THE EFFECT OF MANGANESE ON MAMMALIAN MITOCHONDRIA

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ABSTRACT

Manganese (Mn) is an essential trace element, but excessive inhalation can cause serious disorders of the central nervous system, lungs and liver, and results in the condition known as manganism. The general population is exposed to Mn through its use in the fungicide Maneb and MMT, which is used as an anti-knock agent to replaced lead in petrol. Also there have been a number of reports of Mn contaminated drinking water. Victims of Mn poisoning suffer from serious neurological disorders, such as an intermittent tremor of small amplitude, speech impairments and disruption of postural reflexes, which are caused by damage to certain regions of the brain. After prolonged exposure severe symptoms develop that generally resemble those associated with Parkinson's disease.

The action of Mn on the brain is not well understood, although three possible mechanisms have been proposed:

1. Inhibition of the mitochondrial electron transfer chain following Mn accumulation by mitochondria.
2. Neuronal degradation by free radicals such as $O_2^{\cdot-}$ and ·OH causing lipid peroxidation and damage to DNA and protein.
3. Induction of mutation of the mitochondrial genome, as has previously been shown in both eukaryotes and prokaryotes.

It has been shown in this study that Mn inhibits the mitochondrial electron transfer chain. An overall ionic strength inhibition of the entire electron transfer chain was observed, probably mediated by an interference of the electrostatic interactions between cytochrome *c* and the cytochrome bc_1 complex or cytochrome oxidase. Also a direct inhibition of succinate dehydrogenase, NADH dehydrogenase and cytochrome oxidase was observed. This inhibition would be associated with a decrease the production of ATP and could be sufficient to cause the degradation of brain tissue seen in victims of Mn poisoning.

It seems likely that if Mn can inhibit the mitochondrial electron transfer chain, this inhibition would lead to an increase in the generation of free radical species by the mitochondria. However, this was not shown in this work, due to difficulties with

detector molecules. It was observed that sheep liver mitochondria can oxidise and reduce acetylated cytochrome *c,* which may not have been previously reported.

The effect of Mn on isolated mtDNA showed a decrease in the intensity of PCR products after exposure to Mn, which may have been cause by an interference of the activity of *Taq* polymerase. It has previously been shown that Mn interferes with the activity of both *Taq* polymerase and chicken liver mitochondrial polymerase-γ and, if it could interfere with the activity of mitochondrial DNA polymerase, this would also decrease further both the number of functional mitochondria and the production of ATP.

A decrease in the production of ATP by mitochondria, or a decrease in the production of functional mitochondria, would lead to cellular death of affected cells and could provide an explanation of the symptoms observed in victims of Mn poisoning.

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ABBREVIATIONS

6-OHDA	6-hydroxydopamine
A	adenine
A_{xxx}	absorbance (XXX-wavelength of measurement)
ADP	adenosine 5'-diphosphate
AMProp	2-amino-2-methyl-1 propanol
ATP	adenine triphosphate
bp	base pair
cytochrome bc_1 complex	ubiquinol : ferricytochrome-*c* oxidoreductase (EC 1.10.2.2)
BSA	bovine serum albumin (fraction V powder)
C	cytosine
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
CO1	cytochrome *c* oxidase subunit 1 gene
CR	control ratio (= rate after DNP addition/rate before DNP addition)
cytochrome oxidase	ferrocytochrome *c* : oxygen oxidoreductase (EC 1.9.3.1)
DCPIP	2,6-dichlorophenol-indo-phenol
DMSO	dimethyl sulfoxide
DMPO	5,5-dimethyl-1-pyrroline N-oxide
DNA	deoxyribonucleic acid
DNP	2,4-dinitrophenol
EDTA	ethylene diamine tetra-acetic acid
EGTA	ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetra-acetic acid
EPR	electron paramagnetic resonance
FECN	potassium ferricyanide
G	guanine
GSH	glutathione
H_2O_2	hydrogen peroxide
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
·OH	hydroxyl radical
kb	kilobase

KO_2	potassium superoxide
L-DOPA	L-3,4-dihydroxyphenylalanine
Maneb	[ethylenebis(dithiocarbomato)]manganese
$\Delta\psi$	membrane potential
Mg	magnesium
MMT	methylcyclopentadienyl manganese tricarbonyl
Mn	manganese
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtDNA	mitochondrial DNA
NAD^+	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NADH dehydrogenase	NADH : ubiquinone oxidoreductase (EC 1.5.5.3)
NBT	2,2'-di-p-nitrophenyl-5-5'-diphenyl-3,3'-[3,3'-dimethoxy-4-4'-diphenylene]-ditetrazolium chloride
RAPD	random amplified polymorphic DNA
RCR	respiratory control ratio
RFLP	restriction fragment length polymorphism
SOD	superoxide dismutase (EC 1.15.1.1)
$O_2^{\cdot-}$	superoxide radical
PCR	polymerase chain reaction
PD	Parkinson's disease
succinate dehydrogenase	succinate : ubiquinone oxidoreductase (EC 1.3.5.1)
T	thymine
TE buffer	Tris-HCl (10 mM) EDTA (1 mM) pH 8.0
TAE buffer	Tris (40 mM) acetate (20 mM) EDTA (1 mM) pH 8.0
Taq polymerase	*Thermus aquaticus* DNA polymerase
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
$\Delta\mu_H^+$	transmembrane proton electrochemical potential
tRNA	transfer RNA
U	unit
UQ	ubiquinone
UQH	ubiquinol
UV	ultraviolet
V	volts

CHAPTER 1
INTRODUCTION

Mitochondria are small subcellular organelles approximately 2 μm in length and 0.5 μm in diameter. They have two membranes, an outer membrane, which is permeable to most small molecules and ions, and an inner membrane. The inner membrane is extensively folded into cristae, and contains a large number of selective carriers, it represents a permeability barrier to many ions and polar molecules. The region between the inner and outer membranes is known as the inter-membrane space and the lumen of the inner membrane is called the matrix. The matrix is the site for the reactions of the citric acid cycle and most of the reactions of fatty acid oxidation.

Located on the inner membrane are five enzymes that are involved in energy transduction (Figure 1.1). NADH : ubiquinone oxidoreductase (EC 1.5.5.3 NADH dehydrogenase) catalyses the oxidation of reduced nicotinamide adenine dinucleotide (NADH) to NAD^+ which releases two electrons that reduce the ubiquinone (UQ) pool. Succinate : ubiquinone oxidoreductase (EC 1.3.5.1 succinate dehydrogenase) also contributes to the reduction of the UQ pool, by catalysing the oxidation of succinate to fumarate. The reduced form of UQ is ubiquinol (UQH_2), UQH_2 is oxidised by ubiquinol : ferricytochrome-*c* oxidoreductase (EC 1.10.2.2 cytochrome bc_1 complex) reducing cytochrome *c* which is associated with the exposed surface of the cytochrome bc_1 complex in the inter-membrane space. Cytochrome *c* is then oxidised by ferrocytochrome-*c* : oxygen oxidoreductase (EC 1.9.3.1 cytochrome oxidase), which reduces oxygen to water.

The process of transferring electrons along the electron transfer chain is associated with the translocation of protons across the inner membrane from the matrix to the inter-membrane space. Due to the relative impermeability of the inner membrane to protons a transmembrane proton electrochemical potential ($\Delta\mu_H^+$) is established. The inner membrane does however allow some "leakage" of protons back to the matrix, but this is offset by the rate at which they are transported into the inter-membrane space by electron transfer, establishing a $\Delta\mu_H^+$. The $\Delta\mu_H^+$ that is generated by electron transfer is

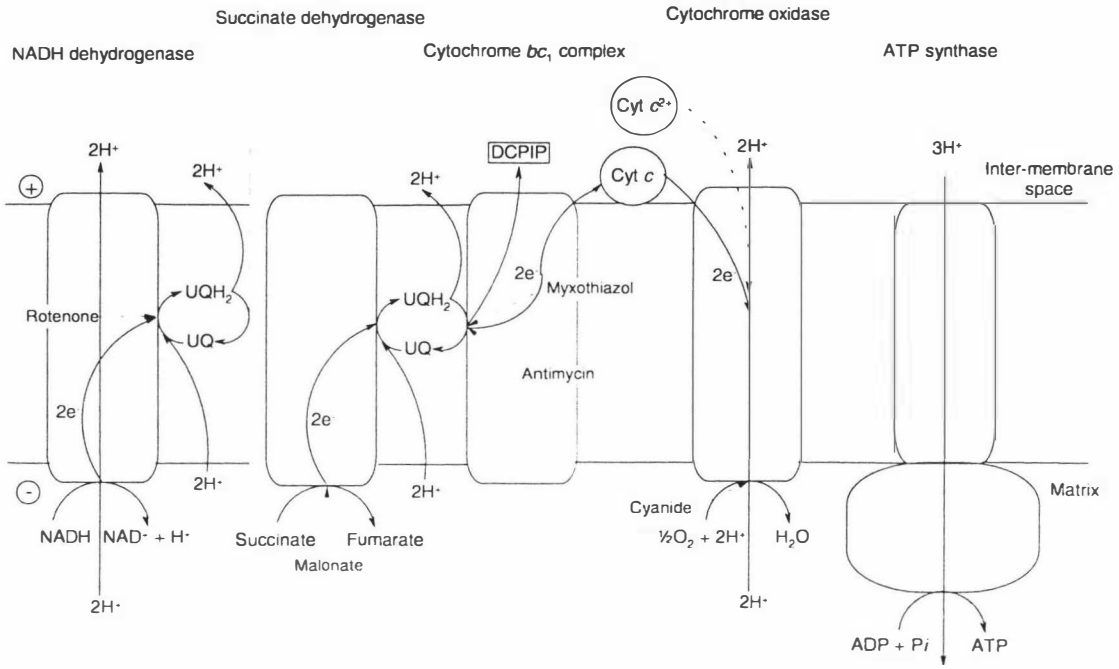

Figure 1.1 The enzymes of the inner mitochondrial membrane involved in energy transduction. Also shown is the site at which 2,6-dichlorophenol-indo-phenol (DCPIP) accepts electrons, the site where reduced cytochrome *c* (cyt c^{2+}) donates electrons and this site of action of several inhibitors. A single pool of UQ is reduced to UQH_2 by both succinate dehydrogenase and NADH dehydrogenase, and is oxidised by the cytochrome bc_1 complex. NADH dehydrogenase NADH : ubiquinone oxidoreductase (EC 1.5.5.3); succinate dehydrogenase succinate : ubiquinone oxidoreductase (EC 1.3.5.1); cytochrome bc_1 complex ubiquinol : ferricytochrome-*c* oxidoreductase (EC 1.10.2.2), cytochrome oxidase ferrocytochrome-*c* : oxygen oxidoreductase (EC 1.9.3.1).

dissipated by ATP synthase in the formation of adenine triphosphate (ATP) from adenosine 5'- diphosphate (ADP) and inorganic phosphate (Pi), a process that is coupled to the transport of protons from the inter-membrane space through ATP synthase back to the matrix. This ATP is then utilised in one of the many reactions carried out in the matrix.

A disruption to this process results in mitochondrial disease. These diseases can be divided into two main groups. The first group consists of mutations to genes encoding components of the electron transfer chain. These mutations cause variations in the structure or function of mitochondrial DNA (mtDNA), and are of seven types

1. Mitochondrial rearrangements, which are large-scale insertions or deletions of the mitochondrial genome.
2. Missense mutations, which are amino acid substitutions that result in miscoded subunits of the mitochondrial electron transfer chain.
3. Transfer RNA mutations, which are nucleotide substitutions in genes coding mtDNA biogenesis components, which are generally more severe than missense mutations.
4. Copy number mutations cause a sharp decline in the total mtDNA within a cell.
5. Nuclear DNA mutations causing multiple mtDNA rearrangements are disruptions to the replication of mtDNA.
6. Mutations to nuclear DNA encoding subunits of the mitochondrial electron transfer chain.
7. Diseases which are believed to be caused by mutations of genes encoding the components required for complete mitochondrial function, however this is yet to be confirmed.

The second main group of mitochondrial diseases is caused by compounds which inhibit the mitochondrial electron transfer chain. These mitochondrial diseases include carbon monoxide and cyanide poisoning as well as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced Parkinsonism. Manganese (Mn) is believed to be capable of inhibiting electron transfer resulting in the putative mitochondrial disease, manganism.

1.1 Manganese

Manganese is a group VII element. It is an essential trace element found in many enzymes including the oxygen evolving complex of higher plants and cyanobacteria (Renger, 1993), the Mn-superoxide dismutase (EC 1.15.1.1 SOD) found in mitochondria (Shimoda-Matsubayashi *et al.*, 1997; Konstantinov *et al.*, 1987) and chloroplasts (Kliebenstein *et al.*, 1998), and arginase (Kanyo *et al.*, 1996), however the inhalation of large amounts of Mn is toxic. Excessive Mn can cause serious disorders of the central nervous system, lung, liver, gastrointestinal and urinary systems, and results in the condition known as manganism (Shukla and Singhal, 1984).

The general population is exposed to Mn through the use of a number of compounds including the fungicide [ethylenebis(dithiocarbomato)]manganese (Maneb) (Ferraz *et al.*, 1988) and methylcyclopentadienyl manganese tricarbonyl (MMT), which has replaced lead as an antiknock agent in petroleum in Canada and parts of the USA. Higher than normal blood concentrations of Mn have been observed in garage workers and taxi drivers in Montreal and Toronto (Zayed *et al.*, 1994; Zayed *et al.*, 1996), it is believed that these elevated levels are due to the inhalation of the Mn_3O_4 aerosol which results from the combustion of MMT. However, at present, this does not represent a health hazard. This aerosol has also been implicated as the cause of a slight increase in the atmospheric concentrations of Mn in intensely populated regions (Zayed *et al.*, 1994). With the increasing use of MMT as a fuel additive, concerns have been raised about the potential public health risks associated with its widespread use (Davis *et al.*, 1998). This could eventually result in higher concentrations of atmospheric Mn, that may lead to more widespread Mn toxicity (Frumkin and Solomon, 1997).

These organic compounds are not the general population's only source of exposure to Mn. The Aboriginal inhabitants of Groote Eylandt, off the northern coast of Australia, suffer from Mn toxicity due to an abundance of Mn ore in their environment (Cawte, 1989; Florence and Stauber, 1989). Also both, Kawamura *et al.* (1941) and Kondakis *et al.* (1989) have reported waterborne epidemics of Mn poisoning with severe neurological effects in victims drinking water containing high concentrations of Mn.

Exposure to Mn is most common amongst individuals involved in the mining or milling of Mn ore. Incidences of manganism in Mn workers have been reported in Africa, Chile, Cuba, India, Italy, Japan, Mexico, Morocco, Russia and the USA, Mena (1979) suggests that as much as 25% of Mn miners or workers at Mn crushing plants suffer from various stages of manganism.

1.1.1 Manganese poisoning

Couper first documented Mn poisoning in 1837 in five workers of a pyrolusite (MnO_2) mill (Mena, 1979; Shukla and Singhal, 1984). The highest number of reported incidences of Mn poisoning still occurs among Mn miners with up to 25% of workers being affected (Mena, 1979; Mena *et al.*, 1967). These workers may be exposed for as

little as four months or as much as twenty years before exhibiting symptoms. The clinical characteristics of Mn poisoning initially observed by Couper have now been well defined. During the early stages of Mn poisoning psychomotor disturbances develop. These disturbances last for 1-3 months whether the miners are removed from the mines or not. The duration of these psychomotor disturbances is relatively constant amongst all individuals, but the manifestations vary in both intensity and type. All victims suffer from nervousness and irritability with most carrying out compulsive acts. For example, Mena *et al.* (1967) reported that one victim was observed to chase passing cars until exhausted, others would only sleep in open air, while others sang and danced compulsively. These individuals were aware of their unusual behaviour, but were unable to control it. Victims also had uncontrollable bouts of crying and laughter, and hallucinations of favourite foods and work tools.

One to two months after the onset of the initial symptoms, more serious symptoms emerge, with the first complaints being generalised muscle weakness and difficulty completing normal tasks. As the condition becomes more established, speech impairments develop with a wide range of intensity, from slight changes in pitch and tone, with others being incomprehensible. Other complaints reported by victims included insomnia, clumsiness of movement, tremors and sexual impotence.

One to two years after the onset of the condition neurological symptoms become permanently established. The most common of these are disorders of gait, speech and postural reflexes, expressionless facies and a fine, intermittent tremor of small amplitude (Mena, 1979). Table 1.1 outlines the neurological signs recorded from 15 patients with Mn poisoning.

Pathological features observed following Mn exposure include lesions of the globus pallidus and the striatum (Mena, 1979). The caudate nucleus and the putamen are severely damaged (Shukla and Singhal, 1984). A transient increase, of ~300 days duration, followed by a significant decrease in the dopamine concentration in the brain has been observed in humans (Mena, 1979; Shukla and Singhal, 1984; Bernheimer *et al.*, 1973; Chandra and Shukla, 1981), rats, mice and monkeys (Shukla and Singhal, 1984; Chandra and Shukla, 1981) with an overall decrease in the concentrations of dopamine. The initial increase in the concentration of catecholamines is believed to be

the cause of the observed increase in monoamine oxidase activity and homovanillic acid concentrations, which are both required to dispose of excess catecholamines (Chandra and Shukla, 1981; Shukla *et al.*, 1976).

Many of these symptoms can be reversed by treatment with the drug $_{L}$-3,4-dihydroxyphenylalanine ($_{L}$-DOPA). If treatment is not carried out with $_{L}$-DOPA symptoms persist until death.

Table 1.1 The neurological effects of Mn poisoning in 15 patients (adapted from Mena, (1979)). Where the symptom was not observed it is indicated by a dash (-) and severity is indicated by pluses (±, +, ++).

Patient No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Signs															
Abnormal gait	++	++	++	++	++	++	++	+	+	++	+	++	+	+	++
Retropulsion	++	++	++	++	++	++	++	+	-	++	+	++	-	-	-
Propulsion	++	++	++	-	++	++	+	+	-	++	-	++	-	-	-
Beam sign	++	++	++	++	++	++	++	+	-	++	-	++	-	-	-
Speech defect	++	++	+	++	++	++	+	+	-	++	+	++	-	++	-
Increased tone in upper extremity	++	-	-	++	++	++	+	-	-	-	-	-	-	++	++
Increased tone in lower extremity	++	-	+	++	++	++	+	-	-	-	+	+	+	+	++
Cogwheel	-	-	-	++	+	-	+	-	-	+	-	-	+	+	++
Expresionless facies	-	++	+	-	++	++	+	-	-	++	+	++	-	++	+
Laughter	+	-	+	-	-	-	+	+	-	+	-	-	-	+	-
Depression	-	++	+	-	-	-	+	+	-	+	-	++	-	+	-
Diminished power in upper extremities	-	-	+	-	-	++	+	-	-	+	-	++	-	+	-
Diminished power in lower extremities	-	+	+	-	-	++	+	-	-	+	-	++	+	-	+
Tremor	-	+	+	-	+	++	+	-	-	-	-	+	-	-	-
Increased deep tendon reflexes, upper extremity	-	++	-	++	+	++	-	+	-	++	+	-	-	-	+
Increased deep tendon reflexes, lower extremity	-	++	±	++	+	++	-	+	-	++	+	+	++	-	+
Babinsky	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Weeping	+	+	-	+	-	-	-	-	-	+	-	-	-	-	-
Salivation	-	++	-	-	-	-	+	-	-	-	-	-	-	+	++
Micrographia	-	-	-	+	-	+	-	-	-	++	-	-	-	+	-

1.1.2 Relationship between manganism and Parkinson's disease

Manganese poisoning and Parkinson's disease (PD) share a number of common characteristics, but they also display some distinct differences, and it has been hypothesised that Mn intoxication could be a factor in at least some cases of PD (Feldman, 1992). Both are associated with the standard extrapyramidal symptoms, including bradykinesia, rigidity, tremor and impairment of postural reflexes. However, Calne *et al.* (1994) concluded that manganism and PD differ in several details. Those with manganism exhibit resting tremors less frequently than those suffering from PD and have a greater propensity to fall backwards. Both conditions can be successfully treated with $_{L}$-DOPA using similar doses, but patients suffering from manganism do not experience the involuntary movements and hallucinations suffered by PD patients during treatment (Mena *et al.*, 1970). Furthermore, victims of manganism do not show a sustained therapeutic response to $_{L}$-DOPA when compared with the long-term benefits seen in PD patients. Manganism patients more often suffer from dystonia than those suffering from PD and a reduction in flurodopa uptake cannot be detected by positron emission tomography in manganism patients.

Nonetheless, victims of both Mn poisoning and PD do show some remarkable similarities (Liccione and Maines, 1988; Donaldson *et al.*, 1982). Victims of both conditions suffer from disruption to REM sleep periods when compared with controls (Mena, 1979), and decreases in the melanin and dopamine concentrations in the brain (Shukla and Singhal, 1984) have also been observed. Furthermore, both have been associated with mitochondrial dysfunction. There is a growing body of evidence (Graeber and Müller, 1998) that supports the association between PD, mitochondrial dysfunction and disruption of cellular energy metabolism. To date, it has been demonstrated that PD patients have reduced activities of mitochondrial electron transfer chain enzymes, alterations in glycolytic activity, mutations of the mitochondrial genome, and exhibit enhanced generation of free radicals (Graeber and Müller, 1998). The overwhelming similarities in the extrapyradimal symptoms observed in both conditions, and the parallels in their biochemical characteristics, prompts the suggestion that they could be caused by the same mechanism.

1.2 Mechanism of Manganese Poisoning

If Mn intoxication is associated with mitochondrial dysfunction, it must be demonstrated firstly that Mn is found in mitochondria and secondly that it disrupts the normal operation of the mitochondria. Three possible mechanisms have been proposed for the disruption of normal mitochondrial activity.

1. Inhibition of the mitochondrial electron transfer chain following Mn accumulation by mitochondria (Galvani *et al.*,1995; Gavin *et al.*, 1992; Taylor, unpublished).
2. Neuronal degradation by free radicals such as $O_2^{\cdot-}$ and ·OH (Donaldson *et al.*, 1982; Cadenas, 1989; Gavin *et al.*, 1992; Liccione and Maines 1988; Shukla *et al.*, 1980; Sloot *et al.*, 1996) cause lipid peroxidation and damage to DNA and protein.
3. Induction of mutation of the mitochondrial genome, as has previously been shown in *Chlamydomonas reinhardtii* (Bennoun *et al.*, 1992), yeast (Putrament *et al.*, 1975a) and bacteria.

1.2.1 Active accumulation of manganese by mitochondria

Manganese is actively accumulated by mitochondria. Manyard and Cotzias (1955) showed that an injection of a tracer dose of $^{54}Mn^{2+}$ became concentrated in rat liver mitochondria within fifteen minutes of administration. It has also been demonstrated that sub-chronic exposure of rats to Mn caused an increase in the intramitochondrial concentration of this ion in the brain (Liccione and Maines, 1989).

Manganese is rapidly removed from the blood stream ($t_{½}$ = 1-2 minutes), but the removal from the liver is much slower ($t_{½}$ = 25 days; Cotzias *et al.*, 1968). The rapid removal of Mn from the blood does not prevent it from accumulating in the brain, especially after inhalation or chronic exposure, because the rate at which it is removed from the brain is extremely slow ($t_{½}$ = 50-60 days; Cotzias *et al.*, 1968). Experimental studies have shown that Mn exposure results in accumulation in both the brain (Liccione and Maines, 1988), particularly in synaptically active tissues, and the liver (Hidiroglou and Shearer, 1976; Shukla and Chandra, 1987). The accumulation of Mn in the liver can result in liver damage (Fell *et al.*, 1996; Devenyi *et al.*, 1994), thereby restricting the rate of excretion of Mn and intensifying Mn toxicity.

It has long been known that Mn is taken into mitochondria by the calcium uniporter (Chance, 1965), however it has been shown that this Mn influx is very slow *in vitro* unless activated by calcium (Gavin *et al.*, 1990). Thus, in synaptically active tissues such as the globus pallidus, striatum, caudate nucleaus and the putamen, which experience frequent calcium spikes, there is an increase of Mn transport into mitochondria. A prolonged transport of Mn occurs, even though these calcium spikes are only short lived, because the calcium uniporter has separate sites for activation and transport, so calcium binds and remains bound for several minutes allowing large amounts of Mn to be transported (Gavin *et al.*, 1990). It has also been demonstrated that brain mitochondria have a very slow rate of Mn efflux, because they do not possess an active Mn efflux mechanism (Gavin *et al.*, 1990) and so Mn clearance is very slow. The fact that Mn influx in the presence of calcium can be relatively fast and that Mn efflux is very slow leads to an accumulation of Mn within the mitochondria.

Gunter *et al.* (1975) showed that the concentration of Mn accumulated in the mitochondrial matrix is increased in the presence of respiratory substrates or ATP, and that Mn efflux is stimulated following dissipation of the $\Delta\mu_H^+$, which implies that Mn accumulation is dependent on mitochondrial energisation (Gunter *et al.*, 1978). Puskin and Gunter (1973) have shown that the concentration of free Mn within the mitochondrial matrix could be at least 500 times greater than the external medium. However, if Mn is in equilibrium with a $\Delta\psi$ of -180 mV, then the matrix concentration could be as much as 10^6 times greater than the extramitochondrial concentration.

In normal conditions the intracellular concentration of Mn is usually about 10 nM and it is probable that most of this is located in the mitochondria. Given the percentage of cell volume that mitochondria make up in the average cell, the usual intramitochondrial concentration of Mn would be no more than about 50 nM. Thus, in normal conditions this concentration of Mn would have no detrimental effects on mitochondrial activity, but during chronic exposure to Mn the intramitochondrial concentration could reach as much as 50 mM, which could have serious repercussions.

1.2.2 Inhibition of mitochondrial electron transfer

It has been demonstrated that Mn is activity accumulated in the mitochondria (Section 1.2.1.). Gunter and Puskin (1975) showed, using electron paramagnetic resonance (EPR) measurements, that Mn is preferentially bound to the inner mitochondrial membrane once it enters the matrix. Respiratory oxygen consumption is catalysed by four enzymes located in the inner mitochondrial membrane (Figure 1.1) NADH dehydrogenase; succinate dehydrogenase; the cytochrome bc_1 complex; and cytochrome oxidase. Together, these enzymes catalyse electron transfer (the oxidation of reduced substrates and the reduction of oxygen to water) and the translocation of protons from the mitochondrial matrix to the inter-membrane space, which generates a $\Delta\mu_H^+$ necessary for ATP synthesis. Inhibition of electron transfer leads to an insufficient supply of ATP to the affected cell which would usually lead to eventual cell death.

A number of *in vivo* studies have shown Mn inhibition of the enzymes of the mitochondrial electron transfer chain. The oxidation of succinate was inhibited following administration of Mn *in vivo* (Singh *et al.*, 1979; Husain *et al.*, 1976) or to cultured human cells (Galvani *et al.*, 1995). Furthermore, Galvani *et al.* (1995) have demonstrated inhibition of both NADH dehydrogenase and NADH : cytochrome *c* reductase (involving both NADH dehydrogenase and the cytochrome bc_1 complex) in PC12 cells, and Husain *et al.* (1976) have shown inhibition of cytochrome oxidase following prolonged intra-peritoneal administration of $MnCl_2$.

In vivo experiments of this type do not allow one to distinguish between a reduction in the mitochondrial concentration of an enzyme and a reduction in the activity of the enzyme. A reduction in enzyme content could arise, for example, from mutations of the mitochondrial genome, resulting in a failure of assembly of electron transfer chain complexes or enhanced rates of degradation of complexes (Section 1.2.3). A reduction in the catalytic activity could arise from a mutation, which does not prevent assembly or affect the stability of the enzyme, but does alter its catalytic rate.

Both organic and inorganic Mn inhibits respiratory electron transfer in isolated mitochondria, which implies that Mn can act directly on those mitochondrial enzymes involved in energy transduction, rather than indirectly, through mutagenesis of the

mitochondrial genome for example. Gavin *et al.* (1992) demonstrated inhibition of ADP-stimulated oxygen consumption (state 3 respiration) by isolated rat liver mitochondria oxidising either succinate or glutamate/malate. However, they were unable to show any inhibition of succinate oxidation in the presence of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and observed only a slight inhibition of glutamate/malate oxidation. They did not observe Mn inhibition of state 4 respiration (that in the absence of ADP) and concluded that Mn interfered directly with the ATP synthase, which implies a specific inhibition of state 3 oxygen consumption, but does not explain the inhibition of NAD^+-linked substrate oxidation in the presence of CCCP. Furthermore, it has been shown that the ATP synthase is not inhibited by millimolar concentrations of Mn (Dorgan *et al.*, 1984) and ATP hydrolysis is not affected by *in vivo* Mn administration (Husain *et al.*, 1976). Therefore it seems unlikely that Mn inhibits the ATP synthase without affecting the activity of the mitochondrial electron transfer chain enzymes.

Galvani *et al.* (1995) showed $MnCl_2$ inhibition of NADH : cytochrome *c* reductase activity in mitochondria isolated from PC12 cells, but did not state whether ADP or uncoupler was present in the assays. Autissier *et al.* (1977) showed that MMT inhibited NAD^+-linked substrate oxidation only in the presence of ADP or an uncoupler. On the basis of these data, and the observations of Gavin *et al.* (1992), it appears probable that only high rates of electron transfer are affected by Mn, as is the case in the presence of ADP or uncoupler.

Nevertheless, these data do not establish which of the electron transfer chain enzymes are affected by Mn. Succinate-dependant oxygen consumption relies on the activity of three separate enzymes (succinate dehydrogenase, the cytochrome bc_1 complex and cytochrome oxidase), and the oxidation of NAD^+-linked substrates relies on two of these three enzymes, as well as the activity of NADH dehydrogenase (Figure 1.1). There are no data that definitively establish which of these enzymes is affected by Mn. However, NADH : cytochrome *c* reductase is usually reported to be more inhibited than either the succinate : cytochrome *c* reductase or the succinate : FeCN reductase activities. This implies that both NADH dehydrogenase and one or more of succinate dehydrogenase, the cytochrome bc_1 complex and cytochrome oxidase is slightly

inhibited following prolonged administration of Mn *in vivo*. Liccione and Maines (1989) observed a generalised 10-20% decline in the haem content of brain mitochondria isolated from rats treated subcutaneously with 1.75 mg kg^{-1} $MnCl_2$ for seven days, implying that the concentration of each of the enzymes could have been affected.

The evidence gathered so far indicates that Mn is actively accumulated by mitochondria and that Mn can inhibit electron transfer, but the site and mechanism are yet to be determined. Furthermore, a direct link between mitochondrial dysfunction and the brain degradation observed in patients is yet to be confirmed. However, this hypothesis may help to explain why the caudate nucleus, globus pallidus, striatum and putamen is preferentially damaged, because this tissue is synaptically active and thus accumulates a large concentration of Mn and has high demand for ATP. For this reason it is very vulnerable to a decrease in available ATP caused by mitochondrial dysfunction which may lead to cell death.

1.2.3 Induction of mitochondrial mutations

Manganese is a well known mutagen. It induces mutations in the mitochondrial genome of *Saccharomyces cerevisiae*, whereas several other divalent cations (including iron, magnesium, zinc and copper) do not (Putrament *et al.*, 1977), and typically only the mitochondrial genome is affected (Putrament *et al.*, 1975a). It has also been shown that Mn induces mutations in the mitochondrial genomes of *Schizosaccharomyces pombe* (Colson *et al.*, 1976) and *Chlamydomonas reinhardtii* (Bennoun *et al.*, 1992), of bacterial genomes (Roberts and Aldous, 1951), of bacteriophage T4 DNA (Orgel and Orgel, 1965) and of isolated DNA *in vitro* (Dube and Loeb, 1975). The concentrations of Mn in the growth medium employed in inducing mutations range from 10 μM in *Escherichia coli* to 4-8 mM in *S. cerevisiae* and *C. reinhardtii*.

It has been shown that Mn causes a decrease in the fidelity of the replication of viral and *E. coli* DNA *in vitro*, perhaps by binding to the DNA (Eichhorn and Shin, 1968) and altering the hydrogen bonding pattern between bases. Certainly, Putrament *et al.* (1975b) have shown that mtDNA replication, but not protein synthesis, is required for Mn mutagenesis. More specifically, Kunkel (1985) showed that Mn increases the

frequency of base substitutions by chicken liver mitochondria DNA polymerase-γ, but did not alter the frequency of base substitution by either DNA polymerase-α or DNA polymerase-β. The same selectivity could operate in mammalian mitochondria.

Furthermore, as mitochondria have less efficient DNA repair mechanisms than the nucleus and mtDNA lacks histones (Richter, 1994), mutations occur more frequently and have a much greater tendency to remain in the genome and be inherited. The mutations induced are of seven types, the two most common being deletion mutations, in which regions of the genome are deleted, and point mutations. In either case, a frequent consequence is an inhibition of the activity of the respiratory electron transfer chain, because one or more subunits of each of the complexes (except for succinate dehydrogenase) are encoded by mtDNA. As each mitochondrion in human cells (about 1700 per hepatocyte, for example) has several copies of the genome, mutant and wild-type genomes coexist within a cell, but until the proportion of mutant genomes is high, there need not be any specific phenotype.

No data demonstrates directly that Mn intoxication is associated with mutations of mammalian mtDNA. However, Itoh *et al.* (1994) have shown that the mtDNA of HTC cells incubated for three hours with 100 μM $MnCl_2$ was not nicked, whereas cells incubated with 10 μM $FeCl_3$ exhibited an increase in the proportion of relaxed super-coiled mtDNA. These data imply that there is little or no change in the extent of nicking of mtDNA in the presence of Mn, but they do not represent evidence for or against the possibility of mutations of mtDNA. Shukla *et al.* (1976) showed decreases in both total DNA and total RNA in the brains of rats treated intraperitoneally with 8 mg $MnCl_2\ kg^{-1}$ for 120 days, consistent with the observation that nuclear DNA replication is inhibited by Mn in yeast (Putrament *et al.*, 1977).

In the absence of any other data, it remains possible that Mn could induce mutations in mammalian mtDNA, perhaps through a direct effect on DNA polymerase-γ, as is the case for other eukaryotes, such as chickens, yeast and *C. reinhardtii*. This possibility merits further investigation.

1.2.4 Influence on levels of free radicals

The mitochondrial electron transfer chain is the single most significant source of oxygen radicals ($O_2^{\cdot-}$, ·OH) in any cell. Mitochondria consume 90% of the cell's oxygen and it has been estimated that the average person could produce more than 2 kg of $O_2^{\cdot-}$ each year (Halliwell, 1994), which implies that much of the $O_2^{\cdot-}$ is produced by the mitochondrial electron transfer chain.

Hydroxyl radical formation, results from $O_2^{\cdot-}$ reacting with hydrogen peroxide (H_2O_2), which is also produced in the mitochondria from ubisemiquinone and UQH_2 of the inner membrane (Boveris *et al.*, 1992). This H_2O_2 then reacts with $O_2^{\cdot-}$ to produce ·OH.

$$O_2^{\cdot-} + H_2O_2 \longrightarrow O_2 + OH^- + \cdot OH \qquad [1.1]$$

These free radical species are extremely reactive and cause lipid peroxidation, mutagenesis of DNA and damage to protein (Chance *et al.*, 1979; Halliwell, 1994; Shukla *et al.*, 1980). In normal conditions the mitochondria are protected from these radicals by the various SOD isoforms, catalase (EC 1.11.1.6), GSH peroxidase as well as a number of other mechanisms (Cadenas, 1989).

Several models have been proposed to describe the role of Mn in influencing the levels of free radicals in the mitochondria.

1. The concentration of free radicals within mitochondria can be rapidly increased by the addition of an inhibitor such as antimycin A (Boveris and Chance, 1973). So, if Mn is an inhibitor of mitochondrial electron transfer (Section 1.2.2) it could significantly increase the levels of free radicals within the mitochondria. This hypothesis implies that the damage to brain tissue observed in patients who have suffered from manganism is not caused directly by Mn but by inhibiting the mitochondrial electron transfer chain this leads to a build up of free radical species which disrupt normal cellular function. This effect is exacerbated by the generation of free radicals which are also known to inhibit mitochondrial electron transfer (Cadenas, 1989).
2. The second hypothesis is that Mn is able to decrease the levels of enzymes and metabolites that are required to protect the cell from oxidative damage. These

antioxidants, such GSH peroxidase, catalase, SOD, ascorbic acid, vitamin E, and β-carotene, scavenge free radical species and protect the cell from damage. Liccione and Maines (1988) demonstrated that Mn is able to decrease the activity of GSH peroxidase and catalase by up to 65% after sub-chronic exposure. This decrease in the presence of these enzymes was most significant in the striatum region of the brain and was accompanied by a decrease in the concentration of dopamine and dopamine metabolites in this region (Liccione and Maines, 1988). The observation of a significant decrease in dopamine concentration in the striatum and damage to this region are classical post mortem symptoms observed in patients suffering from manganism (Section 1.1.1.).

3. Manganese is able to exist in several oxidation states and exhibits both a powerful oxidant and antioxidant properties dependent on its oxidation state (Archibald and Fridovich, 1982).

$$Mn^{(II)} + O_2^{\cdot-} + 2H^+ \longrightarrow Mn^{(III)} + H_2O_2 \qquad [1.2a]$$

$$Mn^{(III)} + H_2O_2 \longrightarrow MnO_2^+ + 2H^+ \qquad [1.2b]$$

$$MnO_2^+ + Mn^{(III)} \longrightarrow (Mn - O - O - Mn)^{+4} \qquad [1.2c]$$

$$[Mn - O - O - Mn]^{+4} \longrightarrow 2Mn^{(II)} + O_2 \qquad [1.2d]$$

The inactivation of $O_2^{\cdot-}$ (reaction 1.2a) is accompanied by the generation of H_2O_2 and $Mn^{(III)}$. Divalent Mn is then regenerated by the reduction of trivalent Mn in the presence of a high concentration of H_2O_2 (reactions 1.2b-1.2d). In these reactions Mn serves a key function in bringing about the inactivation of two major species of active oxygen, $O_2^{\cdot-}$ and H_2O_2, because of its ability to alter between high and lower oxidation states with an appropriate free radical substrate. This results in reduced free radical activity. This hypothesis is supported by some work by Donaldson *et al.* (1982) who observed a decrease in the lipid peroxidation in several regions of rat brain that had been exposed to sub-chronic concentrations of Mn.

However, if there are conditions of inadequate H_2O_2 scavenging and high concentrations of divalent Mn, these could react to produce the highly toxic ·OH, along with the powerful oxidant $Mn^{(III)}$ (Donaldson *et al.*, 1982).

$$Mn^{(II)} + H_2O_2 \longrightarrow \cdot OH + OH^- + Mn^{(III)} \qquad [1.3]$$

This reaction may help to explain why certain regions of the brain are more affected than others, if the constituents of a particular region (eg. high H_2O_2) favour the formation of $Mn^{(III)}$, the ability of the ion to produce selective lesions could be understood (Donaldson *et al.*, 1982).

Variation in valence can also produce ·OH, but it is not Mn that is altering its valency state in this case. In the presence of a catalyst such as $Fe^{(III)}$ and $O_2^{\cdot -}$, H_2O_2 can dismutate to form ·OH and OH^- by the Haber-Weiss reaction.

$$Fe^{(II)} + O_2^{\cdot -} \longrightarrow Fe^{(III)} + O_2 \qquad [1.4a]$$

$$Fe^{(II)} + H_2O_2 \longrightarrow \cdot OH + OH^- + Fe^{(III)} \qquad [1.4b]$$

$$O_2^{\cdot -} + H_2O_2 \longrightarrow \cdot OH + OH^- + O_2 \qquad [1.4c]$$

It is unlikely that Mn is able to catalyse the Haber-Weiss reaction itself (Archibald and Tyree, 1987; Sloot *et al.*, 1996) and accumulation of ·OH has been observed in the stratium following exposure to Mn (Sloot *et al.*, 1996). This could possibly be generated from the iron catalysed dismutation of $O_2^{\cdot -}$.

4. The final hypothesis is that Mn is able to increase the levels of free radicals within the cell by increasing the rate of non-enzymatic autoxidation of catecholamines, which produces toxic quinones and elevated levels of $O_2^{\cdot -}$, ·OH and H_2O_2. It has also been proposed that Mn is capable of catalysing the production of 6-hydroxydopamine (6-OHDA) from dopamine. 6-OHDA is a pro-neurotoxin that is believed to damage vulnerable neurons of the lower brain stem, that are presumed to give rise to PD like symptoms (Linert *et al.*, 1996).

Manganese is capable of acting as an effective scavenger of free radical species (Archibald and Fridovich, 1982), a producer of free radical species, and as a powerful oxidant itself. The determining factor that influences which role it adopts is the oxidation states in which it exists. The literature suggest the most favourable form within the mitochondria is $Mn^{(II)}$ as this is capable of scavenging radicals, and thus acts as an antioxidant. However, I am unaware of any reports of the effects of Mn on the generation of free radicals in isolated mitochondria.

1.2.5 Association of the three proposed mechanisms of manganese toxicity

Given that Mn accumulates specifically in the mitochondrial matrix, there are three principal mechanisms whereby Mn might disrupt mitochondrial function: (1) by inhibition of energy transduction (Section 1.2.2); (2) by the induction of mutations of the mitochondrial genome (Section 1.2.3); and (3) through the enhanced generation of free radicals (Section 1.2.4). There is no information which refutes any of the hypotheses, but the most reasonable explanation appears to be provided by the models involving the inhibition of the mitochondrial electron transfer chain and the generation of free radicals. These hypotheses are linked, as is well established in the literature in connection with PD (Section 1.1.2). For example, it is known that the inhibition of mitochondrial electron transfer enhances the generation of free radicals by the electron transfer chain enzymes (Boveris and Chance, 1973) and that free radicals can induce mutations of the mitochondrial genome. Furthermore, it is well known that free radicals can damage proteins and membranes (Halliwell, 1994) thereby disrupting the operation of the electron transfer chain (Figure 1.2).

1.3 Aims of this Project

Manganese poisoning causes a number of awful symptoms which continue after the removal of the victim from the source of contamination and may worsen, and eventually prove fatal, unless appropriate treatment is given. Victims initially suffer from psychomotor disturbances, followed by more serious symptoms one to three months later. One to two years after the initial exposure, neurological symptoms become permanently established.

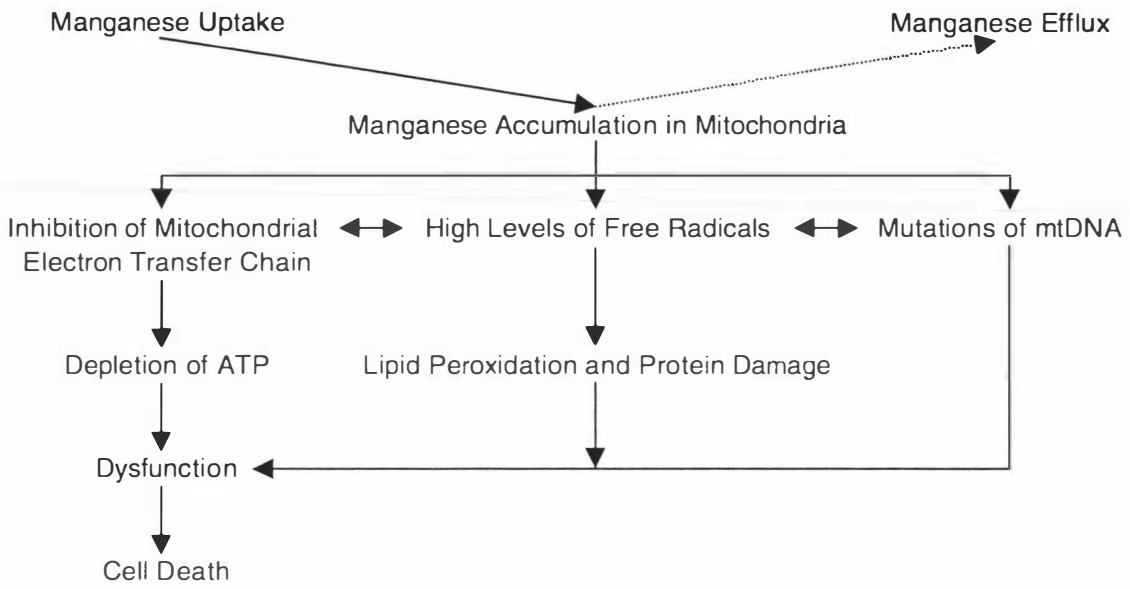

Figure 1.1 The relationship between the three models of Mn neurotoxicity

The mechanism of Mn poisoning is not well understood, however a number of hypotheses have been proposed (Brown and Taylor, 1999).

1. Inhibition of mitochondrial electron transfer chain following Mn accumulation by mitochondria (Galvani *et al.*, 1995; Gavin *et al.*, 1992; Taylor, unpublished).
2. Neuronal degradation caused by free radicals ($O_2^{\cdot-}$ and ·OH) (Donaldson *et al.*, 1982; Cadenas, 1989; Gavin *et al.*, 1992; Liccione and Maines, 1988; Shukla *et al.*, 1980; Sloot *et al.*, 1996) mediated lipid perioxidation and damage to DNA and protein.
3. Induction of mutation of the mitochondrial genome, as has previously been shown in *C. reinhardtii* (Bennoun *et al.*, 1992), yeast (Putrament *et al.*, 1975a), and bacteria.

Each of these could result in cellular death in susceptible regions of the brain such as the globus pallidus, straitum (Mena, 1979), caudate nucleus and the putamen (Shukla and Singhal, 1984). Damage to these regions has been implicated in causing the symptoms observed in victims of Mn poisoning.

The aim of this project is to examine these hypotheses by analysing the effect of Mn on mitochondrial electron transfer of sheep liver mitochondria, *in vitro* (Chapter 3). This will be followed by an examination of the production of free radical species by the mitochondrial electron transfer chain and the effect of Mn on this production (Chapter

4). Finally the effect of Mn on mtDNA will be examined (Chapter 5). These experiments will allow an examination of the possible causes of Mn poisoning.

CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and solvents

All chemicals and reagents used were of analytical grade or better. Most were obtained from Sigma Chemical Company, (St Louis, MO, USA.). Others were purchased from Merck Lab, (Darmstadt, Germany), Aldrich Chemical Company, (Milwaukee, WI, USA.), BDH Laboratory Supplies, (Poole, England), Ajax Chemicals, (Auburn NSW., Australia), ICN Biomedicals Inc., (Aurora, Ohio, USA.) and Boehringer Ingelheim Bioproducts, (Heidelberg, Germany).

All solvents were of analytical grade or better and were purchased from BDH Laboratory Supplies, (Poole, England) and Reidel-de Haën, (Seelze, Germany).

2.1.2 Enzymes

Restriction enzymes *Pst*I, *Hin*dIII, *Pvu*II, *Acc*I and *Hin*cII and enzyme reaction buffers were obtained from New England Biolabs, (MA, USA.). Where required the specified reaction buffer for a particular restriction enzyme was used. *Thermus aquaticus* DNA polymerase (*Taq* polymerase) was purchased from Gibco BRL Life Technologies Ltd, (MD, USA.) SOD (from bovine erythrocytes) and catalase (from bovine liver) were procured from Sigma Chemical Company, (St Louis, MO, USA.).

2.1.3 Primers

Oligonucleotide primers COX1for and COX1rev (Appendix I) were purchased from Gibco BRL Life Technologies Ltd, (MD, USA.).

2.1.4 Miscellaneous products

DNAzol reagent was purchased from Gibco BRL Life Technologies Ltd, (MD, USA.). The tissue press apparatus was designed and constructed in-house (Appendix II).

2.1.5 Sources of liver

Sheep livers were obtained from animals being used for other experimental purposes. These included animals being killed in the Veterinary Science Post Mortem Room, Massey University, Palmerston North; The Animal Physiology Unit, Massey University Palmerston North; or at the Ag Research premises, Bachelor Centre, Palmerston North. The livers were removed as quickly as possible and then transported on ice to the laboratory for mitochondrial preparation.

2.2 General methods

2.2.1 Preparation of reagents

All solutions were prepared using milliQ water or 95% ethanol, depending on the solubility of a particular solute, although water was used preferentially. All eppendorf tubes and disposable plastic as well as enzyme buffers supplied sterile by enzyme manufacturers and water used in PCR and restriction digests reactions was sterilised by autoclaving before use.

2.2.2 Calibration of the Aminco DW2a spectrophotometer

An Aminco DW2a spectrophotometer was used for all spectrophotometric kinetic assays. This instrument was calibrated using a holmium oxide filter, which has a well characterised absorbance spectrum. Firstly the accuracy of the wavelengths of light produced by monochrometer 1 was assessed using the positions of known absorbance peaks of the holmium filter. The accuracy of monochrometer 2 was assesed by taking a derivative spectrum using the holmium filter. During this test the smallest peaks and troughs were sought as an indication that both monochrometers were producing the same wavelengths. The final calibration test carried out was to determine the sizes of the peaks produced by the spectrophotometer and compared these with the published

values. This series of tests allowed me confidently to use the spectrophotometer with the various offsets calculated.

2.2.3 Preparation of reduced cytochrome *c*

Reduced cytochrome *c* (Practical grade: from bovine heart) was prepared by adding 7 mM ascorbate to 3 ml of ~15 mg ml^{-1} cytochrome *c*, this caused a distinct colour change due to the reduction of the haem. This reduced cytochrome *c* was then passed down a small Sephadex G-25 column to remove any excess reductant. The concentration of this product was determined spectrophotometrically (Section 2.2.4) and stored at -70°C for up to three months.

2.2.4 Determination of reduced cytochrome *c* concentration

The final concentration of cytochrome *c* was determined by measuring the absorbance of a diluted sample at 550 nm and 542 nm. The values obtained from these measurements and the extinction coefficients (Margoliash and Frowhirt, 1959) were then used to calculate the final concentration from the equation below.

$$\text{Total reduced cytochrome } c = \frac{A_{550} - 9.0 \times (A_{542} / 9.9)}{18.7}$$

2.3 Preparation and storage of mitochondria

2.3.1 Preparation of mitochondria

Sheep liver mitochondria were prepared by a method based on that of Reid and Husbands (1985). All glassware was cooled to 0-4°C and all centrifugation was carried out within this temperature range. Approximately 50 g of tissue was removed from the extremity of the liver, to avoid most connective and vascular tissue, and rinsed in extraction buffer (225 mM mannitol, 75 mM sucrose, 50 μM ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetra-acetic acid (EGTA), 50 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES)-HCl, pH 7.4). The liver was then chopped into 1-3 mm cubes on a glass plate and minced using a tissue press (Appendix II) by forcing it through 1 mm metal gauze into a Potter-Elvenhjem homogenising tube

containing 40 ml of extraction buffer. This tissue was further disrupted with two passes, by hand, of a 0.12 mm clearance pestle. The homogenate was then diluted by addition of a further 20 ml of extraction buffer and centrifuged in an HB4 rotor at 800 × *g* (2250 rpm) for five minutes. The supernatant was recentrifuged at 4500 × *g* (5250 rpm) for 20 minutes in the HB4 rotor to sediment the mitochondria. The mitochondrial supernatant was discarded, and any fat on the side of the tubes was removed with tissue paper. The mitochondrial pellet was resuspended in 30 ml of extraction buffer and recentrifuged at 4500 × *g* (5250 rpm) for 15 minutes. The supernatant was discarded, and the pellet resuspended in 30 ml extraction buffer and recentrifuged at 4500 × *g* (5250 rpm) for ten minutes. The final pellet was resuspended in 500 µl of extraction buffer and gave a yield of about 4 mg g^{-1} of liver, and the mitochondria were used on the day of preparation or stored (Section 2.3.2).

2.3.2 Preparation of sub-mitochondrial particles

Sub-mitochondrial particles were prepared from thawed sheep liver mitochondria by incubation with 1 mM ATP for ten minutes at 4°C followed by passage through a French press (Aminco Instrument Company, Silver Springs, Ml, USA.) at 3500 p.s.i.. After removal of unbroken mitochondria by centrifugation in an SS-34 rotor at 9000 × *g* (8500 rpm) for ten minutes, the submitochondrial particles were sedimented three times by centrifugation in an SS-34 rotor at 65000 × *g* (24500 rpm) for one hour. The resulting pellet from each spin was washed using extraction buffer (225 mM mannitol, 75 mM sucrose, 50 µM EGTA, 50 mM HEPES-HCl, pH 7.4) and resuspended to 50 ml. After the final centrifugation, the pellet was resuspended in 2 ml of extraction buffer.

2.3.3 Storage of mitochondria

Mitochondria were preferentially made on the day of experimentation, however, when fresh liver was scarce, stored mitochondria were occasionally used in the designing of experimental procedures. When mitochondria with respiratory functions were required, they were fortified in a 1.5 ml Nunc tube with 1% bovine serum albumin (BSA) (Fraction V powder) and 10% dimethyl sulfoxide (DMSO), a cryoprotective substance (Fleisher, 1979). They were then quick-frozen in liquid nitrogen and stored at -70°C for

a period of up to two weeks. When respiratory function was not important (Section 2.7.1) mitochondria were stored in isolation buffer at -70°C.

2.4 Determination of mitochondrial quality

2.4.1 Protein determination of extracted mitochondria

The total protein concentration of extracted mitochondria was determined by the Biuret method described by Layne (1961). A standard curve was created using BSA with concentrations ranging between 1 mg ml^{-1} and 10 mg ml^{-1}. The absorbance values for each sample were measured in triplicate with an Hitachi U1100 spectrophotometer at 550nm. The concentration of the unknowns was then calculated from the standard curve.

2.4.2 Measuring oxygen consumption of mitochondria

Respiratory measurements were carried out in a Clark type electrode (Hansatech Instruments Ltd, King Lynn, U.K.) which was connected to a computer via a data acquisition system (Science Faculty Electronics Workshop, Massey University, Palmerston North, NZ; Brown and Dykstra, 1999). The software used to collect and record the data on the computer was developed in house. The electrode consists of a platinum cathode and a silver anode embedded in epoxy resin. The cathode was covered by a membrane and spacer of cigarette paper, to allow the electrolyte (200 mM KCl, 66 mM K_2HPO_4, 600 mM KNO_3, 70 mM NaOH at pH 11.2-HCl; Walker, 1990) access to both terminals. This was then mounted in a perspex chamber surrounded by a temperature-controlled water jacket. The standard assay buffer used in experiments was 70 mM HEPES-HCl pH 7.4.

All electrode measurements were carried out at 37°C and it was assumed that the oxygen concentration in the buffer was 240 µM. Calibrating the electrode was carried out by adding sodium dithionite to the reaction chamber to remove all oxygen present.

2.4.3 Determining the quality of mitochondria produced

The quality of mitochondria was determined by assaying with succinate and malate/pyruvate to determine the maximum rate of coupled and uncoupled electron transfer. The coupled rate was found by adding either substrate and measuring the resulting stimulation in oxygen consumption and therefore electron transfer. The addition of either ADP or 2,4-dinitrophenol (DNP) after either substrate caused a stimulation of electron transfer due to uncoupling. The buffer used in these assays differed slightly from the standard assay buffer (70 mM HEPES-HCl pH 7.4), this isotonic buffer (100 mM KCl, 2 mM $MgCl_2$, 2 mM KH_2PO_4, 70 mM HEPES-HCl pH 7.4) contained phosphate to allow ADP stimulation and other components that maximise the coupled and uncoupled rate.

2.5 Measurements of mitochondrial electron transfer

2.5.1 Assays of electron transfer chain activities

The standard assay buffer was 70 mM HEPES-HCl pH 7.4 and all experiments were carried out at 37°C. In most assays mitochondria were uncoupled by the addition of 20 μM DNP.

2.5.2 Measurement of electron transfer from succinate to cytochrome *c*

Electron transfer from succinate dehydrogenase to cytochrome *c* was measured using a large pool of exogenous cytochrome *c*. Electrons produced by the oxidation of succinate to fumarate, were passed into the UQ/UQH_2 pool, which transferred the electrons via the cytochrome bc_1 complex, reducing the large pool of excess cytochrome *c*. The reduction of cytochrome *c* was followed spectrophotometrically at 550-542 nm, as the reduced and oxidised forms have different absorbance properties. To prevent the oxidation of the cytochrome *c* by cytochrome oxidase cyanide was added (Figure 1.1).

2.5.3 Measurement of electron transfer from malate/pyruvate to cytochrome c

Measuring electron transfer from NADH dehydrogenase to cytochrome *c* was similar to electron transfer from succinate dehydrogenase to cytochrome *c*. Electrons produced by the oxidation of a combination of malate and pyruvate were passed into the UQ/UQH_2 pool, which transferred the electrons via the cytochrome bc_1 complex, reducing the large pool of excess cytochrome *c*, followed spectrophotometrically at 550-542nm in the presence of cyanide (figure 1.1).

2.5.4 Measurement of succinate dehydrogenase activity

Succinate dehydrogenase activity was measured by the use of the artificial electron acceptor 2,6-dichlorophenol-indophenol (DCPIP). Electrons produced by the oxidation of succinate to fumarate, were passed to the UQ/UQH_2 pool, from this pool they then reduced DCPIP (Figure 1.1). This reduction caused a distinct colour change that could be measured spectrophotometrically at 600 nm. To prevent electrons from entering the cytochrome bc_1 complex and bypassing the DCPIP the inhibitor myxothiazol was added (Figure 1.1).

2.5.5 Measurement of cytochrome *c* oxidase activity

The activity of this enzyme was measured by the addition of a large pool of excess reduced cytochrome *c* (Section 2.2.3) to the isolated mitochondria. Cytochrome oxidase oxidised this pool to catalyse the reduction of oxygen to water resulting in the oxidation of the reduced cytochrome *c* (Figure 1.1). The oxidation of reduced cytochrome *c* could be followed spectrophotometrically at 550-542 nm. This was a direct measurement of the activity of cytochrome oxidase.

2.6 Free radical preparation and measurement

2.6.1 Production of superoxide radicals

Superoxide radicals were produced chemically using the method described by Goldstein and Czapski (1996). A piece (50 mg) of solid potassium superoxide (KO_2) was

dissolved in ice cold NaOH. After approximately thirty seconds, once bubbling had stopped, 300 µl of this solution was added to 3 ml of 50 mM 2-amino-2-methyl-1 propanol-HCl (AMProp-HCl) pH 9.5 buffer and mixed rapidly in a cuvette. The decay of $O_2^{\cdot-}$ was monitored directly at 250-360 nm. Catalase was added to degrade H_2O_2 which is formed by the decay of $O_2^{\cdot-}$.

2.6.2 Measurement of superoxide radicals using NBT

To measure $O_2^{\cdot-}$ production indirectly NBT was used as a detector molecule. In the presence of $O_2^{\cdot-}$ NBT is converted to blue formazan (Goldstein and Czapski, 1996). 300 µl of KO_2 solution containing $O_2^{\cdot-}$ (Section 2.6.1) was added to 3 ml of buffer (70 mM HEPES-HCl pH 7.4) and 90 µM NBT, the formation of formazan was followed at 550 nm. For the indirect measurement of $O_2^{\cdot-}$ in a mitochondrial system NBT was also used. Electron transfer was started by the addition of succinate, from which, in the presence of the inhibitor antimycin A, large amounts of $O_2^{\cdot-}$ were expected. The assays were carried out in 70 mM HEPES-HCl buffer pH 7.4. and were followed spectrophotometrically at 550 nm.

2.6.3 Measurement of superoxide radicals acetylated cytochrome *c*

Superoxide radical production was also measured using the acetylated cytochrome *c* method (Goldstein and Czapski, 1996), a second indirect method that can be used in a mitochondrial system. This method measures the reduction of acetylated cytochrome *c* by $O_2^{\cdot-}$. Once electron transfer from succinate dehydrogenase to oxygen was established and in the presence of the inhibitor antimycin A high rates of $O_2^{\cdot-}$ production were observed at 550-542 nm. The acetylated cytochrome *c* present in the assay was itself not oxidised by cytochrome *c* oxidase due to the acetylation which prevented it from being utilised at any stage of the mitochondrial electron transfer chain.

2.6.4 Production of hydroxyl radicals

Hydroxyl radicals were produced chemically using the Fenton type reaction, with ferrous chloride ($FeCl_2$) acting as the electron donor and H_2O_2 acting as an electron acceptor.

$$Fe^{(II)} + H_2O_2 \longrightarrow {}^{\cdot}OH + OH^- + Fe^{(III)} \qquad [2.1]$$

Hydroxyl radicals were produced by rapid mixing of $FeCl_2$ and H_2O_2 in a cuvette. This was carried out in 70 mM HEPES-HCl pH 7.4 and the formation of ferric chloride ($FeCl_3$) was followed spectrophotometrically at 360 nm.

2.7 Molecular biological techniques

2.7.1 Isolation of mitochondrial DNA

Sheep mtDNA was prepared from isolated mitochondria (Section 2.3.1) using a modification of the method supplied with the DNAzol reagent. 150 µl of a suspension of mitochondria in extraction buffer (225 mM mannitol, 75 mM sucrose, 50 µM EGTA, 50 mM HEPES-HCl, pH 7.4) was mixed by inversion with 1 ml of DNAzol in a 1.5 ml tube and allowed to react at room temperature for five minutes. This mixture was centrifuged at 4°C for ten minutes at 10000 × *g* (10000 rpm) in a bench-top centrifuge. 1 ml of the supernatant was transferred to a clean tube, mixed with 0.5 ml of 100% ethanol and stored at room temperature for five minutes. This mixture was centrifuged at 4°C for 30 minutes at 14000 × *g* (14000 rpm) to pellet the mtDNA. The supernatant was removed using a pipette and the pellet was gently washed twice with 0.5 ml of 95% ethanol, recentrifuged and, after removing the supernatant, dried under reduced pressure. The dried pellet was resuspended in 100 µl of 8 mM NaOH. Once resuspension was complete the solution was adjusted to pH 8.0 by the addition of 11.5 µl of 1 M HEPES (free acid). The solution was centrifuged at 4°C for ten minutes at 10000 × *g* (10000 rpm) and the supernatant stored at -70°C. An aliquot was analysed on an agarose gel to determine the quality and approximate concentration of the preparation (Figure 5.1).

2.7.2 Agarose gel electrophoresis

Electrophoresis of DNA was performed in 0.9% (type 1-A:Low EEO) agarose gels containing 200 ng/ml ethidium bromide according to Sambrook *et al.* (1989). Minigel trays either 10 cm long and 6.5 cm wide or 10 cm long and 13 cm wide were used to

pour and electrophorese all agarose gels. The tank buffer and gel buffer was TAE (Tris (40 mM) acetic acid (20 mM) ethylene diamaine tetra-acetic acid (EDTA) (1 mM) pH 8.0). The size of DNA fragments was determined by comparison with the 1 Kb plus molecular size ladder (Gibco BRL Life Technologies Ltd, MD, USA). DNA fragments separated by gel electrophoresis were visualised using long wavelength ultraviolet (UV) light (366 nm), and gel images captured on an Alphaimager 2000 (version 2.23) system (Alpha Innotech Corporation).

2.7.3 DNA digestion with restriction endonucleases

All restriction endonuclease digests were carried out in 0.5 ml plastic tubes using the buffers supplied with the enzymes. A reaction mix for a typical DNA digest was:

Sterile H_2O	12.75 µl
DNA (~200 ng/µl)	5 µl
Reaction Buffer (10X)	2 µl
Enzyme (10-20 U/µl)	0.25 µl

Reactions mixes were incubated for three hours at the temperature suggested by the enzyme suppliers. All digests were prepared, and the products stored, at 4°C. Where appropriate, positive controls using the plasmid pGEX-2T and the restriction endonuclease *Hin*dIII were carried out to confirm the correct conditions for digestion. Negative controls with no restriction endonucleases were also used to confirm that the resulting products were not formed by non-enzymic DNA degradation.

2.7.4 DNA amplification

The cytochrome *c* oxidase subunit 1 gene (CO1) from isolated sheep mtDNA was amplified by polymerase chain reaction (PCR). Reactions were carried out in sterile 0.5 ml tubes overlaid with a drop of mineral oil. PCR reactions were prepared as follows:

Sterile H_2O	9 µl
10X PCR buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, pH 8.3)	2 µl

dNTPs (2.5 mM of each dNTP)	2 µl
$MgCl_2$ (25 mM)	0.8 µl
COX1for primer (50 ng/µl)	2 µl
COX1rev primer (50 ng/µl)	2 µl
Template mtDNA (~1 ng/µl) (isolated as described in section 2.7.1)	2 µl
Taq polymerase (5 U/µl)	0.2 µl

Initially, the reaction mixture was heated to 95°C for three minutes to separate the strands of template DNA and ensure that all reaction components were mixed and free in solution. The following temperature cycling programme was then applied.

95°C for 1 minute	Denaturing to release single stranded templates
41°C for 0.75 minute	Annealing of primers to single stranded templates
72°C for 2 minutes	DNA synthesis by *Taq* polymerase

This programme was repeated for 30 cycles. In the final cycle DNA synthesis at 72°C was allowed to proceed for five minutes. In addition to each PCR reaction a negative control containing no template DNA was run, to ensure that products were the result of amplification of the added template DNA. Completed reactions were stored at 4°C prior to analysis by agarose gel electrophoresis.

2.7.5 Treatment of mitochondrial DNA with manganese chloride, magnesium chloride and reactive oxygen species

Isolated mtDNA was treated with $O_2^{\cdot-}$ (Section 2.6.1), ·OH (Section 2.6.4), H_2O_2, $MgCl_2$ and various concentrations of $MnCl_2$ (Table 5.1). After three hours at room temperature, 50 µl of 100% ethanol, and 2.5 µl of 3 M sodium acetate (pH 5.5) were added to each sample and the solutions were stored at -70°C for 30 minutes. They were centrifuged at 14000 × *g* (14000 rpm) for thirty minutes at 4°C in a bench-top centrifuge to precipitate the mtDNA. The supernatants were removed using a pipette, and 100 µl of cold 70% ethanol added. The solutions were briefly vortexed and recentrifuged at 14000 × *g* (14000 rpm) for five minutes. The supernatants were removed, the pellets dried under reduced pressure and resuspended in 10 µl TE (Tris-HCl (10 mM) EDTA (1 mM)

pH 8.0) buffer and stored on ice for PCR. PCR reactions were then carried out as outlined in section 2.7.4.

2.7.6 Preparation of PCR products for automated DNA sequencing

The PCR product in 10 µl of a 20 µl PCR mix (Section 2.7.4) was purified using a QIAquick spin column and a microcentrifuge according to the manufacturers' protocol (QIAquick PCR Purification Kit, Qiagen). The purified PCR product was quantified on a 0.8% agarose gel against 20 ng, 50 ng and 100 ng DNA standards. The quantified product was then diluted to 25 ng/µl and submitted to Lorraine Berry (MUSeq) for automated sequencing.

CHAPTER 3
THE EFFECTS OF MANGANESE ON MITOCHONDRIAL ELECTRON TRANSFER CHAIN ENZYMES

Respiratory oxygen consumption is catalysed by four enzymes located in the inner mitochondrial membrane (Figure 1.1). Together, these enzymes catalyse electron transfer and the translocation of protons from the mitochondrial matrix to the intermembrane space, generating a $\Delta\mu_H^+$, which is dissipated in the synthesis of ATP. Manganese is accumulated in the mitochondria by the calcium uniporter (Chance, 1965) and as mitochondria do not possess an active Mn efflux mechanism, Mn clearance is very slow (Gavin *et al.*, 1990). The work described in this chapter examined whether Mn is able to inhibit the normal function of the mitochondrial electron transfer chain. This was achieved by analysing the effects of Mn on whole chain electron transfer and individual enzymes of the mitochondrial electron transfer chain.

3.1 The quality of isolated sheep liver mitochondria

The quality of mitochondria isolated according to the method described in section 2.3.1 was assessed using two related polarographic methods. The first method looked at the coupling between electron transfer and ATP synthesis (Figure 3.1). State 4 respiration, established by the addition of either 10 mM succinate or a combination of 10 mM malate and 2 mM pyruvate, was stimulated by the addition 300 nmoles of ADP (state 3). The phosphorylation of ADP by ATP synthase is associated with the dissipation of $\Delta\mu_H^+$, resulting in an increased rate of electron transfer and therefore of oxygen consumption. Once all the ADP had been exhausted the mitochondria returned to state 4. From these data both the ADP:O ratio and respiratory control ratio (RCR) can be calculated as described by Estabrook (1967). Generally the mitochondria used in this work oxidised succinate at rates of up to 40 nmoles O_2 min^{-1} mg^{-1} protein and malate/pyruvate at rates of up to 11 nmoles O_2 min^{-1} mg^{-1} protein. The succinate-dependant RCRs were between 3 and 4 and the ADP:O ratio was 1.6, and the malate/pyruvate RCRs were slightly lower (between 2 and 3) and the ADP:O ratio was about 2.6. The RCR values reported here were slightly higher than those previously

reported for sheep liver mitochondria (Reid and Husbands, 1985) and the ADP:O values are close to the expected values. The rates observed were dependent on substrate addition and the RCRs were maintained for several additions of ADP (Figure 3.1).

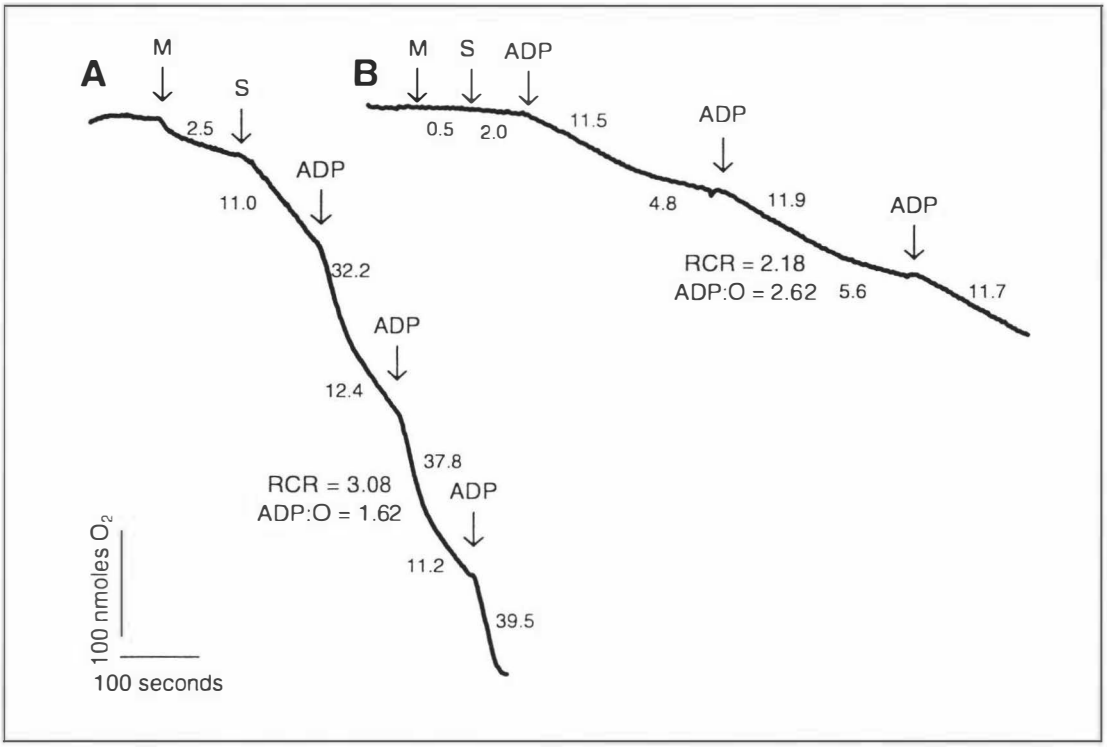

Figure 3.1 Representative oxygen electrode traces showing ADP stimulation of oxygen consumption. Sheep liver mitochondria (M) were added to buffer (100 mM KCl, 2 mM $MgCl_2$, 2 mM KH_2PO_4, 70 mM HEPES-HCl pH 7.4). Substrate (S), either 10 mM succinate (A) or 10 mM malate/2 mM pyruvate (B), and ADP (300 nmoles) were added as indicated. The numbers on the traces represent the rate of oxygen consumption in nmoles O_2 min^{-1} mg^{-1} protein. RCR and ADP:O ratios were calculated by the method described by Estabrook (1967).

The second method of determining quality involved the use of the uncoupler DNP. As with the ADP assay, electron transfer was established by the addition of either substrate, followed by DNP (50 nmoles) which dissipated the $\Delta\mu_H^+$ and removed any constraints on electron transfer. This method allowed measurement of the control ratio (CR = rate after DNP addition/rate before DNP addition), another indicator of mitochondrial quality. The CRs observed for succinate or malate/pyruvate stimulated electron transfer were 3-4 and 6-9, respectively (Figure 3.2). This observation confirmed that the

mitochondria used in this work were tightly coupled and were capable of high rates of electron transfer.

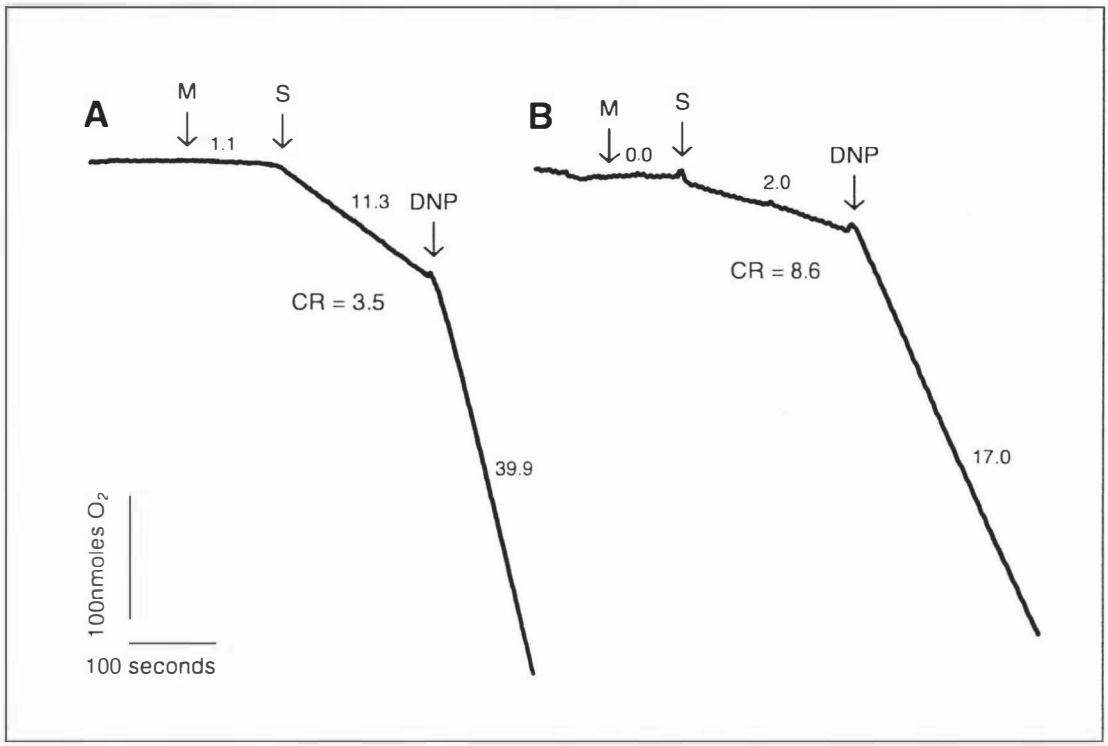

Figure 3.2 Representative oxygen electrode traces showing DNP stimulation of oxygen consumption. Sheep liver mitochondria (M) were added to buffer (100 mM KCl, 2 mM $MgCl_2$, 2 mM KH_2PO_4, 70 mM HEPES-HCl pH 7.4). Substrate (S), either 10 mM succinate (A) or 10 mM malate/2 mM pyruvate (B), and 25 μM DNP were added as indicated. The numbers on the graph represent the rate of oxygen consumption in nmoles O_2 min^{-1} mg^{-1} protein. CR = rate after DNP addition/rate before DNP addition.

3.2 The effect of manganese on whole chain electron transfer

3.2.1 Manganese inhibition of mitochondrial electron transfer in coupled and uncoupled mitochondria

Initially experiments were carried out to determine whether Mn had any effect on whole chain electron transfer (Figure 3.3). The effect of Mn on succinate-stimulated state 4 and state 3 respiration was assayed using $MnCl_2$. Succinate was added to isolated mitochondria to initiate electron transfer and $MnCl_2$ was added, in either state 4 (Figure 3.3 C and D) or in state 3 (Figure 3.3 A and B). DNP-stimulated oxygen consumption

(21.1 nmoles O_2 min^{-1} mg^{-1} protein) was inhibited 65% following the addition of $MnCl_2$ (Figure 3.3A). Similarly, ADP-stimulated oxygen consumption (20.4 nmoles O_2 min^{-1} mg^{-1} protein) was inhibited 40% by the addition of $MnCl_2$ (Figure 3.3B). When the $MnCl_2$ was added before either DNP or ADP no inhibition of state 4 electron transfer was observed (Figure 3.3 C and D respectively). However, upon the subsequent addition of either DNP (Figure 3.3C) or ADP (Figure 3.3D) the expected stimulation of oxygen consumption was not observed. The rate of oxygen consumption before the addition of ADP (6.2 nmoles O_2 min^{-1} mg^{-1} protein) increased by only 53% (Figure 3.3D) in the presence of Mn, compared with 289% after ADP addition (Figure 3.3B) in the absence if Mn. A similar result was observed following DNP addition, an increase of oxygen consumption of only 53% (Figure 3.3C) was observed in the presence of Mn, compared with 353% in the absence of Mn (Figure 3.3A).

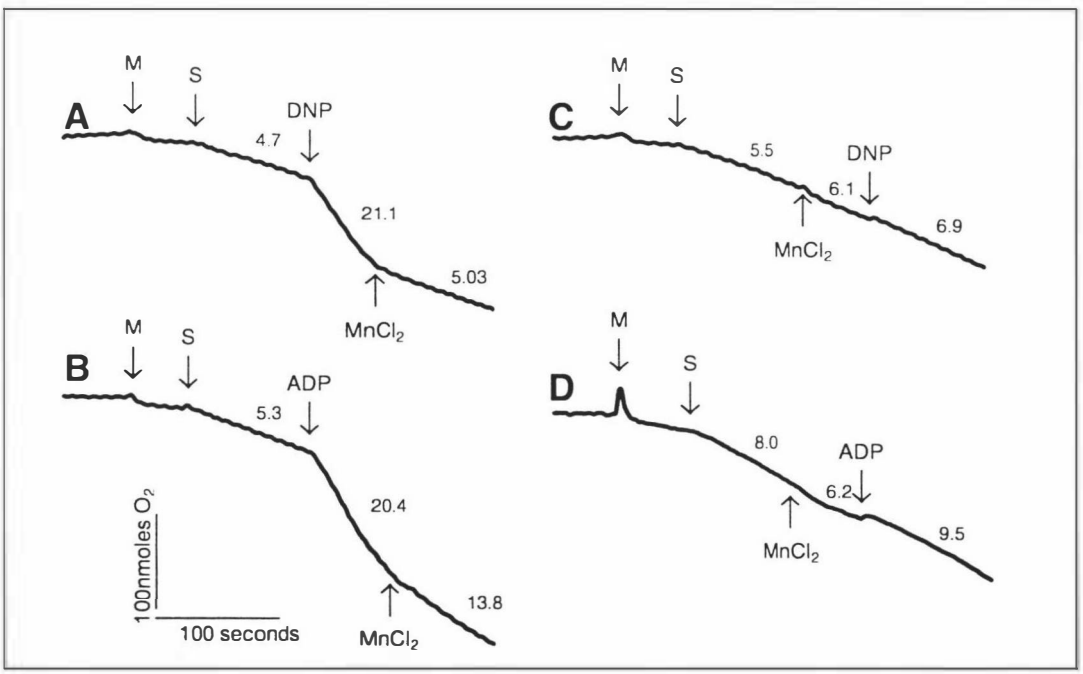

Figure 3.3 Representative oxygen electrode traces showing the effect of Mn on whole chain electron transfer in both state 3 (A and B) and state 4 (C and D). Sheep liver mitochondria (M) were added to buffer (100 mM KCl, 2 mM $MgCl_2$, 2 mM KH_2PO_4, 70 mM HEPES-HCl pH 7.4). Succinate (S) (10 mM), DNP (25 μM) or ADP (300 nmoles) and $MnCl_2$ (25 mM) were added as indicated. The numbers on the graph represent the rate of oxygen consumption in nmoles O_2 min^{-1} mg^{-1} protein.

These data show that, in the presence of $MnCl_2$, mitochondria could not achieve the high rate of electron transfer expected after the addition of ADP or DNP, but that Mn had little effect on coupled rates of oxygen consumption. High rates of electron transfer may be observed in tissues that have a particularly high demand for ATP such as in the brain or in muscle tissue.

3.2.2 The effect of varying the concentration of manganese and magnesium salts on succinate-dependant electron transfer

This series of experiments was carried out to examine the effect of Mn on succinate-dependant whole chain electron transfer. The effect of $MnCl_2$, $MnSO_4$, $MgCl_2$ and $MgSO_4$ was examined by initiating electron transfer by the addition of succinate to a suspension of isolated mitochondria and subsequently adding the appropriate salt at various concentrations. This caused a concentration-dependant inhibition of the rate of oxygen consumption (Figure 3.4). The effect of $MnSO_4$ was examined to ensure that the inhibition observed in the presence of $MnCl_2$ was in fact being cause by the Mn and not the anion. The effect of $MgCl_2$ and $MgSO_4$ was assessed to confirm that inhibition was not caused by ionic strength, and was in fact dependent on the presence of Mn. The inhibition observed with Mn salts ($K_{½}$= 0.2 mmoles mg^{-1} protein) occurred at significantly lower concentrations than that observed with Mg salts ($K_{½}$= 2.1 mmoles mg^{-1} protein). From this observation it could be concluded that the inhibition of oxygen consumption by Mn was independent of the ionic strength of the medium. These data also demonstrated that the inhibition observed with both Mn and Mg was independent of the anion, as the Cl^- and SO_4^{2-} salts had very similar effects for each cation (Figure 3.4). However, during these experiments it was observed that upon the addition of either Mn salt to the oxygen electrode a precipitate was formed, this precipitate was believed to be caused by the binding of Mn to phosphate present in the buffer, since Mn binds Pi (K_a= 380.2) more strongly than does Mg (K_a= 75.9) (Sillen and Martell, 1964).

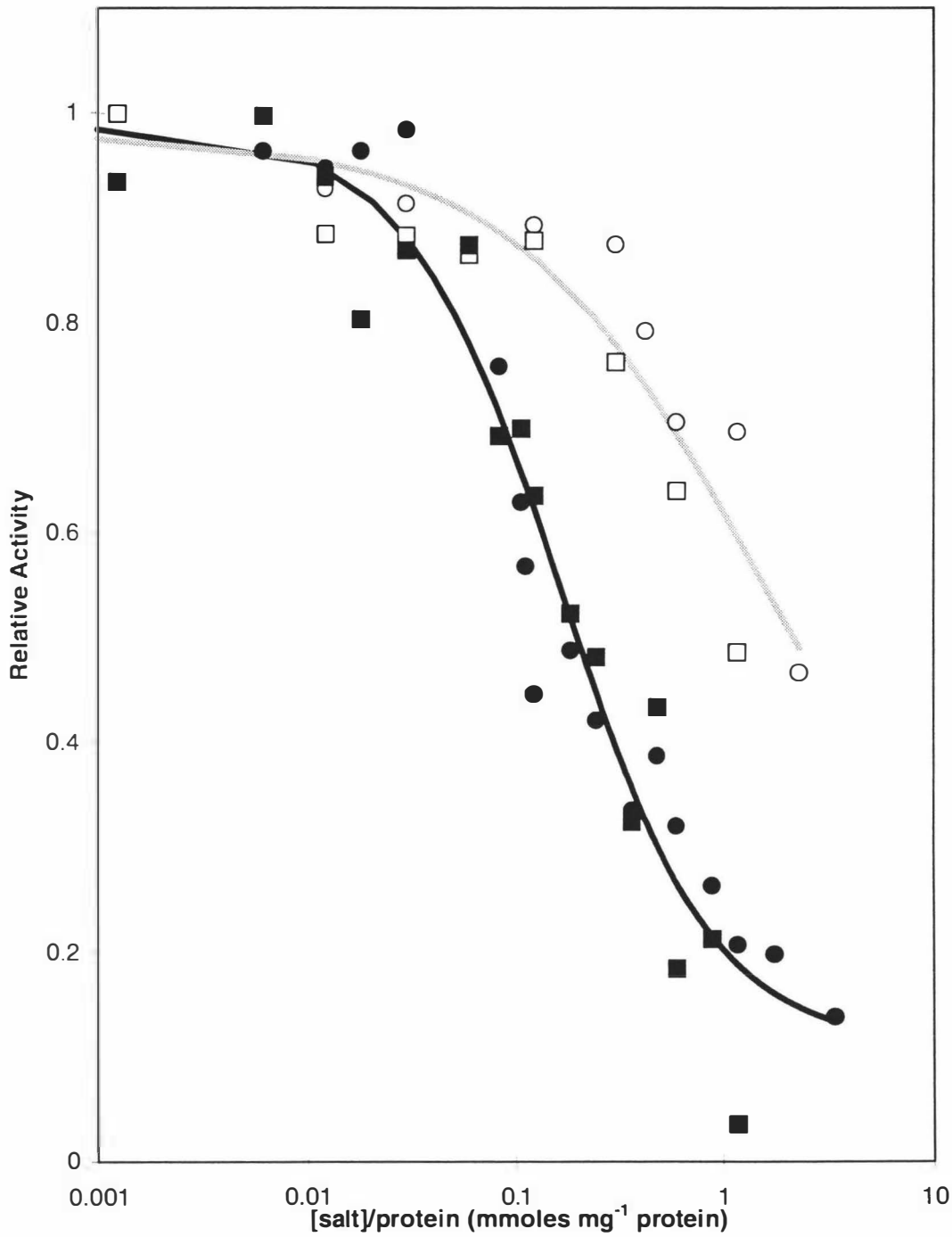

Figure 3.4 The effect of various Mn and Mg salts on the relative rate of whole chain succinate-stimulated electron transfer. Sheep liver mitochondria were added to buffer (100 mM KCl, 2 mM $MgCl_2$, 2 mM KH_2PO_4 70 mM HEPES-HCl pH 7.4) in the presence of 10 mM of succinate and various concentrations of $MnCl_2$ (■), $MgCl_2$ (□), $MnSO_4$ (●) and $MgSO_4$ (O), and the rate of oxygen consumption was measured. The activity is expressed relative to the rate of oxygen consumption of the controls (~30 nmoles O_2 min^{-1} mg^{-1} protein) with no salt addition. Curves were fitted to a pseudo-Hill equation by least squares non-linear regression.

3.2.3 The effect of manganese chloride and magnesium chloride on succinate-dependant electron transfer in a phosphate-free buffer

This set of experiments was prompted by the hypothesis that Mn was precipitating due to the presence of phosphate in the buffer used in previous experiments. The effect of $MnCl_2$ and $MgCl_2$ on whole chain electron transfer was examined in the presence of succinate in a phosphate-free buffer (70mM HEPES-HCl pH7.4) (Figure 3.5). In this buffer there was no significant difference between the inhibition observed with Mn ($K_{½}$= 3.7 ± 0.3 μmoles mg^{-1} protein) and Mg ($K_{½}$= 4.6 ± 0.5 μmoles mg^{-1} protein). The difference between the $K_{½}$ values for Mn in the presence of phosphate ($K_{½}$= 201 μmoles mg^{-1} protein) and in the absence of phosphate ($K_{½}$= 3.7 ± 0.3 μmoles mg^{-1} protein) imply that the phosphate present in the buffer decreased the concentration of Mn available to inhibit electron transfer. These data prompted the hypothesis that the inhibition of oxygen consumption by Mn was a consequence of ionic strength rather than a direct inhibition of electron transfer.

3.2.4 The effect of manganese chloride and magnesium chloride on malate/pyruvate-dependant electron transfer

To examine the effect of Mn on electron transfer from malate/pyruvate to oxygen, isolated sheep liver mitochondria were added to buffer (70 mM HEPES-HCl pH 7.4) in the presence of pyruvate, malate, DNP and various concentrations of $MnCl_2$ and $MgCl_2$ (Figure 3.6). There was no significant difference between the inhibition of NAD^+-linked substrate oxidation observed with Mn ($K_{½}$= >70 μmoles mg^{-1} protein) and Mg ($K_{½}$= >70 μmoles mg^{-1} protein). Similar results were obtained for succinate oxidation (Figure 3.5). It is also important to note that the $K_{½}$ values obtained for the inhibition in this instance were at least 20 times those observed for succinate oxidation (Figure 3.5).

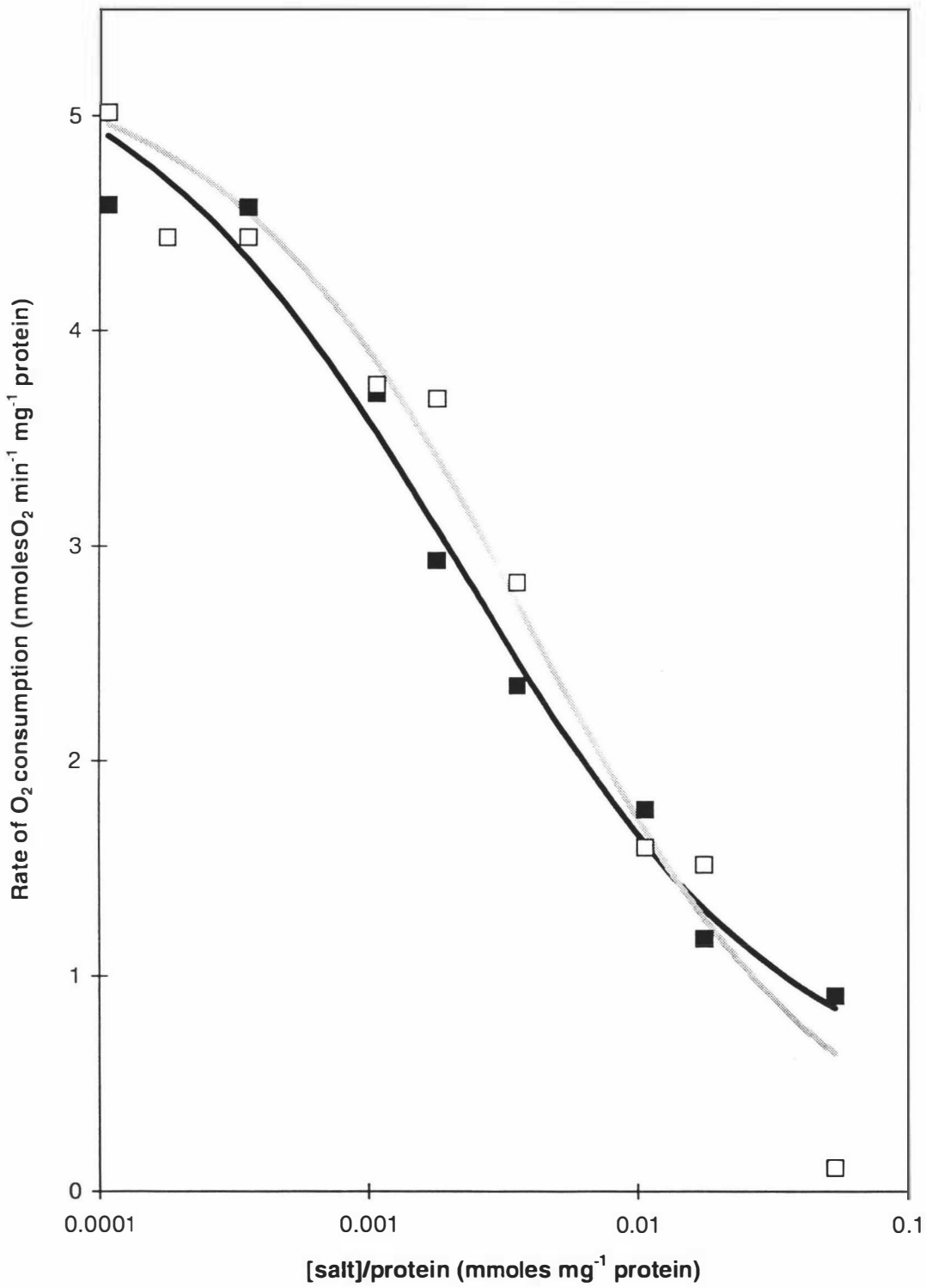

Figure 3.5 The effect of $MnCl_2$(■—■) and $MgCl_2$(□~□) on whole chain succinate-dependant electron transfer. Sheep liver mitochondria were added to buffer (70 mM HEPES-HCl pH 7.4) in the presence of 10 mM of succinate, 25 μM DNP and various concentrations of $MnCl_2$ and $MgCl_2$. Curves were fitted to a pseudo-Hill equation by least squares non-linear regression.

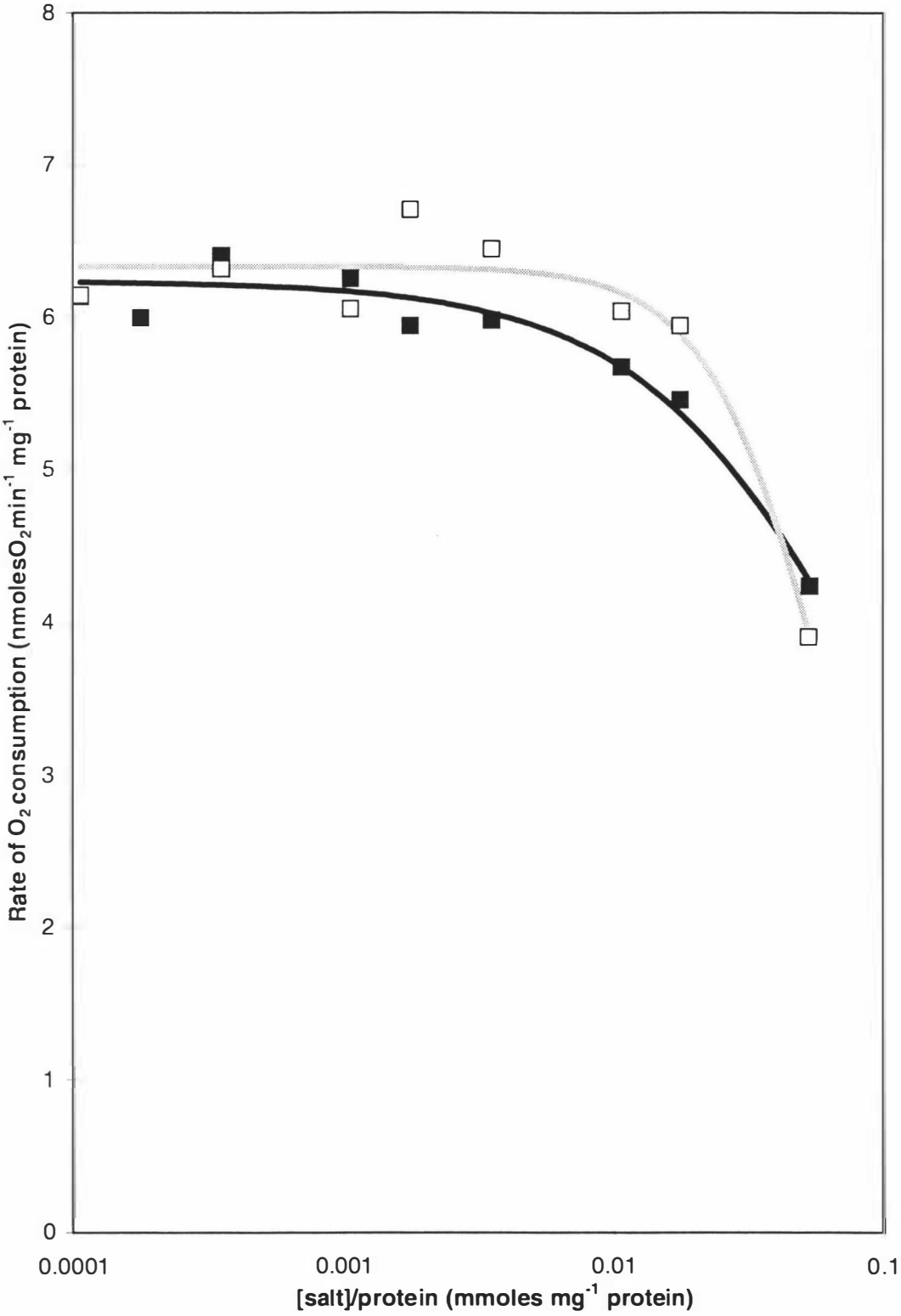

Figure 3.6 The effect of $MnCl_2$(■—■) and $MgCl_2$(□—□) on whole chain malate/pyruvate-stimulated electron transfer. Sheep liver mitochondria were added to buffer (70 mM HEPES-HCl pH 7.4) in the presence of 5 mM pyruvate, 2 mM malate, 25 μM DNP and various concentrations of $MnCl_2$ and $MgCl_2$. Curves were fitted to a pseudo-Hill equation by least squares non-linear regression.

3.3 The effect of manganese on specific partial reactions

3.3.1 The effect of manganese on electron transfer from succinate and malate/pyruvate to cytochrome *c*

Electron transfer from succinate (Figure 3.7) and malate/pyruvate (Figure 3.8) to cytochrome *c* was carried out by adding substrate, DNP, KCN and cytochrome *c* to isolated mitochondria suspended in buffer (70 mM HEPES-HCl pH 7.4). The large pool of exogenous cytochrome *c* was reduced by electrons from the oxidation of either substrate, and cyanide was present to prevent the oxidation of the cytochrome *c* by cytochrome oxidase. The formation of reduced cytochrome *c* was followed spectrophotometrically at 550-542nm.

There was a distinct difference between the inhibition observed for Mn ($K_{½}$= 8 ± 7 μmoles mg^{-1} protein) and Mg ($K_{½}$= 47 ± 6 μmoles mg^{-1} protein) which implied a direct inhibition of succinate to cytochrome *c* electron transfer by Mn (Figure 3.7). However, the $K_{½}$ for Mn was not different from the value observed for whole chain succinate-stimulated electron transfer. While there might have been a direct effect of Mn on electron transfer between succinate dehydrogenase and cytochrome *c*, the dominant effect, as the Mn concentration increased, was due to increasing ionic strength. Control assays were sensitive to malonate and myxothiazol, but were not affected by rotenone.

There was also a distinct difference between the inhibition observed for Mn ($K_{½}$= 16 μmoles mg^{-1} protein) and Mg ($K_{½}$= 42 μmoles mg^{-1} protein) which suggested a direct inhibition by Mn on electron transfer from malate/pyruvate to cytochrome *c* (Figure 3.8). Control assays were sensitive to rotenone and myxothiazol, but were not affected by malonate. This suggested that there could be a direct effect of Mn on NADH dehydrogenase to cytochrome *c* electron transfer, but it was impossible to distinguish between this and the possible effects of Mn on either malate dehydrogenase or pyruvate dehydrogenase. These enzymes, which oxidise their substrates producing NADH which is then oxidised by NADH dehydrogenase. A stimulation in the rate of oxygen consumption was observed at low concentration of Mn as malate dehydrogenase has been shown to be stimulated by the addition of Mn (Brown and Cook, 1981). The

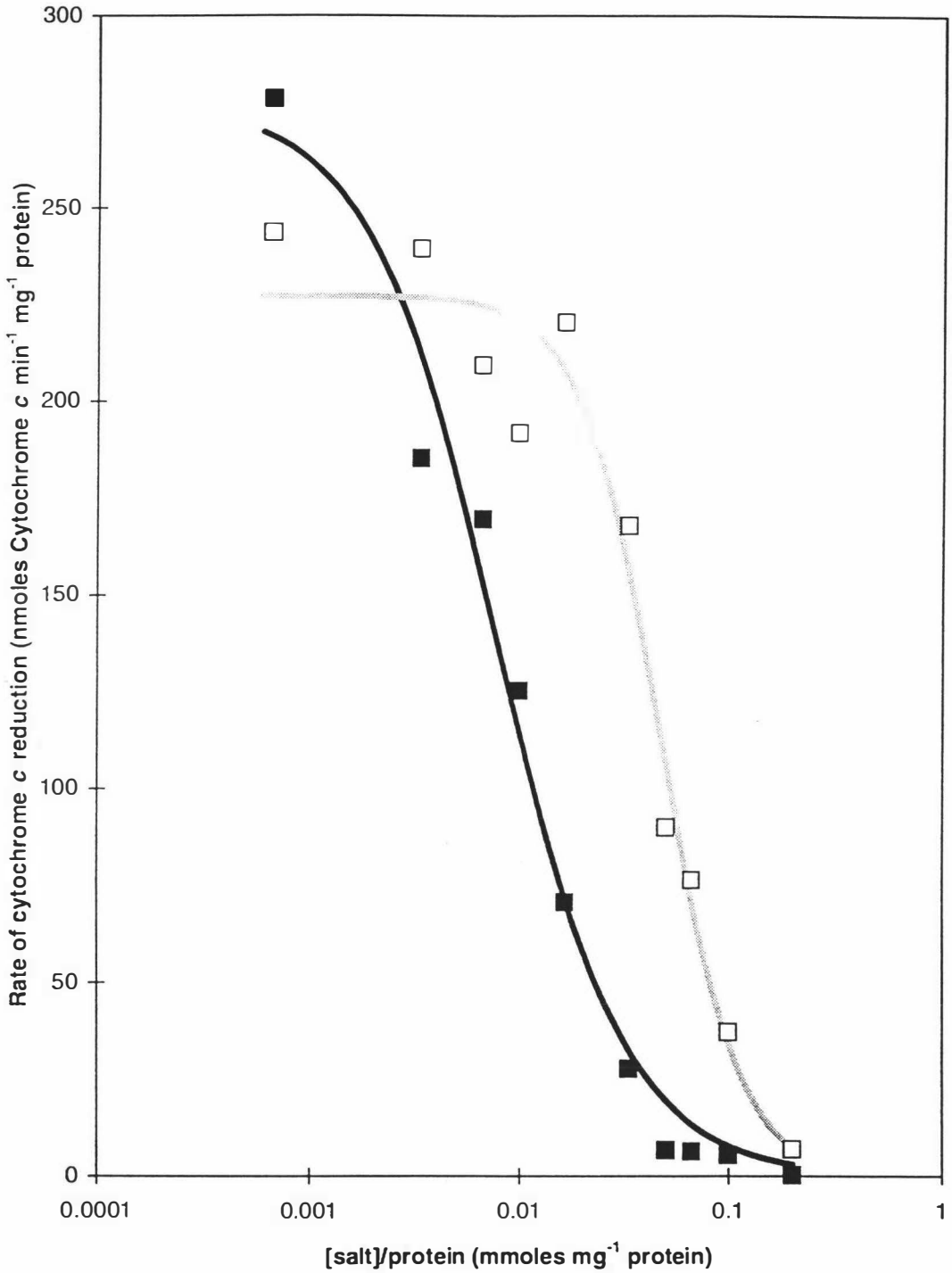

Figure 3.7 The effect of $MnCl_2$(■—■) and $MgCl_2$(□—□) on electron transfer from succinate dehydrogenase to cytochrome *c*. Sheep liver mitochondria were added to buffer (70 mM HEPES-HCl pH 7.4) in the presence of 7 mM succinate, 16 μM DNP, 1 mM KCN, 11 μM cytochrome *c* and various concentrations of $MnCl_2$ and $MgCl_2$. The formation of reduced cytochrome *c* was followed spectrophotometrically at 550-542 nm. Curves were fitted to a pseudo-Hill equation by least squares non-linear regression.

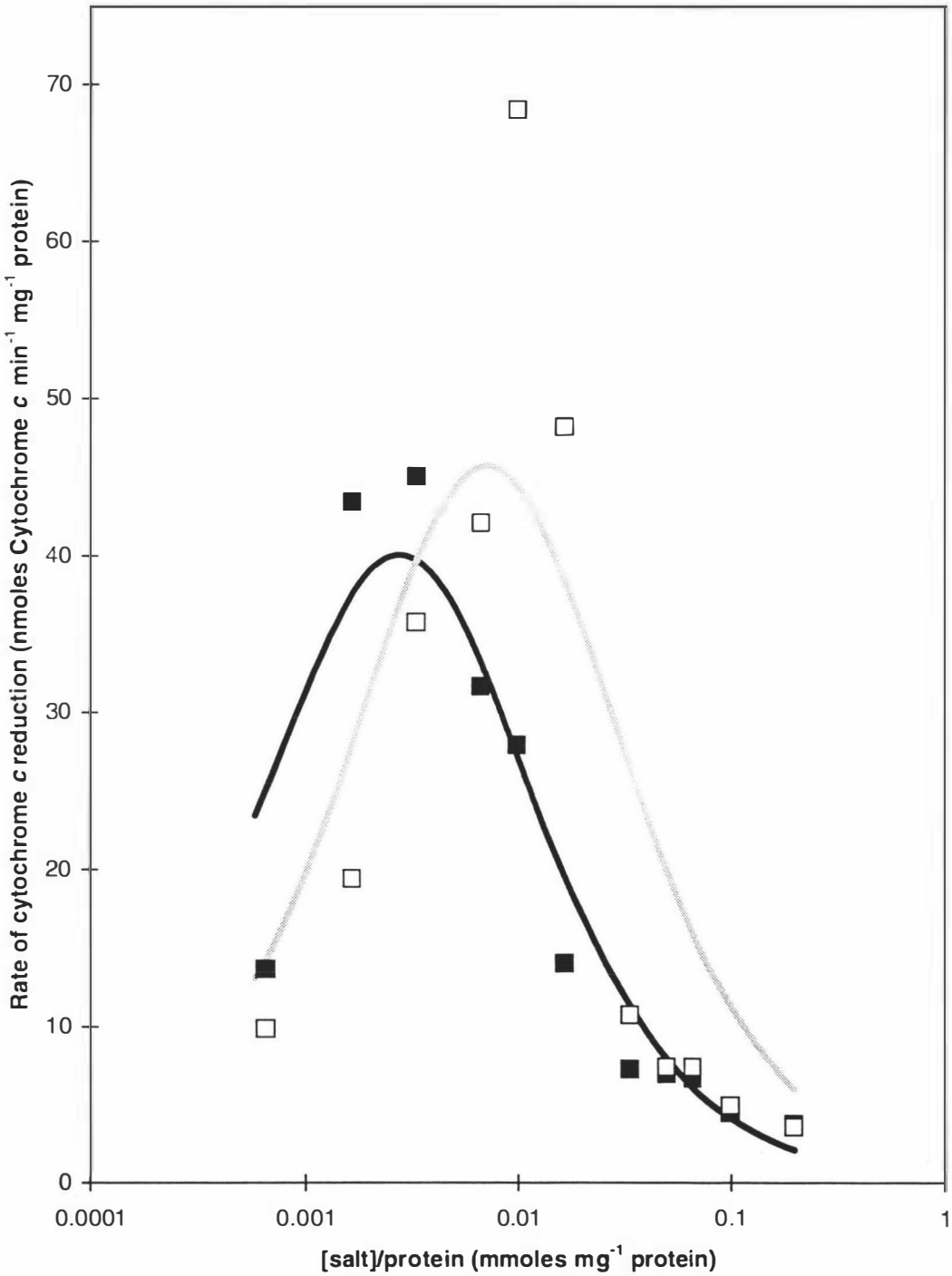

Figure 3.8 Graph showing the effect of $MnCl_2$(■—■) and $MgCl_2$(□---□) on electron transfer from malate/pyruvate to cytochrome *c*. Sheep liver mitochondria were added to buffer (70 mM HEPES-HCl pH 7.4) in the presence of 5 mM pyruvate, 2 mM malate, 16 μM DNP, 1 mM KCN, 11 μM cytochrome *c* and various concentrations of $MnCl_2$ and $MgCl_2$. Cytochrome *c* reduction was measured spectrophotometrically at 550-542 nm. Curves were arbitrarily fitted to equation 2 of Dixon (1973) by least squares non-linear regression in order to facilitate the estimation of $K_{½}$s.

inhibition observed at higher concentrations makes it difficult to distinguish which of at least three possible enzymes were affected in this assay.

3.3.2 The effect of manganese on succinate dehydrogenase activity

The effect of Mn on the activity of succinate dehydrogenase was assayed by following DCPIP reduction. Isolated mitochondria in buffer (70 mM HEPES-HCl pH 7.4) in the presence of succinate, KCN, DNP and DCPIP were exposed to various concentrations of $MnCl_2$ and $MgCl_2$ (Figures 3.9 A and B respectively). Electrons produced from the oxidation of succinate to fumarate were passed from succinate dehydrogenase to the artificial electron acceptor DCPIP. The reduction of DCPIP was followed spectrophotometrically at 600 nm. Control assays were carried out using the inhibitor malonate and the expected inhibition was observed.

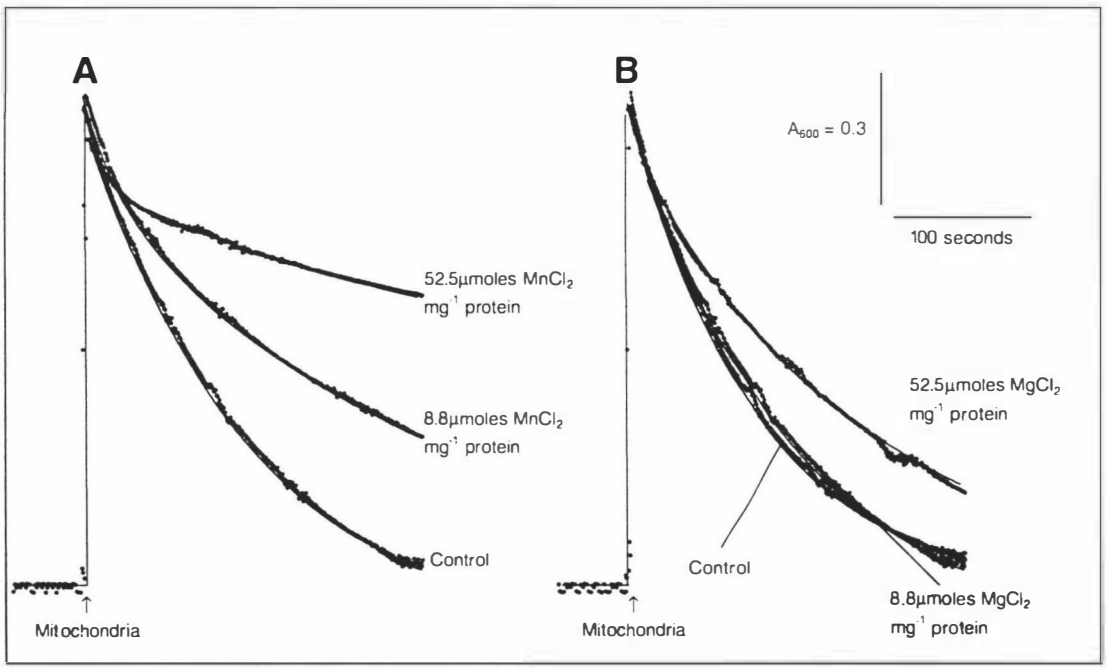

Figure 3.9 Representative traces showing the reduction of DCPIP in the presence of $MnCl_2$ (A) and $MgCl_2$ (B). Sheep liver mitochondria were added to buffer (70 mM HEPES-HCl pH 7.4) in the presence of 7 mM succinate, 1 mM KCN, 4 µM myxothiazol, 16 µM DNP, 85 µM DCPIP and various concentrations of $MnCl_2$ and $MgCl_2$. Reactions were initiated by the addition of mitochondria and DCPIP reduction was measured spectrophotometrically at 600 nm. Curves were fitted to a sum of two exponentials by least squares non-linear regression.

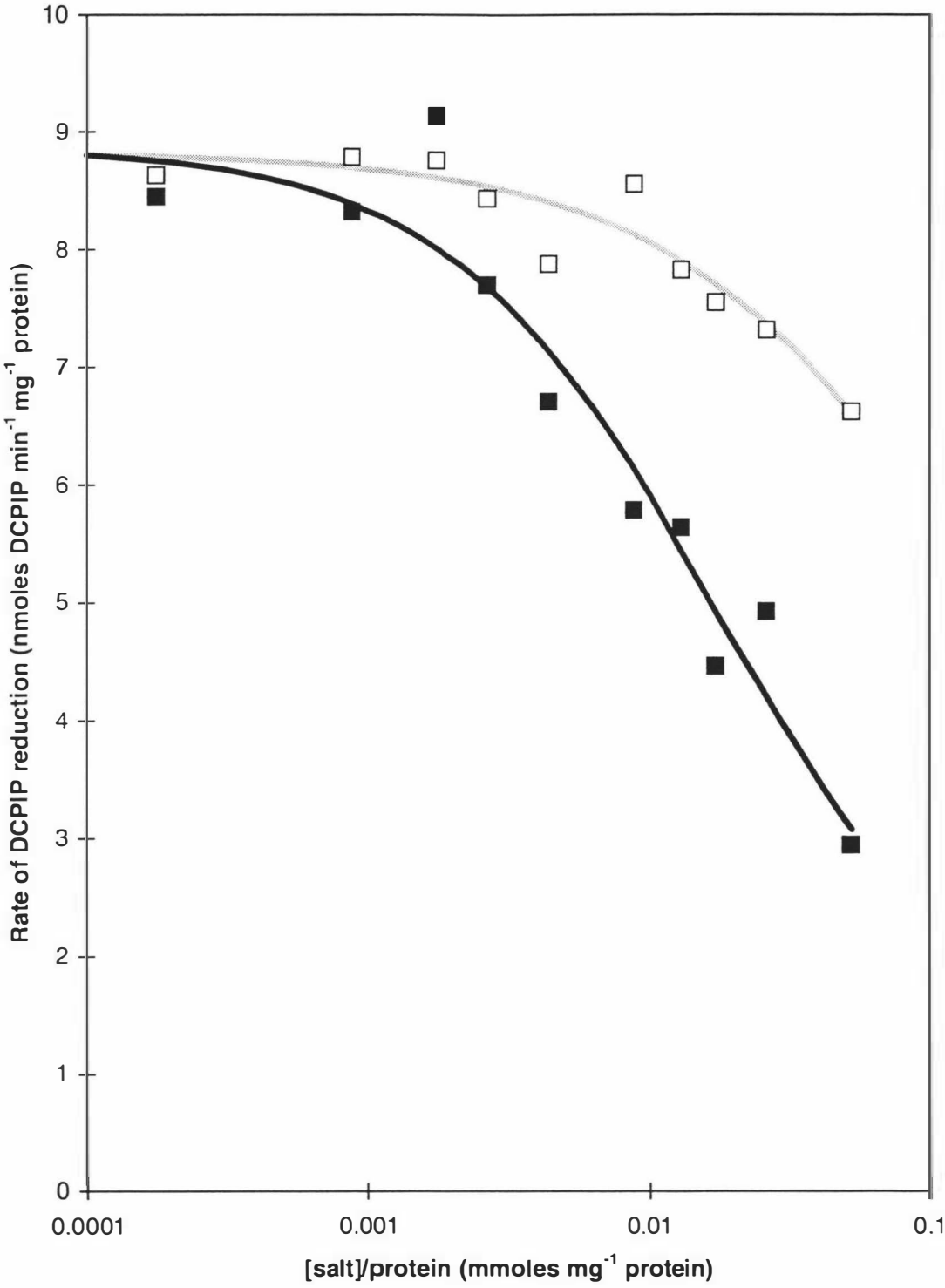

Figure 3.1 The effect of $MnCl_2$(■—■) and $MgCl_2$(□—□) on succinate dehydrogenase activity. Sheep liver mitochondria were added to buffer (70 mM HEPES-HCl pH 7.4) in the presence of 7 mM succinate, 1 mM KCN, 4 μM myxothiazol, 16 μM DNP, 85 μM DCPIP and various concentrations of $MnCl_2$ and $MgCl_2$. Curves were fitted to a pseudo-Hill equation by least squares non-linear regression.

To prevent electrons from by passing the DCPIP and entering the cytochrome bc_1 complex, the inhibitors cyanide and myxothiazol were added, this prevented electrons from travelling down the remainder of the electron transfer chain. Control assays carried out with rotenone showed that there was no need to inhibit NADH dehydrogenase. The data in figure 3.9 demonstrates the dramatic difference between the effects of Mn and Mg on the activity of succinate dehydrogenase. In figure 3.9A 8.8 μmoles mg^{-1} protein $MnCl_2$ significantly decreased the rate of DCPIP reduction compared with the control, whereas in figure 3.9B the control and 8.8 μmoles mg^{-1} protein $MgCl_2$ were almost identical. At high concentrations of Mn the rate of DCPIP reduction was biphasic as it takes a few seconds for the Mn to accumulated in the mitochondria. This implied that Mn does inhibit succinate dehydrogenase. However, the inhibition occurred at a higher concentration ($K_{½}$= 18.5 ± 0.9 μmoles mg^{-1} protein) than the succinate-stimulated whole chain inhibition ($K_{½}$= 3.7 ± 0.3 μmoles mg^{-1} protein) observed previously (Figure 3.5). Therefore, while Mn does inhibit succinate dehydrogenase, as the concentration of Mn increases in the mitochondria, inhibition mediated by ionic strength, would be followed by a direct inhibition of succinate dehydrogenase.

3.3.3 The effect of manganese on cytochrome oxidase activity

The effect of Mn on the activity of cytochrome oxidase was assayed by following the oxidation of reduced cytochrome *c* at 550-542 nm. Sheep liver mitochondria were resuspended in buffer in the presence of reduced cytochrome *c*, myxothiazol and DNP. The large pool of exogenous reduced cytochrome *c* was oxidised by cytochrome oxidase, allowing a measurement of the activity of cytochrome oxidase. To prevent any extraneous reduction of cytochrome *c* from endogenous substrate present within the mitochondria, myxothiazol was added.

The effects of Mn and Mg on the activity of cytochrome oxidase were substantially different (Figure 3.11). At low concentrations of $MnCl_2$ (66 μmoles mg^{-1} protein) $MnCl_2$ significantly decreased the rate of oxidation of cytochrome *c* compared with the control (Figure 3.11A), whereas the same amount $MgCl_2$ had no effect on the rate (Figure 3.11B). This suggested that Mn inhibited cytochrome oxidase. However, the inhibition occurred at a higher concentration ($K_{½}$= 17 ± 2 μmoles mg^{-1} protein) than the

succinate-dependant whole chain inhibition ($K_{½}$= 3.7 ± 0.3 μmoles mg^{-1} protein) observed previously (Figure 3.5). This suggested that while Mn does inhibit cytochrome oxidase, this would occur simultaneously with an inhibition mediated by ionic strength.

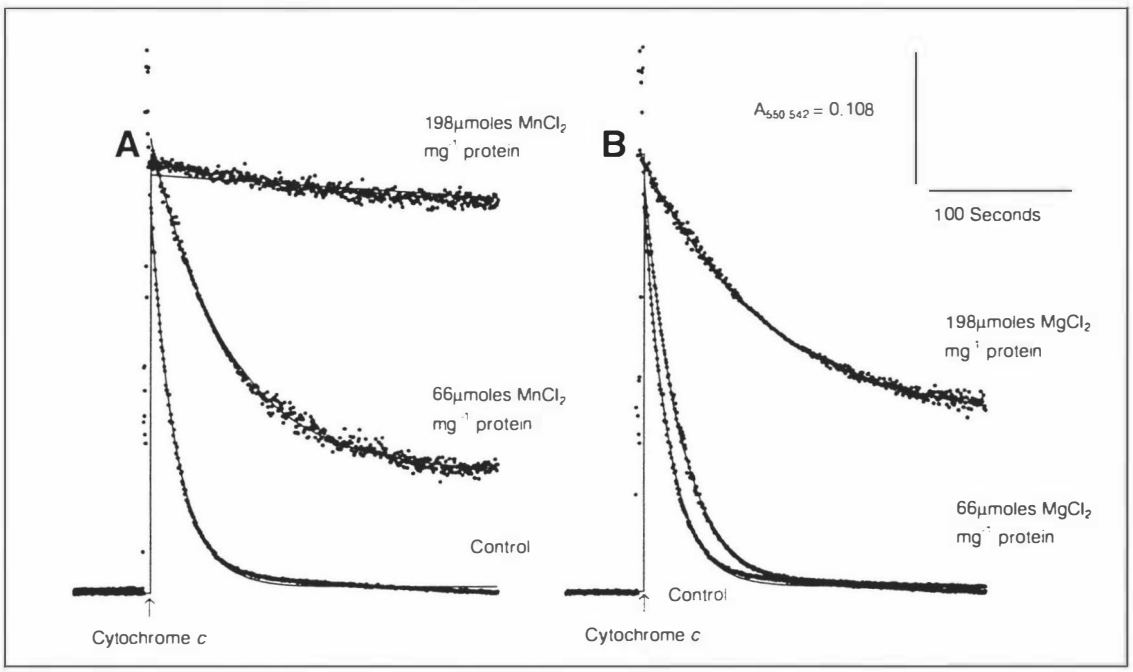

Figure 3.11 Representative traces showing the oxidation of reduced cytochrome *c* in the presence of $MnCl_2$ (A) and $MgCl_2$ (B). Sheep liver mitochondria were added to buffer (70 mM HEPES-HCl pH 7.4) in the presence of 4 μM myxothiazol, 16 μM DNP and 22 μM cytochrome *c* were added to start the reaction. Cytochrome *c* oxidation was measured spectrophotometrically at 550-542 nm. Curves were fitted to sum of two exponentials by least squares non-linear regression.

3.4 Discussion

This section of work was intended to examine the effect of Mn on the operation of the mitochondrial electron transfer chain. It has been shown that Mn is actively accumulated by mitochondria (Manyard and Cotzias, 1955) *via* the calcium uniporter (Chance, 1965) and that its efflux from the body is extremely slow (Cotzias *et al.*, 1968). Therefore, it might be anticipated that, once ingested, Mn would remain concentrated in mitochondria for some time. Once within the mitochondria its effects on electron transfer, which is catalysed by four enzymes located on the inner mitochondrial

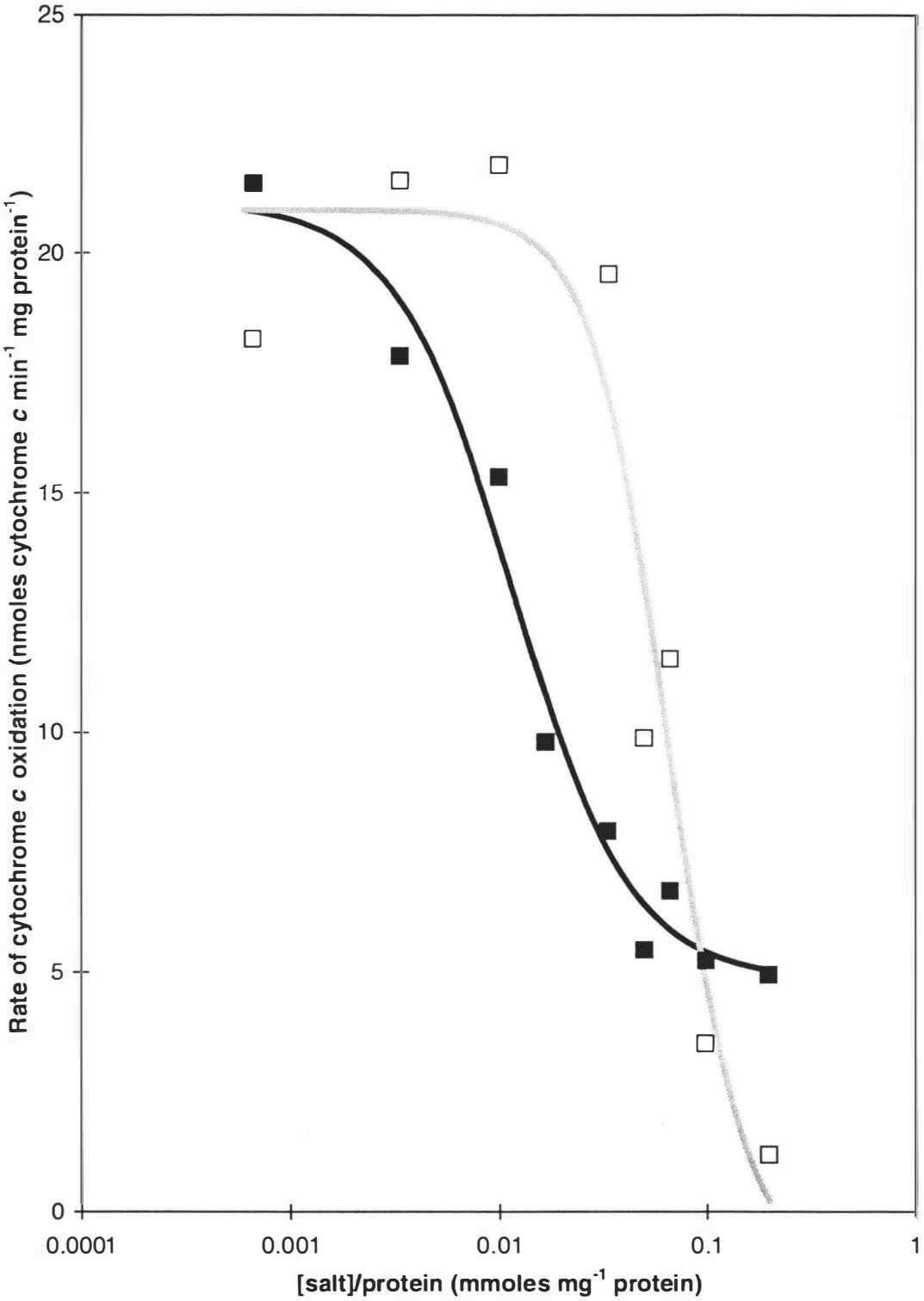

Figure 3.2 The effect of $MnCl_2$(■—■) and $MgCl_2$(□—□) on cytochrome oxidase activity. Sheep liver mitochondria were added to buffer (70mM HEPES-HCl pH 7.4) in the presence of 22 μM cytochrome *c*, 4 mM myxothiazol and 16 μM DNP. Cytochrome *c* oxidation was measured spectrophotometrically at 550-542 nm. Curves were fitted to a pseudo-Hill equation by least squares non-linear regression.

Table 3.1 Inhibition of substrate oxidation by Mn compounds reported in the literature (adapted from Brown and Taylor (1999))

Compound	Concentration[1]	Tissue	Treatment time	Activity (donor → receptor)	Inhibition (%)	Reference[2]
$MnCl_2$	500 μM, *i.v.*	PC12 cells	6 h	NADH → cytochrome c	45	1
				Succinate → cytochrome c	30	1
			48 h	NADH → cytochrome c	45	1
				Succinate → cytochrome c	25	1
	10 μM, *i.v.*	PC12 cell mitochondria	-	NADH → cytochrome c	15	1
				Succinate → cytochrome c	31	1
	16 mg/kg oral	Rat brain homogenates	30 days	Succinate → FeCN	17	2
	4 mg/kg, i.p.	Rat liver homogenates	30 days	Succinate → FeCN	17	3
				Cytochrome c → O_2	16	3
				NADH → cytochrome c	21	3
	15 mg/kg, i.p.	Rat testis homogenates	15 days	Succinate → FeCN	10	4
$MnSO_4$	5 mg/kg, i.p.	Rat testis homogenates	16 days	Succinate → FeCN	44	3
	5 mg/kg, i.p.	Rat testis homogenates	16 days	Succinate → FeCN	39	3
MMT	300 μM, *i.v.*	Rat liver mitochondria	-	Glutamate/malate → O_2	65	5
				Succinate → O_2	20	5

[1]-i.p., intraperitoneal, *i.v., in vitro.* [2]-1- Galvani *et al.* (1995), 2- Singh *et al.* (1979), 3- Husain *et al.* (1976), 4- Chandra and Shukla, (1976), 5- Autissier *et al.* (1977).

membrane (Figure 1.1), was examined. These enzymes contribute to the generation of a $\Delta\mu_H^+$, which is utilised by ATP synthase to generate ATP. Researchers have examined the effects of a number of Mn compounds on various partial reactions of the mitochondrial electron transfer chain and much of this work has been carried out following *in vivo* administration of Mn (Table 3.1). However, no one study has yet examined the effect of a single compound on each of the partial reactions of the mitochondrial electron transfer chain. Furthermore, most of the work previously carried out (Autissier *et al.*, 1977; Galvani *et al.*, 1995; Chandra and Shukla, 1976; Singh *et al.*, 1979) has used phosphate containing buffers in the electron transfer chain assays. Measurements of succinate-dependant electron transfer in the presence and absence of phosphate showed that phosphate can sequester Mn away from the mitochondria and thereby minimise inhibition. The results in section 3.2.2 clearly show that in the presence of phosphate the concentration of Mn required to observe 50% inhibition ($K_{½}$= 201 μmoles mg^{-1} protein) was 50 times that required in the absence of phosphate ($K_{½}$= 3.7 ± 0.3 μmoles mg^{-1} protein). This implies that the concentrations of Mn required for the inhibition observed by those researchers who included phosphate in their assay media may be overestimates of the actual value due to the binding of Mn by the phosphate present.

Gavin *et al.* (1992) have demonstrated inhibition by Mn of ADP-stimulated oxygen consumption (state 3 respiration) by isolated rat liver mitochondria oxidising either succinate or glutamate/pyruvate. Experiments outlined in section 3.2.1 confirm this result, demonstrating inhibition of both succinate and malate/pyruvate stimulated electron transfer in the presence of ADP (state 3 respiration) by sheep liver mitochondria. However, Gavin *et al.* (1992) were unable to show any inhibition of succinate-dependant electron transfer in the presence of the uncoupler CCCP, and observed only a slight inhibition of glutamate/malate oxidation (Gavin *et al.*, 1992). They concluded that Mn interferes directly with the ATP synthase. However, this is inconsistent with the data presented here, which show inhibition of both succinate and malate/pyruvate oxidation in the presence of the uncoupler DNP. It seems likely that Mn does not directly inhibit ATP synthase, but does inhibit the high rates of electron transfer seen during state 3 respiration. Supporting this, it has been shown that ATP synthase is not inhibited by millimolar concentrations of Mn (Dorgan *et al.*, 1984), and

Husain *et al.* (1976) demonstrated that ATP hydrolysis is not affected by *in vivo* Mn administration. These observations suggest that Mn inhibits the high rates of electron transfer seen during state 3 respiration, but that ATP synthase is not involved.

In vivo studies (Table 3.1) have failed to distinguish between three possible effects of the *in vivo* administration of Mn. The inhibition observed could have been caused either by a reduction in the concentration of an enzyme in the mitochondrion or by a decrease in the activity of an enzyme, rather than by direct inhibition. A reduction in the concentration of enzymes of the mitochondrial electron transfer chain could be caused by a disruption of the normal assembly of these enzymes or an enhancement of the rate of their degradation resulting from mutations of the mitochondrial genome (Chapter 5). Mutations to mtDNA could also cause disruptions to the catalytic activity of enzymes without affecting the normal rate of formation or degradation. This could arise from mutations that cause inhibition of one or more enzymes of the mitochondrial electron transfer chain, or by decreasing the binding of one of the mobile electron carriers present in the mitochondria, such as cytochrome *c* or UQ. Here, *in vitro* assays have allowed investigation of the direct inhibition of the mitochondrial electron transfer chain by Mn without the interference of these genetic effects.

Whole chain electron transfer was assayed using both succinate and malate /pyruvate as substrates. Both succinate- and malate/pyruvate-dependant electron transfer were inhibited by Mn ($K_{½} = 3.7 \pm 0.3$ μmole mg^{-1} protein and $K_{½} = >70$ μmole mg^{-1} protein, respectively). However, Mg had a very similar effect on the rates of substrate oxidation ($K_{½} = 4.6 \pm 0.5$ μmole mg^{-1} protein and $K_{½} = >70$ μmole mg^{-1} protein for succinate- and malate/pyruvate-dependant electron transfer, respectively). The similarity between the effects of Mn and Mg implies that Mn does not specifically inhibit electron transfer and prompts the hypothesis that the inhibition is due to the increased ionic strength of the medium. Such an effect could be mediated, for example, by an alteration in the electrostatic interactions involved in the binding of cytochrome *c* to either cytochrome *c* oxidase or cytochrome c_1, the component of the cytochrome bc_1 complex that passes electrons to cytochrome *c* (Sadoski *et al.*, 1999). However, a number of other researchers have reported inhibition of individual enzymes of the mitochondrial electron transfer chain (Table 3.1). Succinate-dependant whole chain electron transfer involves

three separate enzymes (succinate dehydrogenase, the cytchrome bc_1 complex and cytochrome oxidase), and malate/pyruvate oxidation also involves three electron transfer chain enzymes (NADH dehydrogenase, the cytochrome bc_1 complex and cytochrome oxidase) as well as malate dehydrogenase and pyruvate dehydrogenase. To examine this, the activity of each enzyme was determined in the presence of Mn. The $K_{½}$ values obtained are shown in Table 3.2. These data show that there was an inhibition by Mn. However, the concentrations at which the inhibition occurred was slightly higher ($K_{½}$ = 8 to >70 μmole mg^{-1} protein) than those observed for succinate-dependant whole chain electron transfer ($K_{½}$ = 3.7 ± 0.3 μmole mg^{-1} protein). This implies that there is a specific inhibition of succinate dehydrogenase, NADH dehydrogenase and cytochrome oxidase and that the inhibition of these enzymes occurs at comparable Mn concentrations as the ionic strength effect. Galvani *et al.* (1995) observed that Mn elicited a greater inhibition for malate/pyruvate oxidation than for succinate oxidation in contrast to the data shown here (Table 3.2). However, their experiments (Galvani *et al.*, 1995) involved incubating growing PC12 cells in the presence of Mn. As discussed above, such *in vivo* studies fail to address the difference between a specific inhibition and a decrease in the concentration of an enzyme or variations in catalytic the activity of an enzyme.

Table 3.2 Apparent inhibition constants ($K_{½}$) of various partial reactions of the mitochondrial electron transfer chain.

Electron transfer	$K_{½}$ ± S.E. (μmoles Mn mg^{-1} protein)	$K_{½}$ ± S.E. (μmoles Mg mg^{-1} protein)
Succinate → O_2	3.7 ± 0.3	4.6 ± 0.5
Malate/pyruvate → O_2	>70	>70
Succinate → cytochrome c	8 ± 7	47 ± 6
Malate/pyruvate → cytochrome c	16	42
Succinate → DCPIP	18.5 ± 0.9	>70
Cytochrome c→ O_2	17 ± 2	62 ± 2

This series of experiments has shown that Mn inhibits the high rates of electron transfer seen during state 3 respiration and does appear to directly inhibit succinate dehydrogenase, NADH dehydrogenase and cytochrome oxidase ***in vitro***. It has also

been shown that Mn has ionic strength effect on the entire electron transfer chain. This prompts the suggestion that, as the concentration of Mn increases in the mitochondria, electron transfer would be inhibited by the increased ionic strength, and as the concentration increases, individual enzymes in the mitochondrial electron transfer chain would be specifically inhibited. As it has been demonstrated that high rates of electron transfer (state 3) are inhibited, this would occur in tissues which have a particularly high demand for ATP, such as muscle and brain tissue, dysfunction of which could well explain at least some of the symptoms of manganism.

CHAPTER 4
THE ROLE OF FREE RADICAL SPECIES IN MANGANISM

The mitochondrial electron transfer chain is the single most significant source of oxygen radicals ($O_2^{\cdot-}$ and ·OH) in any cell. Mitochondria consume 90% of the cell's oxygen and it has been estimated that the average person could produce more than 2 kg of $O_2^{\cdot-}$ each year (Halliwell, 1994), which implies that much of the $O_2^{\cdot-}$ is produced by the mitochondrial electron transfer chain. Hydroxyl radical formation, results from $O_2^{\cdot-}$ reacting with H_2O_2, which is also produced in the mitochondria from UQ and UQH_2 of the inner membrane (Boveris *et al.*, 1992). The work described in this chapter was to examine the role, if any, of free radical species in manganism. This was carried out by investigating the effects of free radical species on the normal function of the mitochondrial electron transfer chain. The effect of known inhibitors and $MnCl_2$ on the production of free radical species by the mitochondrial electron transfer chain was also examined.

4.1 Generation and measurement of free radical species

4.1.1 Production of superoxide radicals

Superoxide radicals produced using solid KO_2 and NaOH as described in section 2.2.11 were measured by three different methods. Firstly, by direct spectrophotometry at 250-360 nm (Goldstein and Czapski, 1996) and in the other two cases by using a detector molecule to react with the $O_2^{\cdot-}$ present. The detector molecules were necessary because the direct detection of $O_2^{\cdot-}$ in a mitochondrial suspension was not possible because of the absorbance of various components of the suspension.

As $O_2^{\cdot-}$ absorbs in the UV (ε_{250}=2.0mM^{-1}cm^{-1} (Bolann *et al.*, 1992)), its production can be monitored spectroscopically at 250-360 nm (Figure 4.1 A). Upon the addition of KO_2, a large increase (1.83 units) in absorbance was observed (Figure 4.1 A) indicating that $O_2^{\cdot-}$ (~915μM) had been produced, this was rapidly reduced (k_{obs} = 0.5 s^{-1}) to hydrogen peroxide, which also absorbs at 250-360 nm (ε_{240}=43.6 M^{-1}cm^{-1} (Kettle and

Winterbourn, 1994), and catalase was added to remove this (Figure 4.1A). In the presence of SOD (10 U ml^{-1}) the rate of $O_2^{\cdot-}$ reduction was increased consistent with the generation of $O_2^{\cdot-}$ by this technique. This confirmed that the absorbance measured was due to the presence of $O_2^{\cdot-}$ and not something else that absorbs at 250-360 nm. This set of experiments confirmed that this system did actually produce $O_2^{\cdot-}$. However, experiments showed that this method could not be used to measure $O_2^{\cdot-}$ in a mitochondrial system, as there are a number of compounds in mitochondria that absorb at 250-360 nm.

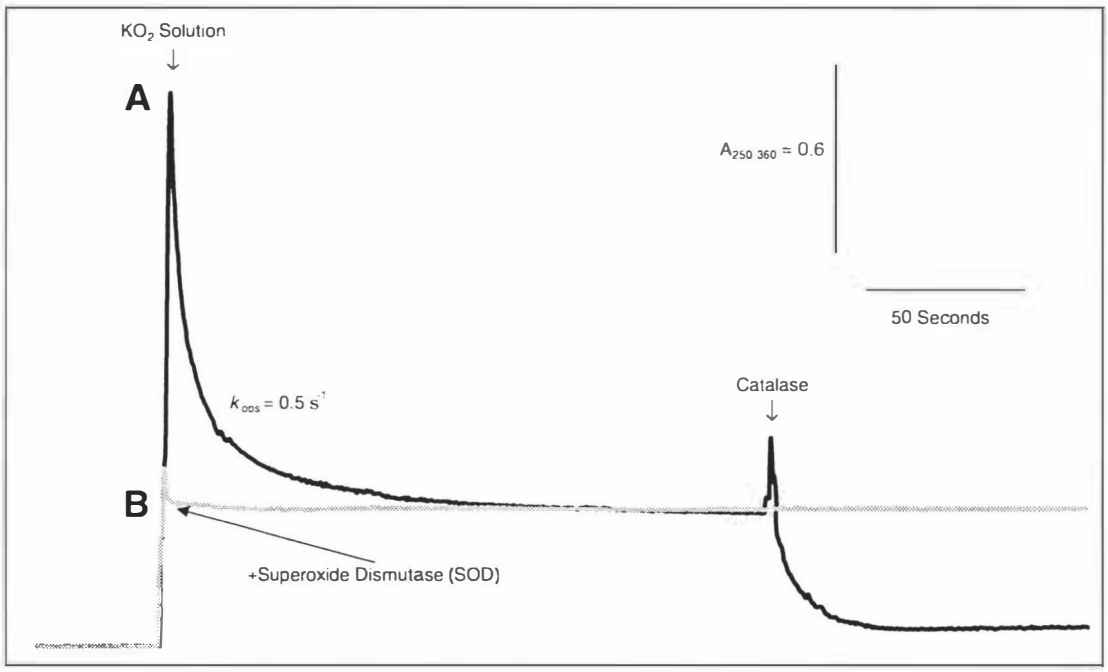

Figure 4.1 Representative traces showing the rapid degradation of $O_2^{\cdot-}$. A solid piece of KO_2 was dissolved in 100 mM NaOH, 300 µl of this solution was then added to 3 ml of buffer (AMProp-HCl pH 9.5). Catalase (26 U ml^{-1}) (A) or SOD (10 U ml^{-1}) (B) were added as indicated.

Indirect methods for measuring $O_2^{\cdot-}$ are based on the reduction of 2,2'-di-p-nitrophenyl-5-5'-diphenyl-3,3'-[3,3'-dimethoxy-4-4'-diphenylene]-ditetrazolium chloride (NBT) or acetylated cytochrome *c*. NBT is reduced to formazan in the presence of $O_2^{\cdot-}$ (Rice-Evans *et al.*, 1991), a process that can be followed spectrophotometrically at 500 nm. Initially, the reaction was tested without mitochondria present, upon the addition of 300 µl (Figure 4.2A, trace I) or 600 µl (Figure 4.2A, trace II) of KO_2 solution to the cuvette large absorbance increases were observed (1.4 units and 2.1 units, respectively) due to the production of formazan. This result was not stoichiometrically correct, because of

the rapid degradation of $O_2^{\cdot-}$. However, the results could be reproduced with reasonable consistency. This confirmed that the $O_2^{\cdot-}$ added reduced NBT to formazan as expected. In this series of experiments the ability of Mn to generate $O_2^{\cdot-}$ itself was also examined by adding it to buffer in the presence of NBT and seeing if it could reduce NBT to formazan. There was no evidence that $MnCl_2$ could cause the formation of formazan, which implied that Mn did not generate an appreciable amount of $O_2^{\cdot-}$ in solution.

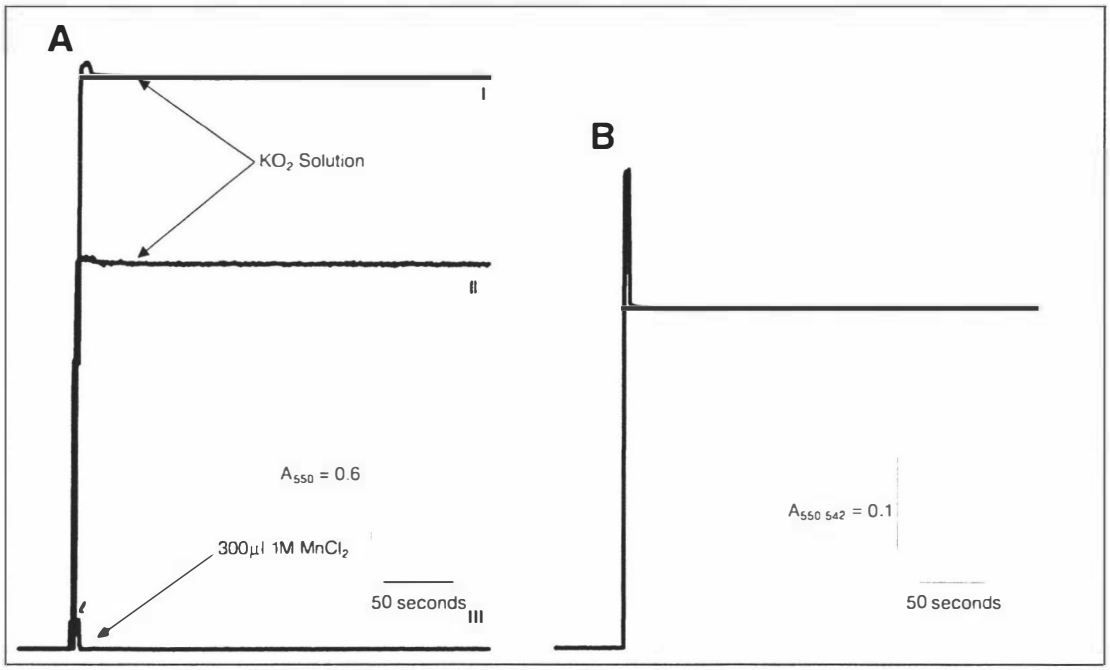

Figure 4.2 Representative traces showing the reduction of detector molecules by $O_2^{\cdot-}$ in the absence of mitochondria. A. Either KO_2 solution (600 µl or 300 µl, traces I and II, respectively) or 300 µmoles $MnCl_2$ (trace III) was added to 3ml of buffer (70mM HEPES-HCl pH 7.4) in the presence of 5.5 mM NBT. (B) 300 µl of KO_2 solution was added to 3 ml buffer in the presence of acetylated cytochrome *c* (40 nmoles).

The use of NBT as a detector molecule for use with mitochondria looked promising at this stage, however initial experiments were carried using mitochondria and it was found that NBT also accepted electrons from the mitochondrial electron transfer chain, and NBT was reduced. Therefore NBT is not suitable for this purpose with isolated mitochondria. An alternative detector molecule which is commonly used to assess the production of $O_2^{\cdot-}$ in mitochondria is acetylated cytochrome *c*. Acetylated cytochrome *c* is reduced by $O_2^{\cdot-}$, but due to its acetylation, is not reduced or oxidised by the mitochondrial electron transfer chain (Turrens and Boveris, 1980). The production of

$O_2^{\cdot-}$ was followed at 550-542 nm corresponding to the reduction of acetylated cytochrome *c* (Figure 4.2B). Experiments carried out using sheep liver mitochondria showed that acetylated cytochrome *c* reduction was not stimulated by the addition of antimycin A, and SOD also had no effect (Figure 4.3A).

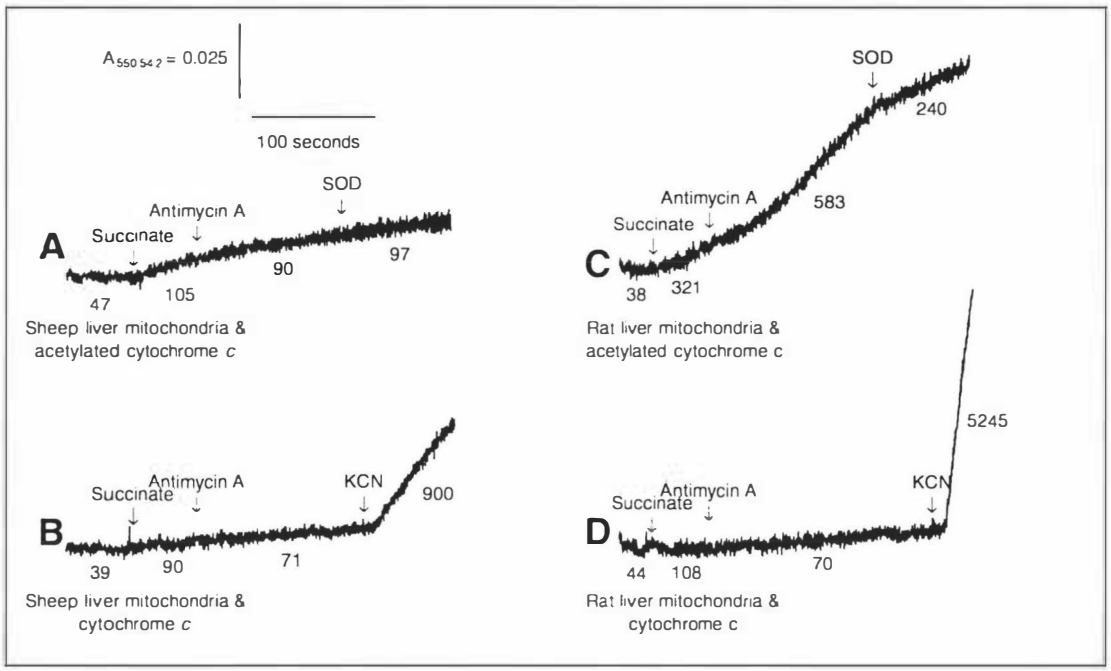

Figure 4.3 Representative traces showing the production of $O_2^{\cdot-}$ by a mitochondrial suspension. Sheep liver mitochondria (traces A and B) or rat liver mitochondria (traces C and D) were placed in buffer (70 mM HEPES-HCl pH7.4) in the presence of 20 μM acetylated cytochrome *c* (traces A and C) or 20 μM non-acetylated cytochrome *c* (traces B and D). At the times indicated 32 nM antimycin A, 7 mM succinate, SOD (10 U ml^{-1}) or 1 mM KCN were added.

Even after extensive washing of mitochondrial preparations to remove any endogenous SODs, and the preparation of sub-mitochondrial particles (Section 2.3.2) which were also extensively washed, no significant $O_2^{\cdot-}$ production by sheep liver mitochondria electron transfer could be detected by this method. To confirm that the detection method worked properly, isolated rat liver mitochondria were prepared in the same way as sheep liver mitochondria and were used to test the assay. In the presence of succinate the reduction of acetylated cytochrome *c* was detectable and it was stimulated by antimycin A addition and inhibited by SOD (Figure 4.3C). Therefore, it appeared either

that sheep liver mitochondria could reduce and oxidise acetylated cytochrome *c* or that there were still high levels of endogenous SODs present in isolated sheep liver mitochondria that were not present in rat liver mitochondria. To determine which of these possibilities was the more likely, rat liver mitochondria were assayed with non-acetylated cytochrome *c* (Figure 4.3D), which can be oxidised and reduced by the electron transfer chain, and compared with sheep liver mitochondria assayed with acetylated cytochrome *c* (Figure 4.3A). These two traces were extremely similar, suggesting that sheep liver mitochondria were capable of oxidising and reducing acetylated cytochrome *c*, a hypothesis supported by the observation of a rapid reduction of cytochrome *c* following the addition of KCN. This was observed in both traces (Figure 4.3, traces B and D) and was due to the inhibition of cytochrome oxidase, preventing the oxidation of cytochrome *c* without affecting its reduction by the mitochondrial electron transfer chain.

The ability of sheep liver mitochondria to oxidise and reduce acetylated cytochrome *c* meant that a new method of detection would need to be found if the effect of Mn on the production of $O_2^{\cdot-}$ production were to be examined in sheep liver mitochondria. Unfortunately, it was not possible to identify a new detector molecule in the time available.

4.1.2 Production of hydroxyl radicals

Hydroxyl radicals were produced using a Fenton type reaction, involving $FeCl_2$ and H_2O_2 (Section 2.6.4). This production was measured by following the formation of $FeCl_3$, one of the products of this reaction, at 360 nm (Figure 4.4). Figure 4.4A shows the formation of $FeCl_3$ after the addition of H_2O_2 and figure 4.4B demonstrates the dependence of this reaction on the presence of $FeCl_2$, since H_2O_2 was added and no change in absorbance was observed. This suggests that ·OH were being produced, although it was not possible to confirm this by EPR (as had been planned) because the spin trap, 5,5-dimethyl-1-pyrroline N-oxide (DMPO), failed to arrive.

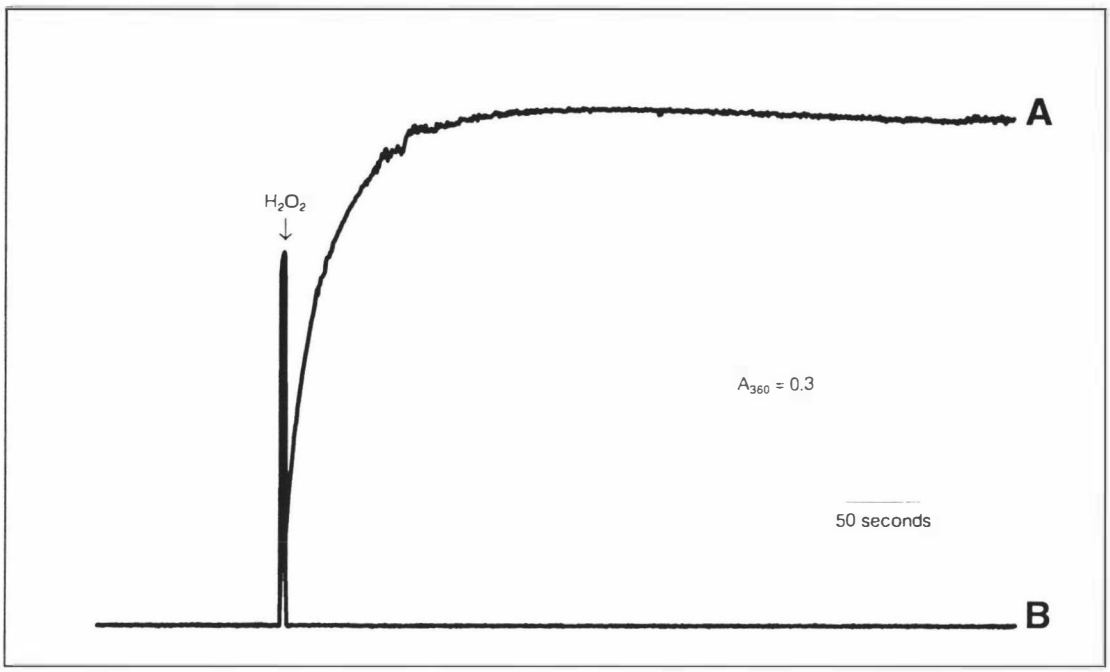

Figure 4.4 Representative spectrophotometric traces showing the production of ·OH. (A) $FeCl_2$ (1 mM) was added to buffer (70 mM HEPES-HCl pH 7.4) and H_2O_2 (1 mM) was added as indicated. The formation of $FeCl_3$ was measured at 360 nm.(B) Control assay (the same as A)in the absence of $FeCl_2$.

4.2 Discussion

The work described in this chapter was intended to examine the effect of Mn on the generation of free radical species by mitochondria. Oxygen radicals, especially ·OH, are particularly reactive and cause lipid peroxidation, mutagenesis of DNA and damage to protein (Halliwell, 1994). The mitochondrial electron transfer chain is the single most significant source of oxygen radicals in any cell (Halliwell, 1994). However, in normal circumstances, the mitochondria are protected from such radicals by SOD and GSH peroxidase, among other mechanisms (Cadenas, 1989). To examine the production of free radical species in mitochondria, a number of experiments were carried out, including the chemical synthesis of radical species, in order to test the detection systems and examining their effect on the normal function of the mitochondrion.

Superoxide radicals were produced chemically and measured by direct spectroscopy (Figure 4.1), the addition of SOD accelerated the decay of the signal, confirming the presence of $O_2^{\cdot-}$. Attempts to measure $O_2^{\cdot-}$ production by the mitochondrial electron

transfer chain were unsuccessful. NBT, a detector molecule which is reduced to formazan in the presence of the $O_2^{\cdot-}$, initially appeared to detect radicals in a mitochondrial system, however further investigation revealed that NBT accepted electrons from the mitochondrial electron transfer chain. This meant that it was impossible to tell whether the NBT was being reduced by the $O_2^{\cdot-}$ present or by the mitochondrial enzymes themselves. To overcome this problem another detector molecule was chosen, again this detected $O_2^{\cdot-}$ in the absence of mitochondria, but it failed to work with sheep liver mitochondria. Sheep liver mitochondria oxidised and reduced acetylated cytochrome *c*. The acetylation prevents this from occurring in rat liver mitochondria (Figure 4.3C) and those of other species (Goldstein and Czapski, 1996), but when compared with sheep liver mitochondria (Figure 4.3A) no $O_2^{\cdot-}$ was detected as the acetylated cytochrome *c* was reduced and oxidised by the mitochondrial electron transfer chain. This observation was confirmed with non-acetylated cytochrome *c* (Figure 4.3, B and D) which showed that sheep liver mitochondria can oxidise and reduce both acetylated and non-acetylated cytochrome *c* in the same way (Figure 4.3, A and B). Currently there are no sequence data available for sheep liver cytochrome c_1, which passes electrons to cytochrome *c* so at this stage, any differences between rat and sheep cytochrome c_1 cannot be determined, however when these data are available this could merit further investigation to explain this observation. These observations prevented the use of acetylated cytochrome *c* as a reliable method of measuring the production of $O_2^{\cdot-}$ by sheep liver mitochondria.

To continue this work a new detector molecule must be found. It must detect $O_2^{\cdot-}$, and not be reduced and oxidised by the mitochondrial electron transfer chain. It would also have to have an absorbance that is not interfered with by components of the mitochondrial electron transfer chain if the reaction is to be followed spectrophotometrically.

Hydroxyl radicals were produced using a Fenton type reaction, involving $FeCl_2$ and H_2O_2. The production of ·OH was measured (Figure 4.4) by detecting the production of $FeCl_3$ at 360 nm. To measure the production of ·OH in a mitochondrial system the spin-trap DMPO would be used to bind to the ·OH which would be detected by EPR. Unfortunately, although ordered in November 1997, DMPO never arrived and it was an essential component of this assay. In a biological system the only way to detect ·OH is

by EPR spectroscopy since ·OH are short lived and must be coupled to a more persistent species, the so-called "spin trap". The spin trap chosen was DMPO, which has been widely used in biological systems as it forms the longest-lived spin adducts with oxygen radicals. The spin trap system would have been tested by adding ·OH (Section 4.2) to the DMPO and taking a spectrum to determine the magnetic field strength at which resonance occurred. This would have been followed by assays with mitochondria in the presence of succinate and the inhibitor antimycin A, and it would have been expected to produced large amounts of ·OH, these would have been detected using DMPO.

Both ·OH and $O_2^{\cdot-}$ were successfully produced and detected by direct methods. However, further advances were prevented by difficulties encountered with measuring $O_2^{\cdot-}$ in a mitochondrial system due to the reduction of both detector molecules by the mitochondrial electron transfer chain. Further advances in the detection of ·OH were prevented by the non-delivery of the spin trap DMPO. Nonetheless these experiments showed that sheep liver mitochondria, unlike those of other species, can reduce and oxidise acetylated cytochrome *c*.

CHAPTER 5
THE EFFECT OF MANGANESE ON MITOCHONDRIAL DNA

Manganese is a well known mutagen. It induces mutations of the mitochondrial genomes of *S. cerevisae* (Putrament *et al.*, 1977), *S. pombe* (Colson *et al.*, 1976) and *C. reinhardtii* (Bennoun *et al.*, 1992), whereas several other divalent cations (including iron, magnesium, zinc and copper) do not (Putrament *et al.*, 1975a). Kunkel (1985) has shown that Mn increased the frequency of base substitutions by chicken liver mitochondria DNA polymerase-γ, but did not alter the frequency of base substitutions by either DNA polymerase-α or DNA polymerase-β. Also, as mitochondria have less efficient DNA repair mechanisms than the nucleus, mutations have a greater tenancy to remain in the genome and be inherited (Richter, 1994). The work described in this chapter was to examine the effect of Mn and reactive oxygen species on mtDNA. Initially, mtDNA was isolated from sheep liver mitochondria and then the gene encoding subunit 1 of cytochrome oxidase (COI) was amplified by PCR. This PCR product was then exposed to various treatments and their effects on the integrity of this product was examined.

5.1 Isolation and treatment of mitochondrial DNA with manganese and free radical species

5.1.1 Isolation of mitochondrial DNA

Mitochondrial DNA was isolated from sheep liver mitochondria produced by the method described in section 2.3.1. Initially, several different published methods (Palva and Palva, 1985; Welter *et al.*, 1989) were tried to isolate mtDNA from this source, but these were either unsuccessful or irreproducible. Eventually, DNAzol, a guanidine-detergent-based reagent used to isolate genomic DNA from tissues and cells, was successfully used to isolate mtDNA from sheep liver mitochondria by a method modified from the instructions included with this product (Section 2.7.1).

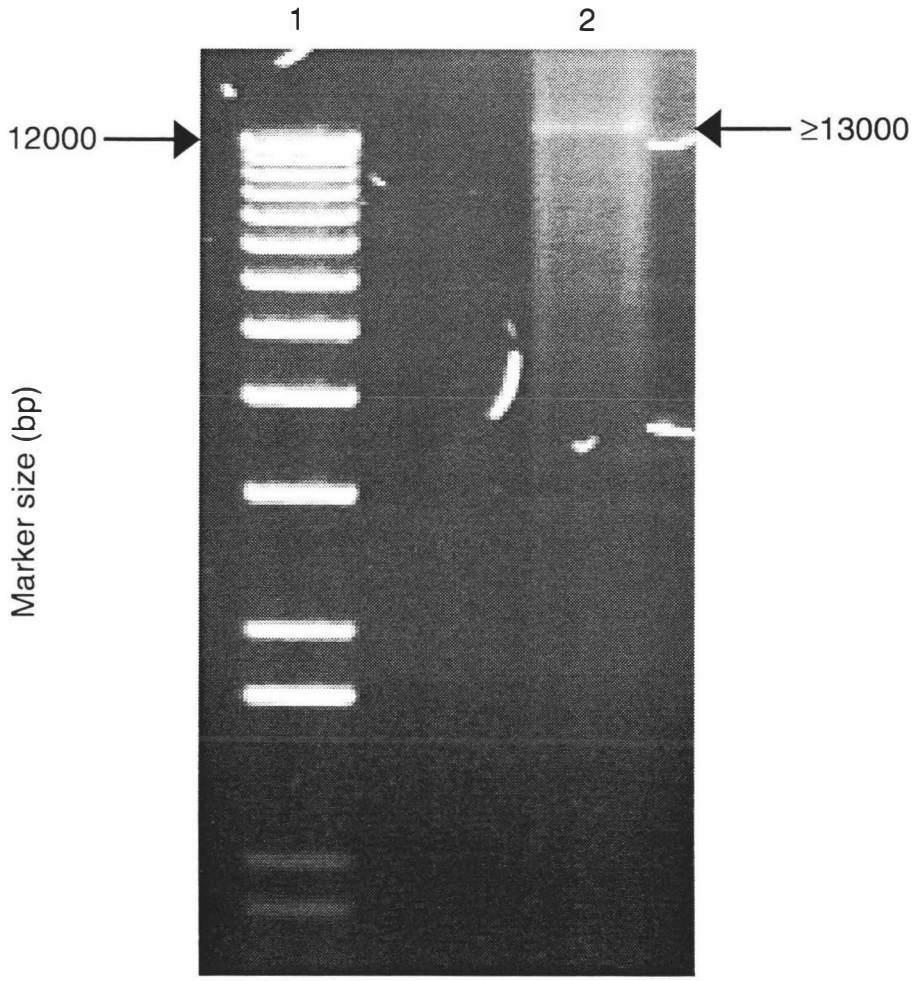

Figure 5.1 Sample of isolated sheep liver mtDNA analysed by electrophoresis in a 0.9% agarose gel containing TAE buffer at 44V for 120 minutes. DNA was stained with ethidium bromide (incorporated into the gel prior to electrophoresis) and visualised using a UV transilluminator.

1: Gibco BRL Life Technologies Ltd 1Kb plus ladder

2: 10 µl of isolated sheep liver mtDNA (Section 2.7.1)

Agarose gel electrophoresis showed that the mtDNA formed a discrete diffuse band of slightly lower mobility than the largest molecular size standard (12000bp) when run of an agarose gel (Figure 5.1). The sheep mitochondrial genome is 16616bp (Hiendleder *et al.*, 1998), so this band could have been intact, or possibly linear, sheep mtDNA. To confirm the identity of this band, several experiments were conducted.

5.1.2 Restriction endonuclease digest of mitochondrial DNA

To confirm that the product seen in Figure 5.1 was amplified sheep mtDNA, restriction digests were carried out, using the enzymes *Hin*dIII, which cuts the mitochondrial genome, and *Pst* I which does not. However, due to the small amount of template mtDNA, the resulting restriction fragments could not be detected after gel electrophoresis and UV transillumination in the presence of ethidium bromide.

5.1.3 Design of PCR primers

Oligonucleotide primers were designed to amplify COI (Appendix III) of the sheep liver mitochondrial genome by PCR. COI was chosen as it was a good length (1.5 kb, (Hiendleder *et al.*, 1998)) for both PCR reactions and restriction digests and most importantly, because it is exclusively mitochondrial (Hiendleder *et al.*, 1998). Two oligonucleotide primers were designed: COX1for and COX1rev (Appendix I). The sequence of these primers was based on the known sequence of the sheep mitochondrial genome (Hiendleder *et al.*, 1998) and the COI coding region. Analysis of the proposed primers using the GCG programme PRIME, (Wisconsin Package version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin) showed that they would not form strong secondary structures or primer dimers. A BLAST search of the Genbank database using these primers as search strings failed to find any significant matches apart from COI in the sheep mitochondrial genome.

5.1.4 PCR of COI using COX1for and COX1rev

COX1for and COX1rev were used to amplify COI using isolated sheep liver mtDNA as the template. The conditions used for PCR are outlined in section 2.7.4 A discrete,

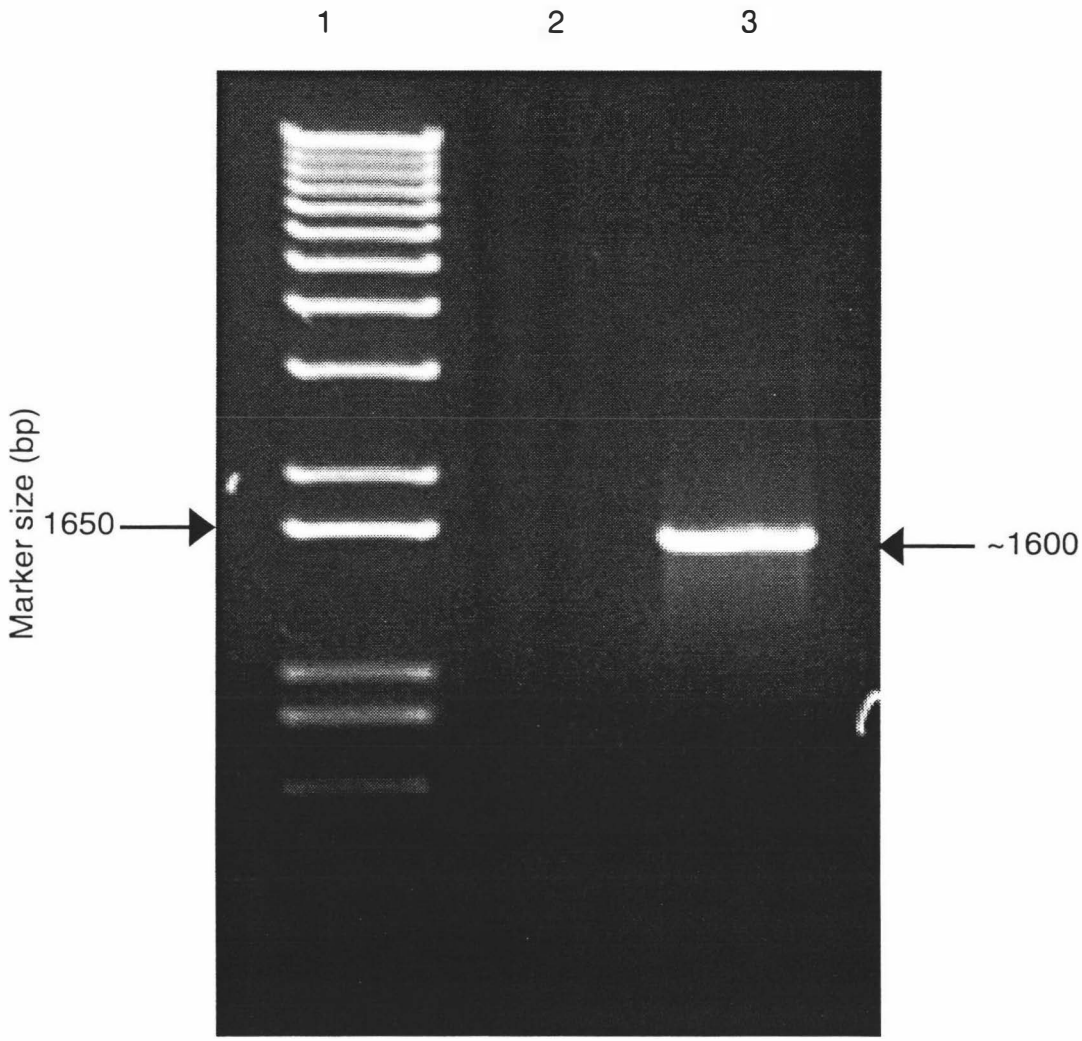

Figure 5.2 The PCR product amplified from sheep liver mtDNA with COX1for and COX1rev primers. A 5 µl aliquot of the PCR mix was analysed by electrophoresis in a 0.9% agarose gel containing TAE buffer at 88 V for 60 minutes. DNA was stained using ethidium bromide and visualised with a UV transilluminator.

1: Gibco BRL Life Technologies Ltd 1Kb plus ladder

2: Negative control

3: PCR product amplified from sheep liver mtDNA using COX1for and COX1rev primers and *Taq* polymerase

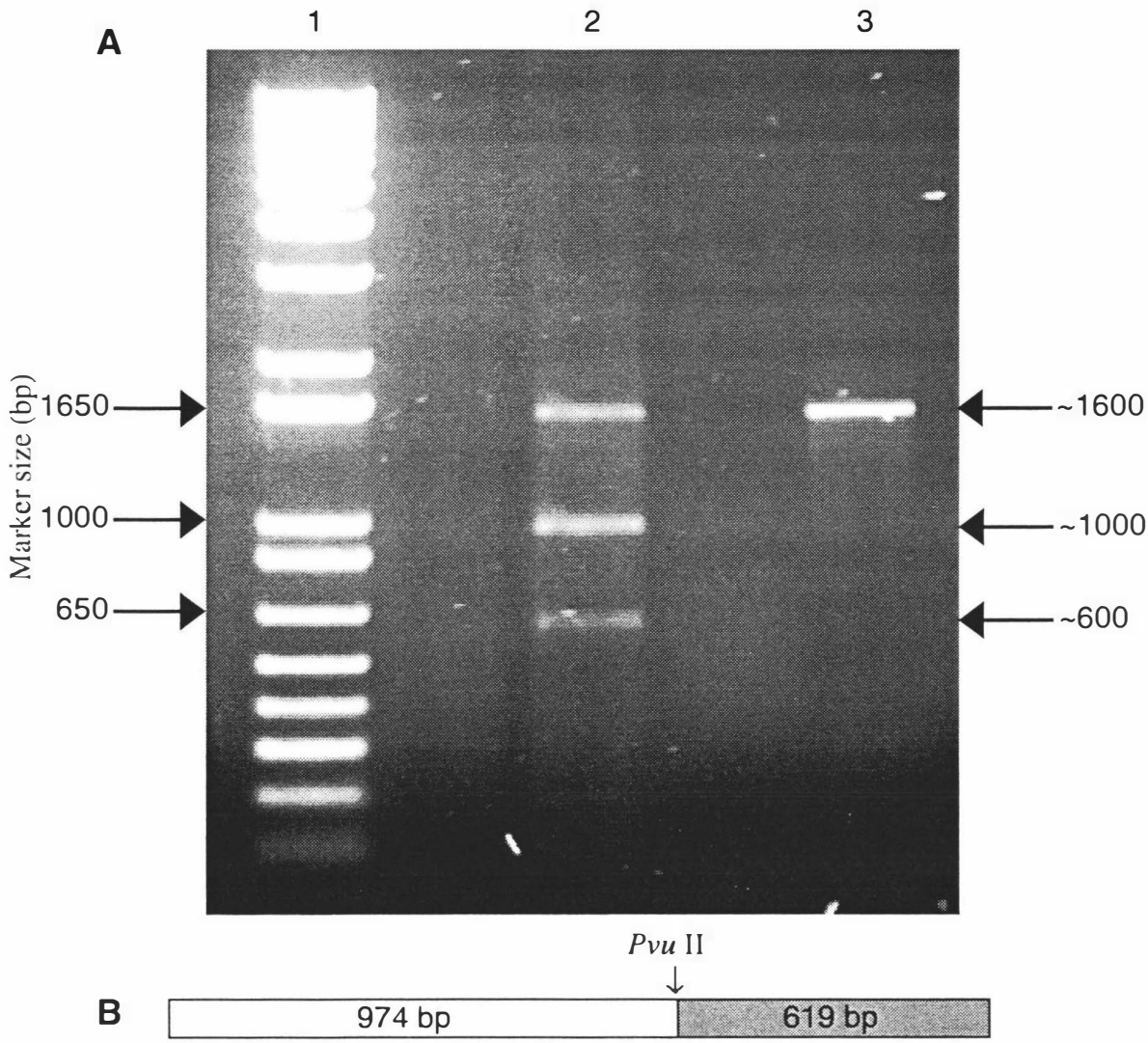

Figure 5.3 Diagnostic digest of ~1600 bp PCR product (A) and a schematic representation of expected digest products (B).

A: Diagnostic digest of the ~1600 bp PCR product

5 µl of the ~1600 bp PCR product was digested and analysed by electrophoresis in a 0.9% agarose gel containing TAE buffer at 88 V for 60 minutes. DNA fragments were stained with ethidium bromide (incorporated into gel prior to electrophoresis) and visualised with a UV transilluminator.

1: Gibco BRL Life Technologies Ltd 1Kb plus ladder

2: ~1600 bp PCR product digested with *Pvu* II

3: Uncut ~1600 bp PCR product

B: Expected digest products of ~1600 bp PCR product

single product of ~1600 bp was generated (Figure 5.2, lane 3) representing 1548 bp of COI and 45 bp of primer sequence. A negative control was also performed without any template DNA, but including all other components of the PCR reaction (Figure 5.2, lane 2). This PCR product was then digested using the restriction enzyme *Pvu* II, giving bands consistent with the predicted sizes of the digestion products (974 bp and 619 bp), (Figure 5.3). This digestion had not gone to completion, as some undigested PCR product (~1600 bp) was still present after three hours. To further confirm the identity of the PCR product, the product was purified (Section 2.7.6) and sequenced using an automated DNA sequencer (ABI Prism model 377, Perkin Elmer Ltd, Vic, Australia). The results of this DNA sequencing are given in Appendix IV.

5.1.5 Treatment of isolated mitochondrial DNA with manganese chloride and reactive oxygen species

Isolated mtDNA was incubated for three hours in the presence of three different concentrations of $MnCl_2$, $MgCl_2$ or reactive oxygen species (Table 5.1). The mtDNA was then precipitated and collected for PCR of the COI region and the products were electrophoresed on an agarose gel (Figure 5.4). The resulting PCR products (excluding sample 7) were then digested using the restriction enzymes *Pvu* II, *Hin*c II and *Acc* I (Figure 5.5) to examine whether base any changes to DNA sequence had occurred during pre-treatment.

Table 5.1 Treatments of mtDNA before amplification by PCR of COI.

Sample	mtDNA	Treatment	H_2O
1	12 µl	Control	13 µl
2	12 µl	5 µl of 1 M $MnCl_2$ (200 mM)	8 µl
3	12 µl	5 µl of 0.1 M $MnCl_2$ (20 mM)	8 µl
4	12 µl	5 µl of 0.01 M $MnCl_2$ (2 mM)	8 µl
5	12 µl	5 µl of 0.1 M $MgCl_2$ (20 mM)	8 µl
6	12 µl	13 µl of KO_2 solution-$O_2^{\cdot-}$	0 µl
7	12 µl	13 µl of H_2O_2 &$FeCl_2$ solution-·OH	0 µl
8	12 µl	13 µl of 323 mM of H_2O_2	0 µl

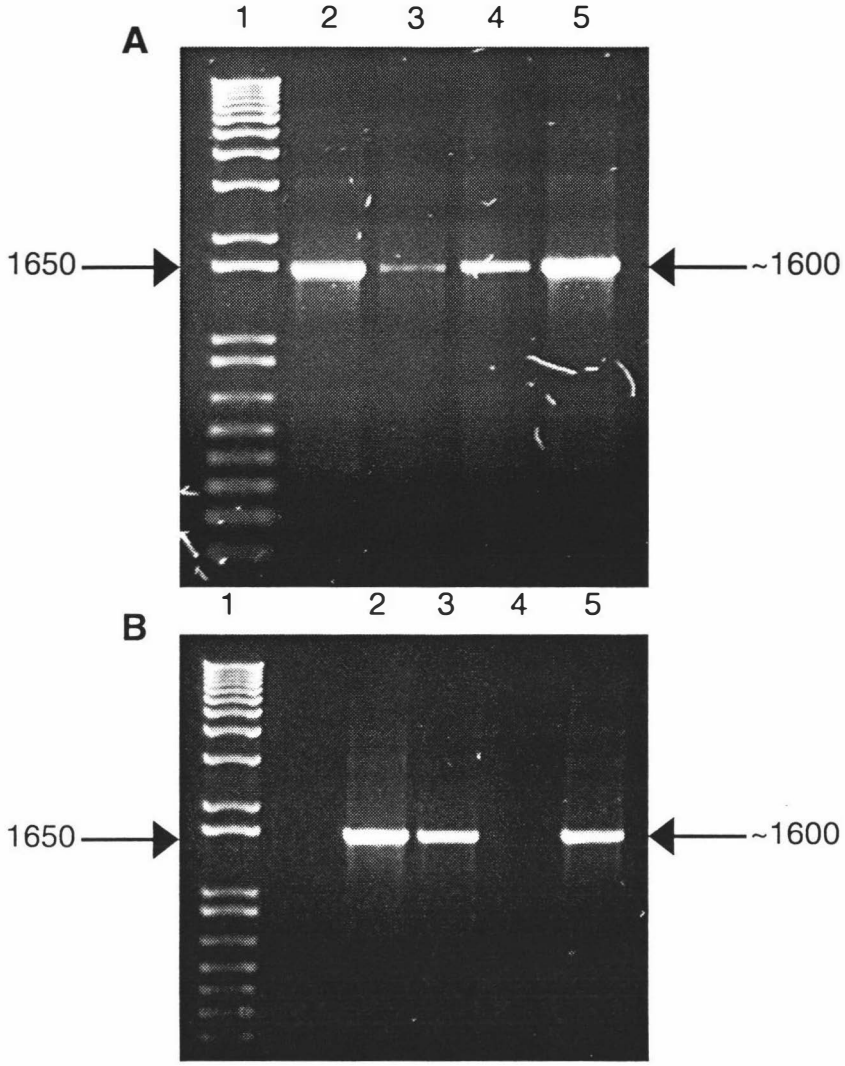

Figure 5.4 The ~1600 bp PCR products produced after various treatments of mtDNA. 10µl of PCR mix, after various pre-treatments, was analysed by electrophoresis at 88 V for 60 minutes in a 0.9% agarose gel containing TAE buffer. DNA fragments were stained using ethidium bromide and visualised with a UV transilluminator.

A: Treatment samples 1-3 and 5

1: Gibco BRL Life Technologies Ltd 1Kb plus ladder
2: PCR product
3: PCR product after mtDNA pre-treatment with 200mM $MnCl_2$
4: PCR product after mtDNA pre-treatment with 20mM $MnCl_2$
5: PCR product after mtDNA pre-treatment with 20mM $MgCl_2$

B: Treatment samples 4, 6-8

1: Gibco BRL Life Technologies Ltd 1Kb plus ladder
2: PCR product after mtDNA pre-treatment with 2mM $MnCl_2$
3: PCR product after mtDNA pre-treatment with $O_2^{\cdot-}$
4: PCR product after mtDNA pre-treatment with ·OH
5: PCR product after mtDNA pre-treatment with H_2O_2

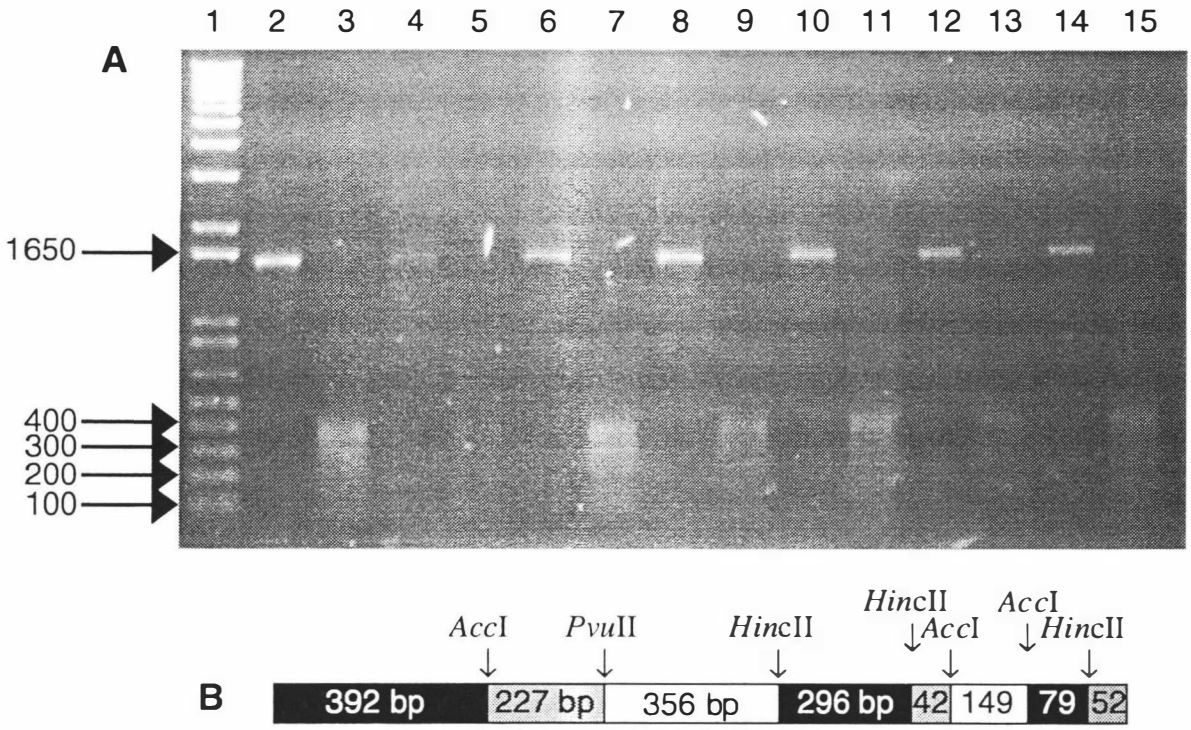

Figure 5.5 Digest of ~1600 bp PCR product amplified from pre-treated mtDNA. (A) and a schematic representation of a restriction map and expected digest products (B)

A: Digest of treated ~1600 bp PCR product

10 µl of PCR mix, was digested and analysed by electrophoresis in a 0.9% agarose gel containing TAE buffer at 88 V for 60 minutes. DNA fragments were stained using ethidium bromide and visualised with a UV transilluminator.

1: Gibco BRL Life Technologies Ltd 1Kb plus ladder
2: Uncut 1593 bp PCR product
3: Digested 1593 bp PCR product
4: Uncut 1593 bp PCR product after pre-treatment with 200mM $MnCl_2$
5: Digested 1593 bp PCR product after pre-treatment with 200mM $MnCl_2$
6: Uncut 1593 bp PCR product after pre-treatment with 20mM $MnCl_2$
7: Digested 1593 bp PCR product after pre-treatment with 20mM $MnCl_2$
8: Uncut 1593 bp PCR product after pre-treatment with 2mM $MnCl_2$
9: Digested 1593 bp PCR product after pre-treatment with 2mM $MnCl_2$
10: Uncut 1593 bp PCR product after pre-treatment with 20mM $MgCl_2$
11: Digested 1593 bp PCR product after pre-treatment with 20mM $MgCl_2$
12: Uncut 1593 bp PCR product after pre-treatment with $O_2^{\cdot-}$
13: Digested 1593 bp PCR product after pre-treatment with $O_2^{\cdot-}$
14: Uncut 1593 bp PCR product after pre-treatment with H_2O_2
15: Digested 1593 bp PCR product after pre-treatment with H_2O_2

B: Expected sizes of digest products of 1593 bp PCR product

None of the treatments prevented the formation of a PCR product except for treatment 7 in which $FeCl_2$ and H_2O_2 were rapidly mixed and added to the isolated mtDNA. However, this reaction formed ·OH (Section 4.1.2) and $FeCl_3$, which precipitated upon its addition to the mtDNA. This precipitate interfered with the subsequent isolation of DNA, and so the absence of a PCR product may not indicate DNA damage. The effect of a treatment of ·OH on DNA before a PCR reaction requires further investigation. Increasing the concentration of Mn present in the treatment decreased the intensity of the PCR band (Figure 5.4A, lanes 3 and 4, and Figure 5.4B, lane 2). There were three possible reasons for this observation. Firstly that Mn was able to modify some of the template DNA reducing the amount of DNA available for primer binding resulting in a less PCR product. The second possibility was that not all the Mn was removed from the sample during the DNA isolation step and that the residual Mn interfered with the activity of *Taq* polymerase. The final possibility was that the Mn was present in the PCR reaction and it interfered with primer binding to the template DNA. The effect of Mn on mtDNA could not be determined from this series of experiments and further investigation is required. The PCR products were digested with *Acc*I, *Hinc*II and *Pvu*II in order to check whether the mtDNA had been mutagenised by the treatment. However, there were no apparent changes to the sequence of the template DNA after various treatments as the banding pattern observed for each treatment group was the same as the control (Figure 5.5). This did not rule out the possibility there were changes to the template sequence, just that they were not detectable by the restriction digests carried out.

5.2 Discussion

This work was intended to examine the effect of Mn and reactive oxygen species on sheep liver mtDNA. Manganese is a well known mutagen, which has been shown to induce mutations in the mitochondrial genomes of *S. cerevisiae* (Putrament *et al.*, 1975a), *S. pombe, C. reinhardtii* (Bennoun et al., 1992), and isolated DNA *in vitro* (Putrament *et al.*, 1975a), Whereas several other divalent cations (including iron, Mg, zinc and copper) do not (Putrament *et al.*, 1977).

Sheep liver mtDNA was isolated from sheep liver mitochondria using DNAzol, a guanidine detergent based isolation product, (Section 2.7.1). The isolation product was run on an agarose gel producing a discrete diffuse band of ≥13000 bp (Figure 5.1, lane 2). The sheep mitochondrial genome is 16616 bp (Hiendleder *et al.*, 1998), but to confirm that the isolated product was sheep liver mtDNA the mitochondrial COI was amplified from the solution containing the ≥13000 bp product. PCR of COI produced a discrete band of the expected size of ~1600 bp (Figure 5.2) which consisted of the predicted 1548 bp of the COI sequence between the primers and 45 bp of primer sequence. The PCR product was then digested using the restriction enzyme *Pvu*II producing the predicted digestion products of the predicted size (Figure 5.3). This suggested that the PCR product was the COI and this was further confirmed by automated sequencing (Appendix IV). These experiments confirmed that sheep liver mtDNA could be isolated successfully using DNAzol.

The sheep liver mtDNA was then treated with different concentrations of $MnCl_2$, $MgCl_2$, $O_2^{\cdot-}$, ·OH and H_2O_2. Unfortunately, the method used to produce ·OH (Section 2.6.4) also produced $FeCl_3$ which precipitated with hydroxide ions, a by-product of this reaction, preventing the subsequent isolation of mtDNA. Consequently, no PCR product could be generated and the analysis of the effect of ·OH on mtDNA was prevented. Another method of producing ·OH would have to be used to assess this, but due to the limited time available, this was not examined.

The PCR products produced from mtDNA exposed to $O_2^{\cdot-}$ and H_2O_2, were less abundant and gave less intense bands on an agarose gel compared with the control (Figure 5.4 B, lanes 3 and 5, and Figure 5.4 A, lane 2, respectively). This implies that these compounds could have decreased the amount of template DNA or disrupted its structure before PCR, or they might have been carried through to the PCR reaction and interfered either with the activity of *Taq* polymerase or disrupted primer binding. These possibilities were not addressed in this study. However, it seems unlikely that that $O_2^{\cdot-}$ was still present three hours after the reaction was initiated, as $O_2^{\cdot-}$ is rapidly degraded forming H_2O_2 (Section 4.1). The similarity in the intensities of the two bands may imply that this was caused by the presence of H_2O_2 in one reaction (Figure 5.4 B, lane 5) and H_2O_2 formed by the degradation of $O_2^{\cdot-}$ in the other (Figure 5.4 B, lane 3). It has been

demonstrated that H_2O_2 can cause damage to DNA (Halliwell, 1994) and thus may have decreased the amount of template DNA available for PCR. To confirm this, different concentrations of H_2O_2 could be used to see whether there is any difference in the intensity of the bands produced. Also, real time or kinetic PCR would allow accurate comparisons of the initial template copy number (Caplin *et al.*, 1999), which would allow one to determine whether H_2O_2 had degraded template DNA. The PCR products produced after pre-treatment of mtDNA were then digested with a cocktail of restriction enzymes in an attempt to determine whether any mutations had occurred after exposure to $O_2^{\cdot-}$ and H_2O_2. However the bands produced were not different from the control, but this does not show that no mutations occurred, rather that they did not occur in the restriction sites of the enzymes chosen for this assay. To determine definitively whether any mutation had occurred each product could be sequenced and compared to the control.

The treatment of mtDNA with $MgCl_2$ produced a PCR product that was very similar to the control band (Figure 5.4 B, lanes 5 and 2, respectively) suggesting that $MgCl_2$ exposure had no effect on the performance of the mtDNA in the PCR assay. This observation is supported by Putrament *et al.* (1977) who observed that Mg did not cause mutations of mtDNA. Furthermore, when this product was digested using the cocktail of restriction enzymes no difference between it and the control could be detected.

Mitochondrial DNA was incubated with three different concentrations of $MnCl_2$, resulting in three PCR products of differing intensities. The highest concentration of Mn produced the least intense band and the lowest concentration of Mn the most intense band (Figure 5.4 A, lanes 2 and 4), similar to the control (Figure 5.4 A, lane 2). As with the treatments involving $O_2^{\cdot-}$ and H_2O_2 this may have occurred by three different mechanisms. The Mn could have decreased the amount or structure of template DNA available for PCR, or been carried through to the PCR reaction and interfered with the activity of *Taq* polymerase or disrupted primer binding. However, in this case it seems unlikely that the Mn would have degraded the template mtDNA as there are no data in the literature which suggest this could occur, but to test this the treated mtDNA could be run on an agarose gel and compared with a control, before PCR. It is possible that some of the Mn was not removed before the PCR reaction and either interfered with the activity of *Taq* polymerase. It has be shown that Mn can modify the kinetic properties

of *Taq* polymerase (Brandis *et al.*, 1996) and it interferes with the activity of chicken liver mitochondria DNA polymerase-γ (Kunkel, 1985), it therefore seems plausible that Mn could be interfering with the activity of *Taq* polymerase. It seems less likely the Mn was interfering with primer binding, as $MgCl_2$, which has similar electrostatic properties as $MnCl_2$, did not have any effect on the intensity of the PCR band. The three PCR products from the treatment with $MnCl_2$ were digested using a cocktail of restriction enzymes, producing a restriction pattern the same as the control, indicating that Mn did not create any mutations in the restriction sites of the enzymes used.

This series of experiments has shown the successful isolation and characterisation of sheep liver mtDNA. The isolated mtDNA was then treated with various concentrations of $MnCl_2$, $MgCl_2$, $O_2^{\cdot-}$, ·OH and H_2O_2. The rapid degradation of $O_2^{\cdot-}$ suggests that the effects seen for this treatment may have in fact been caused by the formation of H_2O_2, and provides an explanation for the similarities between the results obtained with these two treatments. The treatments involving $MnCl_2$ suggest that Mn could possibly be interfering with the activity of *Taq* polymerase, and therefore decreasing the yield of PCR product. However, both the $MnCl_2$ and $O_2^{\cdot-}$ treatments on mtDNA merit further investigation. The effect of $O_2^{\cdot-}$ could be examined by using a system that generates $O_2^{\cdot-}$ over a long period of time, such as the xanthine oxidase system (Goldstein and Czapski, 1996). It was planned to examine the effect of Mn on the synthesis of new mtDNA by culturing mammalian cells in the presence of various concentrations of Mn, but due to the limited time available, this was not done.

However, as Mn has been shown to interfere with the activity of chicken liver mtDNA polymerase-γ (Kunkel, 1985) and *Taq* polymerase (Brandis *et al.*, 1996), it may interfere with the sheep liver mammalian mtDNA polymerase. This series of experiments prompts the suggestion that Mn, and the reactive oxygen species, may interfere with the structure or the production of mtDNA, but this requires further investigation.

CHAPTER 6
DISCUSSION

Manganese is an essential trace element found in many enzymes including the Mn-SOD found in mitochondria (Konstantinov *et al.*, 1987; Shimoda-Matsubayashi *et al.*, 1997) and chloroplasts (Kliebenstein *et al.*, 1998), in the oxygen evolving complex of higher plants and cyanobacteria (Renger, 1993) and in arginase (Kanyo *et al.*, 1996). However, the inhalation of large amounts of Mn causes disorders of the central nervous system, liver and gastrointestinal systems, resulting in the condition known as manganism (Shukla and Singhal, 1984). Manganese poisoning affects up to 25% of workers involved in the mining and milling of Mn ore (Mena, 1979).

The clinical symptoms of Mn poisoning are well defined. During the early stages of manganism psychomotor disturbances develop and continue for 1-3 months, whether or not the workers are removed from exposure. The duration of these psychomotor disturbances is relatively constant amongst all affected individuals, but the manifestations vary in both intensity and type. One to two months after the onset of the initial symptoms, more serious symptoms develop. As the condition becomes established, speech impairments occur. One to two years after the onset of manganism, neurological symptoms become permanent. Table 1.1 outlines the neurological signs recorded from 15 patients with Mn poisoning. Pathological features following exposure to Mn include lesions of the globus pallidus and the striatum (Mena, 1979). The caudate nucleus and the putamen are severely damaged Shukla and Singhal, 1984). The damage to these regions of the brain is believed to be the cause of the symptoms observed in patients suffering from manganism.

If Mn intoxication is associated with mitochondrial dysfunction, it must firstly be demonstrated that Mn is found in mitochondria. Manyard and Cotzias (1955) demonstrated that an injection of a tracer dose of $^{54}Mn^{2+}$ became concentrated in rat liver mitochondria within fifteen minutes of administration. It has also been demonstrated that sub-chronic exposure of rats to Mn causes an increase in the concentration of Mn in brain mitochondria (Liccione and Maines, 1989). Manganese is rapidly removed from the blood stream ($t_{½}$ = 1-2 minutes), but the removal from the

liver and brain is much slower ($t_{½}$ = 25 days and $t_{½}$ = 50-60 days, respectively). Experimental studies have shown that Mn exposure results in accumulation in both the brain (Liccione and Maines, 1988), particularly in synaptically active tissues, and in the liver (Shukla and Chandra, 1987; Hidiroglou and Shearer, 1976). It has long been known the Mn is taken into mitochondria by the calcium uniporter (Chance, 1965), and thus in synaptically active tissues, such as the globus pallidus, striatum, caudate nucleus and putamen, which experience frequent calcium spikes, there is an increase of transport of Mn into the mitochondria. A prolonged transport of Mn occurs, even though these calcium spikes are only short lived, because the calcium uniporter has separate sites for activation and transport, so calcium binds and remains bound for several minutes and large amounts of Mn can be transported. In normal conditions, the concentration of Mn would be no more than about 50 nM and would have no detrimental effects on mitochondrial activity, but during chronic exposure to Mn the intramitochondrial concentration could reach as much as 50 mM which could have serious repercussions.

Three mechanisms have been proposed as the cause of Mn poisoning:

1. Inhibition of mitochondrial electron transfer chain following Mn accumulation by mitochondria (Galvani *et al.*,1995; Gavin *et al.*, 1992; Taylor, unpublished.).
2. Neuronal degradation by free radicals such as O_2^- and ·OH (Donaldson *et al.*, 1982; Cadenas, 1989; Gavin *et al.*, 1992; Liccione and Maines 1988; Shukla *et al.*, 1980; Sloot *et al.*, 1996) cause lipid perioxidation and damage to DNA and protein.
3. Induction of mutation of the mitochondrial genome, as has previously been shown in *Chlamydomonas reinhardtii* (Bennoun *et al.*, 1992), yeast (Putrament *et al.*, 1975a) and bacteria.

6.1 Inhibition of the mitochondrial electron transfer chain

It has been demonstrated that Mn is actively accumulated by mitochondria, specifically in the brain (Liccione and Maines, 1989) and in the liver (Manyard and Cotzias, 1955). Gunter and Puskin (1975) showed, by EPR, that once Mn has entered the mitochondria it preferentially binds to the inner mitochondrial membrane. The enzymes responsible for catalysing respiratory oxygen consumption, NADH dehydrogenase, succinate dehydrogenase, the cytochrome bc_1 complex and cytochrome oxidase are located on the inner membrane of mitochondria (Figure 1.1).

A number of *in vivo* and *in vitro* experiments have examined the effect of various Mn compounds on partial reactions of the mitochondrial electron transfer chain (Table 3.1), but in each case, the experiments have a some inherent problems. *In vivo* experiments, in which animals are injected or fed with Mn, do not allow one to discriminate between a reduction in the mitochondrial concentration of an enzyme and a reduction in the activity of an enzyme. So the results reported from these types of experiments certainly require further investigation and the conclusions drawn could be questioned. To overcome these deficiencies in this study, *in vitro* assays were used as they allow a direct measurement of the activity of an enzyme, or a group of enzymes.

Several *in vitro* studies, using organic and inorganic Mn compounds, have shown Mn inhibition of some of the partial reactions of the mitochondrial electron transfer chain (Autissier *et al.*, 1977; Gavin *et al.*, 1992; Galvani *et al.*, 1995). However, some of these studies used a phosphate-containing buffer for their enzymatic assays. The data presented here has demonstrated that phosphate can bind Mn and thereby reduces the concentration of Mn available for inhibition. For example, Autissier *et al.* (1977) reported a 65% and 20% inhibition by MMT of NAD^+-linked and succinate stimulated whole chain electron transfer, respectively. But due to the phosphate in their assay buffer, the inhibitions may in fact have been significantly greater than that reported if carried out in a buffer free of phosphate. The data presented here represents the first examination of the effect of a single compound on each of the partial reactions of the mitochondrial electron transfer chain *in vitro* in phosphate-free system.

Gavin *et al.* (1992), consistent with the data presented here (Figure 3.3), showed that Mn inhibited ADP-stimulated electron transfer. These authors, concluded that this was due an inhibition of ATP synthase, but this is unlikely to be the case because Mn inhibited both ADP- and uncoupler-stimulated electron transfer (Figure 3.3). If ATP synthase is the site of Mn inhibition, then uncoupled electron transfer would not be expected to be affected by Mn. Furthermore, it has been shown that ATP synthase is not inhibited by millimolar concentrations of Mn (Dorgan *et al.*, 1984), and Husain *et al.* (1976) demonstrated that ATP hydrolysis is not affected by *in vivo* Mn administration. As Mn only inhibits high rates of electron transfer (Figure 3.3), tissues that have a particularly high demand for ATP, such as brain and muscle tissue would be the most affected by the presence of Mn. This is consistent with the observation that victims of

manganism suffer from both lesions and degradation of certain regions of the brain and a weakening of muscles. In brain tissue this would be exaggerated by the frequent increases in calcium transport, and therefore Mn transport into the mitochondria of the brain would exacerbate the inhibition.

The effects of Mn on the whole chain electron transfer in sheep liver mitochondria were examined using both succinate and malate/pyruvate as the substrate. Both succinate-stimulated and malate/pyruvate-stimulated electron transfer showed no significant difference between the inhibition observed for Mn and Mg. This implies that the inhibition was caused by ionic strength. The effect of ionic strength as a cause of inhibition of the mitochondrial electron chain by Mn, does not appear to have been considered previously. Other researchers have assumed that the inhibition was a direct effect on the enzymes of the mitochondrial electron transfer chain, but the data presented here suggest otherwise. The inhibition of electron transfer by ionic strength could be mediated by interfering with the electrostatic interactions of cytochrome *c* with either cytochrome c_1 of the cytochrome bc_1 complex or cytochrome oxidase.

Other researchers have suggested direct inhibition of the partial reactions of the mitochondrial electron transfer chain, and this was also examined here. Inhibition of electron transfer from succinate or malate/pyruvate to cytochrome *c*, as well as the activity of both succinate dehydrogenase and cytochrome oxidase, were inhibited by Mn which is consistent with a number of other studies (Autissier *et al.*, 1977; Chandra and Shukla, 1976; Singh *et al.*, 1979; Galvani *et al.*, 1995; Husain *et al.*, 1976). This implies that succinate dehydrogenase, NADH dehydrogenase and cytochrome oxidase are inhibited by Mn. Thus as the concentration of Mn in the mitochondria increases both an ionic strength effect on whole chain electron transfer and a direct inhibition of certain enzymes would occur. This would result in a less efficient mitochondrial electron transfer chain, and a decrease in the production of ATP. This could be a cause of the lesions and degradation of certain regions of the brain observed in patients suffering from manganism.

This section of work showed that Mn does inhibit several of the partial reactions of the mitochondrial electron transfer chain, as has previously been reported (Autissier *et al.*, 1977; Chandra and Shukla, 1976; Singh *et al.*, 1979; Galvani *et al.*, 1995; Husain *et al.*,

1976), but that there is also an inhibition of electron transfer by ionic strength. This ionic strength effect, may be mediated by an interference of the electrostatic interaction of cytochrome *c* and the enzymes to which it binds, but this is yet to be confirmed. Therefore, it seems plausible that as brain mitochondria accumulate large amounts of Mn as the concentration of Mn increases, an inhibition of electron transfer mediated by both ionic strength and direct inhibition of NADH dehydrogenase, succinate dehydrogenase and cytochrome oxidase would occur. This could cause a decrease in the production of ATP and result in the degradation of the brain tissue observed in victims of manganism.

6.2 Generation of free radical species

The mitochondrial electron transfer chain is the single most significant source of oxygen radicals ($O_2^{\cdot-}$ and ·OH) in any cell. Both $O_2^{\cdot-}$ and ·OH are extremely reactive and cause lipid peroxidation, mutagenesis of DNA and damage to protein (Chance *et al.*, 1979; Halliwell, 1994; Shukla *et al.*, 1980). These species are believed to be the cause of the damage to certain regions of the brain that are observed in victims of manganism. A number of hypotheses (Section 1.2.4) have been proposed to explain how Mn might cause an increase in the level of free radicals species within mitochondria.

The first hypothesis was examined in this project and is based on the observation that the concentration of free radicals within the mitochondria can increase rapidly after the addition of an inhibitor such as antimycin A (Boveris and Chance, 1973). Thus, as Mn has been shown to be an inhibitor of the mitochondrial electron transfer chain it may be expected to increase the rate of production of free radical species within mitochondria. Unfortunately, due to difficulties encountered with the assays used to analyse the production of both $O_2^{\cdot-}$ and ·OH, this question could not be fully addressed.

To continue this work, a new detector for $O_2^{\cdot-}$ must be found, as it was shown that sheep liver mitochondria can oxidise and reduce acetylated cytochrome *c* (Figure 4.3 A), which may not have been previously reported. This does not occur in other species, as was shown when rat liver mitochondria were assayed (Figure 4.3 C). A new detector molecule would allow one to determine whether the inhibition of the mitochondrial electron transfer chain by manganese was affecting the generation of $O_2^{\cdot-}$.

The influence of Mn on the production of ·OH could be assessed in a biological system by EPR spectroscopy. ·OH are short lived and so they must be coupled to a more persistent species, the so-called "spin trap". The spin trap DMPO, is widely used in biological systems as it forms long-lived spin adducts with oxygen radicals.

Both $O_2^{\cdot-}$ and ·OH were produced chemically, but due to difficulties encountered, it was not possible to assess the effect of Mn on the generation of these species in a mitochondrial system. Consequently, it still remains as a possible cause of the degradation of brain tissue seen in victims of Mn poisoning and merits further investigation.

6.3 Mutations of the mitochondrial genome

Manganese is a well known mutagen, it induces mutations in the mitochondrial genomes of *S. cerevisae, S. pombe* (Putrament *et al.*, 1978) *C. reinhardtii* (Bennoun *et al.*, 1992) and in various bacterial genomes, whereas several other divalent cations do not (Putrament *et al.*, 1977). It acts almost specifically on the mitochondrial genome (Putrament *et al.*, 1975a) and it has been shown to interfere with the activity of both *Taq* polymerase (Brandis *et al.*, 1996) chicken liver DNA polymerase-γ (Kunkel, 1985). In the absence of any other data, it appears likely that Mn could induce mutations in mammalian mtDNA, perhaps through a direct effect on DNA polymerase-γ.

Exposure of mtDNA to both $O_2^{\cdot-}$ and H_2O_2 produce less intense PCR products than the control. This could be due to degradation of template DNA before PCR was carried out. However, it seems likely that $O_2^{\cdot-}$ would rapidly decompose to H_2O_2 and so both results are probably due to a H_2O_2 mediated degradation of template DNA. It has previously been demonstrated that H_2O_2 can cause damage to DNA (Halliwell, 1994). To confirm this, samples of template DNA could be exposed to different concentrations of H_2O_2, and its effect on the intensity of the resulting PCR products examined. Real time PCR would allow the determination of the concentration of template DNA before the PCR reaction (Caplin *et al.*, 1999).

Exposure of mtDNA to Mn resulted in less intense PCR products as the concentration of Mn increased. This implies either that Mn could have decreased the amount or structure of template DNA before PCR or that Mn may have have been carried through to the PCR reaction and interfered with the activity of *Taq* polymerase or disrupted primer binding. Currently there are no data that suggest that Mn could have degraded template DNA. To eliminate the possibility that Mn interfered with primer binding, a melting curve with various concentrations of Mn could be constructed. The most plausible possibility is that Mn was carried through to the PCR reaction and interfered with the activity of *Taq* polymerase, as it has previously been demonstrated that Mn can modify the kinetic properties of *Taq* polymerase (Brandis *et al.*, 1996). To confirm this a number of PCR reactions could be carried out with different concentrations of Mn present in the PCR reaction, and its effect on the PCR products could be examined. It was planned, during this study to examine the effect of Mn on the synthesis of new mtDNA. Mammalian cells were to be cultured in the presence of various concentrations of Mn and mtDNA isolated from these cells would have been analysed for mutations by restriction fragment length polymorphism (RFLP) or random amplified polymorphic DNA (RAPD). While RFLP analysis can be used to indicate base substitutions and other alterations in mammalian mtDNA (Ferris *et al.*, 1983), changes in experimental design and alternative approaches would have been tested had time permitted. For example, RAPD analyses have also shown promise in indicating changes to or differences between mtDNA samples (Vanlerberghe-Masutti, 1994).

It appears the Mn was carried through to the PCR reaction and interfere with the activity of *Taq* polymerase and presumably it would also interfere with the activity of sheep liver mitochondrial DNA polymerase-γ, as it has already been shown to interfere with the activity of chicken liver mitochondria DNA polymerase-γ (Kunkel, 1985). This would imply that, in the presence of Mn, a decrease in the concentration of the enzymes encoded by mtDNA would occur. This could lead to a insufficient supply of ATP to the affected cells, where Mn is shown to be accumulated to the greatest extent, such as the brain (Liccione and Maines, 1988) and the liver (Shukla and Chandra, 1987). This insufficient supply could promote the degradation of those regions of the brain observed to be damaged in victims of manganism, and possibly lead to liver damage, which

would slow the removal of Mn from the body and exacerbate the effect of Mn on the brain.

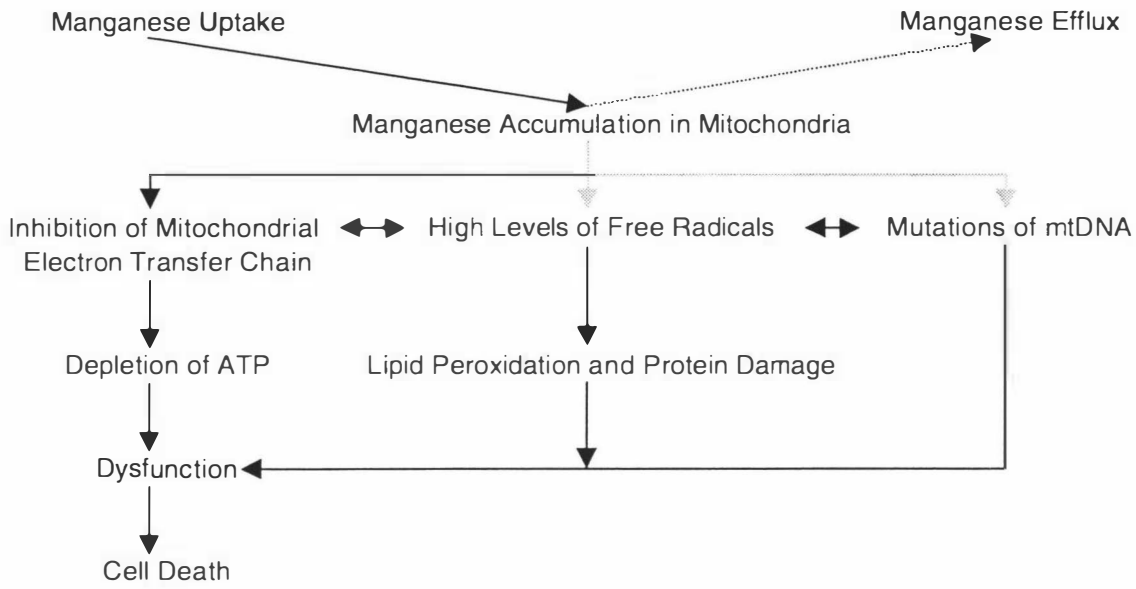

Figure 6.1 The relationship between the three models of Mn neurotoxicity. Black lines indicate known relationships either from the literature or work presented here. Grey lines indicate relationships that are yet to be proven, but seem likely.

6.4 Summary

It has been shown in this study that Mn inhibits the mitochondrial electron transfer chain (Figure 6.1). In itself this inhibition would decrease the production of ATP and could be sufficient to cause the degradation of brain tissue seen in victims of Mn poisoning. However, it seems likely that if Mn is capable of inhibiting the mitochondrial electron transfer chain, it is probable that this inhibition would lead to an increase in the generation of free radical species by the mitochondria. As free radicals are known to cause damage to protein (Halliwell, 1994), and thus could further damage the enzymes of the mitochondrial electron transfer chain, this would further decrease the rate of production of ATP. Furthermore, free radicals species are also known to damage DNA. It therefore seems plausible that they could damage the mtDNA that encodes components of the mitochondrial electron transfer chain. This would further decrease the production of ATP due to a decrease in the production of functional mitochondria. Finally, if Mn can interfere with the activity of mitochondrial DNA polymerase, which

is yet to be shown, this would decrease further both the number of functional mitochondria and the production of ATP. It has been shown here that Mn does inhibit the mitochondrial electron transfer chain, and that it is more likely that Mn does not itself cause mutation to the mitochondrial genome, but in fact it disrupts the activity of mitochondrial DNA polymerase, thereby reducing mitochondrial DNA copy number.

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APPENDICES

Appendix I

Oligonucleotide primers

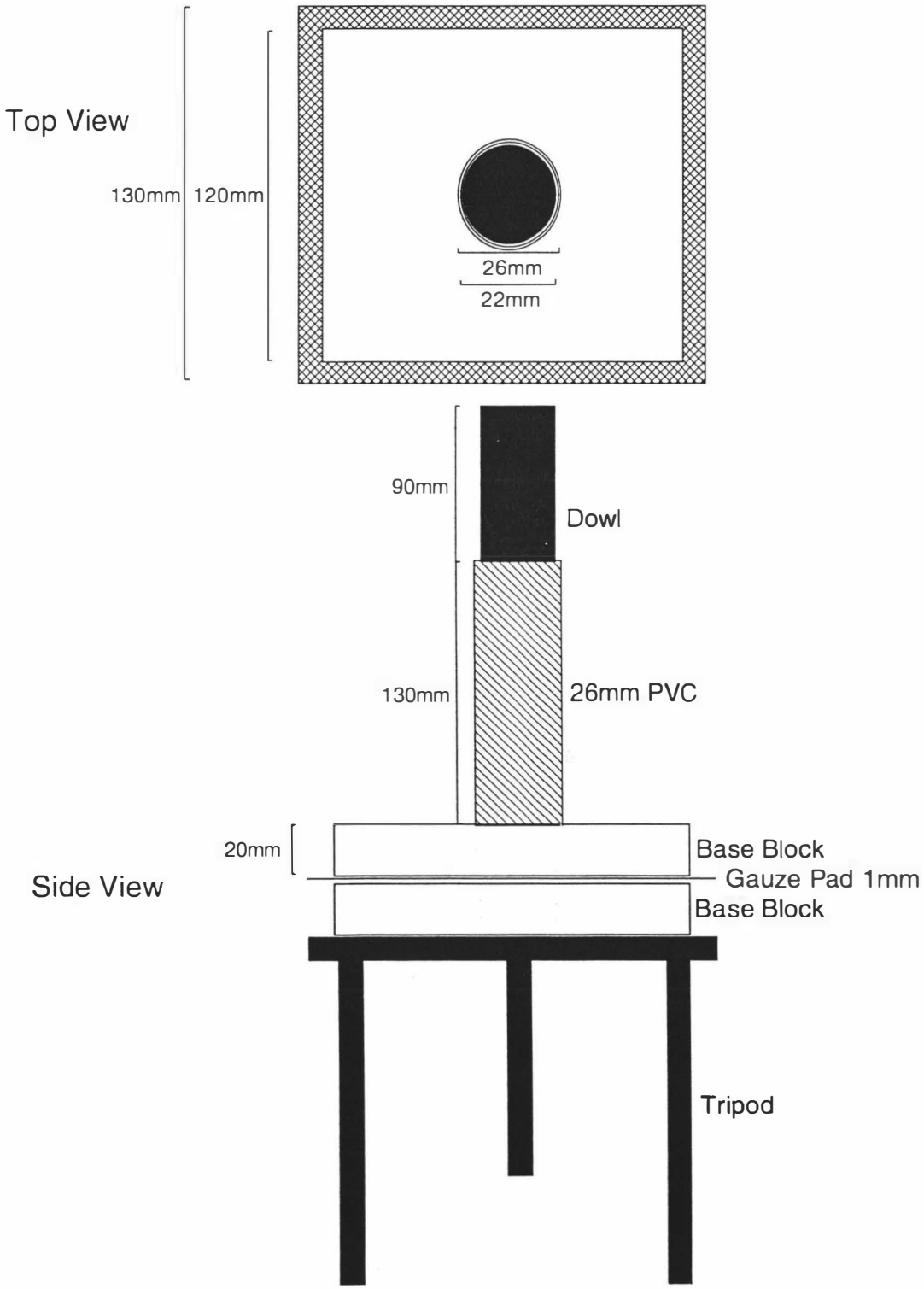
DNA sequence 5' → 3'

COX1for : TTT TAC CCA TGT TCA TCA ACC G

COX1rev : GGC TTG AAA CCA ATA ATA GGA GG

Appendix II

Tissue press



Appendix III

mtDNA sequence of the sheep cytochrome *c* oxidase subunit 1 gene

Primer locations are indicated by arrows. The start (▶) and stop (▶|) codons of CO1 coding sequence are marked.

```
5221 TCTTTGAATT TGCAATTCAA TATGTTAATT CACTACAGGA CCTGGTAAAA AGAGGAATTA
                                                      COX1for
5281 AACCTCTGTT CTTAGATTTA CAGTCTATTG CTTTACTCAG CCATTTTACC CATGTTCATC
                                                      CO1
     →
5341 AACCGCTGAT TATTTTCAAC CAACCACAAA GATATCGGCA CCCTTTACCT TCTATTTGGT
5401 GCCTGAGCTG GTATAGTAGG AACCGCCTTA AGCCTACTAA TTCGCGCCGA ACTAGGCCAA
5461 CCCGGAACTC TACTCGGAGA TGACCAAATC TACAACGTAA TTGTAACCGC ACATGCATTT
5521 GTAATAATTT TCTTTATAGT AATGCCTATT ATAATCGGTG GATTCGGCAA CTGACTAGTT
5581 CCTCTGATAA TTGGAGCCCC TGATATAGCA TTTCCTCGGA TAAATAACAT AAGCTTTTGA
5641 CTTCTTCCCC CATCTTTCCT GTTACTCCTA GCATCCTCTA TGGTTGAGGC CGGAGCAGGA
5701 ACAGGTTGAA CCGTATACCC TCCTCTAGCA GGCAACCTAG CCCATGCAGG AGCCTCAGTA
5761 GATCTAACTA TTTTCTCCCT ACATCTGGCA GGTGTCTCTT CAATTCTAGG AGCCATTAAT
5821 TTTATTACAA CTATTATTAA TATAAAACCC CCTGCGATGT CACAGTATCA AACCCCCTTG
5881 TTTGTATGAT CTGTACTAAT TACTGCCGTA CTTCTCCTTC TCTCACTTCC TGTATTAGCA
5941 GCTGGTATCA CAATACTACT AACGGACCGA AACCTGAATA CAACCTTTTT TGACCCAGCA
6001 GGAGGAGGAG ACCCTATCCT ATATCAACAC CTATTCTGAT TCTTTGGGCA CCCTGAAGTA
6061 TATATTCTTA TTTTACCTGG GTTTGGGATA ATCTCCCATA TTGTGACCTA CTATTCAGGA
6121 AAAAAAGAAC CATTCGGATA TATAGGAATA GTATGAGCCA TAATATCAAT TGGGTTCCTA
6181 GGATTCATTG TATGAGCCCA CCATATATTC ACAGTCGGAA TAGACGTCGA TACACGGGCT
6241 TACTTCACGT CAGCTACTAT AATTATCGCC ATCCCAACAG GAGTAAAAGT ATTCAGTTGA
6301 CTAGCAACGC TTCATGGGGG TAATATCAAA TGATCTCCTG CCATAATATG AGCCCTAGGT
6361 TTCATCTTTC TTTTCACAGT CGGAGGCTTA ACTGGAATTG TTCTAGCCAA CTCCTCCCTT
6421 GACATTGTCC TCCATGACAC ATATTATGTA GTAGCACATT TCCACTACGT ATTATCAATA
6481 GGAGCTGTAT TTGCTATTAT AGGAGGATTT GTACATTGAT TTCCCCTATT CTCAGGCTAT
6541 ACTCTCAATG ATACATGAGC CAAAATCCAC TTTGCAATTA TATTTGTAGG TGTTAACATG
6601 ACTTTCTTTC CACAGCATTT CCTAGGACTA TCCGGTATAC CACGACGATA CTCTGATTAT
6661 CCAGACGCAT ATACAATATG AAATACTATC TCATCTATAG GCTCATTTAT CTCACTAACA
6721 GCAGTAATAC TAATAATCTT CATCATCTGA GAAGCATTTG CATCTAAACG AGAAGTCCTA
6781 ACTGTAGACC TAACCACAAC AAACCTAGAA TGACTAAACG GATGTCCTCC ACCATACCAC
6841 ACATTTGAAG AACCCACATA TGTTAACCTA AAATAAGAAA GGAAGGAATC GAACCTCCTA
                                        ▶|                    ←
6901 TTATTGGTTT CAAGCCAACA CCATAGCCAC TATGACTCTC TCAATAAACG AGATGTTAGT
       COX1rev
6961 AAAACATTAC ATAACCTTGT CAAGATTAAA TTACAGGTGA AAATCCCGTA CATCTCATAT
7021 GGCATATCCC ATACAACTAG GCTTTCAAGA CGCAACATCA CCTATCATGG AAGAACTACT
```

Derived from the complete DNA sequence of the sheep mitochondrial genome, Genbank/EMBL accession number: AF010406.

Appendix IV

Results of automated DNA sequencing

Primer = COX1for

```
  1   CTCCTATTAT TGGTTTCAAG CCNNATAGNA GGAACCGCCT TAAGCCTACT AATTCGCGCC
 61   GAACTAGGCC AACCCGGAAC TCTACTCGGA GATGACCAAA TCTACAACGT AATTGTAACC
121   GCACATGCAT TTGTAATAAT TTTCTTTATA GTAATGCCTA TTATAATCGG TGGATTTGGC
181   AACTGACTAG TTCCTCTGAT AATTGGAGCC CCTGATATAG CATTTCCTCG GATAAATAAC
241   ATAAGCTTTT GACTTCTTCC CCCATCTTTC CTGTTACTCC TAGCATCCTC TATGGTTGAG
301   GCCGGAGCAG GAACAGGTTG AACCGTATAC CCTCCTCTAG CAGGCAACCT AGCCCATGCA
361   GGAGCCTCAG TAGATCTAAC TATTTTCTCC CTACACCTGG CAGGTGTCTC TTCAATTCTA
421   GGAGCCATTA ATTTTATTAC AACTATTATT AATATAAAAC CCCCTGCGAT GTCACAGTAT
481   CAAACCCCCT TGNTTGNATG ATCTGNACTA ATTACTGCCG NACTTCTCCT TCTCTCACTT
541   CCTGNATTAG CAGCTGGNAT CACAATACTA CTAACGGCCG AAACCTGAAT ACAACCTTTT
601   TTTGACCCAC AGGAGGAGGA GACCCTAATC CTATATCAAC ACCTATTCTG NTTCTTTGGG
661   CACCCTGAAG NATATATTCT TATTTTACCC TGGGGTTNGG GATAATCTCC CATATTGGGA
721   CCTCTATTCA NGAAAAAAAN AACCATTCGG ATNTTTAGGA ATAGGATGAG CCCNTATATC
781   AATTGGGGTC CTAGGATTCA TTGGATGANC CCCCCANATT TCNCAGGCNG GAATANACGT
841   NGAAACCCNG GGTTACTTTC CGGNAGNTCT TNAATTATTG GCCTTCCCAC CNGGNANAAA
901   AGNTTCCGNN TGNCTTACCN CCCTTN
```

Primer = COX1rev

```
  1   GAAAATAATC AGCGGTTGAT GAACATGGGT AAAAGAGGAC ATCCGTTTAG TCATTCTAGG
 61   TTTGTTGTGG TTAGGTCTAC AGTTAGGACT TCTCGTTTAG ATGCAAATGC TTCTCAGATG
121   ATGAAGATTA TTAGTATCAC TGCTGTTAGT GAGATAAATG AGCCTATAGA TGAGATAGTA
181   TTTCATATTG TATATGCGTC TGGATAATCA GAGTATCGTC GNGGTATACC GGATAATCCT
241   AGGAAATGTT GTGGAAAGAA AGTCATGTTA ACACCTACAA ATATAATTGC AAAGTGGATT
301   TTGGCTCACG TATCATTGAG AGTATAGCCT GAGAATAGGG GAAATCAATG TACGAATCCT
361   CCTATAATAG CAAATACAGC TCCTATTGAT AATACGTAGT GGAAATGTGC TACTACATAA
421   TATGTGTCAT GGAGGACAAT GTCAAGGGAG GAGTTGGCTA GAACAATTCC AGTTAAGCCT
481   CCGACTGNGA AAAGAAAGAT GAAACCTANG GCTCATATTA TGGCAGGAGA CNTTTGATAT
541   TACCCCCATG AACCGTTGCT AGNCAACTGA ATACTTTTAC TCCTGNTGGG GATGGCAATA
601   ATTATTAGTA GCTGACGTGA AGNAAGCCCG GGGANCGACC GCCTATTCCC ACTGNGAATA
661   TNTTGGGGGG CTCATTCCAT GAATCCTAGA ACCCAAATTG ATTTATNGGG TCATACTATT
721   TCCANNTANT CCGAAAGGGT CCTTTTTTNC TGAATAGAAG NCCCAATNTG GGAGAATTTC
781   CCAAACCCAG GGAAAATAAN ATTTTTNTTT NANGGGGCCC AAAAATCNNA TANGNGGTGT
841   NTTTNGAATN GGGTTCNNCC TCTNGTNGGN NTAAAAAGGG GGTTTCAGGT TNNGCCCCTA
901   TAGGTATTGG AANCCCCTTG TNNNCNAGGN NAGG
```

N represents a space on the electrophoretogram that could not be unambiguously identified by the ABI DNA sequencing software as A, C, G or T.

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ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY
ETAP

Could mitochondrial dysfunction play a role in manganese toxicity?

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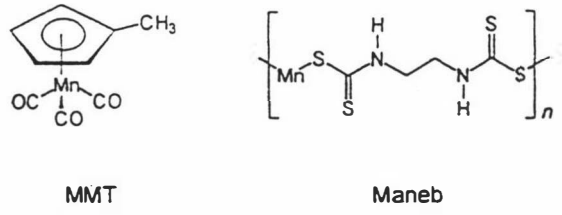
Abstract

Individuals suffering from manganese toxicity exhibit several symptoms, including mitochondrial dysfunction, which are similar to those frequently observed in cases of Parkinson's disease. We review the literature concerning manganese toxicity and mitochondrial function, and propose a simple conceptual model of the aetiology of manganese toxicity which involves an interaction between inhibition of mitochondrial energy transduction, generation of free radicals and mutations of the mitochondrial genome. This conceptual model prompts a number of relatively simple experiments which would provide a test of the model.

Keywords: Manganese; Toxicity; Mitochondria; Energy transduction; Mutagenesis; Free radical

1. Introduction

Manganese (Mn) is an essential trace element which is found in many enzymes, including the oxygen evolving complex of higher plants and cyanobacteria, the Mn-superoxide dismutase found in mitochondria and chloroplasts, and arginase. As is the case for many essential trace elements, excessive Mn is toxic and the syndrome of Mn toxicity is known as manganism. Although the Aboriginal inhabitants of Groote Eylandt, off the northern coast of Australia, suffer from Mn toxicity because of the abundance of Mn ore in their environment, and there are a number of reports of manganese-contaminated drinking water from other parts of the world, the most common source of reports of Mn toxicity arise from mining and milling of Mn ore (Mena, 1979). Increasingly, Mn toxicity is observed during prolonged parenteral nutrition (Fell et al., 1996), often in a clinical context in which the rate of Mn elimination from the body is reduced, such as liver damage (Devenyi et al., 1994). In some countries, common sources of Mn (Fig. 1) include the fungicide Maneb (Ferraz et al., 1988) and methylcyclopentadienyl manganese tricarbonyl (MMT), which has replaced lead as an anti-knock agent in petrol in Canada and parts of the USA. The use of MMT has resulted in a slight elevation of the level of atmospheric Mn, especially in urban areas (Zayed et al., 1994), because its combustion produces a Mn_3O_4 aerosol. It has been shown that those working in continuous contact with fumes from MMT-containing petrol do have elevated tissue levels of Mn (Zayed et al., 1994), but the levels observed are thought not to represent a health hazard. However, concerns have been expressed about the po-



MMT Maneb

Fig. 1. Structures of the petroleum additive methylcyclopentadienyl manganese tricarbonyl (MMT) and the fungicide Maneb (ethylenebis(dithiocarbamato)manganese).

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tential public health risks associated with widespread use of MMT (Davis et al., 1998).

The frequency of manganese poisoning is high amongst those exposed. For example, Mena (1979) suggests that as many as 25% of Mn miners are affected in Chile, India, Russia, Cuba and Africa. However, the introduction of MMT as a fuel additive, especially in major cities, could result in higher levels of atmospheric Mn which would lead to more widespread manganese toxicity (Frumkin and Solomon, 1997).

While the mechanism of manganese toxicity is not known, it has been hypothesised that it is associated with mitochondrial dysfunction because Mn accumulates specifically within mitochondria (see Section 3.1) and adversely affects mitochondrial function both in vivo and in vitro (see Section 3.2). Here, we review Mn toxicity and the literature upon which the hypothesised mitochondrial aetiology is based and we propose a conceptual model for Mn toxicity (see Section 6).

2. Clinical symptoms of manganese poisoning

During the early stages of exposure to manganese, psychomotor disturbances predominate. These frequently include nervousness and irritability, and occasionally involve compulsive acts, such as chasing cars until exhausted (Mena, 1979). These early symptoms are followed by generalised muscle weakness, difficulty in walking, impaired speech and headaches. Neurological symptoms become permanent 1–2 years after onset of poisoning and include disorders of gait, speech and postural reflexes, expressionless faces and a fine, intermittent tremor of small amplitude (Mena, 1979). Pathological features include lesions of the globus pallidus, the striatum, the caudate nucleus and the putamen. A depletion of dopamine from at least the substantia nigra is frequently observed (Bernheimer et al., 1973; Shukla and Singhal, 1984). In addition to the neurological symptoms, manganese-induced hepatotoxicity has also been reported in dogs (Khan et al., 1997).

3. Could Mn toxicity be associated with mitochondrial dysfunction?

If Mn intoxication is associated with mitochondrial dysfunction, it must be demonstrated both that Mn is found in the mitochondria and that it disrupts the normal operation of the organelle. While mitochondria are involved in many aspects of intermediary metabolism, their involvement in energy transduction is probably the single most important aspect of their operation. This process involves the oxidation of reduced substrates (such as malate, glutamate and succinate) and the transfer of reducing equivalents through a sequence of enzymes located in the inner mitochondrial membrane (Fig. 2). The ordinary catalytic cycle of three of these enzymes involves the translocation of protons from the matrix to the intermembrane space, giving rise to a proton electrochemical potential, which is dissipated in the synthesis of ATP by the ATP synthase (Fig. 2).

The common forms of Mn are inorganic salts (such as $MnCl_2$ or $MnSO_4$) or organic compounds (such as MMT). It is probable that the more hydrophobic Mn compounds (such as MMT) are more toxic than the inorganic salts (Table 1), perhaps because they may be accumulated to a much greater extent in living tissue (Garrec and Kudo, 1985).

3.1. Intracellular localisation of manganese

Usually, Mn is rapidly removed from the blood stream ($t_{1/2}$ ↓1–2 min) thereby minimising accumulation in the brain, and then removed from the liver at a much slower rate ($t_{1/2} = 25$ days) (Cotzias et al., 1968). Nevertheless, it does accumulate in the brain, especially following inhalation of Mn or during continuous exposure, because the rate at which it is removed from the brain is extremely slow ($t_{1/2}$ ↓50–60 days) (Cotzias et al., 1968). Experimental studies show that Mn exposure results in accumulation in both the brain (Liccione and Maines, 1988), particularly in the striatum (Shukla and Chandra, 1987) and the hypothalamus (Shukla and Chandra, 1987), and the liver (Shukla and Chandra, 1987). The accumulation of Mn in the liver can result in liver damage (Fell et al., 1996), thereby restricting the rate of excretion of Mn and exacerbating Mn toxicity.

Intracellular Mn is accumulated specifically and substantially in the mitochondrial matrix (Liccione and Maines, 1988), probably using the calcium transporters since Mn enhances the rate of calcium uptake into brain mitochondria and inhibits its efflux (Gavin et al., 1990). Gunter and Puskin (1975) showed that the total Mn accumulated in the mitochondrial matrix is increased in the presence of respiratory substrates or ATP, and that Mn efflux is stimulated following dissipation of the membrane potential ($\Delta\psi$), which implies that Mn accumulation is dependent on mitochondrial energisation (Gunter et al., 1978). Puskin and Gunter have observed accumulations of as much as 20 times the external Mn concentration (Puskin and Gunter, 1972, 1973), although they estimated that the concentration of free Mn in the mitochondrial matrix is about 500 times that in the external medium (Puskin and Gunter, 1973). A 500-fold accumulation means that the concentration of Mn in the matrix could be 10 mM for an extra-mitochondrial concentration of only 20 μM. However, this could be an underestimate if the Mn was in equilibrium across the inner mitochondrial membrane. In this case a $\Delta\psi$ of -180 mV would imply a

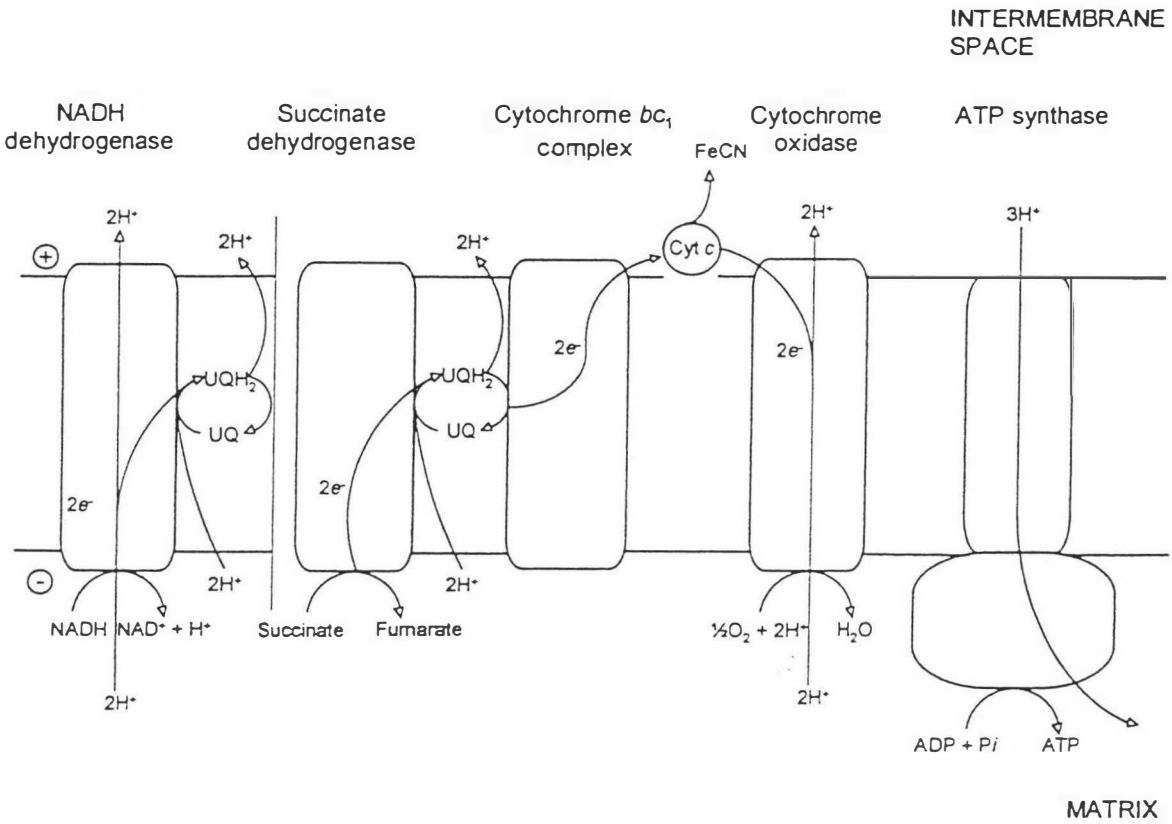

Fig. 2. The enzymes of the inner mitochondrial membrane involved in energy transduction. Also shown is the site at which potassium hexacyanoferrate (FeCN) accepts electrons. A single pool of ubiquinone (UQ) is reduced to ubiquinol (UQH_2) by both succinate dehydrogenase and NADH dehydrogenase, and is oxidised by the cytochrome bc_1 complex. NADH dehydrogenase-NADH → ubiquinone oxidoreductase (EC 1.6.5.3); succinate dehydrogenase-succinate → ubiquinone oxidoreductase (EC 1.3.5.1); cytochrome bc_1 complex-ubiquinol → ferricytochrome-*c* oxidoreductase (EC 1.10.2.2); cytochrome oxidase-ferrocytochrome-*c* → oxygen oxidoreductase (EC 1.9.3.1).

matrix concentration of about 10^6 times the extramitochondrial concentration.

The normal intracellular concentration of Mn is usually about 10 nM and it is probable that much of this is located within the mitochondria. The upper limit of the Mn concentration in the matrix can be estimated by assuming that the mitochondria occupy about 22% of the volume of an hepatocyte and so, even if all the Mn were located in mitochondrial matrix, the usual intramitochondrial Mn concentration would be no more than about 50 nM. Therefore, the usual intramitochondrial Mn concentration is sufficiently small that it would be unlikely to have any detrimental effect on mitochondrial metabolism.

3.2. *Interference with mitochondrial function*

Given that Mn accumulates specifically in the mitochondrial matrix, there are three principal mechanisms whereby Mn might disrupt mitochondrial function: (1) by inhibition of energy transduction; (2) by the induction of mutations of the mitochondrial genome; and (3) through the enhanced generation of free radicals. These parameters are linked, as is well established in the literature in connection with PD (see Section 4). For example, it is known that inhibition of mitochondrial electron transfer enhances the generation of free radicals by the electron transfer chain enzymes (Boveris and Chance, 1973) and that free radicals can induce mutations of the mitochondrial genome. Furthermore, it is well known that free radicals can damage protein and membranes (Halliwell, 1994), thereby disrupting the operation of the electron transfer chain.

3.2.1. *Inhibition of energy transduction*

Respiratory oxygen consumption is catalysed by four enzymes located in the inner mitochondrial membrane (Fig. 2): NADH dehydrogenase; succinate dehydrogenase; the cytochrome bc_1 complex; and cytochrome oxidase. Together, these enzymes catalyse electron transfer (the oxidation of reduced substrates and the reduction of oxygen to water) and the translocation of protons from the mitochondrial matrix to the intermembrane space. The proton electrochemical potential which is generated is dissipated in the synthesis of ATP by the ATP synthase. The effect of Mn on the activity of these enzymes has been assessed using several partial reactions, some of which are indicated in Fig. 2.

Table 1
Toxicity of some organic and inorganic Mn compounds

Compound	Organism	Administration[1]	LD_{50} (mg/kg)	Toxic equivalent (mg Mn/kg)
MMT	Rat	Oral	50	13
	Rat	Oral	58	15
	Mouse	Oral	230	58
	Rat	i.p.	23	6
	Rabbit	Dermal	140–795	35–200
Maneb	Rat	Oral	6750	~1400
Mn acetate	Rat	Oral	3730	836
$MnCl_2$	Rat	s.c.	180–250	50–69
$MnSO_4$	Mouse	Oral	3050	990
$KMnO_4$	Mouse	Oral	1900	660
MnO_2	Rat	i.v.	45	28

[1] i.p., intraperitoneal; s.c., subcutaneous; i.v., intravenous.

A number of in vivo studies have shown Mn inhibition of the enzymes of the mitochondrial electron transfer chain (Table 2). The oxidation of succinate was inhibited following administration of Mn in vivo (Husain et al., 1976; Singh et al., 1979) or to cultured human cells (Galvani et al., 1995). Furthermore, Galvani et al. (1995) have demonstrated inhibition of both NADH dehydrogenase and NADH → cytochrome *c* reductase (involving both NADH dehydrogenase and the cytochrome bc_1 complex) in PC12 cells, and Husain et al. (1976) have shown inhibition of cytochrome oxidase following prolonged intraperitoneal administration of $MnCl_2$.

In vivo experiments of this type do not allow one to distinguish between a reduction in the mitochondrial concentration of an enzyme and a reduction in the activity of the enzyme. A reduction in enzyme content could arise, for example, from mutations of the mitochondrial genome, resulting in a failure of assembly of electron transfer chain complexes or enhanced rates of degradation of complexes (see Section 3.2.2). A reduction in the catalytic activity could arise from a mutation which does not prevent assembly or affect the stability of the enzyme, but does alter the catalytic rate of the enzyme; from inhibition of one or more enzymes; or from a reduction in the affinity of binding of cytochrome *c* to cytochrome oxidase, for example, due to elevated intracellular ionic strength.

Both organic and inorganic Mn inhibit respiratory electron transfer in isolated mitochondria, which implies that Mn can act directly on those mitochondrial enzymes involved in energy transduction, rather than indirectly, through mutagenesis of the mitochondrial genome for example. Gavin et al. (1992) demonstrated inhibition of ADP-stimulated oxygen consumption (state 3 respiration) by isolated rat liver mitochondria oxidising either succinate or glutamate/malate. However, they were unable to show any inhibition of succinate oxidation in the presence of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and observed only a slight inhibition of glutamate/malate oxidation. They did not observe Mn inhibition of state 4 respiration (that in the absence of ADP) and concluded that Mn interfered directly with the ATP synthase, which implies a specific inhibition of state 3 oxygen consumption, but does not explain the inhibition of NAD^+-linked substrate oxidation in the presence of CCCP. Furthermore, it has been shown that the ATP synthase is not inhibited by millimolar concentrations of manganese (Dorgan et al., 1984) and ATP hydrolysis is not affected by in vivo Mn administration (Husain et al., 1976). Therefore, it seems unlikely that Mn inhibits the ATP synthase without affecting the activity of the mitochondrial electron transfer chain enzymes.

Galvani et al. (1995) showed $MnCl_2$ inhibition of NADH → cytochrome *c* reductase activity in mitochondria isolated from PC12 cells, but did not state whether ADP or uncoupler was present in the assays. Autissier et al. (1977) showed that MMT inhibited NAD^+-linked substrate oxidation only in the presence of ADP or an uncoupler. On the basis of these data and the observations of Gavin et al. (1992), it appears probable that only high rates of electron transfer are affected by Mn, as is the case in the presence of ADP or an uncoupler.

However, these data do not establish which of the electron transfer chain enzymes are affected by Mn. Succinate-dependent oxygen consumption relies on the activity of three separate enzymes (succinate dehydrogenase, the cytochrome bc_1 complex and cytochrome oxidase), and the oxidation of NAD^+-linked substrates relies on two of these three enzymes, as well as the activity of NADH dehydrogenase (Fig. 2). There are no data that definitively establish which of these enzymes is affected by Mn. However, NADH → cytochrome *c* reductase is usually reported to be more inhibited than either the succinate → cytochrome *c* reductase or the succinate → FeCN reductase activities (Table 2). This implies that both NADH dehydrogenase and one or

Table 2
Inhibition of substrate oxidation by Mn compounds[a]

Compound	Concentration[1]	Tissue	Treatment time	Activity (donor → receptor)	Inhibition (%)	Reference
$MnCl_2$	500 μM, in vitro	PC12 cells	6 h	NADH → cytochrome *c*	45	Galvani et al., 1995
				Succinate → cytochrome *c*	30	Galvani et al., 1995
			48 h	NADH → cytochrome *c*	45	Galvani et al., 1995
				Succinate → cytochrome *c*	25	Galvani et al., 1995
	10 μM, in vitro	PC12 cell mitochondria	–	NADH → cytochrome *c*	15	Galvani et al., 1995
				Succinate → cytochrome *c*	31	Galvani et al., 1995
	16 mg/kg, oral	Rat brain homogenates	30 days	Succinate → FeCN	17	Singh et al., 1979
	4 mg/kg, i.p.	Rat liver homogenates	30 days	Succinate → FeCN	17	Husain et al., 1976
				Cytochrome *c* → O_2	16	Husain et al., 1976
				NADH → cytochrome *c*	21	Husain et al., 1976
	15 mg/kg, i.p.	Rat testis homogenates	15 days	Succinate → FeCN	10	Chandra and Shukla, 1976
$MnSO_4$	5 mg/kg, i.p.	Rat testis homogenates	16 days	Succinate → FeCN	44	Husain et al., 1976
	5 mg/kg, i.p.	Rat testis homogenates	16 days	Succinate → FeCN	39	Husain et al., 1976
MMT	300 μM, in vitro	Rat liver mitochondria	–	Glutamate/malate → O_2	65	Autissier et al., 1977
				Succinate → O_2	20	Autissier et al., 1977

[a] The enzyme activities are defined in Fig. 2.
[1] i.p., intraperitoneal.

more of succinate dehydrogenase, the cytochrome bc_1 complex and cytochrome oxidase are affected. Husain et al. (1976) have shown that cytochrome oxidase is slightly inhibited following prolonged administration of Mn in vivo. Liccione and Maines (1989) observed a generalised 10–20% decline in the haem content of brain mitochondria isolated from rats treated subcutaneously with 1.75 mg/kg $MnCl_2$ for 7 days, implying that the concentration of each of the enzymes could have been affected. The site(s) of Mn inhibition of the mitochondrial electron transfer chain remain(s) to be identified.

3.2.2. Mutation of the mitochondrial genome

Manganese is a well known mutagen. It induces mutations of the mitochondrial genome of *Saccharomyces cerevisiae*, whereas several other divalent cations (including iron, magnesium, zinc and copper) do not (Putrament et al., 1977), and it acts almost specifically on the mitochondrial genome (Putrament et al., 1975a). It has also been shown that Mn induces mutations of the mitochondrial genomes of *Schizosaccharomyces pombe* and *Chlamydomonas reinhardtii*, of bacterial genomes, of bacteriophage T4 DNA and of isolated DNA in vitro. The concentration of Mn employed in inducing mutations ranges from 10 μM in *Escherichia coli* to 4–8 mM in *S. cerevisiae* and *C. reinhardtii*.

It has been shown that Mn causes a decrease in the fidelity of the replication of viral and *E. coli* DNA in vitro, perhaps by binding to the DNA (Eichhorn and Shin, 1968) and altering the hydrogen bonding pattern between bases. Certainly, Putrament et al. (1975b) have shown that mtDNA replication, but not protein synthesis, is required for Mn mutagenesis. More specifically, Kunkel (1985) showed that Mn increases the frequency of base substitutions by chicken liver mitochondrial DNA polymerase-γ, but did not alter the frequency of base substitution by either DNA polymerase-α or DNA polymerase-β. The same process could operate in mammalian mitochondria.

Furthermore, as mitochondria have less efficient DNA repair mechanisms than the nucleus and mtDNA lacks histones, mutations occur more frequently and have a much greater tendency to remain in the genome and be inherited. The mutations induced are of two types: deletion mutations, in which regions of the genome are deleted, and point mutations. In either case, a frequent consequence is an inhibition of the activity of the respiratory electron transfer chain, because one

or more subunits of each of the complexes (except for succinate dehydrogenase) are encoded by mtDNA. As each mitochondrion in human cells (about 1700 per hepatocyte, for example) has several copies of the genome, mutant and wildtype genomes coexist within a cell, but until the proportion of mutant genomes is high, there need not be any specific mutant phenotype.

We know of no data which demonstrate directly that Mn intoxication is associated with mutations of mammalian mtDNA. However, Itoh et al. (1994) have shown that the mtDNA of HTC cells incubated for 3 h with 100 μM $MnCl_2$ was not affected, whereas cells incubated with 10 μM $FeCl_3$ exhibited an increase in the proportion of linearised mtDNA. These data imply that there is little or no change in the extent of nicking of mtDNA in the presence of Mn, but they do not represent evidence for or against the possibility of mutations of mtDNA. Shukla et al. (1976) showed decreases in both total DNA and total RNA in the brains of rats treated intraperitoneally with 8 mg $MnCl_2$/kg for 120 days, consistent with the observation that nuclear DNA replication is inhibited by Mn in yeast (Putrament et al., 1977).

In the absence of any other data, it would appear very likely that Mn could induce mutations in mammalian mtDNA, perhaps through a direct effect on DNA polymerase-γ, as is the case for other eukaryotes, such as chickens, yeast and *Chlamydomonas* sp. This possibility merits further investigation.

3.2.3. Generation of free radicals

Oxygen radicals, especially the hydroxyl radical ($^{\bullet}OH$), are particularly reactive and cause lipid peroxidation, mutagenesis of DNA and damage to protein (Halliwell, 1994). However, peroxynitrite ($ONOO^-$), formed from superoxide ($O_2^{\bullet -}$) and nitric oxide (NO), is itself highly toxic and can decompose forming $NO_2^{\bullet -}$, $^{\bullet}OH$ and NO_2^+ (Halliwell, 1994). In normal circumstances, the mitochondrion is largely protected from such radicals by Mn-superoxide dismutase (Mn-SOD) and glutathione (GSH) peroxidase, among other mechanisms.

3.2.3.1. Mitochondrial electron transfer. The mitochondrial electron transfer chain is probably the single most significant source of oxygen radicals (superoxide, $O_2^{\bullet -}$ and the hydroxyl radical $^{\bullet}OH$) in any cell. Mitochondria consume 90% of the cell's oxygen and it has been estimated that the average person could produce more than 2 kg $O_2^{\bullet -}$ each year (Halliwell, 1994), which implies that much of the $O_2^{\bullet -}$ is produced by the mitochondrial electron transfer chain. Furthermore, inhibition of the electron transfer chain increases the rate of production of oxygen radicals (Boveris and Chance, 1973).

It might be expected that the inhibition of electron transfer by Mn (Section 3.2.1) would also enhance the rate of radical production, thereby aggravating the effects of Mn-inhibition of electron transfer. Such an increase in radical production would be exacerbated by the Mn-induced reduction of the activity of GSH peroxidase and SOD (Liccione and Maines, 1988). However, Archibald and Fridovich (1982) showed that Mn(II) is an effective scavenger of $O_2^{\bullet -}$ presumably by the reaction

$$2H^+ + O_2^{\bullet -} + Mn^{2+} \rightarrow H_2O_2 + Mn^{3+},$$

and that high concentrations of H_2O_2 can reduce Mn(III)

$$2Mn^{3+} + H_2O_2 \rightarrow 2H^+ + O_2 + 2Mn^{2+},$$

removing both Mn(III) and H_2O_2. On the other hand, Mn(III) is itself a powerful oxidant which is capable of oxidising amino acids, and H_2O_2, in the presence of $O_2^{\bullet -}$, can dismutate to form $^{\bullet}OH$ and OH^- by the Haber–Weiss reaction, which requires a catalyst such as Fe(III)

$$Fe(III) + O_2^{\bullet -} \rightarrow Fe(II) + O_2$$

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + {}^{\bullet}OH$$

the overall effect of which is the production of hydroxyl radicals

$$O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + OH^- + {}^{\bullet}OH.$$

While Mn(II) itself probably does not catalyse the Haber–Weiss reaction (Archibald and Tyree, 1987; Sloot et al., 1996) an accumulation of $^{\bullet}OH$ has been observed in the striatum following Mn exposure (Sloot et al., 1996) which could be a product of peroxynitrite decomposition. These data are inconsistent with the observation that Mn(II) treatment of rats induces a significant decline in lipid peroxidation (Donaldson et al., 1982).

We are unaware of any reports of the effects on Mn on the generation of free radicals by isolated mitochondria. However, the data which are available in the literature imply that Mn could enhance free radical production by mitochondria.

3.2.3.2. Catecholamine metabolism. It has been hypothesised that Mn neurotoxicity is mediated by free radicals via enhanced nonenzymatic autoxidation of catecholamines or production of 6-hydroxydopamine (6-OHDA). Either process might be expected to lead to the production of toxic quinones and elevated levels of H_2O_2, $O_2^{\bullet -}$ and $^{\bullet}OH$. However, Archibald and Tyree (1987) have shown that dopamine is oxidised without the generation of reactive oxygen species. More recently, Linert et al. (1996) have shown that Mn(II) significantly enhances the rate of dopamine autoxida-

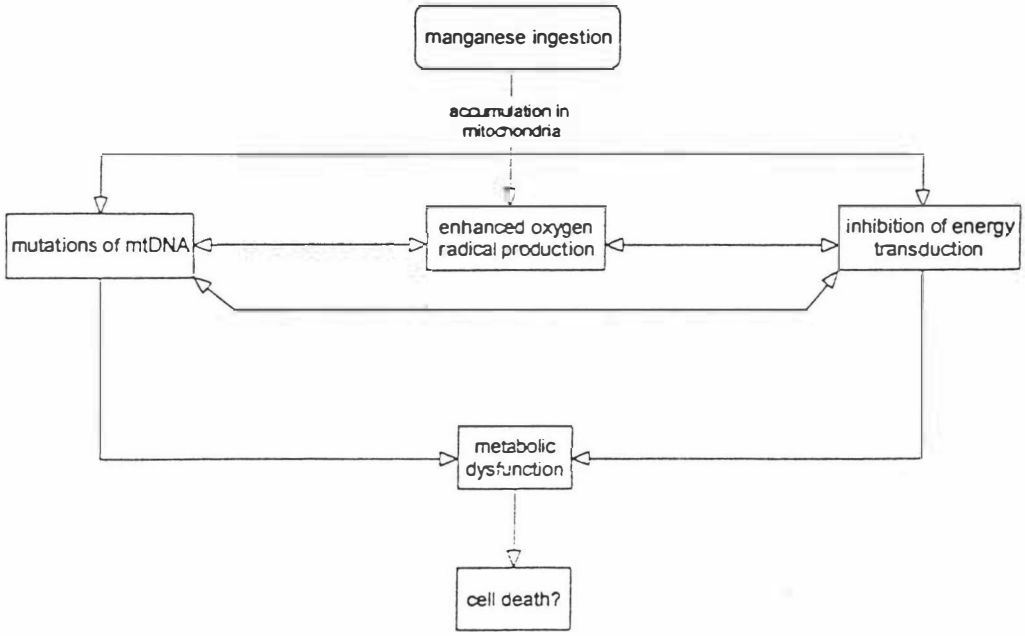

Fig. 3. Conceptual model of the toxic effects of Mn on the mitochondrion.

tion in vitro, transiently yielding a complex containing Mn(III), but that Mn(II) is regenerated (Herlinger and Jameson, 1996). This would explain the observations of dopamine depletion from specific regions of the brain following Mn exposure in vivo (Bernheimer et al., 1973; Liccione and Maines, 1988; Sloot et al., 1996).

4. Relationship between manganism and Parkinson's disease

The symptoms of manganism, particularly in the longer term, resemble those observed in Parkinson's disease (PD). While Calne et al. (1994) have concluded that manganism and PD differ in several details, the major symptoms of these disorders are very similar. Furthermore, both PD and Mn intoxication are associated with depletion of dopamine from the substantia nigra. Finally, as with manganism (Section 3), there is a growing body of data (Graeber and Müller, 1998) which support an association between PD and mitochondrial dysfunction and disruptions of cellular energy metabolism. Specifically, it has been demonstrated that PD patients have reduced activities of mitochondrial electron transfer chain enzymes, alterations in glycolytic activity, mutations of the mitochondrial genome, and exhibit enhanced generation of oxygen radicals (Graeber and Müller, 1998). Therefore, understanding the mechanism of Mn toxicity could help in understanding the role(s), if any, of the mitochondrial defects in the aetiology of PD.

5. Suggestions for further work

The data we have discussed show that Mn inhibits mitochondrial electron transfer and that it could cause mutations of the mitochondrial genome, which would exacerbate any inhibition of energy transduction. However, what remains to be determined includes:

1. the location of the site(s) and mechanism(s) of inhibition of mitochondrial energy transduction;
2. whether or not manganese intoxication is associated with an enhanced frequency of mutations of the mitochondrial genome, and the consequences of any mutations; and
3. the relationship, if any, between Mn-enhanced free radical production and mitochondrial dysfunction.

The results of such experiments cannot demonstrate that mitochondrial dysfunction is the cause of some or all of the symptoms of Mn intoxication, but they might prompt the development of more specific experiments which could do so.

6. A conceptual model of manganese toxicity

Manganese accumulates specifically in the mitochondrial matrix (Section 3.1), where it inhibits mitochondrial electron transfer (Section 3.2.1), is likely to enhance the rate of mutagenesis of mtDNA (Section 3.2.2) and could increase the rate of radical production (Section 3.2.3). These conclusions prompt the hypothesis that Mn ingestion interferes with mitochondrial function in several interdependent ways (Fig. 3): Having accumulated in the

mitochondrial matrix, Mn reduces the rate of substrate oxidation, which enhances the rate of production of radical species, and reduces the fidelity of mtDNA replication by DNA polymerase-γ, which induces either a reduction in the content of electron transfer chain enzymes or a decrease in their catalytic activity. The overall result is the disruption of cellular energy metabolism, potentially leading to cell death.

Manganese intoxication largely affects the nervous system and muscle tissue, both of which have a particularly high energy demand. For example, the brain requires 60% of all the energy input of the average person. Therefore, even a mild disruption of the energy metabolism of these tissues could have profound consequences. This could be exacerbated by the transient increases in calcium concentration observed in synaptic tissue, which would enhance the influx of Mn into the mitochondria (Gavin et al., 1990). At least some of the symptoms of Mn intoxication could be consistent with a disruption of nerve and muscle function.

Such a model of cellular dysfunction would imply severe disruption of energy metabolism. This, in turn, would provide an explanation for the neurological and hepatic dysfunction observed in manganism.

7. Unlinked references

AUTHOR, PLEASE CITE Greger, 1998 AND Feldman, 1992 IN THE TEXT.

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