1993

The Effects of N-Methylated Î²-Carbolinium Analogues of 1-Methyl-4-Phenylpyridinium (Mpp+) on the Cultured Neuron-Like Cell Line-Pc12

Robert Joseph Cobuzzi
Loyola University Chicago

LOYOLA UNIVERSITY CHICAGO

THE EFFECTS OF N-METHYLATED ß-CARBOLINIUM ANALOGUES OF 1-METHYL-4-PHENYLPYRIDINIUM (MPP^+) ON THE CULTURED NEURON-LIKE CELL LINE - PC12

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL OF
LOYOLA UNIVERSITY CHICAGO
IN PARTIAL FULFILLMENT OF THE REQUIREMENT OF THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF MOLECULAR AND CELLULAR BIOCHEMISTRY

BY

ROBERT JOSEPH COBUZZI JR.

CHICAGO, ILLINOIS

JANUARY 1993

ACKNOWLEDGMENTS

I wish to thank my wife, Renata, and my parents, Beverly and Bob, whose tremendous support made this all possible. I would also like to thank my advisor, Dr. Michael Collins, for his support and advice; my co-advisor, Dr. E.J. Neafsey, for his involvement and discourse; Dr. Leonard Erickson and his colleagues, for initiating my interest in molecular biology; my other committee members, Dr. George Battaglia, Dr. Mary Druse-Manteuffel and Dr. William Simmons for their helpful comments and criticisms; and the many members of the Department of Molecular and Cellular Biochemistry who provided me with moral and technical support.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

LIST OF ABBREVIATIONS

α	alpha
ANOVA	analysis of variance
ATP	adenosine triphosphate
ß	beta
B	amount of radioligand bound
B_o	amount of radioligand bound in the absence of inhibitor
BBB	blood-brain barrier
ßC	beta-carboline
BSA	bovine serum albumin
°C	degrees Celcius
cGMP	guanosine 3',5'-cyclic monophosphate
cm	centimeter
CoQ	coenzyme Q
ΔA	change in absorbance
DA	dopamine = 3,4-dihydroxyphenethylamine
DHßC	3,4-dihydro-beta-carboline
DMEM	Dulbecco's Modified Eagle Media
DMI	desipramine = desmethylimipramine
DOPAC	dihydroxyphenylacetic acid
dpm	disintegrations per minute
EC_{50}	concentration of compound producing 50% of total effect

FBS	fetal bovine serum
fmol	femtomole
f.o.	fractional occupancy
g	acceleration due to gravity
GBR 12909	1-(2-[bis-(4-fluorophenyl)methoxy]ethyl)piperazine
h	hour
^3H	tritium ion
[^3H]DA	tritium-labeled dopamine
[^3H]GBR 12935	tritium-labeled 1-(2-diphenylmethoxy)ethyl-4-(3-phenylpropyl)piperazine
H_2O_2	hydrogen peroxide
Ha	harman
Hi	harmine
Hli	harmaline
Hlo	harmalol
Ho	harmol
Homog	homogenate
[^3H]NE	tritium-labeled norepinephrine
HS	horse serum
HVA	homovanillic acid
[I]	concentration of inhibitor
IC_{50}	concentration of inhibitor producing 50% inhibition
K^+	potassium ion
K_d	equilibrium dissociation constant
K_i	equilibrium dissociation constant for a competitive inhibitor

KRP	Kreb's-Ringer phosphate
[*L]	concentration of radiolabeled ligand
LDH	lactate dehydrogenase
L-DOPA	levo-3,4-dihydroxyphenylalanine
M	molar = moles per liter
mA	milliamps
MAO	monoamine oxidase
Me	methyl
2-MeHa$^+$	2-methylharmanium
2-MeHi$^+$	2-methylharminium
2-MeHli$^+$	2-methylharmalinium
2-MeHlo$^+$	2-methylharmalolium
2-MeHo$^+$	2-methylharmolium
2,9-Me_2Ha$^+$	2,9-dimethylharmanium
2,9-Me_2Hi$^+$	2,9-dimethylharminium
2-MeNh$^+$	2-methylnorharmanium
2,9-Me_2Nh$^+$	2,9-dimethylnorharmanium
MeO	methoxy
6-MeO-2-MeHa$^+$	6-methoxy-2-methylharmanium
6-MeO-2-MeHla$^+$	6-methoxy-2-methylharmalanium
2-MeTHßC	2-methyltetrahydro-beta-carboline
mg	milligram
µCi	microcurie
µg	microgram
µl	microliter
µM	micromolar

ml	milliliter
$MPDP^+$	N-methyl-4-phenyl-3,4-dihydropyridinium
MPP^+	N-methyl-4-phenylpyridinium
MPTP	N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
N	normal
Na^+	sodium ion
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide, reduced form of
NAM	N-methyl-nicotinamide
NE	norepinephrine = noradrenaline
Nh	norharman
n_H	Hill coefficient
nm	nanometer
nM	nanomolar
N-5	tissue culture medium
$^{\cdot}OH$	hydroxyl radical
p	probability value
PBS	phosphate-buffered saline
PC12	pheochromocytoma-derived cell line
PLSD	protected least significant difference
PS	penicillin/streptomycin solution
prot	protein
rpm	revolutions per minute
SEM	standard error of the mean
TH	tyrosine hydroxylase
THßC	tetrahyro-beta-carboline

Tris	Tris(hydroxymethyl)aminomethane
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
U	unit of activity of a protein
v	volume
w	weight

CHAPTER I

INTRODUCTION

Parkinson's disease (PD) is a degenerative disorder of the central nervous system (CNS) that develops in individuals primarily between the ages of 50 and 79, and effects up to 1.5% of the population between 70-79 years of age (Martilla, 1987). The clinical characteristics of PD include akinesia, rigidity and tremor (Agid, 1991). These symptoms occur as the result of a profound decrease in nigrostriatal dopamine (DA) content which is concurrent with the loss of pigmented (melanin-containing) DA neurons in the zona compacta of the substantia nigra (Hirsch *et al.*, 1988) (see fig. 1). In addition to the degeneration of the nigral neurons, the presence of eosinophilic cytoplasmic inclusions, termed Lewy bodies, is considered neuropathological confirmation of PD (Agid, 1991). Furthermore, Niznik *et al.* (1991) have observed a reduction in DA uptake sites in parkinsonian postmortem tissues and found that there was no detectable levels of the DA transporter in parkinsonian putamen, and the number of these sites is reduced in the caudate by approximately 65%.

Although the neurochemical and physiological manifestations of PD have been well characterized, the etiology remains enigmatic. Numerous hypotheses have been advanced to explain the selective degeneration of nigrostriatal DA neurons in idiopathic PD. Aging, heredity, stress, trauma, lack of neurotrophic hormone and exposure to toxins have all been proposed as putative pathogenic factors, but none of these have been definitively implicated (Maret *et al.*, 1990; Poirier *et al.*, 1991; Tanner *et al.*, 1987). The clinical onset of PD appears to be preceded by a subclinical period

that may be initiated by one or more of these factors at least 20-30 years prior to the manifestation of clinical symptoms (Martilla, 1987). Consequently, most of the research on the etiology of PD has focused on the search for the elusive precipitating factor(s).

The research described in this dissertation is based upon the hypothesis that one or more of a family of putatively endogenous compounds, N-methylated ß-carbolines (ßC$^+$s) or 3,4-dihydro-ß-carbolines (DHßC$^+$s), may act as causative agents in idiopathic PD (Collins and Neafsey, 1985; Ohkubo *et al.*, 1985; Ramsden and Williams, 1985; Testa *et al.*, 1985). These compounds are structural analogues of N-methyl-2,3-dihydropyridinium (MPDP$^+$) and N-methyl-4-phenylpyridinium (MPP$^+$) which are the oxidation products of the illicit narcotic contaminant, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP produces a syndrome which closely mimics PD in both its clinical and pathological manifestations (Davis *et al.*, 1979; Langston *et al.*, 1983). Consequently, MPTP and its toxic metabolite, MPP$^+$, have become the most widely applied experimental tools in the study of idiopathic PD, and they serve as comparative models for other potential parkinsonian toxins.

The purpose of this dissertation is to examine the direct cytotoxic effects of the putative parkinsonian neurotoxins, N-methylated ßC$^+$s and DHßC$^+$s. To this end, the following specific aims are proposed:

<u>Aim I.</u> Employing the clonal, catecholaminergic cell line (PC12) as a model system, the cytotoxic potential of a series of N-methylated ßC$^+$s and DHßC$^+$s and the relative potencies of the toxic species would be determined. In order to assess toxicity, three parameters would be measured: 1) The release of the cytosolic enzyme lactate dehydrogenase (LDH) into the growth medium by damaged or dead cells, 2) the cell protein concentration in the wells as a measure of cell viability, and 3) the uptake of radiolabeled DA by viable cells.

Aim II. To investigate the hypothesis that N-methylated ßC$^+$s and DHßC$^+$s are accumulated by dopaminergic neurons via the DA uptake system, the specificity of the various compounds for PC12 cell catecholamine uptake sites would be determined. This was done by assessing the abilities of the N-methylated ßC$^+$s and DHßC$^+$s to inhibit the binding or accumulation of radiolabeled ligands specific for the DA and norepinephrine (NE) uptake sites in PC12 cells.

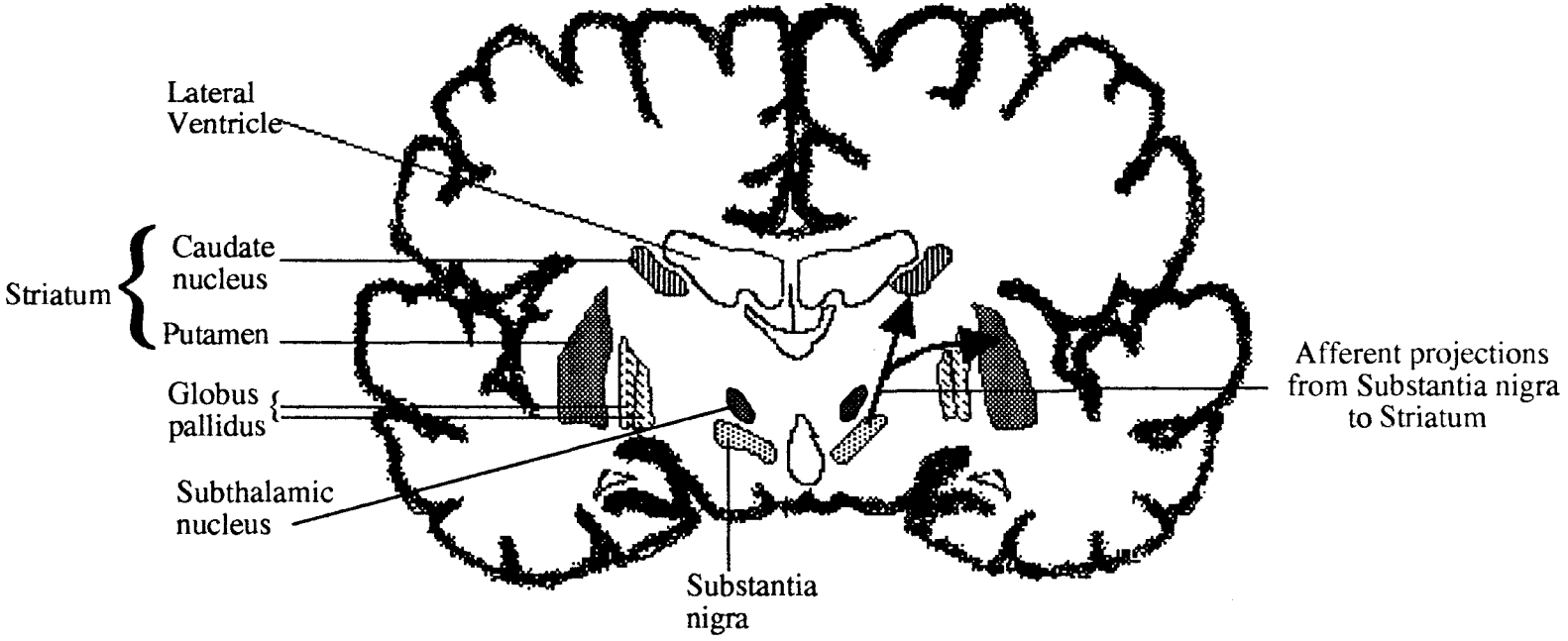


Fig. 1. SCHEMATIC REPRESENTATION OF THE AFFERENTS FROM THE SUBSTANTIA NIGRA ZONA COMPACTA TO THE STRIATUM (CAUDATE NUCLEUS AND PUTAMEN) THAT ARE DESTROYED IN PD. In this coronal section through a human brain, the zona compacta of the substantia nigra comprises the dorsal portion of the nuclei (top portion in this figure), and it contains the pigmented cell bodies. The ventral portion of the substantia nigra, the zona reticulata, is not pigmented. Figure was adapted from Nolte (1981).

CHAPTER II

REVIEW OF RELATED LITERATURE

As mentioned in Chapter I, there are numerous hypotheses concerning the etiology of idiopathic PD, but no single factor has been definitively implicated. The role of heredity in PD has been examined extensively (Calne and Langston, 1983; Duvoisin *et al.*, 1981; Poirier *et al.*, 1991; Tanner *et al.*, 1987; Ward *et al.*, 1983), but the results of these studies were inconclusive. Likewise, no causal relationships have been established for either viral infection or stress factors such as head trauma (Poirier *et al.*, 1991; Tanner *et al.*, 1987). Many of the other proposed posits for the degeneration of the nigral neurons implicate a toxic insult of either endogenous or exogenous origin superimposed on either normal aging or a genetic predisposition. Since this general tenet is the basis for the underlying hypothesis of this dissertation, it will be the focus of the first section of this chapter.

The chapter is divided into three primary sections. The first of these, The Etiology of Parkinson's Disease, discusses various risk factors under consideration for the development of PD. The second section, MPTP and PC12 Cells, summarizes the literature on the mechanism of MPTP neurotoxicity and discusses its effects on PC12 cells. The third section, ß-Carbolines and 3,4-Dihydro-ß-Carbolines, reviews the literature on ßCs and DHßCs, and concludes with a discussion of their neurotoxic effects.

The Etiology of Parkinson's Disease

Aging

Although it does not appear to play a primary role in the development of PD, aging is certainly a prominent factor. It has been reported that an approximately 80-90% decrease in DA, relative to age-matched controls, is necessary before the appearance of the clinical manifestations of PD (Bernheimer *et al.*, 1973; Kish *et al.*, 1992; Pifl *et al.*, 1990; Zigmond and Stricker, 1989). Kish *et al.* (1992) have observed that approximately 60% of the DA is lost in both striatal nuclei throughout the normal aging process, but the pattern of this degeneration differs from that observed in a PD brain, where the loss is more pronounced in the putamen than the caudate nuclei. However, the substantial decrease in striatal DA due to normal, age-related processes may lower a patient's tolerance to insult, thus contributing to the manifestation of PD in the aged.

Metabolic Defects

Under normal conditions, xenobiotic compounds are absorbed and enzymatically metabolized to excretable forms. If such compounds are not so converted, they are retained within the body and often diffuse across the blood-brain barrier (BBB) into the brain where they may induce a toxic effect (Waring *et al.*, 1989). An example of this type of metabolic enzyme is the hepatic cytochrome P450 isozyme, P-450IID1 (Fonne-Pfister *et al.*, 1987; Fonne-Pfister and Meyer, 1988). This and other cytochrome P450 enzymes are responsible for the hydroxylation of a number of drugs including debrisoquine, sparteine, bufuralol, propanolol, nortryptiline, desipramine, 4-methoxyamphetamine and dextromethorphan (Fonne-Pfister and Meyer, 1988). Certain individuals have genetic polymorphisms which impair their ability to metabolize these compounds. In particular, those individuals

who are unable to 4-hydroxylate the anti-hypertensive, debrisoquine are homozygous for an autosomal recessive gene and have been termed poor metabolizers (PM) (Barbeau *et al.*, 1985).

Among PD patients, it has been reported that there is a higher incidence of the PM phenotype, and that those patients tended to have an earlier onset of PD (Barbeau *et al.*, 1985). Because hydroxylation by this enzyme is an important xenobiotic detoxification pathway, Barbeau and colleagues (Barbeau *et al.*, 1985) reasoned that individuals who possessed such a defect would be more susceptible to toxic factors and possibly more likely to develop PD. However, in a later paper (Poirier *et al.*, 1987) the authors noted that they had not controlled for their patients' use of other drugs in their earlier study (Barbeau *et al.*, 1985). Therefore, the observed differences between PD and control patients were a consequence of the extraneous medication (Barbeau *et al.*, 1985). Other groups have also examined debrisoquine metabolism in PD patients (Comella *et al.*, 1987; Steventon *et al.*, 1989a; Steventon *et al.*, 1989b) and found no relationship between the PM phenotype and PD.

Other research indicates that there may in fact be a link between metabolism and PD. Williams and colleagues at the University of Birmingham have examined the roles of other metabolic defects in the etiology of PD (Green *et al.*, 1991; Steventon *et al.*, 1989b; Waring *et al.*, 1989). The enzyme thiolmethyltransferase catalyzes S-methylation of aliphatic sulphydryl compounds, such as the neurotoxin, hydrogen sulphide, which is formed endogenously and is present in the environment (Waring *et al.*, 1989). Waring *et al.* (1989) measured the activity of thiolmethyltransferase in the red blood cells of PD patients and found that activity was decreased in this population. This same group also examined S-oxidation capacity in PD patients by administering the expectorant, S-carboxymethyl-L-cysteine, and measuring the formation of S-oxide metabolites in the urine (Steventon *et al.*, 1989b). Since the endogenous role of S-

oxidation is the cleavage of inorganic sulphate from cysteine, which is the rate-limiting step in the formation of soluble sulphate conjugates, they also measured the formation of sulphate-conjugates of paracetamol (acetaminophen) (Steventon *et al.*, 1989b). As with S-methylation, a decreased level of S-oxidation activity was observed in PD patients relative to controls.

Recently, the Williams group reported increased N-methylation activity in PD patients (Green *et al.*, 1991). They examined the formation of N-methyl-nicotinamide (NAM) from nicotinamide by cytosolic N-methyltransferases. Concurrent with the increase in NAM, there was a decreased formation of the hydroxylated metabolite, N-methyl-2-pyridone. This indicated that there was impaired hydroxylation activity in PD patients, but whether the defect was in the cytochrome P450 system, as discussed previously, or in another oxidative enzyme, such as xanthine or aldehyde oxidase, was not clear (Green *et al.*, 1991). The authors suggest that the increased N-methylation combined with the decreased hydroxylation may make such individuals more susceptible to compounds whose methylated derivatives are more toxic than the parent compound, such as pyridines, isoquinolines and ß-carbolines (Green *et al.*, 1991).

In general, the metabolic studies discussed here support the idea that PD may arise from a toxic insult superimposed on a genetically-derived metabolic impairment. Although this is an appealing hypothesis, the possibility remains that these metabolic dysfunctions occur as a result of PD and are not themselves the cause of the disease. This latter idea is supported by the fact that a large number of compounds, including MPTP and the DHßC, harmaline, have been shown to inhibit the aforementioned cytochrome P450IID1 enzyme (Fonne-Pfister and Meyer, 1988). Consequently, the search for a precipitating parkinsonian factor continues.

Mitochondrial Defect

The finding that MPP^+ elicits its toxic effect by inhibition of complex I of the electron transport chain (discussed in detail later in this chapter) has prompted a search for defective mitochondria in PD patients. Postmortem reductions have been identified in complex I activity that were specific for the nigrostriatal system in PD patients (Mizuno *et al.*, 1989; Schapira *et al.*, 1989). Similar decrements were also reported in the mitochondria from skeletal muscle (Bindoff *et al.*, 1989) and platelets (Parker *et al.*, 1989) of living PD patients, but the validity of these peripheral deficits has been disputed (Bravi *et al.*, 1991). If PD patients do have deficient mitochondrial function, its etiology remains unclear. In a report by Lestienne and colleagues (1990), no demonstrable deletions in the mitochondrial genome of postmortem PD brains were observed, even though the activity of the enzyme was decreased. From these results they concluded that the deletions were either in the subunits encoded by the nuclear genome or the enzyme activity was decreased in response to either metabolites, toxins or increased iron (Lestienne *et al.*, 1990). Similarly, Schapira *et al.* (1990) indicated that they believed the observed complex I deficiency in PD brains was the result of a toxic effect, although they do not exclude the idea of a genetic predisposition to such an effect.

Oxidative Damage

It has been proposed that the nigral DA cell loss associated with PD may occur by a free radical-mediated mechanism resulting in accelerated aging of the dopaminergic neurons in the substantia nigra (Barbeau, 1984; Mann and Yates, 1982). Hydrogen peroxide (H_2O_2) is formed in these neurons due to normal processes such as oxidative deamination of DA by monoamine oxidase (MAO) B, and the formation of the pigment, neuromelanin, by DA auto-oxidation (Youdim *et al.*, 1990). Under

normal conditions, the neurons are protected from peroxide damage primarily by glutathione peroxidase (Youdim *et al.*, 1990). However, decreased levels of reduced glutathione (Perry and Yong, 1986) and another free-radical scavenger, catalase (Ambani *et al.*, 1975), and increased superoxide dismutase activity (Saggu *et al.*, 1989) have been observed in postmortem parkinsonian brains. Under such conditions, high concentrations of H_2O_2 would be generated in the brains of PD patients.

H_2O_2 can react with free ferrous iron (Fe^{2+}), in what is termed a Fenton reaction, to form cytotoxic oxygen species such as the hydroxyl radical ($^{\cdot}OH$) (Youdim *et al.*, 1990). Likewise, the binding of ferric iron (Fe^{3+}) to neuromelanin in the presence of H_2O_2 can also liberate $^{\cdot}OH$ (Youdim *et al.*, 1990). The concentration of iron in brain regions such as the basal ganglia is high under normal conditions, but in PD patients a regionally specific increase in iron content has been reported in the substantia nigra zona compacta (Dexter *et al.*, 1986; Dexter *et al.*, 1989; Earle, 1968; Sofic *et al.*, 1988), particularly within Lewy bodies (Hirsch *et al.*, 1991), and may be the result of decreased ferritin concentrations (Dexter *et al.*, 1990). This, combined with the high concentration of neuromelanin and decreased anti-oxidative capacity of this region, may lead to increased concentrations of $^{\cdot}OH$ resulting in lipid peroxidation and neuronal degeneration (Youdim *et al.*, 1990). Dexter *et al.* (1986 & 1989) have reported an increased level of the lipid peroxidation product, malondialdehyde, specifically in the substantia nigra of postmortem parkinsonian brains, so it is possible that such a mechanism is an underlying factor in PD. However, the questions of how and why iron accumulates in the substantia nigra, and why it is in higher concentrations in PD patients, remain to be answered (Youdim *et al.*, 1990).

Factors other than iron have also been shown to produce potentially destructive oxygen species. Like iron, chronic manganese intoxication may work through an

oxidative mechanism to produce cytotoxic ·OH radicals resulting in an irreversible syndrome closely related to PD (Tanner *et al.*, 1987). It has been posited that the parkinsonian therapeutic drug, L-3,4-dihydroxyphenylalanine (L-DOPA), may form toxic oxygen radicals and thereby contribute to the nigral degeneration (Graham *et al.*, 1978; Wick *et al.*, 1977). Barbeau and Roy (1985) have postulated that toxic oxygen species could result from the accumulation in the brain of environmental chemicals such as paraquat. Although the damage caused by paraquat is more diffuse than that seen in PD (Barbeau and Roy, 1985), it does support the increasingly popular hypothesis that PD develops in response to chronic exposure to an environmental toxin.

Environmental Toxins

A higher prevalence of PD has been reported among the populations of established, industrial nations than among those that are newly industrialized or non-industrialized (Tanner, 1989). Exposure to industrial chemicals, and to pesticides and herbicides in rural areas have been suggested as putative risk factors (Tanner, 1989). To date, however, there have been no environmentally abundant substances that have been demonstrated to produce all of the symptoms of idiopathic PD in either humans or animal models (Tanner, 1989). In fact, in a recent report by Stern *et al.* (1991) the authors indicated that they were unable to implicate well water or exposure to herbicides, pesticides or industrial toxins as causative factors in PD. However, the possibility still exists that an as yet unidentified agent may prove to be the underlying factor in idiopathic PD. Those agents that have been identified as producing syndromes closely related to PD, such as MPTP, have proven to be useful models in identifying possible parkinsonian factors and their toxic mechanisms.

MPTP Neurotoxicity

Introduction In 1983, a paper in *Science* (Langston *et al.*, 1983) reported that MPTP was a contaminating by-product in "synthetic heroin" (1-methyl-4-phenyl-4-propionoxy-piperidine, MPPP) injected by four individuals in northern California. [A similar finding had been reported previously (Davis *et al.*, 1979), but not much interest was generated by that report.] Upon clinical examination, these individuals were found to exhibit the classical symptoms of PD, and their condition showed no signs of remission even after five months, but they were responsive to L-dopa/carbidopa therapy administered in conjunction with DA agonist (bromocriptine or lisuride) (Langston *et al.*, 1983). A postmortem examination of Davis's patient revealed destruction of substantia nigra nerve cell bodies and other pathology similar to that observed in PD patients (Davis *et al.*, 1979; Langston *et al.*, 1983). Langston *et al.* (1983) concluded their paper by stating the following: "Given the pathologically studied case, the relative purity of the clinical syndrome seen in our patients, and its remarkable clinical resemblance to Parkinson's disease, the drug may be of value in producing an animal model of Parkinson's disease." These words proved prophetic as they stimulated an explosion of research on MPTP toxicity and propagated a global search for possible toxic agent(s) of either environmental or endogenous origin involved in the etiology of PD.

Since the report by Langston and colleagues (1983), MPTP has been shown to produce parkinsonian effects in primates similar to those observed in humans (Burns *et al.*, 1983; Collins and Neafsey, 1985; Jenner *et al.*, 1984; Kolata, 1983; Langston *et al.*, 1984a; Neafsey *et al.*, 1986). These effects include destruction of nigral cell bodies and their afferent projections to the caudate nucleus and putamen (Calne and Langston, 1983; Langston *et al.*, 1984a). Also, other pathological hallmarks including

lesions in the locus ceruleus and the presence of Lewy body-like inclusions have been reported in aged primates after chronic MPTP treatment (Forno *et al.*, 1988; Forno *et al.*, 1986; Miyoshi *et al.*, 1988). MPTP has also been shown to produce neuronal damage in mice (Fuller and Hemrick-Luecke, 1985; Hallman *et al.*, 1985; Heikkila *et al.*, 1984a). In contrast, the rat is relatively insensitive to MPTP administered *in vivo*, possibly due to differences in metabolism and in the blood-brain barrier (Johannessen *et al.*, 1985b; Kalaria *et al.*, 1987). However, isolated rat cells grown in culture, both primary and clonal, are susceptible to MPTP/MPP^+-induced toxicity and have been used extensively as experimental models (Basma *et al.*, 1990; Denton and Howard, 1984; Mytilineou and Cohen, 1984; Reinhard *et al.*, 1990a; Sanchez-Ramos *et al.*, 1986; Schinelli *et al.*, 1988; Snyder *et al.*, 1986).

Bioactivation of MPTP. Since MPTP itself is not particularly neurotoxic, it must first be oxidized to its active form, MPP^+. The first step in this process is the conversion of MPTP to the two-electron oxidation product, $MPDP^+$, and subsequently to the four-electron oxidation product, MPP^+ (Chiba *et al.*, 1984; Markey *et al.*, 1984). MPTP is one of the best known substrates for MAO B (Chiba *et al.*, 1984; Salach *et al.*, 1984). It has been shown that purified MAO-A and MAO-B both oxidize MPTP, but the rate of oxidation is 14 fold higher for the B form than for the A form (Singer *et al.*, 1986; Singer *et al.*, 1985). Interestingly, there is very little or no MAO B in the nigrostriatal dopaminergic cells; it is found mostly in glial cells and serotonergic neurons, and neither of these cell types displays toxic sensitivity to the MPTP metabolites which they generate (Westlund *et al.*, 1985). Even though both the A and B forms of the enzyme oxidize MPTP, only MAO-B selective inhibitors (such as deprenyl and pargyline) prevent MPTP-induced neurotoxicity, whereas MAO-A inhibitors such as clorgyline are ineffective (Heikkila *et al.*, 1984b; Langston *et al.*,

1984b; Markey *et al.*, 1984). Chiba *et al.* (1984) have also shown that clorgyline is ineffective in preventing brain mitochondrial oxidation of MPTP.

The exact mechanism of the conversion of the intermediate ($MPDP^+$) to the final product (MPP^+) is not clear. It has been shown that both MAO A & B readily carry out the oxidation of $MPDP^+$ to MPP^+, and $MPDP^+$ can also undergo spontaneous air oxidation (Salach *et al.*, 1984; Singer *et al.*, 1985). DiMonte *et al.* (1987) have suggested that $MPDP^+$ exits the cell where it was formed and then spontaneously oxidizes to form MPP^+. Wu *et al.* (1986) have also shown that synthetic dopamine-melanin and human neuromelanin potentiate the formation of MPP^+ from $MPDP^+$, which would indicate that it is $MPDP^+$ that is accumulated by the dopaminergic neuron and not MPP^+. However, there is not much evidence to support this latter hypothesis.

In rat mesencephalic explant cultures, MPTP selectively destroyed DA neurons (Mytilineou and Cohen, 1984), but was ineffective in dissociated mesencephalic cell cultures (Sanchez-Ramos *et al.*, 1986). Schinelli *et al.* (1988) observed that MPTP was taken up and metabolized to MPP^+ in co-cultures containing astrocytes, and then the MPP^+ was released into the extracellular milieu where it was specifically taken up and concentrated by DA neurons. In contrast, MPTP is not taken up by dopaminergic cultures (Schinelli *et al.*, 1988), which may explain the absence of toxic effects seen by Sanchez-Ramos *et al.* (1986), where the glial cells had been killed by addition of anti-mitotics to the culture medium, thereby preventing the production of MPP^+ (Mytilineou and Friedman, 1988). It has since been demonstrated *in vivo* that destruction of the astroglial cells in the rat substantia nigra prevents MPTP toxicity to the neurons in that region (Takada *et al.*, 1990). Further support for the role of astrocytes comes from a recent paper by Marini *et al.* (1992) which reported that MPTP is accumulated by astrocytes, sequestered to high concentrations within

lysosomes, converted to MPP^+ and released into the local environment where it can be taken up by those cells that efficiently accumulate the compound. Accumulation and oxidation of MPTP by serotonergic neurons is also possible as these neurons also contain MAO-B (Brooks *et al.*, 1988), but all of the data mentioned above indicate that it is probably the astrocytes. It remains to be determined why the cells that carry out the conversion of MPTP to MPP^+ appear to be immune to the toxic effects of MPP^+.

Accumulation of MPP^+. Most of the MPTP that is converted to MPP^+ is accumulated in regions of the brain susceptible to its toxicity, notably the nigral neurons (Irwin and Langston, 1985; Johannessen *et al.*, 1985b). Accumulation of MPP^+ has been shown to occur by an active process in striatal synaptosomal preparations using radiolabeled MPP^+ (Chiba *et al.*, 1985; Javitch *et al.*, 1985), and the kinetic characteristics of MPP^+ uptake were shown to be similar to those for DA (Chiba *et al.*, 1985). The accumulation of MPP^+ by the striatal synaptosomes can be competitively inhibited by DA (Chiba *et al.*, 1985; Javitch *et al.*, 1985) and also by DA uptake blockers such as mazindol and nomifensine, but not by the NE uptake inhibitor, desipramine (Javitch *et al.*, 1985). Mazindol protects against MPTP/MPP^+-induced striatal DA depletion in rodents (Melamed *et al.*, 1985a; Sundstrom and Jonsson, 1985), but there is some discrepancy over the effectiveness of similar compounds in primates (Barnes *et al.*, 1987; Schultz *et al.*, 1986). Javitch *et al.* (1985) have shown that synaptosomal preparations from other brain regions are capable of active accumulation of MPP^+, including those from noradrenergic regions. However, this uptake is only 10-20% of that in striatal synaptosomes (Chiba *et al.*, 1985).

From the above data it appears that the uptake and accumulation of MPP^+ by the DA uptake system of the nigrostriatal cells is essential for the occurrence of neurotoxicity. However, it is not clear what property of the nigral neurons makes them more susceptible to the degenerative effects of MPTP. D'Amato *et al.* (1986) have shown that MPP^+ binds with high affinity to neuromelanin, and that the neuromelanin could be acting to sequester the MPP^+ in the nigrostriatal neurons of primates, which, unlike most rodents, possess high concentrations of neuromelanin in this area. These authors (D'Amato *et al.*, 1986) suggest that there is a good correlation between MPTP-induced neurotoxicity and species that possess neuromelanin in their dopaminergic neurons. Therefore, it has been postulated that binding of MPP^+ to neuromelanin within the cell and its gradual release to its site of action are important factors in the selectivity of this neurotoxin (D'Amato *et al.*, 1986). Further support for this hypothesis was obtained when D'Amato *et al.* (1987) observed that the antimalarial drug chloroquine binds to neuromelanin and prevents the parkinsonian effects of MPTP in primates. However, Marini and colleagues (1992) have reported that chloroquine prevents MPTP-induced toxicity by inhibition of astrocytic accumulation of MPTP. Consequently, the role of neuromelanin, if any, in the susceptibility of nigral neurons to the toxic effects of MPTP remains uncertain.

Mitochondrial toxicity. Nicklas *et al.* (1985) found that MPP^+ (0.5 mM) inhibited oxidation of NADH-linked substrates in the electron transport chain of liver and brain mitochondria, and suggested that this may be the mechanism of cell death. This was the first mechanism proposed, and is still the most widely accepted. It has been observed that both rat liver and brain mitochondria accumulate MPP^+ by an active, energy-dependent uptake system, against the gradient into the mitochondrial matrix (Ramsay *et al.*, 1989; Ramsay *et al.*, 1989; Sayre *et al.*, 1989; Singh *et al.*,

1991). Because of the energy dependence of this uptake system, uncouplers inhibit this action and actually stimulate an efflux of accumulated MPP^+ (Ramsay *et al.*, 1986; Ramsay and Singer, 1986). In contrast, MPTP and 4-phenylpyridine (the desmethylated isomer of MPP^+) are not substrates for the mitochondrial carrier (Ramsay *et al.*, 1986). However, Hoppel *et al.* (1987) have demonstrated that neutral pyridine compounds (including 4-phenylpyridine) are more potent inhibitors of complex I in sub-mitochondrial particle preparations (where the electron transport chain is directly exposed) than the corresponding N-methylated compounds, but the charged species appear to be much more effective inhibitors in intact mitochondria. These authors (Hoppel *et al.*, 1987) suggest that the charged species are better inhibitors because they are accumulated and concentrated within the mitochondria by an energy-dependent process similar to that for MPP^+, whereas the uncharged species are not. From these results it has been suggested that there may be a strict requirement that the responsible toxin be positively charged to be effectively concentrated within mitochondria (Hoppel *et al.*, 1987). Because the MPP^+-induced inhibition does not effect succinate oxidation, it is noted that the inhibition must occur before coenzyme Q (the point where succinate feeds into the pathway), and also that the inhibition is independent of the energy-coupling system (Singer *et al.*, 1987). It has been suggested that the specific site of inhibition is between the Fe-S cluster with the highest oxidation potential and ubiquinone (coenzyme Q_{10}) (Ramsay *et al.*, 1986; Singer *et al.*, 1987). Ramsay *et al.* (1991) have recently reported that the site of inhibition is the rotenone and piericidin binding site, lending further support to the hypothesis that MPP+ elicits its toxic effects by direct inhibition of mitochondrial respiration. The biochemical mechanism by which this inhibition occurs is not well defined, but there is evidence that inhibition of complex I results in the production of free radicals which further and selectively inhibit complex I resulting in the production

of more free radicals (Cleeter *et al.*, 1992). Overall, the effects of inhibition of the NADH dehydrogenase complex are a decrease in cellular ATP levels (DiMonte *et al.*, 1986), a depletion in reduced glutathione (DiMonte *et al.*, 1987), and disruption of intraneuronal calcium homeostasis (Kass *et al.*, 1988). The ultimate result of these processes is cell death (Maret *et al.*, 1990).

Although inhibition of mitochondrial respiration is the most widely accepted mechanism for MPTP-induced neurotoxicity, other mechanisms for the neurotoxicity of MPTP have also been proposed. These include the idea of a toxin induced auto-oxidation of DA, which would produce toxic semi-quinone species (Poirier *et al.*, 1985). However, Schmidt *et al.* (1985) argue against this by showing that manipulations of brain DA levels *in vivo* did not effect MPTP-induced neurotoxicity. The formation of an MPP^+ radical ($MPP^{\cdot}$) by a one-electron reduction, as seen with the herbicide paraquat (a structural analogue of MPP^+), has also been postulated (Johannessen *et al.*, 1985a). However, Sayre *et al.* (1986) have shown that the redox-potential of MPP^+ is insufficient to support this hypothesis. Another posited mechanism of MPTP-induced damage is the disruption of intracellular calcium homeostasis (Kass *et al.*, 1988), which has been implicated in cell death (Boobis *et al.*, 1989; Orrenius *et al.*, 1989; Youdim *et al.*, 1990).

Summary. Based upon the literature reviewed in this section, the following model of MPTP-mediated neurotoxicity has been proposed (fig. 2): MPTP, a lipid soluble molecule, crosses the BBB into the CNS. MPTP then undergoes a series of oxidations to the final toxic product MPP^+ by MAO B in either astrocytes or serotonergic (5-HT) neurons. MPP^+ is then released into the extracellular milieu from which it is taken up into the dopaminergic neurons of the substantia nigra by the high affinity DA uptake system. Once MPP^+ is inside the cytosol, it is transported into the

mitochondrial matrix where it inhibits the NADH dehydrogenase complex (complex I) of the electron transport chain, resulting in decreased cellular ATP levels and eventual cell death.

PC12 Cells

Biochemical properties. PC12 is a clonal cell line derived from rat pheochromocytoma cells that extends neuritic processes in the presence of nerve growth factor and thereby acquires a neuron-like appearance (Greene and Tischler, 1976). These cells synthesize and store DA and NE in granular vesicles, and these vesicles can be induced to secrete catecholamines into the extracellular medium by K^+-evoked depolarization in the presence of Ca^{2+} (Greene and Rein, 1977; Greene and Tischler, 1976; Koide *et al.*, 1986; Rebois *et al.*, 1980; Schubert and Klier, 1977; Takashima and Koike, 1985). Transport of catecholamines into the granules, like chromaffin granules and synaptic vesicles, is driven by a transmembrane proton gradient (acidic inside) powered by a Mg^{2+}-ATPase in the granule membrane (Rebois *et al.*, 1980). Granular accumulation of catecholamines is inhibitable by reserpine, but not by ouabain or desipramine (Rebois *et al.*, 1980). The DA content of PC12 cells is between 3 (Greene and Tischler, 1976) and 20 (Rebois *et al.*, 1980) times the NE content. Intact cells accumulate catecholamines by a saturable process that follows Michaelis-Menten kinetics and is both energy and Na^+-dependent (Greene and Rein, 1977). The apparent K_m of [^{3}H]NE uptake is approximately 2 μM, and it is inhibitable by desipramine and cocaine with approximate IC_{50} values of 10 nM and 1 μM, respectively (Greene and Rein, 1977). Snyder *et al.* (1986) have reported a K_m of approximately 0.6 μM for [^{3}H]DA. Denton and Howard (1984) have suggested that it is the same transport system that accumulates both DA and NE. Banerjee *et al.*

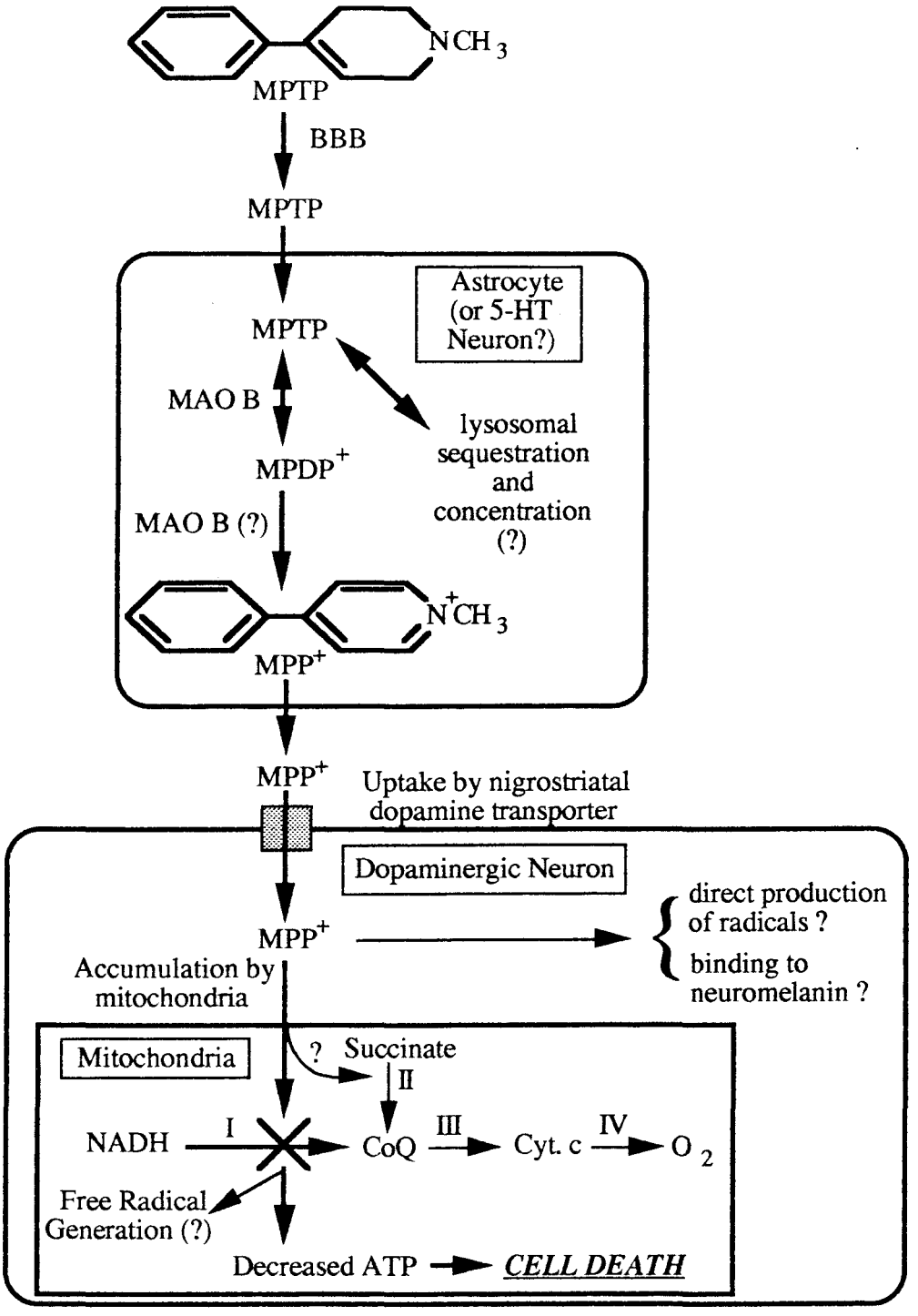


FIG. 2. PROPOSED MECHANISM FOR MPTP-INDUCED DOPAMINERGIC NEURON DEATH. In the mitochondria, the Roman numerals (I, II, III and IV) indicate the enzyme complexes of the electron transport chain. BBB = Blood-brain barrier.

(1987) have also suggested that bovine adrenomedullary chromaffin cells (BAMC, non-transformed equivalents of the rat adrenomedullary PC12 cells) accumulate catecholamines by the same, or closely related, high and low affinity sites on the plasma membrane.

Metabolically, PC12 cells are glycolytic in nature; that is, they derive most of their ATP from glycolysis (Denton and Howard, 1987). Also, a substantial portion of the total cellular ATP is stored in vesicles (Denton and Howard, 1987; Reynolds *et al.*, 1982). Reynolds *et al.* (1982) reported that since the inhibition of oxidative phosphorylation in PC12 cells did not effectively decrease ATP stores, it would be necessary to inhibit both glycolysis as well as oxidative phosphorylation to deplete ATP.

Bioactivation of MPTP. The prevalent form of MAO in PC12 cells is the A form (Youdim *et al.*, 1986) rather than the B form of the enzyme (the active form *in vivo*) (Heikkila *et al.*, 1984a; Langston *et al.*, 1984b; Markey *et al.*, 1984; Salach *et al.*, 1984). However, this does not appear to be a limitation in the use of PC12 cells because it has been shown that the inhibition of MAO-A by clorgyline prevented MPTP toxicity in PC12 cells (Marongiu *et al.*, 1988). Also, MAO-A has been shown to play a significant role in the neurotoxic effects of simple substituted MPTP molecules (Kindt *et al.*, 1988; Sonsalla *et al.*, 1987). 2'Ethylphenyl-MPTP (2'Et-MPTP), which is a good substrate for MAO-A, is a more potent toxin in mice than MPTP (Youngster *et al.*, 1987; Youngster *et al.*, 1989). Basma *et al.* (1990) have shown that this compound (2'Et-MPTP) was considerably more cytotoxic in PC12 cells than MPTP. This is consistent with the idea that it is the oxidized (charged) pyridinium species (MPP^+ or 2'Et-MPP^+) that is toxic to the cells, and not the neutral tetrahydropyridine (MPTP or 2'Et-MPTP).

Accumulation of MPP^+. The catecholamine uptake system in PC12 cells, like that in DA neurons (Chiba *et al.*, 1985; Javitch *et al.*, 1985), has been shown to be the mechanism of MPP^+ accumulation in PC12 cells (Denton and Howard, 1987; Snyder *et al.*, 1986). In fact, the K_m and V_{max} values for the uptake of MPP^+ in these cells were approximately the same as those for DA (Snyder *et al.*, 1986). Snyder *et al.* (1986) also suggested that in PC12 cells both the NE and DA systems are active in the uptake of [^{3}H]MPP^+, and the NE system is the predominant pathway. This latter conclusion was based upon pharmacological data showing that the IC_{50} for desipramine (NE uptake inhibitor) was 30 nM, that the non-specific catecholamine uptake inhibitor, mazindol, blocked the accumulation of [^{3}H]MPP^+ with an IC_{50} of approximately 3 nM, and that the IC_{50} for the selective DA uptake antagonist, GBR 12909, was 700 nM (Snyder *et al.*, 1986). Denton and Howard (1987) also mentioned that desipramine protected PC12 cells from MPP^+-induced toxicity, and these authors had previously suggested that the same system transports both DA and NE in PC12 cells (Denton and Howard, 1984). Reinhard *et al.* (1990a) have reported in BAMC cells that desipramine (50 μM) antagonized the toxic effects of a 3 day exposure to 500 μM MPP^+. These results are interesting, because Javitch *et al.* (1985) have shown that dopamine uptake inhibitors blocked MPP+ accumulation by rat striatal synaptosomes, but desipramine did not. Therefore, it may be that adrenomedulla-derived cells such as chromaffin cells, which are noradrenergic, and PC12 cells, which were initially cloned from a noradrenergic tumor (Greene and Tischler, 1976), possess a general catecholamine uptake site. Whether this is true or not, PC12 cells have been shown to mediate MPP^+ accumulation by a catecholamine uptake system, similar to what has been observed in the brain.

Another interesting point is that inhibition of toxicity with catecholamine uptake blockers effects only a partial attenuation and not a complete inhibition of

toxicity (Denton and Howard, 1984; Reinhard *et al.*, 1990a; Reinhard *et al.*, 1990b). Reinhard *et al.* (1990b) suggest that this is because MPP^+ can delocalize its positive charge through the pyridinium ring to form a neutral species. This could allow neutral MPP to passively diffuse across the cell membrane without aid of a carrier protein. It is also possible that desipramine does not completely block the accumulation of MPP^+ by the chromaffin cells. Banerjee *et al.* (1987) have shown that there are two distinct types of plasma membrane sites for [^{3}H]NE accumulation into BAMC cells. The high affinity site is sensitive to cocaine and tricyclic antidepressants of which desipramine is the most potent (IC_{50} = 130 nM) (Banerjee *et al.*, 1987). In contrast, these compounds have only low potency at the low affinity site even at high concentrations of inhibitors (> 30 μM) (Banerjee *et al.*, 1987). Reinhard *et al.* (1990b) had co-incubated their BAMC cells in the presence of 100 μM MPP^+ and 10 μM desipramine for 3 days. It is possible that MPP^+ can be accumulated by the low affinity uptake site as well as the high affinity site, and as a result at least a portion of the MPP^+ would enter the cell. Consequently, the uptake site(s) does appear to play a role in the accumulation of MPP^+ by catecholaminergic cells.

Mechanism of toxicity. As mentioned previously (Biochemical Properties), one limitation of PC12 cells as an experimental model for the study of compounds like MPTP and MPP^+ is that they are glycolytic, and are thus capable of maintaining vesicular stores of ATP even in the presence of inhibitors of mitochondrial respiration (Denton and Howard, 1987; Reynolds *et al.*, 1982). Snyder *et al.* (1986) demonstrated that 50% of PC12 cells survive a 5 day treatment of 100 μM MPP^+. Denton and Howard (1987) showed that PC12 cells not only survived a 4 day exposure to 100 μM MPP^+ in 'normal' glucose medium (glucose conc. is not indicated, but probably > 10 mM), but they continued to divide at an almost normal rate. However, a marked

reduction in ATP levels was observed and less than 20% of the cells survived the 4 day exposure to 100 μM MPP^+ when they used a low glucose medium (< 1 mM), supplemented with 7 mM pyruvate (Denton and Howard, 1987). Similarly, other inhibitors of oxidative phosphorylation, including oligomycin, antimycin A and rotenone were lethal to PC12 cells in low glucose medium, but not in normal glucose medium (Denton and Howard, 1987). Recently, Basma *et al.* (1992) have also shown that reduction of the glucose concentration in the medium increases the susceptibility of PC12 cells to 2'Et-MPP^+, an MPP^+ analogue. This compound (2'Et-MPP^+) and MPP^+ have also been observed to increase LDH release and reduce ATP levels in a neuroblastoma x glioma hybrid cell line, NG 108-15, and these effects were attenuated by increased high media glucose concentration (Kutty *et al.*, 1991). Reinhard *et al.* (1990a) reported that MPP^+ caused BAMC to utilize glucose in the culture medium at a rate that was 4.6-fold greater than those cells not exposed to MPP^+. This same group (Reinhard *et al.*, 1990a) also observed that the release of LDH from the BAMC cells was not observed until the cells had used up approximately 99% of the media glucose (48 h). Similarly, Henneberry and co-workers (Henneberry, 1989; Novelli *et al.*, 1988) have also reported that the neurotoxic effects of glutamate on cultured rat neurons were potentiated when the cells were grown in a low energy medium.

Another possible reason PC12 cells are much more resistant to the toxic effects of MPP+ can be inferred from work by Reinhard *et al.* (1990b). These authors (Reinhard *et al.*, 1990b) have shown that BAMC cells accumulate and sequester MPP^+ in the cells' catecholamine storage granules. By treating the cells with reserpine or tetrabenazine, which inhibit vesicular accumulation, they potentiated the toxic effects of MPP^+ (Reinhard *et al.*, 1990b). Similar results have been shown with reserpine and tetrabenazine in mice, where these compounds potentiated the neurotoxic effects of MPTP (Melamed *et al.*, 1985b; Reinhard *et al.*, 1988). Reinhard

et al. (1990b) suggest that sequestration of the MPP+ in the granules would reduce their cytosolic concentration and consequently reduce their accumulation by mitochondria thereby protecting the cells. Since PC12 cells have also been shown to possess functional catecholamine storage granules (Rebois *et al.*, 1980), it may be that they also sequester MPP^+; this combined with their glycolytic nature reduces the observed toxicity.

The ultimate mechanism by which MPP^+ elicits its toxic effects appears to be through inhibition of mitochondrial respiration at site I (NADH dehydrogenase complex) of the electron transport chain (Nicklas *et al.*, 1985; Ramsay *et al.*, 1986; Singer *et al.*, 1987), and a similar effect has been reported in PC12 cells (Basma *et al.*, 1992; Denton and Howard, 1987). Denton and Howard (1987) measured the effects of MPP^+ on mitochondrial respiration in intact and digitonin permeabilized PC12 cells, and found that MPP^+ does inhibit oxidative phosphorylation in PC12 cells. However, a lethal effect of MPP^+ was only realized when glycolysis and oxidative phosphorylation were blocked at the same time, thus effectively depleting cellular ATP (Basma *et al.*, 1992; Denton and Howard, 1987). So, although the PC12 cells are not a "perfect" model for the study of the effects of MPTP/MPP^+-like toxins on dopaminergic cells, these cells are ultimately affected in the same way as DA neurons (inhibition of mitochondrial respiration), and so provide a useful system with which to study these compounds provided the proper conditions are employed. They may also provide insight into the mechanism of resistance of those cells that are unaffected by toxins such as MPP^+ (e.g., astrocytes), but that remains to be determined.

ß-Carbolines and 3,4-Dihydro-ß-Carbolines

Introduction

Although MPTP is a good experimental model, it is highly unlikely that a contaminating by-product of synthetic heroin plays a role in the development of idiopathic PD. As mentioned previously, the idea of an environmental toxin as the underlying factor in PD is currently popular (Tanner, 1989). However, it has also been noted that, to date, no environmental factors have been implicated in the development of the disease (Stern *et al.*, 1991; Vieregge *et al.*, 1988). Consequently, the possibility that an endogenously-derived substance may play a role in the pathogenesis of PD has been posited by several groups (Collins and Neafsey, 1985; Ohkubo *et al.*, 1985; Testa *et al.*, 1985). The hypothesis being explored in Dr. Collins' laboratory, and the basis for this dissertation, is that the selective destruction of nigrostriatal neurons that is characteristic of idiopathic Parkinson's disease is the result of the accumulation of endogenously formed alkaloids that are structural analogues of MPP^+ and $MPDP^+$: N-methylated ßC$^+$s (N-MeßC$^+$s) and/or their potential 3,4-dihydro- precursors, N-MeDHßC$^+$s.

Nomenclature

Before discussing the biological effects of ßCs and DHßCs it is first necessary to establish the nomenclature of these compounds. A comparison of the base structures of MPTP and its oxidation products to tetrahydro-ßC and its oxidation products can be seen in fig. 3. The discussion in the following paragraph refers to that figure. For further examples of structures and the abbreviations of the compound names, see figure 4. [To avoid excess confusion, the abbreviations of the compounds names are used sparingly in the literature review.]

Mono-[N]-methylated compounds are methylated only on the 2-nitrogen (piperidine ring nitrogen). Di-[N,N']-methylated species are methylated on the 9-nitrogen (indole ring nitrogen) as well as the 2-nitrogen. The suffix "-ium" attached to the names of the methylated species indicates that they are cationic (i.e., harmaline, becomes 2-methylharmalinium). The "nor-" prefix on certain ßC and DHßC names (i.e., the ßC norharman) indicate that they are not methylated at the 1-carbon. Some of the compounds are oxygenated at the 6-carbon (e.g., 6MeO-2Me-harmanium) or the 7-carbon (e.g., harmine, which has a methoxy group). 3,4-dihydro-beta-carbolines (DHßCs) are similar to MPDP+ (partially reduced piperidine ring); therefore, these are possibly the metabolic precursors of beta-carbolines (ßCs). The insertion of "al" into the compound name indicates the DHßCs. For example, the ßC, harmine, becomes harmaline when it is in the dihydro form.

Biodisposition in Mammals

As previously mentioned, DHßCs and ßCs differ structurally from $MPDP^+$ and MPP^+, respectively, only by the addition of an indole nitrogen bridge (fig. 3). Many DHßCs and ßCs are present in environmental sources, including tobacco smoke, alcoholic beverages, soy sauce, burned or fried tryptophan-rich foods and industrial waste (Airaksinen and Kari, 1981b; Bosin *et al.*, 1988; Reinhart *et al.*, 1987; Wakabayashi *et al.*, 1983). It has also been suggested that ßCs and DHßCs can be formed via spontaneous (Pictet-Spengler) condensation of indoleamines (e.g., tryptamine, tryptophan, or serotonin) with formaldehyde to form tetrahydro-norharman (THßC), or with carbonyl compounds (e.g., acetaldehyde, glyoxylic acid, pyruvic acid) to form tetrahydro-harman (1-methyl-THßC) (Airaksinen and Kari, 1981b; Melchior and Collins, 1982; Rommelspacher *et al.*, 1991). It is possible that these compounds undergo subsequent oxidation/decarboxylation to form DHßCs or

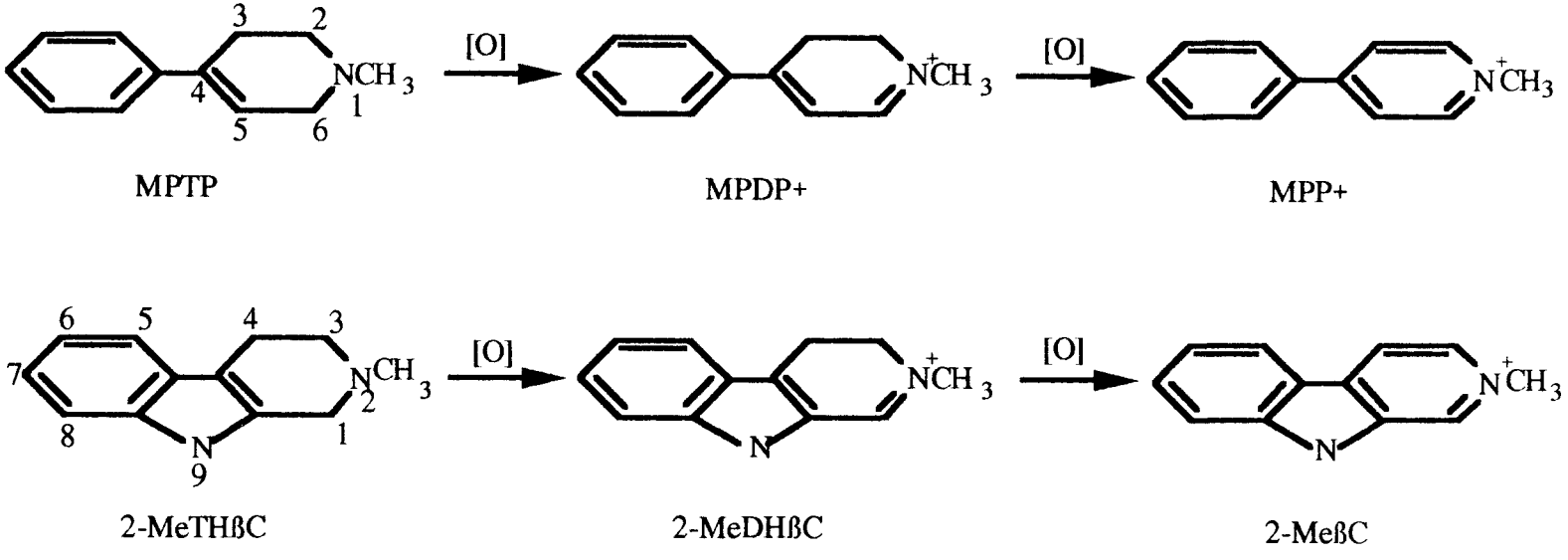


FIG. 3. STRUCTURAL SIMILARITIES BETWEEN MPTP, 2-MeTHßC AND THEIR OXIDATION PRODUCTS. The different numbering conventions of the pyridine and ß-carboline rings are indicated on the parent compounds (MPTP and 2-MeTHßC).

fully oxidized ßCs (Melchior and Collins, 1982; Rommelspacher *et al.*, 1991). The 6- and 7-oxygenated species (hydroxylated or methoxylated), such as harmine, harmaline and harmol, may be generated by condensation of oxygenated indoleamines (i.e., serotonin or 5-methoxy-tryptamine) with carbonyl compounds with subsequent dehydrogenation, or via enzymatic oxidative mechanisms (Drucker *et al.*, 1990; Greiner and Rommelspacher, 1984; Susilo *et al.*, 1987; Uemura *et al.*, 1988). Another possible route to the formation of 7-oxygenated species such as harmine and harmaline is by 7-hydroxylation of THßCs, which has been demonstrated in rodents (Beck *et al.*, 1986; Tweedie and Burke, 1987). Recent reports by Matsubara *et al.* (1992a & c) have indicated that ßCs can be enzymatically mono-2-[N]- and di-2,9-[N,N']-methylated, and THßCs can be mono-2-[N]-methylated, by S-adenosylmethionine-dependent processes in the mammalian brain. Based upon these results, Collins *et al.* (1992) have postulated a brain bioactivation pathway to the formation of potentially neurotoxic 2-[N]-MeßC$^+$s and 2,9-di-[N,N']-MeßC$^+$s.

The presence of non-N-methylated ßC$^+$s, such as norharman and harman, has been reported in mammalian brain (Bosin *et al.*, 1989; Collins, 1983; Rommelspacher and Schmidt, 1985; Schouten and Bruinvels, 1986; Shoemaker *et al.*, 1980). However, the presence of their 2-methyl derivatives (e.g., 2-Me-norharmanium and 2-Me-harmanium, respectively) has not been reported. 2-[N]-methyl-1,2,3,4-tetrahydro-beta-carbolinium (2Me-THßC, the THßC most closely resembling MPTP) is a tryptamine-derived alkaloid that has been found in trace amounts in rat CNS and adrenal gland (Barker *et al.*, 1981). Evidence has also been presented for the presence in the rat brain of 6-OH and 6-MeO substituted THßCs (Collins, 1983; Johnson *et al.*, 1985). In humans, harman has been detected in erythrocytes, platelets and urine (Bidder *et al.*, 1979; Rommelspacher *et al.*, 1990; Rommelspacher *et al.*, 1985; Rommelspacher *et al.*, 1984; Shoemaker *et al.*, 1980). Most recently, the presence of

several 2- and 2,9-dimethylated ßC$^+$s (norharman, 2-Me-norharmanium, 2,9-diMe-norharmanium, harman, 2-Me-harmanium and 2,9-diMe-harmanium) have been identified in the human brain (Matsubara *et al.*, 1992b).

Pharmacology

The neuropharmacological actions of THßCs, DHßCs and ßCs (harmala alkaloids) are many and diverse. The first description of these alkaloids were as derivatives of plant seeds and roots that were used by South American natives as spices or as a hallucinogen, comparable in effect to LSD and mescaline (Naranjo, 1967). Other effects have been ascribed to these compounds in rats and humans, including tremor and tonic-clonic convulsions (Airaksinen and Kari, 1981a; Gunn, 1935; May *et al.*, 1991b; Naranjo, 1979; Sigg *et al.*, 1964). The ßCs, harman and norharman, have been described as inverse agonists of the central nervous system (CNS) benzodiazepine receptor (Airaksinen and Kari, 1981a; Rommelspacher *et al.*, 1980; Rommelspacher *et al.*, 1981), and it has been suggested that a one or both of these compounds may be endogenous ligands for this receptor (Braestrup *et al.*, 1980; Pena *et al.*, 1986). It has also been demonstrated that the DHßC, harmaline, increases cerebellar cGMP levels . This effect appears to be mediated via nitric oxide formation (Wood *et al.*, 1990).

The specific binding to and inhibition of MAO-A in the CNS by harmala derivatives such as harmaline (Burkard and Kettler, 1977; Neff and Fuentes, 1976; Nelson *et al.*, 1979a; Nelson *et al.*, 1979b; Udenfriend *et al.*, 1958) and harman (Airaksinen and Kari, 1981a; Buckholtz and Boggan, 1977; Glover *et al.*, 1982; May *et al.*, 1991a; May *et al.*, 1991b) has been reported, and it has also been suggested that harman may act as an endogenous inhibitor of MAO-A (May *et al.*, 1991b). Tse *et al.*

(1991) have shown that several desmethylated ßCs and DHßCs, including harman, can act as anti-oxidative agents, which may relate to their inhibitory effect on MAO.

DHßCs and ßCs have been shown to be potent inhibitors of Na^+-dependent processes. Harmine, harmaline and harmalol exhibit inhibition of sodium-dependent transport systems by competitive inhibition of membrane bound, Mg^{2+}-dependent ($Na^+ + K^+$)-ATPase in a number of systems including rat brain, squid retinal axon and human erythrocytes (Canessa *et al.*, 1973; Charnock *et al.*, 1976; Dunn and Hunt, 1975; Sastry and Phillis, 1977; Sepulveda and Robinson, 1974; Simonson and Charnock, 1979). The inhibition of the ($Na^+ + K^+$)-ATPase system occurs at the site of sodium activation (Canessa *et al.*, 1973; Dunn and Hunt, 1975) and at the ATP-binding site (Charnock *et al.*, 1976). A number of ßCs and DHßCs, including harmaline, harmine, 2-Me-harmine, harmol and harman, display reserpine-like activity in their inhibition of [^{3}H]NE uptake into isolated catecholamine storage vesicles (Seidler *et al.*, 1977; Slotkin, 1974; Slotkin *et al.*, 1978). Several ßCs and DHßCs, including harmalol, harmine, 2-Me-harminium, harmaline and 2-Me-harmalinium have been shown to inhibit Na^+-dependent [^{3}H]choline uptake into rat brain synaptosomes with K_i values in the range of 3 - 36 µM (Smart, 1981).

Numerous other biological effects have also been ascribed to desmethylated harmala alkaloids. Norharman and harman have been shown to act as comutagens with various compounds including N-nitroso compounds (Nagao *et al.*, 1977a; Nagao *et al.*, 1977b; Ochiai *et al.*, 1986; Suzuki *et al.*, 1987; Wakabayashi *et al.*, 1981). These compounds (norharman and harman) also induce SOS responses and frame shift mutations in bacteria (Oda *et al.*, 1988), sister-chromatid exchanges in human peripheral lymphocytes (Madle *et al.*, 1981) and inhibition of DNA repair and synthesis in human alveolar tumors (Remsen and Cerutti, 1979). Several compounds, including harman, harmaline and harmine have been shown to bind to DNA

(Duportail and Lami, 1975; Madle *et al.*, 1981; Smythies and Antun, 1969). Harmine and several other ßCs are also involved in UV-mediated DNA damage (Hudson *et al.*, 1986a; Hudson *et al.*, 1986b; Larson *et al.*, 1988; McKenna and Towers, 1981; Towers and Abramowski, 1983).

N-methylated ßC$^+$s and DHßC$^+$s as Parkinsonian Agents

As just described, ßCs and DHßCs have a number of diverse biological effects. However, until the last decade, little was known about the effects of N-methylated DHßC$^+$s and ßC$^+$s. Because of the aforementioned structural similarities between these compounds and MPDP$^+$ and MPP$^+$ (fig. 3), investigators have sought to demonstrate that N-methylated DHßC$^+$s and ßC$^+$s are also neurotoxic, and that their mechanisms of toxicity are similar to MPDP$^+$ and MPP$^+$ (i.e., selective destruction of nigral dopaminergic neurons by accumulation via the DA uptake system and consequent inhibition of mitochondrial respiration).

Neurotoxic effects. It has been shown that repeated peripheral administration of 2Me-THßC (an uncharged species which crosses the blood-brain barrier, fig. 3) results in decreased levels of DA and its metabolites (DOPAC and HVA) in owl monkeys (Collins and Neafsey, 1985; Collins *et al.*, 1986) and in C57/Bl mice (Collins *et al.*, 1986). Sayre and colleagues (Arora *et al.*, 1990; Sayre *et al.*, 1991) have shown that intranigral infusion of 2-Me-harminium into rats caused decreases in DA and its metabolites, but these effects were approximately 30-fold less than those of MPP$^+$. When administered via intranigral injections, 2Me-norharmanium produced obvious lesions at the injection site and significant decreases in striatal DA and DOPAC levels (Neafsey *et al.*, 1989). Similarly, intranigral injection of 2-Me-harmalinium in rats caused significant depletion in striatal dopamine levels and gross

lesioning at the site of injection; although it was substantially less potent than MPP^+, 2-Me-harmalinium was considerably more toxic than 2-Me-harminium, 2-Me-norharmanium or 2-Me-harmanium (Neafsey *et al.*, unpublished results). It has also been found that 2Me-norharmanium, when administered using *in vivo* microdialysis, caused irreversible destruction that is selective for the nigrostriatal neurons in the rat brain (Rollema *et al.*, 1988). Similarly, Rollema and colleagues have demonstrated that 2-Me-harmanium and 2,9-diMe-harmanium elicit significant dopamine release, and that the toxic effect of 2,9-diMe-harmanium approached that of MPP^+ (Collins *et al.*, 1992).

Cellular accumulation. Drucker *et al.* (1990) examined the IC_{50} values for inhibition of DA uptake into rat striatal synaptosomes for 15 desmethyl/N-methyl pairs of ßCs, DHßCs and 3-substituted ßCs, to determine which compounds were eligible substrates for the DA transporter, a key step in MPTP/MPP^+ toxicity (Chiba *et al.*, 1985; Javitch *et al.*, 1985). The most potent of these compounds, harmaline (IC_{50} = 12 µM), was observed to be about 30 times less potent than MPP^+ (IC_{50} = 0.4 µM). N-methylation did not appear to improve the effectiveness of the compounds for DA uptake inhibition, and in the case of 2-Me-harmalinium a decrease in inhibitory potency was observed (IC_{50} = 33 µM) (Drucker *et al.*, 1990). In terms of structure-activity relationships, it was reported that the DHßCs were generally more effective inhibitors than the ßCs, and that the 7-oxygenated species were better than the 6-O-substituted compounds; in all cases, the IC_{50} values were between 10 and 150 µM (Drucker *et al.*, 1990). Based upon kinetic analysis of harmine and 2Me-harminium inhibition of [^{3}H]DA uptake, and inhibition of 2-[^{14}C]Me-harminium accumulation by nomifensine, Drucker *et al.* (1990) suggested that at least a portion of the ßCs in the incubation were accumulated by the dopamine uptake system. These data were

consistent with those reported by Arora *et al.* (1990) whose data demonstrated that 2-Me-harminium was a weaker substrate than MPP^+ for the DA uptake system.

Mitochondrial toxicity. The ability of N-methylated βC^+s and $DH\beta C^+$s to inhibit mitochondrial respiration has also been examined. 7-Oxygenated N-Me-βC^+s and $DH\beta C^+$s (e.g., 2-Me-harmalinium, 2-Me-harminium and 2-Me-harmolium) exhibited potencies approaching that of MPP^+ in inhibiting complex I (NADH-linked) respiration in isolated rat liver mitochondria (Albores *et al.*, 1990). Similar results for the IC_{50} of 2-Me-harminium were obtained by Sayre and co-workers (Arora *et al.*, 1990; Hoppel *et al.*, 1987; Sayre *et al.*, 1990; Sayre *et al.*, 1991). It had been previously mentioned that the strongly cationic species, MPP^+, is concentrated in the mitochondria by an energy-dependent process (Ramsay *et al.*, 1989; Ramsay *et al.*, 1989; Sayre *et al.*, 1989; Singh *et al.*, 1991), and it inhibits complex I of the electron transport chain (Ramsay *et al.*, 1986; Singer *et al.*, 1987). Albores *et al.* (1990) have reported, however, that mono-methylated βC^+s probably enter the mitochondria by a passive process in neutral anhydronium base forms. Furthermore, these compounds also inhibit succinate-linked respiration (complex II), to the same extent as complex I, where MPP^+ was a relatively ineffective inhibitor of complex II (Albores *et al.*, 1990; Sayre *et al.*, 1991; Fields *et al.*, 1992). The 2,9-dimethylated compounds are permanently cationic species which cannot deprotonate to neutral anhydronium forms. They resemble MPP^+ in that they were reported to permanently inhibit NADH-linked respiration but not succinate (Fields *et al.*, 1992). Similar to the results of the *in vivo* microdialysis work with 2-Me-harmanium and 2,9-diMe-harmanium (Collins *et al.*, 1992), methylation of the 9-[indole]-nitrogen of 2-Me-norharmanium (a weak inhibitor of NADH-linked respiration) to form the dimethylated βC^+ (2,9-diMe-norharmanium) resulted in a compound that was a more potent inhibitor than MPP^+.

Summary

This literature review has discussed 1) the various hypotheses for the development of idiopathic PD, 2) the neurotoxic mechanism of MPTP and its oxidation products ($MPDP^+$ and MPP^+), 3) the biochemical properties of PC12 cells and the cytotoxic effects of MPTP/MPP^+ upon them, and 4) the biodisposition of ßCs and DHßCs and their pharmacological and toxicological effects. The purpose of this dissertation is to examine the direct cytotoxic effects of the putative parkinsonian neurotoxins, N-methylated $ßC^+$s and $DHßC^+$s in comparison to MPP^+. To this end, as stated previously, the following specific aims are addressed:

Aim I. Employing the clonal, catecholaminergic cell line (PC12) as a model system, the cytotoxic potential of a series of N-methylated $ßC^+$s and $DHßC^+$s and the relative potencies of the toxic species would be determined. In order to assess toxicity, three parameters would be measured: 1) The release of the cytosolic enzyme lactate dehydrogenase (LDH) into the growth medium by damaged or dead cells, 2) the cell protein concentration in the wells as a measure of cell viability, and 3) the uptake of radiolabeled DA by viable cells.

Aim II. To investigate the hypothesis that N-methylated $ßC^+$s and $DHßC^+$s are accumulated by dopaminergic neurons via the DA uptake system, the specificity of the various compounds for PC12 cell catecholamine uptake sites would be determined. This was done by assessing the abilities of the N-methylated $ßC^+$s and $DHßC^+$s to inhibit the binding or accumulation of radiolabeled ligands specific for the DA and norepinephrine (NE) uptake sites in PC12 cells.

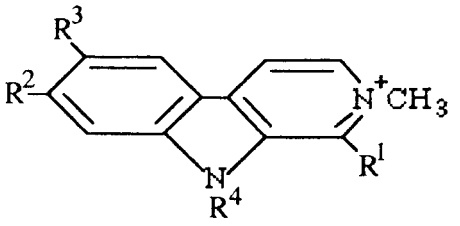
CHAPTER III

MATERIALS AND METHODS

Cell Culture

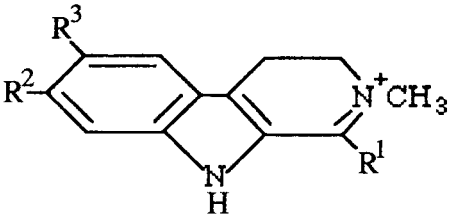
The studies in this dissertation were carried out using PC12 cells, obtained from the American Type Culture Collection (Rockville, MD), grown in two different cell culture media, N-5 or DMEM. The compositions of these two media can be seen in the Appendix (Table 6). For the initial toxicity experiments, cells were grown in N-5 medium supplemented with 10% equine serum (HS), 5% fetal bovine serum (FBS), and 1% penicillin/streptomycin solution (PS; 5000 U/ml x 5 mg/ml). N-5 medium is a modification of Dulbecco's Modified Eagle Media (DMEM) (Kaufman and Barrett, 1983). Kaufman and Barrett (1983) reported that the benefits of the N-5 medium in their primary culture model were the elimination of any putative neurotransmitters (i.e., glutamine and glycine were removed), and that the overall nutrient composition was a better approximation of cerebrospinal fluid than typical cell culture media. In terms of the PC12 cell cultures used in the experiments described in this dissertation, the most significant difference in N-5 medium vs. un-modified DMEM is that N-5 medium contains less than 50% of the available carbon sources found in DMEM. Consequently, N-5 medium can be termed a "low-energy" medium in comparison to DMEM.

For all remaining experiments, PC12 cells were grown in DMEM supplemented as the N-5 medium with 10% HS, 5% FBS, and 1% PS. For cells



2-Methyl-ß-Carbolinium (2-MeßC$^+$)

Name	Abbreviation	R^1	R^2	R^3	R^4
2-Methylnorharmanium	2-MeNh$^+$	H	H	H	H
2,9-Dimethylnorharmanium	2,9-Me$_2$Nh$^+$	H	H	H	CH_3
2-Methylharmanium	2-MeHa$^+$	CH_3	H	H	H
2,9-Dimethylharmanium	2,9-Me$_2$Ha$^+$	CH_3	H	H	CH_3
2-Methylharminium	2-MeHi$^+$	CH_3	CH_3O	H	H
2,9-Dimethylharminium	2,9-Me$_2$Hi$^+$	CH_3	CH_3O	H	CH_3O
2-Methylharmolium	2-MeHo$^+$	CH_3	OH	H	H
6-Methoxy-2-Methylharmanium	6-MeO-2-MeHa$^+$	CH_3	H	CH_3O	H



2-Methyl-3,4-Dihydro-ß-Carbolinium (2-MeDHßC$^+$)

Name	Abbreviation	R^1	R^2	R^3
2-Methylharmalinium	2-MeHli$^+$	CH_3	CH_3O	H
2-Methylharmalolium	2-MeHlo$^+$	CH_3	OH	H
6-Methoxy-2-Methylharmalanium	6-MeO-2-MeHla$^+$	CH_3	H	CH_3O

FIG. 4. NAMES, ABBREVIATIONS AND STRUCTURES OF THE MONO-2-[N]-METHYL-ß-CARBOLINIUM AND 3,4-DIHYDRO-ß-CARBOLINIUM, AND DI-2,9-[N,N']-METHYL-ß-CARBOLINIUM COMPOUNDS.

grown in both media, the medium was changed every 3-4 days and the cells were subcultured every 7-10 days. Cultures were observed at each passage and before each experiment for any indications of variation in gross cell morphology or contamination by bright field microscopy. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

The amount of cells required and the procedure for a given experiment dictated the size and type of culture vessel used for a given experiment. In general, stock cells were grown and maintained in either T-75 cm^2 or T-162 cm^2 flasks (Costar). The type and size of the culture vessel used for the studies are noted in the protocols of the particular experiments listed below. For all experiments where cells were treated *in situ* with compounds, the compounds (fig. 4) were diluted in growth medium to the desired final concentrations, sterile filtered (0.2 μ filters), and supplemented as above with 10% HS, 5% FBS and 1% PS prior to the addition to cultures.

LDH Assay

The release of lactate dehydrogenase (LDH) was quantitated as a measure of non-specific cytotoxicity (Koh and Choi, 1987). The concentration of LDH released by dead or damaged cells into the growth medium was determined on the indicated days (or times) after cells were exposed to various concentrations of either MPP^+, or methylated-ßC$^+$ or -DHßC$^+$. The assay was performed using the Sigma LD-L LDH determination kit, which measures the coupled production of NADH in the oxidation of lactate to pyruvate. At the designated times, the medium was removed from individual culture wells or flasks and centrifuged (Medifuge table-top centrifuge, Baxter Scientific Products, McGaw Park, IL) at 500 x g (2100 rpm) for 5 min. at room T. The supernatant, containing the soluble enzyme, was collected, and the assay was performed by mixing 25 μl of medium with 500 μl of the reconstituted LD-L reagent

(50 mM lactate and 50 mM NAD in a pH 8.9 ± 0.1 buffer). The increase in absorbance at 340 nm was measured for 1.5 min., at 25 °C, using a Gilson Response spectrophotometer. Five samples were measured concurrently, and all samples were run in duplicate. Values of LDH released were expressed as percent of control, where control consisted of parallel groups of cells treated identically (with the exception of drug exposure), and measured at the same time as experimental groups. A significant increase in LDH activity was indicative of cell damage or death.

LDH activity is expressed in units/well (U/well), where a unit of activity is defined as the amount of enzyme catalyzing the production of one micromole of NADH per minute under the defined conditions of the reaction. Linearity of the assay was established periodically using known concentrations of commercial LDH enzyme solution (Lintrol). Calculation of units of LDH activity was carried out using the following formula (eqn. 1):

$$\text{LDH activity (U/well)} = \frac{\Delta A/\text{min.} \times TV \times 1.5}{6.22 \times SV \times LP} \qquad (1)$$

Where:

ΔA/min. = change in absorbance per minute at 340 nm (determined in experiment)

TV = total reaction volume (0.525 ml)

1.5 = converts units per ml to units per well

SV = sample volume (0.025 ml)

6.22 = mM absorptivity of NADH at 340 nm

LP = light path (1 cm)

Inhibition of Toxicity with Mazindol

The DA uptake system appears to be the instrument by which the toxic metabolite, MPP^+, is accumulated by the dopaminergic neurons, and it has been shown that inhibitors of this system prevent neurotoxicity (Javitch *et al.*, 1985; Mayer *et al.*, 1986; Sonsalla *et al.*, 1987). Since PC12 cells possess a DA uptake system, the catecholamine uptake inhibitor, mazindol, was used in an attempt to block the toxic effects of MPP^+, 2-MeHli and 2,9-Me_2Nh. Cells which had been grown in DMEM were plated out the night before the addition of the toxins at a concentration of approximately 150,000 cells/1.5 ml/well in seven 6-well plates. The toxin solutions were prepared, and sterile filtered, the next day in 0.9 volumes of DMEM at the following final concentrations: 500 μM MPP^+ and 2,9-dimethylnorharmanium, and 250 μM 2-methylharmalinium. Mazindol was prepared as 10x concentrated solutions of 300, 100 and 30 μM, sterile filtered, and combined with the sterile toxin solutions or control medium (DMEM). These solutions were supplemented with 10% HS, 5% FBS and 1% PS, and added to the appropriate culture wells. After 2 day incubation, the media was collected and LDH activity was measured as previously described (LDH Assay).

[^{3}H]Dopamine Uptake Assay

Accumulation of [^{3}H]DA by PC12 cells was determined by modification of the method described by Drucker *et al.* (1990). These experiments were performed as a measure of cytotoxicity, where the *a priori* hypothesis was that the energy-dependent nature of the cells' DA uptake system (Greene and Rein, 1977; Koide *et al.*, 1986; Rebois *et al.*, 1980; Schubert and Klier, 1977; Takahashi *et al.*, 1987) would make it a marker of cytotoxicity; that is, dying cells would lose the ability to accumulate DA.

Prior to the addition of compounds to cultures, cells were plated-out in N-5 medium on 6-well plates and grown to near confluency. Cells were then exposed to various compounds for two days in N-5 medium. After this time, the medium was removed from the wells and placed in corresponding test-tubes. The wells were immediately filled with equal volumes of Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) and set aside while the medium was centrifuged at 500 x g for 5 min. at 25 °C to collect any viable cells detached from the surface of the wells during handling of the plates. The supernatants were collected in corresponding test-tubes and put aside for later LDH analysis (as previously described). The cells were then harvested from the individual wells by repeatedly passing the PBS over the surface of the well to loosen the cells, and the resulting cell suspension was used to resuspend the pellet obtained from the initial centrifugation. This suspension was then centrifuged as above. The resulting supernatant was discarded, and the pellet was resuspended in Krebs-Ringer Phosphate (KRP) buffer, pH 7.4, containing 1.7 mM ascorbic acid and 10 μM pargyline. An aliquot was removed for later protein determination (see Protein Determination for procedure).

Test-tubes containing the cell suspensions were placed in a 37 °C water bath with shaking. Non-specific uptake was determined by the addition of 10 μM nomifensine to three of the six tubes in the group prior to addition of [^{3}H]DA. The reaction was initiated by the addition of approximately 3.5 nM [^{3}H]DA in KRP buffer to each tube. After 15 min. incubation, the reaction was terminated and the cells were collected by washing 3 times with ice-cold PBS using a 24 sample Brandel Cell Harvester. The filters (Whatman GF-B filter strips) were transferred into scintillation vials containing 10 ml of Ecoscint scintillation cocktail, and the samples were counted for 10 min. each using a liquid scintillation counter (Beckman LS 7500, Beckman Instruments, Fullerton, CA). Results of these experiments were expressed as percent

of control, where control groups were those not exposed to compounds in culture. All values were adjusted for nonspecific uptake by subtracting the means of the nomifensine-treated tubes from the means of the corresponding 'totals' tubes. 'Totals' tubes were those tubes containing only [^{3}H]DA, KRP buffer and cells.

Variation of the Conditions of [^{3}H]Dopamine and [^{3}H]NorepinephrineUptake

To determine if the accumulation of [^{3}H]DA was indeed actual accumulation by the PC12 cells' catecholamine uptake system, and not a binding phenomenon, the integrity of the cells (intact, homogenized or boiled), the sodium concentration, and the temperature of the incubation were varied. Since PC12 cells accumulate both DA (Rebois *et al.*, 1980) and NE (Greene and Rein, 1977) by mechanisms that are both sodium and temperature dependent, and both neurotransmitters may be taken up by the same system (Denton and Howard, 1984), both [^{3}H]DA and [^{3}H]NE were used in this study.

PC12 cells were grown in DMEM (T-162 cm^2 flasks) in the absence of any toxic compounds. Cells were harvested by removing the growth medium from each flask and replacing it with 10 ml of Hank's Balanced Salts Solution (HBSS) per flask. The cells were gently triturated free from the surface of the flask, the suspensions from each flask were combined in a single flask, and a 0.5 ml aliquot was removed for counting. After counting, the cells were divided among eight 15 ml centrifuge tubes, each containing approximately 0.75 x 10^6 cells. The cell suspensions were centrifuged at 500 x g (Medifuge table-top centrifuge) for 5 min. at room T. After centrifugation, the supernatants were discarded and the cell pellets were resuspended to a final concentration of 5 x 10^4 cells/ml in one of three KRP buffers (components of the various KRP buffers are listed in Table 7, in the Appendix): 4 tubes (3 x 10^6 cells)

in 'normal' KRP buffer; 2 tubes (1.5 x 10^6 cells) in 'sodium control' KRP buffer; 2 tubes (1.5 x 10^6 cells) in 'zero sodium' KRP buffer.

One of the tubes of cell suspension (equal to 0.75 x 10^6 cells) in 'normal' KRP buffer was homogenized (Ultra-Turrax tissue homogenizer, Janke and Kunkel) for 15 seconds at a rheostat setting of '45' (Tissue Mizer High Torque, Tekmar, Cincinnati, OH). The resultant homogenate was then centrifuged (Sorvall RC-5B rotor, SS34 rotor) at 20,000 rpm (47,800 x g) for 20 min. at 4 °C. After centrifugation, the supernatant was discarded and the pellet was resuspended in 10 mls of 'normal' KRP buffer, and centrifuged again as above. After the second centrifugation, the pellet was resuspended to an equivalent concentration of 5 x 10^4 cells/ml in 'normal' KRP buffer. A second tube of cells suspended in 'normal' KRP buffer (equal to 0.75 x 10^6 cells) was boiled for 5 min. in a boiling water bath. After these two treatments, an aliquot was removed from each of the eight tubes for later protein determination (see Protein Determination for procedure).

For the uptake/binding assay, six different groups were run: 1) control (37 °C, 'normal' KRP buffer, intact cells); 2) 4 °C, 'normal' KRP buffer, intact cells; 3) 37 °C, 'normal KRP buffer, homogenized cells; 4) 37 °C, 'normal' KRP buffer, boiled cells; 5) 37 °C, 'sodium control' KRP buffer, intact cells; 6) 37 °C, 'zero sodium' KRP buffer, intact cells. Each group consisted of two sub-groups (6 tubes/sub-group: 4 totals + 2 baseline, containing 5 x 10^4 cells/tube), where one sub-group was incubated in the presence of approximately 3.5 nM [^{3}H]DA, and the other was incubated in the presence of approximately 3.5 nM [^{3}H]NE. Non-specific uptake/binding was determined by the addition of 10 μM nomifensine to the two 'baseline' tubes in each sub-group. For the five assay groups incubated at 37 °C, the reaction tubes were placed in a 37 °C water bath with shaking. The tubes in the assay group incubated at 4 °C were placed on ice. In all cases, the total volume of the reaction was 1.5 ml, and

the reaction was initiated by the addition of 1 ml of the appropriate cell suspension. Samples were then incubated, harvested and counted as previously described ([^{3}H]Dopamine Uptake Assay). Results of these experiments were expressed as femtomoles of radioligand accumulated/bound per mg protein. All values were adjusted for nonspecific uptake/binding by subtracting the 'baseline' value from the 'total'.

[^{3}H]GBR 12935 and [^{3}H]Mazindol Competition Binding Assays

These binding assays were carried out by modification of the methods described by Battaglia *et al.* (1988). The medium was removed from the culture flasks and replaced with 10 mls of ice cold Tris buffer, pH 7.4, containing 50 mM Tris, 120 mM NaCl and 5 mM KCl. The cells were harvested from the surface of the flask by gentle trituration with the Tris buffer. The cell suspensions from the number of flasks required for a final equivalent concentration of 10^5 cells/0.1 mls were combined in a single flask and a 0.5 ml aliquot was removed for cell counting. The cell suspension was homogenized was homogenized as previously described, except that Tris buffer was used here instead of KRP buffer. After the second centrifugation, the pellet was resuspended in a volume of Tris buffer that gave an equivalent concentration of 10^5 cells/0.1 mls.

The [^{3}H]GBR 12935 binding assay was performed using 5 nM of radioligand, and the catecholamine uptake antagonist, mazindol, as a competing ligand at log concentrations from 10^{-10} - 10^{-5} (triplicate concentrations of each). Total binding (consisting of radioligand, buffer and cell homogenate only) was determined in quadruplicate. Nonspecific binding was determined in duplicate tubes by the addition of 10 µM GBR 12909, a specific DA uptake antagonist and a selective inhibitor of [^{3}H]GBR 12935 binding (Andersen, 1989). The reaction was initiated by the addition

of 0.1 ml of cell homogenate to the incubation mixture to give a final reaction volume of 1 ml. Samples were incubated for 60 min. at room T.

Similar to the [^{3}H]GBR 12935 assay, the [^{3}H]mazindol binding assay was carried out using mazindol as a competing ligand at log concentrations from 10^{-10} - 10^{-4} (triplicate concentrations of each) and 6 nM radioligand. As above, total binding was determined in quadruplicate. The NE uptake antagonist desipramine was used (in duplicate tubes) at a concentration of 0.3 μM to describe the nonspecific binding component (Battaglia *et al.*, 1988). The total volume of the incubation mixture was 0.5 mls, and the reaction was initiated by the addition of 0.05 ml of cell homogenate (equivalent of 5 x 10^4 cells/tube). The sample tubes were incubated for 60 min. at 4 °C.

For both the [^{3}H]GBR 12935 and the [^{3}H]mazindol binding assays, the reactions were terminated, the cells were harvested and counted, and the data was analyzed as previously described. In this case, however, washes were performed using cold Tris buffer and samples were counted for only 2 min. each.

IC_{50} and Hill coefficient (n_H) values were calculated by computer-assisted linear regression analysis of the corresponding Hill plot. The Hill equation can be written in the following form (eqn. 2):

$$B = \frac{B_o \cdot [I]^n}{K_i + [I]^n} \qquad (2)$$

and the equation can be transformed to the linear form (eqn. 3):

$$\log \frac{B}{(B_o - B)} = n \log [I] - \log K_i \qquad (3)$$

The Hill plot was generated by plotting the log 'fractional saturation' vs. the log concentration of inhibitor. 'Fractional saturation' is mathematically defined as:

$$\frac{B}{(B_o - B)}$$

Where B is the fraction of ligand bound in the presence of inhibitor, and B_o is the total ligand bound in the absence of inhibitor. In this plot, n_H is equal to the slope of the line and the value of log K_i is equal to the intercept. The IC_{50} value is the x-intercept of the line, so by setting the ordinate value of equation 3 equal to zero:

$$\log \frac{B}{(B_o - B)} = 0$$

equation 3 can be rearranged into the following form (eqn. 4):

$$\log [I] = \frac{\log K_i}{n} \qquad (4)$$

where log [I] is equal to the IC_{50} value of the inhibitor, which in this case is mazindol. For the Hill plots, only those concentrations of competing ligand that inhibited between 10 and 90 percent of specific uptake or binding were included because of the deviation from linearity that occurs at the extremes (Cornish-Bowden and Koshland, 1975).

Once these values were established, it was possible to determine the percent fractional occupancy (% f.o.) of the radioligand at the binding site by the following equation (eqn. 5):

$$\% \text{ f.o.} = \frac{1}{1 + K_i/[*L]} \times 100 \qquad (5)$$

where [*L] is the concentration of radioligand used in the reaction, and where K_i was determined from the Cheng and Prusoff correction (eqn. 6),

$$K_i = \frac{IC_{50}}{1 + [*L]/K_d^*} \qquad (6)$$

where, in the experiments described here, [*L] << K_d*. From the fractional occupancies, the total fmoles of available binding sites in the tube were calculated by the following equation (eqn. 7):

$$\text{fmoles/tube} = 100 / \%\ \text{f.o.} \times ([\text{dpms} / 2.2] / \text{S.A.}) \qquad (7)$$

In this equation: dpms = specific disintegrations per minute, 2.2 x 10^6 = dpms / μCi, S.A. = the specific activity of the radioligand and 10^6 is a conversion factor.

[^{3}H]Dopamine and [^{3}H]NorepinephrineUptake Inhibition Assays

These experiments were performed by modification of the method previously described for [^{3}H]Dopamine Uptake. For these experiments, cells had been grown in DMEM, and were not exposed to toxins. Cells were harvested for the experiment by removing the growth medium from the flask(s) and replacing it with 10 mls of KRP buffer which was used to gently triturate the cells free from the flask surface. The cells were then counted and diluted with the appropriate volume of KRP buffer to a final concentration of 5 x 10^4 cells/ml. A 1 ml aliquot of cells was then removed and frozen at -20 °C for later protein determination.

For the experiment, cells were incubated with 3.5 nM of either [^{3}H]DA or [^{3}H]NE. Concentrations of competing ligand were 10^{-10} - 10^{-5} for DA, NE, desipramine, mazindol and nomifensine, and 10^{-9} - 10^{-4} for MPP^+, 2-methylharmalinium and 2,9-dimethylnorharmanium. For each compound tested, groups were divided as follows: 4 'Totals' tubes (no competing ligand added), 2 'baseline' tubes (containing 10 μM nomifensine to define nonspecific binding), and 3 tubes for each concentration of the particular competing ligand. In all cases the uptake reaction was initiated by the addition of 1 ml of cell suspension (5 x 10^4 cells), and incubated for 15 min. in a 37 °C water bath. The reaction was terminated, the cells

were harvested and counted, and the data was analyzed by the Hill transformation as previously described.

Protein Determination

Protein concentrations were analyzed by the method of Lowry *et al.* (1951). This method was chosen over other similar methods, because it contains a sodium hydroxide digestion step necessary to solubilize the proteins of the intact cells to allow accurate quantitation. In brief: Bovine serum albumin was utilized as the protein standard in the assay at concentrations from 25 - 200 µg protein. A wide concentration range was used due to the variability that was commonly observed among the unknown samples. Standards and samples were solubilized in 100 µl of 1.0 N sodium hydroxide for 30 min. at room T. Then, 1 ml of Lowry reagent (0.1:0.1:10, v/v/v, respectively of, 1% [w/v] cupric sulfate, 2% [w/v] K^+-Na^+ tartrate, and 2% (w/v) sodium carbonate) was added to standards and samples. After 10 min., 100 µl of 1.0 N Folin and Ciocalteu's phenol reagent (Sigma) was added and standards and samples were incubated for 30 min. The absorbance of samples and standards was then measured vs. a water blank at 700 nm.

As a measure of toxicity, the cells that survived exposure to the compounds tested were quantitated by measuring the amount of protein of cells attached to the plate. This method had been employed previously for similar experiments (Andersen *et al.*, 1990; Denton and Howard, 1987), and was chosen here over cell counts for two main reasons: First, PC12 cells grow in loosely attached, grape-like clusters instead of a monolayer that are almost impossible to count using a microscope. By washing away the loose cells and only passing those cells that are strongly attached, one selects for a variant phenotype which is morphologically different (flattened with processes) and grows more slowly than wild-type PC12 cells (personal observation). Second, to

accurately count the cells after drug treatment using a Coulter counter or hemocytometer, one would need to either mechanically or enzymatically (e.g., cell scraper or trypsin, respectively) dissociate the cells attached to the surface of the culture vessel to achieve an accurate quantitation. This would rupture any cells that had been weakened by the toxins (LDH is released because cell membrane integrity is compromised), and these fragmented cells would not be counted, thereby effecting the accuracy of the quantitation. For these reasons, protein levels were quantitated as a measure of cytotoxicity where a significant decrease in protein/well indicated cell death. Protein values were expressed as percent of control, where control consisted of parallel groups of cells treated identically (with the exception of drug exposure), and measured at the same time as experimental groups.

Cell Counting

Based upon the reasons described above, cell counts were used only as a method of estimating the number of cells to assure that approximately the same number of cells were used for repetition of particular experiments. The cells were counted using a Coulter Counter with a Channelyzer 256 (Coulter Electronics, Hialeah, FL). A 0.5 ml aliquot of cell suspension was diluted in 20 mls of Isoton III solution (Coulter Diagnostics, Hialeah, FL). The settings for the instrument were as follows:

current	100
scale	10 mA
polarity	auto
low threshold	6
high threshold	99.9
attenuation	8

gain	1
manometer	500 µl
Channelyzer low cut-off	4.96 µm
Channelyzer high cut-off	36 µm

The low cut-off of 4.96 µm for the Channelyzer was chosen because the size of a PC12 cell has been reported to be 3 - 7 µm (Buskirk *et al.*, 1988). Below 4.96 µm it was too difficult to discriminate between cells and debris. Cell counts were performed three times for each sample counted. The concentration of cells per ml was determined using the following formula (eqn. 8):

$$\text{cells/ml} = \frac{\text{avg. counts}}{\text{vol. counted}} \times \frac{\text{final vol.}}{\text{ml in dilution}} \qquad (8)$$

Where:

average counts = the average of the three readings

volume counted = 0.5 ml (500 µl manometer)

final volume = 20.5 ml (20 ml Isoton + 0.5 ml sample)

ml in dilution = 0.5 ml (0.5 ml sample)

Statistical Analysis

Statistical analyses were carried out using the Statview II statistics software (Abacus Concepts, Inc., Berkeley, CA) on an Apple Macintosh computer. The specific statistical tests used for individual experiments are indicated in the figure and table legends in Chapter IV.

Most of the data were analyzed by performing a one factor analysis of variance (ANOVA) to determine if there were any differences among the means of the groups compared. This particular test was chosen for two reasons: 1) In most cases the groups being compared differed by only one factor (e.g., compound-treated vs.

control), and 2) there were more than two population means that had to be compared, therefore precluding the use of multiple t-tests (Godfrey, 1986). When the ANOVA indicated that at least one of the group means differed from the others ($p < 0.05$), a *post hoc* multiple comparison analysis was carried out to determine which group(s) differed (Godfrey, 1986). Both Scheffé's and Fisher's PLSD (Protected Least Significant Difference) tests were used to do these *post hoc* comparisons, where a p value < 0.05 was considered significant. Since Scheffé's test is a much more conservative test than Fisher's test, those groups found significant for both tests were reported only as having been significant by Scheffé's test. Likewise, there were instances where the less stringent Fisher's test indicated significance where Scheffé's test did not, and these data were reported as having been significant by Fisher's test.

For the time-course LDH release experiment data (fig. 6), a two factor ANOVA model was used because there were two independent variables: treatment (compound-treated vs. control) and time. For these analyses, the ANOVA F test was carried out with respect to both treatment and time, and also for the interaction of these two factors. In each case, the F test was considered significant for values of $p < 0.05$. Significance with respect to treatment indicated that there was a difference between the means of the compound-treated groups relative to the control groups, and significance with respect to time indicated that there was a time-dependent change in LDH released. As it was used in these studies, demonstration of interaction between the two factors ($p < 0.05$) indicated that one group demonstrated an effect with time whereas the other group did not.

In some cases, the purpose of the experiments was only to determine if the means of the individual experimental groups, those treated with compounds, differed from the means of the concurrently run controls. In these cases, the groups were

compared on a one-to-one basis with controls by using an un-paired Student's t-test. For these analyses, a p value < 0.05 was considered significant.

Materials

Acrodisc 0.2 µm Syringe Filter	Gelman Sciences, Ann Arbor, MI
Cell Culture Flasks	
25, 75 and 162 cm^2	Costar, Cambridge, MA
Desipramine HCl	Sigma Chemical Co., St. Louis, MO
DMEM	Sigma Chemical Co., St. Louis, MO
Donor Equine Serum	Biocell, Carson, CA
Dopamine HCl	Sigma Chemical Co., St. Louis, MO
[7,8-^{3}H]Dopamine	
(S.A. 40-48 Ci/mmol)	Amersham, Arlington Heights, IL
Ecoscint	National Diagnostics, Somerville, NJ
Fetal Bovine Serum	Biocell, Carson, CA
Filter Units, 0.2 µm, 500 ml	Costar, Cambridge, MA
Folin & Ciocalteu's Phenol	
Reagent (2.0 N)	Sigma Chemical Co., St. Louis, MO
GBR 12909	RBI, Natick, MA
[propylene-2,3-^{3}H]GBR 12935	
(S.A. 24.4 Ci/mmol)	DuPont NEN Research Products, Boston, MA
LD-L Reagent Kit	Sigma Chemical Co., St. Louis, MO
Lintrol LDH Standardization Soln.	Sigma Chemical Co., St. Louis, MO
Mazindol	RBI, Natick, MA
[4'-^{3}H]Mazindol	
(S.A. 15.8 Ci/mmol)	DuPont NEN Research Products, Boston, MA

† 2-Methylharminium (anhydronium base)	Sigma Chemical Co., St. Louis, MO
MPP^+ (iodide salt)	RBI, Natick, MA
Nomifensine maleate	RBI, Natick, MA
Norepinephrine HCl	Regis Chemical Co., Morton Grove, IL
[7,8-^{3}H]Norepinephrine (S.A. 39 Ci/mmol)	Amersham, Arlington Heights, IL
N-5 Cell Culture Medium	Hazleton, Lenexa, KS - custom preparation
Pargyline HCl	Sigma Chemical Co., St. Louis, IL
Penicillin/Streptomycin Soln.	Sigma Chemical Co., St. Louis, MO
Sterile Pipettes (1, 5, 10 and 25 ml)	Costar, Cambridge, MA
6-Well Cell Culture Plates	Costar, Cambridge, MA
Whatman GF-B filter strips	Whatman Labsales, Hillsboro, OR

† The other methylated-ßC$^+$s and -DHßC$^+$s were prepared in Dr. Collins' laboratory as previously described (Albores *et al.*, 1990; Collins *et al.*, 1992; Drucker *et al.*, 1990).

CHAPTER IV

RESULTS

The results in this chapter are presented in two parts. The first section, Toxicity Studies, reports the toxic effects of the various compounds tested on PC12 cell cultures. The second portion of this chapter, Accumulation and Binding of [^{3}H]Catecholamine Uptake Ligands, summarizes the results from the DA and NE uptake site analyses and the affinity of MPP^+, 2-$MeHli^+$ and 2,9-Me_2Nh^+ for those sites. At the end of the chapter, there is a brief summary of the results from both sections.

Toxicity Studies

Initial Screening of the Compounds

The toxic efficacies of eleven methylated ßCs and DHßCs (fig. 4) and MPP^+ were determined by measuring LDH released by PC12 cells into the growth medium after 2 and 4 day exposure to 500 µM concentrations in N-5 medium. After 2 day exposure (fig. 5, top), seven compounds including MPP^+ were observed to have caused significant LDH release. Most notable of these was the DHßC, 2-$MeHli^+$, which was equipotent with MPP^+. The three 2,9-di[N,N']-methylated $ßC^+$s examined were also toxic, a finding of particular interest because their mono[N]-methylated congeners, with the exception of 2-$MeHi^+$, were significantly less efficacious. In particular, 2-$MeNh^+$ was completely ineffective even after 4 days, whereas 2,9-Me_2Nh^+ elicited a response similar to MPP^+. The remaining compounds tested

displayed no effect at 2 days, and only 6-MeO-2-MeHla$^+$ produced a marginal response (137% of control) after 4 days.

The viable cell protein remaining in the well after 2 day exposure to 500 μM concentration of compounds was also quantitated (fig. 6). The results were similar to those observed for LDH release: 2-MeHli$^+$ and the three di[N]-methylated species (2,9-Me_2Nh$^+$, 2,9-Me_2Hi$^+$ and 2,9-Me_2Ha$^+$) were similar to or equipotent with MPP$^+$. In contrast, 2-MeHi$^+$, which engendered significant release of LDH, produced no significant effect on cell protein. Likewise, the other six compounds tested were ineffective.

From these studies, MPP$^+$, 2-MeHli$^+$, 2-MeHi$^+$ and the three dimethylated species (2,9-Me_2Nh$^+$, 2,9-Me_2Hi$^+$ and 2,9-Me_2Ha$^+$) were found to be the most toxic of the compounds tested. Consequently, the toxic potencies of these six compounds were further examined, and their individual structures are shown in figure 7.

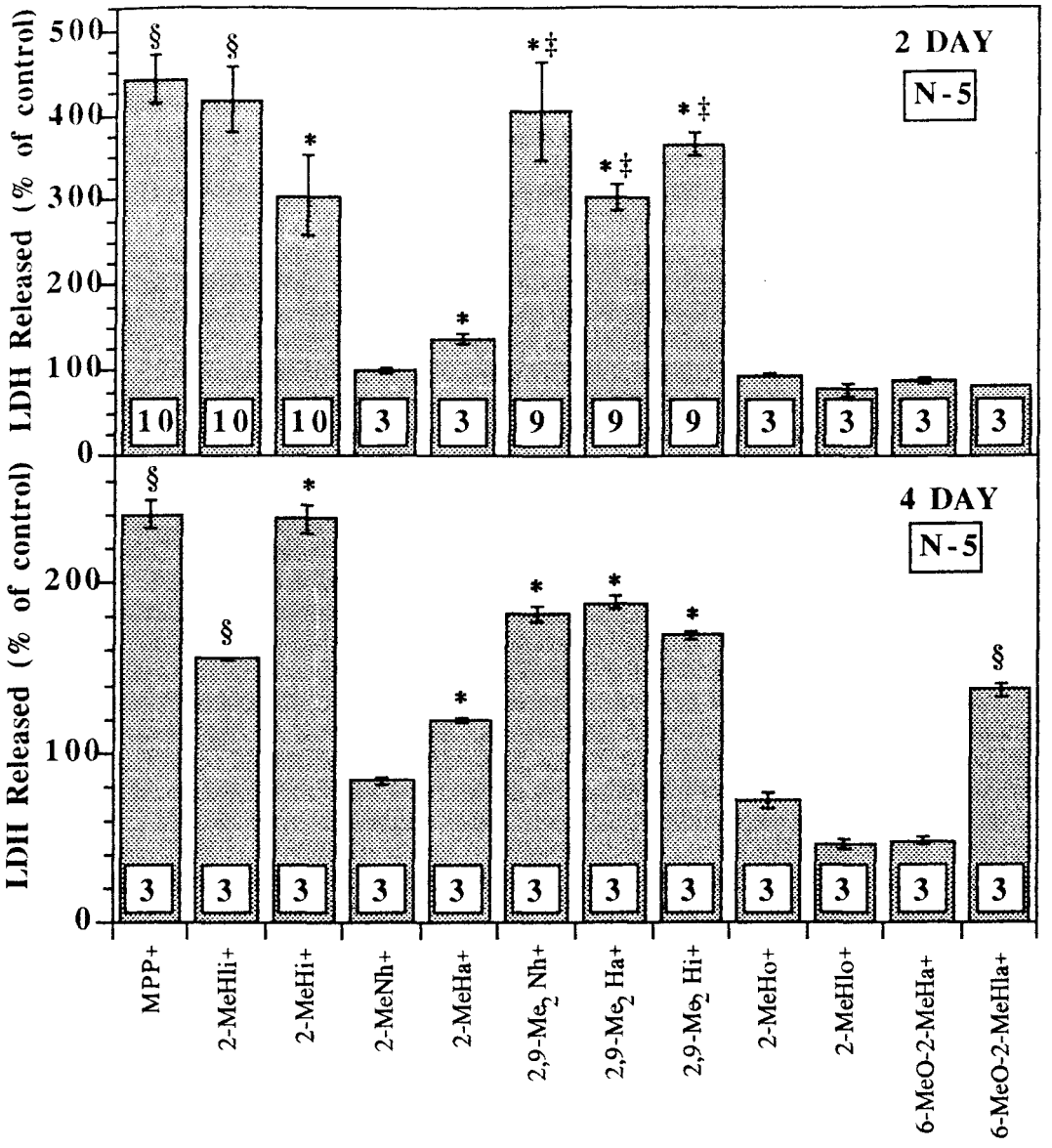


FIG. 5. LDH RELEASED FROM PC12 CELLS AFTER 2 OR 4 DAY EXPOSURE TO 500 μM COMPOUNDS IN N-5 MEDIUM. Results are the means of replicate determinations (n values on the bars) ± SEM with each determination done on triplicate sister wells. Data are expressed as the percent of the corresponding, concurrently run controls, where the overall mean control values were 0.1036 ± .012 U/well at 2 days, and 0.217 ± .003 U/well at 4 days. For mono- and di-methylated pairs, significance was determined relative to the corresponding control and the paired compound by one-factor analysis of variance (ANOVA). ANOVA values were significant for all three pairs ($p < 0.05$), so individual differences within these groups were determined by Scheffé F *post hoc* analysis (* = relative to control, ‡ = dimethyl relative to monomethyl, $p < 0.05$). For the remaining compounds tested, significance was determined relative to the corresponding control by unpaired Student's t-test (§ $p < 0.05$).

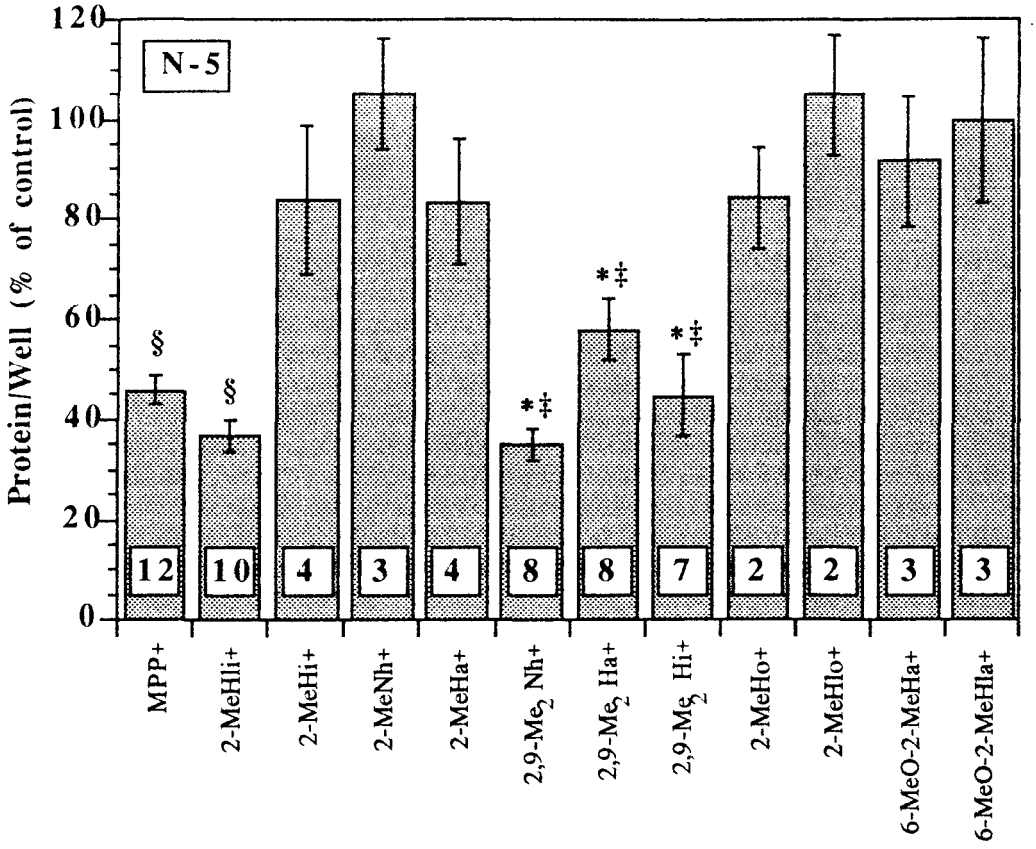
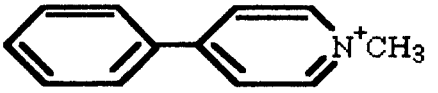
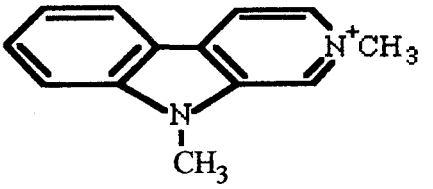


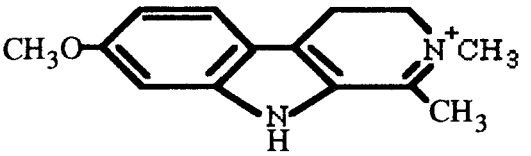
FIG. 6. EFFECTS OF 2 DAY EXPOSURE OF PC12 CELLS TO 500 μM COMPOUNDS IN N-5 MEDIUM ON CELL PROTEIN. Results are the means of replicate determinations (n values indicated on the bars) ± SEM with each determination done on triplicate sister wells. Data are expressed as the percent of the corresponding, concurrently run controls, where the overall mean was 443.00 ± 13.46 μg/well. For mono- and di-methylated pairs, significance was determined relative to the corresponding control and the paired compound by one-factor analysis of variance (ANOVA). ANOVA values were significant for all three pairs ($p < 0.05$), so individual differences within these groups were determined by Scheffé F *post hoc* analysis (* = relative to control, ‡ = dimethyl relative to monomethyl, $p < 0.05$). For the remaining compounds tested, significance was determined relative to the corresponding control by unpaired Student's t-test (§ $p < 0.05$).



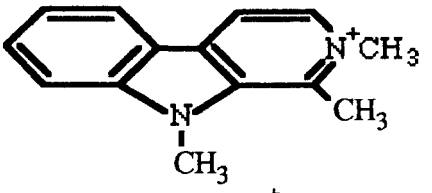
MPP$^+$
(N-methyl-4-phenylpyridinium)



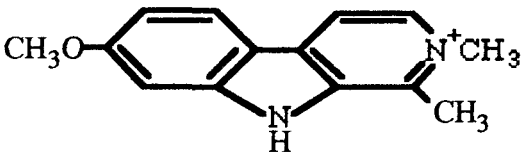
2,9-Me_2Nh$^+$
(2,9-dimethylnorharmanium)



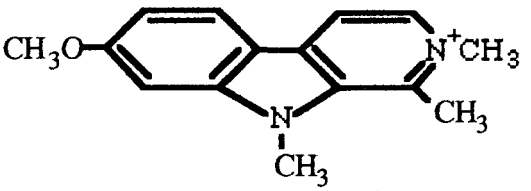
2-MeHli$^+$
(2-methylharmalinium)



2,9-Me_2Ha$^+$
(2,9-dimethylharmanium)



2-MeHi$^+$
(2-methylharminium)



2,9-Me_2Hi$^+$
(2,9-dimethylharminium)

FIG. 7. STRUCTURES OF THE SIX MOST POTENT COMPOUNDS FROM THE INITIAL EXPERIMENTS IN N-5 MEDIUM.

Effects of Compounds on LDH Release

Figure 8 shows the time-course (2-48 h) of LDH released upon exposure to 500 μM compounds in N-5 medium. 2-MeHi$^+$ produced minimal LDH release up through 48 h. This corresponded with the lack of effect on viable cell protein seen in figure 6. Of the other five compounds tested, 2-MeHli$^+$ and MPP$^+$ effected the most robust responses (approximately 660% of control), with peak responses occurring by 24 h. Likewise, 2,9-Me$_2$Hi$^+$ was most effective by 24 h (434% of control). However, neither 2,9-Me$_2$Nh$^+$ nor 2,9-Me$_2$Ha$^+$ appeared to be as effective until at least 36 h. For the three compounds which produced their greatest responses by 24 h (MPP$^+$, 2-MeHli$^+$ and 2,9-Me$_2$Hi$^+$), the subsequent decreases in LDH were due to the relative increases in LDH released in the control wells.

The concentration-dependence of the six compounds shown in figure 7 was also tested in N-5 medium (fig. 9). PC12 cells were exposed to either 50, 100, 250 or 500 μM of compound for 2 days. As with the time-course study, 2-MeHli$^+$ and MPP$^+$ were the two most effective agents. Both showed initial effects at 100 μM and were highly toxic at 250 μM with 2-MeHli$^+$ being slightly more effective. In contrast, 2-MeHi$^+$ produced only a slight effect at the highest (500 μM) concentration. Of the three dimethylated species, 2,9-Me$_2$Nh$^+$ and 2,9-Me$_2$Hi$^+$ effected minimal responses at 250 μM, and displayed significant toxicity, as previously determined (fig. 5 & 8), at 500 μM. 2,9-Me$_2$Ha$^+$ displayed no effect on LDH release at concentrations lower than 500 μM.

In figure 10, cells were also grown and treated in DMEM for 2 days with the same concentrations of the three most potent compounds from figure 9: MPP$^+$, 2-MeHli$^+$ and 2,9-Me$_2$Nh$^+$. Of the three compounds, only 2-MeHli$^+$ evoked a significant release of LDH (325% of control, at 250 μM). In table 1, the results of the LDH release experiments in DMEM (fig. 10) were compared to those obtained in N-5 medium (fig.

9). With the exception of the lowest concentration tested (50 μM), the effects of MPP^+ on LDH release were significantly less in DMEM than in N-5 medium. At concentrations of 250 and 500 μM, 2-$MeHli^+$ was also more potent in N-5 medium than in DMEM. The only difference observed with 2,9-Me_2Nh^+ was at 500 μM, but this was also the only concentration at which 2,9-Me_2Nh^+ produced any significant effect in either medium. Overall, MPP^+, 2-$MeHli^+$ and 2,9-Me_2Nh^+ were each less effective in causing LDH release in DMEM than in the low energy medium, N-5.

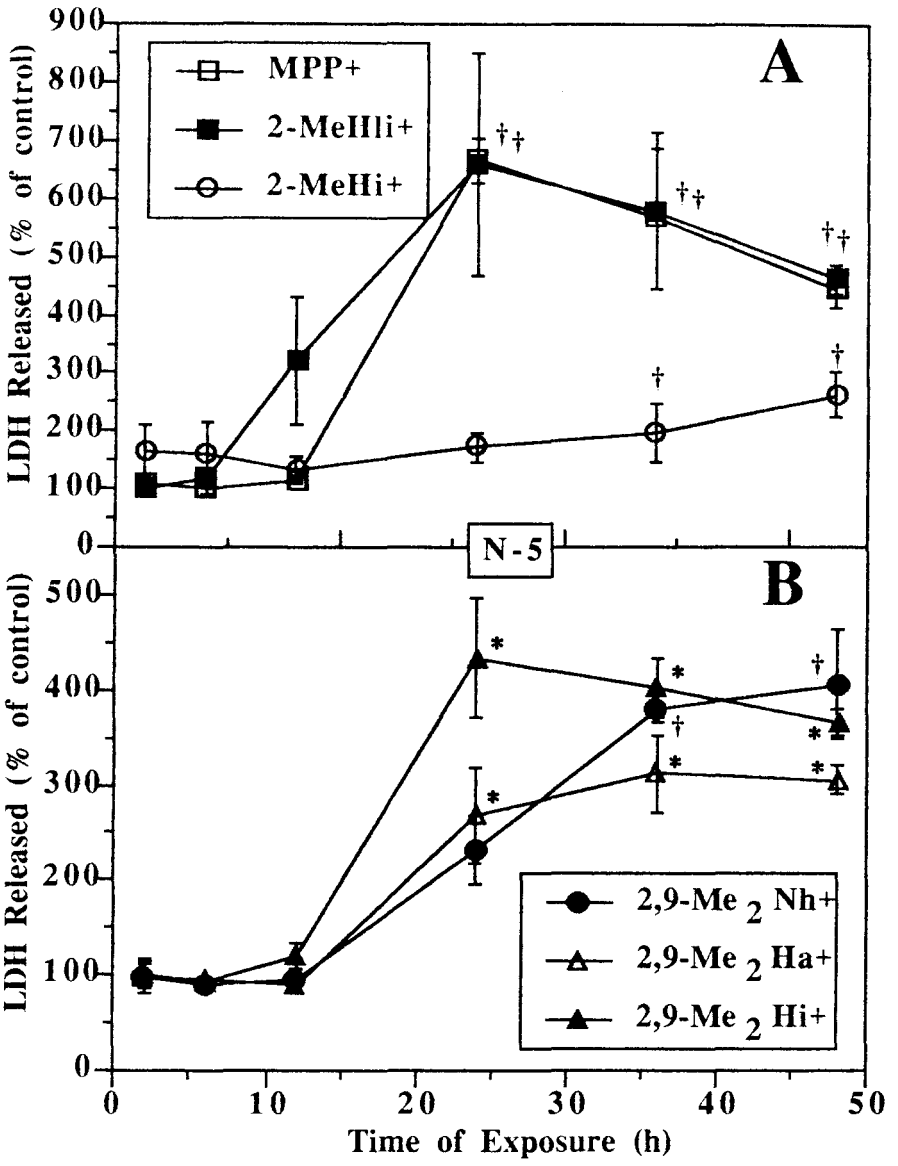


FIG. 8. TIME-COURSE OF LDH RELEASED FROM PC12 CELLS EXPOSED TO 500 μM CONCENTRATIONS OF MPP+ AND SELECTED ß-CARBOLINES IN N-5 MEDIUM. Each time-point is the mean of at least 3 replicate determinations ± SEM with each determination done on triplicate sister wells. Data are expressed as the percent of the corresponding, concurrently run controls, where the mean control values (U/well ± SEM) at the various time points were as follows: 2 h = 0.067 ± 0.005, 6 h = 0.065 ± 0.005, 12 h = 0.072 ± 0.006, 24 h = 0.091 ± 0.013, 36 h = 0.163 ± 0.045, 48 h = 0.104 ± 0.012. Significance was determined by two-factor analysis of variance (ANOVA). Values were significant for all compounds at $p < 0.05$, except for the interaction of time and concentration with 2-MeHIi+ and 2-MeHi+ which were not significant. Significance of individual points relative to the corresponding controls was determined by either Scheffé F (* $p < 0.05$) or Fisher PLSD († $p < 0.05$) *post hoc* analysis.

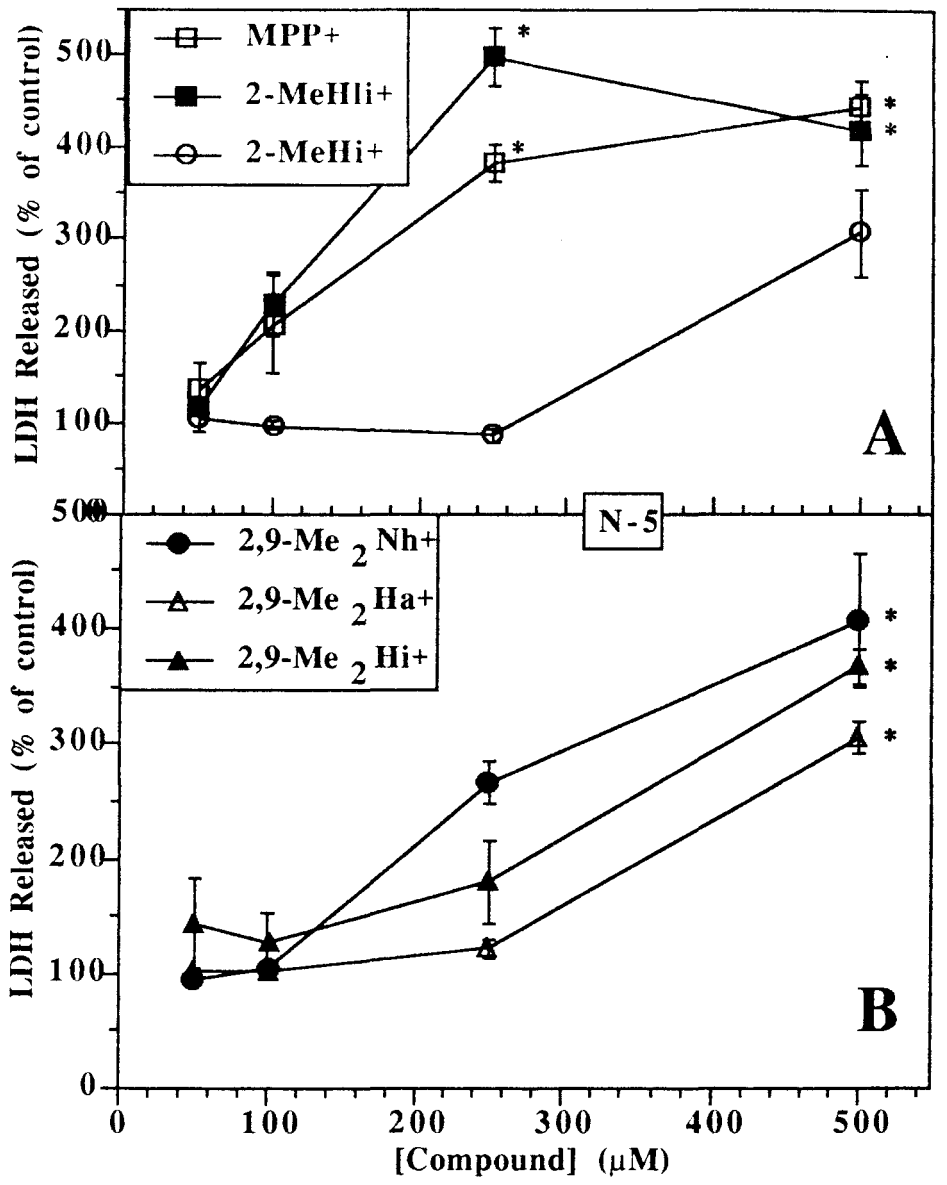


FIG. 9. LDH RELEASED FROM PC12 CELLS AFTER 2 DAY EXPOSURE TO INCREASING CONCENTRATIONS OF MPP+ AND SELECTED ß-CARBOLINES IN N-5 MEDIUM. Each point is the mean of at least 3 replicate determinations ± SEM with each determination done on triplicate sister wells. Data are expressed as the percent of the corresponding, concurrently run controls, where the mean overall control value was 0.104 ± 0.012 U/well. Significance was determined by one-factor analysis of variance (ANOVA). All compounds were significant vs. control at $p < 0.05$. Significance of individual points relative to the corresponding controls was determined by Scheffé F (* $p < 0.05$) *post hoc* analysis.

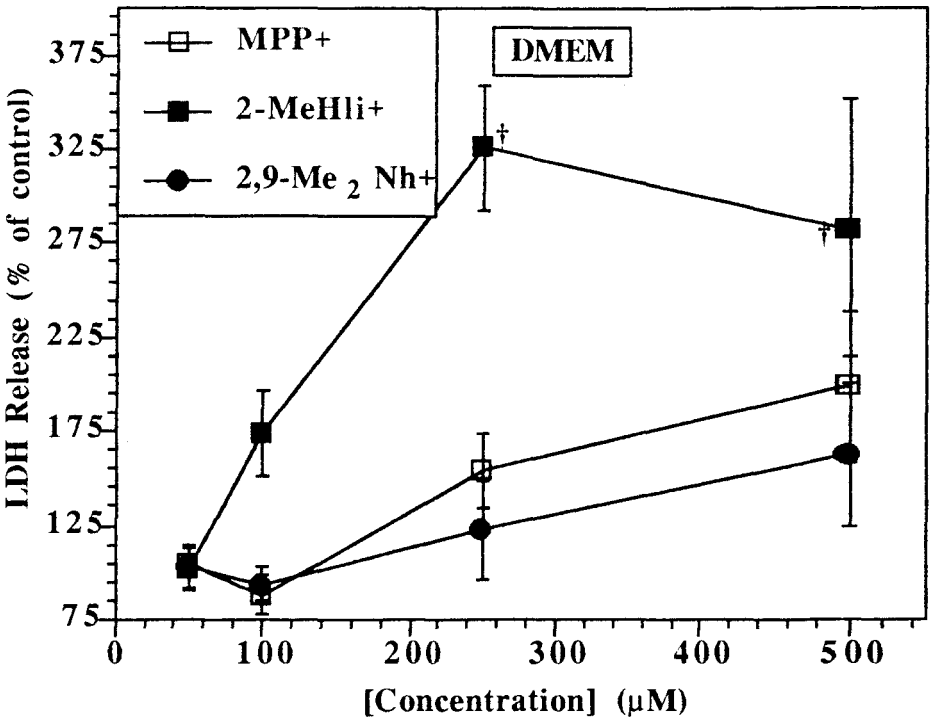


FIG. 10. LDH RELEASED FROM PC12 CELLS AFTER 2 DAY EXPOSURE TO INCREASING CONCENTRATIONS OF MPP$^+$, 2-MeHli$^+$ AND 2,9-Me2Nh$^+$ IN DMEM. Each point is the mean of 5 replicate determinations ± SEM Data are expressed as the percent of the corresponding, concurrently run controls, where the overall mean control value was 0.219 ± 0.43 U/well. Significance was determined by one-factor analysis of variance (ANOVA). Of the three compounds, only 2-MeHli was significant vs. control at $p < 0.05$. † Indicates individual points significant relative to the corresponding controls ($p < 0.05$) by Fisher PLSD *post hoc* analysis.

TABLE 1

COMPARISON OF THE EFFECTS OF MEDIA ENERGY LEVELS (DMEM VS. N-5 MEDIA) ON LDH RELEASED FROM PC12 CELLS BY MPP^+, 2-$MeHli^+$ AND 2,9-Me_2Nh^+

Concentration (μM)	MPP^+	2-$MeHli^+$	2,9-Me_2Nh^+
50	76.05 ± 2.80	89.10 ± 10.59	107.13 ± 11.20
100	42.88 ± 4.92 †	76.32 ± 9.80	90.40 ± 8.88
250	40.37 ± 5.28 *	65.67 ± 6.74 †	46.00 ± 9.82
500	44.91 ± 9.24 *	67.85 ± 16.55 †	40.06 ± 9.40 *

Data are the DMEM values (% of control, fig. 10) expressed as the mean % of the corresponding N-5 values (% of control, fig. 9) ± SEM. [The numerical values from figures 9 and 10 are summarized in Table 8 in the Appendix.] Significance was determined by one-factor analysis of variance (ANOVA), and all of the groups tested were significant ($p < 0.05$). The symbols, * and †, indicate significance ($p < 0.05$) of individual DMEM values relative to the corresponding N-5 values by Scheffé F and Fisher PLSD *post hoc* analysis, respectively.

Effects of Compounds on Cell Protein

The effects of the compounds in N-5 medium on viable cell protein (fig. 11) were consistent with those seen for LDH release (fig. 9). 2-MeHli$^+$ was the most potent of the six agents tested, effecting a 30% reduction in cell protein at 100 μM, and a maximum effect (65% decrease) with 250 μM. MPP$^+$, however, did not approach the maximum effectiveness of 2-MeHli$^+$ until 500 μM. Similarly, 2,9-Me$_2$Nh$^+$ produced a significant protein decrease at 250 μM and was equipotent with MPP$^+$ and 2-MeHli$^+$ at 500 μM. 2,9-Me$_2$Hi$^+$ displayed no significant effect below 500 μM. The other two compounds tested, 2-MeHi$^+$ and 2,9-Me$_2$Ha$^+$, were ineffective in the N-5 medium.

When the three most potent compounds from figure 11 (MPP$^+$, 2-MeHli$^+$ and 2,9-Me$_2$Nh$^+$) were tested for 2 days at the same concentrations in DMEM (fig. 12), 2-MeHli was again the most toxic. However, in this case it produced a significant decrease of cell protein even at 50 μM. Also, 2,9-Me$_2$Nh$^+$ caused a significant decrease at 500 μM, whereas in the N-5 medium it was ineffective even at that high concentration. The results with MPP$^+$ were virtually identical in both media.

A comparison of the effects of MPP$^+$, 2-MeHli$^+$ and 2,9-Me$_2$Nh$^+$ on cell protein in the two different media is shown in table 2, and the corresponding EC_{50} values are listed in table 3. In contrast to the LDH data, 2-MeHli$^+$ was markedly more potent in DMEM (EC_{50} = 25 μM) than in N-5 medium (EC_{50} = 210 μM). This was because the depletion of cell protein was significantly greater at 50 and 100 μM in DMEM compared to N-5 medium. At the higher concentrations of 2-MeHli, and all of the concentrations of MPP$^+$ and 2,9-Me$_2$Nh$^+$, there were no significant differences in the toxicity's of the compounds in the two culture media (Table 2). This is in contrast to the LDH data, where the compounds were observed to be less effective in DMEM (table 1).

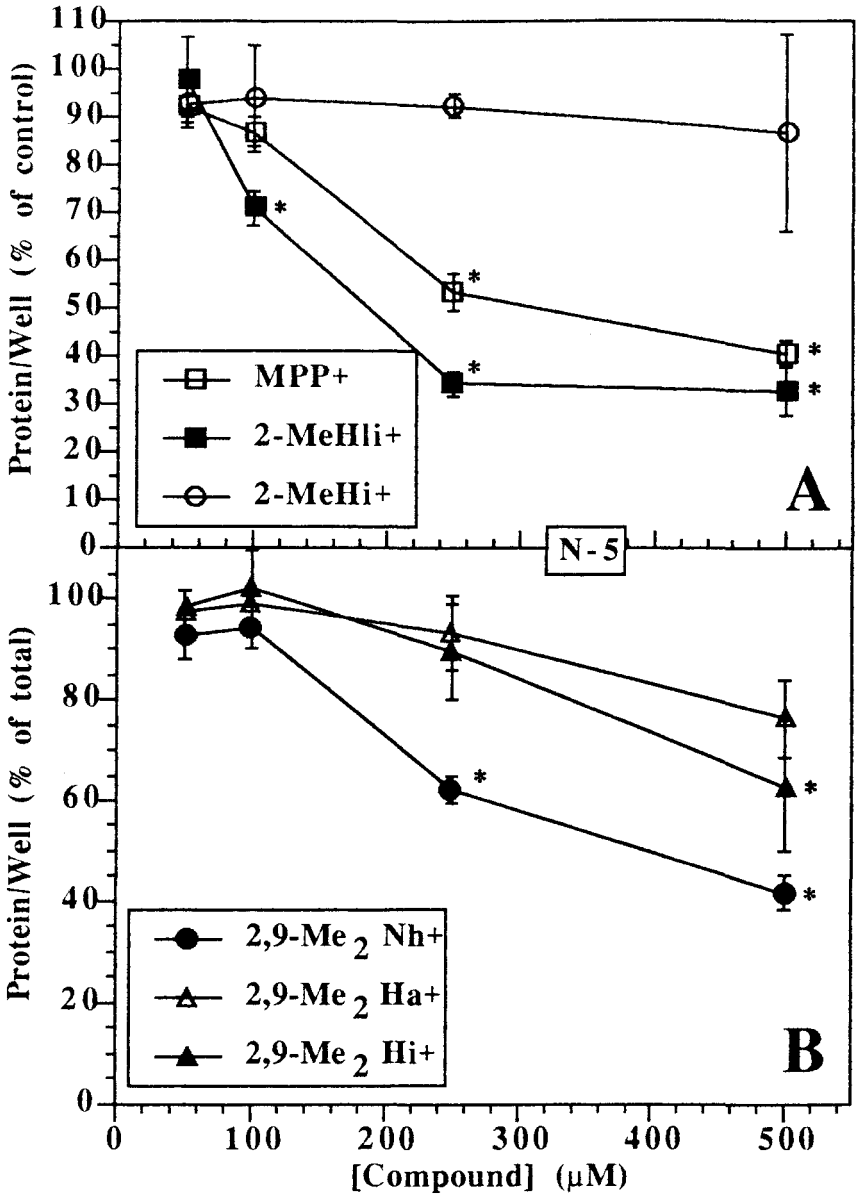


FIG. 11. EFFECTS OF 2 DAY EXPOSURE TO INCREASING CONCENTRATIONS OF MPP^+ AND SELECTED ß-CARBOLINES IN N-5 MEDIUM ON PC12 CELL PROTEIN. Each point is the mean of at least 3 replicate determinations ± SEM with each determination done on triplicate sister wells. Data are expressed as the percent of the corresponding, concurrently run controls, where the overall mean control value was 438.67 ± 18.95 μg/well. Significance was determined by one-factor analysis of variance (ANOVA). MPP^+, 2-$MeHli^+$, 2,9-Me_2Nh^+ and 2,9-Me_2Hi^+ were significant vs. control at $p < 0.05$. Significance of individual points relative to the corresponding control was determined by Scheffé F *post hoc* analysis (* $p < 0.05$).

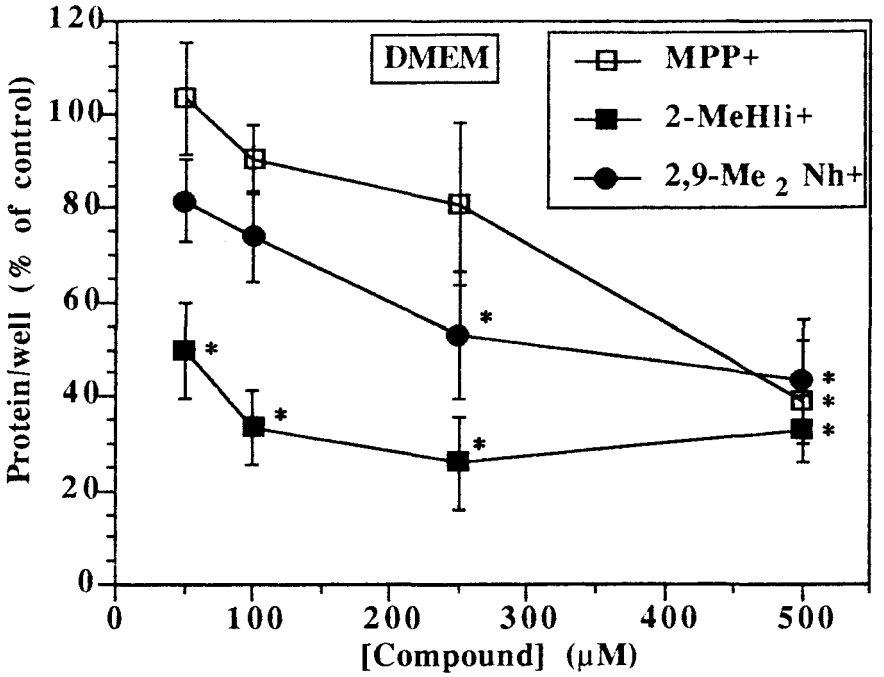


FIG. 12. EFFECTS OF 2 DAY EXPOSURE TO INCREASING CONCENTRATIONS OF MPP^+, 2-$MeHli^+$ AND 2,9-$Me2Nh^+$ IN DMEM ON PC12 CELL PROTEIN. Each point is the mean of 4 replicate determinations ± SEM Data are expressed as the percent of the corresponding, concurrently run controls, where the overall mean control value was 143.9 ± 11.56 μg/well. Significance was determined by one-factor analysis of variance (ANOVA). All three compounds were significant at $p < 0.05$. * Indicates significance of individual points relative to the corresponding controls ($p < 0.05$) by Scheffé F *post hoc* analysis.

TABLE 2

COMPARISON OF THE EFFECTS OF MEDIA ENERGY LEVELS (DMEM VS. N-5 MEDIA) ON MPP^+, 2-$MeHli^+$ AND 2,9-Me_2Nh^+ -INDUCED DEPLETION OF PC12 CELL PROTEIN

Concentration (μM)	MPP^+	2-$MeHli^+$	2,9-Me_2Nh^+
50	111.90 ± 12.79	51.05 ± 10.39 *	87.92 ± 9.46
100	103.98 ± 8.16	47.09 ± 11.07 †	78.44 ± 10.00
250	151.90 ± 32.58	75.45 ± 28.44	85.46 ± 21.44
500	95.61 ± 31.52	100.80 ± 21.84	118.31 ± 31.87

Data are the DMEM values (% of control, fig. 12) expressed as the mean % of the corresponding N-5 values (% of control, fig. 11) ± SEM. [The numerical values for figures 11 and 12 are summarized in Table 8 in the Appendix.] Significance was determined by one-factor analysis of variance (ANOVA), and all of the groups tested were significant ($p < 0.05$). The symbols, * and †, indicate significance ($p < 0.05$) of individual DMEM values relative to the corresponding N-5 values by Scheffé F and Fisher PLSD *post hoc* analysis, respectively.

TABLE 3

LIST OF EC_{50} VALUES FOR THE DEPLETION OF CELL PROTEIN IN DMEM AND N-5 CULTURE MEDIA

Compound	EC_{50} (μM)	
	N-5	DMEM
MPP^+	330	490
2-$MeHli^+$	210	25
2,9-Me_2Nh^+	410	330
2-$MeHi^+$	> 1000	NT
2,9-Me_2Ha^+	> 1000	NT
2,9-Me_2Hi^+	> 1000	NT

The EC_{50} values for the compounds were determined graphically by replotting the data from figures 11 and 12 as the protein/well (% of control) vs. the log concentration of compounds. The numerical values for figures 11 and 12 are summarized in Table 8 in the Appendix. NT = not tested.

Effects of Compounds on [^{3}H]Dopamine Uptake

The capacity of the PC12 cells to accumulate [^{3}H]DA after exposure to various concentrations of compounds was examined as another measure of cytotoxicity (figures 13 & 14). In the case of the mono-methylated compounds tested in N-5 medium (fig. 13A), the results were similar to those seen for cell protein (fig. 11): Both MPP^+ and 2-$MeHli^+$ virtually abolished all DA accumulation at 250 μM (approximately 21 and 13% of control, respectively), and 2-$MeHi^+$ had no significant effect even at 500 μM. Similar results were obtained when the cells were exposed to MPP^+ and 2-$MeHli^+$ in DMEM, where both compounds caused dose-dependent decreases in the accumulation of [^{3}H]DA. MPP^+ did not decrease uptake to the same extent in DMEM as in N-5 medium, but 2-$MeHli^+$ was approximately equipotent (table 4).

The reactions of the cells treated with the dimethylated compounds (fig. 13B) were the opposite of those of the monomethylated species. With the exception of 2,9-Me_2Nh^+, which produced a decrease to control levels at 500 μM, accumulation of [^{3}H]DA increased with dose. At a concentration of 250 μM, each of the compounds produced a significant increase in DA uptake relative to its concurrently run control. When the experiment was repeated on PC12 cells grown and treated in DMEM (fig. 14), the dimethylated compound (2,9-Me_2Nh^+) did not increase the accumulation of [^{3}H]DA. So, similar to what had been previously observed for the effect of 2,9-Me_2Nh^+ on LDH release (table 1), this compound was less effective in producing a response in DMEM than in N-5 medium (table 4).

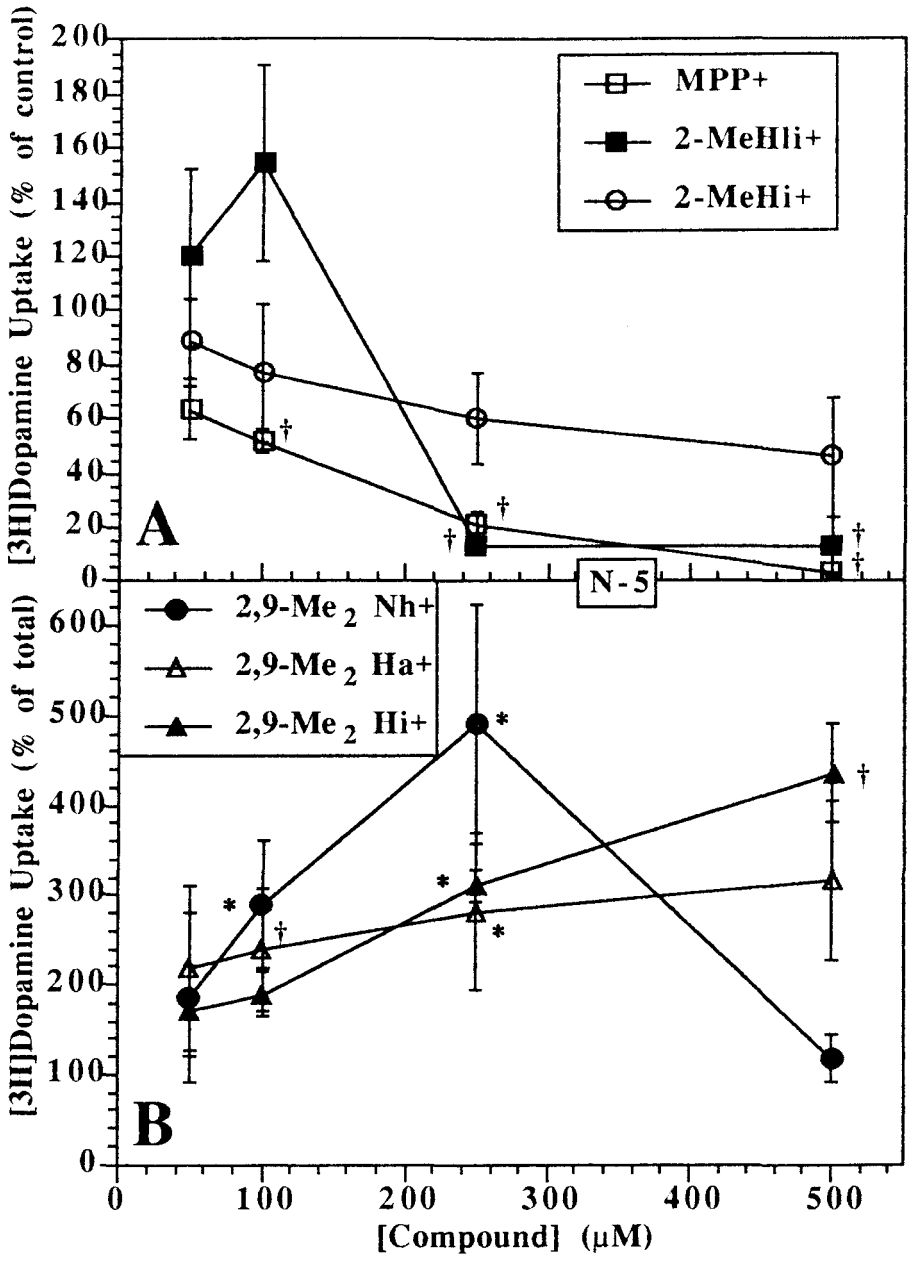


FIG. 13. [^{3}H]DOPAMINE UPTAKE BY PC12 CELLS AFTER 2 DAY EXPOSURE TO INCREASING CONCENTRATIONS OF MPP$^+$ AND SELECTED ß-CARBOLINES IN N-5 MEDIUM. Each point is the mean of at least 3 replicate determinations ± SEM with each determination done on triplicate sister wells. Data are expressed as the percent of the corresponding, concurrently run controls, where the overall mean control value was 268.87 ± 34.09 fmoles/mg protein. Significance was determined by one-factor analysis of variance (ANOVA). All compounds, except 2-MeHi$^+$, were significant vs. control at $p < 0.05$. Significance of individual points relative to the corresponding controls was determined by Scheffé F (* $p < 0.05$) and Fisher PLSD († $p < 0.05$) *post hoc* analyses.

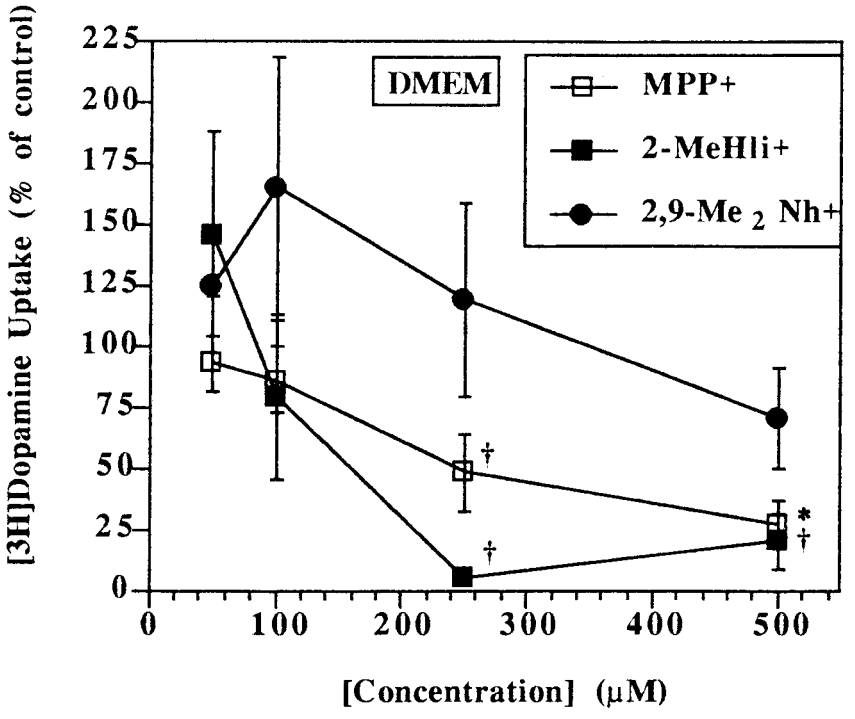


FIG. 14. [^{3}H]DOPAMINE UPTAKE BY PC12 CELLS AFTER 2 DAY EXPOSURE TO INCREASING CONCENTRATIONS OF MPP^+, 2-$MeHli^+$ AND 2,9-$Me2Nh^+$ IN DMEM. Each point is the mean of 4 replicate determinations ± SEM Data are expressed as the percent of the corresponding, concurrently run controls, where the overall mean control value was 350.12 ± 44.58 fmoles/mg protein. Significance was determined by one-factor analysis of variance (ANOVA). MPP^+ and 2-$MeHli^+$, but not 2,9-Me_2Nh^+, were significant vs. control at $p < 0.05$. Significance of individual points relative to the corresponding controls was determined by Scheffé F (* $p < 0.05$) and Fisher PLSD († $p < 0.05$) *post hoc* analysis.

TABLE 4

COMPARISON OF THE EFFECTS OF MEDIA ENERGY LEVELS (DMEM VS. N-5 MEDIA) ON MPP^+, 2-$MeHli^+$ AND 2,9-Me_2Nh^+ -INDUCED CHANGES IN [^{3}H]DOPAMINE UPTAKE

Concentration (μM)	MPP^+	2-$MeHli^+$	2,9-Me_2Nh^+
50	146.83 ± 18.07	122.31 ± 35.21	67.27 ± 2.35
100	166.45 ± 26.34 †	51.36 ± 21.95	57.16 ± 18.65
250	231.36 ± 75.05	39.59 ± 10.48	24.33 ± 8.12 †
500	883.91 ± 329.77	155.51 ± 88.44	60.56 ± 17.41

Data are the DMEM values (% of control, fig. 14) expressed as the mean % of the corresponding N-5 values (% of control, fig. 13) ± SEM. [The numerical values for figures 13 and 14 are summarized in Table 8 in the Appendix.] Significance was determined by one-factor analysis of variance (ANOVA), and all of the groups tested were significant ($p < 0.05$). The symbol, †, indicates significance ($p < 0.05$) of individual DMEM values relative to the corresponding N-5 values by Fisher PLSD *post hoc* analysis.

Effects of Mazindol on Toxicity

PC12 cells were exposed for 2 days to MPP^+, 2-$MeHli^+$ and 2,9-Me_2Nh^+ at their most effective concentrations (500, 250 and 500 μM, respectively), based upon data from previous experiments with these compounds in DMEM (fig. 10). In an attempt to inhibit the toxic effects of these compounds, the cells were concurrently incubated in the presence or absence (control) of 3, 10 or 30 μM of the catecholamine uptake inhibitor, mazindol. LDH released into the extracellular milieu was measured as the indicator of cytotoxicity. Figure 15 shows that mazindol was ineffective at inhibiting any of the toxic effects even at a concentration of 30 μM; at a concentration of 100 μM, mazindol itself was toxic (data not shown).

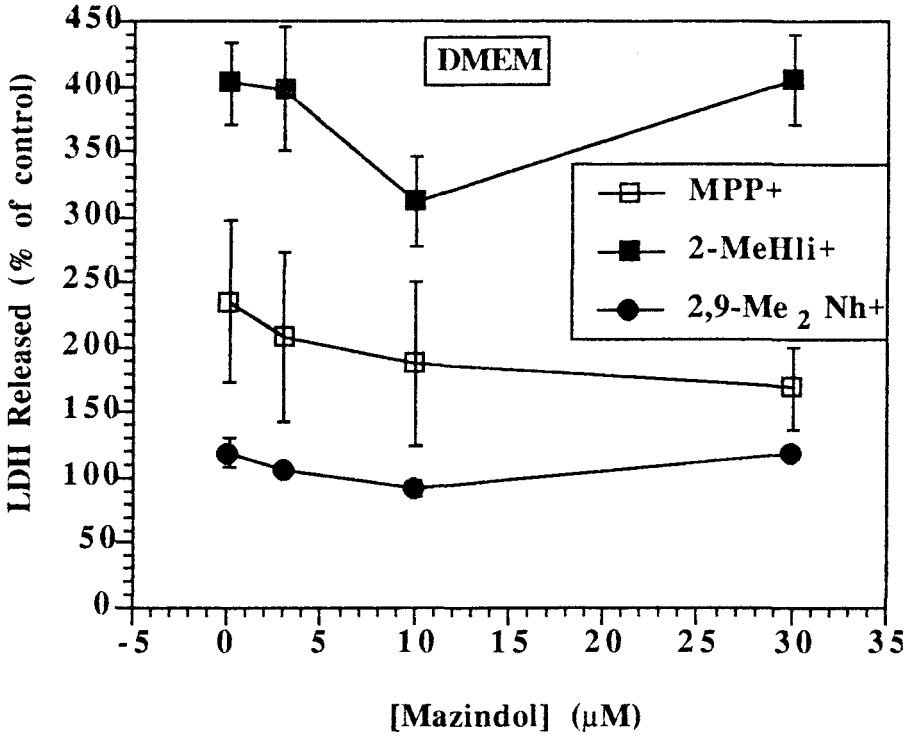


FIG. 15. EFFECTS OF MAZINDOL ON LDH RELEASE FROM PC12 CELLS EXPOSED TO MPP^+, 2-$MeHli^+$ AND 2,9-Me_2Nh^+ IN DMEM. MPP^+ and 2,9-Me_2Nh^+ were both used at a concentration of 500 μM, and 2-$MeHli^+$ was used at a concentration of 250 μM. Each point is the mean of 4 replicate determinations ± SEM. Data are expressed as the percent of the corresponding, concurrently run controls, where the mean control value was 0.082 ± 0.012 U/well. Significance was determined by one-factor analysis of variance (ANOVA). None of the three compounds were significant at $p < 0.05$.

Accumulation and Binding of [^{3}H]CatecholamineUptake Ligands

Effects of Varying Incubation Conditions

The accumulation of NE by PC12 cells had previously been shown to be Na^+ and temperature-dependent (Greene and Rein, 1977), but this had not been demonstrated for DA. To address this question, the temperature of the incubation, the sodium concentration, and the integrity of the cells (intact, homogenized or boiled) were modulated (fig. 16). Reduction of the incubation temperature from 37 °C (control) to 4 °C abolished both [^{3}H]DA and [^{3}H]NE accumulation by intact PC12 cells in unmodified KRP buffer. Likewise, when sodium was removed from the incubation buffer (0 Na^+) intact cells displayed negligible catecholamine uptake. Similarly, when the cells were boiled, uptake was reduced almost to zero. However, homogenization of the cells only reduced the accumulation of [^{3}H]DA and [^{3}H]NE to 40% and 50%, respectively, of the corresponding control values. In the case of [^{3}H]NE this was not a significant decrease.

Catecholamine Uptake Site(s) Analysis

To establish the presence of DA uptake sites in PC12 cells, the selective probe, [^{3}H]GBR 12935, was used. Figure 17 shows the curve for inhibition of [^{3}H]GBR 12935 binding to PC12 cell homogenates by unlabeled mazindol. The specific binding of [^{3}H]GBR 12935 constituted only 35% of total binding, as defined by the presence of 10 μM unlabeled GBR 12909, a selective inhibitor of DA uptake and [^{3}H]GBR12935 binding (Andersen, 1989). From the Hill plot (fig. 17B), the IC_{50} for mazindol inhibition of specific [^{3}H]GBR 12935 binding was determined to be 10 μM. By assuming the IC_{50} was comparable to the K_D for [^{3}H]GBR 12935 at these sites, the theoretical B_{max} was calculated by the Cheng and Prusoff correction (eqn. 6) to be 426 pmol/tube.

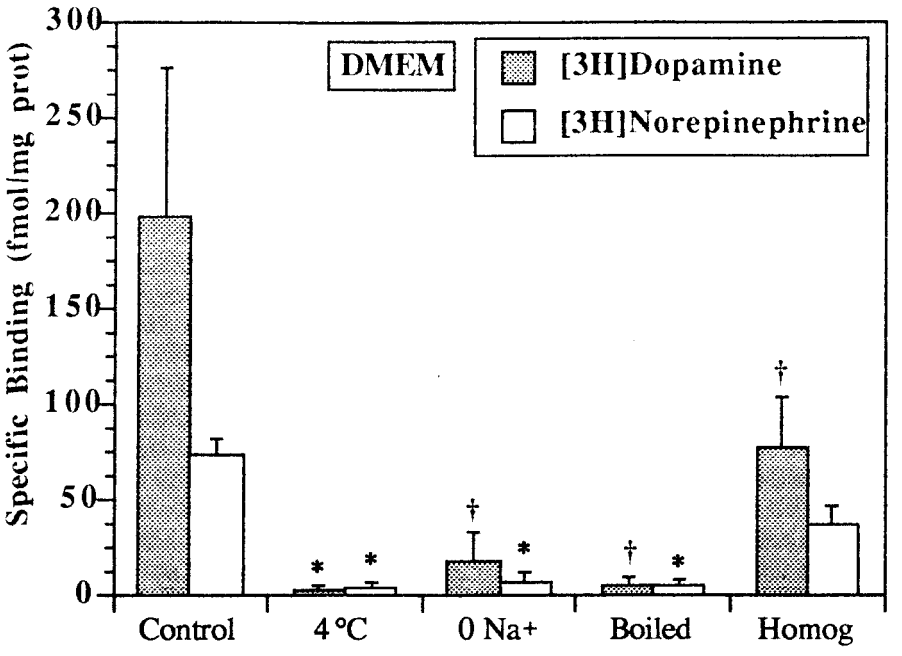


FIG. 16. EFFECTS OF VARYING INCUBATION CONDITIONS ON [^{3}H]DOPAMINE AND [^{3}H]NOREPINEPHRINE UPTAKE INTO PC12 CELLS. Each point is the mean of 3 replicate determinations ± SEM with each determination done on triplicate sister wells. Data are expressed as fmoles/mg protein. Data were analyzed in two groups ([^{3}H]DA and [^{3}H]NE), and significance was determined by one-factor analysis of variance (ANOVA); both groups were significant at $p < 0.05$. Significance of individual points relative to the corresponding controls was determined by Scheffé F (* $p < 0.05$) and Fisher PLSD († $p < 0.05$) *post hoc* analysis. Control = intact cells, in unmodified KRP buffer incubated at 37 °C; 4 °C = incubation at 4 °C; 0 Na^+ = KRP buffer with no sodium; Boiled = boiled cells; Homog = homogenized cells.

To examine the presence and the characteristics of the NE site, [^{3}H]mazindol was used as a probe and an inhibition curve was generated by competition with unlabeled mazindol (fig. 18). To define specific binding to NE sites, [^{3}H]mazindol was incubated in the presence of 0.3 μM desipramine (Battaglia *et al.*, 1988). However, [^{3}H]mazindol binding was virtually unchanged from total in the presence of desipramine (91.68 ± 1.97 % of total) indicating only 10% of the total [^{3}H]mazindol binding was to classical NE sites. Using the GraphPad Inplot program for the IBM PC, the baseline was floated at 21 fmoles, and a Hill plot was generated (fig. 18B) from which the IC_{50} value was

determined to be 5 μM. As previously described for [^{3}H]GBR 12935, the IC_{50} was assumed to be equivalent to the K_D for [^{3}H]mazindol binding and the theoretical B_{max} was calculated (eqn. 6) to be 60 pmol/tube.

The data in figure 18 had demonstrated inhibitable binding of [^{3}H]mazindol to PC12 cell homogenates. Consequently, the abilities of MPP^+, 2-$MeHli^+$ and 2,9-Me_2Nh^+ to inhibit the binding of [^{3}H]mazindol were examined to determine if these compounds had affinities for the catecholamine uptake site in PC12 cells. Of the three compounds, only 2-$MeHli^+$ displayed any effectiveness at inhibiting [^{3}H]mazindol binding, reducing it to 61% of specific binding at 100 μM (fig. 19B). The inhibition curve produced by 2-$MeHli^+$ also appears biphasic indicating two or more binding sites for which [^{3}H]mazindol and 2-$MeHli^+$ may compete. Neither MPP^+ nor 2,9-Me_2Nh^+ reduced [^{3}H]mazindol binding below the level of control (fig. 19A and C). However, all three compounds produced slight increases in [^{3}H]mazindol binding at their lowest concentrations (fig. 19A, B and C), but there was not enough data to determine if these increases were statistically significant.

To determine the relative potencies of MPP^+, 2-$MeHli^+$ and 2,9-Me_2Nh^+ to compete for the catecholamine uptake sites, these compounds and several known substrates and inhibitors were tested as inhibitors of [^{3}H]DA and [^{3}H]NE uptake into intact PC12 cells. The data for these experiments are presented in figures 20-27. For each compound tested (DA, NE, desipramine, mazindol, nomifensine, MPP^+, 2-$MeHli^+$ and 2,9-Me_2Nh^+), IC_{50} values and Hill coefficients (n_H) were determined by transforming the data from the inhibition curves and expressing the results on Hill plots. The results are summarized in Table 5.

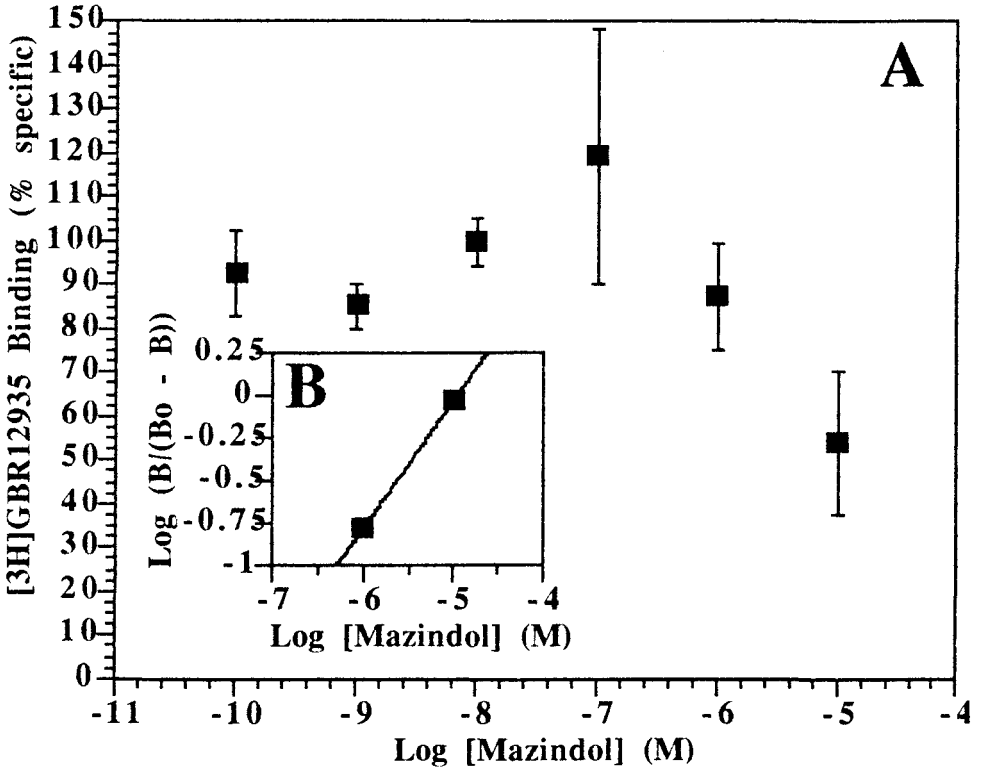


FIG. 17. 5 nM [^{3}H]GBR 12935 BINDING TO PC12 CELL HOMOGENATES IN THE PRESENCE OF UNLABELED MAZINDOL. In the inhibition curve (A), data are expressed as the percent of specific binding, where total binding was 773.19 ± 214.23 fmoles/tube. Specific binding was defined by the addition of 10 μM GBR 12909, and determined to be 35% of total binding. Each point is the mean of 3 replicate determinations ± SEM. From the corresponding Hill plot (B): IC_{50} = 10 μM and n_H = 0.75, fractional occupancy of the binding site(s) was calculated to be 0.05%, and from this the theoretical B_{max} ≈ 426 pmol/tube.

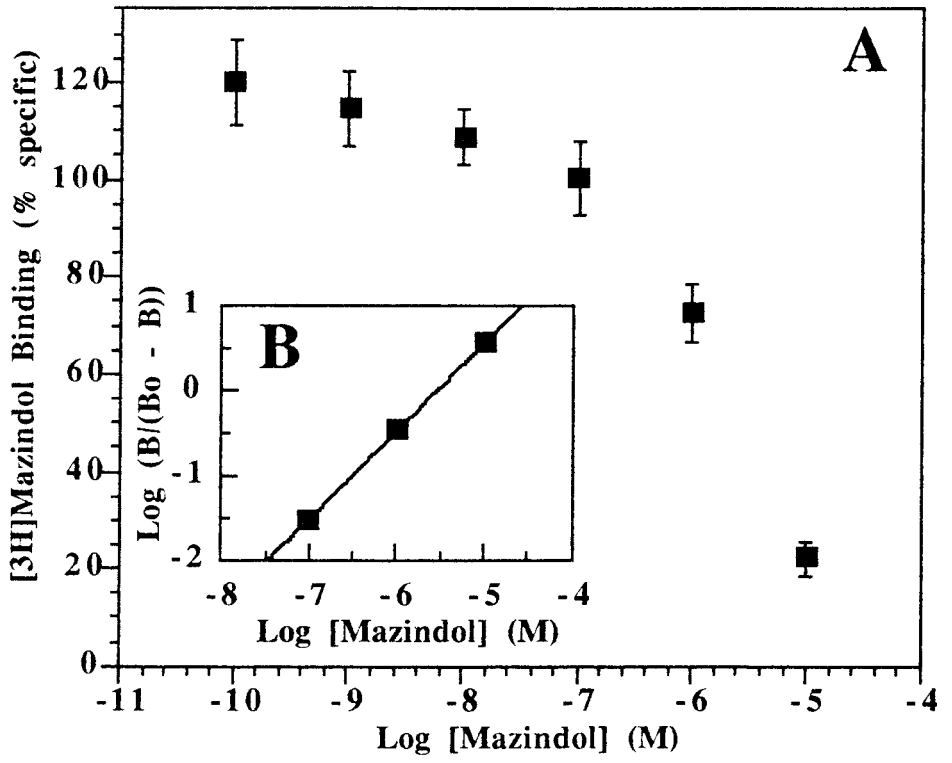


FIG. 18. 6nM [^{3}H]MAZINDOL BINDING TO PC12 CELL HOMOGENATES IN THE PRESENCE OF UNLABELED MAZINDOL. In the inhibition curve (A), data are expressed as the percent of specific binding, where total binding was 92.45 ± 14.92 fmoles/tube. Specific binding was defined by the addition of 0.3 μM desipramine, and determined to be < 10% of total binding. Consequently, the baseline was determined by computer assisted analysis using the GraphPad Inplot program for the IBM PC to float the baseline at 21 fmoles. Each point is the mean of 5 replicate determinations ± SEM. From the corresponding Hill plot (B): IC_{50} = 5 μM and n_H = 1.0, fractional occupancy of the binding site(s) was calculated to be 0.12%, and from this the theoretical B_{max} ≈ 60 pmol/tube.

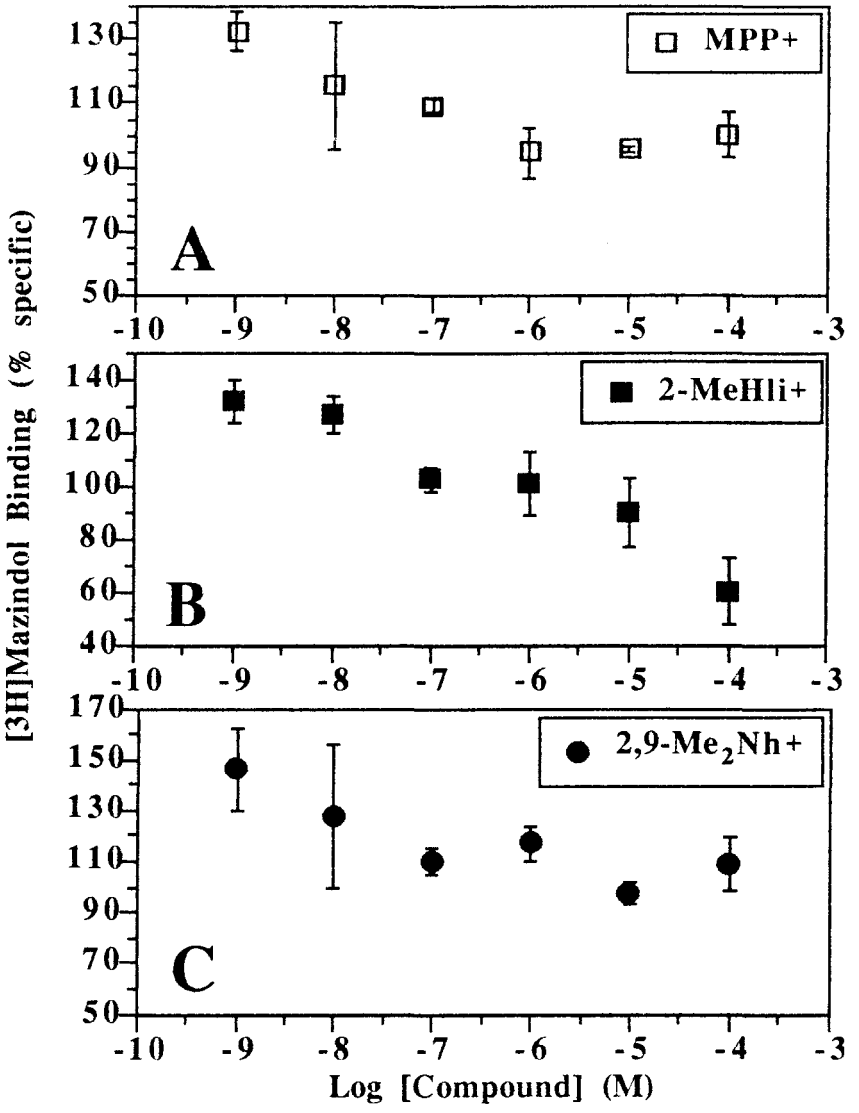


FIG. 19. 6 nM [^{3}H]MAZINDOL BINDING TO PC12 CELL HOMOGENATES IN THE PRESENCE OF UNLABELED MPP^+, 2-$MeHli^+$ AND 2,9-Me_2Nh^+. Cells were grown in DMEM. Non-specific uptake was defined by the presence of 100 μM unlabeled mazindol. Each point is the mean of 2 replicate determinations (each determination in triplicate) ± SEM. Data are expressed as the percent of total specific binding, where the mean control values were as follows: (A) 81.09 ± 22.42 fmoles/tube for MPP^+, (B) 66.71 ± 15.47 fmoles/tube for 2-$MeHli^+$, and (C) 66.51 ± 15.35 fmoles/tube for 2,9-Me_2Nh^+.

The endogenous substrates of the uptake site(s), DA (fig. 20) and NE (fig. 21), were tested only in the presence of their radiolabeled homologues and displayed IC_{50} values of 774 and 331 nM, respectively. The n_H value for NE was less than one (0.28), but that for DA (0.85) approached unity. The curve for NE was biphasic, but the DA curve was sigmoidal.

The known inhibitors of catecholamine uptake, desipramine, mazindol and nomifensine, displayed the highest affinities of the compounds tested for both the DA and NE uptake sites (fig. 22 - 24, respectively). Of these compounds, mazindol was the most potent inhibitor of [^{3}H]DA uptake with an IC_{50} of 0.76 nM, and desipramine was approximately one sixth as effective (IC_{50} = 4.8 nM). The effectiveness of these two compounds in inhibiting [^{3}H]NE uptake were reversed, where the IC_{50} for desipramine was 1.25 nM and that of mazindol was 4.5 nM. Nomifensine displayed approximately equal potency against both [^{3}H]DA (IC_{50} = 10.8 nM) and ^{3}H]NE (IC_{50} = 10.1 nM) binding. The n_H values for all three compounds with respect to both [^{3}H]DA and [^{3}H]NE were less than unity (Table 5).

Each of the toxins tested, MPP^+, 2-$MeHli^+$ and 2,9-Me_2Nh^+ (fig. 25-27, respectively), displayed micromolar potencies in inhibiting [^{3}H]DA with IC_{50} values of 6.5, 13.1 and 16.6 μM, respectively. Similarly, these three compounds had IC_{50} values for [^{3}H]NE uptake by PC12 cells of 1.5 (MPP^+), 13.5 (2-$MeHli^+$) and 61.6 μM (2,9-Me_2Nh^+). In contrast to the other compounds tested (DA, NE, desipramine, mazindol and nomifensine), the n_H values for inhibition of [^{3}H]DA uptake were either greater than one (MPP^+ = 1.43 and 2-$MeHli^+$ = 1.42) or approximately equal to one (2,9-Me_2Nh^+ = 0.92). The n_H values for inhibition of [^{3}H]NE uptake were less than unity for MPP^+, 2-$MeHli^+$ and 2,9-Me_2Nh^+ (0.78, 0.72 and 0.71, respectively), but were not as low as those of the other compounds tested (table 5).

TABLE 5

SUMMARY OF DATA FOR INHIBITION OF [^{3}H]DOPAMINE AND [^{3}H]NOREPINEPHRINE UPTAKE INTO PC12 CELLS BY VARIOUS COMPOUNDS

Competing Ligand	[^{3}H]Dopamine		[^{3}H]Norepinephrine	
	IC_{50} (nM)	n_H	IC_{50} (nM)	n_H
Dopamine	774	0.85	NT	NT
Norepinephrine	NT	NT	331	0.28
Desipramine	4.8	0.49	1.25	0.28
Mazindol	0.76	0.69	4.5	0.32
Nomifensine	10.8	0.47	10.1	0.28
MPP^+	6504	1.43	1495	0.78
2-$MeHli^+$	13,074	1.42	13,491	0.72
2,9-Me_2Nh^+	16,612	0.92	61,625	0.71

Data for the individual compounds were subjected to Hill transformation and plotted as logit-log inhibition plots as previously described. Data were derived and summarized from figures 20 - 27. NT = not tested.

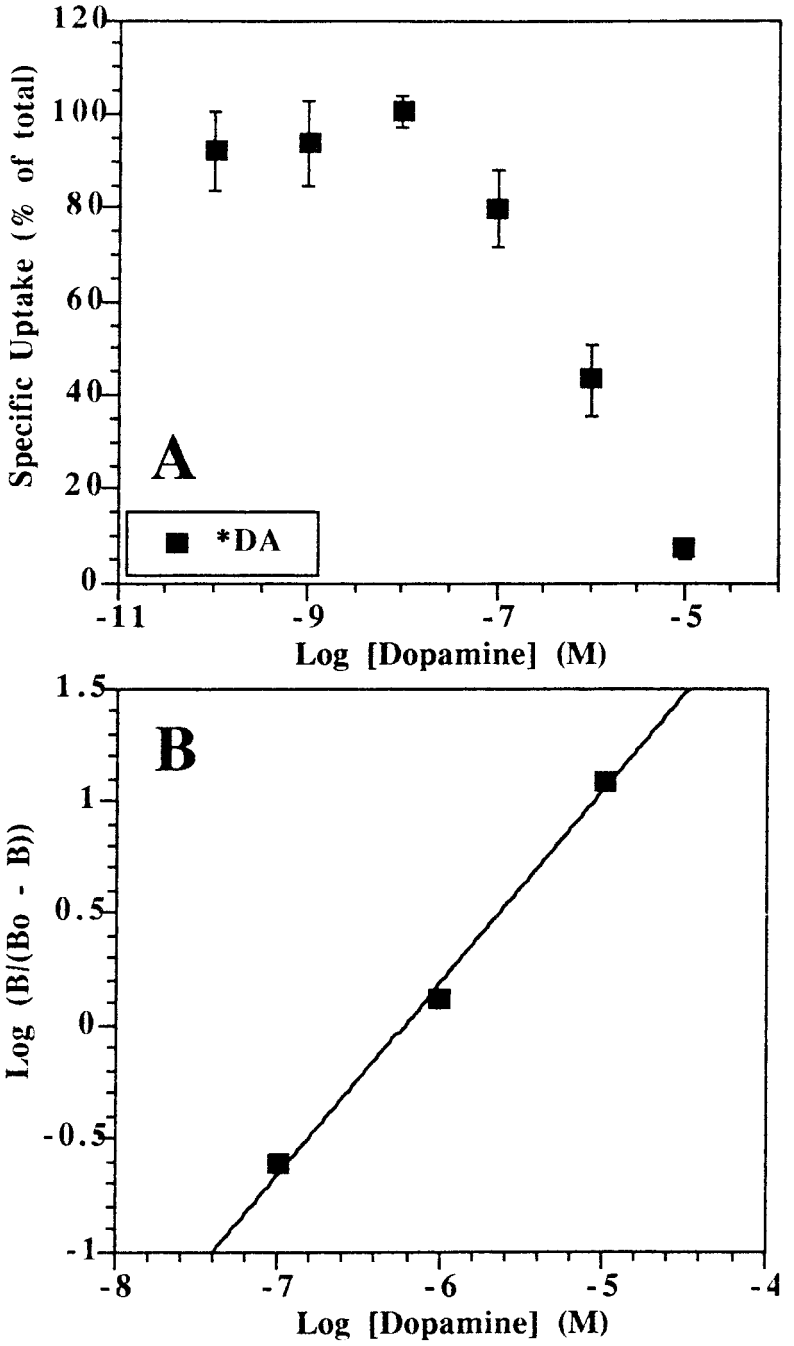


FIG. 20. 3.5 nM [^{3}H]DOPAMINE UPTAKE INTO PC12 CELLS IN THE PRESENCE OF UNLABELED DOPAMINE. (A) is the inhibition curve where specific uptake was defined by the presence of 100 μM unlabeled nomifensine, and (B) is the corresponding Hill plot. Each point is the mean of 3 replicate determinations ± SEM. Data are expressed as the percent of specific uptake, where the mean control value was 26.47 ± 3.39 fmoles/tube. From the Hill plot (bottom): IC_{50} = 774 nM and n_H = 0.85.

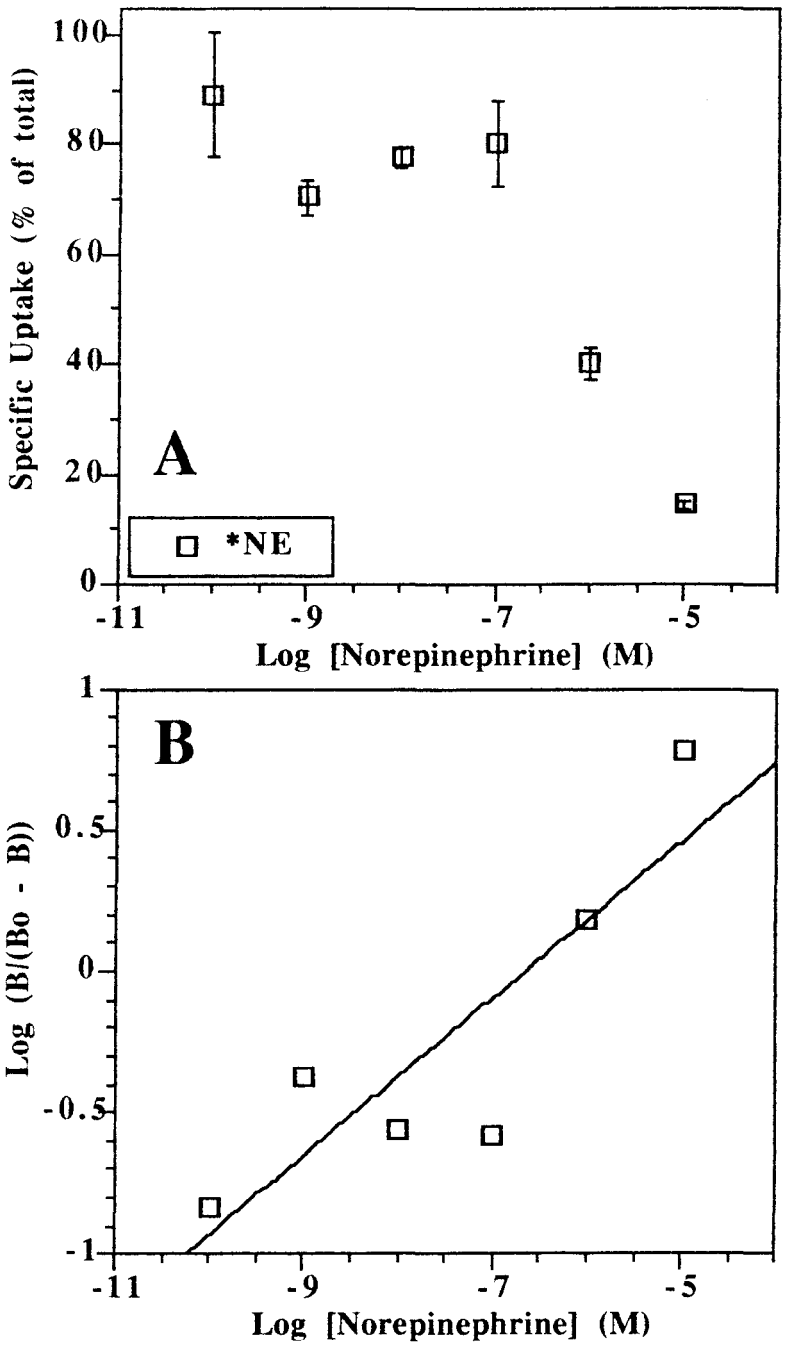


FIG. 21. 3.5 nM [^{3}H]NOREPINEPHRINE UPTAKE INTO PC12 CELLS IN THE PRESENCE OF UNLABELED NOREPINEPHRINE. (A) is the inhibition curve where specific uptake was defined by the presence of 100 μM unlabeled nomifensine, and (B) is the corresponding Hill plot. Each point is the mean of 3 replicate determinations ± SEM Data are expressed as the percent of specific uptake, where the mean control value was 71.64 ± 13.59 fmoles/mg protein. From the Hill plot (bottom): IC_{50} = 331 nM and n_H = 0.28.

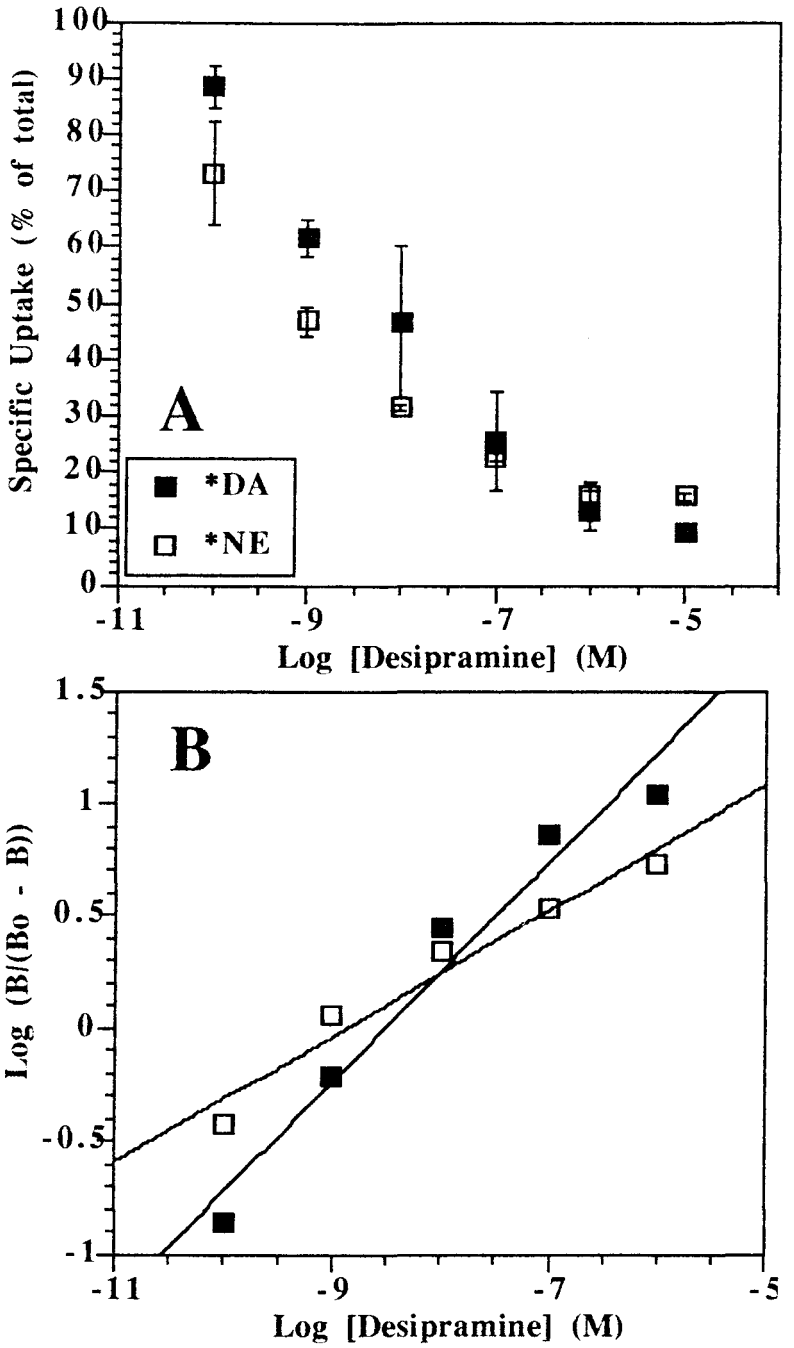


FIG. 22. 3.5 nM [^{3}H]DOPAMINE AND 3.5 nM [^{3}H]NOREPINEPHRINE UPTAKE INTO PC12 CELLS IN THE PRESENCE OF UNLABELED DESIPRAMINE. (A) is the inhibition curve where specific uptake was defined by the presence of 100 µM unlabeled nomifensine, and (B) is the corresponding Hill plot. Each point is the mean of 3 replicate determinations ± SEM Data are expressed as the percent of specific uptake, where the mean control value was 86.16 ± 10.99 fmoles/mg protein for DA and 96.5 ± 2.31 for NE. From the Hill plot (bottom): IC_{50} = 4.8 nM and n_H = 0.49 for DA, and IC_{50} = 1.25 nM and n_H = 0.28 for NE.

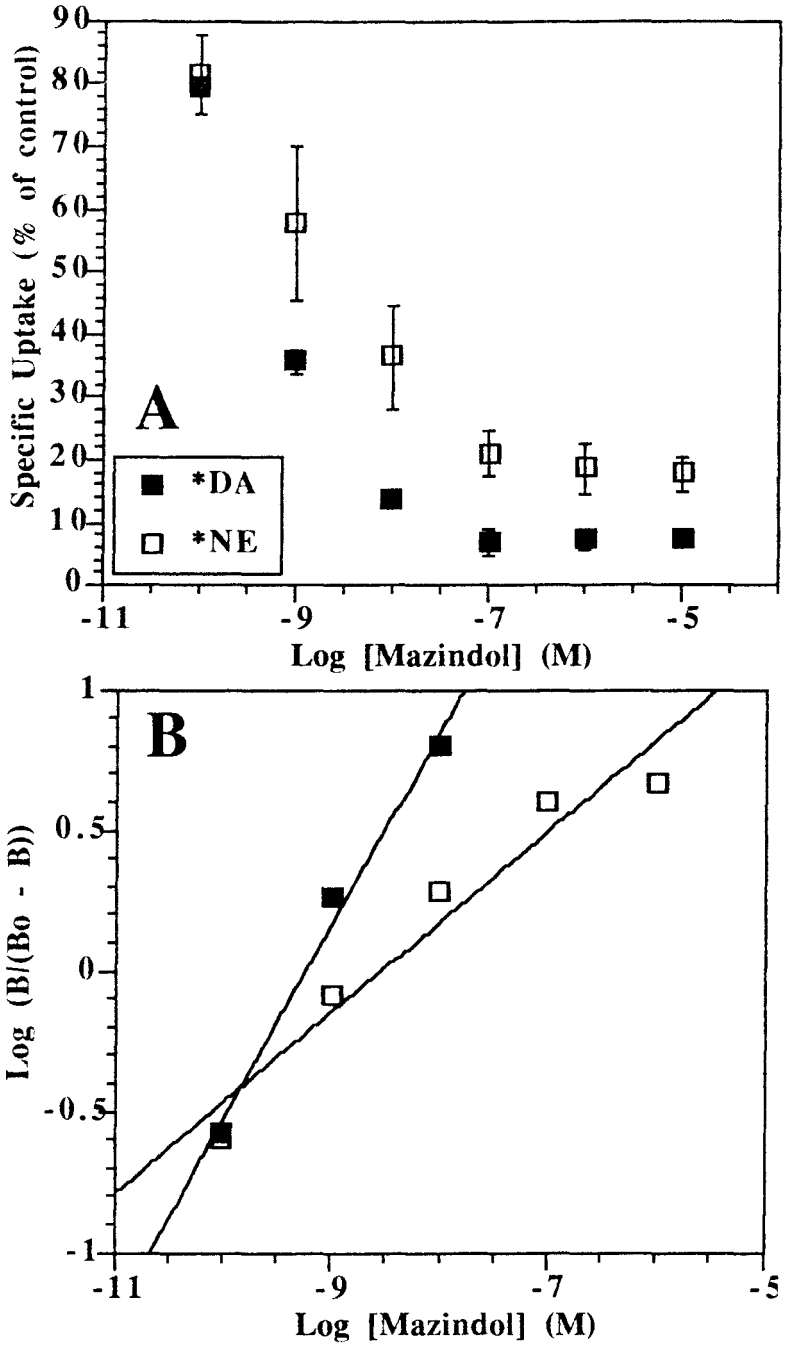


FIG. 23. 3.5 nM [^{3}H]DOPAMINE AND 3.5 nM [^{3}H]NOREPINEPHRINE UPTAKE INTO PC12 CELLS IN THE PRESENCE OF UNLABELED MAZINDOL. (A) is the inhibition curve where specific uptake was defined by the presence of 100 μM unlabeled nomifensine, and (B) is the corresponding Hill plot. Each point is the mean of 3 replicate determinations ± SEM Data are expressed as the percent of specific uptake, where the mean control value was 76.3 ± 3.26 fmoles/mg protein for DA and 82.38 ± 19.38 for NE. From the Hill plot (bottom): IC_{50} = 0.76 nM and n_H = 0.69 for DA, and IC_{50} = 4.5 nM and n_H = 0.32 for NE.

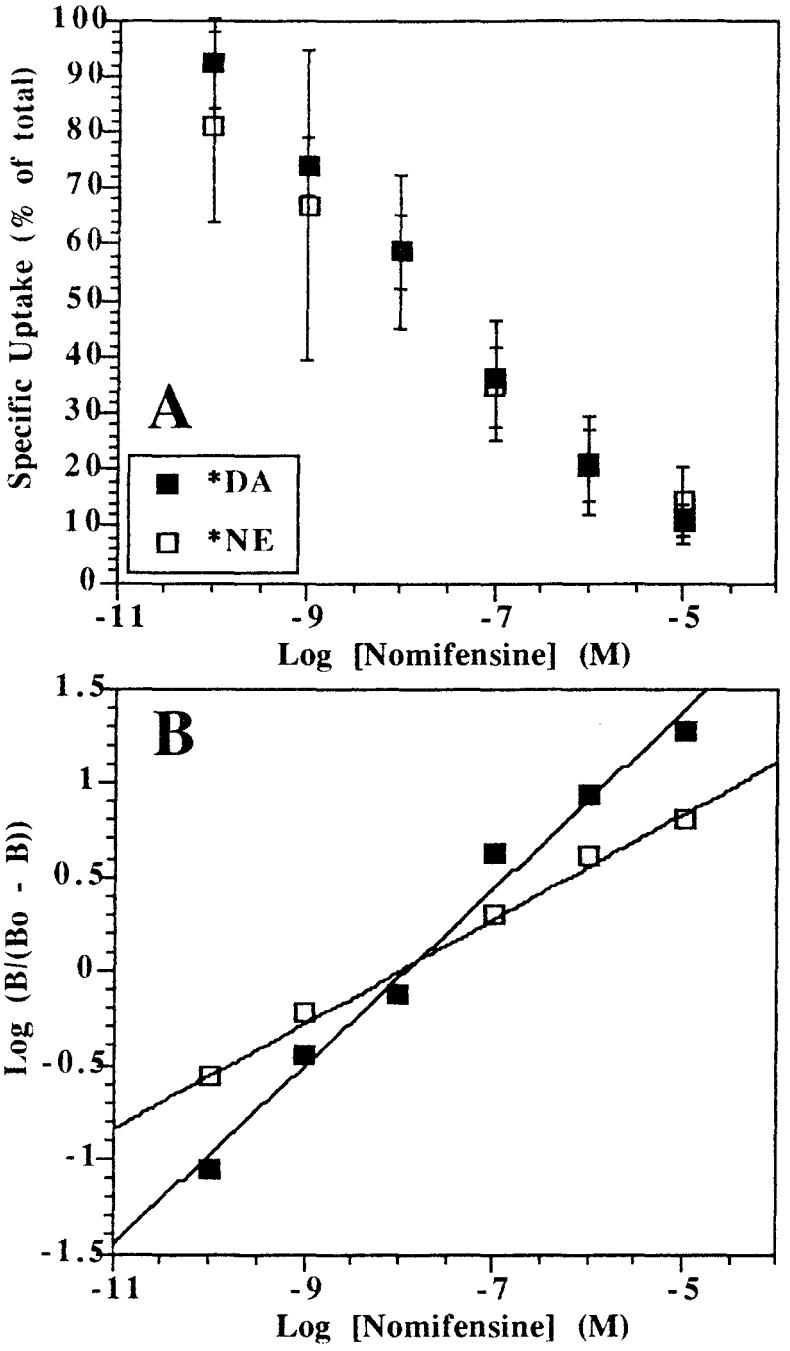


FIG. 24. 3.5 nM [^{3}H]DOPAMINE AND 3.5 nM [^{3}H]NOREPINEPHRINE UPTAKE INTO PC12 CELLS IN THE PRESENCE OF UNLABELED NOMIFENSINE. (A) is the inhibition curve where specific uptake was defined by the presence of 100 μM unlabeled nomifensine, and (B) is the corresponding Hill plot. Each point is the mean of 3 replicate determinations ± SEM Data are expressed as the percent of specific uptake, where the mean control value was 79.83 ± 4.27 fmoles/mg protein for DA and 81.11 ± 12.48 for NE. From the Hill plot (bottom): IC_{50} = 10.8 nM and n_H = 0.47 for DA, and IC_{50} = 10.1 nM and n_H = 0.28 for NE.

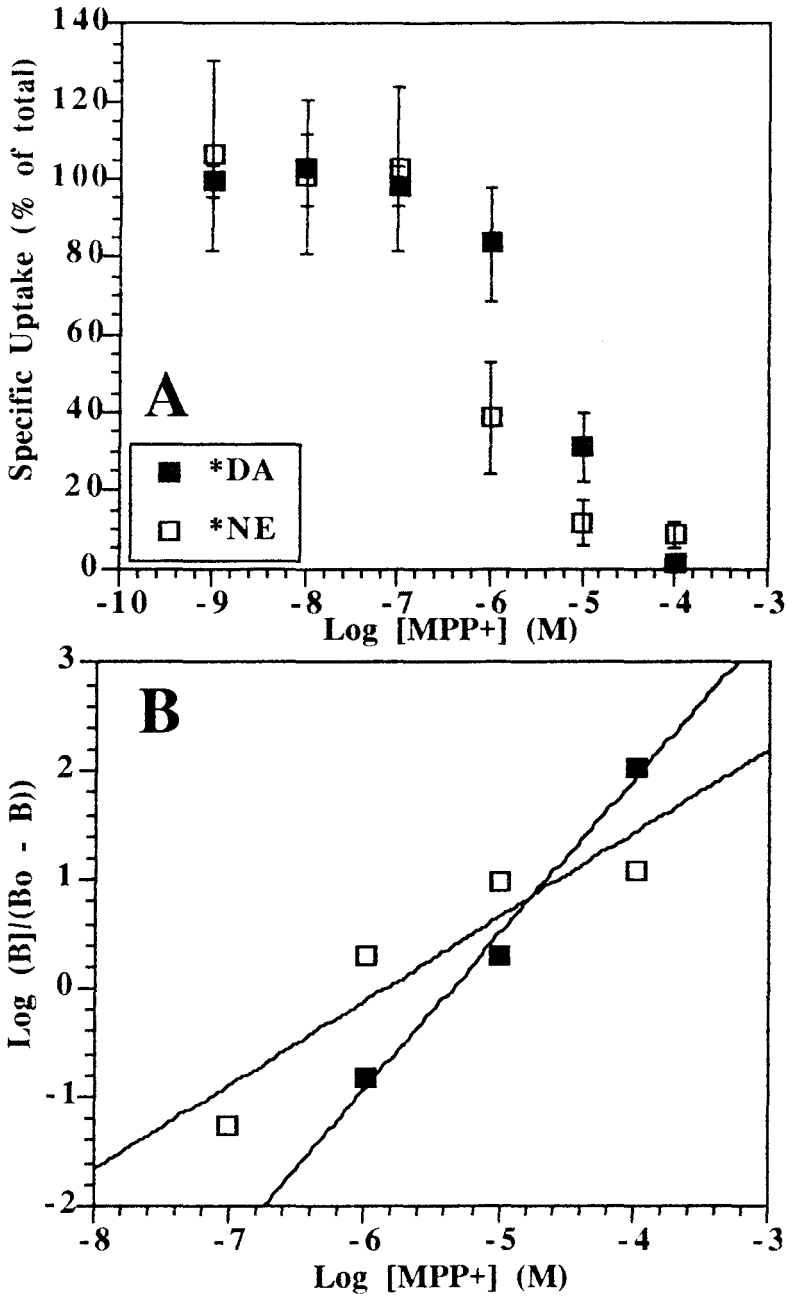


FIG. 25. 3.5 nM [^{3}H]DOPAMINE AND 3.5 nM [^{3}H]NOREPINEPHRINE UPTAKE INTO PC12 CELLS IN THE PRESENCE OF UNLABELED MPP$^+$. (A) is the inhibition curve where specific uptake was defined by the presence of 100 μM unlabeled nomifensine, and (B) is the corresponding Hill plot. Each point is the mean of 3 replicate determinations ± SEM Data are expressed as the percent of specific uptake, where the mean control value was 191.34 ± 4.27 fmoles/mg protein for DA and 68.69 ± 12.50 for NE. From the Hill plot (bottom): IC_{50} = 6504 nM and n_H = 1.43 for DA, and IC_{50} = 1495 nM and n_H = 0.78 for NE.

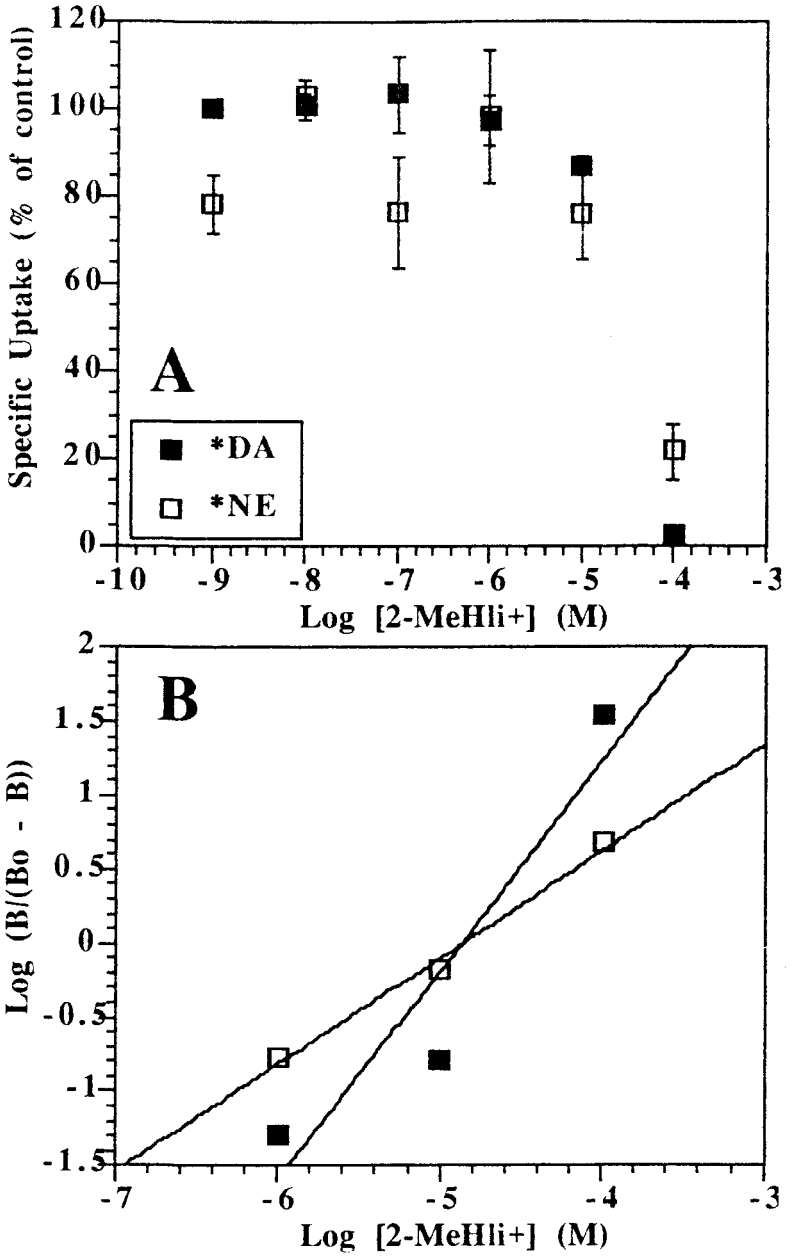


FIG. 26. 3.5 nM [^{3}H]DOPAMINE AND 3.5 nM [^{3}H]NOREPINEPHRINE UPTAKE INTO PC12 CELLS IN THE PRESENCE OF UNLABELED 2-MeHli$^+$. (A) is the inhibition curve where specific uptake was defined by the presence of 100 μM unlabeled nomifensine, and (B) is the corresponding Hill plot. Each point is the mean of 3 replicate determinations ± SEM Data are expressed as the percent of specific uptake, where the mean control value was 186.43 ± 23.45 fmoles/mg protein for DA and 81.97 ± 0.56 for NE. From the Hill plot (bottom): IC_{50} = 13,074 nM and n_H = 1.42 for DA, and IC_{50} = 13,491 nM and n_H = 0.72 for NE.

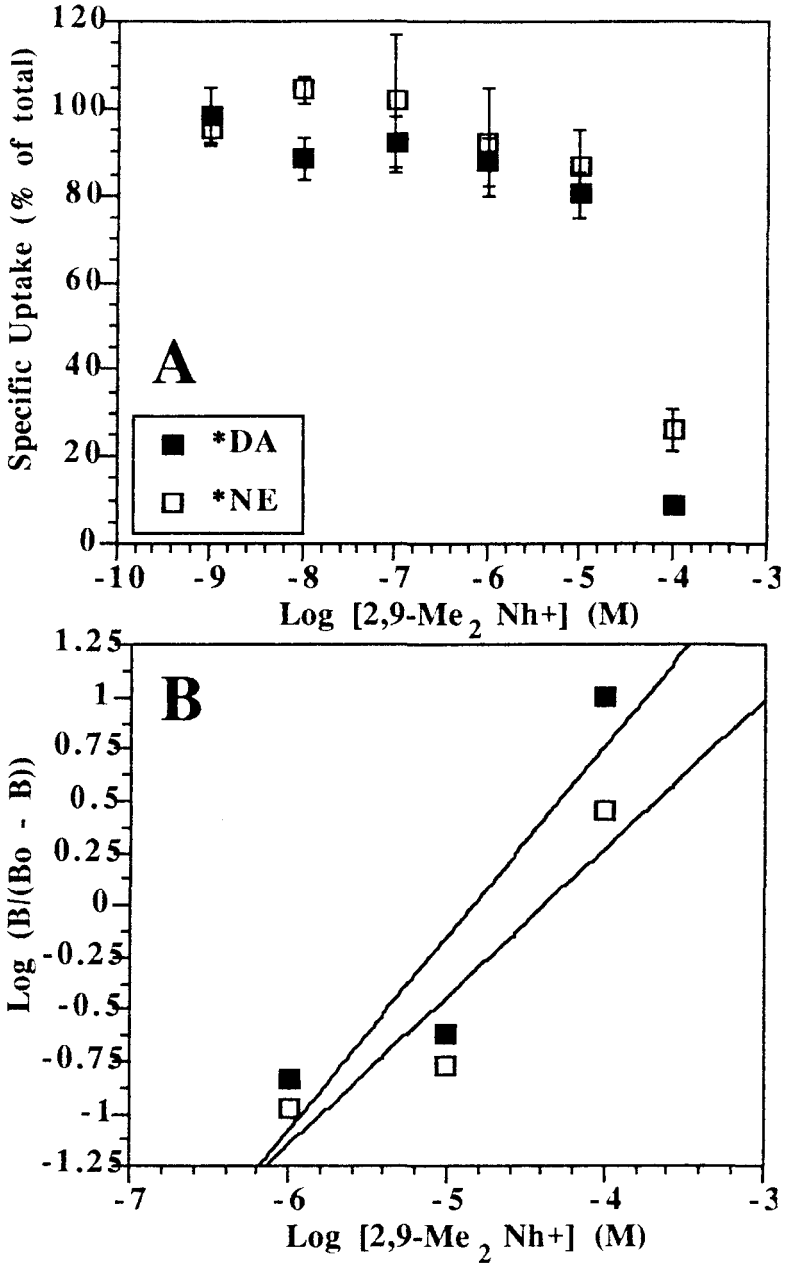


FIG. 27. 3.5 nM [^{3}H]DOPAMINE AND 3.5 nM [^{3}H]NOREPINEPHRINE UPTAKE INTO PC12 CELLS IN THE PRESENCE OF UNLABELED 2,9-Me_2Nh^+. (A) is the inhibition curve where specific uptake was defined by the presence of 100 μM unlabeled nomifensine, and (B) is the corresponding Hill plot. Each point is the mean of 3 replicate determinations ± SEM Data are expressed as the percent of specific uptake, where the mean control value was 199.96 ± 18.06 fmoles/mg protein for DA and 66.20 ± 5.44 for NE. From the Hill plot (bottom): IC_{50} = 16,612 nM and n_H = 0.92 for DA, and IC_{50} = 61,625 nM and n_H = 0.71 for NE.

Summary

1) Six of the twelve compounds initially tested were determined to be toxic to PC12 cells as assessed by LDH release and cell protein concentration: MPP^+, 2-$MeHli^+$, 2-$MeHi^+$, 2,9-Me_2Nh^+, 2,9-Me_2Ha^+ and 2,9-Me_2Hi^+.

2) The time-course and dose-response effects of these six compounds were examined, and 2-$MeHli^+$ and MPP^+ were observed to be the most toxic to PC12 cells in low energy, N-5 medium.

3) The dose-response effects of the three most potent compounds in low energy medium (N-5), MPP^+, 2-$MeHli^+$ and 2,9-Me_2Nh^+, were examined in a culture medium containing a higher concentration of glycolytic substrates, DMEM. Overall, all three compounds were less toxic in DMEM.

4) Co-incubation with the catecholamine uptake inhibitor, mazindol, did not inhibit the toxic effects of either MPP^+, 2-$MeHli^+$ or 2,9-Me_2Nh^+ in DMEM.

5) The accumulation of [^{3}H]DA and [^{3}H]NE by PC12 cells was temperature- and sodium-dependent, and homogenization of the cells reduced uptake by approximately half.

6) The specific binding of [^{3}H]GBR 12935 to PC12 cell homogenates was only 35% of the total signal, as defined by 10 μM GBR 12909. The IC_{50} for mazindol inhibition of specific [^{3}H]GBR 12935 binding was 10 μM, and the theoretical B_{max} was determined to be 426 pmol/tube.

7) Only 10% of total [^{3}H]mazindol binding to PC12 cell homogenates was to classical NE uptake sites as defined by the presence of 0.3 μM desipramine. An IC_{50} value of 5 μM and a theoretical B_{max} of 60 pmol/tube were determined for total [^{3}H]mazindol binding by computer-assisted approximation of a baseline.

8) 2-MeHli$^+$ inhibited 40% of [^{3}H]mazindol binding to PC12 cell homogenates with μM potency, and yielded a biphasic curve. MPP$^+$ and 2,9-Me$_2$Nh$^+$ were ineffective at inhibiting [^{3}H]mazindol binding, even at 100 μM.

9) MPP$^+$, 2-MeHli$^+$ and 2,9-Me$_2$Nh$^+$ inhibited [^{3}H]DA uptake with IC$_{50}$s of 6.5, 13.1 and 16.6 μM, respectively. Similar IC$_{50}$ values were obtained for inhibition of [^{3}H]NE uptake: 1.5 μM (MPP$^+$), 13.5 μM (2-MeHli$^+$) and 61.6 μM (2,9-Me$_2$Nh$^+$). Hill coefficients (n_H) for NE, desipramine, mazindol and nomifensine were approximately ≤ 0.5. The n_H values for DA, MPP$^+$, 2-MeHli$^+$ and 2,9-Me$_2$Nh$^+$ were close to unity.

CHAPTER V

DISCUSSION

Structure-Activity Relationships

In all of the cytotoxicity experiments performed, 2-MeHli$^+$ was consistently the most potent compound tested. The first series of experiments were designed as a selection process. The effects of a high concentration (i.e., 500 μM) of N-methylated ßCs and DHßCs on two non-specific measures of cytotoxicity, LDH release and cell protein concentration, were used to determine which compounds were toxic in PC12 cell cultures. From these experiments, a number of structural characteristics can be identified as important for toxicity.

Methylation of the 9-[indole]-nitrogen produced a dramatic increase in toxicity relative to the less effective 2-MeßC$^+$ congeners. For example, 2-MeNh$^+$ had no effect on either LDH release or cell protein, but the related dimethylated species, 2,9-Me_2Nh$^+$, was equipotent with MPP$^+$ in both measures. Similarly, while 2-MeHi$^+$ and 2-MeHa$^+$ did have significant effects on LDH release, neither compound was as potent as its dimethylated congener at 2 days. The potentiating effect of 9-methylation had been seen previously in both *in vivo* microdialysis studies and mitochondrial respiration experiments (Albores *et al.*, 1990; Collins *et al.*, 1992).

It is also apparent that the methoxyl group on carbon-7 of the cyclohexyl ring increases the toxicity of the compounds in PC12 cell cultures. The two most toxic mono-methylated species were 2-MeHli$^+$ and 2-MeHi$^+$, which are also known as 7-methoxy-2-methylharmalan and 7-methoxy-2-methylharman, respectively. The

equivalent 7-hydroxy compounds, 2-MeHo$^+$ (7-hydroxy-2-methylharman) and 2-MeHlo$^+$ (7-hydroxy-2-methylharmalan), were both non-toxic. Similarly, Slotkin *et al.* (1978) have reported that the 7-methoxyl group is important for ßC inhibition of sodium-dependent vesicular transport, because a hydroxyl group on carbon-7 made the compounds inactive. The importance of the position of the methoxyl group on carbon-7 is also seen because neither 6-MeO-2-MeHa$^+$ nor 6-MeO-2-MeHla$^+$ produced any observable cytotoxicity. A similar structure-activity preference for the 7-oxy versus the 6-oxy substituent has been reported by Sayre *et al.* (1991), where the authors used a rat intranigral infusion model.

The lack of toxicity of both 2-MeHo$^+$ and 2-MeHlo$^+$ (6-hydroxyl compounds) in the present study was also of interest because they had previously been shown to have "toxic" effects in other model systems. Albores *et al.*(1990) had shown that 2-MeHo was equal to or better than 2-MeHli$^+$ as an inhibitor of mitochondrial respiration. Similarly, although 2-MeHlo$^+$ was approximately 4-fold less potent than 2-MeHo$^+$, it was considerably more potent than 2-MeHa$^+$ which did have a small but significant effect on LDH release (fig. 5). Drucker *et al.* (1990) had previously shown that 2-MeHo$^+$ was one of the poorest inhibitors of [^{3}H]DA uptake into striatal synaptosomes, so it may be that this compound was not accumulated by the PC12 cells. However, 2-MeHlo$^+$ was a better uptake inhibitor than 2-MeHa$^+$, so the reason for these disparate results is uncertain.

In summary, it appears that there are at least two structural features that enhance the toxic actions of N-methylated ßC$^+$s/DHßC$^+$s in PC12 cell cultures: 1) Methylation of the 9-[indole]-nitrogen, and 2) the presence of a methoxyl group on carbon-7. Both 2-MeHli$^+$ and 2-MeHi$^+$ are examples of the latter, differing only in the degree of saturation of the pyridyl ring. In PC12 cells, 2-MeHli$^+$ was more toxic. However, 2-MeHi$^+$ has been reported to be a more potent inhibitor of mitochondrial

respiration (Albores *et al.*, 1990), [^{3}H]DA uptake (Drucker *et al.*, 1990) and [^{3}H]choline uptake (Smart, 1981), so it is curious that 2-MeHli$^+$ is much more potent in PC12 cells. It is possible that these two compounds differ in their ability to enter PC12 cells.

Mechanism of Toxicity

Throughout the studies described in this dissertation the effects of N-methylated ßC$^+$s/DHßC$^+$s were compared to those of the known parkinsonian derivative MPP$^+$. As just mentioned, several compounds were observed to be equal to or greater than MPP$^+$ in their toxic effects in PC12 cell cultures. In the following section, the toxic mechanism of these compounds will be discussed in comparison to established aspects of MPP$^+$-induced toxicity. In particular, the effects of PC12 cell metabolism on toxicity and the potential for the compounds to act as substrates for the catecholamine uptake pump are discussed.

The Role of Glycolysis

Similar to MPP$^+$, N-methylated ßC$^+$s/DHßC$^+$s are potent inhibitors of mitochondrial respiration in isolated mitochondrial preparations (Albores *et al.*, 1990; Arora *et al.*, 1990; Fields *et al.*, 1992; Hoppel *et al.*, 1987; Sayre *et al.*, 1990; Sayre *et al.*, 1991). Although the exact loci of the inhibitory effects of these compounds differs from MPP$^+$ (Albores *et al.*, 1990; Fields *et al.*, 1992; Sayre *et al.*, 1991), the end result is still inhibition of mitochondrial respiration and subsequent cell death (Sayre *et al.*, 1991). It has been demonstrated that inhibition of glycolysis is required for inhibitors of oxidative phosphorylation (e.g., MPP$^+$) to be lethal to PC12 cells (Basma *et al.*, 1992; Denton and Howard, 1987; Reynolds *et al.*, 1982). To determine if media glucose concentration influenced the effectiveness of the compounds tested, studies

were performed on PC12 cells grown and treated in two different culture media: 1) N-5 medium, a low-energy medium with respect to concentrations of glycolytic substrates (Kaufman and Barrett, 1983), and 2) DMEM, which contained approximately twice the concentration of metabolic carbohydrates as the N-5 medium (Table 6).

When the time-course of effects was examined in N-5 medium, none of the six compounds tested showed a significant effect on LDH release prior to 24 h (fig. 8). It is possible that the media glucose concentration in these experiments (< 1 mM, Table 6) was depleted in less than 24 h by PC12 cells in the presence of the toxins. Reinhard *et al.* (1990a) have examined the time-course of LDH release from BAMC cells with respect to media glucose concentrations. In their study, no LDH release was observed until approximately 99% of the media glucose had been used up by the cells, and the BAMC cells utilized glucose at 4.6 times the normal rate in the presence of MPP^+ (Reinhard *et al.*, 1990a). So, those compounds which displayed a later effect (2-$MeHi^+$, 2,9-Me_2Nh^+ and 2,9-Me_2Ha^+, fig. 8) may have less effect on glucose utilization. Since PC12 cells, like BAMC cells, can use glycolysis as its source of ATP in the absence of oxidative phosphorylation, this may indicate that those compounds that have an earlier onset of toxicity (MPP^+, 2-$MeHli^+$ and 2,9-Me_2Hi^+) may be better inhibitors of the electron transport chain. However, it may also mean that the earlier acting compounds enter the cell more readily.

The compounds tested in both media (MPP^+, 2-$MeHli^+$ and 2,9-Me_2Nh^+) all had less effect in the higher energy medium (DMEM) on LDH release (Table 1). This was particularly true for MPP^+ and 2,9-Me_2Nh^+ which exhibited no significant effects in DMEM even at 500 μM (fig. 10). Reinhard *et al.* (1990a) have demonstrated that MPP^+ caused an increase in glucose utilization in BAMC cells, and an effect on LDH release was not observed until the media glucose was depleted. Similarly, Basma *et*

al. (1992) observed that untreated PC12 cells utilized 0.47 mM glucose in 48 h, and in the presence of either 2'Et-MPP^+ or rotenone (inhibitor of mitochondrial complex I respiration) the glucose was consumed in 24 h. Concurrent with the decrease in glucose, the lactate concentration of the medium increased two-fold in 24 h in the presence of the toxins indicating inhibition of mitochondrial respiration (Basma *et al.*, 1992). At higher concentrations of glucose, PC12 cells compensated for ATP lost via mitochondrial inhibition by glycolysis, but when the glucose was depleted the cells died (Basma *et al.*, 1992). In the studies described in this dissertation, 2-$MeHli^+$ appears to be a more efficient inhibitor of mitochondrial respiration than MPP^+ or 2,9-Me_2Nh^+, thereby causing the PC12 cells to use the glucose faster. This would be consistent with the results observed for the time-course study (fig. 8), where 2-$MeHli^+$ showed the initiation of effect at 12 h.

The effects of MPP^+ on cell protein concentration were similar to those reported by Denton and Howard (1987) for PC12 cells. They observed that cell protein was reduced by 50% with 100 μM MPP^+ in low glucose medium, and that the cells continued to divide at an almost normal rate in normal glucose medium (Denton and Howard, 1987). In the work described here, MPP^+ had the same effect at 100 μM in N-5 medium, but was ineffective at all but the highest concentration (500 μM) in DMEM (fig. 12). 2,9-Me_2Nh^+ was approximately equipotent in both media (Table 2). 2-MeHli was actually more effective at reducing cell protein at low concentrations in DMEM (EC_{50} = 25 μM) than in N-5 medium (EC_{50} = 210 μM), but there was no difference in the effects at the higher concentrations (Table 2). The unexplained increase in [^{3}H]DA uptake caused by 2,9-Me_2Nh^+ in N-5 medium was diminished in DMEM, as was the effect of MPP^+ (fig. 13 and 14). In contrast, 2-$MeHli^+$ had approximately equal effects on [^{3}H]DA uptake in both media. Overall, the effects of MPP^+ and 2,9-Me_2Nh^+ were diminished in the higher energy medium, whereas those

of 2-MeHli$^+$ were approximately the same in both media. These results indicate that 2-MeHli$^+$ was a more potent PC12 cell toxin than the other two compounds, and that it may elicit its effects via a different mechanism.

The toxic effects of MPP$^+$ were attenuated by increasing the concentration of glycolytic substrate in the media, whereas the effects of 2-MeHli$^+$ were not (fig. 9-14). This indicates that 2-MeHli$^+$ is clearly a more potent toxin than MPP$^+$ in PC12 cell cultures. However, it is possible that a further increase in the media glucose concentrations would protect PC12 cells against the toxic effects of 2-MeHli$^+$. As mentioned, other groups have examined the effects of medium glucose concentration on toxicity in PC12 or like cells (i.e., BAMC cells) , and they have used higher concentrations of glucose for their 'normal' concentration than the 5.55 mM (DMEM) used in this dissertation (Basma *et al.*, 1992; Denton and Howard, 1987; Reinhard *et al.*, 1990a). Basma *et al.* (1992) define a 'normal' glucose concentration in their PC12 cell cultures to be 13.5 mM. They examined the effects of varying glucose concentrations on 2 day exposure to 100 μM 2'Et-MPTP in PC12 cell cultures (Basma *et al.*, 1992). Under these conditions it was observed that cell death was approximately the same in medium containing 0.5 mM glucose (equivalent to N-5) as in medium with 5.5 mM glucose (Basma *et al.*, 1992). A definite attenuation in toxicity was not observed until the glucose concentration of the media was increased to 8.5 mM or higher (Basma *et al.*, 1992). Furthermore, Reinhard *et al.* (1990a) have reported that by changing the medium every 24 h no toxic effects were observed in BAMC cell cultures even at 1 mM MPP$^+$. So, it is possible that if the concentration of glucose in the media were increased, or if the medium was changed more frequently, that the toxicity of 2-MeHli$^+$ could be inhibited completely.

In summary, it appears that 2Me-Hli$^+$, a possible endogenous indole, is more potent than MPP$^+$ as a cytotoxic agent in PC12 cells. However, selected N-

methylated ßC$^+$s tested here also exhibited marked toxic potency. The overall rank order of potency of these compounds in low energy medium was 2-MeHli$^+$ ≥ MPP$^+$ > 2,9-Me$_2$Nh$^+$ > 2,9-Me$_2$Hi$^+$ > 2,9-Me$_2$Ha$^+$ > 2-MeHi$^+$. In the higher energy medium (DMEM), the rank of the first three compounds was 2-MeHli$^+$ > MPP$^+$ ≈ 2,9-Me$_2$Nh$^+$.

The Role of Uptake

It appears that the DA uptake system is the mechanism of MPP$^+$ accumulation in dopaminergic neurons (Chiba *et al.*, 1985; Javitch *et al.*, 1985). This system has also been reported to be responsible for MPP$^+$ accumulation by PC12 cells (Denton and Howard, 1987; Snyder *et al.*, 1986). Since N-methylated ßC$^+$s and DHßC$^+$s may also be substrates for the DA uptake mechanism (Arora *et al.*, 1990; Drucker *et al.*, 1990), the ability of MPP$^+$, 2-MeHli$^+$ and 2,9-Me$_2$Nh$^+$ to inhibit [^{3}H]catecholamine uptake into PC12 cells was examined as a measure of the affinity of the compounds for these sites. In these experiments, the accumulation of both [^{3}H]DA and [^{3}H]NE were examined since the NE and DA systems are both possibly active in the uptake of MPP$^+$, with the NE system being predominant (Snyder *et al.*, 1986). All three compounds yielded monophasic competition curves and Hill coefficients close to unity (Table 5) for both [^{3}H]DA and [^{3}H]NE uptake, suggesting recognition of only single sites. The IC_{50} values (Table 5) and inhibition curves indicated similar inhibitory potencies at both the DA and NE sites for the three compounds. It is possible that these compounds are substrates for either one or both catecholamine uptake site(s) on PC12 cells; however, the inhibitory effects may also occur via a non-competitive mechanism.

In PC12 cells, the accumulation of MPP$^+$ via the dopamine uptake system can be blocked by uptake inhibitors such as desipramine and mazindol (Denton and

Howard, 1987; Snyder *et al.*, 1986). Reinhard and colleagues (Reinhard *et al.*, 1990a; Reinhard *et al.*, 1989) have demonstrated in BAMC cells that administration of a high concentration of desipramine (50 μM) attenuates the toxic effects of a 3 day exposure to 500 μM MPP^+ (Reinhard *et al.*, 1990a). However, only a 20% reduction in toxicity was observed (Reinhard *et al.*, 1990a), and this indicates that much of the MPP^+ was still entering the cell despite the presence of the inhibitor. In the present study, mazindol was added to PC12 cell cultures concurrently with toxins, but did not significantly effect either 2-$MeHli^+$ or MPP^+-induced LDH release (fig. 15). The slight decrease in 2-$MeHli^+$-induced LDH release observed with 10 μM mazindol may indicate that there was an attenuation in toxicity, but the effect may have been masked by the large SEM. It is possible that mazindol (30 μM) may have been contributing to the toxicity, because mazindol alone was toxic at 100 μM (data not shown).

As an alternative, MPP^+ may enter the cell as a neutral, lipophilic species (Reinhard *et al.*, 1990a; Reinhard *et al.*, 1990b). Similarly, 2-$MeßC^+$s/$DHßC^+$s, such as 2-$MeHli^+$, have the ability to lose the 9-[indole]-nitrogen proton forming the neutral anhydro base. The dimethylated ßC$^+$s/DHßC$^+$s, such as 2,9-Me_2Nh^+ (which was not toxic) are permanently charged because they cannot be deprotonated (Albores *et al.*, 1990). Consequently, MPP^+ and 2-$MeHli^+$ as neutral species may enter the PC12 cells via passive diffusion and would be unaffected by the presence of an active uptake inhibitor such as mazindol. These toxins may also be accumulated by another uptake site in PC12 cells. Two NE uptake sites are present in BAMC cells; only the high affinity site is inhibitable by desipramine, but the low affinity site is not (Banerjee *et al.*, 1987). Consequently, the MPP^+ in Reinhard's model (1990a) may be accumulated by the BAMC cells via the low affinity site. This low affinity site would also have a role in the lack of toxicity observed in PC12 cells (fig. 15) if this site were inhibited by mazindol.

The site of entry of the toxins appears to be different than the mazindol site, since [^{3}H]mazindol binding also was not potently inhibited by these toxins. Mazindol has an IC_{50} of >1000 μM for the inhibition of [^{3}H]MPP^+ binding to PC12 cell membranes (Marongiu *et al.*, 1988). In the present study, MPP^+, at concentrations as high as 100 μM, did not inhibit [^{3}H]mazindol binding to PC12 cell homogenates, and 2,9-Me_2Nh^+ was equally ineffective. In contrast, 2-$MeHli^+$ produced a biphasic curve, although only a 50% inhibition of specific binding was observed at the maximum concentration (100 μM). However, this inhibition of specific mazindol binding may be mediated non-competitively. Desmethyl-harmaline, a compound similar to 2-$MeHli^+$, inhibits Na^+-dependent transport processes (Canessa *et al.*, 1973; Sastry and Phillis, 1977; Sepulveda and Robinson, 1974; Smart, 1981). Since [^{3}H]mazindol binding is a sodium-dependent process (Javitch *et al.*, 1983), the inhibition of [^{3}H]mazindol binding observed at high concentrations of 2-$MeHli^+$ (fig. 19B) may have resulted from inhibition of the Na^+-dependent binding process, and not direct competition with [^{3}H]mazindol.

The sodium-dependent nature of catecholamine uptake, and not direct competition with the substrates, may be the mechanism of the aforementioned inhibition of [^{3}H]DA and [^{3}H]NE uptake (Table 5) by 2-$MeHli^+$. PC12 cells accumulate [^{3}H]DA (Denton and Howard, 1984; Rebois *et al.*, 1980; Snyder *et al.*, 1986), and transport of [^{3}H]DA across the plasma membrane is an energy-dependent process, because a toxin-induced decrease in cell viability also decreased uptake (fig. 13; also, Denton and Howard, 1984). However, temperature and sodium-dependence have only been demonstrated for [^{3}H]NE uptake in PC12 cells (Greene and Rein, 1977), and not for [^{3}H]DA uptake. Figure 16 indicates that both [^{3}H]NE and [^{3}H]DA were accumulated by sodium-dependent processes. As mentioned previously, harmaline has been shown to inhibit the plasma membrane (Na^+ + K^+)-ATPase

(Canessa *et al.*, 1973; Sastry and Phillis, 1977; Sepulveda and Robinson, 1974; Smart, 1981), which is the mechanism that drives the catecholamine transporter. Consequently, 2-MeHli$^+$ may also inhibit the (Na^+ + K^+)-ATPase and thereby block [^{3}H]DA and [^{3}H]NE uptake in a non-competitive fashion. Thus, blocking the accumulation of 2-MeHli$^+$ and 2,9-Me_2Nh$^+$ with uptake inhibitors should be preceded by a determination of whether these toxins are accumulated by PC12 cells.

The data thus far have been inconclusive with regard to the interaction of the compounds with the uptake sites of PC12 cells. Therefore, the radiolabeled catecholamine uptake inhibitors, [^{3}H]mazindol and [^{3}H]GBR 12935, were used to further characterize these sites. Mazindol has affinity for both the DA and NE uptake sites, but displays a slight preference for the NE site (Andersen, 1987; Andersen, 1989; Javitch *et al.*, 1983). Desipramine (0.3 μM) can effectively compete for [^{3}H]mazindol binding in rat frontal cortex (Battaglia *et al.*, 1988). However, this concentration of desipramine inhibited less than 10% of the total [^{3}H]mazindol signal in PC12 cell homogenates (fig. 18), suggesting that only this fraction of the total binding sites corresponded to classical NE uptake sites. Furthermore, an approximate IC_{50} value for cold mazindol was determined to be 5 μM, which was considerably higher than the K_i of 22 nM reported for rat striatum (Javitch *et al.*, 1983). Together, these results suggest that the [^{3}H]mazindol binding site(s) in PC12 cells is pharmacologically distinct from that in neuronal membranes.

PC12 cells were also capable of binding the specific DA uptake inhibitor, GBR 12935. [^{3}H]GBR 12935 binds to two sites in striatal membrane preparations and mazindol competes for binding at the high affinity site, which is identified as the classic DA uptake site (Andersen, 1987; Niznik *et al.*, 1990). Mazindol has previously been reported to inhibit [^{3}H]GBR 12935 binding to the DA uptake site with an affinity of 80-93 nM (Andersen, 1987; Niznik *et al.*, 1990). In PC12 cell

homogenates, [^{3}H]GBR 12935 was not binding to the DA uptake site, as an IC_{50} of 10 μM was obtained for mazindol (fig. 17). The second site to which [^{3}H]GBR 12935 binds, identified as cytochrome P450IID1, displays very low affinity for mazindol and is not recognized by DA (Niznik *et al.*, 1990). This site has high affinity for GBR 12909, and is termed the piperazine acceptor site since both [^{3}H]GBR 12935 and GBR 12909 are piperazine compounds (Andersen, 1987; Niznik *et al.*, 1990). [^{3}H]GBR 12935 binds readily to crude PC12 cell homogenates, but only 35% of the total signal is inhibited by 10 μM GBR 12909 (fig. 17). It has previously been demonstrated that GBR 12909 inhibits [^{3}H]GBR 12935 binding with low nanomolar affinity (Andersen, 1987; Niznik *et al.*, 1990). This would indicate that [^{3}H]GBR 12935 binding to the PC12 cell homogenates was not to the piperazine acceptor site. Although [^{3}H]GBR 12935 does bind to PC12 cell homogenates, the identity of this site is not clear since the pharmacology of this site is distinct from that previously described for neuronal membranes.

The catecholamine uptake site(s) of PC12 cells appears to be pharmacologically distinct from the separate and specific DA and NE uptake sites found on neurons in the brain. Desipramine and mazindol inhibit [^{3}H]DA uptake into PC12 cells with IC_{50}'s of 30 nM and 3 nM, respectively (Snyder *et al.*, 1986). In the study (Table 5), the IC_{50}s of 4.8 nM (desipramine) and 0.76 nM (mazindol) were obtained in striatal preparations. Desipramine inhibited both [^{3}H]DA and [^{3}H]NE uptake with IC_{50}s of 4.8 and 1.25 nM, respectively (Table 5). Desipramine ($IC_{50} \approx 10$ μM) is considered a weak inhibitor of [^{3}H]DA uptake (Dubocovich and Zahniser, 1985), whereas mazindol and nomifensine are potent inhibitors with IC_{50}s of approximately 30 and 100 nM, respectively (Andersen, 1989; Dubocovich and Zahniser, 1985). Nomifensine has previously been shown to be a better inhibitor of the NE site ($IC_{50} = 11.2$ nM) than the DA uptake site ($IC_{50} = 134$ nM) in rat brain synaptosomes (Andersen, 1989).

However, like desipramine, nomifensine yielded approximately equivalent IC_{50}s for the inhibition of both [^{3}H]DA and the [^{3}H]NE uptake into PC12 cells (Table 5). These data suggest that the site through which [^{3}H]DA was accumulated by the PC12 cells was pharmacologically distinct from that in the brain.

The uptake of NE by PC12 cells (Greene and Rein, 1977), and the accumulation of DA and NE by PC12 cell catecholamine granules (Greene and Rein, 1977; Rebois *et al.*, 1980) have been well characterized. Although it has been observed that [^{3}H]DA is taken up by PC12 cells (Denton and Howard, 1984; Rebois *et al.*, 1980; Snyder *et al.*, 1986), the pharmacology of this process has not been reported beyond the demonstration that it is inhibited by MPTP (Denton and Howard, 1984). Both DA and NE uptake systems mediate the accumulation of [^{3}H]MPP+ by PC12 cells, and the "norepinephrine system appears to predominate" (Snyder *et al.*, 1986). In addition, accumulation of NE by PC12 cells appears to be mediated by the same transport system that accumulates DA (Denton and Howard, 1984). The data presented in this dissertation suggests that NE recognizes more than one uptake site on PC12 cells, whereas DA appears to recognize only one site. This is evident from the [^{3}H]NE curve (fig. 21) which appears biphasic, and yields a Hill coefficient of less than unity. In contrast, the curve for DA vs. [^{3}H]DA (fig. 20) appears monophasic and has an n_H value of approximately 1. The inhibition curves for desipramine, mazindol and nomifensine all appear similar for their individual effects on both [^{3}H]DA and [^{3}H]NE uptake (each gives nearly identical curves for both substrates). Together these data suggest that both [^{3}H]DA and [^{3}H]NE are accumulated by the same site, and that site is more noradrenergic in nature. Also, NE and the inhibitors (desipramine, mazindol and nomifensine) appear to recognize a second site that is not recognized by DA.

In summary, two conclusions can be drawn from the uptake studies. First, the ability of compounds to inhibit the binding or accumulation of other compounds at the uptake site does not indicate that these compounds are substrates. It also does not even distinguish between competitive and non-competitive inhibition of the accumulated or bound compound. In the case of the N-methylated ßC$^+$s/DHßC$^+$s examined, it is entirely possible that these compounds do not compete for the uptake site but elicit their effects by inhibiting the driving process for uptake, the ($Na^+ + K^+$)-ATPase. Second, it appears that PC12 cells do not have a DA uptake site as characterized pharmacologically in brain. It has been suggested that there may be a general catecholamine uptake system to accumulate both DA and NE in PC12 cells (Denton and Howard, 1984), and a similar observation has been made in BAMC cells (Banerjee *et al.*, 1987). The present data (fig. 17) supports the hypothesis of a common uptake site that more closely resembles the CNS NE uptake system.

A Putative Model

The studies in this dissertation have shown that the N-methylated DHßC$^+$, 2-MeHli$^+$, was the most toxic compound tested in PC12 cell cultures. Throughout these studies, MPP$^+$ served as a model against which the toxic effects of the compounds were compared. Thus, the following model for 2-MeHli$^+$-induced PC12 cell death is described in comparison to the effects of MPP$^+$ (see also fig. 28).

Similar to MPP$^+$ (Denton and Howard, 1987; Snyder *et al.*, 1986), 2-MeHli$^+$ may enter PC12 cells via the catecholamine uptake carrier. 2-MeHli$^+$ can also spontaneously deprotonate to form the neutral anhydro base (Albores *et al.*, 1990), so it may also enter the cell by passive diffusion. Once inside BAMC cells, MPP$^+$ is sequestered by the cells catecholamine vesicles (Reinhard *et al.*, 1990b). Likewise, a

portion of the 2-MeHli$^+$ may be accumulated and sequestered by PC12 cell catecholaminergic vesicles thereby reducing the cytosolic concentration.

From the cytosol, the strongly cationic MPP$^+$ enters the mitochondrial matrix by an active, energy-dependent process and inhibits NADH-linked respiration (Ramsay *et al.*, 1989; Ramsay *et al.*, 1989; Sayre *et al.*, 1989; Singh *et al.*, 1991). However, 2-MeHli$^+$ forms the neutral anhydronium base and passively enters the mitochondria (Albores *et al.*, 1990). Inside the mitochondria, 2-MeHli$^+$ inhibits both complex I (NADH-linked) and complex II (succinate-linked) respiration (Albores *et al.*, 1990; Fields *et al.*, 1992; Sayre *et al.*, 1991). This results in a marked reduction of the cells capacity to generate ATP, but does not necessarily kill the PC12 cell. PC12 cells store a portion of their total ATP in vesicles, and can generate sufficient ATP via glycolysis to maintain viability in the presence of a sufficient concentration of extracellular glucose. However, if the glucose concentration is depleted, the cells eventually consume their ATP stores and succumb to the inhibition of oxidative phosphorylation by 2-MeHli$^+$ (or MPP$^+$).

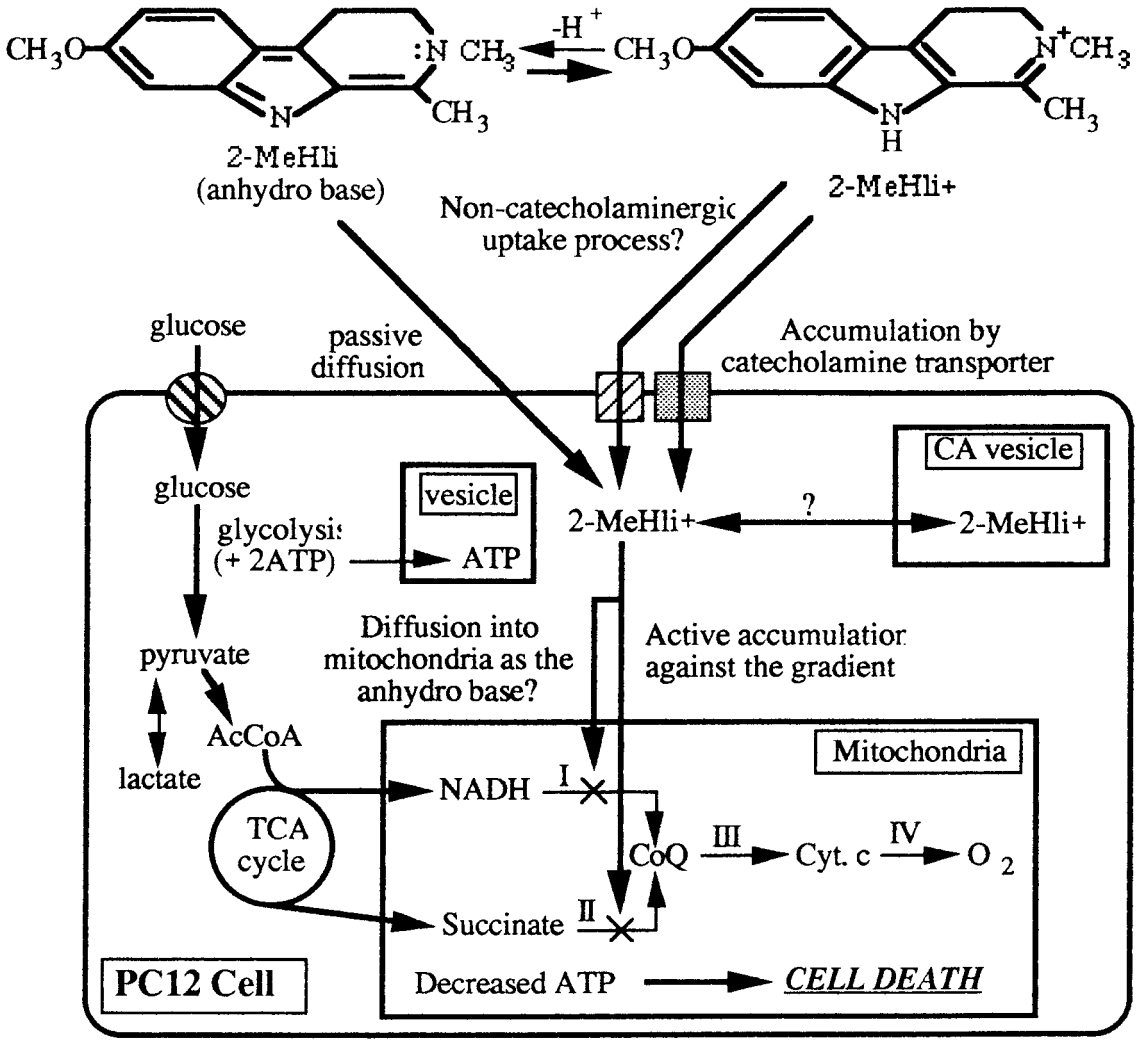


Fig. 28. PROPOSED MECHANISM FOR 2-MeHli$^+$-INDUCED PC12 CELL DEATH. In the mitochondria, the Roman numerals (I, II, III and IV) indicate the enzyme complexes of the electron transport chain. Only one of many possible resonance structures of the anyhdro base of 2-MeHli$^+$ is shown. CA = catecholamine.

ß-Carbolines and Parkinson's Disease

The data in this dissertation demonstrate that select N-methylated ßC/DHßC species approach or surpass the parkinsonian-derivative, MPP^+, as cytotoxic agents in PC12 cell cultures. In particular, the DHßC, 2-$MeHli^+$, was more potent than MPP^+ in both a low energy cell culture medium and in media containing higher concentrations of glycolytic substrates. This compound is a relatively potent inhibitor of mitochondrial respiration (Albores *et al.*, 1990), and also produces significant depletion of striatal dopamine and gross lesions when injected directly into the substantia nigra of rats (Neafsey *et al.*, unpublished results). Furthermore, this compound is worthy of consideration as a putative parkinsonian agent, because the potential exists for its endogenous biosynthesis. 7-Hydroxy-1-methyl-1,2,3,4-tetrahydro-ßC may serve as a precursor as it has previously been shown to be a normal constituent in human and cat urine (Beck *et al.*, 1986). Enzymatic 7-O-methylation and 3,4-dehydrogenation could occur in either the CNS or periphery, resulting in the formation of harmaline, which would be subsequently N-methylated to form the neurotoxic, cationic species (2-$MeHli^+$) in the CNS (Collins *et al.*, 1992).

In addition to 2-$MeHli^+$, the potency of the di-2,9-[N,N']-methylated species tested is also exciting in light of reports by Matsubara *et al.* (1992a & c) that such compounds may be formed enzymatically in mammalian brain. Recently, this same group (Matsubara *et al.*, 1992b) has identified the presence of several 2- and 2,9-dimethylated $ßC^+$s in human brain, including 2,9-Me_2Nh^+ which was demonstrated in this dissertation to be similar in potency to MPP^+ in PC12 cell cultures. In PD patients, such compounds may either be produced at higher levels or metabolized less efficiently than normal.

A number of deficiencies in hepatic detoxification pathways have been described in PD patients (Barbeau *et al.*, 1985; Green *et al.*, 1991; Steventon *et al.*,

1989b; Waring *et al.*, 1989). PD patients have also been shown to have decreased activity of mitochondrial NADH CoQ1 reductase (complex I) (Lestienne *et al.*, 1990; Schapira *et al.*, 1990). Collins *et al.* (1992) have suggested that the hepatic enzyme deficiencies would diminish the peripheral removal of ßC/DHßC protoxins accumulated in the body from either endogenous or environmental sources, thus leading to accumulation and excess formation of the active N-methylated species in the brain and further inhibition of mitochondrial respiration. This lends further support to the hypothesis that ßC or DHßC derivatives may be toxic agents in the pathogenesis of idiopathic Parkinson's disease.

APPENDIX

TABLE 6

COMPONENTS OF TISSUE CULTURE MEDIA

Component	DMEM		N-5 *	
	mg/l	mM	mg/l	mM
Inorganic Salts:				
$CaCl_2$ (anhyd.)	200	1.8	200	1.8
$Fe(NO_3)_3.9H_2O$	0.10	0.0003	---	---
KCl	400	5.4	400	5.4
$MgSO_4$ (anhyd.)	97.67	0.8	97.67	0.8
NaCl	6400	109.4	6400	109.4
$NaHCO_3$	3700	44.05	2200	26.19
$NaH_2PO_4.H_2O$	125	0.9	125	0.9
Other Components:				
Citrate	---	---	20	0.1
Fructose	---	---	100	0.555
Galactose	---	---	200	1.11
D-Glucose †	1000	5.55	40	0.222
α,ß-Glycerophosphate	---	---	100	0.6
Mannose	---	---	50	0.3

Phenol Red	15	0.04	15	0.04
Sodium Pyruvate	110	1.0	---	---
Succinate	---	---	10	0.085
Amino Acids:				
L-Alanine	---	---	150	1.68
L-Cystine.2HCl	62.57	0.2	62.57	0.2
L-Glutamine	584	4.0	---	---
Glycine	30	0.4	---	---
L-Histidine HCl.H_2O	42	0.2	42	0.2
L-Isoleucine	105	0.8	105	0.8
L-Leucine	105	0.8	105	0.8
L-Lysine HCl	146	0.8	146	0.8
L-Methionine	30	0.2	30	0.2
L-Phenylalanine	66	0.4	2.0	0.01
L-Proline	---	---	40	0.35
L-Serine	42	0.4	42	0.4
L-Threonine	95	0.8	95	0.8
L-Tryptophan	16	0.08	2.0	0.001
L-Tyrosine (disodium salt)	103.79	0.45	103.79	0.45
L-Valine	94	0.8	94	0.8
Vitamins:				
D-Ca pantothenate	4.0	0.02	4.0	0.02
Carnitine	---	---	1.0	0.006
Choline Chloride	4.0	0.03	4.0	0.03
Cyanocobalamin (B_{12})	---	---	1.0	0.0007
Folic acid	4.0	0.009	4.0	0.009

i-Inositol	7.2	0.04	7.2	0.04
Nicotinamide	4.0	0.03	4.0	0.03
PABA	---	---	0.5	0.004
Pyridoxal HCl	4.0	0.02	4.0	0.02
Riboflavin	0.4	0.001	0.4	0.001
Thiamine HCl	4.0	0.01	4.0	0.01

* (Kaufman and Barrett, 1983)

† The indicated concentration of glucose is based only upon that in unsupplemented medium. The addition of 15% serum contributes an additional 0.4 - 0.5 mM glucose to the final concentration (Basma *et al.*, 1992).

TABLE 7

CONCENTRATIONS OF COMPONENTS IN UNMODIFIED AND MODIFIED KREBS-RINGER PHOSPHATE BUFFERS

Buffer Components	Concentration (mM)	
	'Normal' Krebs-Ringer Phosphate Buffer	'Zero Sodium'
Sodium Chloride	118	—
Sodium Phosphate, Monobasic	16	—
Sodium Phosphate, Dibasic	16	—
Potassium Phosphate, Monobasic	—	16
Potassium Phosphate, Dibasic	16	16
Potassium Chloride	4.7	4.7
Calcium Chloride	1.8	1.8
Magnesium Sulfate	1.2	1.2
Disodium EDTA	1.2	1.2
D-Glucose	5.6	5.6
L-Ascorbic acid	1.7	1.7
Pargyline HCl	0.08	0.08

TABLE 8

SUMMARY OF THE 2 DAY TOXICITY DATA FOR MPP^+, 2-$MeHli^+$ AND 2,9-Me_2Nh^+ IN N-5 AND DMEM CULTURE MEDIUM

Compound	[μM]	LDH Released (% C ± SEM)	Protein/Well (% C ± SEM)	[^{3}H]Dopamine Uptake (% C ± SEM)
		N-5 MEDIUM		
MPP^+	50	137.40 ± 28.56	92.47 ± 2.17	63.42 ± 11.13
	100	206.10 ± 53.59	86.98 ± 3.08	51.89 ± 4.72 †
	250	382.20 ± 19.74 *	53.33 ± 3.74 *	20.86 ± 4.63 †
	500	442.20 ± 29.26 *	40.71 ± 2.55 *	3.05 ± 0.26 †
2-$MeHli^+$	50	114.88 ± 1.78	97.81 ± 8.82	119.60 ± 32.69
	100	227.67 ± 34.81	70.95 ± 3.65 *	154.10 ± 36.48
	250	496.29 ± 31.40 *	34.30 ± 2.66 *	12.72 ± 1.71 †
	500	417.37 ± 38.32 *	32.80 ± 4.93 *	12.80 ± 11.30 †
2,9-Me_2Nh^+	50	96.05 ± 6.23	92.73 ± 4.74	185.30 ± 94.62
	100	103.56 ± 5.68	94.21 ± 4.25	289.00 ± 71.45 *
	250	266.06 ± 18.97	62.20 ± 2.64 *	490.80 ± 134.60 *
	500	405.89 ± 57.65 *	41.68 ± 3.30 *	116.50 ± 26.79
		DMEM MEDIUM		
MPP^+	50	104.50 ± 3.84	103.48 ± 11.83	93.12 ± 11.46
	100	88.37 ± 10.15	90.44 ± 7.10	86.37 ± 13.67
	250	154.29 ± 20.19	81.01 ± 17.37	48.26 ± 15.66 †
	500	198.60 ± 40.87	38.92 ± 12.83 *	26.96 ± 10.06 *
2-$MeHli^+$	50	102.35 ± 12.16	49.93 ± 10.17 *	146.28 ± 42.11
	100	173.75 ± 22.32	33.41 ± 7.85 *	79.15 ± 33.82
	250	325.91 ± 33.45 †	25.88 ± 9.76 *	5.04 ± 1.33 †
	500	283.19 ± 69.08 †	33.06 ± 7.16 *	19.91 ± 11.32 †
2,9-Me_2Nh^+	50	102.90 ± 10.76	81.53 ± 8.78	124.65 ± 4.35
	100	93.61 ± 9.20	73.90 ± 9.42	165.18 ± 53.89
	250	122.40 ± 26.13	53.16 ± 13.34 *	119.40 ± 39.84
	500	162.58 ± 38.16	43.31 ± 13.28 *	70.55 ± 20.28

Data presented here were compiled directly from figures 9 - 14. All results are expressed as percent of control (% C) ± SEM. The symbols, * and †, indicate that the points are significant vs. the corresponding control values ($p < 0.05$) as determined by the Scheffé F and Fisher PLSD *post hoc* tests, respectively.

REFERENCES

Agid Y. (1991) Parkinson's disease: pathophysiology. *Lancet.* **337**, 1321-1324.

Airaksinen M.M. and Kari I. (1981a) ß-Carbolines, psychoactive compounds in the mammalian body. Part II: Effects. *Med. Biol.* **59**, 190-211.

Airaksinen M.M. and Kari I. (1981b) ß-carbolines, psychoactive compounds in the mammalian body. Part I: Occurence, origin and metabolism. *Med. Biol.* **59**, 21-34.

Albores R., Neafsey E.J., Drucker G., Fields J.Z. and Collins M.A. (1990) Mitochondrial respiratory inhibition by N-methylated B-carboline derivatives structurally resembling N-methyl-4-phenylpyridine. *Proc. Natl. Acad. Sci. USA.* **87**, 9368-9372.

Ambani L.M., Van Woert M.H. and Murphy S. (1975) Brain peroxidase and catalase in Parkinson's disease. *Arch. Neurol.* **32**, 114-118.

Andersen J.K., Zhang M.-B., Zhong X.-H., Rozenberg Y.Y. ad Howard B.D. (1990) 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine-resistant, flat-cell PC12 variants having a partial loss of transformed phenotype. *J. Neurochem.* **55**, 559-567.

Andersen P.H. (1987) Biochemical and pharmacological characterization of [3H]GBR 12935 binding in vitro to rat striatal membranes: Labeling of the dopamine uptake complex. *J. Neurochem.* **48**, 1887-1896.

Andersen P.H. (1989) The dopamine uptake inhibitor GBR 12909: selectivity and molecular mechanism of action. *Eur. J. Pharmacol.* **166**, 493-504.

Arora P.K., Riachi N.J., Fiedler G.C., Singh M.P., Abdallah F., Harik S.I. and Sayre L.M. (1990) Structure-neurotoxicity trends of analogues of 1-methyl-4-phenylpyridinium (MPP+), the cytotoxic metablolite of the dopaminergic neurotoxin MPTP. *Life Sci.* **46**, 379-390.

Banerjee D.K., Lutz R.A., Levine M.A., Rodbard D. and Pollard H.B. (1987) Uptake of norepinephrine and related catecholamines by cultured chromaffin cells: Characterization of cocaine-sensitive and -insensitive plasma membrane transport sites. *Proc. Natl. Acad. Sci. USA.* **84**, 1749-1753.

Barbeau A. (1984) Etiology of Parkinson's disease: A research strategy. *Can. J. Neurol. Sci.* **11**, 24-28.

Barbeau A. and Roy M. (1985) Uneven prevalence of Parkinson's disease in the province of Quebec. *Can. J. Neurol. Sci.* **12**, 169-170.

Barbeau A., Roy M., Paris S., Cloutier T., Plasse L. and Poirier J. (1985) Ecogenetics of Parkinson's disease: 4-hydroxylation of debrisoquine. *Lancet.* **2**, 1213-1216.

Barker S.A., Harrison R.E.W., Monti J.A., Brown G.B. and Christian S.T. (1981) Identification and quantitation of 1,2,3,4-tetrahydro-beta-carboline, and 6-methoxy-1,2,3,4-tetrahydro-beta-carboline as in vivo constituents of rat brain and adrenal gland. *Biochem. Pharmacol.* **30**, 9-17.

Barnes N.J.G., Costall B. and Naylor R.J. (1987) Is the dopamine uptake system required for MPTP to induce neurotoxicity in the primate brain? *Br. J. Pharmacol.* **90**, 241P.

Basma A.N., Heikkila R.E., Nicklas W.J., Giovanni A. and Geller H.M. (1990) 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine- and 1-methyl-4-(2'-ethylphenyl)-1,2,3,6-tetrahydropyridine-induced toxicity in PC12 cells: Role of monoamine oxidase A. *J. Neurochem.* **55**, 870-877.

Basma A.N., Heikkila R.E., Saporito M.S., Philbert M., Geller H.M. and Nicklas W.J. (1992) 1-Methyl-4-(2'-ethylphenyl)-1,2,3,6-tetrahydropyridine-induced toxicity in PC12 cells is enhanced by preventing glycolysis. *J. Neurochem.* **58**, 1052-1059.

Battaglia G., Brooks B.P., Kulsakdinum C. and De Souza E.B. (1988) Pharmacologic profile of MDMA (3,4-methylenedioxymethamphetamine) at various brain recognition sites. *Eur. J. Pharmacol.* **149**, 159-163.

Beck O., Faull K.M. and Repke D. (1986) Rapid hydroxylation of methtryptoline (1-methyltetrahydro-beta-carboline) in rat: identification of metabolites by chiral gas chromatography-mass spectrometry. *Arch. Pharmacol.* **333**, 307-312.

Bernheimer H., Birkmayer W., Hornykiewicz O., Jellinger K. and Seitelberger F. (1973) Brain dopamine and the syndromes of Parkinson and Huntington: clinical, morphological and neurochemical correlations. *J. Neurol. Sci.* **20**, 415-455.

Bidder T.G., Shoemaker D.W., Böttger H.G., Evans M. and Cummins J.T. (1979) Harman in human platelets. *Life Sci.* **25**, 157-164.

Bindoff L.A., Birch-Machin M., Cartlidge N.E.F. and al. e. (1989) Mitochondrial function in Parkinson's disease. *Lancet.* **ii**, 49.

Boobis A.R., Fawthrop D.J. and Davies D.S. (1989) Mechanism of cell death. *TIPS.* **10**, 275-280.

Bosin T.R., Borg S. and Faull K.F. (1989) Harman in rat brain, lung and human CSF: Effect of alcohol consumption. *Alcohol.* **5**, 501-511.

Bosin T.R., Faull K.F. and Barchas J.D. (1988) Harman in alcoholic beverages: pharmacological and toxicological implications. *Alcohol: Clin. Exp. Res.* **12**, 679-682.

Braestrup C., Nielsen M. and Olsen C. (1980) Urinary and brain beta-carboline-3-carboxylates as potent inhibitors of brain benzodiazepine receptors. *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2288.

Bravi D., Anderson J.J., Dagani F., Davis T.L., Ferrari R., Gillespie M. and Chase T.N. (1991) Mitochondrial function in platelets and muscle in Parkinson's disease. *Soc. Neurosci. Abstr.* **17**, 1260.

Brooks W.J., Jarvis M.F. and Wagner G.C. (1988) Attenuation of MPTP-induced dopaminergic neurotoxicity by a serotonin uptake blocker. *J Neural Transm.* **71**, 85-90.

Buckholtz N.S. and Boggan W.O. (1977) Monoamine oxidase inhibition in brain and liver produced by ß-carbolines: structure-activity relationships and substrate specificity. *Biochem. Pharmacol.* **26**, 1991-1996.

Burkard W.P. and Kettler R. (1977) Harmaline and its sulphur analogue: increase of cerebellar cyclic GMP and inhibition of monoamine oxidase. *Biochem. Pharmacol.* **26**, 1303-1306.

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

Buskirk R.G., Gabriels J. and Wagner J. (1988) PC12 cells grown on cellulosic filters differentiate in response to NGF and exhibit a polarity not seen when they are grown on solid substrata. *In Vitro Cell. Develop. Biol.* **24**, 451-456.

Calne D.B. and Langston J.W. (1983) Aetiology of Parkinson's disease. *Lancet.* **2**, 1457-1459.

Canessa M., Jaimovich E. and de la Fuente M. (1973) Harmaline: A competitive inhibitor of Na ion in the (Na+ + K+)-ATPase system. *J. Memb. Biol.* **13**, 263-282.

Charnock J.S., Bashford C.L. and Ellroy J.C. (1976) Effects of ATP and magnesium ions on the fluorescence of harmala alkaloids. Restrictions for the use of harmala alkaloids as fluorescent probes for (Na+ + K+)-ATPase. *Biochim. Biophys. Acta.* **436**, 413-423.

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

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

Cleeter M.W.J., Cooper J.M. and Schapira A.H.V. (1992) Irreversible inhibition of mitochondrial complex I by 1-methyl-4-phenylpyridinium: Evidence for free radical involvement. *J. Neurochem.* **58**, 786-789.

Collins M., Neafsey E.J., Matsubara K., Cobuzzi R. Jr., Albores R. Jr., Fields J. and Rollema H. (1992) Indole-N-methylation of ß-carbolines: The brain's bioactivation route to toxins in Parkinson's disease. *Ann. N.Y. Acad. Sci.* In press.

Collins M.A. (1983) Mammalian Alkaloids, in *The Alkaloids* (Brossi A., eds), pp. 329-358. Academic Press, New York.

Collins M.A. and Neafsey E.J. (1985) ß-carboline analogues of N-methyl-phenyl-1,2,5,6-tetrahydropyridine (MPTP): Endogenous factors underlying idiopathic parkinsonism? *Neurosci. Lett.* **55**, 179-184.

Collins M.A., Neafsey E.J., Cheng B.Y., Hurley-Guis K., Ung-Chhun N.A., Pronger D.A., Christensen M.A. and Hurley-Guis D. (1986) Endogenous analogs of N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: Indoleamine derived tetrahydro-B-carboines as potential causative factors in Parkinson's disease. *Adv. Neurol.* **45**, 179-182.

Collins M.A., Neafsey E.J., Matsubara K., Cobuzzi R.J. Jr. and Rollema H. (1992) Indole-N-methylated ß-carbolinium ions as potential brain-bioactivated neurotoxins. *Brain Res.* **570**, 154-160.

Comella C.L., Tanner C.M., Goetz C.G., Gans S., Rapp D. and Fischer J. (1987) Debrisoquine metabolism in Parkinson's disease. *Neurology.* **37**, Suppl. 1, 261-262.

Cornish-Bowden A. and Koshland D.E. Jr. (1975) Diagnostic uses of the Hill (Logit and Nernst) Plots. *J. Mol. Biol.* **95**, 201-212.

D'Amato R., Alexander G., Schwartzman R., Kitt C., Price D. and Snyder S. (1987) Evidence for neuromelanin involvement in MPTP-induced neurotoxicity. *Nature.* **327**, 324-326.

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

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

Denton T. and Howard B.D. (1984) Inhibition of dopamine uptake by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a cause of parkinsonism. *Biochem. Biophys. Res. Commun.* **119**, 1186-1190.

Denton T. and Howard B.D. (1987) A dopaminergic cell line variant resistant to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *J. Neurochem.* **49**, 622-630.

Dexter D.T., Carayon A., Vidailhet M., Ruberg M., Agid F., Agid Y., Lees A.J., Wells F.R., Jenner P. and Marsden C.D. (1990) Decreased ferritin levels in brain in Parkinson's disease. *J. Neurochem.* **55**, 16-20.

Dexter D.T., Carter C., Agid G., Agid Y., Lees A.J., Jenner P. and Marsden C.D. (1986) Lipid peroxidation as a cause of nigral cell death in Parkinson's disease. *Lancet.* **ii**, 1219-1220.

Dexter D.T., Carter C.J., Wells F.R., Javoy-Agid F., Agid Y., Lees A., Jenner P. and Marsden C.D. (1989) Basal lipid peroxidation in substantia nigra is increased in Parkinson's disease. *J. Neurochem.* **52**, 381-389.

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

DiMonte D., Jewell S.A., Ekstrom G., Sandy M.S. and Smith M.T. (1986) 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (NPTP) and 1-methyl-4-phenyl-pyridine (MPP+) cause rapid ATP depletion in isolated hepatocytes. *Biochem. Biophys. Res. Commun.* **137**, 310-315.

DiMonte D., Sandy M.S. and Smith M.T. (1987) Increase efflux rather than oxidation is the mechanism of glutathione depletion by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Biochem. Biophys. Res. Commun.* **148**, 153-160.

Drucker G., Raikoff K., Neafsey E.J. and Collins M.A. (1990) Dopamine uptake inhibitory capacities of ß-carboline and 3,4-dihydro-ß-carboline analogs of N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) oxidation products. *Brain Res.* **509**, 125-133.

Dubocovich M.L. and Zahniser N.R. (1985) Binding characteristics of the dopamine uptake inhibitor [3H]nomifensine to striatal membranes. *Biochem. Pharmacol.* **34**, 1137-1144.

Dunn M.J. and Hunt W. (1975) The effects of harmaline on sodium transport in human erythrocytes: evidence in favor of action at interior sodium sensitive sites. *J. Pharmacol. Expt. Ther.* **193**, 903-909.

Duportail G. and Lami H. (1975) Studies of the interaction of the fluorophores harmine and harmaline with clf thymus DNA. *Biochim. Biophys. Acta.* **402**, 20-30.

Duvoisin R.C., Eldridge R., Williams A., Nutt J. and Calne D. (1981) Twin study of Parkinson's disease. *Neurology.* **31**, 77-80.

Earle K.M. (1968) Studies in Parkinson's disease including x-ray fluorescent spectroscopy of formalin fixed brain tissue. *J. Neuropathol. Exp. Neurol.* **27**, 1-14.

Fields J.Z., Albores R.R., Neafsey E.J. and Collins M.A. (1992) Inhibition of mitochondrial succinate oxidation - similarities and differences between N-methylated ß-carbolines and MPP+. *Arch. Biochem. Biophys.* **294**, in press.

Fonne-Pfister R., Bargetzi M.J. and Meyer U.A. (1987) MPTP, the neurotoxin inducing Parkinson's disease, is a potent competitive inhibitor of human and rat cytochrome P450 isozymes (P450bufI, P450db1) catalyzing debrisoquine 4-hydroxylation. *Biochem. Biophys. Res. Commun.* **148**, 1144-1150.

Fonne-Pfister R. and Meyer U.A. (1988) Xenobiotic and endobiotic inhibitors of cytochrome P-450db1 function, the target of the debrisoquine/sparteine type polymorphism. *Biochem. Pharmacol.* **37**, 3829-3835.

Forno L.S., Langston J.W., DeLanney L.E. and Irwin I. (1988) An electron microscopic study of MPTP-induced inclusion bodies in an old monkey. *Brain Res.* **448**, 150-157.

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

Fuller R.W. and Hemrick-Luecke S.K. (1985) Mechanisms of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) neurotoxicity to striatal dopamine neurons in mice. *Prog. Neuro-Psychopharmacol. Biol. Psychiat.* **9**, 687-690.

Glover V., Liebowitz J., Armando I. and Sandler M. (1982) ß-Carbolines as selective monoamine oxidase inhibitors: in vivo implications. *J. Neural Transm.* **54**, 209-218.

Godfrey K. (1986) Comparing the means of several groups, in *Medical Uses of Statistics* (Bailar J.C. III and Mosteller F., eds), pp. 205-234. NEJM Books, Waltham, MA.

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

Green S., Buttrum S., Molloy H., Steventon G., Sturman S., Waring R., Pall H. and Williams A. (1991) N-Methylation of pyridines in Parkinson's disease. *Lancet.* **338**, 120-121.

Greene L.A. and Rein G. (1977) Release, storage and uptake of catecholamines by a clonal cell line of nerve growth factor (NGF) responsive pheochromocytoma cells. *Brain Res.* **129**, 247-263.

Greene L.A. and Tischler A.S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA.* **73**, 2424-2428.

Greiner B. and Rommelspacher H. (1984) Two metabolic pathways of tetrahydroharmane (tetrahydro-beta-carboline) in rats. *Arch. Pharmacol.* **325**, 349-355.

Gunn J.A. (1935) Relations between chemical constitution, pharmacological actions, and therapeutic uses, in the harmine group of alkaloids. *Arch. Int. Pharmacodyn.* **50**, 379-396.

Hallman H., Lange J., Olson L., Stromberg I. and Jonsson G. (1985) Neurochemical and histochemical characterization of neurotoxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine on brain catecholamine neurons in the mouse. *J. Neurochem.* **44**, 117-127.

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

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

Henneberry R.C. (1989) The role of neuronal energy in the neurotoxicity of excitatory amino acids. *Neurobiol. Aging.* **10**, 611-613.

Hirsch E., Graybiel A.M. and Agid Y.A. (1988) Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson's disease. *Nature.* **334**, 345-348.

Hirsch E.C., Brandel J.P., Javoy-Agid F. and Agid Y. (1991) Iron and aluminum increase in the substantia nigra of patients with Parkinson's disease: a X-ray microanalysis. *J. Neurochem.* **56**, 446-451.

Hoppel C.L., Greenblatt D., Kwok H.-C., Arora P.K., Singh M.P. and Sayre L.M. (1987) Inhibition of mitochondrial respiration by analogs of 4-phenylpyridine and 1-methyl-4-phenylpyridinium cation (MPP+), the neurotoxic metabolite of MPTP. *Biochem. Biophys. Res. Comm.* **148**, 684-693.

Hudson J.B., Graham E.A., Fong R., Hudson L.L. and Towers G.H.N. (1986a) Further studies on the antiviral activity of harmine, a photoactive ß-carboline alkaloid. *Photochem. Photobiol.* **44**, 483-487.

Hudson J.B., Graham E.A. and Towers G.H.N. (1986b) Antiviral effect of harmine, a photoactive ß-carboline alkaloid. *Photochem. Photobiol.* **43**, 21-26.

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

Javitch J.A., Blaustein R.O. and Snyder S.H. (1983) [3H]Mazindol binding associated with neuronal dopamine uptake sites in corpus striatum membranes. *Eur. J. Pharmacol.* **90**, 461-462.

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

Jenner P., Rupniak N., Rose S., Kelly E., Kilpatrick G., Lees A. and Marsden C.D. (1984) MPTP-induced parkinsonism in the common marmoset. *Neurosci. Lett.* **50**, 85-90.

Johannessen J.N., Adams J.D., Schuller H.M., Bacon J.B. and Markey S.P. (1985a) 1-Methyl-4-phenylpyridine (MPP+) induces oxidative stress in rodents. *Life Sci.* **38**, 743-749.

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

Johnson J.V., Yost R., Beck O. and Faull K. (1985) The use of tandem mass spectrometry for the identification and quantitation of tryptolines (tetrahydro-beta-carbolines) in tissue extracts, in *Aldehyde Adducts in Alcoholism* (Collins M.A., eds), pp. 161. Alan R. Liss, New York.

Kalaria R.N., Mitchell M.J. and Harik S.I. (1987) MPTP neurotoxicity: correlation with blood-brain barrier monoamine oxidase activity. *Proc. Natl. Acad. Sci. USA.* **84**, 3521-3525.

Kass G.E.N., Wright J.W., Nicotera P. and Orrenius S. (1988) The mechanism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity: role of intracellular calcium. *Arch. Biochem. Biophys.* **260**, 789-797.

Kaufman L.M. and Barrett J.N. (1983) Serum factor supporting long-term survival of rat central neurons in culture. *Science.* **220**, 1394-1396.

Kindt M.V., Youngster S.K., Sonsalla P.K., Duvoisin R.C. and Heikkila R.E. (1988) Role for monoamine oxidase-A (MAO-A) in the bioactivation and nigrostriatal dopaminergic neurotoxicity of the MPTP analog, 2'Me-MPTP. *Eur. J. Pharmacol.* **146**, 313-318.

Kish S.J., Shannak K., Rajput A., Deck J.H.N. and Hornykiewicz O. (1992) Aging produces a specific pattern of striatal dopamine loss: Implications for the etiology of idiopathic Parkinson's disease. *J. Neurochem.* **58**, 642-648.

Koh J.Y. and Choi D.W. (1987) Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *J Neurosci Methods.* **20**, 83-90.

Koide M., Cho A.K. and Howard B.D. (1986) Characterization of xylamine binding to proteins of PC12 pheochromocytoma cells. *J. Neurochem.* **47**, 1277-1285.

Kolata G. (1983) Monkey model of Parkinson's disease. *Science.* **220**, 705.

Kutty R.K., Santostasi G., Horng J. and Krishna G. (1991) MPTP-induced ATP depletion and cell death in neuroblastoma x glioma hybrid NG 108-15 cells: Protection by glucose and sensitization by tetraphenylborate. *Toxicol. Appl. Pharmacol.* **107**, 377-388.

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

Langston J.W., Forno L.S., Rebert C.S. and Irwin I. (1984a) Selective nigral toxicity after systemic administration of 1-methyl-4-phenyl-1,2,5,6-tetrahydopyridine (MPTP) in the squirrel monkey. *Brain Res.* **292**, 390-394.

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

Larson R.A., Marley K.A., Tuveson R.W. and Berenbaum M.R. (1988) ß-Carboline alkaloids: mechanisms of phototoxicity to bacteria and insects. *Photochem. Photobiol.* **48**, 665-674.

Lestienne P., Nelson J., Riederer P., Jellinger K. and Reichmann H. (1990) Normal mitochondrial genome in brain from patients with Parkinson's disease and complex I defect. *J. Neurochem.* **55**, 1810-1812.

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

Madle E., Obe G., Hansen J. and Ristow H. (1981) Harman and norharman: induction of sister-chromatid exchanges in human peripheral lymphocytes in vitro and interaction with isolated DNA. *Mut. Res.* **90**, 433-442.

Mann D.M.A. and Yates P.D. (1982) Pathogenesis of Parkinson's Disease. *Arch. Neurol.* **39**, 545-549.

Maret G., Testa B., Jenner P., El Tayar N. and Carrupt P.-A. (1990) The MPTP story: MAO activates tetrahydropyridine derivatives to toxins causing parkinsonism. *Drug Metab. Rev.* **22**, 291-332.

Marini A.M., Lipsky R.H., Schwartz J.P. and Kopin I.J. (1992) Accumulation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in cultured cerebellar astrocytes. *J. Neurochem.* **58**, 1250-1258.

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

Marongiu M.E., Piccardi M.P., Bernardi F., Corsini G.U. and Del Zompo M. (1988) Evaluation of the toxicity of the dopaminergic neurotoxins MPTP and MPP+ in PC12 pheochromocytoma cells: binding and biological studies. *Neurosci. Lett.* **94**, 349-354.

Martilla R.J. (1987) Epidemiology, in *Handbook of Parkinson's Disease* (Koller W.C., eds), pp. 35-50. Marcel Dekker, New York.

Matsubara K., Collins M.A. and Neafsey E.J. (1992a) Mono-N-methylation of 1,2,3,4-tetrahydro-ß-carbolines in brain cytosol: Absence of indole methylation. *J. Neurochem.* In press.

Matsubara K., Neafsey E.J. and Collins M.A. (1992b) The identification of N-methylated beta-carbolines in human brain. *Soc. Neurosci. Abstr.* Submitted.

Matsubara K., Neafsey E.J. and Collins M.A. (1992c) Novel S-adenosylmethionine-dependent indole-N-methylation of ß-carbolines in brain particulate fractions. *J. Neurochem.* In press.

May T., Pawlik M. and Rommelspacher H. (1991a) [3H]Harman binding experiments. II: Regional and subcellular distribution of specific [3H]harman binding and

monoamine oxidase subtypes A and B activity in marmoset and rat. *J. Neurochem.* **56**, 500-508.

May T., Rommelspacher H. and Pawlik M. (1991b) [3H]Harman binding experiments. I: A reversible and selective radioligand for monoamine oxidase subtype A in the CNS of the rat. *J. Neurochem.* **56**, 490-499.

Mayer R.A., Kindt M.V. and Heikkila R.E. (1986) Prevention of the nigrostriatal toxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine by inhibitors of 3,4-dihydroxyphenylethylamine transport. *J. Neurochem.* **47**, 1073-1079.

McKenna D.J. and Towers G.H.N. (1981) Ultra-violet mediated cytotoxic activity of ß-carboline alkaloids. *Phytochem.* **20**, 1001-1004.

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

Melamed E., Rosenthal J., Cohen O., Uzzan A. and Globus M. (1985b) Amphetamine, but not reserpine, protects mice against dopaminergic neurotoxicity of MPTP. *Neuropharm.* **24**, 923-925.

Melchior C.M. and Collins M.A. (1982) The routes and significance of endogenous synthesis of alkaloids in animals. *CRC Crit. Rev. Toxicol.* **10**, 313-356.

Miyoshi R., Kito S., Ishida H. and Katayama S. (1988) Alterations of the central noradrenergic system in MPTP-induced monkey parkinsonism. *Res. Commun. Chem. Pathol. Pharmacol.* **62**, 93-102.

Mizuno Y., Ohta S., Tanaka M., Takamiya S., Suzuki K., Sato T., Oya H., Ozawa T. and Kagawa Y. (1989) Deficiencies in complex I subunits of the respiratory chain in Parkinson's disease. *Biochem. Biophys. Res. Commun.* **163**, 1450-1455.

Mytilineou C. and Cohen G. (1984) 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine destroys dopamine neurons in explants of rat embryo mesencephalon. *Science.* **225**, 529-531.

Mytilineou C. and Friedman L. (1988) Studies on the metabolism and toxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in cultures of embryonic rat mesencephalon. *J. Neurochem.* **51**, 750-755.

Nagao M., Yahagi T., Honda M., Seino Y., Matsushima T. and Sugimura T. (1977a) Demonstration of mutagenicity of aniline and o-toluidine by norharman. *Proc. Jpn. Acad.* **53**, 34-37.

Nagao M., Yahagi T., Kawachi T., Sugimura T., Kosuge T., Tsuji K., Wakabayashi K., Mizusaki S. and Matsumoto T. (1977b) Comutagenic action of norharman and harman. *Proc. Jpn. Acad.* **53**, 95-98.

Naranjo C. (1967) Psychotropic properties of the harmala alkaloids, in *Ethnopharmacological Search for Psychoactive Drugs* (Efron D.H., Holmstedt B. and Kline N.S., eds), pp. 385-392. U.S. Public Health, Washington, D.C.

Naranjo C. (1979) Psychotropic properties of the harmala alkaloids, in *Ethnopharmacological search for psychoactive drugs* (Efron D.H., Holmstedt B. and Kline K.S., eds), pp. 385-391. Raven Press, New York.

Neafsey E.J., Drucker G., Raikoff K. and Collins M.A. (1989) Striatal dopaminergic toxicity following intranigral injection in rats of 2-methyl-norharman, a B-carbolinium analog of N-methyl-4-phenylpyridinium ion (MPP+). *Neurosci. Lett.* **105**, 344-349.

Neafsey E.J., Hurley-Gius K., Cheng B., Ung-Chhun N., Pronger D. and Collins M.A. (1986) Comparison of the effects of MPTP and an endogenous analog, N-Me-1,2,3,4-tetrahydro-beta-carboline, on brain and adrenal monoamines and brain amine metabolites in the owl monkey, in *MPTP: A Neurotoxin Producing a Parkinsonian Syndrome* (Markey S., eds), pp. 495. Academic Press, New York.

Neff N.H. and Fuentes J.A. (1976) The use of selective monoamine oxidase inhibitor drugs for evaluating pharmacological and physiological mechanisms, in *Monoamine Oxidase and its Inhibition* (Ciba Foundation Symposium, eds), Elsevier-Excerpta Medica-North Holland, Amsterdam.

Nelson D.L., Herbet A., Glowinski J. and Hamon M. (1979a) [3H]Harmaline as a specific ligand of MAO A-II. Measurement of the turnover fates of MAO A during ontogenesis in the rat brain. *J. Neurochem.* **32**, 1829-1836.

Nelson D.L., Herbet A., Petillot Y., Pichat L., Glowinski J. and Hamon M. (1979b) [3H]Harmaline as a specific ligand of MAO A-I. Properties of the active site of MAO A from rat and bovine brains. *J. Neurochem.* **32**, 1817-1827.

Nicklas W.J., Vyas I. and Heikkila R.E. (1985) Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenylpyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. *Neurosci. Lett.* **69**, 285-289.

Niznik H.B., Fogel E.F., Fassos F.F. and Seeman P. (1991) The dopamine transporter is absent in parkinsonian putamen and reduced in the caudate nucleus. *J. Neurochem.* **56**, 192-198.

Niznik H.B., Tyndale R.F., Sallee F.R., Gonzalez F.J., Hardwick J.P., Inaba T. and Kalow W. (1990) The dopamine transporter and cytochrome P450IID1 (debrisoquine 4-hydroxylase) in brain: Resolution and identification of two distinct [3H]GBR-12935 binding proteins. *Arch. Biochem. Biophys.* **276**, 424-432.

Nolte J. (1981). The Human Brain: An introduction to its functional anatomy. St. Louis: Mosby.

Novelli A., Reilly J.A., Lysko P.G. and Henneberry R.C. (1988) Glutamate becomes neurotoxic via the N-methyl-D-aspartate receptor when intracellular energy levels are reduced. *Brain Res.* **451**, 205-212.

Ochiai M., Wakabayashi K., Sugimura T. and Nagao M. (1986) Mutagenicities of indole and 30 derivatives after nitrite treatment. *Mut. Res.* **172**, 189-197.

Oda Y., Nakamura S.-I. and Oki I. (1988) Harman and norharman induce SOS responses and frameshift mutations in bacteria. *Mut. Res.* **208**, 39-44.

Ohkubo S., Toshihiko H. and Oka K. (1985) Methyltetrahydro-ß-carbolines and Parkinson's disease. *Lancet.* **i**, 1272-1273.

Orrenius S., McConkey D.J., Bellomo G. and Pierluigi N. (1989) Role of Ca2+ in toxic cell killing. *TIPS.* **10**, 281-285.

Parker W.J. Jr., Boyson S.J. and Parks J. (1989) Abnormalities of the electron transport chain in idiopathic Parkinson's disease. *Ann. Neurol.* **26**, 719-723.

Pena C., Medina J.H., Novas M.L., Paladini A.C. and de Robertis E. (1986) *Proc. Natl. Acad. Sci. USA.* **83**, 4952.

Perry T.L. and Yong V.W. (1986) Idiopathic Parkinson's disease, progressive supranuclear palsy and glutathione metabolism in the substantia nigra of patients. *Neurosci. Lett.* **67**, 269-274.

Pifl C., Bertel O., Schingnitz G. and Hornykiewicz O. (1990) Extrastriatal dopamine in symptomatic and asymptomatic rhesus monkeys treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Neurochem. Int.* **17**, 263-270.

Poirier J., Donaldson J. and Barbeau A. (1985) The specific vulnerability of the substantia nigra to MPTP is related to the presence of transition metals. *Biochem. Biophys. Res. Commun.* **128**, 25-33.

Poirier J., Kogan S. and Gauthier S. (1991) Environment, genetics and idiopathic Parkinson's disease. *Can. J. Neurol. Sci.* **18**, 70-76.

Poirier J., Roy M., Campanells G., Cloutier T. and Paris S. (1987) Debrisoquine metabolism in parkinsonian patients treated with antihistamine drugs. *Lancet.* **2**, 386.

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

Ramsay R.R., Krueger M.J., Youngster S.K., Gluck M.R., Casida J.E. and Singer T.P. (1991) Interaction of 1-methyl-4-phenylpyridinium ion (MPP+) and its analogs with the rotenone/piericidin binding site of NADH dehydrogenase. *J. Neurochem.* **56**, 1184-1190.

Ramsay R.R., Melborn R.J. and Singer T.P. (1989) Enhancement by tetraphenylboron of the interaction of the 1-methyl-4-phenylpyridinium ion (MPP+) with mitochondria. *Biochem. Biophys. Res. Commun.* **159**, 983-990.

Ramsay R.R., Salach J.I., Dadgar J. and Singer T.P. (1986) Inhibition of mitochondrial NADH dehydrogenase by pyridine derivatives and its possible relation to experimental and idiopathic parkinsonism. *Biochem. Biophys. Res. Commun.* **135**, 269-275.

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

Ramsay R.R., Youngster S.K., Nicklas W.J., McKeown K.A., Jin Y.-Z., Heikkila R.E. and Singer T.P. (1989) Structural dependence of the inhibition of mitochondrial respiration and of NADH oxidase by 1-methyl-4-phenylpyridinium (MPP+) analogs and their energized accumulation by mitochondria. *Proc. Natl. Acad. Sci. USA.* **86**, 9168-9172.

Ramsden D.B. and Williams A.C. (1985) Production in nature of compounds resembling methylphenyltetrahydropyridine, a possible cause of Parkinson's disease. *Lancet.* i, 215-216.

Rebois R.V., Reynolds E.E., Toll L. and Howard B.D. (1980) Storage of dopamine and acetylcholine in granules of PC12, a clonal pheochromocytoma cell line. *Biochem.* **19**, 1240-1248.

Reinhard J.F. Jr., Carmichael S.W. and Daniels A.J. (1990a) Mechanisms of toxicity and cellular resistance to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 1-methyl-4-phenylpyridinium in adrenomedullary chromaffin cell culture. *J. Neurochem.* **55**, 311-320.

Reinhard J.F. Jr., Daniels A.J. and Painter G.R. (1990b) Carrier-independent entry of 1-methyl-4-phenylpyridinium (MPP+) into adrenal chromaffin cells as a consequence of charge delocalization. *Biochem. Biophys. Res. Commun.* **168**, 1143-1148.

Reinhard J.F. Jr., Daniels A.J. and Viveros O.H. (1988) Potentiation by reserpine and tetrabenazine of brain catecholamine depletions by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) in the mouse; evidence for subcellular resistance to the toxicant. *Neurosci. Lett.* **90**, 349-353.

Reinhard J.F. Jr., Diliberto E.J. Jr. and Daniels A.J. (1989) Characterization of cellular transport, subcellular distribution and secretion of the neurotoxicant 1-methyl-4-phenylpyridinium in bovine adrenomedullary cell cultures. *J. Neurochem.* **52**, 1253-1259.

Reinhart K. Jr., Kobayashi J., Harbour G.C., Gilmore J., Mascal M., Holt T.G., Shields L.S. and Lafargue F. (1987) *J. Am. Chem. Soc.* **109**, 3378.

Remsen J.F. and Cerutti P.A. (1979) Inhibition of DNA-repair and DNA-synthesis by harman in human alveolar tumor cells. *Biochem. Biophys. Res. Commun.* **86**, 124-129.

Reynolds E.E., Melega W. and Howard B.D. (1982) Adenosine 5'-triphosphate independent secretion from PC12 pheochromocytoma cells. *Biochemistry.* **21**, 4795-4799.

Rollema H., Booth R.G. and Castagnoli N. Jr. (1988) In vivo dopaminergic neurotoxicity of the 2-ß-methylcarbolinium ion, a potential enogenous MPP+ analog. *Eur. J. Pharmacol.* **153**, 131-134.

Rommelspacher H., Damm H., Lutter S., Schmidt L.G., Otto M., Sachs-Ericsson N. and Schmidt G. (1990) Harman (1-methyl-ß-carboline) in blood plasma and erythrocytes of nonalcoholics following ethanol loading. *Alcohol.* **7**, 27-31.

Rommelspacher H., Damm H., Scmidt L. and Schmidt G. (1985) Increased excretion of harman by alcoholics depends on events of their life history and the status of the liver. *Psychopharm. (Berlin).* **87**, 64-68.

Rommelspacher H., Damm H., Strauss S. and Schmidt G. (1984) Ethanol induces an increased excretion of harman in the brain and urine of rats. *Naunyn Schmiedebergs Arch. Pharmacol.* **327**, 107-113.

Rommelspacher H., May T. and Susilo R. (1991) ß-Carbolines and tetrahydroisoquinolines: Detection and function in mammals. *Planta Med.* **57**, S85-S92.

Rommelspacher H., Nanz C., Borbe H.O., Fehske K.J., Müller W.E. and Wollert U. (1980) 1-Methyl-ß-carboline (harmane), a potent endogenous inhibitor of benzodiazepine receptor binding. *Arch. Pharmacol.* **314**, 97-100.

Rommelspacher H., Nanz C., Borbe H.O., Fehske K.J., Müller W.E. and Wollert W. (1981) Benzodiazepine antagonism by harmane and other ß-carbolines in vitro and in vivo. *Eur. J. Pharmacol.* **70**, 409-416.

Rommelspacher H. and Schmidt L. (1985) Tetrahydroisoquinolines and beta-carbolines: putative natural substances in plants and mammals. *Prog. Drug. Res.* **29**, 415-459.

Saggu H., Cooksey J., Dexter D., Wells F.R., Lees A., Jenner P. and Marsden C.D. (1989) A selective increase in particulate superoxide dismutase activity in Parkinsonian substantia nigra. *J. Neurochem.* **53**, 692-698.

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

Sanchez-Ramos J.R., Barrett J.N., Goldstein M., Weiner W.J. and Hefti F. (1986) MPP+, but not MPTP, is toxic to dopamine neurons in cultures of dissociated rat mesencephalic neurons. *Neurosci. Lett.* **72**, 215-220.

Sastry B.S.R. and Phillis J.W. (1977) Antagonism of biogenic amine-induced depression of cerebral cortical neurones by Na+, K+-ATPase inhibitors. *Can. J. Physiol. Pharmacol.* **55**, 170-179.

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

Sayre L.M., Singh M.P., Arora P.K., Wang F., McPeak R.J. and Hoppel C.L. (1990) Inhibition of mitochondrial respiration by analogues of the dopaminergic neurotoxin

1-methyl-4-phenylpyridinium: Structural requirements for accumulation-dependent enhanced inhibitory potency on intact mitochondria. *Arch. Biochem. Biophys.* **280**, 274-283.

Sayre L.M., Wang F., Arora P.K., Riachi N.J., Harik S.I. and Hoppel C.L. (1991) Dopaminergic neurotoxicity in vivo and inhibition of mitochondrial respiration in vitro by possible endogenous pyridinium-like substances. *J. Neurochem.* **57**, 2106-2115.

Sayre L.M., Wang F. and Hoppel C.L. (1989) Tetraphenylborate potentiates the respiratory inhibition by the dopaminergic neurotoxin MPP+ in both electron transport particles and intact mitochondria. *Biochem. Biophys. Res. Commun.* **161**, 809-818.

Schapira A.H.V., Cooper J.M., Dexter D., Clark J.B., Jenner P. and Marsden C.D. (1990) Mitochondrial complex I deficiency in Parkinson's disease. *J. Neurochem.* **54**, 823-827.

Schapira A.H.V., Cooper J.M., Dexter D., Jenner P., Clark J.B. and Marsden C.D. (1989) Mitochondrial complex I deficiency in Parkinson's disease. *Lancet.* **i**, 1269.

Schapira A.H.V., Mann V.M., Cooper J.M., Dexter D., Daniel S.E., Jenner P., Clark J.B. and Marsden C.D. (1990) Anatomic and disease specificity of NADH CoQ1 reductase (complex 1) deficiency in Parkinson's disease. *J. Neurochem.* **55**, 2142-2145.

Schinelli S., Zuddas A., Kopin I.J., Barker J.L. and di Porzio U. (1988) 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine metabolism and 1-methyl-4-phenylpyridinium uptake in dissociated cell cultures from the embryonic mesencephalon. *J. Neurochem.* **50**, 1900-1907.

Schmidt C.J., Bruckwick E. and Lovenberg W. (1985) Lack of evidence supporting a role for dopamine in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity. *Eur. J. Pharmacol.* **113**, 149-159.

Schouten M. and Bruinvels J. (1986) Endogenously formed norharman (beta-carboline) in platelet rich plasma obtained from porphyric rats. *Pharmacol. Biochem. Behav.* **24**, 1219-1223.

Schubert D. and Klier F.G. (1977) Storage and release of acetylcholine by a clonal cell line. *Proc. Natl. Acad. Sci. USA.* **74**, 5184-5188.

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

Seidler F.J., Kirksey D.F., Lau C., Whitmore W.L. and Slotkin T.A. (1977) Uptake of (-) 3H-norepinephrine by storage vesicles prepared from whole rat brain: properties of the uptake system and its inhibition by drugs. *Life Sci.* **21**, 1075-1086.

Sepulveda F.V. and Robinson J.W.L. (1974) Harmaline, a potent inhibitor of sodium-dependent transport. *Biochim. Biophys. Acta.* **373**, 527-531.

Shoemaker D.W., Cummins J.T., Bidder T.G., Boettger H.G. and Evans M. (1980) Identification of harman in the rat arcuate nucleus. *Arch. Pharmacol.* **310**, 227-230.

Sigg E.B., Gyermek L., Hill R.T. and Yen H.C.Y. (1964) Neuropharmacology of some harman derivatives. *Arch. Int. Pharmacodyn.* **149**, 164-180.

Simonson L.P. and Charnock J.S. (1979) The harmala alkaloids: Evidence for their complex inhibition of the K+-acyl phosphatase reaction of membranes. *Mol. Pharmacol.* **15**, 620-626.

Singer T.P., Castagnoli N. Jr., Ramsay R.R. and Trevor A.J. (1987) Biochemical events in the development of parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *J. Neurochem.* **49**, 1-8.

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

Singer T.P., Salach J.I. and Crabtree D. (1985) Reversible inhibition and mechanism-based irreversible inactivation of monoamine oxidases by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Biochem. Biophys. Res. Commun.* **341**, 707-712.

Singh M.P., Wang F., Hoppel C.L. and Syre L.M. (1991) Inhibition of mitochondrial respiration by neutral, monocationic, and dicationic bis-pyridines related to the dopaminergic neurotoxin 1-methyl-4-phenylpyridinium cation (MPP+). *Arch. Biochem. Biophys.* **286**, 138-146.

Slotkin T.A. (1974) Structure-activity relationships for the reserpine-like actions of derivatives of ß-carboline in vitro. *Life Sci.* **15**, 439-454.

Slotkin T.A., Salvaggio M., Seidler F.J. and Whitmore W.L. (1978) Structural characteristics for inhibition of [3H] norepinephrine uptake into rat brain synaptic vesicles by beta-carboline, indolealkylamine, phenethylamine and n-alkylamine derivatives. *Mol. Pharmacol.* **15**, 607-619.

Smart L. (1981) Competitive inhibition of sodium-dependent high affinity choline uptake by harmala alkaloids. *Eur. J. Pharmacol.* **75**, 265-269.

Smythies J.R. and Antun F. (1969) Binding of tryptamine and allied compounds to nucleic acids. *Nature*. **223**, 1061-1063.

Snyder S.S., D'Amato R.J., Nye J.S. and Javitch J.A. (1986) Selective uptake of MPP+ by dopamine neurons is required for MPTP toxicity: studies in brain synaptosomes and PC-12 cells, in *MPTP: A neurotoxin producing a parkinsonian syndrome* (Markey S.P., Castagnoli N., Kopin I.J. and Trevor A.T., eds), pp. 191-201. Academic Press, Inc., New York.

Sofic E., Riederer P., Heinsen H., Beckman H., Reynolds G.P., Hebenstrett G. and Youdim M.B.H. (1988) Increased iron (III) and total iron content in post-mortem substantia nigra of parkinsonian brain. *J. Neural. Transm.* **74**, 199-205.

Sonsalla P.K., Youngster S.K., Kindt M.V. and Heikkila R.E. (1987) Characteristics of 1-methyl-(2'-methylphenyl)-1,2,3,6-tetrahydropyridine-induced neurotoxicity in the mouse. *J. Pharmacol. Expt. Ther.* **242**, 850-857.

Stern M., Dulaney E., Gruber S.B., Golbe L., Bergen M., Hurtig H., Gollomp S. and Stolley P. (1991) The epidemiology of Parkinson's disease. A case-control study of young-onset and old-onset patients. *Arch. Neurol.* **48**, 903-907.

Steventon G.B., Heafield M.T.E., Sturman S.G., Waring R.H., Williams A.C. and Ellingham J. (1989a) Degenerative neurological disease and debrisoquine-4-hydroxylation capacity. *Med. Sci. Res.* **17**, 163-164.

Steventon G.B., Heafield M.T.E., Waring R.H. and Williams A.C. (1989b) Xenobiotic metabolism in Parkinson's disease. *Neurology.* **39**, 883-887.

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

Susilo R., Damm H., Rommelspacher H. and Hofle G. (1987) Biotransformation of 1-methyl-1,2,3,4-tetrahydro-ß-carboline-1-carboxylic acid to harmalan, tetrahydroharman and harman in rats. *Neurosci. Lett.* **81**, 325-330.

Suzuki J., Takahashi N., Kobayashi Y., Miyamae R., Ohsawa M. and Suzuki S. (1987) Dependence on *Salmonella typhimurium* enzymes of mutagenicities of nitrobenzene and its derivatives in the presence of rat-liver S9 and norharman. *Mut. Res.* **178**, 187-193.

Takada M., Li Z.K. and Hattori T. (1990) Astroglial ablation prevents MPTP-induced nigrostriatal neuronal death. *Brain Res.* **509**, 55-61.

Takahashi T., Naoi M. and Nagatsu T. (1987) Uptake of N-methyl-4-phenylpyridinium ion (MPP+) into PC12h pheochromocytoma cells. *Neurochem. Int.* **11**, 89-93.

Takashima A. and Koike T. (1985) Relationship between dopamine content and its secretion in PC12 cells as a function of cell growth. *Biochim. Biophys. Acta.* **847**, 101-107.

Tanner C.M. (1989) The role of environmental toxins in the etiology of Parkinson's disease. *TINS.* **12**, 49-54.

Tanner C.M., Chen B., Wang W.-Z., Peng M.-L., Liu Z.-L., Liang X.-L., Kao L.C., Gilley D.W. and Schoenberg B.S. (1987) Environmental factors in the etiology of Parkinson's disease. *Can. J. Neurol. Sci.* **14**, 419-423.

Testa B., Naylor R., Costall B., Jenner P. and Marsden C.D. (1985) Does an endogenous methylpyridinium analogue cause Parkinson's disease? *J. Pharm. Pharmacol.* **37**, 679-680.

Towers G.H.N. and Abramowski Z. (1983) UV-mediated genotoxicity of furanoquinoline and of certain tryptophan-derived alkaloids. *J. Nat. Prod.* **46**, 576-581.

Tse S.Y.H., Mak I.-T. and Dickens B.F. (1991) Antioxidative properties of harmane and ß-carboline alkaloids. *Biochem. Pharmacol.* **42**, 459-464.

Tweedie D.J. and Burke M.D. (1987) Metabolism of the beta-carbolines, harmine and harmol, by liver microsomes from phenobarbitone- or 3-methylcholanthrene-treated mice. Identification and quantitation of two novel harmine metabolites. *Drug Metab. Dispos.* **15**, 74-81.

Udenfriend S., Witkop B., Redfield B.G. and Weissbach H. (1958) Studies with reversible inhibitors of monoamine oxidase: harmaline and related compounds. *Biochem. Pharmacol.* **1**, 160-165.

Uemura T., Kanashiro M., Yamano T., Hirai K. and Miyazaki N. (1988) Isolation, structure, and properties of the ß-carboline formed from 5-hydroxytryptamine by the superoxide anion-generating system. *J. Neurochem.* **51**, 710-717.

Vieregge P., Kompf D. and Fassl H. (1988) Environmental toxins in Parkinson's disease. *Lancet.* **Feb 13**, 362-363.

Wakabayashi K., Nagao M., Kawachi T. and Sugimura T. (1981) Co-mutagenic effect of norharman with N-nitrosoamine derivatives. *Mut. Res.* **80**, 1-7.

Wakabayashi K., Ochiai M., Saito H., Tsuda M., Suwa Y., Nagao M. and Sugimura T. (1983) Presence of 1-methyl-1,2,3,4-tetrahydro-ß-carboline-3-carboxylic acid, a precursor of a mutagenic nitroso compound, in soy sauce. *Proc. Natl. Acad, Sci. USA.* **80**, 2912-2916.

Ward C.D., Duvoisin R.C., Ince S.E., Nutt J.D., Eldridge R. and Calne D.B. (1983) Parkinson's disease in 65 pairs of twins and in a set of quadruplets. *Neurology.* **33**, 815-824.

Waring R.H., Sturman S.G., Smith M.C.G., Steventon G.B., Heafield M.T.E. and Williams A.C. (1989) S-methylation in motorneuron disease and Parkinson' disease. *Lancet.* **2**, 356-357.

Westlund K.N., Denney R.M., Kochenperger L.M., Rose R.M. and Abell C.W. (1985) Distinct monoamine oxidase A and B populations in primate brain. *Science.* **230**, 181-183.

Wick M.M., Byers L. and Frey E. (1977) L-DOPA: selective toxicity for melanoma cells in vitro. *Science.* **197**, 468-469.

Wood P.L., Emmett M.R., Rao T.S., Cler J., Mick S. and Iyengar S. (1990) Inhibition of nitric oxide synthase blocks N-methyl-D-aspartate-, quisqualate-, kainate-, harmaline-, and pentylenetetrazole-dependent increases in cerebellar cyclic GMP in vivo. *J. Neurochem.* **55**, 346-348.

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

Youdim M.B.H., Ben-Shachar D. and Riederer P. (1990) The role of monoamine oxidase, iron-melanin interaction and intracellular calcium in Parkinson's disease. *J. Neural Transm.* **32**, 239-248.

Youdim M.B.H., Heldman E., Pollard H.B., Fleming P. and McHugh E. (1986) Contrasting monoamine oxidase activity and tyramine induced catecholamine release in PC12 and chromaffin cells. *Neuroscience.* **19**, 1311-1318.

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

Youngster S.K., Sonsalla P.K., Sieber B.A. and Heikkila R.E. (1989) Structure-activity study of the mechanism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine(MPTP)-induced neurotoxicity. 1. Evaluation of the biological activity of MPTP analogs. *J. Pharmacol. Expt. Ther.* **249**, 820-828.

Zigmond M.J. and Stricker E.M. (1989) Animal models of parkinsonism using selective neurotoxins: clinical and basic implications. *Int. Rev. Neurobiol.* **31**, 1-79.

VITA

The author, Robert Joseph Cobuzzi Jr., was born in Waltham, Massachusetts on September 12, 1964.

In September, 1982, Mr. Cobuzzi entered Colby College in Waterville, Maine, and received the degree of Bachelor of Arts in Biochemistry and Art History in May, 1986. That year, he entered the Department of Molecular and Cellular Biochemistry at Loyola University Chicago, Maywood, Illinois. Soon after, he joined the laboratory of Dr. Michael A. Collins, where he initiated his research on the effects of N-methylated ß-carbolines and dihydro-ß-carbolines on cultured PC12 cells. In 1990, Mr. Cobuzzi was awarded a fellowship by the Arthur J. Schmitt Foundation, and in 1991 he was elected to the National Jesuit Honor Society, Alpha Sigma Nu.

Mr. Cobuzzi is currently a post-doctoral fellow in the laboratory of Dr. Terry A. Beerman at Roswell Park Cancer Institute in Buffalo, NY, where he will work on the development of an episomal DNA model for studying the effects of novel anti-cancer drugs.

ABSTRACTS

Cobuzzi R.J., Jr., Neafsey E.J., Rollema H. and Collins M.A. (1992) Endogenous N-methyl-ß-carbolinium ions are potent MPP^+-like neurotoxicants in vitro & in vivo in rats. Poster Abstract, 4th International Trace Amines Conference, Ireland.

Collins M.A., Neafsey E.J., Matsubara K., Cobuzzi R., Jr., Albores R., Fields J. and Rollema H. Unique 2,9-dimethyl-ß-carbolinium ions as possible endogenously activated toxins in Parkinson's disease. Abstract for the New York Academy of Sciences Conference, "Neurotoxins and Neurodegenerative Disorders", Philadelphia, May, 1991.

Cobuzzi R.J., Jr., Neafsey E.J. and Collins M.A. Methylated beta-carbolines and MPP^+: comparable toxicity in PC12 cells. (1990) *FASEB J.*, **4**, A763.

Cobuzzi R.J., Jr., Neafsey E.J. and Collins M.A. Indole methylation of 2-methyl-beta-carbolines dramatically increases their toxic effects in PC12 cells. (1990) *Soc. Neurosci. Abstr.*, **16**, 1342.

Matsubara K., Cobuzzi R.J., Jr., Neafsey E.J. and Collins M.A. Evidence for indole-N- and pyridyl-N-methylation of beta-carbolines in mammalian brain: potential generation of MPP^+-like toxins. (1990) Abstract, Annual Meeting of the Chicago Chapter for Neuroscience.

PUBLICATIONS

Cobuzzi R.J., Jr., Neafsey E.J. and Collins M.A. The cytotoxic effects of methylated beta-carbolinium analogues of 1-methyl-4-phenylpyridinium ion (MPP^+) on PC12 cells. Manuscript in preparation.

Collins M.A., Neafsey E.J., Matsubara K., Cobuzzi R., Jr., Albores R., Jr., Fields J. and Rollema H. Indole-N-methylation of ß-carbolines: The brain's bioactivation route to toxins in Parkinson's disease? (1992) *Annals N.Y. Acad. Sci.*, in press.

Collins M.A., Neafsey E.J., Matsubara K., Cobuzzi R.J., Jr. and Rollema H. Indole-N-methylated-ß-carbolinium ions as potential brain-bioactivated neurotoxins. (1992) *Brain Res.* **570**, 154-160.

APPROVAL SHEET

The dissertation submitted by Robert J. Cobuzzi Jr. has been read and approved by the following committee:

Dr. Michael A. Collins, Ph.D., Director
Professor, Molecular and Cellular Biochemistry
Stritch School of Medicine, Loyola University Chicago

Dr. George Battaglia, Ph.D.
Assistant Professor, Pharmacology
Stritch School of Medicine, Loyola University Chicago

Dr. Leonard C. Erickson, Ph.D.
Professor, Medicine and Pharmacology
Stritch School of Medicine, Loyola University Chicago

Dr. Mary Druse Manteuffel, Ph.D.
Professor, Molecular and Cellular Biochemistry
Stritch School of Medicine, Loyola University Chicago

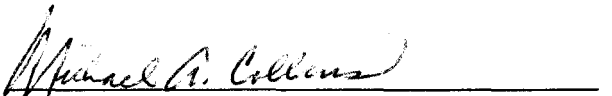
Dr. Edward J. Neafsey, Ph.D.
Associate Professor, Cell Biology, Neurobiology and Anatomy
Stritch School of Medicine, Loyola University Chicago

Dr. William H. Simmons, Ph.D.
Associate Professor, Molecular and Cellular Biochemistry
Stritch School of Medicine, Loyola University Chicago

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

9/21/92
Date


Director's Signature