NEUROCHEMICAL STUDIES OF THE PATHOGENESIS OF FOUR CENTRAL NERVOUS SYSTEM DISORDERS: PARKINSON'S DISEASE, HUNTINGTON'S CHOREA, DIALYSIS ENCEPHALOPATHY, AND HALLERVORDEN-SPATZ SYNDROME

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ABSTRACT

My thesis is divided into 4 chapters, each dealing with a particular central nervous system disorder. The first chapter is devoted to the understanding of the pathogenesis of Parkinson's disease (PD). Several studies with living or dead patients with PD were performed. Animal experiments relied heavily on the use of N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to create an animal model of PD. Two major conclusions of this chapter are firstly, that patients with PD may be exposed up to the time of death to one or more neurotoxins that may act via reactive free radicals, and secondly, that antioxidant compounds such as α-tocopherol may be useful in slowing the progression of neuronal loss in PD.

The second chapter in this thesis tested the hypothesis that the premature neuronal death that occurs in Huntington's chorea (HC) may be the result of a genetically-determined enzymatic failure in the degradation of a circulating neurotoxin of either endogenous or exogenous origin. Two main types of studies were performed: an *in vivo* experiment in which rats were injected repeatedly with serum or serum ultrafiltrate from HC patients or control subjects, and, an *in vitro* study in which rat striatal explants were exposed in tissue culture to serum or CSF from patients or controls. The results from both types of experiments are suggestive for the presence of a neurotoxin in the serum of patients with HC. This putative neurotoxin may either be a small molecule irreversibly bound to serum proteins, or, a molecule larger than 10000 daltons. The identity of the putative neurotoxin is presently unclear.

In the third chapter of this thesis, we examined for neurochemical abnormalities that might be present in the autopsied brains of patients who died with dialysis encephalopathy (DE). A major finding was a deficiency of GABA contents in several regions of autopsied brains of DE patients. Aluminum levels were abnormally high in the frontal cortical gray matter of DE patients. Animal experiments were unsuccessful in clarifying whether or not aluminum is the causative factor in DE, principally because we failed to produce elevation of aluminum content in the brains of rats injected with aluminum hydroxide. The latter was the case even though we employed hemi-nephrectomy, 5/6 nephrectomy, and/or chronic lithium administration in attempts to decrease the renal excretion of aluminum.

Finally, in the fourth chapter, we searched for neurochemical abnormalities in the autopsied brain of 2 patients who died with a rare disease, Hallervorden-Spatz syndrome (HSS). In one patient, contents of cystine and of glutathione-cysteine mixed disulfide in the globus pallidus were elevated 2 SD above those of controls. On the other hand, activity of cysteine dioxygenase, the enzyme that converts cysteine to cysteine sulfinate, was reduced in the globus pallidus of both patients. We propose the hypothesis that cysteine accumulates locally in the globus pallidus in HSS as a result of decreased activity of cysteine dioxygenase. Accumulated cysteine may serve to chelate iron, accounting for the local increase in iron content in the globus pallidus of HSS. The combination of iron and cysteine may generate free radicals that damage neuronal membranes to cause the typical morphological changes observed in HSS.

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DEDICATION

This thesis is dedicated to my wife, Fiona, and our families.

CHAPTER 1. PARKINSON'S DISEASE

ABSTRACT

Speculations as to the etiology of idiopathic Parkinson's disease (PD) abound. A prevailing hypothesis is that the disorder arises as a result of exposure to one or more endogenous or environmental neurotoxins. One support for this contention is the observation that N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a contaminant formed in the synthesis of a meperidine analog, can damage dopaminergic nigrostriatal neurons in humans, monkeys and mice. MPTP itself is not neurotoxic, but requires conversion by the enzyme monoamine oxidase B (MAO-B) to form one or more toxic metabolites. An understanding of the molecular mechanism(s) of the neurotoxic property of MPTP might well lead to clues as to the pathogenesis of PD.

In C57 black mice, a single subcutaneous injection of 40 mg/kg of MPTP produced a loss of 33% of neuronal cell bodies in the zona compacta of the substantia nigra (SN). This was accompanied by a long-lasting reduction (90%) of contents of dopamine and its metabolites in the striatum. The toxicity of MPTP could be partially prevented by pretreatment of mice with any one of four different antioxidants, α-tocopherol, β-carotene, ascorbate or N-acetylcysteine, suggesting the involvement of free radicals in the molecular mechanism of MPTP toxicity. Twenty-four h after an injection of 40 mg/kg of MPTP, the content in the brain stem of the endogenous free radical scavenger, glutathione, was significantly decreased. Furthermore, this loss of glutathione could be prevented by pretreatment of mice with the antioxidants α-tocopherol or β-carotene. These results suggested that glutathione might be critically important in the detoxification of free radicals that might be formed from MPTP. However, prior treatment of mice with

diethylmaleate, a compound that depleted brain stem total glutathione content by 70%, did not potentiate the striatal dopaminergic toxicity of subsequently administered MPTP. This discrepancy remains unexplained.

Although MPTP can produce a parkinsonian lesion, it is certainly not the neurotoxin responsible for most cases of idiopathic PD. To identify a more commonly occurring agent that might be involved in the pathogenesis of PD, various analogs of MPTP were tested in C57 black mice for possible neurotoxic effects on the dopaminergic nigrostriatal system. However, none of the compounds tried, which included paraquat, decreased striatal contents of dopamine.

Several epidemiological studies have reported that PD occurs less frequently among cigarette smokers than among non-smokers. The appparent protective effect of smoking remains unexplained. If compound(s) responsible for causing PD resemble MPTP in first requiring conversion of an inactive precursor to neurotoxic metabolite(s) by MAO-B, and if cigarette smoking inhibits this enzyme, then the protective mechanism of cigarette smoking might be explained. Using platelets as the source of MAO-B, we found that heavy cigarette smokers have a mean decrease of 25% of MAO-B activity when compared to non-smokers. Although interesting, this decrease might not be functionally large enough to account for the protection that is afforded by cigarette smoking.

Using another approach to discern the mechanism of protection against the development of PD that appears to be conferred by cigarette smoking, we pretreated mice with one of 3 compounds (hydrazine, thiocyanate and nicotine) known to accumulate in the physiological fluids and tissues of cigarette smokers, and then gave them an injection of MPTP. We found that hydrazine pretreatment substantially reduced the toxicity of MPTP, suggesting

that the lower incidence of PD among smokers might be due in part to chronic presence of hydrazine in their bodies.

Measurements of MAO-B activity were made in the autopsied brains (SN and frontal cortex) of patients who died with PD. No significant changes were found when compared to controls. It appears unlikely that an overactive MAO-B activity in brain causes excessive conversion of an inactive precursor to neurotoxic metabolite(s), thereby predisposing certain individuals to the development of PD.

Finally, by the inclusion of more PD patients and control subjects, this chapter updates the report by Perry et al. (1982) that glutathione content was decreased in the SN of patients who die with PD. Of 6 regions measured, only the SN in PD brains showed significant reduction in total glutathione content when compared to controls. We suggest the possibilities that the decreased content of glutathione in the SN of PD brains may be the result of its continued consumption in the detoxification of potentially neurotoxic radicals, and that PD patients might well be exposed to such neurotoxins up to the time of death. The results also suggest that antioxidant compounds such as α-tocopherol or β-carotene might be useful in slowing the progression of neuronal death in PD.

I) INTRODUCTION

Idiopathic Parkinson's disease (PD) was first described in 1817 by James Parkinson. Characteristic symptoms include bradykinesia, rigidity, tremor, and a disturbance of posture. In addition, as many as 30% of patients may have an accompanying dementia (Bianchine 1985). PD affects about 200 of 100,000 people in the general population (Kurtzke 1982); however, the incidence increases with age such that a prevalence rate nearing 1 in 40 has been reported in persons above the age of 65 years (Barbeau 1984). In more than 90% of cases, the disease has its onset after the age of 55 years (Bianchine 1985).

The fundamental pathology of PD appears well-defined: degeneration of the dopaminergic nigrostriatal pathway, which originates from neuromelanin-containing neurons in the pars compacta of the substantia nigra (SN). A consequence of this degeneration is a marked decrease in dopamine content in the terminus of the nigrostriatal pathway, the striatum (comprised of the caudate nucleus and putamen) (Ehringer et al. 1960; Hornykiewicz 1973). Symptoms of PD appear only when approximately 80% of nigrostriatal neurons have been lost. Losses of perikarya are also consistently observed, although to a lesser extent, in other neuromelanin-containing nuclei such as the locus coeruleus and the dorsal motor nucleus of the vagus (Forno 1982).

Many neurochemical changes have been described for the parkinsonian brain. These have been succinctly summarised by Marsden (1982) and Hornykiewicz (1982). The significance of some of the changes is uncertain. Many might not be due to death of specific neurons, but might merely be functional changes to compensate for the loss of dopaminergic influence upon the striatum (Marsden 1982). Perhaps the abnormalities of the nigrostriatal pathway are the only important ones for the clinical expression of the dis-

ease. Evidence supporting this contention is supplied by a recently discovered neurotoxin, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which selectively destroys nigrostriatal neurons with a resultant loss of dopamine and its metabolites in the striatum. In sufficient doses, MPTP can produce nearly all the symptoms of PD in humans and monkeys (Davies et al. 1979; Langston et al. 1983; Burns et al. 1983).

An important application of the rather extensive neurochemical studies on autopsied brains of PD patients is that the demonstration of dopamine deficiency in the striatum has led to the rational treatment of patients with the disease. Dopamine itself does not readily cross the blood-brain barrier, but its immediate precursor L-DOPA (L-3,4-dihydroxyphenylalanine) does. Since about 95% of an oral dose of L-DOPA is metabolized outside the brain (Bianchine 1985), combined administration of L-DOPA with a peripheral DOPA decarboxylase inhibitor such as carbidopa or benserazide is beneficial. This combination is now the treatment of choice for PD (Bianchine 1985). The other drugs used to treat PD act via other dopaminergic mechanisms (these include direct dopaminergic agonists such as bromocriptine) or by inhibiting cholinergic activity. The latter class of drugs is useful because in the normal brain the cholinergic interneurons of the striatum are inhibited by the dopaminergic nigrostriatal pathway (Bartholini et al. 1975). Thus, in PD, the striatal dopamine deficiency results in a relative overactivity of cholinergic neurons.

The benefits resulting from the introduction of therapy with L-DOPA (plus carbidopa or benserazide) are remarkable. 90% of patients respond, often dramatically, with a restoration of mobility. However, this response is not maintained indefinitely. After about 3 years of treatment, former

disabilities begin to reemerge in many patients. After 6 years of treatment, most patients have returned to their pretreatment level of disability (Shaw et al. 1980; Curtis et al. 1984). In fact, some studies show that the condition of the majority of patients after several years of treatment is worse than before L-DOPA treatment. Clearly, the loss of nigrostriatal neurons continues unabated even with L-DOPA therapy (Ludin et al. 1976; Marsden et al. 1977). Thus, the clinical effects of the drugs presently used in the treatment of PD are symptomatic in nature and do not cure the underlying disorder.

In recent years, much research has centered on the etiology of idiopathic PD. The expectation is that if the cause of the nigrostriatal neuronal loss can be found, prevention of the disease may be possible by devising appropriate pharmacological strategies. In afflicted patients, the progression of symptoms might be halted or slowed if the molecular basis of nigral cell death could be discerned.

Hypotheses as to the etiology of idiopathic PD abound: heredity, viral infection, accelerated aging of the nervous system, dopamine toxicity, inadequate free radical scavenging mechanisms, and environmental neurotoxins. These hypotheses have relevance for this thesis and merit discussion.

The possible contribution of heredity to the pathogenesis of PD has been debated for many years. An early study by Gowers in 1903 (cited in Duvoisin 1984) noted that 15% of 123 patients reported having a positive family history for the disease. Kurland (1958) reported a familial frequency of 16%. Kondo (1972) concluded that PD follows the multifactorial-threshold model of susceptibilty, with the genetic component being greater than that of the environment. More recently, 2 familial subgroups of PD have been reported: an akineto-rigid subtype transmitted as an autosomal recessive trait, and a

subtype with prominent tremor, dominant inheritance, and a high prevalence of family members with essential tremor (Barbeau et al. 1982). However, other authors have not found such familial subgroups when other disorders that present with rather similar signs and symptoms to PD (for example, 'multisystem atrophies' and benign essential tremor) were excluded from the study (Duvoisin 1982). Perhaps the strongest argument against a significant genetic contribution comes from a recent study of twins. Among 43 monozygotic and 19 dizygotic twin pairs in which the index case had definite PD, only 1 monozygotic pair was concordant for the disease (Ward et al. 1983). Genetic inheritance is thus unlikely to be an important factor in the development of most cases of PD.

Between the years 1916 and 1926, there was an epidemic of encephalitis lethargica. Many affected individuals later developed a condition called postencephalitic parkinsonism (Zeigler 1928; Harris et al. 1937). These patients displayed clinical features resembling those of idiopathic PD. Neuropathological examination of 12 patients (8 of them had a positive history of encephalitis between the years 1920 and 1926) showed severe neuronal loss in the pars compacta of the SN, and neurochemical results indicated over 90% loss of striatal dopamine content (Bernheimer et al. 1973). In fact, the degree of neuronal loss and the dopamine depletion was even more severe than that of the idiopathic disorder (Bernheimer et al. 1973). Although some differences exist between postencephalitic parkinsonism and idiopathic PD (for example, instead of Lewy body inclusions which are found in about 95% of autopsied idiopathic PD brains, postencephalitic parkinsonism showed neurofibrillary tangles) (Bernheimer et al. 1973; Forno 1982), this suggests that some forms of the idiopathic disorder might be caused by a virus. Marttila et al. (1977, 1978) supported this contention by report-

ing that viral antibodies have been found in the sera of patients with idiopathic PD who were not exposed to encephalitis lethargica in the decade between 1916 and 1926. However, all individuals should have various types of viral antibodies in their sera, regardless of whether or not they have PD. Also, the brains of PD patients do not show the histological findings characteristic of infection by either a conventional or a slow virus, and attempted viral isolations from them have been unsuccessful (Calne et al. 1983). It would appear that most cases of idiopathic PD cannot be attributed to a viral infection.

Another hypothesis is that PD is due to accelerated aging of the nervous system. In normal individuals, the number of pigmented neurons in the pars compacta of the SN ordinarily declines with age. In the case of patients with PD, this reduction exceeds that of age-matched controls (McGeer et al. 1977). Similarly, the content of dopamine in the striatum falls with age, and again this decline is much sharper in PD (Hornykiewicz 1966). Studies of neural function reveal the same relationship: reaction time and speed of movement normally become slower with advancing age, but both trends are more pronounced in PD (Evarts et al. 1981). Thus, PD appears to be an exaggerated form of aging. Calne et al. (1983) have put forth 2 arguments against this. Firstly, they reasoned, identical twins should age at the same rate, and thus should show a high concordance for the disease. However, this was not the case (Ward et al. 1983). Secondly, the failure of L-DOPA to correct the declining motor function of normal aging as it does for patients with PD, was taken by Calne et al. (1983) as a pharmacological separation of aging and PD.

The possible toxicity of dopamine, the neurotransmitter of the nigrostriatal tract, is the basis of yet another hypothesis. In vivo and in

vitro, dopamine is cytotoxic to human and murine cancer cells (Wick 1978), and can induce DNA strand breakage in human fibroblasts (Moldeus *et al.* 1983). The mechanism of toxicity has been attributed to reactive free radicals produced during the oxidative metabolism (autoxidation, or via the enzyme monoamine oxidase) of dopamine (Tse *et al.* 1976; Graham *et al.* 1978; Sinet *et al.* 1980; Maker *et al.* 1981; Graham 1984), to species such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), hydroxyl radical ($HO^{\cdot}$), and dopamine semiquinones. Possessing an unpaired electron, which confers on them great reactivity (McCord *et al.* 1978; Halliwell *et al.* 1985), these radicals can disrupt a variety of biomolecules, especially the polyunsaturated phospholipids of cell membranes, by the process of lipid peroxidation (Tappel 1973; Deneke *et al.* 1980; Slater 1982). The result can be loss of membrane integrity and subsequent cell death. Indeed, the neuromelanin pigments in the SN are thought to be a waste product derived from the oxidation of dopamine (Graham 1979).

The potential toxicity of dopamine has led several groups to suggest that PD is a disorder of transport, compartmentalisation, or metabolism of dopamine, resulting in increased shunting of dopamine into the oxidative pathway and thus excessive formation of reactive free radicals (Cohen *et al.* 1976; Graham 1979; Cohen 1982). Support for this hypothesis comes from evidence that the neurotoxin 6-hydroxydopamine, which destroys nigrostriatal neurons when injected into the SN of animals (Thoenen *et al.* 1973), may act via the formation of free radicals, including 6-hydroxydopamine semiquinone, which chemically resembles dopamine semiquinones (Cohen *et al.* 1974; Heikkila *et al.* 1973; Sachs *et al.* 1975). Another line of support comes from studies of manganese toxicity which, in man, can produce a permanent neurological disorder with clinical symptoms and neuropathological and

neurochemical characteristics similar to idiopathic PD (Mena et al. 1967; Bernheimer et al. 1973). Donaldson et al. (1980) have reported that the autoxidation of dopamine was enhanced considerably more by Mn^{2+} ions than by other biologically-important divalent cations such as Cu^{2+}, Zn^{2+}, Ni^{2+}, Ca^{2+}, and Mg^{2+}. This manganese-enhanced autoxidation of dopamine was associated with increased generation of the free radicals $O_2^{\cdot-}$ and HO· (Donaldson et al. 1980).

If dopamine, an endogenous neurotransmitter, is a potential source of reactive free radicals, then it is likely that the body would be equipped with defence mechanisms that can detoxify or scavenge these species. Such natural defence mechanisms exist, and include the enzymes superoxide dismutase (McCord et al. 1969) and catalase (Brannan et al. 1981) which inactivate $O_2^{\cdot-}$ and H_2O_2 respectively. Another enzyme, glutathione peroxidase, detoxifies H_2O_2 and other peroxides by reaction with reduced glutathione (GSH), which becomes oxidised to its disulfide GSSG, during the process (Deneke et al. 1980; Sinet et al. 1980). In this reaction, the levels of GSH are believed to be rate-limiting. GSH is also an important component of the glutathione transferase system which conjugates GSH with a wide variety of electrophiles, including dopamine semiquinone (Habig et al. 1974; Jakoby et al. 1980). In addition to these enzymatic mechanisms, antioxidants such as ascorbate and α-tocopherol probably also may act as radical quenchers in vivo (Tappel 1965; Hafeman et al. 1977; Seregi et al. 1978).

In accordance with the above, it is significant that Perry et al. (1982) have shown that in the normal autopsied human brain, the content of GSH in the SN is significantly lower than in other brain regions analysed, and that in patients with PD, the GSH content of the SN is significantly decreased

from that of normal controls. This observation prompted Perry et al. (1982) to put forth the hypothesis that the loss of nigrostriatal neurons in PD results from a regional GSH deficiency rendering this region vulnerable to oxidative injury. As we shall see later, this observation was the initial point of much of the work reported in this thesis.

Perhaps the most popular current hypothesis as to the etiology of PD is that the disease occurs as a result of exposure to environmental neurotoxins. Earlier, support for this hypothesis came from the ability of chronic manganese intoxication to induce parkinsonian symptoms in man (Mena et al. 1967). More recently, MPTP, a contaminant formed in the synthesis of a meperidine analog, has been found to damage dopaminergic nigrostriatal neurons in humans (Davies et al. 1979; Langston et al. 1983; Wright et al. 1984), several species of monkeys (Burns et al. 1983; Langston et al. 1984a; Jenner et al. 1984), and mice (Heikkila et al. 1984a). These reports suggest the possibility that the environment may contain a selective neurotoxic substance(s) such that exposure can lead to irreversible neuronal damage to the nigrostriatal pathway. Indeed, Calne and Langston (1983) have suggested that PD is due to exposure to one or more environmental neurotoxins many years before the onset of symptoms. This then results in a significant loss of neurons in the pars compacta of the SN and parallel decreases in striatal dopamine content, but not to the extent that is necessary for symptoms to begin to be manifested. With normal aging, and the associated loss of further nigrostriatal neurons, the critical threshold would be reached and symptoms would appear. In other words, after an environmental insult to the nervous system, normal aging provides the 'coup de grace'.

In summary, therefore, the etiology of idiopathic PD remains presently a matter of speculation. The observations that MPTP can induce a selective

lesion of the nigrostriatal pathway and produce nearly all of the symptoms of PD have lent strong support to the hypothesis that environmental (or possibly endogenous) neurotoxic factors are important in the pathogenesis of PD. MPTP could well act via free radical mechanisms, and the amount of GSH or other scavenging systems might be critically important in affording resistance or susceptibility. Some of the experiments to be described in this thesis utilise MPTP to create an animal model of PD, and for this reason, an introduction to some of the highlights of MPTP research done by others is in order.

MPTP itself is not neurotoxic, but requires conversion by the enzyme monoamine oxidase B (MAO-B) to one or more toxic metabolites (Markey et al. 1984; Heikkila et al. 1984b; Langston et al. 1984b). Pretreatment of animals with MAO-B inhibitors such as pargyline and deprenyl, but not the MAO-A inhibitor clorgyline, completely prevents the toxicity of MPTP (Markey et al. 1984; Heikkila et al. 1984b, Langston et al. 1984b). The terminal metabolite of MPTP, after oxidation by MAO-B, is the positively charged N-methyl-4-phenylpyridinium ion (MPP^+) (Castagnoli et al. 1985), which has been shown to destroy dopaminergic neurons when injected into the brain of rats (Heikkila et al. 1985) or when added to explants of rat embryonic substantia nigra in tissue culture (Cohen et al. 1985). In the latter experiment, the neurotoxicity of MPP^+ has been reported to be prevented by deprenyl pretreatment (Mytilineou et al. 1985). In contrast to this, Bradbury et al. (1985) found that intracerebroventricular injections of MPP^+ to mice produced a more extensive neurotoxicity after deprenyl administration.

In addition to MAO-B inhibitors, dopamine uptake blockers such as mazindol (Javitch et al. 1985a), amfolenic acid (Sundstrom et al. 1985), cocaine (Mayer et al. 1985) and GBR 13098 (Pileblad et al. 1985) can completely or

partially prevent the toxicity of MPTP, suggesting the possibility of an active uptake system for MPTP into dopaminergic neurons. However, MPTP is found to be a poor substrate for the dopamine uptake system of rat striatal synaptosomal preparations (Javitch et al. 1985b). MPP^+ on the other hand, is taken up at the same rate as dopamine itself. This suggests that MPTP may be converted extraneuronally (possibly in glial cells which have high concentrations of MAO-B) to MPP^+, which then gains access into the dopaminergic neurons where cell destruction occurs.

A recent publication by Hadjiconstantinou et al. (1985) showed MPTP to increase the acetylcholine and decrease the dopamine contents of mouse striatum. Both responses were blocked by pretreatment with the anticholinergics atropine and trihexyphenidyl. Since atropine was not thought to be an inhibitor either of MAO-B or of the dopamine uptake system, these authors suggested that extensive activation of cholinergic receptors might contribute to the degeneration of dopaminergic nigrostriatal neurons after MPTP administration.

To date, therefore, the molecular mechanism of MPTP toxicity is unknown and uncertainties exist as to the precise identity of the neurotoxic metabolite(s) of MPTP.

II) OUTLINE OF EXPERIMENTS AND METHODS

The following experiments, the rationale of which will be discussed in their respective sections of RESULTS AND DISCUSSION, were performed. They dealt with:

i. The search for an animal model of PD.

ii. The neurotoxic effects of MPTP in C57 black mice.

iii. The possible mechanism(s) of MPTP neurotoxicity.

iv. Attempts to change brain total glutathione content and to determine whether the toxicity of MPTP is altered under such conditions.

v. The search for the natural neurotoxin(s) that might be responsible for most cases of idiopathic PD.

vi. The possible cause(s) of the protective effect of smoking against the development of PD.

vii. The likelihood that increased MAO-B activity in tissues might be a factor in the development of PD.

viii. The status of total glutathione content and glutathione transferase activity in the autopsied brains of PD patients.

Since much of this work has already been published or is in press, it would be redundant to repeat the methodologies in detail here. A series of appendices are attached to the end of this thesis to which the interested reader can refer for experimental details.

In general, the following procedures were common to most experiments:

Animals were sacrificed by cervical dislocation. Relevant brain regions were then dissected according to the instructions of Glowinski et al. (1966). Those brain areas to be used for the various neurochemical analyses were weighed, and then kept frozen at -70° C prior to analysis. Brain stems for histological examination were immersed in buffered 10% formalin upon

excision.

Analyses of striatal contents of dopamine and its metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), noradrenaline and its metabolite 3-methoxy-4-hydroxy-phenylethyleneglycol (MHPG), and serotonin and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), were carried out by HPLC with electrochemical detection as described in Appendix C (Perry et al., Life Sci. 36:1233-1238, 1985). In brief, 25 µl of each striatal supernatant was injected into the HPLC system, which included a reverse-phase chromatographic column (ODS Hypersil) and an amperometric detector with a glassy carbon electrode. The potential was set at +0.7 V with respect to an Ag/AgCl reference electrode. The mobile phase was an aqueous solution of 0.1 M NaH_2PO_4 containing 60 mg Na_2EDTA and 100 mg sodium octyl sulfate per liter. This was at pH 3.6 ± 0.01. The flow rate of the mobile phase was 1 ml/min. External standards were injected after every second to fourth sample of striatum to compensate for slight decreases in sensitivity due to electrode and column changes. The brain contents of the compounds were calculated from the amplitude of the peak deflections they produced on chromatograms.

Measurements of tyrosine hydroxylase activity (the rate-limiting enzyme for the synthesis of dopamine) were by a modification of the method of Waymire et al. (1971), in which dithiothreitol replaced the 2-mercaptoethanol. Total glutathione content was determined either by the spectrophotometric technique of Tietze (1969), or on an amino acid analyser using the procedure of Perry et al. (1968, 1981). The latter was used only for specimens of human brain and of rat brain in the buthionine sulfoximine experiment, and for mouse livers in the glutathione monoethyl ester experiment. While the method of Tietze (1969) allowed multiple analyses, the amino acid

analyser technique gave only 1 result per day. The method of Lowry (1961) was used for the determination of protein content, using bovine serum albumin as standard.

For histological analyses, serial cyrostat sections of 20 µm in thickness were cut from the brain stems fixed in formalin. These sections were then stained with Cresyl violet, and the number of perikarya in the zona compacta of the SN was counted at 2 levels: i) at the level of the interpeduncular nucleus, and ii) at the level of emergence of the third cranial nerve. Cell bodies of about 20 µm in length, moderately stained and fusiform in shape, were counted in each of the 2 sections, and the cell count was averaged for each mouse. These analyses were performed blind.

All other methods (for example, assays of MAO-B and glutathione transferase activities) will either be described in RESULTS AND DISCUSSION, or will be referred to in their respective appendices.

Unless otherwise stated, all statistical analyses were performed using the two-tailed Student's t-test ($P = 0.05$).

III) RESULTS AND DISCUSSION

III.i) Search for an animal model of Parkinson's disease

As described earlier, Perry et al. (1982) found that the mean content of reduced glutathione (GSH) was significantly lower in the SN of autopsied PD brains when compared to controls. Since GSH is a key scavenger of reactive oxidants, Perry et al. (1982) advanced the hypothesis that depletion of GSH in the SN makes this region susceptible to oxidative injury. The result could be marked neuronal loss and consequent symptoms of PD.

If nigral GSH deficiency is a contributing factor in the development of PD, then treatment with antioxidant drugs such as α-tocopherol or ascorbic acid might have value in the prevention of nigrostriatal neuronal loss and subsequent onset of parkinsonian symptoms. Compounds that can elevate brain GSH content might also be useful. In patients already afflicted with the disease, antioxidant drugs might stop further loss of neurons (assuming that the exposure to neurotoxic factor(s) continues even after the symptoms appear), and therefore might slow the progression of the disease. These potential treatment strategies are of importance, since the drugs presently used to treat PD eventually fail.

To test the possibility that the loss of nigral GSH contributes to the development of PD, an animal model that resembles the disease in neuropathological, neurochemical, pharmacological, and perhaps behavioral characteristics, is first required. By manipulating nigral GSH content (depleting or replenishing) in such a model, the etiological importance of this endogenous compound in PD might be determined.

Before the toxicity of MPTP was discovered, the neurotoxin 6-hydroxydopamine was used quite widely to produce animal models of PD (Thoenen et al. 1973; Schultz 1982). However, we decided not to use this neurotoxin

because of the following problems with its use. Injected peripherally, it does not penetrate the blood-brain barrier (Thoenen et al. 1973; Schultz 1982). Injections must be made directly into the brain. Intracerebroventricular injections, which are relatively simple to perform, produce degenerative effects on all catecholamine-containing neurons in addition to the pathway of interest, the dopaminergic nigrostriatal tract. Indeed, dopaminergic neurons appear less susceptible to damage than those containing noradrenaline (Uretsky et al. 1970; Sachs et al. 1972). To achieve greater selectivity with 6-hydroxydopamine, local intracerebral injections (that is, into specific brain nuclei) are necessary. However, these are difficult to perform reliably and accurately in small animals.

Since dopamine may be neurotoxic via the metabolic generation of reactive free radicals (Tse et al. 1976; Wick 1978; Graham et al. 1978; Sinet et al. 1980; Maker et al. 1981; Moldeus et al. 1983; Graham 1984), it seemed possible that if rats were chronically fed large amounts of L-DOPA and carbidopa to produce a sustained elevation of dopamine content in the brain, dopaminergic nigrostriatal neurons might be destroyed. In addition, DL-buthionine-S,R,-sulfoximine (BSO), a compound which blocks GSH synthesis by specifically inhibiting γ-glutamyl-cysteine synthetase, the rate-limiting enzyme for GSH synthesis (Griffith et al. 1979b), was used to test whether or not the prior depletion of GSH could potentiate the toxicity of L-DOPA.

Table 1 shows that treatment for 120 days with a maximally-tolerated oral dose of L-DOPA (approximately 0.94 g/kg/day) and carbidopa, with or without BSO pretreatment, failed to produce neurochemical evidence of damage to the nigrostriatal dopaminergic tract in rats. Normal results were also obtained from behavioral and histological examinations. The methods used are described in detail in Appendix A (Perry et al., J. Neurochem. 43:990-993, 1984).

Table 1. Tyrosine hydroxylase (TH) activity and contents of dopamine and its metabolites in rat striatum after chronic L-DOPA and carbidopa treatment

Drug treatment of animals	TH activity (nmol/h/mg protein)	Dopamine (µg/g wet weight)	DOPAC (µg/g wet weight)	HVA (µg/g wet weight)
L-DOPA, carbidopa, BSO	2.77 ± 0.11 (15)	15.0 ± 0.5 (15)		
L-DOPA, carbidopa	2.63 ± 0.14 (19)	16.4 ± 0.3 (19)	1.96 ± 0.26 (8)	0.82 ± 0.05 (8)
BSO	2.85 ± 0.08 (10)	16.7 ± 0.6 (10)		
Untreated controls	2.70 ± 0.10 (15)	16.5 ± 0.6 (15)	1.79 ± 0.11 (8)	0.85 ± 0.05 (8)

Values are mean ± SEM, with number of animals in brackets. None of the means differ significantly.

Table 2 shows that although administration of oral 20 mM BSO to rats for up to 14 weeks did cause a substantial decrease in total glutathione content in the liver, the brain levels were not different from those of controls. Thus, in the BSO-pretreated rats, the failure of L-DOPA and carbidopa administration to produce a dopaminergic nigrostriatal lesion may reflect the inability of BSO to decrease brain GSH content. The results shown in Table 2 are pooled data from animals sacrificed at 2, 4, 7, 10 or 14 weeks after BSO administration had started. They were pooled because the values obtained at each point of sacrifice did not differ significantly from one another.

Table 2. Effects of BSO on total glutathione contents of brain and liver of rats

	Control	BSO
Whole brain	2.2 ± 0.1 (10)	2.1 ± 0.1 (10)
Liver	2.8 ± 0.7 (5)	0.3 ± 0.1† (6)

Values are μmol/g wet weight, mean ± SEM, with the number of animals analysed shown in parentheses. Values that differ significantly from controls: †$P < 0.01$.

Why was BSO ineffective as a brain glutathione-depleting agent? Possibly, this compound was unable to penetrate the blood-brain barrier. However, a congener of BSO that lacks a methylene group, prothionine sulfoximine, was reported to regularly elicit convulsions in mice (Griffith et al. 1979a), implying that the latter compound was able to gain access into the brain. BSO, with greater lipid solubility conferred by the additional methylene group, should have entered the brain more readily. Unfortunately, a technique to determine BSO concentrations in the rat brain after administration of this drug was not available so that the issue cannot be resolved conclusively at this time.

In another attempt to produce an animal model of PD, rats were injected repeatedly with high doses of methylcyclopentadienyl manganese tricarbonyl (MMT), a compound which has been reported to decrease the striatal content of dopamine in mice after only 11 injections over a 21 day period (Gianutsos et al. 1982). This experiment was of additional interest because in man, chronic manganese administration produces an initial phase of pyschiatric disturbance reminiscent of schizophrenia; the pyschosis then subsides, to be followed by a permanent neurological disorder with symptoms and neuro-

pathological and neurochemical findings similar to those seen in idiopathic PD (Bernheimer et al. 1973; Mena et al. 1967).

For the experimental design, refer to Appendix B (Yong et al., Neurotoxicology 7:19-24, 1986). Table 3 shows that immediately after the period of organic manganese administration (24 injections over 48 days), cerebellar manganese content was elevated more than 2-fold. However, 1 month after the last of 75 injections given over a 5-month period, the brain of manganese-treated animals had a mean manganese content only slightly higher than those of controls. Presumably, this was due to rapid clearing of the manganese compound from the brain.

Table 3. Manganese contents of rat brain after MMT treatment

Animals	Cumulative MMT dose (mg/kg)	Manganese content (µg/g dry wt)	% increase over corresponding controls
Group 1: Controls, 24 injections, killed 24 h after last injection (8)		1.71 ± 0.03	
Group 3: MMT, 24 injections, killed 24 h after last injection (13)	1055	4.59 ± 0.18*	168
Group 2: Controls, 75 injections, killed 1 mo after last injection (10)		2.44 ± 0.15	
Group 4: MMT, 75 injections, killed 1 mo after last injection (11)	3605	2.88 ± 0.11§	18

Values are mean ± SEM, with number of animals shown in parentheses. Brain region used for analysis of Groups 1 and 3 rats was the cerebellum, while that for Groups 2 and 4 animals was occipital cortex. * $P < 0.001$; § $P < 0.05$.

The neurochemical results, tabulated in Table 4, indicate that there was no reduction in striatal dopamine content or in tyrosine hydroxylase activity, either immediately or 1 month after the MMT injections had ended. The latter group of animals was used in order to assess whether any changes which might have occurred during the period of exposure were reversible upon drug withdrawal. Perikarya in the SN, observed histologically, were also normal. Thus, chronic administration of large amounts of organic manganese was not toxic to dopaminergic nigrostriatal neurons of rats.

Table 4. Tyrosine hydroxylase activity and contents of dopamine and its metabolites in rat striatum after MMT treatment

Animals	Tyrosine hydroxylase	Dopamine	DOPAC	HVA
	(nmol/h/mg protein)	(µg/g wet weight)		
Group 1 (8)	2.5 ± 0.1	11.2 ± 0.4	1.4 ± 0.1	0.9 ± 0.0
Group 3 (13)	2.5 ± 0.1	11.4 ± 0.4	1.2 ± 0.1§	0.8 ± 0.0
Group 2 (10)	2.7 ± 0.2	8.5 ± 0.4	3.3 ± 0.1	1.2 ± 0.0
Group 4 (11)	2.8 ± 0.2	8.2 ± 0.3	3.2 ± 0.1	1.2 ± 0.0

Values are mean ± SEM, with number of animals in brackets. SEM less than 0.05 is shown as 0.0. The experimental characteristics of the various groups are the same as those shown on Table 3. Values that differ significantly from their respective controls: § $P < 0.05$.

Possible reasons for the failure to produce a model of PD in the rat by chronic administration of MMT have been discussed in Appendix B. Here, my colleagues and I suggest that the elevated manganese content in the brains of treated rats was of the organic rather than the inorganic form, although our technique of measurement could not differentiate between them. The neurotoxicity of manganese has been suggested to reside in the ability of inorganic Mn^{2+} to oxidise to Mn^{3+} (Graham 1984; Donaldson et al. 1982).

The latter might then oxidise dopamine by one-electron transfer reactions, generating dopamine semiquinones and other free radicals that have the potential to damage neurons. Organic manganese might not have the capability to generate free radicals as has been suggested for inorganic manganese.

As an extension of the above experiments, it would be interesting to test the possibility that simultaneous administration of L-DOPA/carbidopa with an inorganic manganese compound might produce degeneration of the nigrostriatal pathway in rats.

The reports that MPTP was toxic to dopaminergic nigrostriatal neurons of humans and lower primates prompted the next series of experiments. Early in the MPTP saga, before it was demonstrated that MPTP was toxic to nigrostriatal neurons in mice (Heikkila et al. 1984a), efforts to produce a model of PD in small laboratory rodents had been generally unsuccessful (Kolata 1983; Chiueh et al. 1983; Sahgal et al. 1984). A possible exception was the reported 50% reduction of striatal dopamine content in guinea pigs chronically treated with MPTP (Chiueh et al. 1983). Since a smaller, non-primate animal model would offer many advantages, particularly in terms of cost and ease of handling, I attempted to produce a model of PD in guinea pigs made scorbutic (like primates, guinea pigs cannot synthesize their own ascorbate) and then treated with MPTP. The hope was that the lack of ascorbic acid, an antioxidant, might render this species more susceptible to the neurotoxic effects of MPTP. The experimental protocols are described in detail in Appendix C (Perry et al. Life Sci. 36:1233-1238, 1985).

Animals lost about 10% of their initial body weight while on the ascorbate-deficient diet. The rate of weight loss was similar for the MPTP-treated and control scorbutic animals. Their weights began to stabilize or increase only after a rescue ascorbate injection (20 mg/animal) was given.

Clearly, the animals were rendered severely scorbutic during their period on the ascorbate-free diet.

Both normal and scorbutic animals injected with 8 mg/kg MPTP failed to show any neurological signs other than sedation and hypotonia, which lasted for about 4 h after each injection.

The neurochemical results in Tables 5 and 6 indicate that guinea pigs, scorbutic or not, are not highly susceptible to the neurotoxic effects of MPTP.

Table 5. Striatal parameters of non-scorbutic guinea pigs injected with MPTP (8 mg/kg) for 10 days

Parameter	Control	MPTP (killed 2 h after last injection)	MPTP (killed 30 d after last injection)
	(8)	(4)	(9)
Tyrosine hydroxylase	7.5 ± 0.3	6.7 ± 0.3	6.6 ± 0.3
Dopamine	4.8 ± 0.6	6.1 ± 0.6	5.3 ± 0.6
DOPAC	3.4 ± 0.3	1.5 ± 0.3†	2.9 ± 0.3
HVA	3.0 ± 0.2	2.4 ± 0.5	3.0 ± 0.3
Noradrenaline	0.08 ± 0.02	0.07 ± 0.00	0.07 ± 0.02
5-HIAA	0.21 ± 0.02	0.36 ± 0.10	0.27 ± 0.04

Tyrosine hydroxylase activity is expressed in nmol/h/mg protein, while contents of neurotransmitters and metabolites are in µg/g wet weight. All values are mean ± SEM, with number of animals indicated in parentheses. SEM less than 0.005 are shown as 0.0. Significantly different from controls: † $P < 0.01$.

Table 6. Effects of 15 daily injections of MPTP (8 mg/kg) on neurochemical parameters of scorbutic guinea pigs

Parameter	Animals killed 24 h after MPTP injections		Animals killed 60 d after MPTP injections	
	Control (8)	MPTP (8)	Control (15)	MPTP (15)
CAT			11.2 ± 0.5	11.0 ± 0.5
Tyrosine hydroxylase	6.3 ± 0.2	4.9 ± 0.2*	5.5 ± 0.1	4.3 ± 0.1§
Dopamine	9.0 ± 0.4	7.6 ± 0.6§	6.1 ± 0.7	5.0 ± 0.3
DOPAC	1.4 ± 0.1	1.3 ± 0.1	3.4 ± 0.1	2.6 ± 0.1§
HVA	1.9 ± 0.2	2.1 ± 0.3	4.2 ± 0.3	2.9 ± 0.2§
Noradrenaline	0.28 ± 0.03	0.19 ± 0.02§	0.14 ± 0.03	0.17 ± 0.02
5-HIAA	0.11 ± 0.02	0.13 ± 0.02	0.17 ± 0.02	0.13 ± 0.02

Choline acetyltransferase (CAT) activity (nmol/10 min/mg protein) was measured in the frontal cortex while all other analyses were of the striatum. Tyrosine hydroxylase activity is in nmol/h/mg protein. Neurotransmitters and metabolites are expressed in µg/g wet weight. Values are mean ± SEM with the number of animals shown in parentheses. Values that differ from controls killed at the same time: *$P < 0.001$; §$P < 0.05$.

The apparent resistance of guinea pigs (relative to primates, for example) to the neurotoxic effects of MPTP remains presently unexplained. It could be that the metabolic pathway(s) or the localisation and persistence of MPTP are different in guinea pigs. In any event, the guinea pig does not appear to be a suitable small animal for MPTP-induced parkinsonism.

III.ii) Studies of the neurotoxic effects of MPTP in C57 black mice

Following the report by Heikkila et al. (1984a) that C57 black mice were susceptible to the neurotoxic effects of MPTP, various experiments using this strain of mice were pursued. These began with an examination of the effects of MPTP on the dopaminergic nigrostriatal neurons of these mice.

Experiments were designed to assess: 1) the time course of striatal dopamine depletion after a single injection of MPTP, 2) the extent of neuronal loss in the zona compacta of the SN, 3) the degree of striatal dopamine depletion caused by different dosage regimens of MPTP, and 4) the selectivity of MPTP for the depletion of striatal dopamine content as opposed to other neurotransmitter amines. All administrations of MPTP were by the subcutaneous (sc) route.

The experimental protocols used for the determination of 1, 2, and 3 above are described in Appendix D (Perry et al. Neurosci. Lett. 12:321-326, 1985). To assess the selectivity of action of MPTP, C57 black mice were given a single sc injection of 40 mg/kg, and then were sacrificed at 30 min, 2.5 h and 1 month after the injection. In addition to contents of dopamine and its 2 metabolites DOPAC and HVA, striatal contents of serotonin and its metabolite 5-HIAA, and of noradrenaline and its metabolite MHPG, were measured.

Behavioral observations indicated signs of motor impairment as early as 20 minutes after an injection of 40 mg/kg MPTP. These consisted of sedation (though animals moved when disturbed), slight tremors, and difficulty in retracting extended hind limbs. Maximal impairment occurred between 2 and 2.5 h after the injection. At this time, tremors could be readily observed and mice had distinct difficulty in initiating movements even when disturbed. Retraction of extended hind limbs was absent. 24 h after the injection, most mice were still sedated, displaying marked hypokinesia and often tremors. Body posture was hunched, resembling the kyphotic posture that is often seen in PD patients. Thereafter, animals gradually recovered, and by 3 or 4 days after receiving MPTP, they appeared normal. Injections of 20 or 30 mg/kg MPTP caused very little behavioral change. Sedation, when observed,

lasted for only 0.5 to 1 h. 60 mg/kg MPTP, however, was rapidly lethal to 2 of 2 animals so treated.

Our results suggest that a single sc injection of 40 mg/kg of MPTP is the highest dose compatible with survival of most animals. About 10% of animals generally die after such a dose.

The results given in Table 7 show the time course of neurochemical changes in the striatum of mice after a single injection of 40 mg/kg MPTP. At 30 and 60 minutes, dopamine content was modestly increased, and DOPAC content comparably reduced. By 2.5 h after the MPTP injection, striatal dopamine content was markedly reduced, and by 24 h, it was almost as severely reduced as at one month. Thus, 40 mg/kg MPTP in C57 black mice produced long-lasting decrements of dopamine and its metabolites in the striatum. However, in mice allowed to live for 4.5 months after the MPTP injection, there was clear evidence of partial recovery of dopaminergic nigrostriatal neurons. The turnover of dopamine released in the striatum, as judged by the (DOPAC + HVA)/dopamine molar ratio, was increased at 24 h and at one month, but had returned towards normal at 4.5 months.

Histological examinations of 6 control mice showed the pars compacta of the SN to contain 206 ± 5 (mean ± SEM) perikarya per unit area. Those of 7 mice killed 1 month after a single injection of 40 mg/kg MPTP contained only 139 ± 11 perikarya ($P<0.001$).

Why is there such disparity between the magnitude of dopamine reduction (90%) and the loss of neurons (33%) at 1 month after an injection of 40 mg/kg MPTP? The probable reason is that the MPTP injection impaired the function of many dopaminergic neurons, but did not kill them. For instance, the rate-limiting enzyme for the synthesis of dopamine, tyrosine hydroxylase, might have been chronically inhibited by MPTP. This appears likely

since Hirata et al. (1985) have shown that MPTP inhibits tyrosine hydroxylase activity in tissue slices of the rat striatum. Given time, however, the inhibited neurons would recover, as borne out by the dopamine content at

Table 7. Time course of neurochemical changes in striatum of C57 black mice after an injection of 40 mg/kg MPTP

	Controls (26)	30 min (9)	60 min (9)	2.5 h (5)
Dopamine	11.8 ± 0.4	13.8 ± 0.6[§]	14.1 ± 1.1[§]	6.4 ± 0.5[*]
DOPAC	0.96 ± 0.04	0.42 ± 0.04[*]	0.55 ± 0.09[*]	0.46 ± 0.05[*]
HVA	1.22 ± 0.07	1.32 ± 0.06	1.22 ± 0.06	2.11 ± 0.07[*]
(DOPAC + HVA)/ Dopamine	0.16 ± 0.01	0.11 ± 0.01[*]	0.11 ± 0.01[*]	0.35 ± 0.04[*]

	24 h (5)	1 month (14)	4.5 months (8)
Dopamine	1.5 ± 0.4[*]	1.1 ± 0.1[*]	4.4 ± 1.1[*]
DOPAC	0.20 ± 0.03[*]	0.22 ± 0.04[*]	0.34 ± 0.06[*]
HVA	0.63 ± 0.10[*]	0.39 ± 0.02[*]	0.74 ± 0.10[*]
(DOPAC + HVA)/ Dopamine	0.54 ± 0.08[*]	0.52 ± 0.06[*]	0.24 ± 0.02[*]

Values shown (mean ± SEM) are in µg/g wet weight, except that (DOPAC + HVA)/Dopamine ratios are molar. Mice were sacrificed at the time intervals specified after a single injection of 40 mg/kg of MPTP. The number of animals are shown in parentheses. Values that differ significantly from controls: [*]$P < 0.001$; [§]$P < 0.05$.

4.5 months after the single 40 mg/kg injection (Table 7), which was higher than that at 1 month.

Table 8 compares the striatal contents of dopamine and its metabolites in animals given either a single 40 mg/kg injection, or 5 injections of 20 mg/kg. Both groups of animals were sacrificed a month after the single or last injection. The results show that a single insult resulting from a

large dose of MPTP is more damaging to dopaminergic nigrostriatal neurons than multiple injections of lower doses. This is supported by the data in Table 9, which show that a cumulative dose of 390 mg/kg (13 x 30 mg/kg) was still significantly less effective, at 24 h after the last injection, in lowering striatal dopamine contents than that of a single injection of 40 mg/kg. These results suggest a threshold effect such that injury is achieved only when a critical concentration of the neurotoxic species is reached. At sub-threshold concentrations of MPTP (and thus of the neurotoxic species that are formed from MPTP), detoxifying agents may be present in amounts sufficient to reduce the toxicity.

Table 8. Comparison of different dosage schedules of MPTP on the striatal contents of dopamine and its metabolites, in mice sacrificed 1 month after the last injection

Striatal parameters	1 x 40 mg/kg (10)	5 x 20 mg/kg (10)	Level of significance
Dopamine	1.1 ± 0.1	4.6 ± 0.4	$P < 0.001$
DOPAC	0.21 ± 0.05	0.39 ± 0.03	$P < 0.01$
HVA	0.39 ± 0.06	0.83 ± 0.07	$P < 0.001$

Values are expressed in µg/g wet weight, mean ± SEM, with number of mice analysed shown in parentheses. Student's two-tailed t-test was used to determine level of significance. Control values, in µg/g wet weight (mean ± SEM), are: dopamine, 11.8 ± 0.4; DOPAC, 0.96 ± 0.04; HVA, 1.22 ± 0.07.

Table 9. Comparison of different dosage schedules of MPTP on the striatal contents of dopamine and its metabolites, in mice sacrificed 24 h after the last injection

Striatal parameters	1 x 40 mg/kg (5)	13 x 30 mg/kg (10)	Level of significance
Dopamine	1.5 ± 0.4	2.4 ± 0.2	$P < 0.05$
DOPAC	0.20 ± 0.03	0.23 ± 0.02	ns
HVA	0.63 ± 0.10	0.48 ± 0.03	ns

Refer to Table 8 for legend. Not significant: ns.

Table 10 shows the selectivity of action of MPTP on striatal content of various neurotransmitter amines. At 30 minutes after 40 mg/kg MPTP, serotonin and noradrenaline contents, as with dopamine, were increased relative to those of controls. These changes might have been due to MPTP's having acted as a weak MAO inhibitor (Fritz et al. 1985; Singer et al. 1985). At the 1 h or the 1 month mark, only dopaminergic parameters were statistically different from controls. Thus, acutely, MPTP non-selectively affects various amines. At 1 month, only the dopamine system remains selectively affected.

Table 10. Striatal neurochemical parameters of mice sacrificed at various times after a single injection of 40 mg/kg MPTP

	Controls	MPTP		
		30 min	2.5 h	1 mo
Dopamine	11.8 ± 0.4 (26)	13.8 ± 0.6 § (9)	6.4 ± 0.5* (5)	1.1 ± 0.1* (14)
DOPAC	0.96 ± 0.04 (26)	0.42 ± 0.04* (9)	0.46 ± 0.05* (5)	0.22 ± 0.04* (14)
HVA	1.22 ± 0.07 (26)	1.32 ± 0.06 (9)	2.11 ± 0.07* (5)	0.39 ± 0.02* (14)
Serotonin	0.37 ± 0.03 (21)	0.55 ± 0.09 § (9)	0.72 ± 0.07* (5)	0.33 ± 0.04 (14)
5-HIAA	0.24 ± 0.04 (26)	0.25 ± 0.02 (9)	0.19 ± 0.03 (5)	0.22 ± 0.03 (13)
Noradrenaline	0.26 ± 0.04 (7)	0.49 ± 0.06 † (4)	0.34 ± 0.06 (5)	0.17 ± 0.03 (8)
MHPG	0.08 ± 0.01 (10)	0.09 ± 0.02 (5)	0.05 ± 0.00 (5)	0.07 ± 0.02 (8)

Values (mean ± SEM) are in µg/g wet weight, with the number of animals analysed shown in parentheses. SEM less than 0.005 are given as 0.00. Values that differ significantly from controls: * $P < 0.001$; † $P < 0.01$; § $P < 0.05$.

In summary, the effects of MPTP in C57 black mice are as follows: a single injection of 40 mg/kg MPTP produces a loss of about 33% of dopaminergic neurons in the zona compacta of the SN. This is accompanied by a long-lasting reduction of striatal contents of dopamine and its metabolites, but not those of serotonin or noradrenaline. Behavioral impairment is readily seen in the first several days, but this does not persist indefinitely. Thus, a single 40 mg/kg dose of MPTP appears to produce in mice the neurochemical and neuropathological characteristics of human PD, although the magnitude of the neuropathological changes is less than that seen in the

human condition.

III.iii) Possible mechanism of MPTP toxicity

A question that has remained unanswered is whether the damage to dopaminergic nigrostriatal neurons caused by MPTP is exerted directly by metabolites of MPTP, such as the N-methyl-4-phenylpyridinium ion (MPP^+), or by reactive free radicals (for example, dopamine semiquinones, whose formation from dopamine might be increased in the presence of MPTP metabolites). This section deals with the possible molecular mechanism of MPTP neurotoxicity.

Experiments were first performed to assess whether any one of 4 different antioxidants could prevent the neurotoxicity of MPTP. Details of this experiment have been described in Appendix E (Perry et al. Neurosci. Lett. 60:109-114, 1985). In brief, C57 black mice were injected with one of 4 different antioxidants (α-tocopherol, β-carotene, ascorbate or N-acetylcysteine) or saline once daily for 5 days, with 40 mg/kg MPTP administered sc on the third day to all groups. Mice were sacrificed 1 month after the injection of MPTP. The striatum was analysed for contents of dopamine and its metabolites. Histological examinations were performed blind in MPTP mice pretreated with α-tocopherol or saline.

Table 11 shows that although striatal dopamine contents were reduced in MPTP-injected mice pretreated with any one of the 4 antioxidants, when compared to control mice not given MPTP, these were still significantly higher than those of mice given MPTP without a simultaneous antioxidant. Thus, the antioxidant-pretreated mice were partially protected from the toxicity of MPTP. Since the antioxidants share a common property of detoxifying free radicals, the involvement of the latter in the molecular mechanism of MPTP toxicity appeared likely.

Histological examinations indicated an even more impressive result. While 7 mice administered 40 mg/kg MPTP had 139 ± 11 perikarya per unit area (mean ± SEM), those (n = 7) injected with both MPTP and α-tocopherol had 215 ± 2 perikarya ($P<0.001$). Control animals given no MPTP showed 206 ± 5 perikarya per unit area. Therefore, whereas there was a 33% loss of dopaminergic neuronal cell bodies 1 month after a single MPTP injection, this neuronal loss was completely prevented by α-tocopherol.

Additional evidence for the involvement of free radicals in MPTP toxicity came from our studies of MPTP-induced changes in total glutathione content of the brain stem. The rationale here was that inasmuch as glutathione is an endogenous free radical scavenger, total glutathione content might be reduced, if MPTP works via free radicals. Appendix F (Yong et al. Neurosci. Lett. 63:56-60, 1986) provides details of this experiment. Briefly, at various time intervals after an injection of 40 mg/kg MPTP, C57 black mice were sacrificed. Total glutathione contents were measured by the method of Tietze (1969) in the brain stem and striatum. Table 12 gives the results obtained. No changes were observed in the total glutathione content of the striatum. In the brain stem, however, a biphasic response was seen. An initial increase at 1 h was followed by a significant reduction in total

Table 11. Contents of dopamine and its metabolites in the striatum of mice treated with MPTP and protective agents

	Controls (26)	MPTP alone (14)	MPTP + α-tocopherol (12)	MPTP + β-carotene (4)	MPTP + ascorbate (7)	MPTP + N-acetyl-cysteine (7)
Dopamine	11.8 ± 0.4*	1.1 ± 0.1	4.5 ± 0.4*	3.5 ± 0.4*	3.9 ± 0.8*	4.4 ± 0.6*
DOPAC	1.0 ± 0.0*	0.2 ± 0.0	0.4 ± 0.0†	0.3 ± 0.0	0.6 ± 0.2†	0.7 ± 0.2†
HVA	1.2 ± 0.1*	0.4 ± 0.0	0.8 ± 0.0*	0.6 ± 0.0*	0.8 ± 0.1*	0.8 ± 0.1*

Values (mean ± SEM) are expressed in μg/g wet weight, with SEM less than 0.05 listed as 0.0. Number of animals analysed are shown in brackets. Except for controls, all mice were given a single sc injection of 40 mg/kg MPTP, and killed 1 mo after. Protective agents were injected sc daily for 5 days, starting 48 h before MPTP. Daily doses were: α-tocopherol, 2.35 g/kg; β-carotene, 100 mg/kg; L-ascorbate, 100 mg/kg; and N-acetylcysteine 500 mg/kg. Values that differ significantly from MPTP alone: *$P < 0.001$; †$P < 0.01$.

Table 12. Effects of MPTP injections on total glutathione content of striatum and brain stem of mice

Controls	MPTP				
	30 min	1 h	2.5 h	24 h	24h (after 3rd injection)
			Striatum		
1.08 ± 0.07 (19)	0.93 ± 0.12 (10)	1.01 ± 0.05 (14)	1.06 ± 0.05 (15)	1.01 ± 0.03 (18)	1.10 ± 0.04 (15)
			Brain stem		
1.01 ± 0.04 (22)	1.02 ± 0.06 (10)	1.16 ± 0.06§ (16)	1.12 ± 0.07 (14)	0.75 ± 0.05* (18)	0.76 ± 0.04* (15)

Total glutathione values (mean ± SEM) are expressed in µmol/g wet weight, with the number of animals shown in parentheses. Mice were given a single sc injection of 40 mg/kg of MPTP and killed at the time intervals indicated, except for animals in the last column, which were killed 24 h after the last of 3 daily MPTP (40 mg/kg) injections. Values that differ significantly from controls: *P < 0.001; § P < 0.05.

glutathione content at 24 h. This latter reduction was not more pronounced in mice which had been given 3 daily injections of 40 mg/kg MPTP and which were killed 24 h after the third injection.

Because of the minute size of the SN, I was unable to reliably dissect out this area from frozen brain free of contaminating structures. I was therefore forced to study a much larger tissue mass, the brain stem, which included the SN, and for which dissection was easy and reproducible. If the glutathione changes observed in the brain stem were occurring only in the SN, and not in the surrounding non-nigral tissues, then the actual magnitude of change in this structure would probably have been greater than that shown in Table 12 for brain stem.

One plausible intepretation of the results is as follows. The presence of MPTP-induced free radicals may lead to an initial increase in glutathione synthesis and hence of its content in the brain stem. This rise is then followed by glutathione depletion, as it is consumed in the process of detoxification. The observation that striatal glutathione content did not change after the MPTP injection suggests that the free radicals, if any, were formed in the cell bodies of the nigrostriatal tract, and not in the neuronal terminals in the striatum.

Since the antioxidants α-tocopherol and β-carotene had each offered protection against the toxicity of MPTP, the effects of pretreatment with them on MPTP-induced changes of brain stem total glutathione content were determined. Animals were given 3 daily injections of either of these antioxidants. 40 mg/kg MPTP was injected immediately after the third injection. 24 h later the mice were sacrificed. Appendix F provides further details of the experimental design, including the doses of antioxidants used.

The results in Table 13 show that the MPTP-induced loss of total glutathione content in the brain stem was prevented by each of these antioxidants. An interpretation of these data is that by sharing the same free radical-scavenging property, these exogenous antioxidants had substituted for, or aided, glutathione in the detoxification of MPTP-derived free radicals. In the process, the toxicity of MPTP was attenuated (Table 11).

Table 13. Total glutathione contents of brain stem and striatum of mice injected with 40 mg/kg MPTP, with or without antioxidant pretreatment

	Controls (Saline)	No pretreatment (MPTP-alone)	β-carotene and MPTP	α-tocopherol and MPTP
Brainstem	1.01 ± 0.04* (22)	0.74 ± 0.05 (18)	1.07 ± 0.06* (10)	0.98 ± 0.04† (10)
Striatum	1.08 ± 0.07 (19)	1.01 ± 0.03 (18)	1.04 ± 0.05 (10)	1.08 ± 0.04 (10)

Values are in μmol/g wet weight, mean ± SEM, with number of samples analysed shown in brackets. Daily doses of α-tocopherol and β-carotene were 2.35 g/kg and 100 mg/kg respectively. Values that differ significantly from MPTP-alone animals: *$P < 0.001$; †$P < 0.01$.

If free radicals are involved in the mechanism of MPTP toxicity, what then is their identity? Castagnoli et al. (1985) suggested that free radicals derived from dopamine might be involved. To test this, I fed C57 black mice either a normal diet or a diet containing L-DOPA and carbidopa for 3 days. Saline or 20 mg/kg MPTP was then injected sc. Appendix F gives the particulars of the experiment. The L-DOPA treatment, which caused mice to be unusually active and aggressive, substantially increased dopaminergic turnover [(DOPAC + HVA)/dopamine molar ratio] in the striatum (Table 14). Despite this, however, the toxicity of MPTP was not potentiated. This is shown in Table 15, where it can be seen that the dopaminergic parameters in the striata of MPTP-treated animals fed a normal diet were not statistically different from those given the L-DOPA and carbidopa diet. Apparently, the neurotoxicity of MPTP is independent of free radical formation from dopamine.

Table 14. Effects of L-DOPA and carbidopa treatment on striatal contents of dopamine and its metabolites in the mouse

	Controls	L-DOPA and carbidopa diet	
	(normal diet) (26)	1st group[a] (13)	2nd group[b] (10)
Dopamine	11.8 ± 0.4	11.1 ± 0.7	12.0 ± 0.8
DOPAC	0.96 ± 0.04	4.92 ± 0.73*	0.80 ± 0.07
HVA	1.22 ± 0.07	6.68 ± 0.29*	1.26 ± 0.07
(HVA + DOPAC)/ dopamine	0.16 ± 0.01	1.00 ± 0.13*	0.15 ± 0.01

Values for dopamine and its metabolites are expressed in µg/g wet weight, while (DOPAC + HVA)/dopamine values are molar ratios. All values are mean ± SEM, with number of samples shown in brackets. [a]Mice sacrificed immediately after 3 days of the L-DOPA and carbidopa diet, at the time when other similarly-treated mice were given 20 mg/kg MPTP (Table 15). [b]Mice sacrificed 3 weeks after cessation of 11 days of the special diet, when mice given 20 mg/kg MPTP plus L-DOPA and carbidopa diet, were sacrificed (Table 15). Values that differ significantly from controls: *$P < 0.001$.

Table 15. Striatal contents of dopamine and its metabolites in mice given 20 mg/kg MPTP, with or without L-DOPA and carbidopa pretreatment

	Normal diet		L-DOPA + carbidopa
	Controls (26)	MPTP (9)	and MPTP (9)
Dopamine	11.8 ± 0.4*	6.9 ± 0.4	7.0 ± 0.8
DOPAC	0.96 ± 0.04*	0.59 ± 0.04	0.58 ± 0.05
HVA	1.22 ± 0.07§	1.02 ± 0.06	0.98 ± 0.07

Values are mean ± SEM, µg/g wet weight. Number of animals analysed are shown in parentheses. Values that differ significantly from 20 mg/kg MPTP mice on normal diet: *$P < 0.001$; §$P < 0.05$.

To determine whether MPP^+ was responsible for the neurotoxic effects of its parent compound, C57 black mice were injected sc with various doses of MPP^+. Behavioral observations were as follows. As early as 15 minutes after an injection of 10 mg/kg MPP^+, the mice appeared sedated and their body posture was altered ('stretched out'). This differed from the hunched-up kyphotic posture that is seen at 24 h after 40 mg/kg MPTP. However, all animals appeared normal 2 h after the injection. Doses of 20 mg/kg or higher killed all mice rapidly (within 2.5 h), with animals usually exhibiting a generalized seizure just before death. In one group of mice given 40 mg/kg MPP^+, pretreatment with pargyline (which prevents the toxicity of MPTP) failed to prevent death. Thus, MPP^+ is highly toxic, even more so than its parent compound MPTP.

In 3 animals, MPP^+ content of the pooled whole brain 1 h after 100 mg/kg MPP^+ was 14.5 nmol/g wet weight. In the striatum the content was 61.2 nmol/g, while the calculated value of MPP^+ in blood trapped in the brain was 10 nmol/g. The method for analyses of MPP^+ content is described in detail in Appendix D. Hence, when injected subcutaneously, MPP^+ penetrated the blood-brain barrier and was preferentially accumulated in the striatum.

Although MPP^+ penetrated the brain, mice given 10 injections of 10 mg/kg did not suffer any damage to dopaminergic nigrostriatal neurons. This is shown by the neurochemical data in Table 16. Histological examinations confirmed this by indicating that while control mice ($n = 6$) had 206 ± 5 (mean ± SEM) dopaminergic perikarya per unit area, the MPP^+-treated mice ($n = 5$) had 236 ± 3 perikarya. Thus, under the conditions of these experiments, MPP^+ was not neurotoxic to nigrostriatal neurons, although it proved to be more lethal than MPTP.

Table 16. Striatal contents of dopamine and its metabolites in mice given 2 different dosage regimens of MPP^+

	Controls	1 x 40 mg/kg MPP^+	10 x 10 mg/kg MPP^+
	(26)	(5)	(7)
Dopamine	11.8 ± 0.4	10.9 ± 0.6	11.8 ± 0.6
DOPAC	0.96 ± 0.04	0.82 ± 0.09	0.73 ± 0.03§
HVA	1.22 ± 0.07	1.20 ± 0.04	1.20 ± 0.04

Values are expressed in µg/g wet weight, mean ± SEM, with number of samples analysed in parentheses. Values that differ significantly from controls: §P < 0.05.

This experiment has 2 possible interpretations. Either MPP^+ is not the neurotoxic metabolite of MPTP; or, the concentrations of MPP^+ achieved in the SN or striatum after the injection (assuming that MPP^+ is taken into nigrostriatal neurons by neuronal terminals in this latter region) were still below those required to produce damage. The latter appears possible, since the striatal MPP^+ content an hour after an injection of 100 mg/kg MPP^+ was 61.2 nmol/g wet weight. This was an order of magnitude lower than the MPP^+ content (870 nmol/g wet weight) found by Markey et al. (1984) 10 minutes after mice were injected with 10 mg/kg MPTP. Another piece of evidence which suggests that the lack of MPP^+ neurotoxicity might be due to insufficient concentrations at the site of action comes from results that utilized different dosage schedules of MPTP. Tables 8 and 9 show that a single MPTP injection of 40 mg/kg was more neurotoxic to dopaminergic nigrostriatal neurons than multiple injections of lower doses. As already discussed above, this suggests that a threshold concentration must be reached before damage can be acheived.

MPP^+ is widely believed to be the neurotoxic metabolite of MPTP.

MPP^+ is reportedly toxic to nigral explants in tissue culture (Cohen et al. 1985), and, when injected centrally to rats (Heikkila et al. 1985). How might this be reconciled with the involvement of free radicals in MPTP toxicity, as my results suggest? One possibility is that the free radical(s) is derived from MPP^+, perhaps an MPP radical. It would be important to explore the possibility that antioxidants might prevent damage to nigrostriatal neurons using the conditions under which others (Cohen et al. 1985; Heikkila et al. 1985) have reported MPP^+ to be neurotoxic. Since the toxicity of MPP^+ in tissue culture was reportedly prevented by deprenyl pretreatment (Mytilineou et al. 1985), it would be of interest to determine whether or not deprenyl has antioxidant properties.

III.iv) Experiments using butylated hydroxyanisole, glutathione monoethyl ester, buthionine sulfoximine, and diethyl maleate

As discussed earlier, the mechanism of toxicity of MPTP may involve free radicals. Glutathione appears to be critically important in the detoxification of these reactive species. These observations suggest that an increase in normal glutathione contents might reduce the neurotoxicity of MPTP. Butylated hydroxyanisole (BHA) has been reported to have such a glutathione-elevating effect in peripheral tissues. The effect on the brain was not previously determined (Ip 1984; Jaeschke et al. 1985). Another compound, glutathione monoethyl ester, has been shown to increase liver (but not brain) total glutathione content in mice previously treated with buthionine sulfoximine (BSO), an inhibitor of the rate-limiting enzyme in glutathione biosynthesis, γ-glutamylcysteine synthetase (Puri et al. 1983). Experiments described below utilized these compounds in an attempt to produce a sustained increase in brain total glutathione levels. The objective was to deter-

mine whether MPTP was still toxic under such conditions. In addition, another group of mice was given BSO to determine if the prior depletion of liver (but not brain) total glutathione content (Table 2) could accentuate the toxicity of MPTP.

BHA (Sigma) was injected subcutaneously in 2 groups of C57 black mice. The first group (BHA + MPTP) received a total of 8 daily injections of 500 mg/kg BHA, with 40 mg/kg MPTP injected immediately after the 5th BHA injection. These animals were sacrificed a month after the single MPTP injection. The second group of mice (BHA alone) received the same daily dose of BHA for a total of 5 days, and were sacrificed 1, 2, 3, or 5 days after the last injection. This second group of mice served to document the effects of BHA on total glutathione content and glutathione transferase activity in the liver and the brain stem. Total glutathione content was analysed using the Tietze protocol (1969), while glutathione transferase activity was measured using the procedure described in Section III.viii.

As shown in Table 17, BHA produced a substantial elevation of total glutathione content and of glutathione transferase activity in mouse liver 24 h after the last of 5 daily injections. However, both parameters remained unchanged from controls in the brain stem. The increases in both total glutathione content and in glutathione transferase activity in the liver were still evident 120 h after the last injection (total glutathione content was 156% of controls, glutathione transferase activity was 173 % of controls). If BHA had penetrated the blood-brain barrier, and this is likely since it is a very lipid soluble compound, the results would suggest that the glutathione content of the brain is rather resistant to manipulation with a significant change of its content requiring a very potent stimulus. This might have relevance to human idiopathic PD, as will be discussed in Section III.viii.

Table 17. Effects of BHA on total glutathione content and glutathione transferase activity of liver and brain stem of the mouse

	Total glutathione contents (µmol/g wet weight)		GSH transferase activity (µmol/min/mg protein)	
	Control	BHA	Control	BHA
Liver	8.8 ± 0.46 (10)	17.6 ± 0.73* (10)	1.84 ± 0.09 (17)	4.84 ± 0.20* (10)
Brainstem	1.06 ± 0.04 (5)	1.04 ± 0.05 (5)	0.16 ± 0.00 (4)	0.17 ± 0.01 (5)

Values are mean ± SEM, with number of samples analysed shown in brackets. BHA mice were sacrificed 24 h after the last of 5 daily injections of 500 mg/kg. Values that differ significantly from their respective controls: *$P < 0.001$. SEM less than 0.005 are shown as 0.00.

In Table 18, HPLC analysis of the striatal contents of dopamine and its metabolites showed that pretreatment with BHA did not offer any protection from the toxicity of MPTP. This may be a reflection of the failure of BHA to produce an elevation of total glutathione content and of glutathione transferase activity in the brain stem. The substantial and long-term increase in both parameters in the liver only, but not in the brain, appeared an insufficient condition to protect mice against the neurotoxicity of MPTP.

Table 18. Effects of BHA pretreatment on the neurochemical effects of MPTP in mice

	MPTP-alone (22)	BHA and MPTP (6)
Dopamine	1.43 ± 0.19	1.51 ± 0.22
DOPAC	0.17 ± 0.02	0.14 ± 0.01
HVA	0.43 ± 0.03	0.48 ± 0.02

Values are mean ± SEM, in µg/g wet weight. Number of samples in parentheses. None of the means differ significantly.

The next series of experiments involved the use of glutathione monoethyl ester, which was synthesized by the procedure of Anderson et al. (1985). To confirm that it could indeed elevate total glutathione content of the liver, as reported by Puri et al. (1983), C57 black mice were injected sc with 10 mmol/kg of the compound, and were sacrificed at 15 and 30 minutes, 1, 1.5 and 2 h after the single injection. Control saline-injected mice also were killed at the same time intervals. Figure 1 indicates the results obtained using the Tietze method of analysis (Tietze 1969). A small increase in total glutathione content of the liver was noted at 15 and 30 minutes. Thereafter, total glutathione content of the liver remained unchanged, or decreased, from controls. Thus, the results do not replicate those of Puri et al. (1983) who reported a marked increase (2 fold) in total glutathione content of the liver at 2 h after a single injection of the same dose, with a return to control levels at 8 h post-injection. Since these authors used albino mice (Swiss-Webster), the experiment was repeated on CD1 Swiss albino mice. Again, only a small increase in total glutathione content of the liver was observed at 15 and 30 minutes (figure 1).

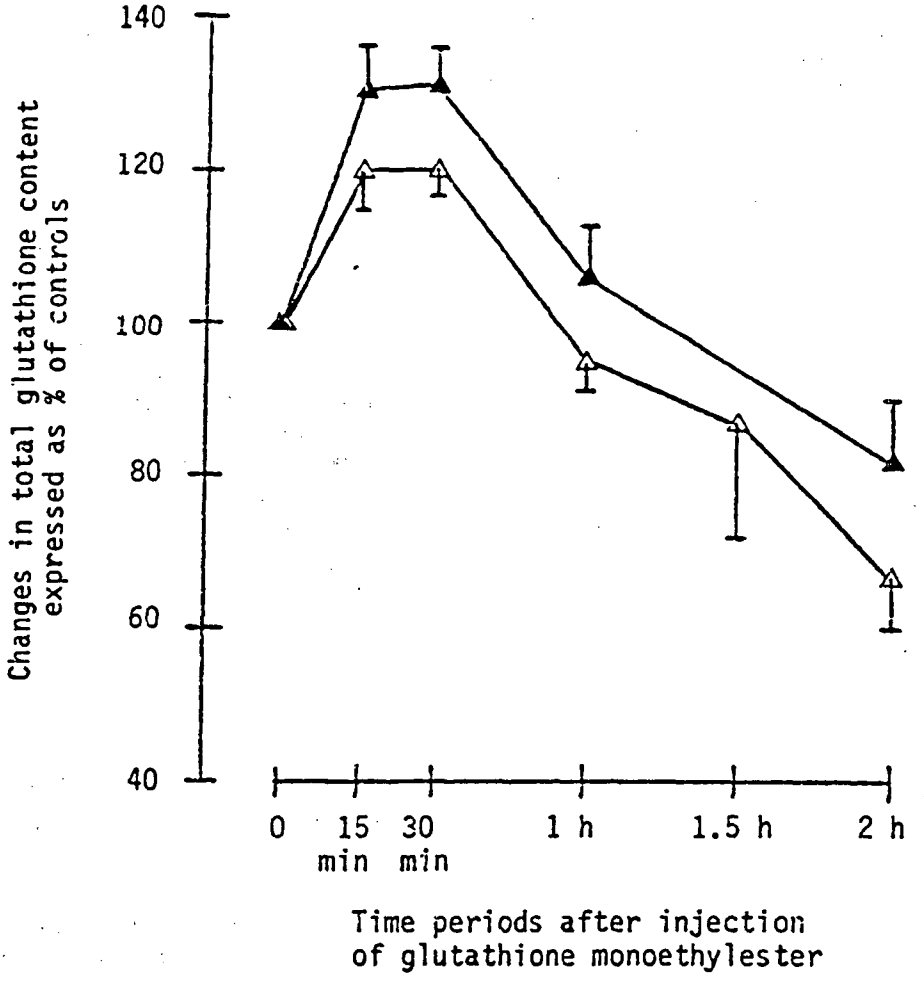

Figure 1. Changes relative to controls of total glutathione content of livers of C57 black or CD1 Swiss albino mice, at various time intervals after an injection of 10 mmol/kg glutathione monoethyl ester. % mean + (or -) SEM are shown. Each point is the average of 3 to 5 mice injected and analysed. Open triangles are of C57 black mice, while closed triangles are of CD1 Swiss albino mice.

There is one important difference between the protocol that I employed, and the method that Puri et al. (1983) used. These authors pretreated their mice with BSO. Thus, the increase in total glutathione content caused by the ester in their experiment probably occurred because the rate-limiting enzyme for glutathione synthesis was inhibited and the liver glutathione content was subnormal. In my experiment where glutathione content and its biosynthetic and degradative enzymes were initially normal, homeostatic mechanisms might have been so effective that any increase in total glutathione content was rapidly returned to normal.

Amino acid analysis using the procedure of Perry et al. (1968, 1981) was performed on the liver of a control mouse, and of a C57 black mouse injected with 10 mmol/kg of glutathione monoethyl ester and sacrificed 2 h after the injection. This was done to assess whether rapid catabolism of glutathione could explain my inability to document its increase.

Table 19 lists liver contents of total glutathione (γ-glutamylcysteinylglycine) and of its component amino acids in the 2 mouse livers. Cysteine and its disulfide, cystine, were not detected in the 2 samples, and are not tabulated. However, 2 products of cysteine metabolism, via the cysteine dioxygenase pathway (Schneider et al. 1983), are shown. These are hypotaurine and taurine. The table shows increased contents of taurine, hypotaurine, and glycine in the liver of the mouse given the ester. Total glutathione content was unchanged, confirming results obtained using the Tietze technique. Another breakdown product of glutathione, glutamic acid, was not increased, probably because transamination to α-ketoglutarate had occurred. Since the latter is not an amino acid, it could not be detected by amino acid analysis. The results listed in Table 19 indicate that liver glutathione content was increased initially in the mouse given the ester, but

glutathione was then rapidly hydrolysed into its component amino acids.

Table 19. Content of amino acids in liver of mouse sacrificed 2 h after 10 mmol/kg glutathione monoethyl ester

Animal	Total glutathione	Taurine	Hypotaurine	Glutamic acid	Glutamine	Glycine
Control	8.44	15.05	0.58	1.60	0.99	2.16
Ester	7.97	18.02	4.03	1.03	1.18	4.18

All values are in μmol/g wet weight.

Despite the failure of glutathione monoethyl ester to produce a sustained increase in total glutathione content of the liver, and presumably of the brain, I injected a group of C57 black mice with 40 mg/kg MPTP (sc), one hour after a sc injection of 10 mmol/kg glutathione monoethyl ester. 9 of 10 mice died after the MPTP injection, and neurochemical or histological analyses of the integrity of the dopaminergic nigrostriatal pathway of these mice was therefore not possible. However, the lethality rate seen in the glutathione monoethyl ester pretreated group does not suggest a protective role of this compound against the toxicity of MPTP.

Another group of C57 black mice were fed BSO (20mM) in their drinking water for 7 days, with 20 mg/kg of MPTP injected sc on the fourth day. When sacrificed a month after the MPTP injection, the striatal contents of dopamine and its metabolites in these animals were not significantly different from those of mice fed ordinary tap water and injected with 20 mg/kg MPTP (Table 20).

To summarise the experiments above, the results suggest that the content of total glutathione in the brain is difficult to alter. That in the liver is more susceptible to change, but in the case of glutathione monoethyl

ester, an initial increase is rapidly returned to normal. The prior alteration of total glutathione content of the liver does not appear to be a sufficient condition to modify the neurotoxicity of MPTP.

Table 20. Effects of BSO pretreatment on the neurochemical effects of 20 mg/kg MPTP in C57 black mice

	20 mg/kg MPTP (10)	BSO and 20 mg/kg MPTP (5)
Dopamine	7.3 ± 0.6	8.4 ± 0.5
DOPAC	0.50 ± 0.03	0.44 ± 0.02
HVA	1.04 ± 0.05	1.03 ± 0.06

Values are mean ± SEM, µg/g wet weight, with the number of samples analysed shown in parentheses. None of the means differ significantly.

More recently, we used diethylmaleate (DEM) (Sigma) in an attempt to deplete total glutathione contents of liver and brain of C57 black mice. DEM is an electrophile that reacts with glutathione in the presence of glutathione transferase (Plummer et al. 1981). Two groups of mice were injected sc with 2 injections, spaced 24 h apart, of 0.8 ml DEM/kg. One group was sacrificed 1 h after the second injection, and the liver and brain stem were obtained for analysis of total glutathione content by the method of Tietze (1969). As shown in Table 21, the 2 injections of DEM produced approximately 90% depletion of total glutathione content of both the liver and the brain stem. In the second group of mice that were allowed to survive after the second injection, rapid loss in body weight was observed over the ensuing few days. Behavioral changes consisting of sedation and tremors were obvious and long-lasting, occurring for at least 7 days after the second injection of DEM. Seven of 10 mice died. We therefore elected to treat

mice with only 1 injection of DEM, to assess whether or not the toxicity of MPTP could be potentiated after DEM pretreatment.

Table 21. Liver and brain stem total glutathione contents of mice injected with DEM

	Control	DEM	
		Two injections	One injection
Liver	9.05 ± 0.54 (14)	1.63 ± 0.32* (10)	3.13 ± 0.19* (5)
Brain stem	1.14 ± 0.03 (12)	0.22 ± 0.02* (10)	0.32 ± 0.03* (5)

Values are mean ± SEM, μmol/g wet weight. DEM mice were sacrificed 1 h after 1 or 2 injections of 0.8 ml DEM/kg. Values that differ significantly from controls: *$P < 0.001$. Total glutathione contents of both the liver and brain stem of DEM mice given 1 injection, differ significantly from those of DEM mice injected with 2 doses of DEM ($P < 0.01$, Student's t-test).

Four groups of C57 black mice were used. The first group received an injection of 0.8 ml DEM/kg and was sacificed 1 h later for analysis of total glutathione content in the liver and brain stem by the Tietze method (1969). As shown in Table 21, these animals had substantial (about 70%) depletions of total glutathione content of the liver and brain stem. A second group of animals received the same dose of DEM, and was sacrificed a month afterwards for analysis of contents of dopamine and its metabolites in the striatum (Table 22). A third group of mice was administered 0.8 ml DEM/kg, followed 1 h later by an injection of 20 mg/kg MPTP. These animals, together with a fourth group that received only 20 mg/kg of MPTP, were killed 1 mo after the MPTP injection. As shown in Table 22, the mean striatal content of dopamine in DEM-pretreated mice given MPTP, was not significantly different from that

of mice that were given only MPTP. However, contents of DOPAC and HVA were slightly elevated in the former group.

Table 22. Contents of dopamine and its metabolites in striatum of mice treated with DEM, DEM plus 20 mg/kg MPTP, or 20 mg/kg MPTP only

Animals	Dopamine	DOPAC	HVA
DEM only (13)	11.2 ± 0.7*	0.58 ± 0.03§	1.08 ± 0.06†
20 mg/kg MPTP (18)	7.3 ± 0.3	0.49 ± 0.02	0.88 ± 0.03
DEM plus 20 mg/kg MPTP (10)	8.4 ± 0.5	0.61 ± 0.02*	0.99 ± 0.05§

Values are mean ± SEM, μg/g wet weight, with the number of samples shown in parentheses. DEM plus 20 mg/kg MPTP mice were given an injection of 0.8 ml DEM/kg 1 h before an injection of 20 mg/kg MPTP. All animals were sacrificed 1 month after the injection of MPTP or DEM. Values that differ significantly from 20 mg/kg MPTP: * $P < 0.001$; § $P < 0.05$; † $P < 0.01$.

Thus, despite a substantial depletion of brain stem total glutathione content in DEM-pretreated mice, the toxicity of MPTP was not potentiated. It is difficult to reconcile the present results with those of Section III.iii which suggest a role for glutathione in the detoxification of neurotoxic species that might be formed from MPTP. This discrepancy cannot be explained presently.

III.v) Experiments that seek to identify the natural neurotoxin(s) responsible for most cases of idiopathic Parkinson's disease

As noted in the introductory section, the currently prevailing hypothesis is that PD is the result of exposure to one or more environmental toxins. These toxins have yet to be identified. Although MPTP produces a parkinson-

ian syndrome, it is unlikely to be the agent responsible for most cases of idiopathic PD. This is because there are no known environmental sources of MPTP, other than its formation as a contaminant during the synthesis of a meperidine analog to which relatively few people have been exposed. If an environmental (or possibly endogenous) parkinsonism-causing toxin were identified, it might be possible to devise strategies to avoid or detoxify it and perhaps prevent the occurrence of PD. For example, if it occurred in certain foods, then simply excluding these from the diet might decrease the incidence of the disease.

For this reason, I attempted to create a model of PD by injecting C57 black mice with chemical analogs of MPTP or MPP^+, some of which are commonly found in nature. These included cinnamaldehyde (which might be converted by Schiff base formation into an MPTP analog *in vivo*); arecoline, a cholinomimetic alkaloid from seeds of the betel nut palm; N,N-dimethylcinnamylamine, an MPTP analog without a complete pyridine ring structure; paraquat, a herbicide that bears a structural resemblance to MPP^+; reduced paraquat [4,4'-bis(1-methyl-1,2,3,6-tetrahydropyridine)] which bears 2 N-methyl-tetrahydropyridine moieties; 2-methyl-1,2,3,4-tetrahydro-β-carboline, a trace endogenous compound (Barker *et al.* 1981) which is formed by intracellular condensation of carbonyl compounds with neuroactive indoleamines (Collins *et al.* 1985); N-methyl-1,2,3,4-tetrahydro-6,7-isoquinolinediol and N-methyl-1,2,3,4-tetrahydroisoquinoline, compounds which might be formed *in vivo* from the condensation of phenylethylamines with carbonyl compounds (Testa *et al.* 1985). The structural formulae of these compounds and their chemical similarity to MPTP and MPP^+, are illustrated in Figure 2. Injections of all the analogs were by the sc route.

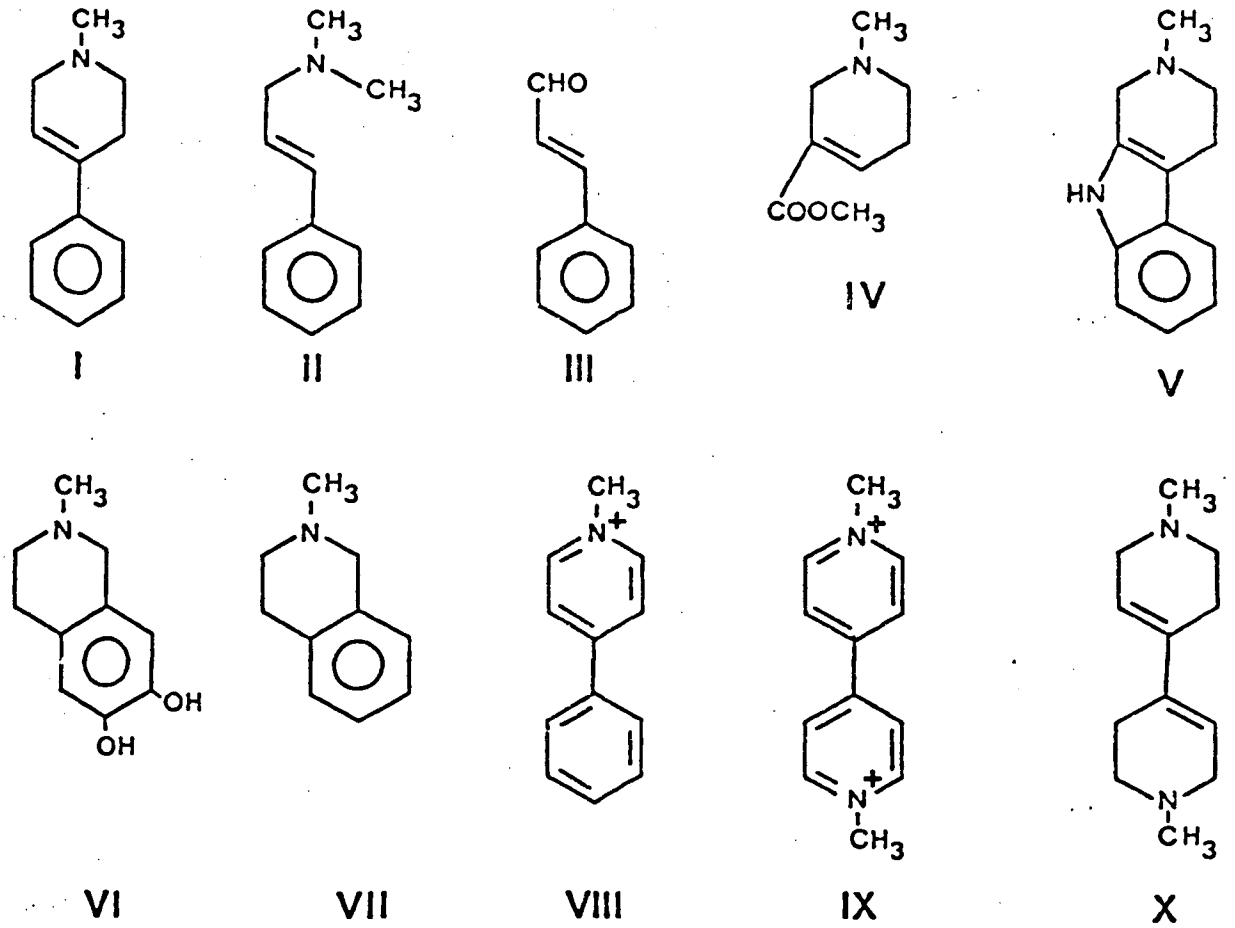

Figure 2. Structural formulae of MPTP, MPP^+, and their analogs. The compounds are:

I) N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP),
II) N,N-dimethylcinnamylamine,
III) Cinnamaldehyde,
IV) Arecoline,
V) 2-methyl-1,2,3,4-tetrahydro-ß-carboline,
VI) N-methyl-1,2,3,4-tetrahydro-6,7-isoquinolinediol,
VII) N-methyl-1,2,3,4-tetrahydroisoquinoline (MTIQ),
VIII) N-methyl-4-phenylpyridinium ion (MPP^+),
IX) Paraquat,
X) Reduced paraquat [4,4'-bis(1-methyl-1,2,3,6-tetrahydropyridine)].

Cinnamaldehyde (Fisher), dissolved in 10% ethanol, was given over a 30 day period in a total of 20 injections. The starting dose was 40 mg/kg and this was increased at each injection until a dose of 900 mg/kg was reached. The last 9 injections were of this latter dose. The total cumulative dose that each animal received was 12 g/kg. Mice were sacrificed 1 h after the last injection, when it appeared that they might not survive any longer.

During the course of the cinnamaldehyde injections, no adverse effects were observed when the dosage was 700 mg/kg or lower. However, when the highest dose (900 mg/kg for the last 9 daily injections) was injected, parkinsonian-like symptoms consisting of akinesia, tremor and kyphosis were seen. These persisted for at least 24 h after each injection. Convulsions were sometimes elicited. 6 of 10 mice died during the period of injection of this highest dose. Despite the obvious behavioral changes, the 4 remaining mice sacrificed 1 h after the last injection did not show any reduction in the striatal contents of dopamine or DOPAC, although HVA levels were slightly higher than controls (Table 23).

Another group of mice were given 29 daily injections of arecoline hydrobromide (Sigma) dissolved in 0.9 % NaCl. Dosage was increased progressively from 5 to 200 mg/kg (expressed as the salt). The last 6 injections were of 200 mg/kg. The total cumulative dose per mouse was 1360 mg/kg. Prior to each arecoline injection the mice were injected with atropine (Sigma), which was also progressively increased from 2 to 14 mg/kg. This atropine cover was necessary to prevent manifestations of excessive muscarinic activity, including excessive salivation and intense tremors. Nine days after the last injection of arecoline the mice were sacrificed. Striatal dopamine and HVA contents did not differ from controls (Table 23). In view of the recent report that anticholinergic pretreatment prevented the toxicity of MPTP (Hadjiconstantinou et al. 1985), it is possible that if arecoline had an

Table 23. Effects of MPTP analogs on striatal contents of dopamine and its metabolites of C57 black mice

Analog	Dopamine	DOPAC	HVA
Saline controls (44)	11.3 ± 0.4	0.95 ± 0.04	1.27 ± 0.05
Cinnamaldehyde (4)	11.6 ± 1.2	0.84 ± 0.32	1.94 ± 0.54†
Arecoline (8)	11.8 ± 0.9	0.66 ± 0.04*	1.04 ± 0.08
N,N-dimethyl-cinnamylamine (7)	11.2 ± 1.3	0.80 ± 0.11	1.41 ± 0.18
N-methyl-1,2,3,4-tetrahydro-6,7-isoquinolinediol (10)	13.1 ± 0.6	0.84 ± 0.03	1.31 ± 0.07
N-methyl-1,2,3,4-tetrahydro-isoquinoline (9)	14.3 ± 0.3*	0.76 ± 0.01§	1.24 ± 0.03
2-methyl-1,2,3,4-tetrahydro-β-carboline (6)	13.8 ± 0.8§	0.84 ± 0.07	1.32 ± 0.07

Values are mean ± SEM, in μg/g wet weight, with the number of mice shown in parentheses. Dosages are described in the text. Values that differ significantly from controls: *$P < 0.001$; †$P < 0.01$; §$P < 0.05$.

MPTP-like mechanism of action and similar neurotoxicity, the atropine protection in my experiment might have prevented its manifestation. However, our attempts to protect mice from MPTP with atropine, using the conditions described by Hadjiconstantinou et al. (1985), failed. Thus, arecoline is unlikely to have neurotoxic effects on the nigrostriatal system.

N,N-dimethylcinnamylamine (synthesized as described in Appendix E) was injected daily for 7 days, in doses increasing from 40 to 200 mg/kg. Total cumulative dose per mouse was 890 mg/kg. Animals showed no signs of drug toxicity when the dose was 40, 80 or 120 mg/kg. However, at 150 or 200 mg/kg, a particular syndrome was observed. Five minutes after the injection, mice began to move continously around their cage. Ataxia and tremors then developed, but the animals continued to move. At 1.5 h post-injection, the constant motion disappeared and was replaced by repetitive chewing

on wood-shavings while standing in one spot. 4 h after the injection, the animals appeared normal again. At sacrifice (10 days after the last injection), striatal dopaminergic parameters were unchanged from control values (Table 23).

N-methyl-1,2,3,4-tetrahydro-6,7-isoquinolinediol HCl (Sigma), dissolved in 0.9% NaCl, was administered to another group of mice. 5 daily injections of 40 mg/kg (expressed as the HCl salt) were given, and animals were sacrificed 20 days after the last injection. No abnormal behavior was observed, and, as shown in Table 23, contents of striatal dopamine and its metabolites remained unchanged from controls.

Since the presence of 2 hydroxyl groups in N-methyl-1,2,3,4- tetrahydro-6,7-isoquinolinediol might hinder the passage of this compound into the brain, an analog that lacks the hydroxyl groups, N-methyl-1,2,3,4-tetrahydroisoquinoline (MTIQ), was tested for possible neurotoxic effects in mice. MTIQ was synthesized by reacting isoquinoline with methyliodide and isopropanol. The product, N-methylisoquinolinium iodide (obtained in 70% yield), was dissolved in water, and 4 moles of excess sodium borohydride were added. Nitrogen gas was bubbled through the solution to exclude oxygen. MTIQ formed as an oily layer and was extracted with ether. The ether solution was dried, a small amount of HCl added, and the hydrochloride salt was evaporated to dryness and recrystallized. Identity of the hydrochloride salt of MTIQ was confirmed using a proton NMR spectrometer.

Eight daily injections of MTIQ were administered sc to a group of C57 black mice. The dosage was increased progressively from 40 to 160 mg/kg (dosage expressed as the HCl salt). The last 5 injections were of 160 mg/kg, giving a total cumulative dose of 1040 mg/kg. After each injection of 160 mg/kg (but not of lower doses), mice became rapidly sedated. This lasted for a period of 3 h, after which animals appeared normal. A

single mouse injected with 200 mg/kg of MTIQ developed generalised seizures and died within 15 minutes. Thus, 160 mg/kg appeared to be the highest dose compatible with survival.

MTIQ animals were sacrificed a month after the last injection. Striatal contents of dopamine and DOPAC were significantly higher than those of controls (Table 23). Although the results are difficult to explain, they nonetheless indicate that MTIQ had no neurodegenerative effects on the nigrostriatal system of mice.

Another group of mice was injected daily for 4 days with 2-methyl-1,2,3,4-tetrahydro-β-carboline (MTHBC) dissolved in 0.9% NaCl. MTHBC was synthesized by reacting norharman (Sigma) with excess methyl iodide in boiling isopropanol. The yellow precipitate was filtered, washed, and recrystallized from isopropanol. The compound, dissolved in a water-methanol solution through which nitrogen was bubbled, was treated with an excess of sodium borohydride. The colorless product formed was filtered, washed, and recrystallized from water-methanol, and its identity as MHTBC was confirmed by mass spectrometry and nuclear magnetic resonance. The first injection of MHTBC was of 40 mg/kg, which did not cause any behavioral change. Subsequent injections were of 80 mg/kg, which produced sedation, ataxia, tremors and occasional clonic convulsions. These lasted for 2 h. When sacrificed 1 month after the last injection, striatal dopaminergic parameters were normal (Table 23).

Another treatment was of paraquat in 0.9% NaCl. Three dosage schedules were used. The first utilized 5 daily injections of the diiodide salt, starting with 6.3 mg/kg, then 8.5 mg/kg, and finally 3 injections of 12.7 mg/kg (doses are expressed here as the free base). Although no abnormalities in behavior were observed, the mice lost weight rapidly and 7 of 10 mice

died within 24 h after the last injection. The remaining mice were sacrificed at this 24 h mark, and they had normal contents of dopamine and HVA in the striatum, although DOPAC levels were slightly decreased (Table 24).

Table 24. Effects of paraquat and reduced paraquat on striatal contents of dopamine and its metabolites of C57 black mice

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	Cumulative doses (mg/kg)	Dopamine (µg/g wet weight)	DOPAC (µg/g wet weight)	HVA (µg/g wet weight)
Controls (44)		11.3 ± 0.4	0.95 ± 0.05	1.27 ± 0.05
Paraquat (3)	52.9, over 5 injections	13.1 ± 0.7	1.03 ± 0.12	0.89 ± 0.14
Paraquat (9)	12.7, single injection	12.5 ± 0.7	0.72 ± 0.04§	1.20 ± 0.06
Paraquat (7)	43.5, over 3 injections	14.5 ± 0.4*	0.76 ± 0.04	1.30 ± 0.07
Reduced paraquat (10)	341.6, over 6 injections	12.7 ± 0.7	0.80 ± 0.03	1.28 ± 0.06

Values for dopamine and its metabolites are mean ± SEM. The number of animals analysed is shown in brackets. Cumulative doses for paraquat and reduced paraquat are expressed as the free base. Values that differ significantly from controls: * $P < 0.001$; § $P < 0.05$.

The second dosage schedule of paraquat diiodide employed a single injection of 12.7 mg/kg (expressed as the free base), and sacrifice was after 1 month. No mortality was encountered. Table 24 shows that these mice had no deficits in striatal contents of dopamine and its metabolites when compared

to normals.

The third dosage schedule of paraquat involved 3 injections of paraquat dichloride. Injections were of 14.5 mg/kg each (expressed as the free base) and were spaced 3 days apart. The latter timing was necessary because in another experiment, 10 of 10 mice given 3 daily injections of the same dose died soon after the third injection. Despite the present design, 3 of 10 animals died. Striatal contents of DOPAC and HVA were normal 1 month after the last injection, although dopamine content was increased (Table 24).

The last treatment group received the dihydrochloride salt of reduced paraquat. This compound was synthesized by first dissolving paraquat in water, then while nitrogen gas was bubbled through the solution to exclude oxygen, excess amounts of sodium borohydride were added. Reduced paraquat formed as an oily layer and was extracted with ether. The ether solution was dried, a small amount of HCl was added, and the dihydrochloride salt of reduced paraquat was evaporated to dryness and recrystallized. Identity of the product as the dihydrochloride salt of reduced paraquat was confirmed by nuclear magnetic resonance and mass spectrometry.

Ten C57 black mice received 6 daily injections of reduced paraquat diHCl, in successive doses of 7.3, 14.5, 29.1, 58.1, 116.3 and 116.3 mg/kg (doses expressed as the free base). Throughout the experimental period, no behavioral abnormalities were evident. When sacrificed 1 month after the last injection, striatal contents of dopamine and its metabolites were normal (Table 24).

In summary, therefore, the various analogs of MPTP or MPP^+ tested were ineffective in damaging the dopaminergic nigrostriatal pathway of C57 black mice. This is in spite of some interesting behavioral changes observed during the course of injections of drugs such as cinnamaldehyde and N,N-di-

methylcinnamylamine. The failure to produce parkinsonian changes in mice does not mean that these compounds might not be neurotoxic to nigrostriatal neurons of primates and man. MPTP itself is toxic to primates at very low doses, but in mice, much higher doses are required (Langston et al. 1984a; Heikkila et al. 1984a). To obtain a less equivocal answer as to the possible toxicity of these MPTP-analogs in man, they should be tested in primates. This is especially important with regard to the herbicide paraquat. Barbeau et al. (cited in Lewin, 1985a) have found an excellent correlation ($r = 0.967$) between the incidence of PD and the level of pesticide use in several regions of Quebec, Canada. In addition, the same investigators (Barbeau et al. 1985) have reported results showing that paraquat could reduce brain dopamine concentrations in frogs, Rana pipiens, although this reduction was more marked with MPTP. Clearly, the possible neurotoxicity of paraquat should be investigated more thoroughly in primates, since this herbicide is in common use. In my experiments, various dosage schedules of paraquat in doses compatible with survival, as well as the reduced form of paraquat which might more readily enter the brain, did not damage dopaminergic nigrostriatal neurons of the mouse.

III.vi) Smoking and Parkinson's disease

An interesting observation in PD research is that the disease has been shown by several epidemiological studies to occur less frequently among cigarette smokers than among non-smokers (Nefzger et al. 1968; Kessler et al. 1971; Baumann et al. 1980; Godwin-Austen et al. 1982). Although the protective mechanism of smoking remains unknown, several theories have been put forth. Perry et al. (1982) suggested that the increased partial pressure of carbon monoxide in brain cells of cigarette smokers might provide a

reducing environment which could partially protect nigrostriatal neurons from oxidant damage. Snyder (1985) postulated that the many pyridine compounds in cigarette smoke might inhibit the uptake of MPTP (or MPP^+), also a pyridine molecule, into nigrostriatal neurons. This hypothesis assumes that the neurotoxin responsible for idiopathic PD is an MPTP-like molecule.

It is possible to formulate at least 3 other hypotheses to explain the protective effect afforded by cigarette smoking. The first is that one or more of the compounds present in tobacco smoke can detoxify MPTP-like molecules. To test this, we used the MPTP mouse model to ascertain if any one of 3 compounds known to accumulate in the physiological fluids and tissues of cigarette smokers can prevent the toxicity of MPTP. The compounds tested were nicotine, sodium thiocyanate, and hydrazine (Liu et al. 1974; Voght et al. 1977). Details of this experiment are described in Appendix G (Yong et al. J. Neurol. Sci. 72:265-272, 1986). In brief, mice were pretreated with one of these 3 compounds for several days before an injection of 40 mg/kg MPTP was given. One month later, mice were sacrificed and the striatum analysed for contents of dopamine and its metabolites.

The results are displayed in Table 25. While thiocyanate offered very slight protection from MPTP-induced damage to dopaminergic nigrostriatal neurons, hydrazine pretreatment offered considerable protection, comparable to that found with antioxidant pretreatment prior to an MPTP injection (Table 11). The mechanism of the protective effect of hydrazine cannot be attributed to MAO-B inhibition since it was not an inhibitor of MAO-B activity in the liver or striatum (Table 26). Mice pretreated for 10 days with nicotine all died within 5 days following the MPTP injection suggesting that, if anything, nicotine intensified the neurotoxic effects of MPTP.

Table 25. Effects of pretreatment with thiocyanate or hydrazine on MPTP-induced depletion of striatal dopamine and its metabolites in mice

	Controls (39)	MPTP (23)	Thiocyanate (8)	Hydrazine (16)
Dopamine	11.3 ± 0.4*	1.3 ± 0.2	2.7 ± 0.7†	5.7 ± 0.5*
DOPAC	0.97 ± 0.05*	0.21 ± 0.03	0.31 ± 0.06	0.51 ± 0.07*
HVA	1.22 ± 0.05*	0.43 ± 0.03	0.62 ± 0.09†	0.89 ± 0.04*

Values are mean ± SEM, in μg/g wet weight, with number of samples indicated in parentheses. Values that differ significantly from mice injected with MPTP alone: *$P < 0.001$; †$P < 0.01$.

Table 26. Failure of hydrazine to inhibit MAO-B activity in liver and striatum of mice

	Controls	Hydrazine
Liver	65.1 ± 3.4 (10)	67.1 ± 5.0 (7)
Striatum	28.0 ± 2.1 (15)	32.7 ± 1.6 (7)

Values are mean ± SEM, nmoles/h/mg protein, with number of samples indicated in parentheses. Hydrazine-treated mice were sacrificed 1 h after the last of 8 daily injections of 11 mg/kg. None of the means are statistically significant.

Thus, if the neurotoxin responsible for most cases of idiopathic PD is an MPTP-like molecule whose toxicity can similarly be reduced by hydrazine, then the apparent protective effect of smoking might be attributable to this compound. Of course, it is possible that there are other substances in cigarette smoke which might be more potent than hydrazine in partially preventing the toxicity of MPTP.

The second hypothesis regarding the protective role of smoking relies on the observation that MPTP requires conversion by MAO-B to become neurotoxic (Markey et al. 1984; Heikkila et al. 1984b; Langston et al 1984b). It is possible that if the agent(s) responsible for most cases of idiopathic PD requires conversion by MAO-B to become neurotoxic, and if cigarette smoking inhibits this enzyme so that the conversion of an inactive precursor(s) to a neurotoxic species is prevented or reduced, then the appparent protective effect of smoking might be explained.

To evaluate this second possibility, the platelet MAO-B activity of heavy cigarette smokers (who each smoked more than 20 cigarettes a day) was compared with that of non-smokers. Details of the enzyme measurements have been described in Appendix G. Platelets were chosen as the source of MAO-B, since they contain only the B isozyme and are the only readily accessible source of the enzyme for routine studies in living persons. Whether or not MAO-B activity in platelets is a reliable index of its activity in the human brain or other tissues is uncertain (Sandler et al. 1981).

Table 27 shows that the mean activity of MAO-B in platelets was significantly lower in cigarette smokers than in non-smokers. This confirms the results of Oreland et al. (1981) who studied a much smaller population. Although interesting, the reduction (25) might not be functionally large enough to account for the protection afforded by smoking. In the treatment of depression, antidepressants that are MAO inhibitors must inhibit platelet MAO-B in excess of 80% before clinical improvement is seen (Youdim et al. 1980).

Table 27. Monoamine oxidase B activity in platelets of human smokers and non-smokers

Subjects	Mean age (years)	Monoamine oxidase B activity (nmol/h/mg protein)
Cigarette smokers (32)	37 ± 2	4.08 ± 0.26*
Non-smokers (41)	34 ± 2	5.46 ± 0.24

Values are mean ± SEM, with number of subjects shown in parentheses. *$P < 0.001$.

What component of cigarette smoke inhibits MAO-B ? To attempt to answer this, I measured rat liver MAO-B activity, using an *in vitro* system described in Appendix G, in the presence of one of 4 constituents of cigarette smoke. These were cotinine (the major metabolite of nicotine), thiocyanate, cyanide, or hydrazine. All 4 compounds, tested at 10^{-6} to 10^{-3}M, were totally ineffective in inhibiting MAO-B activity. Although the cause of the lowered platelet MAO-B activity in cigarette smokers appears not to be due to these compounds, it is certainly possible that *in vivo*, one of these compounds might act as inhibitors of the *synthesis* of molecules of MAO-B, resulting in decreased activity of the enzyme.

A third possible explanation of the protective role of smoking is that an ingredient in cigarette smoke induces the production of other enzymes, perhaps in the liver, which destroy the neurotoxin precursor before it can be converted into the active metabolite by MAO-B. No experiments to test this hypothesis have yet been performed.

III.vii) Status of MAO-B in relation to Parkinson's disease

An important finding in MPTP research is that MPTP itself is not neurotoxic, but requires conversion by MAO-B to one or more toxic metabolites. If the neurotoxin(s) responsible for most cases of idiopathic PD requires such a transformation by the same enzyme, then variations in MAO-B activity in tissues might be a crucial factor in the development of the disease.

The presence of an unidentified endogenous MAO inhibitor has been demonstrated in human urine (Glover et al. 1980; Glover et al. 1982; Bhattacharya et al. 1982). If an endogenous inhibitor were responsible for regulating the activity of MAO-B in human subjects, then a failure to produce it might result in increased MAO-B activity, and thus facilitate conversion of inactive precursors to neurotoxic species, resulting in PD. To test this, the amount of the endogenous MAO inhibitor(s) in the urine of PD patients and control subjects was measured. Platelets were also obtained from some control subjects to ascertain whether or not there was a correlation between urinary MAO-B inhibitory potency and platelet MAO-B activity. Appendix G (Yong et al. J. Neurol. Sci. 72:265-272, 1986) gives details of the method employed, as well as a description of the subjects, PD and controls, who supplied urines and platelets.

Table 28 shows that inhibition of rat liver MAO-B by urine was significantly greater for the PD patients than for control subjects. However, exclusion of PD patients currently being treated with anticholinergic drugs reduced this difference. All but one of the PD patients were receiving some form of drug treatment, while few of the controls were taking any drugs. It is possible that the presence of various drugs and their metabolites in the urine of PD patients might have accounted for their greater inhibition of MAO-B. The difference between the control and PD patients is unlikely to be

due to differing mean ages, since linear regression analysis for the control subjects showed no correlation between age and urinary inhibition of MAO-B ($r = 0.26$, linear regression analysis). In any case, there was no evidence of a decreased urinary excretion of the MAO-B inhibitor(s) by the PD patients, as might have been expected had they failed to synthesize adequate amounts of an endogenous MAO-B inhibitor. Also, the poor correlation between the urinary MAO-B inhibitor(s) and platelet MAO-B activity ($r = 0.13$) in 21 control subjects suggests that the former, whose identity is unknown, might not be an important endogenous regulator of MAO-B activity of tissues. This arguement assumes that platelet MAO-B activity is an accurate reflection of MAO-B activity of other tissues such as the brain.

Direct measurements of MAO-B activity in autopsied brain regions (SN and frontal cortex) from PD patients and control subjects was also performed. The experimental details, as well as descriptions of the PD and control subjects whose post-mortem brains were used, are described in Appendix G.

Table 28. Inhibition of rat liver monoamine oxidase B activity by human urine

Subjects providing urine	Mean age of subjects	% Enzyme inhibition by urine
Control subjects (49)	47 ± 3	20.9 ± 1.3
All Parkinson's disease patients (31)	62 ± 2*	34.5 ± 4.0*
Parkinson's disease patients not receiving anticholinergic drugs (21)	63 ± 2*	27.7 ± 3.4§

Mean ± SEM are shown for age in years, and for urinary MAO-B inhibition in . Number of subjects analysed is shown in parentheses. Values that differ significantly from that of control subjects: *$P < 0.001$; §$P < 0.05$.

In Table 29, it can be seen that MAO-B activity was not significantly increased in the frontal cortex or SN of PD brains when compared to control. MAO-B activity increases with age in most brain regions (Fowler et al. 1980), and the somewhat higher activity in the SN of the PD patients may be due to their greater mean age at death.

In summary, the experiments described showed no significant increase in brain MAO-B activity in the autopsied brains of PD patients. There was no evidence of a decreased excretion of urinary MAO-B inhibitor(s) in living patients. It appears unlikely that an overactive MAO-B activity in tissues causes the excessive conversion of an inactive precursor to a neurotoxic metabolite(s) in PD.

Table 29. Monoamine oxidase B activity in brain of controls and Parkinson's disease patients

Subjects	Mean age (years)	MAO-B activity (nmol/h/mg protein)
	Frontal cortex	
Controls (20)	63 ± 2	24.7 ± 1.8
Parkinson's disease (11)	78 ± 2*	25.2 ± 1.4
	Substantia nigra	
Controls (11)	60 ± 2	41.2 ± 5.2
Parkinson's disease (7)	79 ± 3*	52.9 ± 8.1

Values are in mean ± SEM, with the number of subjects analysed in parentheses. Values that differ significantly from controls: *$P < 0.001$.

III.viii) Glutathione content and glutathione transferase activity in the autopsied brains of Parkinson's disease patients

This research was initiated by the observation of Perry et al. (1982) that glutathione content was reduced in the SN of patients with PD. A small sample of SN (6) from PD brains was used in that study. Since then, there has been opportunity to study more parkinsonian brains. Part of this section updates the observation of Perry et al. (1982).

Measurements of reduced (GSH) and oxidised (GSSG) forms of glutathione, as well as of the mixed disulfide of GSH and cysteine, GS-SCy, were done on an amino acid analyser by the procedure of Perry et al. (1968, 1981). Total glutathione content was taken as the sum of GSH, 2xGSSG, and GS-SCy. The inclusion of GS-SCy enables a more accurate representation of total glutathione content present during life since this compound is not normally detectable in rapidly frozen biopsies. Upon death, and unless the brain is frozen immediately, GS-SCy accumulates, mainly from the autolysis of glutathione.

Brains used for control data were from a heterogeneous group of subjects consisting of persons who died without any signs of neurological disease, and of patients dying with Huntington's chorea (HC), dialysis encephalopathy (DE), olivopontocerebellar atrophy (OPCA), amyotrophic lateral sclerosis, or schizophrenia. In none of these disorders is there any known abnormality of glutathione metabolism. All of the brains studied had a death-to-freezing interval of 8 h or less. For longer intervals, autolysis of glutathione would have been so extensive that quantitation would have become unreliable (Perry et al. 1981). With respect to the age of subjects at death, control brains did not match the PD brains (controls = 57 ± 2 years, PD = 75 ± 3, mean ± SEM, $P < 0.001$). However, linear regression analysis in the control

group showed no correlation of total glutathione content with age (SN, $r = 0.02$; frontal cortex, $r = 0.05$). To confirm this, we analysed 19 cortical biopsied specimens that were frozen immediately after excision. The total glutathione in promptly-frozen biopsies of course gives a more accurate representation of the content present during life. These specimens of biopsied material were from subjects who underwent surgical removal of a deep-seated brain tumour or brain abscess. The surgery required sacrifice of a small area of apparently normal cerebral cortex in order to reach the deeper pathological lesion. The age of subjects ranged from 16 to 64. Again, no linear correlation was found between age and total glutathione content ($r = 0.36$).

Table 30 presents the total glutathione contents of 6 brain regions for PD patients, and for a large group of control subjects. Total glutathione content was significantly reduced only in the SN of PD patients. Although glutathione content was somewhat lower in PD patients than in controls for the other 5 brain regions examined, these reductions did not reach statistical significance (analysed by 2-tailed t-test, $P = 0.05$). Since the mean death-to-freezing intervals were almost identical for the control and PD patients (3.9 ± 0.3 and 4.0 ± 0.5 h respectively, mean ± SEM), the glutathione reduction in the SN of PD patients at autopsy probably represents a nigral glutathione deficiency that was present during life.

Table 30. Total glutathione contents in various regions of rapidly frozen human brains

Brain region	Controls	Parkinson's Disease
Frontal cortex	1.79 ± 0.07 (53)	1.54 ± 0.12 (15)
Occipital cortex	1.67 ± 0.07 (53)	1.44 ± 0.12 (12)
Cerebellar cortex	1.98 ± 0.08 (52)	1.66 ± 0.18 (8)
Caudate nucleus	2.23 ± 0.09 (56)	1.85 ± 0.21 (10)
Putamen	2.13 ± 0.11 (30)	1.72 ± 0.19 (10)
Substantia nigra	1.61 ± 0.07 (53)	1.16 ± 0.11† (15)

Values are mean ± SEM, μmol/g wet weight, with number of subjects shown in parentheses. † $P < 0.01$, as compared to control mean for the same brain region.

Animal experiments described in this thesis (BSO, glutathione monoethyl ester, BHA) have indicated that the total glutathione content of the brain is rather resistant to manipulation. Why then is the total glutathione content of the SN much lower in PD brains when compared to controls ? There are at least 4 possible interpretations. The first is that the lower amount is a reflection of atrophy of this brain region. However, this apppears unlikely since glia, which replaces lost neurons, also contain glutathione. In addition, there is no glutathione deficiency in the striatum in HC or in the cerebellar cortex in OPCA. Large numbers of neurons are lost from these areas in these 2 disorders.

The second possibility is that the reduced total glutathione content in

the SN of PD patients is a result of drug treatment. Most or all of the patients we studied were probably being treated with L-DOPA up to the time of death (we have no clear records of this). Since dopamine or L-DOPA themselves can autoxidise to produce free radicals (Graham et al. 1978; Tse et al. 1976), it is conceivable that the lower levels of glutathione represent an attempt by the SN to detoxify these radicals. However, this possibility appears untenable because the striatum of PD patients should similarly show a significant decrease of total glutathione content. The striatum, being the terminus of the dopaminergic nigrostriatal pathway, would surely have been exposed to high levels of L-DOPA or dopamine. Obviously, one means of confirming that the reduction of total glutathione content in the SN of PD patients was not due to L-DOPA treatment, would be to study autopsied SN of patients who were never treated with this drug. However, such patients are now few and far between.

A third possibility is that the glutathione deficiency of the SN in PD patients is due to decreased synthesis or accelerated breakdown of glutathione. For example, the activity of γ-glutamylcysteine synthetase, the rate-limiting enzyme for the synthesis of glutathione, might be reduced. If this were an inherent enzymatic failure inherited in certain individuals which predisposes them to the development of PD, then the genetic mechanism must be a very complex one, inasmuch as simple genetic predisposition does not appear to be a factor in the susceptibility of individuals to PD (Ward et al. 1983). Also, the lack of significant depletion of glutathione in other brain areas argues against a generalised enzymatic defect.

A fourth possibility which might explain the lower total glutathione content in the SN of PD patients relies on results from our animal experiments which indicate that free radicals might be involved in the molecular

mechanism of MPTP toxicity, and that glutathione appears to be critically important in the detoxification of these radicals. If the cause of nigrostriatal neuronal loss in idiopathic PD is a neurotoxin that forms free radicals, then excessive consumption of glutathione in the process of detoxification, with a consequent decrease in its levels, would be the result. The localised deficiency in the SN as opposed to other brain areas might be a reflection of the localisation of a neurotoxin in this area.

Would the patient still be exposed to the neurotoxin(s) at death? This appears likely since, otherwise, restoration of glutathione levels probably would have occurred. This hypothesis thus differs from that of Calne and Langston (Calne et al. 1983; Calne et al. 1985) who suggested that exposure to the neurotoxin occurs briefly many years before the appearance of symptoms.

What is the relevance of the time and duration of exposure to the putative neurotoxin? A recent review (Lewin 1985b) reported that a multi-centre clinical trial was being planned to determine whether or not α-tocopherol or deprenyl (an MAO-B inhibitor) could slow the progression of symptoms in PD patients at the early stage of the disease. If idiopathic PD is caused by an MPTP-like neurotoxin which destroys neurons possibly via free radicals, and if exposure to it is continous as we speculate, then α-tocopherol or deprenyl would be promising potential candidates to slow or stop the progression of the disease, an effect that none of the drugs currently used can achieve. Similarly, agents that can raise the content of brain glutathione might also be of benefit, although my results from animal experiments indicate that this is indeed a difficult task. On the other hand, if exposure to the neurotoxin occurred many years before the appearance of symptoms, then these potential treatment strategies are unlikely to be of benefit.

Assuming that the decreased total glutathione content in the SN of PD brains was due to an interaction between a neurotoxin (or its derivatives) and GSH, the question that remains is the nature of the reaction. Is it enzymatic in nature, and if so, what is the enzyme involved ? One possibility is the glutathione peroxidase system, which inactivates H_2O_2 or other peroxides by reacting with GSH. In the process, GSH becomes oxidised to GSSG (Deneke et al. 1980; Sinet et al. 1980). However, the latter is efficiently reduced back to GSH by the glutathione reductase system. At any rate, the overall total glutathione content is unlikely to be altered by the action of these enzymes.

Another detoxifying enzyme system involves glutathione transferase, which conjugates GSH with a wide variety of electrophiles (Habig et al. 1974; Jakoby et al. 1980). In the process of detoxification, the levels of GSH are lowered by a process that produces derivatives which cannot be readily transformed back to GSH. It therefore seemed possible that this enzyme could be responsible for the reduced content of total glutathione in the SN of PD patients.

We measured the activity of glutathione transferase in the frontal cortex and SN of patients who had died with a pathologically-confirmed diagnosis of PD. Brains that served as controls were mainly from subjects who died without any evidence of neurological disease; however, some were from patients who died with olivopontocerebellar atrophies (OPCA) or dialysis encephalopathy (DE). It was necessary to include these among the controls because insufficient non-neurological brains were available for the enzyme assay. The OPCA or DE patients had no evidence of any abnormality of glutathione metabolism. The control brains used for glutathione transferase assays matched PD brains with respect to death-to-freezing intervals of the

brain, as well as the duration of storage at -70°C. However, they were not age matched (Table 31).

Analysis of glutathione transferase was done by the modification of Spearman et al. (1984) of the method of Habig et al. (1974). Brain samples were homogenised in 50 volumes (w/v) of 0.25 M sucrose. The homogenates were centrifuged at 10,000 g for 15 minutes, and the supernatants then collected for analysis. Into a reaction cuvet containing 5 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene and 100 mM sodium phosphate buffer at pH 6.5, 100 µl of brain supernatant was added. The final volume of the mixture was 1.0 ml. The reaction was carried out at room temperature and its progress was followed spectrophotometrically at 340 nm every 15 seconds for 90 seconds. The average change in absorbance/15 seconds was corrected for a blank (when no tissue was present), and using a molar absorptivity value of 9.6 $mM^{-1}cm^{-1}$, glutathione transferase activity was calculated and expressed as nmol conjugate formed/min/mg protein. Analysis of each sample was done in duplicate and in a blind fashion. Protein content was determined by the method of Lowry (1951).

The results are shown in Table 31. Mean enzyme activities in the SN and frontal cortex of PD patients did not differ significantly from those of controls. The slightly higher activity in both regions of PD brains could be due to their greater mean age at death. Linear regression analyses of frontal cortex and SN of controls revealed a significant increase in enzyme activity with the age of subject at death ($r = 0.81$ for frontal cortex, and 0.74 for SN).

Table 31. Glutathione transferase activity in the frontal cortex and substantia nigra of PD patients

Subjects	Mean age at death (years)	GSH transferase activity (nmol/min/mg protein)
Frontal cortex		
Controls (23)	60 ± 3	183.1 ± 10.6
Parkinson's disease (17)	77 ± 2*	202.3 ± 13.5
Substantia nigra		
Controls (16)	63 ± 4§	204.2 ± 12.9
Parkinson's disease (12)	77 ± 2§	221.2 ± 15.7

Values (mean ± SEM) that differ significantly from controls: §$P < 0.05$; *$P < 0.001$.

Thus, the results presently available do not clarify the nature of the interaction(s) leading to the decreased total glutathione content in the SN of PD patients. A persistent non-enzymatic interaction of glutathione with oxidants, akin to the direct reaction of α-tocopherol with oxidants (Witting 1980), cannot be excluded.

IV) CONCLUSIONS

Several animal and human experiments have been described in this chapter. How have these aided in the understanding of the pathogenesis of idiopathic PD? My results suggest the following important points about idiopathic PD:

1. If neurotoxin(s) are responsible for most cases of idiopathic PD, they might act, as is possible in the case of MPTP, via a free radical mechanism. Decreased content of glutathione in the SN of PD brains may be the result of its continued consumption in the detoxification of potentially neurotoxic radicals.
2. PD patients might well be exposed to such neurotoxin(s) on many occasions throughout life, even after symptoms of PD have appeared, and perhaps until the time of death.
3. Antioxidants such as α-tocopherol or β-carotene might have value for the therapeutic prevention of further nigral neuronal loss.
4. It appears unlikely that high activity of MAO-B is a predisposing factor in the development of PD.

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Chapter 2 : HUNTINGTON'S CHOREA

ABSTRACT

We tested the hypothesis that the premature neuronal death that occurs in Huntington's chorea (HC) might be the result of a genetically-determined enzymatic failure in the degradation of a circulating neurotoxin of either endogenous or exogenous origin. Two types of studies were performed: an *in vivo* study in which rats were injected repeatedly with serum or serum ultrafiltrate from HC patients or control subjects, and an *in vitro* study in which rat striatal explants were exposed in tissue culture to serum or CSF from patients or controls.

In the *in vivo* study, there was a small but significant reduction in the mean striatal content of GABA in rats treated with whole serum from HC patients, but no striatal GABA deficiency was observed in rats treated with serum ultrafiltrate from HC patients. The results suggested that *if* a circulating neurotoxin contributes to the pathogenesis of HC, it must either be a small molecule that is tightly bound to serum proteins, or a large compound with a molecular weight greater than 10,000 daltons.

In the *in vitro* study, striatal explants exposed to 30% whole serum from adult-onset HC patients consistently showed deficiency of the activity of glutamic acid decarboxylase, the synthetic enzyme for GABA. Attempts to examine some characteristics of the putative neurotoxin in tissue culture indicate, as with the *in vivo* experiments, that the detrimental material may be either a small molecule tightly bound to plasma proteins, or a molecule larger than 10000 daltons. Experiments to delineate these possibilities further met with equivocal results, as did experiments that utilized CSF from HC patients. Possible reasons for the discrepancies are discussed.

The results obtained from both the *in vivo* and *in vitro* experiments provide suggestive but not conclusive evidence for the presence of a neurotoxin in the serum of patients with HC.

I) INTRODUCTION

I.i) Perspectives

Huntington's chorea (HC), also known as Huntington's disease, is a relentlessly progressive neurodegenerative disorder that is inherited in an autosomal dominant fashion with complete penetrance. The causative gene has recently been shown to reside on chromosome 4 (Gusella et al. 1983). In the North American population, the incidence of HC is about 10 in 100,000. In the majority of patients, the onset of symptoms occurs between the ages of 30 and 50 (Martin 1984). About 10% of cases manifest symptoms before the age of 15 and form a distinct clinical subgroup called 'juvenile chorea' or the Westphal variant of HC (Byers et al. 1973; Bird 1980). Remarkably, in most juvenile cases, the disease is transmitted by the father (Went et al. 1984). In contrast, Myers et al. (1983) found that more than twice as many of the late-onset cases they studied inherited the causative gene from an affected mother. Thus, there appears to be a sex-related modifying factor that influences the age of onset of the disorder.

Clinically, HC is characterized by a combination of physical and mental symptoms. Choreiform movements especially of the face, fingers and toes are common signs early in the course of adult-onset patients. As the disease progresses, other parts of the body become affected. Late in the course of HC, dystonia and parkinsonian features emerge, and patients are troubled by dysarthria and difficulty in swallowing.

In contrast, juvenile onset cases typically exhibit muscle rigidity, and chorea is minimal or absent (Byers et al. 1973; Spokes 1981). They may also have muscle spasms, tremor, disturbance of eye movements, and epilepsy.

In addition to the motor disability, mental and emotional problems are observed in both the juvenile and adult-onset varieties of HC. A gradual

intellectual decline, progressing to full-blown dementia, is a regular feature of the disease. Memory deteriorates, and patients have increasing difficulty doing arithmetical problems and carrying out organizational tasks (Caine et al. 1978). Many patients become seriously depressed at some point during the course of the disease, and the risk of their committing suicide is high. About 10% of patients become frankly psychotic, and such patients are frequently misdiagnosed as schizophrenics.

The progression of symptoms is inexorably downhill. The duration of the disease from onset of symptoms to death (unless suicide or accidental death intervenes) usually is about 15 years. Shoulson (1981) has devised a functional capacity rating scale for HC patients. It allots units ranging from a maximum of 13 for patients still retaining full abilities to zero for bedridden ones. In a large group of HC patients receiving conventional drug treatment (see below), he observed a mean loss of 1.8 functional units per year. Patients whose symptoms appeared before the age of 35 years tended to deteriorate more rapidly (Shoulson 1981).

The neuropathological status of many areas of the HC brain has been summarized by Bruyn et al. (1979). Although rather widespread degeneration is observed, neuronal losses are consistently most marked in the caudate nucleus, putamen, and cerebral cortex. Deterioration of the caudate nucleus and putamen, these structures being collectively called the striatum, results in a characteristic enlargement of the lateral ventricles. In the striatum, there is a disproportionate loss of small neurons (which average 8.5 μm in diameter) as compared to large neurons (Lange 1981). Loss of these small neurons may reach 70 or 80 % Dendrites of Type II neurons of the striatum, as studied by Golgi staining techniques, are markedly altered in appearance, showing recurved endings and appendages (Graveland et al.

1985). In the substantia nigra, the degeneration of neurons is usually confined to the more ventral pars reticulata (Bird 1980). The dopaminergic nigrostriatal tract, which originates in the pars compacta of the substantia nigra and terminates in the striatum, is relatively intact in HC (Cross et al. 1983). It is unclear whether or not the corticostriatal glutamatergic pathway, another major projection to the striatum, is atrophied (Shoulson 1984). The nucleus basalis of Meynert, which provides most of the cholinergic input to the cerebral cortex, is reported to be normal in HC (Clark et al. 1983). This contrasts with what is seen in Alzheimer's disease. In general, the brains of juvenile HC patients may have a greater degree of atrophy than that observed in adult-onset cases (Campbell et al. 1961).

Many neurochemical changes have been reported in the autopsied brains of HC patients. A marked deficiency of γ-aminobutyric acid (GABA) is present in many brain areas (Perry et al. 1973; Urquhart et al. 1975; Perry 1982a). These include the caudate nucleus, putamen, globus pallidus and substantia nigra. Activity of glutamic acid decarboxylase (GAD), the enzyme which synthesises GABA, is decreased in the caudate nucleus, putamen, and globus pallidus (Bird et al. 1973 and 1974; Urquhart et al. 1975). It is likely that both the GABA and GAD deficiencies of the afore-mentioned areas represent a loss of striatal GABAergic interneurons, as well as GABAergic neurons in the striatopallidal and striatonigral pathways.

Another neurochemical finding in HC brains is a decrease in the striatal activity of choline acetyltransferase (CAT), the enzyme that synthesises acetylcholine. This occurs in about 50% of patients (Bird et al. 1973; 1974). Substance P contents are reduced in the striatum, globus pallidus and substantia nigra (Kanazawa et al. 1977; Emson et al. 1980). There is also evidence suggesting subnormal concentrations of methionine-enkephalin

in the striatopallidal and striatonigral pathways (Emson et al. 1980). On the other hand, the content of the neuropeptide somatostatin is increased in the striatum and globus pallidus of HC brains (Aronin et al. 1983).

In general, the overall pattern of neuropathological and neurochemical changes suggests that certain populations of neurons within the cerebral cortex and the striatum, and those that extend from the striatum, bear the major brunt of pathology in HC.

Conventional drug therapies of HC patients use antidepressant drugs to treat symptoms of depression, and antipsychotic drugs, such as thioridazine or haloperidol, to reduce choreiform movements (Shoulson 1984). The anti-choreic effects of antipsychotic drugs probably derive from a reduction of nigrostriatal dopaminergic transmission which may be in a state of relative excess due to the loss of striatonigral GABAergic inhibitory influence (Shoulson 1984).

Although useful, antipsychotic and antidepressant drugs have no efficacy in slowing the progressive neuronal loss in HC (Shoulson 1981). Additional evidence supporting this contention is derived from the experience of HC patients belonging to a large kindred living near Lake Maracaibo in Venezuela. Of 65 untreated HC patients studied, the mean rate of decline observed was similar to that of North American drug-treated patients (Young et al. 1986). Thus, conventional drug therapies are palliative in nature, since they treat only the symptoms.

In attempts to provide more effective therapeutic management, efforts have been made to correct the deficiency of GABA content observed in many areas of the HC brain. Administration of GABA itself is ineffective since this amino acid does not readily cross the blood-brain barrier (Roberts et al. 1968). Attempts have been made to slow the enzymatic degradation of

GABA with inhibitors of GABA transaminase such as aminooxyacetic acid (Perry et al. 1980) or γ-vinyl-GABA (Scigliano et al. 1984), but this approach has failed to produce improvement in physical or mental symptoms. GABA receptor agonists such as muscimol (Shoulson et al. 1978) or imidazole-4-acetic acid (Shoulson et al. 1975) have also been tried, but produced no improvements in motor or cognitive functions.

The results of treatment with another brain GABA-elevating agent, isoniazid (INH), deserve mention, because some HC patients may respond favorably to this drug. INH itself is not a GABA-transaminase inhibitor, but is metabolized in the liver into many metabolites, one of which is hydrazine, a potent GABA-transaminase inhibitor (Perry et al. 1981). Elevation of brain GABA was recently demonstrated in the autopsied brain of an HC patient who died while under treatment with INH (Perry et al. 1985). In a non-blind clinical trial of INH, 3 of 6 patients showed significant improvement in mental symptoms (Perry et al. 1979a). However, a later double-blind and placebo-controlled crossover trial by the same investigators (Perry et al. 1982b) showed that only 1 of 9 patients benefited from INH therapy. Negative results were obtained by McLean (1982), who conducted a double-blind INH trial in 6 patients; although another trial of the same agent (non-blind) by Stober et al. (1983) showed that 4 of 11 patients were improved markedly in their functional capacity. Thus, the results are equivocal as to the beneficial effects of INH in the therapy of patients with HC. Clearly, a large scale placebo-controlled trial extending over a long period of time is needed to clarify the role of INH in the treatment of HC. A trial of this sort might answer the question as to whether or not chronic elevation of brain GABA content can prevent or slow the neurological and psychiatric deterioration in HC. Another agent with potential for treatment of

HC, baclofen, will be discussed in the next section.

Thus, although much is known about the neuropathological and neurochemical changes in HC, treatment of patients remains unsatisfactory at the present time. Currently available therapies do not halt or slow the progressive deterioration characteristic of the disease. Improvement of the therapeutic management of patients with HC, or prevention of the onset of symptoms in individuals at risk for the disease, requires a better understanding of the pathogenesis of the neuronal death which occurs most markedly in the striatum and cerebral cortex.

I.ii) Pathogenesis of neuronal death in Huntington's chorea

A key question which needs to be answered is how the mutant gene for HC causes the death of certain populations of neurons. Three main hypotheses have been advanced. The first suggests that the mutant gene may code for an abnormal protein which is present in all cell membranes. The second involves a defective DNA repair mechanism thereby predisposing cells to premature death. The third hypothesis postulates the presence of a neurotoxin which destroys neurons. Because this chapter deals principally with the etiology of HC, these possibilities merit discussion.

The suggestion that a generalized cell membrane defect may be involved in the pathogenesis of HC is based on a series of publications in the 1970s, all claiming to have found membrane abnormalities in cells obtained from peripheral tissues of HC patients. Since brain cells are relatively inaccesible for study, peripheral tissues were used with the hope that the HC gene might be expressed in these cells. Early accounts in the literature dealt with the growth characteristics of skin fibroblasts in tissue culture. Menkes et al. (1973) reported that cultured fibroblasts from HC patients had

a reduced growth potential and decreased life span when compared to those derived from normal controls. In contrast, Goetz et al. (1975) claimed that cultured HC fibroblasts grew to a greater confluent density and had a longer replicative life span than control fibroblasts. Shortly thereafter, Menkes and co-workers (Barkley et al. 1977), using a different culture medium from that of their previous experiment, retracted their original claim and confirmed the observations of Goetz et al. (1975) that HC skin fibroblasts did indeed grow to a greater cell density. This was later corroborated by several groups (Kirk et al. 1977; Leonardi et al. 1978; Goetz et al. 1979). On the other hand, Gray et al. (1979) were unable to detect any difference, either in generation time or in maximum population density, between cultured fibroblasts of HC patients and control samples. Cassiman et al. (1979), in a thoroughly blind-coded study of 22 controls, 22 'at-risk' individuals, and 25 HC patients, provided perhaps the most convincing results. Eleven parameters were tested and statistical discriminant analyses of the resulting data were analysed by computer. No significant differences between the 3 groups could be detected for any of the parameters measured. Meanwhile, Goetz et al. (1981) repeated their original study, this time using blind-coded samples, and found no statistical differences between cultured fibroblasts derived from HC patients and controls.

Thus, the growth characteristics of HC fibroblasts in tissue culture appear to be similar to those of controls. Early reports of a difference came from studies that were either not performed blind, or sample numbers were small in blind studies (two patients in the report of Kirk et al. 1977). Many factors can influence differential growth of cells in tissue culture. Among these are the medium used, the site and size of the skin biopsy, the type of local anesthetic used to obtain the skin biopsy, and the method used

to replenish the culture medium. These have been thoroughly reviewed by Beverstock (1985).

Membrane defects in erythrocytes of HC patients have also been described. Erythrocyte membrane ghosts from HC patients have been reported to have different electron spin resonance (ESR) characteristics from those of control subjects (Butterfield et al. 1977; 1979), although this has been disputed by at least 3 independent laboratories (Comings et al. 1981; Beverstock et al. 1982; Fung et al. 1982).

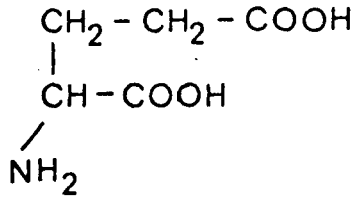
Other techniques have also led to claims of a membrane defect in HC. Cultured fibroblasts from HC patients reportedly differed from controls in their fluorescence spectra after labelling with a fluorescent probe (Pettegrew et al. 1979; 1981). This could not be confirmed by other investigators (Lakowicz et al. 1981; Sumbilla et al. 1982). Lymphocytes from HC patients have been reported to show decreased concanavalin A-induced capping when compared to control lymphocytes (Noronha et al. 1979). This observation has yet to be tested by an independent laboratory (Beverstock 1985).

In summary, present evidence does not support a generalized structural and/or functional defect in the cell membranes of peripheral tissues from HC patients. Claims have either been retracted by their original authors, or have been challenged by others. At any rate, the detection of an abnormal membrane protein in peripheral tissues would not help to explain why the mutant gene in HC affects consistently and markedly only certain populations of neurons.

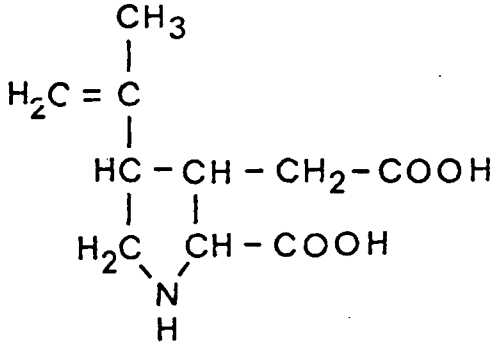
The second hypothesis regarding the etiology of HC is that the mutant gene codes for an abnormal DNA repair mechanism. The inherited neuronal degenerative conditions ataxia-telangiectasia and xeroderma pigmentosum have been shown to have associated defects in their DNA repair mechanisms. Cells

from these patients have an increased mortality rate in response to low doses of radiation or chemical mutagens (Taylor et al. 1975; Andrews et al. 1978; Paterson et al. 1979). In the case of HC, Arlett et al. (1979) reported that 4 peripheral cell lines from HC patients were more sensitive to gamma-irradiation than cells derived from normal controls. In another publication (Arlett 1979), 9 out of 11 HC cell lines tested showed varying degrees of sensitivity to gamma-radiation. These findings were later retracted, when a similar experiment was repeated with blind-coded samples and no evidence of any excess irradiation damage could be detected in the HC cultured fibroblasts (Arlett 1980). The latter negative result was also obtained by other investigators (Beverstock et al. 1982; Evan et al. 1982). Thus, the bulk of the evidence suggests that there is no specific defect in DNA repair mechanisms in HC cells which might render them more sensitive to DNA-damaging agents. Again, even if there were a specific defect in DNA repair, it would be difficult to reconcile this information with the observation that the mutant gene in HC affects markedly only certain populations of neurons.

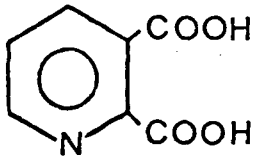
Perhaps the most likely hypothesis for neuronal loss in HC is that it occurs as a result of exposure to a neurotoxin(s), either endogenously formed, or from exogenous sources. The normal allele to the HC gene might code for an enzyme capable of metabolizing and thus detoxifying the putative neurotoxin(s). HC heterozygotes, with one normal and one mutant allele, might have subnormal enzyme activity, rendering them incapable of completely removing the neurotoxin(s). Repeated exposure of susceptible neurons to small amounts of the neurotoxin(s) over many years might eventually cause sufficient neuronal loss to produce the symptoms of HC. The selectivity of neuronal loss might be explained by an exclusive localization of receptors for such toxins on certain types of neurons.



Glutamic acid



Kainic acid



Quinolinic acid

Figure 1. Chemical structures of glutamic acid, kainic acid, and quinolinic acid.

The neurotoxin hypothesis is supported by the observation that at least 3 compounds are capable of producing neurochemical and neuropathological changes that resemble those of HC when injected into the striatum of animals. These are kainic acid (KA), glutamic acid, and quinolinic acid (Figure 1).

KA, a structural analog of glutamic acid, is a product of the Japanese seaweed Digenea simplex. When injected into the striatum of rats, KA produces neurotoxic effects closely resembling those found in HC striatum (Coyle et al. 1976; Coyle et al. 1978a; McGeer et al. 1976; McGeer et al. 1979; Coyle 1983). Intrinsic GABAergic and cholinergic neurons are destroyed, efferent axons of striatal neurons degenerate, while afferents to the striatum, such as the nigrostriatal tract, are spared.

Although KA is not found in the body (Beutler et al. 1981), the mechanism of its toxicity has received much attention in the hope that such studies would help to elucidate the possible cause of neuronal death in HC. KA is an excitant of most mammalian CNS neurons, as is glutamic acid, but it has a potency 300 times greater than that of the latter amino acid (McGeer et al. 1979). KA derivatives devoid of neuroexcitatory effects lack neurotoxic properties when tested on rat striatum (Schwarcz et al. 1978). These observations suggest that excessive neuroexcitation might be involved in the mechanism of toxicity of KA. KA is believed to bind to a subset of glutamate receptors (Coyle et al. 1979), and it does not appear to be transported by the high-affinity uptake system that terminates the synaptic actions of glutamate (Johnston et al. 1979). Thus, KA may persist at receptor sites for a longer period of time than glutamate. These observations have led Olney to propose an 'excitotoxic' mechanism for neuronal death (Olney et al. 1971; Olney 1974; Olney et al. 1979). According to this concept, no particular toxic action would be necessary, other than a persistent activation

of excitatory receptors. Damage would result from ionic shifts exceeding the capacity of membrane pumps to restore and maintain the resting membrane potential. The resulting ionic imbalance would lead to a state incompatible with continued survival of the neuron.

It is now known that for the neurotoxicity of KA to become manifest, an intact glutamatergic corticostriatal pathway is required. Lesions of this pathway in animals reduce the toxicity of KA by at least 2 orders of magnitude (McGeer et al. 1978). In the decorticate striatum, co-administration of glutamic acid and KA, neither of which is neurotoxic alone under such a condition, partially restores toxicity (Coyle et al. 1979). In addition, the rat striatum becomes sensitive to the degenerative effects of KA only when the glutamatergic innervation has matured (Campochiaro et al. 1978). KA-induced morphologic disturbances are observed in tissue culture of striatal cells only in the presence of glutamate-containing cortical neurons (Whetsell 1979).

These observations have led to speculation that glutamate itself may be involved in the pathogenesis of HC (Olney et al. 1978; Olney 1979; Sandberg et al. 1981; Shoulson 1983). In support of this contention is the finding that chronic infusion of L-glutamate produces neurotoxic effects in the rat striatum not unlike that seen in HC (Mangano et al. 1983; McBean et al. 1984; 1985).

To test the glutamate hypothesis, Shoulson and his colleagues (1983) have organized a double-blind and placebo-controlled trial of baclofen in a large group of HC patients. Baclofen preferentially blocks the neuronal release of excitatory amino acids, including glutamate (Potashner 1978; 1979), and its long-term administration might provide chronic pharmacological attenuation of the glutamatergic corticostriatal function. Shoulson's

group intends to continue therapy with either a placebo or baclofen for 3 years in approximately 60 HC patients who will not be permitted to take any other form of anti-choreic drugs during this period. It will be several years (when the code is broken) before it is known whether or not baclofen has helped to slow or prevent the progression of symptoms of HC.

A weakness of the glutamate hypothesis is that it fails to explain why the neurodegenerative process in HC is seen most markedly in only certain neurons. One of the most sensitive areas of the CNS to the neurotoxic effects of glutamate is the neural retina (Coyle et al. 1978b). Yet there appear to be no reports of retinal damage in HC. However, it is possible that susceptible neurons contain a subset of glutamate receptors not found in high quantities elsewhere and that these are specifically involved in HC.

Another problem with the glutamate hypothesis is that the contents of glutamate in the striatum and cerebral cortex of HC patients are not increased from that of controls (Perry et al. 1973). However, it is conceivable that a glutamate abnormality unrelated to the absolute levels of the neurotransmitter might be involved. For instance, a defective uptake mechanism, an increased release of glutamate from synaptic endings, heightened sensitivity of glutamate receptors, or a combination of these, might be present in susceptible neurons of patients with the disease.

As mentioned earlier, there is another agent which can destroy striatal neurons of animals. This is quinolinic acid (QA) (Figure 1), an endogenous metabolite of tryptophan formed in the kynurenine pathway (Stone et al. 1985). QA has been identified in the brains of several mammalian species, including man (Wolfensberger et al. 1983; Moroni et al. 1984), and when injected into the striatum of rats, it produces a kainate-like lesion (Schwarcz et al. 1983a; 1984). Rat brain shows differences in susceptibil-

ity to the neurotoxic effects of QA, with the striatum being one of the most susceptible areas (Schwarcz et al. 1983b). The physiological action of QA is neuroexcitatory. In this regard, the rat CNS shows regional variation in its sensitivity to QA, with the striatum again being one of the more sensitive areas (Perkins et al. 1983). Thus, as is probably the case for KA, the neurotoxic effect of QA appears to be related to its neuroexcitatory action.

It is conceivable that excessive accumulation of QA in the brain could result in the neuronal losses seen in HC patients. However, this appears unlikely, because activity of QA phosphoribosyltransferase, the degradative enzyme for QA, is normal in the putamen and is even slightly elevated in the caudate nucleus of autopsied brains of HC patients when compared to controls (Foster et al. 1985). Content of QA itself needs to be measured in the brains of HC patients before any further conclusions can be drawn. Our preliminary attempt, using a high performance liquid chromatographic system, at quantifying the amounts of QA in human brain has been complicated by the small amounts of QA present, as well as by the co-elution of QA with several other compounds.

Summarizing theories of the etiology of HC, the neurotoxin hypothesis appears less equivocal than those of a generalized membrane defect, or of abnormal DNA repair. The examples of KA and QA serve to highlight the possibility that there may exist an as yet unidentified neurotoxin(s) that might be responsible for selective neuronal death and the consequent symptoms of HC.

II) OUTLINE OF EXPERIMENTS

The preceding section suggests that HC might result from the exposure to a neurotoxin(s), either endogenously formed or of exogenous origin. Direct evidence for the involvement of this neurotoxin(s), as well as its identification, would be useful. If these could be achieved, the therapeutic management of HC patients might be greatly improved. For instance, if the putative neurotoxin proved to be from an exogenous source and was present in certain foods, then simple exclusion of the particular dietary substance(s) might halt or slow the progression of the disease in HC patients. For individuals genetically at risk, this diet-exclusion therapy might help to delay the onset of symptoms for several years, or might even prevent the disease entirely.

If the putative neurotoxin turned out to be endogenously produced, various pharmacological strategies for preventing its formation, or hastening its removal, might become possible. The imminent availability of the restriction fragment polymorphic DNA marker for preclinical identification of carriers of the HC gene (Gusella et al. 1983) makes it especially important to make rapid progress in developing a preventive therapy for HC. It would be helpful to have a practical treatment to offer individuals at risk for the disease, about 50% of whom when tested will receive very bad news.

The first requirement, however, must be proof of the involvement of a neurotoxin(s) in the pathogenesis of HC. During quantitative amino acid analyses of fasting plasma, Perry et al. (Appendix H, Perry et al. J. Neurol. Sci. 67:351-358, 1985) observed in 7 of 28 HC patients trace amounts of an unknown compound not previously found in normal subjects. This unknown compound yielded a yellow color when reacted with ninhydrin, suggesting that it might be an imino acid. It eluted from the chromatographic system very

close to proline and kainic acid, but was clearly not identical with either of them. It seemed possible that it might represent a neurotoxin that was circulating in the blood of HC patients. The *in vivo* rat study in Section III, in which rats were injected repeatedly with control serum or serum obtained from HC patients, represents our initial attempt to investigate this possibility.

The next series of experiments (Section IV) involved the use of tissue culture techniques. In brief, striatal explants from neonatal rats were cultured in a medium that contained either HC or control serum. After a period of incubation, these explants were analysed neurochemically.

The rationale of both the *in vivo* and *in vitro* experiments was that if the striatum of rats, as well as the cultured striatal explants, when exposed to HC serum showed neurochemical changes that resembled those seen in the same brain area in autopsied brain of HC patients, then the results might indicate the presence of a circulating neurotoxin in the blood of HC patients. Neurochemical measurements involved principally that of GABA content and GAD activity. Marked reductions of both GABAergic indices are well documented in the striatum of patients who die with HC (Perry *et al*. 1973; Urquhart *et al*. 1975; Perry 1982a; Bird *et al*. 1973 and 1974).

III) IN VIVO STUDIES

The protocols for this experiment are detailed in Appendix H (Perry et al. J. Neurol. Sci. 67:351-358, 1985) and will be presented briefly here. Non-fasting venous blood was obtained from control subjects or from HC patients with classical symptoms and a proven family history of the disorder. In an effort to avoid any drug effects, none of the patients or control subjects had taken any drugs during the 96 h prior to blood collection. The blood was centrifuged to obtain serum. The latter was left either untreated (henceforth, serum that has not undergone any alteration will be referred to as whole serum) for Experiment 1, or was passed through a filtration membrane of exclusion limit 10,000 daltons, to obtain a serum ultrafiltrate for Experiment 2. The volume of the filtrate obtained was approximately 80% of the volume of the original serum specimen. Some of the HC or control serum filtrates were lyophilized, and were then reconstituted with corresponding unlyophilized filtrates so that the final protein-free filtrate used for animal injection was concentrated 2-fold over that of the original filtrate.

For injections in Experiment 1, randomly-assigned 6 day old litter-mate Wistar rats were injected subcutaneously (sc) with whole serum from either HC patients or control subjects. Injections were administered once daily for 24 consecutive days, in injection volumes of 7 to 9% of body weight. The total volume of HC or control serum injected into each animal was 52 ml.

In Experiment 2, randomly-assigned 1 day old litter-mate Wistar rats were injected sc with a concentrated serum filtrate from either HC patients or control subjects once daily for 37 consecutive days. The total volume of concentrated serum filtrate that each animal received was 76.5 ml (the equivalent of 191 ml of original serum). An additional control group of rats was given 1.8% NaCl, with the injection schedule and volume being identical

to that of the concentrated serum filtrates. This group of animals served as hyperosmolar controls for the serum filtrate-injected rats.

After the final injection, rats from both experiments were left undisturbed until they were 5 months old. Observations during this period revealed no behavioral or movement disorders in the rats injected with HC serum or ultrafiltrate. Sacrifice was by cervical dislocation, and the brains were rapidly removed and frozen in liquid nitrogen. This rapid freezing procedure, within 40 s of death, was necessary to prevent any postmortem increases in GABA content (Perry et al. 1981b). After 20 s immersion in liquid nitrogen, the brains were gradually warmed to -10°C to allow for dissection of both halves of the striatum.

Striatal GABA content was measured on an amino acid analyser using a chromatographic system previously described (Perry et al. 1981b). In Experiment 1, the entire rat striatum was used for GABA analysis. In Experiment 2, only one-half of the striatum was used for GABA determination, while the other half was used for measurements of choline acetyltransferase (CAT) activity by the method of Fonnum (1975).

The results of Experiments 1 and 2 are shown in Tables 1 and 2 respectively. As shown in Table 1, the mean GABA content was slightly but significantly decreased (16%) in rats injected with whole serum from HC patients. In Experiment 2, however, mean striatal GABA content and CAT activity of HC and control serum filtrate-injected groups were similar. These values did not differ significantly from those of the hyperosmolar NaCl group.

Table 1. Striatal GABA content in rats injected with whole serum

Source of whole serum	Age of rats during daily injections (days)	GABA content (μmol/g wet weight)
8 HC patients	5 to 29	2.44 ± 0.09† (18)
8 control subjects	5 to 29	2.90 ± 0.16 (12)

GABA values are in mean ± SEM, with the number of animals examined neurochemically indicated in brackets. †$P < 0.02$.

Table 2. GABA content and CAT activity in striatum of rats injected with concentrated serum filtrates

Source of serum	Age of rats during daily injections (days)	GABA content (μmol/g wet weight)	CAT activity (nmol/10 min/ mg protein)
14 HC patients	1 to 37	2.51 ± 0.13 (16)	38.0 ± 2.1 (15)
19 control subjects	1 to 37	2.27 ± 0.09 (15)	40.0 ± 2.9 (13)
1.8% NaCl	1 to 37	2.55 ± 0.15 (16)	44.4 ± 2.9 (14)

Neurochemical values are shown as mean ± SEM. The number of animals examined is indicated in brackets. None of the means differ significantly ($P = 0.05$).

There are 2 possible interpretations of the results shown in Tables 1 and 2. Firstly, there may have been no neurotoxin present in HC serum. Secondly, if a neurotoxin was present, it may have been either a large molecule of over 10,000 daltons (and therefore removed by the ultrafiltration), or it may have been a small molecule that was so tightly bound to serum proteins that it did not reach the ultrafiltrate. The second interpretation

might explain the finding of a small but significant decrease in GABA content when whole serum was used (Experiment 1), but no difference when serum ultrafiltrate containing only molecules less than 10,000 daltons was used (Experiment 2).

In Experiment 1, it is possible that if the rats had been treated with a larger daily dose of whole serum, or if injections had been continued for a longer period of time, a more impressive reduction in striatal GABA content might have been obtained in animals given the HC serum. However, it was impractical to treat rats for longer periods with whole serum (HC or control) because many deaths occurred. Growth rate was slower in whole serum-exposed rats when compared to uninjected controls, and loss of body fur was extensive. Once the injections were discontinued, animals recovered rapidly and gained weight normally. Animals in Experiment 2 did not show any side effects from the protein-free ultrafiltrate injections.

The adverse effects encountered with whole serum in our in vivo experiment, these being presumably immunological in nature, prompted us to initiate experiments with tissue cultures in vitro.

IV) TISSUE CULTURE STUDIES

There are several advantages to doing striatal tissue culture experiments as opposed to *in vivo* injections of serum. A higher concentration of serum can be used *in vitro* without encountering the presumed immunological toxicities encountered *in vivo* (Section III). The blood-brain barrier, which is known to hinder the passage of some peripherally-injected substances into the brain, is not present in these *in vitro* experiments. Direct exposure of the striatum to large amounts of the putative neurotoxin also is possible *in vitro*.

The experiments described below were designed to test whether or not exposure to serum or CSF from living HC patients would reduce GABA content and GAD activity of rat striatal explants. A decrease (or increase) in GAD activity or GABA content of HC-exposed explants was deemed to be present when these values were reduced (or increased) by at least 25 % from those of the respective control values. Values that did not differ by more than 25% from controls were deemed unchanged. Further, some experiments were designed to elucidate the characteristics of the putative neurotoxin.

Each experiment compared a specimen obtained from a non-medicated HC patient with one from a non-medicated age and sex-matched control subject. All of the HC patients tested had a positive family history of the disorder, and all had symptoms for several years. Relevant descriptions of patients who supplied sera are shown in Table 3, while the patients who supplied CSF specimens are described in Table 8. Except for two juvenile HC patients, all the HC subjects were adult-onset cases. Experiments were always performed blinded with the samples coded.

Table 3. Characteristics of HC patients whose sera were used in tissue culture experiments

Patient	Juvenile or adult-onset	Age (years)	Duration of symptoms (years)	Experiment number
GW	Adult	48	7	1, 9, 14
LB	Adult	32	9.5	2, 5
JT	Adult	38	8.5	3, 16
JC	Adult	45	5	6
DE	Adult	58	3	8
JW	Adult	35	2.5	11
BX	Juvenile	13	μ	12
JS	Juvenile	28	9	15

All the patients listed above are males. Characteristics of HC patients who supplied CSF specimens are shown in Table 8.

IV.i) Methods

Brains were removed from newborn Wistar rats less than 24 h old and were placed in a Petri dish containing Hanks' balanced salt solution. Under the dissecting microscope, the right and left striata were excised. From each half-striatum, about 7 to 10 pieces of tissue were cut, each measuring approximately 1 mm^3. Henceforth, these tissues will be referred to as explants. Three explants were placed on a glass cover slip previously coated with rat tail collagen (Bornstein 1958). The cover slip was then inserted into a Kimax glass test-tube (15 x 160 mm). 0.7 ml of growth medium (see below) was introduced into each test-tube, and the unit was capped with a rubber stopper. All culture tubes were placed stationary at an angle of 5 degrees from the horizontal plane for the first 24 h, and subsequently were placed on a rotating drum where they remained at the same angle. Incubation temperature was maintained at 37° C.

Fifteen to 20 neonatal rats were typically used in each experiment shown in Table 4. This provided enough explants for 60 to 80 culture tubes. The

Table 4. Characteristics of tissue culture experiments

Proportion (v/v) and type of human serum or CSF in medium	Expt number	Name of patient	Duration of exposure of explants to medium (weeks)
15% whole serum	1	GW	1, 2, 5
	3	JT	1, 3
	15	JS	1
	16	JT	1, 3
30% whole serum	2	LB	1, 2, 3
	3	JT	1, 3
	5	LB	1, 3
	6	JC	1, 3
	8	DE	1, 3
	9	GW	1, 3
	11	JW	1, 3
	12	BX	1, 3
	15	JS	1, 3
	16	JT	1, 3
15% deproteinized serum	8	DE	1, 3
	9	GW	1, 3
	11	JW	1, 3
	15	JS	1, 3
	16	JT	1, 3
15% deproteinized and filtered serum	12	BX	1, 3
15% CSF	7	M	1
	10	X	2
	10	Y	2
	13	Z	2

Experiment 14 is not shown on this Table while Experiments 5 and 16 are described further in the text. Patient GW was tested 3 times (Experiments 1, 9 and 14), each time against a different control. Patients LB (Experiments 2 and 5) and JT (Experiments 3 and 16) were tested twice against different controls. There is no Experiment 4.

experiments listed in Table 4 are numbered according to the chronological order in which they were performed.

For the first 7 days of incubation, the nutrient fluid used for all experiments contained 70% by volume of a mixture of Eagle's minimum essential

medium (with Earle's salt, but without glutamine), 0.5% glucose, 20 µg/ml gentamicin, and was 2 mM in added glutamic acid. The remaining 30 % of the medium (v/v) was composed of horse serum. The chemicals and horse serum used were purchased from Gibco Laboratories.

After incubation for 7 days, the state of the explants was determined using a phase-contrast microscope at 10x magnification. Only explants with extended growth processes and insignificant dark necrotic areas were used for the next stage of the experiment.

Culture tubes containing healthy and growing explants were divided into a number of sets, depending on the design of the experiment (Table 4). Each set was then incubated with an appropriate medium containing serum or CSF from HC patients or control subjects. In all cases, the nutrient fluid consisted of 70% by volume of a mixture of Eagle's minimum essential medium (with Earle's salt, but without glutamine), 0.5% glucose, 20 µg/ml gentamicin, and was 2 mM in added glutamic acid. Where 30% by volume of human serum was used, horse serum was excluded from the medium. In experiments that utilised 15 % (v/v) of human serum or CSF, the remaining 15% was made up by horse serum.

After a further 7 days of incubation, explants were either harvested or were incubated with a fresh solution of their respective media once a week until they were ready for harvesting. Harvesting consisted of scraping off each explant with fine forceps from its collagen-coated cover slip, and placing it in Hanks' balanced salt solution for 5 minutes. Explants were then transferred to a fresh Hanks' balanced salt solution for another 5 minutes. These 'washes' were designed to rid the explants of adhering medium. Explants for GABA analysis were then transferred into a homogenization tube containing 0.4 M perchloric acid. Those to be used for GAD analysis were

frozen and stored at -70°C. Seven to 11 explants were pooled for each measurement of GABA content, and 10 to 15 explants were pooled for determination of GAD activity.

All the tissue culture procedures, with the exception of harvesting, were performed under sterile conditions.

Some experiments (Table 4) were done with deproteinized serum in the hope that deproteinization would remove most large molecules from serum samples, or would dislodge small molecules tightly bound to serum proteins. To deproteinize serum, 0.29 ml of 10 N perchloric acid was added in a dropwise manner to 10 ml of serum which was stirred continuously in an ice-bath. The resulting thick homogeneous solution was centrifuged at 10000g for 15 minutes at 4°C. Potassium bicarbonate crystals were then added to the stirred supernatant until the pH was 7.0. This removed excess perchlorate ions (as insoluble potassium perchlorate) in order to avoid detrimental effects on explants in culture. After another centrifugation, the supernatant of the deproteinized serum was mixed with the other ingredients of the culture medium and sterilized by passing it through a Nalgene filter unit (0.20 µm). The sera or CSF's used in the other types of experiment were similarly sterilized.

Other types of experiments performed will be detailed in the next section.

Analysis of GABA content was as described in Perry *et al.* (1982c), by means of a modified amino acid analyser that uses the fluorescence of the reaction products of amino acids with *o*-phthalaldehyde for detection of very small amounts of GABA. GAD activity was determined as described by Perry *et al.* (1979b), except that the specific activity of L-[1-^{14}C]-glutamic acid was increased to 0.1 µCi/µmole. GAD activity of explants was expressed as

μmol/h/g protein, while GABA content was calculated as nmol/explant. It was not possible to express GABA content in terms of the amount of protein present in explants because samples used for GABA analysis had first to be deproteinized. It was hoped that the measurement of GABA content from a pool of explants (7 to 11) similarly exposed to a particular culture medium would offset the difference in sizes between the explants.

IV.ii) Results and Discussion

Table 5 shows the individual values of GAD activity and GABA content of striatal explants obtained in Experiment 8. GAD activities of explants exposed to 15% deproteinized or 30% whole serum from the control subject were higher after three weeks of exposure than after one week. This was indicative of survival and continued growth of the explants, even though human material had been added to the growth medium. On the other hand, GABA contents were the same in explants exposed to 30% control serum for one or three weeks, and actually declined after three weeks of treatment in explants that had been exposed to 15% deproteinized control serum. In other experiments, the GABA contents of different sets of explants exposed to the same control serum similarly varied randomly.

Table 5. Individual values of GAD activity and GABA content of rat striatal explants exposed to 30% whole serum or 15% deproteinized serum of Experiment 8

Serum used	GAD activity (µmol/h/g protein)	GABA content (nmol/explant)
Exposure: 1 week		
Control (30% whole)	47.3	0.102
HC (30% whole)	26.6	0.175
Control (15% deproteinized)	50.5	0.197
HC (15% deproteinized)	35.1	0.127
Exposure: 3 weeks		
Control (30% whole)	66.1	0.102
HC (30% whole)	37.0	0.040
Control (15% deproteinized)	58.3	0.050
HC (15% deproteinized)	39.8	0.016

It should be emphasized that the GABA content of explants was expressed as nmol/explant. The hope was that measurement of GABA from a pool of explants would offset the differences in size between individual explants. However, if explants from a particular pool differed significantly in size from that of another, this would have resulted in erroneous quantitations of GABA values. Another possible source of error in using GABA contents as indicative of the viability of GABAergic neurons in explants is that the absolute level of GABA is subject to a variety of influences. The activity of the catabolic enzyme GABA transaminase, as well as the formation of GABA from putrescine (Seiler 1980), are two factors that could affect the content of GABA in explants.

Very recently (Experiment 15), we discovered another possible source of error in the determination of GABA contents of explants. For the harvesting procedure, earlier experiments had involved two five-minutes washes before the explants were placed in perchlorate. In Experiment 15, two different durations of washing procedure were used for explants exposed to the same medium. One was of ten minutes total duration (as was the case for Experiments 1 to 14), while the other consisted of only a quick rinse, such that the time elapsed between scraping the explants off the coverslip to when they were placed into perchlorate was less than a minute. It was found that there was an increase in GABA content of between 1.5 to 3 fold ($n = 2$) in explants that had undergone the ten minutes washing process, when compared to those explants that had been rinsed quickly. This resembles the situation for postmortem brains, where GABA content rises rapidly with increasing death-to-freezing intervals, reaching a stable maximum 2 h after death (Perry et al. 1981b). Thus, one of the contributing factors to the variability in GABA contents of explants could well have been differences in the duration of the washing process of explants.

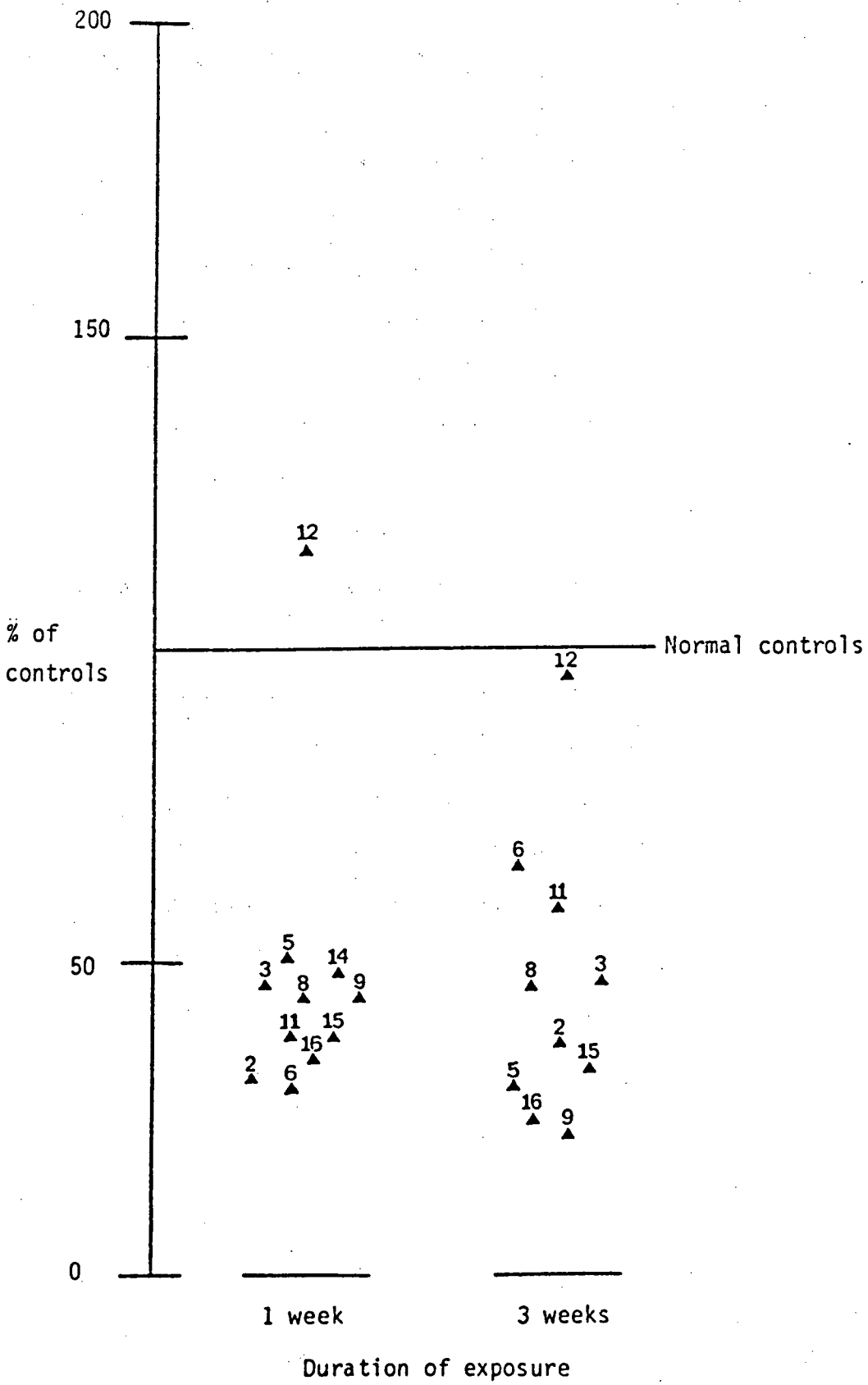
The random variability in GABA content of different pools of control explants precluded any meaningful comparisons of GABA contents between explants exposed to HC or control serum. For this reason, the results and discussion to follow will consist only of the GAD activity of explants.

Figure 2 indicates the GAD activities of striatal explants exposed to 30% whole sera from HC patients, when compared and expressed as % of GAD activities of explants exposed to the appropriate control sera. The results show that 30% HC serum produced a reduction in GAD activity after 1 or 3 weeks of exposure in 10 of 11 experiments. One interpretation of these results is that serum from HC patients contains a substance(s) that impairs the viability of cultured striatal GABAergic neurons of rats (a second possible interpretation is discussed below). The GAD results obtained in Experiment 12 (a juvenile-onset patient) were not consistent with those of the other 10 experiments. The reason is not immediately apparent and is unlikely to be due to the patient's having a juvenile onset, since serum from another juvenile-onset patient (Experiment 15) appeared to contain the putative neurotoxin. It may be that the diagnosis of HC was inaccurate for the patient in Experiment 12.

30 % sera from patients LB, JT and GW were tested twice, each time against a different control (see Table 4 for the respective experiments). Figure 2 shows that GAD activities of explants exposed to their sera were reduced compared to controls at all time periods (one or three weeks) tested.

Table 6 shows the consequence of treating explants with 15% whole serum from HC patients when compared with appropriate controls. In Experiments 1 and 3, GAD activity was initially increased in explants exposed to HC serum, but subsequently returned to normal, or was decreased relative to controls. The results of these two early experiments were interesting, because they were reminiscent of the 'excitotoxic' hypothesis of Olney (see Section I.ii).

Figure 2. GAD activities of explants exposed to 30% whole serum from HC patients for 1 and 3 weeks, expressed as % of their respective controls. Each triangle represents one comparison. The number above each triangle refers to the number of the experiment. There is no 3 weeks exposure for Experiment 14. None of the comparisons in this section has been analysed for statistical significance, since the sampling error for each determination of GAD activity is unknown. However, from previous work unrelated to this thesis, data exist for the coefficient of variation of the analytical technique used. Thus, the coefficient of variation for GAD analyses is 4.3 % (n = 17).


200
150
50
0
% of controls
Normal controls
12
12
6
11
5
14
3
8
9
11
15
16
2
6
8
3
2
15
5
16
9
1 week
3 weeks
Duration of exposure

It was as though initial exposure to low concentrations of the putative neurotoxin in HC serum stimulated GAD activity of striatal explants, while more prolonged exposure resulted in depressed GABAergic neuronal activity. Further experiments with 15% whole serum (Table 6), as well as with 15% serum that had been deproteinized (Table 7), similarly sometimes resulted in increased GAD activity. However, values that were unchanged or decreased from controls were also encountered after one week of incubation. It is possible that these seemingly random results represented a concentration effect, with 15% serum from patients with high concentrations of the putative neurotoxin suppressing GAD activity of explants, while serum from those patients with lower concentrations of the detrimental material either produced no change or an initial increase in GAD activity of explants.

The effects of CSF from HC patients, as 15% by volume of the medium, are shown in Table 8. The CSF from 3 of 4 patients had no effect on the GAD activity of explants. It was not possible to use 30% by volume of CSF (or, for that matter, of 30% by volume of deproteinized serum) in the culture medium, because this entailed the complete exclusion of serum proteins which are apparently required for the growth of explants in tissue culture. If a neurotoxin is present in HC, the GAD results in Table 8 would suggest that this putative neurotoxin is absent from CSF, or alternatively, that it was present in such low concentrations in 3 of the 4 patients that exposure of explants for a period of up to 2 weeks was inadequate to affect GAD activity.

Table 6. Effects of 15% whole serum from HC patients on GAD activity of rat striatal explants

Experiment number	HC patient	Duration of exposure of explants (weeks)	GAD
1	GW	1	=
		2	↑
		5	=
3	JT	1	↑
		3	↓
15	JS	1	↓
		3	n.d.
16	JT	1	↓
		3	↓

A decrease in GAD activity of explants exposed to HC serum of at least 25 %from that of control values is shown as↓ , while an increase of at least 25 % is indicated as ↑. Values that did not differ by more than 25% are shown as =. Non-determined samples are represented by n.d.

Table 7. Effects of 15 % deproteinized serum from HC patients on GAD activity of rat striatal explants

Experiment number	HC patient	Duration of exposure of explants (weeks)	GAD
8	DE	1	↓
		3	↓
9	GW	1	↑
		3	↑
11	JW	1	=
		3	=
15	JS	1	=
		3	↑
16	JT	1	↓
		3	↓

Refer to Table 6 for legend.

Table 8. Effects of 15% CSF from HC patients on GAD activity of rat striatal explants

Experiment number	HC Patient, Age (years)	Duration of symptoms (years)	Duration of exposure of explants (weeks)	GAD
7	M, 49	6	1	+
10	X, 48	Stage I	2	=
10	Y, 29	Stage I	2	=
13	Z, 34	Stage II	2	=

Refer to Table 6 for the legend. I have no information of the duration of symptoms of patients X, Y and Z, except for their stage of the disease. Patients M, Y and Z are females, while patient X is a male.

Experiments 5 and 16 represent our attempts to examine further some characteristics of the putative neurotoxin in serum from HC patients. For experiment 5, some of the serum collected was dialysed at 4° C for 48 h against a dialysate medium of 0.9% NaCl, containing 5% (v/v) of bovine serum albumin. Four changes at 12 h intervals of the dialysate medium were made. Another portion of the original serum was passed through the filter unit described in Appendix H. The filtrate was stored at -70°C prior to use. The protein fraction left behind on the filter was reconstituted to original volume in 0.9 %NaCl. The dialysed serum, as well as the filtrate and reconstituted protein fractions, were applied to explants at a 15% (v/v) concentration in the medium. In Experiment 16, the filtrate and reconstituted protein fractions from the serum of patient JT were also tested at 15% (v/v) concentration in the medium (dialysed serum was not tested).

Figure 3 shows the results. Whole serum, dialysed serum, and the reconstituted protein fraction which failed to pass the filter, collected from HC patients, all reduced GAD activities of explants, while HC serum filtrate

had no effect. Thus, it would appear that the putative neurotoxin present in whole serum from HC patients was absent in the serum filtrate. This result is in accord with that of the *in vivo* rat experiments (Section III). It suggests that if a neurotoxin is present in HC serum, it is either a large molecule of over 10,000 daltons, or it is a small molecule that is so tightly bound to serum protein that dialysis cannot easily dislodge it. A possible means of differentiating between these two alternatives would be to deproteinize serum by chemical denaturation, and to determine whether or not the putative neurotoxin is still present in the deproteinized supernatant from HC patients. This procedure should remove most large molecules, and also free protein-bound small molecules. If GAD activity of explants exposed to deproteinized serum from HC patients were decreased compared to that of controls, the possibility of the putative neurotoxin being a large molecule, or a protein that was perchlorate-*insoluble*, would be reduced. As shown in Table 7, the use of deproteinized serum as 15 % by volume of the medium resulted in GAD activities that were either increased, decreased, or unchanged from controls. If these results were due to a concentration effect of the putative neurotoxin, then it would appear unlikely that the neurotoxin was a large molecule, or a protein that was perchlorate-insoluble.

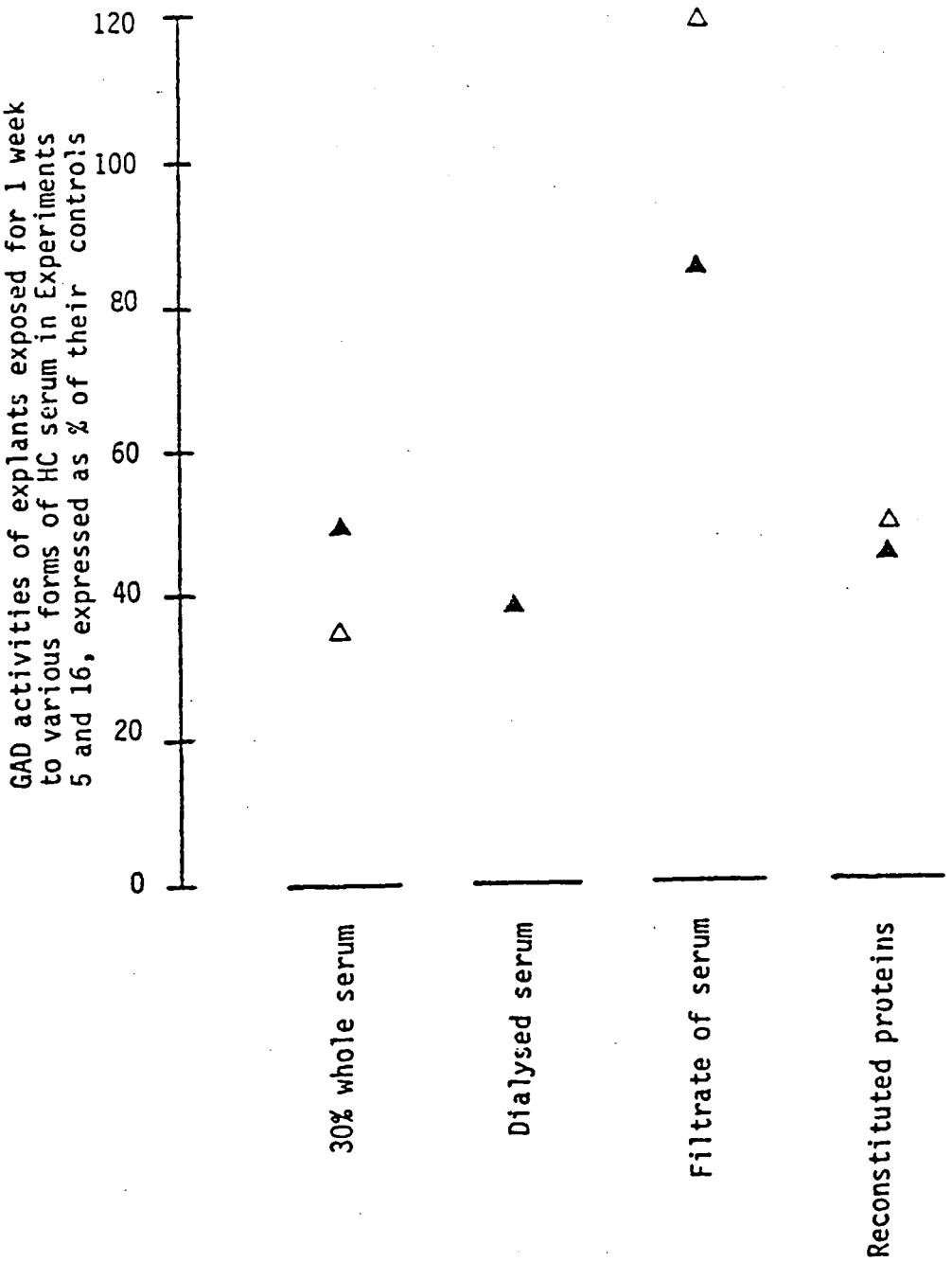


Figure 3. GAD activities of explants exposed to various forms of HC serum for 1 week, expressed as % of their respective controls. Closed triangles are values from Experiment 5, while open triangles are values from Experiment 16. Dialysed serum was not tested in Experiment 16.

Finally, two experiments (14 and 16) were designed to test possible dose-response relationships between HC serum in the medium, and GAD activity of explants. For experiment 14, serum from an HC patient or control subject was filtered as described in Appendix H. The protein fraction left behind on the filter was reconstituted to half its original volume with water, and then deproteinized as mentioned above with perchloric acid. After centrifugation, the pH of the supernatant was brought up to 7.0 with potassium bicarbonate. Another centrifugation removed the potassium perchlorate produced. The supernatant was then lyophilized to 1/12 the volume of the original serum and reconstituted with 0.9 %NaCl. Reconstitution was done in such a manner as to allow final concentrations (v/v) of original serum in the nutrient medium of 10, 20, 30, 50, 80 and 120%. Explants were exposed to these media for 1 week, after which the explants were harvested. However, all the sets of explants treated with these sera from either the control or HC patient had very low GAD activity (they were all around 7 ± 1 μmol/h/g protein, compared to a mean of about 50 μmol/h/g protein in control explants of other experiments). The extensive treatment of the original serum had presumably altered its properties in such a manner that it was no longer capable of sustaining GAD activity of explants. Comparisons between explants exposed to HC and control serum treated in this way were therefore not useful.

In Experiment 16, instead of using sera that had undergone alteration, whole serum was added to the medium as either 7.5%, 15%, or 30% (v/v) concentrations. It was found that the GAD activities of explants exposed to HC serum were equally reduced (over 60%) at one or three weeks of incubation at all three concentrations, when compared to the corresponding control serum media.

In summary, the results of the various experiments suggest the following. Firstly, 30 %whole serum from HC patients appears to contain a material that is detrimental to GABAergic neurons in cultured rat striatal explants. The putative neurotoxin may be either a small molecule tightly bound to protein, or, far-less-likely, a molecule larger than 10,000 daltons which is perchlorate-soluble.

As briefly alluded to above, there is another possible interpretation of our results, especially those obtained with 30% HC serum. Instead of a neurotoxin being present, it is possible that an essential growth factor for GABAergic neurons may be absent from HC serum. As a result, the viability of GABAergic neurons of rat striatal explants might have been impaired, resulting in reduced GAD activity when compared to controls. Although attractive, this appears unlikely, since experiments with 15 %HC serum (whole or deproteinized) sometimes produced increases in GAD activity of HC-serum exposed explants. The lack of a growth factor would certainly not have accounted for such increases.

Further experiments on explants exposed to HC serum might logically include morphological studies using electron microscopy or Golgi staining (Graveland et al. 1985) to identify actual neuronal deterioration. These procedures were not available to us. Experiments should also be performed using serum from patients with other neurodegenerative disorders (for example, Alzheimer's) to exclude the possibility that the GAD reduction in explants exposed to HC serum was due to a factor released into the blood after neurons had undergone degeneration. However, the absence of the inhibitor substance in CSF of HC patients probably argues against this possibility.

V) CONCLUSIONS

The results obtained in the *in vivo* and tissue culture experiments provide suggestive but not conclusive evidence for the presence of a neurotoxin in the serum of HC patients. The use of rat striatal tissues *in vitro* appears to be a useful technique for designing further experiments, particularly if the recommendations in the last paragraph of Section IV can be incorporated.

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CHAPTER 3: DIALYSIS ENCEPHALOPATHY

ABSTRACT

The absence of specific neuropathological abnormalities in dialysis encephalopathy (DE) suggests the possibility that neurochemical changes, unassociated with the loss of neuronal populations, might be present in the brain of patients who die with the disease. We looked for neurochemical abnormalities in the autopsied brains of 10 patients who died with symptoms of DE. Mean GABA contents were significantly reduced in frontal and occipital cortex, cerebellar cortex, dentate nucleus, caudate nucleus, and medial-dorsal thalamus of DE patients. Choline acetyltransferase activity was decreased by 25-35% in 3 cortical regions. Pyridoxal phosphate contents were substantially reduced in brains of the DE patients, but metabolites of noradrenaline, dopamine and serotonin were present in normal amounts. Aluminum levels were abnormally high in the frontal cortical gray matter of patients with DE.

In an effort to clarify the causative role of aluminum in DE, we attempted to produce a model of the disease in rats, using $Al(OH)_3$ to explore the possibility that this metal might produce the same neurochemical changes in rats as those observed in human patients. However, we failed to produce elevation of aluminum in the brains of rats treated with $Al(OH)_3$, even though we employed heminephrectomy, 5/6 nephrectomy, and/or chronic lithium administration in attempts to decrease the renal excretion of aluminum.

Thus, the question as to whether or not aluminum is the causative factor in DE was not clarified by our experiments.

I) INTRODUCTION

I.i) Perspectives

In 1972, Alfrey et al. described a distinct clinical syndrome of a progressive, fatal encephalopathy in 5 chronically hemodialysed patients. This syndrome, termed dialysis encephalopathy (DE) or dialysis dementia, has since been observed in many renal centres throughout the world where it constitutes a significant cause of death for chronically dialysed patients with end-stage renal failure (Platts et al. 1973; Mahurkar et al. 1973; Flendrig et al. 1976a; Chokroverty et al. 1976; Sideman 1982; Prior et al. 1982).

The first noticeable symptom of DE is usually a mild speech abnormality characterized by stammering, hesistancy, and at times, speech arrest. In conjunction with the speech defect, electroencephalographic (EEG) changes occur which include paroxysmal slow waves and sharp and spike activity, especially prominent over the frontal lobes (Alfrey 1978; Hughes et al. 1980). Initially, these symptoms are intermittent and typically more apparent during or immediately following dialysis (Alfrey et al. 1972; Flendrig et al. 1976a; Burks et al. 1976). As the disease progresses, the speech disturbances become continous and increasingly severe, manifesting as a combination of dysarthria, dyspraxia and dysphasia. The EEG abnormalities persist. A progressive dementia develops, and personality changes such as paranoid thinking, psychoses, and delirium are seen. In addition, patients display seizures, asterixis, twitching, motor apraxia, and myoclonic jerks. Death soon ensues, occurring usually a few months after the onset of symptoms; a mean of 6.3 months was reported for 60 cases (Chui et al. 1980).

The incidence of DE appears to be between 0.6 to 0.75% of the chronic renal failure population undergoing dialysis (Lang et al. 1979), and might be related to the duration of dialysis (Alfrey et al. 1976; Schreeder et

al. 1977; Berseth et al. 1978). An analysis of 60 cases revealed that the duration of hemodialysis prior to the onset of symptoms ranged from 6 months to 7.5 years, with a mean of 2.5 years (Chui et al. 1980).

Although usually confined to patients on long-term hemodialysis, DE has also been reported in patients very early in the course of chronic hemodialysis, as well as in patients on peritoneal dialysis (Sideman et al. 1980). In the more recent DE literature, the syndrome has also been reported in patients with renal failure who have never been hemodialysed, but to whom large amounts of oral aluminum hydroxide gels have been administered to reduce intestinal phosphate absorption (Etheridge et al. 1978; Nathan et al. 1980; Griswold et al. 1983).

In spite of the severity of the clinical features, neuropathological studies of DE have been unrewarding. In their original publication, Alfrey et al (1972) commented on the absence of neuropathological abnormalities in the brains of 4 patients. The study included electron microscopic observations. Of the 60 cases surveyed by Chui et al.(1980), 32 had been subjected to autopsy. Diffuse neuronal degeneration and/or gliosis was demonstrated in only 4 cases, but this was considered non-specific for the disease, since they were also observed in uremic patients who had never been hemodialysed, and who did not have DE. Similarly, the neuronal changes (e.g. cytoplasmic and nuclear shrinkage) and astrocyte proliferation reported by Burks et al. (1976) were also considered non-specific changes. To date, histological studies have not discerned any distinctive lesions, and have not clarified the origin of the clinical features of DE. In living DE patients, computed axial tomography scans have been described as normal (Parkinson et al. 1981).

Routine blood and cerebrospinal fluid analyses (e.g. glucose, protein and urea contents, cell counts, etc.) have not yielded results that differ significantly from those of other chronically hemodialysed patients who did

not have DE (Alfrey et al. 1972; Barratt et al. 1975; Alfrey 1978).

Brain neurochemical studies in DE, other than aluminum contents (see Section I.ii), have been lacking. A single report (Perry et al. 1977) found activities of glutamic acid decarboxylase and choline acetyltransferase, the synthetic enzymes for γ-aminobutyric acid (GABA) and acetylcholine respectively, to be low in cerebral cortex and caudate nucleus of 4 DE patients. However, these patients were comatose and had slow anoxic deaths, which can decrease the activity of both enzymes in the brain (McGeer et al. 1976).

The treatment of DE is generally unsatisfactory. Early in the course of the disease, the speech defect can be rapidly, albeit temporarily, improved by intravenous administration of benzodiazepines (Nadel et al. 1976; Sweeney et al. 1985). As the disease progresses, administration of dimercaprol, penicillamine, dexamethasone, levodopa, anticonvulsants, and vitamins, have all failed to alter the course of the disease (Editorial, B. Med. J. 1976). More recently, remission of symptoms has been achieved by the intravenous administration of desferrioxamine (Ackrill et al. 1980; Pogglitsch et al. 1981), although not all patients benefit from the use of this chelating agent (Milne et al. 1982). Renal transplantation has been reported to arrest the progression of symptoms, or even to afford substantial or complete recovery (Parkinson et al. 1981). However, intervention must occur at a very early stage of the disorder.

DE appears to be easier to prevent than to treat. A number of renal centres have reported a lowering of the incidence of DE when efforts were made to decrease the concentration of aluminum in the dialysate fluid (Flendrig et al. 1976a; Platts et al. 1977; Berkseth et al. 1978; Rozas et al. 1978). This observation, as well as others (see section I.ii), has led to aluminum being implicated as the causative agent of DE.

I.ii) Etiology of DE : The possible role of aluminum

Earlier observations (Parsons et al. 1971; Clarkson et al. 1972; Thurston et al. 1972) that serum and/or bone concentrations of aluminum (Al) were increased in renal patients receiving phosphate-binding Al gels orally, led Alfrey et al. (1976) to examine the Al contents in the brains of patients dying with DE. They found that the Al content of gray matter of DE patients was 4 times greater than that of non-demented dialysed patients, and 10 times that of normal controls. These authors also demonstrated a correlation between dialysis duration and the Al content of brain gray matter, muscle and bone in the DE patients. Similar findings of Al accumulation were soon reported by others (Flendrig et al. 1976b; Cartier et al. 1978; Dewberry et al. 1980), leading to the suggestion that Al intoxication might be the cause of DE. This possibility was immediately supported by a previous report of an individual who had worked in an Al powder factory for more than 13 years (McLaughlin et al. 1962), later developing a rapidly progressive encephalopathy with symptoms not unlike those since reported for DE.

Al is the most abundant metal and the third most common element. It comprises approximately 8% of the earth's crust (Boegman et al. 1984). Despite its prevalence, it has not been considered an environmental hazard, primarily because most of the naturally occuring Al compounds were thought to be inert. Also, the intestinal tract has been reported to present a rather formidable barrier to its absorption (Campbell et al. 1957; Poole et al. 1965). In addition, any excess Al in the blood appears to be promptly excreted by the normal kidney (Kaehny et al. 1977a). However, it is now known that the intestinal barrier is by no means impervious to the absorption of Al (Kaehny et al. 1977a; Recker et al. 1977), and more significantly, that in patients with already compromised renal function who are on hemodialysis, Al can enter the circulation from the dialysate (Kaehny et al.

1977b; Kovalchik et al. 1978; King et al. 1982). Indeed, Kaehny et al. (1977b) have measured the rate of Al transfer across the dialysis membrane, and have estimated that an average of 6 mg of Al is taken into the body per dialysis session.

That Al can traverse the dialysis membrane has furnished strong support for the causative role of Al in DE. Epidemiological studies reveal a markedly uneven geographical distribution of cases of DE that appears to correlate with water Al content. In those renal centres with a high incidence of DE, patients have been found to be exposed to dialysate containing a high concentration of Al, usually in excess of 200 µg/L (Flendrig et al. 1976a; Berseth et al. 1978; Elliot et al. 1978). A survey in the United Kingdom showed that DE was almost unknown in those renal centres where the Al concentration of the dialysis fluid was less than 50 µg/L, while the incidence increased progressively with higher water concentration of the metal (Parkinson et al. 1981).

A fascinating article by Flendrig et al. (1976a) reported 6 patients developing DE in an area of ordinarily low water Al content. When the dialysate was analysed, Al concentration was found to be greatly elevated. The source of contamination was traced to the gradual dissolution of 2 Al anodes which were part of a cathodic protection system against corrosion in the dialysate heating tank.

Al is commonly used as a flocculating agent in water purification plants and is not reliably removed by conventional water softening methods (Platts et al. 1977). In a number of centres where outbreaks of DE have occurred, the incidence of the syndrome has been reduced or eliminated when precautions have been taken to restrict oral Al intake and to remove trace metals from the dialysis fluid by means of deionisers or 'reverse osmosis' (Flendrig et al. 1976a; Platts et al. 1977; Berkseth et al. 1978), although this has

not been evident in every instance (Burks et al. 1976; Dewberry et al. 1980).

Oral Al intake is generally considered to be a less important source of intoxication than is dialysis fluid. This is mainly because the universal use of Al gels contrasts with the uneven incidence of DE. Nevertheless, DE has been reported in individuals with impaired renal function who have never been hemodialysed, but who consume large amounts of Al gels (Etheridge et al. 1978; Griswold et al. 1983). The incidence of DE was reported to be reduced in centres where oral Al usage was discontinued (McKinney et al. 1982). At other centres, DE symptoms disappeared when the dialysis Al concentration was reduced and oral Al usage discontinued (Buge et al. 1978). However, when oral Al was reintroduced, symptoms resumed and again abated when this practice was stopped (Buge et al. 1978; Masselot et al. 1978).

Despite much evidence indicating a role of Al in DE, there is still some uncertainty as to its etiological importance. Although many studies have shown the content of brain Al to be elevated in DE, the same finding, but without the symptoms of DE, is also present in Alzheimer's disease (Crapper et al. 1976), and in patients with renal failure, hepatic coma, or metastatic cancer (Arieff et al. 1979). Since the blood-brain barrier might be abnormal in patients with the last three conditions, it has been suggested that the elevated brain Al content seen in DE might be the result of increased permeability of the blood-brain barrier in this condition, rather than the cause of DE (Arieff et al. 1979).

In view of the epidemiological evidence correlating high incidence of DE to high concentrations of Al in the dialysate, and the striking reduction in the incidence of DE when the dialysis fluid has been deionised, Arieff (1981) has suggested that heavy metals might play a role, but pointed out that metals other than Al have not been excluded as the causative factor. Others

(Gacek et al. 1979) have cautioned that factors such as the pH of the dialysate might be important.

Animal experiments have been performed to demonstrate CNS toxicity of Al and its role in DE. Berlyne et al. (1972) reported that fatal intoxication can be produced within 3 to 8 days in partially (5/6) nephrectomised rats when 1 to 2% Al salts were added to the drinking water. Prior to death, these rats developed periorbital bleeding, lethargy, and anorexia. Similarly, fatal intoxication was observed in 5/6 nephrectomised rats, as well as in rats with normal renal function, when Al salts (150 mg/kg as elemental Al) were injected intraperitoneally, but not subcutaneously (Berlyne et al. 1972). In another study, rats with intact renal function administered oral aluminium chloride or aluminium hydroxide ($Al(OH)_3$) by gastric intubation twice daily in doses of 200 to 1650 mg/kg/day, had a high mortality at the higher dose levels, often associated with gastric distension (Bowdler et al. 1979). At the lower doses, the same authors reported modest elevations in serum and brain Al, and stated that the rats showed 'rapid general activity', decreased ability to maintain rotorod activity, and increased sensitivity to flicker. No ill effects were noted by Arieff et al. (1979) when subtotally (15/16) nephrectomised rats were fed for 5 months a diet containing 300 mg/kg $Al(OH)_3$, although brain gray matter Al content increased from less than 1 µg/g dry weight to more than 4 µg/g dry weight. Mayor et al. (1980) demonstrated that higher amounts of Al were retained in the brains of rats fed chow supplemented with aluminum chloride and given parathyroid injections, when compared to rats fed the Al compound alone. Over a 25 day period of injections, these authors observed no behavioral changes in the rats of either group.

In general, the animal experiments described above have not clarified the role of Al in DE. Where behavioral tests were performed on animals

intoxicated with Al compounds, alterations in behavior were either absent, not conspicuous, or were not clearly related to those seen in DE patients. It is possible that studies relying on behavioral alterations, in conjunction with brain Al accumulation, would not be helpful in elucidating the role of this metal in DE, since behavioral changes in animals might not be readily reconciled with the symptomatology of DE patients.

II) OUTLINE OF EXPERIMENTS

The absence of specific neuropathological abnormalities in DE (Alfrey et al. 1972; Burks et al. 1976; Chui et al. 1980) suggests that neurochemical changes unassociated with the loss of major neuronal populations might be present in the brains of DE patients. Since neurochemical studies (other than measurement of Al content) have been lacking in this condition, we decided to examine neurochemically the autopsied brains of DE patients in the hope that some abnormalities might be found. It was a possibility that findings of a specific change(s) might lead to the development of more effective treatments for patients with DE.

After determining some of the neurochemical changes of DE, we attempted to produce a rat model of the disease using $Al(OH)_3$, to explore the possibility that this compound might produce the same neurochemical changes in rats. It seemed likely that neurochemical rather than behavioral end-points might furnish better evidence for or against a causative role of Al in DE. This question is critical since many renal failure patients are currently treated by repeated hemodialysis and are given large amounts of phosphate-binding Al gels.

III) HUMAN STUDIES

III.i) Method

The methodologies used for the neurochemical examination of the brains of DE patients are detailed in Appendix I (Perry et al. J. Neurochem. 45: 1043-1048, 1985). Ten patients were studied: 8 had clear-cut symptoms of DE with seizures, myoclonus, typical EEG changes, dementia, dyspraxia, and the characteristic language disorder. The remaining 2 patients died with neurological symptoms highly suggestive of DE, but they lacked the characteristic language disorder. All 10 subjects had died with chronic renal failure after treatment by repeated hemodialysis, and all had ingested Al phosphate-binding gels. Further information on the patients, as well as on the control subjects, are available in Appendix I.

The various measurements performed included contents of GABA, metabolites of dopamine, noradrenaline and serotonin, and contents of pyridoxal-5-phosphate (PLP), the cofactor for several enzymes, including the synthetic enzyme for GABA, glutamic acid decarboxylase (GAD). Enzyme activity of choline acetyltransferase, the biosynthetic enzyme for acetylcholine, was determined. Brain Al content was also examined.

III.ii) Results and Discussion

Mean GABA contents were significantly reduced in 6 regions of DE brains when compared to control subjects (Table 1), the reduction being at least 40% in frontal and occipital cortex and in the thalamus. This observation is interesting, since the language disorder as well as the EEG changes in DE can often be reversed temporarily by intravenous administration of benzodiazepines, which have recognition sites linked functionally to GABA receptors (Baestrup et al. 1982).

Table 1. Regional GABA contents of autopsied brain in DE and control patients

Patients	Frontal cortex	Occipital cortex	Cerebellar cortex	Caudate nucleus	Putamen
Controls	1.67±0.09 (26)	1.83±0.11 (25)	1.65±0.08 (26)	2.90±0.14 (32)	2.91±0.20 (19)
DE	0.96±0.04* (10)	0.99±0.06* (10)	1.18±0.10† (10)	1.91±0.11* (10)	2.35±0.20 (10)

	Globus Pallidus	Substantia nigra	Nucleus Accumbens	Thalamus	Dentate Nucleus
Controls	7.32±0.40 (16)	6.05±0.26 (28)	4.15±0.29 (23)	2.05±0.12 (35)	4.77±0.23 (18)
DE	6.40±0.35 (10)	5.53±0.37 (10)	3.35±0.30 (9)	1.20±0.10* (10)	3.86±0.28§ (10)

Values (mean ± SEM) are expressed in μmol/g wet weight, with number of brains examined shown in parentheses. Medial-dorsal region of the thalamus was analysed. Values that differ significantly from those of controls: *$P < 0.001$; †$P < 0.01$; §$P < 0.05$.

Why are GABA contents reduced in several brain regions in DE? One possibility is that this neurochemical finding is a result of selective loss of GABAergic neurons, as is the case in Huntington's chorea (Perry et al. 1973). However, examinations of the autopsied brains of DE patients have generally not shown any neuropathological changes. Another possible explanation is that the reduction in GABA content is due to uremia, since 8 of the 10 DE patients had elevated brain urea contents at death. Urea contents can be reliably measured on our amino acid analyser. Our results show that in the two DE patients who died with normal brain urea levels, the reduction of GABA contents were as marked as that of the remaining 8 patients. In addition, we compared GABA contents of the frontal cortex in a group of 10 control patients who died severely uremic (brain urea contents greater than 10

times normal) with a group of 26 patients who died with low to normal brain urea contents. Mean GABA contents in the 2 groups, neither of which had DE, were almost identical (1.64 ± 0.09 μmol/g wet weight in uremic brains, compared to 1.67 ± 0.09 in non-uremic brains, mean ± SEM). It thus appeared that the reduction in GABA content in the brains of DE patients was not due to uremia.

Yet another possibility for the reduction in GABA content in DE is that the activity of GAD, the synthetic enzyme for GABA, is reduced. This possibility cannot be readily tested, since GAD activity is often markedly reduced in brain after prolonged anoxic dying (McGeer et al. 1976). A further likelihood for the decrease in GABA content in the brains of DE patients is that PLP, the cofactor of GAD, is present in deficient amounts in the brain of DE patients. Plasma PLP concentrations have been reported to be reduced by about 50 % in uremic patients, whether or not they have been hemodialysed (Stone et al. 1975; Lacour et al. 1983). If brain PLP contents were reduced in DE, and if greater amounts of this cofactor were required for the optimal activity of GAD than for GABA aminotransferase (the catabolic enzyme for GABA that also requires PLP as a cofactor), this could account for the reduced GABA contents. We did find brain PLP contents to be reduced (Table 2), but this is unlikely to be an explanation for the GABA deficiency, since PLP levels were reduced in both the frontal cortex and putamen, while GABA content was markedly reduced only in the cortex. Also, the normal contents of the metabolites of noradrenaline, dopamine and serotonin in 3 regions of DE brains (Table 3) imply that the DE brain contains reasonable amounts of PLP, since PLP also serves as the cofactor for aromatic L-amino acid decarboxylase, the enzyme essential for the production of these 3 biogenic amines.

Table 2. Pyridoxal phosphate content of autopsied brain of DE patients

Subjects	Frontal cortex	Putamen
Controls	2.96 ± 0.24 (14)	2.10 ± 0.24 (10)
DE	1.53 ± 0.10* (10)	1.41 ± 0.28§ (10)

Values (mean ± SEM) are expressed in nmol/g wet weight, with the number of samples analysed indicated in brackets. Values that differ from control values: *P < 0.001; § P < 0.05.

Table 3. Contents of monoamine metabolites in autopsied brain of DE patients

Patients	Frontal cortex	Caudate nucleus	Putamen
		MHPG	
Controls	0.39 ± 0.07 (13)	0.47 ± 0.10 (8)	0.52 ± 0.12 (13)
DE	0.37 ± 0.17 (10)	0.66 ± 0.14 (10)	0.71 ± 0.14 (9)
		HVA	
Controls	0.79 ± 0.31 (13)	22.3 ± 2.48 (8)	31.5 ± 3.63 (15)
DE	0.95 ± 0.09 (10)	23.4 ± 2.14 (10)	30.8 ± 2.02 (10)
		5-HIAA	
Controls	0.61 ± 0.10 (13)	3.35 ± 0.69 (8)	4.88 ± 0.59 (15)
DE	0.89 ± 0.13 (10)	3.56 ± 0.53 (10)	5.07 ± 0.67 (10)

Values (mean ± SEM) are in nmol/g wet weight, with the number of brains examined indicated in brackets. None of the DE values differ significantly from those of controls. MHPG (3-methoxy-4-hydroxyphenylethylene glycol) is the metabolite of noradrenaline, HVA (homovanillic acid) is the metabolite of of dopamine, while 5-HIAA (5-hydroxy-indoleacetic acid) is the metabolite of serotonin.

Table 4 shows a small decrease (25 to 35%) in mean CAT activity in 3 areas of cerebral cortex in the 10 DE patients. The reduction in enzyme activity was of equal magnitude in the 2 patients with some symptoms of DE, and in the 8 with the clear-cut syndrome. However, the decreased CAT activity in the DE patients was less marked than that observed in Alzheimer's disease (Perry et al. 1980; 1983), or in demented patients with Parkinson's disease (Perry et al. 1983).

Table 4. Choline acetyltransferase activity in cerebral cortex of DE patients

Patients	Frontal cortex	Temporal cortex	Occipital cortex
Controls	1.07 ± 0.05 (16)	1.01 ± 0.08 (15)	0.64 ± 0.08 (12)
DE	0.73 ± 0.09† (10)	0.76 ± 0.15 (9)	0.42 ± 0.07§ (10)

Values (mean ± SEM) are expressed as nmol/10 min/mg protein. Figures in brackets indicate the number of brains examined. Values that differ from controls: § $P < 0.05$; † $P < 0.01$.

The Al content of the gray matter of the frontal cortex of 15 control subjects was 5.6 ± 0.6 µg/g dry weight, with a range of 2.0 to 9.9 µg/g. For the 10 DE patients, the same brain area had a mean Al content of 21.8 ± 4.2 µg/g dry weight ($P < 0.001$ compared to controls, Student's t-test), ranging from 11 to 56.2 µg/g. This increase in the DE patients was of the same magnitude as that found by other investigators (Flendrig et al. 1976; Cartier et al. 1978). Thus, Al accumulation in brains of DE patients seems to be a feature of the disease, although not necessarily an etiological one.

In summary, we found mean GABA contents to be reduced in several brain regions of DE patients. A small decrease in the enzymic activity of CAT was also observed in 3 cortical areas. Normal contents of the metabolites of noradrenaline, dopamine and serotonin, suggest that the synthesis and turnover of these biogenic amines in the DE brain are not different from normal.

IV) ANIMAL EXPERIMENTS

IV.i) Method

5 groups of young adult Wistar rats of either sex were injected subcutaneously once daily for varying periods of time with a continuously agitated suspension of $Al(OH)_3$ in water. The rationale for having 5 groups of Al-treated rats, in which the various experimental characteristics are shown on Table 5, will be clarified in the Results and Discussion section.

Table 5. Treatments given to aluminum-injected animals

Experimental group	$Al(OH)_3$ dosage	Extent of nephrectomy	Duration of LiCl pretreatment
A	150 mg elemental Al/kg daily for 49 days	None	None
B	200 mg elemental Al/kg daily for 14 days	None	None
C	200 mg elemental Al/kg daily for 14 days, starting 14 days after nephrectomy	5/6	None
D	200 mg elemental Al/kg daily for 3 or 5 days, starting 24 h after nephrectomy	5/6	16 weeks
E	200 mg elemental Al/kg daily for 30 days, starting 24 h after nephrectomy	1/2	16 weeks

Prior to injections, some rats underwent surgical nephrectomies, in which only the right kidney (Group E), or the right kidney as well as 2/3 of the left kidney (Platt et al. 1952) were removed (Groups C and D; henceforth, this extent of nephrectomy will be referred to as 5/6). The volume of suspension injected (20 ml/kg) was sufficient to provide a daily dose of

elemental Al of 150 mg/kg in Group A rats, and of 200 mg/kg in Groups B, C, D, or E rats. Appropriate groups of control rats, similarly nephrectomised or non-operated, were injected subcutaneously with equal volumes of 0.9% NaCl for the same periods of time.

After the last $Al(OH)_3$ or saline injection, rats were sacrificed by cervical dislocation, and their brains were removed and immersed in liquid nitrogen within 40s. Brains were frozen immediately to prevent or minimize any postmortem increase in GABA contents (Perry et al. 1981). Brains were then allowed to thaw to -10 C to allow for accurate dissection into discrete areas.

The entire cerebellum and brainstem from each specimen were first removed for assay of Al content. The remaining brain was then bisected midsagitally, and one half was stored at -70 C for subsequent GABA analysis. From the other half, striatum was dissected for measurement of its dopamine content, portions of frontal cortex were removed for GAD assay, and portions of occipital cortex for CAT assay.

At the time rats were sacrificed, blood was also collected for measurement of serum urea and Al concentrations.

All the dissecting instruments and containers for storage of samples for subsequent Al analyses had been previously soaked (for 24 hours) in 0.6% (w/v) disodium EDTA to remove any contaminating Al.

Measurements of GABA content and CAT enzyme activity were performed as described for human brains in Appendix I. Dopamine content was determined using the method employed for rat brain described by Perry et al. (1984). GAD enzyme activity of rat frontal cortices was measured using a method which analysed the rate of formation of $^{14}CO_2$ from L-[1-^{14}C] glutamic acid (Perry et al. 1979).

Two different methods were used to measure Al contents of rat brain. The first (used for Groups A, B and C rats) was as described above for human brains (Appendix I). However, this method yielded a relatively high blank value, which increased with time after reconstitution of the brain, probably due to dissolution of glass which contains relatively large amounts of Al (Sansoni et al. 1980). To avoid this problem, a second method was employed for later brain specimens (Group D and E rats). Samples were dried to constant weight in plastic vials (Fisher linear polyethylene liquid scintillation vials) and were then dissolved in 2 ml of 5% (w/v) tetramethylammonium hydroxide by heating at 90° C for 1 or 2 h. Thus, instead of dry-ashing for 16 h, a tissue dissolution method which employed an alkali and which took a relatively shorter time, was used. The resulting solutions were then diluted by adding 4 volumes of ethanol and were analysed for Al as done with the human specimens. Repeated analyses of Bovine Liver Standard Reference Material No. 1577a from the U.S. National Bureau of Standards with this technique yielded an Al value of 2.8 ± 0.9 μg/g (mean ± SD). A non-certified Al value of 2 μg/g is given for this material by the National Bureau of Standards. Al measurements of rat frontal cortices by either method were done in duplicate.

Serum urea nitrogen concentrations were determined on a routine clinical analyser (Astra 8, Beckman Instruments) with an enzymatic urease-conductivity rate method.

IV.ii) Results and Discussion

Lack of neurochemical changes in rats with normal renal function injected chronically with $Al(OH)_3$ (Group A rats)

In preliminary experiments, the subcutaneous injection of acidic solutions of $AlCl_3$ in rats was followed by severe tissue necrosis. We thus elected to treat rats with daily subcutaneous injections of a neutral sus-

pension of the relatively insoluble $Al(OH)_3$. This offered the advantage of a depot of Al to provide continous exposure to large amounts of the metal.

Young litter-mate female rats were injected subcutaneously with 150 mg elemental Al/kg as $Al(OH)_3$ (Table 5, Group A), or with saline solution, once daily for 49 days. Although the Al-injected rats developed subcutaneous scarring at the injection sites on their backs, they grew normally, their mean weight increasing from 68 to 186 g over 49 days. The saline-injected controls had a corresponding mean weight gain from 70 to 175 g. After 30 days of injections, Al rats became aggressive, and frequently adopted a boxing stance that lasted for about an hour after injections. Saline-injected rats showed no changes in behavior. Before being sacrificed, 24 h after the last of the 49 injections, all rats were observed for gross motor abnormalities in an open field apparatus, and tested on a rotating drum for motor incoordination. No differences were observed between Al-treated and control rats.

Table 6 shows that in Group A rats, the chronic administration of $Al(OH)_3$ did not elevate brain Al content, and that brain GABA content remained unaltered. Quantitation of other amino acids in the brain also revealed no differences between Al-treated and control rats. Cortical activities of GAD and CAT, as well as of striatal dopamine content, similarly did not differ between the Al and control groups.

Table 6. Neurochemical parameters in Group A rats after chronic Al exposure

Animals	Al content (µg/g dry weight)	GABA content (µmol/g wet weight)	GAD activity (µmol/h/g protein)	CAT (nmol/10min/ mg wet weight	Dopamine (µg/g wet weight)
Controls	6.1 ± 1.3 (12)	2.62 ± 0.08 (12)	239 ± 13 (12)	1.13 ± 0.07 (10)	12.7 ± 0.8 (12)
Aluminum-injected	5.0 ± 0.9 (14)	2.52 ± 0.09 (16)	252 ± 11 (16)	1.11 ± 0.06 (10)	12.6 ± 0.7 (12)

Values are mean ± SEM, with number of animals in brackets. Rats were killed 24 h after the last of 49 daily sc injections of $Al(OH)_3$ in a dosage providing 150 mg/kg/day of elemental Al. None of the means for the 2 groups differ significantly (two-tailed Student's t-test, $P = 0.05$). Al content was measured in the cerebellum, GABA content was determined in whole brain minus the cerebellum, dopamine concentration was analysed in the striatum, and GAD and CAT activities were assayed in the frontal and occipital cortices respectively. Brain aluminum was determined by the dry-ashing EDTA method.

Absorption of $Al(OH)_3$ suspension

In view of the failure to obtain Al accumulation in the brains of rats treated with the relatively insoluble $Al(OH)_3$ (Group A rats, Table 5), it was important to prove that Al was indeed absorbed from subcutaneous depots. The lack of absorption of Al might have accounted for our inability to produce neurochemical changes in these rats.

Eighteen rats were given daily subcutaneous injections of either $Al(OH)_3$ equivalent to 200 mg/kg of elemental Al (Table 5, Groups B and C), or of saline, for 14 consecutive days. No mortality was encountered. The dose of elemental Al was increased from 150 to 200 mg/kg in the hope of elevating the body load of Al for this and subsequent planned experiments. In addition, Group C rats had also undergone 5/6 nephrectomies 14 days before $Al(OH)_3$ injections were started.

Table 7 shows that serum Al concentrations were markedly elevated in rats injected with $Al(OH)_3$, regardless of whether or not they had been

nephrectomised. At autopsy, visual inspection showed that only small amounts of the $Al(OH)_3$ injected remained unabsorbed in the subcutaneous space at injection sites. Also, one month after surgery, the remaining 1/3 of the left kidney had hypertrophied to a size as large as an undamaged kidney. Presumably, relatively normal renal function had been regained by this time, this possibility being strenghtened by the observation that serum urea concentrations were elevated only 2 fold in the 5/6 nephrectomised rats (Table 7) at the time of sacrifice (Table 8 shows that 4-6 days after 5/6 nephrectomy, serum urea is about 4-6 times higher than in control rats).

Table 7. Evidence for circulatory absorption of subcutaneously-injected $Al(OH)_3$

Experimental group	Surgical pretreatment	Elemental aluminum dose, 14 injections	Serum aluminum (µg/L)	Serum urea (mmol/L)	Brain aluminum (µg/g dry weight
Controls (6)	None	None	5.8±4.2	9.2±0.6	10.1±1.1
Group B (6)	None	200 mg/kg	69.3±4.6 *	7.0±0.2	9.5±1.1
Group C (6)	5/6 nephrectomy	200 mg/kg	60.8±10.7 *	17.3±1.4 *	11.5±1.2

Values shown are mean ± SEM. The number of rats analysed are indicated in parentheses. Brain aluminum content was measured in cerebellum using the dry-ashing EDTA method. Values that differ from controls: *$P < 0.001$.

Absence of lasting nephrotoxicity in lithium-treated rats

Before commencing Al injections, some rats (Groups D and E in Table 5, and their corresponding controls) were fed large amounts of lithium chloride (LiCl) chronically. Christensen et al. (1983) had reported the production of irreversible chronic renal failure in rats fed LiCl (40 mmol/kg of ground chow) from birth until 16 weeks. Since this procedure appeared to offer a means of impairing renal function, we repeated these investigators' proce-

dure, giving LiCl to newborn rat pups in the dosage used by the foregoing investigators, for the first 10 weeks, and in considerably higher dosage for the last 6 weeks (rats were fed chow containing 60 mmol LiCl/kg from age 10 to 12 weeks, and 80 mmol/kg for the next 4 weeks). Normal rat chow was re-instituted at 16 weeks. There were no deaths during the lithium treatment and the growth of these animals was unimpaired. At the end of 10 and 16 weeks of treatment, blood was collected from the tail vein of rats to measure serum urea content as an indication of the degree of renal impairment. The mean serum urea concentrations in these rats were 10.1 and 7.7 mmol/l at the end of 10 and 16 weeks of LiCl treatment respectively, while control rats had a mean serum urea concentration of 7.9 mmol/l. Thus, the hoped-for renal impairment had not been achieved. We then decided to nephrectomise a group of lithium-treated rats for $Al(OH)_3$ injections.

Rats injected with $Al(OH)_3$ promptly after nephrectomy

Sixty four litter-mate rats of both sexes, all of which had been given high doses of LiCl orally for the first 4 months of life, were placed on a lithium-free diet for 7 to 24 days, and then underwent 5/6 nephrectomies (Table 5, group D rats). The rapidity of the compensatory renal hypertrophy which we had observed in Group C rats led us to conclude that little advantage was likely to be achieved by allowing a substantial period of time to elapse between partial nephrectomy and the initiation of Al injections. Thus, beginning 24 h after surgery, animals were injected subcutaneously once daily with either an $Al(OH)_3$ suspension providing 200 mg/kg of elemental Al, or with saline. Injections continued for only 3 or 5 days. It was not possible to continue further, due to the number of deaths occuring in both groups. The mortality rate in this period was much higher for the

Al-treated rats (58%, 22 of 38) than in saline controls (27%, 7 of 26). Death in the latter group was presumably due to uremia, while death in the Al group was presumably due to combined uremia and Al toxicity.

Seven surviving rats were sacrificed 24h after the third $Al(OH)_3$ injection (i.e. 4 days post-nephrectomy), while 9 were sacrificed 24 h after the fifth injection (i.e. 6 days post-nephrectomy). Because no significant differences were found between these 2 sub-groups, neurochemical data for the 2 groups have been combined in Table 8. The results show that relatively brief exposure to Al immediately after the 5/6 nephrectomy did not elevate Al content in the brain, nor did it have any effect on brain GABA content, even though the brain urea content in nephrectomised rats treated either with Al or saline was more than 14 times that of non-nephrectomised rats.

Table 8. Effects of $Al(OH)_3$ or saline injections started 24 h after 5/6 nephrectomy in LiCl-pretreated rats

Animals	Serum aluminum (µg/L)	Brain aluminum (µg/g dry weight)	Serum urea (mmol/L)	Brain urea (µmol/g wet weight)	Brain GABA (µmol/g wet weight)
Saline injected controls	19.8±5.2 (5)	1.85±0.19 (12)	57.4±13.4 (12)	76.1±17.5 (12)	2.53±0.07 (12)
Aluminum Group D rats	75.2±6.9* (9)	1.63±0.14 (16)	36.8±7.1 (16)	56.0±11.4 (16)	2.61±0.07 (16)

Values shown are mean ± SEM, with number of animals in brackets. Rats were killed 24 h after the last of 3 sc injections of saline, or of $Al(OH)_3$ in a dosage providing 200 mg/kg/day of elemental Al. Brain aluminum content was measured by the tetramethylammonium hydroxide method. Brain urea content in non-nephrectomised rats is < 4 µmol/g wet weight, while serum urea level for non-nephrectomised rats is 9.2 ± 0.6 mmol/L (n = 6). Values that differ from controls: *$P < 0.001$.

It seemed possible that Al treatment might be less lethal in animals with less severe renal impairment and thus make it possible to determine whether or not longer exposure to high serum Al concentrations might lead to substantial brain uptake of the metal. The remaining lithium-pretreated rats (n = 30) therefore underwent unilateral nephrectomy and were injected subcutaneously with either $Al(OH)_3$ suspension sufficient to provide 200 mg/kg/day of elemental Al (Table 5, Group E, n = 16), or with saline (n = 14). Injections started 24 h after nephrectomy, continued daily for 30 days, and the animals were sacrificed 24 h after the last injection. These heminephrectomised rats thrived, and there was no post-operative mortality. Mean cerebellar Al content was only slightly increased ($P < 0.05$, Student's t-test) in the Al-treated group (3.20 ± 0.31 µg/g dry weight) when compared to controls (2.24 ± 0.25) (mean ± SEM). No GABA deficiency was evident in the brains of the Al-treated group.

V) CONCLUSIONS

The question as to whether or not Al is the causative factor in DE was not answered by our animal experiments. We failed to produce elevation of Al in the brains of rats treated with $Al(OH)_3$, even though we employed heminephrectomy, 5/6 nephrectomy, and/or chronic lithium administration in attempts to decrease the renal excretion of Al. This failure was clearly not due to the lack of absorption of the $Al(OH)_3$ suspension deposited subcutaneously. In fact, blood Al concentration was elevated one order of magnitude in rats injected subcutaneously with this Al compound, whether or not these rats were nephrectomised (Table 7).

Other investigators claim to have been more successful in producing Al accumulation in the brains of rats treated with Al compounds. For example, the report by Berlyne et al. (1972) claims that rats with normal renal function, and given an unspecified number of subcutaneous injections of $Al(OH)_3$ (150 mg/kg/day as elemental Al), developed a markedly elevated brain Al content (340 µg/g dry weight), although control values were not given in this study. More modest Al elevations, generally less than 4 µg/g dry weight (from control values of about 1 µg/g dry weight), were reported by others (Arieff et al. 1979; Bowdler et al. 1979; Mayor et al. 1980). It is possible that at least one of these studies encountered technical difficulties which resulted in unreliable measurements of tissue Al levels. Accurate measurements of Al in biological materials are made difficult by sources of contamination, analytical interferences, and lack of appropriate standards. Important sources of contamination include leaching of Al from glass or plastic vials (despite careful cleaning with disodium EDTA), Al contamination of reagents, and Al dust in ambient air (Minczewski et al. 1982). Analytical interferences are both spectral and chemical (Goode et

al. 1977; Slavin et al. 1983). The substantially higher mean brain Al content of control animals which we obtained when Al was determined by the dry-ashing EDTA method (Table 6), as compared to that obtained with the tetramethylammonium hydroxide method (Table 8), was probably due to leaching of Al from heat-activated glass in the vials. This explanation is supported by our finding in Bovine Liver Standard Reference Material, No. 1577a, of an Al content of 8 to 10 µg/g using the dry-ashing EDTA method, but of only 3 to 5 µg/g with the tetramethylammonium hydroxide method.

We assume that we failed to produce Al accumulation in rat brain either because renal function was still adequate to excrete the large amounts of Al injected, or because Al failed to cross the blood-brain barrier and was preferentially deposited in some other tissue, such as bone. Chronic elevation of blood Al concentrations in these rats was clearly insufficient to alter any of the neurochemical parameters we measured. It will be necessary to establish permanent renal failure in the rat, or to use some other laboratory animal, for example the rabbit (Yates et al. 1980; Kosik et al. 1983; Yokel et al. 1983), to answer the question as to whether or not brain Al accumulation in DE patients causes the deficiencies of GABA content and CAT activity, as well as the symptoms of DE. This answer is urgently needed to determine whether the treatment of hemodialysed renal failure patients should be modified, especially with regard to the oral administration of phosphate-binding Al gels. Also, if Al proved to be toxic, then normal individuals who consume Al-containing gels as antacids should perhaps be cautious, although it generally is assumed that the normal renal function in these subjects can offset or prevent the accumulation of Al in the body.

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CHAPTER 4: HALLERVORDEN-SPATZ SYNDROME

ABSTRACT

We examined for neurochemical abnormalities the autopsied brain of 2 patients who died with Hallervorden-Spatz syndrome. In one patient, contents of cystine and of glutathione-cysteine mixed disulfide in the globus pallidus were elevated 2 SD above those of controls. Activity of cysteine dioxygenase, the enzyme that converts cysteine to cysteine sulfinate, was reduced in the globus pallidus of both patients. We propose that cysteine accumulates locally in the globus pallidus in Hallervorden-Spatz syndrome as a result of decreased activity of cysteine dioxygenase. Accumulated cysteine may serve as an iron chelator, accounting for the local increase in iron content in the globus pallidus in this disease. The combination of excessive cysteine and of iron may generate free radicals that damage neuronal membranes to cause the typical morphological changes observed in Hallervorden-Spatz syndrome.

I) INTRODUCTION

First described in 1922 (Hallervorden et al. 1922), Hallervorden-Spatz syndrome (HSS) is a rare autosomal recessive neurological disorder. The symptoms are mainly extrapyramidal, characterized by dystonic postures, muscular rigidity or spasticity, choreoathetosis and other types of hyperkinesias (Dooling et al. 1974; Vakili et al. 1977). In addition, patients can also present with symptoms of Parkinson's disease (Jankovic et al. 1985). Mental changes indicative of dementia are a prominent feature.

The age of onset of symptoms is usually in the first or second decade of life (Hallervorden et al. 1922; Dooling et al. 1974), although one patient with onset at age 55 has been reported (Jankovic et al. 1985). The disease follows a relentless downhill course and culminates in death an average of 11 years after the appearance of symptoms (Elejalde et al. 1979).

At autopsy, the main neuropathological changes are seen in the globus pallidus (GP) (Dooling et al. 1974). In this area, neurons are reduced in number, diffuse gliosis is observed, and round or oval non-nucleated structures ('spheroids') identifiable as swollen axons are scattered throughout. Degeneration of other brain areas can also occur. While the autopsied brain of a patient studied by Perry et al. (1985) had neuronal degeneration and axonal swellings localised only to the GP, others (Dooling et al. 1974; Vakili et al. 1977; Jankovic et al. 1985) have reported involvement of the substantia nigra (both the pars compacta and reticulata), locus coeruleus, substantia innominata, hippocampus, and cerebellum. Loss of neurons in the pars compacta of the substantia nigra probably gives rise to the parkinsonian symptoms seen in some patients.

An interesting neuropathological finding is the accumulation of iron, perhaps of the ferric form (Dooling et al. 1974; Vakili et al. 1977), in

the affected brain areas of patients who die with HSS. For the GP, one report (Vakili et al. 1977) lists 2 patients as having iron contents between 1420 and 2280 µg/g dry weight, while a second report (Goldberg et al. 1979) gives a mean iron content of 1911 µg/g dry weight for 3 HSS patients. These values compare with iron contents of 550 to 1070 µg/g dry weight for GP of normal adult human brain (Volkl et al. 1972). Thus, the iron content of the GP appears to be elevated 2 to 4-fold in HSS. The increased iron content gives this area a dark brown color that is readily observed on gross examination with the naked eye. In addition to iron, contents of copper and zinc are reportedly increased in the GP (Goldberg et al. 1979). Non-protein bound iron may be elevated in the cerebrospinal fluid (Swaiman et al. 1983).

The diagnosis of HSS is based on post-mortem neuropathological findings of the unique combination of neuroaxonal swellings and excessive intra and extracellular deposition of iron pigments in the GP (Park et al. 1975; Seitelberger et al. 1977; Jankovic et al. 1985). Before death, the diagnosis of HSS can be suspected from the clinical presentation, as well as from evidence of high iron retention in the basal ganglia as determined by intravenous injection of ^{59}Fe and a narrow field-of-view collimator (Elejalde et al. 1979; Dooling et al. 1980). Demonstration of lucency in the GP by computed tomography may provide further support of the diagnosis (Dooling et al. 1980).

There is as yet no effective treatment for patients with HSS. Desferroxamine, a heavy metal chelator, was tested for a year in one patient with well-advanced symptoms, but it did not alter the course of the disease, even though serum iron levels decreased 3-fold during the treatment (Dooling et al. 1974). The parkinsonian symptoms of some patients respond initially to L-DOPA and carbidopa therapy, but this response is not maintained (Jankovic

et al. 1985).

Very little is known of the neurochemical changes present in HSS. A single post-mortem subject studied, whose presenting symptoms were mainly parkinsonian, revealed marked loss of dopamine (over 95%) in the nigrostriatal projection, with relative preservation of dopamine in the limbic areas (Jankovic et al. 1985). This was accompanied by parallel reduction of homovanillic acid, one of the 2 major metabolites of dopamine. Such changes are similar to those seen in idiopathic Parkinson's disease.

The biological basis of HSS is unknown. The cause of the marked neuronal loss, especially prominent in the GP, as well as the accumulation of iron, is uncertain.

We had the opportunity to study 2 patients who died with a pathologically-confirmed diagnosis of HSS. We attempted to examine the neurochemical changes that might be present in these 2 subjects. We found localised accumulation of glutathione-cysteine mixed disulfide, and of cystine, in the GP of one patient, and reduction of the activity of the catabolic enzyme cysteine dioxygenase in both patients. We propose the hypothesis that these changes may cause the neuronal loss seen in the GP of patients with HSS.

II) OUTLINE OF EXPERIMENTS

The 2 patients with HSS will be referred to here as HSS Cases 1 and 2. HSS Case 1 has been described in detail in Appendix J (Perry et al. Ann. Neurol. 18:482-489, 1985), while HSS Case 2, whose brain was made available to us through the courtesy of Dr. E. Bird of Massachusetts, is described in Jankovic et al. (1985). Neuropathological examination of HSS Case 1 revealed abnormalities (swollen axons, neuronal loss, iron deposition) confined only to the GP, while in HSS Case 2 (Jankovic et al. 1985), the same abnormalities were more widespread, and included the substantia nigra and caudate nucleus as well as the GP.

Autopsied brains used as controls were mostly from subjects who died without any evidence of neurological diseases, but some were from patients who died with olivopontocerebellar atrophy (OPCA). The OPCA patients had no known abnormalities in the metabolism of sulfur amino acids, γ-aminobutyric acid (GABA), or of iron, in the brain areas that were used for study.

Neurochemical measurements that were performed included that of the contents of 36 amino acids in several brain regions (Table 1), using the chromatographic system detailed in Appendix J. These analyses included those for GABA, cystine, and glutathione-cysteine mixed disulfide. Contents of dopamine and its metabolites in the striatum were quantified by a HPLC system with electrochemical detection (Perry et al. 1984).

Cysteine dioxygenase activity was analysed in the GP, frontal cortex and putamen, using the method of Misra (1979), in which amounts of radioactive cysteine sulfinate formed from L-[^{35}S]-cysteine by brain homogenates are quantified. Assays were done blind in triplicate. For HSS Case 1, 3 areas of the GP were studied for cysteine dioxygenase activity, while for HSS Case 2, only 2 areas of the GP were analysed, due to insufficient quantities of

material being available.

To assess the possible effect of increased iron content on cysteine dioxygenase activity, the concentration of iron in normal GP homogenates was calculated from published values (which were expressed as iron content/dry weight of brain) (Volkl et al. 1972; Vakili et al. 1977) to be 1.43 mM (we assumed that the GP contains 80 % water). We then measured enzyme activity in homogenates of GP from 5 control subjects, adding iron in the form of ferrous ammonium sulfate 2.5, 5, and 10 times the calculated normal value. Although iron deposits in the GP of HSS patients are thought to be mainly of the ferric form, ferrous ammonium sulfate was chosen here as the exogenous form of iron since this compound was already present in the normal reaction mixture as a cofactor for cysteine dioxygenase.

Choline acetyltransferase activity was determined in 0.5% Triton X-100 homogenates of frontal and occipital cortices, using the method of Fonnum (1975). In this method, choline is incubated with [^{14}C]-acetyl-coenzyme A, and radioactivity is measured in the acetylcholine formed. Brain areas other than the cortex could not be analysed because insufficient quantities of tissue were available from both HSS and control subjects.

Protein concentrations in brain homogenates were measured by the method of Lowry et al. (1951), using bovine serum albumin as standard.

III) RESULTS

Quantitative amino acid analyses of the brain samples from HSS Case 1 showed an elevation of the contents of cystine and of glutathione-cysteine mixed disulfide in the GP, and, to a lesser extent, in the putamen (Table 1). The elevations of the amounts of both compounds in these areas were more than 2 SD above the mean control values. In other brain regions analysed (frontal, occipital and cerebellar cortices, dentate nucleus, caudate nucleus, and substantia nigra), contents of these compounds were not significantly different from controls. The death-to-freezing interval of the brain of HSS Case 1 was 3.5 h, which was comparable to the death-to-freezing intervals for control brains. It is critical that measurements of cystine be made only in brains that are rapidly frozen because, after death, glutathione undergoes rapid autolysis to its breakdown products, one of which is cysteine (Perry et al. 1981). Breakdown of proteins also contributes to elevated cystine levels measured after death. A long death-to-freezing interval can therefore result in artifactually high cystine measurements. In fact, neither cystine nor glutathione-cysteine mixed disulfide are normally detectable when rapidly-frozen biopsies of human brain are subjected to amino acid analysis (Perry et al. 1971).

The interval between HSS Case 2's death and freezing of his brain was 7 h, and the body had not been placed at 4°C until 4 h after death. Autolysis of glutathione in this patient's brain had been so extensive that any possible antemortem elevation of cystine or glutathione-cysteine mixed disulfide contents could not be ascertained accurately.

Table 1. Brain cystine and glutathione-cysteine mixed disulfide in Hallervorden-Spatz syndrome

Patients	Caudate	Putamen	Globus Pallidus	Substantia nigra
		Cystine		
Controls	0.05 ± 0.04 (41)	0.05 ± 0.04 (25)	0.10 ± 0.07 (20)	0.12 ± 0.08 (44)
HSS Case 1	0.08	0.14	0.45	0.16
		Glutathione-cysteine mixed disulfide		
Controls	0.32 ± 0.14 (37)	0.34 ± 0.08 (23)	0.41 ± 0.15 (19)	0.43 ± 0.17 (39)
HSS Case 1	0.50	0.65	1.02	0.68

Values are mean ± SD in μmol/g wet weight. The number of control samples is shown in parentheses. The death-to-freezing interval for every control specimen was less than 4.0 h, while that for HSS Case 1 was 3.5 h. HSS values increased > 2 SD of controls are underlined.

The only other amino acid abnormality found in the brains of the 2 HSS patients was a marked reduction in GABA content in the GP and the substantia nigra (Table 2). GABA contents rise rapidly in human brains after death (Perry et al. 1981), reaching a stable maximum after about 2 hours. Since the death-to-freezing intervals of the 2 patients and all of the controls shown in Table 2 were 2 hours or greater, the values could be reliably compared.

The activity of cysteine dioxygenase was found to be low in the GP of both HSS patients. While the mean enzyme activity in the GP of 25 control subjects was 1.77 ± 0.40 μmol/h/mg protein, HSS Case 1 had a mean activity of 0.82 (mean of 3 separate areas of the GP which had values of 0.44, 1.11 and 0.92). HSS Case 2 had cysteine dioxygenase activity of 0.97 μmol/h/mg

Table 2. Brain GABA content in Hallervorden-Spatz syndrome

Patients	Caudate	Putamen	Globus Pallidus	Substantia nigra
Controls	2.90 ± 0.81 (32)	2.91 ± 0.87 (19)	7.32 ± 1.60 (16)	6.05 ± 1.36 (28)
HSS Case 1	4.83	5.28	2.55	0.42
HSS Case 2	1.33	2.79	2.50	1.95

Values (mean ± SD) are in μmol/g wet weight, with the number of controls shown in parentheses. HSS values decreased > 2 SD are underlined.

protein, which was the mean of 2 separate areas of the GP analysed (0.77 and 1.16). Thus, the enzyme activity in the GP of both HSS patients was decreased by more than 2 SD from the mean for the GP of controls. On the other hand, cysteine dioxygenase activities of the frontal cortex and putamen of both patients were within 2 SD of controls (Figure 1).

Control brains had an average death-to-freezing interval of 6.5 ± 7.2 h (mean ± SD), and linear regression analysis of these brains revealed no changes of enzyme activity with increasing death-to-freezing intervals.

In experiments in which up to 10 times the normal iron content (as Fe^{2+}) was added to homogenates of GP, no significant inhibition of cysteine deoxygenase was observed (Figure 2).

Choline acetyltransferase activity was slightly but insignificantly reduced in the cerebral cortex of HSS Case 1, as compared to controls (Table 3). Contents of dopamine and its metabolites in the striatum of HSS case 1 were comparable to those in a small group of control adults in whom the death-to-freezing intervals for the brain were equally short (Table 4). Only tissues from HSS Case 1 were analysed because insufficient material was obtained from HSS Case 2. However, this latter subject had been studied

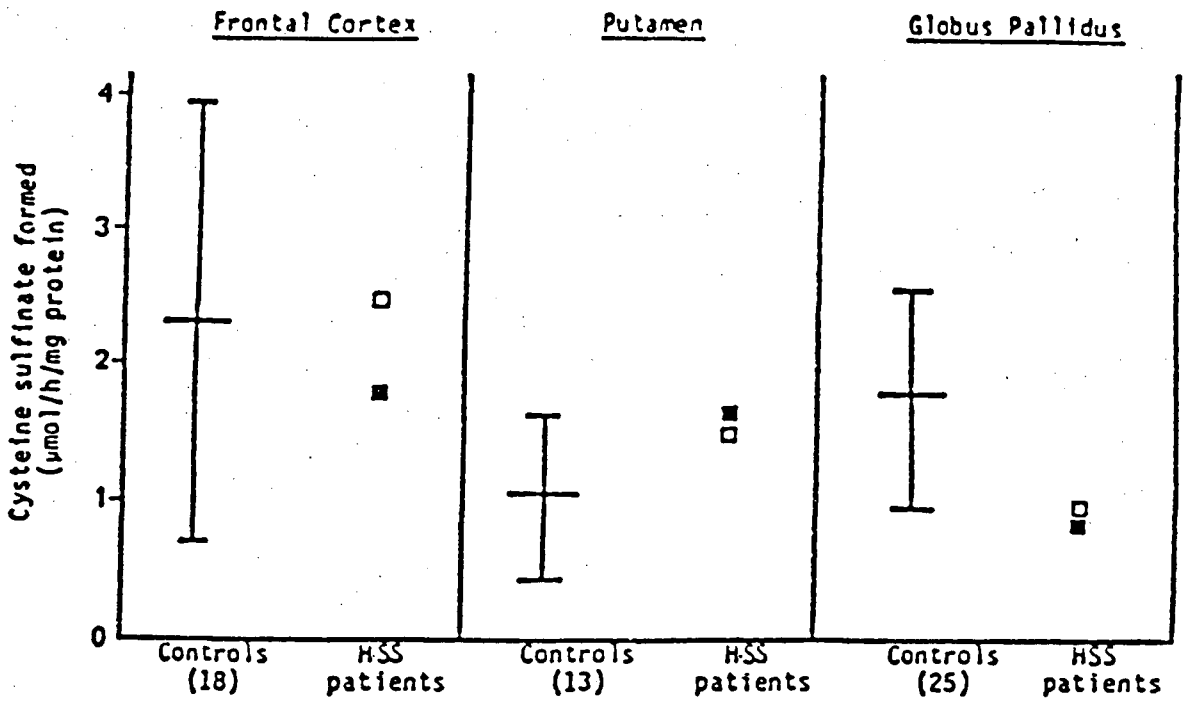

Figure 1. Enzyme activity of cysteine dioxygenase in tissue homogenates from 3 regions of autopsied brain of control adults and HSS patients. Horizontal bars indicate means, and vertical bars indicate ± 2 SD for the controls. The solid square indicates HSS Case 1, and the open square HSS Case 2.

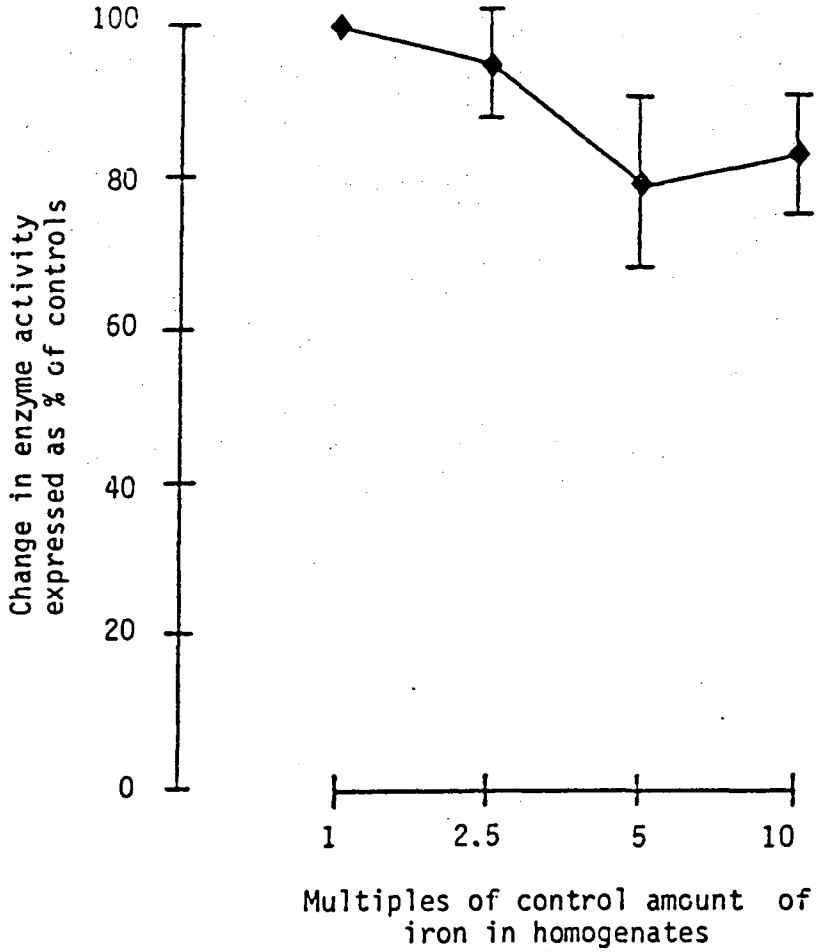

Figure 2. % change in cysteine dioxygenase enzyme activity as a function of increasing brain iron content. Each point represents 5 analyses. Vertical bars shown are mean ± SEM. Analysis of variance shows that none of the points are statistically significant from each other.

(Jankovic et al. 1985), and dopamine and HVA contents were found to be very low in his caudate nucleus and putamen (dopamine contents in both areas were reduced by more than 95%).

Table 3. Choline acetyltransferase activity in Hallervorden-Spatz disease

Patients	Frontal cortex	Occipital cortex
Controls	1.07 ± 0.20 (16)	0.64 ± 0.28 (12)
HSS Case 1	0.79	0.51

Enzyme activity is expressed as nmol/10 min/mg protein. Control values are mean ± SD, with number of controls indicated in parentheses.

Table 4. Dopamine and metabolites in the striatum of HSS Case 1

Patients	Caudate nucleus			Putamen		
	Dopamine	DOPAC	HVA	Dopamine	DOPAC	HVA
Controls (4)	3.34 ± 0.68	0.70 ± 0.70	5.20 ± 1.96	5.22 ± 2.13	0.14 ± 0.05	6.05 ± 1.42
HSS Case 1	2.84	0.73	4.31	6.90	0.74	13.0

Concentrations of dopamine and its metabolites are expressed in µg/g wet weight. Control values are mean ± SD.

IV) DISCUSSION

Little is known about the neurochemical changes or biological basis of HSS except that iron is retained in the GP of patients who have died with the disease. The increase in iron content of the GP is interesting, since iron salts are potent inducers of lipid peroxidation, a process whereby reactive free radicals oxidize the polyunsaturated fatty acids of membrane lipids to form lipid peroxides. The latter, being unstable, undergo decomposition. This can lead to disruption of membrane integrity, and cell death (Slater 1982; Halliwell et al. 1984). Triggs et al. (1984) have demonstrated that injections of ferrous chloride into rat cerebral cortex cause persistent seizures and significant lipid peroxidation, which can be prevented by pretreating the animals with the antioxidant α-tocopherol.

We found that the contents of cystine and of glutathione-cysteine mixed disulfide were significantly elevated in the GP and putamen of HSS Case 1. The reduced form of cystine is cysteine, and in vivo, enzymes such as thiol-disulfide transhydrogenases and nucleotide-dependent disulfide reductases serve to keep cystine in the reduced form (Schneider et al. 1983). It seems highly likely that cysteine had accumulated in the GP of HSS Case 1 during life, resulting in the abnormally high contents of both cystine and the mixed disulfide of cysteine and glutathione when autopsied brain tissue was analysed. The same abnormality might have been present in the GP of HSS Case 2, although delay in freezing his brain after death prevented our demonstrating this possible increase.

If cysteine were increased in HSS during life, some of the neuropathology of the disease might be explained. Firstly, cysteine resembles penicillamine (dimethylcysteine) in chemical structure. Penicillamine is a chelating agent used in the treatment of heavy metal poisoning. Since the struc-

tural elements of penicillamine which allow chelation of metal ions are also present in cysteine, high levels of the latter might act to chelate iron. The resulting complexes, if trapped within the GP, could lead to the characteristic excessive accumulation of iron seen in patients with HSS. Since penicillamine can also chelate other heavy metals such as copper, elevation of cysteine in the GP should also result in the accumulation of metal ions other than those of iron. In this regard, it is significant that Goldberg et al. (1979) reported that copper, zinc, calcium and manganese, in addition to iron, were elevated in the GP of patients with HSS. In the same article, Goldberg et al. (1979) showed that iron contents in non-pallidal areas such as caudate nucleus and substantia nigra of HSS patients were reduced when compared to controls, as though iron were preferentially distributed and localised in the GP.

Another important aspect of cysteine accumulation during life in the GP of HSS patients is that cysteine, although an endogenous amino acid, can be toxic when present in high amounts. Thus, a single injection of cysteine to rats (Karlsen et al. 1981) or mice (Olney et al. 1971) can produce generalized brain atrophy. In vitro, cysteine undergoes autoxidation to generate reactive free radicals, including thiyl and hydroxy radicals detectable by electron spin resonance (Saez et al. 1982). It can also disrupt the integrity of isolated hepatocytes, causing them to release glutathione, ATP and lactate dehydrogenase (Saez et al. 1982).

Malondialdehyde production is an index of lipid peroxidation. Searle et al. (1983) have reported that malondialdehyde production in rat liver microsomes was stimulated by ferrous iron or cysteine. The combination of iron and cysteine became a very potent inducer of lipid peroxidation, and malondialdehyde production induced by the combination grossly exceeded the sum of those resulting from the individual actions of these agents.

The high cysteine content in the GP of HSS Case 1, coupled with iron accumulation, would almost cetainly increase lipid peroxidation in this brain region, possibly by the generation of free radicals. The result could be neuronal death and perhaps the neuroaxonal swellings seen in the GP.

Cysteine dioxygenase is the first of 3 sequential enzymes in the metabolic pathway from cysteine to taurine in brain and other tissues (Schneider et al. 1983). We found the activity of this enzyme to be low in the GP of the 2 HSS patients. Since the action of this enzyme represents the major route by which cysteine is metabolised (Schneider et al. 1983), it is possible that the cysteine accumulation in the brain of HSS Case 1 resulted from the deficient activity of cysteine dioxygenase.

If cysteine dioxygenase deficiency and iron excess are both neurochemical abnormalities in the GP of HSS, it would be important to learn whether either of them is primary. Cysteine dioxygenase requires ferrous ion as a cofactor (Misra 1979), and it is conceivable that excessive iron content might inhibit the enzyme. Although conventional histological staining suggested that the iron in the GP of HSS is mainly of the ferric form (Dooling et al. 1974; Vakili et al. 1977), it is reasonable to presume that there is an equilibrium between the ferrous and ferric forms in the abnormal iron deposits. When enzyme activity was measured in homogenates of GP with the addition of ferrous ion at up to 10 times the expected iron content of the normal GP, there was no significant inhibition of the enzyme. It therefore appears unlikely that the elevated iron content (if it is mainly present in the ferrous form) is itself responsible for the low enzyme activity in the GP in HSS disease. Rather, cysteine dioxygenase deficiency might be primary.

It is useful to look at another disorder in which there is a defect in cysteine metabolism. In the autosomal recessive disease cystinosis (Schneider et al. 1983), cystine accumulates and deposits as cystine crystals in

the kidney, conjunctiva, bone marrow, lymph nodes and leucocytes. We are unaware of any reports of neurological symptoms or neuropathological changes in cystinosis. However, cystinosis is believed to be a disorder of the intracellular compartmentalisation of cystine, rather than an enzymic failure in cystine degradation. Also, the elevated cystine levels in cystinosis are probably associated with storage within lysosomes, and therefore separated from most cellular enzymes or membrane structures (Schneider et al. 1983). The presence of glutathione-cysteine mixed disulfide in excessive quantities in the GP of HSS Case 1, as well as the deficiency in cysteine dioxygenase activity, suggests that in HSS, it is cysteine, rather than cystine, which accumulates in brain.

In Table 1, it is seen that cystine and glutathione-cysteine mixed disulfide contents were elevated in the putamen of HSS Case 1. Yet, the putamen from this patient showed no morphological alterations, and cysteine dioxygenase activity was also normal in this brain region. The probable reason for this inconsistency is that amino acid analysis was performed on a portion of putamen immediately adjacent to the GP. This could have been the pencillate bundles of the putamen, where neuroaxonal swellings and diffuse iron-positive staining were observed histologically. Because of insufficient tissue being available, an area of putamen more distant from the GP could not be analysed.

The GABA reduction found in both the GP and substantia nigra of our 2 HSS patients is probably the result of marked loss of neurons in the GP. This region contains GABAergic cell bodies projecting to the substantia nigra and is also the terminus for GABAergic neurons with cell bodies in the striatum. The increased GABA content of the caudate nucleus and putamen of HSS Case 1 (Table 2) is unexplained. GABA content was normal in other

regions of this patient's brain (frontal, occipital and cerebellar cortex, and dentate nucleus).

HSS Case 1, with early onset and death at the age of 19, had no reduction in striatal dopamine or its metabolites. In this respect, HSS Case 1 did not resemble HSS Case 2, who had an unusually late onset (55 years of age) and symptoms suggestive of parkinsonism (Jankovic et al. 1985). HSS Case 2 was reported to have marked deficiency of dopamine and HVA in the striatum, along with extensive loss of neurons in the substantia nigra (Jankovic et al. 1985). HSS Case 1 showed no neuropathological changes in the substantia nigra.

V) CONCLUSIONS

Following from the findings described in this work, a hypothesis regarding the etiology of the autosomal recessive disorder HSS may be suggested. A primary enzymic defect in cysteine dioxygenase activity in the GP results in accumulation of cysteine, which then binds iron in excessive amounts. High levels of cysteine or iron can be neurotoxic when present individually. The combination of cysteine and iron, however, becomes a lethal mixture, generating free radicals that result in lipid peroxidation and subsequent neuronal death within the GP.

It should be recognized that the changes found at autopsy after many years of the disease may not necessarily reflect the initial pathological lesion, and furthermore, that the above hypothesis is based on data obtained from only 2 patients with HSS, and that more HSS brains need to be examined for similar neurochemical defects. Nevertheless, if confirmed, our findings suggest possible therapeutic approaches to HSS. For instance, when symptoms initially appear, further deterioration might be prevented by treatment with antioxidant drugs such as α-tocopherol or β-carotene, which can serve to detoxify free radicals. Such treatment might also prevent the onset of symptoms in individuals genetically at risk for the disease. The use of the heavy metal chelator desferroxamine might also be useful in the initial stages of the disease, although one report suggested that its use did not alter the course of HSS in a patient with well-advanced disease (Dooling et al. 1974).

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