MITOCHONDRIAL ABNORMALITIES

IN PARKINSON'S DISEASE

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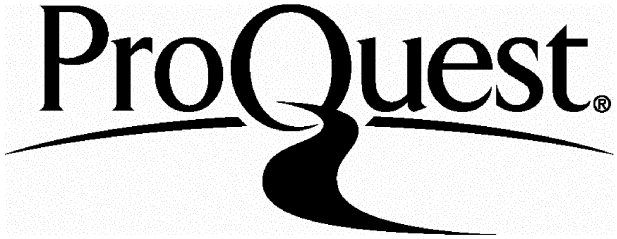

Abstract

The cause of neurodegeneration in the substantia nigra of the brains of Parkinson's disease (PD) patients remains unknown. The presence of a defect of NADH CoQ_1 reductase (complex I of the mitochondrial respiratory chain) in the disease is now well established, although its relevance to disease pathogenesis is unknown. The tissue specificity of the defect is contentious, and was therefore investigated in PD platelets. Complex I activity measured in PD whole platelet homogenates was normal. However when a platelet mitochondrially-enriched fraction (MEF) was prepared, and the complex I assay was modified to maximise activity in platelet MEFs, a slight (16%), but statistically significant decrease in mean complex I activity in PD samples was detected.

The molecular basis of the complex I enzymatic defect in PD is unknown. To determine the presence of any defect in the complex I protein, the structure and subunit composition of the complex in the PD substantia nigra was investigated by two methods; immunoprecipitation of the complex using antibodies raised to the holoenzyme, followed by denaturing electrophoresis and silver staining of the polypeptides; and quantitation of the concentration of the complex by ELISA (enzyme-linked immunosorbent assay), using affinity-purified antibodies to the complex I holoenzyme and to specific complex I subunits. By immunoprecipitation, no major differences were detected between complex I from PD and control substantia nigra. However, by ELISA it was shown that PD substantia nigra samples with low complex I enzymatic activity (40% less than controls) contained low concentrations of complex I protein (40% less than controls).

To explore the possibility that the complex I defect in PD is caused by a defect in the detoxification process of a putative complex I inhibitor, experiments were initiated to correlate complex I activity in the PD substantia nigra with the presence of mutations in the cytochrome P450 debrisoquine hydroxylase gene (CYP2D6). Due to the limited sample number, no samples contained homozygous recessive mutations which would affect the debrisoquine metaboliser phenotype.

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Contents

List of Figures

List of Tables

Abbreviations

mA	milliamp
APS	ammonium persulphate
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BHM	bovine heart mitochondria
BSA	bovine serum albumin
°C	degrees centigrade
cm	centimetre
CS	citrate synthase
CYP2D6	cytochrome P450 2D6 gene
DNA	deoxyribonucleic acid
DDM	n-dodecyl ß-D-maltoside
ddH_2O	double distilled water
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPR	electron paramagnetic resonance spectroscopy
FP	flavoprotein fraction of complex I
g	gram
mg	milligram
µg	microgram
g force	gravity
GSH	reduced glutathione
GSSG	oxidised glutathione
hr	hour
HD	Huntington's disease
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HHM	human heart mitochondria
HP	hydrophobic fraction of complex I
INT	2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium
IP	iron protein fraction of complex I
kDa	kiloDalton
l	litre
LHON	Leber's hereditary optic neuropathy
ml	millilitre
µl	microlitre
min	minute

M	molar
mM	millimolar
μM	micromolar
nM	nanomolar
nmol	nanomoles
nm	nanometres
MAOB	monoamine oxidase B
MEF	mitochondrially-enriched fraction
MELAS	mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
MERRF	myoclonic epilepsy and ragged-red fibres
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP^+	1-methyl-4-phenylpyridinium
MRC	mitochondrial respiratory chain
MSA	multiple system atrophy
mt	mitochondrial
NADH	nicotine adenine dinucleotide (reduced)
NBT	nitro blue tetrazolium
NFR	NADH ferricyanide reductase
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PBS_a	PBS plus sodium azide
PBS_T	PBS plus Tween 20
PD	Parkinson's disease
PET	positron emission tomography
PNS	post-nuclear supernatant
PRP	platelet-rich plasma
Q_1	ubiquinone-1
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylenediamine
TIQ	tetrahydroisoquinoline
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
Tween 20	polyoxyethylenesorbitan monolaurate
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume

Chapter 1. Introduction.

1.1. PARKINSON'S DISEASE

Parkinson's disease (PD) was first described by James Parkinson in 1817 in his "Essay on the Shaking Palsy". The most disabling symptoms of the disease are akinesia (loss of movement) and bradykinesia (extreme slowness of movement), although patients also experience muscular rigidity and rest tremor. The discovery of selective striatal dopamine deficiency and the identification of the nigrostriatal pathway, with the destruction of nigral neurones in the PD brain, led to a new era of treatment. In the late 1960's and early 1970's high dose oral levodopa (L-dopa), the metabolic precursor of dopamine, was established as the most effective therapy for the disease. The clinical definition of Parkinson's disease includes a unilateral onset, classic rest tremor, and a positive response to L-dopa. However, the clinical diagnosis of Parkinson's disease is only correct in 80% of cases; multiple system atrophy, progressive supranuclear palsy and senile dementia of the Lewy body type account for most misdiagnoses. (Hughes *et al*, 1992). PD is one of the commonest neurodegenerative diseases, with a prevalence in the UK of approximately 1/1000, rising to 5/1000 in those aged over 50 years.

1.1.1. Pathology of PD

The cardinal pathological lesion in Parkinson's disease is the degeneration of pigmented, neuromelanin-containing dopaminergic neurones in the zona compacta of the substantia nigra (Figure 1-1). These neurones project mainly to the striatum (putamen and caudate nucleus), and the loss of striatal dopamine is the cause of the motor symptoms of the disease. There is a presymptomatic phase of the disease, and clinical signs do not appear until at least 50% of nigral neurones are lost and the levels of striatal dopamine are reduced by 80% (Bernheimer *et al*, 1973; Marsden, 1990). This phenomenon is a consequence of two systems which compensate for cell loss; dopamine turnover increases significantly in the remaining nigrostriatal dopaminergic neurons, and the

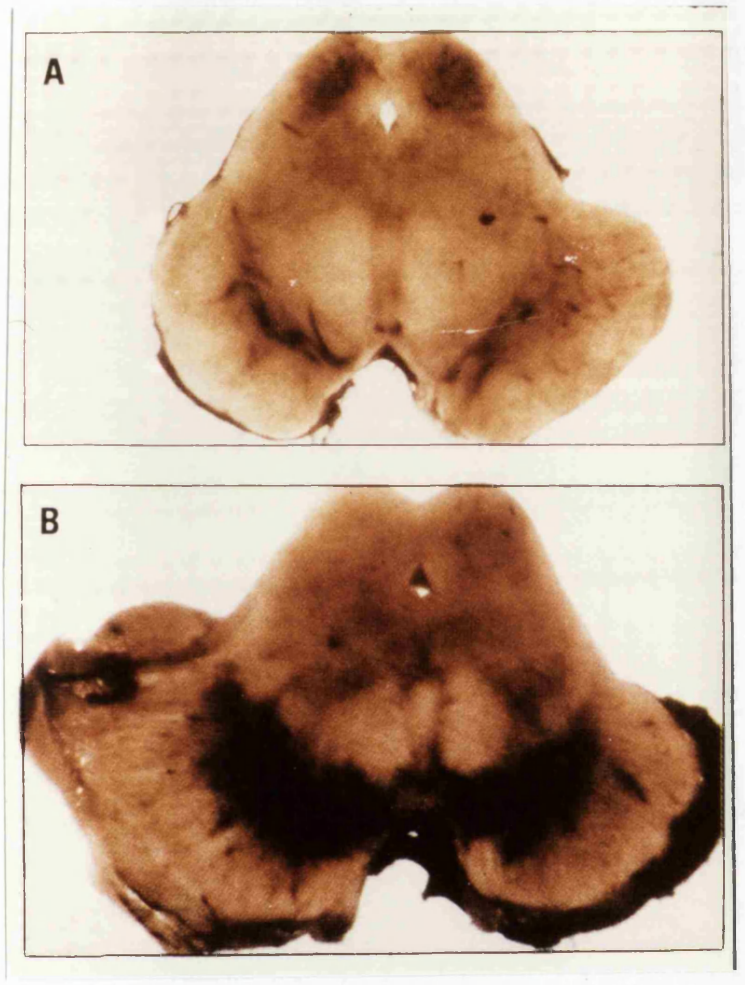


Figure 1-1. Transverse section through the mid-brain showing loss of pigmented neurones in the PD substantia nigra.

A (PD brain); B (Normal brain).

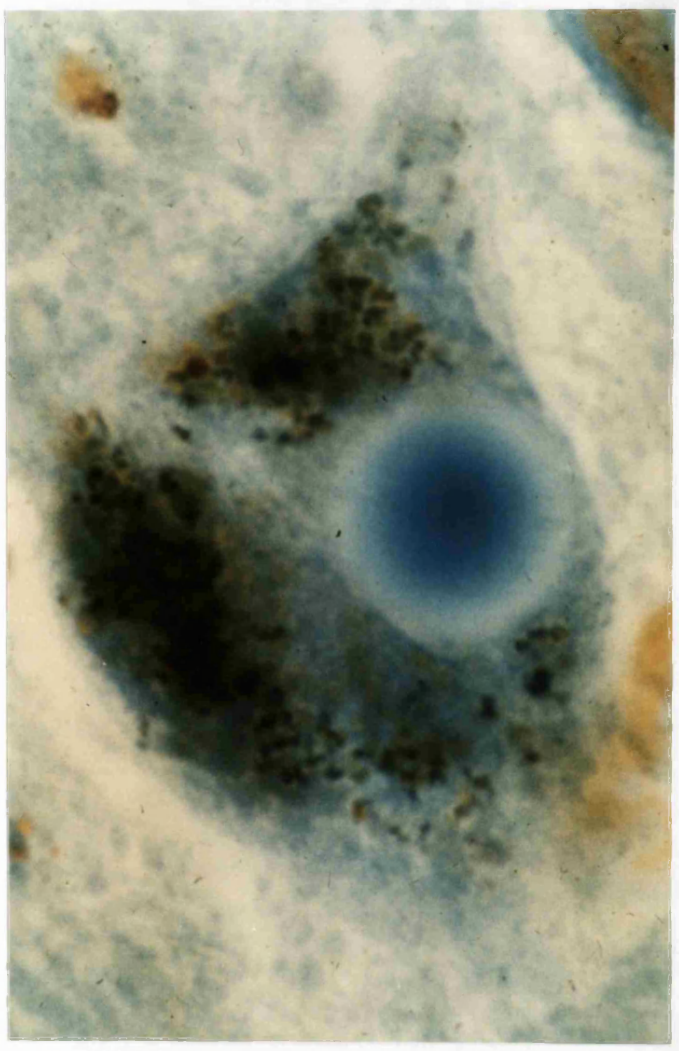


Figure 1-2. The Lewy Body.

postsynaptic dopamine receptors in the striatum become supersensitive (Hornykiewicz and Kish, 1986). The presence of Lewy bodies in the remaining neurones of the substantia nigra is the pathological hallmark of PD (Figure 1-2). These ubiquitin-rich, eosinophilic, intracytoplasmic structures are present in many areas of the PD brain besides the substantia nigra, and their presence is thought to indicate the process of neurodegeneration. Although it is not known how they are formed, they are known to be mainly composed of structurally altered neurofilament. Their significance to any pathological process is unknown.

Within the PD substantia nigra zona compacta cell loss is greatest in the caudal area, and within the caudal nigra cell loss is greatest in the lateral ventral tier (average loss 91%) followed by the medial ventral tier and the dorsal tier (Fearnley and Lees, 1991). Regionally, the pattern of cell loss is the opposite in ageing, the lateral ventral tier being relatively spared and the dorsal tier being most affected. The average cell loss in the caudal substantia nigra zona compacta in normal individuals is 4.7% per decade. However in the PD brain, the rate of cell loss in this region is not linear, but exponential; in the first decade from the onset of neuronal loss, there is a 45% cell loss in this region. The difference in regional selectivity of cell loss and rate of cell loss between PD and ageing suggests that PD is not a form of accelerated ageing. Further evidence that PD is not caused by an accelerated ageing process is that the number of macrophages phagocytosing dopaminergic neurons in the substantia nigra at the time of death is much greater in PD than control brains (McGeer *et al*, 1988). This implies that there is an active neuropathological process occurring up to the time of death in the PD brain.

Cell loss in the PD brain although most marked in the substantia nigra, is not wholly restricted to this area. There is also moderate cell loss in the dopaminergic ventral tegmental area and noradrenergic locus coeruleus, and minor cell loss is often seen in many areas including the dorsal motor vagus nucleus, substantia innominata and raphe nucleus (Forno, 1990).

1.1.2. Treatment of PD

1.1.2.1. L-dopa

L-dopa is now established as the primary therapy for PD patients. After crossing the blood-brain barrier, L-dopa is converted to dopamine by the enzyme dopa decarboxylase. The administration of peripheral dopa-decarboxylase inhibitors (e.g. carbidopa, benserazide) increases the availability of L-dopa to the brain and limits side-effects arising from its peripheral metabolism. Although L-dopa ameliorates the symptoms of PD, it does not halt the progression of the underlying disease and dopaminergic neurons continue to die, so that in the later stages of disease the administration of L-dopa can no longer compensate for the neuronal loss.

1.1.2.2. Selegiline

The MAO-B inhibitor selegiline (deprenyl) has been used in the therapy of PD since 1986. MAO-B is one of the enzymes which metabolises catecholamines, including dopamine; its inhibition therefore increases the concentration of dopamine in the brain. The co-administration of L-dopa and selegiline is reported to enhance the efficacy of L-dopa, and allow a reduction in its dose (Wessel and Szelenyi, 1992). However, selegiline may have other effects which are potentially more important. In the largest clinical trial to date (Parkinson Study Group, 1993), the administration of selegiline to newly diagnosed, untreated PD patients delayed the development of disability requiring L-dopa treatment by an average of nine months, suggesting that selegiline may be having a neuroprotective effect. Although the patients symptoms worsened during the two months after withdrawal of selegiline, they still showed an overall reduction in symptom severity relative to untreated patients. In support of a protective effect of selegiline, Rinne *et al* (1991) showed that preservation of nigral neurons was greater in PD patients treated with selegiline in combination with L-dopa than in those treated with L-dopa alone (although all patients were at the end stage of disease and the selegiline treated patients were on 40% lower L-dopa doses than patients on L-dopa alone).

1.1.3. Genetics of PD

Although the majority of cases of PD are sporadic, there is evidence that genetic factors play some part in the aetiology of the disease. The first is the documentation of families in which PD appears to be inherited in an autosomal dominant manner. The largest of these kindreds has been reported by Golbe *et al* (1990). The inherited disease is similar to the idiopathic disease, except that the age at onset is younger and the course is rather more rapid. Forty four members of this family over four generations have now been shown to be affected, and postmortem histological evidence of typical Lewy body PD has been obtained in two cases (Duvoisin and Johnson, 1992). In this family, and in others reported, it seems that parkinsonism is caused by the autosomal dominant inheritance of a single gene.

1.1.3.1. Twin studies in PD

Evidence of a genetic component in the cause of a disease can be obtained from twin studies; for diseases with a significant genetic component, the degree of concordance is higher in monozygotic than dizygotic twins. Most early twin studies found little difference in concordance rates for PD between monozygotic and dizygotic twins (review; Johnson *et al*, 1990). Positron emission tomography (PET) studies using [^{18}F]dopa as tracer can detect asymptomatic preclinical PD cases; the accumulation of [^{18}F]dopamine in the striatum depends upon the number of surviving nigrostriatal terminals and their dopa decarboxylase activity. Higher concordance rates for PD in monozygotic twins have been found when cases with low [^{18}F]dopa uptake are considered to have preclinical PD (Burn *et al*, 1992). Several genetic models for PD which take account of the low twin concordance rates have been proposed. Two of these are multifactorial threshold inheritance and autosomal dominant inheritance with reduced penetrance. The former assumes the involvement of many genes and environmental factors which act together, and at a particular threshold cause the disease phenotype, while the latter assumes that the disease is caused by a single gene but that its penetrance is affected by other gene loci and/or environmental factors. Two factors complicate the interpretation of genetic data concerning PD. Firstly, the diagnosis of PD is only correct in 80% of cases, and secondly, because PD is a late-onset disease, some cases described as normal may go on to develop PD later in life or may die before developing the disease.

1.1.3.2. PD susceptibility gene

The available evidence suggests that PD is probably a multifactorial disease caused by both genetic and environmental factors. This hypothesis gives rise to the idea of a susceptibility gene, the carriers of which are more susceptible to particular environmental agents. Two methods which may be used in the search for this susceptibility gene are allelic association and linkage mapping. Allelic associations have been found between PD and cytochrome P450 (see Introduction 1.5.3) and monoamine oxidase B (MAO-B) (Kurth *et al*, 1992). However, it is unclear whether or not these MAO-B alleles result in different phenotypic enzyme activities. Several conflicting studies on the activity of MAO-B in PD patients' platelets have been published (review; Sturman and Williams, 1991). In mice of various ages, a direct correlation was observed between striatal dopamine loss after MPTP treatment and brain MAO-B activity (Irwin *et al*, 1992), suggesting that MAO-B activity may be an important factor in the differential toxicity of MPTP in individuals.

An allelic association between PD and a mutation in the mitochondrial $tRNA^{Gln}$ gene has recently been reported by Shoffner *et al* (1993) (see Introduction 1.4.4). The lack of an allelic association between PD and dopamine receptor gene alleles has also been reported (Nanka *et al*, 1993).

Only one study of linkage analysis has been published; Tanaka *et al* (1991) reported no linkage between the familial juvenile parkinsonism locus and the tyrosine hydroxylase gene. The search for a susceptibility gene may be complicated by the fact that PD may be caused by the interplay of a number of genes and environmental factors, and different factors may be differentially important in the cause of the disease in different individuals.

1.2. THE MPTP MODEL OF PD

To date the best model of PD is that of MPTP-induced parkinsonism. That the exogenous chemical MPTP could induce parkinsonism was initially discovered in 1982, when eight individuals developed a permanent parkinsonism caused by a contaminant of a synthetic heroin substitute. This drug, MPPP (1-methyl-4-phenyl-4-pro-pionoxy-piperidine; a meperidine (opiate) analogue), had become contaminated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) during its synthesis. Exposure to MPTP caused the onset of parkinsonian symptoms, including the classic triad of bradykinesia, rigidity and tremor (Langston *et al*, 1983; Ballard *et al*, 1985) in a proportion of those exposed to it. All eight reported patients became symptomatic within one week of taking the drug, and all responded to L-dopa therapy. In the only autopsy case (Davis *et al*, 1979) there was selective cell loss in the zona compacta of the substantia nigra, whereas the rest of the brain was normal, including the locus coeruleus and dorsal motor vagus nerve. In contrast to idiopathic Parkinson's disease there were no Lewy bodies present in the brain.

1.2.1. The MPTP animal model of PD

An animal model of MPTP-induced parkinsonism was first reported in the rhesus monkey by Burns *et al* (1983). The monkeys showed parkinsonian behaviour, severe neuronal loss in the zona compacta of the substantia nigra and decreased striatal dopamine content. The behavioural changes were reversed by the administration of L-dopa and, as in the human autopsy case of MPTP-induced parkinsonism (Davis *et al*, 1979), neuronal degeneration was restricted to the zona compacta of the substantia nigra. MPTP-induced parkinsonism in young-adult primates is therefore not an exact pathological model of the human idiopathic disease. Studies in aged animals have been contradictory; MPTP treated aged squirrel monkeys show degeneration of the locus coeruleus as well as the substantia nigra, and the presence of inclusions resembling immature Lewy bodies (Forno *et al*, 1986). However in MPTP treated aged marmosets cell loss was restricted to the substantia nigra and ventral tegmental area, with no cell loss in the locus coeruleus, and no Lewy bodies present (Rose *et al*, 1993).

MPTP also induces substantia nigra cell loss in mice (Heikkila *et al*, 1984a), which exhibit postural abnormalities and reduced locomotor activity, and it is reported that cell loss occurs in the ventral tegmental area and locus coeruleus as well as in the substantia nigra in older mice (Gupta *et al*, 1986).

1.2.2. MPTP is converted to MPP^+

MPTP itself is not the active neurotoxic agent. MPTP is thought to cross the blood-brain barrier where it is converted to the 1-methyl-4-phenylpyridinium (MPP^+) anion (Markey *et al*, 1984) by monoamine oxidase B (MAO-B) (Salach *et al*, 1984) (Figure 1-3). MAO-B is located mainly in glia, and not in dopaminergic neurons (Levitt *et al*, 1982; Westlund *et al*, 1985), so the conversion of MPTP to MPP^+ occurs extraneuronally (Ransom *et al*, 1975). The two-electron oxidation of MPTP by MAO-B forms the intermediate $MPDP^+$ (1-methyl-4-phenyl-2,3-dihydropyridinium), which is then further oxidised by MAO-B, or spontaneously autoxidises, to form MPP^+. MAO inhibitors block the conversion of MPTP to MPP^+ in rat brain mitochondria *in vitro* (Chiba *et al*, 1984), and pretreatment of mice (Heikkila *et al*, 1984b) or monkeys (Langston *et al*, 1984) with these MAO inhibitors prevents the neurotoxic effects of MPTP. The specific MAO-A inhibitor, clorgyline, does not block the conversion of MPTP to MPP^+ (Chiba *et al*, 1984), providing further evidence that MPTP is converted to MPP^+ by MAO-B. Direct evidence that MPP^+ and not MPTP is the active neurotoxic agent, comes from studies in rats, which are relatively resistant to the neurotoxic effects of MPTP. Infusion of MPTP into the rat substantia nigra has little effect, whereas infusion of MPP^+ causes loss of striatal dopamine and profound motor deficits (Bradbury *et al*, 1986).

Since MAO-B is distributed throughout the brain, MPP^+ is formed from MPTP throughout the brain. The selective accumulation of MPP^+ into dopaminergic neurons of the substantia nigra (Irwin and Langston, 1985) is explained by its high affinity uptake by the dopamine-reuptake system (Javitch *et al*, 1985) (Figure 1-4). There is evidence that MPP^+ is then retrogradely transported from the striatal nerve terminals to the cell bodies of the substantia nigra (Campbell *et al*, 1990); 24hrs after the injection of radioactively labelled MPTP into rat striatum, label was detected in the cell bodies of the substantia nigra zona compacta. MPP^+ binds with high affinity to neuromelanin (D'Amato *et al*, 1986) which may contribute to its anatomical selectivity. Interestingly, there is a positive correlation between the percentage of neuromelanin-pigmented neurons

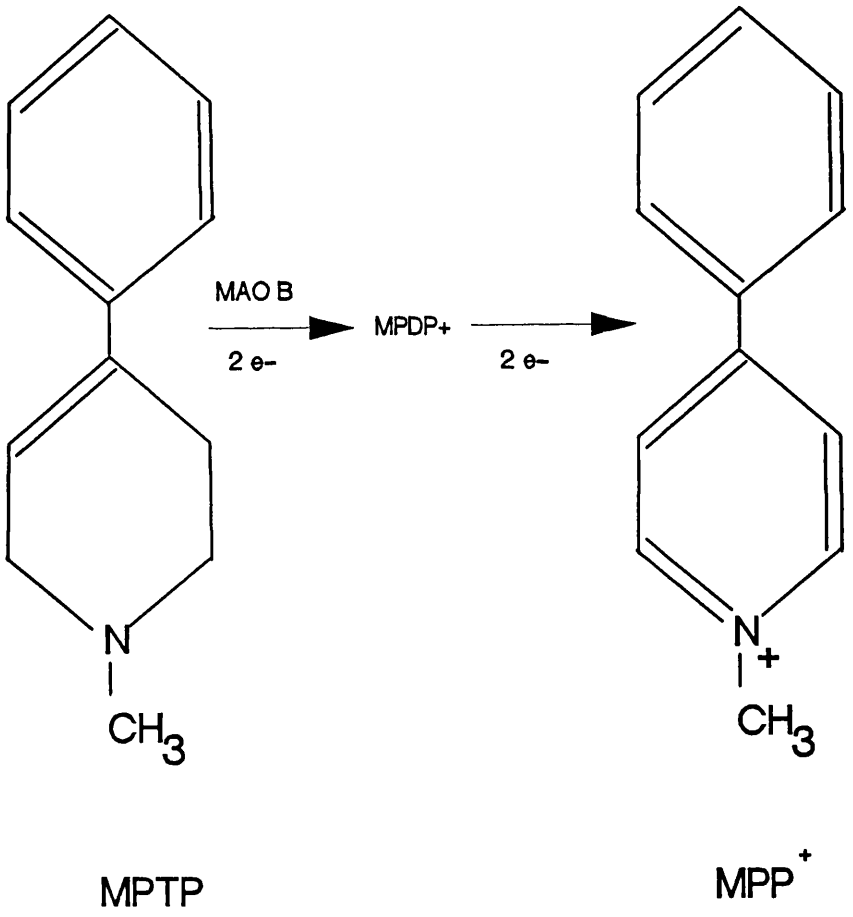


Figure 1-3. The conversion of MPTP to its toxic oxidation product MPP^+ by monoamine oxidase B.

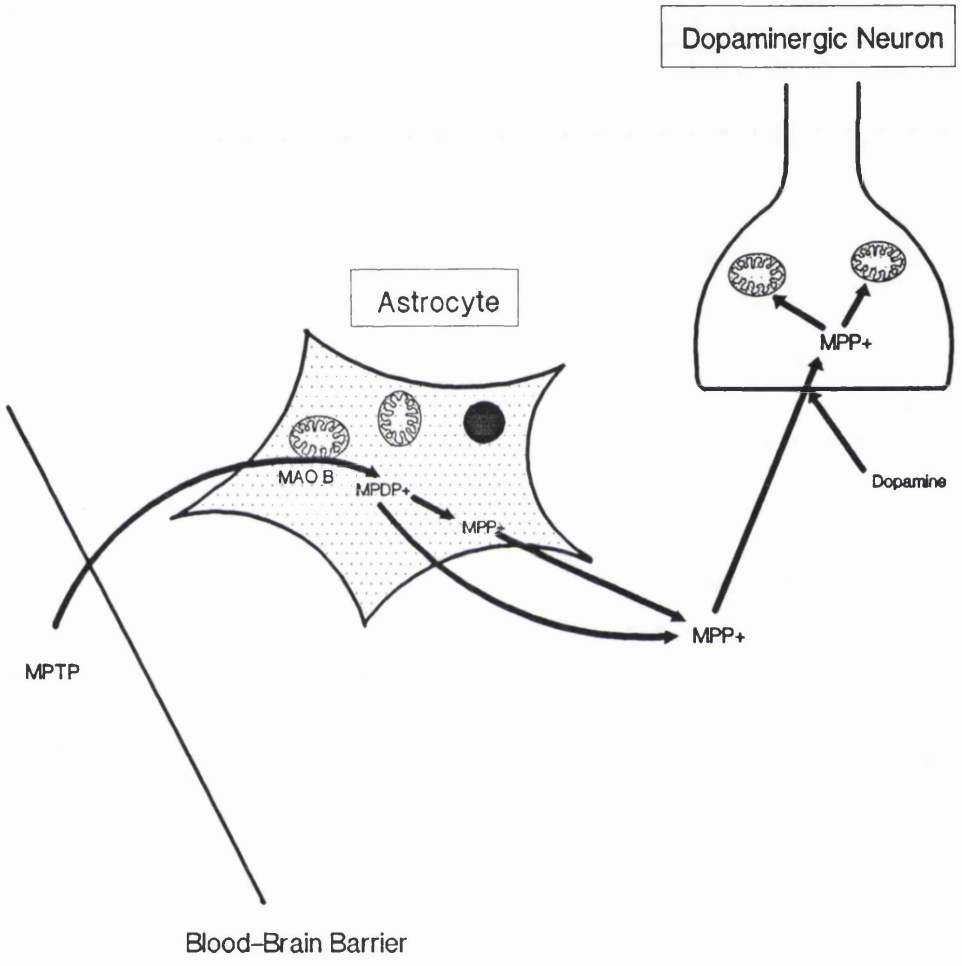


Figure 1-4. The conversion of MPTP to MPP^+ and its uptake by dopaminergic neurones.

in dopaminergic cell groups and their percentage cell loss in idiopathic PD (Hirsch *et al*, 1988). However, degeneration is also seen in some nonmelanized neurons in the PD brain, for example in the substantia innominata. The role of neuromelanin binding in the toxicity of MPP^+ has been questioned by Herkenham *et al* (1991). In monkeys treated with MPTP they observed accumulation and retention of MPP^+ at high concentration in the striatum, and at very low concentration in the substantia nigra.

1.2.3. The dopamine transporter and MPP^+ toxicity

The central role of the dopamine transporter (DAT) in MPP^+ toxicity has been demonstrated in non-neuronal COS (African green monkey kidney) cells. A cloned copy of the DAT gene transfected into these cells allowed them to accumulate dopamine and MPP^+ (Uhl and Kitayama, 1993), and the cytotoxic effect of MPP^+ in these cells was dependent upon the level of DAT expression (Pifl *et al*, 1993). This suggests that differences in the vulnerability of various brain regions to MPP^+ may be related to the density of dopamine uptake sites present. Site directed mutagenesis of the DAT gene has identified several mutations which retain normal dopamine uptake activity but specifically increase MPP^+ uptake (Kitayama *et al*, 1993). These results may influence the treatment of, and the search for a cause of, idiopathic PD. It may be possible to develop drugs which interfere specifically with the uptake of a putative MPP^+-like neurotoxin while sparing DAT dopamine uptake. The DAT gene is therefore a putative susceptibility gene for the development of PD; only individuals carrying mutations in the DAT gene which increase its affinity for MPP^+-like toxin uptake, may develop PD upon exposure to the toxin.

1.2.4. The vesicular amine transporter and MPP^+ toxicity

After both synthesis and reuptake from the synapse, catecholamines are transported from the nerve terminal cytoplasm into storage granules by the vesicular amine transporter (VAT). The bovine VAT gene has been cloned and sequenced (Stern-Bach *et al*, 1992), and is homologous to a gene isolated from rat pheochromocytoma (PC12) cells (which are relatively resistant to the effects of MPP^+). The transfer of this PC12 gene to chinese hamster ovary (CHO) cells, which are more sensitive to MPP^+ than PC12 cells, results in suppression of

MPP^+ toxicity (Liu *et al*, 1992). Uptake into vesicles by the VAT, sequesters MPP^+ and so protects the cell from toxicity. The observed lack of MPP^+ toxicity in platelets (Cesura *et al*, 1987) and adrenal medullary cells (Reinhard *et al*, 1987) may therefore be mediated by toxin uptake into serotonin and chromaffin granules respectively. The VAT in adrenal medullary cells is not identical to that from brain (Liu *et al*, 1992), which may be one explanation for the differential toxicity of MPP^+ in these cell types (although adrenal medullary cells are catecholaminergic, they do not degenerate in PD). Extensive sequence divergence between the human and rat brain VAT genes (Lesch *et al*, 1993) has been proposed as a possible cause of the differential MPP^+ toxicity in these species.

There is evidence from studies in rat striatal slices that at least a proportion of MPP^+ taken up by dopaminergic neurones is accumulated in dopamine vesicles (Keller and DaPrada, 1985). It has been suggested that MPP^+ may only be toxic in cells in which its concentration is so great that some leaks out of the dopamine vesicles and becomes available for mitochondrial accumulation (Tipton and Singer, 1993). The VAT gene is therefore another candidate for the PD susceptibility gene; it can be envisaged that individuals with an abnormal VAT would be more susceptible to the cytotoxic effects of an MPP^+-like toxin, normally sequestered within synaptic vesicles.

1.2.5. Mechanism of MPTP toxicity

The generally accepted mechanism of MPTP toxicity is via inhibition of the mitochondrial respiratory chain (review, Tipton and Singer, 1993). Once inside the cell, MPP^+ is actively accumulated within mitochondria, and concentrated up to 40-fold. This uptake is against a concentration gradient, and is driven by the electrochemical potential of the membrane (Ramsay *et al*, 1986). Within mitochondria MPP^+ specifically inhibits complex I (NADH ubiquinone reductase) of the electron transport chain (Nicklas *et al*, 1985) causing ATP depletion and ultimately cell death (DiMonte *et al*, 1986). The exact mechanism by which MPP^+ inhibits complex I is unclear. There is some evidence for loose binding in the same region as the rotenone binding site (Krueger *et al*, 1990; Ramsay *et al*, 1991), which includes the ND1 mitochondrially encoded subunit (Earley *et al*, 1987). However complex I inhibition via this loose binding can be removed by dilution. The injection of MPP^+ or the mitochondrial complex I inhibitor rotenone into rat brain causes specific damage to the dopaminergic nigrostriatal pathway

(Heikkila *et al*, 1985), directly demonstrating that dopaminergic neurotoxicity can be mediated by inhibitors of mitochondrial respiration.

The production of free radicals may also be one of the mechanisms of MPTP toxicity. Free radicals are produced directly during the oxidation of MPTP, and indirectly via the inhibition of complex I (see Introduction 1.6.8).

1.3. THE MITOCHONDRION

The mitochondrion is an intracellular organelle found in almost all eukaryotic cells. Its name derives from the Greek words mitos (thread) and chondros (granule). The mitochondrion is often referred to as the "power house" of the cell, as its main function is the conversion of energy derived from the oxidation of a range of substrates to the cellular energy supply, ATP. Structurally mitochondria consist of two membranes, an internal matrix bounded by the inner membrane, and an intermembrane space bounded by both the inner and outer membranes. The inner mitochondrial membrane is highly folded which increases its surface area, creating the characteristic folds, or cristae.

1.3.1. Mitochondrial DNA

In mammalian cells mitochondria are unique amongst subcellular organelles in that they contain their own DNA, distinct from that of the nucleus. The mitochondrial DNA (mtDNA) is a double-stranded circular molecule, approximately 16.5kb in length, coding for 13 functional polypeptides, 2 rRNAs and 22 tRNAs. The human mtDNA has been sequenced (Anderson *et al*, 1981), and all 13 polypeptides coded for by mtDNA are components of the respiratory chain complexes located in the mitochondrial inner membrane. Mitochondrial DNA is almost exclusively maternally inherited (Gyllensten *et al*, 1991), so that primary diseases of the mtDNA (except those involving mtDNA deletions) are inherited through the maternal line.

1.3.2. Mitochondria and ATP synthesis

The mitochondrial respiratory chain catalyses the oxidation of NADH and $FADH_2$ and the transport of reducing equivalents via a series of electron acceptors to the terminal electron acceptor, oxygen. The energy loss associated with this transfer of electrons is conserved in the translocation of protons across the inner membrane from the matrix, generating a proton motive force (PMF). The energy from this PMF is harnessed in the generation of ATP from ADP and Pi, by ATP synthase. The importance of the oxidative phosphorylation system is illustrated

by the fact that glycolysis yields two moles of ATP per mole of glucose, whereas 36 moles of ATP are generated per mole of glucose via oxidative phosphorylation.

1.3.3. The mitochondrial respiratory chain

The mitochondrial respiratory chain (MRC) consists of five multi-subunit enzyme complexes, all of which are situated in the inner mitochondrial membrane. The first four complexes form the electron transport chain (ETC); complex I (NADH ubiquinone reductase), complex II (succinate ubiquinone reductase), complex III (ubiquinol cytochrome c reductase) and complex IV (cytochrome c reductase). They are associated with two mobile electron carriers, ubiquinone (Q) and cytochrome c. These four enzyme complexes, together with complex V (ATP synthase) comprise the oxidative phosphorylation system (Figure 1-5).

1.3.3.1. Complex I

1.3.3.1.1. Complex I function

Complex I catalyses the oxidation of NADH and the reduction of ubiquinone. It is enzymatically defined as the rotenone-sensitive NADH-dependent reduction of ubiquinone. The complex contains a number of redox centres, namely flavin mononucleotide (FMN) and 22-24 atoms of iron in the form of iron-sulphur clusters. There is evidence from sequence data for the presence of one binuclear (2Fe-2S) and four tetranuclear (4Fe-4S) clusters (Walker, 1992). The pathway of electron transport from NADH to ubiquinone within the complex is not known. Ubiquinone (Q), is a quinone derivative with an isoprenoid tail (in man, of ten units, Q_{10}). Ubiquinone is reduced by a single electron to the free radical semiquinone ($QH^{\cdot}$), which is subsequently further reduced to ubiquinol (QH_2) by a further electron. Complex I is the first site of proton translocation, from the matrix to the cytosol.

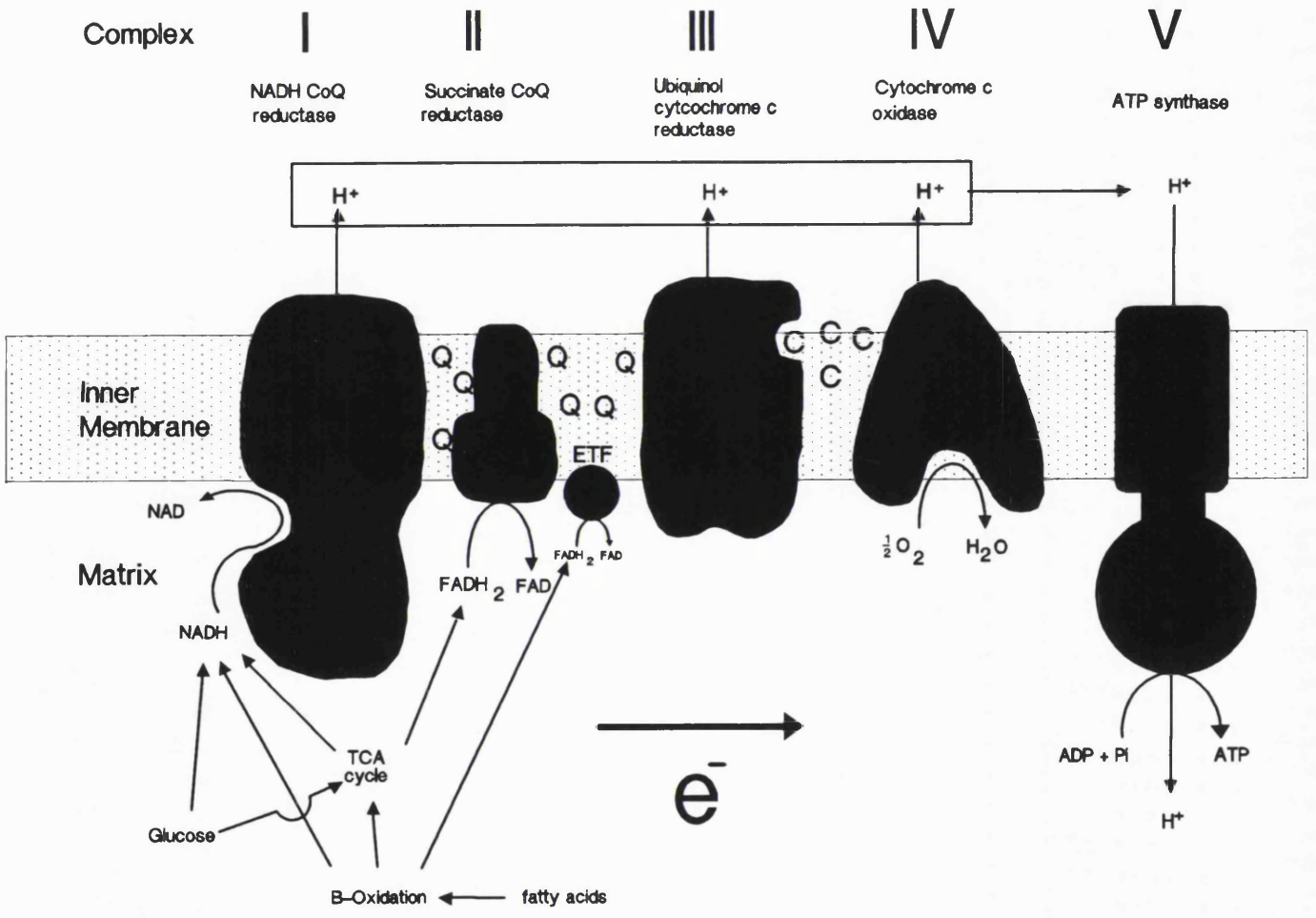


Figure 1-5. The mitochondrial respiratory chain.

1.3.3.1.2. Complex I composition and structure

The complex is believed to contain at least 41 subunits, seven coded for by mitochondrial (mt) DNA (Chomyn *et al*, 1985, 1986) and the remaining 34 by nuclear DNA. The 34 nuclearly encoded subunits of the bovine heart complex have been sequenced (review; Walker *et al*, 1992), and the molecular weight of the complex has been estimated at greater than 900kDa. Table 1-1 lists the subunits of complex I, their nomenclature, assumed molecular weight by gel electrophoresis, actual molecular weight determined from the gene sequence, and their association with subfractions of the complex detailed below.

1.3.3.1.2.1. The "Hatefi" preparation and subfractionation of complex I

Complex I has routinely been purified from bovine heart according to the original method of Hatefi *et al* (1962), by solubilisation in deoxycholate and fractionation by salts (Ragan *et al*, 1987). The complex can be subfractionated with the chaotropic agent perchlorate into three subcomplexes; two soluble fractions, the flavoprotein (FP) and iron protein (IP) fractions, and an insoluble, hydrophobic (HP) fraction (Ragan, 1987).

The FP fraction catalyses the oxidation of NADH by a range of artificial electron acceptors (e.g. ferricyanide) and contains three subunits of 51, 24 and 10kDa. This fraction contains the FMN, and two Fe-S clusters detected by electron paramagnetic resonance spectroscopy (EPR); a binuclear cluster associated with the 24kDa subunit, and a tetranuclear cluster associated with the 51kDa subunit (Ohnishi *et al*, 1985). The 51kDa subunit is also likely to be involved in NAD^+ binding (Chen and Guillory, 1981). The 51kDa subunit gene has been chromosomally mapped to region q13 on chromosome 11 (Ali *et al*, 1993).

The IP fraction, which shows no enzymatic activity contains six subunits of 75, 49, 30, 18, 15 and 13kDa. The 75, 49, 30 and 13kDa subunits are associated with Fe-S clusters (Ohnishi *et al*, 1985), and the 15kDa subunit is involved in Q-binding (Suzuki and Ozawa, 1986). The 75kDa subunit gene has been chromosomally mapped to region q33→q34 on chromosome 2 (Duncan *et al*, 1992).

The HP fraction is an ill-defined fraction containing the remainder of the subunits including, it is presumed, all of the seven mitochondrially encoded subunits,

Subunit	Mol Wt (gel)	Mol Wt (sequence)	Walker prep.	Hatefi prep.
nuclear-encoded				
75	75	77.0	α	IP
51	51	48.4	α	FP
49	49	49.2	α	IP
42	42	36.7	α	HP
39	39	39.1	α	HP
30	30	26.4	α	IP
24	24	23.8	α	FP
B22	22	21.7	ß	
TYKY	23	20.2	α	IP?
PDSW	22	20.8	ß	
PSST	20	20.1	α	IP?
PGIV	19	20.0	α	
ASHI	19	18.7	ß	
SGDH	16	16.7	ß	
B18	18	16.5	ß	
18	18	15.3	α	IP
B17	16.5	15.4	ß	
B15	15	15.1	ß	
B14	14	15.0	α	
B14.5b	14.5	14.1	nd	
B13	13	13.2	α	IP
B14.5a	14.5	12.6	nd	
15	15	12.5	α	IP
B8	8	11.0	α	
B12	12	11.0	ß	
13	13	10.5	α	IP
SDAP	8	10.1	α/ß	
MLRQ	9	9.3	α	
B9	9	9.2	α	
10	10	8.4	α	FP
AGGG	7.9	8.5	ß	
MWFE	7.5	8.1	α	
MNLL	7	7.0	ß	
KYFI	6	5.8	nd	

Subunit	Mol Wt (gel)	Mol Wt (sequence)	Walker prep.	Hatefi prep.
mitochondrially-encoded				
ND5	50	68.3	ß	
ND4	39	52.1	ß	
ND2	30	39.3	*α*	
ND1	30	35.7	nd	HP
ND6	nd	19.1	nd	
ND3	15	13.1	nd	HP
ND4L	10	10.8	nd	HP

Table 1-1. Subunit composition of bovine heart complex I.

Subunit nomenclature: named according to apparent molecular weight (e.g. 75), with modified *α*-amino group (e.g. B22); named according to sequence of amino acids 1-4, with unmodified *α*-amino group (e.g. TYKY); subunits encoded by mtDNA are known as ND (for NADH dehydrogenase) 1-6.

Mol Wt (molecular weight in kDa; gel- by SDS-PAGE (Laemmli, 1970); sequence- from gene sequence; Finel *et al*, 1992, Pilkington *et al*, 1993).

Walker preparation (Finel *et al*, 1992); subunits found in fraction *α* or ß, or nd (not detected in either fraction); subunit SDAP detected in both *α* and ß.

Hatefi preparation (Ragan *et al*, 1987); subunits found in fraction IP or FP; HP (positively identified in HP (Ragan, 1987)); remainder of subunits assumed to be components of HP); IP? (detected in some preparations of IP, Finel *et al*, 1992).

designated ND 1, 2, 3, 4, 4L, 5 and 6. The fraction contains two Fe-S clusters detected by EPR, probably one binuclear and one tetranuclear (Ohnishi *et al*, 1985). Various inhibitors of complex I activity, including rotenone, have been shown to bind to the ND1 subunit of 33kDa (Earley *et al*, 1987), and it has therefore been suggested that this subunit is likely to have an important functional and/or structural role. Furthermore, dicyclohexylcarbodiimide (DCCD) which inhibits proton translocation at sites 1, 2 and 3 of the electron transport chain, binds to the ND1 subunit of complex I, which suggests that this subunit may be involved in proton translocation (Yagi and Hatefi, 1988).

Based on cross-linking studies and hydrophilicity profiles, Ragan (1987) suggested a topography for complex I in which the transmembraneous parts of IP and most of FP are enclosed within a hydrophobic shell created by HP, some of which contacts the lipid bilayer and some of which contacts the aqueous phase on either side of the membrane.

1.3.3.1.2.2. The "Walker" preparation and subfractionation of complex I

An alternative preparation of bovine heart complex I has recently been reported. This preparation involves solubilisation with N-dodecyl-ß-D-maltoside, ammonium sulphate precipitation, and a chromatographic step in the presence of detergent. Treatment of this complex I preparation with the detergent N,N-dimethyldodecylamine N-oxide (LDAO) results in two subcomplexes, Iα and Iß (Finel *et al*, 1992). The Iα subfraction contains 22 detectable subunits including all of the FP (51, 24 and 10kDa) and IP (75, 49, 30, 18, 15, 13kDa and subunit B13) subunits, as well as two subunits found in some preparations of IP (PSST and TYKY). It contains a further nine nuclearly encoded subunits and one mitochondrially encoded subunit, ND2. The Iα subfraction contains FMN and all the Fe-S clusters detectable by electron paramagnetic resonance spectroscopy (EPR). It transfers electrons from NADH to ferricyanide and ubiquinone-1, although the enzyme activity is not sensitive to rotenone. The Iß subfraction contains 13 detectable subunits, including two mtDNA products, ND4 and ND5. The other five mtDNA products have not been detected in either subcomplex. The subfraction contains no redox centres, has no enzymatic activity and its function is unknown.

Based on the proposed structure of the complex in *Neurospora crassa*, gene sequence data and this alternative subfractionation, an alternative model of

bovine complex I topography has been proposed (Pilkington *et al*, 1993). The *N.crassa* complex is L-shaped, composed of a nuclear-encoded peripheral arm and a hydrophobic, nuclear and mtDNA-encoded membrane arm (Hofhaus *et al*, 1991). It is composed of at least 21 different polypeptides, six of which are encoded by mtDNA. Of the fifteen or more nuclear encoded subunits of the *N. crassa* enzyme, nine have so far been identified by sequence comparison as having homologues in the bovine enzyme. The Iα subfraction of the bovine enzyme contains all the redox centres of the complex, and of its 22 or so subunits, four are likely to contain membrane-spanning, hydrophobic segments. It is therefore proposed that the bovine enzyme is also L-shaped, and that Iα forms a hydrophilic, globular domain in the mitochondrial matrix, anchored in the inner membrane by the hydrophobic subunit, ND2. The Iß subfraction is proposed as the hydrophobic membrane domain of the complex, although the role of this domain in complex I function is not known.

1.3.3.1.3. Complex I assembly

Although the details of complex I assembly are not known, it has been shown in cultured rat hepatoma cells that the mtDNA encoded products are inserted into the holoenzyme only after a scaffold of nuclear products has been assembled (Hall and Hare, 1990). If cytoplasmic protein synthesis is inhibited, the mitochondrial gene products do not form a complex, but rather are degraded. Tuschen *et al* (1990) have shown in *N. crassa* that two intermediate complexes are independently assembled, which subsequently join to form the holoenzyme. The 350kDa intermediate contains all of the mtDNA encoded subunits and some nuclearly encoded subunits, and is equivalent to the mammalian HP fraction. The 250kDa intermediate is composed of 13 nuclearly encoded subunits, and is equivalent to the mammalian IP and FP fractions.

Recently, Hofhaus and Attardi (1993) have characterised a mutant human cell line which lacks the ND4 gene product, due to a frame-shift mutation in the ND4 gene. The complex I in this cell line contains most of the nuclear-encoded products, but no ND4 and markedly reduced amounts of ND2, ND3, ND4L and ND6. This implies that the absence of ND4 results in defective incorporation of other mtDNA-encoded subunits.

1.3.3.1.4. Complex I isoforms.

Only one example of the existence of complex I tissue isoforms has been reported (Clay and Ragan, 1988). Complex I immunoprecipitated from rat liver, kidney and lung differed from that present in rat heart, brain and skeletal muscle by the absence of an 18.5kDa subunit and the presence of a 17kDa subunit.

1.3.3.2. Complex II

Complex II catalyses the oxidation of $FADH_2$ and the reduction of ubiquinone (Q) to QH_2. The free-energy change resulting from the transfer of electrons from $FADH_2$ to Q is not sufficient to allow the translocation of protons. Because the oxidation of $FADH_2$ does not include the first proton pump at complex I, the transfer of electrons from $FADH_2$ along the respiratory chain to oxygen yields two molecules of ATP, whereas that from NADH yields three molecules of ATP.

Complex II is the smallest of the respiratory chain complexes, composed of only four subunits. It is the only complex which does not contain mtDNA products, and is not a site of proton translocation, which may suggest that the mtDNA products are important for this function.

1.3.3.3. Complex III

Complex III catalyses the oxidation of ubiquinol, the reduction of cytochrome c and the translocation of protons across the inner membrane. Electron transfer from QH_2 to cytochrome c requires the following redox groups; cytochrome b566, cytochrome b562, cytochrome c_1 and a binuclear iron-sulphur centre, the so-called Rieske iron sulphur-centre. QH_2 transfers one of its electrons to the Fe-S cluster, which passes via cytochrome c_1 to cytochrome c. The other electron in the semiquinone radical ($QH^{\cdot}$) is transferred to cytochrome b566 then b562, which reduces $QH^{\cdot}$ to QH_2. These two pathways of electron transfer allow a two electron carrier (Q) to reduce a one electron carrier (Fe-S cluster).

Complex III contains eleven subunits one of which, cytochrome b, is encoded by mtDNA.

1.3.3.4. Complex IV

Complex IV (cytochrome c oxidase) catalyses the oxidation of cytochrome c, the reduction of oxygen and the translocation of protons . The complex contains the following redox groups; heme a and heme a_3, and two copper atoms, Cu_a and Cu_b. Four electrons completely reduce O_2, the terminal electron acceptor, which is tightly bound to the complex, to H_2O.

Complex IV contains thirteen subunits, three of which are encoded by mtDNA (CO I, II and III). These three subunits contain the catalytic core of the enzyme. In mammals, at least three of the nuclearly encoded subunits exist as tissue-specific isoforms (Capaldi, 1990).

1.3.3.5. Complex V

The transfer of electrons is coupled to the phosphorylation of ADP by a proton-motive force, which is harnessed by complex V (ATP synthase) to synthesise ATP. Protons are translocated at three sites in the electron transport chain; site 1 (NADH ubiquinone reductase), site 2 (ubiquinol cytochrome c reductase) and site 3 (cytochrome c oxidase). ATP is synthesised when protons flow back into the matrix through a channel in complex V.

Complex V consists of 2 domains, the F_1 catalytic centre and the F_0 hydrophobic domain concerned with proton translocation. The human enzyme is composed of fourteen subunits, two of which, ATPase 6 and A6L (ATPase 8), are encoded by mtDNA. Some nuclearly-encoded subunits of complex V have been reported to exist as tissue-specific isoforms (Matsuda *et al*, 1993).

1.3.4. Mitochondrial protein import

The majority of polypeptide constituents of the mitochondrial oxidative phosphorylation system are coded for by nuclear DNA, cytoplasmically synthesised and subsequently transported into mitochondria and assembled into their correct respiratory complex. Approximately 10% of mitochondrial protein import is passive, while the majority is via an energy-requiring import system. The import and assembly process, as understood from work in the fungus *N.*

crassa and in yeast, has four stages (reviews; Segui-Real *et al*, 1992; Schatz, 1993). Many nuclearly-encoded polypeptide precursors are synthesised with 20-80 amino acid N-terminal, cleavable presequences which act as recognition signals for mitochondrial import. Precursor proteins are inserted into the outer membrane and transferred, via import channels at contact sites between the inner and outer membranes, into the matrix. Once in the mitochondrial matrix, N-terminal presequences are cleaved releasing the mature polypeptides. The mechanisms of sorting of polypeptides to their respective mitochondrial compartments and to their respective complexes is not understood. It is also unclear whether inner membrane proteins can be imported directly from the outer membrane, rather than being imported to the matrix, and then re-exported.

1.4. MITOCHONDRIAL RESPIRATORY CHAIN FUNCTION IN PD

It has been proposed that late-onset neurodegenerative diseases may be caused by mitochondrial respiratory chain (MRC) abnormalities. In support of this theory it has been shown that MRC function decreases with age, and that the brain is highly dependent on oxidative phosphorylation for ATP generation (Wallace, 1992). Abnormal MRC function has been associated with a number of neurodegenerative diseases including Alzheimer's disease, Huntington's disease, dystonia and Parkinson's disease.

Following the discovery that the Parkinson-causing neurotoxin MPP^+ is a specific inhibitor of mitochondrial complex I (Nicklas *et al*, 1985), interest has particularly focused on the function of the MRC in idiopathic Parkinson's disease. MRC function has been studied in the PD brain, skeletal muscle platelets and lymphocytes, as detailed below.

1.4.1. MRC function in the PD brain

In the largest study published to date, MRC function was measured in substantia nigra brain homogenate from 22 PD patients and 17 controls (Mann *et al*, 1992a). The activity of complexes II/III and IV were normal, but there was a 37% decrease in mean complex I activity in the PD substantia nigra. Patient and control groups were matched for age, death to refrigeration and death to autopsy times, and there was no correlation between complex I activity and duration of disease, L-dopa treatment or time from death to freezing. The observed complex I defect has been shown to be disease specific (Schapira *et al*, 1990b), in that complex I activity was normal in the substantia nigra of patients who had died with multiple system atrophy (MSA). This disease is characterised by cell death in many areas of the brain, including the substantia nigra, where cell death is observed at levels equal to or greater than that seen in PD. Normal complex I activity in the substantia nigra of MSA patients therefore provides evidence that the complex I defect observed in PD is not simply the result of neuronal degeneration. Patients with MSA are prescribed L-dopa, providing more evidence that the complex I defect in the PD brain is not simply caused by L-dopa therapy. The complex I deficiency has been shown to be anatomically specific to the substantia nigra in the PD brain (Schapira *et al*, 1990b; Mann *et al*, 1992a). Complex I activity was not significantly different in

the caudate nucleus, cerebral cortex or the medial or lateral globus pallidus in four PD patients and five controls, or in the tegmentum (ten samples), cerebellum (sixteen samples) or posterior putamen (twelve samples; Dr JM Cooper, personal communication). These results closely resemble the effects of MPTP in terms of anatomical selectivity (to the substantia nigra) and biochemical specificity (to a deficiency of complex I), suggesting that the cause of cell death in the idiopathic disease may be similar to that in the MPTP model.

Only two other studies of mitochondrial function in the PD brain have been published. Mizuno *et al* (1990) detected a slight but significant decrease in mean complex III activity, and normal complex I activity, in the striatum of five PD patients. Janetzky *et al* (1994) detected a 33% decrease in the citrate synthase corrected activity of rotenone-sensitive NADH cytochrome c reductase (an assay representing complex I activity) in the substantia nigra of seven PD patients compared to seven controls, matched for age and post-mortem parameters.

Two studies have reported on the composition of complex I in the PD brain by Western blotting with complex I antibodies. Schapira *et al* (1990a) using substantia nigra homogenates probed with antibodies to the complex I holoenzyme and specific for the 30, 24 and 13kDa subunits, could detect no differences in molecular weight or staining intensity of any complex I polypeptide. Mizuno *et al* (1989) detected moderate to marked decreases in the staining intensity of polypeptides of 30, 25, and 24kDa in four out of five PD striatum samples compared to controls. Hattori *et al* (1991) have used immunohistochemical techniques to quantify the amount of complex I in PD substantia nigra sections compared to controls. Using antisera to the complex I holoenzyme, they observed that neurons with reduced staining, were far more abundant in the eight PD brain sections than in the control sections.

1.4.2. MRC function in PD skeletal muscle

To date there have been seven reports of MRC function in PD skeletal muscle. Bindoff *et al* (1991) detected statistically significant generalised defects of complexes I, II and IV in five PD patients the mean activities being decreased by 40, 49 and 40% respectively compared to controls. Shoffner *et al* (1991) compared six PD patients and eleven controls. Two of the patients had only complex I defects, two had complex I, II, III and IV defects, one had complex II, III and IV defects and one showed normal MRC function. Relative to the control

mean, the activities of complex I in the four patients showing a complex I defect were 92, 90, 86, and 62% decreased. Nakagawa-Hattori *et al* (1992) using mitochondria prepared from post-mortem samples from four PD patients detected 49, 26 and 21% decreases in the mean activity of complexes I, II and III respectively (only the difference in complex I activity was statistically significant). The activity of complex IV was not significantly different in patients and controls. Mann *et al* (1992a) compared MRC function in nine PD patients and six controls by both spectrophotometry and polarography. No differences were detected. Anderson *et al* (1993) compared MRC function in seven PD patients and six controls by spectrophotometry and polarography. Although some MRC enzyme activities were decreased in the patient group, none of the results were statistically significant, which may have been due to the extremely large spread of data in the control group. Cardellach *et al* (1993) detected statistically significant decreases of 26% and 68% in the mean activities of complexes I and IV respectively in muscle mitochondria from eight PD patients compared to ten controls. DiDonato *et al* (1993) measured MRC enzyme activity in skeletal muscle crude homogenates and purified mitochondria from sixteen PD patients and eight controls (homogenates), and six patients and six controls (purified mitochondria). There were no significant differences in the citrate synthase (CS) corrected activities of any of the MRC enzymes between the two groups, measured in crude homogenates or purified mitochondria.

From the above studies it is clear that the presence of a MRC abnormality in the skeletal muscle of PD patients is controversial. Evidence from ^{31}P magnetic resonance spectroscopy (MRS) studies provides evidence of normal mitochondrial function in PD skeletal muscle (Taylor *et al*, 1994). In a group of mitochondrial myopathy patients, with defined biochemical complex I defects, many of the parameters measured were abnormal; during exercise, stores of high energy phosphate were depleted more rapidly, and during recovery, phosphocreatine and free ADP concentrations returned to basal levels more slowly than in controls. In the PD patients however, none of these parameters were statistically significantly different from controls. Although these results do not rule out the presence of small complex I deficiencies in PD skeletal muscle, they do suggest that any defect present is not as great as that in the mitochondrial myopathy patients, whose mean complex I activity was 60% less than controls. Other evidence against the presence of an MRC defect in PD skeletal muscle is the absence of increased blood lactate levels post-exercise in PD patients (Nakagawa-Hattori *et al*, 1992), and the lack of significant morphological abnormalities in PD skeletal muscle biopsies (Bindoff *et al*, 1991; Shoffner *et al*, 1991; Mann *et al*, 1992a).

1.4.3. MRC function in PD platelets

The main role of platelets in the circulation is the formation of blood clots in response to vascular damage. They adhere to damaged vascular epithelia, and when appropriately stimulated aggregate into clumps sealing the damaged vessel. Platelets are discoid cells, 2-5 μm in diameter, and normal blood contains 150-350,000 platelets per μl. They have a short life span surviving in the circulation for only eight to twelve days. Platelets are released from cytoplasmic projections of the megakaryocyte stem cells of bone marrow. They contain lysosomes, mitochondria, glycogen granules, dense granules and alpha granules which store substances involved in the aggregation process, but have no nucleus. It has been estimated that they only contain four mitochondria per cell, and only one copy of mtDNA per mitochondrion (Shuster *et al*, 1988). Platelets have been considered a useful model system for central serotonergic neurons (Da Prada *et al*, 1988); they have an active transport system for serotonin (5-hydroxytryptamine or 5-HT), which is stored in dense bodies. Their use as a model system for dopaminergic neurons is less well established, although dopamine shows some affinity for the 5-HT uptake and storage systems.

In 1989 Parker *et al* published the first report of MRC function in PD platelets. Mitochondrial fractions were prepared on Percoll density gradients after plateletphoresis from ten PD patients and eight controls. A specific 55% decrease in the activity of complex I in the PD group was detected. Since platelets are a peripheral tissue, this led to the possibility that the detection of a complex I defect in platelets could be used as a diagnostic test for PD. Further studies of PD platelet MRC function are detailed in Chapters 3 and 4.

1.4.4. MtDNA and PD

Mitochondrial DNA codes for 13 functional polypeptides, all of which are constituent subunits of the complexes of the mitochondrial respiratory chain. Since seven of the 41 subunits of complex I are coded for by mtDNA (Chomyn *et al*, 1985, 1986), any complex I defect in PD may be due to a mutation in a complex I gene within mtDNA. As PD is not maternally inherited, a mtDNA mutation is unlikely to be the sole cause of the disease (Zweig *et al*, 1992) (although mtDNA deletions appear to be sporadic and are not maternally inherited). However, mtDNA mutations may be one of many factors contributing to the pathogenesis of PD.

1.4.4.1. MtDNA deletions and PD

The presence of large amounts of deleted mtDNA in the PD substantia nigra (Schapira *et al*, 1990c; Lestienne *et al*, 1990) or skeletal muscle (Shoffner *et al*, 1991; Nakagawa-Hattori *et al*, 1992) has been ruled out by Southern blotting techniques. Initial reports, using the more sensitive polymerase chain reaction (PCR), detected an increased level of mtDNA in the PD brain carrying the 5kb "common" deletion (Ikebe *et al*, 1990; Ozawa *et al*, 1990). This deletion is commonly found in the skeletal muscle of patients with mitochondrial myopathies (Holt *et al*, 1988) and covers the genes encoding four complex I subunits (ND3, 4L, 4 and 5), one complex IV subunit (COIII), two complex V subunits (A6 and A6L) and five tRNAs. Correcting for differences in the efficiency of amplification of normal and deleted mtDNA by PCR, Mann *et al* (1992b) detected similarly low levels (0.01%) of deleted mtDNA in both PD and age-matched control substantia nigra samples. They conclude that the common deletion is not specific to the PD brain, but is rather an age-related phenomenon. This result has subsequently been confirmed by Di Donato *et al* (1993), and the same phenomenon has been observed in platelet mtDNA from PD patients and controls (Sandy *et al*, 1993). Interestingly, DiDonato *et al* (1993) detected higher concentrations (at least a factor of three) of deleted mtDNA in the substantia nigra than in any of the nine other brain regions studied, in a single PD and a single control case.

1.4.4.2. MtDNA point mutations and PD

Mitochondrial DNA polymorphisms have been detected in individual PD patients in many studies (Lestienne *et al*, 1990; Schapira *et al*, 1990c; Johns *et al*, 1991; Ozawa *et al*, 1991) although the relevance of these to disease pathogenicity is not known. Recently, Shoffner *et al* (1993) detected the presence of a homoplasmic point mutation in the mitochondrial $tRNA^{Gln}$ gene in a large group of PD patients. The mutation is a transition (A to G) of a conserved nucleotide (nt4336) in the gene, which connects the amino acid acceptor stem with the TψC stem of $tRNA^{Gln}$ and is presumed to affect its function. The mutation was detected in 5.3% (2/38) of PD samples and in 0.7% (12/1691) of controls; approximately eight times more common in PD patients than controls. The increased frequency of this mutation was not however specific to PD, and was also detected in 3.2% (2/62) of Alzheimer's disease (AD) samples. The authors state that the presence of the mutation in a small proportion of controls

could reflect the prevalence of the presymptomatic stage of these diseases in the general population.

A mutation in a mitochondrial tRNA gene would be expected to affect the translation of all mitochondrial mRNAs, and since mitochondrial polypeptides are constituents of complexes I, III and IV, this $tRNA^{Gln}$ mutation would be expected to affect the activities of all of these complexes. Two mitochondrial diseases caused by mitochondrial tRNA mutations are MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes) and MERRF (myoclonic epilepsy and ragged red fibres). The MELAS syndrome is associated with a mitochondrial $tRNA^{Leu}$ mutation, and the MERRF syndrome with a mitochondrial $tRNA^{Lys}$ mutation. Interestingly, in both syndromes a complex I defect in skeletal muscle mitochondria is most often the principal biochemical abnormality (review; Cooper and Clark, 1994). Therefore, it is possible for a tRNA gene mutation to primarily cause a deficiency of complex I.

1.4.5. MRC function in neurodegenerative diseases other than PD

Alzheimer's disease (AD) pathology includes degeneration of the cerebral cortex, particularly the temporal cortex. Reichmann *et al* (1993) have reported a 40% decrease in the activity of complex II/III in the AD temporal cortex. Cytochrome oxidase (complex IV) activity has been reported as slightly (17%) decreased (Kish *et al*, 1992), or normal (Cooper *et al*, 1993) in the AD temporal cortex , or as 50% decreased in AD platelet mitochondria (Parker *et al*, 1990b).

Huntington's disease (HD) is characterised pathologically by degeneration in the caudate nucleus and putamen. Mann *et al* (1990) have reported a 77% decrease in the activity of complex II/III in the HD caudate nucleus, and Parker *et al* (1990a) have reported a 72% decrease in the activity of complex I in HD platelet mitochondria.

Dystonia is a movement disorder believed to be caused by dysfunction of the basal ganglia. Benecke *et al* (1992) have reported 62% and 37% reductions in the mean activity of complex I in the platelet mitochondria of individuals with either segmental/generalised dystonia or focal dystonia respectively. Only the reduction in segmental/generalised dystonia complex I activity compared to controls reached statistical significance ($p<0.001$).

1.4.6. Mitochondria and ageing

The most important risk factor for many neurodegenerative diseases, including PD, is age. If abnormal mitochondrial function plays a role in the aetiology of PD, decreasing mitochondrial function with age may help to explain the late onset of the disease.

1.4.6.1. Ageing and MRC function

Mitochondrial respiratory chain function has been found to decrease with age in human liver (Yen *et al*, 1989) and skeletal muscle (Trounce *et al*, 1989; Cooper *et al*, 1992), and two studies have shown a decrease in MRC function with age in the primate brain. Bowling *et al* (1993) found a significant negative correlation between complex I and IV activity and age in the frontoparietal cortex of 20 rhesus monkeys, and Di Monte *et al* (1993) found reduced respiratory chain activity with NAD-linked, but not FAD-linked substrates, in the striatum from aged squirrel monkeys.

1.4.6.2. Ageing and mtDNA

Mitochondrial DNA mutations have been shown to accumulate with age in human liver (Yen *et al*, 1991) and skeletal muscle (Cooper *et al*, 1992). The levels of the 5kb "common" deletion in human skeletal muscle increased with age to a maximum proportion of 1 in 5000 (Cooper *et al*, 1992). Such a low level of deleted molecules is unlikely to account for any decrease in respiratory chain function with age. However it is possible that a number of different deletions and point mutations of mtDNA accumulate with age and together account for the decreased respiratory chain function.

Generalised oxidative damage to cellular DNA, measured as the presence of the oxidised nucleoside 8-hydroxy-2'-deoxyguanosine (OH^8dG), increases with age in the human brain (Mecocci *et al*, 1993). This damage was predominantly detected in mtDNA, and may therefore be expected to result in reduced function of the respiratory chain with age.

1.5. NEUROTOXINS AND PD

The discovery that the exogenous chemical MPTP can cause a parkinsonism very similar to the idiopathic disease (Langston *et al*, 1983; Ballard *et al*, 1985) has intensified the search for an environmental cause of the idiopathic disease. PD has repeatedly been associated with a number of environmental factors including rural living, well water drinking, pesticide/herbicide use and exposure to industrial chemicals (review; Tanner, 1989).

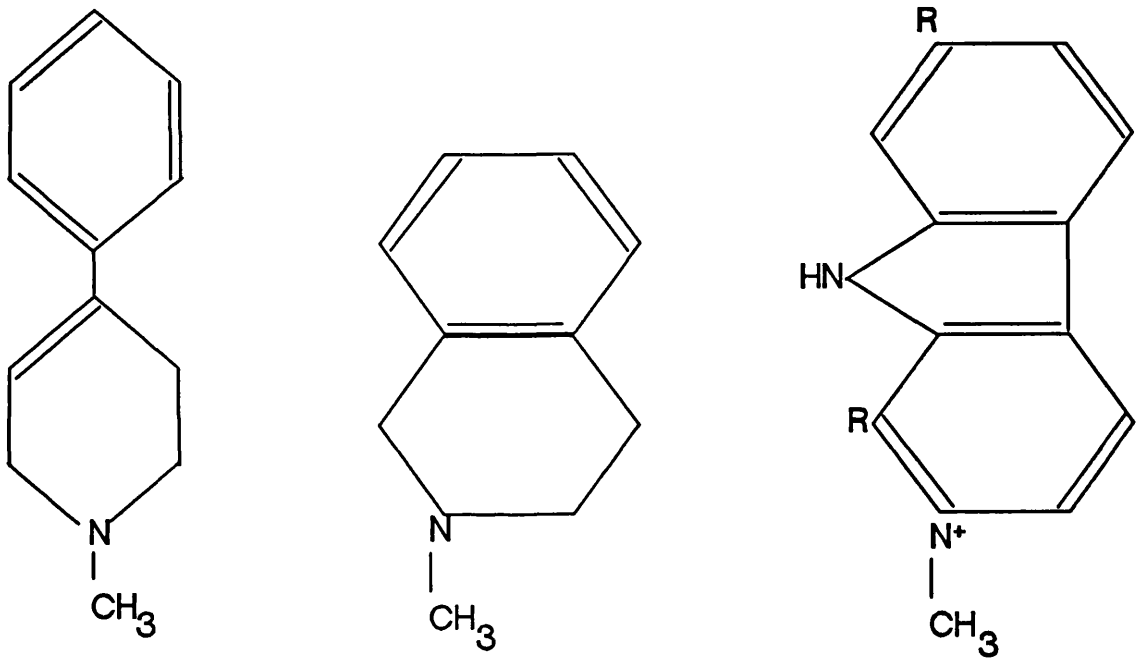
Any potential Parkinson-causing neurotoxin may be rare, such that all those who come into contact with it at significant doses will go on to develop PD, or it may be common, in which case only those individuals who are for some reason susceptible to the toxins effect, will develop the disease. Susceptibility to the toxin may be due to a defect in a detoxification process (for example the cytochrome P450 system) or via a process which enhances the toxicity of a relatively harmless substance (for example, N-methylation). Particular interest has focused on toxins that are MRC inhibitors since the discovery that MPTP inhibits complex I (Nicklas *et al*, 1985), and that the activity of complex I is reduced in the PD substantia nigra (Schapira *et al*, 1990a, 1990b).

1.5.1. Endogenous neurotoxins and PD

The search for compounds structurally similar to MPTP and MPP^+ in the PD brain using polyclonal antibodies to these compounds with wide cross-reactivities has been unsuccessful (Ikeda *et al*, 1992). Nevertheless, based on *in vivo* studies, the endogenous MPTP-like quinoline and carboline alkaloids are of interest as potential Parkinson-causing neurotoxins (See Figure 1-6).

1.5.1.1.Tetrahydroisoquinolines (TIQ's)

Tetrahydroisoquinolines (TIQ's) can be formed endogenously by the condensation of catecholamines, including dopamine, with an aldehyde. They have also been found naturally in certain foods (Makino *et al*, 1988), and both TIQ (1,2,3,4-tetrahydroisoquinoline) and the N-methylated TIQ (N-Me-TIQ) can



MPTP N-Me-TIQ 2-MeBC

Figure 1-6. The structural similarity of MPTP and the potential Parkinson-causing toxins N-Me-TIQ and 2-MeßC.

N-Me-TIQ (N-methyl-tetrahydroisoquinoline); 2-MeßC (2-methyl-ß-carboline).

cross the blood brain barrier (Makino *et al*, 1988) implying that their accumulation in the brain from external sources is possible. Both TIQ and N-Me-TIQ have been detected in the frontal cortex of one control and one Parkinson brain, the level of TIQ being considerably higher in the PD sample than the control (Niwa *et al*, 1987). N-Me-TIQ can be oxidised to the N-methylisoquinolinium ion (N-Me-IQ$^+$) by MAO types A and B (Naoi *et al*, 1989a) and is actively transported into rat PC12 cells by the dopamine uptake system (Naoi *et al*, 1989b), suggesting that it may be able to accumulate in dopaminergic neurones in a similar manner to MPP$^+$.

The administration of TIQ to marmosets induces the clinical characteristics of parkinsonism, and results in depletion of dopamine and reduction in tyrosine hydroxylase activity in the striatum (Nagatsu and Yoshida, 1988). However, its neurotoxicity in mice is contentious (Perry *et al*, 1988; Tasaki *et al*, 1991). N-Me-TIQ is not toxic to dopaminergic nigrostriatal neurons in mice (Perry *et al* , 1986; Tasaki *et al*, 1991) or marmosets (Perry *et al*, 1987), but the N-Me-IQ$^+$ anion produces severe striatal dopamine depletion when intracerebrally administered to rats (Booth *et al*, 1989). TIQ specifically inhibits complex I *in vitro* in isolated rat brain mitochondria (Suzuki *et al*, 1989), with a potency similar to that of MPP$^+$, whereas N-Me-IQ$^+$ inhibits complex I with much higher potency than MPP$^+$ (Suzuki *et al*, 1992b). These compounds could potentially therefore cause the complex I deficiency observed in the PD substantia nigra (Mann *et al*, 1992a).

1.5.1.2. ß-Carbolines (ßC's)

ß-carbolines can be formed endogenously by the condensation of tryptophan with an aldehyde. When methylated on the 2[ß]-nitrogen, the derivatives (2-methyl-ß-carbolines; 2-MeßC's) are structural analogues of MPP$^+$ (see Figure 6-1). Both 2-MeßC and 2,9-Me$_2$ßC (2,9-dimethylated-ß-carbolines) have been detected in human brain (Matsubara *et al*, 1993) and S-adenosylmethionine (SAM)-dependent N-methylation activity towards ßC's has been detected in guinea pig brain (Collins *et al*, 1992). Certain ßC's and their methylated derivatives may be substrates for the dopamine reuptake system of dopaminergic neurons, since they inhibit the uptake of [^{3}H] dopamine by rat striatal synaptosomes (Drucker *et al*, 1990).

The administration of 2-MeßC to rats results in cell loss in the substantia nigra and loss of striatal dopamine (Neafsey *et al*, 1989) although no neurotoxic effects on dopaminergic nigrostriatal neurons have been seen in owl monkeys (Collins and Neafsey, 1985), marmosets (Perry *et al*, 1987) or mice (Perry *et al*, 1986). The 2,9-Me_2ßC's are as neurotoxic as MPP^+ *in vitro* (PC12 cells) and *in vivo*, in rats (Collins *et al*, 1992). Although 2-MeßC's are only weak inhibitors of NAD^+-linked respiration *in vitro* (Albores *et al*, 1990), 2,9-Me_2ßC's have been shown to be even more potent inhibitors of NAD^+-linked mitochondrial respiration than MPP^+ (Collins *et al*, 1992; Krueger *et al*, 1993).

1.5.1.3. N-methylation and PD

The capacity of PD patients to N-methylate pyridines has been investigated. Interest has focused on this reaction because the N-methyl derivatives of TIQ's and ßC's are structurally more similar to MPP^+ and more neurotoxic than their parent compounds. Interestingly, PD patients have an increased capacity to N-methylate pyridines (Green *et al*, 1991), which may make them more susceptible to certain neurotoxins.

1.5.2. Exogenous neurotoxins and induced parkinsonism

As well as MPTP, several other exogenous chemicals can induce parkinsonism, including carbon monoxide (Klawans *et al*, 1982), cyanide (Uitti *et al*, 1985; Rosenberg *et al*, 1989) and manganese (Huang *et al*, 1993). Interestingly, cyanide and carbon monoxide are both inhibitors of cytochrome oxidase, complex IV of the mitochondrial respiratory chain (Haab, 1990).

The most common form of induced parkinsonism is that caused by neuroleptics (antipsychotic medications). Indeed, neuroleptic induced parkinsonism, which can be irreversible, accounts for approximately 7% of all PD cases (Rajput *et al*, 1984). One of the commonly administered neuroleptics, haloperidol, is structurally very similar to MPTP. Burkhardt *et al* (1993) have investigated the effect of haloperidol and other neuroleptics on mitochondrial respiratory chain function in isolated rat brain mitochondria and in platelet mitochondria of individuals receiving neuroleptic treatment. In isolated mitochondria, all neuroleptics tested specifically inhibited complex I at micromolar concentrations.

In patients receiving neuroleptics chronically, platelet mitochondrial complex I activity was specifically affected, the activity being 42% of the control mean.

It has been suggested that there is an hereditary susceptibility to neuroleptic-induced parkinsonism. Negrotti *et al* (1992) noted that patients with calcium-entry blocker drug-induced parkinsonism had a higher occurrence of positive family history for PD, providing a model of a multifactorial disease aetiology, involving both exogenous factors and genetic susceptibility.

1.5.3. Cytochrome P450 and PD

One of the body's major detoxification systems is the cytochrome P450 enzyme system. This consists of a flavoprotein, NADPH cytochrome P450 reductase, and a family of cytochrome P450 hemeproteins. Endogenous substrates, such as fatty acids and steroids, are metabolised by the system, while the hydroxylation of xenobiotics is part of a detoxification process; hydroxylation increases solubility and therefore facilitates excretion. Although the system is predominantly expressed in the liver, its presence has also been detected in the brain, at levels approximately 1% of those found in the liver. Within the brain there are large regional variations, with lowest levels in the substantia nigra (Warner *et al*, 1988), which may make this region more susceptible to xenobiotics.

Although MPTP is predominantly metabolised by MAO-B, it is also a substrate for the cytochrome P450 isoenzyme that hydroxlates the drug debrisoquine (Fonne-Pfister *et al*, 1987). The hydroxylation of MPTP to MPTP N-oxide by this isoenzyme results in its detoxification (Cashman *et al*, 1986). The debrisoquine hydroxylase isoenzyme of the cytochrome P450 system is genetically polymorphic, resulting in the phenotypes of poor and extensive metabolisers of debrisoquine. In rats, the degree of MPTP toxicity, measured as a deterioration in motor activity and reduction in brain dopamine levels, was more severe in species which were poor metabolisers than in species which were extensive metabolisers of debrisoquine (Jimenez-Jimenez *et al*, 1991). This implies that poor metabolisers of debrisoquine may be more susceptible to the neurotoxic effects of MPTP-like toxins.

Tetrahydroisoquinolines (TIQ's) are also hydroxylated by the debrisoquine hydroxylase isoenzyme (Suzuki *et al*, 1992a), and administration of TIQ to a rat

model of a poor metaboliser of debrisoquine, resulted in low level urinary excretion of the hydroxylated TIQ, and high level accumulation of TIQ in the brain (Ohta *et al*, 1990). These results suggest that poor metabolisers of debrisoquine may be more susceptible to the toxic effects of TIQ's as well as MPTP.

1.5.3.1. The CYP2D6 phenotype and PD

The phenotypic expression of the cytochrome P450 isoenzyme which hydroxylates debrisoquine (CYP2D6), has been investigated in PD. Homozygotes for the recessive allele of the isoenzyme are almost completely incapable of the 4-hydroxylation of debrisoquine, and are termed poor metabolisers, whereas heterozygotes and homozygotes for the dominant allele hydroxylate debrisoquine efficiently, and are termed extensive metabolisers. Barbeau *et al* (1985) were the first to suggest that debrisoquine hydroxylation was defective in PD. In their study, significantly more PD patients than controls had partially or totally defective debrisoquine hydroxylase activity and poor metabolisers of debrisoquine were more likely to have young onset PD. These results were subsequently partially retracted when it was discovered that many of the drugs taken by the PD patients interfered with debrisoquine metabolism. Most subsequent studies have detected no differences in debrisoquine hydroxylase activities between PD patients and controls when drug treatments have been taken into account (review; Sturman and Williams, 1991; Steiger *et al*, 1992). However, Benitez *et al* (1990) report a positive correlation between the rate of metabolism of debrisoquine and the age at onset of disease in a group of 44 PD patients free of drugs interfering with debrisoquine metabolism; the lower the hydroxylation activity the younger the onset of disease. However, these results have been contradicted by Steiger *et al* (1992), in a study with similar patient numbers.

1.5.3.2. The CYP2D6 genotype and PD

The potential problems of drug interference with the debrisoquine hydroxylase phenotype can be overcome by studying the genotype directly. The cytochrome P450 dependent debrisoquine hydroxylase enzyme is coded for by the CYP2D6 gene located on chromosome 22. In control populations, 5-10% of individuals

carry autosomal homozygous recessive polymorphisms in the gene which render them poor metabolisers (PM's) of debrisoquine. The detection of just three mutations in the CYP2D6 gene using a PCR (polymerase chain reaction) based assay is 90% predictive of the PM phenotype (Wolf *et al*, 1990).

Several studies have determined the frequency of CYP2D6 genotypes in PD. Armstrong *et al* (1992) report that one mutant allele, a G to A transition mutation at the intron3/exon4 boundary, is twice as common in PD patients as controls. However, this increased frequency is almost entirely due to an increase in heterozygotes (extensive metaboliser phenotype) in the PD group, and the sample size (53 patients, 72 controls) was too small to detect alterations in the frequency of homozygous mutant alleles. A much larger study (229 patients, 720 controls) has been reported by Smith *et al* (1992). Poor metabolisers were classified as individuals homozygous for either the G to A transition mutation at the intron3/exon4 boundary, the base pair deletion within exon5 or the whole gene deletion, or heterozygous for any combination of these mutant alleles. The frequency of the PM genotype was 5% in controls and 11.8% (2.36 times higher) in PD patients. The proportion of heterozygotes, with one mutant and one normal allele, was no different in PD patients and controls.

A mutation in exon 6 of the CYP2D6 gene has also recently been associated with PD (Tsuneoka *et al*, 1993). The nucleotide substitution (C to T) changes a conserved arginine amino acid to a cysteine residue, and is believed to be in the vicinity of the substrate binding site. This mutation was homozygous in 11.1% of PD patients (7/63) and in 2.2% (2/91) of controls; 5.05 times more frequent in PD. Although this difference is highly significant, the allele has not yet been associated with the poor metaboliser phenotype, and therefore its relevance remains uncertain.

1.6. OXIDATIVE STRESS AND PD

A free radical is defined as any species which contains one or more unpaired electrons. Free radicals can be highly reactive, reacting with almost every molecule found in the body, including DNA, protein and lipid, and can cause extensive tissue damage.

The body has a range of antioxidant defences to protect itself against free radical attack. When free radical generation is greater than the antioxidant defences can cope with, the body is said to be under oxidative stress. There is increasing evidence that free radical mediated damage plays a role in the cell death seen in Parkinson's disease. Evidence for conditions of oxidative stress in the PD substantia nigra and circumstances which may make it particularly susceptible to free radical attack are outlined below.

1.6.1. The production of free radicals

Mitochondria are one of the most important sources of free radicals in the cell (Boveris and Chance, 1973), and more than 90% of the oxygen consumed by the human body is used by mitochondrial cytochrome oxidase (complex IV) as the terminal electron acceptor in the electron transport chain (ETC). Although oxygen accepts one electron at a time, it remains tightly bound to the enzyme until it has accepted four electrons and is fully reduced to water ($O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$). However, as electrons pass along the ETC, up to 5% (Halliwell, 1992), leak directly from intermediate electron acceptors to O_2, forming the superoxide radical (O_2^-).

The superoxide radical, continually produced at low levels by the electron transport chain, is dismutated by superoxide dismutase to hydrogen peroxide (H_2O_2) and molecular oxygen ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$). The cytotoxicity of H_2O_2 is due mainly to its ability to decompose in the presence of the reduced form of metal ions (particularly Fe^{2+}), to form the highly reactive hydroxyl radical ($\cdot OH$), by the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$). Furthermore, the superoxide radical (O_2^-) can reduce the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}) thereby augmenting the Fenton reaction, the net result being the production of hydroxyl radicals by the iron-catalysed Haber-Weiss reaction ($O_2^- + H_2O_2 \rightarrow \cdot OH + OH^- + O_2$).

1.6.2. Susceptibility of the substantia nigra to oxidative stress

There are a variety of factors which render the brain susceptible to oxidative stress (Olanow, 1992); it consumes 20% of the total body oxygen, is relatively deficient in protective mechanisms against oxidative stress and contains high levels of iron which, in an appropriate form, may catalyse the formation of the highly reactive hydroxyl radical from H_2O_2. Furthermore, dopaminergic neurones contain neuromelanin which is produced via the autoxidation of dopamine, a process which leads to the production of oxygen free radicals (Graham, 1978). Neuromelanin has been shown to bind iron with a high affinity (Ben-Shacher *et al*, 1991), and the substantia nigra contains very high levels of iron compared to other brain regions (Youdim, 1993).

1.6.2.1. L-dopa and free radicals

L-dopa, the precursor of dopamine, is the primary treatment for PD patients, and since the disease is progressive, the doses of L-dopa administered are increased over time. The oxidation of L-dopa results in the production of toxic free radicals (Cohen, 1990) and it has therefore been suggested that it may be neurotoxic, and that its administration may accelerate cell death in the PD brain. L-dopa is toxic in cell culture systems (Pardo *et al*, 1993), and free radical scavengers partly protect against its toxic effects. Similarly, Przedborski *et al* (1993) have shown that the incubation of rat brain mitochondria with L-dopa leads to a decrease in complex I activity which can be prevented by the addition of free-radical scavengers. Although this suggests that the inhibition of complex I activity was due to free-radical damage, extensive washing of the mitochondria restored complex I activity to normal levels.

Although chronic administration of L-dopa does not cause dopaminergic cell loss in the normal rat brain (Perry *et al*, 1984), its administration subsequent to 6-hydroxydopamine (6-OHDA) induced lesion results in an increased loss of dopaminergic neurons in the ventral tegmental area (VTA) (Blunt *et al*, 1993). This may imply that L-dopa is neurotoxic to neurons already damaged by a previous insult. Nevertheless, L-dopa treatment in PD patients is not associated with increased mortality, but rather with increased survival (Uitti *et al*, 1993). Any pathological effect of L-dopa therapy in the brain is not known.

The oxidative deamination of dopamine by monoamine oxidase (MAO) leads to the formation of H_2O_2 (Cohen, 1990). Since the surviving dopaminergic neurones in the PD substantia nigra are characterised by an increased dopamine turnover (Hornykiewicz and Kish, 1986), this results in an increased production of H_2O_2. In mice, increased presynaptic turnover of dopamine can be induced by the administration of reserpine, which interferes with the storage of dopamine in synaptic vesicles. In these animals a significant increase in the level of oxidised glutathione (GSSG) is observed in the striatum (Spina and Cohen, 1989; Cohen, 1990), which is indicative of conditions of oxidative stress.

1.6.3. Evidence for conditions of oxidative stress in the PD substantia nigra

Direct evidence for oxidative damage in the PD substantia nigra comes from the work of Dexter *et al* (1986, 1989). They detected increased levels of malondialdehyde (MDA), an intermediate in the lipid peroxidation process, in the PD substantia nigra compared to controls. Furthermore, they detected a decrease in the levels of polyunsaturated fatty acids (PUFA), the substrate of lipid peroxidation reactions, in the PD substantia nigra compared to controls. Both of these changes were specific to the substantia nigra and were not seen in any of the other brain regions included in the study. More recently, a ten-fold increase in the level of lipid hydroperoxides, an early marker of lipid peroxidation, has been detected in the PD substantia nigra (Dexter *et al*, 1994b).

1.6.3.1. Iron-induced oxidative stress and PD

1.6.3.1.1. Toxicity of free iron

The toxicity of free iron for dopaminergic neurones has been demonstrated in cultured rat dopaminergic pheochromocytoma (PC12) cells (Hartley *et al*, 1993) and in rat nigrostriatal co-cultures (Mochizuki *et al*, 1993). Furthermore, infusions of iron directly into the rat substantia nigra have been shown to cause dose-dependent reductions in substantia nigra volumes (Sengstock *et al*, 1993), and parkinsonian behaviour (Ben-Shachar *et al*, 1992). The toxicity of free iron results from its ability to catalyse the production of the toxic hydroxyl radical via the Fenton reaction. In the rat PC12 tissue culture system, iron loading resulted

in an increase in the level of MDA, an intermediate of lipid peroxidation (Hartley *et al*, 1993), and iron-induced damage to isolated mitochondria mediated via the hydroxyl radical has been reported (Burkitt and Gilbert, 1989).

The possible importance of iron-induced oxidative stress in the pathogenesis of PD has been shown in the 6-hydroxydopamine (6-OHDA) rat model of the disease. The neurotoxic mechanism of 6-OHDA is believed to involve the generation of free radicals (Heikkila and Cohen, 1973) which may be initiated by the presence of transition metals. Ben-Shachar *et al* (1992) have examined the effect of the iron chelator deferoxamine in rats injected with 6-OHDA. They noted that pretreatment of rats with deferoxamine protected against 6-OHDA induced lesions to dopaminergic neurones, measured as a sharp reduction in the 6-OHDA induced fall in striatal dopamine levels.

1.6.3.1.2. Iron levels in the PD brain

Attention has focused on the role of iron in PD since it is by far the most abundant transition metal in the body. That iron levels are increased in the PD substantia nigra is now well established (review; Youdim *et al*, 1993). However, this increase is not disease specific and has also been observed in the substantia nigra of patients dying with Multiple System Atrophy (MSA) and Progressive Supranuclear Palsy (PSP) both of which exhibit nigral degeneration (Dexter *et al*, 1991). Since these diseases are clinically and pathologically distinct it is unlikely that the increased iron levels alone account for the different pathological changes. The levels of copper are reported to be decreased (Dexter *et al*, 1987) or unchanged (Riederer *et al*, 1989), and manganese levels are reported to be unchanged (Dexter *et al*, 1987) in the PD substantia nigra. The levels of both are reported to be normal in the MSA and PSP substantia nigra (Dexter *et al*, 1991).

1.6.3.1.3. Ferritin levels in the PD brain

Iron bound to the cellular iron storage protein ferritin is considered to be unreactive. Therefore to protect cells from iron-induced oxidative stress, any variations in iron levels should be mirrored by variations in ferritin levels. In the MSA and PSP substantia nigra, increased iron levels are accompanied by increased ferritin levels (Dexter *et al*, 1991). However, data on the ferritin

content of the PD substantia nigra is contradictory; Dexter *et al* (1990) have found levels decreased to between 50 and 75% of control values, not just in the substantia nigra but throughout the PD brain; Riederer *et al* (1989) have reported a 29% increase in ferritin levels in the PD substantia nigra, whilst Mann *et al* (1994) have reported normal levels of ferritin in the PD substantia nigra.

1.6.3.2. Free radical protecting enzymes in PD

Various studies have investigated the activity of protective enzymes in postmortem brain tissue, to determine if there are any factors which may make the PD brain more susceptible to oxidative stress, or if there is any indication that conditions of oxidative stress exist in the PD brain.

The activities of catalase and (non-glutathione dependent) peroxidase, both of which break down H_2O_2 to water, are reported to be decreased in the PD substantia nigra to 64% and 39% of control levels respectively, and are both markedly reduced in other regions of the PD striatum (Ambani *et al*, 1975). This may increase the likelihood of hydrogen peroxide reacting with iron to produce the highly toxic hydroxyl radical in the PD brain. However, Martilla *et al* (1988) reported normal levels of catalase activity throughout the PD brain. Superoxide dismutase (SOD) which catalyses the conversion of the superoxide radical (O_2^-) to H_2O_2 and molecular oxygen, exists in two forms; a copper/zinc dependent cytosolic form (Cu/Zn SOD) and a manganese dependent mitochondrial form (Mn SOD). Saggu *et al* (1989) reported both activities as normal in the PD cerebellum. However, there was a specific increase (32% of control activity) in mitochondrial Mn SOD activity in the PD substantia nigra, which may be a response to conditions of oxidative stress in this tissue. A report of increased cytosolic SOD in the PD substantia nigra and other brain regions (Martilla *et al*, 1988) now appears to be due to a methodological artifact (Jenner *et al*, 1992).

1.6.3.3. Antioxidants in PD

1.6.3.3.1. Zinc

Zinc acts as an antioxidant by an unknown mechanism. The level of zinc has been reported to be approximately 50% higher than controls in the PD substantia nigra, but not other brain regions (to the same extent) and normal in the substantia nigra of MSA and PSP cases (Dexter *et al*, 1991). An increase in the level of zinc in the PD substantia nigra may be an indication of conditions of oxidative stress. However, Riederer *et al* (1989) and Mann *et al* (1994) have reported normal levels of zinc in the PD substantia nigra.

1.6.3.3.2. Antioxidant vitamins

The levels of the antioxidant vitamins ascorbate (vitamin C) and α-tocopherol (vitamin E) have been investigated in the PD brain. Although it has been shown that vitamin E deficiency can lead to the loss of nigrostriatal nerve terminals (Dexter *et al*, 1994c), the levels of α-tocopherol are normal in the PD substantia nigra (Dexter *et al*, 1992). Furthermore, Riederer *et al* (1989) have reported normal levels of ascorbate in the PD substantia nigra. If conditions of oxidative stress exist in the PD substantia nigra, one would expect the level of α-tocopherol to be depleted. Indeed, subjecting isolated rat liver mitochondria to conditions of oxidative stress results in depletion of α-tocopherol (Thomas *et al*, 1989). However, as Dexter *et al* (1992) point out, α-tocopherol and ascorbate can be regenerated (i.e reduced) by glutathione, so that although they may be actively scavenging free radicals, their levels can be preserved at the expense of glutathione.

Vitamin C and E have been tested in large clinical trials with untreated PD patients, based on the hypothesis that oxidative stress plays some part in nigral degeneration. Fahn (1992) gave vitamins C and E at doses of 3000mg and 3200 units per day respectively to 21 untreated PD patients. These patients developed disability which required the introduction of L-dopa therapy 2.5 years later than a control group. The DATATOP study is currently examining the effect of 2000 units per day of vitamin E and 10mg per day of selegiline, a monoamine oxidase B inhibitor, on 800 PD patients not started on L-dopa therapy (Parkinson Study Group, 1993). To date Vitamin E has had no beneficial effect, although a lower dose was used than in the study by Fahn (1992).

1.6.3.4. PD and glutathione

Besides catalase, which is present at very low concentrations in the brain (Chance *et al*, 1979), the other enzyme which detoxifies H_2O_2 and organic peroxides is glutathione peroxidase. This is the most important peroxide detoxification system in the brain (Halliwell and Gutteridge, 1985). Furthermore, catalase is not present in mitochondria, so any H_2O_2 produced within the mitochondria is destroyed by glutathione peroxidase. Indeed, experimentally-induced glutathione deficiency leads to structural damage to mitochondria (Jain *et al*, 1991).

Glutathione is a tripeptide with a free sulphydryl group. The reduced thiol form of glutathione (GSH), acts as the electron donor in a reaction catalysed by glutathione peroxidase, which reduces H_2O_2 to water. The oxidised form (GSSG) in which two tripeptides are linked by a disulphide bond is then rapidly reduced back to GSH by glutathione reductase with NADPH as electron donor, so that a constant tissue ratio of GSH to GSSG is maintained (normally greater than 500).

If conditions of oxidative stress exist in the PD substantia nigra, changes in the levels of glutathione may be expected. Total glutathione (GSH plus GSSG) has been reported to be decreased in the PD substantia nigra but normal in other regions (Perry and Yoon, 1986), and decreased in PD brain regions showing neuronal degeneration (regions not specified) (Riederer *et al*, 1989). Two studies have reported decreased levels of reduced glutathione (GSH) in the PD substantia nigra, to 40% (Sofic *et al*, 1992) and 47% (Jenner *et al*, 1992) of the control mean, with no change in the level of oxidised glutathione (GSSG). Jenner *et al* (1992) further reported that GSH levels were normal in other PD brain regions examined (cerebral cortex, caudate nucleus, putamen, medial and lateral globus pallidus) and normal in the substantia nigra of MSA and PSP cases.

Although the reduction in the levels of reduced glutathione in the PD substantia nigra could be indicative of either conditions of oxidative stress in the PD substantia nigra or an abnormality in the glutathione synthesis pathway, it has been reported that inhibition of the mitochondrial respiratory chain can lead to reductions in the levels of GSH without inducing oxidative stress. Di Monte *et al* (1987) and Mithofer *et al* (1992) incubated cultured hepatocytes with MPTP or MPP^+ and detected a large depletion of intracellular GSH, to approximately 25% of control levels, concomitant with losses of intracellular ATP. Rather than an oxidation of GSH within the cells they noticed an efflux of GSH from the cells

(accounting for approximately 40% of the loss of glutathione), as well as a reduction of the total glutathione pool. They suggest that both of these processes are due to the reduction in the levels of cellular ATP caused by inhibition of the mitochondrial respiratory chain. ATP depletion may cause perturbations in membrane potentials leading to increased efflux of GSH from cells, and in addition the two enzymes involved in glutathione synthesis, γ-glutamylcysteine synthetase and glutathione synthetase, are both ATP dependent; any reduction in cellular ATP levels may therefore lead to a reduced activity of these two enzymes, and thereby lead to reductions in glutathione synthesis.

1.6.3.4.1. Glutathione dependent enzymes and PD

The activity of glutathione reductase is normal in the PD substantia nigra (Martilla *et al*, 1988), and there is no significant decrease in glutathione peroxidase activity, which was reported as normal (Martilla *et al*, 1988) or only slightly decreased (by 19%) in the PD substantia nigra (Kish *et al*, 1985). In the PD brain, glutathione peroxidase positive glial cells are concentrated in areas of dopaminergic neuronal degeneration, suggesting that they may have a protective role, trying to protect the remaining neurons from conditions of oxidative stress (Damier *et al*, 1993).

1.6.4. MPTP and free radicals

Although MPP^+ has been shown to directly inhibit complex I (Nicklas *et al*, 1985), an alternative mechanism of MPTP toxicity is via the induction of oxidative stress. Free radicals are generated during the MAO-B catalysed oxidation of MPTP (Zang and Misra, 1993) and during the autoxidation of $MPDP^+$ (Zang and Misra, 1992), and a redox reaction can occur between MPP^+ and $MPDP^+$ generating the superoxide radical ($O_2^{\cdot}$) (Rossetti *et al*, 1988). There are reports of the protective effects of antioxidants on the toxicity of MPTP (review; Adams and Odunze, 1991), and transgenic mice with increased Cu/Zn superoxide dismutase activity (which catalyses the conversion of $O_2^{\cdot}$ to H_2O_2 thus protecting cells from the superoxide radical), are resistant to MPTP-induced toxicity (Przedborski *et al*, 1992). It has been suggested that any radical species generated as the by-product of redox reactions between MPP^+ and $MPDP^+$ do

not impair mitochondrial respiratory function, since the inhibitory effects of MPP^+ on mitochondrial respiration are not increased by the addition of $MPDP^+$ (Walker *et al*, 1991). However, this does not exclude any other cytotoxic effects of this reaction.

Inhibition of complex I by MPP^+ can itself lead to the generation of free radicals. Cleeter *et al* (1992) have shown that the inhibition of complex I resulting from the incubation of MPP^+ with mitochondria for five minutes could be removed by dilution, and was therefore presumed to be due to a loose binding of MPP^+ to the complex. However, after extended incubation of MPP^+ with mitochondria, complex I was irreversibly inhibited. This inhibition (36% of activity) was prevented by the addition of the free radical scavengers ascorbate and glutathione, implying that it is mediated by free radical damage to the enzyme. The decreased complex I activity in the PD substantia nigra (Mann *et al*, 1992a) may be caused by a similar inhibitory process; a bound toxin or structural alteration of complex I may lead to increased free radical production, leading to further specific complex I damage and so on, in a self-perpetuating cycle.

1.6.5. Oxidative stress and the MRC

At present it is unclear whether the specific complex I defect observed in the PD substantia nigra (Mann *et al*, 1992a) is a primary cause of cell death, or is secondary to some other factor. If conditions of oxidative stress in the PD substantia nigra are the cause of the observed complex I defect, then subjecting mitochondria to conditions of oxidative stress should result in specific reductions in complex I activity.

1.6.5.1. Susceptibility of the MRC to oxidative damage

Free radical induced damage of the mitochondrial respiratory chain (MRC) enzymes could be via a number of mechanisms; oxyradicals may directly oxidise the proteins themselves, they may increase production of lipid peroxides or they may attack mitochondrial DNA. The mitochondrial respiratory chain enzymes are particularly susceptible to free radical damage for a number of reasons;

1. they generate free radicals themselves as a byproduct of their normal function (Boveris and Chance, 1973). Complex I is a particularly important site of superoxide generation (Takeshige and Minakami, 1979), and inhibition of complex I with rotenone (Turrens and Boveris, 1980) or MPP^+ (Hasegawa *et al*, 1990) increases free radical generation at this site. Cleeter *et al* (1992) have shown that incubation of mitochondria with MPP^+ leads to the free-radical mediated impairment of complex I function, but not complex II/III function.

2. mitochondrial membranes contain high levels of PUFA's (Buttriss and Diplock, 1988) and complexes I, III and IV all have an absolute phospholipid requirement for normal function (Fry and Green, 1981).

3. mitochondrial DNA, which codes for a number of subunits in the respiratory chain enzymes, is particularly susceptible to free radical damage, and indeed it has been shown that the steady state level of oxidised bases is sixteen times higher in mitochondrial than nuclear DNA (Richter *et al*, 1988). MtDNA is situated in the mitochondrial matrix close to the free radical generating electron transport chain in the inner membrane; it has little redundancy with few non-coding regions, it is not protected by a histone protein coat and it has an inefficient enzymatic damage repair system (review; Richter, 1992).

1.6.5.2. The effect of oxidative stress on the MRC

Several groups have studied the effects of free radical mediated oxidative damage in isolated mitochondria and submitochondrial particles *in vitro*, using a variety of radical generating systems. Schewe *et al* (1981) reported complexes I and II equally affected; Narabayashi *et al* (1982) reported complex I to be the first enzyme affected, followed by complexes II and III; Hillered and Ernster (1983) reported large reductions in O_2 utilisation in the presence of NAD-linked substrates and a smaller reduction in the presence of FAD-linked substrates; and Zhang *et al* (1990) reported large decreases in the activities of both complexes I and II.

Two *in vivo* studies on the effects of oxidative stress on MRC function have been reported. Benzi *et al* (1991) used 2-cyclohexene-1-one to deplete glutathione in rat brain and detected complex IV inactivation followed by inactivation of complexes I and II. Thomas *et al* (1993) reported decreases in the activities of complexes I, II/III and IV in skeletal muscle mitochondria of vitamin

E deficient rats. Finally, in a cell culture model of iron overload in rat dopaminergic pheochromocytoma (PC12) cells, Hartley *et al* (1993) reported a 15 and 21% decreases in the mean activities of complex I and complex IV respectively.

In every study then, whether it be *in vitro* or *in vivo*, when mitochondria are placed under conditions of oxidative stress, complex I activity is not the only respiratory chain complex to be affected. In the PD substantia nigra the activity of complex I is decreased but the activity of complexes II/III and IV are unchanged (Mann *et al*, 1992a), and therefore the observed complex I defect may not simply be a secondary effect of oxidative stress in this tissue.

1.7. PRESYMPTOMATIC PARKINSON'S DISEASE

The finding that selegiline may have a neuroprotective effect in PD and delay the onset of symptoms (Parkinson Study Group, 1993) added urgency to the search for methods of detecting presymptomatic PD cases. It has been suggested that the presymptomatic phase of PD may be at least five years (Fearnley and Lees, 1991), although subtle clinical signs may be present for much longer (Lees, 1992).

In a proportion of aged brains, Lewy bodies are found in the substantia nigra. These cases make up approximately 10-15% of all subjects over the age of 65 years who die with no evidence of the symptoms of PD and are thought to be presymptomatic PD patients, who in time would have developed the symptoms of the disease. Although it has been argued that the presence of Lewy bodies alone cannot unambiguously identify presymptomatic PD cases, Fearnley and Lees (1991) have shown that these cases exhibit an average 27% loss of pigmented nigral neurones above that seen in the normal aged brain, and show the same regional selectivity of cell loss in the substantia nigra as that seen in the PD brain. These cases may be useful in determining which biochemical changes, if any, are important in the presymptomatic stage of the disease.

1.7.1. Biochemical markers in presymptomatic PD

Dexter *et al* (1994a) found that the levels of both iron and ferritin were normal in the substantia nigra of these presymptomatic PD cases, which argues against either being a primary cause of cell death in the disease. However, mean reduced glutathione (GSH) levels were decreased by approximately 35% in the substantia nigra of these cases (Dexter *et al*, 1994a). Since GSH levels are also decreased in the substantia nigra of symptomatic PD cases (Sofic *et al*, 1992; Jenner *et al*, 1992), this may suggest that this biochemical deficiency is of primary importance in the pathology of PD. Interestingly, the mean activity of complex I was reduced by 17% in these cases, to a level intermediate between the activity in normal controls and in established Parkinson's disease cases (Mann *et al*, 1992).

Chapter 2. Materials and Methods

2.1. Chemicals

All chemicals, unless otherwise stated, were purchased from the Sigma Chemical Co, Poole, Dorset, UK or Merck Ltd, Dagenham, Essex, UK.

2.2. Equipment

2.2.1. Centrifugation

The following centrifuges and rotors were used at the following g forces, unless otherwise stated:
up to 3000 x g_{max}: Beckman GPR bench-top centrifuge with GH-3.7 horizontal rotor (Beckman Ltd, High Wycombe, Bucks, UK);
3000-47000 x g_{max}: Kontron T-124 high-speed centrifuge with A 8.24 8 x 50ml fixed angle rotor (Kontron Instruments Ltd, Watford, Herts, UK);
above 47000 x g_{max}: MSE superspeed 75 ultra centrifuge with 6 x 38ml swing-out rotor (MSE Instruments Ltd, Crawley, Sussex, UK).

2.2.2. Column chromatography

The peristaltic pump (P1), absorbance control unit (UV-1), fraction collector (Frac-100) and chart recorder (Rec1) were supplied by Pharmacia Ltd (Milton Keynes, Bucks, UK).

2.2.3. Electrophoresis

All agarose and polyacrylamide gel electrophoresis was performed with BioRad 200/2.0 constant voltage power packs (BioRad Laboratories Ltd, Hemel Hempstead, Herts, UK);
Horizontal submarine electrophoresis tanks (for agarose gels) were supplied by Uniscience Ltd (Banbury, Oxon, UK);
Vertical electrophoresis tanks (Mini-Protean II system; for acrylamide gels) were supplied by BioRad Laboratories Ltd (Hemel Hempstead, Herts UK);
The PhastSystem horizontal electrophoresis unit was supplied by Pharmacia Ltd (Milton Keynes, Bucks, UK).

2.2.4. Tissue Homogenisation

Uni-form 10ml glass/teflon homogenisers were supplied by Jencons Ltd (Leighton Buzzard, Beds, UK);
The Glas-Col motor was supplied by CamLab Ltd (Cambridge, Cambs, UK);
Dounce type glass/glass homogenisers were supplied by Wheaton Ltd (Millville, NJ, USA);
The cell disruption bomb (45ml volume) was supplied by Parr Instrument Co (Moline, Illinois, USA).

2.2.5. Spectrophotometry

All enzyme assays were performed on Hitachi U-3210 (Hitachi Scientific Instruments, Wokingham, Berks, UK) or Kontron Uvikon 940 (Kontron Instruments, Watford, Herts, UK) split-beam spectrophotometers.

2.3. Patients

All patient studies were performed with the full and informed consent of the patients and with the approval of the Joint Ethics Committee of the National Hospital for Neurology and Neurosurgery and the Institute of Neurology, Queen Square, London, UK.

The diagnosis of Parkinson's disease was based on the presence of an akinetic rigid syndrome with asymmetric onset, a resting tremor and a positive response to L-dopa. Patients who were untreated at the time of sample were followed up to ensure that they subsequently demonstrated a good response to L-dopa.

2.4. Data analysis

Statistical analyses were performed with Oxstat (version 4.10, release 1.10) software, and all significance tests were performed using the Mann-Whitney U test. Figures were drawn using Sigmaplot (version 4.02) graphing system software.

2.5. Platelet preparation and subcellular fractionation

2.5.1. Preparation of platelet homogenates

The preparation of platelet homogenates was performed at room temperature to avoid platelet aggregation. 30ml of venous blood was collected without tourniquet via a 19-gauge butterfly into a plain syringe. The blood was transferred to 25ml plastic universals and mixed 9:1 with 3.8% (w/v) tri-sodium citrate. The blood was centrifuged, to separate the platelet rich plasma (PRP) and red cells, at 200 x g_{max} for 20mins. The PRP was carefully removed to clean universals and platelets pelleted by centrifugation at 1000 x g_{max} for 30mins. The platelet poor plasma was poured off and the sides of the tube wiped clean to remove any serum proteins. The cell pellet was gently resuspended with an equal volume (to the PRP) of a modified Tyrodes buffer (Appendix 1.1) and centrifuged at 1000 x g_{max} for 15mins. The Tyrodes wash was then repeated once more. The washed cell pellet was resuspended with 750μl of ice-cold homogenisation medium (Appendix 1.2), split into two aliquots of 600 and 150μl, snap-frozen in liquid nitrogen and stored at -70°C.

2.5.2. Preparation of platelet mitochondrially-enriched fraction

The preparation of the platelet pellet was as in 2.5.1 except that 60ml of venous blood was required. The washed cell pellet was resuspended with 6ml of ice-cold homogenisation medium (Appendix 1.2) and a 50μl sub-sample removed for calculation of mitochondrial recovery. The cell suspension was transferred to the cell disruption bomb which was charged to 1200psi with oxygen free nitrogen gas (BOC Ltd, Guildford, Surrey, UK) and left on ice for 20mins (Broekman *et al*, 1974). After this time the pressure was released and the cell homogenate collected in a plastic universal. Any nuclei or unbroken cells were pelleted by centrifugation at 1000 x g_{max} for 15mins at 4°C. The Post Nuclear Supernatant (PNS) was transferred to a 30ml centrifuge tube on ice and the Nuclear Pellet (NP) was resuspended with 6ml ice-cold homogenisation medium and re-homogenised in the cell disruption bomb. The second PNS was pooled with the first, and the mitochondrially-enriched fraction (MEF) was pelleted by centrifugation at 8500 x g_{max} for 10mins at 4°C. The supernatant was removed and the MEF was resuspended with 600μl homogenisation medium, split into two aliquots of 500μl and 100μl, snap-frozen in liquid nitrogen and stored at -70°C.

2.5.3. Electron microscopy

Electron microscopy was performed by Dr Jenny Small, Department of Neuroscience, Royal Free Hospital School of Medicine, London, UK. The platelet MEF from the equivalent of 30ml of whole blood was gently resuspended with primary fixative (1ml of PIPES (piperazine -N-N'-bis(2-ethanesulphonic acid)), 2.5% (v/v) glutaraldehyde, 2% (w/v) sucrose) and mixed on a whirly wheel overnight at 4°C. The MEF was pelleted at top speed in a microfuge for 10mins, washed by gentle resuspension with PIPES buffer and re-pelleted. The pellet was then post-fixed by resuspension with 1ml of 1% (w/v) osmium tetroxide and incubated at 4°C for 2 hrs, washed twice with 1ml PIPES buffer and finally resuspended with 100μl of PIPES buffer. This volume was carefully pipetted onto Whatmann No. 1 filter paper, to absorb the buffer, and the mitochondria were collected together with a small spatula. The pellet was split into two, placed onto a 1% agar plate, and then overlayed with a small drop of molten agar. Two small agar blocks containing the mitochondria were cut from the plate and placed in PIPES buffer. These blocks were dehydrated through a graded series of ethanol up to 100%, immersed in propylene oxide and embedded in

araldite epoxy resin, in preparation for sectioning for electron microscopy. Ultra-thin sections, 90nm thick, were cut in a Reiche Ultracut microtome, placed onto 300 mesh copper grids, stained with 50% methanolic uranyl acetate and aqueous lead citrate, and examined with a Jeol 100CX transmission electron microscope.

2.5.4. Blood cell counting

Samples of whole blood, platelet rich plasma, platelet poor plasma and Tyrodes wash supernatants were counted on a Coulter Cell Counter (Coulter Electronics Ltd, Luton, Beds, UK).

2.6. Enzyme assays

All enzyme assays were performed at 30°C unless otherwise stated.

2.6.1. NADH-CoQ_1 oxidoreductase

The assay of NADH CoQ_1 oxidoreductase was used to measure the activity of complex I of the mitochondrial electron transport chain. The assay was performed according to the method of Ragan *et al* (1987). The assay measures the NADH dependent reduction of ubiquinone at 340nm. The assay mixture consisted of identical cuvettes containing a final concentration of 20mM potassium phosphate buffer pH8, 150μM NADH, 1mM KCN and sample in 1ml. The reaction was initiated by the addition of 50μM CoQ_1 (see Methods 2.6.1.1) to the sample cuvette. The rate of NADH oxidation was measured, then rotenone was added to a final concentration of 10μM and the rotenone insensitive rate was measured. The mitochondrial complex I activity was taken as that which was sensitive to rotenone. A molar extinction coefficient for NADH of 6.81×10^3 was used to allow for the contribution of reduced CoQ_1 to the absorbance at 340nm (Ragan *et al*, 1987). The assay required approximately 150μg of platelet homogenate and 100μg of platelet MEF. Enzyme activity was expressed as nmol NADH oxidised per minute per mg of protein.

The assay was later modified to optimise activity in platelet mitochondrial fractions. The modifications were as follows; 20mM potassium phosphate buffer pH7.2; 8mM $MgCl_2$ was included; 2.5mg/ml essentially fatty-acid free BSA was included. In all other respects the assay was performed as before, and required approximately 90μg of platelet homogenate and 60μg of platelet MEF.

2.6.1.1. Calculation of ubiquinone-1 concentration

The ubiquinone-1 (CoQ_1) was a kind gift of the Eisai Chemical Co, Tokyo, Japan. A dilution of the stock was made in ethanol and the absorbance of ubiquinone-1 was read at 275nm. An excess of sodium borohydride was added to the reference cuvette to completely reduce the ubiquinone to ubiquinol, and the absorbance change was noted. The molar extinction coefficient of ubiquinone-1 was taken as 12.25×10^3 (Redfearn, 1967), and the volume of ubiquinone required to give a final cuvette concentration in the assay of 50μM was calculated (usually approximately 10μl).

2.6.2. NADH ferricyanide oxidoreductase

The assay of NADH ferricyanide reductase activity was performed according to the method of King and Howard (1967). The assay measures the oxidation of NADH at 340nm, with potassium ferricyanide as electron acceptor. The assay mixture consisted of identical cuvettes containing final concentrations of 50mM potassium phosphate buffer pH7.4, 0.1% (v/v) Triton X-100, 0.17mM NADH and 0.6mM potassium ferricyanide in 1ml. The reaction was initiated by the addition of sample to the sample cuvette, and the change in absorbance at 340nm was monitored. Enzyme activity was expressed as nmol NADH oxidised per minute per mg of protein, calculated using a molar extinction coefficient for NADH of 6.22×10^3.

2.6.3. Succinate cytochrome c oxidoreductase

The assay of succinate cytochrome c oxidoreductase measures the activity of complexes II and III of the mitochondrial electron transport chain. The assay was performed according to the method of King (1967). The assay measures the succinate dependent reduction of cytochrome c at 550nm. The assay mixture consisted of identical cuvettes containing final concentrations of 0.1M potassium phosphate buffer pH7.4, 0.3mM EDTA (di-K), 0.1mM cytochrome c, 1mM sodium azide, 2.5mg/ml essentially fatty acid free BSA and sample in 1ml. A mixture of sample plus 40μl of 500mM sodium succinate (substrate) and 10μl of 100mM sodium azide was pre-incubated at 37°C for 5mins to activate the enzyme. This mixture was then used to initiate the reaction. The final concentration of sodium succinate in the sample cuvette was 20mM. The mitochondrial complex II/III activity was taken as that which was sensitive to the addition of antimycin A (final concentration of 0.02mM). The assay required approximately 70μg of platelet homogenate and 50μg of platelet MEF. Enzyme activity was expressed as nmol cytochrome c reduced per minute per mg of protein, calculated using a molar extinction coefficient for reduced cytochrome c of 19.2×10^3.

2.6.4. Cytochrome c oxidase

The assay of cytochrome c oxidase measures the activity of complex IV of the mitochondrial electron transport chain. The assay was performed according to the method of Wharton and Tzagoloff (1967). The assay measures the oxidation of reduced cytochrome c at 550nm. The assay mixture consisted of identical cuvettes containing a final concentration of 50mM potassium phosphate buffer pH7.0 and 50μM reduced cytochrome c in 1ml. 1mM potassium ferricyanide was added to the reference cuvette to fully oxidise the reduced cytochrome c, and the reaction was initiated by the addition of sample to the sample cuvette. The assay is first order with respect to cytochrome c, so the pseudo first order rate constant k was calculated. Enzyme activity was expressed as k per minute per mg of protein. The assay required approximately 300μg of platelet homogenate and 200μg of platelet MEF.

2.6.4.1. Preparation of reduced cytochrome c

A 1% (w/v) solution of cytochrome c (from horse heart; Boehringer Mannheim Ltd, Lewes, Sussex, UK) was fully reduced by the addition of an excess of ascorbate. The reduced cytochrome c solution was transferred to size 1 dialysis tubing (Medicell International Ltd, London, UK) which had been boiled in ddH_2O for 3hrs. It was subsequently dialysed against three changes of 5l of 10mM potassium phosphate buffer pH7.0 at 4°C, over at least 36 hrs, to remove excess ascorbate, and stored in aliquots at -20°C. To check that no excess ascorbate remained it was shown that oxidised cytochrome c added to the dialysed reduced sample was not reduced, and to check that the cytochrome c was fully reduced, it was shown that the addition of more ascorbate failed to reduce the cytochrome c further.

2.6.5. Citrate synthase

The assay of citrate synthase, an enzyme of the mitochondrial matrix, was performed according to the method of Coore *et al* (1971). The enzyme catalyses the condensation of acetyl-CoA and oxaloacetate to form citrate. This reaction produces CoenzymeA whose free thiol group combines with DTNB (5-5'-dithio-bis(2-nitrobenzoic acid), resulting in an increase in absorbance at 412nm. The assay mixture consisted of identical cuvettes containing a final concentration of 100mM Tris pH8.0, 200μM acetyl-CoA, 200μM DTNB, 0.1% (v/v) Triton X-100 and sample in 1ml. The reaction was initiated by the addition of 100μM oxaloacetate to the sample cuvette. The assay required approximately 15μg of platelet homogenate and 10μg of platelet MEF. Enzyme activity was expressed as nmol DTNB reduced per minute per mg of protein, and was calculated using a molar extinction coefficient of 13.6×10^3 for the DTNB-CoA-SH complex.

2.6.6. Lactate dehydrogenase

Lactate dehydrogenase activity, a specific marker of the cytosol, was measured according to the method of Clark and Nicklas (1970). This enzyme catalyses the reduction of pyruvate to lactate and the assay measures the oxidation of NADH at 340nm in the presence of pyruvate. The assay mixture consisted of identical cuvettes containing a final concentration of 100mM potassium phosphate buffer

pH7.4, 0.24mM NADH, 0.5% (v/v) Triton X-100 and sample in 1ml. The reaction was initiated by the addition of 1mM sodium pyruvate to the sample cuvette. Enzyme activity was expressed as nmol NADH oxidised per minute per mg of protein, and was calculated using a molar extinction coefficient for NADH of 6.22×10^3.

2.6.7. Esterase

Esterase activity was measured as a specific endoplasmic reticulum marker, according to the method of Beaufay *et al* (1974). The assay measures the production of o-nitrophenol from o-nitrophenyl acetate, at 420nm. The assay mixture consisted of identical cuvettes containing a final concentration of 20mM potassium phosphate buffer pH7.4, 1mM EDTA (di-K), 0.1% (v/v) Triton X-100 and 3mM o-nitrophenyl acetate in 1ml. The reaction was initiated by the addition of sample to the test cuvette. Enzyme activity was expressed as nmol o-nitrophenol produced per minute per mg of protein, using a molar extinction coefficient for o-nitrophenol of 3.06×10^3.

2.6.8. Acid Phosphatase

Acid phosphatase activity was measured as a specific lysosomal marker, according to the method of Hubscher and West (1965) with modifications, at 37°C. Identical samples were pre-incubated in a volume of 625μl in a final concentration of 90mM sodium acetate buffer pH5.0, 1.8mM EDTA (di-Na), 0.2% (v/v) Triton X-100 and 20mM sodium fluoride (blanks only). The reaction mixtures were incubated at 37°C for 10mins to ensure fluoride inhibition of the lysosomal enzyme in the blank assays. The reaction was initiated by the addition of 50μl of 10mM 1-napthyl phosphate to both test and blank samples which were incubated at 37°C for 2hrs. The reaction was terminated by the addition of 400μl of 53mM fast red ITR dye (in 20% (v/v) dimethyl formamide) and 3ml stop mix (35% (v/v) ethanol, 60% (v/v) ethyl acetate, 0.5% (w/v) trichloracetic acid). The samples were centrifuged at 1500 x g_{max} for 20mins in an MSE Centaur 3 centrifuge, and their absorbances read at 540nm against a no-enzyme blank reference. Blank absorbances were subtracted from test. Enzyme activity was expressed as nmol diazo-dye complex formed per minute per mg of protein, using a molar extinction coefficient for the diazo-dye complex of 12.538×10^3.

2.6.9. ß-thromboglobulin

ß-thromboglobulin, a specific marker of platelet alpha granules (Kaplan *et al*, 1979), was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Diagnostica Stago, Asnieres sur Seine, France) by Dr Ron Hutton, Department of Haematology, Royal Free Hospital School of Medicine, London, UK.

2.6.10. Serotonin

Serotonin, a specific marker of platelet dense granules (Tranzer *et al*, 1966), was measured using a commercially available ELISA kit (Immunotech International, Marseille, France) by Dr Manuel Baradas, Royal Free Hospital School of Medicine, London, UK.

2.7. Affinity purification of complex I antibodies

2.7.1. Production of antisera

All procedures were performed by the staff of the CBU, Royal Free Hospital, London, UK. The initial immunisation of adult female New Zealand half-lop rabbits, was by injection at 4 sub-cutaneous sites of 1.5mg purified native bovine heart complex I holoenzyme (kindly provided by Dr JM Cooper, Royal Free Hospital School of Medicine, London, UK) mixed 1:2.5 with Freund's complete adjuvant in a final volume of 1ml. Booster immunisations at approximately 4 week intervals were performed as above except that 0.5mg antigen was injected in Freund's incomplete adjuvant. Up to 20ml of blood was removed at 4 week intervals from the ear vein and animals were killed by cardiac exsanguination under deep terminal anaesthesia. Blood was collected in glass universals, incubated at 37°C for 1hr and then at 4°C overnight. The blood clot was compressed by centrifugation at 3000 x g_{max} for 15mins and the serum removed. Any contaminating red blood cells were removed by centrifugation as above. Serum was stored in aliquots at -20°C, and current stock was maintained at 4°C in the presence of 0.05% (w/v) sodium azide.

2.7.2. Preparation of complex I affinity column

Complex I was covalently coupled to the gel matrix according to the method of Haines *et al* (1992). 10mg of purified bovine heart complex I were resuspended with 3ml of 0.1M $NaHCO_3$, pH8.2 (coupling buffer) and dialysed against 4l of coupling buffer for 3hrs at 4°C (to remove any Tris present). Equal volumes (approximately 5ml bed-volume each) of Affi-Gel 10 and 15 (BioRad Laboratories Ltd, Hemel Hempstead, Herts, UK) were washed five times with ddH_2O and five times with coupling buffer plus 1% Triton X-100. Dialysed complex I was diluted 1:1 with coupling buffer plus Triton X-100, added to the pooled Affi-Gel, and mixed overnight at 4°C. The slurry was blocked with 100μl/ml of gel of 1M ethanolamine-HCl pH8.0 for 1hr at 4°C, and transferred to a C10/20 column (1 x 20cm; Pharmacia Ltd, Milton Keynes, Bucks, UK). The column was equilibrated with PBSa (phosphate buffered saline plus sodium azide; see Appendix 5.1), washed with 3M GuHCl and re-equilibrated with PBSa prior to use.

2.7.3. Affinity purification of antibodies

Neat complex I antiserum (0.5ml) was passed through the column in PBSa at a flow rate of 0.2ml/min. Specifically bound IgG was eluted with 3M GuHCl at the same flow rate and 1ml fractions were collected. Peak absorbance (280nm) fractions were pooled, concentrated and de-salted using Centriprep 30 concentrators (Amicon Ltd, Stonehouse, Glos, UK) and stored at 4°C.

2.8. Immunoprecipitation of complex I

2.8.1. Preparation of human brain mitochondria

Fresh human brain was supplied by the Department of Histopathology, Royal Free Hospital, London, UK and brain mitochondria were prepared by the method of Lai *et al* (1979). Brain samples (approximately 30g) were washed in ice-cold isolation medium (Appendix 1.3) and coarsely chopped. The tissue was suspended in isolation medium at approximately 0.15g/ml, and homogenised in 20ml aliquots by 11 strokes in a Dounce type glass/glass homogeniser with a

0.1mm clearance. The homogenate was centrifuged at 1300 x g_{max} for 5mins at 4°C, and the supernatant was re-centrifuged at 17000 x g_{max} for 10mins. The crude pellet was resuspended to approximately 30ml in a final concentration of 10% Ficoll 400 (Pharmacia Ltd, Milton Keynes, Bucks, UK) in isolation medium and split between 6 thin-walled centrifuge tubes. These were then overlayed with 20ml ice-cold 7.5% Ficoll in isolation medium which was overlayed with approximately 4ml ice-cold isolation medium to fill the centrifuge tubes. The gradients were centrifuged at 99000 x g_{max} for 1hr at 4°C. During centrifugation the mitochondria formed a pellet and the synaptosomes and myelin formed bands at the interfaces of the 10%-7.5% Ficoll and 7.5% Ficoll-isolation medium respectively. The mitochondrial pellet was resuspended with isolation medium and centrifuged at 10000 x g_{max} for 10mins. The final pellet was resuspended with isolation medium at approximately 10mg protein/ml.

2.8.2. Preparation of human and rat skeletal muscle mitochondria

Fresh human skeletal muscle was supplied by the Department of Histopathology, Royal Free Hospital, London, UK. Tissue samples (approximately 40g) were washed in ice-cold high EDTA isolation medium (Appendix 1.4) and coarsely chopped. The tissue was digested for 30mins in a minimal volume of high EDTA isolation medium by the addition of 0.5mg Type III trypsin per gram of tissue. Digestion was stopped by the addition of a 3:1 excess of soyabean trypsin inhibitor. Digested tissue was suspended in high EDTA isolation medium at approximately 0.1g/ml, and homogenised in 60ml volumes by two 5s bursts with an ultra-turrax fitted with an 18N shaft, at full speed. The homogenate was centrifuged at 1500 x g_{max} for 5mins at 4°C, and the supernatant was filtered through muslin and centrifuged at 7000 x g_{max} for 10mins at 4°C. Mitochondrial pellets were resuspended with low EDTA isolation medium (Appendix 1.5) by gentle hand-homogenisation in a loose fit glass/teflon homogeniser and re-centrifuged twice as above. The final mitochondrial pellet was resuspended as above with low EDTA isolation medium at approximately 10mg protein/ml.

Rat skeletal muscle mitochondria were prepared from the gastrocnemius muscle as described above for human skeletal muscle mitochondria.

2.8.3. Immunoprecipitation of complex I from purified mitochondria

Mitochondria were diluted with ddH_2O to 1mg/ml. Dodecyl maltoside (DDM) was added to a final concentration of 0.2% and mitochondria were solubilised by mixing on a whirly wheel at room temperature for 1hr. Insoluble material was pelleted by centrifugation at 47000 x g_{max} for 30mins at 4°C. The precipitating serum was centrifuged as for the mitochondria. 1mg of solubilised mitochondria were mixed with 0.5ml pre-spun serum in 0.2% DDM, and the precipitation reaction was left to mix on a whirly wheel at 4°C overnight. The precipitate was pelleted by centrifugation at 47000 x g_{max} for 30mins at 4°C, washed with 1ml ddH_2O, and resuspended with 10μl gel loading buffer (Appendix 2.1).

2.8.4. Immunoprecipitation of complex I from brain homogenate

The method for immunoprecipitation of complex I from whole brain homogenate was the same as that from purified mitochondria, with the following alterations: brain tissue was homogenised in a nine times volume of brain isolation medium (Appendix 1.3) in a glass/teflon homogeniser (0.15mm clearance) in the presence of protease inhibitors (12.5μg/ml chymostatin, pepstatin A; 25μg/ml antipain, leupeptin; 0.1mM PMSF; 10mM benzamidine); brain homogenate (2mg) was solubilised with 1% DDM and split into two aliquots, to each of which was added 0.5ml of precipitating serum in 1% DDM.

2.8.5. Image analysis of complex I immunoprecipitates

The Kontron VIDAS image analysis system was used. The gel to be analysed was placed on a light box below the system camera and the image was loaded into the computer. The origin of the gel and the positions of the molecular weight markers and their molecular weights were entered manually. The positions of bands in immunoprecipitated samples were entered manually, and their molecular weight and absorbance were calculated automatically.

2.9. Protein electrophoresis and detection

2.9.1. Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gels were prepared using the Mini-Protean II system (BioRad Laboratories Ltd, Hemel Hempstead, Herts, UK). 0.75mm gels (Appendix 2.2) were electrophoresed at 30mA for 45mins in electrode buffer (Appendix 2.3).

2.9.2. Electrophoresis on the Pharmacia PhastSystem

Protein samples were electrophoresed using Phastgels on the Pharmacia PhastSystem electrophoresis unit. The size of the gels (0.45mm thick, 13mm of stacking gel and 32mm of separating gel) allowed the use of very small amounts of sample. The gels consisted of a 7.5% stacking gel and a 20% separating gel. The gels contained 0.112M acetate and 0.112M Tris, pH6.5 and the buffer strips consisted of 0.2M Tricine, 0.55% (w/v) SDS and 0.2M Tris pH8.1 in an inert matrix of 2% agarose. Samples were electrophoresed at 250V for 118Vhrs at 13°C.

2.9.3. Silver staining of polyacrylamide gels

Protein samples electrophoresed in polyacrylamide gels (other than PhastGels) were silver stained according to the method of Heukeshoven and Dernick (1985). Gels were fixed overnight, placed in incubation solution for 30mins, washed three times for 5mins in ddH_2O, placed in silver solution for 40mins and developed for approximately 5-10mins until bands were clearly visible against the background. Development was stopped by the addition of stop solution, and gels were finally rinsed in ddH_2O (for solutions components see Appendix 3.1).

2.9.4. Silver staining PhastGels

PhastGels were silver stained in the PhastSystem development chamber according to the manufacturers recommendations (Appendix 3.2).

2.9.5. Western blotting

Transfer of electrophoresed protein samples onto 0.22μm nitrocellulose membranes (Anderman and Co Ltd, Kingston, Surrey, UK) was performed according to the method of Towbin *et al* (1979). The gel, nitrocellulose membrane and 6 sheets of Whatman No. 1 blotting paper (Whatman Ltd., Maidstone, Kent, UK) were pre-soaked in transfer buffer (Appendix 4.1) for 10mins, and then placed in the Trans-Blot SD semi-dry electrophoretic transfer cell (BioRad Laboratories Ltd, Hemel Hempstead, Herts, UK), the gel and nitrocellulose sandwiched between 3 sheets of blotting paper either side. Electroblotting was performed at 5.5mA per square centimetre of gel (130mA for PhastGels) for 30mins. The nitrocellulose was blocked with 10ml of 5% BSA in PBSa (phosphate buffered saline plus sodium azide; see Appendix 5.1) for 30mins and then washed with 15ml $PBS_{T(0.5\%)}$ (PBS plus 0.5% Tween 20; Appendix 5.1) for 5mins. The membranes were then incubated in 5ml of primary antibody in $PBS_{T(0.5\%)}$ plus 1% (w/v) ovalbumin overnight at 4°C (rabbit sera were diluted 1:100-1:500 in $PBS_{T(0.5\%)}$ for use as primary antibody). The membranes were washed three times with 15ml $PBS_{T(0.5\%)}$ for 5mins, and then incubated for 30mins with Protein G-alkaline phosphatase conjugate (Calbiochem, Nottingham, Notts, UK) diluted 1:1000 in $PBS_{T(0.5\%)}$ plus 1% (w/v) ovalbumin. The membrane was then washed three times with 15ml $PBS_{T(0.5\%)}$ for 5mins and developed in 20ml substrate buffer plus nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Appendix 4.2) for approximately 5mins, until protein bands were detectable. The membranes were finally rinsed with ddH_2O and dried.

2.9.6. ELISA

Antigen samples were diluted in coating buffer (Appendix 5.2) plus 0.01% SDS. Nunc Polysorp 96-well plates (Life Technologies Ltd, Paisley, UK) were coated with 50μl samples of serially-diluted antigen and incubated at 37°C for 1hr. Plates were washed three times with PBS (Appendix 5.1) using a TiterTek M96V microplate washer (ICN-Flow, High Wycombe, Bucks, UK). Primary antibody diluted 1:20 in $PBS_{T(0.05\%)}$ (PBS plus 0.05% Tween 20; Appendix 5.1) plus 1% ovalbumin, was added to each well in 50μl aliquots. Plates were incubated overnight at 4°C. Plates were washed six times with $PBS_{T(0.05\%)}$. The secondary antibody (donkey anti-rabbit Ig, horseradish peroxidase conjugate; Amersham International, Bucks, UK) diluted 1:1000 in $PBS_{T(0.05\%)}$ plus 1%

ovalbumin, was added in 50μl aliquots to each well. Plates were incubated at 37°C for 1hr and washed six times with $PBS_{T(0.05\%)}$. 50μl of freshly prepared substrate buffer (Appendix 5.3) was added to each well and plates were incubated in the dark for 20mins. The reaction was halted by the addition of 100μl 4M sulphuric acid to each well. Plates were read at 492nm using a BioRad 2550 plate reader (BioRad Laboratories, Hemel Hempstead, Herts, UK), and the resulting data was analysed using BioRad Microplate Manager software. The background absorbance of the antibody reaction against coating buffer with no antigen was deducted from the absorbance of the antibody reaction against antigen.

2.10. DNA extraction and PCR analysis

2.10.1. Extraction of genomic DNA from blood

Blood samples were mixed with ddH_2O to lyse red blood cells, and platelets and lymphocytes were pelleted by centrifugation at 3000 x g_{max} for 20mins at 4°C. The supernatant was removed and the pellet was resuspended with 25ml 0.1% (v/v) Nonidet P40 and re-pelleted as above. The supernatant was removed and the pellet was resuspended with 2ml of extraction buffer (Appendix 6.1), plus 2mg/ml Proteinase K and 0.5% (w/v) SDS, and incubated overnight at 37°C shaking at 150rpm on an Infors HT orbital shaker (Infors Ltd, Crewe, Cheshire, UK).

An equal volume of TE-saturated phenol (Appendix 6.2) was added to the sample and the phases mixed on a whirly-wheel for 20mins, then separated by centrifugation at 3000 x g_{max} for 20mins. The top (aqueous) phase was carefully removed to a fresh centrifuge tube, and the phenol extraction repeated twice more with equal volumes of TE-saturated phenol. An equal volume of TE-saturated chloroform:isoamyl alcohol (24:1, v:v) was added to the aqueous phase, to remove any traces of phenol, and the phases were mixed on the whirly-wheel for 5mins and then separated by centrifugation at 3000 x g_{max} for 20mins. The top aqueous phase was removed to a fresh centrifuge tube, and ice-cold 3M sodium acetate was added to a final concentration of 0.3M. Cold 100% ethanol was added to two times the volume of sample plus sodium acetate, and the precipitation left at -70°C for at least 30mins. The DNA was pelleted by centrifugation at 17000 x g_{max} for 30mins at 4°C, the pellet was

washed with 2ml cold 70% ethanol, air dried for at least 30mins and finally resuspended with 200μl of TE buffer (Appendix 6.2). The DNA solution was mixed overnight on a whirly-wheel and stored at 4°C.

2.10.2. Extraction of genomic DNA from brain or skeletal muscle homogenate

The procedure was the same as that for extraction of DNA from blood, with the following alterations; 5mg tissue was crudely homogenised with a glass/teflon hand-held homogeniser prior to the addition of extraction buffer to 2ml final volume; the tissue was proteinase K digested at 56°C for 3hrs shaking at 150rpm, before a second addition of proteinase K was made, to take its final concentration to 4mg/ml and the tissue was subsequently incubated overnight at 37°C shaking at 150rpm.

2.10.3. Calculation of DNA concentration and purity

5μl of the 200μl DNA sample was added to 950μl ddH_2O in silica cuvettes, and the absorbance at 260 and 280nm was read (nucleic acid absorbs at 260nm and protein absorbs at 280nm; a value of A_{260}/A_{280} of ≥ 1.7 was considered to be pure nucleic acid). The sample concentration was adjusted to 1mg/ml by the addition of the appropriate volume of TE buffer (a 1mg/ml solution of nucleic acid has an absorbance at 260nm of 20).

2.10.4. PCR amplification of DNA

DNA primers were synthesised by Oswel DNA Services (Edinburgh, UK); dNTP's were supplied by Pharmacia Ltd (Milton Keynes, Bucks, UK); Taq DNA polymerase was supplied by Promega (Southampton, Hants, UK).

The reaction consisted of 500ng target DNA, 0.5μM forward and reverse primer, 200μM dATP, dGTP, dCTP, dTTP, 2.5 units Taq DNA polymerase and Taq DNA polymerase buffer (10mM Tris, 50mM KCl, 1.5mM $MgCl_2$ pH8.8) in a total volume of 100μl ddH_2O. This reaction mix was overlayed with 80μl liquid paraffin and placed in the thermal cycler (Hybaid Ltd, Teddington, Middx, UK).

The reaction consisted of an initial denaturation step of 94°C for 6mins, at which point the Taq DNA polymerase was added to the reaction. This was followed by 30 cycles of; 1min for primer annealing (temperature depends on the primer composition), 1min at 72°C (primer extension) and 1min at 94°C (denaturation). These cycles were followed by a final extension of 10mins at 72°C.

2.10.4.1. PCR detection of cytochrome P450 gene mutations

The primers used were those described by Smith *et al* (1992).

A guanosine to adenosine base transition at nucleotide position 1934 at the intron3/exon4 boundary within the CYP2D6 gene causes the loss of a BstN1 restriction site. PCR primers (see below) amplified a 334bp DNA fragment which is digested to 105 and 230bp fragments in normal alleles which carry the BstNI restriction site, but is not digested in mutant alleles in which the restriction site is destroyed.

Primers for detection of the G to A transition were;
forward (5'-GCCTTCGCCAACCACTCCG-3');
reverse (5'-AAATCCTGCTCTTCCGAGGC-3').
The primer annealing temperature was 60°C.

Deletion of an adenosine nucleotide at position 2637 within exon5 of the CYP2D6 gene creates a HpaII restriction site. PCR primers amplified a 268bp fragment which contains one HpaII restriction site. The normal allele is therefore digested to 188 and 82bp fragments by HpaII, and the mutant allele, in which a second HpaII site is created is digested to 20, 168 and 82bp fragments by HpaII.

Primers for detection of the base pair deletion were;
forward (5'-GATGAGCTGCTAACTGAGCCC-3');
reverse (5'-CCGAGAGCATACTCGGGAC-3').
The primer annealing temperature was 60°C.

2.10.5. Restriction enzyme digestion of PCR-product DNA

10μl of the PCR reaction was mixed with 1μl of the appropriate restriction enzyme (NBL Ltd, Camlington, Northumberland, UK) and its reaction buffer (supplied) and incubated for 2hrs at the optimum temperature for the particular enzyme.

2.10.6. Agarose gel electrophoresis

Gel loading buffer (Appendix 6.3) was added to the restricted DNA samples. Agarose gels (Life Technologies Ltd, Paisley, UK) were prepared in TBE buffer (Appendix 6.4) containing a final concentration of 1μg/ml ethidium bromide. 500ng of DNA molecular weight standards were included on every gel with DNA samples. The gels were electrophoresed in TBE buffer containing 1μg/ml ethidium bromide at 60V for approximately 1hr, and the DNA visualised on a UV transilluminator (GRI Ltd, Dunmow, Essex, UK).

2.11. Protein assay

Protein concentration was measured according to the method of Lowry *et al* (1951). Protein samples were made up to 1ml in ddH_2O to which was added 5ml solution I (Appendix 7). Samples were mixed and incubated for 20mins, after which time 0.5ml solution II was added and samples were mixed and incubated for 45mins. After this time sample absorbances were read at 750nm against standard samples of 0, 20, 40, 60 and 80μg fatty-acid free BSA.

Chapter 3. Mitochondrial respiratory chain function in platelet homogenates

3.1. Aim

To compare activities of mitochondrial respiratory chain (MRC) enzymes in platelet homogenates of Parkinson's disease (PD) patients and controls.

3.2. Introduction

In 1989, Parker *et al* published data demonstrating a 55% reduction in complex I activity of platelet mitochondria in patients with idiopathic Parkinson's disease compared to normal controls. The method of mitochondrial preparation involved the collection of platelets by plateletphoresis, homogenisation by nitrogen cavitation and preparation of mitochondrial fractions on density gradients. The whole procedure was time consuming, taking up to two days (DiMonte, 1991), and required specialised equipment for platelet collection, homogenisation and fractionation.

If confirmed, this biochemical abnormality may be useful as a diagnostic test for PD, and further may be useful in identifying presymptomatic or at-risk cases. This would be important in terms of treatment since it has been suggested that the monoamine oxidase B inhibitor selegiline may retard the progression of the disease (Parkinson Study Group, 1993). A routine diagnostic test has to be both quick and simple, and for this reason the possibility of detecting the complex I defect in whole cell platelet homogenates was investigated. The method only required 30ml of blood and the platelets were prepared in less than two hours. Since samples were stored frozen at -70°C prior to assay, the effect of storage time on complex I activity was determined. Complex I activity was also compared in platelet homogenate samples after different membrane disruption techniques to maximise its activity. Mitochondrial respiratory chain enzyme activities were compared in platelet homogenates from 14 PD patients and 15 controls.

3.3. Results

3.3.1. Cell counts through the platelet preparation

To determine the purity of platelets prepared from whole blood and to quantify platelet recoveries, the number of blood cells in various samples throughout the platelet preparation were counted using a Coulter cell counter (for details of the platelet preparation see Methods 2.5.1). Nearly 90% of the platelets in whole blood were recovered in the platelet rich plasma (PRP), and 95% of these were pelleted at the first centrifugation. The Tyrodes buffer wash retained greater than 95% of cells in the pellet. This data provides evidence that the preparation achieved good recovery of highly pure platelets from whole blood; the platelets were greater than 99.9% pure and the total recovery from whole blood was approximately 80%.

3.3.2. The effect of sample storage on complex I activity

Platelet homogenate samples from three normal controls were split into two aliquots. Complex I activity was measured on the day of preparation in one aliquot and 21 days later in the other. The mean specific activity of complex I in the samples assayed on the day of preparation was 5.34 ± 0.61 nmol/min/mg protein (mean $\pm$ SD) and in the stored samples was 5.26 ± 0.89. This difference of less than 2% was well within the limits of assay variation. As complex I activity in platelet homogenates was not affected by storage at -70°C for up to 21 days, all samples were subsequently assayed within 21 days of preparation.

3.3.3. MRC function in PD and control platelet homogenates

There were no significant differences between the mean activities of complexes I, II/III, IV or citrate synthase (CS) in the PD patient ($n = 14$) and control ($n = 15$) groups (Table 3-1; Figure 3-1). To correct for any differences in mitochondrial numbers in whole tissue homogenates, all enzyme activities were divided by CS activity. This mitochondrial matrix enzyme is used as an indicator of mitochondrial numbers since its activity is not known to be affected in any disease state; the CS ratio of an enzyme's activity essentially represents that

enzymes activity per mitochondrial unit. There were no significant differences in the CS ratios of any of the MRC enzymes between the patient and control groups (Table 3-1; Figure 3-2).

3.3.3.1. PD patients

Fourteen PD patients were included in the study. The mean age was 59.1 ± 10.2 years (range 45-74 years). The mean age at onset of disease was 48.1 years (range 32-63 years) and the mean duration of disease was 10.9 years (range 2-22 years). Complex I activity did not significantly correlate with age at disease onset, but just reached statistical significance ($p = 0.05$) when correlated with disease duration (Figure 3-3).

Two patients were untreated and had not taken any L-dopa at the time of sample. The specific activity of complex I in these two patients was 3.44 and 4.99 nmol/min/mg protein, as compared to a group mean of 3.51 ± 0.79 nmol/min/mg protein (mean ± SD).

3.3.3.2. Controls

Fifteen controls were included in the study. The mean age was 58.3 ± 9.9 years (range 48-77). Nine of the controls were normal, healthy individuals and six were disease controls who showed no evidence of primary neurodegenerative disease. This group included patients with multiple sclerosis (1), Guillan-Barre syndrome (1), carcinoma (3) and spastic paraperesis due to cord compression (1). Since there was no significant difference in complex I specific activity (3.53 ± 0.72 and 3.65 ± 1.11 nmol/min/mg protein; $p > 0.1$) or the CS corrected ratio of complex I activity (x100; 4.20 ± 1.03 and 4.21 ± 1.32; $p > 0.1$) between the normal control and disease control groups, they were pooled to form one group.

3.3.3.3. Complex I activity and age

Several studies have demonstrated that mitochondrial respiratory chain function decreases with age (see Introduction 1.4.5), and therefore patient and control groups were carefully age-matched in this study. Complex I activity showed no significant correlation with age in platelet homogenate samples of PD patients or controls (Figure 3-4).

3.3.4. MRC function in LHON patients platelet homogenates

Mitochondrial enzyme activities in platelet homogenates from three patients with Leber's hereditary optic neuropathy (LHON) were compared to controls. Two patients with the ND4 gene mutation had complex I activities 89% and 101% of the control mean; one patient with the ND1 gene mutation had a complex I activity 39% of the control mean (Table 3-2).

Specific Activity

	complex I	complex II/III	complex IV	CS
PD	3.51 ± 0.79	9.96 ± 2.00	0.50 ± 0.13	88.13 ± 16.90
C	3.58 ± 0.86	9.22 ± 2.30	0.44 ± 0.14	86.17 ± 12.58

CS corrected ratios

	complex I (x100)	complex II/III (x10)	complex IV (x100)
PD	4.16 ± 1.32	1.14 ± 0.15	0.564 ± 0.108
C	4.21 ± 1.11	1.08 ± 0.27	0.523 ± 0.168

Table 3-1. Mitochondrial enzyme activities in PD and control platelet homogenates.

Activities expressed as nmol/min/mg protein, except complex IV (k/min/mg protein). All activities mean ± SD.

PD (Parkinson's disease, n = 14); C (Controls, n = 15); CS (citrate synthase).

	I/CS (x100)
Controls (n = 15)	4.21 ± 1.11
Patient 1 (ND4)	3.73
Patient 2 (ND4)	4.25
Patient 3 (ND1)	1.63

Table 3-2. Citrate synthase corrected complex I activity in platelet homogenates of LHON patients.

I/CS (citrate synthase corrected complex I activity); ND4/ND1 (patients harbouring LHON-associated point mutations in the ND4(nt11778) / ND1 (nt3460) complex I genes).

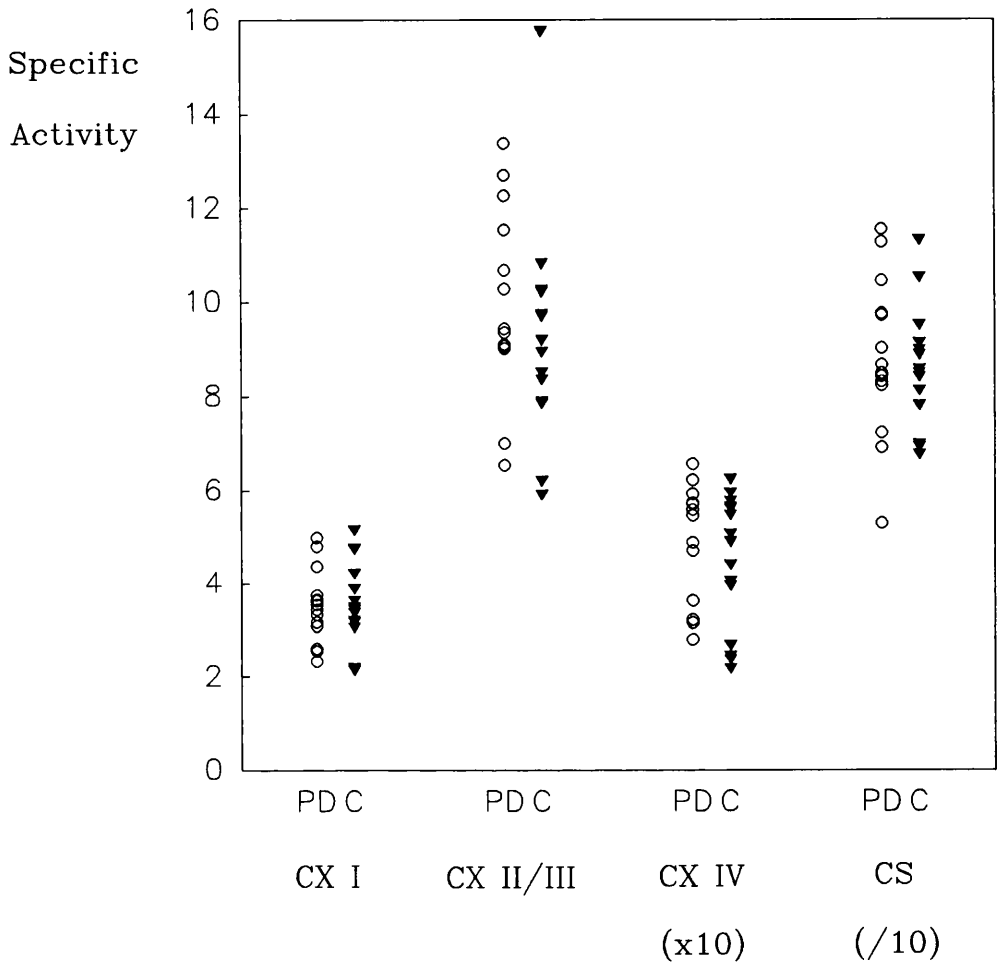


Figure 3-1. Mitochondrial enzyme activities in Parkinson's disease and control platelet homogenates.

Activities expressed as nmol/min/mg protein, except complex IV (k/min/mg protein).

PD (Parkinson's disease; n = 14), Circles; C (controls; n = 15), Triangles; CS (citrate synthase).

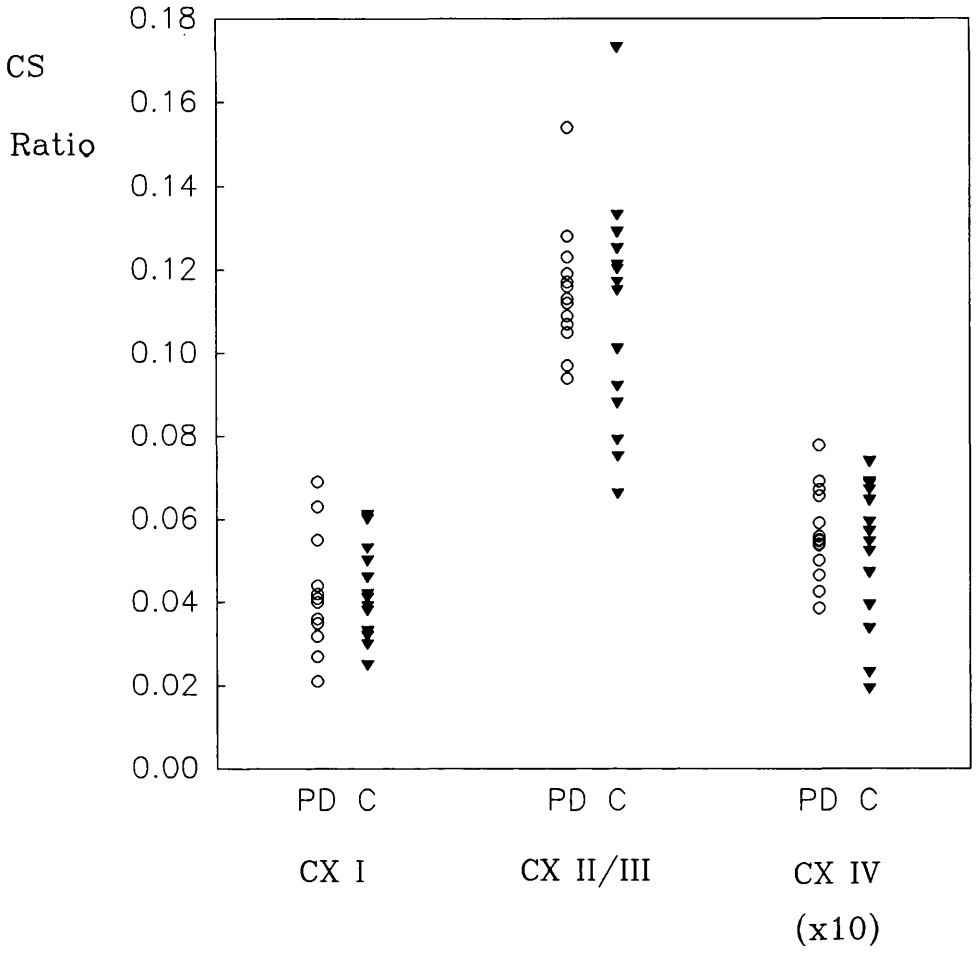
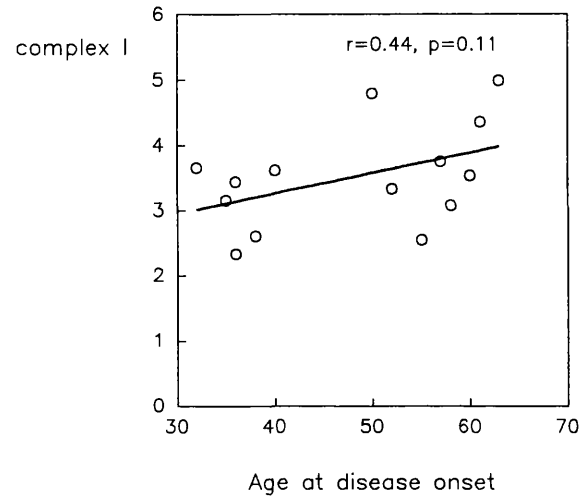


Figure 3-2. Mitochondrial respiratory chain enzyme activities in Parkinson's disease and control platelet homogenates, expressed as ratios of CS activity.

PD (Parkinson's disease; n = 14), Circles; C (controls; n = 15), Triangles; CS (citrate synthase).



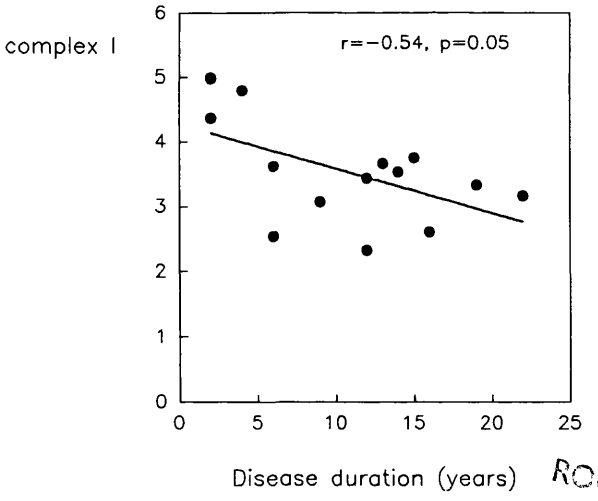
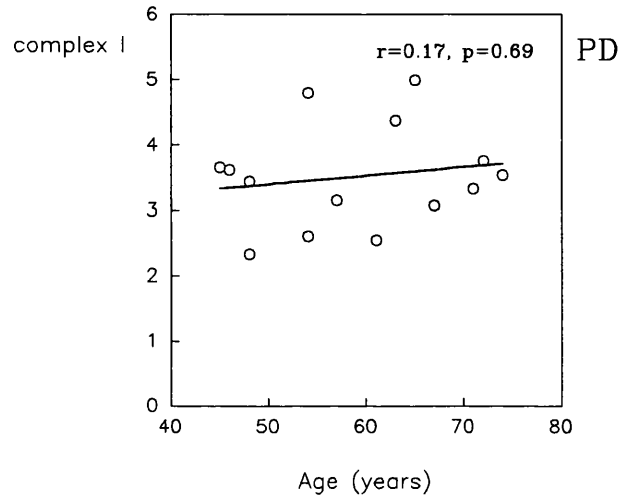


Figure 3-3. Complex I activity versus age at disease onset and duration of disease in Parkinson's disease platelet homogenates.

Complex I activity expressed as nmol/min/mg protein (n = 14).
r (coefficient of correlation).



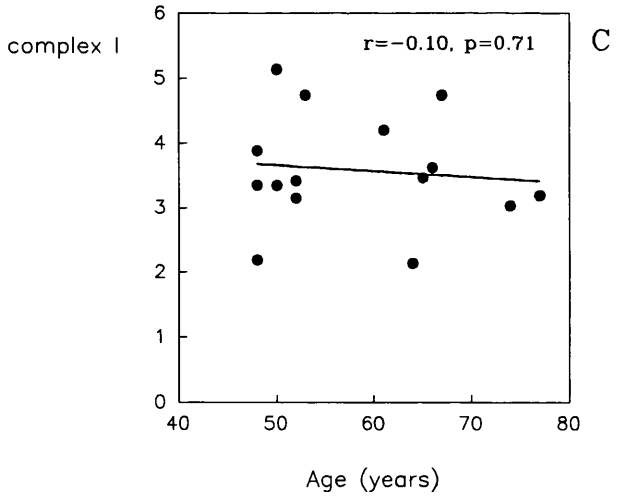


Figure 3-4. Complex I activity versus age in Parkinson's disease and control platelet homogenates.

Complex I activity expressed as nmol/min/mg protein.

PD (Parkinson's disease; n = 14); C (controls; n = 15); r (coefficient of correlation).

3.4. Discussion

The 55% complex I deficiency detected by Parker *et al* (1989) in PD platelet mitochondrial fractions was not detected in whole platelet homogenates. There were no significant differences between patient and control groups when specific enzyme activities were compared, or when CS ratios were compared (Table 3-1). One reason for this difference may have been the difference in complex I specific activity and the rotenone sensitivity of the complex I assay in the two preparations. The rotenone sensitivity of the NADH CoQ_1 reductase assay in platelet homogenates (range 23-37%) was not as sensitive as that in the platelet mitochondrial fractions of Parker *et al* (1989) (mean 75% sensitivity). There was no difference in the rotenone sensitivity of the complex I assay between the patient and control groups in this study. The specific activity of complex I was lower in platelet homogenates in this study than in platelet mitochondrial fractions; approximately 3.5 compared to 19.1 nmol/min/mg protein (Parker *et al*, 1989).

Although it seems unlikely that the reported complex I deficiency of 55% would not be detectable in platelet homogenates from PD patients, a mild enzyme defect may not be detected in this preparation. A mild complex I defect detected in platelet mitochondrial fractions from patients with Leber's hereditary optic neuropathy (LHON) was not detected in whole platelet homogenates (Table 3-2). LHON is associated with a number of mutations in mtDNA. Individuals with LHON harbouring either the ND1 nt3460 or the ND4 nt11778 mutation, have been shown to have a complex I defect, detectable in platelet mitochondrial fractions (Howell *et al*, 1991; Smith *et al*, 1994). The ND1 mutation was associated with a 70% mean complex I deficiency, and the ND4 mutation with a milder 25% complex I defect in platelet mitochondrial fractions. In this study using whole platelet homogenate samples, the CS corrected complex I activities of two patients with the ND4 (nt11778) mutation were 11% below and 1% above the control mean, whilst the activity from a single patient with the ND1 (nt3460) mutation was 61% below the control mean (Table 3-2). Therefore, a complex I defect of approximately 25% detected in platelet mitochondrial fractions was not detectable in whole platelet homogenates, but a complex I defect of 70% detected in mitochondrial fractions was detectable in whole platelet homogenates.

Mitochondrial respiratory chain enzyme activities have also been measured in PD patient and control whole platelet homogenates by Yoshino *et al* (1992). The mean activities of complexes I and II were statistically significantly decreased

by 26% and 20% respectively in the PD patient group (n = 20) relative to controls (n = 17). Two studies have measured MRC enzyme activities in PD lymphocyte homogenate samples. Yoshino *et al* (1992) report normal complex I activity in the PD lymphocyte samples, although in platelet homogenate samples from the same patients the mean complex I activity was decreased by 26% (see above). Barroso *et al* (1993) detected statistically significant mean decreases of 24% and 45% in the activities of complexes I (measured as rotenone-sensitive NADH cytochrome c reductase) and IV respectively, in lymphocytes from PD patients (n = 16) compared to controls (n = 15).

Chapter 4. Mitochondrial respiratory chain function in platelet mitochondrial fractions

4.1. Aim

To prepare mitochondrial fractions from platelets by differential centrifugation, and to compare mitochondrial respiratory chain (MRC) enzyme activities in PD patients and controls.

4.2. Introduction

In Chapter 3 it was shown that the previously reported 55% decrease in complex I activity in PD patients platelets (Parker *et al*, 1989) could not be detected in whole platelet homogenates. Since it is conceivable that a mild defect of complex I would not be detected in platelet homogenates, platelet mitochondria were enriched by differential centrifugation to increase the sensitivity of the complex I assay. Following homogenisation of whole platelets, the homogenate was subjected to a low speed centrifugation which pelleted whole cells and nuclei. The post nuclear supernatant (PNS) was then centrifuged at high speed to pellet mitochondria but leave less dense organelles in the supernatant (see Methods 2.5.2). Platelets have few mitochondria per cell, maybe as few as ten (Shuster *et al*, 1988), and contain various granules whose density is similar to mitochondria (Sixma and Lips, 1978). Therefore any procedure in which mitochondria are isolated on the basis of their density, including differential centrifugation and density gradient centrifugation, will result in co-purification of these granules with mitochondria.

The purity of mitochondria in enriched fractions was determined by the analysis of marker enzymes for other cellular organelles likely to contaminate the fraction, and by electron microscopy of the fraction. The complex I assay developed for use with purified complex I (Ragan *et al*, 1987) was optimised for use in platelet mitochondrial fractions. Mitochondrially-enriched fractions (MEFs) were prepared from 25 PD patients and 15 age-matched controls, and the activities of MRC enzymes were compared in the two groups.

4.3. Results

4.3.1. Electron microscopy of mitochondrial fractions

One indicator of mitochondrial purity is direct visualisation of the fraction by electron microscopy (EM). A typical section through th platelet mitochondrial fraction shows that mitochondria are present in the fraction, but they are far outnumbered by dense granules (Figure 4-1). This finding is in agreement with others (Sixma and Lips, 1978), who observed that platelets contain few mitochondria and many granules of a similar density. It is clear from this data that the fraction did not consist of pure mitochondria, and should therefore more accurately be termed a mitochondrially-enriched fraction (MEF).

4.3.2. Mitochondrial recovery and integrity

Citrate synthase (CS), an enzyme located in the mitochondrial matrix, was assayed in the cell homogenate and MEF and used as an indicator of mitochondrial recovery. Since the enzyme is not membrane-bound, it will only be present in the mitochondrial pellet if the mitochondria are intact. The recovery of CS in the mitochondrial pellet was 27.9% ± 7.6% (mean ± SD; n=6). The percentage of cells not disrupted by nitrogen decompression was calculated as the percentage of CS activity in the nuclear pellet after the second homogenisation relative to that in the whole cell homogenate. Based on this calculation, the percentage of cells not disrupted was 2.3% ± 2.8% (n=6). In other words, over 95% of the platelets were disrupted by the technique of nitrogen decompression. Data has shown that this technique of cell disruption is the most effective for platelet homogenisation (Sixma and Lips, 1978; Lovette *et al*, 1976).

Another consideration of the suitability of the homogenisation technique is the integrity of the subcellular organelles released by cellular homogenisation. In the case of mitochondria this can be measured as the latency of CS. Because the enzyme is located in the matrix surrounded by two membranes, if CS activity is assayed in fresh, intact mitochondria, substrates cannot reach the enzyme and no activity is detectable. However, in the presence of detergent (Triton X-100), the membranes are solubilised, substrates can reach the enzyme, and activity is detectable. The percentage of total activity released in the presence of detergent

is referred to as the latency of the enzyme. In the above preparation, the latency of CS in the post-nuclear supernatant was 70.3% ± 4.3% (n=3). So homogenisation disrupted over 95% of the platelet cell membranes, but only disrupted approximately 30% of the mitochondrial membranes. This is also in agreement with reports that the homogenisation method of nitrogen decompression in platelets preserves intracellular platelet membranes (Sixma and Lips, 1978; Lovette *et al*, 1976).

4.3.3. Purification factors of mitochondrial enzymes

In order to assess the extent of mitochondrial enrichment in the MEF, the activities of mitochondrial enzymes were measured in both whole cell homogenates and MEFs (Table 4-1); specific activity in the latter divided by that in the former gives a purification factor for that enzyme. The purification factors revealed that the activities of complex II/III and complex IV were purified approximately six-fold but complex I activity was purified less than two-fold. The activity of CS was only purified approximately three-fold; because the enzyme is located in the mitochondrial matrix, some will be lost to the soluble PNS during mitochondrial preparation if the mitochondria do not remain intact.

One possible explanation why complex I activity did not have as great a purification factor as the other membrane-bound respiratory chain enzymes, was that the enzyme lost activity upon homogenisation. To test this hypothesis, respiratory chain enzyme activities were compared in platelets before and after nitrogen cavitation (Table 4-2). The activities of complexes II/III, IV and CS did not change appreciably after nitrogen cavitation, however complex I activity decreased. This decrease, of approximately 50%, was specifically of the rotenone-sensitive mitochondrial activity. This would explain the lower purification factor of complex I in platelet MEFs relative to homogenate (Table 4-1).

Based on the hypothesis that the specific loss of mitochondrial complex I activity upon nitrogen cavitation was due to some specific structural instability of the enzyme, three techniques were employed to try and protect the activity from any destruction; the pressure of nitrogen in the bomb was lowered from 1200psi to 600psi, 0.35% BSA was added to the platelets prior to homogenisation and the volume of platelet homogenate in the nitrogen bomb was increased from 3ml to 20ml. None of these measures protected the enzyme from any loss of activity upon nitrogen cavitation.

Homogenisation of platelets was attempted using glass/teflon homogenisers to try and circumvent the loss of complex I activity during nitrogen bomb homogenisation. However, even after long periods of homogenisation using tight fitting homogenisers (0.005" clearance) the number of cells broken was much lower than that achieved by nitrogen cavitation (59.0% ± 6.6%; n=3), based on the release of CS activity to the PNS. The recovery of CS in the MEF was also much lower (16.3% ± 6.4%; n=3), and therefore it was clear that glass/teflon homogenisation of platelets was not a suitable technique.

4.3.4. Optimisation of the complex I assay for use in platelet MEFs

The complex I assay as described by Ragan *et al* (1987) was initially used to measure the activity of complex I in platelet MEFs. The specific activity of complex I in these fractions from controls was only 5.42 ± 1.00 nmol/min/mg protein (n=5) and the percentage rotenone sensitivity of the assay only 36.5% ± 3.5% (n=5). These figures compared with mean specific activities of complex I in the mitochondrial fraction prepared by Parker *et al* (1989) of 19.1 nmol/min/mg protein and rotenone sensitivity of 75% (Parker *et al*, 1988). In an attempt to improve the rotenone sensitivity and specific activity of complex I in platelet MEFs, various components of the assay were optimised. These included the pH of the potassium phosphate buffer, the addition of $MgCl_2$ and the addition of bovine serum albumin (BSA). These modifications were based on the results of Lowerson *et al* (1992) of complex I assays in human fibroblast mitochondria.

Complex I was initially assayed in the presence of 20mM potassium phosphate pH8.0 according to the method of Ragan *et al* (1987). However the assay composition suggested by Lowerson *et al* (1992) was 20mM potassium phosphate pH7.2 with the addition of 5mM $MgCl_2$ and 2.5mg/ml BSA. Complex I activity was maximal in platelet mitochondrial fractions between pH7.0 and pH7.6 and was significantly less at pH8.0 (Figure 4-2). The complex I assay was subsequently carried out at pH7.2. The addition of $MgCl_2$ to the complex I assay in platelet mitochondrial fractions more than doubled the specific activity. The activity was maximal between 8 and 16mM $MgCl_2$, but was inhibited at 40mM (Figure 4-2). The complex I assay in platelet MEFs was subsequently performed in the presence of 8mM $MgCl_2$. The effect of the addition of BSA to the complex I assay in platelet MEFs was maximal at a concentration of 2.5mg/ml (Figure 4-2), and therefore this concentration of BSA was subsequently added to

the complex I assay. Figure 4-3 shows the additive effect of these factors on the complex I assay in platelet MEFs. The rotenone insensitive NADH CoQ_1 reductase non-mitochondrial activity was essentially unaffected by the assay conditions (Figure 4-3).

The optimal complex I assay conditions in platelet mitochondrial fractions, 20mM potassium phosphate, pH7.2, 8mM $MgCl_2$ and 2.5mg/ml BSA, resulted in a specific activity of 21.39 ± 2.45 nmol/min/mg protein and rotenone sensitivity of 73.8% ± 1.79% (Figure 4-3). These results were comparable with those of Parker *et al* (1988, 1989).

The effect of nitrogen cavitation on the activity of complex I assayed under these conditions was determined (Table 4-3). In contrast to complex I activity measured by the method of Ragan *et al* (1987), complex I activity measured using the modified assay conditions was not affected by nitrogen cavitation.

4.3.5. Relative mitochondrial purification

To determine the degree of mitochondrial purification in MEFs relative to platelet homogenates, the activities of various cellular fraction markers were assayed in both fractions (Table 4-4, Figure 4-4). The markers used were lactate dehydrogenase (cytosol), esterase (endoplasmic reticulum), acid phosphatase (lysosomes), ß-thromboglobulin (*α*-granules), serotonin (dense granules) and complex I, II/III, IV and citrate synthase (mitochondria),

There was some enrichment of mitochondria in the MEFs; the purification factors of the mitochondrial enzymes complex I, II/III and CS were approximately two-fold, whilst that of complex IV was almost five-fold. However, dense and alpha granules and lysosomes co-purified with mitochondria. Esterase and LDH, did not co-purify, resulting in purification factors of less than one. These results are in agreement with EM data (see Results 4.3.1).

Since enzyme activity in the mitochondrial fraction is measured per milligram of total protein, it is important that the extent of mitochondrial purification, and contamination with other organelles, is consistent between preparations. The relatively small standard deviations of the mean purification factors, of both the mitochondrial and other cellular fraction markers, suggest that this was indeed the case.

4.3.6. MRC function in PD and control platelet MEFs

The activity of complex I was significantly different in the two groups (Table 4-5). The mean complex I specific activity was 14% decreased in the PD patient group relative to controls (16.57 ± 3.24 and 19.34 ± 5.22 nmol/min/mg protein respectively) and the mean CS corrected ratio of complex I activity was decreased by 16% in the patient group. The difference in the CS corrected complex I ratio was highly statistically significant ($p=0.017$) while the difference in complex I specific activity just failed to reach statistical significance ($p=0.063$). The specific activities and CS ratios of complexes II/III and IV were not significantly different. Figures 4-7 and 4-8 show the spread of individual data points for the specific activities and CS corrected ratios of the mitochondrial respiratory chain enzymes.

4.3.6.1. PD patients

Twenty five patients were included in the study. The mean age was 62.7 ± 6.7 years (range 48-73 years). The mean age at onset of disease was 56.7 ± 8.4 years (range 31-72 years). The mean duration of disease was 5.9 ± 5.2 years (range 0.08-17 years). Four of the patients were untreated and had not taken any L-dopa at the time of sample. Complex I activity was not significantly different in the treated and untreated patients; 16.30 ± 3.05 and 15.62 ± 1.19 nmol/min/mg protein respectively ($p>0.1$) (CS ratios x100; 8.19 ± 1.28 and 8.37 ± 1.20 respectively ($p>0.1$)). Of the 21 treated patients, data on drug ingestion was available for 19; the mean total amount of L-dopa ingested since treatment began was 969 ± 1374 mg (range 10-4464mg). Complex I activity in these PD patients did not show any correlation with age, age at onset of disease, duration of disease or total L-dopa ingested (Figure 4-5, 4-6).

Smith *et al* (1993) reported mean complex I activity in platelet MEFs 24% decreased in smokers compared to non-smoking controls. In this study, fourteen of the PD patients were non-smokers and four were current smokers (no information available for the other seven); there was no significant difference in complex I activity between the PD smoking and non-smoking groups (19.38 ± 4.91 and 16.12 ± 2.14 nmol/min/mg protein respectively ($p>0.1$)).

4.3.6.2. Controls

Fifteen control subjects were included in the study. The group were age matched with the PD group; the mean age was 65.7 ± 6.9 years (range 50-76 years). Four of the controls were normal healthy individuals and eleven were disease controls who showed no evidence of neurodegenerative disease. This group included patients with various peripheral neuropathies, stroke and carcinoma. Since there was no significant difference in complex I specific activity between the normal and disease control groups, they were pooled to form one control group. Complex I activity did not correlate with age in this control group (Figure 4-5). Six of the controls were non-smokers, three were current smokers and no information was available for the other six. There was no significant difference in complex I activity between the smoking and non-smoking groups (17.64 ± 5.39 and 18.50 ± 4.14 nmol/min/mg protein respectively ($p > 0.1$)).

4.3.7. MRC function in platelet MEFs of mitochondrial myopathy patients

Mitochondrial enzymes activities were determined in platelet MEFs from two patients with mitochondrial myopathy. Patient JH had an as yet unidentified molecular defect, whilst patient VA carried the MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes) mutation at nucleotide 3243 in the mitochondrial $tRNA^{Leu}$ gene (Dr JM Cooper, personal communication). Both patient JH and patient VA had complex I and complex IV enzyme defects in skeletal muscle (Table 4-6). The skeletal muscle mitochondrial complex I defect (3% of age-matched control mean) in patient JH was detectable in the platelet MEF (51% of control mean), whilst that in patient VA (26% of age-matched control mean) was not (105% of control mean) (Table 4-6). The skeletal muscle mitochondrial complex IV defect in both patients was not detected in the platelet MEFs.

	Homogenate (n = 9)	Mitochondrial fraction (n = 5)	Purification factor
complex I	3.53 ± 0.72	5.42 ± 1.00	1.53
complex II/III	8.92 ± 1.27	53.2 ± 7.60	5.96
complex IV	0.39 ± 0.16	2.26 ± 0.93	5.79
CS	85.7 ± 16.4	235.6 ± 29.7	2.75

Table 4-1. Mitochondrial enzyme activities in control platelet homogenates and MEFs.

Activities expressed as nmol/min/mg protein, except complex IV (k/min/mg protein). All activities mean ± SD.
CS (citrate synthase); Purification factor is the specific activity in mitochondrially enriched fractions divided by that in homogenates.

Homogenate and mitochondrial fractions were prepared from separate groups of controls.

	Pre bomb	Post bomb	Post/Pre
complex I			
Total	11.76 ± 1.95	10.27 ± 2.70	0.87
Insensitive	7.19 ± 1.40	7.91 ± 2.25	1.10
Sensitive	4.57 ± 0.70	2.36 ± 0.54	0.52
% Sensitivity	38.1 ± 3.4	23.4 ± 3.49	---
complex II/III	14.00 ± 3.85	12.62 ± 4.00	0.90
complex IV	0.576 ± 0.149	0.494 ± 0.171	0.86
CS	104.6 ± 25.1	105.9 ± 34.8	1.01

Table 4-2. Mitochondrial enzyme activities in platelet homogenates pre and post nitrogen bomb homogenisation.

All activities expressed as nmol/min/mg protein, except complex IV (k/min/mg protein). All activities mean ± SD (n = 10).

Complex I activity expressed as rotenone sensitive, insensitive and total (insensitive plus sensitive) NADH CoQ_1 reductase activity; CS (citrate synthase).

	Pre bomb	Post bomb	Post/Pre
NADH CoQ_1 reductase			
Total	16.02 ± 1.22	13.22 ± 2.23	0.82
Insensitive	7.14 ± 0.56	6.22 ± 0.92	0.87
Sensitive	8.88 ± 1.46	7.00 ± 1.32	0.79
% Sensitivity	55.2% ± 5.6%	52.8% ± 1.2%	---

Table 4-3. NADH CoQ_1 reductase activities in platelet homogenates pre and post nitrogen bomb homogenisation using the modified assay.

Activities expressed as nmol/min/mg protein (mean ± SD; n = 4).
Activity expressed as rotenone sensitive (complex I), insensitive and total (insensitive plus sensitive) NADH CoQ_1 reductase activity.

For details of the modified complex I assay see Methods 2.6.1.

	Homogenate	Mitochondrial fraction	Purification factor
Serotonin	307.1 ± 150.0	546.4 ± 305.7	1.74 ± 0.13
ß-TG	907 ± 559	1960 ± 1130	2.27 ±0.58
EST	161.7 ± 54.0	64.0 ± 9.0	0.42 ± 0.13
AP	6630 ± 410.	10590 ± 1860	1.59 ± 0.21
LDH	1050 ± 282.3	109.9 ± 27.3	0.12 ± 0.06
CXI	8.88 ± 1.46	17.68 ± 2.52	2.02 ± 0.33
CXII/III	18.46 ± 2.73	53.91 ± 15.24	2.93 ± 0.70
CXIV	0.41 ± 0.16	1.76 ± 0.58	4.69 ± 1.57
CS	110.8 ± 11.4	204.3 ± 27.8	1.86 ± 0.30

Table 4-4. Platelet enzyme activities and marker concentrations in platelet homogenates and MEFs.

All activities expressed as nmol/min/mg protein (mean ± SD; n=4), unless otherwise stated.
Serotonin (ng/mg protein; n=3); ß-TG (ß-thromboglobulin, pmol/mg protein; n=3); EST (esterase); AP (acid phosphatase); LDH (lactate dehydrogenase); CXI (complex I); CXII/III (complex II/III); CXIV (complex IV, k/min/mg protein); CS (citrate synthase).
Purification factor is the specific activity in mitochondrial fractions divided by that in homogenates.

Specific Activity

	complex I	complex II/III	complex IV	CS
PD	16.57 ± 3.24	53.98 ± 10.55	1.89 ± 0.59	202.40 ± 31.99
C	19.34 ± 5.22	47.96 ± 12.31	1.92 ± 0.62	197.26 ± 41.40

CS corrected ratios

	complex I (x100)	complex II/III (x10)	complex IV (x100)
PD	8.26 ± 1.34*	2.69 ± 0.47	0.939 ± 0.257
C	9.88 ± 2.14	2.44 ± 0.44	0.988 ± 0.250

Table 4-5. Mitochondrial enzyme activities in Parkinson's disease and control platelet MEFs.

Activities expressed as nmol/min/mg protein, except complex IV (k/min/mg protein). All activities mean ± SD.
PD (Parkinson's disease, n = 25); C (controls, n = 15); CS (citrate synthase).

* p = 0.017 (Mann Whitney U-test).

A. Skeletal muscle mitochondria

	Age	I	II/III	IV	CS
CON (n=4)	26.5±1.3	232±59	371±128	37.3±11.6	1270±156
JH	20	6.0	166.0	12.9	1420
CON (n=6)	63.2±11.9	97±38	204±72	25.2±11.4	1768±641
VA	58	25.0	230.0	5.8	1866

B. Platelet mitochondrially-enriched fractions

	Age	I/CS (x100)	(II/III)/CS	IV/CS (x1000)
CON (n=23)	62.1±7.8	7.44±1.04	0.202±0.058	9.00±2.64
JH	20	3.82	0.386	9.60
VA	58	7.81	0.234	12.97

Table 4-6. Mitochondrial enzyme activities in skeletal muscle mitochondria and platelet MEFs from two patients with mitochondrial myopathy.

Specific activities in skeletal muscle mitochondria expressed as nmol/min/mg protein, except complex IV (k/min/mg protein). Activities in platelet MEFs expressed as a ratio of citrate synthase activity. Control activities mean ± SD.

CON (controls); I (complex I); II/III (complex II/III); IV (complex IV); CS (citrate synthase).

All skeletal muscle mitochondria assays and control platelet MEF assays were performed by Dr JM Cooper and Mrs MT Gash.

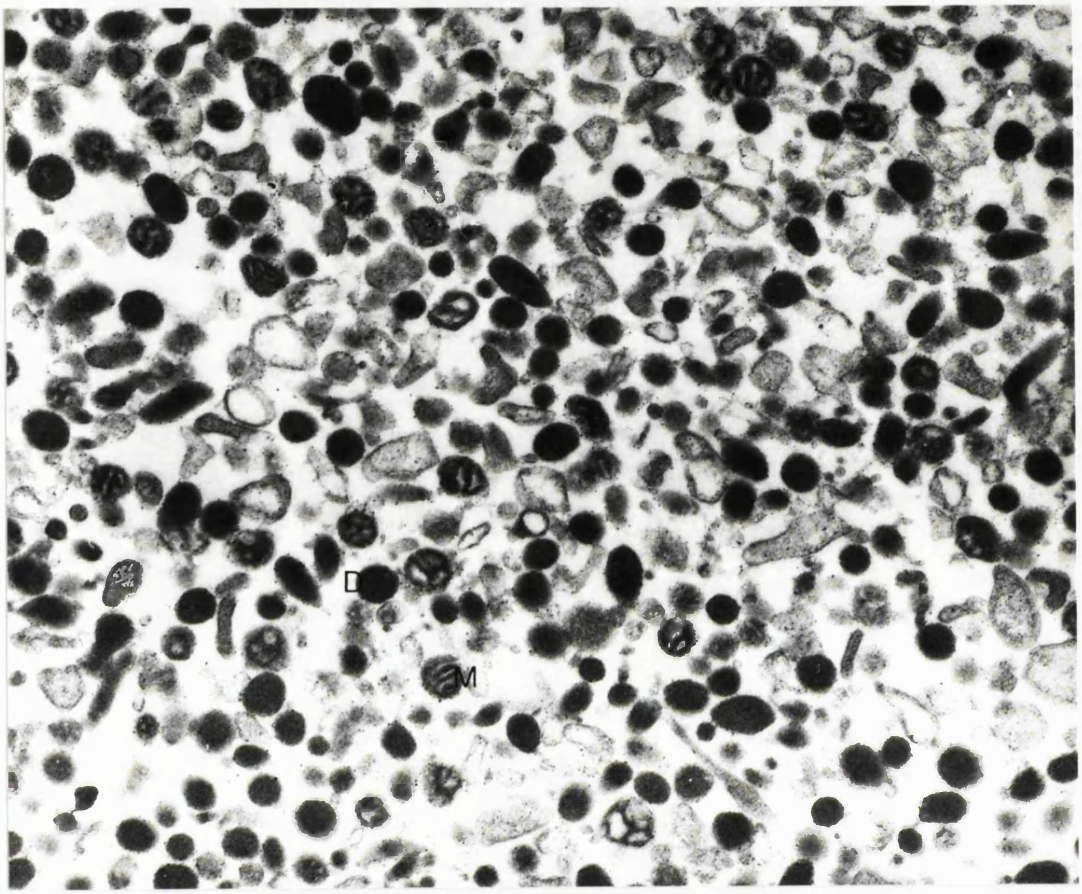


Figure 4-1. Electron micrograph of the platelet mitochondrially-enriched fraction.

M (mitochondria); D (dense granule).

Magnification x 22000.

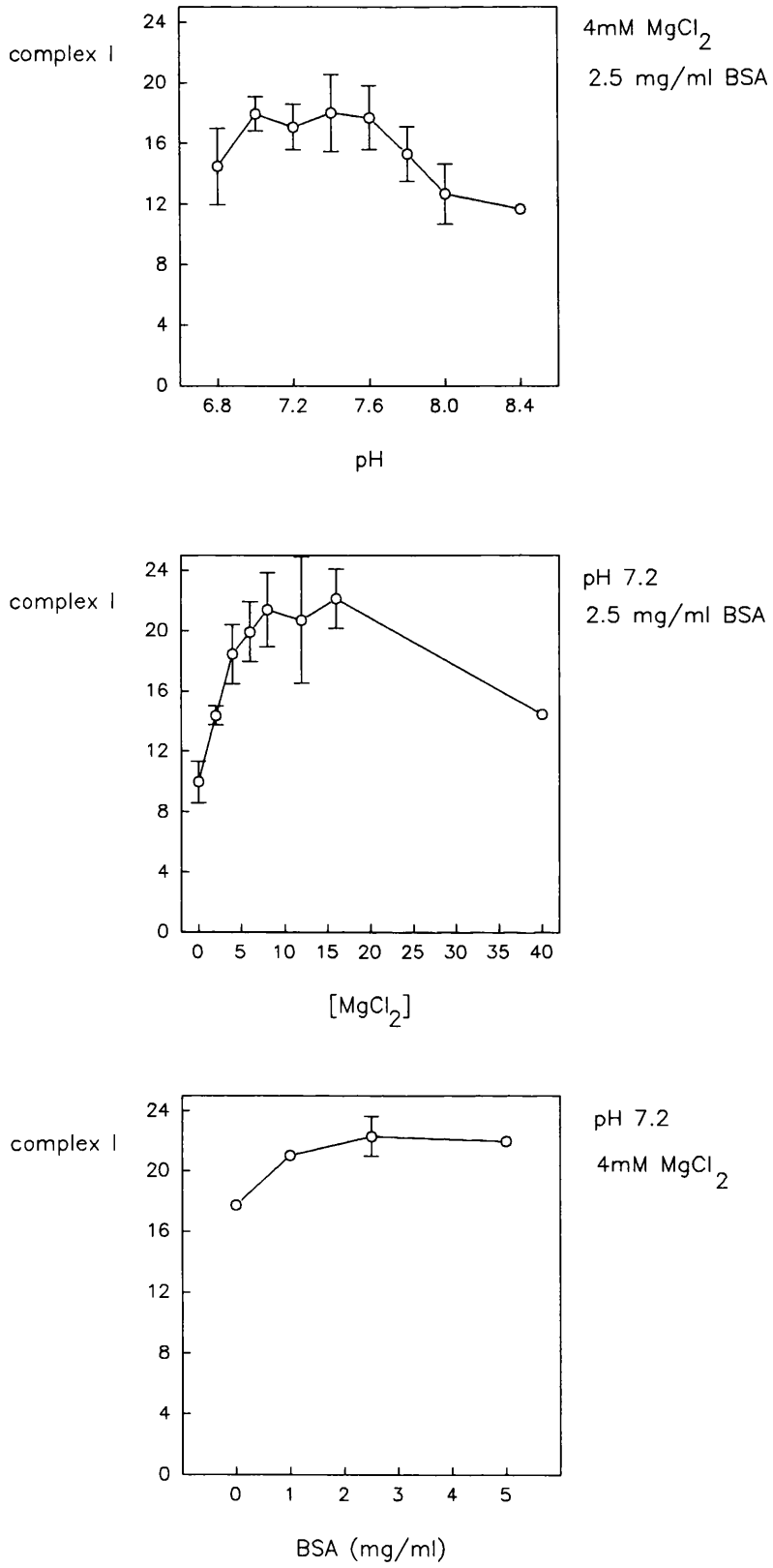


Figure 4-2. Complex I activity in platelet MEFs under varying assay conditions of pH, concentration of $MgCl_2$ and concentration of BSA.

Complex I activity measured as rotenone-sensitive NADH CoQ_1 reductase (nmol/min/mg protein).

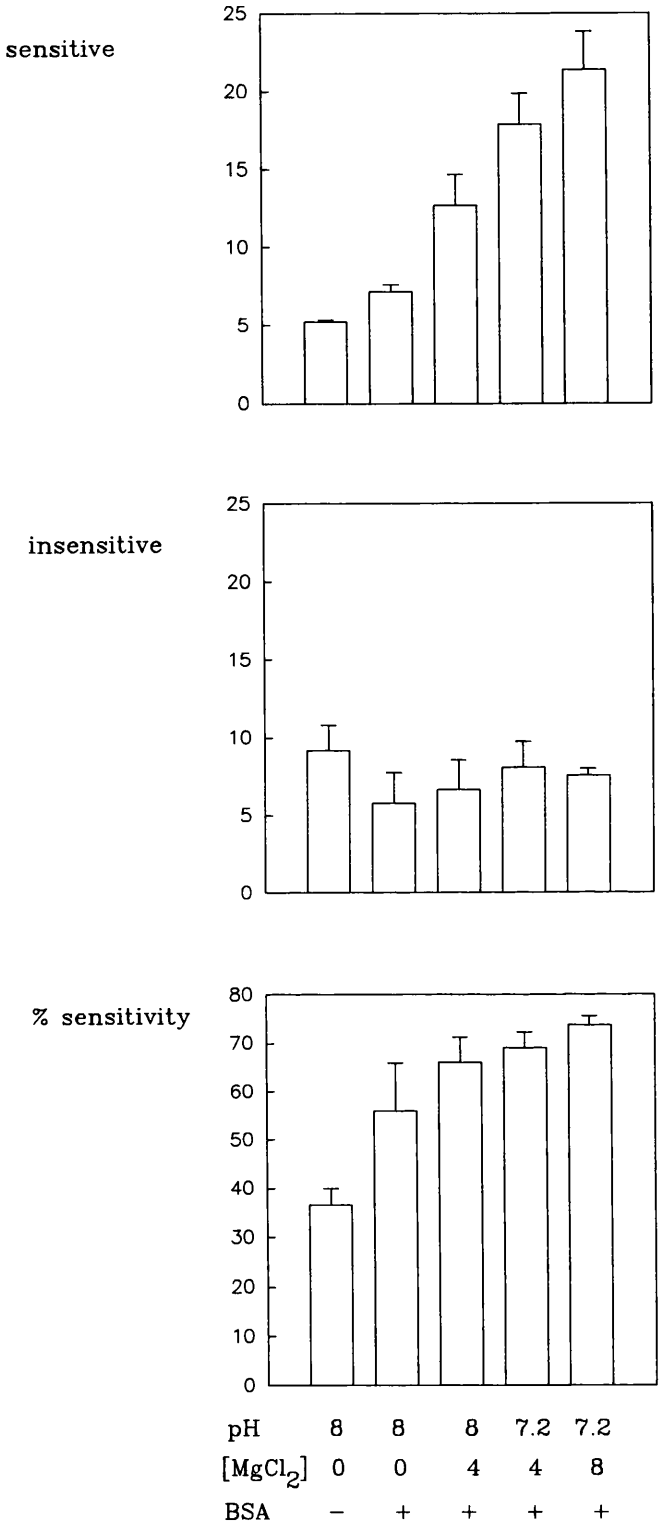


Figure 4-3. Rotenone sensitive and insensitive NADH CoQ_1 reductase activity in platelet MEFs under varying assay conditions of pH, concentration of $MgCl_2$ and concentration of BSA.

sensitive/insensitive (rotenone sensitive/insensitive NADH CoQ_1 reductase activity); % sensitivity (% rotenone sensitivity of the NADH CoQ_1 reductase assay). All activities expressed as nmol/min/mg protein.

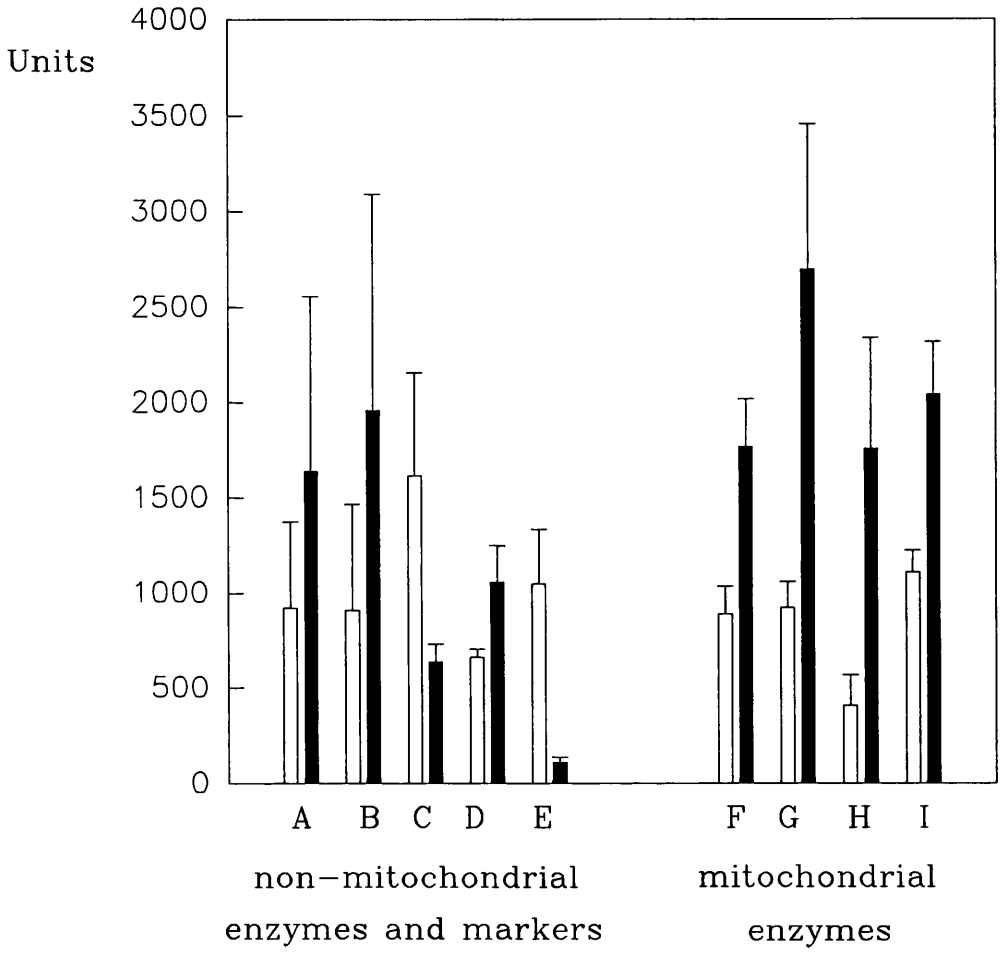
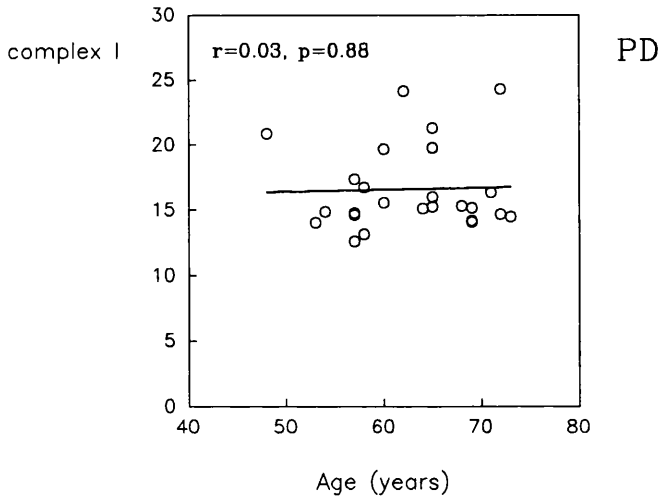


Figure 4-4. Platelet enzyme activities and marker concentrations in platelet homogenate (open boxes) and MEFs (solid boxes).

All activities mean ± SD (bars represent SD). All units as Table 4-4.
A (serotonin x3); B (ß-thromboglobulin); C (esterase x10); D (acid phosphatase /10); E (lactate dehydrogenase); F (complex I x100); G (complex II/III x50); H (complex IV x1000); I (citrate synthase x10).



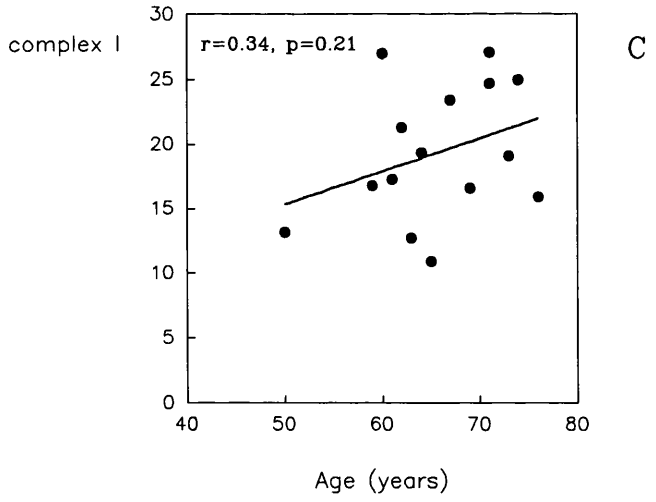


Figure 4-5. Complex I activity versus age in Parkinson's disease and control platelet MEFs.

Complex I activity measured as rotenone-sensitive NADH CoQ_1 reductase (nmol/min/mg protein).

PD (Parkinson's disease; n = 25); C (controls; n = 15); r (coefficient of correlation).

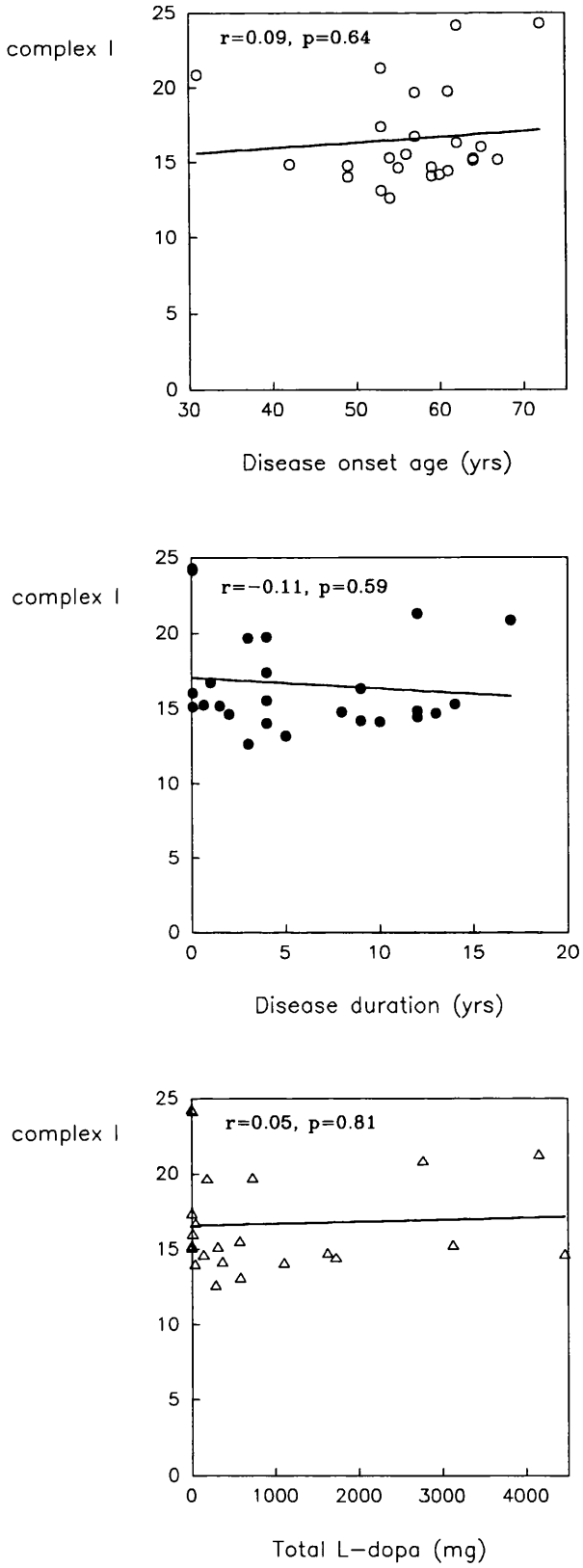


Figure 4-6. Complex I activity versus age at disease onset, duration of disease and total l-dopa ingested in Parkinson's disease platelet MEFs.

Complex I activity measured as rotenone-sensitive NADH CoQ_1 reductase (nmol/min/mg protein; n = 25). r (coefficient of correlation).

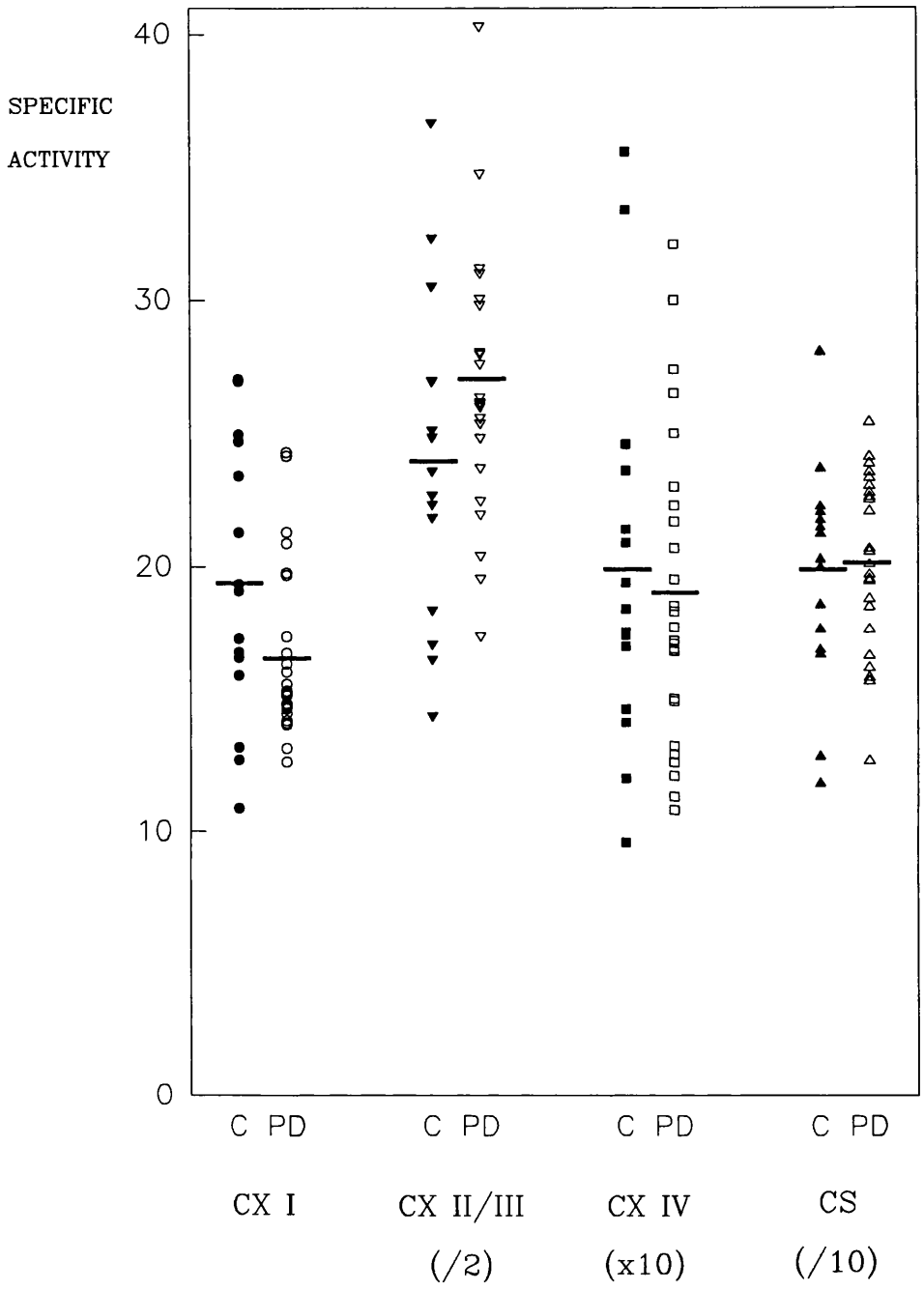


Figure 4-7. Mitochondrial enzyme activities in Parkinson's disease and control platelet MEFs.

Activities expressed as nmol/min/mg protein, except complex IV (k/min/mg protein). Bars represent the mean.

PD (Parkinson's disease; n = 25); C (controls; n = 15); CS (citrate synthase).

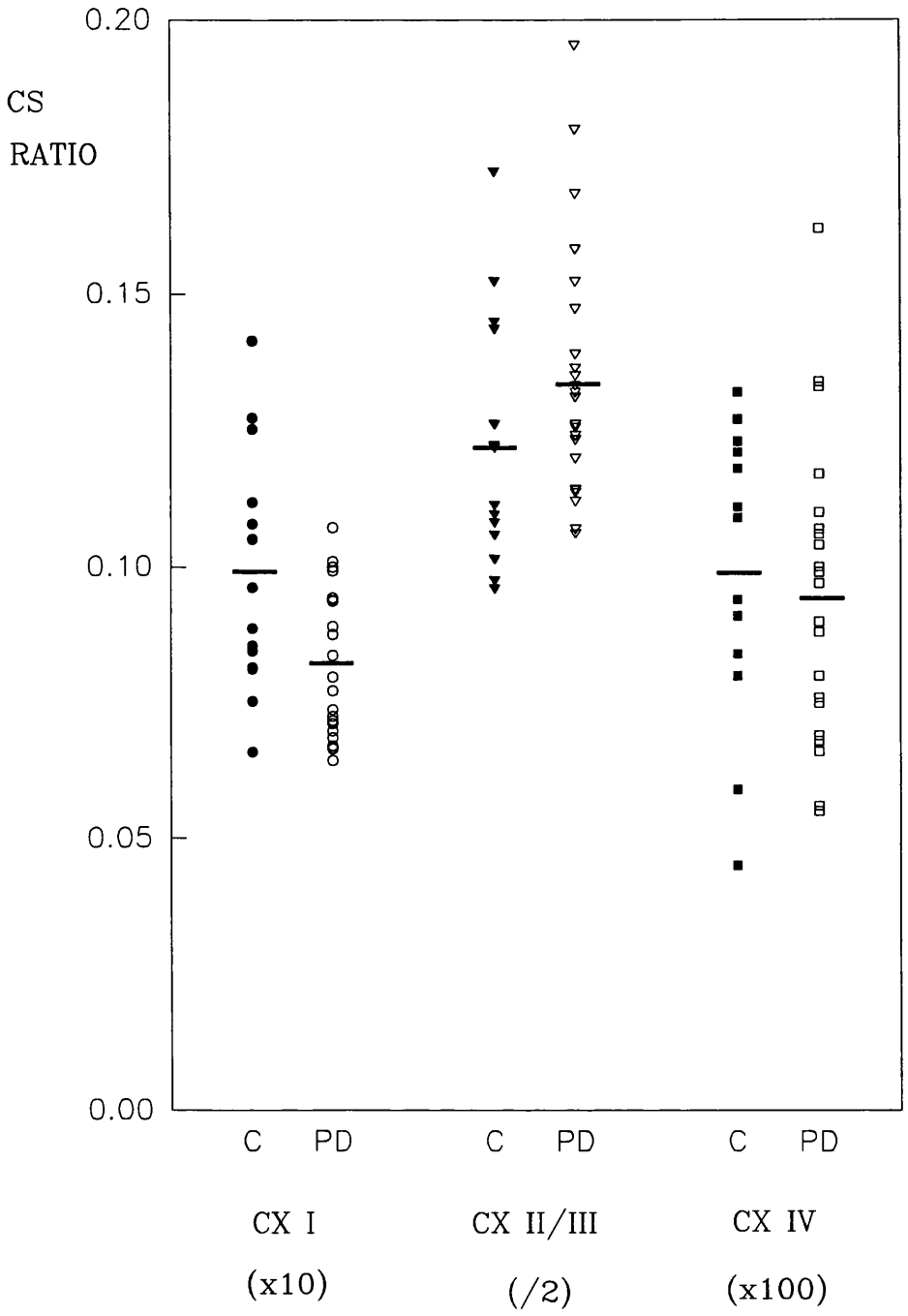


Figure 4-8. Mitochondrial respiratory chain enzyme activities in Parkinson's disease and control platelet MEFs, expressed as ratios of CS activity.

Bars represent the mean.

PD (Parkinson's disease; n = 25); C (controls; n = 15); CS (citrate synthase).

4.4. Discussion

The data presented above demonstrated the presence of a complex I defect in PD patients platelets (Table 4-5), but not of the magnitude of that detected by Parker *et al* (1989). Whole platelet homogenates may not be a sensitive enough preparation to detect mild decreases in complex I activity; data presented in Chapter 3 supports this. However, the preparation of enriched mitochondria should be sensitive enough to detect deficiencies of the magnitude reported by Parker *et al* (1989).

There is no obvious explanation as to why nitrogen bomb homogenisation of platelets resulted in the loss of complex I activity when the assay of Ragan *et al* (1987) was employed (Table 4-1), but not when the assay was modified to optimise activity in platelet mitochondrial fractions (Table 4-3). Since complex I activity in PD and control platelet MEFs was measured using the modified assay, this question was not pursued further.

Besides the original paper of Parker *et al* (1989) and the work presented in this thesis, two other papers have described studies of mitochondrial function in PD platelet mitochondrial fractions. Bravi *et al* (1992) measured the activities of complexes I (as rotenone sensitive NADH cytochrome c reductase), II/III and IV in 17 PD patients and 13 age-matched controls. Although the mean activity of complex I was 32% decreased in the patient group, this result was not statistically significant, possibly due to the large spread of data points in both patient and control groups. More recently, Benecke *et al* (1993) have detected substantial deficiencies of complex I and complex IV in PD patients platelet mitochondrial fractions. The mean activity of complex I was 50% decreased (14 versus 29 nmol/min/mg protein) and that of complex IV was 30% decreased (58 versus 83 nmol/min/mg protein) in 27 PD patients versus 44 controls. The activity of complex III was similar in the two groups. Additionally they reported that the mean complex I activity was normal in platelet mitochondrial fractions from eight patients with multiple system atrophy.

The data presented in this thesis does not agree with that of Parker *et al* (1989) or Benecke *et al* (1993) in terms of the magnitude of the complex I defect in PD platelet mitochondrial fractions. These discrepancies may not simply be due to differences in mitochondrial purity arising from the different preparations used, as the mean specific activity of complex I in the control samples of Parker *et al* (1989) and Benecke *et al* (1993) were similar to those of the control sample data in this study (19.1, 29 and 19.3 nmol/min/mg protein respectively).

However since complex I assay conditions were different in each study, direct comparisons of absolute activities between the studies may not be appropriate. Since different mitochondrial preparations were used in each study, it is possible that the degree of mitochondrial purification was different for each method. To verify this, complex I activity in samples of mitochondrial fraction from each method would have to be assayed under identical conditions.

A further explanation for the different results in each study is that different patient groups were used; Figure 4-7 shows a significant overlap in complex I activity in patient and control groups in this study, and Benecke *et al* (1993) report that complex I activity in 30% of their PD patients fell within the control range (Parker *et al* (1989) did not publish individual control enzyme activities). If PD patients are a heterogeneous group, with some exhibiting severe platelet complex I defects while others have normal platelet complex I activity, then the mean complex I activity in a group of PD patients will largely depend on the individual patients included. Furthermore, since the clinical diagnosis of PD during life is only correct in 80% of cases (based on post-mortem pathology; Hughes *et al*, 1992), all of these studies are likely to include patients that do not have idiopathic PD; if different clinicians use different diagnostic criteria for PD, then the number of misdiagnosed cases may vary between studies.

Although the activity of complex II/III was normal in PD platelet MEFs in this study, the presence of a slight defect of complex III cannot be ruled out. The flow of electrons through complex II has been shown to be the rate-limiting step in the combined complex II/III assay, antimycin A sensitive succinate cytochrome c reductase (Taylor *et al*, 1993). Therefore slight complex III defects may not be detected by the assay, although Benecke *et al* (1993) demonstrated normal platelet complex III activity in the PD patients they studied.

The data presented in this thesis does not support the view that the complex I deficiency in PD platelet mitochondria may be used as a diagnostic test for the disease. The defect detected was slight, and there was almost total overlap of enzyme activities in the two groups (Figure 4-7, 4-8). The majority of the patient complex I data points fell towards the bottom end of the control range, but within it, and it was this skewing of data points that accounted for the differences in mean activities. Another reason why this complex I deficiency cannot be used as a diagnostic test for PD is that it is not disease specific. Complex I defects have also been detected in the platelets of patients with Huntington's disease (HD), dystonia and Leber's Hereditary Optic Neuropathy (LHON). Parker *et al* (1990a) have reported mean complex I activity to be 72%

decreased in HD patients platelets; Benecke *et al* (1992) reported 37% and 62% decreases in mean complex I activity in focal and segmental/generalised dystonia patients platelets respectively, and complex I defects in LHON patients platelets of 25 or 70% depending on the mtDNA mutation they carry have been reported (Howell *et al*, 1991; Smith *et al*, 1994)

It has been shown (Table 4-6) that a systemic complex I defect in a patient with mitochondrial myopathy can be detected in platelet MEFs. Patient JH had a skeletal muscle complex I activity 3% of age-matched control mean, and a platelet MEF complex I activity 51% of control mean. Therefore if there is a systemic complex I defect in PD patients, this should be detectable in platelet MEFs. However a less severe (26% of age-matched control mean) skeletal muscle complex I defect in the mitochondrial myopathy patient VA was not detectable in the platelet MEF. The detection of a skeletal muscle complex I defect in platelet MEFs may therefore depend upon the severity of the complex I defect.

It seems unlikely that the observed complex I defect in the platelets of PD patients has any pathological significance; PD patients have no apparent clotting abnormalities and their platelets appear to be unaffected. Whether or not a mild defect in complex I activity could lead to mitochondrial dysfunction severe enough to adversely affect platelet aggregation, is uncertain. Patients with LHON carrying the mtDNA nt3460 mutation have a 70% decrease in complex I activity in platelets (Howell *et al*, 1991; Smith *et al*, 1994), and yet the tissue appears unaffected. Rather, it may be that the platelet complex I defect in PD is indicative of the underlying cause of the defect in the substantia nigra, which is exacerbated by other factors.

As in the substantia nigra (Schapira *et al*, 1990b) and skeletal muscle (Mann *et al*, 1992a) of patients with PD, platelet mitochondrial complex I activity was not affected by L-dopa treatment. It has been suggested (see Introduction 1.6.3.1.) that L-dopa may be toxic due to the production of free radicals during its oxidation. Przedborski *et al* (1993) have shown that L-dopa and dopamine both specifically inhibit complex I *in vitro*, in rat brain mitochondria, and *in vivo*, in the striatum and substantia nigra of rats treated chronically with the compounds. Although the complex I inhibition *in vitro* was prevented by the addition of free radical scavengers, it was also removed by washing the mitochondria. Furthermore the inhibitory effects *in vivo* were fully reversible, disappearing seven days after the last injection. The chronic treatment of rats with L-dopa has been shown to have no effect on skeletal muscle mitochondrial complex I

activity (Dagani *et al*, 1991), in agreement with Mann *et al* (1992a) who saw no difference in complex I activity between three L-dopa treated and five untreated PD patients skeletal muscle. Furthermore there is evidence that the observed complex I deficiency in the PD substantia nigra is not due to L-dopa treatment (see Introduction 1.4.2.1.).

Why the complex I defect which is anatomically specific in the PD brain for the pathologically affected area, the substantia nigra (Schapira *et al*, 1990b), is also present in platelets is unknown. Two hypotheses, involving toxic and genetic mechanisms are discussed;

a. Toxic.

Although in the brain MPP^+ is specifically accumulated by the dopaminergic neurons of the substantia nigra, Cesura *et al* (1987) demonstrated that human platelets also actively accumulate [^{3}H]-MPP^+. They provided evidence that the MPP^+ uptake process is energy requiring and is via the serotonin uptake mechanism; accumulation was inhibited by metabolic poisons (KCN and ouabain), and by specific serotonin uptake inhibitors (cianopramine and paroxetine). Furthermore, platelets contain high levels of monoamine oxidase-B (MAO-B) (Da Prada *et al*, 1985) which converts MPTP to MPP^+. Inhibition of MAO-B in platelets incubated with [^{3}H]MPTP stops the accumulation of radioactive label; MPTP, which can enter platelets by passive diffusion, is converted to MPP^+ and thereby trapped within cells.

However, at low concentrations (50nM) [^{3}H]-MPP^+ does not accumulate in platelet mitochondria but in serotonin granules (Cesura *et al*, 1987). This vesicular uptake may protect other platelet subcellular structures from the toxic effects of MPP^+, as has been proposed for bovine adrenomedullary chromaffin cells (Reinhard *et al*, 1987). Indeed, no ultrastructural damage was observed in human platelets incubated with 5μM MPTP (Da Prada *et al*, 1985), or in the platelets of rats injected intravenously with 10mg/kg MPP^+ twice daily for four days (Da Prada *et al*, 1988). It has been suggested that MPP^+ may only be toxic in cells in which its concentration is so great that some leaks out of vesicles and becomes available for mitochondrial accumulation (Tipton and Singer, 1993).

If the observed complex I defect in PD is due to inhibition of the enzyme by an MPTP-like toxin, with similar uptake and conversion characteristics, then this may explain the anatomic specificity of its effect in the substantia nigra **and** platelets. Another possible explanation for this anatomic selectivity of the complex I defect is a genetic one.

b. Genetic.

Of the forty or so complex I subunits, seven are encoded by mitochondrial DNA (mtDNA) (Chomyn *et al*, 1985,1986) and the remainder by nuclear DNA. MtDNA segregates randomly during cell replication, which can give rise to the phenomenon of heteroplasmy in mitochondrial diseases whereby different tissues contain heterogeneous populations of mtDNA. Therefore, an individual may contain very high levels of mutant mtDNA in some tissues and 100% normal mtDNA in others. Heteroplasmy, along with the fact that the effect of the level of mutant mtDNA in any particular tissue may depend on that tissues dependence on oxidative phosphorylation, are two factors which possibly explain the tissue specificity of many mitochondrial diseases.

One explanation of the tissue specificity of the complex I defect in PD for the substantia nigra and platelets is that the both the substantia nigra and bone marrow megakaryocytes contain high levels of mtDNA carrying mutations in one or several complex I genes. This theory assumes that the level of mutant mtDNA in, for example, regions of the PD brain besides the substantia nigra are lower than those in the substantia nigra. However, there is no evidence for variation in mtDNA mutation levels in discrete brain areas (Macmillan *et al*, 1993; Tanno *et al*, 1993, who quantitated the levels of $tRNA^{Lys[8344]}$ and $tRNA^{Leu[3243]}$ mutations respectively). Furthermore, the levels of mtDNA carrying the "common" deletion in the PD substantia nigra are no higher than those in the aged brain (Mann *et al*, 1992b; DiDonato *et al*, 1993). Sandy *et al* (1993) have detected low levels of deleted mtDNA in the platelets of PD patients, but similar levels were also present in the platelet mtDNA of aged controls. These results do not however rule out the possibility that point mutations or small deletions may be present in the complex I genes of mtDNA in PD patients.

Tissue specific isoforms of complex IV are well documented (Capaldi, 1990) and one study has suggested that tissue specific isoforms of complex I may also exist (Clay and Ragan, 1988). Therefore it is possible that tissue specific expression of nuclear complex I genes may explain the anatomic specificity of the observed complex I defect, although again there is no evidence for this.

Another possible explanation as to why the complex I defect should be present in the substantia nigra and platelets is that it is in fact systemic, present throughout all tissues of the body at low levels (as observed in platelets), but is amplified in the substantia nigra by other factors specific to that tissue. The presence of a complex I defect in PD skeletal muscle is controversial (see Introduction 1.4.2); four studies have detected reduced activity of complex I,

and other respiratory chain complexes, in PD skeletal muscle mitochondria (Bindoff *et al*, 1991; Shoffner *et al*, 1991; Nakagawa-Hattori *et al*, 1992; Cardellach *et al*, 1993). However, three studies have observed similar complex I activity in PD and control skeletal muscle mitochondria (Mann *et al*, 1992a; Anderson *et al*, 1993; DiDonato *et al*, 1993).

Besides the substantia nigra, six other brain regions have been analysed for MRC enzyme activity (Schapira *et al*, 1990b; Mann *et al*, 1992a). In four regions, caudate nucleus, cerebral cortex, globus pallidus (medial) and globus pallidus (lateral), five control and four PD samples were analysed. Complex I activity was not significantly different in any of these regions, and the mean CS corrected activity was actually higher in PD cases in all four regions. In the posterior putamen twelve samples were analysed and the mean CS corrected complex I activity was 15% increased in the PD samples (Dr JM Cooper, personal communication); in the tegmentum ten samples were analysed and the mean CS corrected complex I activity was 15% decreased in PD cases; and in the cerebellum sixteen samples were analysed and mean CS corrected complex I activity was 9% decreased in PD cases.

Although it is possible that the analysis of an increased number of samples of tegmentum and cerebellum may result in a statistically significant decrease in complex I activity in the PD samples, there is not a consistent decrease in complex I activity in PD brain areas besides the substantia nigra. This argues against the complex I deficiency in PD being systemic. The use of tissue homogenates may not be suitable to detect small differences in complex I activity between groups; this was the case with platelets in that although PD platelet mitochondrially-enriched fractions revealed a complex I deficiency, this was not detected in platelet homogenates (see Chapter 3). However, the small tissue samples available from brain regions (approximately 100-200mg wet weight) are not sufficient for the preparation of mitochondria.

Chapter 5. Studies on the structure of complex I in the PD substantia nigra by immunoprecipitation

5.1. Aim

To optimise conditions for immunoprecipitation of complex I from purified mitochondria from a range of tissues of human, bovine and rat sources. To identify any species-specific or tissue-specific differences in complex I polypeptides by SDS-PAGE (SDS-denaturing polyacrylamide gel electrophoresis). To purify complex I from PD and control substantia nigra brain homogenates by immunoprecipitation and to compare polypeptide profiles by SDS-PAGE.

5.2. Introduction

The underlying cause of the complex I deficiency in the PD substantia nigra is unknown. If the defect is a primary event, it may be caused by toxic inhibition of the enzyme, or by an abnormality of the protein complex itself. One approach to determining the structure of complex I in the PD substantia nigra is using complex I antibodies for Western blotting, immunohistochemistry or ELISA (enzyme-linked immunosorbent assay). Results of previous studies to determine the composition of complex I in the PD substantia nigra by Western blotting and immunohistochemistry have been contradictory (see Introduction 1.4.2.1). In this study, complex I was purified by immunoprecipitation from PD and control substantia nigra tissue homogenate samples and visualised directly by silver stained SDS-PAGE.

Complex I had only previously been immunoprecipitated from purified mitochondria (Cleeter and Ragan, 1985; Moreadith *et al*, 1987; Clay and Ragan, 1988 and others), and therefore the reaction conditions were initially optimised using purified bovine heart mitochondria. The polypeptide profiles of immunoprecipitated complex I were visualised by silver stained SDS-PAGE, and Western blotting with complex I antibodies was used as evidence that the precipitated material was complex I. Complex I was immunoprecipitated from human heart, skeletal muscle, kidney, cortex and cerebellum mitochondria;

tissue-specific differences in human complex I may help to explain the tissue specificity of the complex I defect observed in PD (Mann *et al*, 1992a). Following from a previous report of tissue specific differences in rat complex I (Clay and Ragan, 1988), the complex was immunoprecipitated from rat liver and skeletal muscle mitochondria and the polypeptide profiles compared by silver stained SDS-PAGE.

The conditions for immunoprecipitation of complex I from brain homogenate samples were initially optimised using control caudate nucleus tissue homogenate samples. Complex I was immunoprecipitated from a number of caudate nucleus homogenate samples to ensure that the technique was reproducible between samples. Complex I was subsequently immunoprecipitated from substantia nigra homogenates of three PD and three control brains, and silver stained SDS-PAGE profiles were compared visually and with the use of a computerised image analysis system. Western blotting with affinity-purified subunit specific complex I antibodies was used as evidence that the immunoprecipitated material was complex I.

5.3. Results

5.3.1. Optimisation of conditions for immunoprecipitation of complex I from purified mitochondria

5.3.1.1. Solubilisation of complex I

The conditions for immunoprecipitation of complex I from purified mitochondria were optimised using bovine heart mitochondria (BHM), as the complex I antibodies available were raised against bovine heart complex I. Because the activity of rotenone-sensitive NADH CoQ_1 reductase (the defining activity of mitochondrial complex I) was known to be sensitive to the presence of detergents (Smith and Ragan, 1980), NADH ferricyanide reductase (NFR; an activity associated with the flavoprotein (FP) fraction of complex I) activity was used as a measure of the proportion of complex I solubilised in the presence of detergents.

BHM were solubilised in a range of detergents, at a range of concentrations, to determine which would solubilise complex I most efficiently (Table 5-1). The eight detergents tested were N-dodecyl-ß-D-maltoside, decyl-ß-D-maltoside, octyl ß-D-glucopyranoside, octyl ß-D-thioglucopyranoside and Triton X-100 (all non-ionic detergents), sodium deoxycholate (anionic) and CHAPS (3-[(3-choloamidopropyl)-dimethylammonio]-1-propane-sulfonate) and Zwittergent 3-14 (Zwitterionic). To calculate the optimal concentration of detergent required for solubilisation of the complex, BHM at 1mg protein/ml was mixed with a range of concentrations of each detergent (0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 1.0%). The proportion of complex I solubilised was calculated as the proportion of NFR activity remaining in the supernatant after a centrifugation step (at 47000 x g_{max} for 30mins) to remove insoluble material. The concentration of detergent required to maximally solubilise complex I ranged from 0.05-0.5% depending on the detergent, and the proportion of NFR activity solubilised ranged from 14-99% (Table 5-1). Detergents A, B, E and H solubilised greater than 85% NFR activity, whilst detergents C, D, F and G solubilised less than 50% NFR activity.

5.3.1.2. Effect of the solubilising detergent on complex I activity

Proof that the material immunoprecipitated by complex I antibodies was indeed complex I, would be that it exhibited rotenone-sensitive NADH CoQ_1 reductase activity (the defining activity of mitochondrial complex I). The activity of rotenone-sensitive NADH CoQ_1 reductase was assayed in the presence of each detergent at the concentration at which it solubilised the maximum proportion of NFR activity, and compared to that in the absence of detergent, as a measure of the inhibition of its activity in the presence of detergent. Greater than 80% of the activity of rotenone-sensitive NADH CoQ_1 reductase was inhibited by the optimal solubilising concentration of detergent for seven of the eight detergents tested (Table 5-1). Although detergent G only inhibited 42% of the rotenone-sensitive NADH CoQ_1 reductase activity, it only maximally solubilised 50% of the NFR activity (Table 5-1); since only very limited substantia nigra tissue samples were available for subsequent immunoprecipitation studies, the proportion of complex I immunoprecipitated had to be maximal, making this detergent unsuitable. None of the detergents tested therefore solubilised the majority of NFR activity without inhibiting rotenone-sensitive NADH CoQ_1 reductase activity. Of the four detergents that solubilised almost all of the NFR activity (A, B, E and H), detergent A (N-dodecyl-ß-D-maltoside) at 0.2% was

chosen for use in future experiments, as it had previously been shown to retain all subunits of the complex detectable by SDS-PAGE during solubilisation (Haines *et al*, 1992).

5.3.1.3. Titration of antibody with antigen

To calculate the amount of antibody required to immunoprecipitate complex I from 1mg protein of BHM and human heart mitochondria (HHM), a range of volumes (0.05-1ml) of complex I holoenzyme antiserum were incubated with mitochondria solubilised with 0.2% DDM (see Methods 2.7.1 for production of complex I antiserum). The proportion of complex I immunoprecipitated was calculated as the percentage of NFR activity lost from the supernatant to the pellet after the antibody:antigen complex had been pelleted by centrifugation (47000 x g_{max} for 30mins). The addition of complex I holoenzyme antiserum to BHM or HHM solubilised with 0.2% DDM and incubated overnight at 4°C did not inhibit NFR activity (Table 5-2).

Approximately 80% of BHM NFR activity was precipitated by 0.5ml of complex I holoenzyme antiserum, with a slight inhibition of the reaction at higher volumes of serum (Figure 5-1). It has been shown that both bovine and human mitochondria contain an NFR activity not associated with complex I (Moreadith *et al*, 1987), which is not precipitated by complex I antibodies. Control serum (pre-immune) as expected, did not precipitate any NFR activity (Figure 5-1), providing evidence that the complex I antiserum was able to immunoprecipitate complex I from solubilised mitochondria because of a specific reaction with the complex. NFR activity was also precipitated from HHM, demonstrating that the antibody, which was raised against highly purified bovine complex I, cross reacts with human complex I (Figure 5-1). The results were similar in HHM and BHM, except that the amount of NFR activity precipitated was less in HHM (60% by 0.5ml serum). The addition of greater volumes of serum did not precipitate any more NFR activity from HHM (Figure 5-1). The proportion of NFR activity precipitated from mitochondria of other human tissues (skeletal muscle, kidney, cortex and cerebellum) ranged from 41-71% (Table 5-3).

5.3.2. Immunoprecipitation of complex I from purified mitochondria

5.3.2.1. Polypeptide profile of bovine and human immunoprecipitated complex I

Complex I was purified by immunoprecipitation from 1mg of BHM and human heart, skeletal muscle, kidney, cortex and cerebellum mitochondria (see Methods 2.8.3). The BHM, human heart and human kidney mitochondria were provided by Dr JM Cooper (Royal Free Hospital School of Medicine, London, UK). Skeletal muscle and cerebellum mitochondria were prepared post-mortem from a 78 year old male, and cortex mitochondria were prepared from a 38 year old male (see Methods 2.8.1, 2.8.2).

Based on the amount of sample required for equal staining intensities on acrylamide gels, the amount of complex I immunoprecipitated from BHM was approximately four times that immunoprecipitated from human mitochondria. The polypeptide profile of complex I immunoprecipitated from beef heart mitochondria (BHM) was almost identical to that of purified bovine heart complex I (Figure 5-2). The only differences were the presence of immunoglobulin heavy and light chains and two extra polypeptides of approximately 48kDa in the immunoprecipitated samples. The heavy chains of immunoglobulin appeared above and below the 75kDa subunit of complex I and the light chains appeared diffuse, creating hazy staining in the 20 to 30kDa range of the gel. The two polypeptides of approximately 48kDa, visible just below the 49kDa subunit of complex I, were shown by Western blotting with complex III antiserum to be core I and II of complex III (See Results 5.3.2.2). Since all polypeptides visible in purified bovine heart complex I were also present in the immunoprecipitate from BHM, this provided good evidence that the immunoprecipitated material was indeed complex I. However, purified bovine heart complex I was itself used as the antigen to produce the immunoprecipitating serum. Therefore any impurities present in this antigen are likely to be immunoprecipitated by its antiserum. This problem could potentially be overcome by using antiserum to single complex I subunits to immunoprecipitate complex I. However, none of the subunit specific antibodies tested (raised against the 75, 51, 49 and 39kDa subunits of complex I) immunoprecipitated complex I from BHM (Table 5-4) (complex I subunit specific antibodies, prepared by elution from denaturing polyacrylamide gels of purified bovine heart complex I, were provided by Dr JM Cooper, Royal Free Hospital School of Medicine, London, UK). Nevertheless, the complex I holoenzyme antiserum used to immunoprecipitate complex I reacted with approximately 10

major polypeptides in purified bovine complex I (Figure 5-3), yet immunoprecipitated greater than 20 polypeptides from BHM visible by silver stained SDS-PAGE (Figure 5-2). This provided evidence that the immunoprecipitated material was associated in a complex.

Visual examination of silver stained SDS-PAGE gels of complex I immunoprecipitated from various human tissues revealed no tissue-specific differences in polypeptide profiles (Figure 5-2). However, differences between human and bovine complex I were detectable; a subunit of 39kDa in bovine complex I had a lower molecular weight in human complex I (Figure 5-2). Western blotting using an antiserum to the 39kDa bovine complex I subunit identified these as the same subunit (see Results 5.3.2.2). Western blotting with complex I subunit specific antibodies was used to detect other differences and to positively identify subunits of bovine and human complex I (see Results 5.3.2.2).

5.3.2.2. Western blotting of bovine and human immunoprecipitated complex I

To provide further evidence that the immunoprecipitates were indeed complex I, and to determine any species-specific or tissue-specific differences in bovine and human complex I, samples from the above tissues were separated by SDS-PAGE on 20% Phastgels, transferred to nitrocellulose membranes and probed with antibodies raised against specific complex I subunits and subfractions (provided by Dr JM Cooper, Royal Free Hospital School of Medicine, London, UK). The antibody reactivities against purified bovine heart complex I are shown in lane 2 of the Western blots in Figure 5-4, and their reactivities against the immunoprecipitates from bovine and human mitochondria in lanes 3-8.

Antibody 75 reacted with a single subunit of 75kDa in all samples. Antibody 49 reacted with a subunit of 49kDa in all samples and cross reacted with several other subunits less strongly. Antibody 39 recognised a complex I subunit of 39kDa in the bovine samples. The antibody reacted weakly in the human samples with a subunit of lower molecular weight than the bovine subunit. This antibody also cross-reacted with a subunit of approximately 15kDa. Antibody FP, raised against the flavoprotein fraction of bovine complex I, which includes the 51, 24 and 10kDa subunits, reacted with the 51 and 24kDa subunits in all samples. Antibody IP, raised against the iron protein fraction of bovine complex I, which includes the 75, 49, 30, 18, 15 and 13kDa subunits, predominantly

reacted with three subunits of 49, 30 and 13kDa in the bovine samples. In the human samples the antibody reacted with a subunit of 49kDa, but reacted with a subunit of lower molecular weight than the bovine 30kDa subunit, and a subunit of higher molecular weight than the bovine 13kDa subunit. The observation that the 30 and 13kDa subunits of human complex I have lower and higher molecular weights respectively than the bovine complex, has been made by others (Cleeter and Ragan, 1985).

Western blotting with antibodies raised against subunits I and II (the core proteins) of complex III, identified the presence of these subunits in immunoprecipitated complex I. These subunits, of 47 and 45kDa, were visible in immunoprecipitated complex I samples on silver stained SDS-PAGE gels just below the 51 and 49kDa complex I subunits (Figure 5-2). The complex III antiserum reacts specifically with the cores of complex III and does not cross-react with any complex I subunits (Dr JM Cooper, personal communication; demonstrated by Western blotting of mitochondrial proteins separated according to the method of Schagger and von Jagow, 1991). This antiserum also detected the core proteins of complex III in purified heart bovine complex I by Western blotting (Figure 5-4). Since purified bovine heart complex I contains the core subunits of complex III and is used as the antigen to raise complex I antibodies, complex I antiserum is likely to contain antibodies to these complex III subunits. Therefore complex I immunoprecipitated by complex I antiserum is likely to be contaminated with the complex III core subunits.

5.3.2.3. Immunoprecipitation of complex I from purified rat mitochondria

Complex I was immunoprecipitated from rat skeletal muscle mitochondria (prepared as described in Methods 2.8.2) and rat kidney mitochondria (provided by Dr GM Gibb, Royal Free Hospital School of Medicine, London, UK). The immunoprecipitates were electrophoresed on Phastgels, which were silver stained to detect any tissue-specific subunit differences (Figure 5-5). Based on the amount of sample required for similar staining intensities on polyacrylamide gels, the amount of complex I immunoprecipitated from rat mitochondria was similar to that immunoprecipitated from human mitochondria. No subunit differences were detectable between rat skeletal muscle and kidney complex I.

5.3.3. Optimisation of conditions for immunoprecipitation of complex I from caudate nucleus brain homogenate

5.3.3.1. Solubilisation of complex I

Based on the results obtained in purified mitochondria (see Results 5.3.1.1), the detergent used to solubilise complex I in brain tissue homogenate samples was N-dodecyl-ß-D-maltoside (DDM). To determine the optimum concentration of DDM for solubilisation, control caudate nucleus (CN) brain homogenate was incubated with a range of detergent concentrations. At 10mg protein/ml 1% DDM maximally solubilised approximately 70% NFR activity in CN homogenate; increasing DDM concentrations did not increase the proportion of NFR activity solubilised (Figure 5-6). Therefore in future experiments, 10mg brain homogenate protein/ml was solubilised with 1% DDM.

5.3.3.2. Titration of antibody with antigen

Maximum immunoprecipitation of complex I from 1mg protein of caudate nucleus brain homogenate required 0.5ml of complex I antiserum (Figure 5-7), which immunoprecipitated approximately 25% NFR activity. This figure could not be increased by using more antiserum (Figure 5-7).

5.3.3.3. Immunoprecipitation of complex I from caudate nucleus homogenate

To analyse the reproducibility of the immunoprecipitation reaction between samples, complex I was immunoprecipitated from five control caudate nucleus samples (see Methods 2.8.4). The proportion of NFR activity immunoprecipitated in each sample was very similar, ranging from 25-34%. Visually, the polypeptide profile of immunoprecipitated complex I by silver stained SDS-PAGE was similar in each sample (Figure 5-8); each had the same number of visible subunits at the same molecular weights. Using a computerised image analysis system (see Methods 2.8.5), the absorbance of two pairs of subunits in each sample were compared as internal controls, to correct for any differences in protein loading on the gel (Table 5-5). In complex I immunoprecipitated from control caudate nucleus homogenate samples, the

ratios of the two subunit pairs (51:49kDa and 75:39kDa) ranged from 1.01-1.28 and 0.66-0.98 respectively. In purified bovine heart complex I and complex I immunoprecipitated from human heart mitochondria, the ratios were 1.00 and 1.28, and 1.01 and 1.17 respectively (Table 5-5).

5.3.4. Immunoprecipitation of complex I from PD and control substantia nigra homogenate

5.3.4.1. Polypeptide profile of complex I immunoprecipitated from PD and control substantia nigra homogenate

Figure 5-9 shows the silver stained SDS-PAGE mini-gel of complex I immunoprecipitated from three PD and three control substantia nigra homogenates. No differences in subunit molecular weight or staining intensity between samples were visible by eye. Therefore the gel was analysed using a Kontron VIDAS image analysis system (see Methods 2.8.5). The system detected 25 subunits in purified bovine heart complex I and 18 subunits in the immunoprecipitated samples. To avoid differences in the absolute amount of complex I loaded onto the gel from different samples (due to possible differences in the efficiency of the immunoprecipitation reaction), the absorbances of subunits were compared as ratios to one another. Tables 5-6 and 5-7 show the mean absorbances in PD and control samples of every subunit as a ratio of the absorbance of subunits 1 and 3 respectively. Subunit 1 is stained dark orange and has a molecular weight of approximately 51kDa, and subunit 3 is stained dark orange and has a molecular weight of approximately 42kDa (see Figure 5-9). The major difference between the PD and control samples was in the absorbances of bands 6 and 7. Compared to the absorbance of subunit 1, the mean absorbance of band 6 (30.8kDa) was decreased by 77% in the PD samples, and the mean absorbance of band 7 (24.8kDa) was decreased by 81% in the PD samples (Table 5-6). Compared to the absorbance of subunit 3, the mean absorbance of subunits 6 and 7 were 80 and 89% respectively decreased in PD samples (Table 5-7). Both bands have corresponding subunits in purified complex I (Figure 5-9), and are therefore likely to be complex I subunits. The absorbances of other bands were similar in both PD and control samples (Table 5-6, 5-7).

5.3.4.2. Western blotting of complex I immunoprecipitated from control substantia nigra homogenate

Western blots of complex I immunoprecipitated from control substantia nigra homogenate and probed with subunit specific complex I antibodies are shown in Figure 5-10. The antibodies were affinity purified against purified bovine heart complex I by column chromatography (see Methods 2.8.3) prior to use, to remove any cross reactivities to other proteins present in crude brain homogenate. All the antibodies reacted with bands of expected molecular weight in the immunoprecipitates, as well as a protein of approximately 100kDa, which is likely to be non-dissociated IgG (Figure 5-10).

5.3.4.3. Mitochondrial enzyme assays in substantia nigra homogenate

Complex I, measured as rotenone-sensitive NADH CoQ_1 reductase, and citrate synthase activities were measured in the substantia nigra homogenate samples used for immunoprecipitation. There were no significant differences in the complex I specific activity or the citrate synthase corrected complex I activity between the PD and control groups (Table 5-8).

Detergent	Detergent conc. (%)*	NFR activity solubilised (%)	NADH CoQ_1 reductase activity inhibition (%)
A	0.2	99	95
B	0.1	91	86
C	0.5	20	98
D	0.5	41	98
E	0.5	99	98
F	0.1	14	94
G	0.4	50	42
H	0.05	87	100

Table 5-1. The effect of various detergents on complex I solubilisation and activity in BHM.

* the detergent concentration shown is that at which the solubilisation of NFR activity was maximal.

NFR (NADH ferricyanide reductase); BHM (bovine heart mitochondria).

Detergents: A (N-dodecyl-ß-D-maltoside); B (decyl-ß-D-maltoside); C (octyl-ß-D-glucopyranoside); D (octyl-ß-D-thioglucopyranoside); E (Triton X-100); F (sodium deoxycholate); G (CHAPS; (3-[(3-choloamidopropyl)-dimethylammonio]-1-propane-sulfonate)); H (Zwittergent 3-14).

Volume of antiserum (ml)	NFR activity in BHM	NFR activity in HHM
0.00	4029	1764
0.25	4212	1821
0.50	3756	1619
0.75	3820	1836
1.00	4187	1739

Table 5-2. The effect of complex I holoenzyme antiserum on NFR activity in solubilised BHM and HHM.

NFR (NADH ferricyanide reductase); BHM (bovine heart mitochondria); HHM (human heart mitochondria).

NFR activity expressed as nmol/min/mg protein.

Source of mitochondria		% NFR activity immunoprecipitated
Bovine	heart	76
Human	heart	60
	skeletal muscle	71
	kidney	55
	cortex	41
	cerebellum	48

Table 5-3. The proportion of NFR activity immunoprecipitated from bovine and human mitochondria by complex I holoenzyme antiserum.

Mitochondria at 1mg protein/ml solubilised with 0.2% N-dodecyl-ß-D-maltoside; complex I immunoprecipitated with 0.5ml of complex I holoenzyme antiserum.

NFR (NADH ferricyanide reductase).

Complex I antiserum	% NFR activity immunoprecipitated
holoenzyme	76
75kDa subunit	0
51kDa subunit	3
49kDa subunit	2
39kDa subunit	5

Table 5-4. The proportion of NFR activity immunoprecipitated from BHM by complex I holoenzyme and subunit-specific antiserum.

Mitochondria at 1mg protein/ml solubilised with 0.2% N-dodecyl-ß-D-maltoside; complex I immunoprecipitated with 0.5ml of complex I antiserum.

NFR (NADH ferricyanide reductase).

Sample	Absorbance ratio 51:49	75:39
I	1.00	1.01
CN 1	1.11	0.86
CN 2	1.01	0.72
CN 3	1.15	0.98
CN 4	1.28	0.66
CN 5	1.17	0.91
HHM	1.28	1.17

Table 5-5. Ratio of absorbance of 51:49kDa and 75:39kDa subunits of complex I immunoprecipitated from control caudate nucleus homogenate samples.

I (purified bovine heart complex I); CN 1-5 (complex I immunoprecipitated from control caudate nucleus samples 1-5); HHM (complex I immunoprecipitated from human heart mitochondria).

Absorbance calculated by computerised image analysis system. For silver stained SDS-PAGE gel see Figure 5-8.

Band	MW(kDa)	PD	Control
2	49.0	0.743 ± 0.220	0.693 ± 0.220
3	42.0	1.980 ± 0.714	1.767 ± 1.116
4	38.1	0.520 ± 0.410	0.386 ± 0.283
5	34.3	0.270 ± 0.235	0.156 ± 0.117
6	30.8	0.117 ± 0.093	0.400 ± 0.347
7	24.8	0.083 ± 0.076	0.433 ± 0.184*
8	23.1	0.563 ± 0.479	0.240 ± 0.205
9	21.6	1.433 ± 0.831	1.165 ± 0.361
10	20.3	1.883 ± 0.726	1.530 ± 0.575
11	18.0	0.843 ± 0.459	0.943 ± 0.540
12	17.2	2.843 ± 2.088	2.347 ± 1.325
13	16.5	2.963 ± 2.035	2.263 ± 1.182
14	15.4	1.570 ± 0.697	1.476 ± 0.912
15	14.5	4.766 ± 2.973	3.706 ± 2.654
16	13.2	9.686 ± 5.280	8.063 ± 5.515
17	10.6	1.110 ± 0.785	0.770 ± 0.487
18	9.5	1.763 ± 1.253	1.450 ± 1.075

Table 5-6. Mean ratio to Band 1 of absorbance of bands in immunoprecipitated complex I from PD and control substantia nigra.

Data expressed as mean ± SD (n=3); * p=0.039 by Mann Whitney U-test.

MW (molecular weight calculated by image analysis system); PD (Parkinson's disease).

For silver stained SDS-PAGE gel see Figure 5-9.

Band	MW(kDa)	PD	Control
1	51.0	0.547 ± 0.169	0.713 ± 0.358
2	49.0	0.393 ± 0.146	0.437 ± 0.176
4	38.1	0.313 ± 0.260	0.337 ± 0.280
5	34.3	0.170 ± 0.147	0.140 ± 0.130
6	30.8	0.073 ± 0.063	0.367 ± 0.394
7	24.8	0.040 ± 0.036	0.350 ± 0.270
8	23.1	0.307 ± 0.298	0.220 ± 0.233
9	21.6	0.697 ± 0.170	0.585 ± 0.163
10	20.3	0.950 ± 0.052	0.953 ± 0.230
11	18.0	0.410 ± 0.069	0.540 ± 0.178
12	17.2	1.320 ± 0.504	1.367 ± 0.540
13	16.5	1.407 ± 0.485	1.333 ± 0.136
14	15.4	0.823 ± 0.415	0.843 ± 0.388
15	14.5	2.283 ± 0.601	2.010 ± 0.374
16	13.2	4.697 ± 0.835	4.433 ± 0.797
17	10.6	0.513 ± 0.214	0.433 ± 0.035
18	9.5	0.820 ± 0.288	0.773 ± 0.254

Table 5-7. Mean ratio to Band 3 of absorbance of bands in immunoprecipitated complex I from PD and control substantia nigra.

Data expressed as mean ± SD (n = 3); no statistically significant differences by Mann Whitney U-test.

MW (molecular weight calculated by image analysis system); PD (Parkinson's disease).

For silver stained SDS-PAGE gel see Figure 5-9.

	Complex I	Citrate Synthase	I/CS (x100)
PD (n=3)	5.19 ± 2.30	115.29 ± 20.37	4.41 ± 1.39
C (n=3)	5.13 ± 0.73	115.85 ± 19.23	4.47 ± 0.60

Table 5-8. Mitochondrial enzyme activities in PD and control substantia nigra homogenate.

Activities expressed as nmol/min/mg protein (mean ± SD).

Complex I (rotenone-sensitive NADH CoQ_1 reductase); I/CS (citrate synthase corrected ratio of complex I activity); PD (Parkinson's disease); C (control).

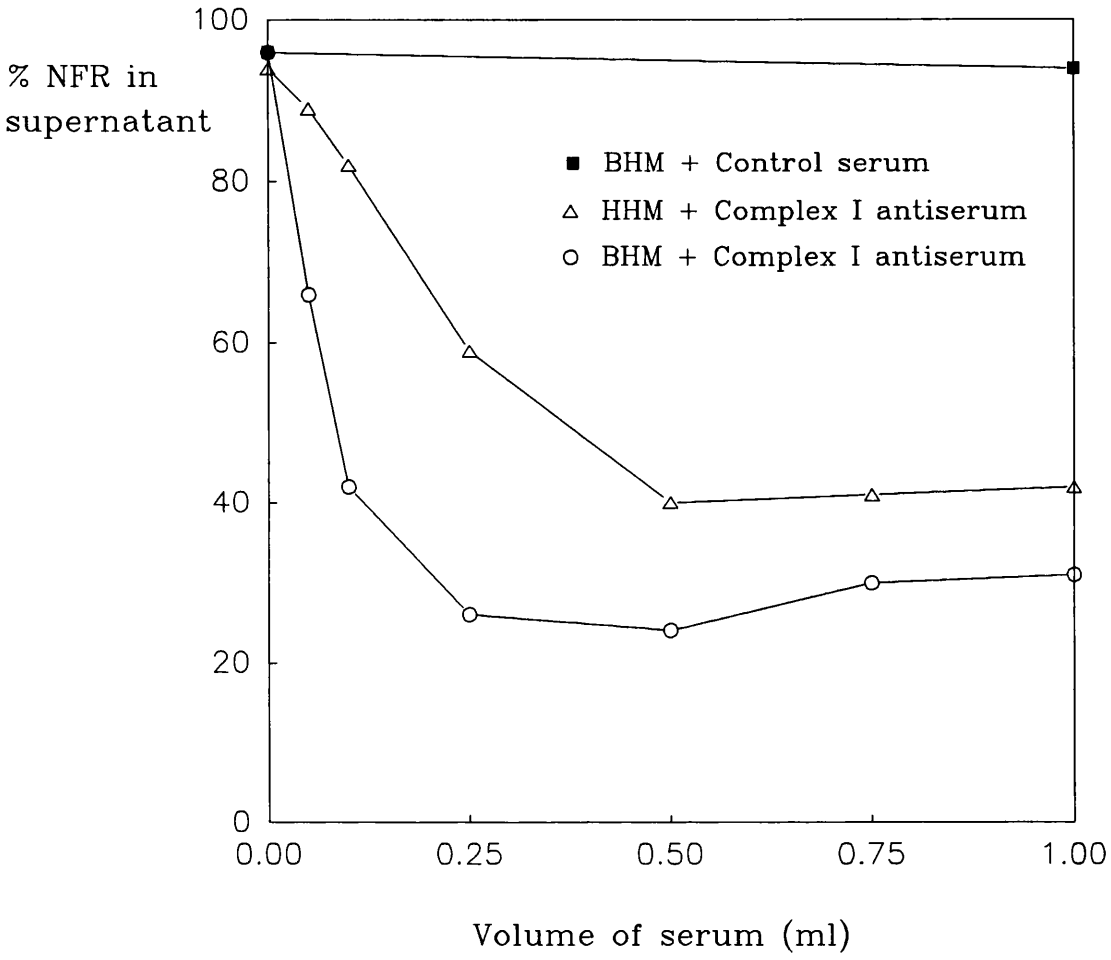


Figure 5-1. Titration of complex I holoenzyme antiserum with 1mg protein of BHM and HHM; effect on immunoprecipitation of NFR activity.

NFR (NADH ferricyanide reductase); BHM (bovine heart mitochondria); HHM (human heart mitochondria); % NFR in supernatant (% NFR activity remaining in supernatant after centrifugation at 47000 x g_{max} for 30mins; a measure of complex I not immunoprecipitated).

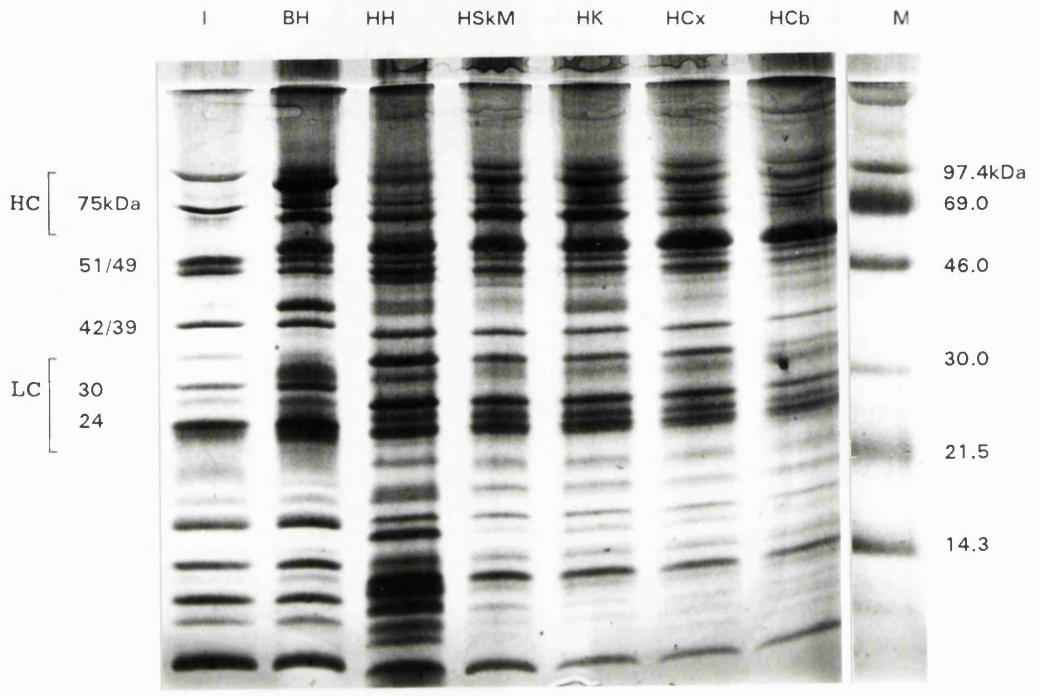


Figure 5-2. Silver stained SDS-PAGE Phastgel of complex I immunoprecipitated from bovine and human mitochondria.

M (molecular weight markers); I (purified bovine heart complex I); Complex I immunoprecipitated from BH (bovine heart mitochondria), HH, HSkM, HK, HCx, HCb (human heart, skeletal muscle, kidney, cortex, cerebellum mitochondria); HC/LC (immunoglobulin heavy/light chains).

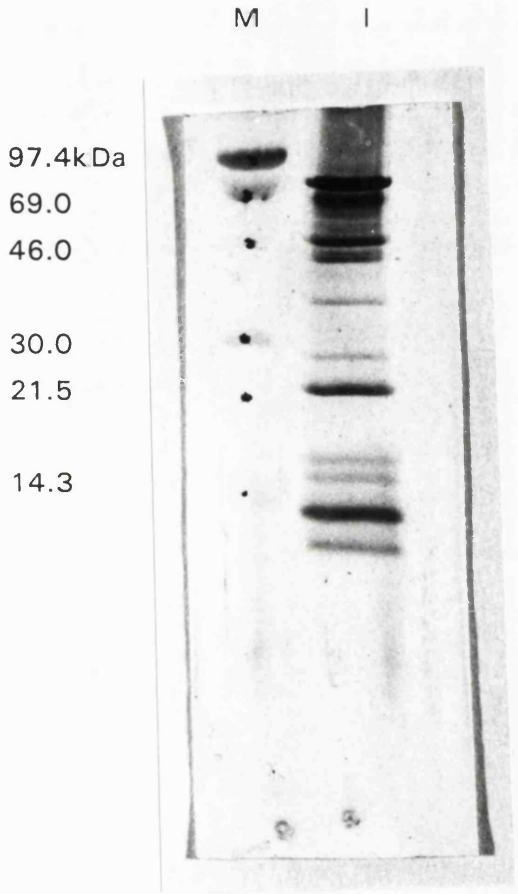


Figure 5-3. Western blot of purified bovine heart complex I probed with the complex I holoenzyme antiserum used for immunoprecipitation.

M (molecular weight markers); I (purified bovine heart complex I).

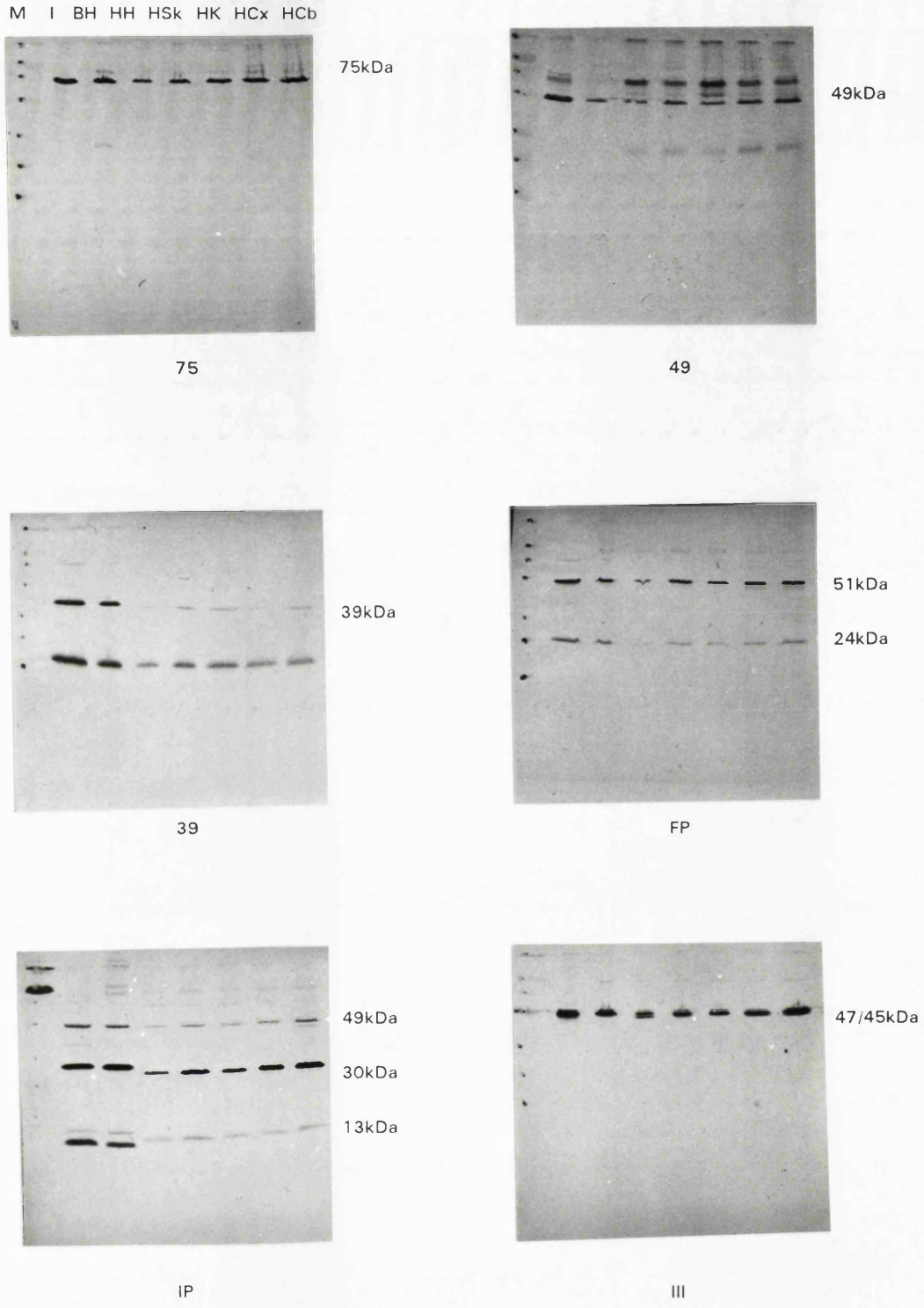


Figure 5-4. Western blots against complex I immunoprecipitated from bovine and human mitochondria.

M (molecular weight markers); I (purified bovine heart complex I); Complex I immunoprecipitated from BH (bovine heart mitochondria), HH, HSk, HK, HCx, HCb (human heart, skeletal muscle, kidney, cortex, cerebellum mitochondria).

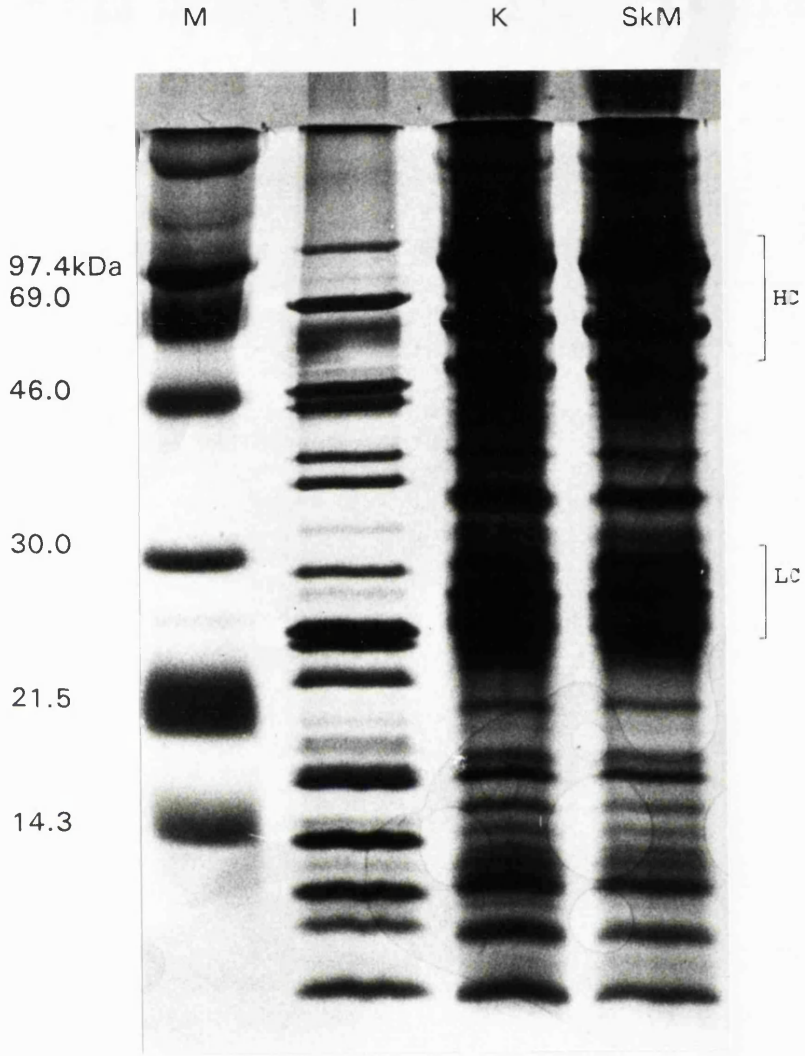


Figure 5-5. Silver stained SDS-PAGE Phastgel of complex I immunoprecipitated from rat kidney and skeletal muscle mitochondria.

M (molecular weight markers); I (purified bovine heart complex I); K, SkM (complex I immunoprecipitated from rat kidney and skeletal muscle mitochondria); HC/LC (immunoglobulin heavy/light chains).

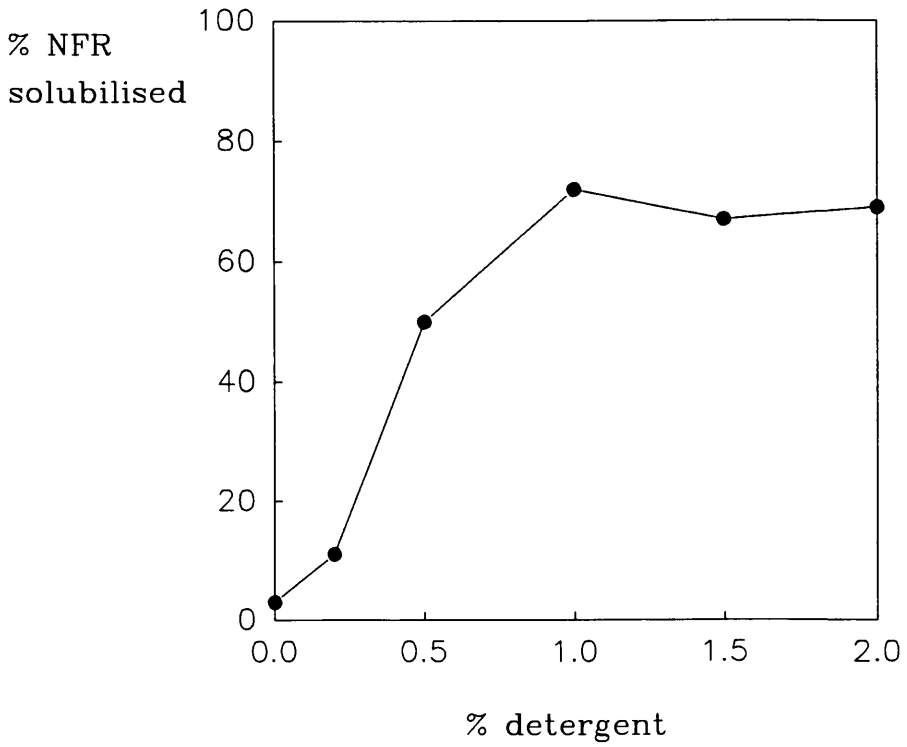


Figure 5-6. Titration of N-dodecyl-ß-D-maltoside with caudate nucleus brain homogenate; effect on the proportion of NFR solubilised.

NFR (NADH ferricyanide reductase); % NFR solubilised (% NFR activity remaining in the supernatant after centrifugation at 47000 x g_{max} for 30mins).

Caudate nucleus homogenate at 10mg protein/ml.

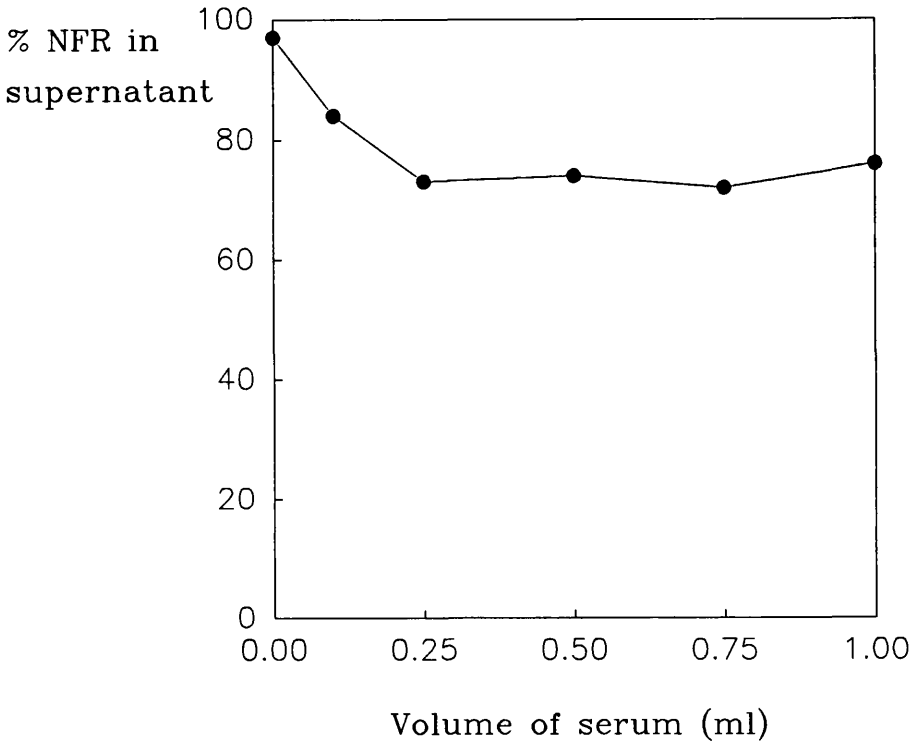


Figure 5-7. Titration of complex I holoenzyme antiserum with 1mg protein of caudate nucleus brain homogenate; effect on immunoprecipitation of NFR activity.

NFR (NADH ferricyanide reductase); % NFR in supernatant (% NFR activity remaining in supernatant after centrifugation at 47000 x g_{max} for 30mins; a measure of complex I not immunoprecipitated).

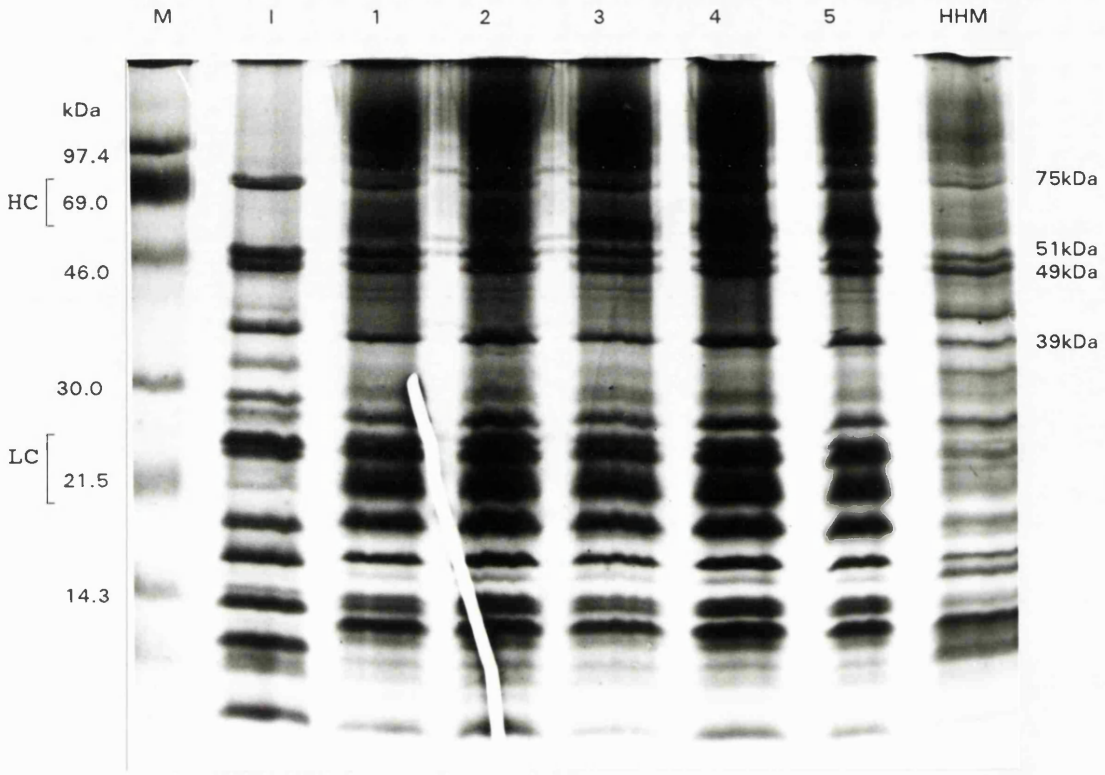


Figure 5-8. Silver stained SDS-PAGE Phastgel of complex I immunoprecipitated from five control caudate nucleus homogenate samples.

M (molecular weight markers); I (purified bovine heart complex I); 1-5 (complex I immunoprecipitated from control caudate nucleus homogenate samples 1-5); HHM (complex I immunoprecipitated from human heart mitochondria); HC/LC (immunoglobulin heavy/light chains).

The absorbance ratios of subunits 75:39kDa and 51:49kDa are compared in Table 5-5.

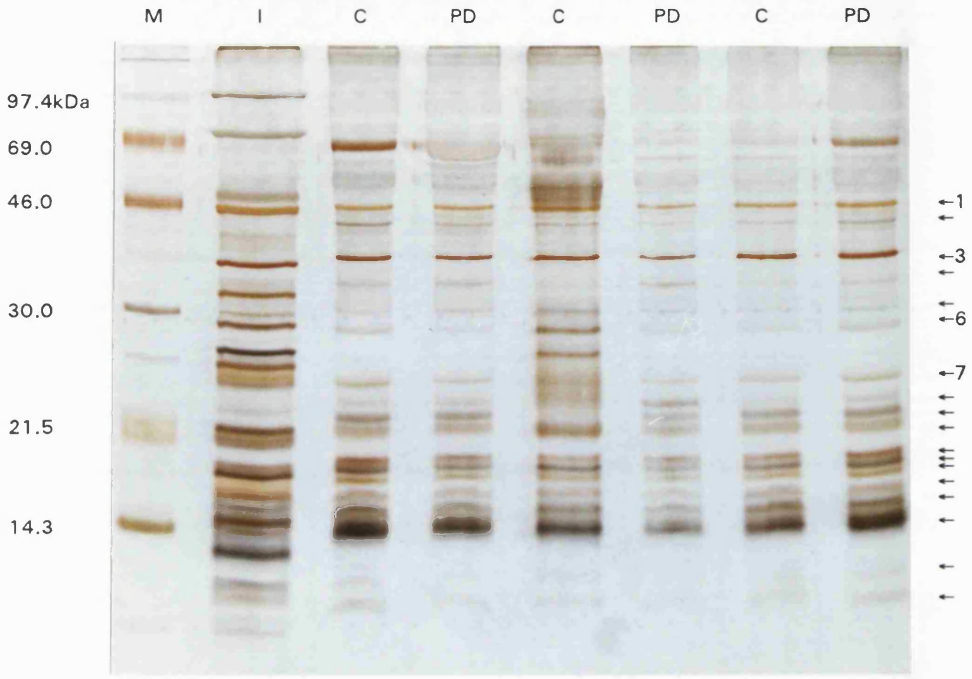


Figure 5-9. Silver stained SDS-PAGE minigel of complex I immunoprecipitated from three PD and three control substantia nigra homogenate samples.

M (molecular weight markers); I (purified bovine heart complex I); PD/C (complex I immunoprecipitated from Parkinson's disease/control substantia nigra homogenate); ← (subunits detected in complex I immunoprecipitates).

Subunits 1 and 3 were used as standards to which the absorbance of all other subunits were compared (see Table 5-6, 5-7). The mean absorbance of subunits 6 and 7 were decreased in PD samples.

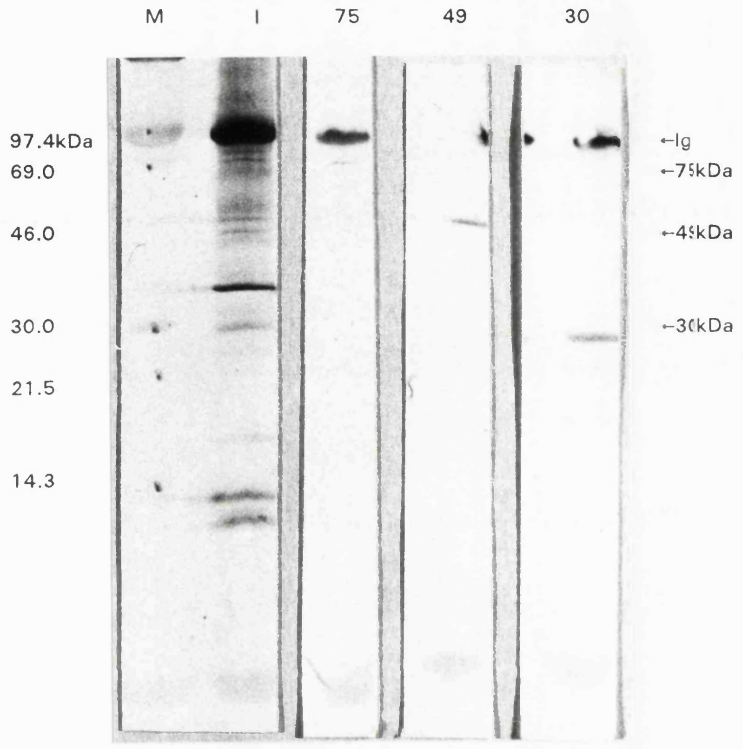


Figure 5-10. Western blots of complex I immunoprecipitated from control substantia nigra brain homogenates probed with affinity purified complex I antibodies.

M (molecular weight markers); I (complex I holoenzyme antibody); 75 (75kDa subunit antibody); 49 (49kDa subunit antibody); 30 (30kDa subunit antibody); Ig (non-dissociated immunoglobulin).

5.4. Discussion

Evidence that material immunoprecipitated by antiserum to the complex I holoenzyme contained complex I has been provided. Firstly, at least in purified mitochondria, the antiserum immunoprecipitated NFR activity, the enzymatic activity of the FP fraction of complex I (Figure 5-1). The definitive proof of the immunoprecipitation of complex I would be the immunoprecipitation of rotenone-sensitive NADH CoQ_1 reductase activity. However, none of the detergents tested solubilised sufficient complex I without destroying this enzyme activity (Table 5-1). In crude brain homogenate, the antiserum only precipitated approximately 25% of the NFR activity (Figure 5-7). This may have been because only 25% of the complex I was precipitated, or because there was an NFR activity in the tissue homogenate not associated with mitochondrial complex I which would not be precipitated by the complex I antiserum. It has been shown that mitochondria contain an NFR activity not associated with complex I (Moreadith *et al*, 1987).

Further evidence that the material immunoprecipitated from purified mitochondria and from tissue homogenates contained complex I was that the polypeptide profiles on silver stained SDS-PAGE gels were almost identical to purified bovine heart complex I (Figures 5-2, 5-5, 5-8, 5-9). All of the subunits detectable in purified bovine heart complex I were also present in complex I immunoprecipitated from BHM (Figure 5-2). Since complex I has not been purified from human material, a direct comparison with immunoprecipitated human complex I is not possible. In complex I immunoprecipitated by Cleeter and Ragan (1985), a polypeptide of 42kDa was absent, which they argued suggests that this polypeptide is not a constituent of complex I. However in this study, complex I immunoprecipitated from purified mitochondria appeared to include a polypeptide of 42kDa (Figure 5-2, 5-5), whilst those from brain tissue homogenate did not (Figure 5-8, 5-9). One explanation for these differences in results may be differences in solubilisation conditions; Cleeter and Ragan (1985) solubilised mitochondria with sodium deoxycholate and Triton X-100, whereas solubilisation in these experiments was with N-dodecyl-ß-D-maltoside. Another possible explanation is that the reactivities of the antibodies used to precipitate complex I may have been different. Since the 42kDa subunit is not consistently present in immunoprecipitates of complex I, this suggests that it may be only loosely associated with the complex. This is supported by the observation that in the preparation of complex I by solubilisation of mitochondria with DDM followed by a chromatographic step in the presence of detergent (Finel *et al*, 1992), the 42kDa subunit is present at lower levels than the other

subunits. Immunoprecipitated complex I also contained some proteins that were not complex I subunits; the heavy and light chains of IgG from the precipitating serum were present (Figure 5-2) as were the core proteins of complex III (Figure 5-4).

Western blotting with antibodies to complex I also provided evidence that the precipitated material was predominantly comprised of complex I subunits. Antibodies raised against complex I subunits cross-reacted with subunits of the same molecular weight in purified and immunoprecipitated complex I from bovine material (Figure 5-4) and, with the exception of the 39, 30 and 13kDa subunits, with subunits of the same molecular weight in purified bovine complex I and immunoprecipitated human complex I (Figure 5-4).

As mentioned above, species-specific differences between bovine and human complex I were observed by silver stained SDS-PAGE polypeptide profiles and by Western blot (Figure 5-2, 5-4). The bovine 30kDa subunit had a lower molecular weight in human complex I, and the bovine 13kDa had a higher molecular weight in human complex I. These results are in agreement with the earlier study of Cleeter and Ragan (1985). However an additional difference was detected in this study; the bovine 39kDa subunit of complex I had a lower molecular weight in human complex I. Cleeter and Ragan (1985) did not have an antibody cross-reacting with the 39kDa subunit available for Western blotting studies. Since not all of the 41 subunits of complex I (Walker *et al*, 1992) were detectable by SDS-PAGE, there may be other differences between bovine and human complex I that were not detected. Furthermore, not all subunit changes will result in mobility changes resolvable on SDS-PAGE gels. Changes in charge may be detected by IEF (isoelectric focusing), which separates proteins according to charge rather than molecular weight.

No tissue-specific differences in human complex I were observed by polypeptide profiles on SDS-PAGE (Figure 5-2) or by Western blotting (Figure 5-4). An attractive hypothesis for the cause of the tissue-specific complex I defect observed in PD (Mann *et al*, 1992a (substantia nigra); Results 4.3.6.3 (platelets)), is that isoforms of complex I exist in the affected tissues. However, complex I was not immunoprecipitated from substantia nigra or platelets in these studies. Tissue-specific differences in rat complex I were not observed (Figure 5-5) by silver stained SDS-PAGE. Clay and Ragan (1988) detected the presence of a 17kDa subunit and absence of an 18kDa subunit in rat liver, kidney and lung, compared to rat heart, brain and skeletal muscle complex I. No antibodies to these polypeptides were available so it is possible that they were not

components of complex I. If this was the case, then the differences may not have been observed in the above studies because these contaminating polypeptides were not present in the immunoprecipitates. However, the differences may be due to differences in complex I subunits resolved by different electrophoresis conditions in the two studies.

No difference in the molecular weights of the 18 detectable complex I subunits immunoprecipitated from PD and control substantia nigra tissue homogenates were resolved by SDS-PAGE (Figure 5-9). However, the mean staining intensities, measured using a computerised image analysis system (see Methods 2.8.5), of bands 6 and 7 of 30.8 and 24.8kDa respectively, were decreased in PD samples relative to controls (Table 5-6, 5-7). It is unclear whether these two subunits are nuclearly or mitochondrially encoded; because antibodies to mitochondrial subunits are not available, they have not been positively identified by electrophoresis. Staining intensity was measured as a ratio to two other subunits (1 and 3) to control for any differences in protein loadings on the gels. Although the mean ratios of staining intensity were different in the PD and control groups, there was an overlap of values in the two groups; the ratios in two of the PD samples were higher than in one of the control samples. Measurements of staining intensity of complex I subunits immunoprecipitated from five control caudate nucleus homogenate samples showed large inter-sample variation (Table 5-5). The same was true in the substantia nigra samples, shown by the large standard deviations of the mean staining intensities in both the PD and control groups (Table 5-6, 5-7); for example the staining intensity of subunit 1 as a ratio of that of subunit 6 ranged from 0.01-0.18 in the three PD samples and from 0.08-0.77 in the three control samples. Because of the large variation of staining intensity within the groups, it is difficult to conclude that there were any significant differences between the groups.

Two studies have compared the polypeptide composition of complex I in PD and control substantia nigra samples by Western blotting. Mizuno *et al* (1989) detected decreased staining intensities of polypeptides of 30, 25 and 24kDa in PD striatum samples compared to controls. However, Schapira *et al* (1990a) detected no difference in the staining intensities of the 75, 51, 49, 42, 39, 30, 15 and 13kDa subunits of complex I in PD substantia nigra samples compared to controls by Western blot.

Changes in the molecular weight of a subunit or complete absence of a subunit may be the consequence of a mutation in the gene coding for that subunit. However, changes in the relative level of a subunit in the complex are harder to

interpret. The reduction in the level of the complex I subunits of 31.1 and 28.8kDa, if not artefactual, may be caused by decreased synthesis of the subunits, increased degradation of the subunits, inefficient incorporation of these subunits into the holoenzyme or, if they are nuclearly-encoded, by reduced import of the subunits into mitochondria. A mutation in the leader sequence of a mitochondrial protein, methylmalonyl-CoA mutase, has been reported, which disrupts import of this protein into the mitochondrial matrix from the cytosol (Ledley *et al*, 1990). Furthermore, a deficiency of the Rieske protein of complex III in muscle mitochondria of a patient with mitochondrial myopathy is believed to be due to a defect in mitochondrial import (Schapira *et al*, 1990d). The subunit was almost undetectable in skeletal muscle mitochondria, but was present at normal levels in muscle homogenate from the patient. These two reports demonstrate that defects of mitochondrial protein import can lead to deficiencies of single nuclear-encoded polypeptides.

The PD substantia nigra samples used in the immunoprecipitation experiments described above did not have a complex I deficiency (Table 5-8). Although as a group PD substantia nigra samples have a 40% complex I deficiency compared to controls, the complex I activities in some PD substantia nigra samples lie within the control range (Mann *et al*, 1992a), and therefore on an individual basis not all PD substantia nigra samples show a complex I deficiency. Since the PD substantia nigra samples used did not have reduced complex I activity, the differences observed in the levels of subunits 6 and 7 between PD and control immunoprecipitated complex I, may have been artefactual. However, it is possible that these differences did not affect the activity of the enzyme. These results do not exclude the cause of the complex I defect in some PD substantia nigra samples being a structural abnormality in complex I; unfortunately no PD substantia nigra samples in which a complex I defect had been demonstrated were available for immunoprecipitation experiments.

Chapter 6. Quantitation of complex I in the PD substantia nigra by ELISA

6.1. Aim

To quantitate the levels of complex I holoenzyme and three complex I subunits in PD and control substantia nigra homogenate samples by ELISA (enzyme linked immunosorbent assay), using affinity purified complex I antibodies. To determine any relationship between complex I protein levels and enzymatic activity in the substantia nigra.

6.2. Introduction

Complex I immunoprecipitated from PD and control substantia nigra homogenate samples has been shown to be identical in terms of both subunit number and subunit molecular weight based on electrophoretic mobility. Based on staining intensities of silver stained SDS-PAGE gels, two subunits were present in decreased amounts in some PD samples (see Results 5.3.2). However, the PD samples used in the immunoprecipitation study did not have a complex I deficiency (see Results 5.3.4.3).

Since staining of SDS-PAGE gels is not an accurate quantitative method, an ELISA method was used to more accurately quantify the amount of complex I holoenzyme and specific complex I subunits in PD and control substantia nigra homogenate samples. Antibodies to the complex I holoenzyme were prepared as described (see Methods 2.7.1); antibodies to the 30 and 13kDa subunits of complex I were prepared by Dr JM Cooper (Dept of Neuroscience, Royal Free Hospital School of Medicine, London, UK) and antibodies to the 24kDa subunit were provided by Dr JE Walker (MRC Laboratory of Molecular Biology, Cambridge, UK). These four antibodies were affinity purified against purified bovine heart complex I as described (see Methods 2.7.2) and were used to quantify the levels of complex I in two groups of PD and control substantia nigra samples; the Group A PD samples showed a complex I enzymatic deficiency, and the Group B PD samples had normal complex I enzymatic activity.

6.3. Results

6.3.1. Specificity of the affinity purified complex I antibodies

The reactivities of the four affinity purified antibodies to purified bovine heart complex I were determined by Western blot (Figure 6-1). The holoenzyme antibody reacted with at least 10 complex I subunits with molecular weights of approximately 75, 51, 49, 39, 20, 18, 17, 15, 13 and 10kDa. The antibody also cross reacted with a protein of approximately 110kDa, which is likely to be pyridine dinucleotide transhydrogenase, a recognised contaminant of complex I preparations. The 30kDa antibody reacted specifically with the 30kDa subunit; the 24kDa antibody reacted with the 24kDa subunit and less strongly with the 51kDa subunit; and the 13kDa antibody reacted predominantly with the 13kDa subunit, less strongly with a subunit of approximately 15kDa and very weakly with subunits of approximately 50kDa and above.

6.3.2. Optimisation of assay conditions.

Three variables, coating buffer, choice of detergent and detergent concentration, were examined to determine optimal antigen-specific absorbance values in the assay. The absorbance of antigen (0.5μg control substantia nigra homogenate) in coating buffer plus detergent was determined, and the background absorbance (coating buffer plus detergent but no antigen) was subtracted, giving an absorbance value specific for antigen. Three antigen coating buffers were examined to determine which would result in the highest absorbance values in the assay. The coating buffer is the medium in which the antigen, in this case substantia nigra brain homogenate, is solubilised and applied to the wells of the plate (see Methods 2.9.6). Carbonate buffer (see Appendix 5.2) produced optimal absorbance values (Table 6-1A).

Four detergents were assessed, in carbonate coating buffer; CHAPS (3-[3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate), DDM (N-dodecyl-ß-D-maltoside), NaDOC (sodium deoxycholate) and SDS (sodium dodecyl sulphate). SDS produced optimal absorbance values (Table 6-1B). The optimal concentration of SDS in the assay was 0.01% (Table 6-1C). To summarise, for optimal antigen-specific absorbance values in the assay, antigen was solubilised with 0.01% SDS in carbonate buffer.

6.3.3. Complex I activity in PD and control substantia nigra

Complex I and citrate synthase assays were performed as described (see Methods 2.6.1; 2.6.5). The assays were performed in two sets of PD and control substantia nigra homogenate samples. In one set (A), mean complex I activity was decreased in the PD samples by approximately 40%, and in the other set (B), mean complex I activity was similar in PD and control samples (Table 6-2). Enzyme assays in Group A samples were performed by Dr VM Mann (Dept of Neuroscience, Royal Free Hospital School of Medicine, London, UK). The difference in CS corrected complex I activity between PD and control samples in Group A just failed to reach statistical significance ($p=0.056$; Mann-Whitney U test).

PD and control samples in each group were matched for age, death to refrigeration and time to freezing of brain following post mortem. Furthermore, these parameters were similar in Groups A and B. However, disease duration was significantly different in the two PD groups, 16.8 ± 2.8 years in Group A and 8.6 ± 4.5 years in Group B ($p=0.016$; Mann-Whitney U test).

6.3.4. Quantitation of complex I protein in PD and control substantia nigra

The 96-well ELISA plates were coated with 0.01, 0.02, 0.04 and 0.08μg of each sample in duplicate as described (see Methods 2.9.6). The mean antibody reactivities at four antigen concentrations for Groups A and B are shown graphically; Figure 6-2 (holoenzyme antibody), Figure 6-3 (30kDa antibody), Figure 6-4 (24kDa antibody) and Figure 6-5 (13kDa antibody). The assay response (absorbance) for each antibody over the range of antigen concentrations used was linear, indicating that each antibody was at an excess concentration relative to antigen at all antigen concentrations.

Antibody reactivity (absorbance) results were expressed as the slope of the linear regression of absorbance (492nm) versus amount of antigen. In the Group A samples, with a 40% complex I deficiency in the PD samples, the mean reactivities of all the antibodies tested were reduced in the PD samples compared to controls (Table 6-3) by 40% (holoenzyme antibody), 43% (30kDa antibody) and 30% (13kDa antibody). The difference in the 30kDa antibody reactivity in Group A PD and control samples was statistically significant (Table 6-3); the difference in reactivities of the holoenzyme and 13kDa antibodies in

Group A failed to reach statistical significance ($p > 0.1$, Mann-Whitney U test). In the Group B samples with normal PD complex I activity, the mean reactivities of all four antibodies were similar in PD and control samples (Table 6-3). Complex I enzymatic activity and complex I protein content showed some correlation in PD and control samples from both groups (Figures 6-6, 6-7, 6-8, 6-9), although these correlations did not reach statistical significance ($p > 0.1$, Mann-Whitney U test).

A. Coating buffer	Absorbance (492nm)
Carbonate	0.504
PBS	0.275
Water	0.015

B. Detergent	Absorbance (492nm)
---	0.285
CHAPS	0.190
DDM	0.003
NaDOC	0.300
SDS	0.504

C. SDS concentration (%)	Absorbance (492nm)
0.001	0.637
0.01	0.694
0.1	0.504
1.0	0.250

Table 6-1. The effect of coating buffer, detergent and detergent concentration on quantitation of complex I in substantia nigra homogenates by ELISA.

Antigen (0.5μg control substantia nigra homogenate); Antibody (1:100 affinity purified complex I holoenzyme antibody). See Methods 2.9.6 for general ELISA assay protocol. For detergent abbreviations see Results 6.3.2.

Table 6-1A. Antigen in various coating buffers plus 0.1% SDS.
Table 6-1B. Antigen in carbonate buffer plus 0.1% various detergent.
Table 6-1C. Antigen in carbonate buffer plus various concentrations of SDS.

	Complex I	Citrate Synthase	I/CS (x100)
Group A			
PD	2.15 ± 1.23	155.00 ± 29.02	1.38 ± 0.68
C	3.42 ± 0.85	147.20 ± 10.03	2.32 ± 0.22
Group B			
PD	5.53 ± 1.80	117.77 ± 6.63	4.64 ± 1.12
C	5.41 ± 1.09	131.33 ± 25.36	4.20 ± 0.85

Table 6-2. Mitochondrial enzyme activities in PD and control substantia nigra homogenates.

Enzyme assays in Group A performed by Dr VM Mann (Dept Neuroscience, Royal Free Hospital School of Medicine, London, UK). Activities expressed as nmol/min/mg protein (all mean ± SD; n=5). No statistically significant differences by Mann Whitney U-test.

Complex I (measured as rotenone-sensitive NADH CoQ_1 reductase); I/CS (citrate synthase corrected ratio of complex I activity).

	holoenzyme	30	24	13
Group A				
PD	1.32 ± 0.88	1.61 ± 0.59*		3.90 ± 1.56
C	2.21 ± 0.70	2.82 ± 1.09		5.61 ± 0.90
Group B				
PD	4.07 ± 0.48	3.57 ± 0.27	2.98 ± 0.31	6.59 ± 0.68
C	4.14 ± 0.91	3.40 ± 0.56	2.98 ± 0.36	6.10 ± 1.01

Table 6-3. Reactivities of affinity purified complex I antibodies with PD and control substantia nigra homogenates by ELISA.

Data expressed as slope of regression line of absorbance (492nm) versus protein concentration (mean ± SD, n = 5; except Group A holoenzyme antibody reactivity, n = 4).

* $p = 0.032$ by Mann Whitney U-test.

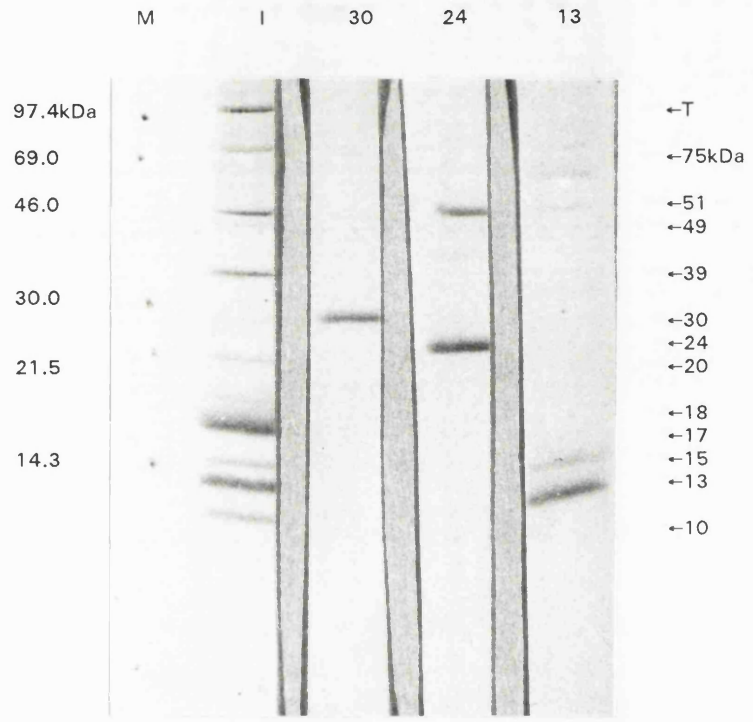


Figure 6-1. Western blots of purified bovine heart complex I probed with affinity purified complex I antibodies used for ELISA.

M (molecular weight markers); I (complex I holoenzyme antibody); 30 (30kDa subunit antibody); 24 (24kDa subunit antibody); 13 (13kDa subunit antibody); T (pyridine dinucleotide transhydrogenase).

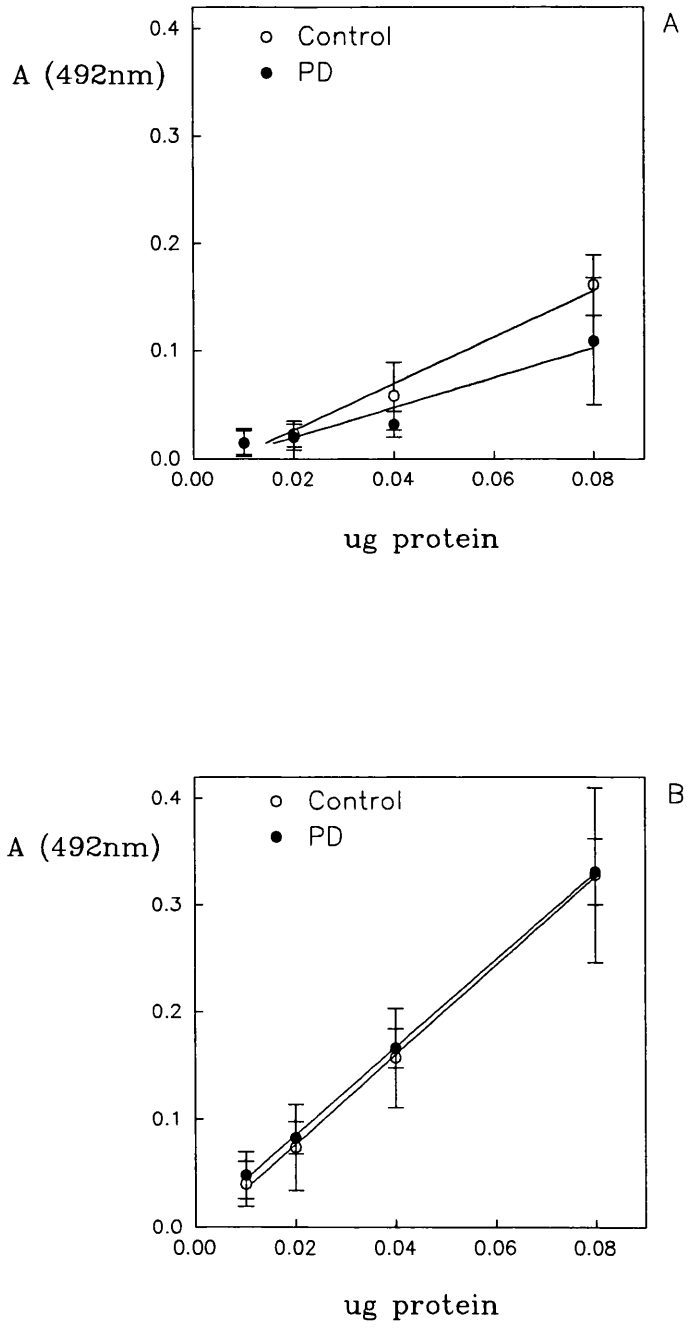
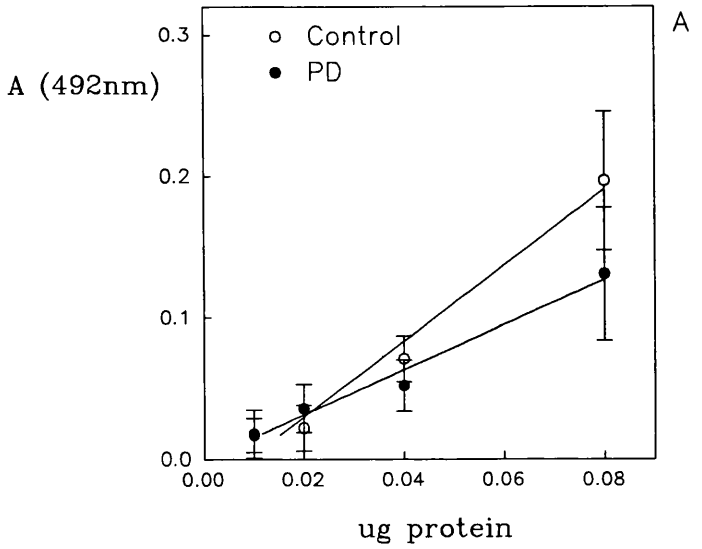


Figure 6-2. Quantitation of complex I in control and PD substantia nigra homogenates by ELISA using an affinity purified holoenzyme antibody.

Points at each antigen concentration represent the mean (Group A, n=4; Group B, n=5). Bars represent standard deviation of the mean. Lines of best fit are drawn through the data. A (Group A); B (Group B).



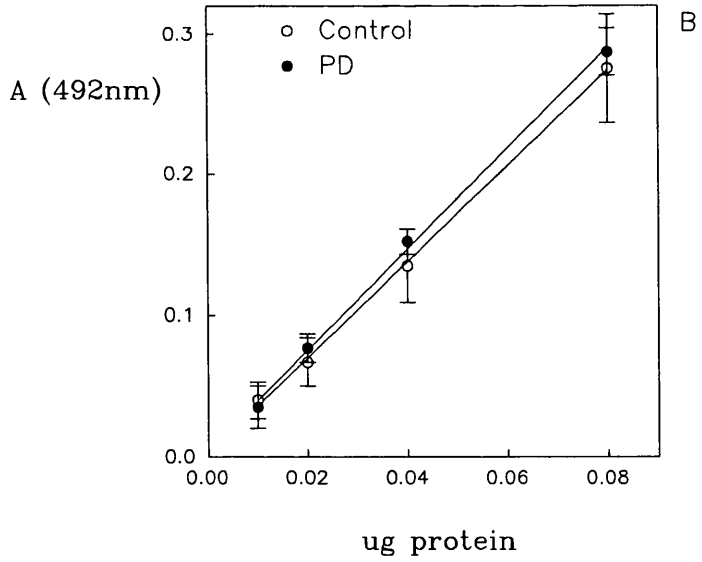


Figure 6-3. Quantitation of complex I in control and PD substantia nigra homogenates by ELISA using an affinity purified 30kDa subunit antibody.

Points at each antigen concentration represent the mean (n = 5). Bars represent standard deviation of the mean. Lines of best fit are drawn through the data.
A (Group A); B (Group B).

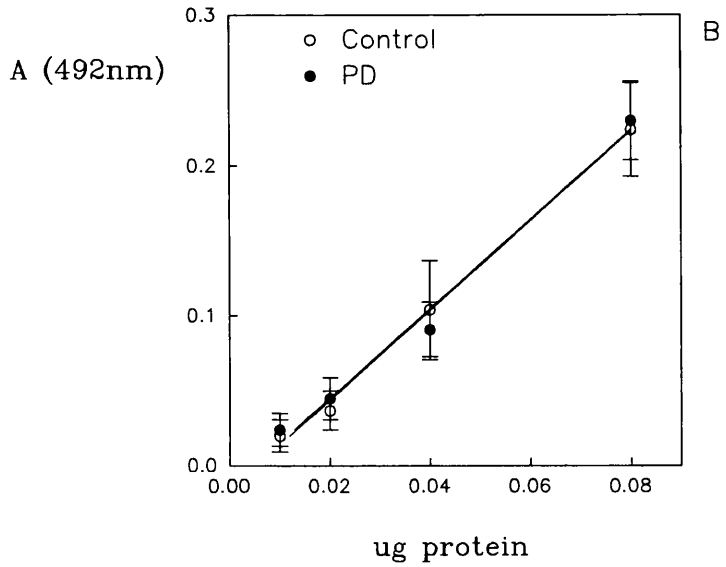
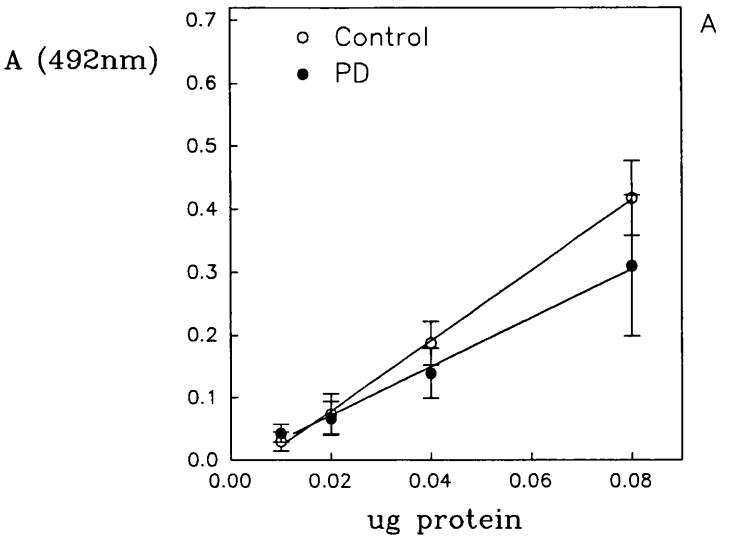


Figure 6-4. Quantitation of complex I in control and PD substantia nigra homogenates by ELISA using an affinity purified 24kDa subunit antibody.

Points at each antigen concentration represent the mean (n = 5). Bars represent standard deviation of the mean. Lines of best fit are drawn through the data.
B (Group B).



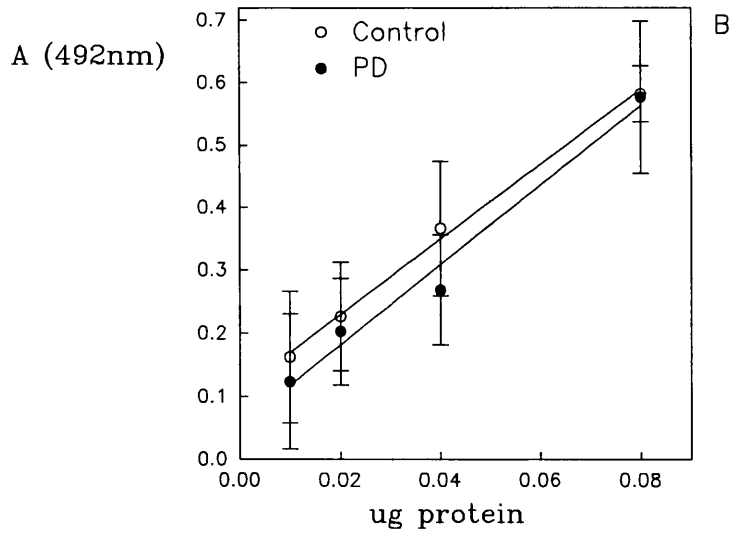


Figure 6-5. Quantitation of complex I in control and PD substantia nigra homogenates by ELISA using an affinity purified 13kDa subunit antibody.

Points at each antigen concentration represent the mean (n = 5). Bars represent standard deviation of the mean. Lines of best fit are drawn through the data. A (Group A); B (Group B).

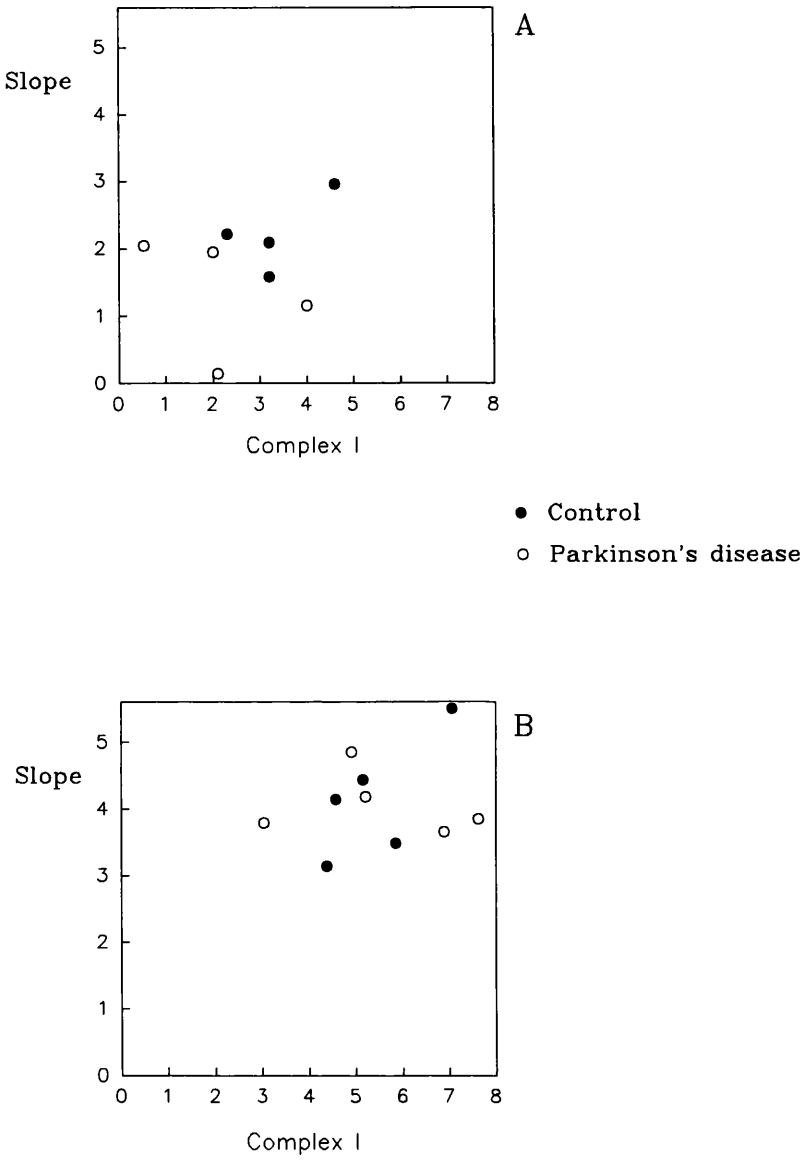


Figure 6-6. Scatterplot of complex I enzyme activity versus complex I holoenzyme antibody reactivity by ELISA.

Slope (slope of the linear regression of absorbance (492nm) versus amount of antigen; a measure of antibody reactivity); Complex I (rotenone-sensitive NADH CoQ_1 reductase activity; nmol/min/mg protein).

A (Group A); B (Group B).

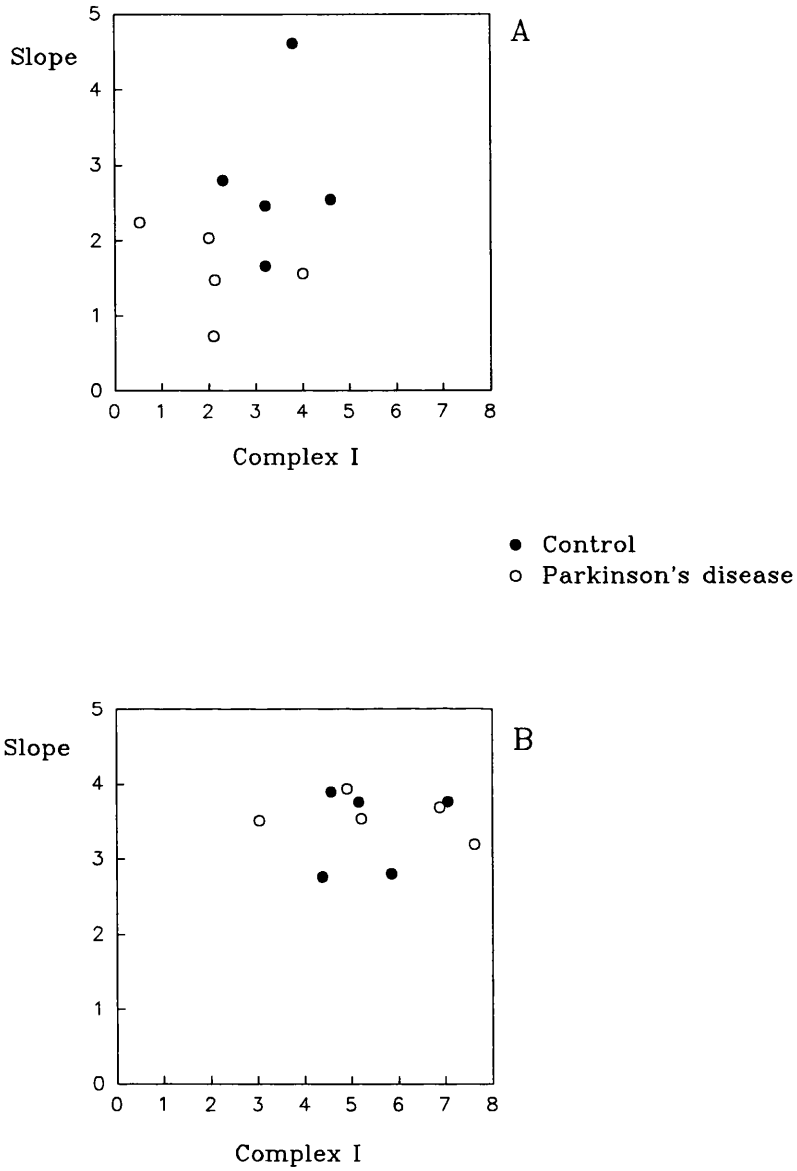


Figure 6-7. Scatterplot of complex I enzyme activity versus complex I 30kDa subunit antibody reactivity by ELISA.

Slope (slope of the linear regression of absorbance (492nm) versus amount of antigen; a measure of antibody reactivity); Complex I (rotenone-sensitive NADH CoQ_1 reductase activity; nmol/min/mg protein).

A (Group A); B (Group B).

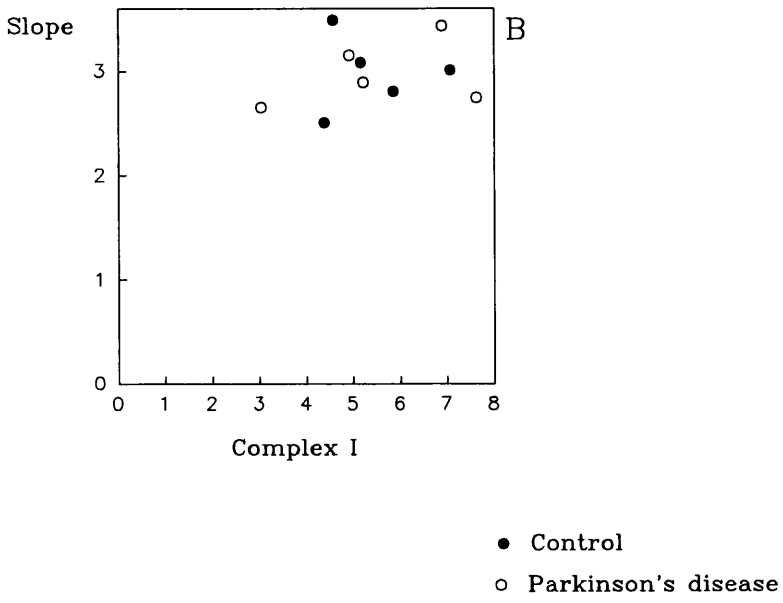


Figure 6-8. Scatterplot of complex I enzyme activity versus complex I 24kDa subunit antibody reactivity by ELISA.

Slope (slope of the linear regression of absorbance (492nm) versus amount of antigen; a measure of antibody reactivity); Complex I (rotenone-sensitive NADH CoQ_1 reductase activity; nmol/min/mg protein).

B (Group B).

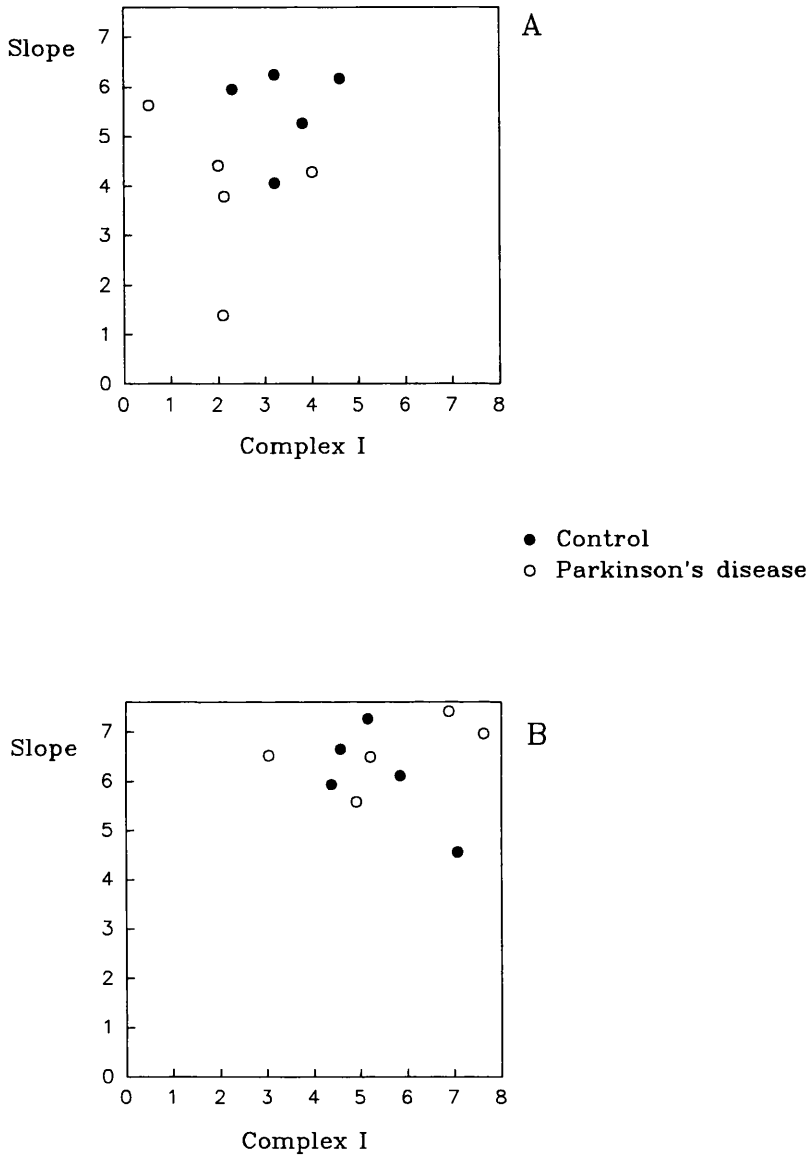


Figure 6-9. Scatterplot of complex I enzyme activity versus complex I 13kDa subunit antibody reactivity by ELISA.

Slope (slope of the linear regression of absorbance (492nm) versus amount of antigen; a measure of antibody reactivity); Complex I (rotenone-sensitive NADH CoQ_1 reductase activity; nmol/min/mg protein).

A (Group A); B (Group B).

6.4. Discussion

The relative concentration of complex I holoenzyme and three specific complex I subunits has been determined in two groups of PD and control substantia nigra samples, using affinity purified antibodies in an ELISA assay. Ten complex I subunits were detectable with the holoenzyme antibody, and three single subunits were detectable with the subunit specific antibodies (Figure 6-1). It is unclear whether any of the subunits detected by the holoenzyme antibody were mitochondrially encoded; because no antibodies are available which react with single mitochondrially encoded subunits, they have not been positively identified by electrophoresis.

In Group A, the PD samples showed a complex I enzymatic deficiency, and in Group B they showed normal complex I activity compared to controls. Although in larger group to group comparisons PD substantia nigra samples have a 40% complex I enzymatic defect (Mann *et al*, 1992a), there is an overlap of activities so that not all PD samples have lower complex I activity than control samples. This explains how it was possible that the Group B PD samples did not show a complex I deficiency when compared to Group B control samples. In Group A, complex I activity was approximately 40% decreased in the PD samples, in agreement with previous studies (Mann *et al*, 1992a). Based on the reactivities of the holoenzyme antibody and two subunit specific complex I antibodies in the ELISA, these PD samples contained approximately 40% less complex I protein than the control samples. PD substantia nigra samples with similar complex I activity to controls (Group B), contained similar levels of complex I protein to control substantia nigra samples (Table 6-2). The complex I content of PD and control substantia nigra samples, measured as the reactivities of complex I antibodies in an ELISA, therefore showed some correlation with complex I enzyme activities (Figures 6-6, 6-7, 6-8, 6-9); PD samples in Group A, with reduced complex I activity, contained reduced levels of complex I protein compared to Group A controls; and PD samples in Group B, with normal complex I activity, contained similar levels of complex I protein to Group B controls. However, there was no statistically significant correlation between antibody reactivity and complex I activity within any group. This may have been due to the small number of samples in each group ($n = 5$).

Since the Group B PD samples had normal complex I enzymatic activity compared to Group B controls, it was not surprising that the levels of complex I protein in these samples were not decreased relative to controls. The same group of samples was used in the immunoprecipitation experiments; by SDS-

PAGE there were no significant differences in the complex I polypeptide profile of PD and control samples (see Figure 5-9). Due to a lack of material, Group A PD substantia nigra samples with a complex I deficiency were not available for the immunoprecipitation experiments (ELISA experiments only required 0.4μg protein in contrast to immunoprecipitation experiments which required 2mg protein). It is interesting to note that Group A PD patients had a significantly longer disease duration than Group B patients (16.8 ± 2.8 vs 8.6 ± 4.5 years), which could imply that complex I enzyme activity and protein concentration decrease with disease duration. However, in a study including a large number of samples (n = 17) there was no correlation between substantia nigra complex I activity and PD disease duration (Mann *et al*, 1992a).

It remains unclear why the control samples in each group (A and B) had different complex I activities and contained different amounts of complex I protein (Table 6-2; Table 6-3) . Although the enzyme assays in each group were performed by different operators (Dr VM Mann, Group A; D Krige, Group B), the assays were performed under identical conditions. The difference in enzyme assay operator cannot explain the difference in the level of complex I protein between the two groups, and furthermore, the complex I protein concentration showed some correlation with the complex I enzyme activity in each group (Figures 6-6, 6-7, 6-8, 6-9). Both groups of control and PD samples were matched for age and post-mortem parameters, and all brain samples were supplied by the Parkinson's Disease Society Brain Bank. The ELISA experiments in each group were performed separately, which could potentially explain the difference in antibody reactivities in the two groups. However as already stated, these reactivities showed some correlation with complex I enzyme activity in the two sets of PD and control samples (Figures 6-6, 6-7, 6-8, 6-9).

The only other study that has attempted to quantify the levels of complex I in the PD substantia nigra is that of Hattori *et al* (1991). By immunohistochemical techniques using antibodies to the complex I holoenzyme, they showed that weakly staining nigral neurons were more common in the PD sections than control sections, 36% in PD sections and 14% in control sections. Staining for complexes III and IV was normal in the PD sections, although some PD samples (3/8) showed reduced staining for complex II.

The results from the ELISA experiments described above and the immunohistochemical studies of Hattori *et al* (1991) favour the hypothesis that complex I protein levels are reduced in the PD substantia nigra, and that it is this reduction which is the cause of the complex I deficiency observed (Mann *et al*,

1992a). The cause of the observed decrease in complex I protein concentration in PD substantia nigra samples with a complex I deficiency is itself unknown. A toxin bound to the complex, or oxidative damage to the complex may reduce complex I enzymatic activity without grossly affecting complex I protein levels. The reduction in the reactivity of complex I in PD substantia nigra samples with a complex I enzymatic defect was observed not only for single subunits but for the holoenzyme. However it cannot be concluded from this result that the reduction in complex I enzymatic activity is directly due to a reduction in the concentration of complex I holoenzyme; the loss of a single subunit may cause incorrect assembly or increased degradation of the complex, and thereby indirectly lead to reduced levels of the holoenzyme.

The only study which has determined the effect of the loss of a single subunit on the assembly of complex I is that of Hofhaus and Attardi (1993). They showed that in a human cell line lacking the mitochondrially encoded subunit ND4, nuclear encoded subunits assembled normally (NADH ferricyanide reductase activity was normal), but many of the other mitochondrially encoded subunits besides ND4 did not assemble into the complex. To determine whether or not the reduced level of complex I holoenzyme in the PD substantia nigra is due to a lack of assembly of the whole complex caused by the absence of a single subunit, it would be necessary to quantitate the level of every single complex I subunit in the samples, to determine whether one specifically was absent or present at much lower levels than the others. This approach is feasible using the technique of ELISA, the only constraint being the availability of antibodies to single subunits.

To determine whether or not the observed decrease in the concentration of complex I in the PD substantia nigra is specific to complex I, it would be necessary to quantify the levels of one or more of the other respiratory chain complexes. A reduction in the number of mitochondria in the PD substantia nigra is unlikely to account for the reduced levels of complex I protein, since the activity of citrate synthase, a marker of the mitochondrial matrix, was similar in PD and control groups (Table 6-1).

The finding that PD substantia nigra samples with complex I activity similar to controls contained normal levels of complex I protein, and that PD substantia nigra samples showing a complex I enzymatic defect contained lower levels of complex I protein may have important implications. If the cause of the complex I deficiency in some PD substantia nigra samples is a decrease in the levels of complex I protein present, and if this complex I defect is relevant to the

pathogenesis of the disease (whether it be a primary or secondary phenomenon), then these PD cases may have a different disease aetiology to those with normal complex I enzymatic activity and normal complex I protein levels.

Chapter 7. Cytochrome P450 gene mutations and PD

7.1. Aim

To determine the frequency of three cytochrome P450 gene mutations in PD, the presence of which can detect 90% of poor metabolisers of debrisoquine. To correlate the presence of these mutations with mitochondrial complex I activity in substantia nigra brain homogenates.

7.2. Introduction

The hypothesis that abnormal xenobiotic metabolism is associated with the cause of PD is supported by the fact that the exogenous neurotoxin MPTP causes a parkinsonism very similar both clinically and pathologically to the idiopathic disease (Langston *et al*, 1983; Ballard *et al*, 1985). It is postulated that exposure to an environmental toxin, with similar characteristics to MPTP, causes PD in susceptible individuals. This susceptibility may be genetically determined and could involve abnormal detoxification system phenotypes, for example the cytochrome P450 system.

Three studies (Armstrong *et al*, 1992; Smith *et al*, 1992; Tsuneoka *et al*, 1993) have demonstrated an increased frequency of mutant alleles of the CYP2D6 debrisoquine hydroxylase gene in PD. Armstrong *et al* (1992) and Smith *et al* (1992) classified individuals as poor metabolisers of debrisoquine if they were homozygous for either a G to A transition at the intron3/exon4 boundary, a base pair deletion within exon5 or a whole gene deletion, or heterozygous for any combination of these mutant alleles. The detection of these three mutations can identify 90% of poor metaboliser individuals. These mutant alleles were used to define poor metabolisers in this study. The mutant allele detected in PD patients by Tsuneoka *et al* (1993) has not been associated with the poor metaboliser phenotype and was therefore not included in this study.

To examine any relationship between mitochondrial dysfunction and cytochrome P450 mutations, the presence of the three CYP2D6 mutant alleles was

determined in 24 PD patient samples by PCR (polymerase chain reaction) amplification and restriction enzyme digestion and where available, correlated with nigral complex I activity.

7.3. Results

7.3.1. Frequency of mutant alleles

Figure 7-1 shows a schematic representation of the PCR-based method for the detection of the CYP2D6 gene mutations (for a detailed protocol see Methods 2.10.4.1). The whole gene deletion is detected by the lack of a PCR product. The transition mutation was heterozygous in 9/24 samples from PD patients. The other 15 PD samples were homozygous normal with respect to this mutation. The base pair deletion and whole gene deletion mutations were not detected in the homozygous or heterozygous state in any of the PD samples.

Table 7-1 shows the allele frequencies of the G to A transition and the base pair deletion in this study and in two earlier reports which detected these mutations (Armstrong *et al*, 1992; Smith *et al*, 1992). Since all individuals have two copies of each gene, the number of alleles is twice the number of samples. The frequencies were similar in all three studies, although the sample number in this study was not sufficient to detect any base pair deletion mutations.

7.3.2. Relationship of transition mutation to nigral complex I activity

The G to A transition mutation was heterozygous in 9/24 PD samples. Nigral complex I activities were available for eight heterozygous and eight homozygous normal PD samples (assays performed by Dr VM Mann, Royal Free Hospital School of Medicine, London, UK). The mean complex I specific activity and CS corrected ratio for both homozygous normal and heterozygous samples are shown in Table 7-2. There was no difference in complex I activity between the two groups.

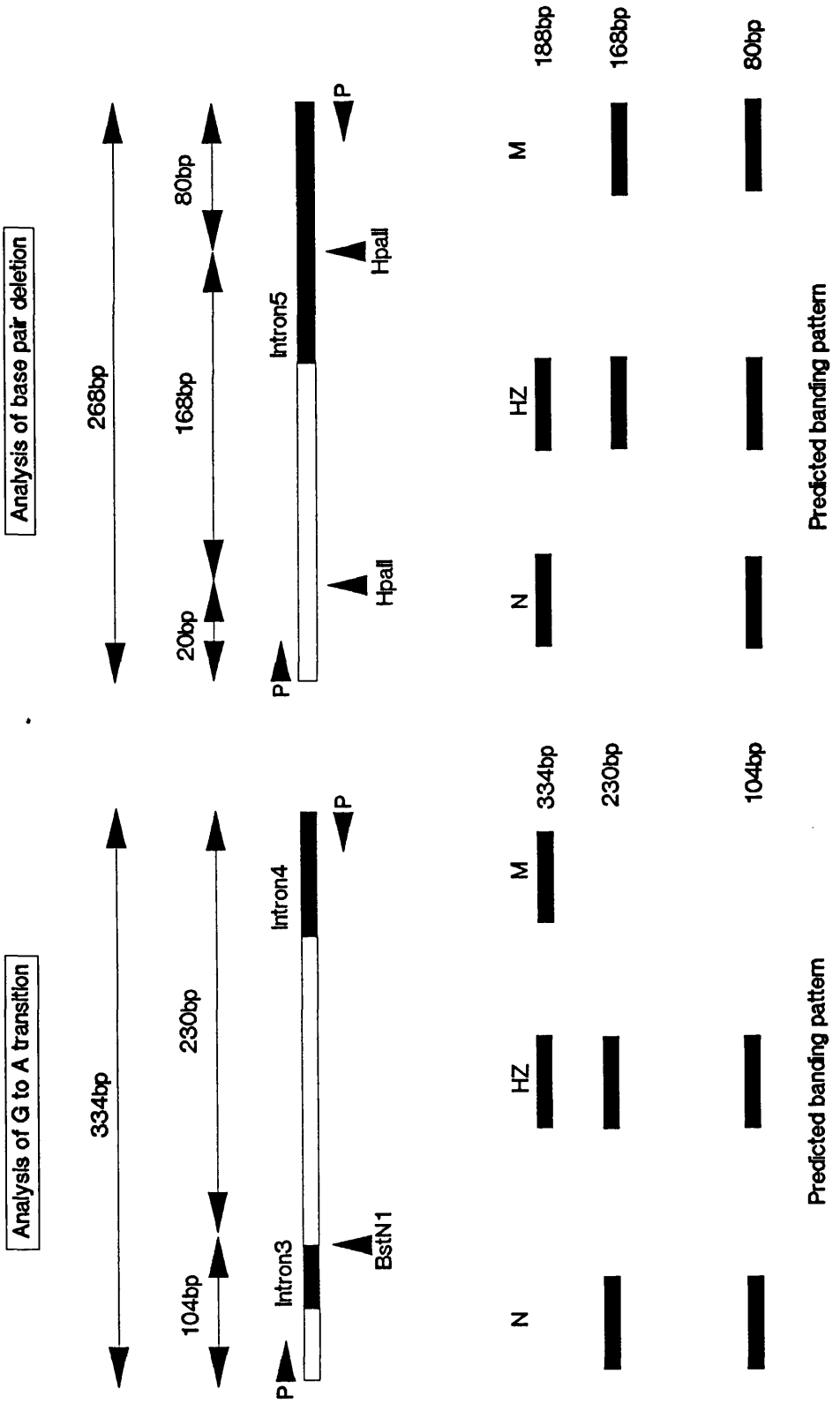


Figure 7-1. Analysis of mutant CYP2D6 alleles by PCR.

The banding pattern of amplified DNA is that expected after separation of restriction fragments by gel electrophoresis.

P► (primers); BstN1, Hpall (restriction sites); N (normal); HZ (heterozygous); M (mutant); PCR (polymerase chain reaction).

	This study	Armstrong *et al* (1992)	Smith *et al* (1992)
Number of Alleles	**n=48**	**n=106**	**n=458**
Allele frequency			
transition	0.187	0.217	0.225
deletion	0.000	0.009	0.030

Table 7-1. Frequency of CYP2D6 mutant alleles in Parkinson's disease patients.

n=number of alleles.

	Heterozygous	Homozygous Normal
complex I	2.54 ± 1.01	2.41 ± 0.98
I/CS (x100)	2.58 ± 1.02	2.33 ± 1.23

Table 7-2. Complex I activity in the substantia nigra of Parkinson's disease patients homozygous normal or heterozygous for the transition mutation in the CYP2D6 gene.

Activity expressed as nmol/min/mg protein (mean ± SD).

Heterozygous (n = 8); Homozygous normal (n = 8); complex I (rotenone-sensitive NADH CoQ_1 reductase); I/CS (citrate synthase corrected complex I activity).

7.4. Discussion

The studies of Armstrong *et al* (1992) and Smith *et al* 1992) detected 4% (3/53) and 12% (27/229) of PD cases as poor metabolisers of debrisoquine respectively based on their genotype. The small number of samples (n=24) in this study was not sufficient to detect any mutation in the homozygous state, nor the base pair deletion mutation or gene deletion in the heterozygous state. Therefore none of the 24 PD samples included in the study carried the poor metaboliser genotype. Although the transition mutation was heterozygous in 9/24 PD samples, the poor metaboliser phenotype is only expressed when both alleles in an individual are mutant, so that heterozygotes are phenotypically normal. It is therefore not surprising that no relationship was detected between nigral complex I activity and heterozygosity of the transition mutation.

As more samples become available it should be possible to detect the poor metaboliser genotype and determine if this phenotype is associated with low nigral complex I activity in PD brains. If the poor metaboliser phenotype affects nigral complex I activity, then it is possible that a sub-set of PD patients have very low nigral complex I activity because they are poor metabolisers of debrisoquine; in support of the existence of a heterogeneous PD population, the spread of complex I activities in individual PD substantia nigra homogenates is fairly broad, ranging from 0.53 to 4.00 nmol/min/mg protein (n=24; Dr JM Cooper, personal communication).

Chapter 8. General Discussion

There is increasing evidence for the presence of a defect of mitochondrial complex I in Parkinson's disease (PD). However, the tissue specificity of the defect remains unclear. Within the brain, the defect appears to be restricted to the substantia nigra (Schapira *et al*, 1990b; Mann *et al*, 1992a), the principal area of cellular degeneration. However, the presence of a complex I defect in PD skeletal muscle remains contentious (review; Schapira, 1994). Work presented in this thesis has supported the presence of a complex I defect in PD platelets, a finding which now appears to be consistent (see Chapter 4). A slight (16%), but statistically significant complex I deficiency was detected in PD platelet mitochondrially-enriched fractions, but not whole platelet homogenates.

It is still unclear whether the complex I defect in the PD substantia nigra is primary and causes some of the other abnormalities observed, or is a secondary phenomenon. The tissue specificity of the complex I defect, its possible causes and any possible secondary effects of a complex I enzymatic deficiency are discussed below.

8.1. Tissue specificity of the complex I defect in PD

8.1.1. MRC function in the PD brain

Three groups have now published data on mitochondrial respiratory chain enzyme activities in the PD brain (Table 8-1). Mann *et al* (1992a) have reported a 30% mean decrease ($p<0.02$) in the citrate synthase (CS) corrected activity of complex I in the PD substantia nigra (Controls, $n=22$; PD, $n=17$). The activities of complex II/III and complex IV were not different from controls. However since the flow of electrons through complex II is the rate-limiting step in the complex II/II assay (Taylor *et al*, 1993) the presence of a slight complex III defect cannot be ruled out. Enzyme activities were expressed as a ratio of CS activity to correct for differences in mitochondrial numbers between samples; since CS is a mitochondrial matrix enzyme it will not be affected by an abnormality of the respiratory chain. Although unnecessary in samples of highly purified mitochondria, this is important in tissue homogenates or in impure

mitochondrial preparations (from platelets for example). The same group has detected no MRC abnormality in any other brain region besides the substantia nigra, including the caudate nucleus, cerebral cortex, medial and lateral globus pallidus, tegmentum, cerebellum or posterior putamen (Schapira *et al*, 1990b; Mann *et al*, 1992a; Dr JM Cooper, personal communication). Recently, Janetzky *et al* (1994) detected a 33% mean decrease ($p<0.05$) in the CS corrected activity of complex I in the PD substantia nigra (Controls, $n=7$; PD, $n=7$).

Mizuno *et al* (1990) measured the activities of complexes I, II, III and IV in PD ($n=5$) and control ($n=5$) mitochondrial preparations from post-mortem frozen striatum. They detected a 35% mean decrease ($p<0.05$) in the activity of complex III in the PD samples, which is at odds with the studies described above which detected no MRC abnormalities in any brain region besides the substantia nigra. Although only the activity of complex III was statistically significantly different between PD and control samples, the activities of the other complexes were reduced by between 17 and 29% in PD samples. Since none of these activities were CS corrected, these differences may reflect differences in mitochondrial numbers in the mitochondrial preparations.

It seems unlikely that a 37% complex I deficiency (Mann *et al*, 1992a) alone could account for the cell death observed in the PD substantia nigra. However, enzyme assays are, by necessity, performed on brain tissue homogenate, which will include both neurones and glia. Since neurones represent less than 3% of the total nigral cell population in PD (Schapira, 1994), and since neurones are more dependent on oxidative metabolism for ATP than glia (Schapira, 1994), it may be that the complex I defect in the neurones of the PD substantia nigra is much greater than 37%.

Schapira *et al* (1990b)		**Tissue homogenate***
SN	C = 6 PD = 7	42% deficiency of complex I ($p = 0.004$) 39% deficiency of CS corrected complex I Complexes II-IV not different from controls
CN	C = 5 PD = 4	Complexes I-IV not different from controls
CCTX	C = 5 PD = 4	Complexes I-IV not different from controls
CBL	C = 5 PD = 4	Complexes I-IV not different from controls
GP(m)	C = 5 PD = 4	Complexes I-IV not different from controls
GP(l)	C = 5 PD = 4	Complexes I-IV not different from controls
Mizuno *et al* (1990)		**Mitochondrial fractions***
STR	C = 5 PD = 5	35% Complex III deficiency ($p < 0.05$) Complexes I, II and IV not different from controls
FCTX	C = 5 PD = 5	Complexes I-IV not different from controls
Mann *et al* (1992a)		**Tissue homogenate***
SN	C = 22 PD = 17	37% deficiency of complex I ($p < 0.001$) 30% deficiency of CS corrected complex I ($p < 0.02$) Complexes II-IV not different from controls
TEG	C = 10 PD = 10	Complexes I-IV not different from controls
CBL	C = 16 PD = 16	Complexes I-IV not different from controls

Janetzky *et al* (1994)		**Tissue homogenate****
SN	C = 7 PD = 7	34% deficiency of complex I 33% deficiency of CS corrected complex I ($p < 0.05$)
CTX	C = 3 PD = 3	Complex I not different from controls
PUT	C = 3 PD = 3	Complex I not different from controls
Dr JM Cooper (1994[1])		**Tissue homogenate***
PPUT	C = 12 PD = 12	Complexes I-IV not different from controls

Table 8-1. Mitochondrial respiratory chain function in the PD brain.

* Complex I activity measured as rotenone-sensitive NADH CoQ_1 reductase;
** Complex I activity measured as rotenone-sensitive NADH cytochrome c reductase; [1] personal communication.

Not different means not statistically significantly different.

C (control); PD (Parkinson's disease); CS (citrate synthase); CBL (cerebellum); CCTX (cerebral cortex); FCTX (frontal cortex); CN (caudate nucleus); GP(l/m) (lateral/medial globus pallidus); PUT (putamen); PPUT (posterior putamen); SN (substantia nigra); STR (striatum); TEG (tegmentum).

8.1.2. MRC function in PD platelets

Since the original report of Parker *et al* (1989) of a 55% decrease in complex I activity in PD platelet mitochondrial fractions, there have been a further six reports of MRC function studies in platelet and lymphocyte homogenates and platelet mitochondrial fractions (Table 8-2). Studies described in this thesis (see Chapter 3) detected normal specific activities and CS corrected activities of complexes I, II/III and IV in platelet homogenates from 15 controls and 14 PD patients. Since the specific activities of MRC enzymes are rather low in platelet homogenates, it is possible that a slight MRC enzyme defect may be missed by measuring enzyme activities in these preparations. However, it seems unlikely that a 55% complex I defect, as reported by Parker *et al* (1989) would not be detected. Yoshino *et al* (1992) detected a 26% complex I defect and a 20% complex II defect in PD whole platelet homogenates, and in the same study detected a 13% complex II defect in PD lymphocyte homogenates (a complex I defect was not detected in PD lymphocyte homogenates). Barroso *et al* (1993) detected a 24% complex I defect and a 45% complex IV defect in PD lymphocyte homogenates. However, MRC enzyme activities were not corrected for variations in mitochondrial numbers (by expressing them as ratios of CS activity) in either of these studies (Yoshino *et al*, 1992; Barroso *et al*, 1993).

Three further studies of MRC function in PD platelet mitochondrial fractions have been published. Studies described in this thesis (see Chapter 4) detected a slight (16%) but statistically significant ($p=0.017$) reduction in the CS corrected activity of complex I in the platelet mitochondrially-enriched fractions from 25 PD patients compared to 15 controls. The activities of complexes II/III and IV were not different from controls. However this does not rule out the presence of a slight complex III defect since the flow of electrons through complex II is the rate-limiting step in the complex II/III assay (Taylor *et al*, 1993). Bravi *et al* (1992) measured MRC function in platelet mitochondrial fractions from 17 PD patients and 13 controls, and detected no differences in MRC function between groups. Lastly, Benecke *et al* (1993) detected 52% complex I and 30% complex IV deficiencies in platelet mitochondrial fractions from 27 PD patients compared to 44 controls.

Because platelets contain various granules whose density is similar to mitochondria, any procedure in which mitochondria are isolated on the basis of their density, will result in co-purification of these granules (Sixma and Lips, 1978). Electron microscopy of the mitochondrial fraction obtained from platelets by differential centrifugation has shown this to be the case (Figure 4-1). Since

the platelet mitochondrial fraction is not composed of pure mitochondria it remains important to correct for any differences in mitochondrial numbers between samples by expressing MRC enzyme activities as ratios of CS activity; neither Parker *et al* (1989) nor Benecke *et al* (1993) did this. Furthermore, Benecke *et al* (1993) measured complex I as rotenone-sensitive NADH DB (decylubiquinone) reductase activity; Estornell *et al* (1994) have shown that although this assay yields a high activity it shows non-linear rates with time, suggesting that it is not a suitable assay of complex I activity.

Besides the reports of Parker *et al* (1989) and Benecke *et al* (1993), every other study has found either normal or mildly reduced complex I activity in PD platelet homogenates or mitochondrial fractions. However, since PD is a clinically heterogeneous disease it may be caused by different factors in different individuals, and it is therefore possible that some PD patients do have gross MRC defects in their platelets. Another consideration of studies in PD platelets, and indeed skeletal muscle, is that the clinical diagnosis of PD can only be made with 80% accuracy (Hughes *et al*, 1992). Therefore a percentage of patients with parkinsonian neurodegenerative diseases are likely to be included in all these studies. A further potential difficulty of using platelets as a source of tissue to study enzyme function is that because they are present in the circulatory system they are immediately exposed to any circulating toxins, which may affect enzyme function.

The presence of a complex I deficiency in PD platelets may at first seem at odds with the apparent tissue specific nature of the complex I deficiency in the PD brain (Schapira *et al*, 1990b; Mann *et al*, 1992a). However platelets actively accumulate MPP^+ (Cesura *et al*, 1987), and contain high concentrations of MAO-B (Da Prada *et al*, 1985), the enzyme that converts MPTP to its toxic metabolite MPP^+. If the complex I deficiency in PD is caused by a toxin with similar uptake and conversion characteristics to MPTP, then this may explain the specificity of the defect to dopaminergic neurones in the substantia nigra and platelets.

Because there is a large overlap between individual complex I activities in PD and control platelet mitochondrial fractions (Figures 4-7, 4-8), the defect cannot be used as a diagnostic test for the disease. However, if PD is a heterogeneous disease, then patients with low platelet complex I activity may represent a subpopulation with increased susceptibility to some external factor. If this is indeed the case, then these patients may be of use in determining the molecular basis of this deficiency.

Platelet homogenates

This thesis*

C = 15 PD = 14	Complexes I-IV not different from controls

Yoshino *et al* (1992)*

C = 17 PD = 20	26% deficiency of complex I ($p < 0.001$) 20% deficiency of complex II ($p < 0.01$) Complexes III and IV not different from controls

Lymphocyte homogenates

Yoshino *et al* (1992)*

C = 17 PD = 20	13% complex II deficiency ($p < 0.05$) Complexes I, III and IV not different from controls

Barroso *et al* (1993)**

C = 15 PD = 16	24% complex I deficiency ($p < 0.05$) 45% complex IV deficiency ($p < 0.05$) Complexes II and III not different from controls

Platelet mitochondrial fractions

This thesis*	
C = 15 PD = 25	14% complex I deficiency 16% CS corrected complex I deficiency (p = 0.017) Complexes II, III and IV not different from controls
Parker *et al* (1989)*	
C = 8 PD = 10	55% complex I deficiency (p = 0.0009) Complexes II, III and IV not different from controls
Bravi *et al* (1992)**	
C = 13 PD = 17	Complexes I-IV not different from controls
Benecke *et al* (1993)***	
C = 44 PD = 27	52% complex I deficiency (p < 0.001) 30% complex IV deficiency (p < 0.001) Complex III not different from controls

Table 8-2. MRC function in PD platelets and lymphocytes.

* Complex I activity measured as rotenone-sensitive NADH CoQ_1 reductase; ** Complex I activity measured as rotenone-sensitive NADH cytochrome c reductase; *** Complex I activity measured as rotenone-sensitive NADH DB (decylubiquinone) reductase.

C (control); PD (Parkinson's disease); CS (citrate synthase). Not different means not statistically significantly different.

8.1.3. MRC function in PD skeletal muscle

Seven reports of MRC function in PD skeletal muscle have now been published (Table 8-3). Three of these reported normal MRC function in PD skeletal muscle (Mann *et al*, 1992a; Anderson *et al*, 1993; DiDonato *et al*, 1993). Bindoff *et al* (1991) detected generalised defects of complexes I, II and IV in five PD patients. Shoffner *et al* (1991) reported two patients with gross complex I defects (14 and 38% of the control mean), two patients with complex I-IV defects, one patient with complex II-IV defects and one patient with normal MRC function. Patient and control groups in this study were not age-matched; skeletal muscle MRC activity has been reported to decrease with age (Trounce *et al*, 1989; Cooper *et al*, 1992), although Shoffner *et al* (1991) detected no correlation between MRC enzyme activity and age. Cardellach *et al* (1993) reported 26% and 68% reductions in the activities of complex I and IV respectively in skeletal muscle mitochondria from eight PD patients compared to ten age-matched controls. Both the study of Shoffner *et al* (1991) and Cardellach *et al* (1993) measured complex I activity as rotenone-sensitive NADH DB (decylubiquinone) reductase activity; Estornell *et al* (1993) have shown that although this assay yields a high activity it shows non-linear rates with time, suggesting that it is not a suitable complex I assay. Nakagawa-Hattori *et al* (1992) detected a 49% complex I deficiency in the skeletal muscle mitochondria from four PD patients compared to six controls, although the source of skeletal muscle mitochondria was frozen, post-mortem tissue.

Although the presence of an MRC defect in PD skeletal muscle is controversial, available evidence suggests that the majority of PD patients do not have gross skeletal muscle MRC defects. However, since PD is clinically heterogeneous, it may be caused by different factors in different individuals, and it is therefore possible that some patients do have MRC defects in their skeletal muscle. Another consideration of these studies, as with those in platelets, is that the clinical diagnosis of PD is only 80% accurate (Hughes *et al*, 1992). Therefore a percentage of patients with parkinsonian neurodegenerative diseases are likely to be included in these studies. Since muscle weakness is not a clinical feature of PD it seems unlikely that gross MRC abnormalities would be present in skeletal muscle. Evidence against the presence of gross skeletal muscle MRC defects includes the lack of morphological abnormalities in PD skeletal muscle at biopsy (Bindoff *et al*, 1991; Shoffner *et al* 1991; Mann *et al*, 1992a), the lack of a resting or exercise-associated lactic acidosis (Bravi *et al*, 1992; Nakagawa-Hattori *et al*, 1991), and the finding of normal PD skeletal muscle energetics by ^{31}P magnetic resonance spectroscopy (MRS) (Taylor *et al*, 1994).

Bindoff *et al* (1991)	**Mitochondrial fractions***
C = 4 PD = 5	40% deficiency of complex I ($p < 0.004$) 49% deficiency of complex II ($p < 0.001$) 40% deficiency of complex IV ($p < 0.002$)
Shoffner *et al* (1991)	**Mitochondrial fractions*****
C = 11 PD = 6	Two patients with complex I deficiency (86% and 62% deficiencies) Two patients with complexes I-IV deficiency One patient with complexes II-IV deficiency One patient with complexes I-IV not different from controls
Mann *et al* (1992a)	**Mitochondrial fractions***
C = 6 PD = 9	Complexes I-IV not different from controls
Nakagawa-Hattori *et al* (1992)	**Post-mortem mitochondrial fractions***
C = 6 PD = 4	49% deficiency of complex I ($p < 0.01$) Complexes II-IV not different from controls
Anderson *et al* (1993)	**Mitochondrial fractions****
C = 6 PD = 7	Complexes I-IV not different from controls
Cardellach *et al* (1993)	**Mitochondrial fractions*****
C = 10 PD = 8	26% deficiency of complex I ($p = 0.004$) 68% deficiency of complex IV ($p = 0.00001$) Complexes II, III and IV not different from controls

DiDonato *et al* (1993)	**Muscle homogenates***
C = 8 PD = 16	CS corrected complexes I-IV not different from controls
DiDonato *et al* (1993)	**Mitochondrial fractions***
C = 6 PD = 6	Complexes I-IV not different from controls

Table 8-3. MRC function in PD skeletal muscle.

* Complex I activity measured as rotenone-sensitive NADH CoQ_1 reductase; ** Complex I activity measured as rotenone-sensitive NADH cytochrome c reductase; *** Complex I activity measured as rotenone-sensitive NADH DB (decylubiquinone) reductase.

C (control); PD (Parkinson's disease); CS (citrate synthase).

Not different means not statistically significantly different.

8.2. Possible causes of the complex I defect in PD

The cause of the complex I defect observed in the PD substantia nigra remains unknown. Possible causes include inhibition of the enzyme by an environmental toxin, free-radical mediated damage to the enzyme, or a genetic mutation in either nuclear or mitochondrial DNA. Each of these is discussed in turn below.

8.2.1. Environmental toxin

The discovery that the Parkinson-causing neurotoxin MPTP is a specific inhibitor of mitochondrial complex I (Nicklas *et al*, 1985), raises the possibility that the complex I deficiency observed in the idiopathic disease may be caused by an environmental toxin. If this putative toxin showed similar uptake and conversion characteristics to MPTP, it may explain the specificity of the complex I defect to the substantia nigra and platelets, as both dopaminergic neurones and platelets are able to convert MPTP to MPP^+ and actively accumulate MPP^+.

Epidemiological studies have correlated an increased incidence of PD with a number of environmental factors including rural living, well water drinking, pesticide/herbicide use and exposure to industrial chemicals (review; Tanner, 1989). Furthermore, defects in the cytochrome P450 detoxification system have been detected in PD at both the phenotypic (Benitez *et al*, 1990) and the genotypic (Armstrong *et al*, 1992; Smith *et al*, 1992) level. Both these pieces of evidence support the theory that PD may be caused by an increased susceptibility to a common environmental toxin due to an ineffective xenobiotic metabolising system. To determine any relationship between complex I activity in the PD substantia nigra and cytochrome P450 genotype, studies described in this thesis have begun to correlate various cytochrome P450 genotypes with complex I activity in the PD substantia nigra (see Chapter 7). Due to the small sample size and low frequency of mutant genotypes no correlation has yet been established. However it is interesting to speculate that a subpopulation of PD patients with low nigral complex I activity may have defective detoxification systems, allowing the accumulation of a complex I inhibitor.

8.2.2. Oxidative damage

Direct evidence of oxidative damage in the PD substantia nigra is accumulating; an increase in the levels of malondialdehyde and lipid hydroperoxides, and a decrease in the concentration of polyunsaturated fatty acids, the substrate of lipid peroxidation reactions, have all been observed (Dexter *et al*, 1986, 1989, 1994b). Although the levels of iron are increased in the PD substantia nigra, the levels of the iron-storage protein ferritin are contentious (see Introduction 1.6.3.1). The levels of reduced glutathione (GSH) are decreased in the PD substantia nigra (Sofic *et al*, 1992; Jenner *et al*, 1992), as well as in the substantia nigra of presymptomatic PD cases (Dexter *et al*, 1994a), which suggests that oxidative stress may be an important pathological process in the early stages of the disease. Conversely, the levels of iron and ferritin are normal in the substantia nigra of presymptomatic PD cases (Dexter *et al*, 1994a), which suggests that these factors may not be important in disease pathogenesis. Interestingly, Mann *et al* (1994) detected no correlation between complex I activity and iron levels or iron/ferritin ratios in the PD substantia nigra, which may suggest that the complex I defect in the PD substantia nigra (Mann *et al*, 1992a) is not caused by an increase in the levels of free iron.

Although there is good evidence for conditions of oxidative stress in the PD substantia nigra, there is little evidence to suggest that oxidative damage can specifically impair the activity of complex I of the mitochondrial respiratory chain (see Introduction 1.6.5.2). Using isolated mitochondria, *in vitro* models of oxidative stress have reported reductions in the activity of complexes I, II and III (Schewe *et al*, 1981; Narayabashi *et al*, 1982; Hillered and Ernster, 1983; Zhang *et al*, 1990), whilst *in vivo* models report complexes I, II, III and IV to be affected (Benzi *et al*, 1991; Thomas *et al*, 1993). One cell culture model of iron overload reported the activities of complexes I and IV reduced (Hartley *et al*, 1993). Since studies to date have detected a specific complex I deficiency and normal activities of the other respiratory chain enzymes in the PD substantia nigra (Mann *et al*, 1992a; Janetzky *et al*, 1994), this suggests that oxidative damage may not be the cause of this enzyme deficiency. Nevertheless, it may be that specific conditions exist within the substantia nigra, not accurately replicated in any *in vitro* or *in vivo* model, which cause a specific decrease in complex I activity as a result of oxidative damage.

8.2.3. Genetic defect

Although the majority of cases of PD are sporadic, autosomal dominant inheritance of the disease has been described in a few pedigrees (Golbe *et al*, 1990; and see Introduction 1.1.3), indicating that the disease can be caused by a primary genetic mutation. Recently, Lazzarini *et al* (1994) surveyed PD cases with and without affected parents and determined the frequency of affected siblings of probands. The proportion of probands with affected siblings was higher in the patients with affected parents, suggesting that PD may be dominantly inherited in a subset of patients. Using positron emission tomography (PET) with [^{18}F]dopa as a tracer to detect asymptomatic, preclinical PD cases has increased the concordance rates for monozygotic over dizygotic twins (Burn *et al*, 1992), providing further evidence that there is a strong genetic component to PD. Although in the majority of PD cases the disease is not dominantly inherited this does not imply that there is not a genetic component in all cases of PD. The theory that PD is a multifactorial disease, gives rise to the idea of a PD susceptibility gene, which could mask any inheritance pattern. For example, the gene may confer susceptibility to an environmental toxin, so that only individuals who carry the gene and who come into contact with the toxin go on to develop the disease. Presymptomatic PD cases may provide a valuable population in which to search for any PD susceptibility gene, since the presence of the gene may not necessarily lead to clinical signs of PD until other factors exert their effect.

Since complex I subunits are coded for by both nuclear and mitochondrial genes, a mutation in either of these genomes could account for the observed complex I defect. PD is not dominantly inherited, or maternally inherited (Zweig *et al*, 1992), in the majority of cases. Therefore a nuclear or mtDNA mutation cannot alone account for the disease in the majority of patients. However this does not necessarily imply that a complex I gene mutation does not contribute to the cause of PD. A mutation in a complex I gene may make the enzyme more susceptible to toxic inhibition or free radical mediated damage, or may itself lead to increased free radical production at the site, which in turn could further inhibit the enzyme. Alternatively, a complex I gene mutation may directly affect the structure and/or the function of the enzyme. The structure of the enzyme in the PD substantia nigra has been studied by immunoprecipitation and visualisation of the purified complex by SDS-denaturing polyacrylamide gel electrophoresis (SDS-PAGE) (see Chapter 5); no gross differences between PD and control samples were observed, although the mean staining intensities of two subunits were decreased in the PD samples. However, the PD samples available for this

particular part of the study did not have a complex I enzymatic defect relative to the controls used for comparison.

The levels of the complex I holoenzyme and three specific complex I subunits have been quantitated in the PD substantia nigra by ELISA (enzyme-linked immunosorbent assay) using affinity-purified antibodies (see Chapter 6). In a group of PD samples with a 40% complex I enzymatic deficiency, the levels of complex I holoenzyme and specific complex I subunits were decreased by approximately 40%, which suggests that in these samples the complex I enzymatic deficiency is caused by a reduced concentration of complex I protein.

8.3. Relationship between the complex I defect and other abnormalities in PD

The primary effect of a mitochondrial complex I deficiency is a decrease in ATP synthesis, with reduced cellular energy-requiring activity and ultimately cell death. However, there are a number of secondary effects of complex I dysfunction, which may also be contributory factors whose ultimate consequence is cell death (review; Olanow, 1993).

8.3.1. Oxidative stress

The mitochondrial respiratory chain is a major source of free radicals within the cell (Takeshige and Minakami, 1979), and inhibition of complex I increases free radical generation at this site (Turrens and Boveris, 1980; Hasegawa *et al*, 1990; and see Introduction 1.6.5.1). It is therefore possible that a complex I defect, initially caused by perhaps a bound toxin or an inherent genetic defect, could lead to increased free radical production, which could in turn damage a range of cellular components, including complex I. Oxidative damage to this enzyme may lead to increased free radical production at this site, which may damage the enzyme further, and so on in a self-perpetuating cycle. However, at present the interrelationship between the complex I defect and the observed conditions of oxidative stress in the PD substantia nigra remains unclear.

8.3.2. Calcium cytotoxicity

Since extracellular calcium concentrations are 1000-fold higher than cytosolic concentrations, the cell requires an efficient calcium extrusion pump to remove calcium which continually enters the cell. Calcium is extruded from the cell against this concentration gradient by an energy requiring plasma membrane calcium pump. Under normal conditions, cytosolic Ca^{2+} is sequestered primarily by endoplasmic reticulum (ER), but if it rises to abnormally high levels mitochondria have the ability to uptake the excess. Mitochondrial Ca^{2+} uptake is driven by the membrane potential. Cytosolic Ca^{2+} overload can therefore result from increased cellular influx, decreased cellular efflux or impairment of sequestration within ER or mitochondria. The cytotoxic effects of an increased cytosolic Ca^{2+} are mediated via a number of mechanisms; nuclear Ca^{2+} uptake and DNA fragmentation, mitochondrial accumulation and impaired mitochondrial function and activation of calcium-dependent proteases, lipases and endonucleases.

Mitochondrial respiratory chain dysfunction leading to a decrease in ATP generation would deprive the cell of its capacity to extrude or sequester calcium via these ATP-dependent mechanisms. Indeed, hepatocytes treated with MPP^+ exhibit depletion of mitochondrial Ca^{2+} and marked and sustained elevation of cytosolic Ca^{2+}, leading to cell death (Kass *et al*, 1988). Therefore, one of the mechanisms of MPP^+ cytotoxicity may be via an elevation of cytosolic Ca^{2+} levels. In the PD substantia nigra, surviving dopaminergic neurones contain high concentrations of the calbindin calcium binding proteins (Yamada *et al*, 1990), which may suggest that increased intracellular Ca^{2+} binding is protecting these cells from Ca^{2+} cytotoxicity.

8.3.3. Excitotoxicity

Excitatory amino acids (EAAs), for example glutamate and aspartate, have excitatory effects on neurones, but at high doses are neurotoxic or "excitotoxic" (review; Beal *et al*, 1993a). This excitotoxicity is thought to be mediated via persistent stimulation of EAA receptors, which opens voltage-dependent calcium channels and allows the influx of calcium into the cell. As discussed above, increased cytosolic levels of Ca^{2+} are cytotoxic.

Inhibition of mitochondrial ATP generation causes normal endogenous levels of

EAAs to become excitotoxic (Novelli *et al*, 1988). It is thought that decreased ATP levels reduce the plasma membrane resting potential, which in turn removes the voltage-sensitive Mg^{2+} block on NMDA (N-methyl-D-aspartate; a glutamate agonist) receptors. Removal of this block allows stimulation of the receptors, which then become permeable to calcium.

Although the role of excitotoxic mechanisms in MPP^+-induced damage is still rather controversial (review; Schapira, 1993), it has been demonstrated in both rats (Storey *et al*, 1992) and primates (Zuddas *et al*, 1992) that the toxicity of MPP^+ to dopaminergic neurones of the substantia nigra can be blocked by co-administration of the NMDA antagonist MK-801. Furthermore, the administration of the irreversible mitochondrial complex II inhibitor 3-nitropropionic acid (3-NP) or the reversible complex II inhibitor malonate in rats, causes striatal lesions (Brouillet *et al*, 1993; Beal *et al*, 1993b), and the neurotoxic effects of both can be blocked by co-administration of the NMDA receptor antagonist MK-801. Interestingly, the neurotoxic effects of both 3-NP and malonate have been shown to be highly age-dependent, aged animals being more susceptible than young animals (Brouillet *et al*, 1993; Beal *et al*, 1993b). These results demonstrate not only that mitochondrial respiratory chain toxins can cause excitotoxic cell death, but that their effects may be age-dependent. This may be of relevance if excitotoxic mechanisms are involved in the process of cell death in PD, since PD, and many other neurodegenerative diseases, are diseases of late-onset, and mitochondrial function has been shown to decrease with age (see Introduction 1.4.6.1).

8.3.4. Programmed cell death

Programmed cell death or "apoptosis" is distinct from necrosis, cell death initiated by cell injury, in which cells swell and lyse (review; Altman, 1992). By contrast apoptosis is an active process during which the cell nucleus and cytoplasm shrink and fragment, and cells are phagocytosed by neighbours without eliciting an inflammatory response. The extreme view of apoptosis is that all cells are genetically programmed to undergo a programmed cell death, and that factors exist which suppress this intrinsic programme (Raff, 1992). During development of the nervous system a huge proportion of unwanted cells undergo apoptotic death, due to a lack of trophic factors, and neuronal cells in culture undergo apoptotic death when deprived of growth factors. This raises the possibility that cell death in neurodegenerative diseases may be apoptotic.

Current research is directed towards identifying factors that can precipitate, or protect against, apoptosis. The proto-oncogene bcl-2 protects against apoptosis and necrosis in a variety of systems, and there is now evidence that it may be functioning as an antioxidant. The overexpression of bcl-2 can prevent cell death mediated by H_2O_2, which is morphologically similar to an apoptotic cell death (Hockenberry *et al*, 1993). Similarly the overexpression of bcl-2 protected a neural cell line from a necrotic cell death resulting from glutathione depletion and increased free radical damage (Kane *et al*, 1993).

Factors which have been shown to induce apoptosis are increased cytosolic Ca^+ (Richter, 1993), the excitatory amino acid glutamate (Kure *et al*, 1991) and oxidative stress (Hockenberry *et al*, 1993). The toxic effects of MPP^+ may also be partly mediated by apoptotic processes, since the administration of MPP^+ to cultured neurones can induce apoptosis (Dipasquale *et al*, 1991; Mochizuki *et al*, 1994). Furthermore, the complex I inhibitor rotenone and the complex III inhibitor antimycin A have both been shown to induce apoptosis in cultured human lymphoblastoid cells (Wolvetang *et al*, 1994). These results suggest that inhibition of the MRC can lead to apoptotic cell death, and raise the possibility that MRC deficiencies in the PD substantia nigra may cause some cells to die by apoptosis.

Chapter 9. Further work

The results of experiments presented in Chapters 3-7 have begun to address the issues of the tissue specificity and molecular basis of the complex I defect in Parkinson's disease (PD). Further experiments, which would extend the above results, are outlined below.

9.1. Platelet enzymatic analysis

Results presented in Chapter 4 describe a 16% decrease in the activity of complex I detectable in platelet mitochondrially-enriched fractions (MEFs) from PD patients. There was no difference in the mean complex I activity between treated and untreated patients, although only four of the 25 PD patients recruited for the study were untreated, and had not taken any l-dopa at the time of sample. Furthermore there was no correlation of complex I activity with the amount of l-dopa ingested in the 21 treated patients . Nevertheless, to confirm that the complex I deficiency in platelets from PD patients is not due to l-dopa therapy more untreated patients would be recruited.

To determine the disease specificity of the platelet complex I defect, platelet MEFs would be prepared from patients with neurological diseases other than PD, and the activities of mitochondrial respiratory chain (MRC) enzymes would be determined. These disease controls would include patients with multiple system atrophy (MSA) and Huntington's disease (HD). The former have been used as a control for MRC enzyme assays in the PD substantia nigra (Schapira *et al*, 1990b), as this disease is characterised by nigral cell loss at least as severe as that seen in PD. HD is a neurodegenerative disease not involving nigral cell loss, and Parker *et al* (1990a) have detected a complex I defect in the platelets of patients with HD.

There was a large spread of platelet complex I activities from individual PD patients (see Figures 4-7, 4-8). This variability may be due to individual variation within a heterogeneous population. However, if PD is a multifactorial disease caused by different factors in different individuals, complex I deficiency may be the primary abnormality in some subgroups of PD patients. These subgroups

may have lower platelet complex I activities than any others. To identify any subgroups of PD patients with lower platelet complex I activity, patients with familial PD and young-onset PD would be recruited and their platelet MRC enzyme activities measured.

9.2. Identification of a circulating toxin

If the platelet complex I deficiency in PD patients is caused by a circulating toxin, the toxin will be present in serum and/or platelets of PD patients. To test this hypothesis, PD patients serum and whole platelet homogenates would be incubated with control platelet mitochondria, and the effect on complex I activity determined. Initially, patients with low platelet complex I activity would be chosen as the donors of serum and platelets. One potential difficulty of these experiments is that the complex I deficiency in PD patients platelet mitochondria is rather small (16%). Therefore any effect of a circulating toxin in PD patients serum or platelets on complex I activity in control platelet mitochondria may be difficult to detect.

9.3. Immunoprecipitation of complex I

Eighteen subunits were detectable in complex I immunoprecipitated from PD substantia nigra homogenate and subjected to SDS-denaturing polyacrylamide gel electrophoresis (PAGE) (see Chapter 5). No gross changes in concentration or molecular weight were detected. However, the PD samples available for immunoprecipitation experiments did not have a complex I enzymatic deficiency compared to the control group, and therefore would not necessarily be expected to contain abnormal complex I protein.

Complex I would be immunoprecipitated from a group of PD substantia nigra samples which had a complex I enzymatic deficiency relative to controls, and the polypeptide profiles compared. As well as SDS-PAGE, complex I immunoprecipitates would be subjected to isoelectric focusing (IEF) electrophoresis, which separates proteins according to charge rather than mass, and may be able to detect changes in the protein not detected by SDS-PAGE. If there was enough sample, complex I subunits would also be separated by two-dimensional electrophoresis, which has a much greater resolving capacity

than one-dimensional SDS-PAGE or IEF alone. If any abnormality of complex I from PD substantia nigra was detected, the study would be extended to include other regions of the PD brain (to determine its tissue specificity within the brain), to PD skeletal muscle and platelet mitochondria (since complex I deficiencies have been reported in both; see Introduction 1.4.2 and Chapter 4 respectively), and to the substantia nigra of other neurodegenerative diseases (to determine its disease specificity). Since the gene sequences of all bovine complex I subunits are now known (review; Walker *et al*, 1992), it is potentially possible to produce specific antibodies to every subunit by immunising with peptides specific to each. These antibodies could be used to positively identify every complex I subunit in immunoprecipitated material.

A two-dimensional electrophoretic technique has been described which potentially allows the visualisation of all respiratory chain complexes from either purified mitochondria or tissue homogenate (Schagger and von Jagow, 1991). The method relies on coomassie dyes to induce a charge shift in the protein complexes and aminocaproic acid to solubilise the complexes, allowing the separation of intact respiratory chain complexes during first-dimension non-denaturing electrophoresis. The individual polypeptides of each complex are then separated during second-dimension SDS-denaturing electrophoresis. Assuming that the technique can be applied to brain homogenate samples, the respiratory chain complexes of PD and control substantia nigra would be compared using this technique.

9.4. Quantitation of complex I by ELISA

Studies described in Chapter 6 detected reduced concentrations of complex I protein in PD substantia nigra samples with a complex I enzymatic defect. To determine whether or not this reduction in protein concentration is specific to complex I, the concentration of another mitochondrial protein, for example ubiquinol cytochrome c reductase (complex III), would be determined in PD and control substantia nigra samples. To determine whether the concentration of one or more specific complex I subunits are reduced, antibodies specific to more complex I subunits would be affinity purified for use in the ELISA. The only constraint to the number of individual complex I subunits which can be quantitated by ELISA is the availability of specific antibodies; to date for example, no antibodies to mitochondrially encoded complex I subunits are available.

As with the immunoprecipitation experiments, if further ELISA studies confirmed a reduction in complex I protein concentration in PD substantia nigra samples with low complex I activity, the study would be extended to include other regions of the PD brain (to determine tissue specificity within the brain), to PD skeletal muscle and platelet mitochondria (since complex I deficiencies have been reported in both; see Introduction 1.4.2 and Chapter 4 respectively), and to the substantia nigra of other neurodegenerative diseases (to determine the disease specificity of the defect).

9.5. Correlation of complex I activity with genetic mutations

Chapter 7 describes initial experiments to correlate nigral complex I activity with mutations in the cytochrome P450 debrisoquine hydroxylase gene (CYP2D6). These results would be extended to include more substantia nigra samples, and to include samples from PD patients whose platelet complex I activity had been measured. The presence of the $tRNA^{Gln}$ mutation in mtDNA (Shoffner *et al*, 1993) would also be correlated with complex I activity in these samples. Although these mutations are found at increased frequencies in PD patients, their frequency is still rather low, so that large sample numbers would be required to gain any meaningful correlations between the presence of mutations and complex I activity.

Any newly discovered gene mutations which may be regarded as potential PD susceptibility genes (for example the dopamine transporter gene and vesicular amine transporter genes; see Introduction 1.2.3, 1.2.4), or which may affect complex I activity, would also be correlated with complex I activity in PD samples.

Appendix

1. Preparation of mitochondria

1.1. Modified Tyrodes Hepes Buffer

(Clare and Scrutton, 1984)

150mM	NaCl
5mM	HEPES
0.55mM	NaH_2PO_4
7mM	$NaHCO_3$
2.7mM	KCl
0.5mM	$MgCl_2$
5.6mM	Glucose
1mM	EDTA (di-K)
pH7.4	

1.2. Homogenisation medium (platelets)

0.25M	Sucrose
10mM	Tris
1mM	EDTA (di-K)
pH7.4	

1.3. Isolation medium (brain)

0.32M	Sucrose
10mM	Tris
1mM	EDTA (di-K)
pH7.4	

1.4. High EDTA isolation medium (skeletal muscle)

225mM	Mannitol
75mM	Sucrose
10mM	Tris
10mM	EDTA (di-K)
pH7.4	

1.5. Low EDTA isolation medium (skeletal muscle)

225mM	Mannitol
75mM	Sucrose
10mM	Tris
100μM	EDTA (di-K)
pH7.4	

2. SDS-PAGE

2.1. Gel loading buffer

10mM	Tris
1mM	EDTA (di-Na)
2.5% (w/v)	SDS
0.01% (w/v)	Bromophenol blue
0.002% (v/v)	Pyronin Y
5% (v/v)	Mercaptoethanol
pH8.0	

2.2. Acrylamide gel composition (2x 0.75mm mini-gels)

(Laemmli, 1970)

	Running gel (15%)	Stacking gel (5%)
Stock concentrations		
29.2% Acrylamide:0.8% Bisacrylamide	5.0ml	---
7.6% Acrylamide:0.6% Bisacrylamide	---	5.0ml
1.5M Tris pH8.8	2.5ml	---
0.5M Tris pH6.9	---	2.0ml
10% (w/v) SDS	0.1ml	0.1ml
10% Ammonium persulphate	0.1ml	0.1ml
ddH_2O	2.3ml	0.8ml
	10.0ml	8.0ml

Polymerisation was initiated by the addition of 20μl TEMED

2.3. Electrode buffer

25mM	Tris
194mM	Glycine
0.1% (w/v)	SDS

3. Silver staining SDS-PAGE gels

3.1. Silver staining mini-gels

(Heukeshoven and Dernick, 1985)

Fix

40% (v/v)	Ethanol
10% (v/v)	Acetic acid

Incubation solution

30% (v/v)	Ethanol
0.5M	Sodium acetate
8mM	Sodium thiosulphate
0.13% (v/v)	Glutaraldehyde

Silver solution

0.1% (w/v)	Silver nitrate
0.008% (v/v)	Formaldehyde

Developing solution

236mM	Sodium carbonate
0.004% (v/v)	Formaldehyde

Stop solution

40mM	EDTA (di-Na)

3.2. Silver staining PhastGels

Step	Solution	Time(mins)	Temp(°C)
1	Wash I	2	50
2	Wash I	5	50
3	Sensitisation	6	50
4	Wash I	3	50
5	Wash I	5	50
6	Wash II	2	50
7	Wash II	2	50
8	Stain	7	40
9	Wash II	0.5	30

10	Wash II	0.5	30
11	Developer	0.5	30
12	Developer	7	30
13	Background reducer	1	30
14	Stop	5	50

Wash solution I

10% (v/v)	Ethanol
5% (v/v)	Acetic acid

Wash solution II

ddH_2O

Sensitisation solution

5% (v/v)	Glutaraldehyde

Staining solution

0.4% (w/v)	Silver nitrate

Developing solution

235mM	Tris.HCl
100mM	Sodium thiosulphate

Background reducer

2.5% (w/v)	Sodium carbonate
0.0133% (v/v)	Formaldehyde

Stop solution

10% (v/v)	Glycerol

4. Western blotting SDS-PAGE gels

4.1. Transfer buffer

(Towbin *et al*, 1979)

25mM	Tris
194mM	Glycine
20% (v/v)	Methanol
pH8.4	

4.2. Substrate buffer (alkaline phosphatase)

0.1M	Tris
0.1M	NaCl
50mM	$MgCl_2$
pH9.0	

NBT:	dissolved in 200μl 70% (v/v) dimethyl formamide (DMF)
BCIP:	dissolved in 200μl 100% DMF

5. Enzyme-linked immunosorbent assay (ELISA)

5.1. Phosphate Buffered Saline (PBS)

8.3mM	Na_2HPO_4
1.5mM	KH_2PO_4
154mM	NaCl
2.7mM	KCl
pH7.4	

PBS_T:	plus Tween 20 (variable concentration; detailed)
PBSa:	plus 0.0002% (w/v) Sodium azide

5.2. Coating buffer

0.1M	Sodium carbonate
pH9.6	

5.3. Substrate buffer (horseradish peroxidase)

0.05M	Phosphate-citrate buffer
0.03% (w/v)	Sodium perborate
0.4mg/ml	O-phenylenediamine (OPD) dihydrochloride
pH5.0	

6. Extraction of DNA/agarose gel electrophoresis

6.1. Extraction buffer

75mM	NaCl
50mM	EDTA (di-K)

6.2. TE buffer

10mM	Tris
1mM	EDTA (di-K)
pH7.4	

6.3. Gel loading buffer (DNA)

5% (w/v)	Sucrose
5mM	EDTA (di-Na)
0.01% (w/v)	Bromophenol blue
0.001% (w/v)	SDS

6.4. TBE buffer

90mM	Tris
90mM	Boric acid
2.5mM	EDTA (di-Na)

7. Protein assay

(Lowry *et al*, 1951)

Solution A

2% (w/v)	Na_2CO_3
0.1M	NaOH

Solution B

1% (w/v)	$CuSO_4$

Solution C

2% (w/v) K Na Tartrate

Solution I

100ml Solution A
+ 1ml Solution B
+ 1ml Solution C

Solution II

1:2 dilution of Folin and Ciocalteau's phenol reagent in ddH_2O

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BRAIN, SKELETAL MUSCLE AND PLATELET HOMOGENATE MITOCHONDRIAL FUNCTION IN PARKINSON'S DISEASE

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SUMMARY

The recent discovery of mitochondrial complex I deficiency in the substantia nigra of patients with idiopathic Parkinson's disease has provided new understanding into the possible mechanisms that may underlie this neurodegenerative disorder. The biochemical defect is identical to that induced in humans, primates and mice exposed to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. We have studied mitochondrial respiratory chain function in various brain regions, in skeletal muscle and in blood platelets from patients with idiopathic Parkinson's disease and from matched controls. We provide evidence suggesting that the complex I deficiency in Parkinson's disease is limited to the brain and that this defect is specific for the substantia nigra. The tissue specificity of the complex I deficiency in Parkinson's disease and its localization to the substantia nigra support the proposition that complex I deficiency may be directly involved in the cause of dopaminergic cell death in Parkinson's disease. An understanding of the molecular basis of this biochemical defect will provide valuable insight into the cause of Parkinson's disease. Our finding of normal mitochondrial function in platelet homogenates suggests that this tissue cannot be used to develop a 'diagnostic test' for Parkinson's disease.

INTRODUCTION

The recent discovery of NADH CoQ_1 reductase (complex I) deficiency in Parkinson's disease (Schapira *et al.*, 1989, 1990*a*) has focused attention on the possible role of abnormal mitochondrial function in the pathogenesis of this disease. The first indication that abnormal mitochondrial function may be involved in Parkinson's disease came from analysis of the mechanism of action of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP produces parkinsonism in humans (Davis *et al.*, 1979; Langston *et al.*, 1983) and other primates (Burns *et al.*, 1983; Langston *et al.*, 1984) by inducing death of dopamine containing cells in the substantia nigra. MPTP is converted to its active metabolite 1-methyl-4-phenylpyridium (MPP^+) by glial monoamine oxidase B (MAO-B) (Salach *et al.*, 1984), an enzyme of the outer mitochondrial membrane. MPP^+ is accumulated within dopaminergic neurons by the dopamine re-uptake system (Javitch *et al.*, 1985) and is then concentrated into mitochondria by a specific-energy-dependent system capable of producing millimolar intramitochondrial concentrations of MPP^+ (Ramsay *et al.*, 1986). MPP^+ has been

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found to be a specific inhibitor of complex I both *in vivo* (Mizuno *et al.*, 1988) and *in vitro* (Nicklas *et al.*, 1985). The inhibition of complex I leads to a fall in adenosine triphosphate (ATP) production which ultimately results in cell death (Denton and Howard, 1987).

The demonstration of complex I deficiency in Parkinson's disease substantia nigra has provided a direct link between the idiopathic disease and the MPTP model. The recent demonstration that complex I deficiency is anatomically specific to the substantia nigra and disease specific for Parkinson's disease (Schapira *et al.*, 1990*c*) enhances the likelihood that this biochemical defect may be involved in the pathogenesis of Parkinson's disease. We have extended our initial studies on complex I deficiency in Parkinson's disease substantia nigra and have also examined mitochondrial function in skeletal muscle and platelet homogenates from parkinsonian patients.

METHODS

Patients

Muscle biopsies and platelet studies were performed with the full and informed consent of the patients and with the approval of the Joint Ethics Committee of the National Hospital for Neurology and Neurosurgery and the Institute of Neurology, Queen Square, London. The diagnosis of Parkinson's disease was based on the presence of an akinetic rigid syndrome with asymmetric onset, a resting tremor and a positive response to apomorphine or L-dopa. Control muscle biopsies and blood for platelet studies were obtained from patients with no clinical evidence of neurodegenerative disease. Disease controls used in the platelet studies were also age-matched patients with no clinical evidence of any neurodegenerative disorder. This group included patients with multiple sclerosis, Guillian-Barré syndrome and carcinoma. Post-mortem brain samples were provided by the Parkinson's Disease Society Brain Bank. Parkinson's disease was confirmed pathologically in the contralateral fixed half brain by degeneration of the pars compacta of the substantia nigra and by the presence of Lewy bodies in nigral neurons. Control brain samples were removed from age-matched patients who had died without evidence of neurological or psychiatric disease and without pathology in the substantia nigra.

Brain samples

Samples of substantia nigra, tegmentum and cerebellum were removed from liquid nitrogen storage, weighed, homogenized in 9 vols of ice-cold medium (320 mM sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4) using a glass-glass homogenizer. Protein concentration was measured as previously described (Lowry *et al.*, 1951). After three cycles of freeze-thawing, mitochondrial enzymes were assayed blind and in triplicate in a final volume of 1 ml at 25°C. NADH CoQ_1 reductase (complex I), succinate cytochrome *c* reductase (complexes II and III), cytochrome oxidase (complex IV) and citrate synthase were assayed by standard techniques (Schapira *et al.*, 1990*a*,*c*). Rotenone sensitive values were 40–65% of total NADH CoQ_1 reductase rates. For the succinate cytochrome *c* reductase assay the protein sample was maximally activated in the presence of succinate for 5 min at the assay temperature.

Muscle biopsies

Muscle biopsies were removed from left vastus lateralis under a brief, light general anaesthetic. Samples were processed for routine histochemistry and biochemical studies. Mitochondria were isolated within 90 min of biopsy as previously described (Holt *et al.*, 1989). Purified mitochondrial preparations were used immediately for polarographic determination of oxygen uptake and enzyme analysis. Assays of respiratory chain enzyme activities from purified mitochondria were performed as described for the brain samples. Rotenone sensitive rates for purified mitochondria were 64–82% of total NADH CoQ_1 reductase rates.

Platelet studies

Platelet homogenates were prepared from 30 ml of venous blood taken without tourniquet. Blood was mixed 9:1 with 3.8% (w/v) tri-sodium citrate, and platelet-rich plasma (PRP) separated at 12°C by

centrifugation at 200 *g* for 20 min. The top three-quarters of the PRP was removed and centrifuged at 1000 *g* for 30 min. All subsequent steps were performed at 4°C. The resulting platelet pellet was gently resuspended in 15 ml Tyrodes buffer (pH 7.4) with 1 mM EDTA added, and the platelets pelleted by centrifugation at 1000 *g* for 15 min. The platelets were washed once more. The platelet pellet was gently resuspended on 0.75 ml ice-cold medium (250 mM sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4) and disrupted with a glass-glass homogenizer. Mitochondrial respiratory chain enzyme activities were measured as for brain samples, but at 30°C. Rotenone sensitive rates were 23–37% of total NADH CoQ_1 reductase rates.

RESULTS

Brain samples

Table 1 summarizes the age, death to refrigeration and death to autopsy times of the various cohorts of control and parkinsonian patients. All patient groups were well matched to their controls for all these parameters. An additional 16 control and 10 parkinsonian substantia nigra, and 11 control and 12 parkinsonian cerebellum samples have been analysed and added to those studied previously (Schapira *et al.*, 1990*c*). The results of tegmentum analysis have not been reported previously. The only significant difference from control for any of the respiratory chain enzymes in any brain area examined was complex I activity in parkinsonian substantia nigra, which decreased by 37% of the control mean. Table 2 shows the correction of enzyme activities by citrate synthase activities to account for any variation in mitochondrial numbers between tissues. Complex I activity in parkinsonian substantia nigra remains the only enzyme activity significantly different from control. There was no correlation of complex I activity with the duration of the patients' Parkinson's disease (n = 14, r = 0.36), L-dopa treatment (n = 12, r = 0.12), death to freezing of brain (n = 17, r = 0.38). Controls also failed to show any correlation to complex I activity for storage parameters.

Muscle samples

Histochemical analysis of muscle biopsies from patients with Parkinson's disease showed no significant morphological abnormality. The substrates used in polarographic

TABLE 1. REGIONAL BRAIN ACTIVITIES OF MITOCHONDRIAL RESPIRATORY CHAIN ENZYMES

		Age (yrs)	*Death to refrigeration (min)*	*Death to autopsy (h)*	*NADH CoQ_1 rotenone sensitive*	*SCcR antimycin a sensitive*	*COX (k/min/mg)*	*CS*
Substantia nigra	C (n = 22)	72.4±13.4	151±76	16.6±7.2	3.90±1.24	11.86±2.54	1.09±0.38 (n = 19)	115.2±27.8
	PD (n = 17)	75.4±7.8	127±48	15.4±9.5	2.48±0.93*	11.01±2.08	1.02±0.31 (n = 13)	111.0±37.5
Tegmentum	C (n = 10)	75.9±8.2	187±71	16.3±8.5	2.59±0.86	8.65±1.73	0.76±0.17	92.1±16.3
	PD (n = 10)	75.8±8.2	133±50	14.7±8.1	2.55±0.48	8.46±1.85	0.89±0.20	108.0±26.4
Cerebellum	C (n = 16)	73.9±13.3	140±85	17.5±8.0	3.98±2.08	15.88±4.69	1.05±0.27	200.7±36.9
	PD (n = 16)	74.5±7.2	130±48	16.1±9.3	3.61±1.06	15.90±4.26	1.01±0.30	200.4±34.7

C = control; PD = Parkinson's disease; NADH CoQ_1 = NADH ubiquinone oxidoreductase (complex I); SCcR = succinate cytochrome *c* reductase (complexes II and III); COX = cytochrome oxidase (complex IV); CS = citrate synthase. Results are expressed as mean±SD. Enzyme activities are in nmol/min/mg total protein, unless stated; k is the first order rate constant. Significance relative to control by Student *t* test and Mann-Whitney U test. * $P = < 0.001$.

TABLE 2. REGIONAL BRAIN ACTIVITIES OF MITOCHONDRIAL RESPIRATORY CHAIN ENZYMES CORRECTED FOR CITRATE SYNTHASE ACTIVITIES

		NADH CoQ_1/CS (×100)	*SCcR/CS (×10)*	*COX/CS (×100)*
Substantia nigra	C (n = 22)	3.56 ± 1.33	1.06 ± 0.28	1.02 ± 0.56 (n = 19)
	PD (n = 17)	2.50 ± 1.08*	1.09 ± 0.38	1.05 ± 0.51 (n = 13)
Tegmentum	C (n = 10)	2.87 ± 1.00	0.96 ± 0.26	0.83 ± 0.17
	PD (n = 10)	2.43 ± 0.53	0.79 ± 0.13	0.83 ± 0.13
Cerebellum	C (n = 16)	1.99 ± 0.97	0.82 ± 0.31	0.55 ± 0.19
	PD (n = 16)	1.81 ± 0.45	0.80 ± 0.21	0.51 ± 0.16

*$P < 0.02$ Student t test and Mann-Whitney U test. Abbreviations as for Table 1.

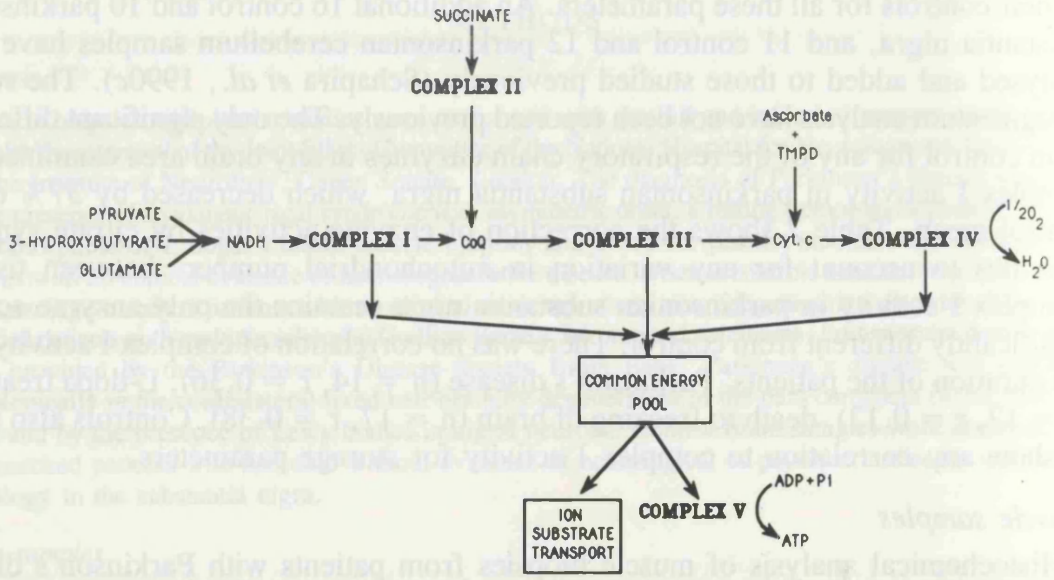

FIG. 1. The mitochondrial respiratory chain. TMPD denotes *NNN'N'*-tetramethyl-*p*-phenylenediamine, CoQ coenzyme Q, Cyt. c cytochrome *c*, ADP adenosine diphosphate, and P_i inorganic phosphate. [Reprinted by permission of the *New England Journal of Medicine*, **323**, 37–42 (1990)].

analysis test oxygen utilization by each part of the respiratory chain (Fig. 1). Results of polarography of freshly isolated mitochondria from eight patients with Parkinson's disease (five untreated, three treated) did not show any significant difference in mitochondrial function when compared with age-matched controls (Table 3). In addition, spectrophotometric analyses of respiratory chain enzyme activities of muscle mitochondria from nine parkinsonian patients (five untreated, four treated) failed to show any difference when compared with age-matched controls (Table 4). There was no significant difference in mitochondrial function between treated and untreated patients by polarography or enzyme analysis.

Platelet studies

Two of the patients with Parkinson's disease were untreated. There was no difference in any of the mitochondrial respiratory chain activities between controls, disease controls

TABLE 3. POLAROGRAPHIC ANALYSIS OF SKELETAL MUSCLE MITOCHONDRIA

	Age	*Pyruvate*	*Glutamate*	*Succinate*	*Ascorbate/TMPD*
C (n = 5)	61.2±6.9	85±13	76±27	131±59	338±127
PD (n = 8)	62.4±7.6	83±12	85±29	119±19	227±113

TMPD = *NNN'N'*-tetramethyl-phenylenediamine. Results expressed as nmol 0/min/mg mitochondrial protein.

TABLE 4. MITOCHONDRIAL RESPIRATORY CHAIN ACTIVITIES OF SKELETAL MUSCLE MITOCHONDRIA

	Age (yrs)	*NADH CoQ_1 rotenone sensitive*	*SCcR antimycin a sensitive*	*COX (k/min/mg)*
C (n = 6)	63.2±1.9	95.6±37.9	204±72	25.2±11.4
PD (n = 9)	62.4±7.8	90.5±21.1	258±130	36.5±13.8

Results expressed as nmol/min/mg mitochondrial protein unless stated.

TABLE 5. PLATELET MITOCHONDRIAL RESPIRATORY CHAIN ENZYME FUNCTION, ABSOLUTE AND CITRATE SYNTHASE CORRECTED

	Age (yrs)	*NADH CoQ_1 rotenone sensitive*	*SCcR antimycin a sensitive*	*COX (k/min/mg)*	*CS*	*NADH CoQ_1/CS (×100)*	*SCcR/CS (×10)*	*COX/CS (×100)*
Control n = 9	59.10±10.2	3.53±0.72	8.92±1.27	0.39±0.16	85.74±16.4	4.20±1.03	1.06±0.20	0.476±0.194
Disease control n = 6	57.17±10.4	3.65±1.11	9.67±3.43	0.52±0.09	86.82±3.81	4.21±1.32	1.11±0.38	0.595±0.095
PD n = 14	58.90±10.1	3.51±0.79	9.96±2.00	0.50±0.13	88.13±16.9	4.16±1.32	1.14±0.15	0.564±0.108

Results expressed as nmol/min/mg total protein unless stated. Abbreviations as for Table 1.

and Parkinson's disease patients (Table 5). Correction of activities for citrate synthase levels also failed to show any significant difference between the three groups.

DISCUSSION

These results support the anatomic specificity of complex I deficiency of the substantia nigra in Parkinson's disease. The results of this and a previous study (Schapira *et al.*, 1990*c*) have demonstrated normal mitochondrial function in parkinsonian cerebral cortex, cerebellum, caudate nucleus, putamen, medial and lateral globus pallidum, and tegmentum. Our finding of normal mitochondrial function in parkinsonian skeletal muscle is not surprising, therefore, bearing in mind the anatomic specificity of complex I deficiency in the CNS. Recent reports have indicated a decline of mitochondrial function in skeletal muscle with age (Trounce *et al.*, 1989) and so control and patient groups have been carefully matched in this respect in all our studies. Polarographic and enzyme analyses of respiratory chain activity in the skeletal muscle of our controls confirmed a decline with age, especially involving complex I (data not shown). Failure to use accurately age-matched controls could therefore produce misleading results.

Bindoff *et al.* (1989) studied muscle in five parkinsonian patients (one untreated) and

four controls by enzyme analysis and found decreased levels of complexes I, II and IV in the parkinsonian group. However, our study using both polarographic and enzyme analysis does not support this finding. The deficiency of complexes I, II and IV observed in the previous study indicates a more comprehensive and non-specific decline in respiratory chain function that may be related to age and the relative immobility of parkinsonian patients. The controls used in our skeletal muscle study included orthopaedic patients matched for the relative immobility of our parkinsonian patients.

A recent study of skeletal muscle mitochondrial function in six patients with Parkinson's disease found normal respiratory chain function in one patient, pure complex I defects in two, complex I–III defects in a further two patients and low activity of complexes II–IV in one patient (Shoffner *et al.*, 1991). The level of complex I activity measured was 8%, 10%, 14% and 38% of control mean in affected patients. In patients with mitochondrial myopathy, muscle mitochondrial complex I activities below 20% of control, in our experience, have always been associated with severe clinical weakness, fatigue and a resting lactic acidosis. It is therefore surprising to see such low activities in parkinsonian patients, since in Parkinson's disease neither muscle weakness nor lactic acidosis are part of the normal clinical spectrum. The 16 controls used in their study varied in age from 6 yrs to 69 yrs, only four were 60 yrs or older, and the mean age of the group was 35 yrs. They found no decline in respiratory chain enzyme activity with age. This contrasts with the studies of Trounce *et al.* (1989) and Yen *et al.* (1989), as well as with the results of our own studies, in which a clear decline of complex I activity with age was shown.

The immediate assessment of respiratory chain function by both polarography and enzyme analysis in our study circumvented any effect that storage may have on enzyme function. In addition, the close age matching of our controls ensured against the production of an apparent age related complex I defect.

The results of our study on platelet homogenate mitochondrial function in 14 patients with Parkinson's disease are at variance with a previous study of 10 parkinsonian patients (Parker *et al.*, 1989). We used platelet homogenates to determine respiratory chain enzyme activities, whereas Parker *et al.* (1989) used mitochondrially enriched preparations obtained by platelet-phoresis and disruption using a nitrogen bomb. Nevertheless, the degree of complex I deficiency suggested in parkinsonian platelets by the latter group (about 50%) should be detectable by our techniques. Although the complex I assay was on average only 31% rotenone-sensitive, triplicate assays of each sample fell within 10% of each other, reflecting the reproducibility of the analysis. Our adjustment of variations in citrate synthase activity allows for correction of any differences in mitochondrial content between samples. The use of platelet homogenate activities corrected for citrate synthase activities circumvents the potential problems associated with enriched mitochondrial preparations which may have variable degrees of contamination by other subcellular compartments. Nevertheless it is still conceivable that a mild to moderate defect of platelet complex I activity in patients with Parkinson's disease may not be detected in homogenates.

Our findings of normal skeletal muscle and platelet mitochondrial function in Parkinson's disease corresponds with the specificity of the complex I defect to the substantia nigra within the brain, and with the concept of Parkinson's disease as a neurodegenerative disorder. Our failure to demonstrate complex I deficiency in platelet

homogenates indicates that this tissue could not be easily used, if at all, as a 'diagnostic test' for Parkinson's disease.

The relationship of the substantia nigra complex I deficiency to the cause of Parkinson's disease is as yet unclear. This defect may be a primary phenomenon, or the end result of endogenous or exogenous toxic activity. In either case, the complex I deficiency will result in decreased ATP synthesis leading to cell death.

Current hypotheses for the etiology of Parkinson's disease must take account of data suggesting contributions from both genetic and environmental factors. The recent description of autosomal dominantly inherited Parkinson's disease in a large American-Italian family (Golbe *et al.*, 1990) implies that this disorder can potentially be induced by a nuclear gene defect. The MPTP model clearly demonstrates the ability of an environmental agent to cause Parkinson's disease. The recent identification of abnormal xenobiotic metabolism in patients with Parkinson's disease (Steventon *et al.*, 1989) suggests that this disorder may develop as a result of an inherited defect involving abnormal metabolism of environmental chemicals.

Complex I deficiency may play a pivotal role in the chain of events culminating in nigral cell death. Complex I comprises 26 polypeptides, seven of which are encoded by mitochondrial DNA (mtDNA). Mitochondrial DNA is maternally inherited and so a mitochondrially encoded defect could not be directly involved in a disorder inherited in an autosomal pattern. In addition, Parkinson's disease itself does not exhibit maternal inheritance. In agreement with this, two studies have examined mtDNA in parkinsonian brain by restriction fragment length polymorphism (RFLP) analysis and failed to show any significant deletions in any region including substantia nigra (Lestienne *et al.*, 1990; Schapira *et al.*, 1990*b*). Deletions of mtDNA have been seen after amplification by the polymerase chain reactions in parkinsonian striatum and cortex, but similar deletions were present in age-matched controls (Ikebe *et al.*, 1990) and so are likely to be related simply to ageing. Although mtDNA may not play any part in the inheritance of Parkinson's disease, mutations of mtDNA may be induced by certain toxic processes. Mitochondrial DNA lacks the protection of histones and repair enzymes and so is susceptible to damage from free radicals (Richter *et al.*, 1988) which could lead to defects of mtDNA encoded products. The fact that more than half the polypeptides encoded by mtDNA are part of complex I, renders this protein of the respiratory chain potentially the most vulnerable to such an effect. In addition, the mitochondrially encoded products of complex I are important functional elements of complex I, in particular the NDI gene product which is involved in the binding site of the mitochondrial inhibitors rotenone (Earley *et al.*, 1987), a pethidine analogue (Werner, 1989) and possibly MPP^+ (Ramsay *et al.*, 1991). There is evidence of an active toxic process involving oxidative damage in the substantia nigra of patients with Parkinson's disease (Dexter *et al.*, 1989) and this could induce mtDNA abnormalities not detected by RFLP analysis. The mitochondrial respiratory chain is an important source of superoxide radicals and this production is stimulated by inhibitors such as rotenone (Takeshige and Minakami, 1979). Thus complex I inhibition itself may lead to the oxidative damage seen in parkinsonian substantia nigra. However, there is some evidence that free radicals themselves can impair complex I activity (Hillered and Ernster, 1983). This close association between complex I inhibition and free radical production has been confirmed in the MPTP/MPP^+ model (Cleeter *et al.*, 1992). It has been shown that the irreversible

inhibition of complex I function induced by MPP^+ can generate free radicals which in turn irreversibly and specifically inhibit complex I. This suggests that a primary defect in complex I could produce a cycle of free radical production and complex I inhibition, and account for the findings of complex I inhibition and oxidative stress in parkinsonian substantia nigra.

The finding of complex I deficiency in Parkinson's disease and its anatomic selectivity for the substantia nigra provides a direct link between the idiopathic disease and the toxin (MPTP) model. The recent description of normal complex I activity in multiple system atrophy (MSA, a disorder involving severe degeneration of nigral neurons) indicates that this biochemical defect is not simply a secondary phenomenon of cell death (Schapira *et al.*, 1990*c*). Furthermore, the fact that the MSA patients studied were taking L-dopa in amounts equivalent to those of parkinsonian patients shows that complex I deficiency is not a side-effect of treatment. All these factors enhance the possibility that complex I deficiency plays an important role in the pathogenesis of Parkinson's disease. Further work needs to be focused on the molecular basis of this defect and the possible mechanisms that lead to it. The relationship between the complex I deficiency and oxidative stress also needs to be explored further.

The results of this study suggest that neither muscle nor platelet homogenates from patients with Parkinson's disease are suitable for the investigation of complex I deficiency in this disorder.

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Platelet Mitochondrial Function in Parkinson's Disease

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There is increasing evidence that defective function of the mitochondrial enzyme NADH CoQ reductase (complex I) is involved not only in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity, but also in idiopathic Parkinson's disease (PD). Complex I deficiency has been identified in PD substantia nigra and appears to be disease-specific and selective for the substantia nigra within the central nervous system. We describe a method for preparation of an enriched mitochondrial fraction from 60 mL blood. Using this technique, we analyzed respiratory chain function in 25 patients with PD and 15 matched control subjects. We confirm a previous report of a specific complex I deficiency in PD platelet mitochondria. Although there was a statistically significant decrease in complex I activity in the PD group compared with the control group ($p = 0.005$), the defect was mild (16%); it was not possible to distinguish PD from control values on an individual basis. This deficiency is not detectable in platelet whole-cell homogenates, presumably reflecting the relative insensitivity of this preparation and the limited decrease in complex I activity in PD. The presence of a mild complex I defect in platelets together with a more severe defect in substantia nigra suggests either that the pharmacological characteristics shared by these two tissues render them susceptible to a particular toxin or toxins, or that the defect is widely distributed and other biochemical events enhance the deficiency in substantia nigra. Evidence for a complex I defect in platelet mitochondria from patients with PD supports the proposition that this biochemical deficiency may have some role in the cause of dopaminergic cell death in this disorder.

Krige D, Carroll MT, Cooper JM, Marsden CD, Schapira AHV. Platelet mitochondrial function in Parkinson's disease. Ann Neurol 1992;32:782–788

Parkinson's disease (PD) is a neurodegenerative disorder characterized clinically by bradykinesia, rigidity, and tremor, and pathologically by the death of dopaminergic neurons in the substantia nigra with Lewy bodies in some surviving neurons. The cause of neuronal death in PD is uncertain and may involve both genetic and environmental factors.

The first clue to the possibility of mitochondrial dysfunction in PD came with an understanding of the mechanism of action of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP is converted to 1-methyl-4-phenylpyridinium (MPP^+) by monoamine oxidase B (MAO-B) [1], probably in glia. MPP^+ is then actively taken up into dopaminergic neurons [2] and concentrated into mitochondria [3], where it specifically inhibits NADH CoQ_1 reductase (complex I) [4], the first enzyme of the respiratory chain. The resulting decrease in adenosine triphosphate synthesis is thought to account for the death of the dopamine-containing neurons [5]. The first description of complex I deficiency in the substantia nigra of patients with PD provided a direct biochemical link between the idiopathic disease and the neurotoxin model [6]. The subsequent report of complex I deficiency in platelet mitochondria from patients with PD promised to provide a more accessible tissue for the study of the molecular basis of this defect [7]. The presence of the complex I defect in platelets would, if confirmed, have important implications for our understanding of the distribution of the defect and for its potential contribution to the pathogenesis of PD. For this reason, we undertook a large and detailed study of mitochondrial respiratory chain function in PD.

The original method for platelet mitochondrial enrichment described by Parker and colleagues [7] took 2 days and required plateletpheresis and separation of mitochondria on a Percoll gradient. Platelet homogenates are more easily prepared but do not show any complex I defect in PD [8], presumably because of the relative insensitivity of this preparation. We therefore developed a technique for platelet mitochondrial isolation that requires only 60 mL venous blood and can

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be completed in 3 hours. This technique has been applied to the study of mitochondrial respiratory chain function in PD.

Materials and Methods

Patients

All studies were performed with full and informed consent of the patients and with the approval of the Joint Ethics Committee of the National Hospital for Neurology and Neurosurgery and the Institute of Neurology, Queen Square, London. The diagnosis of idiopathic PD was based on the presence of an akinetic rigid syndrome with asymmetrical onset, a resting tremor, and a positive response to apomorphine or L-DOPA. Blood was collected from 25 patients with PD with Hoehn and Yahr gradings between I and III; 21 were receiving L-DOPA at the time of sampling, 4 were untreated, and only 4 were smokers. Those patients who were untreated at the time of sampling subsequently demonstrated a good response to L-DOPA. The results from the patients with PD were compared with 15 age-matched control subjects who had no evidence of neurodegenerative disease, only 2 of whom were known to be smokers.

Mitochondrial Preparation

Sixty milliliters of venous blood were collected without tourniquet and mixed 9:1 with 3.8% (w/v) trisodium citrate, and the platelet fraction was prepared at room temperature. A platelet-rich plasma (PRP) was separated by centrifugation at 200 *g* for 20 minutes. The top three fourths of the PRP was removed and centrifuged at 1,000 *g* for 30 minutes to obtain a platelet pellet. The platelets were washed twice by resuspending the pellet in a volume (equal to the PRP volume) of a modified Tyrode's buffer (150 mM NaCl, 5 mM HEPES, 0.55 mM NaH_2PO_4, 7 mM $NaHCO_3$, 2.7 mM KCl, 0.5 mM $MgCl_2$, 5.6 mM glucose, 1 mM EDTA(di-K), pH, 7.4), and pelleted by centrifugation at 1,000 *g* for 15 minutes. All subsequent steps were performed at 4°C. The washed platelet pellet was gently resuspended in 6 mL ice-cold medium (250 mM sucrose, 1 mM EDTA, 10 mM Tris, pH, 7.4) and transferred to the cell disruption bomb (Parr Instrument Co, Moline, IL; 45 mL volume). Platelets were homogenized by equilibration with nitrogen at 1,200 psi for 20 minutes before explosive decompression [9]. The homogenate was centrifuged at 1,000 *g* for 15 minutes, the postnuclear supernatant (PNS) was removed, and the nuclear pellet was homogenized and centrifuged as described. This method of cell disruption by nitrogen cavitation consistently gave more than 95% cell breakage, based on the proportions of citrate synthase activity in supernatant and pellet. There was minimal damage to mitochondria: typically, 85% of the total citrate synthase activity was released only after the addition of Triton-X100. The PNSs were pooled and mitochondria were pelleted by centrifugation at 8,650 *g* for 10 minutes. The mitochondrial pellet was gently resuspended in 600 μL ice-cold medium to a protein concentration of approximately 3 mg/mL, snap-frozen in liquid nitrogen, and placed at −70°C for storage before assay within 2 weeks. The percentage recovery of citrate synthase activity in the mitochondrially enriched fraction relative to homogenate was 27.9 ± 7.6% (mean ± SD; n = 6).

Mitochondrial Enzyme Assays

Control and PD samples were analyzed simultaneously in batches. After 3 cycles of freeze-thawing, mitochondrial enzymes were assayed blind and in triplicate in a final volume of 1 mL at 30°C on Hitachi U3210 and Kontron 940 dual-beam spectrophotometers. Assays were accepted only if triplicate values were within 15% of one another. Succinate cytochrome *c* reductase (complex II/III), cytochrome oxidase (complex IV), and citrate synthase were assayed by standard techniques [10], except that a final concentration of 2.5 mg/mL bovine serum albumin (BSA) was included in the complex II/III assay. NADH CoQ_1 reductase (complex I) was measured according to the method of Ragan and associates [11] with some modifications to optimize activity in platelet mitochondrial fractions. The assay mixture contained 20 mM potassium phosphate, 8 mM $MgCl_2$ (pH, 7.2), 150 μM NADH, 1 mM KCN, 50 μM CoQ_1, and 2.5 mg/mL BSA (essentially fatty-acid free). Mitochondrial complex I activity was taken as that sensitive to rotenone (10 μM). Samples were between 60 and 80% sensitive to rotenone.

Organelle Marker/Enzyme Assays

Lactate dehydrogenase activity (cytosolic marker) was assayed at 30°C according to the method of Clark and Nicklas [12]. Esterase activity (endoplasmic reticulum marker) was assayed at 30°C according to the method of Beaufay and colleagues [13]. Acid phosphatase activity (lysosome marker) was assayed at 37°C using a modification of the method of Hubscher and West [14]. β-Thromboglobulin (β-TG) (alpha-granule marker) levels were measured using a commercially available enzyme-linked immunosorbent assay kit (Diagnostica Stago, Asnieres sur Seine, France).

Results

Mitochondrial Purity

Electron microscopy showed that the mitochondria obtained from platelets using this method were contaminated with other granules (data not shown). This contamination has been noted by others [15, 16] and is to be expected because platelets are rich in alpha and storage granules, which have similar densities to mitochondria. The platelet endoplasmic reticulum and the cytosol did not copurify with mitochondria in our protocol (Fig 1). Figure 1 shows that the purification factor (specific activity in the "mitochondrial fraction" relative to the homogenate) is similar for β-TG (a marker of alpha granules), acid phosphatase (a lysosomal marker), and mitochondrial complex I. The specific activity of complex I in our "mitochondrial fraction" from control subjects (19.34 ± 5.22 nmol/min/mg total protein; Table) is comparable to those of Parker and associates [7, 17] (19.1 ± 5.6 nmol/min/mg total protein), who used Percoll density gradients to purify platelet mitochondria. In the absence of data concerning the purity of the latter preparation, we conclude that the purification factor is similar in the two techniques.

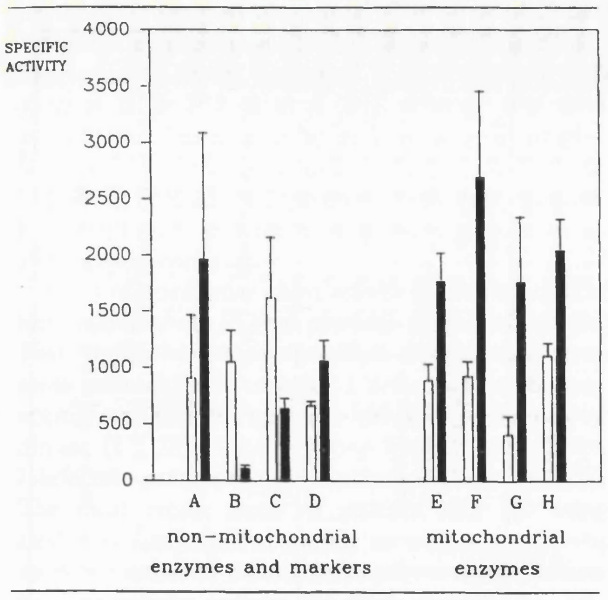

Fig 1. Comparison of the activities of specific subcellular fraction enzymes and markers and respiratory chain enzymes in control platelets before (open boxes) *and after* (solid boxes) *mitochondrial enrichment. All boxes represent mean ± SD; n = 4 (except A, n = 3). (A) β-Thromboglobulin (pmol/mg). (B) Lactate dehydrogenase (nmol/min/mg). (C) Esterase (nmol/min/mg × 10). (D) Acid phosphatase (μmol/min/mg × 100). (E) Complex I (nmol/min/mg × 100). (F) Complex II/III (nmol/min/mg × 50). (G) Complex IV (k/min/mg × 1,000). (H) Citrate synthase (nmol/min/mg × 10). Lanes E to H indicate the purification of mitochondrial enzymes in the mitochondrial fraction relative to the platelet homogenate, which shows a mean enrichment factor of 2.8 in the mitochondrial platelet preparation from control subjects. This preparation has been used in subsequent analyses.*

Mitochondrial Function

UNCORRECTED. The control and patient groups were accurately age-matched. The platelet mitochondrial complex I (NADH CoQ_1 reductase) activity in the patients with PD, expressed per mg total protein, was lower than in control subjects (14% deficiency) but barely reached statistical significance ($p = 0.044$). The control group's complex I values overlapped those of the PD group (Fig 2A). There was no difference between control and PD groups for complexes II/III and IV; PD values for these enzymes spread equally throughout the control ranges.

CORRECTED. Correction of each individual enzyme result for the respective citrate synthase activity allows for any difference in mitochondrial mass and purity between samples. When this correction is applied to PD and control platelet data (Fig 2B), there is a small (16%) but significant ($p = 0.005$) decrease in complex I activity in patients with PD. As in the results expressed per mg total protein, there is overlap of the PD values by the control group. Once again, there was no difference in complexes II/III and IV activities between PD and control subjects, with equal spread of the PD values throughout the control ranges.

We found no correlation of platelet complex I activity with age in either the control (n = 15; $r = 0.34$) or the patient group (n = 25; $r = 0.03$). Four patients with PD were untreated at the time of sample; there was no difference in specific or citrate synthase–corrected complex I activity between treated and untreated patients (data not shown). In addition, there was no correlation of complex I activity with severity of disease, total L-DOPA ingested (n = 19; $r = 0.14$), duration of disease (n = 25; $r = -0.11$), or age at onset of disease (n = 25; $r = 0.10$).

Platelet Mitochondrial Respiratory Chain Enzyme Function[a]

	Age (yr)	NADH CoQ_1 Rotenone Sensitive	SCcR Antimycin A Sensitive	COX (k/min/mg)	CS
Specific activities					
Control (n = 15)	65.67 ± 6.90	19.34 ± 5.22	47.96 ± 12.31	1.92 ± 0.62	197.26 ± 41.40
PD (n = 25)	62.72 ± 6.75	16.57 ± 3.24*	53.98 ± 10.55 (n = 23)	1.89 ± 0.59	202.40 ± 31.99
Citrate synthase–corrected		NADH CoQ_1 × 100	SCcR × 10	COX × 100	
Control (n = 15)		9.88 ± 2.14	2.44 ± 0.44	0.988 ± 0.250	
PD (n = 25)		8.26 ± 1.34**	2.69 ± 0.47 (n = 23)	0.939 ± 0.257	

[a]Results are expressed as mean ± SD. Enzyme activities are expressed as nmol/min/mg total protein, unless stated; k is the first order rate constant. Significance relative to control by student's t test.
*$p = 0.44$. **$p = 0.005$.

NADH CoQ_1 = NADH ubiquinone reductase (complex I); SCcR = succinate cytochrome *c* reductase (complex II/III); COX = cytochrome oxidase (complex IV); CS = citrate synthase; PD = Parkinson's disease.

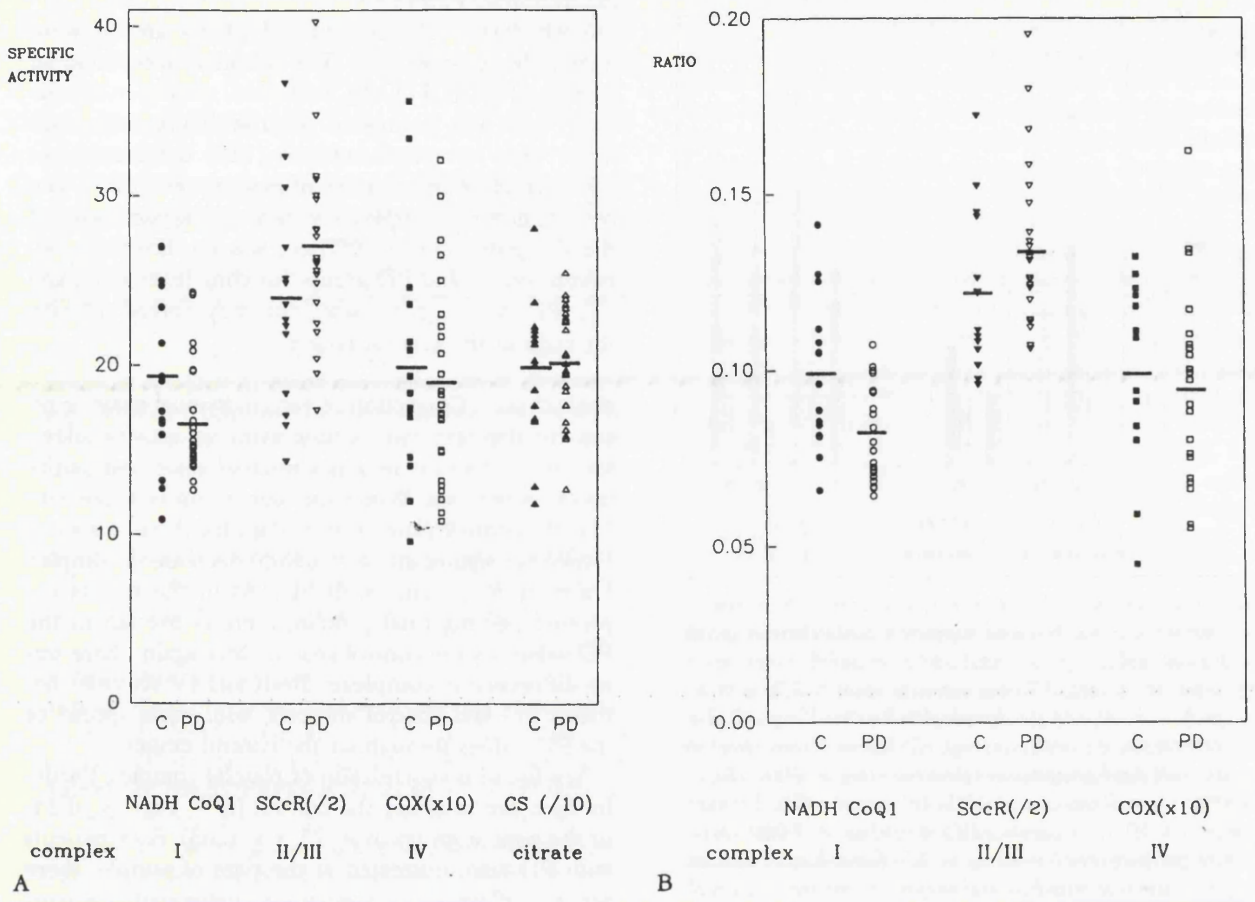

Fig 2. (A) Specific activities of mitochondrial enzymes in platelet mitochondrial fraction. C = control (n = 15); PD = Parkinson's disease (n = 25). Abbreviations and activities as in the Table. Bars *represent mean activities. (B) Ratio of mitochondrial respiratory chain enzyme activities to citrate synthase activity in platelet mitochondrial fractions. Abbreviations as in the Table.* Bars *represent mean activities.*

There was no obvious relationship between platelet complex I activity and smoking in the 4 patients with PD and the 2 control subjects; however, numbers are too few to make any relationship clear between potential toxins in cigarettes and complex I activity. Nevertheless, exclusion of the smokers from the data does not alter the significance of the results.

Discussion

Several abnormalities that might contribute to dopaminergic cell death in PD substantia nigra have now been identified. The decrease in polyunsaturated fatty acids [18], the increase in superoxide dismutase activity [19], and the recent description of a decrease in reduced glutathione [20] all suggest active free-radical damage in the substantia nigra at time of death. The increase in the iron content of PD substantia nigra in association with a decrease in brain ferritin levels [21] would enhance such oxidative damage. Autooxidation of dopamine may also contribute to such a process [22].

Discovery of a complex I deficiency in PD substantia nigra provides another mechanism by which neuronal death may be induced. Analysis of respiratory chain activity in PD brain has shown a 37% decrease in complex I activity in PD substantia nigra, with normal activities of complexes II to IV [8, 10]. The activities of complexes I to IV were normal in PD cerebral cortex, cerebellum, caudate, globus pallidum, and tegmentum [8, 23]. These results suggests that the mitochondrial defect is specific for complex I and highly selective within the central nervous system for the substantia nigra. A study of mitochondrial function in the substantia nigra from patients with (MSA) did not show any defect of respiratory chain activity [23]. Because patients with MSA have severe neuronal degeneration in the substantia nigra, the normal level of complex I activity in this group would suggest that the defect in PD is not a secondary effect of degeneration. These patients with MSA received L-DOPA in similar quantities as patients with PD and suffered similar agonal states before death. These two factors could therefore also

be discounted as possible causes of the substantia nigra complex I deficiency in PD. In contrast to these findings, another report suggested a mild deficiency of complex III in PD striatum [24], although this same group found decreased complex I but normal complex III levels by both immunohistochemistry and immunoblotting [25, 26]. In agreement with other studies, they also found no defect in mitochondrial function in PD cerebral cortex.

Study of respiratory chain activity in skeletal muscle from patients with PD has provided conflicting results. Two studies have shown multiple enzyme defects in some patients, pure complex I defects in others, and normal activities in 2 patients with PD with advanced disease [27, 28]. Another group found pure complex I defects in patients with PD studied post mortem [29]. The most recent study of patients with PD using freshly isolated skeletal muscle mitochondria showed no defect either by polarography (direct oxygen utilization) or enzyme analysis [8]. Such discrepancies may be due to differences in patient selection, age, mobility, treatment, sample handling, and possibly biochemical heterogeneity within the PD population.

Until recently, the study by Parker and associates [7] remained the only full report of isolated platelet mitochondrial function in PD. The results of our study also show that there is a small but significant and specific decrease in platelet mitochondrial complex I activity in our group of 25 patients with PD. This finding has important implications for our understanding of the importance of mitochondrial dysfunction in PD.

The severity of the defect in our studies was mild (16%) and contrasts with the previous study [7], in which a mean decrease of 55% was found in 10 patients with PD. Because the control rates for the rotenone-sensitive complex I enzyme assays were almost identical in both studies, such a discrepancy is unlikely to be attributable to differences in mitochondrial enrichment. Although control and patient samples might be treated identically, a preexisting defect in complex I might render it more susceptible to degradation during an extended preparation. The method described in our study produced a mitochondrial sample within 3 hours of venesection and therefore limited any changes in enzyme activity related to prolonged separation and purification procedures.

The presence of complex I deficiency in the platelets of patients with PD might suggest that this biochemical deficiency could serve as a diagnostic, or even a preclinical, marker for PD. There are, however, 3 immediate problems with this concept.

Platelet Mitochondrial Preparation

The method of Parker and colleagues [7] for preparation of platelet mitochondria involves plateletpheresis and takes 2 days to complete. Although our method uses only 60 mL blood and is completed in 3 hours, access to a nitrogen cavitation bomb and a high-speed centrifuge is still required.

Disease Specificity

Although the substantia nigra complex I defect in PD is not seen in patients with MSA, we have not as yet determined the disease specificity of the platelet defect. Indeed, a severe complex I defect (72% deficiency) has been described in platelet mitochondria from patients with Huntington's disease (HD) [30], although we found no evidence for such a defect in HD caudate nucleus [31].

Sensitivity of the Defect

The range of complex I activities of the control group almost completely overlaps the PD group, even in the citrate synthase–corrected data (see Fig 2). An individual's complex I activity therefore cannot discriminate between control and PD populations.

The platelet mitochondrial defect exactly mirrors that in the substantia nigra in terms of its selectivity to complex I. The severity of the deficiency in the substantia nigra, however, is much greater than in platelets—a 37% deficiency was demonstrable in substantia nigra homogenates compared with a 16% deficiency in a platelet mitochondrial preparation. Nevertheless, the presence of complex I deficiency in both substantia nigra and platelets raises many intriguing questions as to the cause of the defect and its relationship to the pathogenesis of PD. The complex I defect may be due to a genetic abnormality (nuclear or mitochondrial), an environmental agent (endogenous or exogenous), or an interaction between the two.

GENETIC. Autosomal inheritance, usually dominant with variable penetrance, has been identified in only a small proportion of patients with PD [32]. Twin studies of PD provided inconclusive results [33]. No evidence for maternal (mitochondrial) inheritance in PD was found in a recent survey of familial PD [34]. Although there were some initial reports of an increase in the proportion of mitochondrial DNA (mtDNA) molecules bearing the "common" 5-kb deletion [35] in PD striatum, subsequent studies have not confirmed this finding [36, 37] and have shown that there is no increase in deleted mtDNA over and above that expected for age [38]. The proportion of mtDNA with the common deletion has been calculated to be only 1 in every 5,000 to 10,000 molecules in aged patients; it is therefore very unlikely to contribute to any biochemical deficiency [38]. Nevertheless this finding does not exclude other deletion sites or point mutations contributing to the biochemical defect; however, the spontaneous mutation rate of mtDNA is high and polymorphisms are common. Care must therefore be

exercised in interpreting the relevance of homoplasmic "point mutations" of mtDNA in patients with PD that do not segregate with the disease and do not result in alteration in phylogenetically conserved or clearly important functional amino acids.

A putative genetic defect in PD is likely either to be relevant in only a small proportion of patients or to be a general susceptibility factor on which another mechanism must be superimposed to precipitate the disease. Such a two-step process would mask any clear inheritance pattern, even though a genetic abnormality may be present. In this respect, the 2 recent reports [39, 40] of an increase in the incidence of mutant alleles of the cytochrome P450 gene CYP2D6 in PD provide an interesting and direct link between potential environmental neurotoxins, abnormal metabolism, and increased susceptibility to development of PD.

The biochemical consequences of a systemically distributed genetic defect of complex I genes may depend on other local factors; for instance, any underlying but mild deficiency of complex I may be accentuated in substantia nigra by increased free-radical formation. There is already some evidence that an underlying defect of complex I function may render it particularly susceptible to the effects of free radicals generated by the respiratory chain [41].

In platelets, the lower end of the control group's complex I activity overlapped with the majority of patients with PD. Although this overlap prevents the complex I assay being used as a diagnostic test for PD, it is interesting to speculate that those individuals of the control group who had complex I activities below the mean of the PD group might carry PD susceptibility. Such individuals may not have as yet developed the clinical features of PD, or do not have, nor have been exposed to, the additional factor or factors that precipitate the disease. The PD group itself may be heterogenous and include those in whom complex I deficiency is relevant, as well as those in whom it is not. Studies of platelet mitochondrial function in specific PD groups (e.g., young onset and familial cases) may help answer this question.

toxic. MPTP is a useful model to demonstrate how an environmental agent can target specific neurons and induce cell death through selective uptake and conversion characteristics. In this respect, blood platelets have similar pathways to dopaminergic neurons, which allow them to concentrate MPTP and to convert it to MPP^+ [42]. The parallel of the complex I defect between substantia nigra and platelets in PD could therefore support the exposure of both to a common toxin. The recent description of abnormal xenobiotic metabolism in patients with PD raises the possibility of chronic exposure to an endogenously generated toxin contributing to dopaminergic cell death [43, 44]. In particular, the enhanced *N*-methylation of compounds observed in patients with PD could produce compounds that would follow similar metabolic pathways to MPTP and therefore target both dopamine-containing neurons and platelets.

Assuming that the complex I defects in substantia nigra and platelets have the same underlying cause, confirmation of a complex I defect in PD platelet mitochondria has important implications for the direction of future research into understanding the molecular basis of this defect and any role it might have in the pathogenesis of PD. The mitochondrial preparation described herein should be readily reproducible in other laboratories and should provide material for identifying the cause of complex I inhibition at the polypeptide level.

The relationship of the platelet complex I defect described herein to common, as yet undetermined, nonspecific respiratory chain toxins has not yet been established. Further work must be directed toward investigating this relationship, as well as the reproducibility and disease specificity of the defect.

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