MOLECULAR BASIS OF MPTP-INDUCED PARKINSON'S DISEASE

By

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Abstract

Self-administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has resulted in irreversible symptoms of Parkinson's disease in several young drug abusers. It was found that this neurotoxicant selectively destroys neuronal cells in the substantia nigra of humans and other primates. Although the mechanism of action of MPTP is not fully understood, it is now generally believed that the crucial species for MPTP neurotoxicity is not MPTP itself, but rather some of its metabolites. $MPDP^+$, an intermediate in the metabolism of the neurotoxin MPTP, was found to generate superoxide radical ($\cdot O_2^-$) during its autoxidation process. The generation of $\cdot O_2^-$ was detected by their ability to reduce ferricytochrome *c*. Superoxide dismutase (SOD) inhibited this reduction in a dose-dependent manner. The rate of reduction of ferricytochrome c was dependent not only on the concentration of $MPDP^+$, but also on the pH of the system. Thus, the rate of autoxidation of $MPDP^+$ and the sensitivity of this autoxidation to superoxide dismutase inhibitable ferricytochrome *c* reduction were both augmented as the pH was raised from 7.0 to 10.5. The rate constant (k_c) for the reaction of superoxide radical with ferricytochrome *c* to form ferrocytochrome *c* was found to be $3.48 \times 10^5\ M^{-1}\ S^{-1}$. The rate constant ($k_{MPDP^+}$) for the reaction of $MPDP^+$ with ferricytochrome *c* was found to be $4.86\ M^{-1}S^{-1}$. The generation of $\cdot O_2^-$ was further confirmed by spin-trapping in combination with EPR techniques using 5, 5-dimethyl-1-pyrrolonine-N-oxide (DMPO) as the spin trapping agent. The rate of formation of spin adduct (DMPO-O_2^-) was dependent not only on the concentrations of $MPDP^+$ and oxygen but also on the pH of the system. Superoxide dismutase inhibited the spin adduct formation in a dose-dependent manner. The ability of DMPO to trap superoxide radicals, generated during the autoxidation of $MPDP^+$, and of SOD to effectively compete with this reaction for the available $\cdot O_2^-$, was used as a

convenient competition reaction to quantitatively determine various kinetic parameters. Using this technique, the rate constant for scavenging of superoxide radicals by superoxide dismutase was found to be 7.56×10^9 $M^{-1}S^{-1}$. The maximum rate of superoxide generation at a fixed spin trap concentration using different amounts of $MPDP^+$ was found to be 4.48×10^{-10} $M \cdot S^{-1}$. The rate constant (k_1) for $MPDP^+$ making superoxide radical was found to be 3.97×10^{-6} Sec^{-1}. The second order rate constant (k_{DMPO}) for DMPO trapping superoxide radicals was found to be 10.2 $M^{-1}S^{-1}$. The life time of superoxide radical at pH 10.0 was calculated to be 1.25 seconds. These data indicate that superoxide radicals are produced during spontaneous oxidation of $MPDP^+$ and that EPR spin trapping techniques can be used to determine the rate constants and life time of free radicals generated in aqueous solution.

Monoamine oxidase type B (MAO-B), an enzyme present in mitochondrial membranes, is known to metabolize MPTP to $MPDP^+$, which then spontaneously oxidizes to MPP^+. In the studies of MAO-B catalyzed oxidation of MPTP, the neurotoxicant was found to generate reactive oxygen species during its interaction with the enzyme. The kinetic parameters, K_m and V_{max}, for MAO-B catalyzed oxidation of MPTP to the corresponding species $MPDP^+$ were found to be 0.194 mM and 0.335 μM/min, respectively. The generation of $\cdot O_2^-$ and hydroxyl ($\cdot$OH) radicals was detected as the DMPO spin adduct by spin trapping in combination with EPR techniques. Addition of Fe^{2+} (10 μM) to this system caused a 5-fold enhancement in EPR signal intensity of the DMPO-OH adduct. Catalase, a scavenger of hydrogen peroxide (H_2O_2), inhibited the DMPO-OH spin adduct formation in a dose-dependent manner, indicating that H_2O_2 is produced in the MAO-B catalyzed oxidation of MPTP. Ethanol, a well known scavenger of hydroxyl radical, rapidly produced an alpha-hydroxyethyl radical signal. SOD inhibited the formation of DMPO-O_2^- and DMPO-OH spin adducts in a dose-dependent fashion.

These data suggest that $\cdot O_2^-$ are produced during the oxidation of MPTP by MAO-B and that the generation of H_2O_2 and $\cdot OH$ was secondary to the production of $\cdot O_2^-$.

MPTP and its metabolites, $MPDP^+$ and MPP^+, were found to inhibit the activity of acetylcholinesterase (AChE). The kinetic parameter, K_m for the substrate (acetylthiocholine), was found to be 0.216 *m*M and K_i values for MPTP, $MPDP^+$ and MPP^+ to inactivate AChE were found to be 2.14, 0.265 and 0.197 *m*M, respectively. The inactivation of AChE by these neurotoxicants was found to be dose-dependent. It was found that MPTP, $MPDP^+$ and MPP^+ are neither substrates of AChE nor the time-dependent inactivators. The studies of reaction kinetics indicate that the inactivation of AChE by these inactivators is via a mixed-type inhibition. The dilution of the enzyme-inhibitor complex completely reversed the MPTP inhibition but only partially reversed the MPDP+ and MPP+ inhibition. These data indicate that MPTP and its metabolites can inactivate AChE and thereby increase ACh level in the basal ganglia of the brain, leading to potential cell dysfunction.

These results suggest that once MPTP enters the basal ganglia of the brain, it can be catalyzed by MAO-B to generate a series of reactive species, including $\cdot O_2^-$, H_2O_2 and $\cdot OH$, which are known to destroy cell membranes, enzymes and other important biological molecules. The nigrostriatal toxicity by MPTP leading to Parkinson's disease-like syndrome may largely be due to the reactivity of these reactive oxygen species in combination with the inactivation of the AChE enzyme in the brain, leading to potential cell dysfunction.

With Love and Appreciation

to

my wife and son
Yun Zhang & Nan Bernie Zang
my parents
Jin-Shen & Su-Zheng Zang
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to

my advisor & friend

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freedom and an independent environment
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DECLARATION

This research dissertation was performed completely by this author from start to finish which included proposal ideas, experimental design, all techniques and manuscript preparation as well.

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Table of Contents

Abstract	ii
Dedication	iv
Acknowledgements	vii
Declaration	viii
List of Figures and Scheme	xiii
List of Tables	xvii
List of Abbreviations	xviii

CHAPTERS

I: Introduction

The neurotoxicants, MPTP and its metabolites	1
Spin trapping and EPR	4
The proposed mechanism of MPTP neurotoxicity	7
Our hypothesis of MPTP neurotopxicity	10
Significance of this study	13
References	14

II: Superoxide radical produced during the autoxidation of 1-methyl-4-phenyl-2,3-dihydropyridinium perchlorate ($MPTP^+$).

Abstract	19
Introduction	20
Materials and Methods	21

Results 22

Discussion 28

References 32

III: **EPR Kinetic studies of superoxide radical generated during the autoxidation of 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$), A bioactivated intermediate of a Parkinsonian inducing neurotoxin MPTP.**

Abstract 46

Introduction 47

Materials and Methods 49

Results 50

Discussion 58

References 64

IV: **Generation of reactive oxygen species during the monoamine oxidase-catalyzed oxidation of the neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.**

Abstract 78

Introduction 79

Materials and Methods 80

Results 81

Discussion 89

References 96

V: **INACTIVATION OF ACETYLCHOLINESTERASE BY THE NEUROTOXICANT, 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDRO-PYRIDINE HYDROCHLORIDE.**

Abstract 113

Introduction 114

Materials and Methods 115

Results 117

Discussion 119

References 124

VI: ACETYLCHOLINESTERASE INHIBITION BY 1-METHYL-4-PYRIDINIUM, A BIOACTIVATED METABOLITE OF MPTP.

Abstract 134

Introduction 135

Materials and Methods 136

Results 138

Discussion 140

References 145

VII: Characteristics of acetylcholinesterase inhibition by 1-methyl-2,3-dihydropyridinium ion, a metabolite of MPTP.

Abstract 157

Introduction 158

Materials and Methods 159

Results 160

Discussion 164

References 169

VIII: Interaction of the neurotoxicants, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and its metabolites on mitochondria of guinea pig and dog brains.

Abstract 180

Introduction 181

Materials and Methods 182

Results 186

Discussion 191

References 195

IX. Summary 207

X. Bibliography 209

Vita 227

List of Figures and Schemes

Figure			*Page*
Chapter I			
1.	Scheme I.	Comparison of structures between MPP^+ and Paraquat	3
2.	Scheme II.	Proposed site of inhibition of mitochondrial electron transport chain by MPP^+	9
3.	Scheme III.	Proposed mechanisms of MPTP action	12
Chapter II			
1.		Kinetics of $MPDP^+$ oxidation	36
2.		Decay kinetics of $MPDP^+$	37
3.		The effect of pH on the rate of autoxidation of $MPDP^+$	38
4.		The initial rates of reduction of ferricytochrome c as a function of $MPDP^+$ concentration	39
5.		Reduction of ferricytochrome c as a function of its concentration	40
6.		Inhibition of ferricytochrome c reduction by superoxide dismutase	41
7.		Effect of pH on the rate of autoxidation of 2,3-$MPDP^+$, measured in terms of accumulation of ferricytochrome c	43
8.		Possible mechanism of autoxidation of $MPDP^+$ to form MPP^+	44
Chapter III			
1.		EPR spectra of spin adducts of free radicals observed during the autoxidation of $MPDP^+$	69
2.		Kinetics of spin adduct formation	70

3. The rate of spin-adduct formation as a function of $MPDP^+$ 71
4. Inhibition of EPR spin adduct signal intensity by superoxide dismutase 72
5. The initial rate of formation of spin adduct as a function of DMPO concentration 73
6. Effect of pH on the rate of spin adduct formation during the autoxidation of $MPDP^+$ 74
7. Proposed mechanisms for superoxide radicals generation during the autoxidation of $MPDP^+$ 75

Chapter IV

1. Kinetics of MPTP oxidation catalyzed by MAO-B 103
2. Effect of MPTP concentration on the rate of $MPDP^+$ formation at a fixed MAO-B concentration 105
3. EPR spectra of spin adducts of superoxide radicals observed during MAO-B catalyzed oxidation of MPTP 106
4. EPR spectra of spin adducts of free radicals observed during MAO-B catalyzed oxidation of MPTP 107
5. The rate of spin-adduct formation as a function of MAO-B concentration in air-saturated DMSO solution 108
6. Inhibition of EPR spin adduct signal intensity by superoxide dismutase in air-saturated DMSO solution 109
7. Proposed mechanisms for the generation of superoxide radicals during MAO-B catalyzed oxidation of MPTP 110

Chapter V

1. Effect of MPTP concentration on the activity of acetylcholinesterase 129
2. Effect of incubation time on the acetylcholinesterase activity 130
3. Reversibility of MPTP inhibition of AChE 131
4. Lineweaver-Burk plot on the inhibition of the acetylcholine hydrolytic activity of acetylcholinesterase by MPTP 132
5. The secondary plot derived from the slopes of primary double reciprocal plots at different concentrations of MPTP 133

Chapter VI

1. Effect of MPP^+ concentration on the activity of acetylcholinesterase 150
2. Effect of incubation time on the acetylcholinesterase activity 151
3 Reversibility of MPP^+ inhibition of AChE
(a) Effect of dilution of enzyme-inhibitor complex on AChE activity
(b) Effect of gel exclusion of enzyme-inhibitor complex on AChE activity 152
4. Lineweaver-Burk plot on the inhibition of the acetylcholine hydrolytic activity of acetylcholinesterase by MPP^+ 155
5. The secondary plot derived from the slopes of primary double reciprocal plots at different concentrations of MPP^+ 156

Chapter VII

1. Kinetics of AChE catalyzed-hydrolysis of acetylthiocholine 174
2. Effect of $MPDP^+$ concentration on the activity of acetylcholinesterase 175
3. Effect of incubation time on acetylcholinesterase activity 176
4. Reversibility of $MPDP^+$ inhibition of AChE
(a) Effect of dilution of enzyme-inhibitor complex on AChE activity

CHAPTER I

INTRODUCTION

Parkinson's disease was first described by James Parkinson, a physician, in 1817 as "shaking palsy" (Mehler, 1992). Although this condition was observed over a century and a half ago, the actual cause of this devastating disease remains obscure. Tremendous amounts of money and efforts have been spent in Parkinson's research, unfortunately with minimal results. The recent discovery of MPTP-induced Parkinson's disease provides a clue for exploring this secret and suggests that the cause of Parkinson's disease may be one or more environmental pollutants.

The neurotoxicants, MPTP and its metabolites:

Self-administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), along with several other agents including 1-methyl-4-phenylpropionoxy piperidine (MPPP), has resulted in irreversible symptoms of Parkinson's disease in several young drug abusers (Langston *et al.*, 1983). These abusers were attempting to simulate the actions of heroin with the potent agent MPPP. The clinical features that were observed in those affected by MPTP, include the tremor, rigidity and akinesia, are commonly observed in Parkinson's patients. Based on the observed lack of concordance of Parkinsonism in identical twins, most investigators now believe that there are no genetic causes for the disease (Heikkila, 1988) and the discovery of a causative agent remains a major research goal.

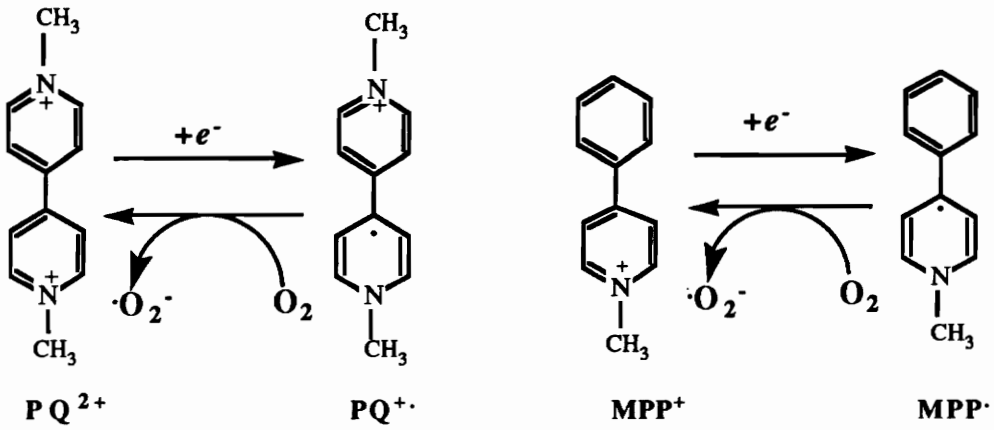
The discovery of MPTP and MPPP in early 1980's as the primary constituents of illicit drug mixtures that lead to Parkinson's disease, raised certain intriguing questions. It was found that primates and dogs treated with rather low doses of MPTP exhibited

extensive neurobiogical, neurochemical and behavioral deficits, similar to those observed in humans with idiopathic Parkinson's disease (Burns et al., 1983). In contrast, rats (Boyce *et al.*, 1984; Chiueh *et al.*, 1984; Heikkila *et al.*, 1984 and Sahgal *et al.*, 1984), guinea pigs (Heikkila *et al.*, 1984 and Chiueh *et al.*, 1984) and gerbils (Heikkila *et al.*, 1984) were relatively insensitive to the neurotoxic actions of MPTP (Chiueh *et al.*, 1984). Pure MPPP, however, caused no deleterious effects in either rodents or primates (Burns *et al.*, 1983; Chiueh *et al.*, 1984 and Heikkila *et al.*, 1984).

Although the mechanism of action of MPTP is not fully understood, most investigators working in this field do agree that the culprit is not MPTP itself, but rather its metabolite, 1-methyl-4-phenylpyridinium (MPP^+). Since MPP^+, being a positively charged molecule, is unable to cross the blood-brain barrier, it must be formed within the brain to induce tissue injury. It was reported (Markey *et al.*, 1984 and Langston *et al.*, 1984) that the MPP^+ is the major metabolite of MPTP found in the brains of experimental animals. This led to the suggestion that MPP^+ formation plays an important role in MPTP-induced neurotoxicity (Markey *et al.*, 1984). In the studies of MPTP-metabolism, it has been demonstrated that monoamine oxidase-B (MAO-B) catalyzes the 2-electron oxidation of MPTP to a dihydropyridinium intermediate ($MPDP^+$), which then spontaneously oxidizes to form MPP^+ or disproportionates to form MPTP and MPP^+ (Chiba *et al.*, 1984 and 1985). The administration of MAO-B inhibitor, L-deprenyl, affords full protection against the neurotoxic actions of MPTP (Heikkila *et al.*, 1984) in mice. In parallel experiments, it was shown that animals treated with MAO-B inhibitor prior to MPTP administration have decreased brain levels of MPP^+ compared with mice treated with MPTP alone (Markey *et al.*, 1984). Moreover, MAO-B inhibitors, given to primates prior to administration of MPTP, protect against MPTP-induced neurotoxicity (Langston *et al.*, 1984 and Cohen *et al.*, 1984). This shows that the MAO-B mediated bioactivation of MPTP to MPP^+ plays a critical role in the neurotoxic actions of MPTP. Since MAO-B in

the brain is located mainly in the astrocytes (Singer *et al.*, 1988), the question then arises as to how MPP^+ generated extraneuronally reaches the nigrostriatal cells. Javitch *et al* (1985) have clearly shown and later confirmed by Mayer *et al* (1986), Sundatrom *et al* (1986) and Ricaurte *et al* (1985) that the dopamine reuptake system of dopaminergic neurons binds MPP^+ very tightly, but not MPTP. This was based on the findings that Mazindol, a reuptake inhibitor, protects against MPTP-induced toxicity. Subsequently, it was demonstrated that MPP^+ is a good substrate for the neostriata dopamine transport system. The K_m and V_{max} values for MPP^+ transport are very similar to the values for dopamine (Javitch *et al.*, 1985).

The structural features which render MPTP a neurotoxic substance remain obscure and the mechanism by which MPTP/MPP^+ destroys the dopaminergic neurons is yet to be elucidated. Based on the similarity of chemical structures between MPP^+ and paraquat, speculation has centered on redox cycling reactions between MPP^+ and dopamine or other cellular constituents (Castagnoli *et al.*, 1985 and Kopin *et al.*, 1985). It is possible that the toxicity of MPP^+, like paraquat (Bus *et al*, 1976), may be mediated via the generation of superoxide free radical ($\cdot O_2^-$) by redox cycling reactions as follows (Scheme I):



Scheme I. Comparison of structures between MPP^+ and Paraquat.

The generation of reactive oxygen species could overwhelm the protective antioxidants (such as glutathione, superoxide dismutase and catalase), and can react with essential polyunsaturated lipids resulting in accumulation of membrane lipid peroxides. It is generally believed that the generation of free radicals or reactive oxygen species during metabolism of various toxins results in cell damage and issue injury (Freeman and Crapo, 1982; Trush *et al.*, 1982). Superoxide dismutase (SOD), catalase, and glutathione-peroxidase and -reductase as well as antioxidant vitamins (vitamin A, E and C) can break the free radical mediated chain reactions and protect the tissue against oxidative injury. However, if the rate of the reactive species generation is accelerated, such defense mechanisms will become inadequate, leading to injury and cell death. Cohen (1984) has reported that catecholamine neurons are sensitive to oxy-radical attack. Graham (1984) has suggested that formation of dopamine quinone, its semiquinone products and reactive species of oxygen ($\cdot O_2^-$, $\cdot OH$, H_2O_2) are factors in the pathogenesis of Parkinson's disease. However, the mechanism of generation of these reactive species responsible for the injury of these neurons is not known. Although postulated by several investigators, neither the generation of reactive species of oxygen nor the formation of $MPP^{\cdot}$ radical, one key intermediate in the redox cycling of $MPP^+/MPP^{\cdot}$, have yet been identified.

Spin Trapping and EPR:

Since its discovery, about forty-five years ago, electron paramagnetic resonance (EPR) has proven to be a useful tool for studying the chemical, physical, and biological properties of biomolecules. The ability of EPR to detect low concentrations of free radicals and its sensitivity to change in environment and molecular motions have greatly augmented its use. The application of EPR to biological studies has been limited because of the small quantities of reactive free radicals produced for the direct EPR detection. Various methods have been employed to overcome this problem including high-energy radiolysis, high-

intensity photolysis, and rapid-flow techniques. However, these techniques are rather expensive and/or cumbersome and do not appear to be generally applicable to in vivo conditions. The introduction of spin trapping techniques by Janzen (1973) overcame most of the above problems.

The spin trapping technique makes use of a diamagnetic compound (the spin trap) which reacts with a free radical (the spin) giving rise to a relatively stable , EPR-observable free radical (the spin adduct) as follows:

Radical + Spin Trap ----------> Spin Adduct

In favorable cases, the free radicals and the spin adducts can be identified from EPR parameters (hyperfine splitting constant, g-factor). Thus, spin trapping extends the capabilities of EPR in that previously unobservable free radicals (or at least, radicals observable only with difficulty) can now be studied as their respective spin adducts in a somewhat more leisurely fashion. The spin traps that have been most commonly employed are those that upon reaction with a free radical produce a stable nitroxide. Typically, spin traps are either nitroso compounds or nitrones.

The actual experimental procedure employed in spin-trapping experiments depends on a number of factors such as the manner of radical production, the inertness of the solvent and reagents with respect to the spin trap, the lifetime of the spin adducts, and presence of molecular oxygen. Usually, deoxygenation by bubbling purified nitrogen or argon gas through the solution is sufficient for spin-trapping studies. In some cases degassing by the freeze-thaw vacuum pump technique is necessary if a very low oxygen level is required or if volatile reagents are involved. The most popular nitroso compound used for spin trapping has been 2-methyl-2-nitroso-propane (MNP) or, nitroso-tert-butane (NtB). Nitroso compounds have an inherent advantage over nitrones for free radical identification in that the added group lies immediately adjacent to the nitroxide center and therefore can easily give rise to additional hyperfine splitting and helps to identify the

radical trapped. The nitrone which is used in most spin-trapping studies is phenyl-N-tert-butyl nitrone (PBN). This is probably due to the fact that it has a good shelf stability, has been commercially available for a long time, and was the first nitrone to be used in this manner. However, PBN does not distinguish between alkyl radicals particularly well. Its spin adducts generally consist of triplet of doublets with a relatively small variation in the doublet splitting as a function of trapped radical. However, a nitrone which has shown more sensitivity to the structure of the radical is 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), introduced by Janzen (1973). It is interesting to consider the origin of the variation in the proton hyperfine splitting observed as a function of structure of the trapped free radical. The magnitude of this interaction is governed by the Heller-McConnell (1960) equation as following:

$$^{\beta}A_H = (B_o + B_2\cos^2\emptyset)\varphi$$

Where B_o and B_2 are constants ($B_o = 0$ and $B_2 = 26$ Gauss for nitroxides) and $\emptyset$ is the dihedral angle formed by the C-N p-orbital and the N-C βH planes; φ = spin density on the atom in question. Thus, each group R· added to the spin trap will have different stereo-electronic characteristics and will therefore give rise to a different value for $\emptyset$. The spin trap DMPO is structured so that the conformation of its adducts places the ß-hydrogen in a nearly eclipsing relationship with the nitrogen p-orbital ($\emptyset$ is small and $^{\beta}A_H$ is large). As a result, small changes in the bulk of R· give rise to relatively large variations in $^{\beta}A_H$ and it helps to identify the trapped free radicals. However, each spin trap for the application of spin trapping has its advantages and disadvantages. The following is the comparison of the typical spin traps:

Table 1. Comparison of spin traps on their advantages and disadvantages.

	Advantages	Disadvantages
MNP	Spin-adduct spectrum more definitive;	Only slightly soluble in H_2O; Photolabile to give $(Me_3C)_2N$-O; No stable oxyl spin adducts.
PBN	Fast trapping rate constant (for some R.); Relatively stable compound; Spin adduct relatively long-lived;	Spin adduct spectrum less definitive; Slower trapping rate constant (for some R·);
DMPO	Spectrum very sensitive to radical trapped; Fast trapping rate constant.	Fairly reactive compound; Spin adduct relatively short-lived.

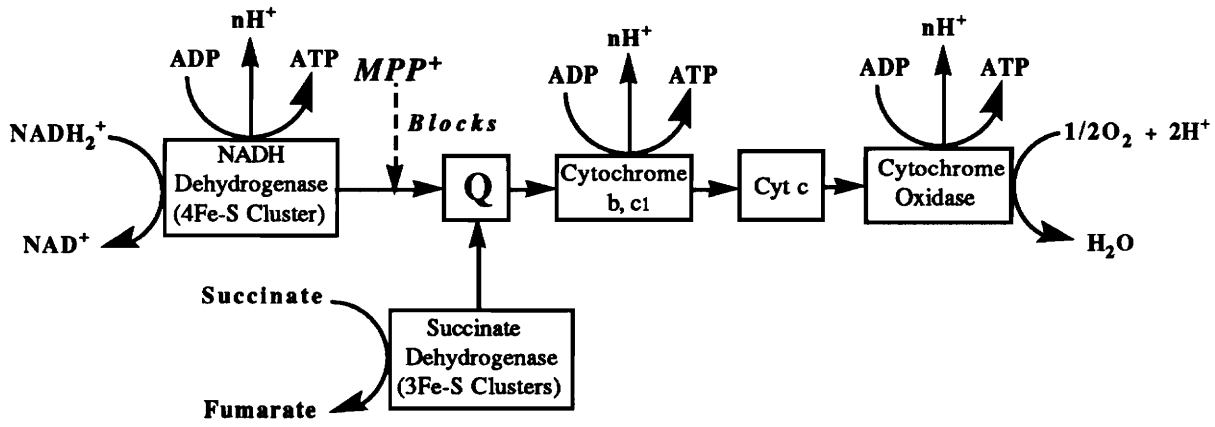
The proposed mechanisms of MPTP neurotoxicity:

Considering the fact that neuromelanin is absent in rodent brains but present in primates and maximum concentration is found in human brain (Marsden, 1961), and that the MPP^+ can be rapidly removed from rodent brain within few hours, but persists for up to 20 days in monkey brain (Johannessen *et al.*, 1985), it is possible that MPTP toxicity is related to the neuromelanin content of brain tissues. One major theory of MPTP-induced Parkinson's disease, first introduced in 1986 by R. J. D'Amato, Z. P. Lipman and S. H. Snyder of the Johns Hopkins University, is that MPP^+ causes accumulation of intracellular neuromelanin in the substantia nigra of primates and subsequent release of neuromelanin induces damage to the neurons of the substantia nigra. On the other hand, neuromelanin accumulations may reflect a lower capacity of the cellular defense mechanisms that limit levels of reactive oxygen molecules ($\cdot O_2^-$, H_2O_2, $\cdot OH$). These reactive species in turn can promote quinone formation from catechols (Fridovich 1975). Accumulation of MPP^+ and the rate of its redox cycling in the cytoplasm may play a critical role in accelerating superoxide formation to a level which exceeds the capacity of the neuron to dispose of

oxidative radicals. The low level of glutathione, an antioxidant, in human substantia nigra (Perry *et al.*, 1982) has been implicated in the pathogenesis of Parkinson's disease and may contribute to the susceptibility to MPTP toxicity. Neuromelanin may be a participant in the redox cycling of MPP^+ which generates free radicals because neuromelanin is a redox polymer which can form stable free radicals (Van Woert and Ambanic, 1984), *i.e.*, it can, depending upon the state of oxidation, serve as an electron donor or acceptor.

Since the neuromelanin is sequestered in particles, there is little opportunity for this compound to react with mitochondrial electron transport system. Although MPP^+ was proposed by some investigators to react with the reduced quinone and a "MPP^+-$MPP^\bullet$ redox cycle" may act as an electron shuttle enhancing free radicals formation in neuronal mitochondria, it has been questioned by others which led to negation of the above hypothesis. Hart (1987) thought that MPP^+ is not neurotoxic in animal models because it, like paraquat, is a charged hydrophilic molecule which cannot readily cross the blood-brain barrier and will therefore not be available in sufficient amounts to affect the substantia nigra cells. Based on the observations that MPP^+ induces a very little amount of oxygen radicals (Sinha *et al.*, 1986) and causes very little lipid peroxidation in subcellular fractions (Corongin et al., 1987), Smith *et al.* (1987), it was concluded that MPP^+-induced Parkinsonism is unlikely to involve oxygen radicals or lipid peroxidation. However, Javitch *et al.* (1985) and D'Amato *et al* (1986) reported that MPP^+ can enter the neuronal cells by the dopamine uptake system and its concentration in the dopaminergic neurons may become several thousand-times greater than in the extracellular concentration.

An alternate approach to explain MPTP neurotoxicity has recently been postulated based on inhibition of the mitochondrial electron transport chain by MPP^+. Similar to the action of rotenone, and piericidin A, MPP^+ blocks mitochondrial electron transport at site I (Nicklas *et al*, 1985; Vyas *et al.*, 1986; Ramsay *et al.*, 1991). This process is shown in scheme II as follows:



Scheme II

Proposed site of inhibition of mitochondrial electron transport chain by MPP+.

The first clue (Nicklas *et al.*, 1985) came from the observations that the incubation of rat liver or brain mitochondria with relatively high concentrations (0.5 *mM*) of MPP+ caused progressive inhibition of the ADP-stimulated oxidation of NAD-linked substrates, without affecting succinate oxidation. This suggested a block of NADH dehydrogenase between substrate and ubiquinone (Q). Other studies confirmed this observation and demonstrated an associated decrease in ATP formation within the dopaminergic neuron (Heikkila *et al.*, 1985). MPP+ was found to accumulate in the mitochondria of liver and midbrain to 20-30 *mM* levels which are more than enough to block electron transfer (and associated ATP synthesis) from NADH dehydrogenase to Q_{10} (Ramsay and Singer, 1986; Ramsay *et al.*, 1986). However, the mechanism of inhibition of the electron transport chain of neuronal mitochondria by MPP+ has not yet been resolved. Although such a process would partially interfere with ATP synthesis, it probably would not kill cells because complex II can provide sufficient electrons to the respiratory chain to maintain cell function. More recently, it has been reported that the generation of free radicals induced by MPP+ in submitochondrial particles may be responsible for the neurotoxicity (Hasegawa *et al*, 1990 and Cleeter *et al*, 1992). However, the inability of MPP+ to be bio-reduced or to

stimulate the production of superoxide radicals during aerobic reduction ruled out this hypothesis (Frank *et al*, 1987).

In 1990, researchers from the National Institute of Mental Health (NIMH) and the National Institute of Neurological Disorder and Stroke (NINDS) hypothesized that MPP^+ kills the axons, leading to cell death (NIH News, Nov., 1990). Other researchers found swelling of axons, followed by degeneration at their terminals, degeneration of the axon and separation of the axon from its parent cell body, and finally; the death of the cell body. However, these results merely show that the axons die first; they do not provide a molecular mechanism of cellular death. The axons and the cell terminals being dependent upon the cell body, any problem in the cell body can compromise neuron respiration and axoplasmic flow as well. Thus, any injury to the cell body may first be reflected on cell terminals or the axons (Turner, 1990). Therefore, further studies are necessary to investigate the primary events of neuronal cell injury caused by MPP^+.

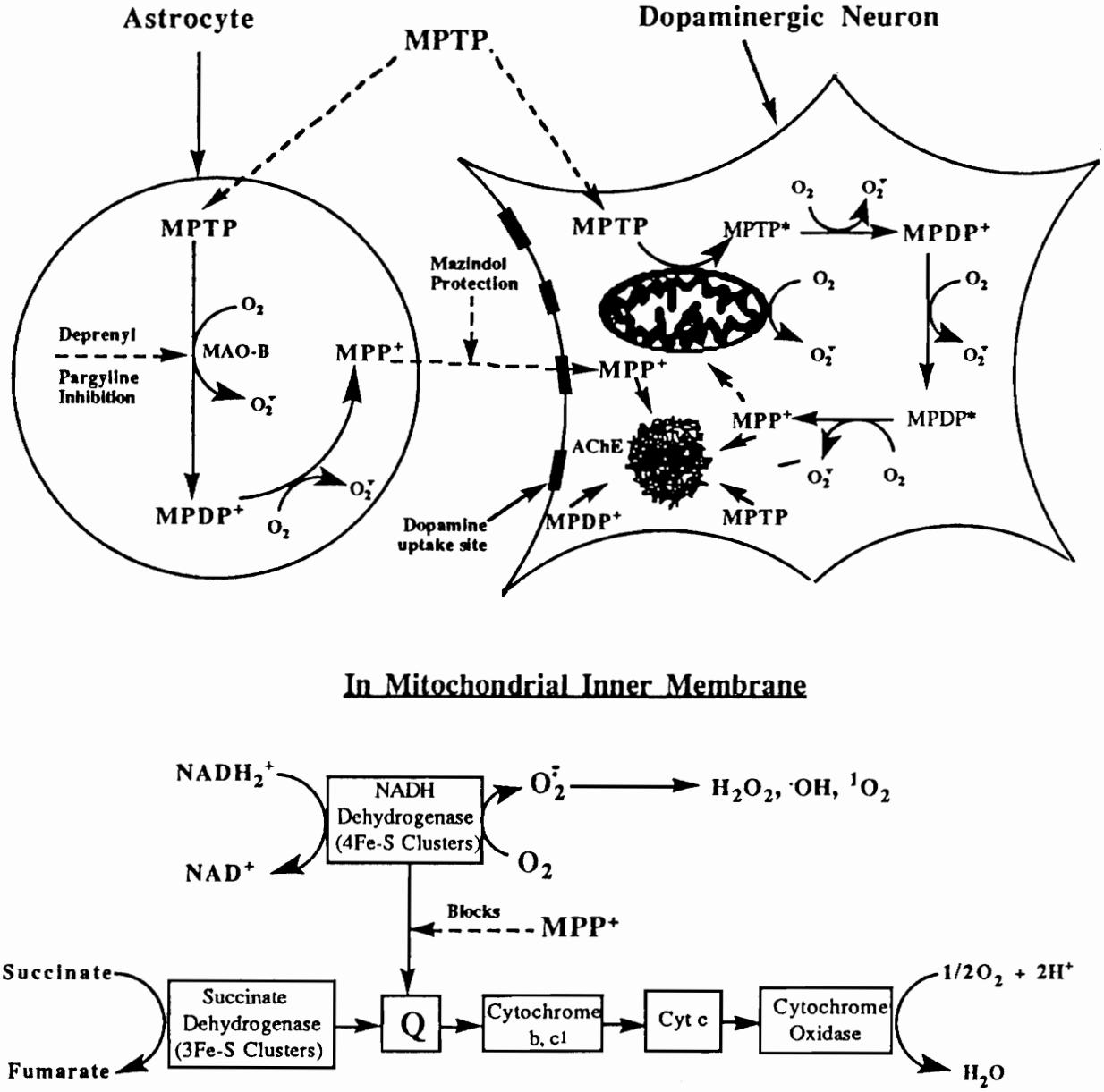
Our hypothesis of MPTP neurotoxicity:

According to the information described above, it is evident that MPP^+ can play an important role in MPTP-induced Parkinson's disease. However, the neuronal cell damage caused by MPTP and its metabolites cannot entirely be ascribed to MPP^+ action on NADH dehydrogenase alone because MPTP inhibits the activity of NADH dehydrogenase more powerfully than MPP^+ does (Mizuno *et al*, 1987). The interaction of MPP^+ with NADH dehydrogenase (Ramsay *et al*, 1991) would partially interfere with ATP synthesis but probably not kill the cell because the complex II can provide sufficient electrons to the respiratory chain for cellular functions. It was reported that MPTP and MPP^+ can induce oxidative stress in the lung in a manner similar to that of paraquat (Johannesson *et al*, 1986 and Adams *et al*, 1986). More recently, several reports have indicated that inhibition of complex I by rotenone or MPP^+ and inhibition of complex III by antimycin A enhances the

production of free radicals by the respiratory chain (Hasegawa et al, 1990, Minagawa et al., 1992 and Cleeter et al, 1992). Recently we have found that reactive oxygen species are produced in MAO-B catalyzed oxidation of MPTP (Zang and Misra, 1993). $MPDP^+$, a reactive intermediate produced during the process of MPTP metabolism, was found to decay to MPP^+ accompanied by with the generation of superoxide radicals (Zang and Misra, 1992). Any one of these reactive species are capable of causing cell injury and cell dysfunction. Therefore, it was hypothesized that the free radicals generated during MPTP metabolism may be the primary events in cellular injury.

Parkinson's disease has been recognized as the most common of the basal-ganglion disorders (Burnhan, 1989). Closely associated with the basal ganglia are the substantia nigra and the subthalamus. The caudate-putamen of the basal ganglia contains a number of neurotransmitter substances including norepinephrine (NE), serotonin (5-HT), glutamate (Glu), gamma-aminobutyric acid (GABA), dopamine (DA) and acetylcholine (ACh). Normal function of the caudate-putamen depends on the balance of these transmitters, particularly on the balance of DA and ACh (Burnhan, 1989). DA has been described as the "**go**" system and ACh the "**no go**" system (Burnhan, 1989). Thus, an excess of DA produces an excess of movement while an excess of ACh produces immobility. ACh can be rapidly broken down by the enzyme acetylcholinesterase (AChE), which occurs in high concentration on both pre- and post-ganglion membranes within autonomic ganglia and on the membranes of parasympathetic nerve terminals where ACh also functions as a neurotransmitter (Francis, 1988 and Flattery *et al*, 1989). Inactivation of AChE by a neurotoxicant will certainly result in the accumulation of an excess amount of ACh leading to not only immobility, but also may cause neuronal cell injury (Ballinger *et al*, 1990, Fest *et al*, 1973 and Eto, 1979). Although the effect of MPTP and its metabolites on the MAO enzyme system and mitochondria has been extensively studied, no reference has been made

to the "**no go**" system. Therefore, it was hypothesized that excess amounts of free radicals are produced during metabolism of MPTP and its metabolites and that these neurotoxicants may alter the activity of AChE; any one of these processes would lead to Parkinson's like syndrome. The overall mechanisms of the proposed research may be illustrated by the following scheme:



Scheme III. Proposed Mechanisms of MPTP Action.

Significance of this study:

Since James Parkinson first described the disorder , a century and a half ago, the cause of Parkinson's disease has been a mystery. The recent discovery of MPTP-induced Parkinson's disease may provide a clue for exploring this secret. Since some environmental pollutants such as paraquat, NO, NO_2, Ozone, *etc* are known to induce free radicals and free radicals are thought to cause cell injury and cell dysfunction, it is reasonable to attribute the MPTP-induced Parkinson's to these reactive radicals. Our proposed studies will focus on this important point. The results of the proposed studies will permit not only identification of the types of reactive species formed during the metabolism of MPTP, but also quantitate the rate of their production with the conditions imposed. Fundamental knowledge derived from these studies could lead to enhanced functional improvement of patients and may further the cause of defeating this devastating disease.

The Specific Aims:

1. To examine the possibility of the generation of the reactive oxygen species during autoxidation of MPTP and its metabolites, using spin trapping in combination with EPR techniques;
2. To detect the types of reactive species produced during the interaction of MPTP, $MPDP^+$ and MPP^+ with NADH-linked mitochondrial respiratory chain;
3. To investigate the effect of MPTP, $MPDP^+$ and MPP^+ on NADH dehydrogenase;
4. To investigate the generation of free radicals during the interaction of MPTP, $MPDP^+$ and MPP^+ with MAO-B using purified MAO-B and intact mitochondria as the models;
5. To determine the effect of MPTP, $MPDP^+$ and MPP^+ on acetylcholinesterase.

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CHAPTER II

SUPEROXIDE RADICAL PRODUCTION DURING THE AUTOXIDATION OF 1-METHYL-4-PHENYL-2,3-DIHYDROPYRIDINIUM PERCHLORATE ($MPDP^+$)

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Abstract

$MPDP^+$, an intermediate in the metabolism of the neurotoxicant MPTP, was found to generate superoxide radical during its autoxidation process. The generation of superoxide radicals was detected by their ability to reduce ferricytochrome *c*. Superoxide dismutase (SOD) inhibited this reduction in a dose-dependent manner. The rate of reduction of ferricytochrome c was dependent not only on the concentration of $MPDP^+$, but also on the pH of the system. Thus, the rate of autoxidation of $MPDP^+$ and the sensitivity of this autoxidation to superoxide dismutase inhibitable ferricytochrome *c* reduction were both augmented, as the pH was raised from 7.0 to 10.5. The rate constant (k_c) for the reaction of superoxide radical with ferricytochrome *c* to form ferrocytochrome *c* was found to be $3.48 \times 10^5\ M^{-1}S^{-1}$. The rate constant ($k_{MPDP^+}$) for the reaction of $MPDP^+$ with cytochrome^{3+} *c* was found to be only $4.86\ M^{-1}S^{-1}$.

These results, in conjunction with complexities in the kinetics, lead to the proposal that autoxidation of $MPDP^+$ proceeds by at least two distinct pathways, one of which involves the production of superoxide radicals and hence is inhibitable by superoxide dismutase. It is possible that the free radicals so generated could induce oxidative-injury which may be central to the MPTP/$MPDP^+$-induced neuropathy.

INTRODUCTION

Self-administration of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) made manifest the irreversible symptoms of Parkinson's disease in several young drug abusers (1). It was found that this neurotoxin selectively destroys neuronal cells in the substantia nigra of humans and other primates (2). Although the mechanism of action of MPTP is not fully understood, it is now generally believed that the crucial species for MPTP neurotoxicity is not MPTP itself, but rather some of its metabolites (3, 4). In studies of MPTP-metabolism, it has been demonstrated that monoamine oxidase-B catalyzes the two-electron oxidation of MPTP to a dihydropyridinium intermediate ($MPDP^+$), which in turn spontaneously oxidizes to form methyl phenylpyridinium (MPP^+) or disproportionate to form MPTP and MPP^+ (5-7). The generation of oxygen radicals during metabolism of MPTP has been proposed to explain its neurotoxicity (8-15), but no direct evidence has been available to confirm this hypothesis. It was reported that $MPDP^+$ autoxidizes to generate hydrogen peroxide (H_2O_2) in a reaction promoted by chelated iron (16). This demonstration, that $MPDP^+$ could cause divalent reduction of oxygen to form H_2O_2, gave no indication of the relative importance of the univalent reduction of oxygen to generate superoxide radicals. It appeared desirable to investigate the univalent reduction of oxygen by $MPDP^+$ as a function of pH and partial pressure of oxygen. The present report describes such measurements.

MATERIALS AND METHODS

1-methyl-4-phenyl-2, 3-dihydropyridinium perchlorate ($MPDP^+$), 1-methyl-4-phenyl-pyridinium (MPP^+) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) were purchased from Research Biochemicals Inc. Superoxide dismutase (SOD), xanthine oxidase and ferricytochrome *c* (type III) were obtained from Sigma Chemical Company. All other chemicals were obtained at the highest obtainable purity.

Spectrophotometric assays were performed in a Shimadzu UV-visible recording spectrophotometer UV-160 at 25 *°C*. The extent of univalent reduction of O_2 by $MPDP^+$ was measured with the use of ferricytochrome *c* as an indicator. The rate of ferricytochrome *c* reduction was followed at 550 *nm* using $\varepsilon_{550nm} = 2.1 \times 10^4\ M^{-1}cm^{-1}$ between the oxidized and reduced states of cytochrome *c* (17).

Anaerobic samples were made in cuvettes which allowed purging the reaction volume with purified N_2. The reactions were started by adding the given amount of $MPDP^+$. The $MPDP^+$ stock was prepared in 0.05 *M* acetate buffer, pH 5.0 and was found to be stable at this pH for at least 24 hours. All data shown in the results were the mean values of measurements repeated at least three times.

RESULTS

Absorption Spectra and Decay Kinetics of MPDP+:

Figure 1 shows the absorption spectra of $MPDP^+$ autoxidation over time in air-saturated 0.05 *M* sodium borate-carbonate buffer, pH 10.5. It is evident that the absorbance at 345 *nm* diminished in intensity whereas the absorbance at 290 nm intensified with increasing incubation time. The 290 *nm* absorbing species appears to be a stable product, since it accumulated to a plateau after a short initial lag (Fig. 1). However, the 240 *nm* absorption species accumulated linearly for 2 *min* and then decayed gradually. Since $MPDP^+$ has an absorption maximum at 345 *nm* and MPTP and MPP^+ have absorption maximums at 240 and 290 *nm*, respectively (18), it is possible that the MPDP+ decays rapidly to produce MPP^+ and MPTP *via* a dismutation reaction: $2MPDP^+ \rightarrow MPP^+ + MPTP$, which is consistent with the previously proposed notion (7). The molar concentrations of $MPDP^+$, MPTP, and MPP^+ were determined utilizing the molar extinction coefficients of 1.74×10^4, 1.2×10^4 and 1.2×10^4 $M^{-1}cm^{-1}$, respectively at the above wavelengths(18). The loss of [$MPDP^+$] was found to be not equal to the sum of accumulation of MPTP and MPP^+. Thus, the total accumulation of MPTP and MPP^+ was found to be equivalent to ~80% of the total loss of $MPDP^+$. The ratio of the accumulation of [MPP^+] to [MPTP] at 60 minutes incubation was ~1.5. This may partly be due to the unstable properties of MPTP under these conditions. It is also possible that besides dismutation reaction, a competing alternate reaction occurs during the autoxidation of $MPDP^+$.

The kinetics of the $MPDP^+$ oxidation reactions show mixed second- and first-order decays for $MPDP^+$ oxidation. As shown in Figure 2, the first-order component was evident in the beginning of the reaction where the inverse plot is linear and at the later stage of the reaction the inverse plot is no longer linear (Figure 2b). In order to further identify

the orders of the reaction, the derivative (*-dx/dt*) was used to analyze the experimental results (19). If the slope of the plot of log v against log C equals to 1, the reaction should be first order; and if the slope is close to 2, it should be a second-order reaction (19). As shown in Figures 2c and d of the plots of log v against log C, the slopes were found to be 1.2606 for the first two minutes reaction time and 2.0297 at the later stage of the reaction. These results indicate that initially there is a first-order decay but once the $MPDP^+$ decay products appear, a second-order component begins to dominate. Superoxide dismutase at 1 *μg/ml* had no effect on the ability of $MPDP^+$ autoxidation as measured at 345 *nm*. If superoxide anions are generated during the oxidation of $MPDP^+$, their rate of dismutation should have no influence on the rate of decay of $MPDP^+$ unless the superoxide radical propagates a chain reaction. The inability of superoxide dismutase to inhibit this assay was therefore anticipated. EDTA up to 2×10^{-4} *M* had a trivial effect on the rate of autoxidation of $MPDP^+$. Metal ions such as Fe^{2+} and Fe^{3+} up to 10^{-5} *M* had also little effect on the rate of $MPDP^+$ oxidation indicating that the trace metals do not play an important role in this autoxidation process.

Effects of pH:

The pH of the reaction mixture plays an important role in the rate of autoxidation of $MPDP^+$. Thus, as shown in Figure 3, the rate of depletion of $MPDP^+$ in air-saturated buffer was found to increase with increasing pH. As shown in this figure, the rate of $MPDP^+$ autoxidation at pH 10.5 was over 100 times as high as the one at pH 8. No spontaneous oxidation of $MPDP^+$ was detected at low pH.

Since the first-order decay for $MPDP^+$ was evident in the beginning of the reaction (Figure 2b), the half-life ($t_{1/2}$) and the first-order decay rate constant (k_1) of $MPDP^+$ could be estimated by monitoring the initial and final concentrations of $MPTP^+$ during

spontaneous oxidation for no more than two minutes and using the first-order kinetic equation:

$$\ln C_o/C = k_1 \cdot t \quad (1)$$

where k_1 is the first-order decay-rate constant of $MPDP^+$; t is the incubation time; and C_o/C is the ratio of concentrations of $MPDP^+$ at the initial and given reaction time, respectively.

If C is identical to one-half of C_o, we can obtain the following equation for the half-life of $MPDP^+$, $t_{1/2}$. Thus,

$$t_{1/2} = ln\ 2/k_1 = 0.693/k_1 \quad (2)$$

The half-life time and decay rate of $MPDP^+$ at different pH values are listed in Table I. Thus, as shown in this table, the k_1 value at pH 7.0 is almost four orders of magnitude less than that at pH 10.5 and the half-life time at pH 7.0 is three orders larger than one at pH 10.5.

Reduction of Ferricytochrome c by MPDP+:

$MPDP^+$ was found to transfer electrons to ferricytochrome *c* either directly or via oxygen. The direct transfer occurs under anaerobic conditions and is not affected by superoxide dismutase, whereas the indirect route plays an important role in the presence of oxygen and can be inhibited by superoxide dismutase. Thus, as shown in Figure 4, the rate of accumulation of reduced cytochrome c in the presence or absence of oxygen at a fixed ferricytochrome c concentration (15 μM) increased with increasing $MPDP^+$ concentration. It is apparent that the rate of formation of reduced cytochrome *c* in the absence of oxygen was ~50% of one in the presence of oxygen (Figure 4, line A and B). Addition of EDTA (0.25 *m*M) into the reaction mixture did not result in significant changes on the rate of reduction of ferricytochrome *c*. The direct transfer of electrons from $MPDP^+$ to ferricytochrome *c* occurs in the absence of oxygen and was not inhibitable by superoxide

dismutase (1 $\mu g/ml$). Under identical conditions, but in solutions equilibrated with air, the rate of cytochrome *c* reduction was inhibited 50% by 1.0 $\mu g/ml$ of superoxide dismutase, indicating that $\cdot O_2^-$ are generated during the autoxidation of $MPDP^+$.

Since ferricytochrome *c* (Cyt^{3+} *c*) accepted an electron directly from $MPDP^+$ (or its metabolites) and indirectly via $\cdot O_2^-$, the observed rate of cytochrome *c* reduction should be a sum of the rates of $\cdot O_2^-$ and $MPDP^+$ reducing ferricytochrome *c* as shown in the following equation:

$$V_{obs} = V_{\cdot O_2^-} + V_{MPDP^+} = k_C[Cyt^{3+}\ c]\cdot[\cdot O_2^-] + k_{MPDP^+}[Cyt^{3+}\ c]\cdot[MPDP^+] \qquad (3)$$

Since V_{obs} increased as a function of $[MPDP^+]$, k_{MPDP^+} could be calculated by running a series of experiments varying $[MPDP^+]$ and $[Cyt^{3+}\ c]$ and solving the simultaneous linear equations. Using the rate constant of $\cdot O_2^-$ ($6 \times 10^5\ M^{-1}S^{-1}$) with ferricytochrome *c* as determined by Simic *et al.* (20), we have obtained the k_{MPDP^+} to be 4.86 $M^{-1}S^{-1}$ in this system. The rate of ferricytochrome c reduction by $\cdot O_2^-$ could now be calculated from V_{obs} with the above values of k_C and k_{MPDP^+}. As shown in Figure 4, the rate of $MPDP^+$ directly reducing ferricytochrome *c* was proportional to $[MPDP^+]$, whereas the rate of $\cdot O_2^-$ with ferricytochrome *c* increased linearly with increasing $[MPDP^+]$ up to 40 μM and then gradually to a plateau (Figure 4D). At the linear range, the rate of $\cdot O_2^-$-dependent ferricytochrome *c* reduction is close to two-thirds of the one of $MPDP^+$ directly reducing ferricytochrome *c*.

Effect of Ferricytochrome c Concentration on the Rate of Cytochrome c Reduction:

The rate of trapping of superoxide radical by an acceptor, under a given condition, is dependent upon the concentration of the acceptor in the system. If the concentration of the acceptor does not change appreciably in the reaction and if the reaction is not a chain

event, the plot of the inverse velocity of ferrocytochrome *c* formation against $[Cyt^{3+}\ c]^{-1}$ should be a straight line with a constant slope and intercept. That this was the case is shown in Figure 5, where the rate of accumulation of ferrocytochrome *c* increased as a function of ferricytochrome *c* concentration. The ratio of slope to intercept can give directly k_d/k_c, the ratio of the decay of superoxide radical to the rate of its reaction with ferricytochrome *c*. This ratio value was found to be $1.15 \times 10^{-5}\ M$. Using k_d ($4\ Sec^{-1}$ at pH 8.12) reported by Rabani and Nielsen (21), k_c was calculated to be $3.48 \times 10^5\ M^{-1}S^{-1}$ which is very well in accord with the published value of $6 \times 10^5\ M^{-1}S^{-1}$ (20).

Effect of Superoxide Dismutase on the Rate of Reduction of Ferricytochrome c During Autoxidation of MPDP+:

Since the incubation of $MPDP^+$ in both the absence and presence of oxygen has resulted in the reduction of ferricytochrome *c*, we have attempted to monitor the contribution of superoxide radicals in this reaction. Addition of superoxide dismutase, a specific and efficient scavenger for superoxide radicals ($k = 2 \times 10^9\ M^{-1}S^{-1}$) (22), should inhibit the $\cdot O_2^-$-dependent ferricytochrome *c* reduction. As shown in Figure 6a, superoxide dismutase was found to inhibit the rate of reduction of ferricytochrome *c* in a dose-dependent manner. Thus, the inhibition of ferricytochrome c reduction increased with increasing concentration of SOD and the maximum inhibition, both actual and extrapolated, was only 50% of control rate. At higher pH, however, the ability of superoxide dismutase to inhibit $MPDP^+$-dependent ferricytochrome c reduction was higher than at pH 8.0. These results further indicate that superoxide radicals were generated during the autoxidation of $MPDP^+$. The partial inhibition by SOD may be related to the availability of alternate and competing pathways for ferricytochrome *c* reduction. Since there exists a competition between ferricytochrome *c* and SOD for the available $\cdot O_2^-$, the overall rate of the reaction (*V*), at steady state, may be written as follows:

$$V = V_c + V_{sod} \quad \text{or} \quad V_{sod} = \Delta V = V - V_c \tag{4}$$

where V and V_c are the rates of reduction of ferricytochrome *c* in the absence and presence of SOD, respectively; and V_{sod} is the rate of SOD scavenging $\cdot O_2^-$ and assumed to be as follows:

$$V_{sod} = k_{sod}\,[SOD]\cdot[\cdot O_2^-] \tag{5}$$

where k_{sod} is the rate constant of SOD with $\cdot O_2^-$.

At a given condition, the values of V_{sod} can be obtained at different levels of [SOD]. As shown in Figure 6b, the velocity of SOD scavenging $\cdot O_2^-$ increased as a function of [SOD]. The plot of $1/V_{sod}$ against 1/[SOD] gave a straight line with a constant slope and intercept (Figure 6b). The ratio of slope to intercept can give directly k_d/k_{sod}, and this ratio was estimated to be 1.82×10^{-9} *M*. Thus, using $k_{sod} = 2 \times 10^9\ M^{-1}S^{-1}$ (22), k_d value was calculated to be 3.64 Sec^{-1}, which is close to the k_d value (4 Sec^{-1}) reported by Rabani and Nielsen (21) for the decay of $\cdot O_2^-$. These data strongly support the notion that $\cdot O_2^-$ are generated during aerobic autoxidation of $MPDP^+$ and that ferricytochrome *c* was able to detect these radicals effectively.

Effect of pH on Rate of Reduction of Cytochrome c During Autoxidation of $MPDP^+$:

The pH plays an important role in the reduction of ferricytochrome *c* during the autoxidation of $MPDP^+$. As shown in Figure 7, the rate of formation of $Cyt^{2+}c$ was found to increase with increasing pH. It is evident that the rate of ferricytochrome *c* reduction at pH 10.5 was over 10 times as high as the one at pH 8. Superoxide dismutase inhibited this reaction at any tested pH. The inhibitory ability of SOD was also slightly higher at high pH. Thus, at pH 10.5, the maximum inhibition obtained by a saturated concentration of SOD (0.4 $\mu g/ml$) was about 70-74%, whereas the maximum inhibition was only ~60, 53, and 50% at pH 9.5, 9.0, and 8.0, respectively.

DISCUSSION

The results of these studies provide the following new information: (1) evidence for the generation of superoxide radicals during the autoxidation of $MPDP^+$, (2) the decay of $MPDP^+$ is a multi-order complex kinetic which includes a mixed first- and second-order reaction, and (3) the disproportion of $MPDP^+$ proceeds through a free radical adduction reaction.

Although the decay kinetics of $MPDP^+$ have been studied (6, 14, 19, and 24), no further work has been done to ascertain the nature of its kinetics. Our studies provide evidence that $MPDP^+$ autoxidation is mainly a second-order reaction with an initial first-order reaction. This was indicated by the findings (Figure 2b) that the inverse plot of $MPDP^+$ decay kinetics is linear at the initial stage up to 2 *min* and at the later stage of the reaction the curve is no longer linear. This was confirmed from the slopes (Fig. 2c and d) of the plots of log v against log C, which was 1.26 in the beginning of the reaction and 2.0296 at the later stage of the reaction. These values are close to 1 for first-order and 2 for second-order kinetic reactions.

$MPDP^+$ autoxidation reduced ferricytochrome *c*, a detector for superoxide radicals, in a dose dependent manner (Figure 4) and superoxide dismutase inhibited this reaction (Figure 6) indicating that superoxide radicals are produced during this process.

Although the generation of superoxide anions during the autoxidation of $MPDP^+$ has been qualitatively confirmed by the evidence above, a quantitative analysis would help us in understanding the presence of other reactive species. Ferricytochrome c was found to be accepting electrons from $MPDP^+$ directly or from its decay products in the absence of oxygen. Superoxide dismutase, a well known specific scavenger for superoxide radicals, was found to eliminate the reduction of ferricytochrome *c* at a maximum ~50% of control rate at pH 8.0. The rate constant of the reaction of ferricytochrome c with superoxide

anion, as determined by our kinetic analysis was found to be $3.48 \times 10^5\ M^{-1}S^{-1}$ which is in accord with the published value of $6 \times 10^5\ M^{-1}S^{-1}$ (20). The rate constant of reaction between $MPDP^+$ with ferricytochrome c was found to be $4.86\ M^{-1}S^{-1}$ which is almost five orders of magnitude less sensitive than the rate constant for $\cdot O_2^-$ reaction with ferricytochrome *c*. Nevertheless 50% of the electrons derived from $MPDP^+$ did not reduce molecular oxygen under the univalent pathway. This implies that the reactive species produced during the autoxidation $MPDP^+$ converting ferricytochrome *c* to ferrocytochrome *c*, appears to be not only $\cdot O_2^-$ but also other species such as $MPDP^+$ itself and /or its reactive intermediates during the pathway of its oxidation.

Metal ions such as Fe^{2+} or Fe^{3+} (up to $10^{-5}\ M$) had trivial effects on the rate of decay of $MPDP^+$. EDTA up to $2 \times 10^{-4}\ M$ had no inhibitory effects in the above process. EDTA had also no effect on the rate of ferricytochrome c reduction. Therefore we have ruled out the role of metals during the process of autoxidation of $MPDP^+$.

The autoxidation of $MPDP^+$ and the half-life time of $MPDP^+$ was dependent on the pH of the medium. Thus, the half-life time for $MPDP^+$ was decreased with increased pH (Table 1) and the rate of decay of $MPDP^+$ increased with increased pH (Figure 3). Moreover, the rate of reduction of ferricytochrome c and sensitivity of this reduction to superoxide dismutase were both augmented as the pH was raised from 7.0 to 10.5. It is possible that $MPDP^+$ readily donates a H^+ at higher pH to form an unstable intermediate [MPDP*] during its oxidation to yield MPP^+. The [MPDP*] so generated could donate an electron and/or hydrogen atom to molecular oxygen to generate $\cdot O_2^-$ or $\cdot OOH$. This intermediate, MPDP*, could also react with ferricytochrome c to yield ferrocytochrome c.

In this way, one initial event, as shown in Scheme I, occurs as the univalent oxidation of an $MPDP^+$. $\cdot O_2^-$ does not participate in oxidizing $MPDP^+$ to initiate a chain reaction because superoxide dismutase did not inhibit the rate of decay of $MPDP^+$. It is

however possible that $MPDP^+$ could react with MPDP* to not only generate MPP^+, as shown in Scheme I, but also generate MPTP. This was observed (Figure 1) where the $MPDP^+$ decay is followed by the generation of MPP^+, which has absorption maximum at 290 *nm*, and probably MPTP which has an absorption maximum at 240 *nm* (18). It is also possible that MPDP* could dismute to generate MPTP and MPP^+ in which case superoxide dismutase or oxygen would have little effects.

Although the oxidation pathways for 2, 3-$MPDP^+$ have been previously proposed (27, 28), no direct evidence was available for the generation of free radical species of oxygen during this process. We have confirmed the generation of superoxide radical during the autoxidation of $MPDP^+$. Thus, as shown in Scheme I, after a proton abstraction, $MPDP^+$ forms compound **II** which has three available pathways to form MPP^+. In the first pathway, as described in the catalyzed reaction (27, 28), the resonance-stabilized carbon-centered radical species **IV** ↔ **V** could be formed after a single electron oxidation followed by a proton abstraction. Loss of a second electron from **IV** or **V** would generate MPP^+. In the second pathway, compound **II** loses a hydrogen atom to form the resonance structure of **IV** and **V** which in turn loses an electron to from MPP^+. Both the first and second pathways include the formation of resonance structure carbon-centered radical species **IV** ↔ **V**. However, it is possible to have an alternate pathway, without forming a carbon-centered radical species, to form MPP^+. In this pathway (pathway 3), the compound **II** undergoes an electron loss to form compound **III** which directly forms MPP^+ after the loss of a hydrogen atom.

In the presence of molecular oxygen the e_{aq}^- and ·H so generated, in any or all of the above three pathways of $MPDP^+$ oxidation (Scheme I), could react with diatomic oxygen to generate $\cdot O_2^-$ and ·OOH, respectively (29, 30):

$$O_2 + e_{aq}^- \xrightarrow{k = 2 \times 10^{10}\, M^{-1}S^{-1}} \cdot O_2^- \qquad (6)$$

$$O_2 + \cdot H \xrightarrow{k = 2 \times 10^{10}\, M^{-1}S^{-1}} \cdot OOH \xrightarrow{pKa = 4.8} H^+ + \cdot O_2^- \qquad (7)$$

Since $MPDP^+$ could accept an electron and a hydrogen atom to form MPTP (31-33), there could exist a competition between molecular oxygen and $MPDP^+$ for the available electrons and hydrogen atoms. Ferricytochrome c could be reduced directly by some of the intermediates of $MPDP^+$ oxidation products or indirectly by $\cdot O_2^-$. This may be the reason why the generation of superoxide radicals (ferricytochrome *c* reduction) was quenched by high concentrations of $MPDP^+$ (Figure 4D) and superoxide dismutase was able to partially inhibit the ferricytochrome *c* reduction.

We are now forced to think of the biological effects of $MPDP^+$ in a new light. Thus $MPDP^+$ now not only produce $\cdot O_2^-$ during spontaneous oxidation but also may yield MPDP radicals and possibly other, as yet undetected, reactive intermediate(s). It is tempting to attribute some of the pathological effects of $MPDP^+$/MPTP-induced neurotoxicity to intermediates produced during its oxidation.

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Table I. Effect of pH on the decay rate and half-life of $MPDP^+$:

pH	k_1 $min.^{-1}$	$t_{1/2}$ $min.$
7.0	2.30×10^{-4}	3.14×10^{3}
8.0	4.32×10^{-4}	1.61×10^{3}
9.0	1.41×10^{-3}	4.92×10^{2}
10.0	1.36×10^{-2}	5.10×10
10.5	1.93×10^{-1}	3.59

Note: $[MPDP^+] = 20\ \mu M$.

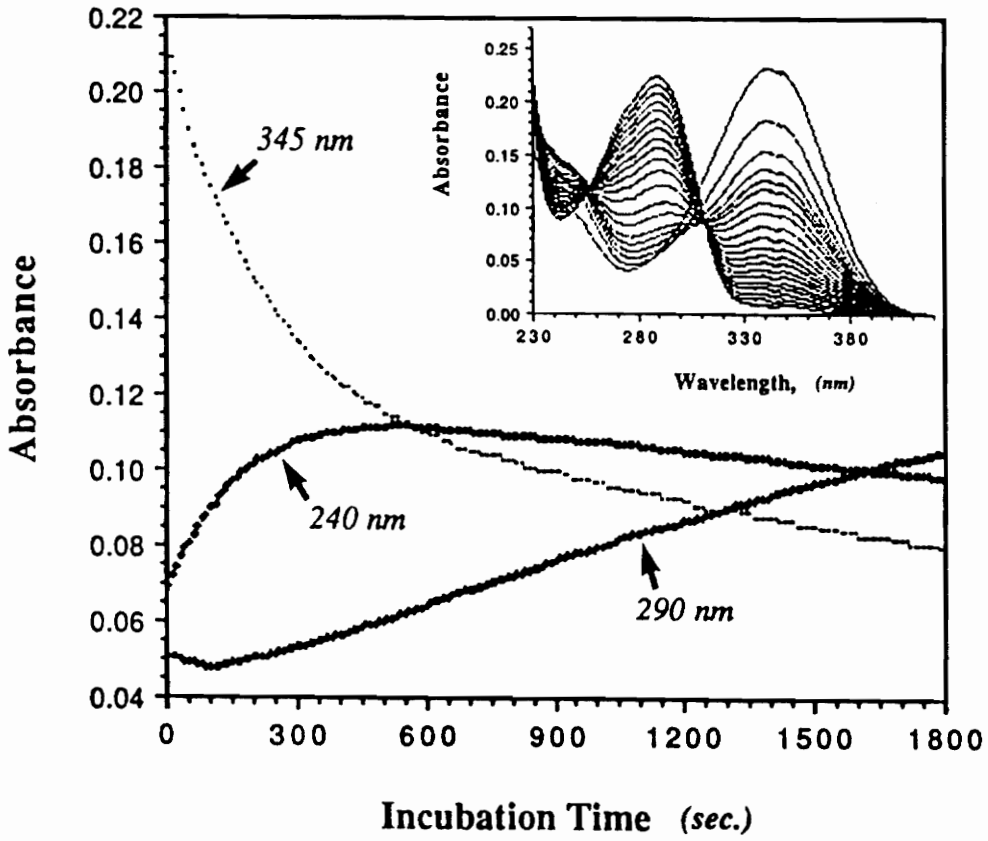


Figure 1. Kinetics of $MPDP^+$ oxidation. The reaction mixture contained 20 μM $MPDP^+$ in air-saturated 0.05 *M* sodium borate-sodium carbonate buffered at pH 10.5 and at 25 *°C*. The rate of decay of $MPDP^+$ at 345 *nm* and rate of accumulation of MPP^+ and MPTP at 290 and 240 *nm*, respectively, were recorded. Inset: Changes in absorption spectra during the autoxidation of 2, 3-$MPDP^+$.

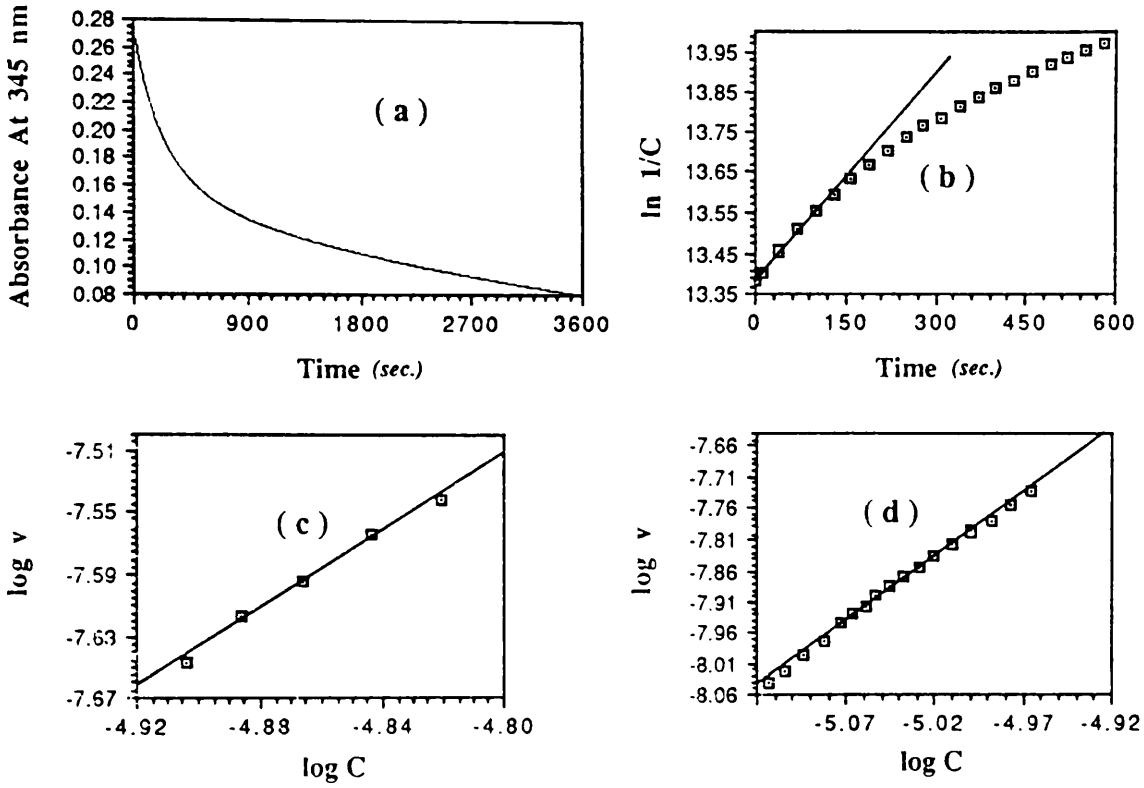


Figure 2. Decay kinetics of $MPDP^+$. (a) Time course of decomposition of 2, 3-$MPDP^+$ (20 μM) in 0.05 M sodium borate-sodium carbonate buffered pH 10.5; (b) plot of pseudo-first order; (The data points were as in Figure 2a.); (c) plot of log of initial velocity versus log concentration of $MPDP^+$; (d) plot of log of initial velocity against log concentration at the later stage of the reaction. $V = -d(C_o-C)/dt = dC/dt$. The concentration of 2, 3-$MPDP^+$ was determined by the use of extinction coefficient at 345 *nm* with 1.74×10^4 M^{-1} cm^{-1}. C_o = initial concentration of $MPDP^+$; C = concentration of $MPDP^+$ at time t; and V = velocity of $MPDP^+$ decay.

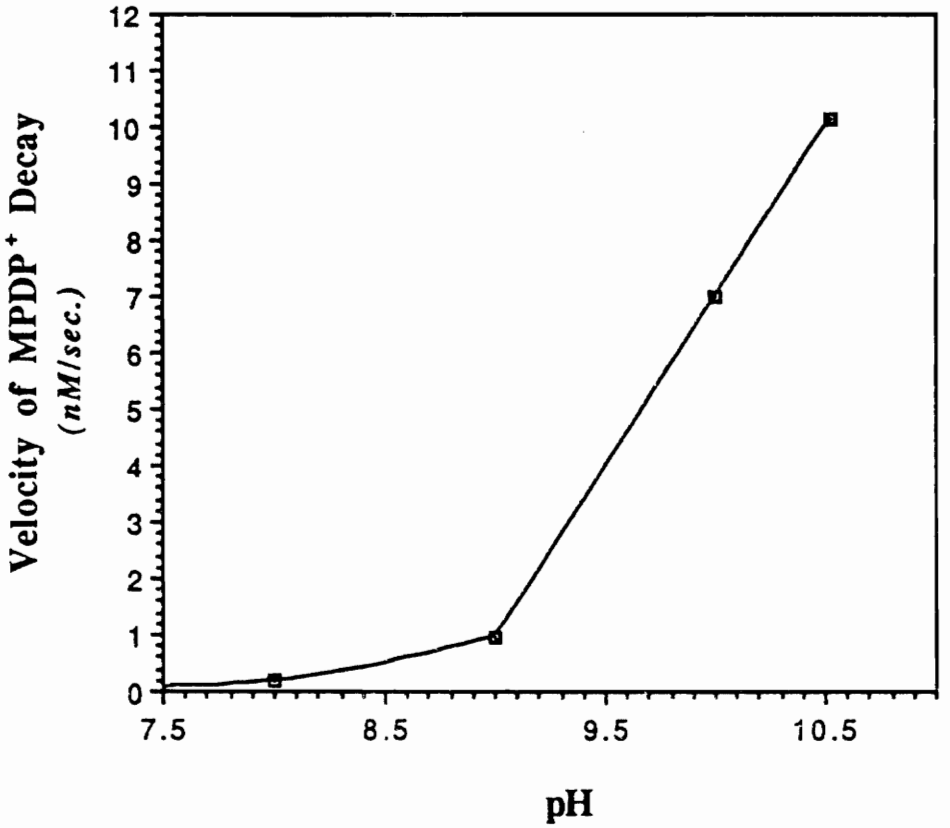


Figure 3. The effect of pH on the rate of autoxidation of $MPDP^+$, measured in terms of loss of $MPDP^+$ absorbance at 345 *nm*. The reaction mixtures consisted of 20 μM 2,3-$MPDP^+$ in the following aerobic 0.05 M buffer systems: sodium-potassium phosphates pH 7.0 and 8.0; potassium phosphate-sodium borate, pH 9.0 and 9.5; sodium borate-sodium carbonate, pH 10.0 and 10.5.

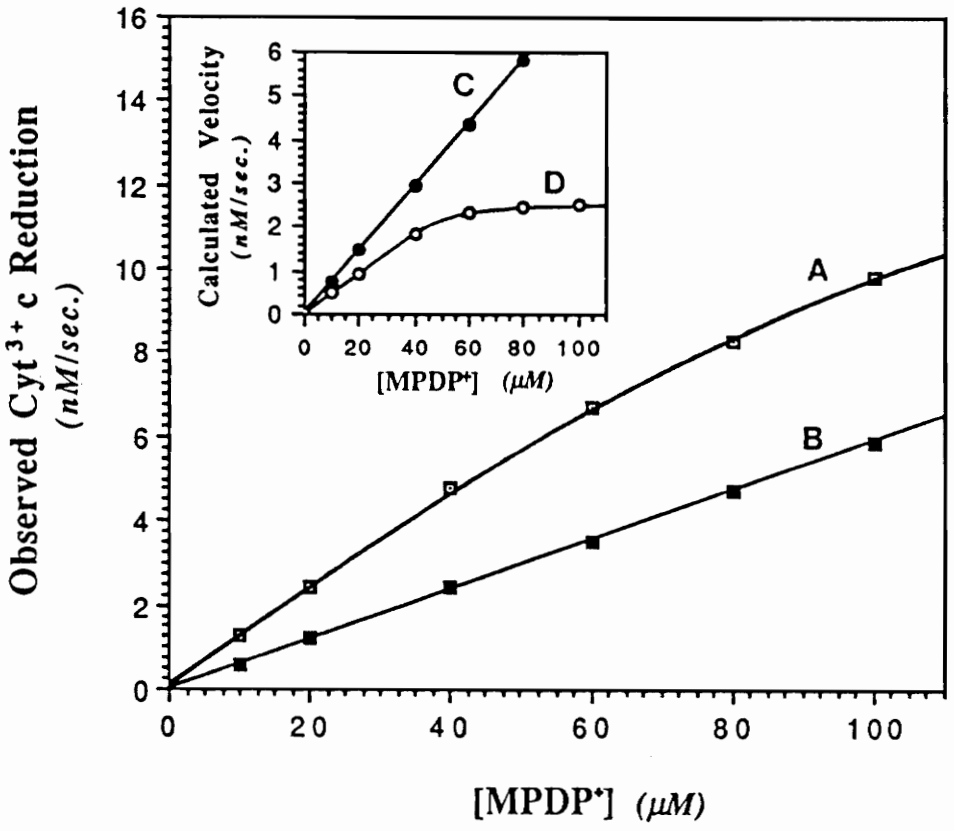


Figure 4. The initial rates of reduction of ferricytochrome c as a function of $MPDP^+$ concentration. $MPDP^+$ at indicated concentrations was added to 15 μM ferrocytochrome *c* in 0.05 M phosphate-buffer at pH 8.0. Line (A) air-saturated; (B) N_2-bubbled for 10 minutes. Inset: The plots represent the rates of reduction of ferricytochrome c calculated to be due to directly by $MPDP^+$ (line C) or *via* $\cdot O_2^-$ (line D) as a function of [$MPDP^+$]. These calculations are as described in the results section.

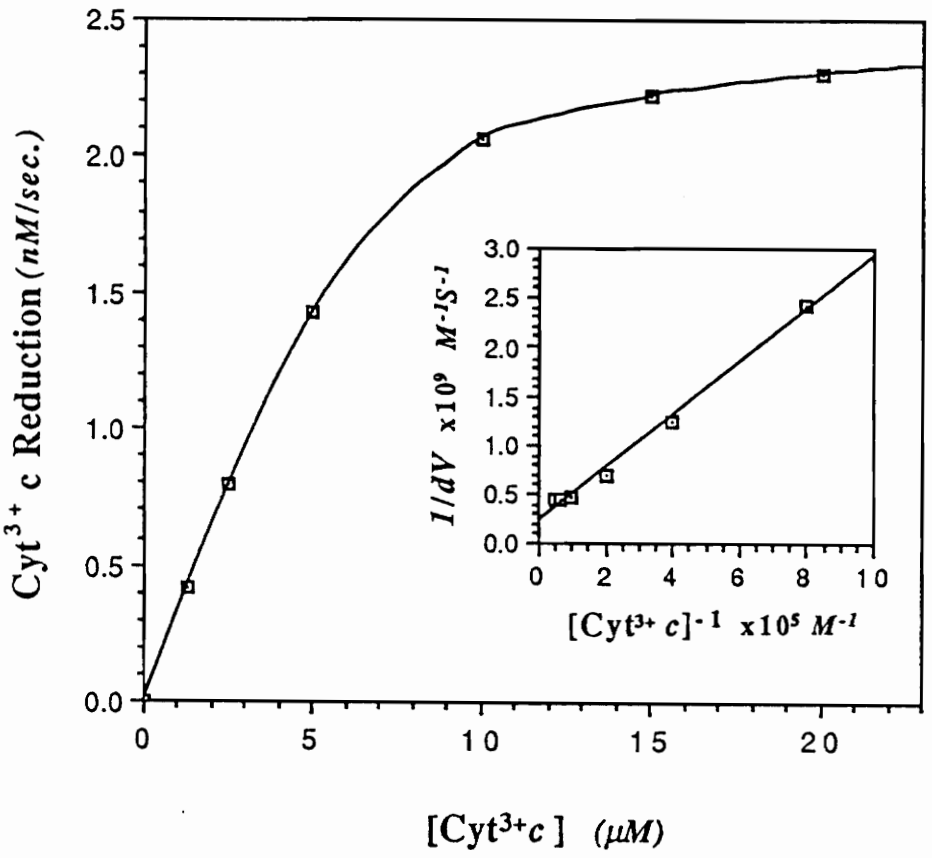
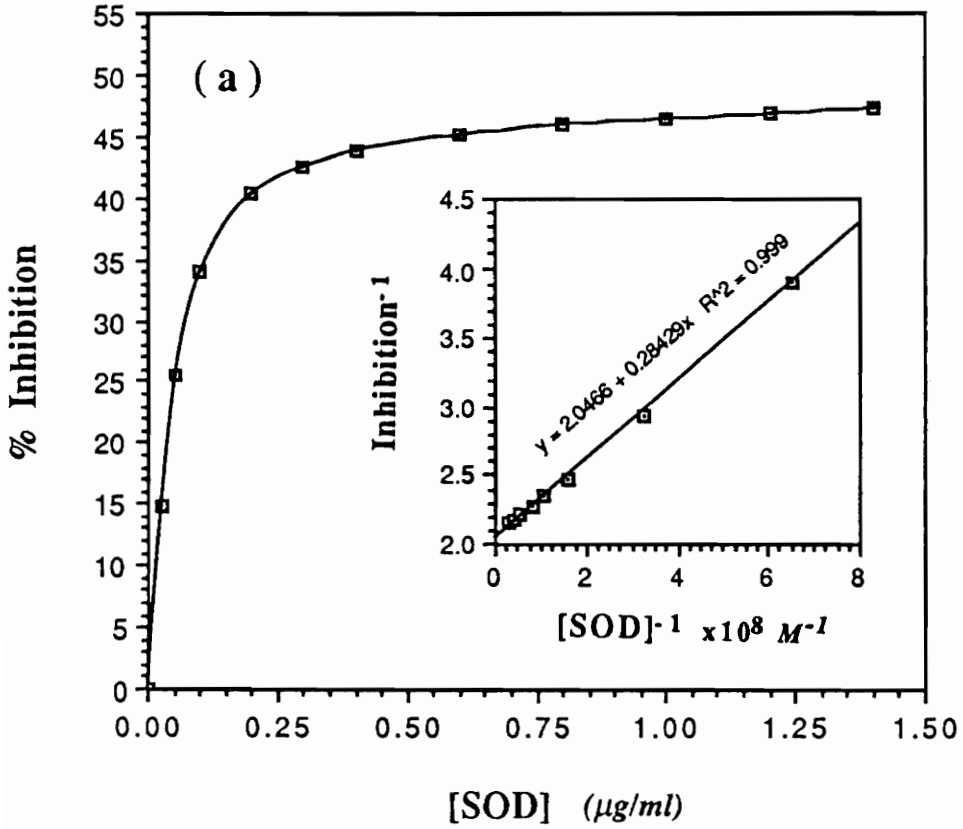


Figure 5. Reduction of ferricytochrome *c* as a function of its concentration. The initial rate of formation of ferricytochrome *c* was observed at 550 *nm*. Reaction mixtures contained 20 μM 2,3-MPDP$^+$ and various amounts of ferrocytochrome *c* in 0.05 *M* phosphate-buffered solution, pH 8.0, at 25 *°C*.

Fig. 6.


(a)
% Inhibition
[SOD] (µg/ml)
Inhibition-1
y = 2.0466 + 0.28429x R^2 = 0.999
[SOD]-1 x10^8 M-1

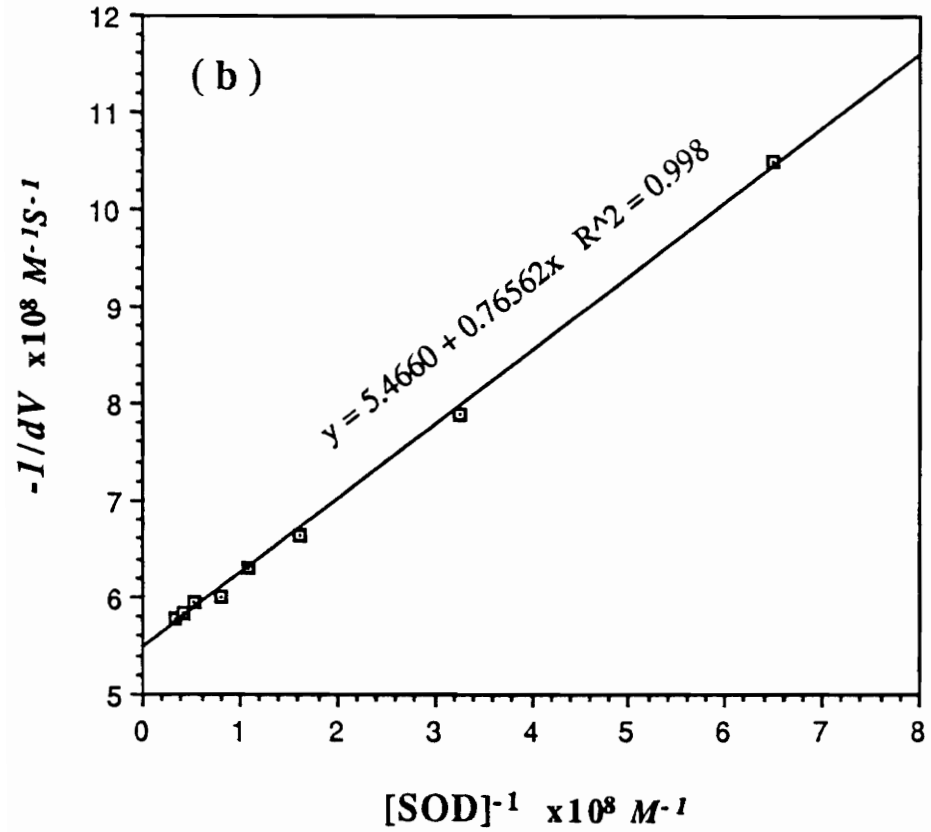

(b)
-1/dV x10^8 M-1S-1
y = 5.4660 + 0.76562x R^2 = 0.998
[SOD]-1 x10^8 M-1

Figure 6. Inhibition of ferricytochrome c reduction by superoxide dismutase in air-saturated 0.05 *M* phosphate buffer, pH 8.0. The samples contained 20 μM 2,3-MPDP$^+$, 15 μM ferricytochrome c and indicated concentrations of SOD. (a): The data obtained are presented on linear and on reciprocal coordinates. (b): The double reciprocal plot of velocity of SOD scavenging $\cdot O_2^-$ as a function of [SOD]

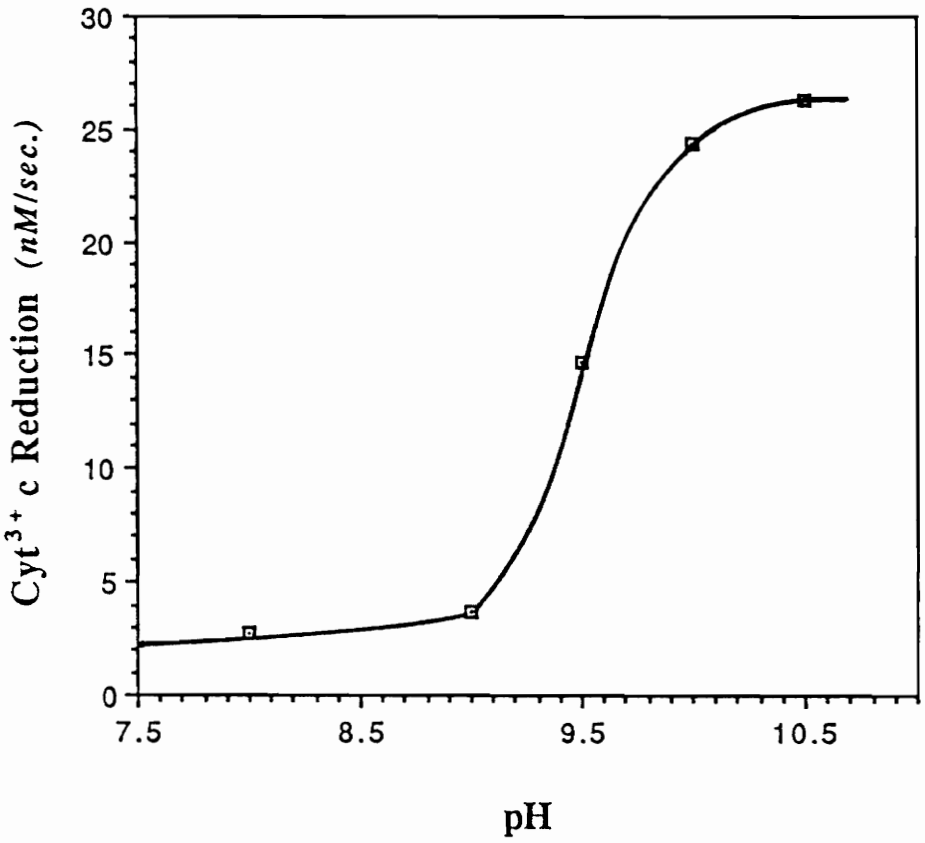
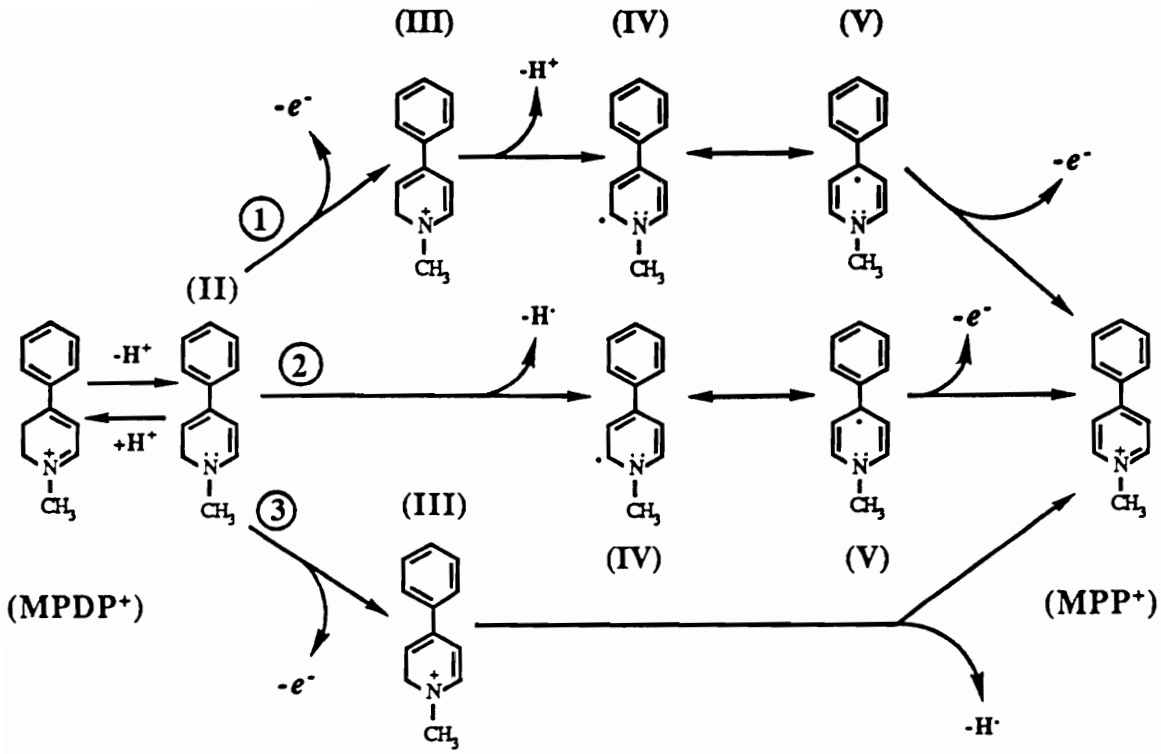


Figure 7. Effect of pH on the rate of autoxidation of 2, 3-MPDP$^+$, measured in terms of accumulation of ferrocytochrome *c*. The reaction mixture consisted of 20 μM MPDP$^+$ and 15 μM ferricytochrome *c*. The conditions of buffering were the same as indicated in Figure 3 legend.



Scheme I

Possible Mechanism of Autoxidation of $MPDP^+$ To Form MPP^+.

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Author(s): L-Y Zang and H P Misra

Title: Superoxide radical production during autoxidation of MPDP+

Comments to the Author:

THis is a well written experimental paper that provides much needed information on the biochemistry of MPTP neurotoxicity focusing on the autoxidation of the MAO-derived product MPDP+. The analysis of the autoxidation process has been very completely characterized as to the possible products and the kinetics of their formation. THis reviewer agrees that the information should aid in our understanding of the in vivo mechanism of MPTP toxicity to dopamine neurons.

Please indicate recommendation 1 [X] Accept 2 [ ] Accept after revision 3 [ ] Decline

If box 1 or 2 is checked please summarize the reasons why this is an important contribution and should be published in the JBC

AS noted above, this is a well designed and well written paper that is conistent with the scope and quality of JBC. I was not able to locate any significant flaws. I urge its acceptance in its present form.

CHAPTER III

EPR KINETIC STUDIES OF SUPEROXIDE RADICALS GENERATED DURING THE AUTOXIDATION OF 1-METHYL-4-PHENYL-2, 3-DIHYDROPYRIDINIUM (MPDP+), A BIOACTIVATED INTERMEDIATE OF PARKINSONIAN INDUCING NEUROTOXIN MPTP

*(Published in the Journal of Biological Chemistry, **267**, 23016-23123, 1992)*

Lun-Yi Zang and Hara P. Misra

Abstract

MPDP$^+$, a metabolic product of the nigrostriatal toxin MPTP, has been shown to generate superoxide radicals during its autoxidation process. The generation of superoxide radicals was detected as a DMPO-O_2^- spin adduct by spin-trapping in combination with EPR techniques. The rate of formation of spin adduct was dependent not only on the concentrations of MPDP$^+$ and oxygen but also on the pH of the system. Superoxide dismutase inhibited the spin adduct formation in a dose-dependent manner. The ability of DMPO to trap superoxide radicals, generated during the autoxidation of MPDP$^+$, and of superoxide dismutase to effectively compete with this reaction for the available $\cdot O_2^-$, has been used as a convenient competition reaction to quantitatively determine various kinetic parameters. Thus, using this technique the rate constant for scavenging of superoxide radical by superoxide dismutase was found to be 7.56×10^9 $M^{-1}S^{-1}$. The maximum rate of superoxide generation at a fixed spin trap concentration using different amounts of MPDP$^+$ was found to be 4.48×10^{-10} $M \cdot S^{-1}$. The rate constant (k_1) for MPDP$^+$ making superoxide radical was found to be 3.97×10^{-6} Sec^{-1}. The secondary order rate constant (k_{DMPO}) for

DMPO trapping superoxide radicals was found to be 10.2 $M^{-1}S^{-1}$. The life time of superoxide radical at pH 10.0 was calculated to be 1.25 seconds. These values are in close agreement to the published values obtained using different experimental techniques. These results indicate that superoxide radicals are produced during spontaneous oxidation of $MPDP^+$ and that EPR spin trapping can be used to determine the rate constants and life time of free radicals generated in aqueous solutions. It appears likely that the nigrostriatal toxicity of MPTP/$MPDP^+$ leading to Parkinson's disease may largely be due to the reactivity of these radicals.

INTRODUCTION

Bioactivation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to corresponding 2,3-dihydropyridium intermediates $MPDP^+$ and subsequently to MPP^+ is known to be an obligatory process for the manifestation of the nigrostriatal toxicity of this Parkinsonian inducing agent (1-5). This bioactivation is catalyzed by monoamine oxidase B (MAO-B), which removes the two electrons from the parent tertiary amine MPTP to form $MPDP^+$ (5, 6). The $MPDP^+$ so formed then undergoes further two-electron oxidation to the pyridium species MPP^+ (3) or disproportionates to form MPTP and MPP^+ (5-7). It was found that this neurotoxin selectively destroys neuronal cells in the substantia nigra of humans and primates (2). All of the proposed mechanisms of action focus on MPP^+ as the mediator of the toxic effects and the role of chemically reactive dihydropyridinium species $MPDP^+$ has not been thoroughly investigated. It was reported that $MPDP^+$ can readily cross the cell membrane and reach the extracellular space (8).

Therefore it is possible that $MPDP^+$ could be transported from astrocyte cells to intraneuronal sites where conditions may render it suscept towards spontaneous oxidation, yielding free radicals. The generation of oxygen radicals during the metabolism of MPTP has been proposed to explain its neurotoxicity (9-11), but no direct evidence is available to confirm this hypothesis. It was reported that $MPDP^+$ autoxidizes to generate hydrogen peroxide (H_2O_2) in a reaction promoted by chelated iron (12). This evidence, that $MPDP^+$ could cause divalent reduction of oxygen to form H_2O_2, gave no indication of the relative importance of the univalent reduction of oxygen to generate superoxide radicals. Such oxidation is likely to involve two successive single electron transfer steps with the generation of free radical species as intermediates (13). Recently we have demonstrated that autoxidation of $MPDP^+$ reduced ferricytochrome c which was inhibit-able by superoxide dismutase and proposed that reactive species of oxygen may be generated during this process which, in turn, may play a role in the process of degeneration of nigrostriatal cells (14). It appeared desirable, however, to confirm the above findings using a different technique and to quantitate the rate of production of these radicals as a function of pH and concentration of $MPDP^+$.

Spin trapping in combination with electron paramagnetic resonance (EPR) provides an effective tool for detecting these reactive species. If free radicals are generated during the autoxidation of $MPDP^+$, the characteristic signal of trapped radicals can be measured by EPR spectroscopy and specific scavengers of free radicals should diminish the EPR signal. Using this kinetic competition reaction, the rate of production of free radicals can be measured. Here, we report the generation of superoxide radicals during autoxidation of $MPDP^+$ and the rate of their production using EPR spin-trapping techniques.

MATERIALS AND METHODS

1-methyl-4-phenyl-2,3-dihydropyridinium perchlorate ($MPDP^+$), 1-methyl-4-phenyl- pyridinium iodide (MPP^+) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP) were purchased from Research Biochemicals, Inc. Natick, MA. The spin trap, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from Aldrich Chemical Compony, Inc., Milwaukee, WI. Bovine erythrocyte superoxide dismutase, 3-cyano-proxyl free radical and diethylenetriaminepentaacetic acid (DTPA) were obtained from Sigma Chemicals Company, St. Louis, MO. All other chemicals were obtained at the highest obtainable purity.

Since commercial DMPO contains colored impurities which have additional absorption maxima between 200 to 227 *nm* and which give EPR signals similar to the spin adducts of carbon-centered radicals, nitroxide species (without β- hydrogen splitting) and hydroxyl radical (·OH) (15, 16), it was purified by eluting through an activated charcoal column. Thus, the commercial DMPO (8.7 M) was diluted to ~1.0 M with nitrogen bubbled triple distilled water containing 1.0 mM DTPA and passed by vacuum through a prewashed activated charcoal column (5 cm × 1 cm) under a nitrogen atmosphere. The filtered solution of DMPO was then divided into several 1 *ml* aliquots, sealed under nitrogen in small dark brown vials afterwards and stored at -20 °C until used. The purified DMPO solution gave no free radical signal seen by EPR and had only one absorption peak in the UV region of the spectrum at 227 *n*m in aqueous systems with an extinction coefficient of 8.0×10^3 $M^{-1}cm^{-1}$ as reported earlier (17). Thus, the purified DMPO concentration can be measured by optical spectroscopy. All spectrophotometric assays were performed at 25 °C in a Shimadzu *UV-Visible* recording spectrophotometer

Since trace metals present in buffered solutions interfere in spin trapping experiments, diethylenetriaminepentaacetic acid (DTPA), a metal chelating agent was used

in our system. Anaerobic samples were made in sealed cuvettes which allowed purging the reaction volume with dried purified N_2. The reactions were started by adding the given amount of $MPDP^+$ stock which was prepared in triple distilled water containing 1.0 *m*M DTPA. The stock solution was found to be stable for at least 24 hours in triple distilled water. Individual samples were placed in the EPR TM cavity using an EPR aqueous flat cell (60 *mm* × 17 *mm* × 0.25 *mm* i.d.) for the measurement of free radical generation at a given incubation time at room temperature. The commercial 3-cyano-proxyl free radical was used as the nitroxide standard for determining relative concentrations of free radicals. The recordings of EPR spectra were performed with a Bruker D-200 ER spectrometer. Unless otherwise stated, the EPR parameters were set at: 100 *KHz*, X-band; microwave frequency, 9.78 *GHz*; microwave power, 20 mW; modulation amplitude, 0.71 *G*; time constant, 0.64 *sec.*; scan time, 500 *sec.* and receiver gain, 1×10^6.

RESULTS

EPR Spectra of Free Radical Spin Adducts Formed During Autoxidation of $MPDP^+$:

A DMPO-superoxide anion radical adduct was generated by incubation of air-saturated solutions of $MPDP^+$ in buffered DMSO solution at pH 10.0 containing DMPO, (Fig. 1b). In the absence of $MPDP^+$, oxygen or DMPO, no detectable EPR signal was observed (Fig. 1a). The EPR spectrum in Figure 1b exhibits a $3 \times 2 \times 2$ splitting which usually results from the interactions of an unpaired electron with a primary nitrogen atom along with the secondary *b*- and *g*- protons. The hyperfine splitting constants of the signal (a_N =13.1 *G*, $^{\beta}a_H$ =10.4 *G* and $^{\gamma}a_H$ =1.3 *G*) are consistent with previously reported values

for DMPO-O_2^- in dimethylsulfoxide (18-23). Superoxide dismutase was used in this system in an attempt to verify the role of $\cdot O_2^-$ in the formation of the above adduct. The $\cdot O_2^-$, generated by the oxidation of $MPDP^+$, could either dismute to $H_2O_2 + O_2$ or could react with DMPO to form a DMPO-O_2^- adduct. Superoxide dismutase, by catalyzing the former reaction at a second order rate constant of 2×10^9 $M^{-1}S^{-1}$ (24), should inhibit the $\cdot O_2^-$-dependent DMPO-O_2^- adduct formation. That this was the case is shown in Figure 1c. Superoxide dismutase inhibited the adduct formation in a dose dependent manner and at 50 *n*g/ml, the enzyme inhibited the EPR signal intensity ~94% of control rate and at 200 *n*g/ml the EPR signals were completely suppressed. It follows that autoxidation of $MPDP^+$ generates $\cdot O_2^-$ and DMPO is capable of reacting with this primary species to form the spin adduct with the characteristic EPR signal as shown in Figure 1b.

When $MPDP^+$ was incubated in an air-saturated aqueous solution of 0.05 *M* sodium borate-carbonate buffer (pH 10.0) in the presence of DMPO, the resulting EPR spectrum exhibited four splitting lines in an intensity ratio of 1:2:2:1 (Fig.1d). The hyperfine splitting constants of the signal for this EPR spectrum were found to be $a_N = a_H$ = 14.9 *G*, consistent with previously reported values for DMPO-OH in aqueous systems (25, 26). In the absence of oxygen (Fig. 1a) or presence of 50 *n*g/ml superoxide dismutase the EPR signal of DMPO-OH adduct was completely suppressed (Fig. 1e). Since DMPO-O_2^- spin adduct is known to decompose to DMPO-OH in aqueous solution and decomposition of $\cdot O_2^-$ to $\cdot OH$ could be complete within one second in an aqueous solution (27), the appearance of DMPO-O_2^- adduct and the ability of superoxide dismutase to inhibit its formation is therefore understandable. It does, however, establish that $\cdot O_2^-$ radicals are produced during the autoxidation of $MPDP^+$ in aqueous solution and the resulting DMPO-OH adduct was secondary to the $\cdot O_2^-$ formation.

The mechanism of formation of DMPO-OH adduct was further investigated. Thus, ethanol, a well known scavenger of hydroxyl radical ($k = 1 \times 10^9$ $M^{-1}S^{-1}$) (28) can

rapidly react with ·OH to produce a unique carbon-centered radical which in turn can react readily with DMPO to form a spin adduct. The EPR signal of this carbon-centered radical spin adduct is quite different from that of the hydroxyl radical spin adduct. If hydroxyl radicals were generated in this system, by either the dismutation of superoxide or the decomposition of DMPO-O_2^-, ethanol should scavenge these radicals to give a carbon-centered spin adduct EPR signal. The results of our study indicate that this was not the case. Thus, when ethanol up to 200 *m*M was added to the aqueous system, it neither affected the intensity of the DMPO-OH signal nor did it exhibit any additional signal (data not shown). These results indicate that the production of DMPO-OH was not due to trapping of primary ·OH but due to the reduction of DMPO-O_2^- adduct. It is clear, however, that the intensity of this DMPO-OH signal can be utilized to estimate the relative amounts of superoxide radicals generated in this aqueous system.

Kinetics of Spin-adduct Formation:

The rate of accumulation of DMPO-OH spin adduct during autoxidation of $MPDP^+$ is shown in Figure 2. Thus, when 0.3 *m*M $MPDP^+$ was added to an aqueous buffered solution (pH 10.0) containing 80 *m*M DMPO, a linear initial rate of increase of signal intensity of the spin adduct was observed for at least 2 mins. Since the recording was initiated 2 min after the addition of all reactants, the formation of spin adduct in the first 120 seconds can be obtained by extrapolating to zero reaction time (broken line in Fig. 2). As shown in Figure 2 inset, the initial rate of accumulation of the spin adduct during the first 4 minutes did not change significantly, and beyond this incubation time, the rate decreased gradually with time. The rate of spin adduct formation, thus, can be calculated directly from the initial slope of the accumulation of nitroxide radical EPR signal. The rate under these condition was found to be 3.512×10^{-10} M·S^{-1}.

Effect of MPDP$^+$ Concentration on the yield of $\cdot O_2^-$:

In a given reaction system, MPDP$^+$ is known to undergo a disproportionation to yield MPTP and MPP$^+$ (29) or spontaneously oxidize to MPP$^+$ (30). The disproportionation and autoxidation pathways are dependent upon the initial concentration of MPDP$^+$ in the reaction system. Thus, at $\geq$ 50 μM MPDP$^+$ the disproportionation reaction is prevalent whereas at $\leq$ 50 μM the autoxidation reaction predominates (29, 30). Therefore, we have investigated the effect of MPDP$^+$ concentration on the rate of generation of $\cdot O_2^-$ in an aqueous system. As shown in Figure 3, the rate of generation of $\cdot O_2^-$, as measured by the formation of spin adduct, exhibits a biphasic curve with increasing MPDP$^+$ concentration indicating the existence of alternate and competing pathways which predominates beyond 50 μM MPDP$^+$. However, when the rate of accumulation of "spin adduct/μM MPDP$^+$" was plotted against [MPDP$^+$], the rate of spin adduct formation was found to decrease with increasing MPDP$^+$ concentration (Fig. 3 inset a). Since the generation of the spin adduct was totally inhibitable by superoxide dismutase at any of the above concentrations of MPDP$^+$ (data not shown), we conclude that $\cdot O_2^-$ are produced during MPDP$^+$ autoxidation which in turn reacts with DMPO to give rise to the EPR signal and this autoxidation reaction predominates at $\leq$ 50 μM MPDP$^+$ in the reaction system.

In order to quantitate the rate of generation of radicals and spin adduct formation by the spin trapping technique (31), the following assumptions were made:

$$\text{MPDP}^+ + O_2 \xrightarrow{k_1} \cdot O_2^- + \text{Products} \qquad (1)$$

$$\cdot O_2^- + \text{DMPO} \xrightarrow{k_{DMPO}} \text{DMPO-}O_2^- \qquad (2)$$

where MPDP$^+$ serves as a source of $\cdot O_2^-$, DMPO as the spin trap and k_1 is the first order rate constant of superoxide generation during autoxidation of MPDP$^+$. The increase in concentration of spin adduct could be expressed as :

$$d[\text{DMPO-}O_2^{\cdot-}]/dt = k_{DMPO}[\text{DMPO}]\cdot[{}^{\cdot}O_2^{-}]. \quad (3)$$

Under steady state-conditions for the $^{\cdot}O_2^{-}$,

$$d[{}^{\cdot}O_2^{-}]/dt = 0 = k_1[\text{MPDP}^+] - k_{DMPO}[\text{DMPO}]\cdot[{}^{\cdot}O_2^{-}]. \quad (4)$$

Then,

$$d[\text{DMPO-}O_2^{\cdot-}]/dt = k_1[\text{MPDP}^+], \quad \text{or} \quad k_1 = dV/[\text{MPDP}^+], \quad (5)$$

where, dV is the rate of formation of spin adduct.

Thus, the EPR signal intensity is directly proportional to the number of superoxide radicals produced during $MPDP^+$ oxidation. As shown in Figure 3 inset b, the inverse plot of the rate of spin adduct formation against $MPDP^+$ concentration gave a straight line with a correlation coefficient of 0.994 indicating a stoichiometric relationship between $MPDP^+$ oxidation and $^{\cdot}O_2^{-}$ generation. The intercept of the plot, $1/V_{max}$, the maximum rate of $^{\cdot}O_2^{-}$ produced in this system was found to be 4.48×10^{-10} M·S^{-1}. The rate constant (K_1) of superoxide radical generation can be obtained directly from 1/slope of the plot and was found to be 3.97×10^{-6} S^{-1}.

Effect of Superoxide Dismutase on the Rate of Spin Adduct Formation:

Superoxide dismutase was found to inhibit the rate of formation of spin adduct in a dose-dependent manner. Thus, as shown in Figure 4, superoxide dismutase did powerfully inhibit the EPR signal intensity and as little as ~5 *n*g/ml of the enzyme caused 50% inhibition. Superoxide dismutase at 200 ng/ml completely eliminates the EPR signals of spin adducts when 80 mM DMPO was used to trap these radicals, (Fig. 1c and e). Addition of BSA (up to 200 μg/ml) or 400 *n*g/ml of denatured superoxide dismutase (obtained by boiling it in 1 *N* HCl for at least 20 min.) to the reaction mixture had trivial effects on EPR signal intensity indicating that the inhibition by superoxide dismutase was not a non-specific protein effect. These results strongly support the contention that superoxide radicals are generated during the autoxidation of $MPDP^+$.

Since there exists a competition between DMPO and superoxide dismutase (SOD) for the available superoxide radicals, the rate constant of SOD, as the competitive inhibitor (27, 32) in the above system, can be obtained. In a typical reaction, the autoxidation of $MPDP^+$ generates $\cdot O_2^-$, which in turn either spontaneously dismute ($2\ \cdot O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$) or react with DMPO to form DMPO-O_2^- adduct. Superoxide dismutase inhibits the adduct formation by scavenging $\cdot O_2^-$. Under steady state-conditions, in the presence of SOD and DMPO, for superoxide radical,

$$\Phi_{\cdot O_2^-} = k_{DMPO}[\text{DMPO}]\cdot[\cdot O_2^-] + k_{SOD}[\text{SOD}]\cdot[\cdot O_2^-] + k_d[\cdot O_2^-],$$

or

$$\Phi_{\cdot O_2^-} = [\cdot O_2^-]\cdot(k_{DMPO}[\text{DMPO}] + k_{SOD}[\text{SOD}] + k_d), \qquad (6)$$

where $\Phi_{\cdot O_2^-}$ is the yield of superoxide radical and k_d is the rate constant for spontaneous decay of $\cdot O_2^-$.

Let $$\Phi = k_{DMPO}[\text{DMPO}]\cdot[\cdot O_2^-]; \qquad (7)$$

then dividing equation 7 by equation 6, we obtain:

$$\Phi = \Phi_{\cdot O_2^-}\cdot\frac{k_{DMPO}[\text{DMPO}]}{k_{DMPO}[\text{DMPO}] + k_d + k_{SOD}[\text{SOD}]}. \qquad (8)$$

In the absence of scavenger (SOD),

$$\Phi_{\cdot O_2^-} = k_{DMPO}[\text{DMPO}]\cdot[\cdot O_2^-] + k_d[\cdot O_2^-] = [\cdot O_2^-]\,(k_{DMPO}[\text{DMPO}] + k_d), \qquad (9)$$

then,
$$\Phi_o = \Phi_{\cdot O_2^-}\cdot\frac{k_{DMPO}[\text{DMPO}]}{k_{DMPO}[\text{DMPO}] + k_d}. \qquad (10)$$

Dividing equation 10 by equation 8, we obtain:

$$\Phi_o/\Phi = \frac{k_{DMPO}[\text{DMPO}] + k_d + k_{SOD}[\text{SOD}]}{k_{DMPO}[\text{DMPO}] + k_d}, \qquad (11)$$

or
$$\Phi_o/\Phi - 1 = \frac{k_{SOD}[\text{SOD}]}{k_{DMPO}[\text{DMPO}] + k_d}, \qquad (12)$$

where, Φ_o/Φ is the ratio of the rates of spin trapping (or the EPR signal intensity of spin adduct) in the absence and presence of SOD; k_{SOD} and k_{DMPO} are the rate constants for SOD and DMPO scavenging $\cdot O_2^-$, respectively; and k_d is the spontaneous decay rate constant for superoxide radical. From this equation it follows that:

$$k_{SOD} = \frac{(\Phi_o/\Phi - 1)}{[\text{SOD}]} \cdot (k_{DMPO}[\text{DMPO}] + k_d). \qquad (13)$$

Under a given condition, item (k_{DMPO}[DMPO] + k_d) is constant and a plot of (Φ_o/Φ -1) against the [SOD] should give a straight line. That this was the case is shown in Figure 4 inset, where a simple *Stern-Volmer* behavior for scavenging of $\cdot O_2^-$ by SOD was observed with a correlation coefficient of 0.999. The ratio of (Φ_o/Φ -1) to [SOD] can be directly obtained from the slope of this plot and was found to be 9.443×10^9 M^{-1}. The constant item, (k_{DMPO}[DMPO] + k_d), was calculated to be 0.802 Sec^{-1}, using the rate constant (k_{DMPO}) for DMPO trapping $\cdot O_2^-$ of 10 $M^{-1}S^{-1}$ (33) and the rate of superoxide radical decay (k_d) at pH 9.7 of 2×10^{-3} Sec^{-1} (34). The rate constant, k_{SOD}, for SOD scavenging $\cdot O_2^-$ was found to be 7.56×10^9 $M^{-1}S^{-1}$ in our aqueous system, pH 10.0.

Since K_d is a pH-dependent constant whose value decreases with increasing pH, at pH 10.0 its value (2×10^{-3} Sec^{-1}) is almost negligible. Thus, equations 11 and 12 can be expressed as follows:

$$\Phi_o/\Phi - 1 = \frac{k_{SOD}}{k_{DMPO}} \cdot \frac{[\text{SOD}]}{[\text{DMPO}]}, \quad \text{or} \quad \frac{k_{SOD}}{k_{DMPO}} = \frac{\Phi_o/\Phi - 1}{[\text{SOD}]/[\text{DMPO}]}, \qquad (14)$$

Thus, when (Φ_o/Φ -1) value were plotted against the [SOD]/[DMPO] (data not presented) a ratio of (Φ_o/Φ -1) to [SOD]/[DMPO], which equals to k_{SOD}/k_{DMPO}, can be obtained directly from the slope of this plot and was found to be 7.5×10^8. Thus, k_{SOD} was calculated to be 7.5×10^9 $M^{-1}S^{-1}$ which is almost identical to the value (7.56×10^9 $M^{-1}S^{-1}$) obtained by the kinetic competition method described above.

However, at lower pH such as pH 7.12, the k_d value, is reported to be 11.0 Sec^{-1} (34). Taking into account this k_d value, the k_{SOD} value was calculated to be 1.14×10^{11} $M^{-1}S^{-1}$ at pH 7.12 which is 15 times higher than one at pH 10.0.

The life-time of $\cdot O_2^-$ in this medium was estimated utilizing the *Sterm-Volmer* quenching equation (35):

$$k_{sv} = \frac{\Phi_o/\Phi - 1}{[\text{SOD}]}. \qquad (15)$$

Since $k_{sv} = k_{SOD} \cdot t$ (16)

where t is the life-time of $\cdot O_2^-$ in a medium, the equation 16 could be substituted for equation 15 to obtain:

$$t = \frac{\Phi_o/\Phi - 1}{k_{SOD}[\text{SOD}]}, \qquad (17)$$

Using the k_{SOD} value of 7.765×10^9 $M^{-1}S^{-1}$, calculated above, we obtained a life-time of 1.25 second for $\cdot O_2^-$ in this aqueous solution (pH 10.0).

Effect of Varying Concentrations of DMPO on the Rate of Trapping:

Since $\cdot O_2^-$ is responsible for the conversion of DMPO to DMPO-O_2^- adduct, the rate of trapping of superoxide radical by the spin trap, under a given condition, should depend on the concentration of the trap in the system. If the reaction is not a chain event, increasing the concentration of spin trap should result in the interception of an ever increasing proportion of the $\cdot O_2^-$ generated and should therefore yield a saturation curve, and the plot of the inverse rate of spin adduct formation against [Spin Trap]$^{-1}$ should give a straight line with a constant slope and intercept. That this was the case is shown in Figure 5, where the rate of accumulation of spin adduct increased as a function of DMPO concentration. The maximum velocity of DMPO trapping superoxide radical generated in

this system can be obtained from the inverse of the intercept of the plot and was calculated to be 8.85×10^{-10} M·S^{-1}. The ratio of slope to intercept can give directly k_d/k_{DMPO}, the ratio of the spontaneous decay of superoxide radical to the rate constant of its reaction with DMPO. This ratio value was found to be 1.96×10^{-4} M. Using k_d value of 2×10^{-3} Sec^{-1}, at pH 9.7 reported by Rabani and Nielsen (34), k_{DMPO} was calculated to be 10.2 M^{-1}S^{-1} which is in accord with the published value of 10.0 M^{-1}S^{-1} (33).

Effect of pH on the Rate of Generation of Superoxide Radicals During Autoxidation of MPDP$^+$:

As shown in Figure 6, the rate of formation of superoxide spin adduct with DMPO, taken as the initial rate of increase in EPR signal intensity, increased with increasing pH. Below pH 7.0 the rate of accumulation of the spin adduct was minimum. These results indicate that the free radicals formation is facilitated by ionization of MPDP$^+$ at higher pH as discussed below.

DISCUSSION

The results reported here clearly demonstrate, for the first time, that superoxide radicals are produced during the autoxidation of MPDP$^+$. The EPR-spin trapping techniques used here have proven useful in analyzing complex reactions for the detection and quantitation of these radicals during the autoxidation process in aqueous solutions.

The characteristic EPR spectrum of the superoxide radical spin adduct with DMPO as spin trap, exhibiting $3 \times 2 \times 2$ lines (Fig. 1b), was observed when MPDP$^+$ was incubated with DMPO in a buffered-DMSO solution. The hyperfine parameters (a_N =13.1

G, $^{\beta}a_H$ =10.4 *G* and $^{\gamma}a_H$ =1.3 *G*) for this EPR spectrum are similar to those previously reported values for DMPO-O_2^- (18-23), indicating that $\cdot O_2^-$ spin adduct with DMPO is responsible for the EPR signal. Superoxide dismutase inhibited the EPR signal intensity in a dose-dependent manner, further confirming the generation of $\cdot O_2^-$ during this process.

In air-saturated aqueous solutions, the $MPDP^+$ autoxidation gave rise to free radicals which upon reacting with DMPO gave a spin adduct whose EPR spectrum exhibited four splitting lines in an intensity ratio of 1:2:2:1 (Fig. 1d) with hyperfine splitting constants of $a_N = a_H = 14.9$ G, consistent with DMPO-OH adduct characteristics (25, 26). Since superoxide dismutase inhibited this signal in a dose-dependent manner and as little as 50 *n*g/ml of this enzyme virtually eliminated the signal, we believe that the primary species manifested as DMPO-OH adduct is $\cdot O_2^-$. This view was further confirmed by the use of ethanol. Thus, ethanol, a good hydroxyl radical scavenger (28), up to 200 mM had trivial effect on the DMPO-OH signal. It is possible that the intermediates produced during $MPDP^+$ oxidation (such as MPDP* or MPDP·) were able to directly reduce the DMPO-O_2^- to form DMPO-OH by a hydroxyl radical-independent pathway, in which case the rate of reduction of the spin adduct may be much faster than the decomposition of DMPO-O_2^- and spontaneous dismutation of $\cdot O_2^-$. This reasoning is further supported by the following observations: (a) the estimated life-time of $\cdot O_2^-$ in our system was found to be 1.25 sec which is in close agreement with the published value of 1.11 sec (36), (b) the rate constant, k_{DMPO}, was found to be 10.2 $M^{-1}S^{-1}$, which is also in close agreement with the published value of 10.0 $M^{-1}S^{-1}$ (33) for DMPO trapping $\cdot O_2^-$, (c) superoxide dismutase, a specific and efficient scavenger of superoxide radical ($K = 2 \times 10^9$ $M^{-1}S^{-1}$; Refs.24, 37), was found to virtually eliminate the EPR signal (Figs. 1c and 4), and (d) the secondary order rate constant for superoxide dismutase scavenging $\cdot O_2^-$ was calculated to be 7.56×10^9 $M^{-1}S^{-1}$, which closely approximates the value reported (24, 37). Therefore, in spite of the limitations of our methods, all the above results provide

confidence that the primary radical produced during the autoxidation of $MPDP^+$ was indeed $\cdot O_2^-$ and that the intensity of the EPR signal of the spin adduct can be utilized to estimate the relative amounts of $\cdot O_2^-$ generated in an aqueous system.

The generation of $\cdot O_2^-$ was quantitatively determined by a kinetic competition analysis technique. Here $MPDP^+$ was the source of $\cdot O_2^-$ and DMPO at saturated concentrations effectively trapped these radicals. Superoxide dismutase, an efficient scavenger of $\cdot O_2^-$, inhibited this reaction completely. Under these conditions the maximum rate of $\cdot O_2^-$ produced in this system was found to be 4.48×10^{-10} $M \cdot Sec^{-1}$ and the first order rate constant (k_1) for $\cdot O_2^-$ generated in the system was found to be 3.97×10^{-6} Sec^{-1}. The maximum velocity of DMPO trapping $\cdot O_2^-$ in this system was calculated to be 8.85×10^{-10} $M \cdot Sec^{-1}$. The rate constant of DMPO trapping (k_{DMPO}) was found to be 10.2 $M^{-1}S^{-1}$.

The rate of generation of $\cdot O_2^-$ was found to increase with increasing concentrations of $MPDP^+$ up to 50 μM $MPDP^+$. Subsequent increase of the $MPDP^+$ concentration generated lower levels of $\cdot O_2^-$ (Fig. 3). The rate of superoxide generation per-unit amount of $MPDP^+$, however, decreased with increasing $MPDP^+$ concentration (Fig. 3 inset a) giving rise to the possibility that high concentrations of $MPDP^+$ or some of its redox cycling products may quench superoxide radical. Since $MPDP^+$ could accept a hydrogen atom and an electron to form MPTP (38, 39), or donate an electron/hydrogen atom to generate $\cdot O_2^-$, there exists a competition between molecular oxygen and $MPDP^+$ for the available electrons or hydrogen atoms. In the absence of oxygen, no reactive oxygen species could be produced and the predominant reaction, therefore, could be the disproportionation of $MPDP^+$ to yield equimolar amounts of MPTP and MPP^+:

$$MPDP^+ \underset{+ H^+}{\overset{- H^+}{\longrightarrow}} MPDP^* \qquad (15)$$

$$MPDP^+ + MPDP^* \text{ ----------> } MPTP + MPP^+ \quad (16)$$

In the presence of oxygen, the competition between dissolved oxygen and $MPDP^+$ will be dependent on the $MPDP^+$ concentration because the oxygen concentration in the reaction mixture open to atmospheric air is virtually constant. At lower concentration of $MPDP^+$ such as 50 μM, the dissolved oxygen (240 μM oxygen in air-saturated aqueous solution at room temperature) would effectively compete for the available electrons or hydrogen atom. Although hydrogen atom can be quickly converted to hydrated electron (e_{aq}^-) at a rate constant of 2×10^7 $M^{-1}S^{-1}$ at high pH (34), it should be captured by molecular oxygen at a rate of 2×10^{10} $M^{-1}S^{-1}$ to form superoxide radical (40). The two possible pathways for the generation of $\cdot O_2^-$ are: (i) ionization, in which the hydrated electron or hydrogen atom ejected by the ionized MPDP* react with dissolved oxygen to form $\cdot O_2^-$, and (ii) transfers of an electron or hydrogen atom directly from the intermediates produced during the autoxidation of $MPDP^+$ to oxygen. Since, DMPO at moderate concentration can rapidly react with both hydrogen atom and the hydrated electron at a rate of 1.5×10^{10} $M^{-1}S^{-1}$ to form the DMPO-H spin adduct (41), the hydrated electrons and hydrogen atoms formed in the autoxidation of $MPDP^+$ should give an additional EPR spectrum consisting of a triplet of triplets with each triplet arranged in a ratio of 1:2:1 pattern (a_N = 16.69 *G* and $^{\beta}a_H$ = 22.5 *G*) (42, 43) corresponding to the DMPO-H spin adduct. As this DMPO-H signal was not observed, the generation of $\cdot O_2^-$ is most likely to occur by a direct electron or hydrogen atom transfer from the intermediates of $MPDP^+$ to dissolved oxygen. At higher concentrations of $MPDP^+$ such as above 100 μM, the rates of $MPDP^+$ competing for electron and hydrogen atom to form MPTP could be enhanced. As a result, the amount of oxygen radical would decrease proportionately with increasing concentrations of $MPDP^+$. This may be the reason why the rate of generation of superoxide radical was, but not the same proportion to the increased concentrations of $MPDP^+$ beyond 50 μM (Fig. 3).

The pH dependency of the rate for spin adduct formation under a given condition is an important consideration in explaining the mechanism of autoxidation of $MPDP^+$. Thus, $MPDP^+$ can readily donate a H^+ at higher pH to form an unstable intermediate, MPDP*. The MPDP* so generated could then release an electron or a hydrogen atom which in turn would react with molecular oxygen, at a rate constant of $2 \times 10^{10}\ M^{-1}S^{-1}$, to form $\cdot O_2^-$ or HOO·, respectively (39, 43). In alkaline pH, however, the HOO· would give up a H^+ to yield $\cdot O_2^-$. Since the superoxide radicals are more stable in alkaline aqueous solutions ($t_{1/2}$ = 50 Sec at pH 14) and their stability decreases with decreasing pH ($t_{1/2}$ = 0.2 Sec at pH 10) (45), it renders the apparent concentration of superoxide radical at high pH to be greater than at low pH under a given condition. Furthermore, at lower pH, the rate constant for DMPO trapping superoxide radicals increases with a concomitant increase in the spontaneous dismutation of superoxide (46). Therefore, at lower pH, any increase in EPR signal intensity due to the higher rate of trapping is offset by the increased rate of spontaneous dismutation of $\cdot O_2^-$. At higher pH, however, the spontaneous dismutation rate decreases and the change of the rate constant of spin trapping reaction becomes less sensitive to pH (33). Thus, the ability of DMPO to compete with the spontaneous dismutation of available superoxide is actually enhanced at higher pH. The enhanced rate of formation of the spin adduct at higher pH, however, may mostly be attributed to the increased levels of MPDP* produced by the deprotonation of $MPDP^+$ generating increased levels of $\cdot O_2^-$ as discussed above.

Although the oxidation pathways for 2, 3-$MPDP^+$ have been previously proposed (13, 47), no direct evidence was available for the generation of free radical species of oxygen during this process. We have confirmed the generation of superoxide radical during the autoxidation of $MPDP^+$. The mechanism(s) of generation of these radicals is presented in Scheme I where, after a proton abstraction, $MPDP^+$ forms compound **II** (MPDP*) which has three available pathways to form MPP^+. In the first pathway, as

described in the catalyzed reaction (13, 47), the resonance-stabilized carbon-centered radical species **IV** ↔ **V** could be formed after a single electron oxidation from compound **II** to form **III** followed by a proton abstraction. Loss of a second electron from **IV** or **V** would generate MPP^+. In the second pathway, compound **II** loses a hydrogen atom to form the resonance structure of **IV** and **V** which in turn loses an electron to form MPP^+. Both the first and second pathways include the formation of resonance structure carbon-centered radical species **IV** ↔ **V**. However, it is possible to have an alternate pathway, without forming a carbon-centered radical species, to form MPP^+. In this pathway (pathway 3), the compound **II** undergoes an electron loss to form compound **III** which directly forms MPP^+ after the loss of a hydrogen atom. The electrons or hydrogen atoms released in these steps could react with molecular oxygen ($k = 2 \times 10^{10}\ M^{-1}S^{-1}$; Ref. 40) to generate superoxide radicals. Since $MPDP^+$ is capable of accepting an electron and a hydrogen atom to form MPTP (38, 39), there exists a competition between molecular oxygen and $MPDP^+$ for the available electrons or hydrogen atoms, and this may be the reason why the intensity of spin adduct signal was quenched at high concentrations of $MPDP^+$.

We are now forced to think of the biological effects of $MPDP^+$ in a new light. Thus, $MPDP^+$ not only generates $\cdot O_2^-$ during spontaneous oxidation but also may yield MPDP radicals and possibly other, as yet undetected, reactive intermediate(s). It is tempting to attribute some of the pathological effects of $MPDP^+$/MPTP-induced neurotoxicity to intermediates produced during its oxidation. Although $MPDP^+$ was shown to exist in the astrocytes as an intermediate of the MAO-catalyzed oxidation product of MPTP, it can readily cross the cell membrane and reach the extracellular space (8). Since (i) the rate of decay of $MPDP^+$ is relatively slow at pH 7.6 (48), (ii) its decay rate was shown to be independent of MAO-catalyzation (29), various transition metals (14, 30) and

hydrogen peroxide (48), and (iii) its stability is pH-dependent (29), $MPDP^+$ could cross the cell membrane and enter the nigrostriatal cells where conditions may be favorable for an accelerated autoxidation to generate superoxide radical and the final stable species, MPP^+. The superoxide radical so generated can be protonated to form a much more reactive species, the ·OOH, at or near the cell surface where the cell membranes are H^+-rich. These reactive species are known to cause cell injury and cell dysfunction and may be associated with the neurodegenerative process of MPTP.

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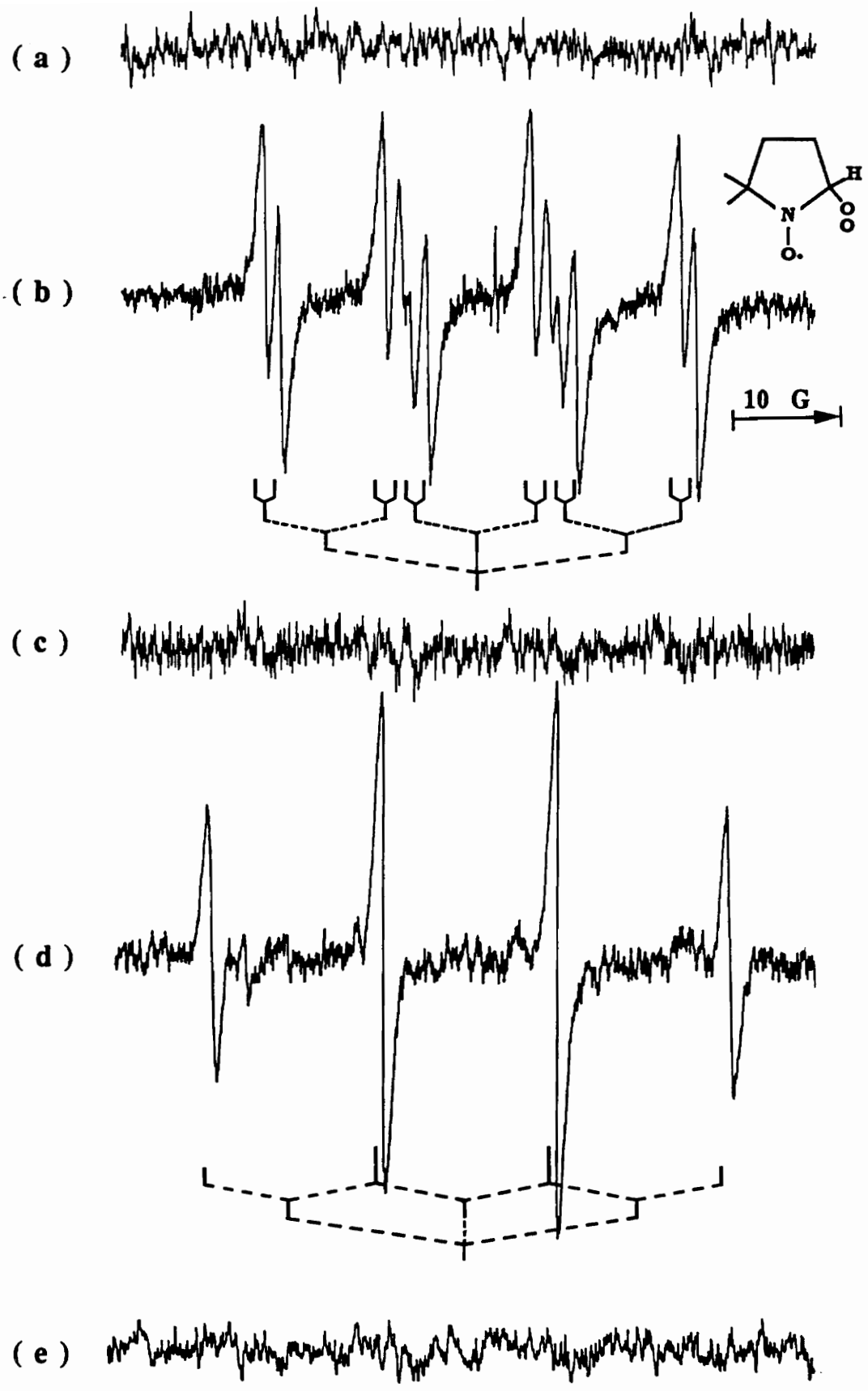
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Figure 1. EPR spectra of spin adducts of free radicals observed during the autoxidation of $MPDP^+$. The reaction mixtures consisted of $MPDP^+$ (0.3 mM) and DMPO (80 mM) in air-saturated DMSO solution (containing 10% buffered aqueous solution of 0.05 M sodium borate-carbonate buffer, pH 10.0) or 0.05 M sodium borate-sodium carbonate buffered solution (pH 10.0) without any DMSO. EPR spectra of reaction mixture: (a) bubbled with N_2 in the presence of DMSO, or in the absence of either DMPO or $MPDP^+$ in air-saturated DMSO solution incubated for 20 mins; (b) $MPDP^+$ and DMPO incubated for 10 minutes in DMSO solution (containing 10% buffered aqueous solution, pH 10.0); (c) similar conditions as "b " but in the presence of SOD (50 ng/ml); (d) $MPDP^+$ and DMPO incubated for 10 minutes in buffered aqueous solution (pH 10.0) without DMSO; and (e) conditions similar to "d" but in the presence of SOD (50 ng/ml). EPR spectroscopy settings were the same as described under "Materials and Methods", except that (a) the sensitivity of signal amplitude was reduced by changing the modulation amplitude to 0.63 *G*, time constant to 0.32 sec; and receiver gain to 5×10^5; (b) the time constant was fixed at 0.32 Sec.; and (d) reciever gain was slightly reduced to 8×10^5.

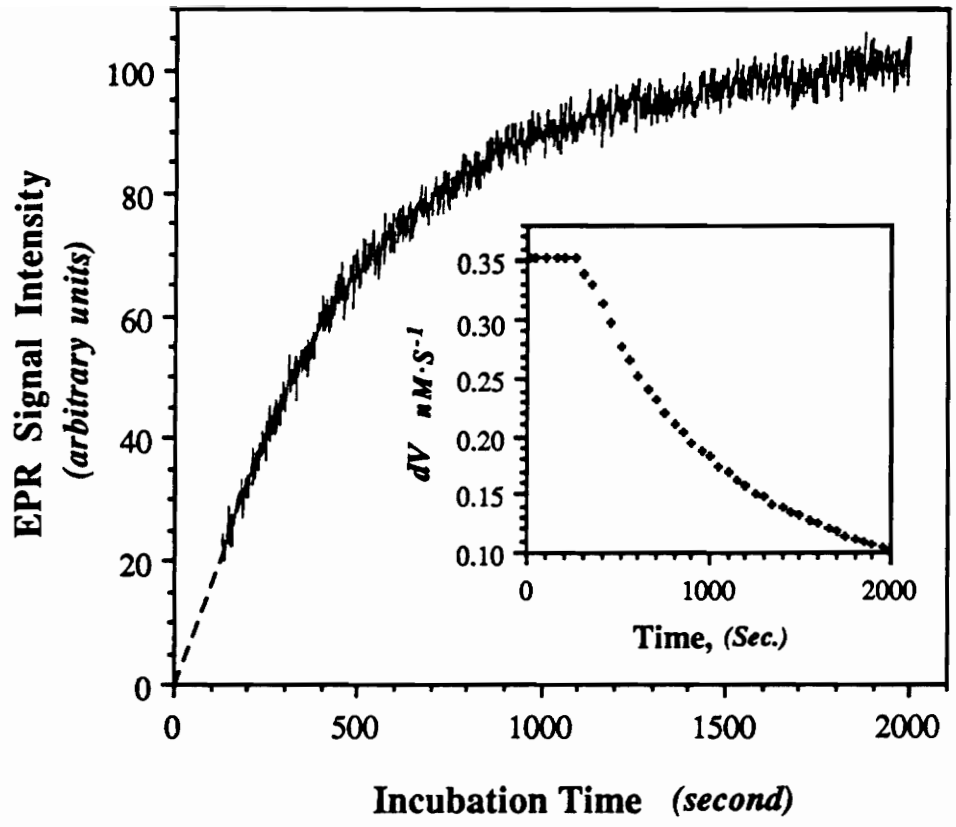


Figure 2. Kinetics of spin-adduct formation. The reaction mixture contained 0.3 mM $MPDP^+$ in air-saturated sodium borate-carbonate buffer, pH 10.0, in the presence of 80 mM DMPO. EPR signal intensity with incubation time was traced at set magnetic field of 3453.9 *G* to record the intensity of the first peak of DMPO-OH adduct. Inset: the change of velocity of spin adduct formation with the incubation time. The parameters were set at: microwave power, 20 mW; modulation amplitude, 2.0 *G*; time constant, 1.25 Sec.; receiver gain, 2×10^6; and scan time, 2 Ksec.

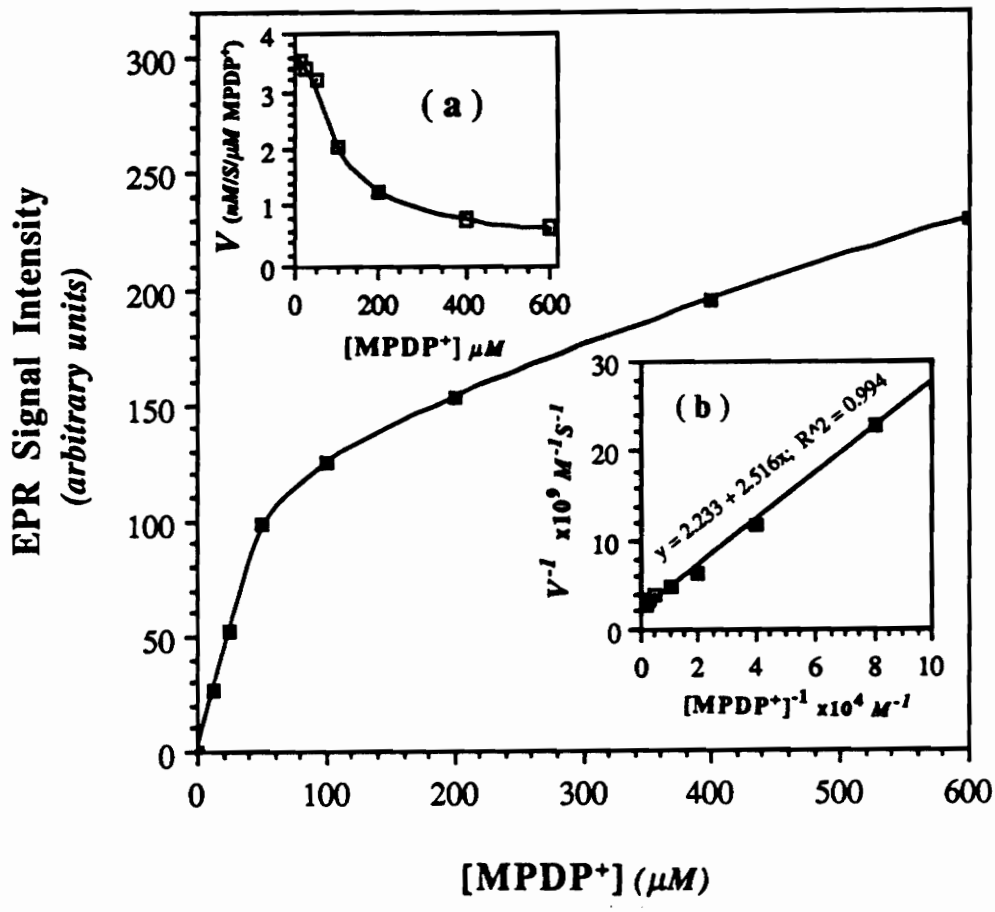


Figure 3. The rate of spin-adduct formation as a function of $MPDP^+$ concentration in the buffered solution. Inset: (a) effect of $MPDP^+$ concentration on the rate of spin adduct formation per-micromolar $MPDP^+$; and (b) the inverse plot of the rate of spin adduct formation *vs* $MPDP^+$ concentration. The reaction mixtures contained 80 mM DMPO and the indicated amounts of $MPDP^+$. EPR spectroscopy was set at: microwave power, 20 mW; modulation amplitude, 1.0 *G*; time constant, 0.64 Sec.; receiver gain, 1×10^6; and scan time, 500 Sec.

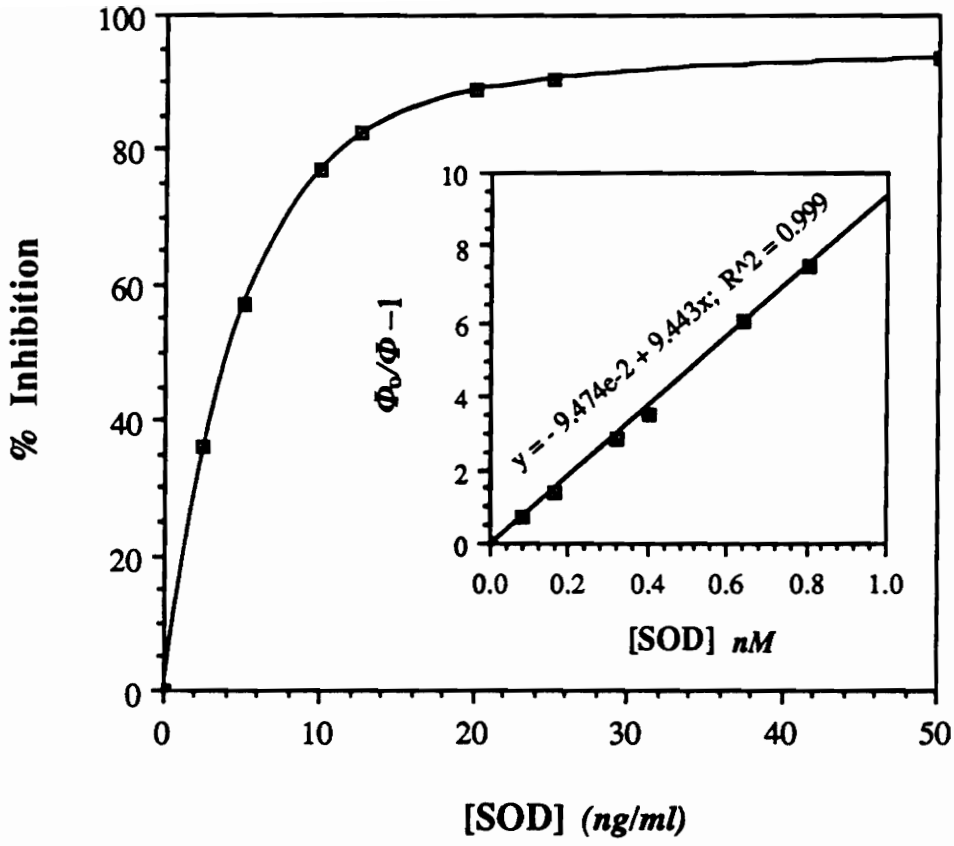


Figure 4. Inhibition of EPR spin adduct signal intensity by superoxide dismutase in air-saturated 0.05 M borate-carbonate buffered solution, pH 10.0. The reaction mixture contained 0.3 mM $MPDP^+$, 80 mM DMPO and indicated concentrations of SOD. Inset: *Sterm-Volmer* plot. The Φ_o/Φ is the ratio of the rates of increase of EPR signal intensity in the absence and presence of SOD. EPR spectroscopy was set at: microwave power, 20 mW; modulation amplitude, 2.0 *G*; time constant, 0.64 Sec.; receiver gain, 1×10^6; and scan time, 500 Sec.

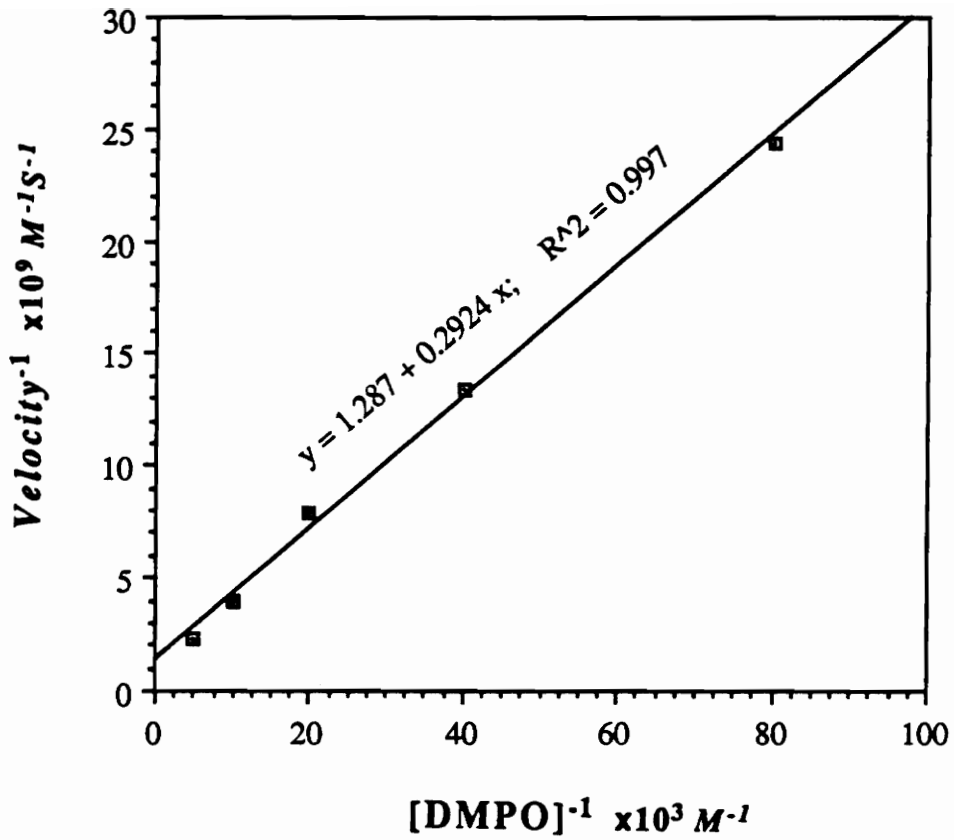


Figure 5. The initial rate of formation of spin adduct as a function of DMPO concentration. The reaction mixture contained 0.4 *m*M $MPDP^+$ and the indicated concentration of DMPO in 0.05 M borate-carbonate buffered solution (pH 10.0). Inset: Double-reciprocal plot. EPR parameters were set at: microwave power, 20 mW; modulation amplitude, 1.0 *G*; time constant, 0.64 Sec; receiver gain, 1×10^6; and scan time, 500 Sec.

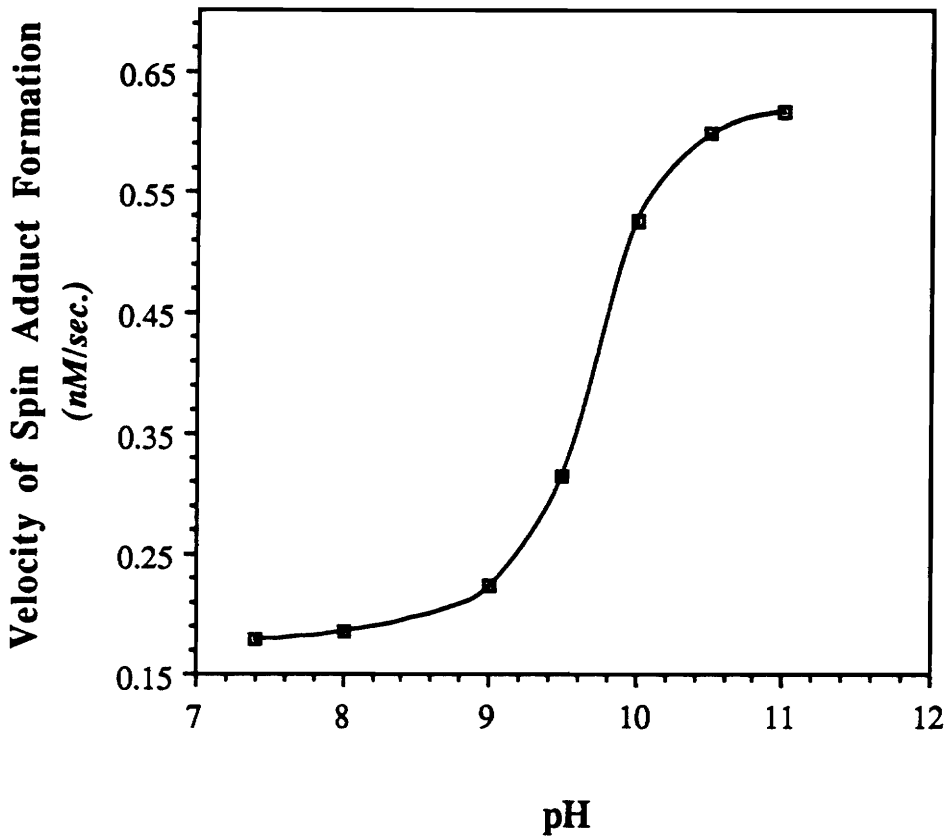
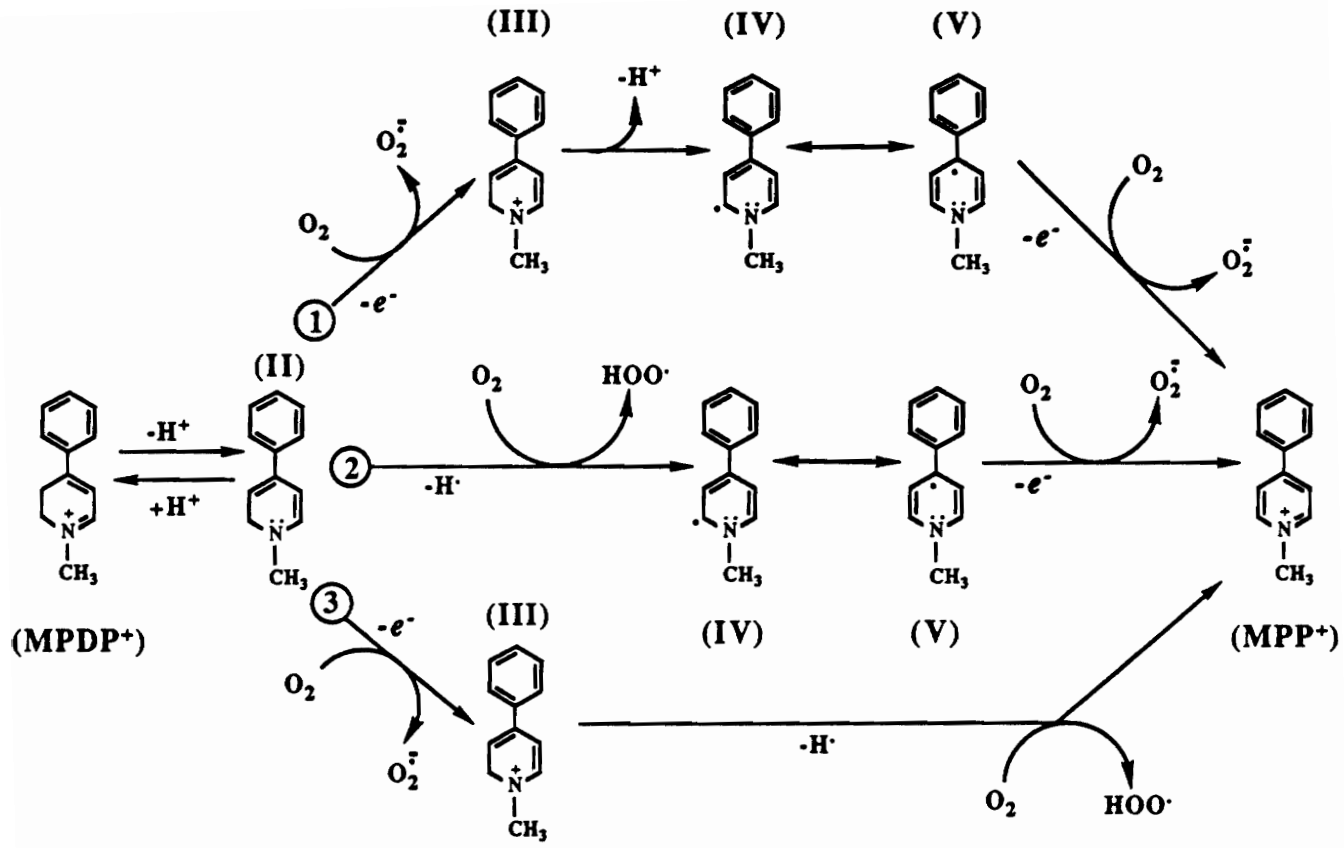


Figure 6. Effect of pH on the rate of spin adduct formation during the autoxidation of $MPDP^+$. The reaction mixture contained 0.3 *m*M $MPDP^+$ and 80 *m*M DMPO in the indicated pH buffered solutions. The pH of the reaction mixture were achieved by different 0.05 M bufferred solutions (Sodium-potassium phosphates, pH 7.4 and 8.0; Potassium phosphate-Sodium borate, pH 9.0 and 9.5; and Sodium borate-Sodium carbonate, pH 10.0, 10.5 and 11.0). EPR spectroscopy was set at: microwave power, 20 mW; modulation amplitude, 0.71 *G*; time constant, 0.64 Sec; receiver gain, 1×10^6; and scan time, 500 Sec.



Scheme I. Proposed Mechanisms for Superoxide Radicals Generation During the Autoxidation of $MPDP^+$.

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FROM: Hedy Grant - Asst. to QUENTIN H. GIBSON - J.BIOL.CHEM.

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Dear Dr. Misra:

Happy to tell you that your manuscript M2-3318 with Dr. Lun-Yi Zang is acceptable for publication without any revision! Thought you would like to see the comments from a reviewer.

Please send me ASAP a computer disk to facilitate the editing and printing process that is required by the Redactory Office.

This paper very effectively demonstrates that the autoxidation of the dopaminergic neurotoxin MPTP (actually MPDP) can generate superoxide radicals. This is an important piece of mechanistic information in regards to this widely used tool for the experimental induction of Parkinson's disease. The methods and experimental design are excellent. The manuscript and study are quite consistent with the standards of JBC. This reviewer could not make any substantive criticisms.

CHAPTER IV

GENERATION OF REACTIVE OXYGEN SPECIES DURING THE MONOAMINE OXIDASE-CATALYZED OXIDATION OF THE NEUROTOXICANT, 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE

(Journal of Biological Chemistry, July Issue, 1993, In press)

Lun-Yi Zang and Hara P. Misra

Abstract

The neurotoxicant, MPTP has been shown to generate reactive oxygen species during its interaction with monoamine oxidase type B (MAO-B). The kinetic parameters, K_m and V_{max}, for MAO-B catalyzed oxidation of MPTP to the corresponding species $MPDP^+$ were found to be 0.194 *m*M and 0.335 μM/min, respectively. The generation of superoxide ($\cdot O_2^-$) and hydroxyl ($\cdot OH$) radicals was detected as the 5, 5-dimethyl-1-pyrroline-N-oxide (DMPO) spin adduct by spin trapping in combination with EPR techniques. Addition of Fe^{2+} (10 μM) to this system caused a 5-fold enhancement in EPR signal intensity of the DMPO-OH adduct. Catalase, a scavenger of hydrogen peroxide (H_2O_2), inhibited the DMPO-OH spin adduct formation in a dose-dependent manner, indicating that H_2O_2 is produced in the MAO-B catalyzed oxidation of MPTP. Ethanol, a well known scavenger of hydroxyl radical, rapidly produced an alpha-hydroxyethyl radical signal. Superoxide dismutase (SOD) inhibited the formation of DMPO-O_2^- and DMPO-OH spin adducts in a dose-dependent fashion. These data suggest that superoxide radicals are produced during the oxidation of MPTP by MAO-B and that the generation of H_2O_2 and $\cdot OH$ was secondary to the production of $\cdot O_2^-$. It appears likely that the nigrostriatal toxicity of MPTP leading to Parkinson's disease-like syndrome may in part be mediated via these reactive oxygen species.

INTRODUCTION

Self-administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been reported to produce irreversible symptoms of Parkinson's disease in several young drug abusers (1). It was found that this neurotoxicant selectively destroys neuronal cells in the substantia nigra of humans and other primates (2). Although the mechanism of action of MPTP is not fully understood, it is generally believed that the crucial species for MPTP neurotoxicity is not MPTP itself, but rather some of its metabolites (3,4). In studies of MPTP-metabolism, it has been demonstrated that monoamine oxidase-B (MAO-B) catalyzes the two-electron oxidation of MPTP to a dihydropyridinium intermediate ($MPDP^+$), which in turn spontaneously oxidizes to form methyl phenylpyridinium (MPP^+) or disproportionates to form MPTP and MPP^+ (5-7). The generation of oxygen radicals during metabolism of MPTP has been proposed to explain its neurotoxicity (8-13), but no direct evidence is available to confirm this hypothesis. It was reported that MAO-B catalyzes MPTP oxidation to generate hydrogen peroxide (H_2O_2) during the incubation of MPTP and MAO-B (14). This demonstration, that MAO-B catalyzed-oxidation of MPTP could cause divalent reduction of oxygen to form H_2O_2, gave no indication of the relative importance of the univalent reduction of oxygen to generate other reactive oxygen species. Since O_2 can not accept two electrons simultaneously (spin restriction) to form H_2O_2, the generation of H_2O_2 may be secondary to the production of superoxide radical, a univalent reduction product of O_2. For these reasons, we have investigated potential univalent reduction of oxygen by MAO-B catalyzed oxidation of MPTP. In addition, H_2O_2 in the presence of Fe^{2+} may be converted to the hydroxyl radical, giving rise to lipid peroxidation and oxidative damage to membranes, protein and nucleic acids (15). The present report describes measurements of the generation of reactive oxygen species during MAO-B catalyzed-oxidation of MPTP.

MATERIALS AND METHODS

1-methyl-4-phenyl-2,3-dihydropyridinium perchlorate ($MPDP^+$), 1-methyl-4-phenyl- pyridinium iodide (MPP^+), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP) and deprenyl were purchased from Research Biochemicals, Inc. Natick, MA. The spin trap, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from Aldrich Chemical Company, Inc., Milwaukee, WI and was purified and measured quantitatively as previously described (16). Bovine erythrocyte superoxide dismutase, catalase, 3-cyano-peroxyl free radical and diethylenetriaminepentaacetic acid (DTPA) were obtained from Sigma Chemical Company, St. Louis, MO. All other chemicals were obtained at the highest obtainable purity.

Monoamine oxidase (MAO-B) from bovine liver mitochondria was a generous gift from Dr. Castagnoli from the Department of Chemistry, Virginia Tech. It was prepared by the method of Salach (17) and stored in phosphate buffer pH 7.2 at -20 *°C* containing 50% (vol/vol) glycerol. Before use, glycerol was removed by diluting the stock solution with 20-40 volumes of 50 *m*M phosphate buffer, pH 7.4 and centrifuging at 200,000 *g* at 0-4 *°C* for 60 *min*. The supernatant was discarded and the pellet containing the enzyme was resuspended with a minimum volume of buffer. The activity of the enzyme was determined spectrophotometrically at 250 *nm* using the initial rate of oxidation of 3.3 mM benzylamine. One unit of enzyme activity is defined as the amount of the protein required to convert 1 *μMole* of benzylamine to benzaldehyde per min at pH 7.4 at 30 *°C* (18). Protein concentration was determined by the dye-binding assay of Bradford (19) using bovine serum albumin as a standard. The specific activity was calculated to be 1.03 *units/mg protein*. All above operations were performed in the dark because of the photosensitivity of the flavoprotein (20).

Spectrophotometric assays were performed in a Shimadzu UV-visible recording spectrophotometer UV-160 at indicated temperature. The MAO-B catalyzed oxidation of MPTP was determined by detecting the formation of $MPDP^+$ at 345 *nm* or by monitoring the decay of MPTP absorption at 242 *nm*. The extent of univalent reduction of O_2 during the oxidation of MPTP was measured with the use of DMPO as a spin trap in combination with EPR techniques.

Because trace metals such as iron interfere in spin trapping experiments, the metal chelator diethylenetriaminepentaacetic acid (DTPA) was used in our system. The reactions were started by adding appropriate amounts of MAO-B stock in 50 *mM* phosphate buffer, pH 7.4. The enzyme was found to be stable for at least 2 days in the dark at 0-4 *°C*. Individual samples were placed in the EPR TM cavity using an EPR aqueous flat cell (60 *mm* ¥ 17 *mm* ¥ 0.25 *mm* i.d.) for the measurement of free radical generation at a given incubation time at room temperature. The commercial 3-cyano-peroxyl free radical was used as the nitroxide standard for determining relative concentrations of free radicals. The recordings of EPR spectra were performed with a Bruker D-200 ER spectrometer. Unless otherwise stated, the EPR parameters were set at: 100 *KHz*, X-band; microwave frequency, 9.78 *GHz*; microwave power, 20 mW; modulation amplitude, 1.0 *G*; time constant, 0.64 *sec.*; scan time, 500 *sec.* and receiver gain, 1×10^6.

RESULTS

Formation of $MPDP^+$ During MAO-B Catalyzed-Oxidation of MPTP:

Figure 1 shows the courses of MPTP oxidation and $MPDP^+$ formation in the air-saturated 0.05 *M* phosphate buffer, pH 7.4 containing 0.05 *unit/ml* of MAO-B, at 37 *°C* and the indicated incubation time. No oxidation of MPTP and formation of $MPDP^+$ were observed without MAO-B in the reaction mixture, indicating that the oxidation of MPTP is

MAO-B dependent. It is evident that the absorption maximum of MPTP at 242 *nm* diminished in intensity (Fig. 1a), whereas the absorbance of $MPDP^+$ at 345 *nm* intensified with increasing incubation time (Fig. 1b). The rate of formation of $MPDP^+$ was found to be equal to 75~80% of the amount of MPTP lost using extinction coefficients of 1.74×10^4 $M^{-1}cm^{-1}$ for $MPDP^+$ and 1.2×10^4 $M^{-1}cm^{-1}$ for MPTP at 345 nm and 242 nm, respectively, which were based on the calculations from the results of five experiments.

The formation of $MPDP^+$ was found to be dependent upon the pH within the tested range of pH 7.0-11.0. Thus, in a computer traced plot (Fig. 1c), the optimum pH for $MPDP^+$ formation was found to be ~7.6. The optimum activity of MAO-B using phenylethylamine as substrate (21) approximates to this pH value. The final stable metabolite, MPP^+, was measured by monitoring the change at 290 *nm*. MPP^+ was not observed at low pH but was found to appear only at pH > 8.5, indicating that the oxidation of $MPDP^+$ to MPP^+ may not be a consequence of MAO-B mediated oxidation, but instead may be dependent upon the autoxidation of $MPDP^+$ at its favoring conditions.

The rate of formation of $MPDP^+$ was found to be dependent upon the initial concentration of MPTP in the reaction system. Figure 2 shows the rate of formation of $MPDP^+$ as a function of [MPTP]. The hyperbolic nature of the curve indicates saturation of enzyme by the substrate. The Lineweaver-Burk plot (Fig. 2. inset) shows a straight line with a correlation coefficient of 0.997. The reciprocal of the intercept of the plot, V_{max}, the maximum rate of $MPDP^+$ produced in this system was found to be 0.335 $\mu M/min.$, and the Michaelis constant (K_m), obtained directly from the ratio of slope to intercept of the plot, was found to be 0.194 *m*M. Also, the rate constant (k_1) of $MPDP^+$ formation, calculated directly from 1/slope of the plot was found to be 2.89×10^{-5} sec^{-1}. The kinetic parameters for MAO-B catalyzed oxidation of MPTP reported by other researchers (18, 22, 23, 24) are compared in Table 1. As shown in this table, the K_m values for both MPTP and

benzylamine obtained in our system are in accord with the published values. All these results provide confidence that the oxidation of MPTP in our system is MAO-B dependent.

EPR Spectra of Free Radical Spin Adducts Formed During the MAO-B Catalyzed Oxidation of MPTP:

The incubation of an air-saturated reaction mixture of MPTP (50 μM) and MAO-B (0.05 unit/ml) in the presence of DMPO (80 *m*M) in buffered DMSO solution (pH 7.4), generated a DMPO-superoxide anion radical adduct (Fig. 3b). In the absence of MAO-B, MPTP, O_2 or DMPO, no detectable EPR signal was observed (Fig. 3a). The hyperfine splittings of the spin adduct consists of $3 \times 2 \times 2$ splitting lines which usually result from the interactions of an uncoupled electron with a primary nitrogen atom along with the secondary β- and γ- protons. The hyperfine splitting constants of the signal (a_N =13.3 *G*, $^{\beta}a_H$ =10.5 *G* and $^{\gamma}a_H$ =1.3 *G*) are consistent with previously reported values for DMPO-OOH in buffered dimethylsulfoxide solutions (25, 26). Since trapping of oxygen-centered radicals other than superoxide would result in an EPR spectrum very similar to that of DMPO-OOH (27), superoxide dismutase was used in this system in an attempt to verify the role of ·OOH in the formation of the above adduct. The superoxide radicals generated by the MAO-B catalyzed-oxidation of MPTP could either dismute to $H_2O_2 + O_2$ or could react with DMPO to form a DMPO-OOH adduct. Superoxide dismutase, by catalyzing the former reaction at a second order rate constant of $2 \times 10^9\ M^{-1}S^{-1}$ (28, 29), should inhibit the ·OOH-dependent DMPO-OOH adduct formation. That this was the case is shown in Figure 3c. Superoxide dismutase inhibited the adduct formation in a dose dependent manner. At ~25 ng/ml, superoxide dismutase inhibited the EPR signal intensity to ~50% of the control rate (Fig. 3c),while at 1.0 μg/*ml* the EPR signals were virtually suppressed (data shown and discussed later, Fig. 6). It follows that MAO-B catalyzed oxidation of

MPTP generates $\cdot O_2^-$ and that DMPO is capable of reacting with this primary species to form the spin adduct with the characteristic EPR signal shown in Figure 3b. L-deprenyl, a specific inhibitor of MAO-B (30), inhibited the superoxide spin adduct formation in a dose-dependent manner (data not shown) and at 10 μM it suppressed the signal intensity to ~50% of the control (Fig. 3d).

When MPTP and MAO-B were incubated in an air-saturated aqueous solution of 0.05 *M* phosphate buffer (pH 7.4) in the presence of DMPO, the resulting EPR spectrum exhibited four splitting lines with an intensity ratio of 1:2:2:1 (Fig.4a), as well as small but detectable amounts of superoxide radicals. The hyperfine splitting constants of the signal for this EPR spectrum were found to be $a_N = a_H = 15.0$ *G*, consistent with previously reported values for DMPO-OH in aqueous systems (31, 32). In the presence of 50 *ng/ml* superoxide dismutase (Fig. 4b) the intensity of the EPR signal of DMPO-OH adduct was suppressed to about 30% of control. Since the DMPO-OOH spin adduct is known to decompose to DMPO-OH with a half-life of approximately 2 min in aqueous solution (pH 7.4) and dismutation of ·OOH to ·OH could be completed within one second in an aqueous solution (33), the appearance of DMPO-OH adduct and the ability of superoxide dismutase to inhibit its formation is therefore understandable. It does however establish that superoxide radicals are produced during the MAO-B catalyzed-oxidation of MPTP in aqueous solution and that the resulting DMPO-OH adduct was secondary to the ·OOH formation.

Since DMPO-OOH could be directly reduced by certain biological reductants to form DMPO-OH (16), the mechanism of formation of DMPO-OH adduct was further investigated. Ethanol, a well known scavenger of hydroxyl radical (34), can rapidly react with ·OH at a rate constant of $k = 1 \times 10^9\ M^{-1}S^{-1}$ to produce an alpha-hydroxyethyl radical (CH_3·CH-OH) which in turn can react readily with DMPO to form a spin adduct. The EPR signal of this alpha-hydroxyethyl radical spin adduct is quite different from that of the

hydroxyl radical spin adduct. The hydroxyl radicals generated by the spontaneous dismutation of superoxide radicals could be scavenged by ethanol giving rise to the carbon-centered spin adduct EPR signal. The results of our study indicate that this was indeed the case. Thus, when ethanol (100 *mM*) was added to the aqueous system, the EPR spectrum exhibited 2 × 3 splitting lines with identical intensity (Fig. 4d) which is a typical carbon-centered radical spin adduct with DMPO. The hyperfine splitting constants for this spin adduct (a_N = 15.8 *G* and a_H = 22.8 *G*) are consistent with previously reported values for DMPO-$CH(OH)CH_3$ in an aqueous system (35, 36). These results indicate that the production of DMPO-OH is the result of trapping of ·OH and is not due to the reduction of DMPO-OOH adduct.

Since H_2O_2 is believed to be formed as an intermediate during the univalent pathway of oxygen reduction, addition of Fe^{2+} to this system should yield ·OH in a Fenton-type reaction ($Fe^{2+} + H_2O_2$ --------> $Fe^{3+} + \cdot OH + OH^-$). As shown in Fig.4c (please note the changes in EPR settings), addition of Fe^{2+} (10 μM) into the mixture of MAO-B and MPTP in the presence of DMPO enhanced the EPR signal intensity of DMPO-OH to about 5-times of control. In this Fe^{2+} catalyzed system, SOD (up to 1.0 $\mu g/ml$) had trivial effect on the DMPO-OH signal intensity (data not shown), indicating that the enhancement of the EPR signal of DMPO-OH by Fe^{2+} is contributed by a Fenton-type reaction. In order to further investigate the mechanism of the formation of ·OH, Catalase, a specific scavenger for H_2O_2, was used in this system. If the formation of ·OH is due to the reduction of H_2O_2, Catalase should remove this reactive species (H_2O_2) at a rate constant of $1.7 \times 10^7 M^{-1}S^{-1}$ (37) and block the formation of hydroxyl radical spin adduct with DMPO. The results shown in Fig. 4e indicates that this was indeed the case. Addition of Catalase (10.0 $\mu g/ml$) completely suppressed the EPR signal of DMPO-OH, indicating that hydrogen peroxide was produced in this system and that the formation of ·OH is indeed *via* the intermediate, H_2O_2. These results further confirm the findings of

Sandri *et al.* (14) who reported the production of H_2O_2 in the reaction of MAO-B with MPTP. It appears that H_2O_2 can be produced in this reaction system by directly reducing O_2 by two electrons and/or by the dismutation of superoxide radicals released by the reduced MAO-B. Nevertheless, a small concentration of H_2O_2 could accumulate during the catalytic action of MAO-B.

Effect of MAO-B Concentration on the Yield of Superoxide radicals:

Since generation of $\cdot O_2^-$ was dependent upon the presence of MPTP, O_2 and MAO-B, the rate of generation of $\cdot O_2^-$, under a given condition and fixed MPTP and O_2 concentration, should depend on the concentration of MAO-B in the system. In order to quantitate the rate of generation of radicals and spin adduct formation by the spin trapping technique (38), the following assumptions were made:

$$\text{MAO-B}_{(red)} + O_2 \xrightarrow{k_g} \cdot O_2^- + \text{MAO-B}_{(ox)} \qquad (1)$$

$$\cdot O_2^- + \text{DMPO} \xrightarrow{k_{DMPO}} \text{DMPO-}O_2^- \qquad (2)$$

where $\text{MAO-B}_{(red)}$ serves as a source of $\cdot O_2^-$, DMPO is the spin trap and k_g is the first order rate constant of superoxide generation during MAO-B catalyzed oxidation of MPTP. The increase in concentration of spin adduct could be expressed as :

$$d[\text{DMPO-}O_2^-]/dt = k_{DMPO}[\text{DMPO}]\cdot[\cdot O_2^-]. \qquad (3)$$

Under steady state-conditions for the $\cdot O_2^-$,

$$d[O_2^{-}\cdot]/dt = 0 = k_g[\text{MAO-B}] - k_{DMPO}[\text{DMPO}]\cdot[\cdot O_2^-]. \qquad (4)$$

Then, $d[\text{DMPO-}O_2^-]/dt = k_g[\text{MAO-B}]$, or $k_g = dV/[\text{MAO-B}]$, (5)

where, dV is the rate of formation of spin adduct.

If the reaction is not a chain event, increasing the concentration of MAO-B should result in the interception of an ever-increasing proportion of the $\cdot O_2^-$ generated, and should therefore yield a saturation curve, in which the plot of the inverse rate of spin adduct

formation against $[MAO\text{-}B]^{-1}$ should give a straight line with a constant slope and intercept. That this was the case is shown in Figure 5, where the rate of accumulation of spin adduct increased as a function of MAO-B concentration and the inverse plot (inset) gave a linear line with a correlation coefficient of 0.999. The maximum velocity of MAO-B converting O_2 to $\cdot O_2^-$ in this system can be obtained from the inverse of the intercept of the plot and was calculated to be 8.0×10^{-9} $M \cdot S^{-1}$. The rate constant (k_g) of superoxide radical generation can be obtained directly from 1/slope of the plot and was found to be 9.75×10^{-5} s^{-1}.

Effect of Superoxide Dismutase on the Rate of Spin Adduct Formation:

As described above (Fig. 3c), superoxide dismutase inhibits the formation of superoxide radicals spin adduct with DMPO. Figure 6 shows the percent inhibition of the spin adduct formation as a function of SOD concentration. As shown in this figure, the generation of the spin adduct was powerfully inhibited by SOD and as little as ~25 *ng*/ml of the enzyme caused 50% inhibition. Superoxide dismutase at ~1.0 *μg/ml* almost eliminated the EPR signals of spin adducts when 80 mM DMPO was used to trap these radicals. Addition of 1.0 μg/ml of denatured superoxide dismutase (obtained by boiling for 30 min) to the reaction mixture had negligible effects on EPR signal intensity indicating that the inhibition by superoxide dismutase was not a non-specific protein effect. These results strongly support the contention that superoxide radicals are generated during the MAO-B catalyzed oxidation of MPTP and will be discussed below.

In order to quantitate the generation of superoxide radicals in this system, we assumed that the rate of inhibition of the generation of spin adduct by SOD is equal to the rate of formation of the spin adduct, when SOD suppressed the EPR signal intensity of the DMPO-superoxide spin adduct by 50%. Thus, according to the methods of Buettner *et al.* (39):

$$k_{sod} \cdot [SOD] \cdot [\cdot O_2^-] = (k_{\cdot O_2^-} \cdot [\cdot O_2^-] + k_{\cdot OOH} \cdot [\cdot OOH]) \cdot [DMPO], \quad (6)$$

where $k_{\cdot O_2^-}$ (10.0 $M^{-1}S^{-1}$) and $k_{\cdot OOH}$ (6.6 ¥ 10^3 $M^{-1}S^{-1}$) are the rate constants for DMPO trapping $\cdot O_2^-$ and ·OOH, respectively.

Since $k_{app} \cdot([\cdot OOH] + [\cdot O_2^-]) = k_{\cdot O_2^-} \cdot [\cdot O_2^-] + k_{\cdot OOH} \cdot [\cdot OOH]$,

then,

$$k_{SOD} \cdot [SOD] \cdot [\cdot O_2^-] = k_{app} \cdot ([\cdot OOH] + [\cdot O_2^-]) \cdot [DMPO]. \quad (7)$$

Dividing equation (7) by [·OOH] yields:

$$k_{SOD} \cdot [SOD] \cdot ([\cdot O_2^-]/[\cdot OOH]) = k_{app} (1 + [\cdot O_2^-]/[\cdot OOH]) \cdot [DMPO]. \quad (8)$$

According to the Henderson-Hasselbalch Equation:

$$\cdot O_2^- + H^+ \overset{pKa = 4.88}{<----------->} \cdot OOH \quad (9)$$

$$\log [\cdot O_2^-]/[\cdot OOH] = pH - pKa \quad (10)$$

From these equations, the $[\cdot O_2^-]/[\cdot OOH]$ and k_{app}, at pH 7.4, can be calculated to be 398 and 26.5 $M^{-1}S^{-1}$, respectively. Since the values of terms, ($[\cdot O_2^-]/[\cdot OOH]$) and ($1 + [\cdot O_2^-]/[\cdot OOH]$) are calculated to be 398 and 399, respectively, the two terms could be considered identical and thus can be omitted. Thus, equation 8 can be written as

$$k_{SOD} = k_{app} \cdot [DMPO]/[SOD] \quad (11)$$

where k_{SOD} is the rate constant of SOD scavenging $\cdot O_2^-$, and k_{app} is the apparent rate constant of DMPO trapping superoxide radicals which is the pH-dependent parameter. Using the value of [SOD] (25 ng/ml) described above and the calculated k_{app} of 26.5 $M^{-1}S^{-1}$, we obtained a rate constant of 3.3×10^9 $M^{-1}S^{-1}$ for SOD scavenging $\cdot O_2^-$ in this aqueous solution (pH 7.4) which is in accord with the published value of 2×10^9 $M^{-1}S^{-1}$ (28, 29).

Because there exists a competition between DMPO and SOD for the available superoxide radicals, the rate constant of SOD in this system can be obtained by using a previously reported method (40). Under steady-state conditions,

$$\Phi_o/\Phi - 1 = k_{sod} \cdot [SOD]/k_{app} \cdot [DMPO] \quad (12)$$

where Φ_o/Φ represent the ratio of the rates of spin trapping (or the EPR signal intensity of spin adduct) in the absence and presence of SOD; k_{sod} is the rate constant for SOD scavenging $\cdot O_2^-$; and k_{app} is a pH-dependent apparent rate constant which includes the contribution of both $\cdot O_2^-$ and ·OOH trapping. From this equation it follows that

$$ksod = \frac{\Phi_o/\Phi - 1}{[SOD]} \cdot k_{app} \cdot [DMPO]. \quad (13)$$

Under a given condition, item k_{app}·[DMPO] is constant and a plot of (Φ_o/Φ -1) against [SOD] should give a straight line. That this was the case is shown in Fig. 6 inset, where a simple Stern-Volmer behavior for scavenging of $\cdot O_2^-$ by SOD was observed with a correlation coefficient of 0.99. The ratio of (Φ_o/Φ -1) to [SOD] can be directly obtained from the slope of this plot and was found to be 2.629×10^9 M^{-1}. The constant item k_{app}·[DMPO], was calculated to be 2.12 sec^{-1}, using the apparent rate constant k_{app}, for DMPO trapping superoxide radicals of 26.5 $M^{-1}S^{-1}$ at pH 7.4. The rate constant, *ksod*, for SOD scavenging was found to be 5.57×10^9 $M^{-1}S^{-1}$ in our system at pH 7.4, which is close to our above value (3.3×10^9 $M^{-1}S^{-1}$) obtained by the method described by Buettner *et al.* (39).

DISCUSSION

The present studies provide strong evidence for the production of the reactive oxygen species ($\cdot O_2^-$, H_2O_2 and ·OH) during MAO-B catalyzed oxidation of MPTP. Superoxide radicals were detected as a primary reactive species, H_2O_2 as an intermediate

and ·OH as a secondary radical species produced in MAO-B dependent oxidation of MPTP. These informations are further discussed below.

Oxidation of MPTP is MAO-B Dependent:

Although MAO-B catalyzed oxidation of MPTP has been studied, most of the work in this area has involved under high concentrations of MPTP, which has led to severe inactivation of MAO-B (39, 40). Since inactivation of MAO-B increases with increasing MPTP concentration (23, 42), low concentrations of MPTP are required to study the catalytic activity of MAO-B. The present studies were therefore performed at low MPTP concentrations, in which loss of MPTP and the formation of $MPDP^+$ were found to be incubation-time dependent. No decrease of MPTP and formation of $MPDP^+$ were observed in the absence of MAO-B in the reaction mixture (Fig. 1). It is evident that the oxidation of MPTP is MAO-B dependent. Additional support for this conclusion comes from kinetic studies indicating that the oxidation of MPTP by a fixed concentration of MAO-B to yield $MPDP^+$ is dependent upon the initial concentration of MPTP (Fig. 2). The kinetic parameters are well in accord with previously reported values for MAO-B catalyzed oxidation of benzylamine and MPTP. These results further confirm that the oxidation of MPTP is indeed MAO-B dependent under low concentrations of MPTP.

Generation of Superoxide Radicals As A Primary Reactive Species:

The results reported here clearly demonstrate for the first time that superoxide radicals are produced during MAO-B catalyzed oxidation of MPTP. The characteristic EPR spectrum of the superoxide radical spin adduct with DMPO as spin trap, exhibiting $3 \times 2 \times 2$ lines (Fig. 3b), was observed when MPTP and MAO-B were incubated with DMPO in a buffered-DMSO solution. The magnetic parameters (a_N =13.3 *G*, $^{\beta}a_H$ =10.5 *G* and $^{\gamma}a_H$ =1.3 *G*) for this EPR spectrum are similar to those previously reported values for DMPO-O_2^- (25, 26), indicating that $\cdot O_2^-$ spin adduct with DMPO is responsible for the EPR

signal. This view was further confirmed by the following observations: (a) superoxide dismutase, a specific and efficient scavenger of superoxide radical ($k = 2 \times 10^9$ $M^{-1}S^{-1}$; Refs.28, 29), was found to virtually eliminate the EPR signal (Figs. 3c and 6), and (b) the second order rate constant for superoxide dismutase scavenging $\cdot O_2^-$ was calculated to be 3.3×10^9 $M^{-1}S^{-1}$, which closely approximates the values reported by other investigators (28, 29). Therefore, all the above results provide confidence that the primary radical produced during MAO-B catalyzed oxidation of MPTP was indeed $\cdot O_2^-$, and that the intensity of the EPR signal of the spin adduct can be utilized to estimate the relative amounts of $\cdot O_2^-$ generated in MAO catalyzed reaction system.

The generation of $\cdot O_2^-$ was quantitatively determined by a kinetic analysis technique. Here the reaction catalyzed by MAO-B was the source of $\cdot O_2^-$, and DMPO at saturated concentrations effectively trapped these radicals. Under these conditions the maximum rate of $\cdot O_2^-$ produced in this system was found to be 8.0×10^{-9} $M \cdot Sec^{-1}$ and the first order rate constant (k_g) for $\cdot O_2^-$ generated in the system was found to be 9.75×10^{-4} Sec^{-1}.

Production of Hydrogen Peroxide as an intermediate:

Sandri *et al.* (14) have fluorometrically detected the production of H_2O_2 in MAO-B catalyzed oxidation of MPTP by the scopoletin and horseradish peroxidase method. Here, we further confirmed the production of H_2O_2 in this enzymatic reaction by EPR in combination with spin trapping techniques. Since $\cdot O_2^-$ is the precursor of H_2O_2 and can spontaneously dismutate to H_2O_2 at the rate constant of $7.6\text{-}8.5 \times 10^7$ $M^{-1}S^{-1}$ (43) and the rate of breakdown of H_2O_2 to $\cdot OH$ by Haber-Weiss reaction is only 0.13-0.5 $M^{-1}S^{-1}$ (44, 45, 46), a small but detectable concentration of H_2O_2 may accumulate under certain conditions. Ferrous iron can rapidly reduce H_2O_2 by Fenton-type reaction to form $\cdot OH$ which in turn can be trapped by DMPO at a rate constant of 3.4×10^9 $M^{-1}S^{-1}$ (27) to form

DMPO-OH adduct. Therefore, the EPR signal intensity of DMPO-OH spin adduct should be enhanced by the addition of Fe^{2+} to the reaction mixture. As shown in figure 4c, as little as 10 μM of Fe^{2+} enhanced the EPR signal to about 5-times of control, while SOD (1.0 μg/ml) had trivial effect on the DMPO-OH signal, confirming that the enhancement of the EPR signal is due to the Fenton-type reaction. The demonstration that Catalase (at 10.0 μg/ml), a specific scavenger for H_2O_2 at a rate constant of 1.7×10^7 $M^{-1}S^{-1}$ (37), virtually eliminated the signal further confirms that H_2O_2 was produced in MAO-B catalyzed oxidation of MPTP.

Formation of Hydroxyl Radical by Iron Catalyzed Haber-Weiss Reaction:

In air-saturated aqueous solutions, the MAO-B catalyzed oxidation of MPTP gave rise to free radicals which, upon reacting with DMPO, gave a spin adduct whose EPR spectrum exhibited four splitting lines in an intensity ratio of 1:2:2:1 (Fig. 4a) with hyperfine splitting constants of $a_N = a_H = 15.0$ G, consistent with DMPO-OH adduct characteristics (31, 32). Ethanol, a scavenger of hydroxyl radical with a rate constant of 1×10^9 $M^{-1}S^{-1}$ (34) to produce an alpha-hydroxyethyl (CH_3·CH-OH) radical, was readily trapped by DMPO to form DMPO-CH(OH)CH_3 spin adduct, further confirming the formation of ·OH in aqueous system. Since 50 ng/ml superoxide dismutase was effective in inhibiting this signal to about 30% of control rate, we believe that the primary species in aqueous solution manifested as DMPO-OH adduct is $\cdot O_2^-$. This belief is based on the anticipated competition for $\cdot O_2^-$ between SOD and the Haber-Weiss reaction. SOD scavenges $\cdot O_2^-$ to form H_2O_2 at a rate constant of 2×10^9 $M^{-1}S^{-1}$ whereas the rate constant for the Haber-Weiss reaction ($H_2O_2 + \cdot O_2^- \rightarrow \cdot OH + O_2 + OH^-$ or $H_2O_2 + \cdot OOH \rightarrow \cdot OH + O_2 + H_2O$) is only 0.13 to 0.5 $M^{-1}S^{-1}$ (44, 45, 46). It appears that the capacity of SOD competition for $\cdot O_2^-$ is 4×10^9 times faster than the Haber-Weiss reaction. Therefore the $\cdot O_2^-$ generated in MAO-B catalyzed processes will be scavenged by SOD to quickly

produce H_2O_2, blocking the pathway of generation of ·OH by the Haber-Weiss reaction. However, in the presence of Fe^{2+} in this system, SOD had only a trivial effect on the DMPO-OH signal, indicating that SOD cannot effectively compete with the Fenton-type reaction. It does establish however that hydroxyl radicals are produced by both Haber-Weiss and iron catalyzed reactions following the generation of primary ($\cdot O_2^-$) and intermediate (H_2O_2) species during MAO-B catalyzed oxidation of MPTP. Since neurons are rich in iron (47), the Fenton reaction should predominate in generating the most potent oxidant, ·OH, which in turn could destroy these cells when exposed to MPTP.

Overall Mechanism For the Formation of Reactive Oxygen Species:

Although oxidative pathways for MPTP catalysis by MAO-B have been previously proposed (13, 48, 49), no direct evidence was available for the generation of the oxyradicals during this enzymatic process. We report here the generation of reactive oxygen species which include superoxide radicals, hydrogen peroxide and hydroxyl radical during the MAO-B catalyzed oxidation of MPTP. The mechanism(s) of generation of these reactive oxygen species is presented in Scheme I where, (a) after an electron transfer, MPTP forms compound **B** which has two possible routes to form $MPDP^+$. In the first route, the carbon-centered radical species **C** could be formed after a proton abstraction from compound **B**. Loss of a second electron from the carbon centered radical would generate $MPDP^+$. In the second route, compound **B** loses a hydrogen atom to directly form $MPDP^+$. Alternately, using pathway (b), MPTP transfers a hydrogen atom to MAO-B and forms the carbon centered radical **C**. The carbon centered radical, so generated in both pathways (a) and (b), could be the electron donor which transfers an electron to oxygen to form superoxide radicals. Concomitant with MPTP oxidation, MAO-B bound oxidized flavin coenzyme is converted to enzyme-bound reduced flavin, an anion (60), which is reoxidized by oxygen to regenerate the free enzyme and superoxide radicals. It should be

made clear that these pathways suggest several different possibilities for the formation of superoxide and that possibly only one of these is actually relevant. Although we have not done the study on the kinetic mechanism of the enzymatic reaction, from the above scheme we could propose that MAO-B catalyzed oxidation of MPTP may obey a ping-pong kinetic mechanism (scheme I. c), in which MPTP binds to a reactive site of MAO-B to form a complex; after transferring an electron or a hydrogen atom, the complex converts to a MPTP reactive intermediate and a reduced form of the enzyme. This enzyme form, in which the flavin component of the enzyme is reduced, then interacts with O_2 to give a complex, which breaks down to yield oxidized enzyme and superoxide radicals.

Superoxide radicals, so generated, then can be converted to form the other reactive species through the following reaction scheme (43, 44, 45, 46):

$$\cdot O_2^- + \cdot O_2^- \xrightarrow[+2H_2O]{k< 100\ M^{-1}S^{-1}} H_2O_2 + O_2 + 2OH^- \quad (14)$$

$$\cdot OOH + \cdot O_2^- \xrightarrow[+H_2O]{k = 8.5 \times 10^7\ M^{-1}S^{-1}} H_2O_2 + O_2 + OH^- \quad (15)$$

$$\cdot OOH + \cdot OOH \xrightarrow{k = 7.6 \times 10^7\ M^{-1}S^{-1}} H_2O_2 + O_2 \quad (16)$$

$$H_2O_2 + \cdot O_2^- \xrightarrow{k = 0.13\ M^{-1}S^{-1}} \cdot OH + O_2 + OH^- \quad (17)$$

$$H_2O_2 + \cdot OOH \xrightarrow{k = 0.5\ M^{-1}S^{-1}} \cdot OH + O_2 + H_2O \quad (18)$$

In the presence of ferrous iron, eqs. 15 and 16 will be replaced by a Fenton type reaction:

$$H_2O_2 + Fe^{2+} \longrightarrow \cdot OH + OH^- + Fe^{3+} \quad (19)$$

Since an increase in MAO-B activity and a lower level of dopamine (DA) (22) were found in the substantial nigra of patients with severe Parkinsonism, investigations in the past seem to have focused on the irreversible inhibition of MAO-B. Recent studies have shown that selective irreversible MAO-inhibitors, like L-deprenyl, result in mild to

moderate improvement in Parkinson's disease disability. L-deprenyl in combination with L-dopa therapy prolongs the life span of Parkinson's disease patients compared to L-dopa alone. However, the mechanism remains unclear by which MPTP, an irreversible inactivator of the MAO-B (41, 42), induces Parkinson's disease. It is known that the inactivation of MAO-B increases with increasing MPTP concentration (23) and MPTP, at low concentration, is a good substrate of MAO-B (50). Since reactive species of O_2 are shown to be produced by MAO-B catalyzing low concentrations of MPTP, it strongly implicates the generation of the reactive intermediates in the neuronal injury. Although MAO-B rich glial cells have no anatomical contacts with neurons (51, 52) and MPTP metabolism has been proposed to occur predominantly in glial cells, it is possible that superoxide radicals are produced in glial cells and because of their longer $t_{1/2}$ can then migrate to adjacent neuron cells. It is, however, possible that MPTP could enter the nigrostriatal cells, where MAO-B could catalyze the oxidation of MPTP to produce the reactive oxygen species ($\cdot O_2^-$, $\cdot OOH$, H_2O_2 and $\cdot OH$) and the final stable species, MPP^+. From kinetic considerations, SOD should rapidly catalyze $\cdot O_2^-$ to form H_2O_2 which, in turn, can be metabolized by GSH peroxidase to form H_2O. Although GSH peroxidase has a lower K_m (1-10 μM) for H_2O_2 (53) than Catalase (37), cells may avoid GSH depletion and GSSG accumulation by depending more on Catalase during increased rates of cellular H_2O_2 production (54). However, unlike other cells, brain cells possess relatively poor Catalase as well as peroxidase activities, and high monoamine oxidase activity for neurotransmitter metabolism (55-58). Under the normal condition, the generation of the reactive oxygen species can be controlled under a lower level ($< 8 \times 10^{-12}$ M) (59) by the cellular defense system. However, once MPTP enters the neurons, the rate of production of the reactive species could be greatly enhanced which, in turn, could overwhelm the defense system causing neuron cell dysfunction and possibly cell death.

The results of this investigation force us to consider the potential biological effects of MAO-B catalyzed oxidation of MPTP in a new light. Thus, MAO-B catalyzed oxidation of MPTP not only generates reactive oxygen species ($\cdot O_2^-$, $\cdot OOH$, H_2O_2 and $\cdot OH$), but also may yield MPTP-related radicals and possibly other, as yet undetected, reactive intermediate(s). It is, therefore, tempting to attribute some of the pathological effects of MPTP-induced neurotoxicity to these reactive intermediates. Although MPTP was shown to be a good substrate of the MAO-B in glial cells, it may also readily cross the cell membrane and reach the nigrostriatal cells, where it might be metabolized by MAO-B to generate superoxide radical and the final stable species, MPP^+. The superoxide radical so generated can be protonated to form a much more reactive species, the $\cdot OOH$, or react with cellular iron to form $\cdot OH$. All of these reactive species are known to cause cell injury and cell dysfunction, and may be associated with the neurodegenerative process of MPTP.

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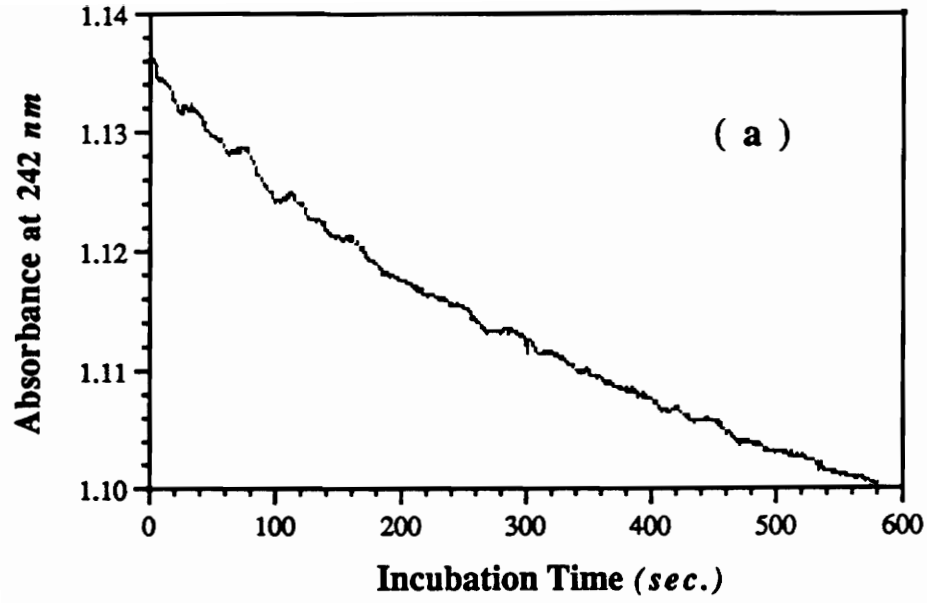
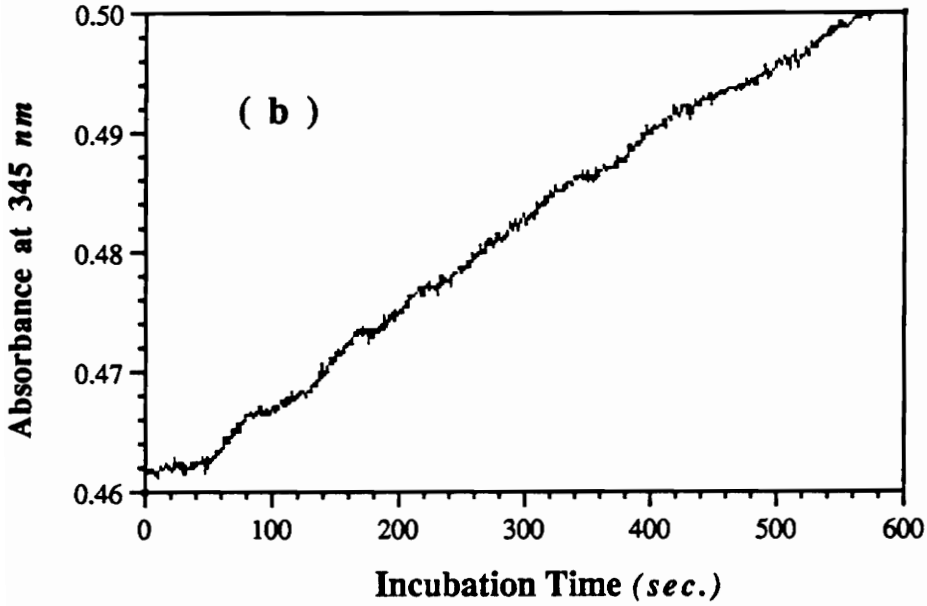
Table 1. Comparison of some kinetic parameters of MAO:

Substrate	K_m (mM)	V_{max} (μM/min)	Ref.
MPTP	0.194	0.335	*
	0.200	---	(22)
	0.390	---	(23)
	0.480	0.0182	(18)
	0.300	---	(21)
Benzylamine	0.053	5.08	*
	0.390	---	(23)

Note: * our results.


(a)
Absorbance at 242 nm
1.14
1.13
1.12
1.11
1.10
0
100
200
300
400
500
600
Incubation Time (sec.)


(b)
Absorbance at 345 nm
0.50
0.49
0.48
0.47
0.46
0
100
200
300
400
500
600
Incubation Time (sec.)

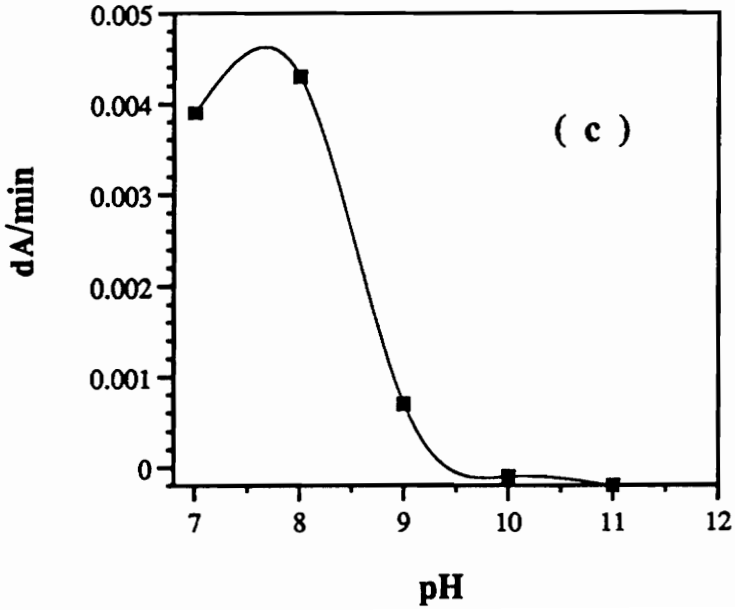

(c)
dA/min
0.005
0.004
0.003
0.002
0.001
0
7
8
9
10
11
12
pH

Figure 1. Kinetics of MPTP oxidation catalyzed by MAO-B. (a) time course of loss of MPTP, monitored at 242 *nm*; (b) formation of $MPDP^+$ at 345 *nm*; and (c) the effect of pH on the rate of formation of $MPDP^+$, measured in terms of increased rate of $MPDP^+$ absorbance at 345 *nm* up to 10 minuts incubation time. The reaction mixture consisted of 50 μM MPTP, MAO-B (48.8 μg protein/ml) in the phosphate buffered solution, pH 7.4 and incubated at 37 °C.

Fig. 2.

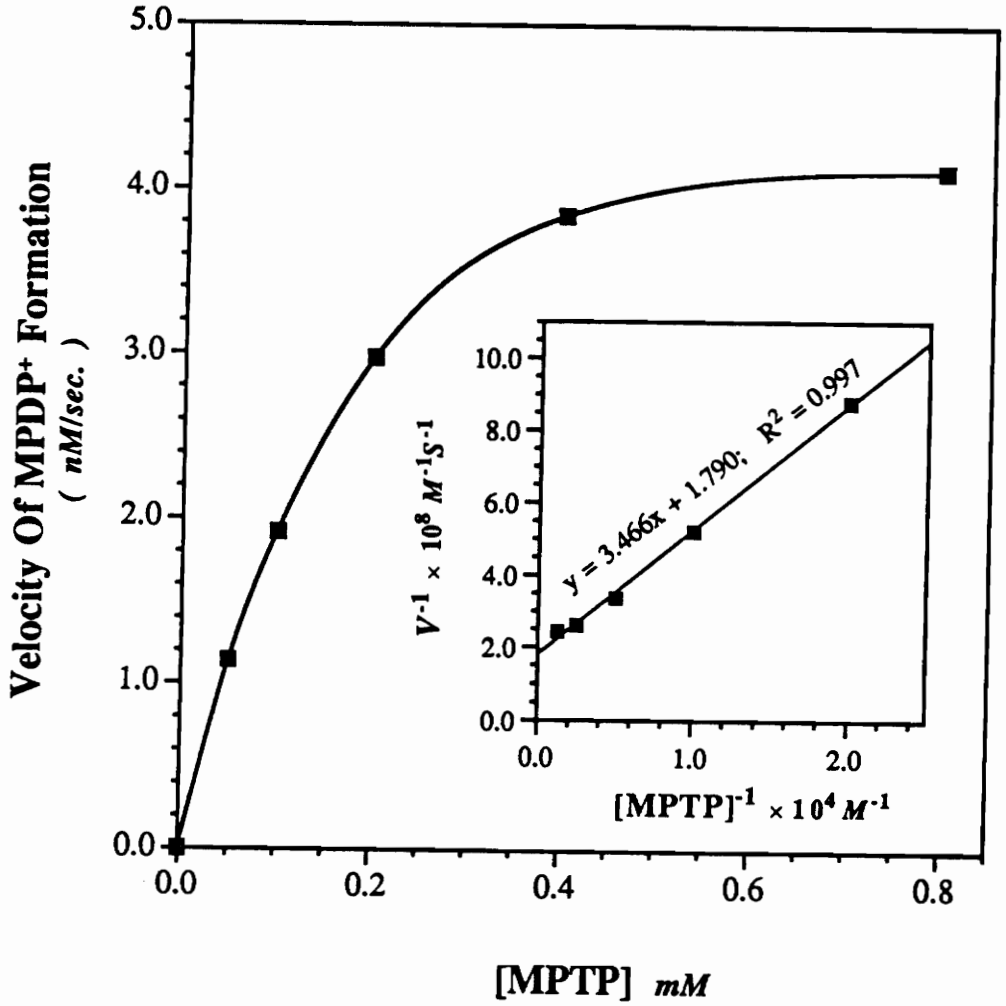


Figure 2. Effect of MPTP concentration on the rate of $MPDP^+$ formation at a fixed MAO-B concentration. The reaction mixture contained MAO-B (48.8 μg protein/ml) and the indicated amounts of MPTP in the phosphate buffer, pH 7.4 at 37 °C. Inset, the double reciprocal plot of velocity of $MPDP^+$ formation as a function of [MPTP].

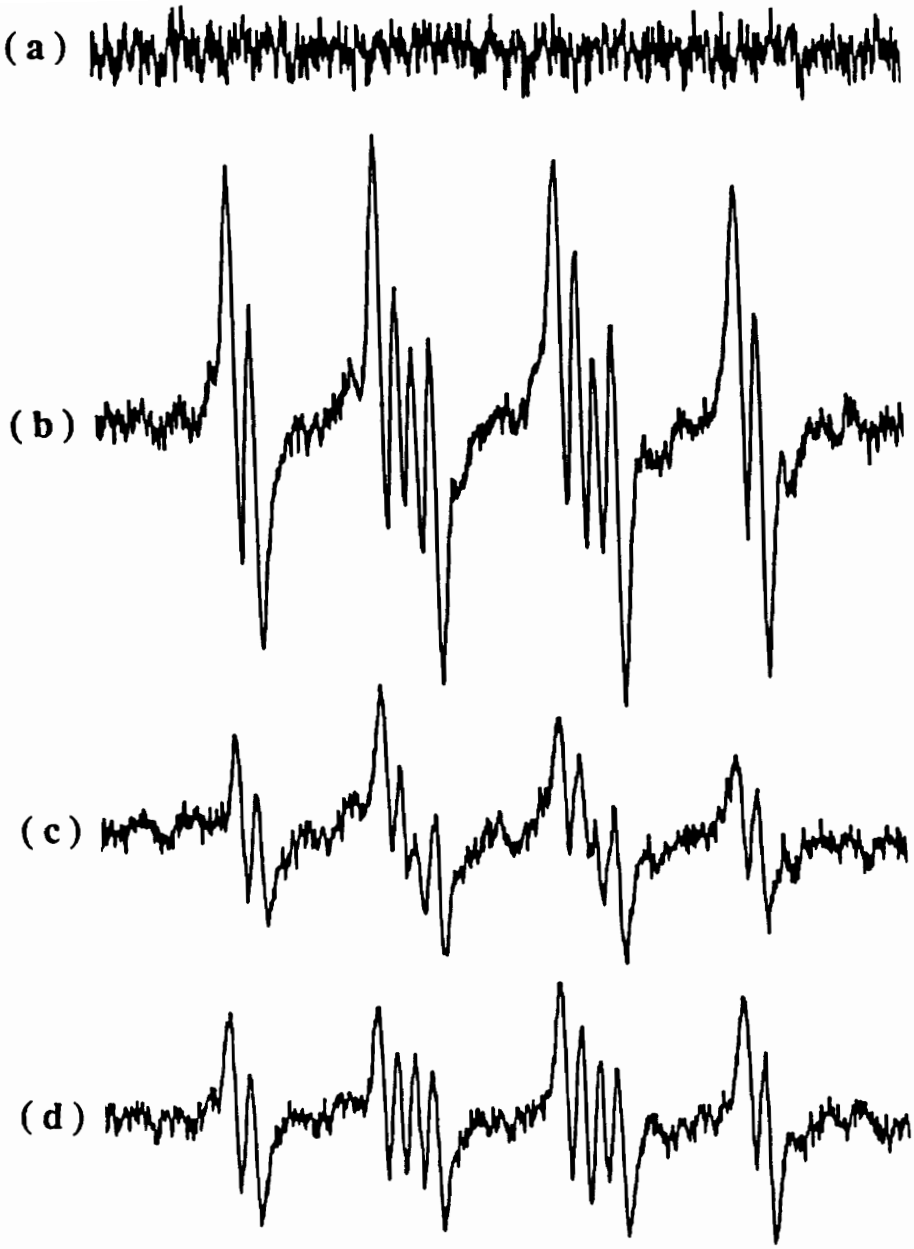


Figure 3. EPR spectra of spin adducts of superoxide radicals observed during MAO-B catalyzed oxidation of MPTP. The reaction mixtures consisted of MPTP (100 μM), DMPO (80 *m*M) and MAO-B (48.8 μg protein/ml) in air-saturated DMSO solution (containing 20% buffered aqueous solution of 50 mM phosphate buffer, pH 7.4). The reaction mixtures: (a) bubbled with N_2, or in the absence of either DMPO, MAO-B or MPTP in air-saturated DMSO solution incubated for 10 mins; (b) MPTP, DMPO and MAO-B incubated for 6 minutes; (c) similar conditions as "b " but in the presence of 25 *ng/ml* SOD; (d) similar conditions as "b " but in the presence of 10 μM L-Deprenyl. EPR spectroscopy settings were the same as described under "Materials and Methods", except that (a) modulation amplitude, 2.0 *G*, time constant, 0.32 sec; and receiver gain to 1.0×10^6; (b) the time constant, 0.32 Sec.; modulation amplitude, 0.8 G; receiver gain, 5×10^5; (c) and (d) modulation amplitude, 0.63 G.

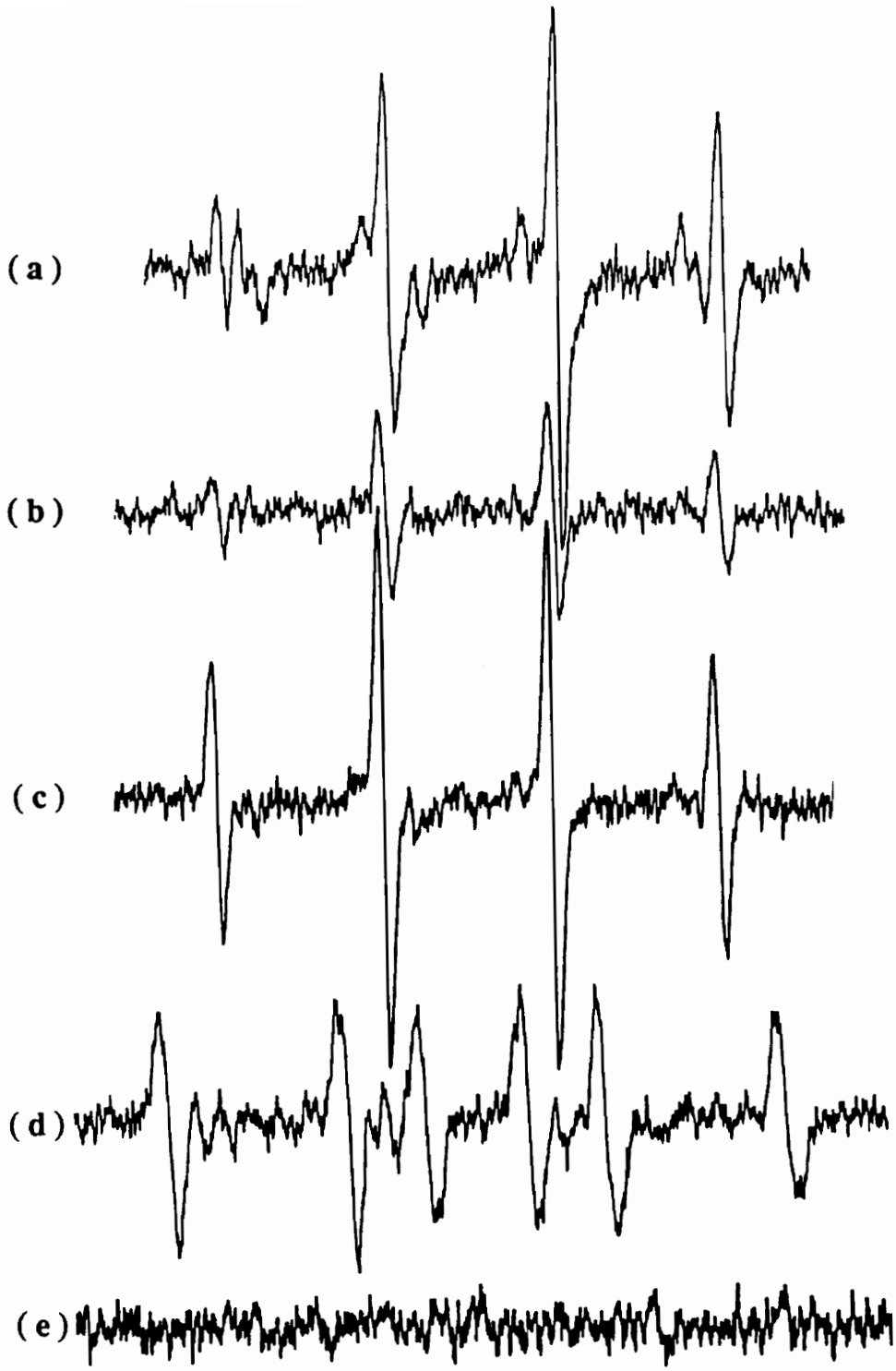


Figure 4. EPR spectra of spin adducts of free radicals observed during MAO-B catalyzed oxidation of MPTP. The reaction mixtures consisted of MAO-B (48.8 μg protein/*m*l), MPTP (100 μM) and DMPO (80 *m*M) in air-saturated aqueous solution of 50 mM phosphate buffer, pH 7.4, and were incubated for 4.5 minuts at 37 °C. (a) MPTP, DMPO and MAO-B; (b), (c), (d) and (e) conditions similar to "a" but in the presence of SOD (50 *ng/ml*) for (b); Fe^{2+} (10 μM) for (c); ethanol for (d); and catalase (10 μg) for (e). EPR spectroscopy settings were: modulation amplitude, 2.0 G; time constant, 0.64 sec; and receiver gain to 1.0×10^6 for (a), (b), (d) and (e); Please note the change of EPR setting: (c) modulation amplitude, 0.8 G; time constant, 0.32 sec; and receiver gain to 5.0×10^5.

Fig. 5

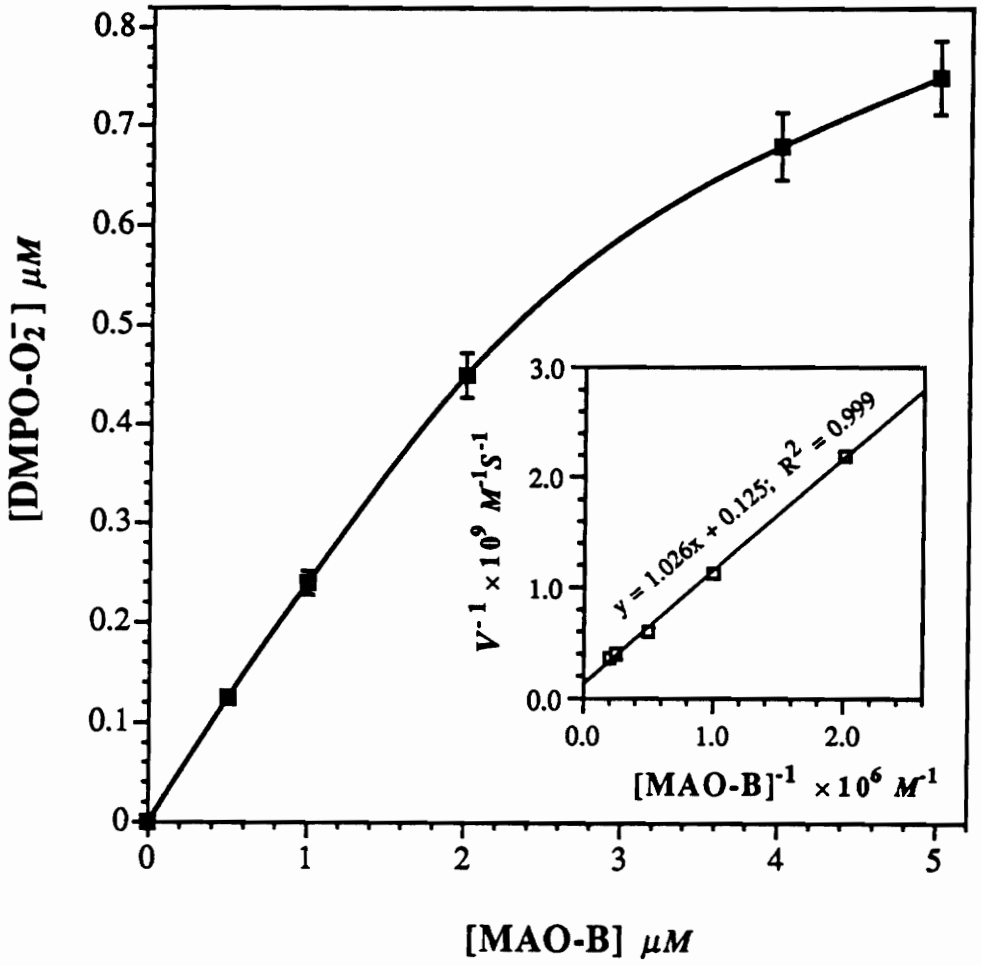


Figure 5. The rate of spin-adduct formation as a function of MAO-B concentration in air-saturated DMSO solution (containing 20% buffered aqueous solution of 50 mM phosphate buffer, pH 7.4). The reaction mixtures contained 80 mM DMPO and the indicated amounts of MAO-B in the presence of 100 μM MPTP. Inset: the inverse plot of the rate of spin adduct formation *vs* MAO-B concentration. EPR spectroscopy was set at: microwave power, 20 mW; modulation amplitude, 1.0 *G*; time constant, 0.64 Sec.; receiver gain, 1×10^6; and scan time, 500 Sec.

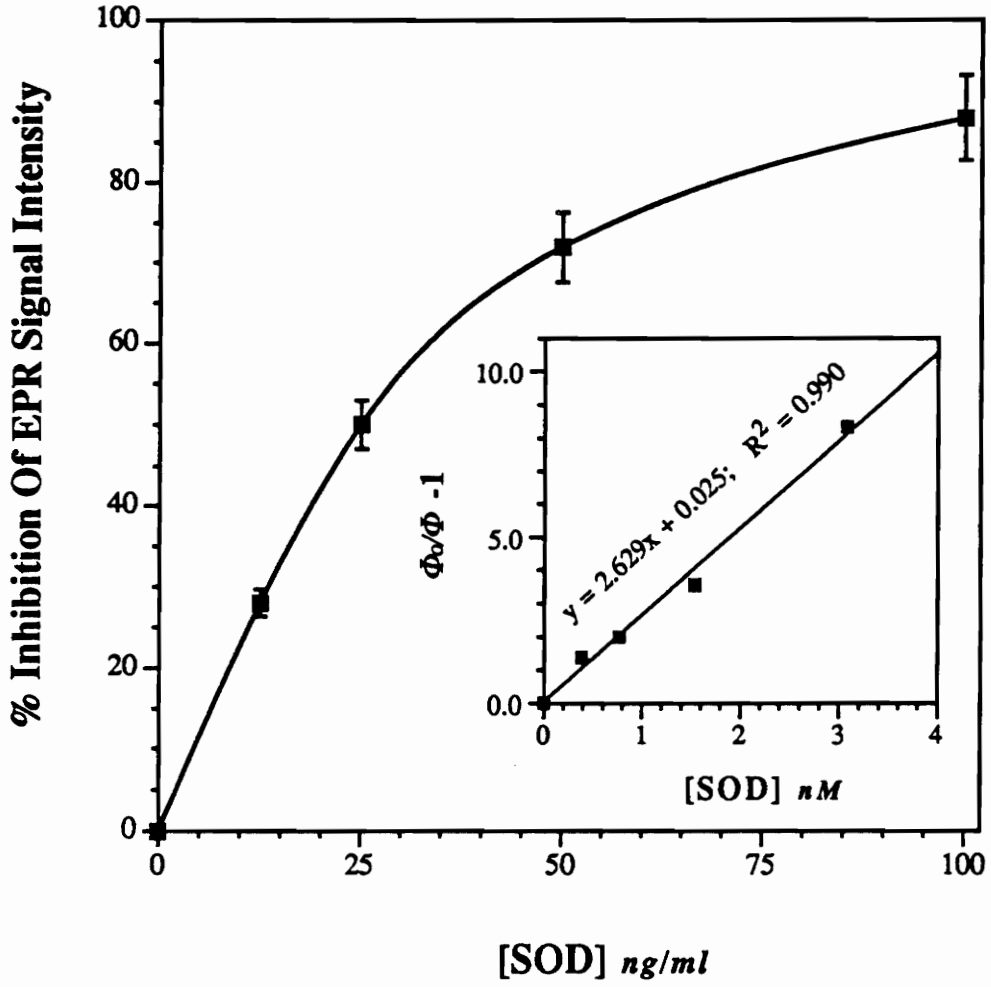
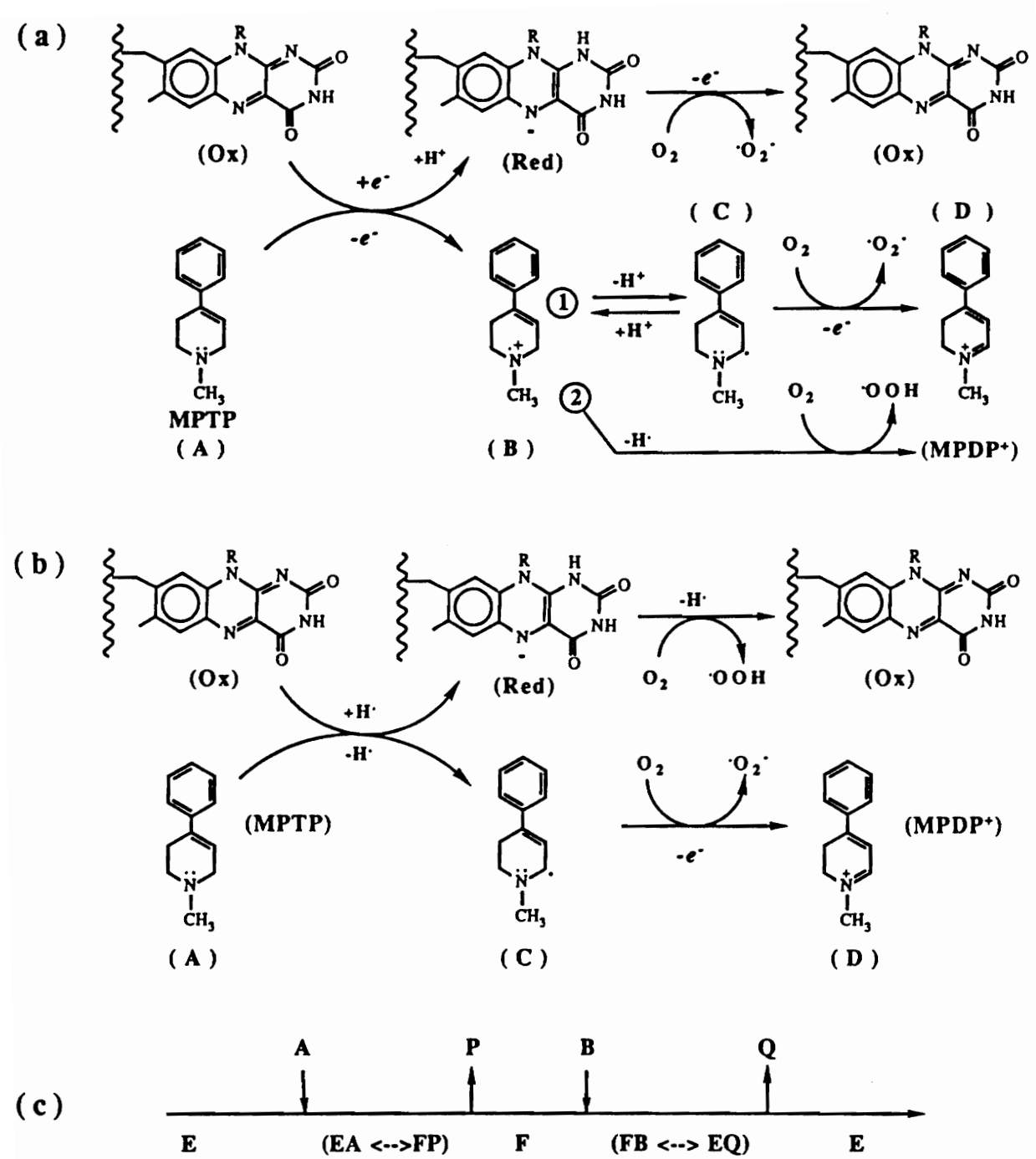


Figure 6. Inhibition of EPR spin adduct signal intensity by superoxide dismutase in air-saturated DMSO solution (containing 20% buffered aqueous solution of 50 *m*M phosphate buffer, pH 7.4). The reaction mixture contained 100 μM MPTP, MAO-B (48.8 μg protein/ml), 80 *m*M DMPO and indicated concentrations of SOD. Inset: *Sterm-Volmer* plot. TheΦ_o/Φ is the ratio of the rates of increase of EPR signal intensity in the absence and presence of SOD. EPR spectroscopy was set at: microwave power, 20 mW; modulation amplitude, 2.0 *G*; time constant, 0.64 Sec.; receiver gain, 1×10^6; and scan time, 500 Sec.



Scheme I. **Proposed mechanisms for the generation of superoxide radicals during MAO-B catalyzed oxidation of MPTP.**

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April 16, 1993 M3-0678

Dr. Hara F. Misra
Dept.of Biomedical Sciences
Virginia Tech. & State University
Phase II Duckpong Drive
Blacksburg, VA 24061-0442

Dear Dr. Misra:

Your manuscript: "*Generation of reactive oxygen species during the monoamine oxidase-catalyzed oxidation of the neurotoxicant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine*" has been accepted for publication and is tentatively scheduled for a July/August issue. You will receive both galley and page proof; both should be returned promptly to expedite publication. You will be billed after publication for page charges, reprints you order, half-tones, electron micrographs and authors' alterations as applicable.

Sincerely,

Alton Meister

Alton Meister
Associate Editor

AM:ehp

I

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Referee's Comments

MS #3-0678 Date: 3/15/93
Authors: Misra et al.

Title: "Generation of Reactive Oxygen Species During the Monoamine Oxidase-catalyzed Oxidation of the Neurotoxin, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine."

This manuscript provides kinetic analysis of the production of superoxide and hydroxyl radicals upon metabolism of MPTP by monoamine oxidase (MAO-B). Evidence, derived mostly by spin-trapping of intermediates, is presented that in the course of the MAO-B catalyzed reaction, a variety of reactive oxygen species are produced: these include hydrogen peroxide in addition to the compounds mentioned above. The authors suggest that these highly reactive species underlie the toxicity of MPTP, giving rise to Parkinson's-like symptoms in intoxicated individuals. The experimental data supporting the existence of these intermediates appears solid, and while portions of the discussion (notably the last page) are clearly speculative, this paper makes a solid contribution and is worthy of publication in the J. Biol. Chem. Its presentation would be improved, however, by being edited into more grammatical English, and the authors should be given the opportunity to do this.

CHAPTER V

INACTIVATION OF ACETYLCHOLINESTERASE BY THE NEUROTOXICANT, 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE HYDROCHLORIDE

(Submitted to *J. Biol. Chem.* in April, 1993)

Lun-Yi Zang and Hara P. Misra

Abstract

The neurotoxicant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been shown to reversibly inhibit the activity of acetylcholinesterase. The inactivation of the enzyme was detected by monitoring the accumulation of yellow color produced from the reaction between thiocholine and dithiobisnitrobenzoate ion. The kinetic parameter, K_m for the substrate (acetylthiocholine), was found to be 0.216 *m*M and K_i for MPTP to inactivation of acetylcholinesterase was found to be 2.14 *m*M. The inactivation of enzyme by MPTP was found to be dose-dependent. It was found that MPTP is neither a substrate of AChE nor the time-dependent inactivator. The studies of reaction kinetics indicate the inactivation of AChE to be a linear mixed-type inhibition. The dilution assays indicate that MPTP is a reversible inhibitor for AChE. These data suggest that once MPTP enters the basal ganglia of the brain, it can inactivate the acetylcholinesterase enzyme and thereby increase the acetylcholine level in the basal ganglia of brain, leading to potential cell dysfunction. It appears likely that the nigrostriatal toxicity by MPTP leading to Parkinson's disease-like syndrome may, in part, be mediated *via* the acetylcholinesterase inactivation.

INTRODUCTION

Self-administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) manifests the irreversible symptoms of Parkinson's disease in several young drug abusers (1). It was found that this neurotoxicant selectively destroys neuronal cells in the substantia nigra of humans and other primates (2). Although the mechanism of action of MPTP is not fully understood, it is generally believed that the crucial species for MPTP neurotoxicity is not MPTP itself, but rather some of its metabolites (3,4). In studies of MPTP-metabolism, it has been demonstrated that monoamine oxidase-B (MAO-B) catalyzes the two-electron oxidation of MPTP to a dihydropyridinium intermediate ($MPDP^+$), which in turn spontaneously oxidizes to form methyl phenylpyridinium (MPP^+) or disproportionate to form MPTP and MPP^+ (5-7). Since an increase in MAO-B activity and lower levels of dopamine (DA) (8) were found in the substantia nigra of patients with severe Parkinsonism, the investigations in the past seem to be have focused on the irreversible inhibition of MAO-B (9). It was found that selective irreversible MAO-inhibitors, like L-deprenyl, improve Parkinson's disease disability in mild to moderate degree, and L-deprenyl in combination with L-dopa therapy prolongs the life span of Parkinson's disease patients compared to L-dopa alone. However, the mechanism by which MPTP, which is known to be an irreversible inactivator of the MAO-B (10, 11), induces Parkinson's disease remains unclear.

Parkinson's disease has been recognized to be the most common of the basal-ganglion disorders (12). The basal ganglia includes the caudate-putamen and the globus pallidus. Closely associated with the basal ganglia are the substantia nigra and the subthalamus. The caudate-putamen contains a number of neurotransmitter substances including norepinephrine (NE), serotonin (5-HT), glutamate (GLu), gamma-aminobutyric acid (GABA), DA and acetylcholine (ACh). Normal function of the caudate-putamen

depends on the balance of these transmitters, and particularly on the balance of DA and ACh (12). DA has been described as the "**go**" system and ACh is the "**no go**" system (12). Thus, an excess of DA produces an excess of movement while an excess of ACh produces immobility. Acetylcholine can be rapidly broken down by the enzyme acetylcholinesterase (AChE), which occurs in high concentration on both pre- and post-ganglion membranes within autonomic ganglia and on the membranes of parasympathetic nerve terminals where acetylcholine also functions as a neurotransmitter (13, 14). If this enzyme system is destroyed by a neurotoxicant, the accumulation of an excess amount of acetylcholine will result, not only in immobility, but also may lead to neuronal cell injury (15-18). It is evident that AChE, like MAO-B, should play an important role in toxin-induced Parkinson's disease. To date, the drugs used in the treatment of Parkinson's disease act to correct the DA/ACh imbalance. Raising DA levels and inhibiting MAO enzyme activity seem to be the major therapeutic intervention for Parkinson's disease disorder today. Although the inactivation of MAO-B by MPTP has been studied quite extensively, no information has as yet been reported to relate inactivation of acetylcholinesterase by MPTP. The present report describes such measurements for the inactivation of the acetylcholinesterase by MPTP.

MATERIALS and METHODS

Acetylcholinesterase (EC 3.1.1.7) type III (from electric eel), Acetylthiocholine iodide (ACh) and 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma Chemical Company, St. Louis, MO. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP) was purchased from Research Biochemicals, Inc. Natick, MA.

Sephadex G-25 was obtained from Pharmacia Fine Chemicals, Division of Pharmacia Inc., Piscataway, New Jersey. All other chemicals were obtained at the highest obtainable purity.

Measurement of the Enzyme Activity:

The activity of acetylcholinesterase (AChE) was measured by following the accumulation of yellow color produced from thiocholine when it reacts with dithiobisnitrobenzoate ion (19). The assay is based upon the coupling of the following reactions:

$$(CH_3)_3N^+CH_2CH_2SCOCH_3 + H_2O \xrightarrow{AChE} (CH_3)_3N^+CH_2CH_2S^- + CH_3COO^- + 2H^+$$

$$(CH_3)_3N^+CH_2CH_2S^- + RSSR \longrightarrow (CH_3)_3N^+CH_2CH_2SSR + RS^-$$

where RSSR is 5,5'-dithiobis(2-nitrobenzoate ion) and RS^- is the yellow anion of 5-thio-2-nitro-benzoic acid. Spectrophotometric assays were performed in a Shimadzu UV-visible recording spectrophotometer UV-160 at 25 °C. The extent of inactivation of acetylcholinesterase by MPTP was measured with the use of DTNB as an indicator. The rate of the yellow anion accumulation was measured at 405 nm using $\varepsilon_{405\,nm} = 1.36 \times 10^4$ $M^{-1}cm^{-1}$ (20).

Unless otherwise stated, the enzyme activity was monitored following the sequential addition of 20 μl inactivator stock solutions (different concentration), 0.5 *m*l acetylcholinesterase (0.5 unit/ml) and incubated for 5 minutes at 25 °C. The reaction was started by adding 2.5 *m*l mixture of acetylthiocholine iodide (75 *m*M) and DTNB (0.01 M) solutions. The rate of accumulation of yellow RS^- was monitored immediately for 2 min against a blank which consists of all components except acetylcholinesterase. The acetylcholinesterase stock (0.5 unit/*m*l) was prepared in 0.005% Gelatin distilled water solution; DTNB (0.01 M) was made in 10 *m*l 0.1 *M* phosphate buffer (pH 7.0) containing

15 *m*g $NaHCO_3$; and acetylthiocholine iodide (75 *m*M) was prepared in distilled water. The assay mixture was made by mixing 40 μl acetylthiocholine, 100 μl DTNB stocks, 0.5 *m*l of AChE and 2.5 *m*l 0.1 M phosphate buffer (pH 8.0). All operations were performed in the dark because of the photosensitivity of the acetylcholinesterase (21).

RESULTS

Effect of MPTP on Acetylcholinesterase Activity:

The effect of varying concentrations of MPTP on the inactivation of AChE was investigated under a fixed concentration of enzyme (0.5 units/*m*l). MPTP was found to inactivate the AChE in a dose-dependent manner. Thus, as shown in Figure 1, the inhibition of AChE activity increased with increasing MPTP concentrations and at 4 *m*M MPTP, ~50% of AChE activity was inhibited. The maximum inhibition extrapolated was found to be 100% (Figure 1, inset a). No inactivation of AChE was observed in the reaction mixture incubated under identical conditions in the absence of MPTP, indicating that the inactivation of the enzyme is MPTP-dependent.

MPTP was found to be not a substrate for AChE. No significant changes on the absorption spectrum of MPTP were observed (Figure 1, inset b) when MPTP (50 μM) was incubated with AChE (0.083 unit/ml) for 60 min, indicating that MPTP is a stable compound under the experimental conditions, and not a substrate for acetylcholinesterase. In a time course assay, the inactivation of AChE by MPTP was found to be independent of incubation time. As shown in figure 2, the inhibition was effective immediately with no significant difference in the rate of thiocholine formation observed when AChE was incubated with fixed concentrations of MPTP up to 60 min at 25 °C. As shown in this figure, there was a small variation (within ±5%) in the beginning of reaction, and the

percent inhibition subsequently remained unchanged from 5 to 60 min incubation. It is evident that the degree of inhibition depends on the inhibitor concentration and is independent of incubation time.

Identification of Inhibitor Type:

Since the reversible inhibition is dependent upon an equilibrium between enzyme and inhibitor (22), the activity of the enzyme can be recovered by removing or diluting the inhibitor using dialysis, gel exclusion chromatography or simple dilution techniques. We have utilized the gel (Sephadex G-25) exclusion chromatographic technique to distinguish the type of MPTP inhibition. The investigation for the effect of dilution on the rate of thiocholine formation was also performed under a fixed concentration of enzyme (0.5 unit/*m*l) in the presence of 8.0 *m*M MPTP. After 5 minutes incubation in the dark at 25 °C, the reaction mixtures were either diluted or eluted through Sephadex G-25 column (1 ¥ 5 cm) and assayed for AChE activity. If MPTP is a reversible inhibitor, the rate of AChE catalyzed-hydrolysis of acetylthiocholine should increase with increased dilution because a new equilibrium between AChE and MPTP was reached. The rate of AChE-catalyzed hydrolysis of acetylthiocholine was found to increase with increased dilution and a complete recovery of the enzyme activity was achieved after eluting through a Sephadex G-25 column (Figure 3), indicating that MPTP does not covalently interact with enzyme.

Kinetics of Inactivation of Acetylcholinesterase by MPTP:

Effects of substrate concentration on the rate of AChE-catalyzed hydrolysis of acetylthiocholine were investigated under a fixed concentration of the enzyme in the presence of different concentrations of the inactivator. The extent of inactivation of AChE was found to be dependent upon the initial concentration of the inactivator in the reaction system. As shown in Figure 4, the Lineweaver-Burk plot gave a number of computer

traced straight lines with varying slopes and K_m corresponding to individual concentration of MPTP. Since both the apparent K_m and V_{max} of the enzyme (22) was changed at different concentrations of the inhibitor (Fig. 4), it is apparent that MPTP exhibited a mixed competitive-noncompetitive inhibition. The reciprocal of the intercept of the plot (V_{max}), the maximum rate of thiocholine produced by AChE catalyzation in this system, was found to be 0.125 *m*M/sec, and the Michaelis constant (K_m), obtained directly from the ratio of the slope to intercept of the plot, was found to be 0.216 *m*M which is in accord with previously reported values (24).

The kinetic inhibition constant (K_i) was determined from the secondary plot derived from Figure 4 (25). When the slopes from figure 4 were plotted against MPTP concentration, a straight line with a correlation coefficient of 0.998 was obtained (Fig. 5) indicating a stoichiometric relationship between MPTP concentration and AChE inactivation. The inhibition rate constant, K_i can be obtained directly from the ratio of the intercept and the slope of the secondary plot and was found to be 2.14 *m*M which is ten-times more than the K_m (0.216 *m*M) value. The affinity of AChE-MPTP was calculated as reciprocal of K_i value and found to be 4.67×10^4 M^{-1}.

DISCUSSION

The results reported here clearly demonstrate, for the first time, that the neurotoxicant, MPTP is able to inhibit acetylcholinesterase activity. The kinetic assay has proven that the inactivation of acetylcholinesterase is dependent upon the concentration of MPTP and is independent of incubation time during their interaction. MPTP was found to be a reversible inhibitor and is not a substrate for acetylcholinesterase. The significance of this new information is discussed below:

Although the interaction of MPTP with MAO-B has been extensively studied, no reference made to the inactivation of acetylcholinesterase by MPTP. Our studies provide evidence that acetylcholinesterase was indeed inactivated by MPTP. This is confirmed by comparing the rates of acetylcholinesterase-catalyzed hydrolysis of acetylthiocholine in the absence and presence of this neurotoxicant. Thus, the rate of thiocholine formation decreased with increased MPTP concentration (Fig. 1). It is evident that the inactivation of acetylcholinesterase is MPTP-dependent. Additional support for this conclusion comes from the study of time-course of acetylcholinesterase inactivation at a fixed [AChE] and different [MPTP], in which the rate of AChE-catalyzed hydrolysis of acetylthiocholine was found to be dependent upon the initial concentration of MPTP rather than incubation time. The kinetic study further provided powerful support for this, in that the computer-generated Lineweaver-Burk plots resulted in straight lines with varying slopes which were increased with increased MPTP concentration (Fig. 4). The secondary plot, derived from slopes of primary double reciprocal plots at different concentrations of the inhibitor, gave a linear line with a correlation coefficient of 0.998, indicating that there is a stoichiometric relationship between MPTP concentration and acetylcholinesterase inactivation. The inhibition rate constant, K_i was found to be 2.14 mM which is ten times of K_m (0.216 mM) value, indicating that MPTP virtually eliminated the activity of AChE. These results strongly suggest that MPTP inactivates acetylcholinesterase in this reaction system.

Inactivation of Acetylcholinesterase is reversible:

Since a reversible inhibition is characterized by an equilibrium between enzyme and inhibitor, the activity of enzyme could be recovered on merely removing free inhibitor by dialysis, gel exclusion chromatography or by simple dilution of the inhibitor (26). In contrast, with irreversible inhibitors no such equilibrium is achieved and the activity of enzyme does not return to normal by removing or diluting the inhibitor (22). We have

utilized Sephadex G-25 gel exclusion chromatographic techniques and the effect of dilution on the rate of AChE-catalyzed hydrolysis of acetylthiocholine to determine the reversibility of MPTP inhibition. Since irreversible inhibitors usually inactivate enzyme by covalently bonding, the rate of product formation by residual active enzyme could not be significantly affected by the dilution of reaction mixture under a given reaction condition and the activity of enzyme could not be recovered by gel exclusion chromatography. If MPTP is an irreversible inhibitor, the rate of thiocholine formation could not vary significantly with a newly reached equilibrium between MPTP and AChE once the reaction was diluted, and the activity of AChE could not be recovered by the gel exclusion chromatographic technique. Our studies show that diluting the reaction mixture (Fig. 3a) or eluting through a Sephadex G-25 gel column recovered the enzyme activity, indicating that MPTP is a reversible inhibitor for AChE.

It is known that the reversible inhibition system is characterized by a definite degree of inhibition, depending on the inhibitor concentration, which is usually reached relatively rapidly and thereafter is independent of incubation time, provided that the inhibitor is stable (22, 27). On the other hand, irreversible inhibition is characterized by a progressive increase in inhibition with time, ultimately reaching complete inhibition even with a very dilute concentration of inhibitor, provided that the inhibitor is in excess of the amount of enzyme present (22, 27). According to Schrader's rules (16), an irreversible mechanism is related to the presence of a labile bond in the inhibitor molecule. Since MPTP is a stable compound in this medium, a reversible mechanism was expected. This view was further confirmed by the following observations: (a) the time course of AChE inactivation by MPTP has shown that MPTP is neither a time-dependent inhibitor nor a substrate of AChE and the inhibition of AChE is dependent upon the concentration of MPTP which was effective within a few seconds; (b) the Lineweaver-Burk plot has shown linear mixed competitive-noncompetitive inhibition kinetics for MPTP with a K_i of 2.14 mM. The

AChE-MPTP affinity, the reciprocal of the inhibition constant ($1/K_i$), was calculated to be 4.67×10^4 M^{-1}. All the above results provide confidence that MPTP is indeed a reversible inhibitor similar to sulfur, selenium heterosubstituted isomers of N, N-diethylcarbamyl choline and carbaryl (23).

The Possible Mechanism for the Inactivation of Acetylcholinesterase:

Although inactivation of AChE has been extensively studied (28-35), no reference was available for the inhibition of AChE by MPTP, its metabolites ($MPDP^+$ and MPP^+) and their analogs. We report here the inactivation of AChE by MPTP, which was found to inhibit AChE in a manner similar to that of various amphiphilic drugs and to affect AChE activity through a mixed type inhibition kinetics (36-38). The mechanism(s) for MPTP interaction with AChE may be speculated below:

Since the active site of AChE contains two subsites, the esteratic and anionic subsites (39) which correspond to the catalytic machinery and the choline-binding pocket, MPTP and substrate could combine with the enzyme at the same site when the acetyl-enzyme and Michaelis complex are formed. At this situation, the anionic site undoubtedly participates in this combination. In the acetyl-enzyme, the anionic site is certainly free, whereas in the Michaelis complex it is not free. Therefore, MPTP may be attached to the acetyl-enzyme and show a mixed type of inhibition.

Considering the three-dimensional structure of AChE, since the enzyme active site is located at the bottom of a deep and narrow hydrophobic "gorge" that reaches halfway into the protein (39), and since the choline-binding anionic subsite contains at most one negative charge (39), the neutral charged MPTP could hardly get access to these sites. Therefore, an alternative explanation comes from the theoretical consideration (40) and the experimental results reported previously (41-43) that the quaternary moiety of choline appears to bind chiefly through interaction with the p electrons in the aromatic residues on

AChE. Thus, the aromatic groups may interact with the tertiary amine of MPTP because of the polarizability of the ion (40) and thereby modulate the enzyme activity. In addition, the gorge is so deep and its aromatic surface is so extensive that there may be many different ways and sites for substrate and MPTP to bind AChE.

The Significance of This Finding and Necessity of Further Studies:

The results of the investigation clearly demonstrate that MPTP inhibits AChE activity by mixed-type inhibition kinetics. Further, these results provide important new information regarding the inactivation of AChE by MPTP, a mechanism of action that may be involved in the MPTP-induced Parkinson's disease. The reason for this is that Parkinson's disease is the most common of the basal-ganglion disorders (12). The function of basal ganglia depends mainly on the balance of the transmitters, DA and ACh in which DA is the "Go" system and ACh is the "No Go" system (12). An excess ACh could thus produce immobility. AChE is a specific enzyme for ACh catalysis to form choline. However, once MPTP enters the basal ganglia, it will inactivate AChE and lead to the accumulation of an excess amount of ACh, which in turn will cause neuronal cell injury with resultant immobility (15-18). Since the amount of MPTP in basal ganglia of monkey brain was found to be higher than the other sections in brain (4), and since the most serious clinical symptom observed in Parkinson's disease is the akinesia where the patient gradually freezes into immobility and becomes dependent on others for survival (12), our results may explain why MPTP selectively destroys neuronal cells in the brain. Knowledge of AChE inactivation by MPTP is therefore essential for understanding the mechanism behind environmentally-induced Parkinson's disease and for developing therapeutic intervention as well. Furthermore, information about the MPTP-binding site of AChE should contribute to understanding the molecular basis for the neurotoxicity of these pyridine species.

Since the tertiary amine MPTP is lipid soluble, it can be rapidly taken up by the brain where it is catalyzed by MAO-B in neuronal cells of the substantia nigra to form $MPDP^+$ which then undergoes a further two-electron autoxidation to the pyridinium species MPP^+ (3, 44, 45) or disproportionates to form MPTP and MPP^+ (5-7). We have recently reported that MPTP is used as a substrate for MAO-B, generating reactive oxygen species (46). Since these reactive species of oxygen are known to cause cell injury, we proposed that MAO-B in combination with MPTP produces reactive oxygen species, which in turn may play an important role in destroying neuronal cells of the substantia nigra. The results of the present study force us to think in a new light regarding the possibility that MPTP is actually acting as a "double edged sword" in that it not only generates reactive oxygen species by interacting with MAO-B but also causes Ach to accumulate by inactivating AChE. Both phenomena would lead to neuronal cell death and induce Parkinson's syndrome.

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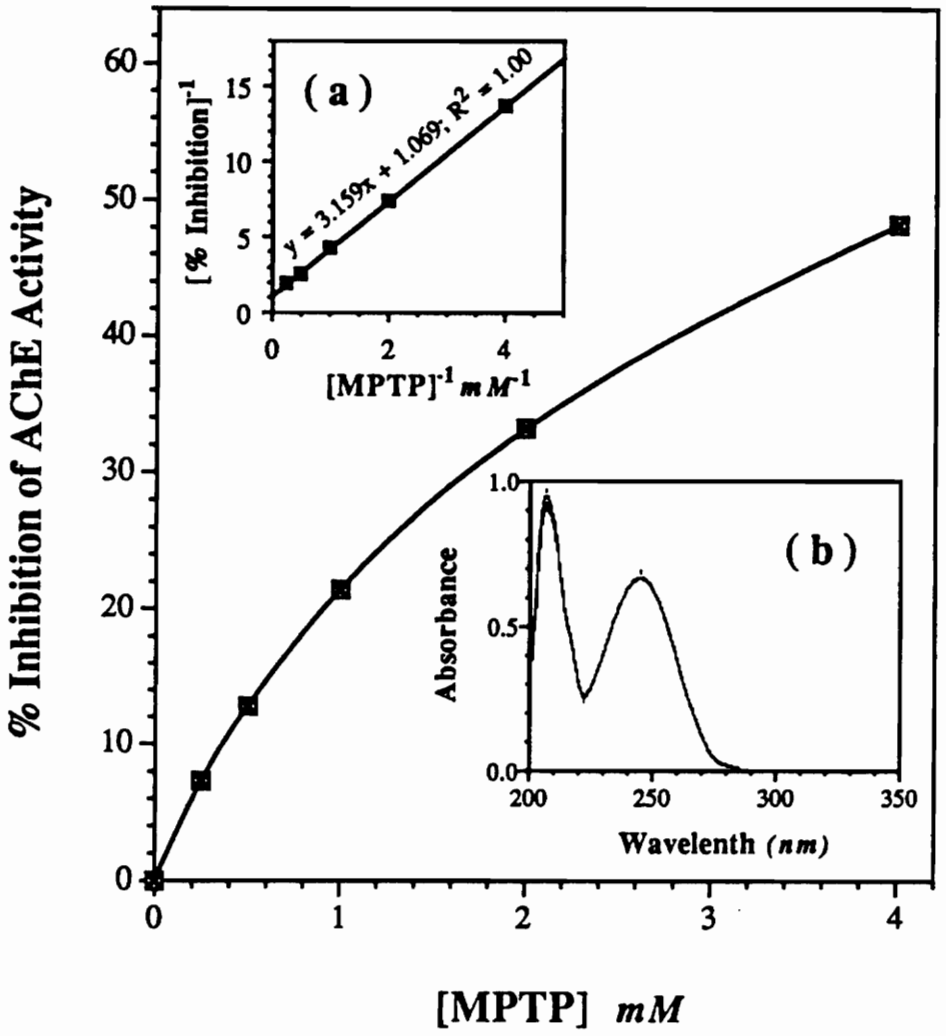

Figure 1. Effect of MPTP concentration on the activity of acetylcholinesterase. The reaction mixture (0.5 *m*l) consisted of acetylcholinesterase (0.5 unit/*m*l) and indicated amount of MPTP and incubated for 5 minutes at 25 °C in the dark followed by adding the assay mixture (2.5 *m*l) of acetylthiocholine (0.118 *m*M) and DTNB (0.35 *m*M). The rate of accumulation of yellow color at 405 *nm* (dA/min) was monitored up to 2 minutes. Inset: (a) the data obtained are presented on reciprocal coordinates; and (b) the absorption spectra obtained during the incubation of MPTP (50 μM) solution in the presence of AChE (0.083 unit/*m*l) in phosphate buffer, pH 7.0 at 25 °C.

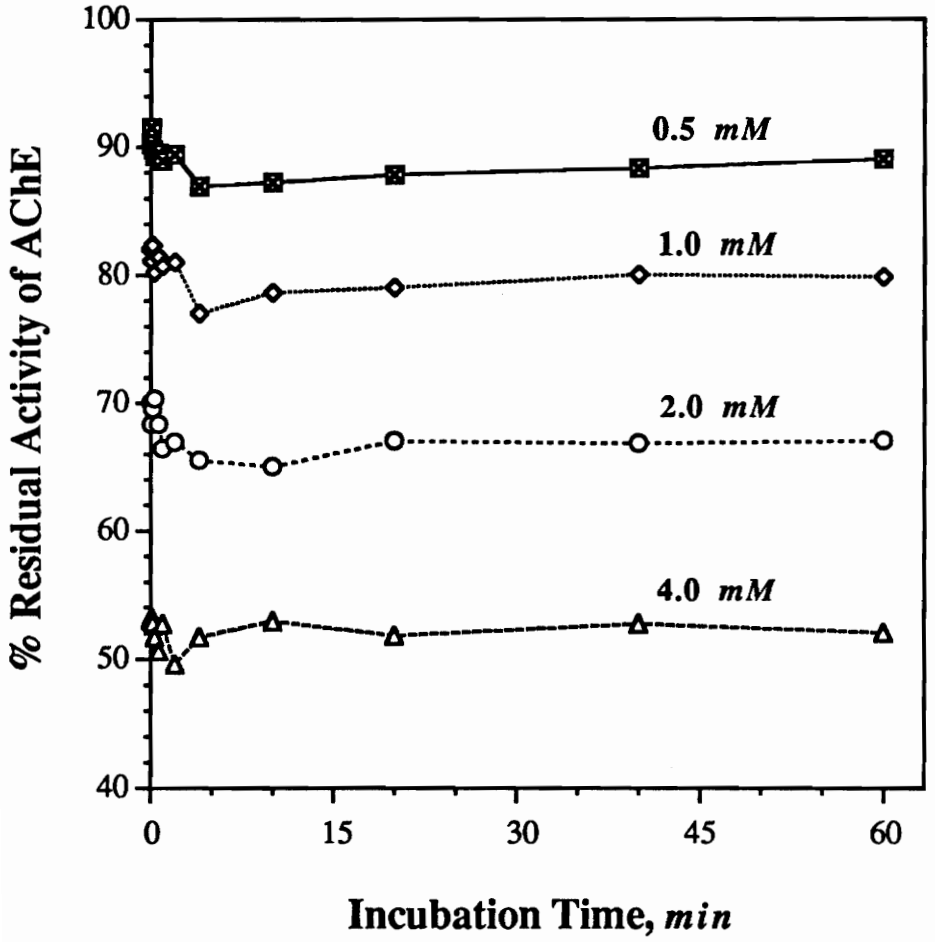

Figure 2. Effect of incubation time on the acetylcholinesterase activity. The reaction mixture contained AChE (0.5 units/*m*l) and the indicated concentrations of MPTP. The assay mixture consisted of substrate (0.94 *m*M) and DTNB (0.35 *m*M).

Figure 3

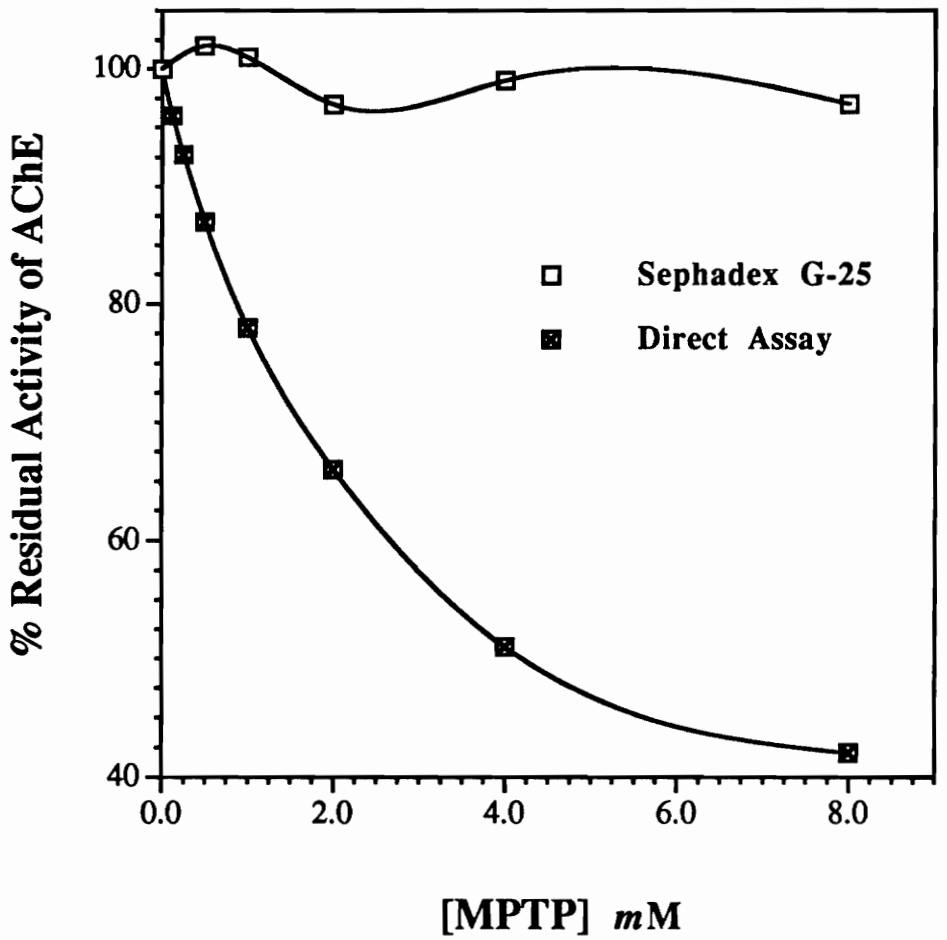

Figure 3. Reversibility of MPTP inhibition of AChE. Indicated concentrations of MPTP were incubated with 0.5 units/ml of AChE for 5 min. The reaction mixture was divided into two equal aliquates, one aliquate was eluted through a 1 x 5 cm Sephadex G-25 column and the other half was directly used for AChE activity. Similar concentrations of enzyme, without MPTP, were also eluted through a column as control.

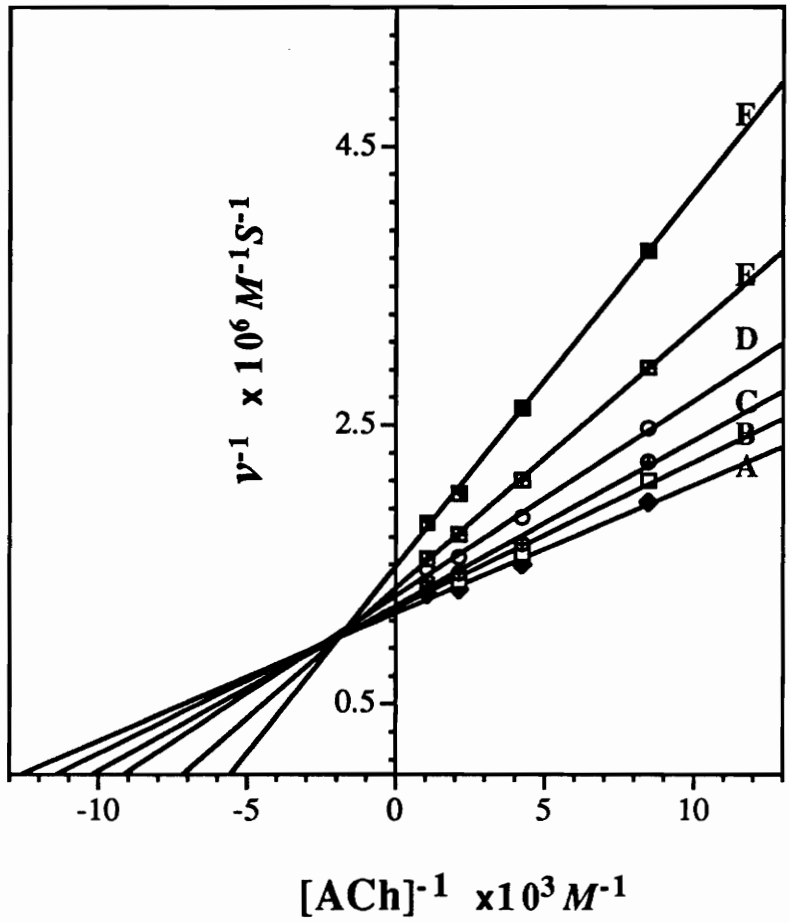

Figure4. Lineweave-Burk plot on the inhibition of the acetylthiocholine hydrolytic activity of acetycholinesterase by MPTP. The reaction mixture contained varing amounts of ACh at fixed amount of AChE (0.5 unit/ml) in the presence of MPTP: (A) none; (B) 0.25 mM; (C) 0.5 mM; (D) 1.0 mM; (E) 2.0 mM and (F) 4.0 mM. All experiments were performed in the dark at 25 °C.

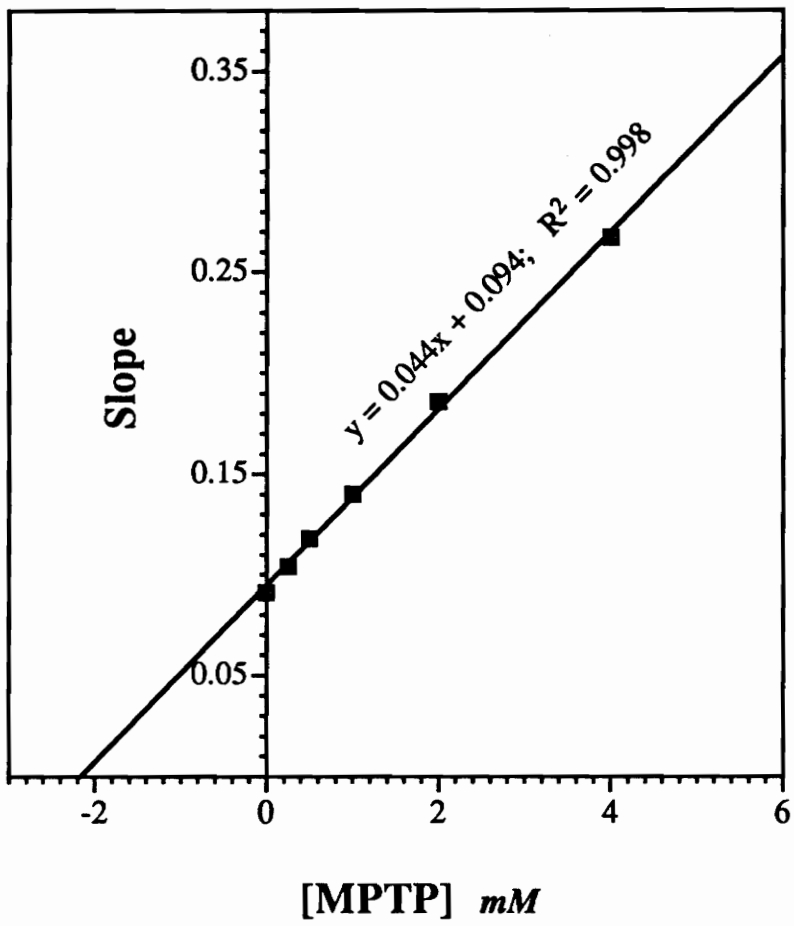

Figure 5. The secondary plot derived from primary double reciprocal plots of activity *vs* substrate concentration at different concentrations of MPTP. Experimental conditions were same as in Figure 4 legend.

CHAPTER VI

ACETYLCHOLINESTERASE INHIBITION BY 1-METHYL-4-PHENYLPYRIDINIUM ION, A BIOACTIVATED METABOLITE OF MPTP

(*Molecular and Cellular Biochemistry, 1993, In Press*)

Lun-Yi Zang and Hara P. Misra

Abstract

The effect of the neurotoxicant, 1-methyl-4-phenylpyridinium ion (MPP^+) on acetylcholinesterase (AchE) activity was investigated. The MPP^+ was found to inactivate the enzyme in a dose dependent manner. The kinetic parameter, K_m for the substrate (acetylthiocholine), was found to be 0.216 *m*M and K_i for MPP^+ for the inactivation of AChE was found to be 0.197 *m*M. It was found that MPP^+ is neither a substrate of AChE nor the time-dependent inactivator. The studies of reaction kinetics indicate the inactivation of AChE to be a linear mixed-type inhibition. The inactivation of AchE by MPP^+ was partially recovered by either dilution or gel exclusion chromatography. These data suggest that once MPP^+ enters the basal ganglia of the brain, it can inactivate the AChE and thereby increase the acetylcholine level in the basal ganglia, leading to potential cell dysfunction. It appears likely that the nigrostriatal toxicity by MPP^+ leading to Parkinson's disease-like syndrome may, in part, be mediated *via* the AChE inactivation.

INTRODUCTION

Parkinson's disease has been recognized as the most common of the basal-ganglion disorders (1). Closely associated with the basal ganglia are the substantia nigra and the subthalamus. The caudate-putamen of the basal ganglia contains a number of neurotransmitter substances including norepinephrin (NE), serotonin (5-HT), glutamate (GLu), gamma-aminobutyric acid (GABA), dopamine (DA) and acetylcholine (ACh). Normal function of the caudate-putamen depends on the balance of these transmitters, particularly on the balance of DA and ACh (1). DA has been described as the "**go**" system and ACh is the "**no go**" system (1). Thus, an excess of DA produces an excess of movement while an excess of ACh produces immobility. ACh can be rapidly broken down by the enzyme acetylcholinesterase (AChE), which occurs in high concentration on both pre- and post-ganglionic membranes within autonomic ganglia and on the membranes of parasympathetic nerve terminals where ACh also functions as a neurotransmitter (2, 3). Inactivation of this enzyme by a neurotoxicant will result in the accumulation of an excess amount of ACh leading to not only immobility, but also may cause neuronal cell injury (4-6).

It has been reported that self-administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) manifests the irreversible symptoms of Parkinson's disease in several young drug abusers (7). This neurotoxicant selectively destroys neuronal cells in the substantial nigra of humans and other primates (8). Although the mechanism of action of MPTP is not fully understood, it is generally believed that the crucial species for MPTP neurotoxicity is not MPTP itself, but rather some of its metabolites (9, 10). In studies of MPTP-metabolism, it has been demonstrated that monoamine oxidase-B (MAO-B) catalyzes the two-electron oxidation of MPTP to a dihydropyridinium intermediate ($MPDP^+$), which in turn spontaneously oxidizes to form 1-methyl-4-phenylpyridinium

(MPP^+) or disproportionate to form MPTP and MPP^+ (11-13). Because the administration of MAO-B inhibitor, L-deprenyl, affords protection against the neurotoxic action of MPTP in mice (14), and because an increase in MAO-B activity and lower levels of DA (15) are found in the substantia nigra of patients with severe Parkinsonism, the investigations in the past seem to be have focused on the irreversible inhibition of MAO-B (16). Although MPP^+ does not affect MAO-B activity (17) and can not cross the blood/brain barrier because of its high polarity, introducing it directly into the neostriatum of mice causes the same kind of dopamine reduction as seen with MPTP administration (18). It has been reported that the selective irreversible MAO-B inhibitor, L-deprenyl, improves Parkinson's disease disability in mild to moderate degree, and L-deprenyl in combination with L-dopa (a precursor of DA) therapy prolongs the life span of Parkinson's disease patients compared to L-dopa alone (19). However, the mechanism by which MPP^+ induces Parkinson's disease remains unclear. Although MPP^+ does not affect MAO-B, it was hypotherized that MPP^+ may affect AChE thereby causing an imbalance in DA/ACh ratio in caudat-putamen and leading to the neuronal cell dysfunction. We have investigated the effects of MPP^+ on AChE and report here for the first time that MPP^+ is a powerful inhibitor of AchE.

Materials and methods

Acetylcholinesterase (EC 3.1.1.7) type III (from electric eel), acetylthiocholine iodide (ACh) and 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma Chemical Company, St. Louis, MO. 1-methyl-4-phenylpyridine hydrochloride (MPP^+) was purchased from Research Biochemicals, Inc. Natick, MA. Sephadex G-25 was

obtained from Pharmacia Fine Chemicals, Division of Pharmacia Inc., Piscataway, New Jersey. All other chemicals were obtained at the highest obtainable purity.

The activity of acetylcholinesterase (AChE) was measured by following the accumulation of yellow color produced from thiocholine when it reacts with dithiobisnitrobenzoate ion (20). The assay is based upon the coupling of the following reactions:

$$(CH_3)_3N^+CH_2CH_2SCOCH_3 + H_2O \xrightarrow{AChE} (CH_3)_3N^+CH_2CH_2S^- + CH_3COO^- + 2H^+$$

$$(CH_3)_3N^+CH_2CH_2S^- + RSSR \longrightarrow (CH_3)_3N^+CH_2CH_2SSR + RS^-$$

Where RSSR is 5, 5'-dithiobis(2-nitrobenzoate ion) and RS^- is the yellow anion of 5-thio-2-nitro-benzoic acid. Spectrophotometric assays were performed in a Shimadzu UV-visible recording spectrophotometer UV-160 at 25 °C. The extent of inactivation of AChE by MPP^+ was measured with the use of DTNB as an indicator. The rate of the yellow anion accumulation was measured at 405 nm using $\varepsilon_{405\,nm} = 1.36 \times 10^4\ M^{-1}cm^{-1}$ (21).

Unless otherwise stated, the enzyme activity was monitored following the sequential addition of 20 μl inactivator stock solutions (different concentration) and 0.5 ml acetylcholinesterase (0.5 unit/ml), followed by incubation for 5 minutes at 25 °C. The reaction was started by adding 2.5 *m*l mixture of acetylthiocholine iodide (75 *m*M) and DTNB (0.01 M) solutions. The rate of accumulation of yellow RS^- was monitored immediately for 2 min against a blank which consists of all components except AChE. The AChE stock (0.5 unit/*m*l) was prepared in 0.005% Gelatin distilled water solution; DTNB (0.01 M) was made in 10 *m*l 0.1 *M* phosphate buffer (pH 7.0) containing 15 *m*g $NaHCO_3$; and AChE (75 *m*M) was prepared in distilled water. The assay mixture was made by mixing 40 μl ACh, 100 μl DTNB stocks, 0.5 *m*l of AChE and 2.5 *m*l 0.1 M phosphate buffer (pH 8.0). All operations were performed in the dark because of the photosensitivity of the AChE (22).

RESULTS

Effect of MPP^+ on Acetylcholinesterase Activity:

MPP^+ was found to inactivate the AChE in a dose-dependent manner. Further, this enzyme inhibition was found to be powerful, with as little as 0.18 mM of MPP^+ causing ~50% inhibition and 2 mM MPP^+ causing ~92% inhibition of AChE activity (Figure 1). The maximum inhibition extrapolated was found to be 100% (Figure 1, inset a). No inactivation of AChE was observed in the reaction mixture incubated under identical conditions in the absence of MPP^+, indicating that the inactivation of the enzyme is MPP^+-dependent.

MPP^+ was found not to be a substrate for AChE. No significant changes on the absorption spectrum of MPP^+ were observed (Figure 1, inset b) when MPP^+ (50 μM) was incubated with AChE (0.083 unit/*m*l) for 60 min, indicating that MPP^+ is a stable compound under the experimental conditions, and not a substrate for AChE. In a time course study, the inactivation of AChE by MPP^+ was found to be independent of incubation time. As shown in figure 2, the inhibition was effective immediately with no significant difference in the rate of thiocholine formation observed when AChE was incubated with fixed concentrations of MPP^+ for up to 100 min at 25 °C. As shown in this figure, there was a small variation (within ±5%) in the reaction, and the velocity of thiocholine formation remained virtually unchanged from beginning up to 100 min incubation. The degree of inhibition was found to be dependent on the inhibitor concentration and not on incubation time.

Binding of MPP^+ to AchE:

The inhibitory action of MPP^+ on AChE was partially reversed by dilution. Since the reversible inhibition is dependent upon an equilibrium between enzyme and inhibitor

(23), the activity of the enzyme can be regained by removing or diluting the inhibitor. The investigation for the effect of dilution on the rate of thiocholine formation was performed under a fixed concentration of enzyme (0.5 unit/*m*l) in the presence of 4.0 *m*M MPP^+. If MPP^+ is a reversible inhibitor, the rate of AChE catalyzed-hydrolysis of ACh should increase with increased dilution because a new equilibrium between AChE and MPP^+ is reached after dilution. This indeed proved to be the case, as shown in Figure 3a. In separate trials utilizing the gel exclusion chromatographic technique it was found that only 50% enzymatic activity was regained after AChE (0.5 unit/ml) samples, which had been incubated with 2 mM MPP^+ for 5 min in dark, were eluted through Sephadex G-25 column (Figure 3b), indicating that inactivation of AChE by MPP^+ is only partially reversible and probably accompanied by electrostatic interaction of MPP^+ with the enzyme.

Kinetics of Inactivation of Acetylcholinesterase by MPP^+:

The effects of substrate concentration on the rate of AChE-catalyzed hydrolysis of acetylthiocholine were investigated under a fixed concentration of the enzyme in the presence of different concentrations of MPP^+. MPP^+ was found to inhibit the AChE activity in a dose dependent manner and the extent of inactivation of AChE was found to be dependent upon the initial concentration of MPP^+ in the reaction system. As shown in Figure 4, the Lineweaver-Burk plot gave a number of computer traced straight lines with varying slopes and K_m corresponding to individual concentration of MPP^+. Both the apparent K_m and V_{max} of the enzyme (23) changed with varying concentrations of the inhibitor (Figure 4), indicating that MPP^+ exhibits a mixed competitive-noncompetitive inhibition. The reciprocal of the intercept of the plot (V_{max}), the maximum rate of thiocholine produced by AChE catalyzation in this system, was found to be 0.125 *m*M/sec, and the Michaelis constant (K_m), obtained directly from the ratio of the slope to intercept of

the plot, was found to be 0.216 *m*M, which is in accord with previously reported values (24).

The inhibition constant (K_i) was determined from the secondary plot (25) derived from Figure 4. When the slopes from figure 4 were plotted against MPP^+ concentration, a straight line with a correlation coefficient of 0.998 was obtained (Figure 5) indicating a stoichiometric relationship between MPP^+ concentration and AChE inactivation. The K_i can be obtained directly from the ratio of the intercept and the slope of the secondary plot and was found to be 0.197 *m*M which is similar to the K_m (0.216 *m*M) value. The affinity of AChE-MPP^+ was calculated as the reciprocal of the K_i value and found to be $5.08 ¥ 10^3$ M^{-1}.

DISCUSSION

Since AChE plays an important role in neurotransmission, regulation of its activity by various effectors has been studied extensively. Altered levels of AChE activity have been reported in various clinical conditions, including neuromuscular dysfunction (26), hereditary muscular dystrophy (27), prenatal neuronal tube defects (28) and senile dementia of the Alzheimer type (29). The results reported here clearly demonstrate, for the first time, that the neurotoxicant, MPP^+ is able to inhibit acetylcholinesterase activity. The kinetic assay further demonstrated that the inactivation of acetylcholinesterase is dependent upon the concentration of MPP^+ and is independent of incubation time during their interaction. MPP^+ was found to be a mixed type inhibitor and is not a substrate for acetylcholinesterase.

Although the interaction of MPP^+ with mitochondria and sub-mitochondrial particles has been extensively studied (30-33), no reference was made to the inactivation of

AChE by MPP^+. Our studies provide evidence that AChE was indeed inactivated by MPP^+. This is confirmed by comparing the rates of acetylcholinesterase-catalyzed hydrolysis of ACh in the absence and presence of this neurotoxicant. Thus, the rate of thiocholine formation decreased with increased MPP^+ concentration (Fig. 1). Additional support for this conclusion comes from the study of time-course of AChE inactivation at a fixed concentration of AChE and different concentrations of MPP^+, in which the rate of AChE-catalyzed hydrolysis of ACh was found to be dependent upon the initial concentration of MPP^+ rather than incubation time (Fig. 2). The kinetic study further provided powerful support for this, in that the computer-generated Lineweaver-Burk plots resulted in straight lines with varying slopes that were increased with increased MPP^+ concentration (Fig. 4). The secondary plot, derived from slopes of primary double reciprocal plots at different concentrations of the inhibitor, gave a linear line with a correlation coefficient of 0.998, indicating that there is a stoichiometric relationship between MPP^+ concentration and AChE inactivation. The inhibition rate constant, K_i was found to be 0.197*m*M which is similar to the K_m (0.216 *m*M) value, indicating that MPP^+ is a powerful AChE inhibitor capable of virtually eliminating the activity of AChE.

Since a reversible inhibition is characterized by an equilibrium between enzyme and inhibitor, the activity of enzyme could be recovered on merely removing the free inhibitor (34). In contrast, with irreversible inhibitors no such equilibrium is achieved and the activity of enzyme does not return to normal by removing or diluting the inhibitor (23). We have utilized Sephadex G-25 gel exclusion chromatographic techniques and the effect of dilution on the rate of AChE-catalyzed hydrolysis of ACh to determine the reversibility of MPP^+ inhibition. If MPP^+ is an irreversible inhibitor, the rate of thiocholine formation would not vary significantly with a newly reached equilibrium between MPP^+ and AChE once the AChE-MPP^+ compact was diluted, and the activity of AChE would not be

recovered by removing the free inhibitor using a gel exclusion chromatography. Our studies show that diluting the reaction mixture (Fig. 3a) or eluting it through a Sephadex G-25 gel column (Fig. 3b) recovered only 50% of the enzyme activity, indicating that MPP^+ is a mixed inhibitor for AChE, with ability to inhibit the enzyme reversibly and irreversibly.

The time course of AChE inactivation by MPP^+ has shown that MPP^+ is neither a time-dependent inhibitor nor a substrate of AChE, and that the inhibition of AChE is dependent upon the concentration of MPP^+ which was effective within a few seconds The Lineweaver-Burk plots demonstrate linear mixed competitive-noncompetitive inhibition kinetics for MPP^+ with a K_i of 0.197 *m*M. The AChE-MPP^+ affinity, the reciprocal of the inhibition constant ($1/K_i$), was calculated to be 5.08×10^3 M^{-1}.

Although inactivation of AChE has been extensively studied (35-42), no reference was available for the inhibition of AChE by MPP^+ and its analogs. We report here the inactivation of AChE by MPP^+, which was found to inhibit AChE in a manner similar to that of various amphiphilic drugs and to affect AChE activity through mixed type inhibition kinetics (43-45). The mechanism(s) for MPP^+ interaction with AChE could be speculated as below:

The active site of AChE contains two subsites, the esteratic and anionic subsites (46) which correspond to the catalytic machinery and the choline-binding pocket. MPP^+ and substrate may combine with the AChE enzyme at the same site during formation of the acetyl-enzyme and Michaelis complex. In such a situation, the anionic site would also participate in this combination. In the acetyl-enzyme, the anionic site is free, whereas in the Michaelis complex it is not free. Therefore, MPP^+ may be attached to the acetyl-enzyme and show a mixed type of inhibition.

Considering the three-dimensional structure of AChE, in which the enzyme active site is located at the bottom of a deep and narrow hydrophobic "gorge" that reaches halfway into the protein (46), and in which the choline-binding anionic subsite contains at most one

negative charge (46), MPP^+ would not be expected to gain access to these sites although it is a positively charged ion. Therefore, an alternative explanation comes from theoretical considerations (47) in conjunction with the experimental results reported previously (48-50), that the quaternary moiety of choline appears to bind chiefly through interaction with the p electrons in the aromatic residues on AChE. Thus, the aromatic groups may interact with the tertiary amine of MPP^+ because of the polarizability of the ion (47) and thereby modulate the enzyme activity. However, it must be considered that there may be many different ways and sites for substrate and MPP^+ to bind the extensive aromatic surface of the gorge AChE (46).

The results of this investigation clearly demonstrate that MPP^+ inhibits AChE activity by mixed-type inhibition kinetics. Further, these results provide important new information regarding the inactivation of AChE by MPP^+, a mechanism of action that may be involved in the MPTP-induced Parkinson's disease. The function of basal ganglia is largely dependent upon the balance of the neuro transmitters, DA and ACh in which DA. (1) Further, excess ACh is thought to be involved in basal-ganglion related immobility disorders. The results reported here indicate that, once MPP^+ enters or is formed in the basal ganglia, it may inactivate AChE and lead to the accumulation of an excess amount of ACh, which in turn may cause neuronal cell injury with resultant immobility (4-6). Since the amount of MPP^+ in basal ganglia of monkey brain was found to be much higher than the other sections in brain (11), and since the most serious clinical symptom observed in Parkinson's disease is the depletion of DA leading to akinesia in which the patient gradually freezes into immobility and becomes dependent on others for survival (1), our results may explain why MPP^+ selectively destroys neuronal cells in the substantia nigra of the brain. In vivo neuropharmacologic studies have shown that systemic administration of the irreversible AChE inhibitor, soman, produces a rapid and profound depletion of NE in the olfactory and forebrain (51). Therefore it is reasonable to argue that depletion of substantia

nigra DA after systemic administration of MPTP is the result of tonic hypercholinergic stimulation of these DA-containing neurons. Knowledge of AChE inactivation by MPP^+ is therefore essential for understanding the mechanism behind environmentally-induced Parkinson's disease, as well as for development of therapeutic interventions. Furthermore, information about the MPP^+-binding site of AChE is critical to a more complete understanding of the molecular basis for the neurotoxicity of these pyridine species.

Since the tertiary amine MPTP is lipid soluble, it can be rapidly taken up by brain where it is catalyzed by MAO-B in neuronal cells of the substantia nigra to form $MPDP^+$ which then undergoes a further two-electron autoxidation to the pyridinium species MPP^+ (10, 52) or disproportionate to form MPTP and MPP^+ (12-14). On the other hand, MPP^+ formed in glial cells can be taken up by dopamine containing neuronal cells *via* the DA uptake system in the terminals of the neurons (53). As a result, the concentration in the dopaminergic neurons may become several thousand-times greater than in the extracellular concentration (48, 49). In addition, MPP^+ so generated has been shown to inhibit NADH dehydrogenase (complex I) in mitochondrial respiratory chain, thereby cutting off neuronal ATP supply (50, 52, 54, 55). We have recently reported that $MPDP^+$ undergoes an autoxidation process producing superoxide radicals (56, 57) that immediately decompose to form hydroxyl radicals (58). Furthermore, MPTP was found to be a substrate for MAO-B generating reactive oxygen species (59). Because these reactive oxygen species are known to cause cell injury, we proposed that MAO-B in combination with MPTP produces the reactive intermediate species, including superoxide, hydrogen peroxide and hydroxyl radical, which in turn may play an important role in destroying neuronal cells of substantial nigra. The results of the present study force us to think in a new light regarding the possibility that MPTP is actually acting as a "double edged sword" in that it not only generates reactive oxygen species by interacting with MAO-B but also causes Ach to

accumulate by one of its metabolites, MPP^+, inactivating AChE. The consequences of a combined hypercholinergic effect through inactivation of AChE and the generation of reactive oxygen species by reacting with MAO-B, following exposure to these neurotoxicants, is likely sufficient to induce neuronal cell death within the substantia nigra, and thereby produce Parkinson's disease-like syndrome, in MPTP-exposed individuals.

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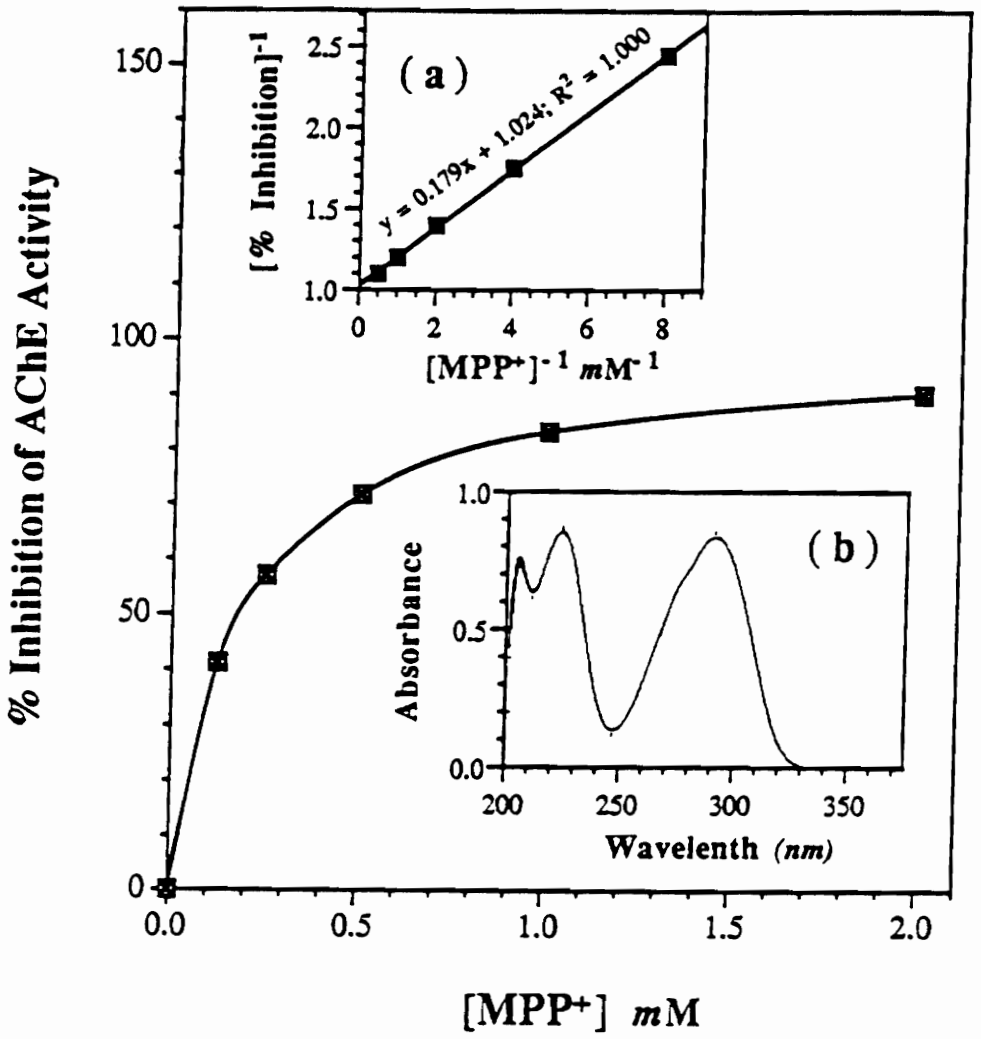


Figure 1. Effect of MPP^+ concentration on the activity of acetylcholinesterase. The reaction mixture (0.5 *ml*) consisted of acetylcholinesterase (0.5 unit/*ml*) and indicated amount of MPP^+, followed by incubated for 5 minutes at 25 °C in the dark and then addition of the assay mixture (2.5 *ml*) of acetylthiocholine (0.118 *m*M) and DTNB (0.35 *m*M). The rate of accumulation of yellow color at 405 *nm* (dA/min) was monitored up to 2 minutes. Inset: (a) the data obtained are presented on reciprocal coordinates; and (b) the absorption spectra obtained during the incubation of MPP^+ (50 μM) solution in the presence of AChE (0.083 unit/*ml*) in phosphate buffer, pH 7.0 at 25 °C.

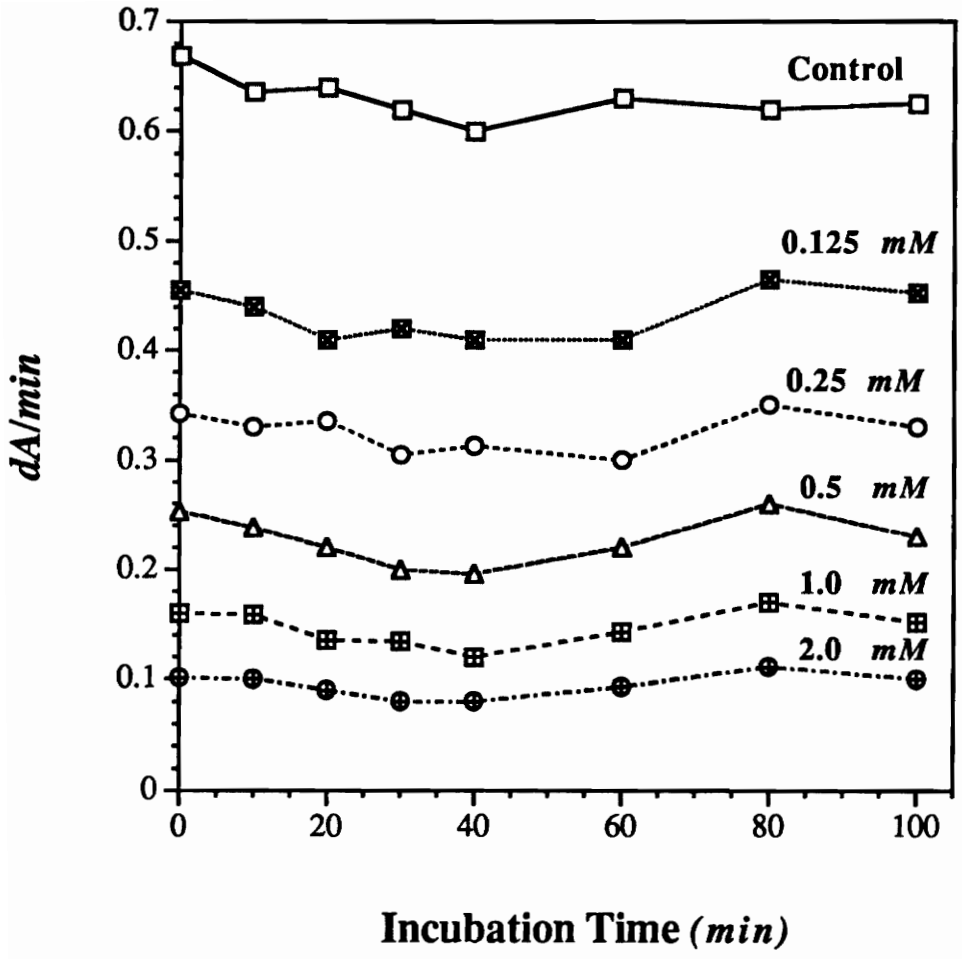
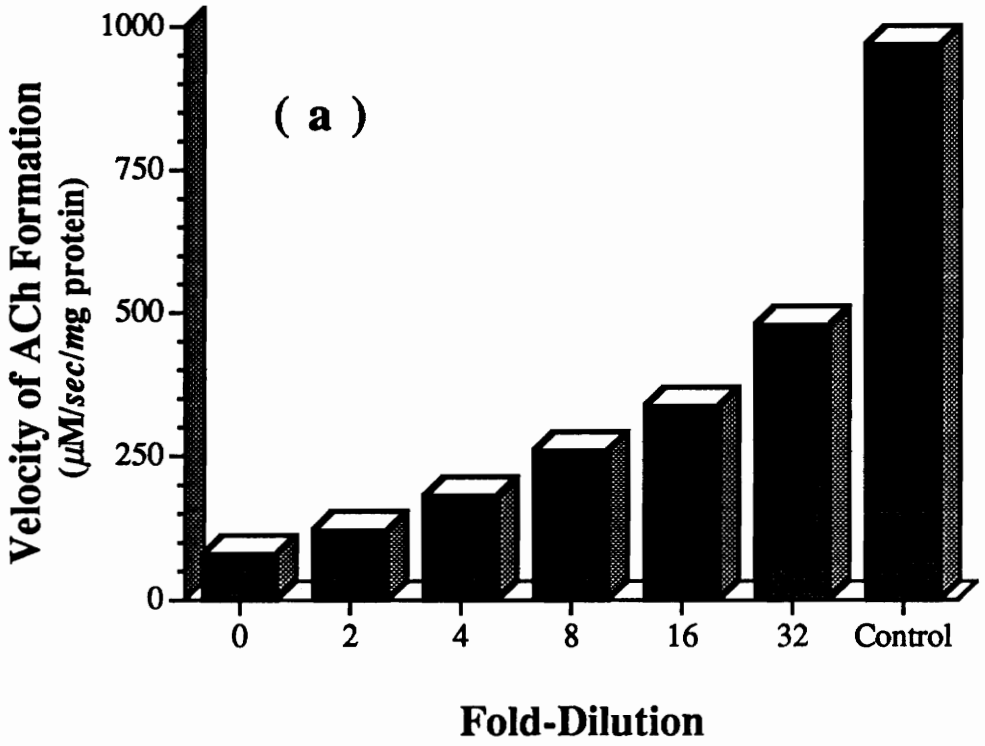
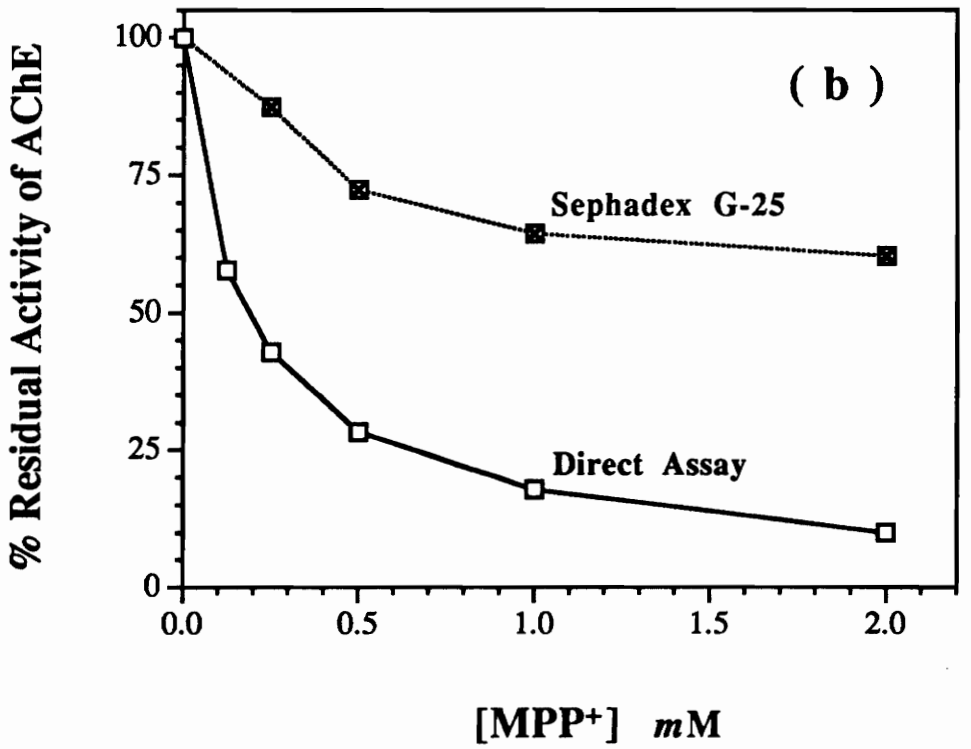


Figure 2. Effect of incubation time on the acetylcholinesterase activity. The reaction mixture contained AChE (0.5 unit/ml) and the indicated concentrations of MPP^+. The assay mixture consisted of substrate (0.94 *m*M) and DTNB (0.35 *m*M).

Figure 3


(a)
Velocity of ACh Formation
(μM/sec/mg protein)
1000
750
500
250
0
0
2
4
8
16
32
Control
Fold-Dilution


(b)
% Residual Activity of AChE
100
75
50
25
0
Sephadex G-25
Direct Assay
0.0
0.5
1.0
1.5
2.0
[MPP+] mM

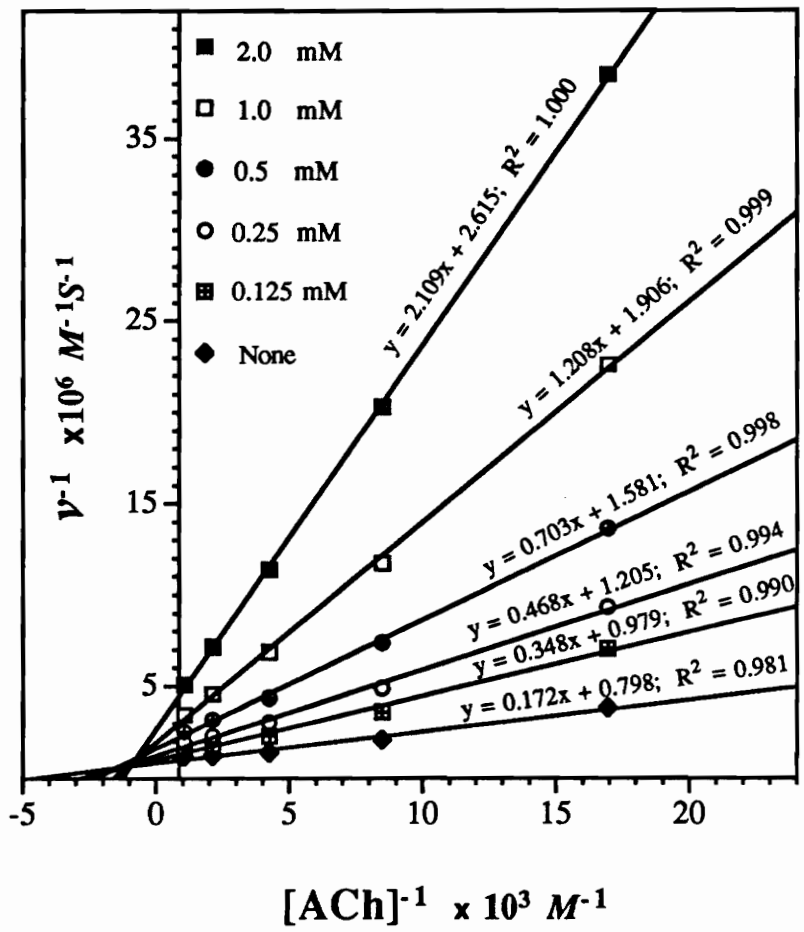


Figure 4. Lineweaver-Burk plot on the inhibition of the acetylcholine hydrolytic activity of acetylcholinesterase by MPP^+. The reaction mixture contained varying amounts of AChE at fixed amount of AChE (0.5 unit/ml) in the presence of indicated [MPP^+]. All experiments were performed in the dark at 25 °C.

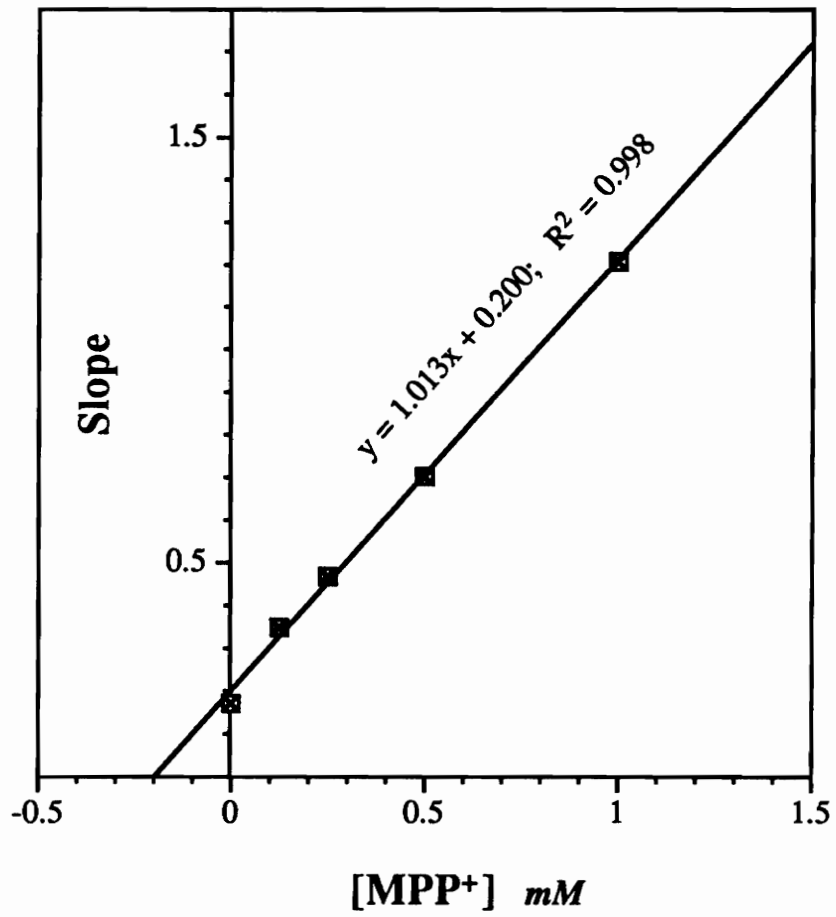


Figure 5. The secondary plot derived from the slopes of primary double reciprocal plots at different concentrations of MPP^+. Experimental conditions were same as in Figure 4 legend.

CHAPTER VII

CHARACTERISTIC OF ACETYLCHOLINESTERASE INHIBITION BY 1-METHYL-4-PHENYL-2,3-DIHYDROPYRIDINIUM ION, AN INTERMEDIATE OF MPTP METABOLISM

(Submitted to Proc. Natl. Acad. Sci., USA)

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Abstract

The effect of the neurotoxicant, 1-methyl-4-phenylpyridinium ion ($MPDP^+$) on acetylcholinesterase (AchE) activity was investigated. The $MPDP^+$ was found to inactivate the enzyme in a dose dependent manner. The kinetic parameter, K_m for the substrate (acetylthiocholine), was found to be 0.22 *m*M and K_i for $MPDP^+$ for the inactivation of AChE was found to be 0.265 *m*M. It was found that $MPDP^+$ is neither a substrate of AChE nor a time-dependent inactivator. The studies of reaction kinetics indicate the inactivation of AChE to be a linear mixed-type inhibition. The inactivation of AchE by $MPDP^+$ was partially reversed by either dilution or gel exclusion chromatography. These data suggest that once $MPDP^+$ enters the basal ganglia of the brain, it can inactivate the AChE and thereby increase the acetylcholine level in the basal ganglia, leading to potential cell dysfunction. It appears likely that the nigrostriatal toxicity by $MPDP^+$ leading to Parkinson's disease-like syndrome may, in part, be mediated *via* the AChE inactivation.

INTRODUCTION

Parkinson's disease has been recognized as the most common of the basal-ganglion disorders (1). Closely associated with the basal ganglia are the substantia nigra and the subthalamus. The caudate-putamen of the basal ganglia contains a number of neurotransmitter substances including norepinephrine (NE), serotonin (5-HT), glutamate (Glu), gamma-aminobutyric acid (GABA), dopamine (DA) and acetylcholine (ACh). Normal function of the caudate-putamen depends on the balance of these transmitters, particularly on the balance of DA and ACh (1). DA has been described as the "**go**" system and ACh is the "**no go**" system (1). Thus, an excess of DA produces an excess of movement while an excess of ACh produces immobility. ACh can be rapidly broken down by the enzyme acetylcholinesterase (AChE), which occurs in high concentration on both pre- and post-ganglionic membranes within autonomic ganglia and on the membranes of parasympathetic nerve terminals where ACh also functions as a neurotransmitter (2, 3). Inactivation of this enzyme by a neurotoxicant would result in the accumulation of an excess amount of ACh leading to not only immobility, but also may cause neuronal cell injury (4-6).

It has been reported that self-administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) manifests the irreversible symptoms of Parkinson's disease in several young drug abusers (7). This neurotoxicant selectively destroys neuronal cells in the substantia nigra of humans and other primates (8). Although the mechanism of action of MPTP is not fully understood, it is generally believed that the crucial species for MPTP neurotoxicity is not MPTP itself, but rather some of its metabolites (9, 10). In studies of MPTP-metabolism, it has been demonstrated that monoamine oxidase-B (MAO-B) catalyzes the two-electron oxidation of MPTP to a dihydropyridinium intermediate ($MPDP^+$), which in turn spontaneously oxidizes to form 1-methyl-4-phenylpyridinium

(MPP^+) or disproportionate to form MPTP and MPP^+ (11-13). Because the administration of MAO-B inhibitor, L-deprenyl, affords protection against the neurotoxic action of MPTP in mice (14), and because an increase in MAO-B activity and lower levels of DA (15) are found in the substantia nigra of patients with severe Parkinsonism, the investigations in the past seem to be have focused on the irreversible inhibition of MAO-B (16). Although $MPDP^+$ was shown to generate superoxide radicals during its autoxidation (53, 54), no reference was available to relate its role in basal ganglia. We hypothesized that $MPDP^+$ may affect AChE thereby causing an imbalance in DA/ACh ratio in caudate-putamen and leading to the neuronal cell dysfunction. We have investigated the effects of $MPDP^+$ on AChE and report here for the fist time that $MPDP^+$ is a powerful inhibitor of AchE.

Materials and methods

Acetylcholinesterase (EC 3.1.1.7) type III (from electric eel), acetylthiocholine iodide (ACh) and 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma Chemical Company, St. Louis, MO. 1-methyl-4-phenylpyridine hydrochloride ($MPDP^+$) was purchased from Research Biochemicals, Inc. Natick, MA. Sephadex G-25 was obtained from Pharmacia Fine Chemicals, Division of Pharmacia Inc., Piscataway, New Jersey. All other chemicals were obtained at the highest obtainable purity.

The activity of acetylcholinesterase (AChE) was measured by following the accumulation of yellow color produced from thiocholine when it reacts with dithiobisnitrobenzoate ion (17). The assay is based upon the coupling of the following reactions:

$$(CH_3)_3N^+CH_2CH_2SCOCH_3 + H_2O \xrightarrow{AChE} (CH_3)_3N^+CH_2CH_2S^- + CH_3COO^- + 2H^+$$

$(CH_3)_3N^+CH_2CH_2S^-$ + RSSR ----------> $(CH_3)_3N^+CH_2CH_2SSR$ + RS^-

Where RSSR is 5, 5'-dithiobis(2-nitrobenzoate ion) and RS^- is the yellow anion of 5-thio-2-nitro-benzoic acid. Spectrophotometric assays were performed in a Shimadzu UV-visible recording spectrophotometer UV-160 at 25 °C. The extent of inactivation of AChE by $MPDP^+$ was measured with the use of DTNB as an indicator. The rate of the yellow anion accumulation was measured at 405 nm using $e_{405\,nm} = 1.36 \times 10^4\ M^{-1}cm^{-1}$ (18).

Unless otherwise stated, the enzyme activity was monitored following the sequential addition of 20 μl inactivator stock solutions (different concentration) to 0.5 ml acetylcholinesterase (0.5 unit/ml), and incubated for 5 minutes at 25 °C. The reaction was started by adding 2.5 *m*l mixture of acetylthiocholine iodide (75 *m*M) and DTNB (0.01 M) solutions. The rate of accumulation of yellow RS^- was monitored immediately for 2 min against a blank which consists of all components except AChE. The stock solutions of AChE (0.5 unit/*m*l) was prepared in 0.005% Gelatin distilled water solution; DTNB (0.01 M) was made in 0.1 *M* phosphate buffer (pH 7.0) containing 15 *m*g $NaHCO_3$; and AChE (75 *m*M) was prepared in distilled water. The assay mixture was made by mixing 40 μl ACh, 100 μl DTNB stocks, 0.5 *m*l of AChE and 2.5 *m*l 0.1 M phosphate buffer (pH 8.0). All operations were performed in the dark because of the photosensitivity of the AChE (19).

RESULTS

Kinetics of AChE Catalyzed-Hydrolysis of Acetylthiocholine:

Figure 1 shows the kinetics of AChE-catalyzed hydrolysis of acetylthiocholine over time in air-saturated 0.05 *M* phosphate buffer, pH 7.0. The absorbance at 226.5 *nm*

diminished in intensity with increasing incubation time, indicating that the AChE catalyzes acetylthiocholine to produce thiocholine and the loss of [Acetylthiocholine] was found to be linear for at least 2 min of the initial reaction. The thiocholine so generated can react with DTNB to form a yellow anion of 5-thio-2-nitrobenzoic acid which can be detected at 405 nm. As shown in the inset of Figure 1, the accumulation of 5-thio-2-nitrobenzoic acid was linear for at least two minutes with a correlation coefficient of 1.000 when 0.083 mM AChE was allowed to act on 50 μM acetylthiocholine at 25 °C. The rate of thiocholine formation, thus, can be calculated directly from the initial slope using an extinction coefficient $e_{405\ nm} = 1.36 \times 10^4\ M^{-1}cm^{-1}$.

Effect of MPDP$^+$ on Acetylcholinesterase Activity:

MPDP$^+$ was found to inactivate the AChE in a dose-dependent manner. Further, MPDP$^+$ was found to be a powerful inhibitor of AChE, with as little as 0.2 mM of MPDP$^+$ causing ~50% inhibition and 2 mM MPDP$^+$ causing ~90% inhibition of AChE activity (Fig. 2). When $(1-\Phi/\Phi_o)^{-1}$ was plotted against $[MPDP^+]^{-1}$ where $(1-\Phi/\Phi_o)$ is the percent inhibition of AChE activity and Φ/Φ_o is the ratio of the rates of AChE catalyzed-hydrolysis of acetylthiocholine or absorption intensity per-minute (dA/min) at 405 *nm* in the presence and absence of inhibitor, a straight line with a correlation coefficient of 0.999 was obtained (Fig. 2 inset) and the maximum inhibition extrapolated was found to be 100%. No inactivation of AChE was observed in the reaction mixture incubated under identical conditions in the absence of MPDP$^+$, indicating that the inactivation of the enzyme is MPDP$^+$-dependent.

MPDP$^+$ was found not to be a substrate for AChE. No significant differences on the absorption spectra of MPDP$^+$ were observed (data not shown) when MPDP$^+$ (50 μM) was incubated in the reaction mixtures under identical conditions in the absence and presence of AChE (0.083 unit/*m*l) for 60 min, indicating that MPDP$^+$ is not a substrate for

AChE. In a time course study, the inactivation of AChE by $MPDP^+$ was found to be independent of incubation time. As shown in figure 3, the inhibition was effective immediately with no significant difference in the rate of thiocholine formation observed when AChE was incubated with fixed concentrations of $MPDP^+$ for up to 100 min at 25 °C. As shown in this figure, the velocity of thiocholine formation remained virtually unchanged (±5%) from beginning up to 100 min incubation. The degree of inhibition was found to be dependent on the inhibitor concentration and not on incubation time.

Binding of $MPDP^+$ to AchE:

The inhibitory action of $MPDP^+$ on AChE was partially reversed by dilution. Since the reversible inhibition is dependent upon an equilibrium between an enzyme and an inhibitor (20), the activity of the enzyme should be regained by removing or diluting the inhibitor. The effect of dilution on the rate of thiocholine formation was performed under a fixed concentration of enzyme (0.5 unit/*m*l) in the presence of 4.0 *m*M $MPDP^+$. If $MPDP^+$ is a reversible inhibitor, the rate of AChE-catalyzed hydrolysis of ACh should increase with increased dilution because a new equilibrium between AChE and $MPDP^+$ is reached after dilution. This indeed proved to be the case, as shown in Figure 4a in which 50% of AChE activity was found to be recovered by diluting the reacted mixture (incubated for 5 min) thirty-two fold of original volume. In separate trials utilizing the gel exclusion chromatographic technique it was found that ~75% of enzymatic activity was regained after AChE (0.5 unit/ml) samples, which had been incubated with 2 mM $MPDP^+$ for 5 min in dark, were eluted through Sephadex G-25 column (Figure 4b), indicating that inactivation of AChE by $MPDP^+$ is partially reversible and partly accompanied by covalent interaction of $MPDP^+$ with the enzyme.

Kinetics of Inactivation of Acetylcholinesterase by $MPDP^+$:

The effects of substrate concentration on the rate of AChE-catalyzed hydrolysis of acetylthiocholine were investigated under a fixed concentration of the enzyme in the presence of different concentrations of $MPDP^+$. $MPDP^+$ was found to inhibit the AChE activity in a dose dependent manner and the extent of inactivation of AChE was found to be dependent upon the initial concentration of $MPDP^+$ in the reaction system. As shown in Figure 5, the Lineweaver-Burk plot gave a number of computer traced straight lines with varying slopes and K_m values corresponding to individual concentration of $MPDP^+$. Both the apparent K_m and V_{max} of the enzyme (20) changed with varying concentrations of the inhibitor (Figure 5), indicating that $MPDP^+$ exhibits a mixed competitive-noncompetitive inhibition. The reciprocal of the intercept of the plot (V_{max}), the maximum rate of thiocholine produced by AChE catalyzation in this system, was found to be 0.13 *m*M/sec, and the Michael's constant (K_m), obtained directly from the ratio of the slope to intercept of the plot, was found to be 0.22 *m*M, which is in accord with previously reported values (21).

The inhibition constant (K_i) was determined from the secondary plot (22) derived from Figure 5. When the slopes from figure 5 were plotted against $MPDP^+$ concentration, a straight line with a correlation coefficient of 0.999 was obtained (Fig. 6) indicating a stoichiometric relationship between $MPDP^+$ concentration and AChE inactivation. The K_i can be obtained directly from the ratio of the intercept and the slope of the secondary plot and was found to be 0.265 *m*M which is close to the K_m (0.22 *m*M) value. The affinity of AChE-$MPDP^+$ was calculated as the reciprocal of the K_i value and found to be $3.77 ¥ 10^3$ M^{-1}.

DISCUSSION

Since AChE plays an important role in neurotransmission, regulation of its activity by various effectors has been studied extensively. Altered levels of AChE activity have been reported in various clinical conditions, including neuromuscular dysfunction (23), hereditary muscular dystrophy (24), prenatal neuronal tube defects (25) and senile dementia of the Alzheimer type (26). The results reported here clearly demonstrate, for the first time, that the neurotoxicant, $MPDP^+$ is able to inhibit AChE activity. The kinetic studies further demonstrated that the inactivation of AChE is dependent upon the concentration of $MPDP^+$ and is independent of incubation time during their interaction. $MPDP^+$ was found to be a mixed type inhibitor and is not a substrate for AChE.

Although the interaction of $MPDP^+$ with mitochondria and sub-mitochondrial particles has been extensively studied (27-30), no reference was made to the inactivation of AChE by $MPDP^+$. Our studies provide evidence that AChE was indeed inactivated by $MPDP^+$. This is confirmed by comparing the rates of acetylcholinesterase-catalyzed hydrolysis of ACh in the absence and presence of this neurotoxicant. Thus, the rate of thiocholine formation decreased with increased $MPDP^+$ concentration (Fig. 1). Additional support for this conclusion comes from the study of time-course of AChE inactivation at a fixed concentration of AChE and different concentrations of $MPDP^+$, in which the rate of AChE-catalyzed hydrolysis of ACh was found to be dependent upon the initial concentration of $MPDP^+$ rather than incubation time (Fig. 2). The kinetic study further provided powerful support for this, in that the computer-generated Lineweaver-Burk plots resulted in straight lines with varying slopes that were increased with increased $MPDP^+$ concentration (Fig. 5). The secondary plot, derived from slopes of primary double reciprocal plots at different concentrations of the inhibitor, gave a linear line with a correlation coefficient of 0.998, indicating that there is a stoichiometric relationship

between $MPDP^+$ concentration and AChE inactivation. The inhibition rate constant, K_i was found to be 0.197*m*M which is similar to the K_m (0.216 *m*M) value, indicating that $MPDP^+$ is a powerful AChE inhibitor capable of virtually eliminating the activity of AChE.

Since a reversible inhibition is characterized by an equilibrium between enzyme and inhibitor, the activity of the enzyme could be recovered on merely removing the free inhibitor (31). In contrast, with irreversible inhibitors no such equilibrium is achieved and the activity of the enzyme does not return to normal by removing or diluting the inhibitor (20). We have utilized Sephadex G-25 gel exclusion chromatographic techniques and the effect of dilution on the rate of AChE-catalyzed hydrolysis of ACh to determine the reversibility of $MPDP^+$ inhibition. If $MPDP^+$ is an irreversible inhibitor, the rate of thiocholine formation would not vary significantly with a newly reached equilibrium between $MPDP^+$ and AChE once the AChE-$MPDP^+$ complex was diluted, and the activity of AChE would not be recovered by removing the free inhibitor using a gel exclusion chromatography. Our studies show that diluting the reaction mixture (Fig. 4a) or eluting it through a Sephadex G-25 gel column (Fig. 4b) recovered only part of the enzyme activity, indicating that $MPDP^+$ is a mixed inhibitor for AChE, with ability to inhibit the enzyme reversibly and irreversibly.

$MPDP^+$ was found to be neither a time-dependent inhibitor nor a substrate of AChE, and the inhibition of AChE is dependent upon the concentration of $MPDP^+$ which was effective within a few seconds The Lineweaver-Burk plots demonstrate linear mixed competitive-noncompetitive inhibition kinetics for $MPDP^+$ with a K_i of 0.197 *m*M. The AChE-$MPDP^+$ affinity, the reciprocal of the inhibition constant ($1/K_i$), was calculated to be $5.08 \text{ ¥} 10^3\ M^{-1}$.

Although inactivation of AChE has been extensively studied (32-40), no reference was available for the inhibition of AChE by $MPDP^+$ and its analogs. We report here the

inactivation of AChE by $MPDP^+$, which was found to inhibit AChE in a manner similar to that of various amphiphilic drugs and to affect AChE activity through mixed type inhibition kinetics (40-42). The mechanism(s) for $MPDP^+$ interaction with AChE could be speculated as below:

The active site of AChE contains two subsites, the esteratic and anionic subsites (43) which correspond to the catalytic machinery and the choline-binding pocket. $MPDP^+$ and substrate may combine with the AChE enzyme at the same site during formation of the acetyl-enzyme and Michaelis complex. In such a situation, the anionic site would also participate in this combination. In the acetyl-enzyme, the anionic site is free, whereas in the Michaelis complex it is not free. Therefore, $MPDP^+$ may be attached to the acetyl-enzyme and show a mixed type of inhibition.

Considering the three-dimensional structure of AChE, in which the enzyme active site is located at the bottom of a deep and narrow hydrophobic "gorge" that reaches halfway into the protein (43), and in which the choline-binding anionic subsite contains at most one negative charge (43), $MPDP^+$ would not be expected to gain access to these sites due to electrostatic interaction. Therefore, an alternative explanation comes from theoretical considerations (44) in conjunction with the experimental results reported previously (45-47), that the quaternary moiety of choline appears to bind chiefly through interaction with the p electrons in the aromatic residues on AChE. Thus, the aromatic groups may interact with the tertiary amine of $MPDP^+$ because of the polarizability of the ion (44) and thereby modulate the enzyme activity. However, it must be considered that there may be many different ways and sites for substrate and $MPDP^+$ to bind the extensive aromatic surface of the gorge AChE (43).

The results of this investigation clearly demonstrate that $MPDP^+$ inhibits AChE activity by mixed-type inhibition kinetics. Further, these results provide important new information regarding the inactivation of AChE by $MPDP^+$, a mechanism of action that may

be involved in the MPTP-induced Parkinson's disease. The function of basal ganglia is largely dependent upon the balance of the neuro transmitters, DA and ACh (1). Further, excess ACh is thought to be involved in basal-ganglion related immobility disorders. The results reported here indicate that, once $MPDP^+$ enters or is formed in the basal ganglia, it may inactivate AChE and lead to the accumulation of an excess amount of ACh, which in turn may cause neuronal cell injury with resultant immobility (4-6). Since the amount of $MPDP^+$ in basal ganglia of monkey brain was found to be much higher than the other sections in brain (11), and since the most serious clinical symptom observed in Parkinson's disease is the depletion of DA leading to akinesia in which the patient gradually freezes into immobility and becomes dependent on others for survival (1), our results may explain why $MPDP^+$ selectively destroys neuronal cells in the substantia nigra of brain. In vivo neuropharmacologic studies have shown that systemic administration of the irreversible AChE inhibitor, soman, produces a rapid and profound depletion of olfactory and forebrain NE (48). Therefore it is reasonable to argue that depletion of substantia nigra DA after systemic administration of MPTP is the result of tonic hypercholinergic stimulation of these DA-containing neurons. Knowledge of AChE inactivation by $MPDP^+$ is therefore essential for understanding the mechanism behind environmentally-induced Parkinson's disease, as well as for development of therapeutic interventions. Furthermore, information about the $MPDP^+$-binding site of AChE is critical to a more complete understanding of the molecular basis for the neurotoxicity of these pyridine species.

Since the tertiary amine MPTP is lipid soluble, it can be rapidly taken up by the brain where it can be catalyzed by MAO-B in neuronal cells of the substantia nigra to form $MPDP^+$ which then undergoes a further two-electron autoxidation to the pyridinium species MPP^+ (10, 49) or disproportionate to form MPTP and $MPDP^+$ (12-14). On the other hand, $MPDP^+$ formed in glial cells can be taken up by dopamine containing neuronal cells

via the DA uptake system in the terminals of the neurons (50). As a result, the concentration of $MPDP^+$ in the dopaminergic neurons may become several thousand-times greater than in the extracellular concentration (45, 46). In addition, $MPDP^+$ so generated has been shown to inhibit NADH dehydrogenase (complex I) in mitochondrial respiratory chain, thereby cutting off neuronal ATP supply (48, 49, 51, 52). We have recently reported that $MPDP^+$ undergoes an autoxidation process producing superoxide radicals (53, 54) that immediately decompose to form hydroxyl radicals (55). Furthermore, MPTP was found to be a substrate for MAO-B generating reactive oxygen species (56). Because these reactive oxygen species are known to cause cell injury, we proposed that MAO-B in combination with MPTP produces the reactive intermediate species, including superoxide, hydrogen peroxide and hydroxyl radical, which in turn may play an important role in destroying neuronal cells of the substantia nigra. The results of the present study force us to think in a new light regarding the possibility that MPTP is actually acting as a "double edged sword" in that it not only generates reactive oxygen species by interacting with MAO-B but also causes Ach to accumulate by one of its metabolites, $MPDP^+$, inactivating AChE. The consequences of a combined hypercholinergic effect through inactivation of AChE and the generation of reactive oxygen species by reacting with MAO-B, following exposure to these neurotoxicants, is likely sufficient to induce neuronal cell death within the substantia nigra, and thereby produce Parkinson's disease-like syndrome, in MPTP-exposed individuals.

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Table I. Comparison of potency of neurotoxicants on AChE:

Inactivator	K_i	Affinity	% Reversible
MPP^+	0.197 *m*M	5.1×10^3 M^{-1}	50
$MPDP^+$	0.265 *m*M	3.8×10^3 M^{-1}	50
MPTP	2.14 *m*M	4.7×10^2 M^{-1}	100

*K_m for substrate acetylthiocholine is 0.22 *m*M.

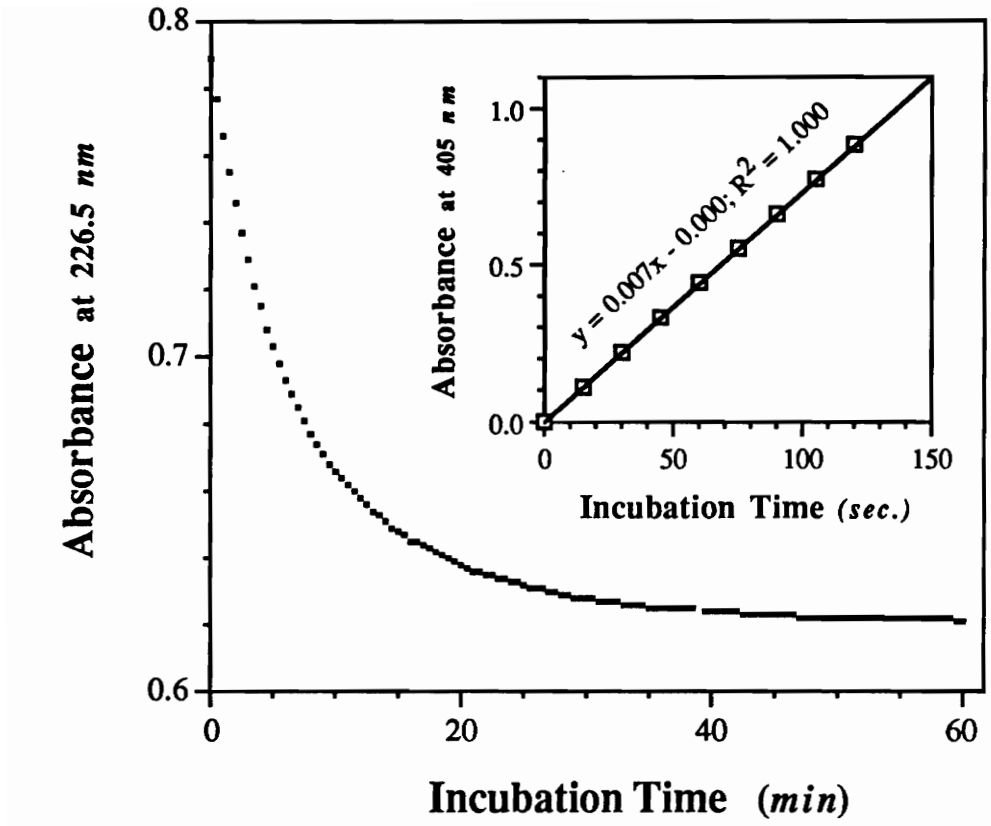

Figure 1. Kinetics of AChE catayzed-hydrolysis of acetylcholine. The reaction mixture contained 50 μM acetylthiocholinesterase (0.083 unit/ml) in air-saturated 50 *m*M phosphate buffer, pH 7.0 at 25 °C. The rate of AChE catalyzed-hydrolysis of acetylthiocholine was monitored at 226.5 *nm*. Inset, thiocholine formation measured as a yellow anion of 5-thio-2-nitrobenzoic acid produced in the reaction DTNB and the formed thiocholine.

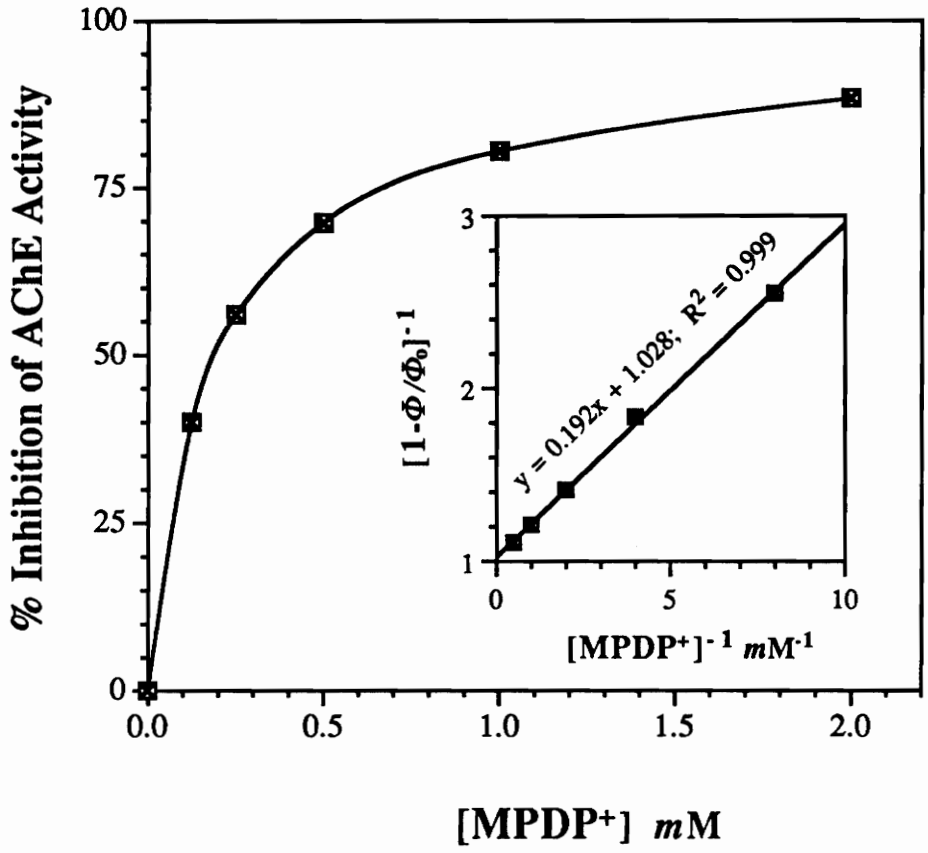

Figure 2. Effect of $MPDP^+$ concentration on the activity of acetylcholinesterase. The reaction mixture (0.5 *m*l) contsisted of acetylcholinesterase (0.5 unit/ml) and indicated amount of $MPDP^+$, followed by incubated for 5 minutes at 25 oC in the dark and then addition of the assay mixture (2.5 ml) of acetylthiocholine (0.118 *m*M) and DTNB (0.35 *m*M). The rate of accumulation of yellow color at 405 *nm* (dA/min) was monitored up to 2 minutes. Inset: the data obtained are presented on reciprocal coorrdinates.

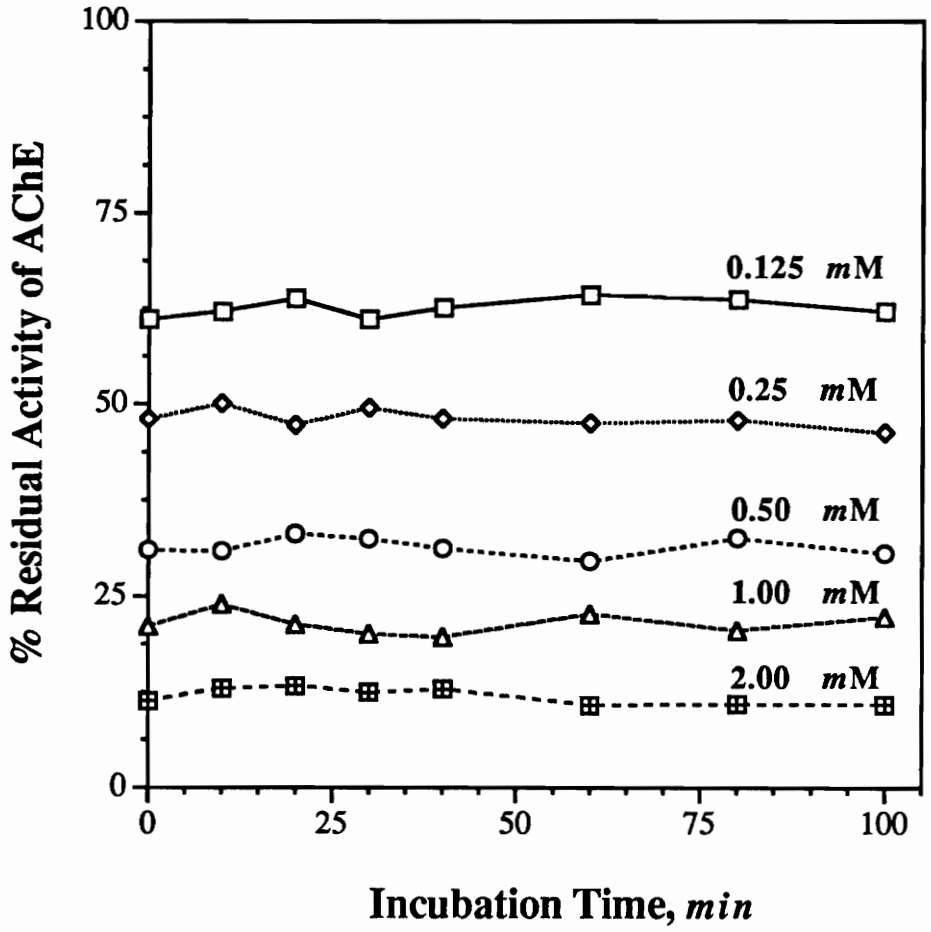

Figure 3. Effect of incubation time on the acetylcholinesterase activity. The reaction mixture contained AChE (0.5 unit/ml) and the indicated concentrations of $MPDP^+$. The assay miture consisted of substrate (0.118 *m*M) and DTNB (0.35 *m*M).

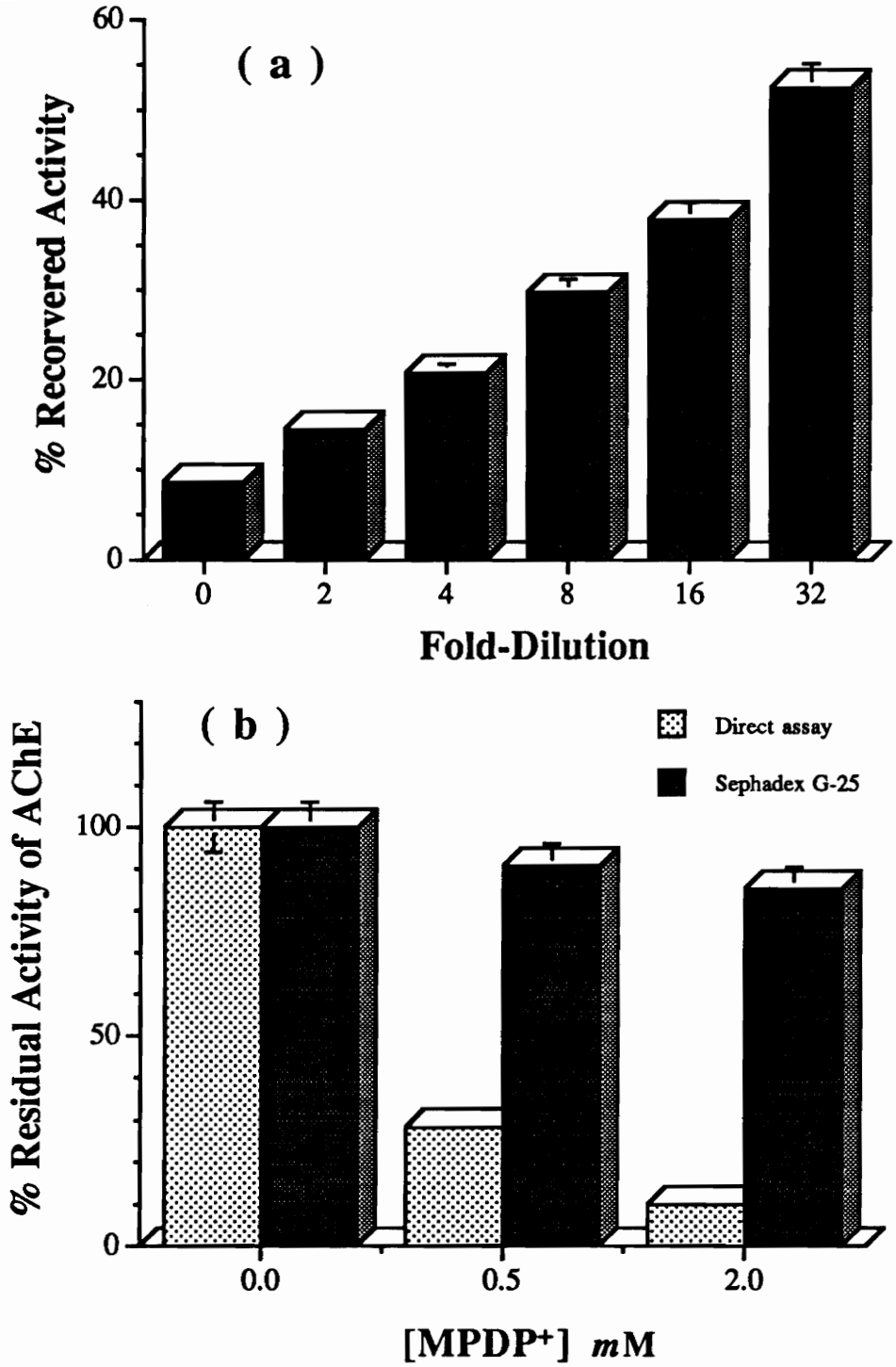

Figure 4. Reversibility of $MPDP^+$ inhibition of AChE. (a) Effect of dilution of enzyme-inhibition mixture on AChE activity. The reaction mixture consisted of $MPDP^+$ (4 *m*M) and AChE (0.5 unit/ml), and was diluted to indicated fold-dilution after 5 min incubation: (b) Effect of gel exclusion of enzyme-inhibition mixture on AChE activity. Indicated concentrations of $MPDP^+$ were incubated with 0.5 units/ml of AChE for 5 min. The reaction mixture was divide into two equal aliquotes, one aliquote was eluted through a 1× 5 cm Sephadex G-25 column and the other half was directly used for AChE activity assay. Similar concentrations of enzyme, without $MPDP^+$, were also eluted through a column as control.

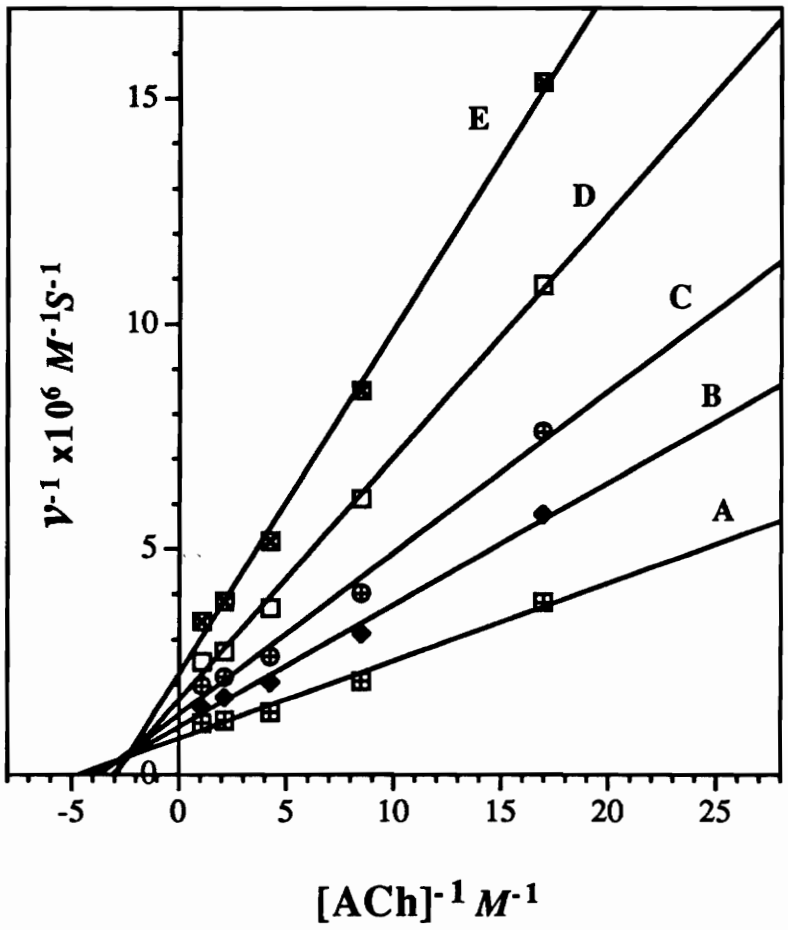

Figure 5. Lineweaver-Buek plot on the inhibition of the acetylthiocholine hydrolytic activity of acetylcholinesterase by $MPDP^+$. The reaction mixture contained varying amounts of ACh at a fixed amount of AChE (0.5 unit/ml) in the presence of [$MPDP^+$]: (A) 0; (B) 0.125 *m*M; (C) 0.25 *m*M (D) 0.5 *m*M and (E) 1.0 *m*M. All experiments were performed in the dark at 25 °C.

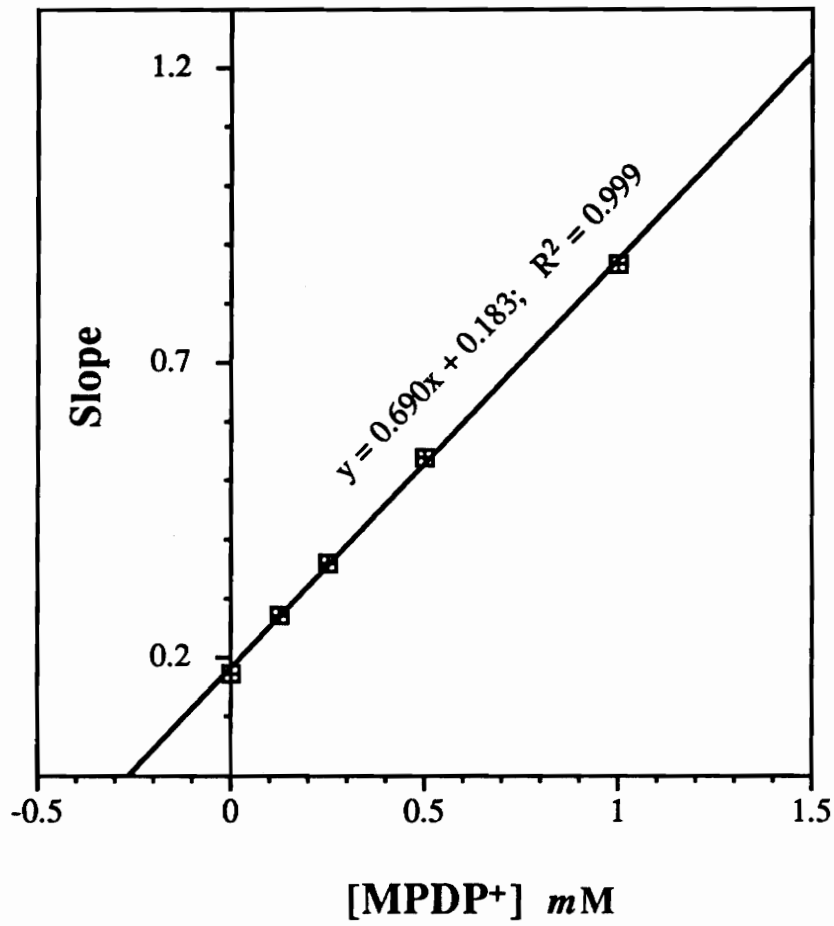

Figure 6. The secondary plot derived from the slope of primary double reciprocal plots at different concentrations of $MPDP^+$. Experimental conditions were same as in Figure 5 legend.

CHAPTER VIII

EFFECTS OF THE NEUROTOXICANT, 1-METHYL-1,2,3,6-TETRAHYDROPHENYLPYRIDINE AND ITS METABOLITES ON THE GENERATION OF FREE RADICAL DURING THEIR INTERACTION WITH BRAIN MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES

(Submitted to J. Biol. Chem.)

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Abstract

Mitochondria and submitochondrial particles from brains of dog and guinea pig were found to generate superoxide and hydroxyl radicals when treated with MPTP and NADH-linked substrates of electron transport chain, respectively. MPTP, $MPDP^+$ and MPP^+ were found to inhibit the activity of NADH dehydrogenase in a dose-dependent manner in which MPTP was more powerful than $MPDP^+$ and MPP^+. MPTP was found to increase mitochondrial oxygen uptake whereas MPP^+ strongly inhibited mitochondrial respiration when treated with NADH-linked substrate glutamate/malate. Both MPTP and MPP^+ were found to enhance the EPR signal of H atom and alpha-hydroxyethyl spin adducts in the mitochondrial particle system. However, the oxygen radical related signals were inhibited by these neurotoxicants in both SMPs and purified NADH dehydrogenase systems. The neurotoxicants MPTP and its metabolites inhibit the mitochondrial respiration by blocking the electron transport at certain unknown sites between NADH dehydrogenase and coenzyme Q. These results not only provide evidence for the production of oxyradicals by mitochondria treated with MPTP but also by submitochondrial particles using complex I of the electron transport chain.

INTRODUCTION

Chronic Parkinsonism in humans due to a product of meperidine-analog, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was discovered in several young street drugs-abusers in early 1980s (1, 2) and since then the mechanism of its neurotoxicity has been extensively studied. It was thought that MPTP is oxidized to 1-methyl-4-phenylpyridinium (MPP^+) by monoamine oxidase B (MAO-B) *via* an intermediate, 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) (3, 4) and MPP^+ was believed to be the culprit for the neurotoxicity. It was proposed that the neurotoxic action of these pyridinium compounds probably ensues from ATP depletion, initiated by the inhibition of mitochondrial respiration on NAD^+-linked substrates (5-7). The evidence supporting this has come from the fact that the oxidation products of MPTP and its analogues inhibit mitochondrial respiration as well as NADH oxidation in submitochondrial particles (8). Further support for the proposed hypothesis comes from the fact that barbiturates ameliorate the cytotoxic actions of MPP^+ on dopaminergic neurons in culture (9). Barbiturates were also found to prevent mitochondrial abnormalities in MPTP-treated monkeys and complex I deficiency in idiopathic Parkinsonian patients (10, 11).

Although the site of MPP^+ action was considered to be NADH oxidase, between NADH dehydrogenase and co-enzyme Q (12, 13), such a process would only partially interfere with ATP synthesis and probably not kill the cells because complex II should provide sufficient electrons to the respiratory chain for the maintenance of essential functions of neuronal cells. It was reported that MPTP and MPP^+ can induce oxidative stress in the lung in a manner similar to that of paraquat (14, 15). In contrast, some other reports indicated that MPP^+ is unable to undergo redox cycling in biological systems, as the electrochemical potential for its reduction to the free radical is too large (16). However, more recently, several reports have indicated that inhibition of complex I by rotenone or

MPP^+ and inhibition of complex III by antimycin A enhances the production of free radicals by the respiratory chain (17-19).

The controversy on MPP^+-$MPP^\cdot$ redox cycling to produce free radicals has not seen resolved due to the lack of direct evidence for free radical production. Recently, we have reported that purified MAO-B catalyzes the oxidation of MPTP to generate superoxide free radicals (20). In the present study, we have investigated the mechanism of action of MPTP, $MPDP^+$ and MPP^+ on brain mitochondrial complex I and detected the generation of free radicals in both intact mitochondria and submitochondrial particles using spin trapping in combination with EPR techniques.

MATERIALS AND METHODS

NADH-FMN oxido-reductase (EC 1. 6. 99. 3) and b-NADH were purchased from Sigma Chemical Company, St. Louis, MO. 1-methyl-4-phenyl-2,3-dihydropyridinium perchlorate ($MPDP^+$), 1-methyl-4-phenylpyridinium iodide (MPP^+) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP) were purchased from Research Biochemicals, Inc. Natick, MA. The spin traps, 5, 5-dimethyl-1-pyrroline-N-oxide (DMPO), 2-methyl-2-nitroso propane (MNP) and ethanol (^{13}C) were purchased from Aldrich chemical Co., Milwaukee, MI. All other chemicals were obtained at the highest obtainable purity.

Preparation of Mitochondria and its particles:

The brain mitochondria of dog or guinea pig was isolated according to the published protocol (21-25) with following modifications: The animals were anaesthetized with pentobarbital. The brain was removed within one minute and was placed in an ice-cold homogenization medium (medium A) which consisted of sucrose (25 *mM*), Mannitol (0.3 M), EGTA (1.0 *mM*) MOPS (5.0 *mM*), KH_2PO_4 (5.0 *m*M) and BSA (0.1%, w/v), and adjusted to pH 7.4 with KOH. The tissue was chopped into small cubes and washed in ice-cold medium A to remove as much blood as possible. The chopped brain was transferred to a pre-cooled Teflon-glass homogenizer and homogenized with 2 ml/gram medium A using six up- and down-strokes of the pestle rotating at 800-1,000 r.p.m. The homogenate was transferred to pre-cooled centrifuge tubes and centrifuged for 8 min at 600 ¥ g (24) at 24 °C. The supernatant was decanted carefully and recentrifuged for 10 min at 1,000 × g at 4 °C and the pellet was discarded. The supernatant was centrifuged for 10 min at 10,000 × g at 4 °C and the mitochondrial pellet was collected.

The pellet, consisted of a tan lower layer and a white fluffy upper layer, was resuspended in Medium A and centrifuged for 10 min at 5,000 × g at 4 °C. The loosely packed white material on the top of the pellet was removed by carefully shaking several times with small volumes of the medium A. The crude mitochondrial pellet was resuspended to 10 volumes of medium A using a cooled glass rod. This suspension was carefully layered onto the medium B, which was made by adding 6% Ficoll-400 (molecular weight of approximately 4×10^5) in medium A, and centrifuged at 10,000 ¥ g for 30 min. Because Ficoll interferes with some biochemical reactions, all fractions were washed twice by medium A without EDTA (to prevent the permeability of the mitochondrial membrane for water (26)) and centrifuged at 5, 000 × g for 10 min.

The submitochondrial particles (SMPs) were prepared by sonicating the mitochondria suspension (27). These particles were found to be suitable for studying

electron transfer and were essentially devoid of matrix enzymes. The preparative steps were as follows:

The above mitochondria from guinea pig or dog brains were resuspended at a concentration of approximately 15 *mg/ml* in the medium A. They were sonicated on ice with a probe sonicator for six 5 sec bursts at the maximum energy setting interspersed with 30 sec cooling periods. The samples were diluted with equal volumes of cold buffer and centrifuged again at 15,000 × g for 10 min at 4 °C. The pellets were resuspended in 10 times its volume of cold buffer (medium A) and centrifuged again at 100,000 × g. This process was repeated twice. The pellet was resuspended in same buffer after the final washing, in half the original volume. The activity of the matrix marker enzyme malate dehydrogenase was measured in the first washing and compared with the final preparation to check the effectiveness of the sonication and subsequent washing.

Polarography:

Oxygen consumption was measured polarographically at 25 °C with a Clarke-type oxygen electrode (Yellow Springs, Co). The reaction mixture contained mitochondria (approx. 2 *m*g protein/ml) and indicated concentrations of inhibitor in air-saturated 0.05 M phosphate buffer, pH 7.4, giving a final volume of 1.5 ml. After 5 min incubation in the polarography chamber, glutamate/malate were added to the pre-incubation mixture giving a final concentration of 5 *m*M. Then, ~3-4 min later, ADP (5 μl) was added to a final concentration of 0.25 *m*M. O_2 consumption in state 3 respiration was recorded for ~1-2 min and then DNP (2 μM)was added to uncouple the reaction. The rate of O_2 uptake was calculated from the slope of state 3 respiration. The concentration of mitochondrial protein was determinated by the dye-binding assay of Bradford (28) using bovine serum albumin as a standard.

Enzyme Assay:

NADH-ubiquinone oxidoreductase activity was measured spectrophotometrically by monitoring the changes in absorbance at 340 *nm* at 25 °C, using an extinction coefficient of 6.23×10^3 $M^{-1}cm^{-1}$ (29). The reaction mixture contained commercial NADH dehydrogenase (0.01 unit/*m*l) or sub-mitochondrial particles (0.6 mg of protein/ml) in 1.0 *m*l of the above buffer in the presence of different concentrations of MPTP or MPP^+. After 5 min incubation, the assay reaction was started by the addition of NADH giving a final concentration of 0.25 *m*M. Spectrophotometric assays were performed in a Shimadzu UV-visible recording spectrophotometer UV-160 at 25 °C.

EPR and Spin Tapping:

In these experiments, MNP, PBN and DMPO were used as the spin traps. The reaction mixture consisted of spin trap and mitochondria or mitochondrial particles. The reactions were started by adding appropriate amounts of mitochondria or its particles stock in 50 *m*M phosphate buffer, pH 7.4. Individual samples were placed in the EPR TM cavity using an EPR aqueous flat cell (60 *mm* × 17 *mm* × 0.25 *mm* i.d.) for the measurement of free radical generation at a given incubation time at room temperature. The recordings of EPR spectra were performed with a Bruker D-200 ER spectrometer. Unless otherwise stated, the EPR parameters were set at: 100 *KHz*, X-band; microwave frequency, 9.78 *GHz*; microwave power, 20 mW; modulation amplitude, 1.0 *G*; time constant, 0.64 *sec.*; scan time, 500 *sec.* and receiver gain, 1×10^6.

RESULTS

Effects of MPTP, $MPDP^+$ and MPP^+ on Mitochondrial Respiration and Submitochondrial Particles:

The effects of MPTP and its metabolites ($MPDP^+$ and MPP^+) on the respiration of isolated intact mitochondrial at state 3 are shown in Figure 1. As shown in this Figure, incubation of MPTP with dog brain mitochondrial preparations did not inhibit the ADP-stimulated O_2 uptake when NAD-linked substrates glutamate/malate were used, rather MPTP was found to slightly increase (15-20%) the respiration of the mitochondria compared to the control rate on the oxygen uptake (Figure 1). Addition of $MPDP^+$ caused a dose-dependent inhibition of the oxygen uptake and at 1 *m*M inhibited ~20% of the rate of oxygen uptake (Figure 1). MPP^+ also caused a dose-dependent inhibition of the ADP-stimulated respiration of the mitochondria and ~80% of O_2 uptake was inhibited at 0.5 *mM* level of MPP^+ (Figure 1). These results are in accord with previously reported results (30-32). Because MAO-B is inlaid in the outer membrane of mitochondria, the small increase of O_2 uptake by MPTP could be due to the MAO-B catalyzed oxidation of MPTP generating the reactive oxygen species (33) and in part increasing O_2 uptake. MPTP and its two metabolites had similar effects on Guinea pig brain mitochondria O_2 uptake, indicating that there is no species difference on the effects of these neurotoxicants on mitochondrial function between dog and guinea pig.

Figure 2 shows the inhibition of NADH dehydrogenase activity by these neurotoxicants. This was based on the spectrophotometric measurements of the initial rate of NADH oxidation at 340 nm by SMPs (Fig. 2a) or purified NADH dehydrogenase (Fig. 2b). As shown in these Figures, the metabolites $MPDP^+$ and MPP^+ had similar inhibitory effects on the NADH oxidation and MPTP inhibited powerfully in both systems. These

results seem to be in contrast to that obtained by intact mitochondria (13) but in accord with previously reported results using NADH-FMN oxido-reductase (34-36).

EPR Spectra of Free Radical Spin Adducts Formed During the Incubation of MPTP With Intact Mitochondria:

The incubation of an air-saturated reaction mixture of MPTP (500 μM) and mitochondria (2 *m*g protein/*m*l) in the presence of DMPO (80 *mM*) in 50 *m*M phosphate buffered (pH 7.4), generated a DMPO-OH spin adduct (Fig. 3a) in the absence and presence of glutamate/malate. In the absence of mitochondria, MPTP, O_2 or DMPO, no detectable EPR signal was observed (data not shown). Also, no signal was obtained in the reaction mixture of mitochondria and MPP^+ (500 μM). The EPR spectrum in Figure 3a consists of four splitting lines with an intensity ratio of 1:2:2:1 which usually result from the interactions of an unpaired electron with a primary nitrogen atom along with a secondary β- proton. The hyperfine splitting constants of the signal ($a_N = a_H = 15$ *G*) are consistent with previously reported values for DMPO-OH in aqueous solutions (39-41). We have previously reported that $\cdot O_2^-$ is produced as a primary radicals species in the MAO-B catalyzed-oxidation of MPTP (33). Since the spin adduct of superoxide with DMPO is known to decompose to DMPO-OH with a half-life of approximately 2 min in aqueous solution (pH 7.4) and dismutation of superoxide radicals to hydroxyl radical could be completed within one second in aqueous solution (42), superoxide dismutase was used in this system in an attempt to verify the role of superoxide radicals in the formation of the above adduct. If superoxide radicals were generated during the MPTP oxidation catalyzed by MAO-B inlaid in the outer membrane of mitochondria, they could either dismute to $H_2O_2 + O_2$ or could react with DMPO to form a DMPO-OOH adduct. Superoxide dismutase, by catalyzing the former reaction at a second order rate constant of 2×10^9 M^{-1} S^{-1} (43, 44), should inhibit the superoxide-dependent DMPO-OH adduct formation.

Superoxide dismutase used up to 10 μg/ml did not inhibit the spin adduct formation in this system.

Because DMPO-OOH adduct could be reduced by certain biological reductants to form DMPO-OH (20), the mechanism of formation of DMPO-OH adduct was further investigated. Ethanol, a well known scavenger of hydroxyl radical (45), can rapidly react with ·OH at a rate constant of $k = 1 \times 10^9$ $M^{-1}S^{-1}$ to produce an alpha-hydroxyethyl radical (CH_3·CH-OH) which in turn can react readily with DMPO to form a spin adduct. The EPR signal of this alpha-hydroxyethyl radical spin adduct is quite different from that of the hydroxyl radical spin adduct. The hydroxyl radicals generated by the spontaneous dismutation of superoxide radicals could be scavenged by ethanol giving rise to the carbon-centered spin adduct EPR signal. The results of our study indicate that this was indeed the case. Thus, when ethanol (2%) was added to the aqueous system, the EPR spectrum exhibited 2 × 3 splitting lines with identical intensity (Fig. 3b). The hyperfine splitting constants for this spin adduct ($a_N = 15.8$ *G* and $a_H = 22.7$ *G*) are consistent with previously reported valuces for DMPO-CH(OH)CH_3 in an aqueous system (46, 47). These results indicate that the production of DMPO-OH is the result of trapping of ·OH and is not due to the reduction of DMPO-OOH adduct.

Because H_2O_2 can be formed as an intermediate during the univalent pathway of oxygen reduction, addition of Fe^{2+} to this system should yield ·OH in a Fenton-type reaction ($Fe^{2+} + H_2O_2$ --------> $Fe^{3+} + \cdot OH + OH^-$). As shown in Fig.3c, addition of Fe^{2+} (10 μM) into the mixture enhanced the EPR signal intensity of DMPO-OH to about 3.5-times of control. In this Fe^{2+} catalyzed system, SOD (up to 10 units/*m*l) had trivial effect on the DMPO-OH signal intensity (data not shown), indicating that the enhancement of the EPR signal of DMPO-OH by Fe^{2+} is contributed by a Fenton-type reaction. In order to further investigate the mechanism of the formation of ·OH, catalase, a specific scavenger for H_2O_2, was used in this system. If the formation of ·OH is due to the reduction of

H_2O_2, catalase should remove this reactive species (H_2O_2) at a rate constant of 1.7×10^7 $M^{-1}S^{-1}$ (48) and block the formation of hydroxyl radical spin adduct with DMPO. The results shown in Fig. 3d indicates that this was indeed the case. Addition of catalase (10.0 $\mu g/ml$) suppressed the EPR signal of DMPO-OH, indicating that hydrogen peroxide was produced in this system and that the formation of ·OH is indeed *via* the intermediate, H_2O_2. These results further confirm the findings of Sandri *et al.* (49) who reported the production of H_2O_2 in the reaction of MPTP with the intact mitochondrial MAO-B. It appears that H_2O_2 can be produced and accumulated in this reaction system by directly reducing O_2 by two successive steps of single electron transfer and/or by the dismutation of superoxide radicals released by the reduced MAO-B inlaying in the outer membrane of mitochondria.

EPR Spectra of Spin trapped Free Radicals during the interaction of MPTP and MPP^+ with Submitochondrial Particles:

MNP was used as a spin trap in this experiment because nitroso compounds have an inherent advantage over nitrones for free radical identification in that the added group lies immediately adjacent to the nitroxide center and therefore can easily give rise to additional hyperfine splitting and helps to identify the radical trapped (50). As shown in Figure 4a, an EPR spectrum resulting from two different types of spin trapped radicals was detected during incubation of submitochondrial particles (SMPs) with NADH in the dark in the presence of the spin trap (MNP) containing 150 *m*M ethanol in air-saturated 50 mM phosphate buffer (pH 7.4) at 25 °C. In the absence of SMPs, NADH or spin trap, no detectable EPR signal was observed, indicating that the free radicals were produced in the enzyme catalyzed oxidation of NADH. Similar results were obtained in the system consisting of purified NADH dehydrogenase and β-NADH in the presence of MNP under

above conditions. An enhancement of EPR signal intensity was observed when SMPs were treated with MPP^+ or MPTP for 5 min (Fig. 4b and c).

It is apparent that the observed spectra were due to two spin adducts, each species exhibiting 2 × 3 splitting lines which usually results from the interactions of an unpaired electron with a primary nitrogen atom along with the secondary β-proton. The hyperfine splitting constants for the spectral lines marked "H" were found to be a_N = 14.6 G and $^{\beta}a_H$ = 14.4 G which is in accord with previously reported values for the spin adduct formed by one electron reduction of MNP followed by a proton addition (49, 50). The EPR spectral lines marked "C" possesses the characteristics of the MNP-$CH(CH_3)OH$ radical (51, 52) and its hyperfine splitting constants were found to be a_N = 15.7 G, $^{\beta}a_H$ = 2.0 G which is similar to the previously reported values for this spin adduct. The latter species also was confirmed by the isotope effect on the EPR spectrum. As shown in Figure 4d, 2 × 3 splitting lines of the EPR spectrum of MNP-$CH(CH_3)OH$ was converted to 3 × 2 × 2 lines when CH_3CH_2OH was replaced by $CH_3{}^{13}CH_2OH$. It is evident that this EPR spectrum results from the interactions of an unpaired electron with a primary nitrogen atom along with ^{13}C and the secondary β-proton, indicating that the spin adduct was from trapping of the alpha-hydroxyethyl radical (CH_3·CH-OH).

Since ethanol does not react with e_{aq}^- (53), the alpha-hydroxyethyl radical (CH_3·CH-OH) was assumed to be produced during the interaction of hydroxyl radical with ethanol. In order to further identify the primary species, SOD and catalase were used to the above SMPs catalytic reaction system without inhibitor. SOD was found to inhibit, not only the EPR signal of MNP-$CH(CH_3)OH$, but also MNP-H. Thus, SOD at 10 units/ml inhibited ~90% of both EPR signals, indicating that superoxide radicals are generated as primary species in this reaction. Whereas catalase (20-25 μg/ml) completely suppressed the EPR signal of MNP-$CH(CH_3)OH$, indicating hydrogen peroxide was produced in the NADH dehydrogenase catalyzed oxidation of NADH.

Although both EPR signal of MNP-H and MNP-$CH(CH_3)OH$ were enhanced by MPTP (data not shown) or MPP^+ treatment using SMPs, the EPR signal intensity of only MNP-$CH(CH_3)OH$ produced in purified NADH dehydrogenase system was found to be inhibited by these neurotoxicants (Fig. 5). The rate of MNP-$CH(CH_3)OH$ formation was faster than that of MNP-H in the absence of MPTP or MPP^+ (see the lower two curves in Fig. 5a). In a parallel experiment but with purified NADH dehydrogenase, neither MPTP nor MPP^+ had significant effect on the EPR signal intensity of MNP-H. The signal intensity from MNP-$CH(CH_3)OH$ was, however, inhibited about 35% of the control at 0.5 mM MPP^+ (Fig. 5b). Rotenone (10 μM), a specific inhibitor of complex I, inhibited 50-60% of the EPR signal of MNP-$CH(CH_3)OH$, indicating that the mechanism of MPTP or MPP^+ inhibition may be similar to that of rotenone.

DISCUSSION

The present investigations were designed to examine the possibility of the neurotoxicants MPTP and its metabolites stimulating the generation of free radicals during their interaction with isolated intact mitochondria and submitochondrial particles. It was found that the reactive oxygen species ($\cdot O_2^-$, H_2O_2 and $\cdot OH$) were produced in reaction systems containing NADH and submitochondrial particles, or with purified NADH dehydrogenase and NADH, and using intact mitochondria with NADH-linked substrates glutamate/malate. MPTP and its metabolites were found to inhibit the generation of these reactive species in SMPs and purified NADH dehydrogenase systems. No species difference between guinea pig and dog brain mitochondria was observed. This imformation will be further discussed below.

The reactive oxygen species were produced during the interaction of MPTP with intact mitochondria:

It was reported that H_2O_2 was produced during the oxidation of MPTP by MAO-B inlaid in the outer membrane of mitochondria (55). The present study further confirmed the production of H_2O_2 in this enzymatic reaction by EPR in combination with spin trapping techniques. Since $\cdot O_2^-$ is the precursor of H_2O_2 and can spontaneously dismutate to H_2O_2 at the rate constant of 7.6-8.5 $\times 10^7$ $M^{-1}S^{-1}$ (56) and the rate of breakdown of H_2O_2 to $\cdot OH$ by Haber-Weiss reaction is only 0.13-0.5 $M^{-1}S^{-1}$ (57-59), a small but detectable concentration of H_2O_2 may accumulate under certain conditions. Ferrous iron can rapidly reduce H_2O_2 by Fenton-type reaction to form $\cdot OH$ which in turn can be trapped by DMPO at a rate constant of 3.4 $\times 10^9$ $M^{-1}S^{-1}$ (60) to form DMPO-OH adduct. Therefore, the EPR signal intensity of DMPO-OH spin adduct should be enhanced by the addition of Fe^{2+} to the reaction mixture. As shown in figure 3b, as little as 10 μM of Fe^{2+} enhanced the EPR signal to about 3.5-times of the control, while SOD (1.0 μg/ml) had trivial effect on the DMPO-OH signal, confirming that the enhancement of the EPR signal is due to the Fenton-type reaction. The demonstration that catalase (at 10.0 μg/ml), a specific scavenger for H_2O_2 at a rate constant of 1.7 $\times 10^7$ $M^{-1}S^{-1}$ (48), virtually eliminated the signal further confirms that H_2O_2 was produced during the oxidation of MPTP probably during MAO-B (inlayed in the outer membrane of mitochondria) catalyzed oxidation of MPTP (20).

The incubation of the mixture of MPTP and isolated intact mitochondria in air-saturated aqueous solutions gave rise to free radicals which, upon reacting with DMPO, gave a spin adduct whose EPR spectrum exhibited four lines in an intensity ratio of 1:2:2:1 (Fig. 3b) with hyperfine splitting constants of $a_N = {}^{\beta}a_H$ = 15.0 G, consistent with DMPO-OH adduct characteristics (39-41). Ethanol, a scavenger of hydroxyl radical with a rate constant of 1 $\times 10^9$ $M^{-1}S^{-1}$ (45) produced an alpha-hydroxyethyl ($CH_3\dot{C}H$-OH) radical, which was readily trapped by DMPO to form DMPO-$CH(OH)CH_3$ spin adduct, further

confirming the formation of ·OH in this aqueous system. Although superoxide dismutase (10 units/ml) did not inhibit the EPR signal of DMPO-OH, we believe that the primary species in aqueous solution manifested as DMPO-OH adduct is $\cdot O_2^-$. This belief is based on the powerful inhibition of SOD (as little as 1 μg/ml) on the EPR signal of DMPO-OH produced in the purified MAO-B catalytic reaction (20). The inability of SOD inhibiting the EPR signal in intact mitochondria may be due to the inaccessibility of SOD to the superoxide radical generating site. However, in the presence of Fe^{2+}, SOD had trivial effect on the DMPO-OH signal, indicating that SOD cannot effectively compete with the Fenton-type reaction. It does establish however that hydroxyl radicals are produced by both Haber-Weiss and iron catalyzed reactions following the generation H_2O_2. Since the EPR signals were generated in the air-saturated reaction mixture of MPTP and intact mitochondria in the absence and presence of NADH-linked substrates glutamate/malate and since no detectable EPR signal was observed in the absence of the intact mitochondria, the generation of these reactive oxygen species may be related to MAO-B catalysis of MPTP as reported before (20). Since neurons are rich in iron (61), the Fenton reaction is expected to predominate in nerve cells exposed to MPTP generating the most potent oxidant, ·OH, which in turn could destroy these cells.

The reactive oxygen species were produced in the NADH dehydrogenase catalytic reaction system:

We have observed the formation of the spin adducts of hydrogen atom and alpha-hydroxyethyl radical with MNP when NADH dehydrogenase was allowed to act on NADH. Since there is an equilibrium between ·H and e_{aq}^- ($H_2O + e_{aq}^- \leftrightarrow \cdot H + OH^-$), and since MNP can rapidly trap the ·H at a rate of 1×10^9 $M^{-1}S^{-1}$, our data suggest that the MNP-H adduct is formed from ·H. However, oxygen can react with e_{aq}^- or ·H to form superoxide radicals at a rate of 1×10^{10} $M^{-1}S^{-1}$ (43). The superoxide radicals so produced

can dismutate spontaneously to H_2O_2 followed by the formation of ·OH *via* the Haber-Weiss or iron catalyzed reactions in which this process could be completed within one second in an aqueous system (42). SOD inhibited both EPR signals of MNP-H and MNP-$CH(CH_3)OH$ and catalase removed MNP-$CH(CH_3)OH$ only, further confirming that superoxide radicals were indeed produced in the NADH dehydrogenase catalyzed reaction system.

"Redox cycling" of $MPP^+/MPP^·$ could be negated:

Although the "redox cycling" reactions between MPP^+ and dopamine or other cellular constituents has been proposed based on the similarity of chemical structures between MPP^+ and paraquat (63, 64), no evidence was presented to confirm its existence. Indirect evidence suggests (65) that MPP^+ induces a small amount of oxygen radicals but this has been questioned by others (16). The redox cycling of $MPP^+/MPP^·$ is unlikely because (i) we were not able to detect $MPP^·$ apices in our reaction system, and (ii) $MPP^+/MPP^·$ redox cycling is thermodynamically unfavorable because of the high negative redox potential (-1.09 V). Although paraquat and MPP^+ have similar bipyridium structure, the mechanism of redox cycling of $MPP^+/MPP^·$ is quite different them $PQ^{2+}/PQ^{+·}$ because of the high negative redox potential (-1.09 V) for MPP^+ compared to PQ^{2+} (-0.45 V). Therefore, we propose that the enhanced EPR signal by MPP^+ was not due to redox cycling of MPP^+ but probably due to its blocking of electron transport chain between NADH dehydrogenase and coenzyme Q.

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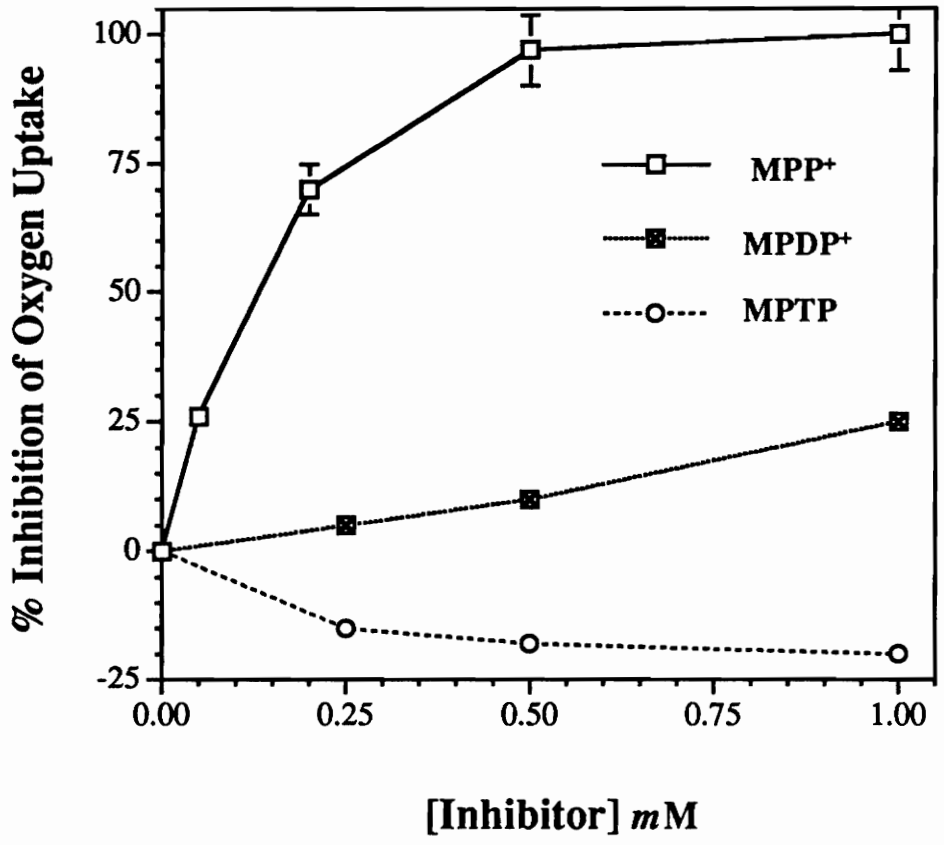

Figure 1. The effect of inhibitor concentrations on state 3 respiration of dog brain mitochondria. The mitochondria (2 mg protein/ml) was incubated with indicated concentrations of MPTP, $MPDP^+$ and MPP^+ for 5 min at 25 °C followoed by the addition of 5 *m*M glutamate/2.5 *m*M malate and 0.25 *m*M ADP. Inhibition was determined as a percentage of the value for the corresponding control which is the same above assay without inhibitors. The each point is the mean value from 3-5 measurements from five preparations.

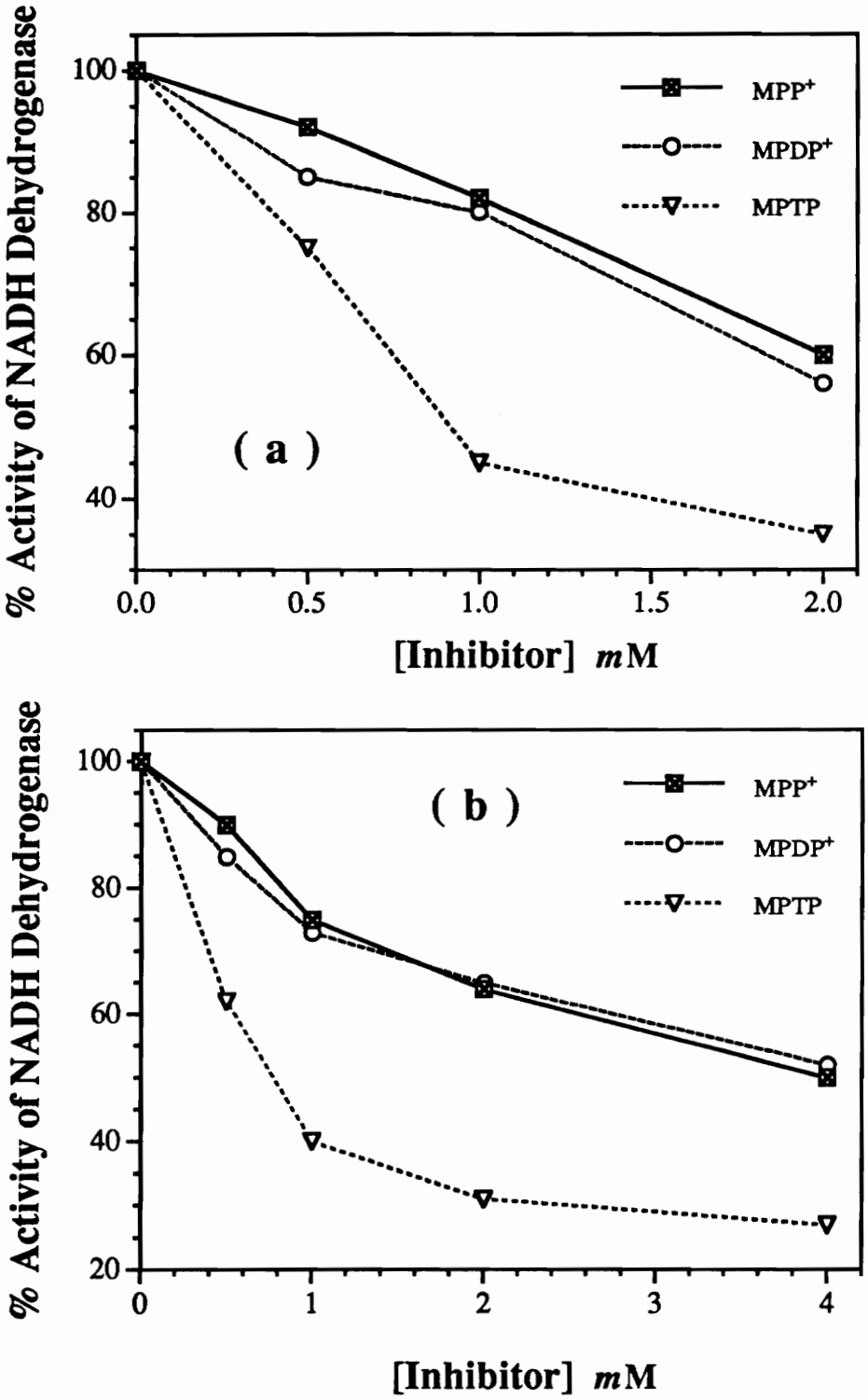

Figure 2. The effect of inhibitors concentrations on the NADH dehydrogenase activity. The sonicated dog brain mitochondrial particles (0.5 *m*g protein/ml) or purified enzyme (0.005 unit/ml) were incubated in 50 mM phosphate buffer (pH 7.4) containing 0.25 *m*M NADH in the absence and presence of indicated amounts of inhibitors. The data on Fig. a and b were obtained using SMPs and purified NADH dehydrogenase, respectively.

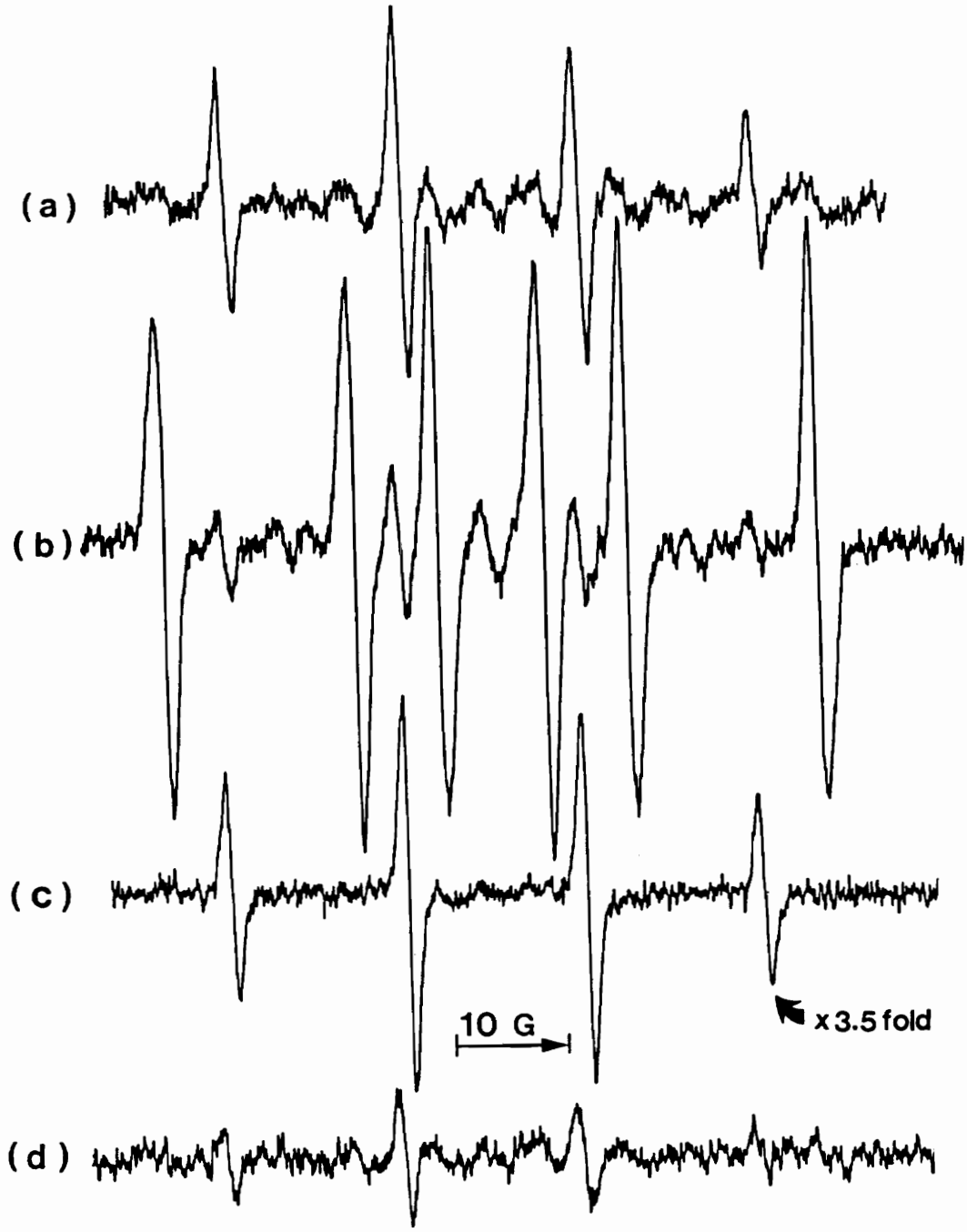

Figure 3. EPR spectra of spin adducts of free radicals observed during the incubation of the intact mitochondria of dog brain and NADH-linked substrate glutamate/mlate in the absence and presence of MPTP in 50 *m*M phosphate buffer (pH 7.4). The reaction mixtures consisted of isolated intact mitochondria (2 *m*g protein/ml), MPTP (500 μM) and DMPO (80 *m*M) in air-saturated aqueous solution of 50 *m*M phosphate buffer, pH 7.4, and were incubated for 5 minuts at 25 °C. (a) MPTP, DMPO and mitochondria in the absence and presence of 5 *m*M glutamate/2.5 *m*M malate; (b), (c) and (d) conditions similar to "a" but in the presence of 2% ethanol for (b); Fe^{2+} (10 μM) for (c); and catalase (10 $\mu g/ml$) for (d). EPR spectroscopy settings were: modulation amplitude, 2.0 G; time constant, 0.64 sec; and receiver gain to 1.0 $\times 10^6$ for (a), (b) and (d); Please note the change of EPR setting: (c) modulation amplitude, 0.8 G; time constant, 0.32 sec; and receiver gain to 5.0 $\times 10^5$ to decrease the amplitude by 3.5 fold.

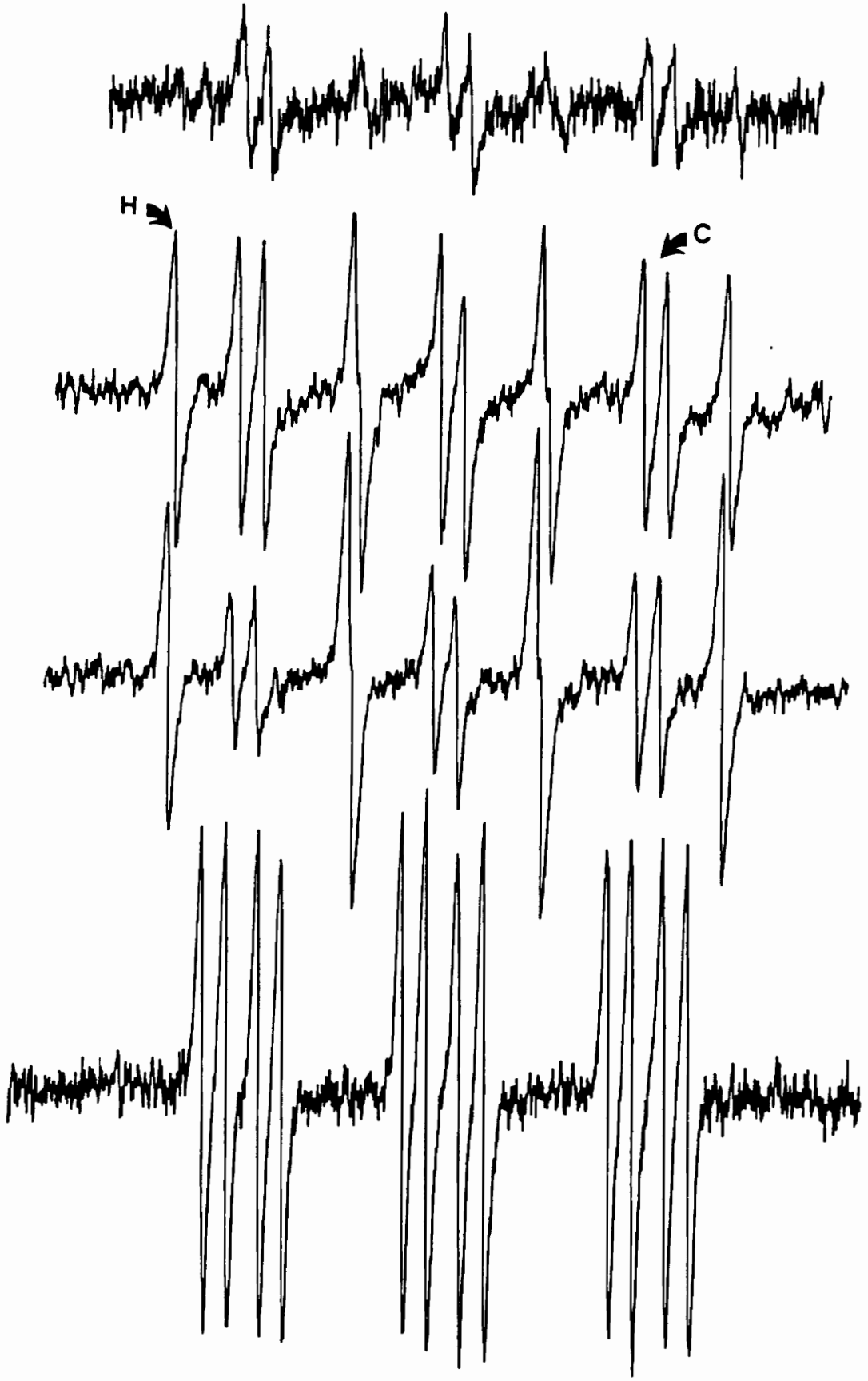
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Figure 4. The EPR spectra of spin trapped free radicals obtained in the NADH dehydrogenase cataytic reactions in the absence and presence of MPTP or MPP^+. The reaction mixtures consisted of SMPs (1 mg protein/ml), NADH (0.6 mM) and MNP (10 mM) in 50 mM phosphate buffer, pH 7.4 containing 10% ethanol. (a) no inhibitor; (b) with 1 mM MPP^+; (c) 1 mM MPTP; and (d) reaction mixture consisted of purified NADH dehydrogenase (0.01 unit/ml) and NADH (0.6 mM) containing 10% $CH_3{}^{13}CH_2OH$. All experiments were performed in the dark at room temperature. EPR spectroscopy settings were microwave power, 20 mW; modulation amplitude, 0.8 G; time constant,0.64 sec; receiver gain, 8×10^5 and scan time, 500 sec, except that (a) receiver gain to 1.0×10^6; and (d) time constant, 0.32 sec.

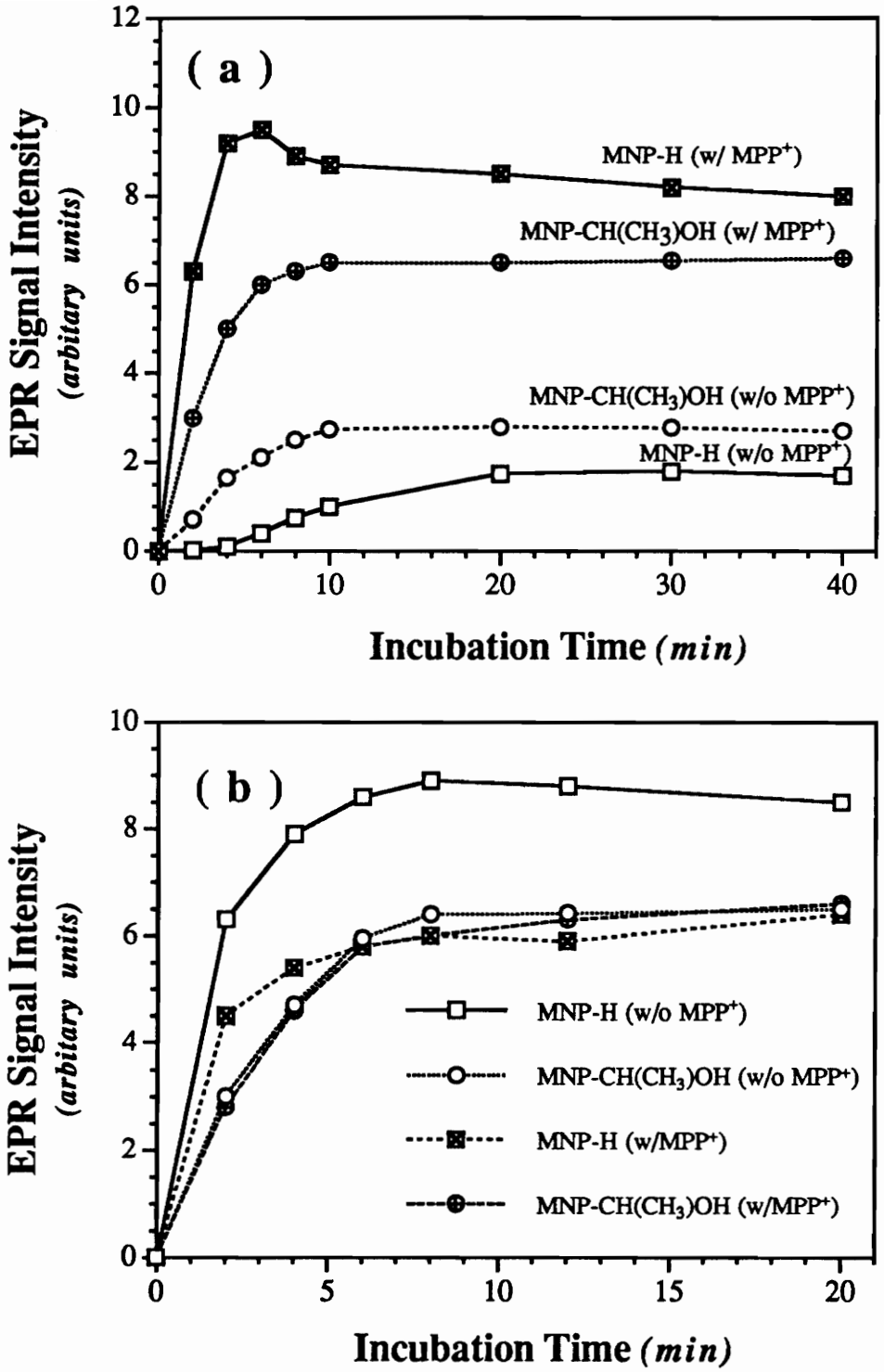

Figure 5. Effect of incubation time on the EPR signal intensity of spin adducts in the absence and presence of MPP^+ (2 mM). (a) the reaction mixtures contained SMPs (1 mg protein/ml) and NADH (0.6 mM) in the presence of MNP (10 mM) in 50 mM phosphate buffer (pH 7.4) with and without MPP^+; (b) The mixture consisted of NADH dehydrogenase (0.005 unit/ml) and NADH (0.6 mM) in the presence of MNP (10 mM) in phosphate buffer pH 7.4 with and without MPP^+. For the effect of inhibitor on spin aducts formation, both reaction mixtures were preincubated with MPP^+ for 5 min followed by the addition of other reagents.

CHAPTER IX

SUMMARY

The mechanism(s) of action of the neurotoxicant MPTP in inducing Parkinson's disease-like syndrome was investigated. Using EPR spin trapping techniques and enzymological manipulations we found that:

(1) MPTP and its metabolites $MPDP^+$ and MPP^+ were able to inhibit acetylcholinesterase activity. The inhibition by MPTP was completely reversible where as by $MPDP^+$ and MPP^+ were only partially reversible. The kinetic parameter, K_m for the substrate acetylthiocholine was found to be 0.216 *m*M. The studies of reaction kinetics indicate that the inactivation of acetylcholinesterase to be a linear mixed type inhibition with K_i for MPTP, $MPDP^+$ and MPP^+ determined to be 2.14, 0.265 and 0.197 mM, respectively. We conclude that the neurotoxic action of these toxicants may be due in part to the inhibition of AChE leading to the accumulation of ACh levels which inturn can imbalance dopamine/ACh ratio and destroy the nerve cells.

(2) Monoamine oxidase B (MAO-B) generates reactive oxygen species during its interaction with MPTP. The kinetic parameters, K_m and V_{max} for MAO-B catalyzed oxidation of MPTP to the corresponding $MPDP^+$ species were determined to be 0.194 *m*M and 0.34 μM/min, respectively. Generation of $\cdot O_2^-$ and $\cdot OH$ was detected by EPR-spin trapping techniques using DMPO as a spin trap. We propose that the neuro-toxicity of MPTP leading to Parkinson's disease-like syndrome may in part be mediated via these reactive oxygen species.

(3) $MPDP^+$, a metabolic product of MPTP generates superoxide radicals during its autoxidation process. The maximum rate of $\cdot O_2^-$ generation at a fixed spin trap concentration was found to be 4.48×10^{-10} $M \cdot S^{-1}$. The rate constant (k) for $MPDP^+$ making $\cdot O_2^-$ was found to be 3.97×10^{-6} sec^{-1}. We conclude that the nigrostriatal toxicity of MPTP may largely be due to the reactivity of these radicals produced during its metabolism.

(4) Mitochondria and submitochondrial particles from brains of dog and guinea pig generate reactive oxygen species when treated with MPTP and NADH-linked substrates of electron transport chain, respectively. Using MNP as spin trap a H atom and alpha-hydroxyelthyl radical species were detected in submitochondrial particles when treated with NADH. Similar spin adducts were obtained when submitochondrial particles were replaced with NADH dehydrogenase. Both MPTP and MPP^+ were found to enhance the intensity of these signals.

The above *in vitro* findings challenge us to think in a new light regarding the possibility that MPTP is actually acting as a "double edged sword". It not only appears to generate free radicals by interacting with MAO-B and mitochondrial electron transport system, it also causes acetylcholine to accumulate by inactivating the AChE. If occurs *in vivo*, both phenomena would lead to neuronal cell dysfunction.

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