langston *et al.*, 1987a).

Like paraquat, MPP+, when administered, causes severe toxic damage in the lungs and increases the plasma levels of glutathione disulfide, indicating an oxidative stress (Johannessen *et al.*, 1986a). Sinha *et al.* (1986), however, could not demonstrate by electron spin resonance free radical formation from MPP+ under conditions in which paraquat free radicals were apparent. In isolated hepatocytes, both MPP+ and paraquat cause rapid depletion of ATP (DiMonte *et al.*, 1986b). On the other hand, MPDP+ is more toxic than both MPTP

or MPP+ in the depletion of intracellular ATP, which preceeds the onset of hepatocyte toxicity (DiMonte *et al.*, 1987). In the presence of BCNU (an inhibitor of glutathione reductase) the toxicity of paraquat, but not of MPP+, is enhanced (DiMonte *et al.*, 1986b). Nor is MPP+ cytotoxicity diminished by the antioxidant N,N-diphenyl-p-phenylenediamine. Furthermore, DiMonte *et al.* (1986b) have shown with isolated hepatocytes that MPP+ does not produce the membrane lipid peroxidation or free radical damage which is characteristic of paraquat. Moreover, current evidence indicates that MPP+ is very stable. A comparison of the reduction potential of MPP+ (-1.18 V) to that of paraquat (-0.44 V) suggests that MPP+ is unlikely to undergo redox cycling (Sayre *et al.*, 1986).

As described below, the presence of DDC appears to affect the oxidation of MPTP by partially purified bovine liver MAO B. These results together with our other findings from a recent study (Irwin *et al.*, 1987a) on the effects of DDC on the biotransformation and distribution of MPTP may provide an alternative explanation for the role of DDC in potentiating MPTP neurotoxicity.

<u>Results</u>

A.   Effect of DDC on the oxidation of MPTP by partially purified MAO B

In the absence of DDC, purified MAO B catalyzed the α-carbon oxidation of MPTP (3.3 mM) to form its two-electron oxidation product, the 2,3-MPDP+ species, at an initial rate of 300 nmol/min per mg protein. As shown in Fig. 7, the formation of MPDP+ was not linear with time, and it reached a plateau at approximately 60 min due to mechanism-based inactivation of the enzyme (Singer *et al.*, 1985). In a concentration-dependent manner, preincubation with DDC increased the rate of formation of MPDP+. Ideally, the disappearance of MPTP should have been monitored; however, the high concentration required (3.3 mM) precluded this. By monitoring the stoichiometric formation (1:1) of the first metabolite, MPDP+, the metabolism of MPTP by purified MAO B and the effect of DDC on this biotransformation could be indirectly calculated. When incubated with 100 μM MPDP+ in buffer, but without MAO B, DDC (0.5 mM) had no effect on the oxidation of the dihydropyridinium metabolite (Fig. 8). In experiments (n = 4) on MAO B inactivation by 3.3 mM MPTP, the half-life of

enzyme inactivation was about 35.3 min. (Fig. 9). The addition of DDC (0.25 mM) did not significantly alter the half-life (34.1 min) for the inactivation of the enzyme by the neurotoxin. Thus, the effect of DDC on the MAO B mediated oxidation of MPTP does not appear to involve protection against the mechanism-based inactivation of the enzyme caused by MPTP or the prevention of nonenzymic oxidation of MPDP+.
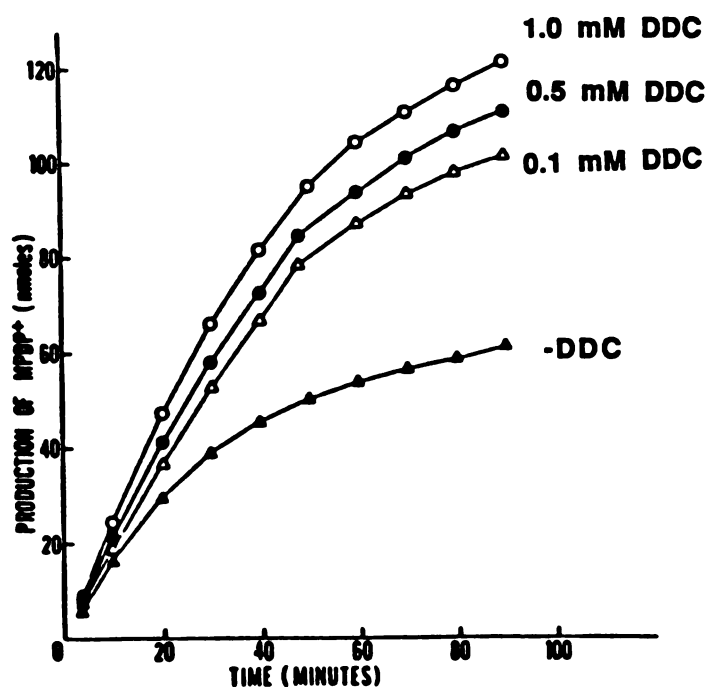
Fig. 7. Effect of DDC on oxidation of MPTP by partially purified MAO B. The oxidation of MPTP by MAO B in the absence of (solid triangles) and presence of 0.1 mM (open triangles), 0.5 mM (solid circles) and 1.0 mM (open circles) DDC. Results represent the means of six determinations at each time point.
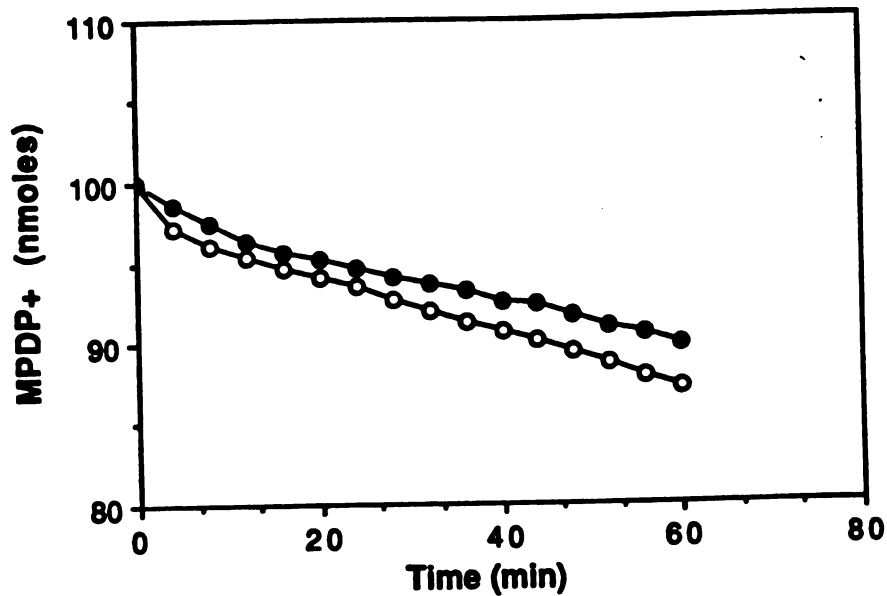
Fig. 8. Effect of DDC on autoxidation of MPDP+ (100 μM). The concentration of MPDP+ measured at 343 nm in the absence (open circles) and presence (solid circles) of 0.5 mM DDC.
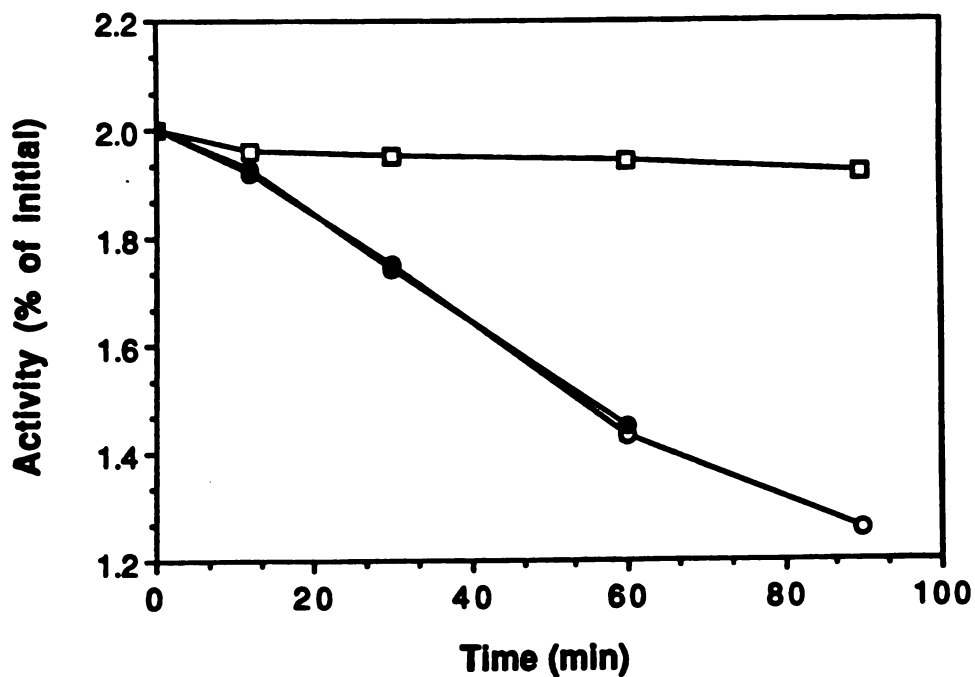
Fig. 9. Effect of DDC on the inactivation of MAO B by MPTP. The activity of MAO B at different time points when incubated with no MPTP (control, open squares), with 3.3 mM MPTP (open circles), and with 3.3 mM MPTP and 0.25 mM DDC (solid circles).

[Note: The following results in sections B. through D. are from studies conducted by I. Irwin and L.E. DeLanney at The Institute for Medical Research, San Jose, CA. These experiments are published in a paper (Irwin *et al.*, 1987a) of which I am a coauthor.]

B.   Effect of DDC on MPTP-induced striatal dopamine depletion in mice

MPTP alone, given as a single i.p. injection to young mature C57 Bl mice (male, 6-8 weeks old), did not produce statistically significant dopamine depletions at doses less than 30 mg/kg (Table I). Although DDC alone (400 mg/kg) had no statistically significant effect on striatal dopamine (12.8 $\pm$ 0.3 ng/mg tissue), the dopamine-depleting effect of MPTP was greatly enhanced when DDC was given prior to MPTP.

TABLE I. Striatal Dopamine Concentrations in C57 Bl Mice (6-8 wk) Receiving Increasing Dosages of MPTP Alone and n Combination with DDC

DDC (400 mg/kg) was administered i.p. 0.5 h prior to MPTP injection; MPTP was given as a single i.p. injection. Animals were killed by cervical dislocation 1 week following drug treatment. Striata were rapidly removed and assayed for dopamine as previously described (Ricaurte *et al.*, 1986). Dopamine concentrations are in ng/mg $\pm$ S.E.M. Values for saline control and for DDC alone were not significantly different from each other.

MPTP Dose	Control	DDC alone	MPTP alone	MPTP + DDC
0	13.0 $\pm$ 0.3	12.8 $\pm$ 0.3	--	--
10 mg/kg	--	--	12.6 $\pm$ 0.3 (n = 5)	9.5 $\pm$ 0.5 (n = 5) [a,b]
20 mg/kg	--	--	12.1 $\pm$ 0.5 (n = 5)	3.1 $\pm$ 0.2 (n = 5) [a,b]
30 mg/kg	--	--	6.1 $\pm$ 0.2 (n = 10)[a]	0.4 $\pm$ 0.1 (n = 10)[a,b]

[a]Significantly different than control (P < 0.001). [b]Significantly different from MPTP alone (P < 0.001).

C.   Effect of DDC on MPTP and MPP+ concentrations in mouse striata, ventral mesencephalon and frontal cortex after MPTP administration

MPTP and MPP+ were assayed by a gas chromatography/mass spectrometry method previously described (Irwin *et al.*, 1987b). MPTP was detected in all three brain regions (striata = STR, ventral mesencephalon = VME, frontal cortex = FC) only in treated young mature animals killed at the 30 min time point. However, at 30 min MPTP concentrations were much higher in the mice which were also pretreated with DDC (Fig. 10). MPP+ was detected in all three brain regions at every time point examined. Pretreatment with DDC produced higher concentrations of MPP+ compared to treatment with MPTP alone (Fig. 11).
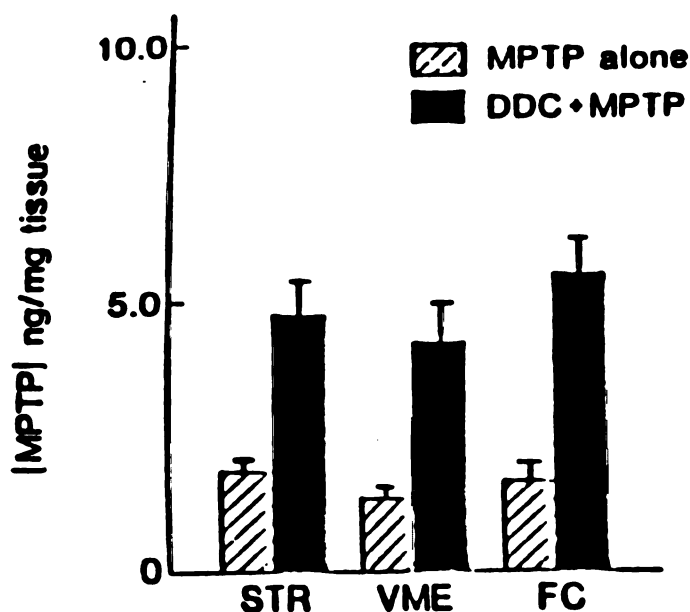
Fig. 10. Effect of DDC on [MPTP] in various regions of C57 BI mouse (male, 6-8 wk) brain following MPTP treatment. [MPTP] in various brain regions 0.5 h following a single 30 mg/kg i.p. dose of MPTP alone (striped bars) or after pretreatment (0.5 h prior to MPTP) with 400 mg/kg DDC (solid bars). Values are means ±S.E.M. for each group (n = 5 per group). Concentrations of MPTP in DDC-pretreated animals differed significantly from those animals given MPTP alone as follows: STR, $P < 0.001$; VME, $P < 0.005$; FC, $P < 0.001$.

31
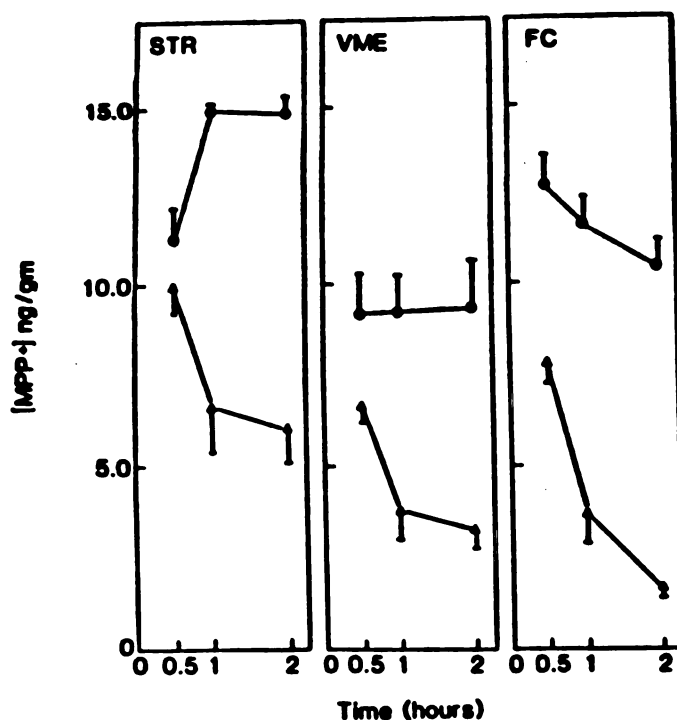
Fig. 11. Concentrations of MPP+ at 0.5, 1, and 2 h in STR, VME, and FC of C57 Bl mice (male, 6-8 wk) given 30 mg/kg MPTP alone (triangles) or after pretreatment (0.5 h prior to MPTP) with 400 mg/kg DDC (circles). Values are means ±S.E.M. for each group (n = 5 per group). Concentrations of MPP+ in DDC-pretreated animals differed significantly from those in animals given MPTP alone as follows: 0.5 h, FC only (P < 0.005); 1 h, STR (P < 0.001), VME (P < 0.005), FC (P < 0.001); 2 h, STR (P < 0.001), VME (P < 0.005), FC (P < 0.001).

D. Effect of DDC on the biotransformation of MPTP by mouse brain homogenates

The oxidation of MPTP (100 μM) by young mature C57 Bl mouse brain whole homogenates produced MPP+ at a rate of 0.06 nmol/min per mg protein. MPP+ was assayed by HPLC as previously described (Langston *et al.*, 1984c). Preincubation of the homogenates with DDC resulted in a significant increase in the rate of MPP+ production (Fig. 12), which was dependent on the

32

concentration of DDC. DDC itself does not catalyze the oxidation of MPTP to MPP+ since the addition of DDC to boiled homogenates did not result in the conversion of MPTP to MPP+. In a separate incubation MPP+ was found to be stable in the presence of brain homogenate and DDC. When brain homogenate incubations contained only MPTP, the addition of deprenyl (10 $^{-6}$ M) reduced MPP+ production by 90 percent (0.006 nmol/min per mg protein). The addition of 100 μM DDC to these deprenyl-containing incubations did not result in the production of any additional MPP+. Thus, the mechanism of the DDC effect on the production of MPP+ probably involves non-inhibited, viable MAO B.
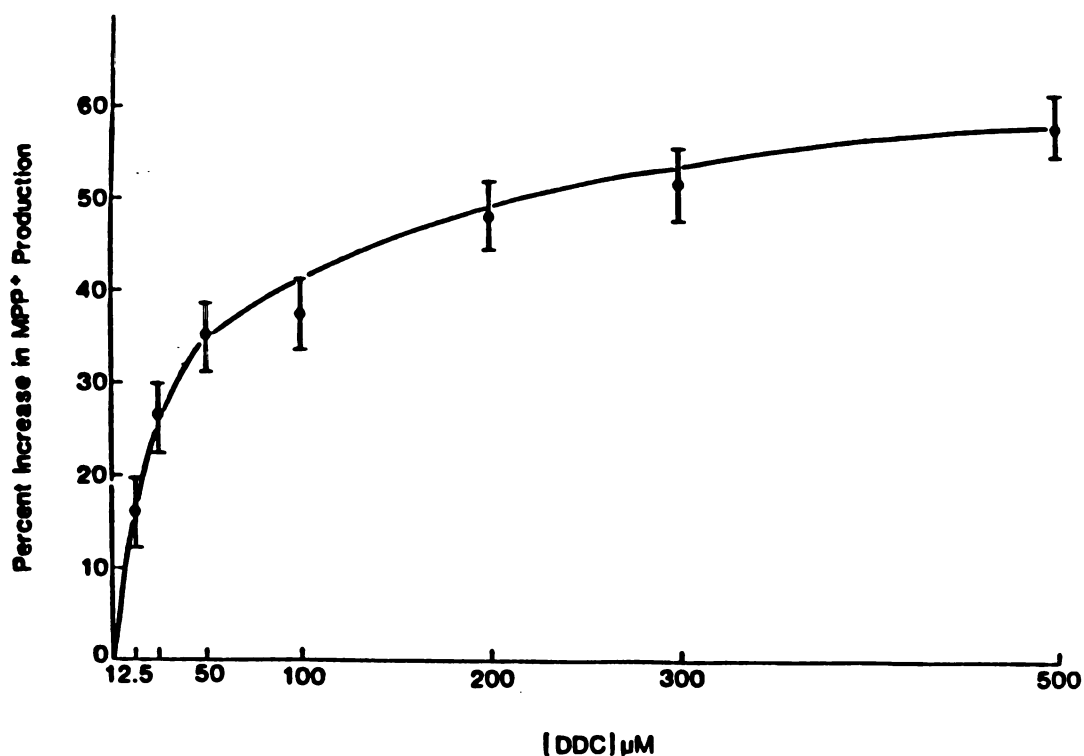
Fig. 12. *In vitro* enhancement of MPP+ production by DDC. The effect of increasing concentrations of DDC on the production of MPP+ in whole brain homogenates prepared from C57 Bl mice (male, 6-8 wk) and incubated with MPTP. Values are means ±S.E.M. of three separate experiments.

33

Discussion

These studies have confirmed earlier findings (Corsini *et al.*, 1985) that DDC potentiates MPTP neurotoxicity in young mature mice. The DDC-induced enhancement of toxicity is accompanied by significant increases in brain concentrations of MPP+. Although the methods of analysis used here did not allow the detection of the metabolite MPDP+, it seems likely--judging from the work by Shinka *et al.* (1987; see pp. 44-54)--that this intermediate would also be present in the brain at least at the earlier time points. The increase in mouse brain MPP+ levels due to DDC pretreatment were detectable 30 min after drug treatment and by 2 h MPP+ concentrations were three to eight times higher than in animals given MPTP alone.

Three factors have been suggested to increase the CNS concentrations of the MAO B catalyzed metabolites of MPTP in animals receiving DDC prior to MPTP treatment. These include the increased delivery of MPTP to the CNS, increased biotransformation of MPTP to MPP+, and reduced elimination of MPP+ from the CNS. A fourth suggestion that DDC may potentiate MPTP-induced dopamine depletion by enhancing uptake of MPP+ into dopaminergic terminals has been made unlikely by recent observations that DDC actually inhibits, not potentiates, MPP+ uptake (DiMonte, personal communication). That DDC may increase the delivery of MPTP to the CNS is supported by the finding that 30 min after its administration, the concentration of MPTP in the brain regions studied was two to three times higher in DDC pretreated animals (Fig. 10). A possible mechanism to explain this effect is that DDC may reduce the peripheral biotransformation or elimination of MPTP, thereby allowing more MPTP to reach the brain. For example, DDC is an inhibitor of the copper containing enzyme, plasma amine oxidase, which oxidizes monoamines such as benzylamine and phenylethylamine (Petterson, 1985) but may also account for some peripheral metabolism of MPTP.

The results also suggest that DDC may alter the elimination of MPP+. The concentration of MPP+ decreases in a time-dependent manner in the STR, VME, and FR of animals given MPTP alone (Fig. 11). Pretreatment with DDC, however, significantly decreases the rate of disappearance of MPP+ from the CNS. The persistence of higher MPP+ levels over time is difficult to attribute to continued production of MPP+ because MPTP was no longer present at the 1 or

34

2 h time points. However, not fully explored is the possibility that continued production of MPP+ may come from some "sequestered" source of MPDP+, which is not detected as such by the assay methods used here. It may be that DDC inhibits alternate pathways in the metabolism of MPDP+ and thus allows more of the compound to be oxidized to MPP+. The administration of DDC has been shown to result in the formation of mixed disulfides with plasma and tissue proteins (Stromme, 1965). The possibility also exists that DDC may compete with or inhibit, perhaps by its interaction with protein thiols, some "carrier" system which normally facilitates the clearance of MPP+ from the CNS.

In a concentration-dependent manner, DDC also increased the rate of MAO B catalyzed biotransformation of MPTP in brain homogenates. At the highest concentration used DDC (0.5 mM) produced nearly a 60 percent increase in the rate of MPP+ production (Fig. 12). It appears that DDC may somehow interact with MAO B to result in apparent enzyme activity enhancement since deprenyl prevented the DDC-induced apparent increase in activity. Incubations of partially purified MAO B with DDC and MPTP confirmed that DDC increased the rate of oxidation of MPTP to MPDP+ (Fig. 7) . The results from these experiments suggest that DDC enhancement of MPDP+ formation does not appear to involve protection against the irreversible inactivation of MAO B by MPTP (Fig. 9) or the prevention of nonenzymic MPDP+ oxidation (Fig. 8). To confirm that protection of MAO B against irreversible inhibition by MPTP is not involved, additional work may include the investigation of MAO B activity in brain homogenates of animals treated with MPTP in the presence and absence of DDC. If MAO B activity is similar in DDC-pretreated animals as in animals given MPTP alone, this would provide further evidence that protection against enzyme inhibition is not a factor in DDC mediated potentiation of MPTP neurotoxicity.

Although DDC appears to stimulate MAO B activity in MPTP biotransformation and neurotoxicity, the mechanism of this interaction remains unknown. DDC is a known chelating agent. It has been suggested that the removal of metals by chelation could reduce the formation of free radicals resulting in increased levels of peroxide. This action could be important in view of the recent demonstration that hydrogen peroxide in the absence of catalase inhibition appears to stimulate MAO B activity (Konradi et al., 1986). From a few determinations in our laboratory, the addition of 10, 100, and 500 μM H_2O_2 to incubations of purified MAO B and MPTP did not seem to affect the rate of

MPDP+ production (data not shown; rate difference from control was insignificant). However, no attempt was made to determine the amount of catalase (if any) present in the partially purified enzyme preparation. Determining whether or not the ability to increase MAO activity is shared by other chelating agents or free radical scavenger may help clarify the role of metals and free radical species in MAO activity and regulation.

Changes in the biodisposition and neurotoxicity of MPTP after DDC pretreatment parallel those which are seen in the natural process of aging (Ricaurte et al., 1987; Langston et al., 1987b). The administration of MPTP to older animals (8-12 months) results in greater DA depletion and higher concentrations of MPP+ in the CNS compared to results from equivalent doses to young animals (6-8 weeks). Furthermore, homogenates of brain tissue from older animals exhibit enhanced metabolism of MPTP to MPP+ (likely due to higher levels of brain MAO) and this can account, at least in part, for the increased susceptibility of older animals to MPTP. In view of these results, the effects of DDC appear to make a young mouse as sensitive as an old mouse to the neurotoxic effects of MPTP. It is possible, therefore, that DDC may also provide an useful tool for studying the age-related effects of MPTP.

INHIBITION OF SYNAPTOSOMAL DOPAMINE UPTAKE BY THE 3,4-DIHYDRO-2-METHYL-9-H-INDENO[2,1-C]PYRIDINIUM (DMIP+) SPECIES, A RIGID ANALOG OF THE 1-METHYL-4-PHENYL-2,3-DIHYDROPYRIDINIUM (MPDP+) SPECIES

Introduction

Recent studies have shown that MPP+, the MAO B catalyzed four-electron oxidation product of MPTP, is selectively accumulated in the dopamine (DA) containing nerve terminals of the nigrostriatal system (Chiba *et al.*, 1985a; Javitch *et al.*, 1985; Heikkila *et al.*, 1985c). The uptake of DA and MPP+ have been reported to have similar K_m and V_{max} values (Javitch and Snyder, 1985; Shen *et al.*, 1985). Furthermore, DA is a potent and competitive inhibitor of the uptake of [^{3}H] MPP+ (Chiba *et al.*, 1985a). These common characteristics plus the fact that other neurotransmitters do not share these same properties have led to the conclusion that MPP+ and DA are transported by the same neuronal DA uptake system and that the neuronal specificity seen in MPTP toxicity may be due to the specific accumulation of MPP+ by this uptake system.

Previous results have shown that the dihydropyridinium species MPDP+ is more than just a transient intermediate *in vivo* after MPTP administration (Shinka *et al.*, 1987). It has been suggested that the potentially reactive MPDP+, in common with other known iminium species (Castagnoli *et al.*, 1985; Ward *et al.*, 1982), may alkylate specific proteins in brain cells to form drug-protein covalent adducts and result in neurotoxicity. Radiolabeled MPTP has been observed to covalently bind to tissue proteins in rat and monkey brain homogenates, apparently through the action of MAO B and the subsequent alkylation of the metabolic product to specific proteins (Corsini *et al.*, 1986). MPDP+ has also been shown to interact with the pigment neuromelanin found in dopaminergic substantia nigra neurons to form MPP+ (Wu *et al.*, 1986). Because MPDP+ is a potentially reactive metabolite (with a measurable half life), which may be directly or indirectly involved in the mechanism of MPTP neurotoxicity, it would be useful to evaluate its role as a substrate for the the dopamine uptake system. However, since MPDP+ itself is subject to autoxidation, disproportionation, and self-condensation at high concentrations, an analog of the dihydropyridinium species was used.

The methylene bridged 3,4-dihydro-2-methyl-9-H-indeno[2,1-C]pyridinium species (DMIP+, 18) is the MAO B catalyzed two-electron oxidation product from the tetrahydroindeno derivative, the 1,2,3,4-tetrahydro-2-methyl-9-H-indeno[2,1-C]pyridine (19) (Booth *et al.*, in press). DMIP+ is formed from the tetrahydroindenopyridine at about 1 percent the rate observed for the corresponding formation of MPDP+ from MPTP. Unlike MPDP+ which undergoes autoxidation and disproportionation at concentrations greater than 200 μM, the DMIP+ species is remarkably stable. A 100 μM solution of DMIP+ in pH 7.2 phosphate buffer showed no evidence of decomposition at 25° C after 15 h and a 10 mM solution was stable under the same conditions for at least 4 h. The enhanced stability of DMIP+ compared to MPDP+ in part may be due to the ring strain present in the dihydroindenopyridine free base 20 which is likely to be an intermediate in the oxidation of DMIP+ to 2-methyl-9-H-indeno[2,1-C]pyridinium (MIP+, 21).
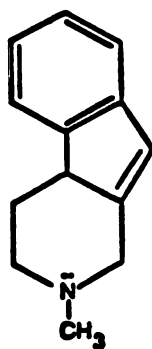
19
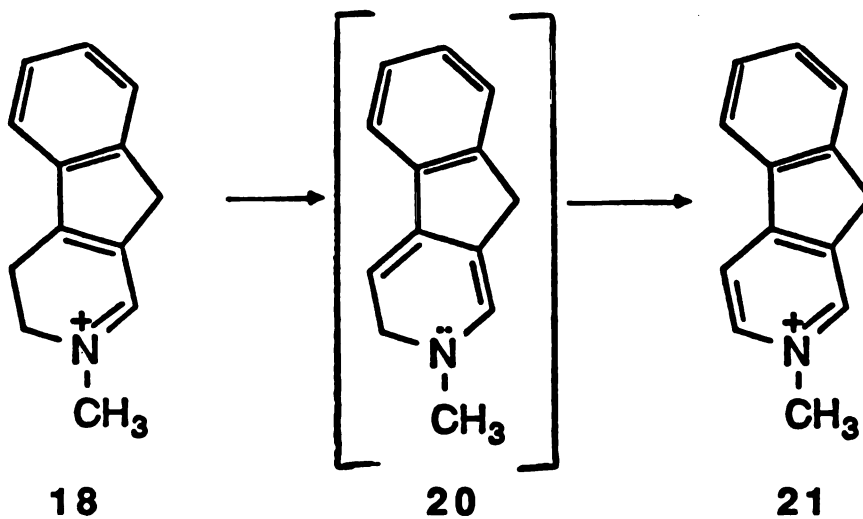
18   20   21

The direct assessment of the substrate properties of a compound for the mouse striatal synaptosomal uptake system would require a radiolabel(s) on the compound. Hence, the capability of DMIP+ and MIP+ to act as substrates was indirectly assessed by measuring their ability to inhibit the synaptosomal uptake of [^{3}H] DA and [^{3}H] MPP+. As part of these studies the parameters for the striatal synaptosomal uptake of these two radiolabeled ligands were first established.

Results

The following experiments were conducted with E. A. Johnson.

A variety of problems were initially encountered in running the striatal synaptosomal uptake assay. The following points are detailed as most critical for the success of the assay: (1) Fiberglass (Whatman GF/B) filters gave minimal nonspecific binding of radiolabeled substrate to the filter compared to other filters tried. (2) Freshly prepared synaptosomal suspensions (from the brains of male C57 Bl mice, 10-12 weeks old) were required for each new experiment to ensure reproducibility. (3) Because of suspension problems (settling of protein), the synaptosomal preparations required gentle resuspension prior to the withdrawal of a sample for incubation. (4) Control active uptake incubations (see Experimental section) were conducted throughout each experiment to monitor any loss of uptake activity. Control active uptake values (n = 15-18) were averaged for an entire 4-6 h experiment and used in the final calculations of uptake parameters and IC_{50} values.

Under the above conditions we were able to obtain the uptake parameters for [^{3}H] DA and [^{3}H] MPP+ seen in Table II and Fig. 13. These results are in good agreement with values previously reported (Javitch *et al.*, 1985; Chiba *et al.*, 1985a; Shen *et al.*, 1985).

A.

B.

Double Reciprocal Plot of the Specific Uptake of [³H] Dopamine
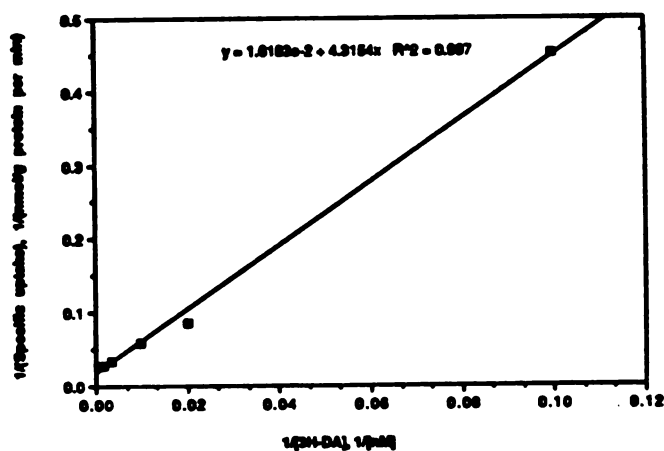
Double Reciprocal Plot of the Specific Uptake of [³H] MPP⁺
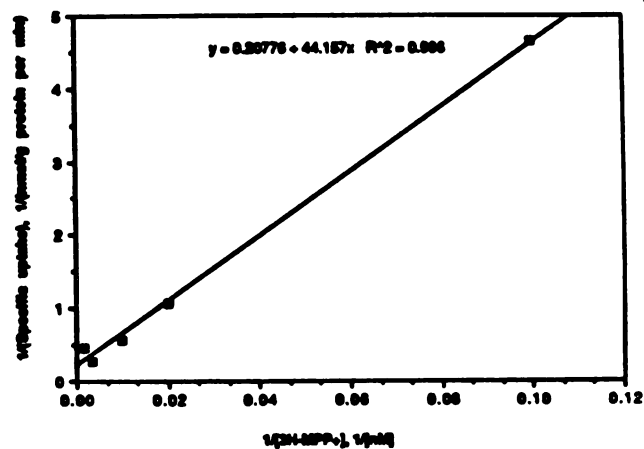
Fig. 13. Double reciprocal plot of the uptake of **A.** [³H] Dopamine and **B.** [³H] MPP⁺ by brain synaptosomal preparations from the C57 Bl mouse (male, 10-12 wk) . Both radiolabeled compounds (0.01-1.0 mM) were incubated at 37° C for 5 min with synaptosomal protein (~0.3 mg). "Background binding" was determined by separate incubations containing 10 mM mazindol as well as the radiolabeled compound and synaptosomes. Each point is the mean of three experiments. Kinetic parameters K_m and V_{max} are given in Table II.

TABLE II. **Kinetic Parameters for the Mouse Striatal Synaptosomal Uptake of [³H] Dopamine ([³H] DA) and [³H] MPP⁺**

	[³H] DA	[³H] MPP⁺
K_m	264 nM	212 nM
V_{max}	61 nmol/g·min	4.8 nmol/g·min

40

Table III summarizes the IC$_{50}$ values for the inhibition of the uptake of [3H] DA and [3H] MPP+ by DMIP+ and MIP+. Unlabeled DA and MPP+ are included as reference compounds. Unlabeled DA is the most potent inhibitor of [3H] DA uptake while unlabeled MPP+ is the most potent inhibitor of [3H] MPP+ uptake. DMIP+ does not appear to be an extremely effective inhibitor of either [3H] DA or [3H] MPP+ synaptosomal uptake. MIP+ is a somewhat good inhibitor of [3H] MPP+ uptake but not of [3H] DA uptake.

TABLE III. Inhibition of Mouse Striatal Synaptosomal Uptake of [3H] Dopamine ([3H] DA) and [3H] MPP+

Compound	[3H] DA Uptake Avg IC$_{50}$ (± S.E.M.)	Inhibition of [3H] MPP+ Uptake Avg IC$_{50}$ (±S.E.M.)
DA	0.27 (0.21) μM	1.23 (0.37) μM
MPP+	2.70 (0.21)	0.28 (0.03)
MIP+	51.5 (11.9)	4.33 (0.56)
DMIP+	144 (19.3)	178 (84.0)

Each average IC$_{50}$ value is calculated as the average of six determinations at each of four different concentrations of the test compound.

Discussion

Unlabeled DA and MPP+ were included as test compounds in the inhibition of striatal synaptosomal uptake of [3H] DA and of [3H] MPP+. Addition of unlabeled substrate to the same but radiolabeled substrate in the incubation mixture dilutes the specific activity of [3H] DA and [3H] MPP+. This results in an overall apparent competitive inhibition of the synaptosomal uptake of the labeled compound when compared to control. When competitive kinetics are obeyed, the unlabeled compound interacts at the same site as labeled compound, *ie.*, the uptake site. On the other hand, addition of unlabeled substrate to the same but radiolabeled substrate increases the total substrate concentration in the incubation mixture and therefore increases the overall rate of synaptosomal uptake. The combined results of these competing effects are expressed in the following equation (Segel, 1975):

$$a = \frac{V_I}{V_o} = \frac{K_m + [S^*]}{K_m + [S^*] + [S]}$$

where V_I is the velocity of uptake in the presence of unlabeled plus labeled substrate, V_o is the velocity of uptake in the presence of undiluted labeled substrate or the "control active uptake" value, $[S^*]$ is the concentration of labeled substrate, and $[S]$ is the concentration of unlabeled plus labeled substrate. Since the K_m for the uptake of both [3H] DA and [3H] MPP+ can be determined and $[S^*]$ is known, the theoretical IC_{50} ($[S]$ required for apparent 50 percent uptake inhibition) can be calculated by setting a equal to 0.5.

For the synaptosomal uptake of [3H] DA a theoretical IC_{50} of 274 nM for unlabeled DA was calculated with the above equation. The actual IC_{50} determined from experimental data was 270 nM (S.E.M 50). In the case of [3H]MPP+ synaptosomal uptake, the theoretical IC_{50} value for uptake inhibition by cold MPP+ was 213 nM and the actual IC_{50} value was 280 nM (S.E.M. 30). The good agreement of experimental data with theoretically predicted values implies that, in our hands, the synaptosomal uptake systems obeys Michaelis-Menten competitive kinetics. Not all other reported results, however, agree with the theoretically predicted values. For example, Shen *et al.* (1985) reported a K_m value of 200 nM for [3H] MPP+ uptake. The theoretical IC_{50} value for inhibition of synaptosomal [3H] MPP+ uptake by unlabeled MPP+ can be

42

calculated for their system to be 200.5 nM. Their experimental IC_{50} value, however, was reported to be 20 nM--an order of magnitude lower than the theoretical value.

The tricyclic dihydroindenopyridinium compound, DMIP+ (a rigid analog of MPDP+), was used to probe the possible role of MPDP+ in synaptosomal uptake since DMIP+ is stable whereas MPDP+ is not. The results from the inhibition of [^{3}H] DA and [^{3}H] MPP+ synaptosomal uptake by the dihydroindeno compound indicate that DMIP+ is not a very good substrate for the dopamine uptake system. The additional steric bulk of the methylene bridge at the position beta to the nitrogen in the ring is possibly one of the factors which makes the indenopyridinium (MIP+) species significantly less potent than MPP+ as an inhibitor of [^{3}H] DA uptake and the steric bulk may also cause DMIP+ to be an even less effective inhibitor. The dihydro-oxidation state may also be a factor in making DMIP+ less than half as potent as MIP+ in the inhibition of [^{3}H] DA and [^{3}H] MPP+ uptake. These two compounds share the same general steric characteristics but differ in the number of hydrogens on the pyridinium ring. This difference in oxidation state allows the dihydroindeno compound to lose a proton and form a non-charged structure, the dienamine compound **20**. It may be that the permanent positive charge of the indenopyridinium (MIP+) species is important for optimal inhibitor-uptake site interaction. This has important implications on whether MPDP+ is transported by the dopamine uptake system as it can also lose a proton to adopt a non-charged enamine form (**13**).

If DMIP+ is truly a good structural analog of MPDP+, then the results from this study suggest that MPDP+ most likely does not serve as a substrate for the dopamine uptake system. It should be noted, however, that the lipophilic nature of the free base MPDP could allow it to passively diffuse across a neuronal membrane and thereby gain access to dopaminergic neurons without the assistance of the dopamine uptake system. Once in the dopaminergic neuron, the MPDP (or MPDP+) may interact with specific proteins or other macromolecules. Whether or not MPDP+ is a substrate for the dopamine uptake system, its role in the metabolism and neurotoxicity of MPTP should not be overlooked.

BIODISPOSITION AND NEUROTOXICITY FOLLOWING INTRAVENOUS ADMINISTRATION OF MPTP AND MPDP+ IN THE MOUSE

Introduction

The blockade of MPTP toxicity by MAO B inhibitors such as pargyline and deprenyl demonstrate that either the process or a product of oxidation is required for the expression of neurotoxicity. However, the biochemical events which take place prior to and subsequent to the biotransformation of MPTP as well as the molecular mechanism of its toxicity remain poorly understood. For reasons discussed above a major effort in this field has focused on characterizing the toxic properties of the chemically stable four-electron oxidation product, the pyridinium metabolite, MPP+. On the one hand, intracerebral injections of MPP+ have been shown to cause dopamine depletion and certain behavioral effects similar to parkinsonism (Heikkila et al., 1985b; Bradbury et al., 1986); on the other hand, MPP+ selectivity for dopaminergic neurons is not exclusive (Bradbury et al., 1985; Markey et al., 1984; Johannnessen et al., 1985; Irwin and Langston, 1985). By contrast, little is known about MPDP+, the primary two-electron MAO B generated metabolite of MPTP. Its inherent chemical instability (Chiba et al., 1985b) suggests that this dihydropyridinium species may only be a transient intermediate in the overall conversion of MPTP to MPP+. Yet, at physiological pH, MPDP+ should exist in part as its lipophilic conjugate free base, 1-methyl-4-phenyl-1,2-dihydropyridine (MPDP, 13), which can diffuse across membranes unlike MPP+. Therefore, the biodisposition characteristics and potential interactions of MPDP+ with membrane-bound macromolecules may influence the sequence of events leading to nigrostriatal cell damage. These considerations led us to undertake a series of studies designed to evaluate the in vivo behavior of metabolically generated MPDP+ as well peripherally administered MPDP+ and to measure the resulting neurotoxicity in the mouse model. By studying the biodisposition of MPTP and its metabolic oxidation products in the brain, we can arrive at a better understanding of the "kinetic profile" of which species are present at various time points after MPTP exposure. By studying the biodisposition characteristics and the neurotoxicity of intravenously (i.v.) administered MPDP+

itself, any possible influence on the sequence of events leading to nigrostriatal neuronal death via initial MAO catalyzed two-electron oxidation of MPTP is excluded.

The development of useful animal models of Parkinson's disease is perhaps one of the most important consequences of the discovery of MPTP. In all species of subhuman primates tested so far a parkinsonian syndrome develops within several days after low doses (1-10 mg/kg) of MPTP (Kopin and Markey, 1988). The acute motor impairment effects of MPTP are short-lived (< 1 day); however, movement disorders gradually increase in severity for several weeks and persist for up to nine months. Substantia nigra cell death has been characterized in MPTP-treated monkeys (Langston *et al.*, 1984a; Jacobowitz *et al.*, 1984) and the specificity of the dopaminergic lesion has also been confirmed by extensive measures of neurotransmitters and their synthetic enzymes (Markey *et al.*, 1986). After MPTP treatment there is an initial increase in caudate and putamen dopamine levels which then drop markedly (to less than 10 percent of normal values) by 8 weeks after MPTP administration, concomitant with the disappearance of cell bodies in the substantia nigra (Burns *et al.*, 1983; Forno *et al.*, 1986).

While primates are sensitive to relatively low doses of MPTP, much higher doses are required to produce a lasting (5 months) depletion of dopamine in the mouse brain. In particular, the C57 black strain of mice has been found to be the most sensitive to MPTP-induced neurotoxicity (Heikkila *et al.*, 1984; Hallman *et al.*, 1985). Using the regimen of intraperitoneally (i.p.) injected 30 mg/kg/day of MPTP for 10 days, Heikkila *et al.* (1984b) produced marked cell loss in the mouse substantia nigra, comparable to that observed in primates. Ricaurte *et al.* (1985b, 1986), using the same dosing regimen, determined that in the young mature mouse (6-8 weeks old) MPTP destroys a large number of striatal dopaminergic nerve terminals but, for the most part, does not affect the cell bodies in the substantia nigra. On the other hand, older mice (8-12 months of age) do develop substantia nigral cell loss as well as terminal damage when treated with MPTP. Furthermore, the effects of MPTP on dopaminergic nerve terminals in the young mature mice appear to be transient since decreased striatal dopamine levels are gradually restored starting from 3 weeks after MPTP treatment. This increase in dopamine levels may be due to regeneration of the dopaminergic nerve fiber projections from the cell body.

45

Thus, the use of the older C57 black mouse in MPTP neurotoxicity studies is likely more appropriate.

In undertaking the investigation of the biodisposition of MPDP+ as well as MPTP, we (Shinka *et al.*, 1987) developed a cation-exchange high-performance liquid chromatography (HPLC) diode-array assay which directly can measure MPDP+ extracted from mouse brain tissues--a task not accomplished by other reported assays for MPTP and its metabolites: gas chromatography-mass spectrometry (GC-MS) (Langston *et al.*, 1984c; Shih and Markey, 1986); reversed-phase HPLC with UV (Chiba *et al.*, 1984; Castagnoli *et al.*, 1985), electrochemical (Rollema *et al.*, 1985), or radiochemical (Markey *et al.*, 1984) detection. In evaluating the neurotoxicity of i.v. injected MPDP+ and MPTP, the levels of striatal dopamine in treated mice were measured using an HPLC electrochemical detection assay (Mefford *et al.*, 1980; Heikkila *et al.*, 1984a).

Results and Discussion

A.   HPLC diode-array assay of brain levels of MPTP and its metabolites

The UV and chromatographic characteristics of MPTP, MPDP+, and MPP+ are illustrated with a mixture of standards in Fig. 14 and Fig. 15A. Under these chromatographic conditions, MPP+ and MPDP+ have overlapping retention volumes. As shown in Figs. 14 and 15B, however, MPP+ does not absorb light at 345 nm (the λ_{max} value for MPDP+) and therefore the height of the peak monitored at 345 nm provides a direct measure of MPDP+ extracted from the tissue. Since the chromophore for MPDP+ extends below 295 nm (the λ_{max} value for MPP+) (Fig. 15C), estimations for MPP+ at 295 nm must be corrected by subtracting 20 percent of the height of the 345 nm peak from the height of the 295 nm peak. Although the internal standard, 4-phenylpyridine (4-PP, **22**) and MPTP have similar retention times (Fig. 15A), no UV significant overlap of these two species occurred under these conditions. The lower limits of detection for on-column injections of standard MPP+, MPDP+, and MPTP were found to be 5, 5, and 10 ng, respectively. Additionally, we found that the flavin monooxygenase catalyzed metabolite, MPTP N-oxide (**10**), and the

46

cytochrome P-450 catalyzed product, PTP (11), have retention times of 0.35 and 0.22, respectively (Fig. 15A), and do not interfere with the present assay.
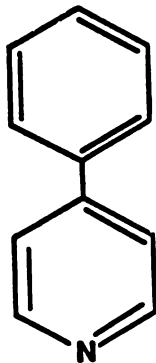
22
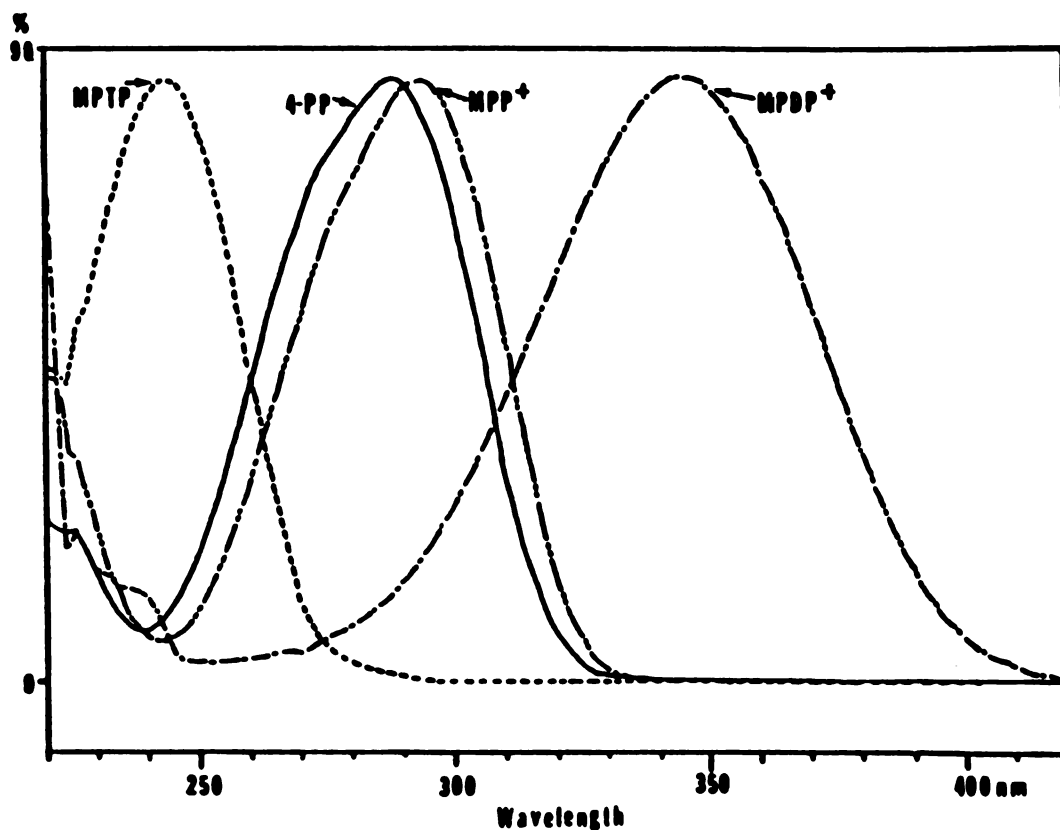
Fig. 14. A composite of the UV spectra obtained with the four analytes by HPLC diode-array analysis.
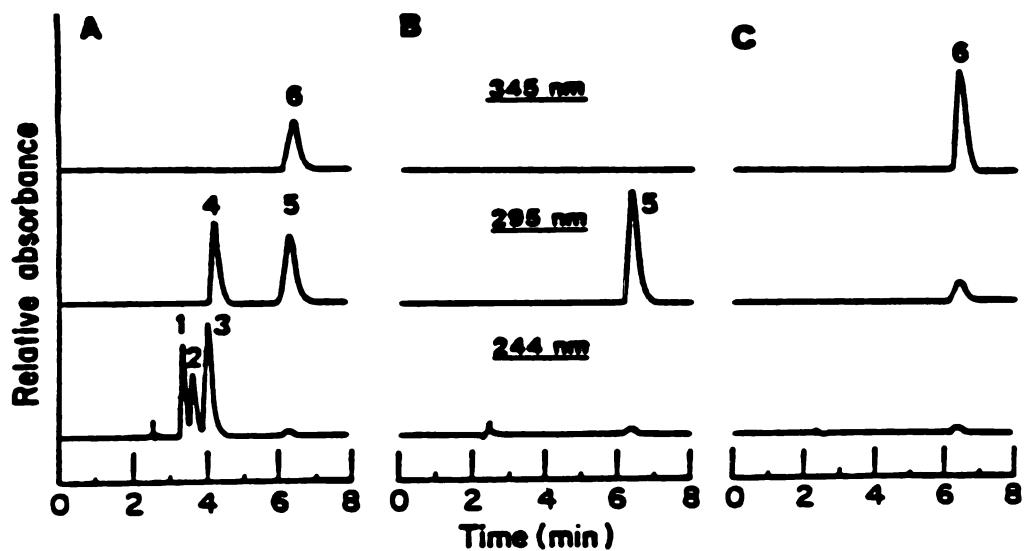
Fig. 15. Liquid chromatographic trace from the injection of a mixture of MPTP and its metabolites. Precolumn: Ultrasil SCX (10 μm, 4 x 10 mm); column: Ultrasil SCX (10 μm, 4.6 x 250 mm); eluent: 90 % 0.1 M acetic acid, 10 % acetonitrile, 0.075 M TEA-HCL, adjusted to pH 2.3 with formic acid; flow-rate: 1.5 mL/min. (A) mixture of PTP (1), MPTP N-oxide (2), MPTP (3), 4-PP (4, internal standard), MPP+ (5), MPDP+ (6). (B) MPP+. (C) MPDP+.

48

A typical chromatographic tracing of a brain extract obtained from a C57 Bl mouse (male, 8-12 months old) treated with MPTP is shown (Fig. 16A). The corresponding "background" tracing obtained with a brain sample isolated from an untreated animal (Fig. 16B) is included to show that no interfering substances are extracted from the tissue.
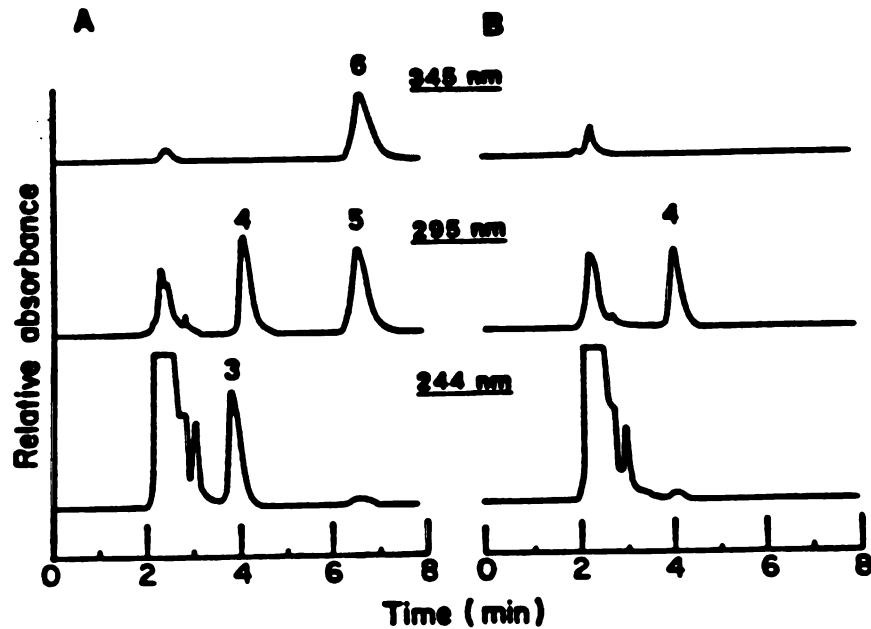
Fig. 16. Liquid chromatographic trace from the injection of an extract from C57 Bl mouse (male, 8-12 mo) brain tissue isolated 30 min after the i.v. administration of 10 mg/kg MPTP (A), or of saline (B). Chromatographic conditions were as described in Fig. 15.

MPDP+ is an unstable compound which, at neutral pH, undergoes autoxidation, concentration-dependent disproportionation, and self-condensation. At low pH, however, MPDP+ is relatively stable. Thus, the 5 percent trichloroacetic acid (TCA) used to precipitate protein in the tissue extract also stabilizes the MPDP+ present. Analysis of extracts of fresh brain homogenates prepared with TCA and spiked with the perchlorate salt of MPDP+ showed no MPP+ or MPTP even after standing at room temperature for 1 h.

The assay was standardized with the aid of calibration curves (Fig. 17) for MPTP, MPDP+, and MPP+, which displayed good linearity. Recovery values for all three compounds averaged about 90 percent. Using this method, 0.1 ng/mg of tissue (0.6 pmol/mg) of MPDP+ and MPP+, and 0.5 ng/mg tissue (3 pmol/mg) of MPTP could be estimated accurately using pooled brain sections isolated from two treated mice.
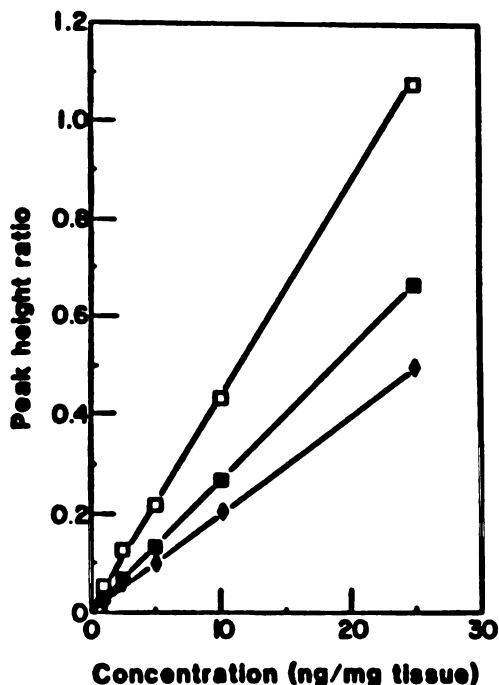
Fig. 17. Calibration curves for MPTP (open squares), MPDP+ (solid triangles), and MPP+ (solid squares). See legend to Fig. 15 for chromatographic conditions.

Table IV lists the time-dependent changes of the concentrations of MPTP, MPDP+, and MPP+ in the various brain regions examined after i.v. administration of MPTP (Table IV A) or MPDP+ (Table IV B) to C57 Bl mice (male, 8-12 months old). In Table IV A the concentrations of MPTP and MPP+ are similar to those published by Rollema *et al.* (1985) and Shih and Markey (1986). MPTP is detectable only at the earliest time point while MPP+ concentrations peak at about 60 min. Also of interest is the apparent localization of metabolically generated MPP+ in the dopamine-containing neostriatum and substantia nigra regions of the brain compared to the cortex.

The results from Table IV B demonstrate that a much smaller amount of MPP+ is found in the brain after the i.v. administration of MPDP+. The levels of MPP+, which were highest at the earliest time point taken (10 min), never reached the levels of MPP+ seen after i.v. MPTP. This may be due to the difficulty of peripheral MPDP+ to enter the brain. Although at the earliest time point very little MPDP+ is detectable in the neostriatum and cortex, a significant amount of MPP+ is seen in all three brain regions, indicating at least partial entry of the injected MPDP+ into the brain. It may be that after tail (i.v.) injections of MPDP+ much of the dihydropyridinium compound is rapidly metabolized before reaching the brain. The neuroglial cells and the endothelial cells surrounding the brain capillaries form tight junctions which severely restrict exchange of water soluble molecules into the central nervous system from the periphery (Bloom, 1985). The result is a blood-brain barrier that allows only slow diffusion of organic acids and bases into the brain and effectively keeps permanently charged species such as the quarternary MPP+ compound out of the central nervous system. On the other hand, possible covalent interactions with macromolecules (peripheral or central) may effectively make MPDP+ unavailable if these interactions are not disturbed by the extraction procedure of the brain tissue samples. Similar to the results in Table IV A, Table IV B shows that there is an apparent localization of MPP+ in the neostriatum and substantia nigra compared to the cortex after i.v. MPDP+.

TABLE IV. Quantitative analysis of MPTP, MPDP+, and MPP+ (pmol/mg tissue) in C57 Bl mouse (8-12 mo) brain neostriatum (NS), substantia nigra (SN), and cortex (C) following i.v. administration of A. MPTP (10 mg/kg), and B. MPDP+ (10 mg/kg).

Time (min)	MPTP			MPDP+			MPP+		
	NS	SN	C	NS	SN	C	NS	SN	C
A.[a]									
10	19.5	21.5	19.2	35.1	32.1	24.4	18.1	19.4	13.5
	21.1	19.1	17.7	35.7	33.9	24.7	14.4	17.1	10.9
30	0	0	0	8.2	4.6	3.5	35.8	29.4	16.8
	7.7	4.5	6.0	16.8	7.0	7.6	38.8	35.9	24.0
60	0	0	0	1.2	0	0	37.9	29.7	17.8
	0	0	0	4.0	1.6	1.5	47.1	35.0	22.4
120	0	0	0	0	0	0	22.2	13.2	6.8
B.									
10	--	--	--	3.5	0	3.0	6.5	27.1	5.3
30	--	--	--	0	0	0	8.5	7.4	4.1
60	--	--	--	0	0	0	5.00	3.5	2.1

[a]The values from two separate experiments are given for the t = 10, 30, and 60 min time points in part A.

Results from these studies establish that MPDP+ is not a transient metabolite of MPTP. Although it appears that neither metabolically generated nor peripherally introduced MPDP+ accumulates in the mouse brain, the relatively high brain concentrations found at 10 min after i.v. MPTP suggests that the rate limiting step in the conversion of MPTP to MPP+ is that involving the

oxidation of MPDP+ to MPP+. This is consistent with the reported relative rates (about 30:1) (Trevor *et al.*, 1986) of MAO B catalyzed conversion of MPTP to MPDP+ versus MPDP+ to MPP+ . It is also of interest to note that the half lives of all three compounds in the mouse brain appear to be quite short, which may account for the high doses of MPTP required to cause nigrostriatal lesions in this species (Johannessen *et al.*, 1985). Comparison of these biodisposition characteristics with those in animals known to be very sensitive to MPTP could lead to a better understanding of the remarkable species selectivity associated with the neurotoxic properties of this compound.

B.   HPLC electrochemical assay of striatal dopamine levels after intravenous injection with MPTP and MPDP+

The effects of tail i.v. administration of MPTP and MPDP+ on striatal dopamine levels in mice are summarized in Table V. Consistent with earlier findings (Heikkila *et al.*, 1984), a significant reduction in striatal dopamine was observed at 24 h after the final injection of MPTP. In contrast, a similar dosing regimen using MPDP+ did not significantly affect the dopamine levels.

TABLE V.   Levels of Dopamine ±S.E.M. (ng/mg tissue) in Mouse Striatum [a] After Administration of MPTP or MPDP+

CONTROL	MPTP	MPDP+
14.07 ± 2.29	2.75 ± 0.41	13.91 ± 0.50

[a] C57 black mice (male, 8-12 mo) were injected intravenously with 10 mg/kg MPTP (n = 6) or MPDP+ (n = 6) dissolved in Intralipid-ethanol (90:10) once daily for 3 d. Control mice (n = 12) were injected or not injected with vehicle only. Since dopamine concentrations in these animals did not differ, both groups were combined and considered as controls. All animals were killed 24 h after the final injection.

In light of the above biodisposition characteristics (see section A) of peripherally administered MPDP+, these neurotoxicity (or rather, lack of) results are not surprising. It had been hoped that i.v. injections (compared to intraperitoneal injections where no MPDP+ or MPP+ was detected in the brain; data not shown) would help "conserve" MPDP+ from peripheral oxidation and allow it to enter the brain. However, these results indicated that, at these doses, apparently not enough MPDP+ reaches the brain to cause damage to the striatal dopamine terminals. A higher dose of MPDP+ may be tried but that would lead to problems, such as autoxidation and disproportionation, seen with high concentrations. It would appear, then, that a better method for the administration of MPDP+ to the brain is required to evaluate the neurotoxicity of MPDP+. *In vivo* brain dialysis, a technique which allows the intracerebral administration of known amounts of compounds for specified time periods and the simultaneous monitoring of their effect on neurotransmitters in discrete brain areas (Ungerstedt *et al.*, 1987; Westerink *et al.*, 1987), may provide the answer. However, the high concentrations required for the perfusion solutions may provide complications as the resulting autoxidation/disproportionation/self-condensation of MPDP+ may make interpretation of the data difficult (Rollema, personal communication).

IN VITRO METABOLIC STUDIES ON THE NIGROSTRIATAL TOXIN MPTP AND ITS MAO B GENERATED DIHYDROPYRIDINIUM METABOLITE MPDP+

Introduction

It has been well-documented that MAO B catalyzes the α-carbon two-electron oxidation of MPTP to form the dihydropyridinium MPDP+ species, which then undergoes a second two-electron oxidation to the pyridinium species MPP+ (Chiba *et al.*, 1984; Castagnoli *et al.*, 1985). At the time of our studies, however, relatively little was known about other enzyme systems which may contribute to the biological fate of the nigrostriatal toxin MPTP. Using gas chromatography-electron impact mass spectrometry techniques (GC-EIMS), Baker *et al.* (1984) identified the metabolites 1-methyl-4-phenyl-5,6-dihydro-2-pyridone (**23**) and 1-methyl-4-phenyl-2-pyridone (**24**) in mouse liver 10,000 x g supernatant incubation mixtures containing 1 mM MPTP and an NADPH-generating system. The authors suggested that these lactam species may be formed from the aldehyde oxidase (a soluble liver enzyme) catalyzed oxidation of MPDP+ and MPP+, respectively. However, since MAO is localized in mitochondria which sediment at 10,000 x g during tissue fraction preparation (Schnaitman and Greenawalt, 1968), their suggestion implies that enzymes other than mitochondrial MAO may catalyze the conversion of MPTP to MPDP+. Yet, results from our previous studies demonstrated that rodent liver microsomes do not convert low concentrations (100 μM) of MPTP to MPDP+ (Weissman *et al.*, 1985). Under these conditions the NADPH-dependent liver microsomal metabolism of MPTP leads to the cytochrome P-450 demethylated product, the 4-phenyl-1,2,3,6-tetrahydropyridine (PTP, **11**) and the flavin monooxygenase catalyzed product, the MPTP N-oxide (**12**).

55
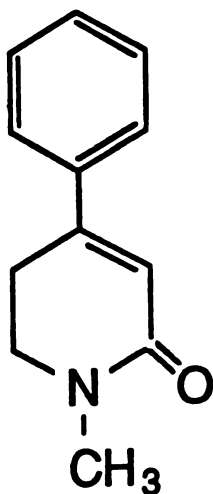
23
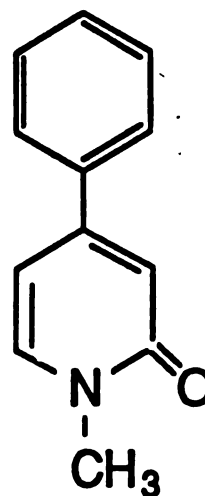
24

DiMonte *et al.* (1987) reported that isolated rat hepatocytes convert MPTP to MPDP+, MPP+, PTP, and MPTP N-oxide. In the hepatocytes MPDP+ is converted exclusively to MPP+ and this transformation is not inhibited by MAO A or MAO B inhibitors (DiMonte *et al.*, 1988). The principal fate of MPDP+ in the brain also appears to be its two-electron oxidation to MPP+ (Markey *et al.*, 1984; Shinka *et al.*, 1987). While we have shown that the pigment neuromelanin (present in the target cells of the primate substantia nigra) oxidizes MPDP+ to MPP+ (Wu *et al.*, 1986), apparently via direct electron transfer from the dihydropyridine free base **13** (Peterson *et al.*, 1985), enzyme systems which may catalyze this conversion have not been well characterized. Although MPDP+ may be a substrate for MAO B (Singer *et al.*, 1986) estimation of the catalytic rate of this interaction is complicated by the slow turnover of the substrate, its mechanism-based inactivation of MAO (Singer *et al.*, 1986), and the tendency for MPDP+ to undergo autooxidation (Korytowski *et al.*, 1987), disproportionation (Peterson *et al.*, 1985), and self-condensation (Leung *et al.*, in press).

Initially, it had been hypothesized that the unique species selectivity of MPTP was due to species differences in peripheral metabolism with the resultant failure of free MPTP to reach the brain. However, studies with radiolabeled MPTP indicated that MPTP reached the brain in all species very rapidly (Markey *et al.*, 1984; Johannessen *et al.*, 1985). It was in the relative

56

rates of disappearance of radioactivity from brains of animals administered [³H] MPTP that species differed (Johannessen *et al.*, 1985). There is also much specificity in the tissue localization of radioactivity after [³H] MPTP administration. Within the brain the highest concentrations of radioactivity lie within the locus coeruleus, caudate, putamen, and nucleus accumbens (Markey *et al.*, 1984). Although the major metabolite found in the brains of MPTP-treated animals has been MPP+, a small amount of unidentified MPTP metabolites isolated from brain tissues has been detected (Johannessen *et al.*, 1986b). This discovery along with the unusual cell, tissue, and species selectivity of MPTP argued for a more thorough characterization of its metabolic profile. In the following experiments (Wu *et al.*, 1988) the fates of MPTP and MPDP+ with a variety of mouse brain and liver tissue preparations were quantitatively studied. MPP+ proved to be stable in all tissue preparations examined. The effect of substrate concentration on the metabolism of MPTP in mouse liver and brain homogenates was also investigated.

Results

A. Chemical Decomposition of MPDP+

In preparation for our metabolic studies on MPDP+, we examined the stability of this dihydropyridinium species in pH 7.6 buffer so that enzyme-mediated and spontaneous chemical reactions could be differentiated. Kinetic and product analysis using the published HPLC-diode array assay (Shinka *et al.*, 1987) established that 5 mM MPDP+ is converted via a combination of disproportionation and autoxidation to yield a 3 to 1 mixture of MPP+ and MPTP (Fig. 18A). The stability of MPDP+ was found to increase with decreasing pH. At pH 7.6 the half life was 25 min while at 6.8 it was 70 min and at 6.3 about 220 min. At 50 μM only autoxidation occured since no MPTP could be detected in the incubation mixture (Fig. 18B). The extrapolated half life for 50 μM MPDP+ was about 240 min at pH 7.6 and > 300 min at pH 6.8. Kinetic studies on the metabolism of MPDP+ were conducted at a substrate concentration of 50 μM to minimize the contribution of spontaneous chemical transformations.

CONVERSION OF 5 mM MPDP+ TO MPP+ AND MPTP AT pH 7.6

CONVERSION OF 50 μM MPDP+ TO MPP+ AND MPTP AT pH 7.6
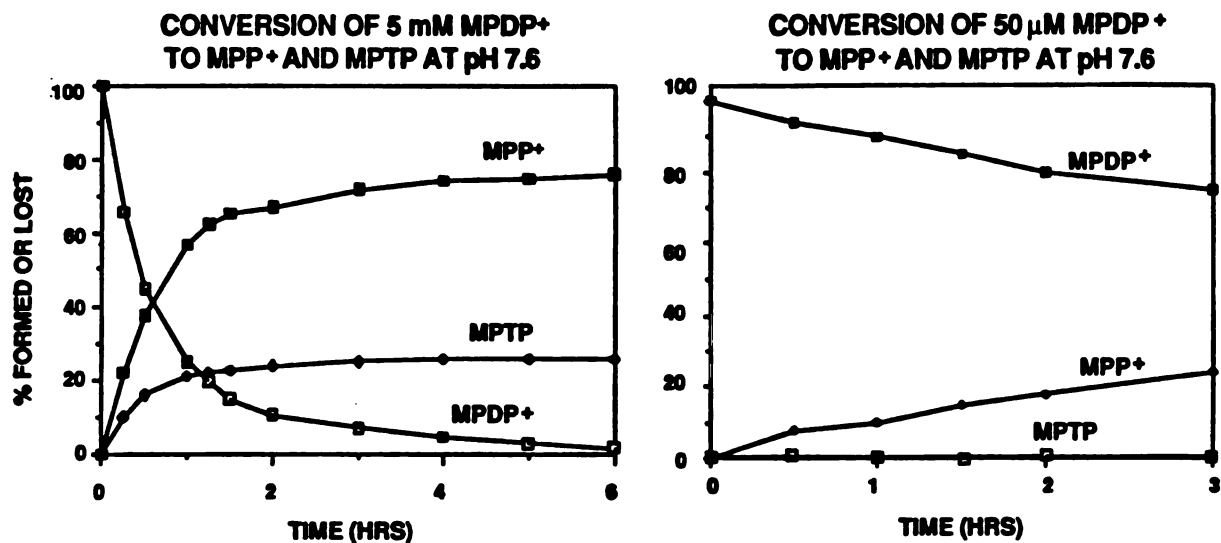
Fig. 18. Time course of the chemical decomposition of MPDP+ at 5 mM (left panel) and at 50 μM (right panel).

B. Brain Studies

Table VI summarizes the results of our studies on the metabolism of MPTP by various C57 Bl mouse (male 10-12 weeks old) brain preparations. Only MPDP+ and MPP+ were detected in the HPLC tracings of the isolates after a 60 min incubation. The mass balance values ranged between 89 and 99 percent. Following the 1 h incubation period, the levels of MPDP+ and MPP+ were comparable in all preparations. The post-mitochondrial supernatant (S_2), microsomal (P_3), and post-microsomal supernatant (S_3) fractions did not metabolize MPTP to any detectable extent.

Table VI. **Percent Yield (± SEM) of MPTP Metabolites Formed with Various C57 Bl Mouse (10-12 wk) Brain Tissue Fractions**

Fraction	Substrate Conc.	%MPTP	%MPDP+	%MPP+	Mass Balance
WholeTissue					
-NADPH	50 µM	65 (5.1)	13 (0.58)	15 (1.5)	93 (4.5)
+NADPH	50 µM	69 (2.6)	17 (1.2)	9 (1.2)	96 (4.5)
-NADPH	1 mM	92 (1.5)	4 (1.5)	3 (0)	99 (1.0)
+NADPH	1 mM	91 (4.0)	2 (0)	3 (1.5)	96 (2.6)
10,000 x g (S₂)					
-NADPH	50 µM	94 (4.6)	ND	ND	94 (4.6)
+NADPH	50 µM	92 (8.1)	ND	ND	92 (8.1)
-NADPH	1 mM	94 (1.2)	ND	ND	94 (1.2)
+NADPH	1 mM	95 (0.58)	ND	ND	95 (0.58)
Mitochondria (P₂)					
-NADPH	50 µM	21 (2.3)	25 (2.3)	44 (3.5)	90 (6.7)
Microsomes (P₃)					
-NADPH	50 µM	89 (3.2)	ND	ND	89 (3.2)
+NADPH	50 µM	93 (3.0)	ND	ND	93 (3.0)
-NADPH	1mM	97 (3.2)	ND	ND	97 (3.2)
+NADPH	1mM	98 (2.1)	ND	ND	98 (2.1)
100,000 x g (S₃)					
-NADPH	50 µM	97 (4.2)	ND	ND	97 (4.2)

Incubations (n = 3) were conducted at 37° C for 60 min at the indicated concentrations in the presence of 2 mg protein/mL of the indicated mouse brain fractions. ND = not detected.

The results summarized in Table VII indicate that MPDP+ is converted exclusively to MPP+ in mouse brain preparations. Whole tissue homogenates and all membrane-containing fractions (S₂, P₂, and P₃) significantly increased the rate of this oxidation (half life *ca.* 60 min) compared to incubations in buffer (half life 240 min). Enzymic activity appears to be involved as heat denaturation of microsomal and mitochondrial fractions reduced significantly the oxidation of

MPDP+ to MPP+. Although purified MAO B is reported to catalyze the oxidation of MPDP+ (Singer *et al.*, 1986), pretreatment of the mitochondria with 30 μM pargyline, a concentration which should inhibit both MAO A and MAO B activity, did not affect the conversion of MPDP+ to MPP+. NADPH was not required for this oxidation and in some preparations was inhibitory. The stability of MPDP+ in mouse brain 100,000 x g supernatant (S_3) fractions is essentially the same as that observed in buffer alone.

Table VII. Percent Yield (± SEM) of MPDP+ Metabolites Formed with Various C57 Bl Mouse (10-12 wk) Brain Tissue Fractions

Fraction	%MPDP+	%MPP+	Mass Balance
Whole Tissue			
-NADPH	32 (7.0)	61 (3.1)	93 (4.5)
+NADPH	47 (1.7)	45 (4.6)	93 (6.2)
10,000 x g (S_2)			
-NADPH	52 (12)	38 (0.58)	89 (12)
+NADPH	60 (8.4)	26 (0)	86 (8.4)
Mitochondria (P_2)			
-NADPH	23 (1.2)	75 (2.3)	97 (3.1)
+Pargyline (30 μM)	25 (1.5)	72 (1.5)	96 (3.1)
Denatured, -NADPH	57 (5.3)	34 (0.58)	91 (5.9)
Microsomes (P_3)			
-NADPH	59 (9.9)	32 (5.5)	91 (4.4)
+NADPH	66 (8.3)	25 (7.8)	91 (2.5)
100,000 X g (S_3)			
-NADPH	76 (13)	15 (11)	98 (1.0)
pH 7.6 Buffer			
-NADPH	83 (5.3)	15 (5.8)	98 (1.2)
+NADPH	85 (3.8)	14 (5.2)	99 (5.1)

Incubations (n = 3, 50 μM) were performed at 37° C for 60 min in the presence of 2 mg protein/mL of the indicated mouse brain fractions or pH 7.6 buffer.

C. Liver Studies

The metabolic profile of MPTP in C57 Bl mouse (male, 10-12 weeks old) liver preparations is considerably more complicated (Table VIII) than that

observed in the brain fractions. The HPLC tracings obtained with whole liver homogenates lacking NADPH revealed a new peak with a retention time of 2.91 min. Diode array analysis displayed a chromophore with λ_{max} 284 nm. The extent of MPTP metabolism by whole liver homogenates and formation of MPP+ were suppressed by NADPH. The 2.91 min peak also was observed in the corresponding NADPH-containing samples although the UV maximum was shifted to 268 nm. Subsequent studies (see below) established that whole liver homogenates convert MPTP to the lactam **23** which, in the presence of NADPH, is oxidized partially to the co-eluting 2-pyridone **24**. These metabolites were not detected in any of the other liver preparations except when 1 mM MPTP was incubated with pargyline-pretreated 10,000 x g supernatant (S$_2$) fractions containing NADPH. Low levels of MPDP+ and MPP+ also were observed in these incubation mixtures as well as in the pargyline-pretreated and NADPH-supplemented 1 mM MPTP (but not 50 μM) microsomal incubation mixtures. The known MPTP metabolites PTP (**11**) and N-oxide **12** were formed in the 1 mM MPTP (NADPH-supplemented) microsomes-containing incubation mixtures but only **12** was found with the 50 μM MPTP (NADPH-supplemented) mixtures.

Similar to the results with corresponding brain preparations, only MPDP+ and MPP+ were detected in mouse liver microsomal and mitochondrial incubation mixtures of MPDP+ (Table IX). Also as found with brain membrane containing preparations, the extent of oxidation of MPDP+ to MPP+ was greater in microsomal and mitochondrial incubation mixtures than in buffer. The liver microsomal and mitochondrial oxidation of MPDP+ to MPP+ may be enzymatically mediated since MPDP+ is more stable when incubated with heat-denatured preparations. The catalase inhibitor 3-amino-1,2,4-triazole (Margoliash and Novogrodsky, 1958), diamine oxidase (Mondovi et al., 1967) and amino acid oxidase inhibitor semicarbazide (Edlbacher et al., 1946) and amino acid oxidase inhibitor sodium benzoate (Klein and Kamin, 1941) had no detectable effect on the process. Liver preparations which contained soluble enzymes--whole tissue, 10,000 x g (S$_2$) and 100,000 x g (S$_3$) fractions-- catalyzed the conversion of MPDP+ to the product(s) which had been observed in whole liver incubation mixtures of MPTP.

Table VIII. Percent Yield (± SEM) of MPTP Metabolites Formed with Various C57 Bl Mouse (10-12 wk) Liver Tissue Fractions

Fraction	Substrate Conc.	%MPTP	%MPDP+	%MPP+	%23 [& 24]ᵃ	%11	%12	Mass Balance
WholeTissue								
-NADPH	50 μM	2 (0.58)	ND	5 (1.0)	83 (5.5)	ND	ND	89 (4.2)
+NADPH	50 μM	32 (9.7)	ND	Trace	[22] (3.8)	ND	38 (11.8)	92 (2.3)
-NADPH	1 mM	77 (8.7)	5 (0.58)	8 (0.58)	4 (0)	ND	ND	93 (8.7)
+NADPH	1 mM	85 (4.2)	2 (0.58)	1 (0.58)	[4] (1.5)	5 (2.9)	3 (1.7)	99 (1.0)
10,000 x g (S₂)								
-NADPH	50 μM	92 (2.1)	ND	ND	ND	ND	ND	92 (2.1)
+NADPH	50 μM	45 (7.2)	ND	ND	ND	ND	51 (5.1)	96 (2.1)
-NADPH	1 mM	97 (2.1)	ND	ND	ND	ND	ND	97 (2.1)
+NADPH	1 mM	86 (4.4)	ND	Trace	[1] (0)	6 (1.0)	4 (1.0)	97 (5.2)
Mitochondria (P₂)								
-NADPH	50 μM	ND	ND	93 (1.5)	ND	ND	ND	93 (1.5)
-NADPH	1 mM	69 (7.2)	11 (5.5)	16 (11)	ND	ND	ND	96 (2.6)
Microsomes (P₃)								
-NADPH	50 μM	96 (3.8)	ND	ND	ND	ND	ND	96 (3.8)
+NADPH	50 μM	Trace	ND	ND	ND	Trace	97 (0.58)	97 (0.58)
-NADPH	1 mM	93 (1.7)	ND	ND	ND	ND	ND	93 (1.7)
+NADPH	1 mM	79 (4.4)	1 (0)	2 (0)	ND	8 (2.9)	9 (2.6)	99 (1.5)
100,000 x g (S₃)								
-NADPH	50 μM	97 (2.3)	ND	ND	ND	ND	ND	97 (2.3)

Incubations (n = 3) were carried out at 37° C for 60 min at the indicated concentrations and in the presence of 2 mg protein/mL of the indicated mouse liver fraction. ND = not detected. ᵃ Numbers in brackets denote rough estimates of the combined percentages of lactam **23** and pyridone **24**.

Table IX. Percent Yield (± SEM) of MPDP+ Metabolites Formed with Various C57 Bl Mouse (10-12 wk) Liver Tissue Fractions

Fraction	%MPDP+	%MPP+	%23 [& 24][a]	%11	%12	Mass Balance
Whole Tissue						
-NADPH	ND	ND	98 (1.5)	ND	ND	98 (1.5)
+NADPH	ND	ND	[85] (7.0)	ND	ND	78 (7.0)
10,000 x g (S2)						
-NADPH	ND	ND	96 (3.5)	ND	ND	96 (3.5)
+NADPH	ND	ND	[87] (2.5)	ND	ND	87 (2.5)
Mitochondria (P2)						
-NADPH	2 (1.2)	93 (7.1)	ND	ND	ND	93 (6.0)
+Pargyline (30 μM)	8 (0.58)	88 (1.5)	ND	ND	ND	96 (1.0)
Denatured, -NADPH	42 (6.7)	47 (3.8)	ND	ND	ND	89 (6.0)
Microsomes (P3)						
-NADPH	8 (5.6)	80 (5.1)	ND	ND	ND	88 (2.1)
+NADPH	14 (6.1)	68 (4.2)	ND	ND	ND	82 (10)
Denatured, -NADPH	72 (3.1)	23 (1.2)	ND	ND	ND	95 (4.7)
100,000 x g (S3)						
-NADPH	ND	ND	95 (3.0)	ND	ND	95 (3.0)
+NADPH	ND	ND	93 (4.2)	ND	ND	93 (4.2)
+Menadione	84 (1.0)	13 (1.5)	ND	ND	ND	98 (1.5)
pH 7.6 Buffer						
-NADPH	83 (5.3)	15 (5.8)	ND	ND	ND	98 (1.2)
+NADPH	85 (3.8)	14 (5.2)	ND	ND	ND	99 (5.1)

Incubations (n = 3, 50 μM) were performed at 37° C for 60 min in the presence of 2 mg protein/mL of the indicated mouse liver fractions or pH 7.6 buffer. ND = not detected. [a] Numbers in brackets denote rough estimates of the combined percentages of lactam **23** and 2-pyridone **24**.

HPLC analysis of a methylene chloride extract of the whole liver homogenate incubation mixture displayed a single peak with diode array UV spectral characteristics (Fig. 19a) consistent with a conjugated eneone such as that present in lactam **23**. The direct insertion probe CI mass spectrum of this HPLC fraction displayed a prominant ion at *m/z* 188 as required for the protonated molecular ion of lactam **23**. The diode array spectra of the corresponding HPLC peak observed in whole liver and 10,000 x g incubation mixtures supplemented with NADPH were shifted slightly to shorter wavelengths (Fig. 19b). NADPH had no effect on the UV characteristics of the metabolic peak generated in the liver 100,000 x g (S₃) fractions.
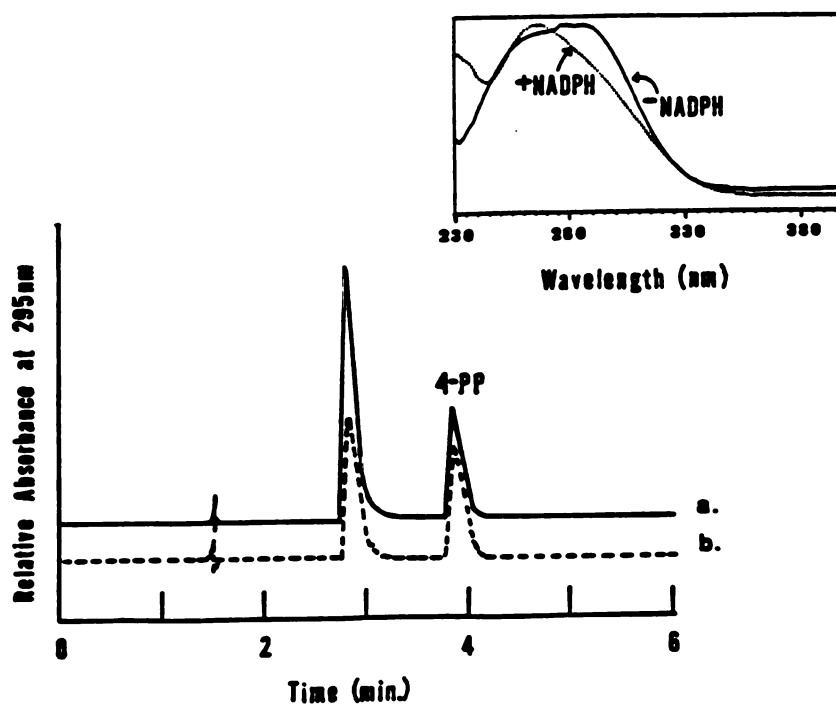
Fig. 19. (a) HPLC diode-array analysis of the methylene chloride extract obtained following a 10-min incubation period of MPDP+ perchlorate (50 μM) with a C57 Bl mouse (10-12 wk) liver homogenate (2 mg protein/mL). (b) The same scan obtained from the corresponding incubation containing NADPH. The internal standard was 4-phenylpyridine (4-PP).

Preparative scale incubations of MPDP+ (500 μM) with rabbit liver 100,000 x g (S₃) fractions led to the formation of the same product which, as expected at these comparatively high concentrations of MPDP+, was accompanied by the formation of MPP+ and MPTP. The metabolite could be extracted from the incubation mixture with ethyl acetate and following centrifugal chromatographic purification was obtained as a colorless, crystalline product. The high resolution probe EI mass spectrum of this product (Fig. 20) displayed the parent ion (M·+) at m/z 187.0997 (calcd. for $C_{12}H_{13}NO$: 187.10159) as the base peak and intense fragment ions at m/z 144 (M-NC_2H_5)+, 116 (M-C_3H_5NO)+ and 115 (M-C_3H_6NO)+ which we have assigned to species I, II, and III, respectively.
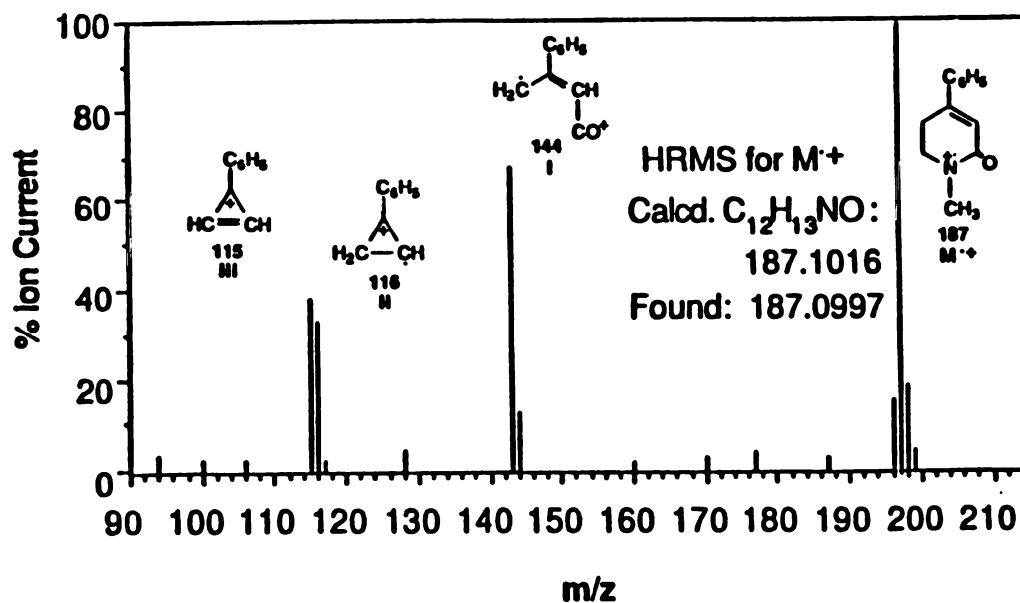
Fig. 20. The EI mass spectrum of the MPDP+ metabolite isolated from the New Zealand White rabbit (3.5 kg) liver 100,000 x g supernatant (S₃) fraction incubation mixture.

65

The 500 MHz ^1H NMR spectrum (Fig. 21) displayed two coupled triplets centered at δ 2.80 and 3.60 ppm as required for the vicinal C-5 and C-6 methylene proton signals of lactam **23**. These spectral characteristics eliminate the 2,6-dihydro-2-pyridone isomer **26** which could result from the oxidation of the corresponding 2,5-dihydropyridinium species **25**, a possible rearrangement product of MPDP+ (Peterson *et al.*, 1985). The metabolically derived product from the incubation of MPDP+ with liver 100,000 x g supernatant fractions gave physicochemical and spectroscopic properties identical to the synthetic 1-methyl-4-phenyl-5,6-dihydro-2-pyridone (**23**).
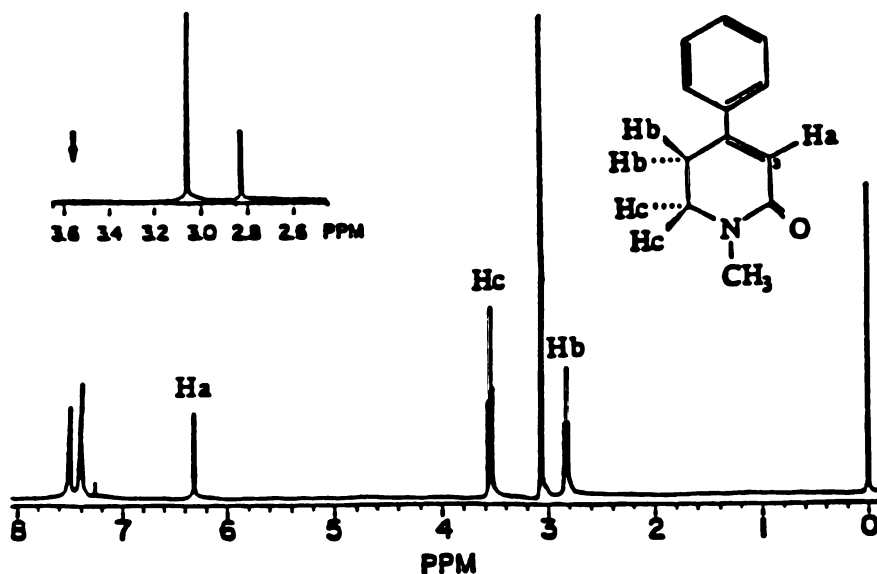
Fig. 21. The 500 MHz ^1H NMR spectrum of the MPDP+ metabolite isolated from the New Zealand White rabbit (3.5 kg) liver 100,000 x g supernatant (S_3) fraction incubation mixture.

66
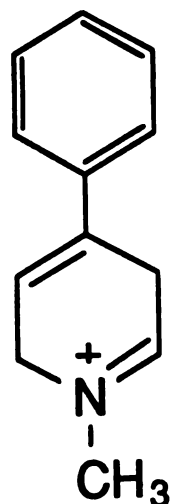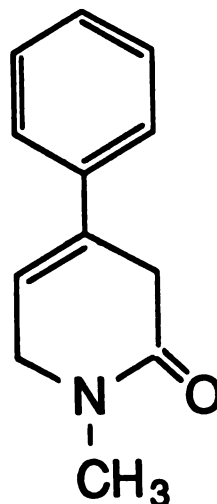
25   **26**

Quantitative estimations of the conversion of MPDP+ to lactam **23** in the 100,000 x g supernatant (S_3) fraction of the mouse liver preparation established that product formation was essentially stoichiometric (Fig. 22). A similar rate of oxidation was observed for the formation of 3-aminocarbonyl-1-methyl-2-pyridone (**28**) from the 3-aminocarbonyl-1-methylpyridinium species (**27**), a known substrate for the liver cytosolic enzyme aldehyde oxidase (Rajagopolan *et al.*, 1962). Furthermore, our results indicate that the addition of 25 μM menadione, a known inhibitor of this enzyme (Rajagopolan *et al.*, 1962), blocked the oxidation of both **27** to **28** and MPDP+ to lactam **23** (Table IX). Therefore, as originally suggested by Baker *et al.* (1984), this transformation is likely to be catalyzed by the molybdenum containing enzyme aldehyde oxidase, the same enzyme which other workers (Brandage and Lindblom, 1979) have described as an iminium ion oxidase based on its ability to catalyze the conversion of the $\Delta^{1',5'}$-iminium metabolite **29** of (S)-nicotine to the lactam (S)-cotinine (**30**).
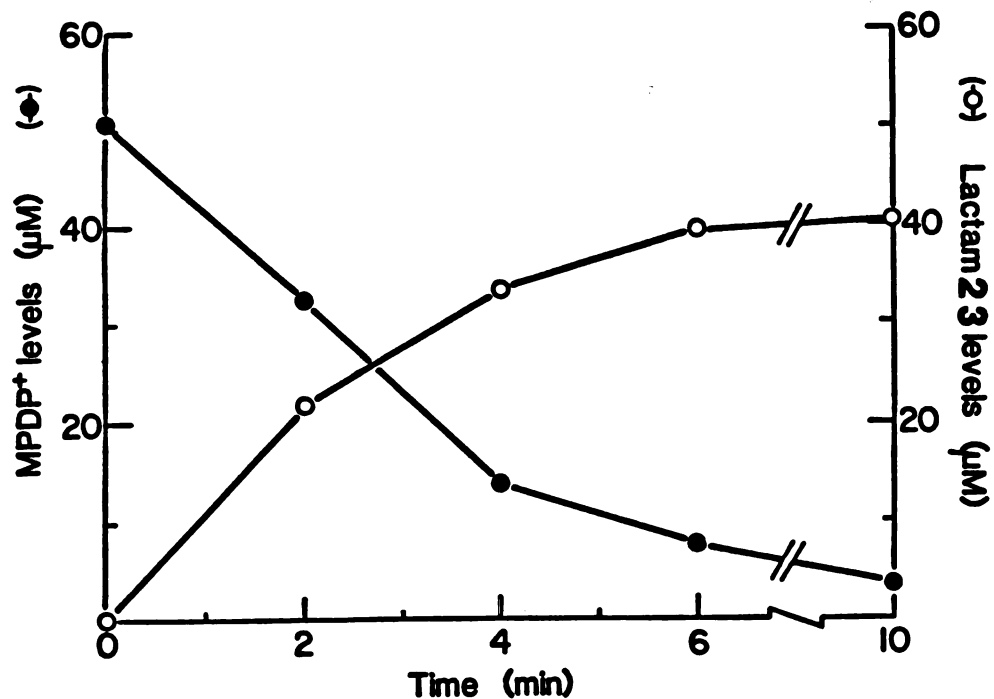
Fig. 22. Kinetic analysis of the metabolic conversion of MPDP+ to lactam **23** by the C57 Bl mouse (10-12 wk) liver 100,000 x g supernatant (S₃) fraction.

27   **28**   **29**   **30**
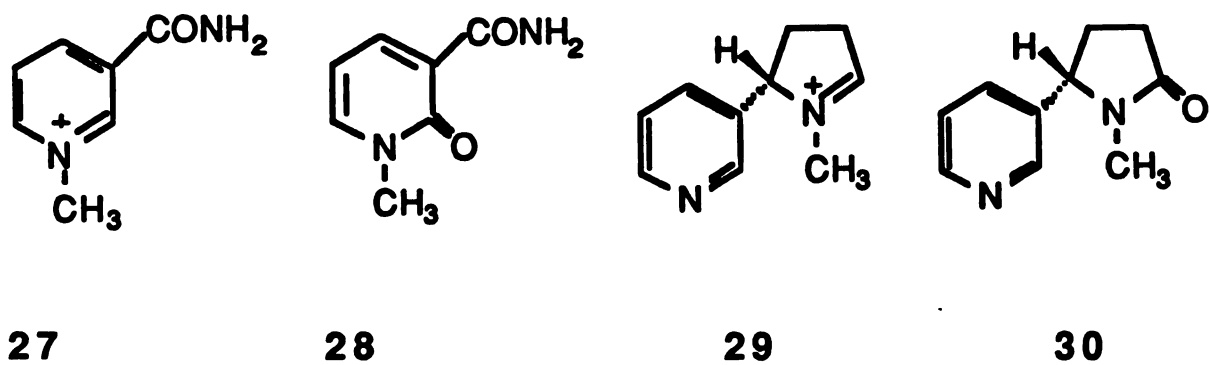
An alternative HPLC system was developed which resolved what proved to be a mixture of lactam **23** and the 2-pyridone species **24** in the NADPH supplemented whole tissue and 10,000 x g incubation mixtures. The structure of the new metabolite was identified by comparison of its HPLC retention time (9.83 min), diode array UV spectral characteristics [λ_{max} 266 and 312 (sh) nm] and CI mass spectrum with the corresponding data obtained with the synthetic 2-pyridone **24**. Since 2-pyridone **24** was not detected in 100,000 x g incubations of MPDP+ or MPP+ and only lactam **23** was detected in the whole tissue and 10,000 x g incubation mixtures lacking NADPH, we examined the fate of this dihydropyridone in mouse liver microsomal incubation mixtures in the absence and presence of NADPH. The results (Fig. 23) document the NADPH dependent conversion of lactam **23** to the 2-pyridone **24**, a reaction which may be catalyzed by cytochrome P-450. In the absence of NADPH, lactam **23** was recovered quantitatively.
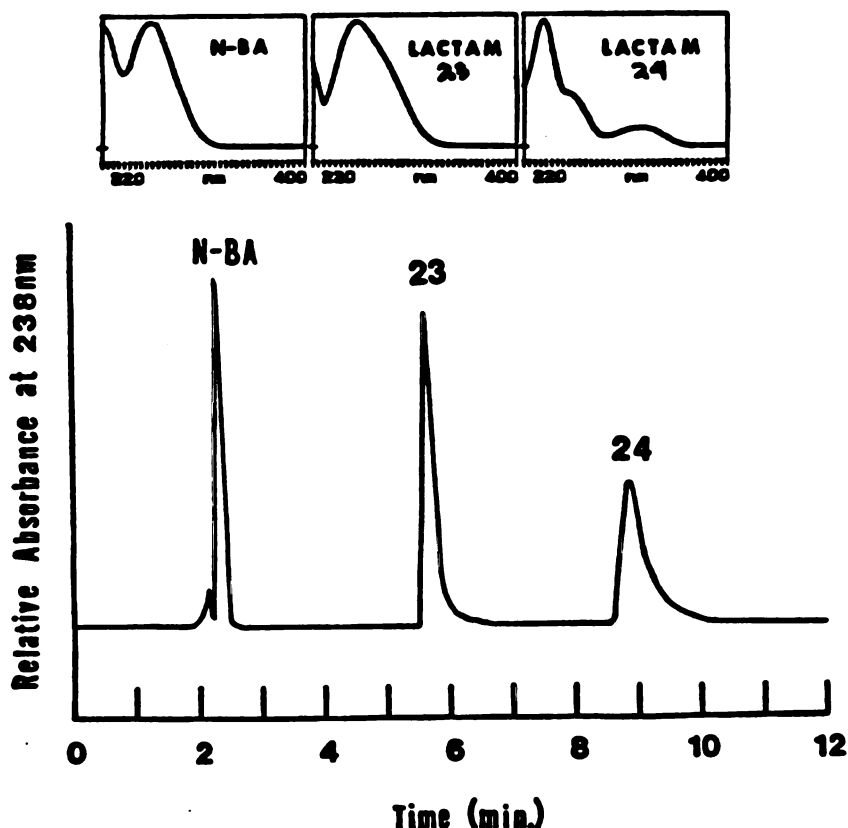
Fig. 23. HPLC diode-array analysis of the hexane extract obtained following a 10-min incubation period of lactam **23** (50 μM) with a C57 Bl mouse (10-12 wk) liver microsomal (P_3) fraction (2 mg of protein/mL) in the presence of NADPH. The internal standard was N-benzoylaniline (N-BA).

Discussion

These studies provide a quantitative picture of the *in vitro* metabolism of MPTP and MPDP+ in mouse brain and liver tissues. In general, the metabolic profile of MPTP is relatively simple in brain tissue as only MPDP+ and MPP+ appear to be produced. The fairly good recoveries suggest that any undetected metabolic or spontaneous chemical pathway will be quantitatively minor. The data are consistent with the generally held view that the conversion of MPTP to MPP+ via MPDP+ is the principal and perhaps exclusive metabolic pathway for this neurotoxin in the central nervous system. These data also show that after the 60 min incubation period the concentration of MPDP+ is comparable to that of MPP+, indicating that the dihydropyridinium compound is not just a transient intermediate in the conversion of MPTP to MPP+. Although current theories focus on the role of MPP+ as the mediator of the toxic effects of MPTP and the toxic effects of MPDP+ are not known, a potential role (such as in transport from glial cells to intraneuronal sites) for the chemically reactive dihydropyridinium species probably should not be overlooked.

Chemical stability studies have confirmed the tendency of MPDP+ to undergo disproportionation and autoxidation. Both of these processes, however, are relatively slow at 50 μM and pH 7.6, the conditions used in our metabolic studies. The data presented in Table VII and IX indicate that the oxidation of MPDP+ to MPP+ may be catalyzed by an enzyme or group of enzymes present in membrane containing structures of the brain and the liver since the amount of MPP+ formed in the 100,000 x g soluble fractions of these tissues is comparable to that formed in buffer. It is possible that the conversion of MPDP+ to MPP+ may be due in part to the presence of non-protein dependent oxidizing species since the dihydropyridinium species is less stable in heat denatured microsomes than in buffer. We also have found that 1 mM hydrogen peroxide does not increase the rate of oxidation of MPDP+ to MPP+ so that this conversion is unlikely to be a consequence of other enzyme mediated oxidations which produce hydrogen peroxide as a by-product. Examination of the effects of NADPH and various known inhibitors of monoamine oxidase, catalase, diamine oxidase and amino acid oxidase have failed thus far to identify an oxidase which might catalyze the conversion of MPDP+ to MPP+.

Variations in the rate of this conversion could contribute to differences in the susceptibility of different tissues and species to the toxicity of MPTP.

The behavior of MPTP in mouse liver preparations is considerably more complex than in the corresponding brain preparations. The present studies have confirmed previous findings that MPTP is converted by NADPH-supplemented liver microsomes to the desmethyl (Weissman *et al.*, 1985) and N-oxide (Cashman and Zeigler, 1986) metabolites. The preferential formation of the N-oxide at the 50 μM concentration of MPTP relative to the comparable levels of the two metabolites formed at the 1 mM concentration indicates that the cytochrome P-450 isozyme(s) which catalyzes the N-demethylation of MPTP may have a relatively high K_m value compared to the flavin monooxygenase which catalyzes the formation of the N-oxide. From these studies we have evidence that NADPH-dependent and pargyline-insensitive liver microsomal enzymes also catalyze the oxidation of MPTP to MPDP+ though, again, only at relatively high substrate concentrations of MPTP. Recently, it was shown that purified cytochrome P-450 isozymes catalyze the oxidation of MPTP at the allylic α-carbon position to form MPDP+ (Ottoboni, personal communication). The possibility that cytochrome P-450 isozymes or related oxidases may catalyze this class of reaction is of interest both in terms of the associated bioorganic reaction mechanisms and the potential for bioactivation of potentially neurotoxic xenobiotics which are structurally related to MPTP.

Our studies have provided unambiguous documentation of the efficient and preferential formation of 1-methyl-4-phenyl-5,6-dihydro-2-pyridone (**23**) from low concentrations (50 μM) of MPTP in whole liver cell homogenates and from MPDP+ (50 μM) in several liver preparations. In the absence of NADPH, the conversion of MPDP+ to the lactam **23** is efficient and quantitative in liver preparations which contain the soluble enzyme fraction. This suggests that the biotransformation of MPDP+ to the lactam **23** is a major hepatic metabolic pathway which will compete with its conversion to MPP+. The stability of MPDP+ in the menadione treated liver 100,000 x g (S_3) fractions confirms the suggestion by Baker *et al.* (1984) that liver aldehyde oxidase catalyzes the conversion of MPDP+ to lactam **23**. Since MPDP+ is stable in mouse brain 100,000 x g (S_3) fractions, this potential detoxification pathway cannot protect the central nervous system against the formation of MPP+.

The suggestion by Baker and colleagues that MPP+ also may be a substrate for liver aldehyde oxidase has not been confirmed. MPP+ proved to

be stable in all of the incubation mixtures examined in this study. The stability of MPP+ in the aldehyde oxidase-containing liver 100,000 x g (S_3) fraction is noteworthy in view of the excellent substrate properties of the 3-aminocarbony-1-methylpyridinium species **27**.

We found that only lactam **23** was present in microsomal-containing incubation mixtures which lacked NADPH whereas both lactam **23** and the 2-pyridone **24** were formed in the presence of NADPH. The possibility that lactam **23** was oxidized to **24** by a microsomal NADPH-dependent oxidase was confirmed by independent studies with synthetic **23** (synthesis conducted by P. Caldera as described in Wu *et al.*, 1988) and mouse liver microsomal preparations. Based on these results and the microsome catalyzed formation of MPDP+ from MPTP, the sequence of events observed by Baker and colleagues is likely to have involved the NADPH-dependent microsomal (not MAO) catalyzed oxidation of 1 mM MPTP to MPDP+ which then is converted to lactam **23** by aldehyde oxidase. Subsequently, lactam **23** will be oxidized by an NADPH-dependent microsomal enzyme to the 2-pyridone **24**.

A summary of the *in vitro* metabolic fate of MPTP is provided in Scheme I. MPTP is an excellent substrate for MAO B which catalyzes its oxidation to the 1,2-dihydropyridinium species MPDP+ both in the liver and in the central nervous system. MPDP+ then undergoes further oxidation to MPP+, possibly via MAO, but also via an unknown, pargyline-insensitive microsomal and mitochondrial heat-sensitive process. Thus far, these are the only oxidative pathways which have been documented in the central nervous system. Liver microsomal enzyme systems, including the cytochrome P-450 and the microsomal flavin-containing monooxygenase system, also catalyze the oxidative N-demethylation and N-oxidation of MPTP to yield the desmethyl metabolite **11** and the N-oxide **12**. Apparently the K_m value of the putative cytochrome P-450 mediated N-demethylation reaction is considerably higher than the corresponding value for N-oxidation. An NADPH-dependent microsomal enzyme also catalyzes the conversion of MPTP to MPDP+ and this process seems to proceed with a high K_m value as well since MPDP+ (and MPP+) are observed only at a high concentration (1 mM) of MPTP. The fate of MPDP+ in liver homogenates is dominated by the enzyme aldehyde oxidase which catalyzes the oxidation of the dihydropyridinium species to the lactam **23**. This dihydropyridone then is further oxidized to the 2-pyridone **24** by a microsomal NADPH-dependent oxidase.

Due to the combined activities of cytochrome P-450 N-demethylase, microsomal flavin-containing monooxygenase, and aldehyde oxidase, it was not surprising that very little MPP+ was observed in whole liver cell homogenate incubations with low concentrations (50 μM) of MPTP or MPDP+. However, earlier studies (DiMonte *et al.*, 1987) using incubations of freshly prepared rat hepatocytes with high concentrations (1.5 mM) of MPTP or MPDP+ resulted mainly in MPP+ production with no lactam **23** or 2-pyridone **24** detected. These observations suggest that the metabolic fate of MPDP+ is highly dependent upon the intracellular environment to which it is exposed and that the metabolic reactions involving MPTP and MPDP+ observed in subcellular fractions may not be the same as those pathways followed in the intact cell. In our studies relatively low yields of **23** and **24** were observed in incubations of NADPH-supplemented whole liver cell homogenates with high concentrations (1 mM) of MPTP compared to low concentrations of the compound (Table VIII). This difference may reflect an important concentration dependency on the *in vivo* metabolism of MPTP by liver enzymes. Furthermore, the competition of enzymes for MPTP (MPDP+) may be different in the hepatocyte (where all necessary cofactors are available) compared to the isolated subcellular fractions. Work utilizing *in vivo* studies on the biotransformation of MPTP in the mouse and other species (see pp. 75-86) will help elucidate the overall metabolic fate of this neurotoxic amine.

Scheme I. Proposed Overall Scheme Summarizing the *In Vitro* Metabolic Fate of MPTP with C57 Bl Mouse (10-12 wk) Liver Preparations[a]
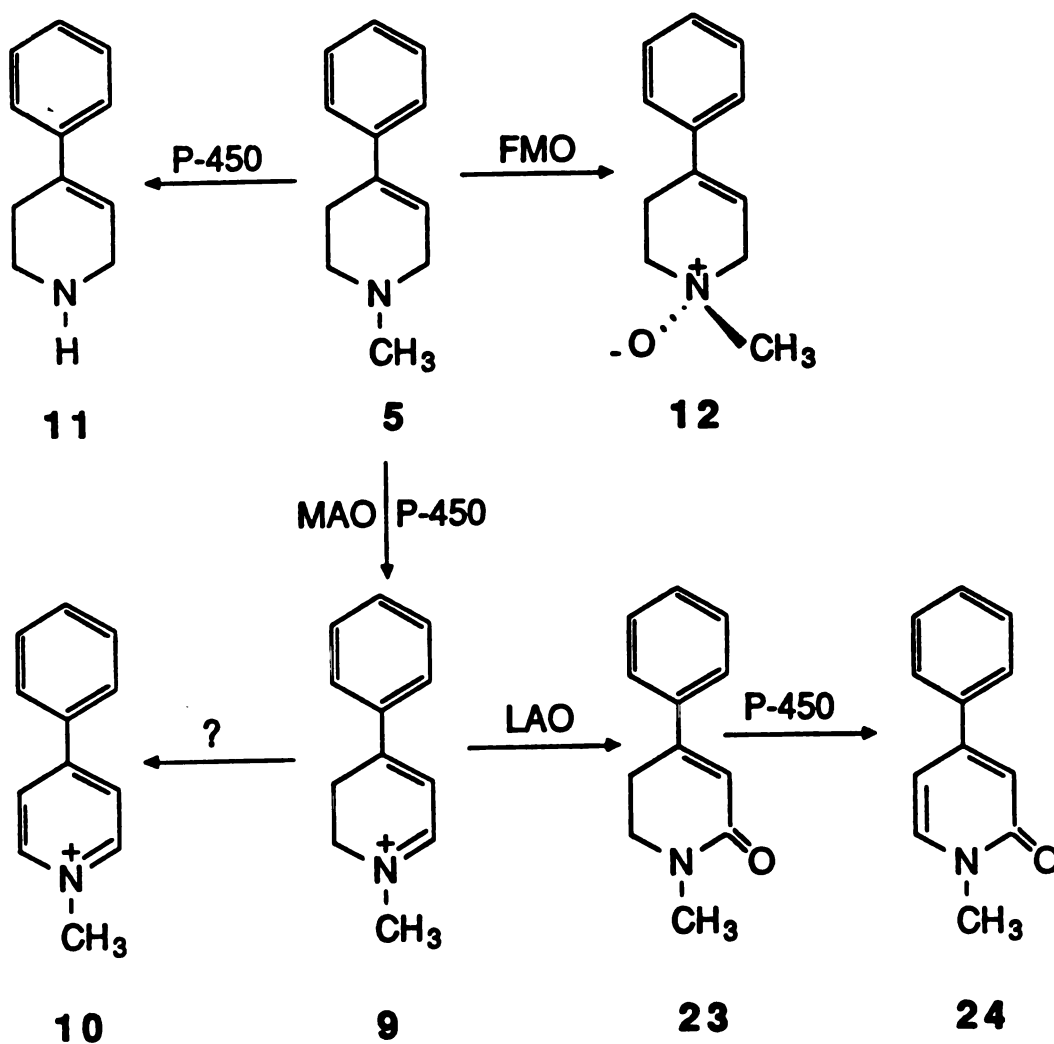
a P-450 = cytochrome P-450; FMO = microsomal flavin monooxygenase; MAO = monoamine oxidase; LAO = liver aldehyde oxidase.

COMPARATIVE STUDIES ON THE *IN VITRO* AND
IN VIVO METABOLISM OF MPTP AND MPDP+:
THE ISOLATED PERFUSED RAT LIVER
AND *IN VIVO* METABOLISM IN THE RAT

Introduction

The metabolic fates of the nigrostriatal toxin MPTP and its two-electron oxidation product MPDP+ have been examined in mouse brain and liver tissue fractions (Wu *et al*, 1988). Incubations of MPTP (50 μM and 1 mM) with mouse brain preparations resulted in the expected MAO B catalyzed formation of MPDP+ and MPP+. The oxidation of 50 μM MPDP+ to MPP+ in the same preparations appears to be mediated by unidentified components present in membrane containing fractions although the requirement for enzyme catalysis in this oxidation is not well defined. The behavior of MPTP in mouse liver subcellular fractions is considerably more complex. NADPH-supplemented liver microsomes convert MPTP to the N-oxide (12) and desmethyl (11) metabolites, the latter being a cytochrome P-450 catalyzed reaction with a relatively high K_m value. At high (1mM) initial concentrations of MPTP, NADPH-dependent, pargyline-insensitive liver microsomal enzymes also catalyze the oxidation of MPTP to MPDP+, again with a relatively high K_m. Cytosolic liver aldehyde oxidase catalyzes the oxidation of MPDP+ to the corresponding lactam, the 1-methyl-4-phenyl-5,6-dihydro-2-pyridone (23), which in turn may be oxidized to the 2-pyridone derivative, the 1-methyl-4-phenyl-2-pyridone (24), in a reaction catalyzed by an NADPH-dependent microsomal enzyme. The lactam 23 (in the absence of NADPH) and the lactam 23/2-pyridone 24 mixture (in the presence of NADPH) were the only products detected in the whole liver cell homogenates of MPDP+. These results, however, do not appear to be consistent with those obtained with freshly prepared rat hepatocytes which convert MPTP (1.5 mM) and MPDP+ (1.5 mM) exclusively to MPP+ (DiMonte *et al.*, 1987).

In recent studies Arora *et al.* (1988) reported the characterization of the lactam 23 and the 2-pyridone 24 in brain and liver tissues isolated from young

mature Wistar rats administered MPTP. The rats were given 50 mg/kg i.p. of the neurotoxin and sacrificed 45 min after the injection. The metabolites present in brain and liver extracts were identified through multiple analytical methods and were found to be unreacted MPTP, large amounts of MPP+, and a mixture of three nonpolar lactams: the lactam **23**, the 2-pyridone **24**, and a previously unreported metabolite, the 1-methyl-4-phenyl-2-piperidinone (**31**). Whereas MPP+ was more prevalent in the brain than in the liver, the lactam metabolites were predominant in the liver. We have obtained similar results in the investigation of the *in vivo* metabolism of MPTP in the Sprague-Dawley rat.
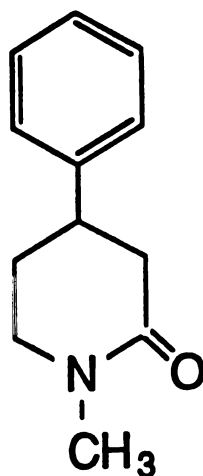
31

We have also examined the metabolism of MPTP and MPDP+ in isolated perfused Sprague-Dawley rat livers. The technique of isolated liver perfusion offers a convenient model of an integrated metabolic system where single reactions and their kinetics can be easily followed. As a first approach, the perfused liver can be viewed as a multi-compartment system composed of tissue, blood, and bile (Bartosek *et al.*, 1973). The isolated perfused liver has

been widely used for metabolism studies (Garattini *et al.*, 1973). By using this technique it is possible to investigate the role of liver metabolism without the interference of other organs which may contribute to the general metabolism of a drug. Also, whereas various *in vitro* preparations such as tissue homogenates and slices may be useful for the observation of single steps, the isolated liver preserves integral cell structure and permits examination of the overall metabolism of a drug. By using the isolated perfused liver, one can also avoid the "diluting" effect of *in vivo* metabolism in which a drug is usually transformed into a hydrosoluble metabolite that is easily excreted into the urine. Although blood can be monitored for various compounds, metabolites are normally present in very low concentrations. These difficulties can be partially overcome by the use of the isolated perfused liver in which concentrations of the added drug may be higher, and therefore the chance of detecting metabolites increases. In this case, the use of the isolated perfused liver to study the metabolism of MPTP and MPDP+ provides another means of examining the metabolic profile of this neurotoxic tertiary amine.

Results

A.   Isolated Perfused Sprague-Dawley Rat Liver

These experiments were conducted with B. Hoener.

In the single-pass perfusion experiment a 1.0 mM solution of MPTP was perfused through the isolated liver from a Sprague-Dawley rat (male, 370-400 g) at 26 mL/min for 30 min and approximately 0.8 L of perfusate was collected. In the recirculated perfusion experiments, an isolated liver was perfused at 45 mL/min with a recirculated 70 mL solution of 0.1 mM MPTP which was collected after the 30 min perfusion. After perfusing with buffer for 15 min to "wash out" the liver, a 70 mL solution of 1.0 mM MPTP was recirculated at 45 mL/min for 30 min through the same isolated liver. In a separate experiment, a 70 mL solution of 0.05 mM MPDP+ was recirculated at 45 mL/min for 30 min through an isolated liver. Each of the perfusates was treated with 5 percent TCA and

analyzed by HPLC diode-array techniques. The liver itself from each of the perfusion exercises was analyzed for its content of MPTP and metabolites. Each perfusate and liver sample extract was also treated once with ethyl acetate (1:1, v/v) to extract all uncharged, nonpolar compounds such as the lactam 23/2-pyridone 24 products. HPLC diode-array analysis (not shown) of the TCA extracts of perfusate or liver or of the ethyl acetate extracts, however, revealed no lactam or pyridone metabolites.

Table X summarizes the results of A) the metabolism of MPTP (1.0 mM) in the single-pass and B) of MPTP (0.1 mM and 1.0 mM) and MPDP+ (0.05 mM) in the recirculated isolated perfused rat liver. In the perfusate, following the 1 mM MPTP single-pass perfusion of the liver (Table X A), much MPTP remains unchanged and the major metabolites are PTP (3.3 percent) and MPTP N-oxide (3.5 percent), the products of MPTP oxidation by the liver cytochrome P-450 and flavin monooxygenase systems, respectively. Relatively small amounts of MPDP+ and MPP+ are detected (0.65 and 0.94 percent, respectively). In the liver itself, the only metabolite detected is MPP+ (0.31 percent). The total mass balance recovery in the perfusate and liver is approximately 73 percent of the amount actually perfused through the liver.

Analysis of the perfusate following recirculation of a 0.1 mM MPTP solution through the liver [Table X B 1)] reveals a large percentage of the MPTP N-oxide (43 percent) with no evidence of the PTP metabolite. These results confirm earlier findings (Wu *et al.*, 1988) that the K_m value for the demethylase must be higher than the value for the flavin monooxygenase. The other metabolite detected is MPP+ (26 percent). In the perfusate, following recirculation of a 1.0 MPTP solution [Table X B 2)], some MPTP (16 percent) remains unchanged but a relatively large percentage is converted to MPP+ (10.5 percent) and the N-oxide (7.3 percent). In analysis of the liver itself, we see that much of the metabolite MPP+ has been retained within the organ (27 percent). The total mass balance recovery for this recirculation exercise is about 68 percent of the original amount actually perfused. (Note: It was assumed that any residual metabolites from the 0.1 mM MPTP perfusion would have little effect on the 1.0 mM perfusion.)

Analysis of the perfusate from the recirculation of a 0.05 mM MPDP+ solution indicates that no MPDP+ remains and the only metabolite detected is MPP+. Analysis of the liver itself reveals the presence of no metabolites. The total mass balance recovery is only 34 percent. To check whether the lactam

78

23 is further metabolized or derivatized in the isolated liver, a 0.05 mM solution of this compound was perfused for 30 min at 45 mL/min. Analysis of the perfusate (data not shown) indicated that the species now existed as a mixture of lactam **23**/2-pyridone **24** as would be expected from the action of cytochrome P-450 on the lactam **23** (Wu *et al.*, 1988). Estimations from peak heights indicated a fairly good recovery of the lactams and no other metabolites were detected. (Note: Exact quantification of the lactam **23**/2-pyridone **24** species was not attempted. See p. 69).

TABLE X.   **Metabolism of MPTP and MPDP+ in the Isolated Perfused Sprague-Dawley Rat Liver**

A.   **Metabolism of 1 mM MPTP in the single-pass (26 mL/min for 30 min) isolated perfused rat liver (n = 1)**

	Metabolite	Concentration	Relative Mole %
Perfusate (0.8 L)	(MPTP)	(637) μM	(64)
	MPDP+	6.5	0.65
	MPP+	9.4	0.94
	PTP	33.2	3.3
	N-oxide	34.9	3.5
Liver (10.01 g)	(MPTP)	(0.65) nmol/mg	(0.65)
	MPP+	0.31	0.31

B.   **Metabolism of MPTP and MPDP+ in the recirculated (70 mL at 45 mL/min) isolated perfused rat liver (n = 1 each)**

	Metabolite	Concentration	Relative Mole %
1) 0.1 mM MPTP:			
Perfusate (0.07 L)	(MPTP)	(< 10) μM	(< 10)
	MPP+	26.3	26
	N-oxide	42.5	43
2) 1 mM MPTP:			
Perfusate (0.07 L)	(MPTP)	(161) μM	(16)
	MPDP+	20.6	2
	MPP+	105	10.5
	PTP	32.6	3.3
	N-oxide	72.3	7.3
Liver (24 g)	(MPTP)	(0.054) nmol/mg	(1.9)
	MPP+	0.80	27
3) 0.05 mM MPDP+:			
Perfusate (0.07 L)	(MPDP+)	(0)	(0)
	MPP+	17 μM	34
Liver (12.5 g)	(MPDP+)	(0)	(0)
	MPP+	(0)	(0)

B. *In Vivo* Metabolism in Rat Brain and Liver

In a preliminary trial the administration of 50 mg/kg MPTP i.p. to a Sprague-Dawley rat (male, 370-400 g) was lethal so the dose was lowered to 25 mg/kg. The animals were killed at 15, 30, 60, and 120 min after MPTP administration and their brains and livers examined for MPTP and metabolites. The tissues were homogenized with 5 percent TCA to extract the compounds. A portion of each TCA extract was then treated once with ethyl acetate (1:1, v/v) to extract uncharged, nonpolar species from the tissue "debris." All the TCA and ethyl acetate extracts were analyzed by HPLC diode-array techniques and it was found that a "new" metabolite, previously undetected in *in vitro* studies, was present in the aqueous layer after extraction with ethyl acetate. This species (Fig. 24, λ_{max} 266 and 314 nm) elutes at a similar retention time as lactam **23** and 2-pyridone **24** and thus would not have been detected if the ethyl acetate extraction had not been performed.

Table XI summarizes the metabolite species identified in the brain and liver at various times after MPTP administration. At 15 min after MPTP administration the brain was found to contain 14 pmol/mg tissue of MPP+ and about half that much MPDP+. A mixture of the lactam **23**/2-pyridone **24** species was detected but only in relatively small amounts (estimated by peak heights < 5 pmol/mg tissue each). In the liver, a larger concentration of the lactam **23**/2-pyridone **24** species was present (estimated < 10 pmol/mg tissue each). No trace of the "unknown" was detected in the brain and none at the 15 min time point in the liver. At 30 min after MPTP administration, MPP+ levels reached their highest measured in both the brain (51 pmol/mg tissue) and liver (266 pmol/mg tissue). The brain no longer contained detectable levels of lactam **23** or 2-pyridone **24**, and the levels of these species also decreased in the liver (estimated < 2 pmol/mg tissue each). At 30 min and more so at 60 min after MPTP administration, the "unknown" metabolite was present in appreciable (but unquantifiable) amounts. By 120 min, the brain contained only MPP+ (11 pmol/mg tissue) and the liver contained MPP+ (21 pmol/mg tissue) with a trace of the "unknown" metabolite still present.

Neither PTP nor the N-oxide was detected in appreciable amounts in either brain or liver at any of the time points studied. It is interesting to note that MPDP+ was not detected in the liver at any of the time points. Also, at 30 min

when both the brain and liver contained their highest level of the pyridinium species, the brain had one fifth the amount of hepatic MPP+ per mg tissue . At 120 min, MPP+ levels in hepatic tissues had dropped, but the brain had half the level of MPP+ per mg tissue.
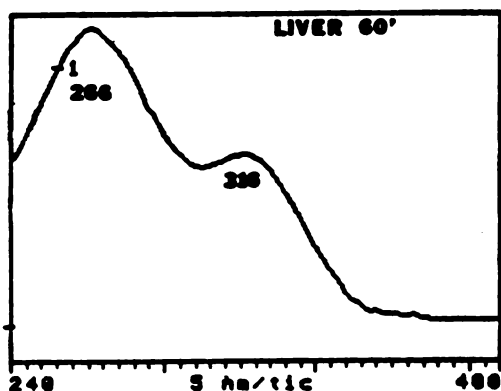
Fig. 24. UV spectra of the "unknown" metabolite, isolated by ethyl acetate extraction from rat whole liver homogenate at 60 min after i.p. MPTP administration.

TABLE XI.   *In Vivo* Metabolism[a] of 25 mg/kg i.p. MPTP in the Sprague-Dawley Rat Brain and Liver

Time After Injection (min.)	Rat Brain (pmol/mg tiss.)				Rat Liver (pmol/mg tiss.)				
	MPTP	MPDP+	MPP+	Lac-Pyr[b]	MPTP	MPDP+	MPP+	Lac-Pyr[b]	"Unknown"[b]
15	49	7.5	14	+	118	-	93	++	-
30	53	9.1	51	-	72	-	266	+	+
60	trace	-	36	-	36	-	71	-	++
120	-	-	11	-	trace	-	21	-	+

[a] n = 1 for each time point

[b] The lactam, pyridone, and the "unknown" metabolite eluted at similar retention times. Relative amounts were determined after extraction of the lactam/pyridone mixture by ethyl acetate.

82

Discussion

Results from both single-pass and recirculation of 1 mM MPTP through the isolated rat liver are similar to the results from previous *in vitro* liver subcellular fraction studies (Wu *et al.*,1988) in which the incubation of mouse whole liver homogenates with 1 mM MPTP and NADPH yielded appreciable amounts of the N-oxide, PTP, MPDP+, and MPP+. Furthermore, the recirculation of 0.1 mM MPTP throught the isolated liver gave results similar to those from the incubation of 0.05 mM MPTP and NADPH with mouse whole liver homogenates which produced the N-oxide but no PTP. In a separate experiment (data not shown), Sprague-Dawley rat (male, 380 g) liver whole homogenate converted 50 μM MPTP and 50 μM MPDP+ to lactam **23** in the absence of NADPH and to a mixture of lactam **23**/2-pyridone **24** in the presence of NADPH. Similarly, the rat liver 100,000 x g supernatant fraction converted 50 μM MPDP+ to lactam **23**. Where the difference arises between the liver homogenate incubations and the liver perfusion studies, however, is that no trace of the lactam **23**/2-pyridone **24** products is detected in the latter.

It is difficult to explain the discrepancy between the liver fraction studies and the isolated perfused liver studies, although the latter results are from preliminary studies. The lactam **23** is formed very rapidly from MPDP+ by liver fractions that contain the cytosolic aldehyde oxidase (Wu *et al.*, 1988). MPDP+ is detected in the perfusate of both single-pass and recirculated 1 mM MPTP perfusions [Table X A and B 2)] as well as being in the perfusion medium itself for one experiment [Table X B 3)]. MPTP presumably must enter the liver cell and reach the outer mitochondrial membrane to be oxidized by MAO B into MPDP+. In order for the dihydropyridinium species to be detected in the perfusate, it must have crossed through the cytosol (containing aldehyde oxidase) before passing out of the cell and into the perfusate. The conversion of MPDP+ to lactam **23** is rapid in both the mouse (Wu *et al.*, 1988) and rat whole liver homogenate. The lack of *in vivo* formation of lactam **23** from MPDP+ in the rat liver may be due to conditions in the liver which prevent aldehyde oxidase from interacting with the dihydropyridinium species. Moreover, it may be that much of the MPDP+ formed within the liver cell is oxidized to MPP+ which remains trapped in the cell due to its charged nature [Table X B 2)]. The lactam **23** and 2-pyridone **24** are fairly stable in the whole

83

liver (see above results from the perfusion of lactam **23** through the isolated liver). Thus, if formed *in vivo* in appreciable quantities from MPTP or MPDP+, the lactam **23** and the 2-pyridone **24** should not have been further biotransformed and should have been detected.

The somewhat low recoveries for the MPTP perfusions and the low (34 percent) recovery for the MPDP+ perfusion may be partially explained by the possibility that other metabolites are formed which escape detection by HPLC diode-array techniques. For example, Arora *et al.* (1988) recently reported the *in vivo* detection of the piperidinone compound **31** which has little UV absorption. It should be noted that unlike perfusions with MPTP, the perfusion of MPDP+ resulted in no retention of MPP+ in the liver. The pyridinium species is detected only in the perfusate. A much smaller concentration of MPDP+ (0.05 mM) was used compared to MPTP (1.0 mM), but this lack of MPP+ retention in the liver brings up the possibility that perfused MPDP+ may interact with membrane surfaces, perhaps endothelial, outside of the cell which could oxidize it to MPP+ without ever entering the liver cell. In such a case, no lactam **23** or 2-pyridone **24** would be formed during the perfusion of MPDP+ through the isolated liver. Yet, according to DiMonte *et al.* (1987) the incubation of 1.5 mM MPDP+ with isolated hepatocytes results in the rapid intracellular accumulation of MPP+. The high concentrations of MPDP+ (1.5 mM) used in this incubation, though, may make it more likely that the compound would enter the cell. Although a 1.5 mM MPDP+ isolated liver perfusion has not been performed, a 0.1 mM MPDP+ perfusion (data not shown) also resulted in no formation of the lactam **23**/2-pyridone **24** species and little accumulation of MPP+ in the liver itself.

Elimination of the interference from other organs in the isolated perfused rat liver system may assist in the analysis of the total hepatic metabolism of MPTP. However, it should be remembered that the general conditions of the perfused liver present an altered homeostasis which may be quite different from the conditions *in situ*. By isolating the liver, one may prevent the "dilution" effects on the test compound seen in the intact animal. However, one also introduces the accompanying, and possibly confounding factors of a "closed" system. During the recirculation of a compound through the liver, the parent drug may be transformed in a manner similar to that *in vivo* . However, the continuous increase in metabolite levels as well as possible further biotransformation may affect the metabolism of the parent compound. In the

isolated liver, with the exception of biliary excretion (which does not appear to play an appreciable role here. Data not shown.), there is no other excretion route. Substrate and product(s) concentrations, then, are attained at levels likely to be much different from those in the whole animal. Due to the low recovery numbers in these preliminary isolated perfused liver studies, a firm conclusion cannot be made as to the value of using this method to study the hepatic metabolism of MPTP. In our hands, isolated rat liver perfusion studies on MPTP and MPDP+ gave results which should probably be considered quantitatively rather than qualitatively in relation to other methods used in studying the biodisposition and metabolic profile of MPTP and its metabolites.

To complement the *in vitro* studies which used brain and liver tissue homogenate incubations and the isolated perfused liver, we also examined the metabolism of MPTP in the intact animal. Results from the *in vivo* metabolism of MPTP show that neither the MPTP N-oxide nor PTP was present at any time in detectable amounts in either the brain or the liver. This suggests that the flavin monooxygenase and the cytochrome P-450 systems play only a minor role in the *in vivo* metabolism of MPTP, at least in the rat brain and liver and at this i.p. dose of MPTP. By contrast, the prevalence in both brain and liver extracts of MPP+ and the lactams, all of which derive from MPDP+, indicate the dominant *in vivo* role of MAO in the primary metabolism of MPTP. The finding of more lactams in liver than in brain is consistent with the localization of aldehyde oxidase in the liver (Rajagopalan *et al.*, 1964). In fact, it is possible that the presence of the lactams in the brain represents the entry of these lipid-soluble metabolites into the brain following liver metabolism of MPTP--a suggestion first made by Arora *et al.* (1988). Furthermore, the apparent retention of MPP+ in the brain compared to the liver may partially account for the greater sensitivity of the CNS versus peripheral organs to the neurotoxic actions of MPTP.

The identity of the "new" metabolite is unknown. Recently, however, Arora *et al.* (1988) reported their identification of 1-methyl-4-phenyl-2-piperidinone (**31**) in both the brain and liver of rats by the use of a combination of chromatographic (HPLC and TLC) retention times using both UV and radiometric detection coupled with ^{1}H NMR chemical shift analysis and EI mass spectrometry. They speculated that this species might arise from the enzymatic reduction of lactam **23**. Their reported λ_{max} for this piperidinone **31** is 253 and 257 nm whereas the λ_{max} for our "unknown" peak is 266 and 314 nm. However, the extraction and "purification" of our unknown metabolite were quite

rudimentary and complete rigorous characterization steps have not been carried out. Nevertheless, the discovery of this "unknown" metabolite which elutes at a similar retention time as the lactam **23** and 2-pyridone **24** species in our HPLC system does elicit concern over whether our previous studies are quantitatively correct. Closer inspection of the HPLC diode-array chromatographic traces from the incubation of MPTP or MPDP+ with mouse whole liver homogenates (see pp. 55-74) reveals no peak present at the 314 nm detection wavelength. On the other hand, the chromatographic trace of an extract of the rat liver (60 min after MPTP i.p. administration) shows a distinct peak eluting at the approximate time of the lactams (~ 3.4 min) at the 314 nm detection wavelength. Examination of the other mouse tissue fraction incubations also yielded no detectable trace of the "unknown" metabolite. Therefore, either the metabolite is not formed in the mouse or our HPLC diode-array assay is unable to detect this metabolite in mouse subcellular fraction incubations of MPTP or MPDP+. A third possibility is that this "unknown" metabolite is only formed in the intact animal. It may be that the enzyme(s) involved in its formation do not survive or are masked or removed during the preparation of the various tissue fractions. Furthermore, it may be that a certain concentration of the "parent" drug has to be reached before this particular "unknown" is produced. In this preliminary *in vivo* study, we used only a single injection of one dose of MPTP.

Although *in vivo* studies may be complicated by unknown pharmacokinetic and distribution variables as well as certain analytical considerations, the results of the *in vivo* metabolism of MPTP put the results of our *in vitro* studies in a different perspective. *In vitro* studies can sometimes misrepresent the metabolic activity of intact tissue, particularly in the case of subcellular fractions which may involve non-physiologically relevant enzymatic exposures. It is important, therefore, to look at the complementary *in vivo* studies. For the most part, the results from the *in vivo* studies here, as well as our previous efforts in the mouse brain (Shinka *et al.*, 1987), are consistent with the results from our brain and liver subcellular fraction studies. What has been particularly apparent is that the relatively lower amounts of MPP+ (due to relatively more rapid clearance) and the presence of other non-toxic metabolites (*i.e.*, the lactams) in the rat liver versus the brain point to the important detoxification role of liver enzymes which metabolize MPDP+ to products other MPP+.

GENERAL CONCLUSIONS

The studies described in this dissertation define some of the possible roles of the dihydropyridinium species MPDP+ in the series of events following MPTP ingestion. Our studies have shown that despite its inherent chemical instability, MPDP+ is not just a transient intermediate in the brains of mice and rats after the peripheral administration of MPTP. By 10 min after the i.v. administration of MPTP to mice, the concentration of MPDP+ is considerably higher in all brain regions analyzed than both the parent compound itself and MPP+. Although the dosage of the drug and the route of administration to the two species differed, the results from the *in vivo* metabolism of MPTP indicate that both MPDP+ and MPP+ appear to be retained in the brain of the mouse longer than in the brain of the rat. Though the half-lives of all three compounds (MPTP, MPDP+, MPP+) appear to be relatively short in both species compared to primates, the differences in the biodisposition of the metabolites of MPTP between the mouse and the rat may account for differences in the vulnerability of these species to the neurotoxin. This consideration may be important in light of the findings that certain factors or compounds, such as DDC, can be involved in potentiating the neurotoxicity of MPTP. DDC appears to either increase the rate of production of MPDP+ (and thus of MPP+) or may reduce the rate of elimination of MPP+ from the CNS--or both. It makes sense that the longer the brain is exposed to larger quantities of MPTP metabolites the greater is the manifestation of the neurotoxic effects.

Direct administration of MPDP+ intravenously to the mouse did not result in neurotoxicity as measured by striatal dopamine levels. The lack of neurotoxicity, however, appears to be due to problems of the unstable MPDP+ species reaching the brain. Yet because of the dihydropyridinium's tendency to undergo disproportionation, self-condensation, and autoxidation, other methods of direct administration of MPDP+ (*e.g.*, *in vivo* brain cannulation and microdialysis) may also result in problems of disposition and data interpretation due to the high concentrations of MPDP+ required. In an attempt to circumvent the unstable nature of MPDP+, we studied the behavior of a rigid analog of MPDP+, the methylene bridged dihydroindenopyridinium species (18), which is remarkably stable. This MPDP+ analog was found not to be a very effective inhibitor of synaptosomal uptake of either [^{3}H] DA or [^{3}H] MPP+. If the steric

hindrance and structure rigidity of **18** do not interfere with substrate requirements for the dopamine uptake system, the results suggest that MPDP+ itself is likely not a good substrate, either, for synaptosomal uptake. However, the ability of MPDP+ to form the enamine free base **13** should allow it to cross the neuronal membrane and gain access to the dopaminergic neuron wherein the dihydropyridinium species may interact with specific proteins or endogenous substances such as neuromelanin which could act as a depot for MPP+.

The neurotoxic action of MPTP may be governed by the competition of enzyme systems which could metabolize the tertiary allylamine to species that do not cross the blood brain barrier or are non-neurotoxic. We have examined the metabolic fate of MPTP and MPDP+ in the brain and the liver with both *in vitro* and *in vivo* methods. The MAO B enzyme predominates in the brain and MPDP+ and MPP+ are the only consistently identified metabolites. The mechanisms involved in the oxidation of MPDP+ to MPP+ are still not identified although it appears that some component in membrane containing fractions may be involved. In the liver the metabolism is more complex with the MAO-, the cytochrome P-450-, the flavin monooxygenase-, and the aldehyde oxidase-systems all playing different roles, depending on substrate concentration and the subcellular fraction being studied. Preliminary isolated perfused rat liver studies gave results similar to those from mouse liver subcellular fraction studies with the exception that the lactam **23** and 2-pyridone **24** species were not detected in the perfusates or in the perfused liver itself (even though rat liver subcellular fractions gave results similar to mouse liver fractions). However, further studies with the isolated perfused rat liver are required before firm conclusions can be made. The *in vivo* metabolism of MPTP gave results in which the lactam **23** and 2-pyridone **24** species were detected but the N-oxide **12** and PTP (**11**) species were not. These findings point to the fact that the metabolic fates of MPTP and MPDP+ are highly dependent upon their intracellular concentrations as well as the intracellular environment to which they are exposed. *In vitro* studies may yield results which lead to the misinterpretation of the "real world" due to possibly unnatural exposure of compounds to certain enzyme systems. On the other hand, interpretation of results from *in vivo* studies may be difficult due to unknown pharmacokinetics and distribution variables. However, the examination of both types of experiments probably give a better overall view of what might be really

88

occurring in the metabolism of MPTP. With a better understanding of its overall metabolic profile, the mechanisms of the neurotoxic action of MPTP on the dopaminergic neurons in the nigrostriatal tract may become better defined.

Like some other diseases, parkinsonism may have multiple causes. What MPTP provides for the neurologist, biochemist, and pharmacologist is a clue to the etiology of this disease. Although many questions such as those concerning the selectiveness of the neurotoxicity remain, many other questions have been answered or at least partially addressed. The role of MAO and other enzymes, the significance of the dopamine reuptake and the mitochondrial transport systems as well as the inhibitory actions of MPP+ on mitochondrial respiration have provided one interpretation of the events leading to parkinsonian symptoms elicited by MPTP. In light of these discoveries, the possibility that the chronic exposure to toxic environmental and/or endogenous agents similar to MPTP might be involved in the etiology of Parkinson's disease has been proposed. It has been suggested that since humans are born with a relatively small number of nigrostriatal cells which "naturally" declines with age, an assault(s) on the nigrostriatum by environmental MPTP-like compounds would speed up this natural aging process and push the number of surviving neurons below the crucial 20 percent of control threshold level for manifestation of parkinsonian symptoms (Snyder and D'Amato, 1986). The discovery of animal models vulnerable to MPTP toxicity has provided a useful means of screening compounds which may produce similar toxic changes (Youngster *et al.*, 1987) and also a means by which to evaluate new therapeutic interventions. Thus, the tragic yet serendipitous discovery of MPTP and the resulting useful animal models have stimulated many new studies on the pathogenesis and treatments of Parkinson's disease. With the availability of MPTP, prospects for the prevention, arrest, or even reversal of this debilitating disease are greatly improved.

EXPERIMENTAL

Chemicals

The hydrochloride salt of MPTP was obtained from Research Biochemicals, Inc. P. Caldera-Munoz synthesized the following compounds: the iodide salt of MPP+ was prepared from 4-phenylpyridine and iodomethane as previously described (Wu *et al.*, 1986); the perchlorate salt of MPDP+ was synthesized from 6-cyano-1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine according to the method of Chiba *et al.* (1985b); 1-methyl-4-phenyl-5,6-dihydro-2-pyridone and 1-methyl-4-phenyl-2-pyridone were prepared by procedures previously reported (Wu *et al.*, 1988); and MPTP N-oxide was synthesized according to the method of Weissman *et al.* (1985). The perchlorate salt of 3,4-dihydro-2-methyl-9-H-indeno[2,1-C]pyridinium was synthesized by R.G. Booth according to previously described methods (Booth *et al.*, in press).

4-Phenylpyridine, 4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride, N-benzoylaniline, sodium acetate, trichloroacetic acid, dopamine hyrochloride, dihydroxybenzylamine hydrochloride, benzylamine hydrochloride, 3-amino-1,2,4-triazole, semicarbazide hydrochloride, and sodium benzoate were purchased from Aldrich Chemical Company (Milwaukee, WI). Diethyldithiocarbamate (disodium salt), D-glucose-6-phosphate, glucose-6-phosphate dehydrogenase, glucose, thymol-free catalase, glucose oxidase, β-NADP+, and menadione were obtained from Sigma Chemical Company (St. Louis, MO). Mazindol (Sandoz Pharmaceutical, East Hanover, NJ), 10 percent Intralipid (Kabivitrum, Alameda, CA), triethylamine hydrochloride and methanesulfonic acid (Eastman Kodak, Rochester, NY), 3-(aminocarbonyl)-1-methylpyridinium chloride (W.A. Taylor and Co., Baltimore, MD), ketamine hydrochloride (Bristol, Syracuse, NY), and acepromazine (Tech America, Elwood, KS) were purchased from the identified vendors. [3H] Dopamine (15 Ci/mmol) was obtained from Amersham (Arlington Heights, IL) and [3H] MPP+ (85.5 Ci/mmol) from New England Nuclear (Boston, MA). All other chemicals were of reagent grade, or in the case of HPLC solvents, "HPLC" grade.

<u>Analytical Methods</u>

Proton NMR spectra were obtained by P. Caldera-Munoz on a custom-built 240 MHz SP instrument or a GE 500 MHz instrument linked to a Nicolet 1180 computer; chemical shifts are reported in parts per million (ppm) relative to Me_4Si as an internal standard and spin multiplicities are given as s (singlet), d (doublet), t (triplet), and m (multiplet). Low resolution CI mass spectra were obtained by T. Shinka on a modified AEI 902S instrument at 8 kV using 2-methylpropane (*ca.* 1 Torr) as reagent gas. High resolution EI mass spectra were obtained on a Kratos MS 50S by the Bio-organic, Biomedical Mass Spectrometry Resource at the University of California, San Francisco (A.L. Burlingame, Director). HPLC analyses of MPTP and its metabolites were performed with a Beckman Model 114M chromatograph equipped with a Rheodyne 7125 injection valve, a Beckman Ultrasil SCX precolumn (10 μm, 4 x 10 mm), and an Altex Ultrasil SCX column (10 μm, 4.6 x 250 mm) according to the procedure reported by Shinka and colleagues (Shinka *et al.*, 1987). The mobile phase used with this cation exchange column consisted of a mixture of 90 percent 0.1 M acetic acid and 0.075 M triethylamine hydrochloride (pH adjusted to 2.3 with formic acid) and 10 percent acetonitrile and was delivered at a rate of 1.5-2.0 mL/min, depending on the experiment. HPLC effluents were analyzed with the aid of a Hewlett-Packard Model 1040a diode-array detector. The retention times (with a flow rate of 2.0 mL/min) and detection wavelengths in this system together with the UV characteristics of the analytes and the internal standard, 4-phenylpyridine (4-PP), were as follows: PTP: 2.65 min and 295 nm (λ_{max} 244 nm, ε 14,300 M-1 cm-1); lactams **23** and **24**: 2.91 min and 295 nm (λ_{max} 284 nm, ε 11,500 M-1 cm-1 and λ_{max} 232 nm, 262, 315, ε 24,200, 13,600, 4,500 M-1 cm-1, respectively); MPTP N-oxide: 3.03 min and 244 nm (λ_{max} 244 nm, ε 10,200 M-1 cm-1); MPTP: 3.45 min and 244 nm (λ_{max} 244 nm, ε 12,000 M-1 cm-1); 4-PP (IS): 3.89 min and 295 nm (λ_{max} 288 nm, ε 17,000 M-1 cm-1); MPDP+: 6.66 min and 345 nm (λ_{max} 345 nm, ε 17,000 M-1 cm-1); and MPP+: 6.66 min and 295 nm (λ_{max} 295 nm, ε 18,000 M-1 cm-1). Compounds **23** and **24**, which did not separate under the above conditions, were analyzed by H. Yoshizumi with the same HPLC system equipped with an Alltech direct-connect precolumn assembly (5 μm absorbasphere cartridge) and a Chemco silica column (3 μm, 4.6 x 250 mm). In this case the mobile phase, acetonitrile, was delivered at 1.5 mL/min. The retention times and detection wavelengths of the

analytes in this system and the UV characteristics of the internal standard N-benzoylaniline (N-BA) are as follows: lactam **23**: 5.57 min and 260 nm; 2-pyridone **24**: 9.83 min and 238 nm; N-BA (IS): 2.28 min and 260 nm (λ_{max} 265 nm, ε 12,700 M-1 cm-1).

Brain dopamine levels were determined by HPLC-electrochemical detection. Analyses of dopamine was performed by a Beckman Model 110A LC pump equipped with a Rheodyne 7125 injection valve, an Alltech C-18 precolumn (10 μm, self-packed, 4 x 10 mm), and an Alltech C-18 column (5 μm, 4.6 x 250 mm) according to the procedures previously reported (Mefford *et al.*, 1980; Heikkila *et al.*, 1984a). The mobile phase used with this reverse-phase column consisted of a mixture of 0.03 M phosphoric acid, 0.06 M methanesulfonic acid, and 0.05 M EDTA (pH 3.5 adjusted with sodium hydroxide) delivered at the rate of 1.5 mL/min. HPLC effluents were analyzed with the aid of a Bioanalytical System Model LC-3 amperometric detector (sensitivity set at 5 nA/V), equipped with Ag/AgCl and glassy carbon electrodes, and the results recorded on a Shimadzu integrator/recorder. The retention times for dopamine and the internal standard, dihydroxybenzylamine, on this system were 5.2 min and 8.8 min, respectively.

Preparative chromatographic separations were carried out on a Harrison Research Model 7924T Chromatotron centrifugal chromatographic device. Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Spectrophotometric measurements were carried out on a Cary Model 15 spectrophotometer or a Beckman Model DU-50 spectrophotometer equipped with a heating unit. Radioactivity was measured by a Beckman Model LS7800 liquid scintillation counter.

Interactions of MPDP+ with Synthetic Dopamine-melanin

Preparation of dopamine-melanin and isolation of human neuromelanin

All aqueous phosphate buffers were prepared from doubly distilled water and were subsequently passed through a column of Chelex 100 (sodium form, 50-100 mesh) to remove trace metals. Dopamine-melanin (D-M) was prepared

according to the procedure of Lyden *et al.* (1982). Dopamine hydrochloride (1 g) was added to 400 mL 0.05 M sodium phosphate buffer (pH 8.0). An air stream--previously passed through concentrated sulfuric acid, 40 percent potassium hydroxide, and water--was bubbled through the reaction mixture at room temperature. After three days, the melanin was precipitated by the addition of 1 mL of concentrated hydrochloric acid. The pigment was then isolated by suction filtration, washed with 1 L distilled water, and dried to constant weight at 75^0 C. The yield was 20 percent. Similar polymers also were generated in the presence of 10 μm ferrous, magnesium, manganese, and zinc sulfate and 10 μm ferric chloride. The extent to which these ions were incorporated into the polymers was not determined. These synthetic pigments (5 mg/mL) were suspended in sodium phosphate buffer (0.1 M, pH 7.4) and the resulting mixtures were sonicated for 10 min to disperse the particles prior to storing at - 4^0 C.

Neuromelanin was isolated from post-mortem, frozen human substantia nigra tissue provided by J.W. Langston (The Institute for Medical Research, San Jose, CA). Approximately 60 g of the thawed tissue was homogenized with 9 N hydrochloric acid (125 mL) and the resulting mixture was stirred at room temperature for 48 h. After the addition of 250 mL of 6 N hydrochloric acid the mixture was heated gently under reflux for 48 h to digest tissue proteins. The cooled mixture then was extracted with a total of 300 mL chloroform (4 times 75 mL) to remove lipids. The jet black solid that had sedimented into the lower chloroform layer was collected by suction filtration and was allowed to dry overnight before being resuspended in chloroform (300 mL) and refiltered. The black particulate material was washed with 100 mL chloroform and 10 mL ether and then was dried to constant weight at 75^0 C to yield 137 mg of product. The pigment, suspended in 0.1 M pH 7.4 sodium phosphate buffer (5 mg/mL), was sonicated and stored as above.

Incubation of melanin pigments with MPDP+

As required, the frozen suspensions were thawed and were 'vortexed' to disperse the particles. Incubation mixtures contained 100 μg polymer/mL 0.1 M sodium phosphate buffer (pH 7.4) in a final volume of 15 mL. After preincubation with constant shaking for 15 min at 37^0 C, appropriate volumes of

93

stock solutions of MPTP, MPDP+, or MPP+ (0.01 M in 0.1 M sodium phosphate buffer, pH 4.5) were added. In some instances metal salts (ferrous, magnesium, manganese, and zinc sulfate or ferric chloride) were added to the incubation mixtures at final concentrations of 10 to 100 μM. Aliquots (1.5 mL) were withdrawn at appropriate time points and placed in 60 mL of concentrated hydrochloric acid to stop further reaction and to displace any bound material. After filtration through acetate filters (Millipore, 0.65 μ) the solutions were analyzed by scanning from 210 to 410 nm. To confirm the identity of the product (MPP+) bound to the pigment after the incubation of MPDP+ with D-M, the incubation mixture was extracted with four equal volumes of chloroform. The combined extracts were dried over anhydrous magnesium sulfate and the solvent removed under vacuum. The crude product was characterized by ^{1}H NMR (in $CDCl_3$) and HPLC diode-array analysis as described above using the cation exchange column and a mobile phase flow rate of 1.5 mL/min.

Studies on the interactions of MPDP+ with D-M in an atmosphere of 100 percent oxygen or 100 percent nitrogen were conducted in scintillation vials which were closed with a serum cap. The gases were bubbled through the solution via a glass capillary tube and exited via a second glass capillary tube. The MPDP+ was introduced via a 22-gauge needle and the sample aliquots were withdrawn through a second 22-gauge needle. The D-M containing mixtures were purged with the appropriate gas for 15 min prior to the addition of a stock solution of MPDP+ perchlorate (0.01 M). Each incubation mixture then was maintained under a stream of oxygen or nitrogen gas for the duration of the incubation period. Additional anaerobic studies with MPDP+ were performed under a nitrogen atmosphere in suspensions of D-M which contained 5 mM D-glucose-6-phosphate, 13.2 μg/mL (activity 150 μmol/min-mg) thymol-free catalase, and 20 μg/mL glucose oxidase (activity 125 μmol/min-mg) to remove traces of oxygen (Trudell *et al.*, 1982). Following preincubation periods of up to 180 min, MPDP+ was added and its rate of oxidation to MPP+ determined spectrophotometrically. All studies were conducted at 37° C in a temperature controlled water bath with constant shaking.

Effects of DDC on MPTP Oxidation by Semi-purified MAO B

MAO B purification

MAO B was extracted and purified from bovine liver mitochondria by J.I. Salach according to methods previously described (Salach, 1979). Enzyme activities were determined spectrophotometrically at 30° C by initial rate measurements with benzylamine as substrate. The final enzyme preparation was estimated to be >50 percent homogeneous, based on its specific activity of 1 μM of benzylamine oxidized /min·mg protein, or 1 Unit/mg (Weyler and Salach, 1981).

MPTP oxidation by MAO B: Effect of DDC on MPDP+ formation

Samples of partially purified MAO B (0.006 U) in 0.05 M sodium phosphate buffer containing 0.2 percent (w/v) Triton X-100, pH 7.2, were preincubated with DDC (0.10 to 1.0 mM) at 30° C for 5 min. MPTP (3.3 mM) was added and the formation of MPDP+ was monitored spectrophotometrically at 343 nm at time points up to 90 min. The concentration of MPTP (3.3 mM) was chosen to be about ten times the K_m value for the MAO B oxidation of MPTP (K_m 0.30 mM) and benzylamine (K_m 0.38 mM). To assess the effects of DDC on the inactivation of MAO B by MPTP, 0.6 U of enzyme was incubated at 30° C in the standard assay mixture with 3.3 mM MPTP and either 0.05, 0.25, or 0.50 mM DDC. Control samples were similarly incubated with 3.3 mM MPTP alone, DDC alone, and in the absence of both MPTP and DDC. Portions (10 μL) were periodically removed and added to 0.99 mL of the assay mixture containing 3.3 mM benzylamine and initial rates of benzaldehyde formation were measured spectrophotometrically at 250 nm.

Inhibition of Synaptosomal Dopamine Uptake by the DMIP+ Species

Preparation of mouse neostriatal synaptosomes

Male C57Bl mice (Bantin-Kingman, 25-30 g, 10-12 weeks old) were killed, the brains rapidly removed, and the neostriata dissected on ice as previously reported (Glowinski and Iversen, 1966). The striata from 25-35 mice were combined in sets of 5-6 and each set homogenized in 2 mL cold 0.32 M sucrose solution (pH 7.4) with a motor driven glass pestle-glass homogenizer (0.15 mm clearance). Combined striatal homogenates were centrifuged at 1,000 x g for 15 min in a refrigerated Eppendorf microcentrifuge (Model 5415). The supernatant was decanted and centrifuged at 17,500 x g for 22 min. The resulting pellet was washed by homogenization with 10 mL of 0.32 M sucrose solution (pH 7.4) followed by centrifugation at 17,500 x g for 22 min. The pellet thus obtained was suspended in 15 volumes (w/v) of a buffered physiological Tris [50 mM Tris-HCl (pH 7.4), 125 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM glucose]. Protein in the synaptosomal preparations was measured by previously reported methods (Lowry *et al.*, 1951).

Synaptosomal uptake studies

A 1 mL incubation mixture containing radiolabeled compound (10 nM [^{3}H] dopamine or 1 nM [^{3}H] MPP+) and uptake buffer (0.02 percent ascorbic acid and 50 μM pargyline in physiological Tris) was preincubated in test tubes with or without the test compound at 37º C for 5 min. The uptake of radiolabeled compound was initiated by the addition of freshly prepared synaptosomes (~0.3 mg protein/mL) followed by incubation of the mixture with shaking at 37º C for 5 min. The uptake was terminated by placing the test tubes in ice water and adding 4 mL of ice-cold uptake buffer to each tube. The diluted incubates were then filtered through presoaked fiberglass filters (Whatman GF/B). After two consecutive 2.5 mL washes with ice-cold uptake buffer, the filters were placed in scintillation vials and 10 mL of Ecolite scintillation fluid (West Chem, San Diego, CA) was added. After vigorous vortexing of each vial for 15 sec, the radioactivity which had been collected on the filters was

measured by liquid scintillation spectrometry at a 40 percent average counting efficiency. "Total control" samples were incubated under the conditions above to give the total dpm accumulated. To correct for passive diffusion and adsorption to membranes and filters, "control background binding" samples were incubated in the presence of 10 mM mazindol, a specific dopamine uptake inhibitor. "Control active uptake" was calculated by subtracting from the "total control" dpm the number of dpm accumulated in the presence of 10 mM mazindol ("control background binding") and was expressed as nmol per g protein per min.

The kinetic parameters (K_m and V_{max}) of uptake were calculated from substrate saturation data using at least five concentrations (triplicate samples, 10-1000 nM) of radiolabeled substrate. Separate incubations containing 10 mM mazindol were also run at each radioactive substrate concentration to determine the level of "background binding" since this parameter increases linearly with the concentration of radioactive ligand.

In experiments in which IC_{50} values were to be calculated, at least four concentrations of each test compound were chosen based on preliminary experiments to give between a 15 and 90 percent inhibition of uptake relative to control incubations. Incubations at each inhibitor concentration were run in triplicate in each of two separate experiments to give a total of n = 6 data points for a given inhibitor concentration. A set of controls ("total control binding" and "control background binding") were run between each set of inhibitor concentrations. The values for "total control binding" and "control background binding" were averaged (n = 15-18) for a given experiment. The "control background binding" was assumed to be independent of inhibitor concentration so that uptake in the presence of a given inhibitor could be calculated by subtracting the "control background binding" in dpm (calculated for that particular experiment) from the total dpm accumulated in the presence of the inhibitor. Percent control values were calculated for each inhibitor concentration. These values were then plotted versus the log of the concentration of the inhibitor. The data was then fitted to a line by standard linear regression and IC_{50} values were calculated. The average IC_{50} values and S.E.M values reported in Table III are from the averages of six independent IC_{50} values each calculated from four inhibitor concentrations.

Measurement of Biodisposition and Neurotoxicity Following Intravenous Administration of MPTP and MPDP+ in the Mouse

Biodisposition studies

Male C57 Bl mice (Jackson, 8-12 months old) were treated intravenously with 10 mg/kg MPTP hydrochloride or MPDP+ perchlorate dissolved in Intralipid-ethanol (90:10, v/v). The animals were killed at various times after injection and the brains removed rapidly. The following three brain regions were dissected according to literature procedures (Glowinski and Iversen, 1966) and placed immediately on dry ice: two 25-mg sections (right and left brain halves) containing the neostriatum; a 50-mg section containing the substantia nigra; and a 50-mg section of cortex. The corresponding tissue isolated from two mice were combined and homogenized in five volumes of cold 5 percent TCA. To each homogenate was added 4-PP (25 ng/mg tissue) as internal standard. After standing 1 h at 0-2° C to allow complete precipitation of protein, the homogenate was centrifuged at 8,000 x g for 5 min. The supernatant fraction was filtered through a 0.2 μ nylon filter and 50-200 μL of this filtrate was analyzed by the HPLC diode-array system described above where the mobile phase was delivered 1.5 mL/min through the cation exchange column. The assay was standardized with the aid of calibration curves which were constructed by analyzing 0.5 mL of drug-free brain tissue homogenates (10 percent in 0.32 M sucrose) spiked with known amounts of the analytes and processed as described above. Peak height ratios of analytes (1-25 ng/mg tissue) versus the 4-PP internal standard (25 ng/mg tissue) were used. Tissue concentrations of the three analytes in the unknown samples were determined by comparing measured peak height ratios to those of the calibration curves. Recoveries were determined by comparing peak height ratios of spiked brain samples with samples prepared in an identical way but without the addition of brain homogenate.

Neurotoxicity studies

Male C57 Bl mice (Jackson, 8-12 months old) were treated intravenously with 10 mg/kg MPTP hydrochloride or MPDP+ perchlorate (dissolved in Intralipid-ethanol [90:10, v/v]) once daily for 3 days. Control mice were injected or not injected with vehicle only. Since dopamine concentrations in these animals did not differ, the two groups were combined and considered as controls. All animals were killed 24 h after the final injection and the brains rapidly removed. The two 25-mg sections of each brain neostriatum were dissected as described above and placed immediately on dry ice. Samples were stored less than 2 weeks at -80° C. Thawed tissue samples were spiked with 3 ng/mg tissue of 3,4-dihydroxybenzylamine (DHB) as an internal standard and then were homogenized with 1.0 mL of cold 0.2 M perchloric acid. After centrifugation of the homogenate at 8,000 x g for 15 min, a 500 mL aliquot of the supernatant was added to 20 mg of acid-washed aluminum oxide (Al_2O_3, pH 3.4) in a 1.5 mL polypropylene microcentrifuge tube. To this was added 1.0 mL of 1.0 M Tris buffer (pH 8.6); the mixture was then 'vortexed' for 5 sec and shaken mechanically for 15 min. The supernatant was discarded and the Al_2O_3 washed with 1.5 mL of cold distilled water. The catechols were eluted by the addition of 1.0 mL of cold 1.0 M perchloric acid/sodium perchlorate followed by vigorous 'vortexing' for 15 sec. This supernatant was filtered through a 0.2 μm nylon filter and 50-100 μL were analyzed by the HPLC-electrochemical detection system described above. Catechols were oxidized at an applied potential of +750 mV at room temperature. The concentrations of dopamine were determined by peak height ratio analysis (internal standard/dopamine) using linear standard calibration curves prepared from spiked samples (0 to 150 ng dopamine/mg versus 3 ng DHB/mg) in buffer. Recoveries averaged 53 percent.

In Vitro Metabolic Studies on MPTP and MPDP+ in the Mouse

Preparation of tissue fractions

The livers and brains of male C57 Bl mice (Bantin-Kingman, 25-30 g, 10-12 weeks old) and the livers of male New Zealand White rabbits (Animal West, 3.5 kg) were used for the preparation of tissue fractions. All buffers were bubbled with N_2 just prior to use. Animals were euthanized by exposure to CO_2. The mouse and rabbit livers were perfused *in situ* with 15 or 250 mL, respectively, of 250 mM sucrose-50 mM Tris buffer, pH 7.6. The tissues were minced and homogenized in three volumes of this buffer with a Teflon-glass homogenizer and the resulting homogenates were either used as whole tissue homogenates or were subjected to centrifugation at 700 x g for 10 min. The resulting supernatant (S_1) fractions were centrifuged at 10,000 x g for 20 min. The 10,000 x g (P_2) pellet was resuspended in three volumes of the sucrose-Tris buffer and recentrifuged at 10,000 x g for 20 min. The P_2 pellet was then resuspended in three volumes of 0.1 M phosphate buffer (pH 7.6) and stored at -80° C for up to one week as the crude mitochondrial fraction. The 10,000 x g supernatant (S_2) fraction was used as such or was subjected to centrifugation at 100,000 x g for 75 min. The resulting P_3 pellet was resuspended in two volumes of 0.1 M phosphate buffer and stored at -80° C for up to two weeks as the microsomal fraction. The 100,000 x g supernatant (S_3) preparation was recentrifuged at 100,000 x g for 75 min and stored at -80° C for up to one month. Mouse brain fractions were prepared similarly. Protein concentrations were measured by previously reported methods (Lowry *et al.*, 1951).

Metabolic incubations

MPTP (50 μM and 1 mM), MPDP+ (50 μM), MPP+ (50 μM and 1 mM), or lactam **23** (50 μM) was incubated with mouse liver or brain fractions (2 mg protein/mL) in 1 mL of 100 mM Hepes buffer (pH 7.6) in the presence and absence of an NADPH-generating system [(0.5 mM NADP+, 8 mM glucose-6-phosphate, 1 unit/mL glucose-6-phosphate dehydrogenase, 4 mM $MgCl_2$)]. In some cases, brain and liver mitochondrial and microsomal fractions were

100

denatured at 85° C for 30 min before use in incubation mixtures. Microsomal and 10,000 x g fractions were preincubated with 30 μM pargyline to suppress any contaminating MAO activity. In some cases pargyline was added to mitochondrial incubations to assess the role of MAO in the oxidation of MPDP+ to MPP+. Studies to assess aldehyde oxidase activity in 100,000 x g supernatant fractions (S$_3$) were performed in the presence of 25 μM menadione. Studies to assess the potential contribution of other enzymic actions in liver microsomal incubations were performed in the presence of 0.5 mM 3-amino-1,2,4-triazole, semicarbazide, and sodium benzoate. With the exception of the microsomal studies on lactam **23**, 4-phenylpyridine (4-PP, 1.25 - 2.5 μg/mL) was added to the mixtures as an internal standard (IS) after a 60 min incubation period and the reactions were terminated by the addition of 0.5 mL cold 5 percent trichloroacetic acid (TCA). After standing on ice for 1 h, the mixtures were centrifuged at 8,000 x g for 5 min and the resulting supernatant fractions filtered through a 0.2 μ nylon filter. A 50 - 200 μL aliquot of each filtrate was analyzed on the ion exchange column by the HPLC diode-array techniques described above where the mobile phase was delivered at 2.0 mL/min. The microsomal incubations of lactam **23** were treated in a similar way except that N-benzoylaniline (N-BA, 5 μg/mL) was used as an internal standard and the supernatant fractions were extracted with hexane. The hexane layers were dried over Na$_2$SO$_4$, filtered through a 0.2 μ nylon filter and the filtrates analyzed by HPLC on the silica column as described above. The assays were standardized with the aid of calibration curves for all the detected metabolites. Peak height ratios of analytes (1-25 ng/mg tissue) versus internal standard (1.25-2.5 μg/mL 4-PP or 5 μg/mL N-BA) were used to determine tissue concentrations of analytes in unknown samples.

Isolation of metabolically-generated lactam **23**

Preparative scale incubations containing MPDP+ (500 μM) and rabbit liver 100,000 x g supernatant (S$_3$) fractions (3 mg/mL protein) were run in 500 mL of 0.1 M phosphate buffer, pH 7.6. After shaking in l L beakers for 3 h at 37° C, the mixtures were allowed to cool to room temperature and then were extracted three times with 125 mL ethyl acetate. The combined organic extracts were dried over anhydrous MgSO$_4$, filtered, and the solvent removed under

vacuum. The residue was subjected to centrifugal chromatography on 1 mm thick silica gel discs with $CHCl_3$:CH_3OH (9.5:0.5). Twenty 1 mL fractions were collected. The metabolite was detected in fractions 6-11 and MPTP in fractions 12-20. The residue obtained from the combined fractions 6-11 was recrystallized from benzene/hexane to yield 10.4 mg (56.1 nmol, 4.1 percent) of pure product: mp 94-95° C; CIMS 188 (MH^+); ^1H NMR ($CDCl_3$) δ 2.82 [t, 2H, J = 7.2 Hz, C(5)-H_2], 3.05 [s, 3H, CH_3], 3.57 [t, 2H, J = 7.2 Hz, C(6)-H_2], 6.32 [s, 1H, C(3)-H], 7.4-7.5 ppm [m, 5H, ArH]; UV (H_2O) λ_{max} 284 nm (ϵ 11,500). HR EIMS Calcd. for $C_{12}H_{13}NO$: 187.10159. Found: 187.0997.

In Vitro Metabolism in the Isolated Perfused Rat Liver and *In Vivo* Metabolism in the Rat Brain and Liver

Liver perfusion

Liver isolation and perfusion studies were conducted by B. Hoener according to previously described methods (Hoener, 1988). Experiments were begun between 10:00 and 11:00 a.m. in order to minimize diurnal variation. Each rat (male Sprague-Dawley, Bantin-Kingman, 370-400 g) was anesthetized with about 1 mL of a mixture containing ketamine (80 mg/mL) and acepromazine (2 mg/mL). The abdominal cavity was opened and the bile duct, portal vein, and the thoracic inferior vena cava were cannulated. The liver was cut free and placed in a humidified and thermoregulated plexiglass cabinet. The perfusate was pumped (Masterflex, Cole-Palmer) through a filter to a membrane lung (Silastic medical grade tubing, Dow-Corning) where it was oxygenated with 95 percent oxygen/5 percent carbon dioxide. The perfusate then continued over a thermal probe (YSI), to a bubble trap, then to a pressure gauge, an in-line pH probe, and a sampling port before reaching the liver. In the recirculating mode, the perfusate exited through the inferior vena cava cannula and dropped back into the glass reservoir before returning to the pump. In the single-pass mode, the effluent left the liver and was collected into a separate container.

102

The perfusate consisted of a modified Krebs-Henseleit bicarbonate buffer [24.8 mM sodium bicarbonate (bubbled for 1 h with 95 percent O_2/5 percent CO_2), 120 mM sodium chloride, 4.75 mM potassium chloride, 1.70 mM calcium chloride, 1.20 mM dihydrate potassium phosphate, and 1.12 mM magnesium chloride] containing 1 mg/mL of glucose. Before each perfusion exercise the desired compound was dissolved to the appropriate concentration in the perfusate. The flow rate was adjusted to about 3 mL/min/g liver assuming a liver weight of 4.5 percent of total body weight. Oxygenation of the perfusate held the pH at approximately 7.4 throughout the experiment. Bile production, collected at 15-min intervals throughout each experiment, was as a rough assessment of the condition of the liver.

After an equilibrium period of 15 min in the recirculating mode, the liver was perfused for an additional 15 min in the single-pass mode with blank perfusate. In the single-pass experiment, the liver was perfused for 30 min with 1.0 mM MPTP. A final 15 min of blank perfusate in the single-pass mode followed. In the recirculation experiments, after the initial 15-min blank perfusion, the liver was perfused in the recirculation mode for 30 min with 70 mL of 0.10 mM MPTP and the perfusate was collected. After a 15-min blank perfusion in the single-pass mode, the same liver was perfused in the recirculation mode for 30 min with 1.0 mM MPTP and this perfusate was also collected. A final 15 min of blank perfusate followed. In other recirculation experiments on separate livers, the same steps were taken as described above except a 70 mL of 0.05 or 0.10 mM MPDP+ or 0.05 mM lactam **23** was used. An aliquot from each perfusate sample was immediately withdrawn and placed in an equal volume of 5 percent TCA. The liver itself from each perfusion experiment was analyzed for content of MPTP and metabolites. The entire liver was weighed and homogenized in two volumes of 5 percent TCA. Internal standard (4-PP, 2.5 μg/mL or 25 ng/mg tissue) was added to the perfusate aliquots and the liver homogenates and these samples were treated as described above. The final TCA-extracted samples were analyzed on an HPLC diode-array system as described above where the mobile phase was pumped through the cation exchange column at 2.0 mL/min. Each of the TCA-extracted samples was also extracted once with equal volume (1:1,v/v) ethyl acetate to extract any uncharged nonpolar compounds and these extracts were analyzed by HPLC diode-array techniques.

In vivo metabolism of MPTP

Male Sprague-Dawley rats (Bantin-Kingman, 370-400 g) were treated intraperitoneally with 25 mg/kg MPTP dissolved in saline. The animals were killed at 15, 30, 60, and 120 min after MPTP administration and their brains and livers rapidly removed. The tissues were weighed and homogenized in two volumes of 5 percent TCA. Internal standard (4-PP, 25 ng/mg tissue) was added and the homogenates were treated and analyzed by HPLC diode-array techniques as described above where the mobile phase was pumped through the cation exchange column at 2.0 mL/min. Each TCA-extracted sample was also treated once with equal volume of ethyl acetate as described above and these ethyl acetate extracts were analyzed by the above HPLC diode-array assay.

REFERENCES

Alvares, A.P., Leigh, S., Kappas, A., Levin, W., and Conney, A.H. (1973). Induction of aryl hydrocarbon hydroxylase in human skin. *Drug Metab. Dispos.* **1**, 386-390.

Arora, P., Riachi, N.J., Harik, S.I., and Sayre, L.M. (1988). Chemical oxidation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its *in vivo* metabolism in rat brain and liver. *Biochem. Biophys. Res. Commun.* **152**, 1339-1349.

Baker, J.R., Borne, R.F., Davis, W.M., and Waters, I.W. (1984). Metabolism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mouse liver preparations. *Biochem. Biophys. Res. Commun.* **125**, 484-490.

Barden, H. and Levine, S. (1983). Histochemical observations on rodent brain melanin. *Brain Res. Bull.* **10**, 847-851.

Bartosek, I., Guaitani, A., and Garattini, S. (1973). Prolonged perfusion of isolated rat liver. In *Isolated Liver Perfusion and its Applications* (Bartosek, I., Guaitani, A., and Miller, L.L., eds.). Raven Press, New York, New York, 63-72.

Bend, J.R., Hook, G.E.R., and Gram, T.E. (1973). Characterization of lung microsomes as related to drug metabolism. *Drug Metab. Dispos.* **1**, 358-367.

Bloom, F.E. (1971). Fine structural changes in brain after intracisternal injection of 6-hydroxydopamine. In *6-Hydroxydopamine and Catecholamine Neurons* (Malforms, R. and Thoenen, H., eds.). North Holland, Amsterdam, Netherlands, 135-150.

Bloom, F.E. (1985). Neurohumoral transmission and the central nervous system. In *The Pharmacological Basis of Therapeutics, 7th Ed.* (Gilman, A.G., Goodman, L.S., Rall, T.W., and Murad, F., eds.). MacMillan Publishing Co., New York, New York, 236-259.

Booth, R.G., Trevor, A., Singer, T.P., and Castagnoli, N., Jr. (1988). Studies on semirigic tricyclic analogs of the nigrostriatal toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *J. Med. Chem.*, in press.

Born, P., Mingels, M.J., Hulshoff, A., Frankhuyzen-Sierevogel, A., and Noordhoek, J. (1983). Rapid formation of N-hydroxymethylmelamine by mitochondria from rat small intestinal epithelium. *Life Sci.* **33**, 2113-2119.

Bradbury, A.J., Costall, B., Domeney, A.M., Jenner, P., Keller, M.E., Marsden, C.D., and Naylor, R.J. (1986). 1-Methyl-4-phenylpyridine is neurotoxic to the nigrostriatal dopamine pathway. *Nature* **319**, 56-57.

Bradbury, A.J., Costall, B., Domeney, A.M., Jenner, P., Marsden, C.D., Naylor, R.J., and Tan, C.C.W. (1985). The neurotoxic actions of MPP+ in the rat are not confined to dopamine and the substantia nigra. *Br. J. Pharmac.* **86**, 691.

Brandange, S. and Lindblom, L. (1979). The enzyme "aldehyde oxidase" is an iminium oxidase. Reaction with nicotine $\Delta^{1',5'}$-iminium ion. *Biochem. Biophys. Res. Commun.* **91**, 991-996.

Breese, G.R. and Traylor, T.D. (1970). Effect of 6-hydroxydopamine on brain norepinephrine and dopamine: evidence for selective degeneration of catecholaminergic neurons. *J. Pharmac. Exp. Ther.* **174**, 413-420.

Burns, R.S., Chiueh, C.C., Markey, S.P., Ebert, M.H., Jacobowitz, D.M., and Kopin, I.J. (1983). A primate model of parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Proc. Natl. Acad. Sci.* **80**, 4546-4550.

Burns, R.S., Markey, S.P., Phillips, J.M., and Chiueh, C.C. (1984). The neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in the monkey and man. *Can. J. Neurol. Sci.* **11** (1 suppl.), 166-168.

Bus, J.S., Aust, S.D., and Gibson, J.E. (1974). Superoxide- and singlet oxygen-catalyzed lipid peroxidation as a possible mechanism for paraquat (methyl viologen) toxicity. *Biochem. Biophys. Res. Commun.* **58**, 749-755.

Cashman, J.R. and Ziegler, D.M. (1986). Contribution of N-oxidation to the metabolism of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) by various liver preparations. *Molec. Pharmacol.* **29**, 163-167.

Castagnoli, N., Jr., Chiba, K., and Trevor, A.J. (1985). Potential bioactivation pathways for the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Life Sci.* **36**, 225-230.

Chiba, K., Peterson, L.A., Castagnoli, K.P., Trevor, A.J., and Castagnoli, N., Jr. (1985b). Studies on the molecular mechanism of bioactivation of the selective nigrostriatal toxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Drug Metab. Dispos.* **13**, 342-347.

Chiba, K, Trevor, A., and Castagnoli, N., Jr. (1984). Metabolism of the neurotoxic tertiary amine, MPTP, by brain monoamine oxidase. *Biochem. Biophys. Res. Commun.* **120**, 574-578.

Chiba, K., Trevor, A.J., and Castagnoli, N., Jr. (1985a). Active uptake of MPP+, a metabolite of MPTP, by brain synaptosomes. *Biochem. Biophys. Res. Commun.* **128**, 1228-1232.

Chiueh, C.C., Johannessen, J.N., Sun, J.L., Bacon, J.P., and Markey, S.P. (1986). Reversible neurotoxicity of MPTP in the nigrostriatal dopaminergic system in mice. In *MPTP: A Neurotoxin Producing a Parkinsonian Syndrome* (Markey, S.P., Castagnoli, N., Jr., Trevor, A.J., and Kopin, I.J., eds.). Academic Press, Inc., New York, New York, 473-479.

Chiueh, C.C., Markey, S.P., Burns, R.S., Johannessen, J.N., Jacobowitz, D.M., and Kopin, I.J. (1984). Neurochemical and behavioral effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in rat, guinea pig, and monkey. *Psychopharmac. Bull.* **20**, 548-553.

Christensen, E., Moller, J.E., and Faurbye, A. (1970). Neuropathological investigation of 28 brains from patients with dyskinesia. *Acta Psychiat. Scand.* **46**, 14-23.

Corsini, G.U., Pintus, S., Bocchetta, A., Piccardi, M.P., and Del Zompo, M. (1986). *In vitro* MAO type-B dependent formation of an alkylating metabolite from [^{3}H] MPTP in rat and monkey brain. In *MPTP: A Neurotoxin Producing a Parkinsonian Syndrome* (Markey, S.P., Castagnoli, N. Jr., Trevor, A.J., and Kopin, I.J., eds.). Academic Press, New York, New York, 371-376.

Corsini, G.U., Pintus, S., Chiueh, C.C., Weiss, J.F., and Kopin, I.J. (1985). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity in mice is enhanced by pretreatment with diethyldithiocarbamate. *Eur. J. Pharmacol.* **119**, 127-128.

Cotzias, G.C., Papavasiliou, P.J., van Woert, M.H., and Sakamoto, A. (1964). Melanogenesis and extrapyramidal disease. *Fed. Proc.* **23**, 713-718.

D'Amato, R.J., Benham, D., and Snyder, S.H. (1987). Characterization of the binding of N-methyl-4-phenylpyridine, the toxic metabolite of the parkinsonian neurotoxin N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, to neuromelanin. *J. Neurochem.* **48**, 653-658.

D'Amato, R.J., Lipman, Z.P., and Snyder, S.H. (1986). Selectivity of the parkinsonian neurotoxin MPTP: toxic metabolite MPP+ binds to neuromelanin. *Science* **231**, 987-989.

Davis, G.C., Williams, A.C., Markey, S.P., Ebert, M.H., Calne, E.D., Reichert, C.M., and Kopin, I.J. (1979). Chronic parkinsonism secondary to intravenous injection of meperidine analogues. *Psychiat. Res.* **1**, 249-254.

Demarest, K.T., Smith, D.J., and Azzaro, A.J. (1980). The presence of the type A form of monoamine oxidase within nigrostriatal dopamine containing neurons. *J. Pharmacol. Exp. Ther.* **215**, 461-468.

DiMonte, D., personal communication.

DiMonte, D., Ekstrom, G., Shinka, T., Smith, M.T., Trevor, A.J., and Castagnoli, N., Jr. (1987). Role of 1-methyl-4-phenylpyridinium ion formation and accumulation in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity to isolated hepatocytes. *Chem.-Biol. Interactions* **62**, 105-116.

DiMonte, D., Jewell, S.A., Eckstrom, G., Sandy, M.S., and Smith, M.T. (1986a). 1-Methyl-4-phenyltetrahydropyridine (MPTP) and 1-methyl-4-phenylpyridine (MPP+) cause rapid ATP depletion in isolated hepatocytes. *Biochem. Biophys. Res. Commun.* **137**, 310-315.

DiMonte, D., Sandy, M.S., Ekstrom, G., and Smith, M.T. (1986b). Comparative studies on the mechanisms of paraquat and 1-methyl-4-phenylpyridine (MPP+) cytotoxicity. *Biochem. Biophys. Res. Commun.* **137**, 303-309.

DiMonte, D., Shinka, T., Sandy, M.S., Castagnoli, N., Jr., and Smith, M.T. (1988). Quantitative analysis of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine metabolism in isolated rat hepatocytes. *Drug Metab. and Dispos.* **16**, 250-255.

Donaldson, J., Labella, F.S., and Gesser, D. (1981). Enhanced autooxidation of dopamine as a possible basis of manganese neurotoxicity. *Neurotoxicol.* **2**, 53-64.

Edlbacher, S., Wiss, O., and Walser, A. (1946). The degeneration of amino acids in the animal organism. IX. Additional information concerning the effectors of d-amino acid oxidase. *Helv. Chem. Acta* **29**, 162-174.

Farley, I.J., Price, K.S., and Hornykiewicz, O. (1977). Dopamine in the limbic regions of the human brain: normal and abnormal. In *Advances in Biochemical Pharmacology, Vol. 16* (Costa, E. and Gessa, G.L., eds.). Raven Press, New York, New York, 57-64.

Forno, L.S. (1982). Pathology of Parkinson's disease. In *Movement Disorders* (Marsden, C.D. and Fahn, S., eds.). Butterworth Scientific, London, England, 25-40.

Forno, L.S., Langston, J.W., DeLanney, L.E., Irwin, I., and Ricaurte, G.A. (1986). Locus ceruleus lesions and eosinophilic inclusions in MPTP-treated monkeys. *Ann. Neurol.* **20**, 449-455.

Frei, B. and Richter, C. (1986). 1-Methyl-4-phenylpyridine (MPP+) together with 6-hydroxydopamine or dopamine stimulates Ca^{+2} release from mitochondria. *FEBS Lett.* **198**, 99-102.

Fridovich, I. (1979). *Oxygen Free Radicals and Tissue Damage*, Excerpta Medica, Amsterdam, Netherlands.

Garattini, S., Guaitani, A., and Bartosek, I. (1973). Use of isolated perfused liver in the study of drug metabolism. In *Isolated Liver Perfusion and its Applications* (Bartosek, I., Guaitani, A., and Miller, L.L., eds.). Raven Press, New York, New York, 225-234.

Glowinski, J. and Iversen, L.L. (1966). Regional studies of catecholamines in the rat brain I: the disposition of [3H] norepinephrine, [3H] dopamine, and [3H] dopa in various regions of the brain. *J. Neurochem.* **13**, 655-669.

Goodchild, N.T., Kwock, L., and Lin, P.S. (1981). Melanin: a possible cellular superoxide scavenger. In *Oxygen and Oxyradicals in Chemistry and Biology* (Rodgers, M.A.J. and Powers, E.L., eds.). Academic Press, New York, New York, 645-648.

Graham, D.G. (1978). Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. *Molec. Pharmacol.* **14**, 633-643.

Graham, D.G., Tiffany, S.M., Bell, W.R., Jr., and Gutknecht, W.F. (1978). Autooxidation versus covalent binding of quinones as the mechanism of toxicity of dopamine, 6-hydroxydopamine, and related compounds toward C1300 neuroblastoma cells *in vitro*. *Molec. Pharmacol.* **14**, 644-653.

Hallman, H., Lange, J., Olson, L., Stromberg, I., Jonsson, G. (1985). Neurochemical and histochemical characterization of neurotoxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine on brain catecholamine neurons in the mouse. *J. Neurochem.* **44**, 117-120.

Heikkila, R.E., Cabbat, F.S., and Cohen, G. (1976). *In vivo* inhibition of superoxide dismutase in mice by diethyldithiocarbamate. *J. Biol. Chem.* **251**, 2182-2185.

Heikkila, R.E. and Cohen, G. (1971). Inhibition of amine uptake by hydrogen peroxide: a mechanism for the toxic effects of 6-hydroxydopamine. *Science* **172**, 1257-1258.

Heikkila, R.E., Hess, A, and Duvoisin, R.C. (1984b). Dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in mice. *Science* **224**, 1451-1453.

Heikkila, R.E., Hess, A., and Duvoisin, R.C. (1985a). Dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the mouse: relationship between monoamine oxidase, MPTP metabolism, and neurotoxicity. *Life Sci.* **36**, 231-236.

Heikkila, R.E., Manzino, L., Cabbat, F.S., and Duvoisin, R.C. (1984a). Protection against the dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine by monoamine oxidase inhibitors. *Nature* **311**, 467-469.

Heikkila, R.E., Nicklas, W.J., and Duvoisin, R.C. (1985b). Dopaminergic toxicity after stereotaxic administration of the 1-methyl-4-phenylpyridinium ion (MPP+) to rats. *Neurosci. Lett.* **59**, 135-140.

Heikkila, R.E., Youngster, S.K., Manzino, L., Cabbat, F.S., and Duvoisin, R.C. (1985c). Effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and related compounds on the uptake of [³H] 3,4-dihydroxyphenethylamine and [³H] 5-hydroxytryptamine in neostriatal synaptosomal preparations. *J. Neurochem.* **44**, 310-313.

Hinson, J.A. (1980). Biochemical toxicology of acetaminophen. In *Reviews in Biochemical Toxicology, Vol. 2* (Hodgson, F., Bend, J.R., and Philpot, R.M., eds.). Elsevier/North Holland, New York, New York, 103-130.

Hoener, B. (1988). Nitrofurazone: kinetics and oxidative stress in the singlepass isolated perfused rat liver. *Biochem. Pharmacol.* **37**, 1629-1636.

Hornykiewicz, O. (1966). Dopamine (3-hydroxytyramine) and brain function. *Pharmac. Rev.* **18**, 925-964.

Irwin, I. and Langston, J.W. (1985). Selective accumulation of MPP+ in the substantia nigra: a key to neurotoxicity? *Life Sci.* **36**, 207-212.

Irwin, I., Langston, J.W., and DeLanney, L.E. (1987b). 4-Phenylpyridine (4-PP) and MPTP: the relationship between striatal MPP+ concentrations and neurotoxicity. *Life Sci.* **40**, 731-740.

Irwin, I., Wu, E.Y., DeLanney, L.E., Trevor, A., and Langston, J.W. (1987a). The effect of diethyldithiocarbamate on the biodisposition of MPTP: an explanation for enhanced neurotoxicity. *Eur. J. Pharmacol.* **141**, 209-217.

Jacobowitz, D.M., Burns, R.S., Chiueh, C.C., and Kopin, I.J. (1984). N-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes destruction of the nigrostriatal but not the mesolimbic dopamine system in the monkey. *Psychopharmacol. Bull.* **20**, 416-422.

Javitch, J.A., D'Amato, R.J., Strittmater, S.M., and Snyder, S.H. (1985). Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: uptake of the metabolite N-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. *Proc. Natnl. Acad. Sci. USA* **82**, 2173-2177.

Javitch, J.A. and Snyder, S.H. (1985). Uptake of MPP+ by dopamine neurons explains selectivity of Parkinsonism-inducing neurotoxin MPTP. *Eur. J. Pharmacol.* **106**, 455-456.

Johannessen, J.N., Adams, J.D., Schuller, H.M., Bacon, J.P., and Markey, S.P. (1986a). 1-Methyl-4-phenylpyridine (MPP+) induces oxidative stress in the rodent. *Life Sci.* **38**, 743-749.

Johannessen, J.N., Chiueh, C.C., Burns, R.S., and Markey, S.P. (1985). Differences in the metabolism of MPTP in the rodent and primate parallel differences in sensitivity to its neurotoxic effects. *Life Sci.* **36**, 219-224.

Johannessen, J.N., Chiueh, C.C., Herkenham, M.A., Markey, S.P., Burns, R.S., Adams, J.D., and Schuller, H.M. (1986b). Relationship of the *in vivo* metabolism of MPTP to toxicity. In *MPTP: A Neurotoxin Producing A Parkinsonian Syndrome* (Markey, S.P., Castagnoli, N., Jr., Trevor, A.J., and Kopin, I.J., eds.). Academic Press, Inc., New York, New York, 173-189.

Juchau, M.R., Namkung, M.J., Berry, D.L., and Zachariah, P.K. (1975). Oxidative biotransformation of 2-acetylaminofluorene in fetal and placental tissue of humans and monkeys. *Drug Metab. Dispos.* **3**, 494-501.

Klein, J.R. and Kamin, H. (1941). Inhibition of the *d*-amino acid oxidase by benzoic acid. *J. Biol. Chem.* **138**, 507-512.

Konradi, C., Riederer, P., and Youdim, M.B.H. (1986). Hydrogen peroxide enhances the activity of monoamine oxidase type-B but not of type-A: a pilot study. *J. Neural Transm.* (suppl) **22**, 61-73.

Kopin, I.J. and Markey, S.P. (1988). MPTP toxicity: implications for research in Parkinson's disease. *Ann. Rev. Neurosci.* **11**, 81-96.

Korytowski, W., Felix, C.C., and Kalyanaraman, B. (1987). Mechanism of oxidation of 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP+). *Biochem. Biophys. Res. Commun.* **144**, 692-698.

Kostrzewa, R. and Jacobowitz, D. (1974). Pharmacological actions of 6-hydroxydopamine. *Pharmac. Rev.* **26**, 199-287.

Langston, J.W., personal communication.

Langston, J.W., Ballard, P., Tetrud, J.W., and Irwin, I. (1983). Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* **219**, 979-980.

Langston, J.W., Forno, L.S., Robert, C.J., and Irwin, I. (1984a). Selective nigral toxicity after systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the squirrel monkey. *Brain Res.* **292**, 390-495.

Langston, J.W., Irwin, I., and DeLanney, L.E. (1987b). The biotransformation of MPTP and disposition of MPP+: the effects of aging. *Life Sci.* **40**, 749-754.

Langston, J.W., Irwin, I., Langston, E.B., and Forno, L.S. (1984b). Pargyline prevents MPTP-induced parkinsonism in primates. *Science* **225**, 1480-1482.

Langston, J.W., Irwin, I., Langston, E.B., and Forno, L.S. (1984c). 1-Methyl-4-phenylpyridinium ion (MPP+): identification of a metabolite of MPTP, a toxin selective to the substantia nigra. *Neurosci. Lett.* **48**, 87-92.

Langston, J.W., Irwin, I., and Ricaurte, G.A. (1987a). Neurotoxins, parkinsonism, and parkinson's disease. *Pharmac. Ther.* **32**, 19-49.

Larsson, B. and Tjalve, H. (1979). Studies on the mechanism of drug binding to melanins. *Biochem. Pharmacol.* **28**, 1181-1187.

Leung, L., Ottoboni, S., Oppenheimer, N., and Castagnoli, N., Jr. (1988). Characterization of a product derived from the 1-methyl-4-phenyl-2,3-dihydropyridinium ion, a metabolite of the nigrostriatal toxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *J. Org. Chem.*, in press.

Lindquist, N.G. (1973). Accumulation of adrenaline, dopamine, noradrenaline, and serotonin on melanin. *Acta Radiol.* (suppl.) **325**, 62-77.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.

Lyden, A., Bondesson, U., Larsson, B., and Lindquist, N. (1983). Melanin affinity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, and inducer of chronic parkinsonism in humans. *Acta Pharmacol. Toxicol.* **53**, 424-432.

Lyden, A., Bondesson, U., Larsson, B.S., Lindquist, N.G., and Olsson, L.I. (1985). Autoradiography of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): uptake in the monoaminergic pathways and in melanin containing tissues. *Acta Pharmacol. Toxicol.* **57**, 130-135.

Lyden, A., Larsson, B., and Lindquist, N.G. (1982). Studies on the melanin affinity of [^{3}H] haloperidol. *Arch. Int. Pharmacodyn.* **25**, 230-243.

Margolish, E. and Novogrodsky, A. (1958). Inhibition of catalase by 3-amino-1,2,4-triazole. *Biochem. J.* **68**, 468-475.

Marietta, M.P., Vessel, E.S., Hartman, R.D., Weisz, J., and Dvorchik, B.H. (1979). Characterization of cytochrome P-450-dependent aminopyrine N-demethylase in rat brain: comparison with hepatic aminopyrine N-demethylation. *J. Pharmacol. Exp. Ther.* **208**, 271-279.

Markey, S.P., Castagnoli, N., Jr., Trevor, A.ZJ., and Kopin, I.J., eds. (1986). *MPTP: A Neurotoxin Producing A Parkinsonian Syndrome*. Academic Press, Inc., New York, New York.

Markey, S.P., Johannessen, J.N., Chiueh, C.C., Burns, R.S., and Herkenham, M.A. (1984). Intraneuronal generation of a pyridinium metabolite may cause drug-induced parkinsonism. *Nature* **311**, 464-467.

Marsden, C.D. (1969). Brain melanin. In *Pigments in Pathology* (Wolman, E., ed.). Academic Press, Inc., New York, New York, 395-420.

Marsden, C.D. (1982). Neurotransmitters and disease. Basal ganglia disease. *Lancet* II, 1141-1147.

Marsden, C.D., Parkes, J.D., and Quinn, N. (1982). Fluctuations of disability in Parkinson's disease: clinical aspects. In *Movement Disorders* (Marsden, C.D. and Fahn, S., eds.). Butterworth Scientific, London, England, 96-122.

McBrien, D.C.H. and Slater, T.F., eds. (1982). *Free Radicals, Lipid Peroxidation, and Cancer.* Academic Press, Inc., London, England.

Mefford, I.N., Gilberg, M., and Barchas, J.D. (1980). Simultaneous determination of catecholamines and unconjugated 3,4-dihydroxyphenylacetic acid in brain tissue by ion-pairing reverse-phase high-performance liquid chromatography with electrochemical detection. *Anal. Biochem.* **104**, 469-472.

Melamed, E., Rosenthal, J., Cohen, O., Globus, M., and Uzzan, A. (1985). Dopamine but not epinephrine or serotonin uptake inhibitors protect mice against neurotoxicity of MPTP. *Eur. J. Pharmacol.* **116**, 179-181.

Menon, I.A., Leu, S.L., and Haberman, H.F. (1977). Electron transfer properties of melanin. Optimum conditions and the effects of various chemical treatments. *Can. J. Biochem.* **55**, 783-787.

Mondovi, B., Costa, M.T., Finazzi, A., and, Rotilio, G. (1967). Pyridoxal phosphate as a prosthetic group of pig kidney diamine oxidase. *Arch. Biochem. Biophys.* **119**, 373-381.

Mytilineou, C., Cohen, G., and Heikkila, R.E. (1985). 1-Methyl-4-phenylpyridine (MPP+) is toxic to mesencephalic dopamine neurons in culture. *Neurosci. Lett.* **57**, 19-24.

Nicklas, W.J., Vyas, I., and Heikkila, R.E. (1986). MPP+ inhibits NADH-linked mitochondrial oxidation: role in the mechanism of MPTP neurotoxicity. In *MPTP: A Neurotoxin Producing A Parkinsonian Syndrome* (Markey, S.P., Castagnoli, N., Jr., Trevor, A.J., and Kopin, I.J., eds.). Academic Press, Inc., New York, New York, 591-596.

Orrenius, S., Ellin, A., Jakobsson, S., Thor, H., Cinti, D.L., Schenkman, J.B., and Estabrook, R.W. (1973). The cytochrome P-450 containing monooxygenase system of rat kidney cortex microsomes. *Drug Metab. Dispos.* **1**, 350-357.

Ottoboni, S., personal communication.

Parisi, J.E. and Burns, R.S. (1986). The neuropathology of MPTP-induced parkinsonism in man and experimental animals. In *MPTP: A Neurotoxin Producing A Parkinsonian Syndrome* (Markey, S.P., Castagnoli, N., Jr., Trevor, A.J., and Kopin, I.J., eds.). Academic Press, Inc., New York, New York, 141-148.

Paul, S.M., Axelrod, J., and Diliberto, E.J. (1977). Catechol estrogen-forming enzyme of brain: demonstration of a cytochrome P-450 monooxygenase. *Endocrinology* **101**, 1604-1610.

Peterson, L.A., Caldera, P.S., Trevor, A.J., Chiba, K., and Castagnoli, N., Jr. (1985). Studies on the 1-methyl-4-phenyl-2,3-dihydropyridinium species 2,3-MPDP+, the monoamine oxidase catalyzed oxidation product of the nigrostriatal toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *J. Med. Chem.* **28**, 1432-1436.

Petterson, G. (1985). Plasma amine oxidase. In *Structure and Functions of Amine Oxidases* (Mondovi, B., ed.). CRC Press, Inc., Boca Raton, Florida, 105-120.

Poirier, J. and Barbeau, A. (1985). A catalyst function for MPTP in superoxide formation. *Biochem. Biophys. Res. Commun.* **131**, 1284-1289.

Rajagopolan, K.V., Fridovich, I., and Handler, P. (1962). Hepatic aldehyde oxidase. I. Purification and properties. *J. Biol. Chem.* **237**, 922-928.

Ramsay, R.R., Dadgar, J.I., Trevor, A., and Singer, T.P. (1986). Energy-driven uptake of N-methyl-4-phenylpyridine by brain mitochondria mediates the neurotoxicity of MPTP. *Life Sci.* **39**, 581-588.

Ramsay, R.R. and Singer, T.P. (1986). Energy-dependent uptake of N-methyl-4-phenylpyridinium, the neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, by mitochondria. *J. Biol. Chem.* **261**, 7585-7587.

Ricaurte, G.A., Irwin, I., Forno, L.S., DeLanney, L.E., Langston, E.B., and Langston, J.W. (1987). Aging and MPTP-induced degeneration of dopaminergic neurons in the substantia nigra. *Brain Res.* **403**, 43-51.

Ricaurte, G.A., Langston, J.W., DeLanney, L.E., Irwin, I., Peroutka, S.J., and Forno, L.S. (1985a). The MPTP mouse: a model for exploring strategies to promote recovery of nigrostriatal dopaminergic neurons after injury. *Ann. Neurol.* **18**, 117.

Ricaurte, G.A., Langston, J.W., DeLanney, L.E., Irwin, I., Peroutka, S.J., and Forno, L.S. (1986). Fate of nigrostriatal neurons in young mature mice given 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: a neurochemical and morphological reassessment. *Brain Res.* **376**, 117-124.

Ricaurte, G.A., Langston, J.W., Irwin, I., DeLanney, L.E., and Forno, L.S. (1985b). The neurotoxic effect of MPTP on dopaminergic cells in the substantia nigra of mice is age-related. *Soc. Neurosci. Abstr.* **11**, 631.

Riederer, P. and Woketich, S.S. (1976). Time course of nigrostriatal degeneration in Parkinson's disease. *J. Neural. Transm.* **39**, 277-301.

Rollema, H., personal communication.

Rollema, H., Damsma, G., Horn, A.S., DeVries, J.B., and Westerink, B.H.C. (1986). Brain dialysis in conscious rats reveals an instantaneous massive release of striatal dopamine in response to MPP+. *Eur. J. Pharmacol.* **126**, 345-346.

Rollema, H., DeVries, J.B., Damsma, G., Westerink, B.H.C., Kranenborg, G.L., Kuhr, W.G., and Horn, A.S. (1988a). The use of *in vivo* brain dialysis of dopamine, acetylcholine, amino acids, and lactic acid studies on the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Toxicology* **49**, 503-511.

Rollema, H., Fries, D.S., DeVries, J.B., Mastebrock,D., and Horn, A.S. (1985). HPLC-assay with electrochemical detection for the neurotoxin MPTP, its metabolite MPP+ and MPTP-analogues in biological samples after purification over Sephadex G10. *Life Sci.* **37**, 1633-1640.

Rollema, H., Kuhr, W.G., Kranenborg, G., DeVries, J.B., and Van Den Berg, C. (1988b). MPP+-induced efflux of dopamine and lactate from rat striatum have similar time courses as shown by *in vivo* brain dialysis. *J. Pharmacol. Exp. Ther.* **245**, 858-866.

Salach, J.I. (1979). Monoamine oxidase from beef liver mitochondria: simplified isolation procedure, properties, and determination of its cysteinyl flavin content. *Arch. Biochem. Biophys.* **192**, 128-137.

Salach, J.I., Singer, T.P., Castagnoli, N., Jr., and Trevor, A. (1984). Oxidation of the neurotoxic amine 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) by monoamine oxidases A and B and suicide inactivation of the enzymes by MPTP. *Biochem. Biophys. Res. Commun.* **125**, 831-835.

Saner, A. and Thoener, H. (1971). Model experiments on the molecular mechanisms of action of 6-hydroxydopamine. *Molec. Pharmacol.* **7**, 147-154.

Sayre, L.M., Arora, P.K., Feke, S.C., and Urbach, F.L. (1986). Mechanism of induction of Parkinson's disease by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Chemical and electrochemical characterization of a geminal-dimethyl-blocked analogue of a postulated toxic metabolite. *J. Am. Chem. Soc.* **108**, 2464-2466.

Schnaitman, C. and Greenawalt, J.W. (1968). Enzymatic properties of the inner and outer membranes of rat liver mitochondria. *J. Cell Biol.* **38**, 158-175.

Schneider, J.S., Yuwiler, A., and Markham, C.H. (1986). Production of a Parkinson-like syndrome in the cat with N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Behavior, histology, and biochemistry. *Exp. Neurol.* **91**, 293-307.

Schultz, W., Scarnate, E., Sundstrom, E., Tsutsumi, T., and Jonsson, G. (1986). The catecholamine uptake blocker nomifensine protects against MPTP-induced parkinsonism in monkeys. *Exp. Brain Res.* **63**, 216-220.

Sealy, R.C., Felix, C.C., Hyde, J.S., and Schwartz, H.M. (1980). Structure and reactivity of melanins: influence of free radicals and metal ions. In *Free Radicals in Biology, Vol. 4* (Pryor, W.A., ed.). Academic Press, Inc., New York, New York, 209.

Segel, I.H. (1975). *Enzyme Kinetics Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems.* John Wiley and Sons, New York, New York, 118-120.

Shen, R.S., Abell, C.W., Gessner, W., and Brossi, A. (1985). Serotonergic conversion of MPTP and dopaminergic accumulation of MPP+. *FEBS* **189**, 225-229.

Shih, M. and Markey, S.P. (1986). Quantification of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 1-methyl-4-phenylpyridinium ion in brain tissue by gas chromatography/mass spectrometry. *Biomed. Environ. Mass Spectrom.* **13**, 85-89.

Shinka, T., Castagnoli, N., Jr., Wu, E.Y., Hoag, M.K.P., and Trevor, A. (1987). Cation-exchange high-performance liquid chromatography assay for the nigrostriatal toxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and its monoamine oxidase B generated metabolites in brain tissues. *J. Chromatog.* **398**, 279-287.

Singer, T.P., Salach, J.I., and Crabtree, D. (1985). Reversible inhibition and mechanism-based irreversible inactivation of monoamine oxidases by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Biochem. Biophys. Res. Commun.* **127**, 341-346.

Singer, T.P., Salach, J.I., Castagnoli, N., Jr., and Trevor, A.J. (1986). Interactions of the neurotoxic amine 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine with monoamine oxidases. *Biochem. J.* **235**, 785-789.

Sinha, B.K., Singh, Y., and Krishna, G. (1986). Formation of superoxide and hydroxyl radicals from 1-methyl-4-phenylpyridinium ion (MPP+): reductive activation by NADPH cytochrome P-450 reductase. *Biochem. Biophys. Res. Commun.* **135**, 583-588.

116

Slawinska, D., Stawinski, J., and Ciesla, L. (1983). The inhibition of peroxyradical-induced chemiluminescence by melanins. *Physiol. Chem. Phys.* **15**, 209-222.

Snyder, S.H. and D'Amato, R.J. (1986). MPTP: a neurotoxin relevant to the pathophysiology of Parkinson's disease. *Neurology* **36**, 250-258.

Stromme, J.H. (1965). Metabolism of disulfiram and diethyldithiocarbamate in rats with demonstration of an *in vivo* ethanol-induced inhibition of the glucuronic acid conjugation of the thiol. *Biochem. Pharmacol.* **14**, 393-410.

Sundstrom, E. and Jonsson, G. (1985). Pharmacological interference with the neurotoxic actions of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on central catecholaminergic neurons in the mouse. *Eur. J. Pharmacol.* **110**, 293-299.

Tranzer, J.P. and Thoenen, H. (1968). An electron microscopic study of selective acute degeneration of sympathetic nerve terminals after administration of 6-hydroxydopamine. *Experientia* **24**, 155-156.

Thoenen, H. and Tranzer, J. (1973). The pharmacology of 6-hydroxydopamine. *Ann. Rev. Pharmacol.* **132**, 169-180.

Trevor, A.J., Caldera, P.S., Castagnoli, K.P., Castagnoli, N., Jr., Chiba, K., Peterson, L.A., Salach, J.I., Singer, T.P., and Wu, E.Y. (1986). Metabolism of MPTP *in vitro*: the intermediate role of 2,3-MPDP+ and studies on its chemical and biochemical reactivity. In *MPTP: A Neurotoxin Producing A Parkinsonian Syndrome* (Markey, S.P., Castagnoli, N., Jr., Trevor, A.J., and Kopin, I.J., eds.). Academic Press, Inc., New York, New York, 161-172.

Trudell, J.R., Bosterling, B., and Trevor, A.J. (1982). Reductive metabolism of carbon tetrachloride by human cytochromes P-450 reconstituted in phospholipid vesicles: mass spectral identification of trichloromethyl radical bound to dioleoyl phosphatidylcholine. *Proc. Natnl. Acad. Sci. USA* **79**, 2678-2682.

Ungerstedt, U. (1968). 6-Hydroxydopamine-induced degeneration of central monoaminergic neurons. *Eur. J. Pharmacol.* **5**, 107-110.

Ungerstedt, U. (1971). Stereotaxic mapping of the monoamine pathways in the rat brain. *Acta Physiol. Scand.* (suppl.) **367**, 1-48.

Ungerstedt, U. and Hallstrom, A. (1987). *In vivo* microdialysis: a new approach to the analysis of neurotransmitters in the brain. *Life Sci.* **41**, 861-864.

Van Woert, M.H. and Ambani, L.M. (1974). Biochemistry of neuromelanins. In *Advances in Neurology, Vol. 5* (McDowell, F.H. and Barbeau, A., eds.). Raven Press, New York, New York, 215-223.

Ward, D.P., Trevor, A.J., Adams, J.P., Baillie, T.A., and Castagnoli, N., Jr. (1982). Metabolism of phencyclidine: the role of iminium ion formation in covalent binding to rabbit microsomal protein. *Drug Metab. Dispos.* **10**, 690-695.

Weissman, J., Trevor, A., Chiba, K., Peterson, L.A., Caldera, P., Castagnoli, N., Jr., and Baillie, T. (1985). Metabolism of the nigrostriatal toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine by liver homogenate fractions. *J. Med. Chem.* **28**, 997-1001.

Westerink, B.H.C., Damsma, G., Rollema, H., DeVries, J.B., and Horn, A.S. (1987). Scope and limitations of *in vivo* brain dialysis: a comparison of its application to various neurotransmitter systems. *Life Sci.* **41**, 1763-1776.

Westlund, K.N., Denney, R.M., Kochenperger, L.M., Rose, R.M., and Abell, C.W. (1985). Distinct monoamine oxidases A and B populations in primate brain. *Science* **230**, 181-183.
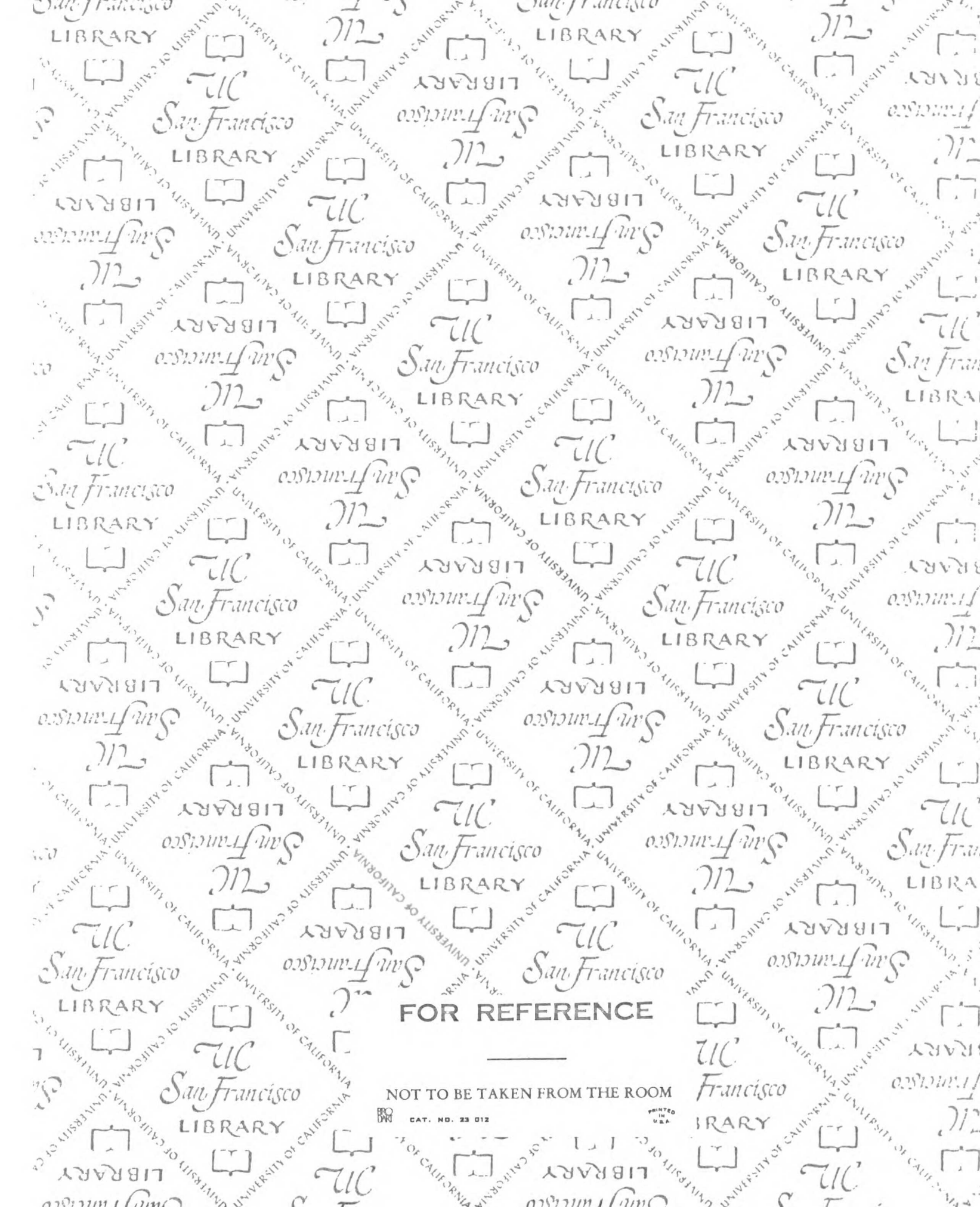
Weyler, W. and Salach, J.I. (1981). Iron content and spectral properties of highly purified bovine liver monoamine oxidase. *Arch. Biochem. Biophys.* **212**, 147-153.

Wu, E.Y., Chiba, K., Trevor, A.J., and Castagnoli, N., Jr. (1986). Interactions of the 1-methyl-4-phenyl-2,3-dihydropyridinium species with synthetic dopamine-melanin. *Life Sci.* **39**, 1695-1700.

Wu, E.Y., Shinka, T., Caldera-Munoz, P., Yoshizumi, H., Trevor, A., and Castagnoli, N., Jr. (1988). Metabolic studies on the nigrostriatal toxin MPTP and its MAO-B generated dihydropyridinium metabolite MPDP+. *Chem. Res. Toxicol.* **1**, 186-194.

Young, S.K., Sonsalla, P.K., and Heikkila, R.E. (1987). Evaluation of the biological activity of several analogues of the dopaminergic neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *J. Neurochem.* **48**, 929-934.