AN INVESTIGATION INTO THE POSSIBLE NEUROPROTECTIVE OR NEUROTOXIC PROPERTIES OF METRIFONATE

THESIS

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ABSTRACT

Alzheimer's disease is a progressive neurodegenerative disorder, in which there is a marked decline in neurotransmitters, especially those of the cholinergic pathways. One of the approaches to the symptomatic treatment of Alzheimer's disease is the inhibition of the breakdown of the neurotransmitter acetylcholine, using an acetylcholinesterase inhibitor. One such drug tested, is the organophosphate, metrifonate. Any drug used for the treatment of neurodegenerative disorders should preferably not induce further neurological damage. Thus, in the present study, we investigated whether or not metrifonate is neuroprotective.

The *in vivo* and *in vitro* effect of this drug on free radicals generation shows that metrifonate increases the level of these reactive species. Lipid peroxidation induced using quinolinic acid (QA) and iron (II) and show that metrifonate increased the peroxidative damage induced by using quinolinic acid. Metrifonate is also able to induce lipid peroxidation both *in vivo* and *in vitro*. This was reduced *in vitro* in the presence of melatonin. Using iron (II), *in vitro*, there was no significant difference in the level of lipid peroxidation in the presence of this drug. An investigation of the activity of the mitochondrial electron transport chain and complex I of the electron transport chain in the presence of metrifonate revealed that metrifonate reduces the activity of the electron transport chain at the level of complex I. The activity of the mitochondrial electron transport chain was restored in the presence of melatonin.

Pineal organ culture showed that metrifonate does not increase melatonin production. Histological and apoptosis studies show that tissue necrosis and apoptosis respectively, occur in the presence of this agent, which is reduced in the presence of melatonin.

Metal binding studies were performed using ultraviolet spectroscopy, and electrochemical analysis to examine the interaction of metrifonate with iron (II) and iron (III). No shift in the peak was observed in the ultraviolet spectrum when iron (II) was added to metrifonate. Electrochemical studies show that there may be a very weak or no ligand formed between the metal and drug.

This study shows that while drugs such as metrifonate may be beneficial in restoring cognitive function in Alzheimer's disease, it could also have the potential to enhance neurodegeneration, thus worsening the condition, in the long term.

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LIST OF ABBREVIATIONS

ACh:	Acetylcholine
AChE:	Acetylcholinestrase
AChEI:	Acetylcholinesterase inhibitor
AD:	Alzheimer's Disease
ADP:	Adenosine Diphosphate
Ag:	Silver
AgCl:	Silver chloride
aHT:	N-acetylserotonin
AIF:	apoptosis inducing factor
APES:	Aminopropyl triethoxysilane
ASV:	Adsorptive stripping voltammograms
ATP:	Adenosine triphosphate
Bcl:	apoptosis inhibiting factor
BHT:	Butylated Hydroxytoluene
BuChE :	Butyrylcholinesterase
^{0}C:	Degrees celcius
Ca^{2+}:	Calcium
cAMP:	cyclic Adenosine monophosphate
Ch:	Choline
ChAT:	Choline acetyl transferase
Cl^{-} :	Chloride
CNS:	Central nervous system
CPM:	Counts per minute
CoQ:	Coenzyme-Q

DDVP:	2-dimethyl dichlorovinyl phosphate (dichlorvos)
DNA:	Deoxyribonucleic acid
DPI:	2,6 – diclorophenolindophenol
EAA:	excitatory amino acids
EDTA:	Ethylenediaminetetraacetic acid
ETC:	Electron transport chain
FAD:	Flavin adenine dinucleotide
FDA:	Food and Drug Administration
Fe^{2+}:	Iron (II)
Fe^{3+}:	Iron (III)
FMN:	flavin mononucleotide
GABA:	γ-aminobutyric acid
GCE:	Glassy carbon electrode
H^+:	Proton
HA:	5-hydroxyindole acetic acid
HIOMT:	Hydroxyindole-O-methyltransferase
HL:	5-hydroxytryptophol
H_2O:	Water
H_2O_2:	Hydrogen peroxide
HT:	serotonin
K^+:	Potassium
KCN:	Potassium cyanide
KP:	Kyurenine pathway
kg:	Kilogram
LD_{50}:	fifty percent of the lethal dose
M:	Molar
MA:	5-methoxyindole acetic acid
mCi/ml:	millicurrie per milliliter
MDA:	Malondialdehyde
MEL:	Melatonin
MET:	Metrifonate

Mg^{2+}:	Magnesium
μl:	microlitre
ml:	millilitre
μM:	micromolar
μmol:	micromoles
mM:	millimolar
Mn:	Manganese
mg:	milligram
mg/L:	milligrams per litre
ML:	5-methoxytryptophol
MT:	5-methoxytryptamine
mtDNA:	mitochondrial DNA
MTP:	mitochondrial transition pore
mV:	millivolt
Na^{+}:	Sodium
NADPH:	Nicotinamide adenine dinucleotide phosphate
NADH:	Nicotinamide adenine dinucleotide
NAT:	Serotonin N-acetyltransferase
NBT:	nitroblue tetrazolium
NBD:	nitroblue diformazan
NDA:	New Drug Application
NO:	Nitric oxide
NOS :	Nitric oxide synthase
nm:	Nanometre
NMDA:	*N*-methyl-D-aspartate
nmol:	Nanomoles
NTE:	Neuropathy target esterase
O_2:	Oxygen
$O_2^{\cdot-}$:	Superoxide anion radical
$OH^{\cdot}$:	hydroxyl radical
$ONOO^{-}$:	Peroxynitrite

OPIDN:	Organophosphate Induced Delayed Neurotoxicity
OXPHOS:	Oxidative phosphorylation system
PARS:	poly-ADP ribose polymerase
PBS:	phosphate buffered saline
PD:	Parkinson's Disease
PTP:	permeability transition pore
PUFA:	polyunsaturated fatty acids
P450:	Cytochrome P450
QA:	Quinolinic acid
ROS:	Reactive oxygen species
RNS:	Reactive nitrogen species
SCN:	Suprachiasmatic nucleus
SD:	Standard deviation
SEM:	Standard error of mean
SOD:	Superoxide dismutase
TBA:	2- Thiobarbituric assay
TCA:	Trichloroacetic acid
TEAP:	Tetraethylammonium phosphate
TLC:	Thin layer chromatography
UV:	Ultraviolet
V:	Volts
$V.s^{-1}$:	Volts per second
w/v:	Weight per volume

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CHAPTER ONE – LITERATURE REVIEW

1.1 Introduction to Neuroscience

Throughout the history of neuroscience, one of the most contentious and puzzling investigations was the localization of function to specific parts of the human brain. The first Hippocratic writings assigned thought to the brain, which in spite of Aristotle's view that the mind resided in the heart, the Galenists, too, placed the soul in the brain and gradually transposed "soul" to intellect, or "anima rationalis" (Marshall and Magoun, 1998). Renaissance figures, such as Da Vinci and Vesalius identified brain tissue and over a century later, ideas on localization of Gall and Magendie emerged. After it was established that the convolutions are arranged in lobes with sulci between them, the roles of the separate eight lobes (four in each cerebral hemisphere) were gradually determined by observational and experimental studies.

Neuroscience is one of the basic biological sciences, which refers to the knowledge of how brain and behaviour interact and is studied by scientists of many different disciplinary backgrounds from time immemorial. It combines the neural, behavioural and communicative sciences. Due to the multidisciplinary nature of neuroscience, the methodology necessary for problem solving has become progressively formidable. (Marshall and Magoun, 1998)

Neuroanatomy

In the brain, neurons accumulate and transmit electrical activity. Shown in fig.1.1 is the basic structure of the neuron which is the building block of the human brain. Approximately 100 billion neurons in a human brain exist which at any given moment the simultaneous activity of these neurons, enable signals to flow in many directions at once. As electricity flows through the synaptic connections of the neurons, another set of neurons is activated and the brain shifts to another mental state. The activation level of a neuron is a continuous variable, enabling incredibly subtle variations and shading in mental states. If patterns of neural activity result in a particular mental state then

"knowledge" may be defined as that which drives cognitive flow from one mental state to another, and must be encoded in the neural connections. That means learning is achieved by growth of new synapses, or the strengthening or weakening of existing ones. Evidence exists for both mechanisms, and any new long term knowledge in a brain requires a modification of its anatomy (http://www.sdu.dk/sdub/hum/paedagogik/910202)

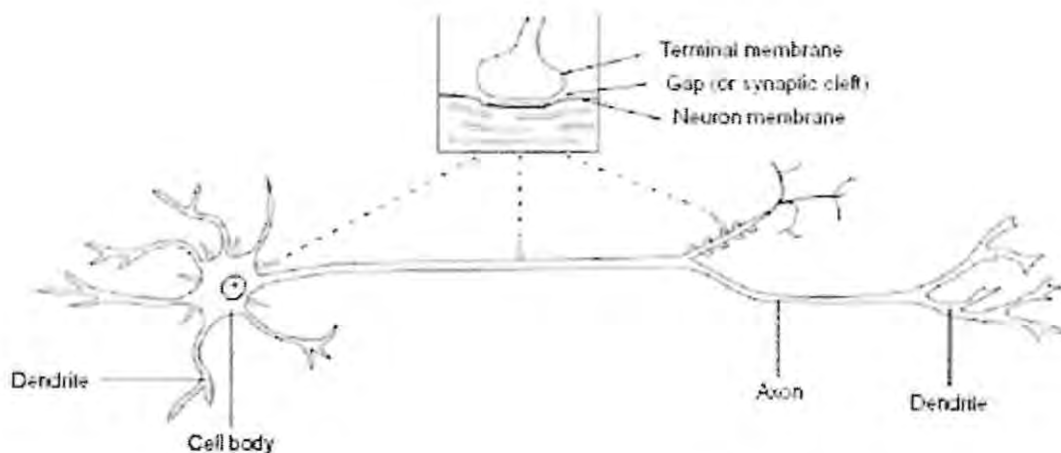


Fig. 1.1. Represents the basic building block of the central nervous system (Souteyrand, J-P., 2002 - http://www.sdu.dk/sdub/hum/paedagogik/910202).

Different parts of the brain are responsible for different information processing tasks. The brain is located at the top of the spinal cord and is divided into the lower structures. These are responsible for the co-ordination of the basic bodily functions (*e.g.*, breathing, digestion, and voluntary movement), expressing basic drives (*e.g.*, hunger, sexual arousal) and processing primary emotions (*e.g.*, fear). The higher structures which evolved later and on top of the lower ones, and are more developed in humans compared to any other animal. The brain is divided into two hemispheres, left and right and joined by a neural band of fibers called the corpus callosum which acts as bridge, enabling the exchange of information between the two hemispheres. There is further division of lobes as shown in fig 1.2. below, which specializes in co-ordination of different tasks. The frontal lobes co-ordinate planning and action, and the temporal lobes are responsible for audition, memory, and object recognition. The parietal lobes co-ordinate sensation and

spatial processing, while the occipital lobes deal with vision. These are general characterizations; as each lobe is further subdivided into groups of neuronal pathways specialized for very specific processing of information. Complex skills, depends on co-ordination of several of these specialized neural networks in different areas of the brain. Damage to any of the neuronal networks or to the connections will disrupt the function it co-ordinates, and to each possible irregularity corresponds a specific discrepancy in brain function. No two brains are identical as there are individual differences found in size, and in the number of neurons designed to carry out certain functions, or even in the organization and localization of functional modules. Due to the inter-changeable functional nature of most neurons, the same neuron can be assigned to one task, and reassigned to another task at a later stage, which means that nature, and learning makes every brain unique, and a work in progress throughout the human lifetime. (http://www.sdu.dk/sdub/hum/paedagogik/910202).

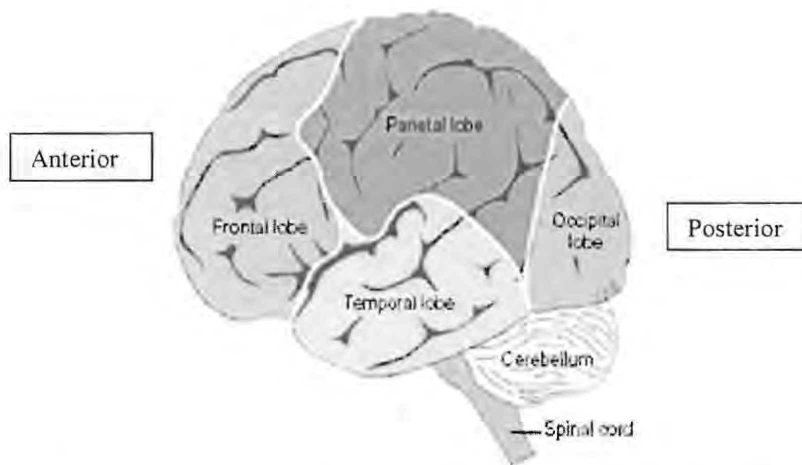


*Fig.1.2 Represents the basic structure of the human brain with its major divisions of the cerebral cortex (*Souteyrand, *J.*, 2002 -http://www.sdu.dk/sdub/hum/paedagogik/910202)

1.2 Neurodegenerative disorder – Alzheimer's disease- mechanism of pathogenesis

With the industrialized world's population growing increasingly older, it does so with the increases in both life expectancy and age-related neurodegenerative disorders such as Alzheimer's and Parkinson's Diseases (AD, PD respectively). In America, approximately 15% of the population over age 65 years are afflicted with AD (Wolozin, 1996), and 1% by PD (Hauser and Zesiewicz, 1996) both aging and age-associated neurodegenerative diseases are associated with various degrees of behavioral impairments that significantly decrease the quality of life. In the aging process, there is an intracellular generation of free radicals which result in the progressive accumulation of defective molecules in the cells, causing an alteration in the physiological function as well as the accumulation of lipofuscins of the cell. There is also a correlation between the age and the specific onset of diseases in which free radicals play an important role, thus supporting the hypothesis that free radicals are closely involved in the aging process (Fantone and Ward, 1985)

The most likely cause in the production of neuronal changes which mediate these behavioral deficits, appear to be free radicals and the generation of oxidative stress. The free radical hypothesis of aging suggests that age-related changes occur as a result of an inability to cope with oxidative stress which occurs throughout the human lifetime. It has been utilized to explain the increased incidence of heart disease and cancer, yet the brain is more vulnerable to the oxidative stress, as it exhibits high oxygen utilization rates and a decrease in free radical scavenging ability (Cadet and Brannok 1998; Ames *et al.*, 1993, Benzi and Moretti, 1995; Fariello, 1990; Halder and Bhaduri, 1998; Harman, 1994; Wolozin *et al.*, 1996).

Normal and pathological aging (Harman, 1994), AD (Benzi and Moretti, 1995; Smith *et al.*, 1991), and PD (Poirier and Thiffault, 1993) have been associated with an increased sensitivity to reactive oxygen species, perhaps the result of pro-oxidant mediators (e.g. iron) and the decrease in antioxidants (Artur *et al.*, 1992; Dexter *et al.*, 1992; Sofic *et al.*,

1988). The damage may include lipid and protein peroxidation (Dubey *et al.*, 1995; Wagner *et al.*, 1996) increases in DNA oxidation products (Shigenaga and Ames 1991; Ames *et al.*, 1993) and deficits in calcium regulatory mechanisms that may eventually lead to cell death (Orrenius *et al.*, 1991; Joseph *et al.*, 1997). A large amount of evidence has accumulated over the years which implicate oxidative stress as being closely involved in the deficits observed in aging and age-related neurodegenerative diseases, and there have been a great number of studies which have examined the positive effects of antioxidants in minimizing, reversing, or forestalling these neuronal or behavioral decrements, with varying degrees of success (Launer and Kalmijn ,1998; Lynch, 1998; Butterfield *et al.*, 1998; Pitchumoni and Doraiswamy, 1998; Reiter *et al.*, 1998).

Alzheimer's disease is a neurodegenerative dementia affecting mainly the cortical brain areas. Characterized by a slow progressive decline of cognitive functions, it is accompanied by a deterioration of behavior and social adaptation. AD exists in two forms i.e. a genetically determined form, known as the familial form (with an autosomal dominant character), and a sporadic form. Postmortem studies of brains from AD patients show cortical atrophy with a loss from 8 to 10% of the brain weight every 10 years of disease progression and histopathologic lesions of two types: senile plaques and neurofibrillary tangles (Hauw *et al.*, 1996). The senile plaques are extracellular deposits mainly localized in the cortex, containing degenerated neuritis and a 39–43 amino acid peptide called β-amyloid (Aβ) which has a natural tendency to form insoluble fibrils and the neurofibrillary tangles are intraneuronal filamentous deposits which contain hyperphosphorylated tau proteins, accumulated mostly in pairs of helical filaments and associated are with ubiquitin (Vickers *et al.*, 2000).

Lesions accompanying the deterioration of the neuronal system, and their distribution are correlated with multiple deficits of the neurotransmitters implicated in cholinergic, monoaminergic, and peptidergic systems (Moller, 1999; Sirvio, 1999). There is a marked decline in neurotransmitters, especially those of the cholinergic pathways (Brodarty, 1999). Changes in cholinergic function are implicated in the pathogenesis of learning and memory alterations occuring in adult onset dementia disorders (Amenta *et al.*, 2001;

Avery et al., 1997; Muir, 1997; Bowen *et al.*, 1995). A correlation between the loss of cortical cholinergic synapses and cognitive decline has been demonstrated as well as close relationships between this loss and the decrease of high affinity cholinergic receptors (Amenta *et al.*, 2001; Whitehouse *et al.* 1982; Gray *et al.*, 1989; Nordberg, 1992; Bowen *et al.*, 1995; Hellstrom-Lin-dahl *et al.*, 1999).

Since there is no cure for AD, the currently available pharmacological treatments are only symptomatic. One of the approaches to the symptomatic treatment of Alzheimer's disease is cholinergic replacement therapy, one of the methods being the inhibition of the breakdown of the neurotransmitter acetylcholine (ACh), via inhibition of the acetylcholinesterase (AChE) using an acetylcholinesterase inhibitor (AChEI). This increases the half-life of ACh in the synapse and thereby augments the receptor mediated post-synaptic signal (Gauthier, 1997; Giacobini, 1998).

The certainty of a diagnosis of AD, in its sporadic form, can be established at the present time only by a postmortem anatomopathologic examination, while during the life of the patient, only a diagnosis at best probable, can be established after a neuropsychological and behavioral evaluation (Amouyel, 1998; Growdon, 1999; Ritchie and Touchon, 2000), followed by symptomatic treatment.

1.3 Cholinergic transmission system

There is considerable evidence that ACh arrived with the evolutionary scheme long before the design of the nervous system and functional synapses (Taylor and Brown, 1989). Storage, possession and degradative capacities for ACh turnover exist in bacteria, fungi, protozoa and plants. In higher animals, ACh distribution is far wider than the nervous system i.e. it is found in the cornea, certain ciliated epithelia, spleen of ungulates and the human placenta (Rama-Sastry and Sadavongvivad, 1979).

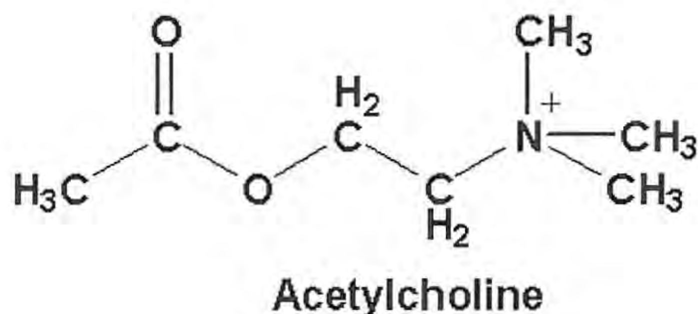


Fig 1.3. Illustrates the of Structure of Acetylcholine
(http://www.awbc.com/mathews/AB/ACETCHOL.GIF)

The molecular structure of acetylcholine, shown above in fig. 1.3, consists of three planes: (1) the plane of the ester oxygen carboxyl group (2) the plane of the one methyl carbon atom, the nitrogen atom and the two CH_2 group carbon atoms and (3) the plane of the ester oxygen-CH_2 bond and one methyl carbon-nitrogen bonds (Canepa *et al.,* 1966)

Acetylcholine plays a role in physiological processes such as memory, learning, attention and motivation. It is synthesized from choline and acetyl-CoA, by choline acetyltransferase (ChAT), as shown below in figure 1.4. in the axonal terminal bulbs of nerve cells.

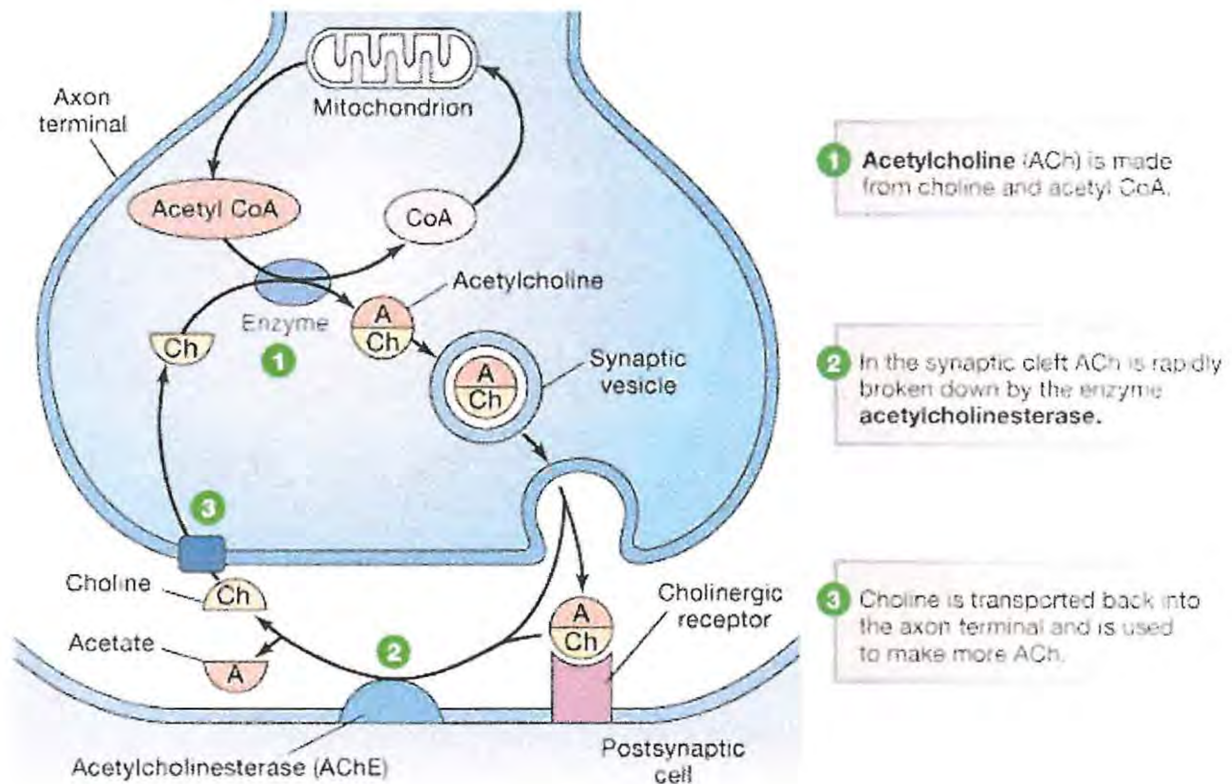


Fig 1.4. Indicates the production, release, degradation and re-uptake of ACh in the axonal terminal bulbs.
(http://mcb.berkeley.edu/courses/mcb136/topic/Tissue_Cells_Membranes/SlideSet3/AP%20review_files/endshow.htm)

Neural pathways that use ACh as their neurotransmitter are referred to as cholinergic i.e. nerve fibres are termed cholinergic when there is a release of ACh when a nerve action potential passes (Bowman and Rand, 1980)

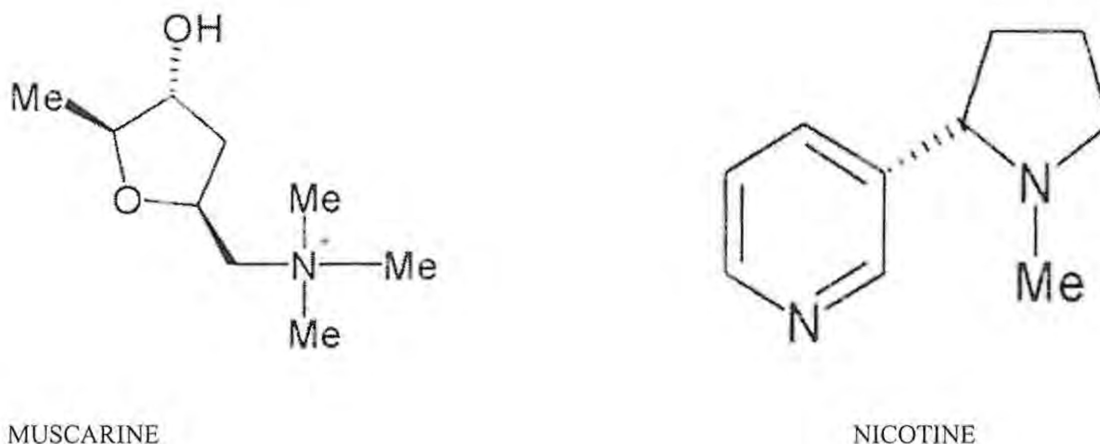
The interaction of acetylcholine with the postsynaptic receptor induces configurational changes in receptor molecules. This leads to an opening of the ionic channels and so to the flow of ions across the postsynaptic terminal. Large quantities of ACh can be detected at the binding sites of peripheral synapses (Lopez *et al.*, 1993). Once ACh has been released and has acted on the postsynaptic membrane receptors, it rapidly dissociates from it. The ACh is then broken down the enzyme, AChE to produce choline and

acetate. Most of the choline is taken up into the presynaptic neuron to be re-utilised in the synthesis of ACh.

Acetylcholine was first identified as a possible mediator of cellular function in 1907 by Hunt, and its action was found to mimic the response of the parasympathetic nerve stimulation (Dale, 1914). Experiments with separate receptors explained the variety of actions of acetylcholine (Dale, 1914).

1.4 Acetylcholine receptors

Sub typing of the cholinergic nervous system was initially based on the pharmacological activity of two alkaloids : nicotine and muscarine as shown below in fig.1.5. The effects of nicotine and muscarine are similar to those of ACh, therefore ACh is said to have two groups of properties (Lopez *et al.*, 1993) and each stimulates different categories of cholinergic function i.e. nicotinic and muscarinic (Bradford, 1986). Cholinergic neurotransmission is primarily nicotinic in the spinal cord, whereas both muscarinic and nicotinic responses are found in cortical and subcortical areas of the brain. (Taylor and Brown, 1989). Depending on the individual cell, muscarinic responses and high affinity mucarinic receptors predominate in the corpus striatum, hippocampus and cerebral cortex, however some areas of the brain do rely on nicotinic responses e.g. the optic tectum. The mapping of cholinergic pathways in the brain continues to be actively studied and relies on several techniques (Adams *et al.*, 1982).



MUSCARINE NICOTINE

Fig1.5. Illustrates the structure of muscarine and nicotine respectively. (http://www.medicine.emory.edu/pulm/labs/roman/images/Muscarine.jpg and http://www.medicine.emory.edu/pulm/labs/roman/images/Nicotine.jpg)

1.5 Acetylcholinesterase

This allosteric enzyme is synthesized in the rough endoplasmic reticulum and transported to the terminals by axoplasmic flow. The function of this enzyme is the termination of action of the neurotransmitter acetylcholine after it has exerted its action on the postsynaptic membrane by the breakdown of ACh into acetate and choline. (Lopez *et al.*, 1993). Hydrolysis of ACh by this enzyme occurs within seconds (Bradford, 1986).

Cholinesterases are widely distributed throughout the body and based on the substrate specificity, can be subdivided into acetylcholinesterases, butyryl and psuedocholinesterases (Massoulie and Bon, 1982).

Cholinesterases are found in serum, erythrocytes, placenta etc. and AChE is a globular protein, which is specific for acetylcholine, exists in several molecular forms and is present in vertebrate nerves, muscle and erythrocytes (Bradford, 1986).

Acetylcholinesterases, in their several molecular forms, differ in their solubility and mode of membrane attachment rather than in catalytic activity. This allosteric enzyme binds its substrate and other ligands in a complex oligomeric form. This enzyme is a complex molecule with two to four or more different subunits. The smallest subunit has a molecular weight of 62000 to 64000 daltons, thus the complete complex has a very high molecular weight (Lopez *et al.*, 1993; Bradford, 1986). Depending on the form of solubility, AChE can be isolated in various molecular forms of different molecular weights (Lopez *et al.*, 1993). Two main forms of molecular AChE exist : a homologous assembly of catalytic subunits; or a heterologous assembly of subunits (Taylor and Brown, 1989).

The homologous assembly form:

Solubilization of AChE with high ionic strength buffers produces several of the homologous globular forms that are monomers, dimers and tetramers of catalytic subunits (Lopez *et al.*, 1993). The dimers are linked by a single disulfide bridge and two dimers form the tetramer. The tetramers are usually joined to three stranded "tails" by disulfide bonds. This is likely to be the basic unit of the enzyme with the molecular weight of around 330000 daltons (Bradford, 1986). These differ in degrees of hydrophobicity, which is dependent on a post translation addition of glycophospholipid on the C-terminal carboxyl group. The glycophospholipid is responsible for the enzyme attaching to the external surface of the cell membrane. The hydrophobic form can be found in excitable and non-excitable tissue (Taylor and Brown, 1989).

The heterologous assembly form:

This form of catalytic subunits (up to twelve) is linked by disulfide bonds to filamentous collagen containing structural subunits. These are asymmetric due to the tail unit imparting substantial dimensional asymmetry to the molecule. The asymmetry species appear on synapse formation and are localised on synaptic areas. The collegenase tail unit is responsible for this molecular form being associated with the basal lamina of the synapse instead of the plasma membrane. The basal lamina is a mesh of connective tissue present in the gap between the pre and postsynaptic membranes at neuromuscular

junctions (Kandel and Schwartz, 1986). The asymmetric form is particularly abundant in the neuromuscular junction (Taylor and Brown, 1989).

The active sites of the AChE have two substrates 4-5Å apart and are termed the anionic and esteratic sites. The anionic site is thought to consist of a carboxylate ion derived from the side chain of a glutamic or aspartic acid residue that attracts the positive charge of the nitrogen atom in the choline portion of acetylcholine. The esteratic site is more complex. There is a serine hydroxyl present at this site. Acylation occurs on this active-serine site, when it interacts with the carbonyl carbon of the acetate portion of acetylcholine which is rendered nucleophilic by a charge-relay system involving a histadine. The acetyl enzyme that is formed is short-lived, which accounts for the high catalytic activity of the AChE (Taylor and Brown, 1989).

There is no precise biological reason for the existence of all these forms of the same enzyme, yet the different arrangement in synapses, neuromuscular junctions and ganglionic or central cholinergic synapses provide versatility in the control and efficacy of interaction with ACh and its receptors in the synaptic cleft (Bradford, 1986)

Distribution of AChE in the human brain is widespread, yet the general distribution is not well understood (Goodman-Gilman, 1996). Acetylcholinesterase has weak activity in the cerebral cortex, in comparison to the basal ganglia. It has an uneven distribution in the cortex. The AChE activity is strong the motor area, premotor area, auditory area, temporal lobe and hippocampus, and has weak activity in the areas of the visual cortex. In the cerebrum, activity of AChE is predominantly of the specific type (Goodman-Gilman, 1996).

1.6 Acetylcholinesterase inhibitors

The function of AChE is the termination of the action of ACh at the junctions of the various nerve endings. Acetylcholinesterase inhibitors inhibit the action of esterases, leading to the accumulation of ACh at the cholinergic receptor sites, and are potentially

capable of producing effects equivalent to excessive stimulation of cholinergic receptors throughout the central and peripheral nervous system (Taylor, P., 1996).

Inhibition of AChEs occurs by several different mechanisms (Taylor and Brown., 1989). The mechanism of action allows for the classification of AChEIs into the different classes.

Reversible enzyme inhibition due to the combination at the anionic site only thereby preventing the substrate (ACh) from attaching e.g. edrophonium. Other reversible inhibitors, such as gallamine and propodium, bind to a peripheral site on the enzyme.

Transition state analogue inhibitors are effective inhibitors of serine enzymes (Nair *et al.*, 1993), whose potency arises from a reversible covalent interaction with the serine residue of the active site of the enzyme to form a tetrahedral hemiketal adduct which structurally resembles the transition states in the catalytic mechanisms of the enzyme.

This class includes substances such as the trifluoromethylketones e.g. *m-(N,N,N* trimethylammonio)trifluoroacetophenone and the more lipophilic zifrosilone, which is currently being developed as a possible treatment of Alzheimer's disease due to it's usefulness in inhibiting central AChE (Camps and Muñoz-Torrero, 2002).

Pseudo-irreversible enzyme inhibition is a class that includes a group of carbamates which form a carbamoylated complex with the serine residue of the catalytic triad of AChE that is hydrolysed at a slower rate than the acylated form resulting from the interaction with the substrate Ach (Camps and Muñoz-Torrero, 2002). The carbamoylating agents such as neostigmine and physostigmine, form the carbamoyl enzyme by reacting with the serine residue. The carbamoyl enzymes are more stable than the acetyl enzyme; as this deacylation occurs over several minutes.

Irreversible enzyme inhibition is the class which accounts for the reaction between AChE and most organophosphorous agents (alkylphosphates and alkylphosphonates) e.g.

echothiophate. This occur only at the esteratic site. The organophosphates form a stable phosphorylated complex with the serine residue of the active site of AChE, whose dephosphorylation is even slower than the decarbamoylation (Camps and Muñoz-Torrero, 2002). The formation of extremely stable bonds with the serine active site on the enzyme occurs. The time that is required for the hydrolysis of these bonds exceed that for the biosynthesis and turnover of the enzyme. Accordingly, inhibition with the alkylphosphates is often irreversible (Taylor and Brown, 1989).

The consequences of AChE inhibition differ from synapse to synapse. At postganglionic parasympathetic effector sites, AChE inhibition enhances or potentiates the action of the administered or nerve stimulated release of ACh. This is a consequence of receptor stimulation extending over a large area from the point of transmitter release. Similarly ganglionic transmission is enhanced by cholinesterase inhibitors. Although the behavioural and physiologic action of cholinesterase inhibition in the CNS has been well studied, its precise action on individual cholinergic pathways is not well understood; however since atropine and other muscarinic antagonists are effective antidotes of the toxicity of acetylcholinesterases (Wiener and Taylor, 1985), implies that the CNS manifestations are a result of excessive muscarinic stimulation.

By prolonging the residence time of ACh, in the synapse, the AChE inhibition in the neuromuscular junction promotes a persistent depolarisation in the motor-end plate. The decay of the end-plate currents or potentials resulting from a spontaneous release of ACh is prolonged from 1 to 2 milliseconds to 5 to 10 milliseconds. This indicates that the transmitter activates multiple receptors before diffusing from the synapse. Excessive depolarisation of the end-plate and a diminished capacity to initiate co-ordinated action potentials follow. In a fashion similar to depolarising blocking agents, fasiculations and muscle twitching are initially observed with AChE inhibition, followed by flaccid paralysis. Some AChE inhibitors are useful therapeutically e.g. pyridostigmine in myasthenia gravis, whereas others have proven useful as insecticides.

Still others have been manufactured for a more insidious use in chemical warfare (Taylor and Brown, 1989). The main focus of drug development in Alzheimer's disease has been on strategies that enhance central cholinergic function, based on the cholinergic hypothesis which proposes that the decline in cognitive function is linked to the loss of cholinergic neurotransmission in the hippocampus and cortex of the human brain (Perry *et al.*, 1978). The use of acetylcholinesterase inhibitors is the only therapy to have shown consistent positive results in the treatment of Alzheimer's disease (Holden and Kelly, 2002). There are two main types of central cholinesterases: acetylcholinesterase and butylcholinesterase. Acetylcholinesterase is predominant, exists in several forms i.e. AChE can be separated into multiple molecular forms. The globular tetrameric G4 form and the monomeric G1 form are predominant in the human brain. Both are required for neuronal function and the G4 form is diminished in Alzheimer's disease. The enzyme has also been demonstrated in the plaques and neurofibrillary tangles that are pathological markers of the disease (Holden and Kelly, 2002).

1.7 Neurotoxicity and it's biological markers

The history of the Free Radical or Oxidative Stress Theory of Aging dates back to the when it was discovered that Fe(II) catalyzed the oxidation of tartaric acid by hydrogen peroxide (Fenton., 1894). Later on it was proposed that hydroxyl radicals, hydrogen peroxide, and superoxide anions undergo a chain reaction that results in a net conversion of hydrogen peroxide into water (Haber and Willstätter, 1931; Haber and Weiss, 1932). This became known as the Haber–Weiss reaction and is now believed to be at the heart of cascades that generate most reactive oxygen species in the cell.

Around this time, although the toxicity of oxygen was known (Binger *et al.*, 1927), its connection to free radicals was not yet understood. However this has become much clearer in recent times.

The brain is a complex organ responsible for mood, behaviour, memory, learning and thought processes, just to name a few. The brain and nervous system are especially vulnerable to oxidative damage due to the high concentration of polyunsaturated fat

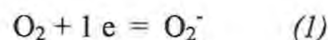
content which is particularly susceptible to lipid peroxidation (Yu *et al.*, 1992), it's high oxygen utilization (Hoyt, *et al.*, 1997) and the gradual accumulation of pro-oxidant iron in brain tissue and spinal cord, which is worsened by the low endogenous antioxidant content (Dawson and Dawson, 1996). The key role players thought to elicit various neurological disorders are oxidative stress and excitotoxicity (Launer and Kalmijn, 1998; Lynch, 1998; Butterfield *et al.*, 1998; Pitchumoni and Doraiswamy, 1998; Reiter *et al.*, 1998)

1.7.1 Oxidative stress and free radicals

Biological systems and living organisms depend on molecular oxygen in order to sustain life. Free radicals are an unstable highly reactive species which have a characteristic unpaired electron in its outermost shell (Schipper, 1998). Free radicals, which have oxygen involved, may be referred to as reactive oxygen species (ROS). This class includes molecules such as the peroxynitrite ($ONOO^-$), nitric oxide (NO), hydrogen peroxide (H_2O_2), the hydroxyl radical (OH^-) and the superoxide anion radical (O_2^-). These oxygen radicals are constantly produced in the body as a natural consequence of aerobic metabolism. (Ames *et al.*, 1993).

1.7.1.1 The Superoxide Radical

The formation of a superoxide anion occurs when the molecular oxygen accepts an additional electron forming a more reactive form of oxygen (Cheeseman and Slater, 1993) as shown below in equation 1.

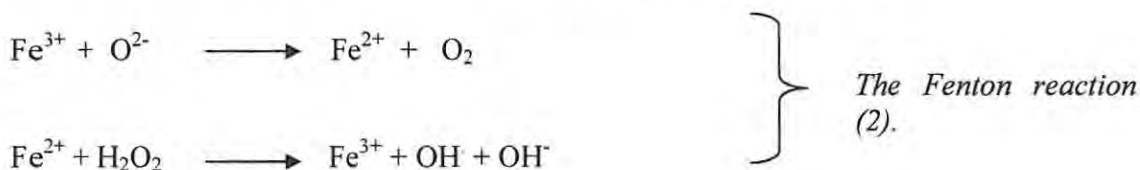


$$O_2 + 1\,e = O_2^- \qquad (1)$$

The superoxide anion appears to be the first oxygen reaction product generated under physiological conditions, generated by the mitochondria via the monoelectric reduction of oxygen of the electron transport chain (Alicia, 1999). The major site of superoxide generation is the mitochondrial electron transport chain, within the respiratory complex I, the NADH-ubiquinone oxidoreductase, and complex III, the ubiquinol-cytochrome *c* oxidoreductase. It is due to the ability of the flavin mononucleotide (FMN) of complex I

and ubiquinone of complex III to exist in a semiquinone anion form, which contains an unpaired electron that can be donated to molecular oxygen to form a superoxide anion. (Bailey *et al.*, 2002). The semiquinone generated within complex I has been identified as the cause of damaging superoxide generation (Robinson, 1998).

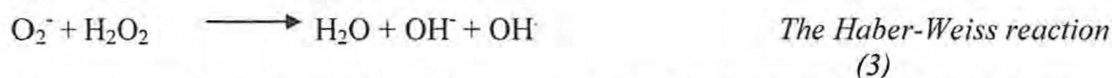
Inactivation of iron-sulphur centre containing proteins result in superoxide toxicity due to the ferrous iron released during the activation, which is an important reactant in the Fenton reaction as shown below in equation (2). (Raha *et al.*, 2000).



$$Fe^{3+} + O^{2-} \longrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

The Fenton reaction (2).



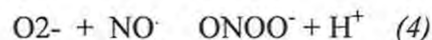
$$O_2^{-} + H_2O_2 \longrightarrow H_2O + OH^{-} + OH^{\cdot}$$

The Haber-Weiss reaction (3)

Generation of H_2O_2 results from the dismutation of the superoxide and the H_2O_2 formed may react with Fe^{2+} to produce the highly toxic hydroxyl radical, $OH^{\cdot}$. This hydroxyl radical may also be formed via the Haber-Weiss reaction, as shown above in equation (3). The $OH^{\cdot}$ Is probably the most reactive of the ROS (Poeggler *et al.*, 1993; Dawson and Dawson, 1996) since it can react with almost all molecules in living cells (Fridovich, 1996). In a pH 7.4 environment, superoxides partially protonate to form a more reactive oxidising species, i.e. the hydroperoxyl radical ($HO_2^{\cdot}$) (Rao and Haydon, 1973).

Nitric oxide (NO) is mainly released by vascular endothelial cells and phagocytes (Moncada *et al.*, 1991) and is formed by nitric oxide synthase (NOS) during the conversion of L-arginine to L-citrulline (Knowles and Moncada, 1994), which then reacts with superoxide anions to form the nitric oxide radical ($NO^{\cdot}$), which goes on to form the peroxynitrite radical. Superoxides also have the potential to react with $NO^{\cdot}$ to produce the peroxynitrite anion $ONOO^{-}$ which can oxidatively damage mitochondrial respiration due to further production of O_2^{-} resulting in a damaging cascade of events (Radi et al., 1994). The interaction of NO with the superoxide radical leads to the formation of the

peroxynitrite radical which is three-fold faster than the rate of superoxide dismutation by superoxide dismutase, as shown below in equation (4) (Beckman *et al.*, 1993).



$$O2- + NO^{\cdot} \quad ONOO^{-} + H^{+} \quad (4)$$

Oxygen free radicals and the oxidative stress induced are contributing factors in a number of chronic conditions in humans including artherosclerosis, vascular diseases, mutagenesis, cancer, neurodegeneration, immunologic disorders and aging (Beyer, 1998).

1.7.1.2 The Hydroxyl Radical

The hydroxyl ($OH^{\cdot}$) radical is probably the most reactive oxygen species, formed via the Fenton reaction or Haber-Weiss reaction as mentioned earlier, and even though are short lived, can induce damage to proteins, carbohydrates, DNA and lipids (Volterra *et al.*,1994; Dawson and Dawson, 1996). The damage to DNA includes strand breakage, and chemical alterations of the deoxyribose and of the purine and pyrimidine bases. The polyunsaturated fatty acids of cell membranes are the major target of free radicals and result in degradation or alteration in membrane structure and function (Viani *et al.*, 1991).

1.7.1.3 The Peroxynitrite Radical

In the CNS, NO-synthase isoforms are present in three states: neuronal (type I), inducible (type II), and endothelial (type III). Neuronal NO-synthase can be found in the cerebellum, hippocampus, striatum, hypothalamus, and medulla oblongata. It is responsible for catalysis in the formation of nitric oxide (NO), the neurotransmitter, which starts from l-arginine, NADPH, and oxygen, in the presence of five cofactors: FMN, FAD, calmodulin, tetrahydrobiopterin, and heme (Gorren and Mayer, 1998). The NO radical reacts almost instantaneously with other radicals and can form a strong cytotoxic oxidant, the peroxynitrite. This occurs by addition to superoxide anions produced by the mitochondrial respiratory chain i.e. the NADPH-oxidase of phagocytes or auto-oxidation of flavoproteins. In the inflammatory hypothesis of AD, peroxynitrite

production has been identified as a factor worsening the neuronal degeneration (Estévez *et al.*, 1998; Torreilles *et al.*, 1999).

1.7.2 Lipid Peroxidation

A major cause in human disease and toxicology, are lipid peroxidation and other free radical reactions (Gutteridge and Halliwell, 1990). Oxygen in cells are required for biochemical reactions however lead to the formation of highly reactive oxygen species capable of producing irreversible and reversible damage to macromolecular targets such as proteins, nucleic acids and cellular membranes (Volterra *et al.*, 1994; Ottino and Duncan., 1997). The oxidative damage to the polyunsaturated lipids is lipid peroxidation. (Halliwell, 1992). Membrane lipids, due to the high level of polyunsaturated fatty acids, are most susceptible. Extensive lipid peroxidation results in the loss of membrane integrity, impairment of ion channel functioning and disruption of cellular ion homeostasis. This finally results in the eventual cell rupture with the subsequent release of cell contents (Mattson, 1997).

1.7.2.1 Reaction Steps

Initiation of lipid peroxidation occurs by the initial attack of a reactive species which abstracts a hydrogen atom from a methylene group i.e. the polyunsaturated fatty acids are attacked by a ROS. This result in the removal of a hydrogen atom and the formation of a lipid derived radical. The propagation step is caused by the addition of molecular oxygen to the derived radical which results in the production of a lipid peroxyl radical. This radical has the potential to attack other lipids and generate lipid radicals and lipid hydroperoxides. This step is self-perpetuating and can undergo a number of cycles. The final step is the termination step, which results in two radicals joining to form a non-radical product. Thus, limiting lipid peroxidation in cells by the removal of ROS is critical. The sequence of events is depicted in fig.1.6 (Gutteridge and Halliwell,1990).

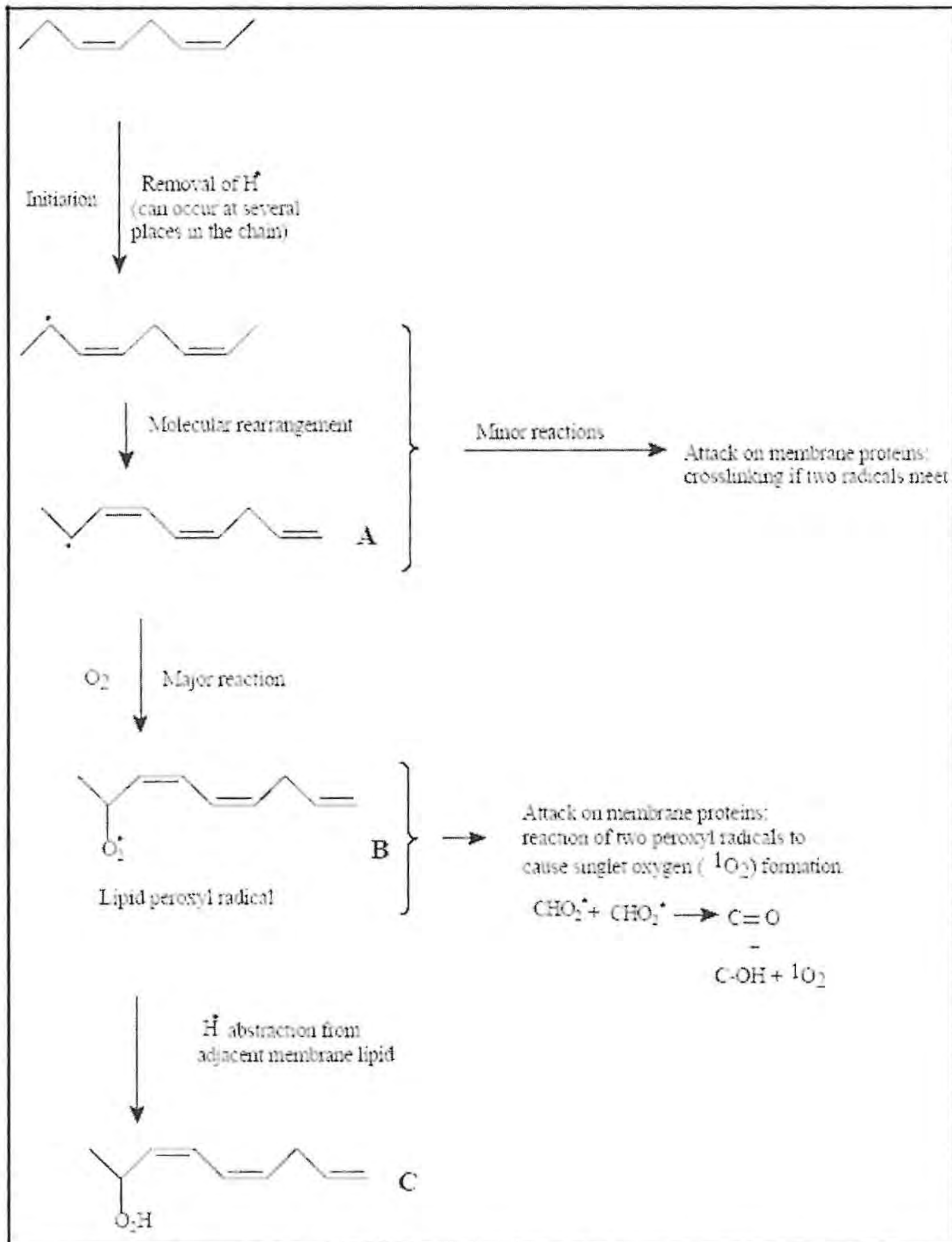


Fig1.6. Mechanism of lipid peroxidation (Gutteridge &Halliwell; 1990).

1.7.2.2 Iron and Lipid Peroxidation

Oxidative stress, along with high levels of iron has been observed in neurodegenerative disorders including AD and PD (Grunewald and Beal, 1999; Campbell, 2001). Due to its participation in the Fenton reaction (Štipek *et al.*, 1996; Viani *et al.*, 1991) and the resultant ROS, iron is detrimental to the well being of a cell (Liochev and Fridovich, 1994). Iron is a good promoter of free radical reactions due to its transitional state. It is also the most abundant transition metal in the brain and the accumulation of iron (Dawson and Dawson, 1996) in the brain adds to the potential toxicity of this transition metal ion. Iron contributes to lipid peroxidation in two ways i.e. it catalyzes the formation ROS as well as the decomposition of hydroperoxides to peroxyl and alkoxyl radicals (Halliwell and Gutteridge, 1990). The large amounts of polyunsaturated fatty acids present in the brain are extremely sensitive to iron-induced oxidative stress and readily undergo lipid peroxidation in the presence of iron (Chiueh, 2001). Iron also potentiates an increase in oxidative events leading to cellular damage which is controlled by a series of iron binding proteins (Campbell, 2001). Levels of lactoferrin, which has been shown to protect against severe inflammation, are increased in the brain of patients with neurodegenerative diseases (Fillebeen *et al.*, 1999). It has also been shown that lipid peroxidation in rat brain homogenate increases significantly with the addition of ferrous ions with ascorbate, and this significant increase in inhibited in the presence of an iron chelator, desferoxamine (Zaleska and Floyd, 1985).

1.7.3 Mitochondria and Oxidative Stress

Mitochondria are the site of energy production in the cell and therefore, the source of oxidative species (Beal *et al.*, 1993; Beal,1995). Thus due to the endogenous production of ROS by the mitochondria oxidative stress is common in most mammalian cells (Bowling and Beal., 1995).

1.7.3.1 Superoxide Anion Generation

Superoxide and hydrogen peroxide production is produced by the continuous transfer of electrons from the respiratory chain of the mitochondria to molecular oxygen. In the

respiratory chain of the mitochondria, complex I and complex III have been suggested as the major source of the ROS (Barja *et al.*, 1999). The cell relies on energy for its functioning and once the energy production becomes dysfunctional and fails the cell, an energy deficit arises. This deficit leads to neuronal depolarisation, activation of excitatory amino acid receptors (i.e. the N-methyl-D-aspartate receptors) and increases intracellular calcium which are buffered by the mitochondria (Beal, 1995). Once this occurs and ROS production increases, damage to the cell is often inevitable (Sheehan *et al.*, 1997a; Sheehan *et. al.*, 1997b).

1.7.3.2 Electron transport chain and Complex I

The electron transport chain accounts for the majority of oxygen consumed within cells and been recognized as a major intracellular source of ROS (Chance *et al.*, 1979). Complex I is an integral membrane protein complex, and is the first electron transport energy conserving enzyme complex of the mitochondrial transport chain, which is responsible for the oxidation of NADH. It transfers the electrons via a flavin mononuleotide cofactor and several iron-sulphur clusters to ubiquinone (Q) (Walker *et al.*, 1992). Electron flow through the mitochondrial respiratory can be blocked by site-specific inhibitors. Several neurodegenerative disorders, including PD and Leber's optic neuropathy, have been associated with the aging process and complex I dysfunction (Schapira *et al.*, 1990; Betarbet *et al.*, 2000; Brown *et al.*, 1992; Jun *et al.*, 1994).

The impairment of NADH oxidation, impairment of proton pump activity and the resultant decrease in adenosine triphosphate (ATP) synthesis, and increased production mitochondrial DNA mutations, lipid peroxidation and protein denaturation arising from excess production of ROS, are some of the problems presented with complex I dysfunction (Boo *et al.*, 1998).

1.7.4 Excitotoxicity

Excitotoxicity is the property exhibited by excitatory amino acids (EAA) and glutamate, the main excitatory neurotransmitter in the mammalian brain, has important roles in several physiological and pathological events. Glutamatergic neurotransmission is achieved through ionotropic (ligand-gated ion channels) and metabotropic (G protein-coupled) receptors (Ozawa *et al.*, 1998). Over-stimulation of the glutamatergic system, as observed when glutamate concentration in the synaptic cleft increases, may be neurotoxic (Izquierdo & Medina, 1997; Ozawa *et al.*, 1998).

Excitatory amino acids cause neurotoxicity by two mechanisms, based on the differences in time course and ionic dependence. The first process is termed the acute process which is characterized by a rapid influx of sodium ions into the neuron, causing a passive entry of chloride ions and water into the cell through osmotic pressure. Due to the excessive depolarization, ion influx and water entry, swelling of the neurons result (Tilson & Mundy, 1995). Histological studies may be used to visualize such changes of the cell.

The second process is one that involves calcium ion (Ca^{2+}) influx, mediated by the NMDA receptor, and is a delayed toxicity. The increased concentration of Ca^{2+}within cells can trigger a number of neurotoxic enzymatic and metabolic processes. The Ca^{2+} activates phospholipases, which can break down cell membranes and liberate arachidonic acid. This can lead to the breakdown of Ca^{2+} stores within the cells and thus further amplify the excitotoxic response. Moreover, arachidonic acid liberation leads to synthesis of prostaglandins, the mechanism of the anti-inflammatory response. Inflammatory cells have shown to produce ROS and reactive nitrogen species (RNS) as a mechanism for attacking opsonised targets (Akiyama *et al.*, 2000).

Also among these processes, it was also discovered that by increasing the expression of neuronal nitric oxide synthase (NOS) in culture, leads to an increased glutamate neurotoxicity. Thus, neuronally originated nitric oxide was discovered to play a critical role in behavioral function but also act as a trigger in the excitotoxic mechanism (Dawson

et al., 1993). As mentioned earlier, excess nitric oxide generation within the neuron can, in the presence of superoxide, lead to the production of the peroxynitrite radical. Production of superoxide radicals can also lead to increased H_2O_2 production, which can react with transition metals such as iron (II) resulting in damage to cellular macromolecules like DNA, which leads to the activation of poly-ADP ribose polymerase (PARS). Due to PARS activation, ATP and NAD are depleted, leading to cell death. Both mitochondrial accumulation of Ca^{2+} and oxidative damage leads to the activation of the permeability transition pore (PTP) that is linked to excitotoxic cell death (Bernardi and Petronelli, 1996). The release of cytochrome C, caspase and apoptosis inducing factor (AIF) by the mitochondria is also linked to increased mitochondrial $Ca^{2+}$and free radical production. These mediators lead to the activation of a caspase cascade, and finally apoptotic death (Du *et al.*, 1997; Kluck *et al.*, 1997; Zamzani *et al.*, 1997; Cassarino and Bennett, 1999)

1.7.5 Melatonin

1.7.5.1 Antioxidant properties

Melatonin (MEL) has been demonstrated to be a powerful antioxidant and free radical scavenger (Tan *et al.*, 1993) preventing oxidative damage to the cell membranes, cytosol organelles and nuclear and mitochondrial DNA. It has shown to possess the ability to scavenge highly reactive reactants including the hydroxyl radical and hydrogen peroxide, nitric acid, peroxynitrite and peroxynitrous acid (Reiter *et al.*, 2002). Melatonin is a scavenger of both $OH^{\cdot}$ and H_2O_2, with the resulting products being cyclic 3-hydroxymelatonin and N1-acetyl-N2-formyl-5-methoxy-kynuramine, respectively (Reiter *et al.*, 2002). These products are also effective scavengers that contribute to the highly efficacious reduction in oxidative damage by melatonin (Reiter *et al.*, 2002).

Melatonin also stimulates the synthesis of antioxidant enzymes including superoxide dismutase, glutathione peroxidase and glutathione reductase (Pablos *et al.*, 1998; Albarran *et al.*, 2001) which suggests that melatonin may act not only directly against

free radicals, but also indirectly as an enzyme stimulator, although the specific induction mechanism is unknown (Albarran *et al.*, 2001).

1.7.5.2 Structure and Synthesis

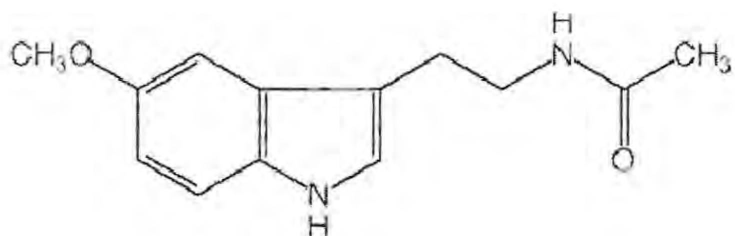


Fig 1.7. Illustratration of the structure of melatonin (http://leda.lycaeum.org/Images/Melatonin_2D_Structure.3519.gif)

Melatonin (5-methoxy-N-acetyl-tryptamine) is an indoleamine synthesized from tryptophan predominantly in the pineal gland and other organs in vertebrates (Reiter *et al.*, 2000). The structure of melatonin is shown above in fig.1.7. The starting point of melatonin synthesis is L-tryptophan which is first converted to 5-hydroxytryptophan by the enzyme tryptophan 5-hydroxylase. The 5-hydroxytryptophan is then converted to 5-hydroxytryptamine (serotonin) with the enzyme aromatic amino acid decarboxylase. N-acetyltransferase then converts the serotonin to N-acetylserotonin which is the rate-limiting product for melatonin. This is then converted to melatonin by the enzyme hydroxyindole- O – methyltransferase (Young and Silman, 1982)

The synthesis of the hormone is under rhythmic control (Reiter, 1987) with peak levels produced at night in darkness.

1.8 Neuroprotective Strategies

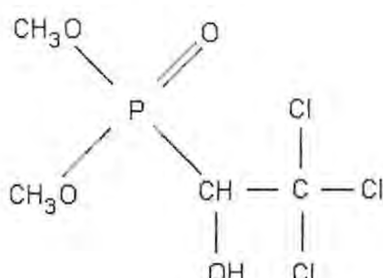
As mentioned before, the electron transport chain in the mitochondria accounts for the majority of the total oxygen metabolized by the cell. Potential sources of oxidative damage to the mitochondrion itself and to other cellular compartments are the by-products produced by the electron transport chain (e.g., superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals). There are also pathways which produce ROS as their primary biological function, e.g., NADPH oxidase in macrophages.

Due to the continuous generation of superoxide anions in the mitochondria, the mitochondria possess an efficient antioxidant system. These complex antioxidant defense systems include both small molecules (tocopherols, vitamin C, glutathione, etc.) (Frei *et al.*, 1989; Burton and Ingold, 1989; Behl *et al.*, 1997; Murphy *et al.*, 1989) and antioxidant enzymes (the superoxide dismutases (SOD), the glutathione peroxidases, catalase, etc.). These are some of the ROS scavenging systems in place in the human body (Halliwell and Gutteridge, 1989; Cheeseman & Slater, 1993). The principal defense systems in place in order to deal with the oxidative stress against oxygen free radicals are SOD, reduced form of glutathione, glutathione peroxidases, glutathione reductase, catalase (a haem enzyme), and antioxidant nutrients. Steroid hormones, especially oestrogen, have been reported to have antioxidant neuroprotective properties (Goodman *et al.*, 1996; Behl *et al.*, 1997). It has been reported to be a more potent inhibitor of iron-catalyzed lipid peroxidation in brain tissue than vitamin E (Behl *et al.*, 1992; Hall *et al.*, 1991). With regard to AD, it has been demonstrated *in vitro* that 17-β-estradiol protects neurons from Aβ-mediated cytotoxicity (Behl *et al.*, 1995) and from oxidative insults attenuating membrane lipid peroxidation and stabilising neuronal calcium homeostasis (Goodman *et al.*, 1996; Keller *et al.*, 1997).

Ultimately it is the balance between the production and the scavenging of ROS which determines the absolute level of oxidative stress.

1.9 Metrifonate – introduction

Shown below in fig.1.8. is the structure of metrifonate also known as trichlorfon.



STRUCTURE OF METRIFONATE

Fig 1.8 Illustration of the structure of Metrifonate (http://www.inchem.org/documents/hsg/hsg/v066hs01.gif)

Recently, more commonly known under the generic name of metrifonate, trichlorfon is an organophosphorus insecticide that has been in use since the early 1950s. In agriculture, it is mainly used against insect pests in field and fruit crops, used to control forest insects and for the control of parasites in domestic animals. It is a broad-spectrum insecticide that is particularly effective against *Diptera* and is also used to control forest insects, in public health, and for the control of endo- and ectoparasites in/on domestic animals and fish.

Under the generic name of metrifonate, trichlorfon is also used as an antihelminthic in humans and is one of the treatments of choice for infestation by *Schistosoma haematobium,* primarily in Africa (Snellen, 1981; Davis, 1986; Aden Abdi *et al.*, 1987; Wilkins & Moore, 1987; Aden Abdi & Gustafsson, 1989; Yakoub, 1990; Aden Abdi,

1990). The usual regimen consists of three doses of 7.5 or 10 mg/kg, given at intervals of 14-21 days. Because of the lower costs in comparison with other treatments, metrifonate is particularly attractive for mass treatments. It has been given to millions of patients with Schistosomiasis with only occasional mild side effects (Nordgren, 1981). In order to obtain better patient compliance, Aden Abdi (1990) recently proposed a regimen of 3 x 5 mg, administered in one day.

The major transformation product of metrifonate in mammals, including human beings, is dichlorvos, the cholinesterase inhibiting activity of which is at least 100 times that of metrifonate (Hofer, 1981). Metrifonate can be said to act in the mammalian body as a "slow release source" of dichlorvos, which may be of essential importance for, among others, its schistosomicidal effect (Nordgren, 1981; Nordgren *et al.*, 1978).

Metrifonate is unique as a cholinesterase inhibitor in that it is a prodrug that is non-enzymatically transformed into its active metabolite 2,2-dimethyl dichlorovinyl phosphate (DDVP) (Nordgren *et al.*, 1978). The clinical effect of metrifonate is mediated exclusively via the formation of DDVP (Hinz *et al.*, 1966,a). Metrifonate is non-enzymatically transformed to DDVP at a very slow rate so that there is only about 1/100 the amount of DDVP. The DDVP concentration is only approximately 1% of the metrifonate concentration at any time. Hence, metrifonate acts as a slow- or controlled-release formulation giving rise to the active DDVP metabolite. Very low concentrations of DDVP steadily released from metrifonate lead to levels that are sufficient to inhibit cholinesterases *in vivo*. By comparison, if DDVP was to be administered alone, high doses would be required because of its very short plasma elimination half-life (Beiber *et al.*, 1997).

Thus, metrifonate can be viewed as a drug delivery system or reservoir that provides steady, titrated administration of DDVP. The controlled release of DDVP in the brain and slow inhibition kinetics of DDVP for cholinesterase may contribute to the low acute cholinergic toxicity of metrifonate. Alzheimer's disease patients receiving metrifonate generally develop only short-lived and mild cholinergic toxicity (Beiber *et al.*, 1997).

Metrifonate has now undergone phase II and phase III trials, and a New Drug Application (NDA) was submitted to the United States Food and Drug Administration (FDA). Previous reviews have addressed aspects of its pharmacology and acute tolerability in early clinical trials (Crimson, 1998; Cummings et al., 1998; Schmidt and Hewing, 1998).

Previously used in the treatment of schistosomiasis, trials have shown beneficial effects on cognitive and non-cognitive (behavioural and psychiatric disturbance) features of Alzheimer's disease (Morris et al., 1997). However, despite it efficacy, reports of muscle weakness have resulted in suspension of clinical development and use of this agent (Knopman, 2003; Lamb and Faulds, 1997)

1.9.1 Chemical formula and physical properties of Metrifonate

Metrifonate is an organic phosphorous compound which was synthesized in 1955 (Lorenz et al., 1955) as a condensation reaction of dimethyl hydrogen phosphite with chloral. It is a racemic mixture of dimethyl 2,2,2-trichloro-1- hydroxyethylphosphonate.
It is a white crystalline hygroscopic substance soluble in water, alcohols and hydrocarbons. Shown below is the structure a summary of its chemical properties (Schneider and Giacobini, 1999).

Chemical formula: $C_4H_8Cl_3O_4P$

Relative molecular mass: 257.44

Common name: trichlorfon

Chemical name: dimethyl 2,2,2-trichloro-1- hydroxyethylphosphonate

Synonyms: chlorofos, DEP, DETF, dipterex, dimethyl 1- hydroxy, 2,2,2-trichloro ethanephosphonate, *O, O*-dimethyl

(2,2,2-trichloro-1-hydroxyethyl) phosphonate, metrifonate, foschlor, trichlorofon, trichlorphon.

1.9.2 Mode of action of Metrifonate

In the *in vitro studies* of metrifonate, it was found that this agent does not significantly inhibit cholinesterases. Rather, the inhibitory activity of metrifonate on cholinesterase is mediated by its degradation product 2,2-dimethyl dichlorovinyl phosphate (Hinz *et al.*, 1966a) small amounts of which are sufficient to inhibit acetylcholinesterase and butylcholinesterase for several weeks, thus being the anticholinesterase with the longest duration of action (Camps and Muñoz-Torrero, 2002). DDVP inhibits the enzyme AChE in a biphasic manner. It first interacts with AChE as a competitive inhibitor, and then, once bound, inhibits this enzyme noncompetitively. The latter inhibition correlates with the progressive phosphorylation of the AChE esteratic site. As AChE binds to the active site, it forms a stable phosphorylated enzyme complex (Hinz *et al.*, 1966a). Phosphorylating agents such as DDVP react covalently with the enzyme to form an inactive phosphoryl enzyme. DDVP forms a dimethoxyphosphoryl enzyme with the phosphate conjugated to the active center serine (Taylor, 1998).

Metrifonate inhibits both AChE and butyrylcholinesterase (BuChE) activities in rodent and human brain cortex (Giovannini *et al.*, 1998; Hallak and Giacobini,1989; Pettigrew *et al.*, 1998). DDVP inhibits AChE and BuChE approximately equally in rat brain but more potently inhibits AChE in erythrocytes compared to BuChE activity in human plasma (Giacobini, 1997).

Mammalian brain AChE can be separated into multiple molecular forms. The globular tetrameric G4 form and the monomeric G1 form are predominant in the human brain. G1 is almost exclusively intracellular, whereas G4 is extracellular and almost entirely membrane-bound. Both G1 and G4 forms are important for neuronal function and ACh regulation. The presynaptic G4 form is directly involved in the regulation of ACh

transmission. AChE and its membrane- associated G4 form are selectively decreased in AD, whereas BuChE and the G1 form are unchanged or only slightly decreased in the brain of AD patients (Ogane *et al.*, 1992 a). In rat brain, administration of metrifonate inhibits both monomeric (G1) and tetrameric (G4) molecular forms of AChE (Ogane *et al.*, 1992 b). The non-selectivity of metrifonate for AChE forms may represent a therapeutic advantage over more selective compounds because, by inhibiting both enzymes forms the drug may act throughout the disease process (Ogane *et al.*, 1992a; Pacheco *et al.*, 1995). Considering the progressive decrease in AChE activity taking place in the brain of AD patients and the unvaried level of BuChE activity, a nonselective AChE inhibitor may offer the advantage of a more prolonged effect compared to a more selective AChE inhibitor. In addition, DDVP inhibits equally both G1 and G4 forms of rat and human brain AChE (Ogane *et al.*, 1992 a,b).

Metrifonate and DDVP do not bind to 61 out of 62 mammalian neurotransmitter receptor subtypes, neuropeptide receptors, or ion channel binding sites investigated (Hinz et al., 1966 b). There is only a slight interaction of DDVP with the nicotine receptor binding site (35 %) was observed(Hinz *et al.*, 1966b). This supports the assumption that the therapeutic effect of metrifonate is mediated primarily through a selective activation of the cholinergic system (Mori *et al.*, 1995a). According to data, metrifonate inhibits AChE by increasing acetylcholine (ACh) levels, and stimulate muscarinic receptors (mainly M1) (Mori *et al.*, 1995 b).

Metrifonate, administered through various routes, begins to inhibit cholinesterase activity in various regions of rat brain within minutes (Hallak and Giacobini, 1987; Hallak and Giacobini,1989; Hinz *et al.*, 1966 c). Concomitantly and in relation to this enzymatic inhibition, total levels of brain ACh measured with microdialysis are significantly elevated for up to 6 h after a single dose of metrifonate (Hallak and Giacobini, 1987; Hallak and Giacobini,1989). Consecutive doses of metrifonate will continue to maintain high ACh levels (Mori *et al.*, 1995 a) without interfering with ACh accumulation, release, or synthesis in brain (Hallak and Giacobini, 1989). Using transcortical microdialysis (without adding a cholinesterase inhibitor in the probe), it was demonstrated that

metrifonate increases cortical ACh levels in a dose-dependent manner, the increase lasts for several hours and can be reproduced after consecutive doses of the drug. In addition to ACh, metrifonate also elevates norepinephrine and dopamine (but not serotonin) levels in rat cortex (Mori *et al.*, 1995a).

The effects of metrifonate and DDVP on brain cholinesterase activity have been confirmed after oral administration in both young and old rats. Older animals exhibited 30 to 50% higher levels of inhibition and higher levels of ACh (Hinz *et al.*, 1966c Scali *et al.*, 1997).

1.9.3 Clinical Applications:

Metrifonate is used as an alternative to praziquantel to treat *Schistosoma haematobium* infections. Its use in Alzheimer's disease appears to be efficacious.

(a) Dosing information

The usual dose to treat *Schistosoma haematobium* is 7.5 to 10 milligrams/kilogram orally every other week for a total of 3 doses. The recommended dose of metrifonate in these infections in children is 7.5 to 10 milligrams/kilogram every other week for 3 doses (Anon, 1982a; Reynolds, 1995).

In a dose finding study, the maximum tolerated dose of metrifonate for the treatment of Alzheimer's disease consisted of a loading dose of 2.5 milligrams/kilogram (mg/kg) for 14 days followed by a once-daily maintenance dose of 1.5 mg/kg. This dosing resulted in a more favourable adverse event profile while still inhibiting acetylcholinesterase at a level of 89% to 91% (Cutler *et al*, 1998).

For Alzheimer's disease, loading doses have varied from 0.5 to 4 mg /kilogram/day given for 5 to 14 days. Maintenance doses have varied from 0.2 to 1 mg/kilogram given daily to

2.9 mg/kilogram given weekly (Morris *et al*, 1998; Becker *et al*, 1998; Pettigrew *et al*, 1998).

1.9.4 Storage and stability

Metrifonate has a shelf-life of approximately 2 years in the tropics. Once the package has been opened, the drug should be used as soon as possible. All metrifonate packages should be inspected for the exact expiration date (Shekhar, 1991).

1.9.5 Pharmacokinetics

(a) Distribution

The drug is well absorbed from the gastrointestinal tract with peak plasma levels occurring 1 hour after administration; protein binding is less than 15%. Dichlorvos is an active metabolite and the amount formed is 1% of metrifonate levels. Times to peak concentrations were: Orally, 0.5 to 2 hours (Schmidt and Heinig, 1998; Pettigrew *et al*, 1998; Edwards and Breckenridge, 1988; Nordgren *et al*, 1980). Time to peak concentration of the active metabolite dichlorvos (DDVP) is 0.7 to 1.2 hours (Schmidt and Heinig, 1998).

(b) Metabolism

The risk of drug interactions is thought to be low with metrifonate since neither the drug nor its active metabolite, dichlorvos, are substrates for cytochrome P450 enzymes (Schmidt and Heinig, 1998; Elwood, 1998).

The predominant metabolic pathway of metrifonate is glucuronidation. Its principal metabolite, dichlorvos, is metabolized via O-demethylation and cleavage of the phosphate bond (Pettigrew *et al.*, 1998). Dichlorvos concentrations were approximately 2% of the parent compound concentrations (Schmidt and Heinig, 1998; Pettigrew *et al*,

1998). Shown in fig.1.9. below, is a schematic representation of the metabolic pathway of metrifonate. Metrifonate (1) rearranges readily to form dichlorvos (DDVP) (2,2-dichlorovinyl dimethyl phosphate) (2) via dehydrochlorination (Lorenz *et al.*, 1955; Metcalf *et al.*, 1959; Nordgren *et al.*, 1978; Hofer, 1981; Nordgren, 1981). This transformation occurs under physiological conditions (Miyamoto, 1959). However, dichlorvos could not be detected very often and only its degradation products, such as demethyl dichlorvos (2,2-dichlorovinyl methyl hydrogen phosphate)(4) were found, as evidence of the formation of dichlorvos *in vivo* (Arthur and Casida, 1957; Bull and Ridgway, 1969; Miyata and Saito, 1973; Otto *et al.*, 1980). Following the administration of metrifonate, dichlorvos was found in erythrocytes and plasma at levels corresponding to 0.2-1% of the metrifonate concentrations (Nordgren, 1981; Aden Abdi, 1990). The main metabolites of ^{32}P-metrifonate found in mammals were demethyl metrifonate (6), demethyl dichlorvos (4), dimethyl hydrogen phosphate (5), methyl hydrogen phosphate (7) and phosphoric acid (8) (Hassan *et al.*, 1965; Bull and Ridgway, 1969; Miyata and Saito, 1973).

Fig. 1.9 Represents the proposed metabolic pathway of metrifonate in vivo
(www.inchem.org/documents/ ehc/ehc/ehc132.htm)

(c) Elimination and Excretion

Metrifonate is rapidly eliminated, primarily via the urine i.e. the major route is renal excretion (Edwards and Breckenridge, 1988). Only 0.6% to 1.9% of a dose is excreted renally as unchanged drug (Dingemanse *et al*, 1999). However, even though the parent compounds half life is 2 to 4.6 hours (Pettigrew *et al*, 1998; Edwards and Breckenridge, 1988) the recovery half-life of red blood cell cholinesterase may be as long as 26 to 54 days (Crismon, 1998).

1.9.6 Cautions and Contraindications

Depression of blood cholinesterase is the most serious adverse effect; other effects include headache, vertigo, muscle weakness, abdominal pain, nausea, vomiting, and diarrhoea. Persons treated with metrifonate should not receive neuromuscular blocking agents or be exposed to insecticides with anticholinesterase effects for at least 48 hours. Contraindicated with concomitant exposure to other organophosphates, in patients with decreased plasma or erythrocyte cholinesterase and in patients with hypersensitivity to metrifonate. Precautionary measures must be taken to avoid neuromuscular blocking agents, insecticides, or other agricultural chemicals with anticholinesterase activity within 48 hours after metrifonate therapy (Mandell *et al*, 1990; Reynolds, 1995)

1.9.7 Adverse effects

(a) Haematological effects

The only significant adverse effect of metrifonate therapy that has been reported is the depression of blood cholinesterase. Decreases observed in plasma cholinesterase are immediate, while there is a gradual decline in erythrocyte cholinesterase levels (Plestina *et al*, 1982). One study reports inhibition of erythrocyte cholinesterase by 50% to 60% of normal levels (Nordgren *et al*, 1980).

(b) Central nervous system effects

Metrifonate can cause headache and vertigo (Reynolds, 1995). Fainting has been reported with metrifonate in the treatment of a *Schistosoma haematobium* infection (Aden-Abdi *et al*, 1987). Seizures occurred in two women, 58 and 66 years of age, who were stabilized on metrifonate 60 mg/day for Alzheimer's disease. Neither had a history of seizures. A contributing factor, based on the temporal relationship, was thought to be the sudden withdrawal of antimuscarinic agents, doxepin in one case and hyoscyamine in the other case. Lowering of the seizure threshold may have resulted (Piecoro *et al*, 1998). Metrifonate has been reported to cause fatigue (Shekhar, 1991).

(c) Gastrointestinal effects

Metrifonate may cause colic, diarrhoea, nausea, vomiting, and abdominal pain (Cummings, 1998; Reynolds, 1995; Shekhar, 1991). Gastrointestinal events have been reported in Alzheimer's disease patients receiving loading doses of metrifonate (Cutler *et al*, 1998).

(d) Respiratory Effects

Metrifonate has been reported to cause bronchospasm (Shekhar, 1991).

(e) Sweating

Metrifonate has been reported to cause sweating (Shekhar, 1991).

(f) Muscular effects

Metrifonate has been reported to cause muscle weakness and tremors (Shekhar, 1991). Muscle cramps, especially in the legs, have been reported in Alzheimer's disease patients receiving loading doses of metrifonate (Cutler *et al*, 1998).

1.9.8 Toxicity

Trichlorfon is a moderately toxic organophosphorus ester insecticide. Over-exposure from handling during manufacture or use and accidental or intentional ingestion may cause serious poisoning. The acute toxicity of metrifonate is due to the inhibition of acetylcholinesterase at the nerve endings by the degradation product dichlorvos, leading to accumulation of endogenous acetylcholine. The effects are manifested by muscarinic and central nervous system signs and symptoms (Taylor, 1980).

In the rat, the toxic effects produced by metrifonate are characteristic of organophosphorus poisoning, i.e. muscular fibrillation, salivation, lacrimation, incontinence, diarrhoea, respiratory distress, prostration, gasping, tonic and clonic convulsion, coma, and death. Metrifonate caused rapid onset of poisoning, effects occurring within 5 min at approximate LD_{50} doses (Edson and Noakes, 1960).

Several cases of acute poisoning from intentional (suicide) or accidental exposure have occurred. Signs and symptoms of intoxication characteristic of AChE inhibition, are exhaustion, weakness, confusion, excessive sweating and salivation, abdominal pains, vomiting, pinpoint pupils, and muscle spasms. In severe cases of poisoning, unconsciousness and convulsions developed and death usually resulted from respiratory failure. In cases where victims survived due to medical intervention, a delayed polyneuropathy (Hayes, 1982), associated with weakness of the lower limbs (Shiraishi *et al.*, 1977; Bátora *et al.*, 1988), sometimes occurred a few weeks after exposure. In fatal cases, autopsy findings showed ischaemic changes in the brain, spinal cord, and vegetative ganglia, damage to the myelin sheath in the spinal cord and brain peduncles, and structural changes in the axons of peripheral nerves (Akimov and Kolesnichenko, 1985)

Summarized reports of neurotoxicity studies on trichlorfon in hens as follows: The acute maximum tolerated cholinergic dose (200 mg/kg subcutaneously) produced no marked neuropathy in hens; however, moderate neuropathy was observed when a further dose of

100 mg/kg was given subcutaneously, 3 days later. Histological signs of severe degeneration were seen in the sciatic nerve and spinal cord of the ataxic bird at autopsy after 3 weeks. Slight changes were reported in sections of the brain stem of the birds given 200 mg/kg, orally, or 100 mg/kg, subcutaneously (Olajos *et al.*, 1979). However, this did not appear to be entirely typical of organophosphorus neuropathy and no lesions were reported for the spinal cord or sciatic nerve. Inhibition of NTE in the spinal cord was only measured in later studies (Hierons and Johnson, 1978) and lagged markedly behind that in the brain.

Rats, which are much less sensitive to agents that cause delayed neurotoxicity, exhibited electrophysiological signs of neurotoxicity without accompanying histological changes when given oral doses of 30 mg/kg for 3 weeks (Lehotzky, 1982) or intraperitoneal injections of trichlorfon at 200 mg/kg per day for 5-15 days (Averbook and Anderson, 1983). The major transformation product of trichlorfon in mammals, including human beings, is dichlorvos, which is at least 100 times more active as a cholinesterase inhibitor than trichlorfon (Hofer, 1981). Dichlorvos is moderately to highly toxic when administered in single doses to a variety of animals species by several routes. It directly inhibits acetylcholinesterase (AChE) activity in the nervous system and in other tissues (WHO, 1989). The slow conversion of trichlorfon to dichlorvos results in the inhibition of both central and peripheral cholinergic nerve acetylcholinesterase, with the accumulation of the neurotransmitter, acetylcholine, at nerve endings. and the generation of characteristic signs and symptoms of toxicity. The muscarinic, nicotinic, and the central nervous system-induced signs and symptoms observed in humans have been described extensively (Ecobichon *et al.*, 1977; Hayes, 1982; Hayes and Laws, 1991).

In other *in vivo* studies on mice receiving a diet containing 100 mg trichlorfon/kg, the cholinesterase activities in the brain, erythrocytes, and plasma were, respectively, 72.1, 115.7, and 92.7% respectively of control values after one-day (24 h) and 79.9, 83.8, and 81.0%, respectively, after 20 days (Tsumuki *et al.*, 1970).

In rats given trichlorfon intraperitoneally at 150 mg/kg, considerable increases in the activities of superoxide dismutase (× 1.94) and microsomal cytochrome P-450 (× 2.09), and lipid peroxidation (× 1.44) in the liver were observed, 2.5 hours after treatment (Matkovics *et al.*, 1980), thus indicative of oxidative stress.

Metrifonate (MET), also known as trichlorfon, or chlorofos, is one of the organophosphorus compounds for which not only acutely toxic effects have been described, but also delayed neurotoxicity in humans (WHO, 1986).

1. 10 Organophosphate Induced Delayed Neurotoxicity

The use and easy availability of organophosphate compounds has lead to a noted increase in poisoning and mortality with these agents, in developing countries (Singh *et al.*, 1997; Siwatch and Gupta, 1995). Apart from the distinct symptoms described earlier of cholinergic over-stimulation due to the inhibition of AChEs, many organophosphate compounds have been shown to induce a delayed neurotoxic effect.

This delayed neurotoxic effect is characterized by the appearance of muscular in-coordination, prolonged motor ataxia and eventual paralysis two to three weeks after a single exposure to the compound (Abou-Donia, 1981). The histopathological lesions associated with the delayed neurotoxic effect, include axonal degeneration with secondary breakdown of myelin, muscle denervation and muscle degeneration (Jortner and Ehrich, 1987).

An initial biochemical event associated with the development of organophosphate induced delayed neurotoxicity (OPIDN), is the inhibition of the neuronal neuropathy target esterase (NTE) (Johnson *et al.*, 1985) which is a sensitive marker, used in the assessment of the potential of an organophosphate to induce OPIDN. However, the exact order of events between pesticide exposure and clinical manifestation has yet to be precisely defined (Johnson, 1987; Randall *et al.*, 1997).

The hallmark of OPIDN, the accumulation of cytoskeletal proteins, including microtubules and neurofilaments might impair axonal transport and thus result in axonal degeneration. Calcium and calmodulin (a calcium regulating protein) have been found to regulate microtubule assembly *in vitro* (Weisenberg, 1972) and is also involved in calcium dependent regulation of microtubule dynamics (Deery *et al.*, 1984). Apart from calcium and calmodulin, microtubule assembly has also been known to be regulated by other endogenous protein kinases e.g. calcium or calmodulin kinase and cyclic adenosine monophosphate (cAMP). It has been suggested that calcium and calmodulin regulated phosphorylation may play an important role in the pathogenesis of OPIDN.

Therefore the aberrant phosphorylation of the microtubule proteins by these kinases following organophosphate exposure may alter the physicochemical properties hence destabilizing the microtubule assembly. The hyperphosphorylation of microtubule proteins like MAP-2, tubulin and tau-factor has been proposed to be the underlying biochemical mechanism for the impairment fast axonal transport resulting in axonal degeneration which may be observed in neurodegenerative disorders as well as OPIDN (Choudhary *et al.*, 2001). This may result in the accumulation and subsequent axonal degeneration seen in OPIDN (Patton *et al.*, 1985).

Dichlorvos, the active metabolite of metrifonate, is a commonly used pesticide in India, yet it's delayed neurotoxic potential is controversial since the first case of human exposure and delayed neurotoxcity was reported (Wadia *et a*l., 1985; Lotti *et al.*, 1998) whereas the same was denied by an earlier study (Johnson, 1978). In a report (Sarin and Gill, 2000) diclorvos was noted to be capable of inducing delayed neurotoxicity in rats. Another investigation into the potential neurotoxicity of dichlorvos, indicated that at a dose of 200mg/kg/day produces delayed neurotoxicity in terms of early brain NTE inhibition.

It also showed enhanced phosphorylation by microtubule associated protein kinases with concomitant increases in calmodulin and cAMP, confirming the involvement of these protein kinases in the etiopathology of OPIDN.

The study suggests that dichlorvos-induced hyperphosporylation and resultant disruption of microtubules is the underlying mechanism of dichlorvos induced-delayed neurotoxicity in rats i.e. OPIDN (Choudhary *et al.*, 2001).

CHAPTER TWO – SUPEROXIDE RADICAL GENERATION STUDIES

2.1 INTRODUCTION

Oxidative stress has been implicated in playing a central role in ageing and its associated neurodegenerative diseases (Markesbery, 1997; Mattson *et al.*, 1997; Zorov, 1996; Beal, 1995; Olanow, 1993 ; Le Bel and Bondy, 1992).

Evidence supports the involvement of ROS and iron-induced oxidative stress in Alzheimer's disease and Parkinson's disease (Cormier *et al.,* 2001; Olanow and Arendash, 1994). There have been a number of reports which indicate that ROS may play an important role in the processes leading to neuronal cell damage successive to hypoxia or reoxygenation (Traystman *et al.*, 1991; Kirsch *et al.,* 1991; Halliwell 1992). Superoxide ($O_2^{\cdot-}$) is considered the principal ROS generated in the cell and is precursor to hydrogen peroxide, hydroxyl radical, and lipid peroxyl radicals (Gutteridge and Halliwell, 1989). It also reacts at a diffusion-limited rate with NO to form peroxynitrite ($ONOO^-$) which, upon protonation or reaction with CO_2; decomposes to radical species (Wink *et al.,* 2001; McPherson et al 2002). Strong oxidants are produced from superoxide anions as shown in fig. 2.1.

Oxygen radicals may serve as effectors of cell death, resulting in oxidative damage of DNA, lipids, and proteins; and also as signaling molecules susceptible to participation in the apoptotic process (Bredesen, 1995; Suzuki, *et al.*, 1997).

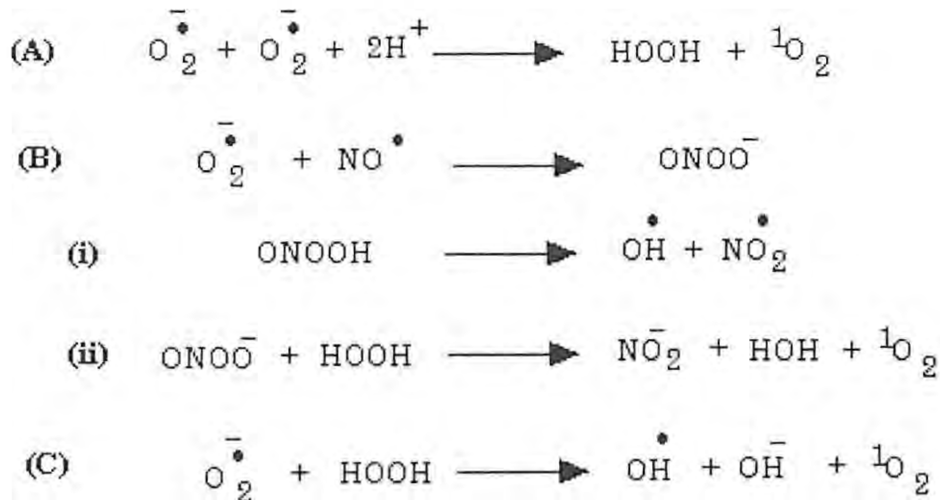


Fig. 2.1. Represents resultant strong oxidants which are produced from superoxide anions. (Juurlink and Patterson 1998; Halliwell and Gutteridge, 1989) (www.mcmaster.ca/.../ juurlink0216/two.html)

The central nervous system is the principal organ affected by cyanide toxicity (Way, 1984) and the dopaminergic system exhibits a selective susceptibility to cyanide (Cassel and Persson, 1992; Matsumoto *et al.*, 1993; Kanthasamy *et al.*, 1994). Thus the neuropathological similarities between cyanide induced dystonia and Parkinson's disease has led to use of cyanide as a model neurotoxin (Mills *et al*, 1999).

It is reported that cyanide toxicity results primarily from inhibition of cytochrome oxidase, the terminal enzyme of the electron transport system (Way, 1984).
Johnson *et al.* (1987) proposed that cyanide raises intracellular calcium through activation of voltage- sensitive calcium channels, direct redox modulation and enhancement of *N*-methyl-D-aspartate (NMDA) receptor function (Patel *et al.,* 1992; Sun *et al.,* 1997), and mobilization of intracellular calcium stores (Yang *et al.,* 1997). Due to the disruption of the Ca^{2+} pump, this set off a series of reactions, which result in free radical generation that will affect lipids, proteins and DNA (Southgate and Daya, 1999).

It is also believed that cyanide inhibits a number of antioxidant enzymes and therefore oxidative stress plays an important role in cyanide induced neurotoxicity (Ardelt *et*

al., 1989). Hence in this study, cyanide was used to generate superoxide anions an investigation into its effect and MET on it in rat brain homogenate.

2.2 EFFECT OF KCN AND METRIFONATE *IN VITRO* ON SUPEROXIDE RADICAL GENERATION IN RAT BRAIN HOMOGENATE

2.2.1 INTRODUCTION

Since the brain is the primary target organ for cyanide toxicity (Gunesekar *et al.*, 1996), the effect of cyanide in the absence and presence of MET (*in vivo* and *in vitro* were investigated. The following experiments were conducted to determine the extent of toxicity that cyanide exerts in rat brain homogenate with respect to the generation of superoxide free radicals and to investigate the effect of MET thereon.

2.2.2 MATERIALS AND METHODS

2.2.2.1 Animals

Adult male albino rats of the Wistar strain, weighing between 250 and 300g were purchased from the South African Institute for Medical Research (Johannesburg, South Africa) and used in this study. All animals were housed in a well ventilated animal room under a light: dark cycle of 12:12 (lights on from 06:00 to 18:00) with the temperature in the animal house was maintained at 20-24°C. Food and water were provided *ad libitum*. The experimental protocol was approved by the Rhodes University animal ethics committee.

2.2.2.2 Chemicals and Reagents

All reagents were of the highest quality available. Potassium cyanide (KCN), nitroblue tetrazolium (NBT) and nitroblue diformazan (NBD) were purchased from Sigma Chemical Corporation, St. Louis, U.S.A. Glacial acetic acid was purchased

from Saarchem (PTY) Ltd, Krugersdorp, South Africa. The KCN was dissolved in Milli-Q water for the purposes of the experiment. The NBT reagent (0.1%) was prepared by dissolving the NBT in ethanol before making up the solution to the required volume with Milli-Q water.

2.2.2.3 Brain Removal

Rats were sacrificed by cervical dislocation and rapidly decapitated. The brains were surgically removed and either used immediately or stored at –70 ° C.

2.2.2.4 Preparation of Homogenate

The brains were weighed and rapidly homogenized in 0.02 M phosphate buffer saline (PBS) at pH 7.4, so as to give a final concentration of 10 % w/v. The use of PBS at pH 7.4 is necessary to prevent lysosomal damage to the tissue.

2.2.2.5 Preparation of Standard Curve

Using NBD as a standard, a series of reaction tubes containing known concentrations of NBD in glacial acetic acid were prepared. The absorbance of each solution at 20μM intervals was read at 560 nm and a standard curve was generated (appendix 1).

2.2.2.6 Nitro Blue Tetrazolium Assay

The NBT assay is generally accepted as a simple and reliable method for assaying the superoxide free radical (Das *et al.*, 1990; Sagar *et al.*, 1992; Ottino and Duncan; 1997). The assay involves the reduction of the NBT ion to the insoluble diformazan form, which can be extracted with glacial acetic acid and the absorbance read at 560nm. The extent of superoxide radical produced induces NBT reduction thus resulting in the reduced form of the compound NBD being produced. Homogenate (1ml) containing varying concentrations of toxin or drug was incubated for one hour at 37^0C to which 0.4 ml 0.1% NBT in an oscillating water bath for 1 hour at 37°C. Termination of the assay and extraction of reduced NBT was carried out by

centrifugation of the samples at 2000g and the resuspension of the pellet with 2 ml glacial acetic acid. The absorbance of the glacial acetic acid fraction was measured at 560 nm and converted to μmol diformazan using a standard curve generated from NBD. Results were then presented as μmol diformazan/mg tissue.

2.2.2.7 *In vitro* exposure to KCN

A modification of the assay described by Das *et al.*, 1990, Sagar *et al.*, 1992 and Ottino and Duncan; 1997 was used in the following set of experiments. Homogenate (1ml) containing varying concentrations of KCN (0, 0.25, 0.5, 1mM) were incubated with 0.4 ml 0.1% NBT in an oscillating water bath for 1 hour at 37°C. Termination of the assay and extraction of reduced NBT was carried out by centrifugation of the samples at 2000g and the resuspension of the pellet with 2 ml glacial acetic acid. The absorbance of the glacial acetic acid fraction was measured at 560 nm and converted to μmol diformazan using a standard curve generated from NBD.

2.2.2.8 *In vitro* exposure to Metrifonate only

Homogenate (1ml) containing varying concentrations of MET (0.125mM, 0.25mM, 0.5mM and KCN 1mM) was incubated for one hour to which 0.4 ml 0.1% NBT in an oscillating water bath for 1 hour at 37°C. Termination of the assay and extraction of reduced NBT was carried out by centrifugation of the samples at 2000g and the resuspension of the pellet with 2 ml glacial acetic acid. The absorbance of the glacial acetic acid fraction was measured at 560 nm and converted to μmol diformazan using a standard curve generated from NBD.

2.2.2.9 *In vitro* exposure to Metrifonate and KCN

Homogenate(1ml) containing varying concentrations of MET (0,0.125, 0.25, 0.5, 1mM) and 1mM KCN was incubated in an oscillating water bath for 1 hour at 37°C to which 0.4 ml 0.1% NBT was added and incubated for 1hour. Termination of the assay and extraction of reduced NBT was carried out by centrifugation of the samples at 2000g and the resuspension of the pellet with 2 ml glacial acetic acid. The absorbance of the glacial acetic acid fraction was measured at 560 nm and converted to μmol diformazan using a standard curve generated from NBD.

2.2.3 RESULTS

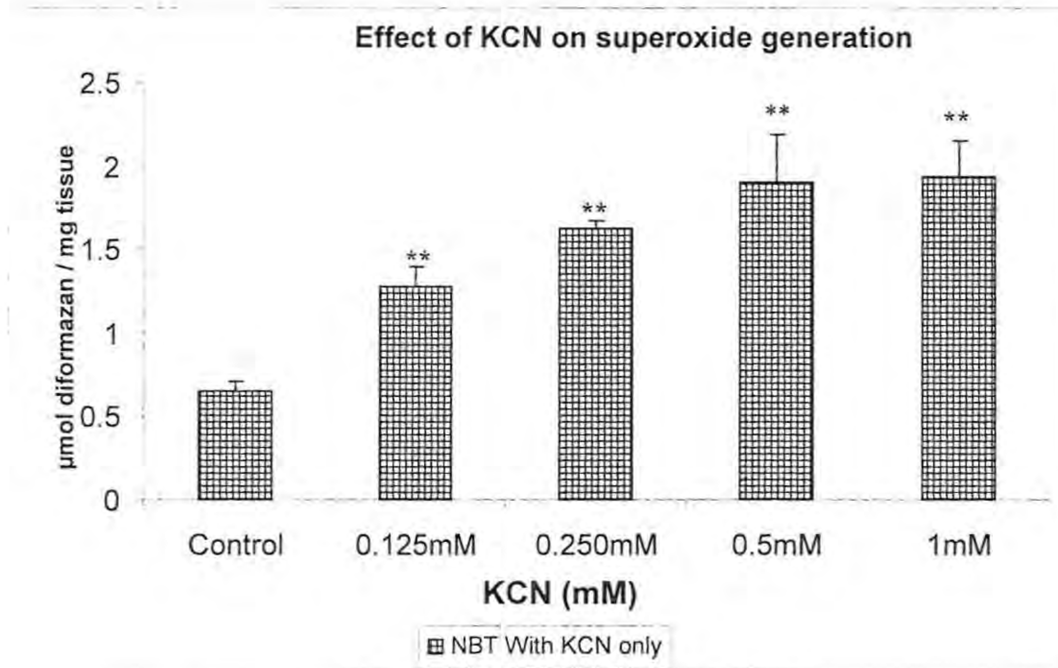


*Fig. 2.2 The effect of increasing the doses of KCN only on diformazan levels formed in rat brain homogenate. Each bar represents the mean ± SEM; n=5. Student-Newman-Keuls Multiple Range Test applied. (** p< 0.01 in comparison to the control).*

Exposure of the whole rat brain homogenate to varying concentrations of KCN significantly increased superoxide anion production in comparison to the control in a concentration dependent manner in comparison to the control. This production of superoxide anions in rat brain homogenate was significantly increased as expected by exposure to KCN (Pillay et al, 2002).

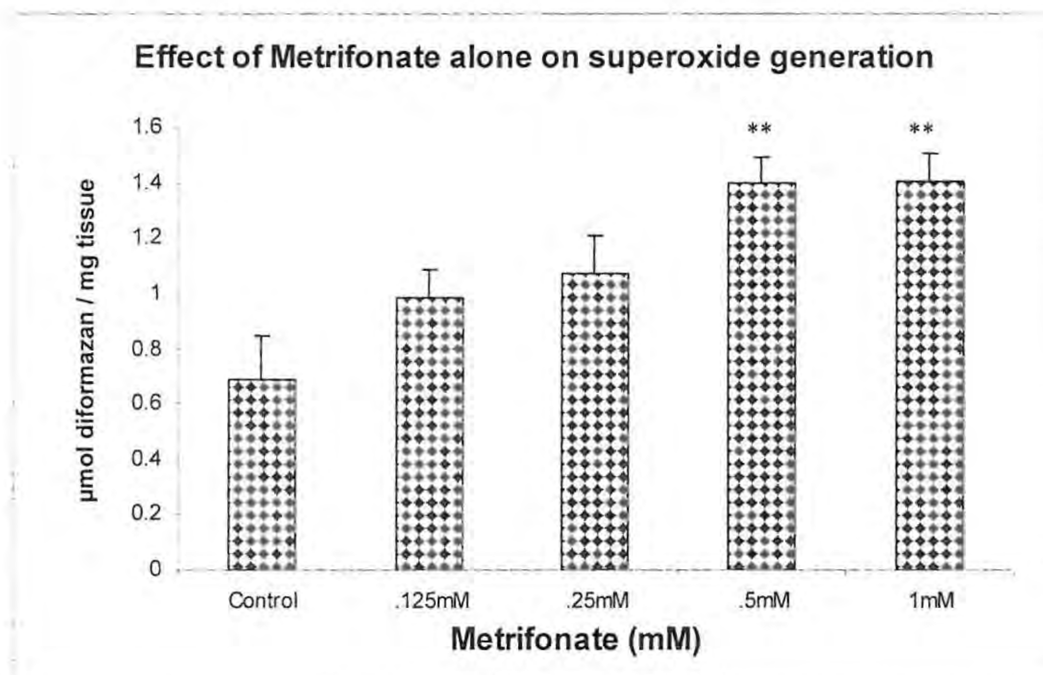


*Fig 2.3 The effect of increasing high doses of MET only on diformazan levels formed in rat brain homogenate with the above concentrations of MET. Each bar represents the mean ± SEM; n=5. Student-Newman-Keuls Multiple Range Test applied. (** $p < 0.01$ in comparison to the control).*

In the presence of MET only, there was a significant increase in the production of superoxide anions at the higher concentrations of 0.5mM and 1mM in comparison to the control. Thus the increase in the levels of diformazan formed implies that MET could play a role in superoxide generation.

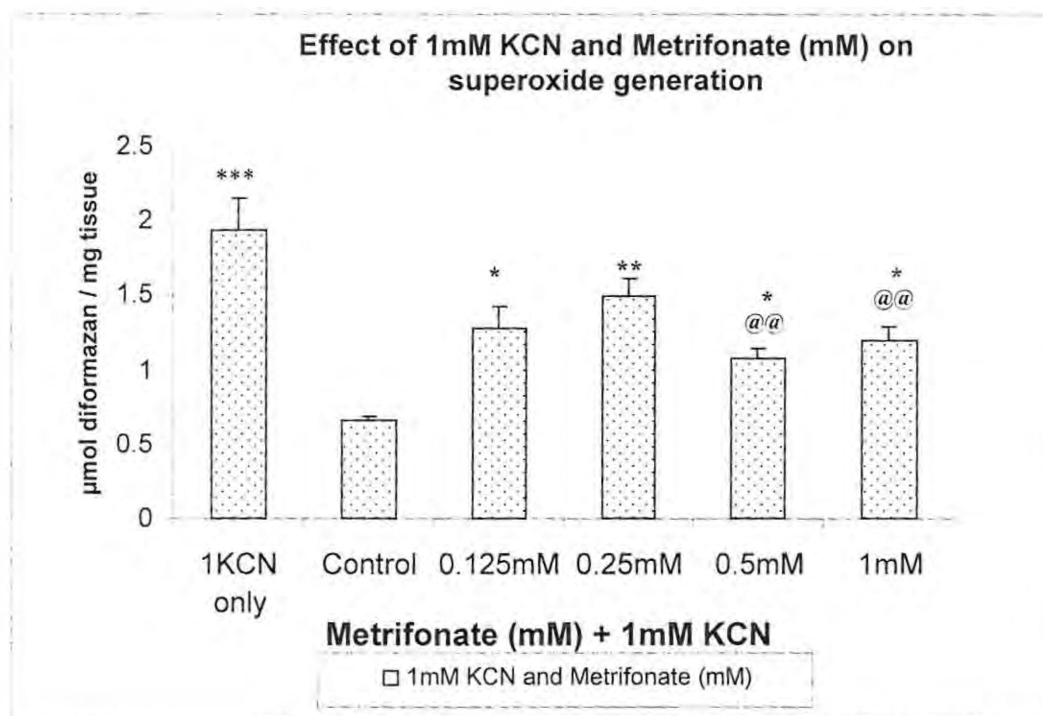


*Fig.2.4 The effect of increasing high doses of MET and 1mM KCN on diformazan levels in rat brain homogenate. Each bar represents the mean $\pm$ SEM; n=5. Student-Newman-Keuls MultipleRange Test applied. (***$p<0.001$, ** $p<0.01$ and *$p<0.05$ in comparison to the control and @@ $p<0.01$ in comparison to the toxin-1mM KCN only).*

In comparison to the control there is a significant increase in the formation of NBD in the 1mM KCN only, as well as in the varying doses of MET and 1mM KCN. However, in comparison to the 1mM KCN only, there is a significant reduction in the formation of NBD in the homogenate treated with 0.5mM and 1mM MET in the presence of 1mM KCN.

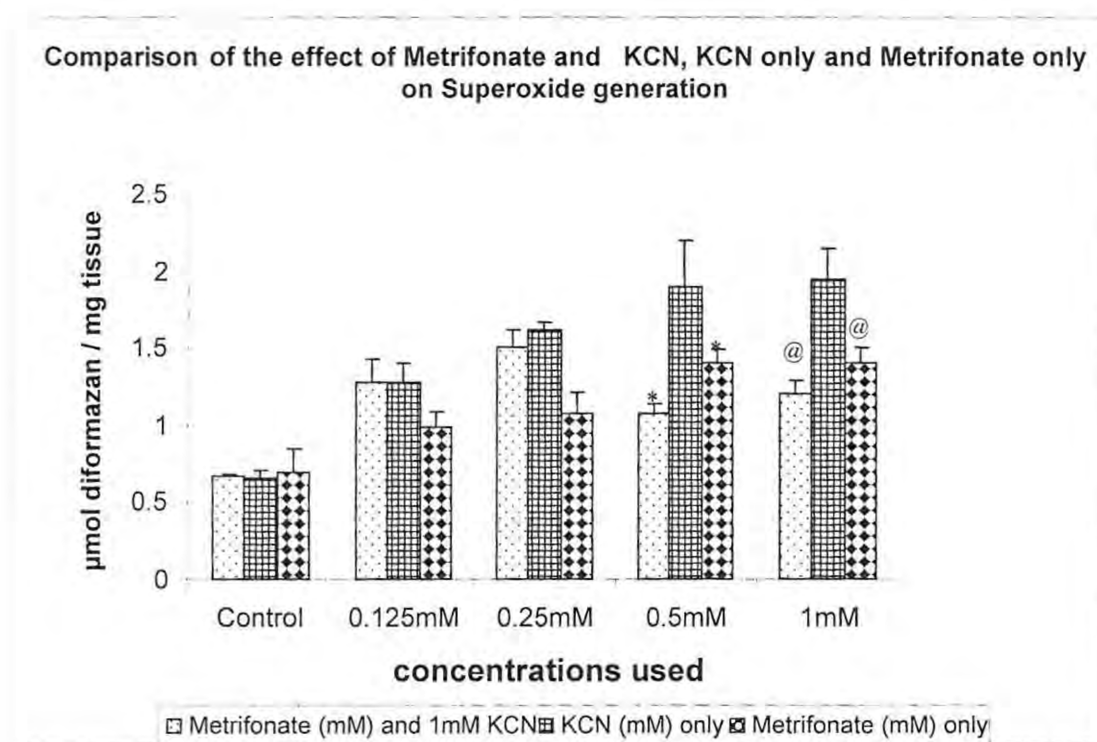


Fig.2.5.The Comparison of the effect of MET alone, KCN alone and KCN and MET in combination on levels of diformazan formed in rat brain homogenate. Each bar represents the mean ± SEM; n=5. Student-Newman-Keuls MultipleRange Test applied ($p < 0.05$ in comparison to the 0.5mM KCN only and @ $p < 0.05$ in comparison to the 1mM KCN only).*

When compared, there were no significant differences between the controls. However, there is a significant difference between the level of superoxide generation between the 0.5mM MET only and the 0.5mM MET with KCN in comparison to the 0.5mM KCN only. Here, the 0.5mM KCN caused the most NBD formation, and the 0.5mM MET with KCN produced the least amount of NBD formation. The same applies for the 1mM MET only and 1mM MET with KCN versus the 1mM KCN only, i.e. there is a significant difference. The 1mM MET with KCN produced the least amount of superoxides. Thus, 1mM KCN produces less superoxide radical formation in the presence of at concentrations of 0.5mM and 1mM MET.

2.2.4 DISCUSSION

KCN disrupts the mitochondrial electron transport chain, which leads to the production of free radicals (Way, 1984). Due to the increase of NBD formation with MET only, it is possible that MET also has this capability. However further investigation into the electron transport chain and Complex I has to be undertaken in order to positively conclude this possibility. In combination, there was a reduction in NBD formation. MET is an acetylcholinesterase inhibitor and therefore leads to an increase in ACh. ACh is a known neuroprotectant against KCN toxicity (Pillay *et al.*, 2000). This could explain the slight reduction in NBD formation in the homogenate which was exposed to both KCN and MET, and not due to a direct neuroprotective effect of MET.

2.3 EFFECT OF METRIFONATE *IN VIVO* ON SUPEROXIDE RADICAL GENERATION IN RAT BRAIN HOMOGENATE

2.3.1 INTRODUCTION

Metrifonate alone *in vitro* was able to produce an increase in NBD formation in rat brain homogenate. However, as seen in the results above, tissue exposed to both MET and KCN lead to a decrease of superoxide generation when compared to the homogenate in the presence of 1mM KCN only, and therefore an *in vivo* investigation was undertaken.

2.3.2 MATERIALS AND METHODS

2.3.2.1 Animals

Adult male Wistar rats weighing between 250and 300 g were used in this experiment, and were housed and maintained under the conditions described in section 2.2.2.1.

2.3.2.2 Chemicals and Reagents

All reagents were of the highest quality available. Potassium cyanide (KCN), and nitroblue tetrazolium (NBT) were purchased from Sigma Chemical Corporation; St. Louis, U.S.A. Glacial acetic acid was purchased from Saarchem (PTY) Ltd, Krugersdorp, South Africa. KCN was dissolved in Milli-Q water for the purposes of the experiment. 0.1% NBT reagent was prepared by dissolving the NBT in ethanol before making up the solution to the required volume with Milli-Q water.

2.3.2.3 Brain Removal

The rats were sacrificed by cervical dislocation and the brains were removed as described in section 2.2.2.3.

2.3.2.4 Preparation of Homogenate

The tissue was homogenized in the same manner described in section 2.2.2.4.

2.3.2.5 Nitro Blue Tetrazolium Assay

The experimental procedure used in the following set of experiments as described earlier in section 2.2.2.6.

2.3.2.6 *In vivo* exposure to Metrifonate only

For the *in vivo* study, animals were divided into four groups (I - IV), each group containing 4 animals:

Group I: Control received 0.02M phosphate buffered saline (PBS), pH 7.40;

Group II: 0.65 mg/kg/day MET;

Group III: 0.85mg/kg/day of MET;

Group IV: 1mg/kg/day of MET.

MET was dissolved in PBS (pH 7.4) administered via intraperitoneal injections daily. The animals were treated for a period of seven days after which they were sacrificed by decapitation on the eighth day. Homogenate (1mL) was prepared as described in section 2.2.2.4. The homogenate was allowed to incubate at 37°C for one hour, after which the NBT assay was performed as described in section 2.2.2.7.

2.3.2.7 *In vivo* exposure to Metrifonate and KCN

For the *in vivo* study, animals were divided into four groups (I - IV), each group containing 4 animals:

Group I: Control received 0.02M phosphate buffered saline (PBS), pH 7.40;

Group II: 0.65 mg/kg/day MET;

Group III: 0.85mg/kg/day of MET;

Group IV: 1mg/kg/day of MET

MET was dissolved in PBS (pH 7.4) administered via intraperitoneal injections daily. The animals were treated for a period of seven days after which they were sacrificed by decapitation on the eighth day. Homogenate (1mL) was prepared as described in section 2.2.2.4.The homogenate was allowed to incubate for one hour with KCN 1mM at 37°C for one hour, after which the NBT assay was performed as described in section 2.2.2.7.

2.3.3 RESULTS

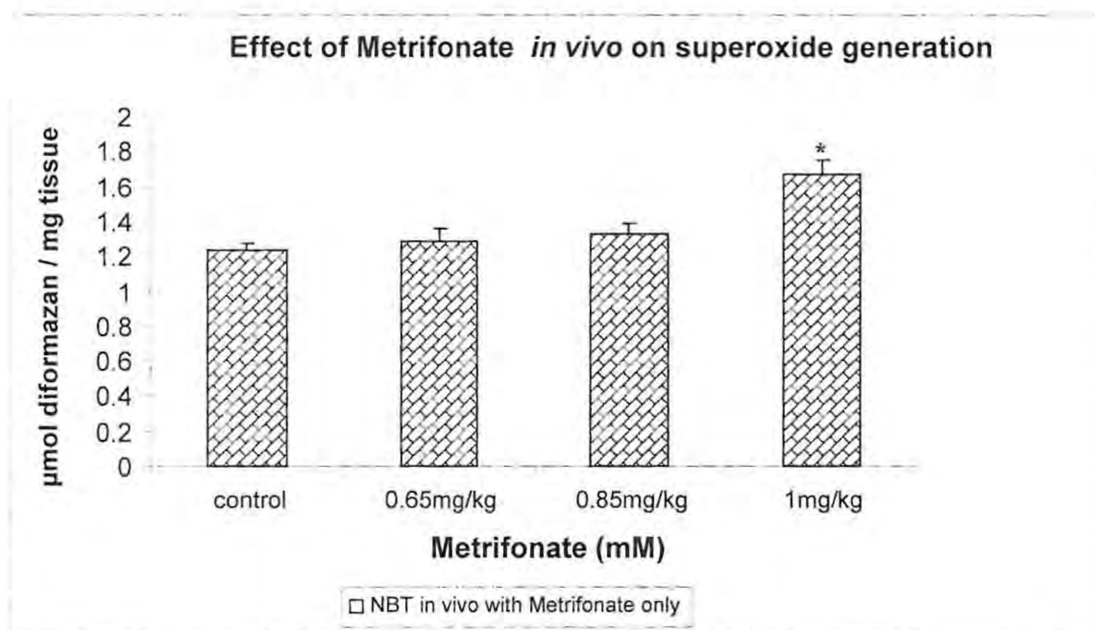


*Fig.2.6 The effect of increasing doses of MET on levels of diformazan formed in rat brain homogenate obtained from rats pretreated with 0.65 and 1 mg/kg/day i.p for 7 consecutive days. Each bar represents the mean ± SEM; n=5. Student-Newman-Keuls MultipleRange Test applied. (* $p < 0.05$ in comparison to the control).*

When NBD levels in vivo were measured with the administration of MET only, the results indicate that only at the highest dose, i.e. 1mg/kg/day, was there a significant increase in NBD formed in comparison to the control group.

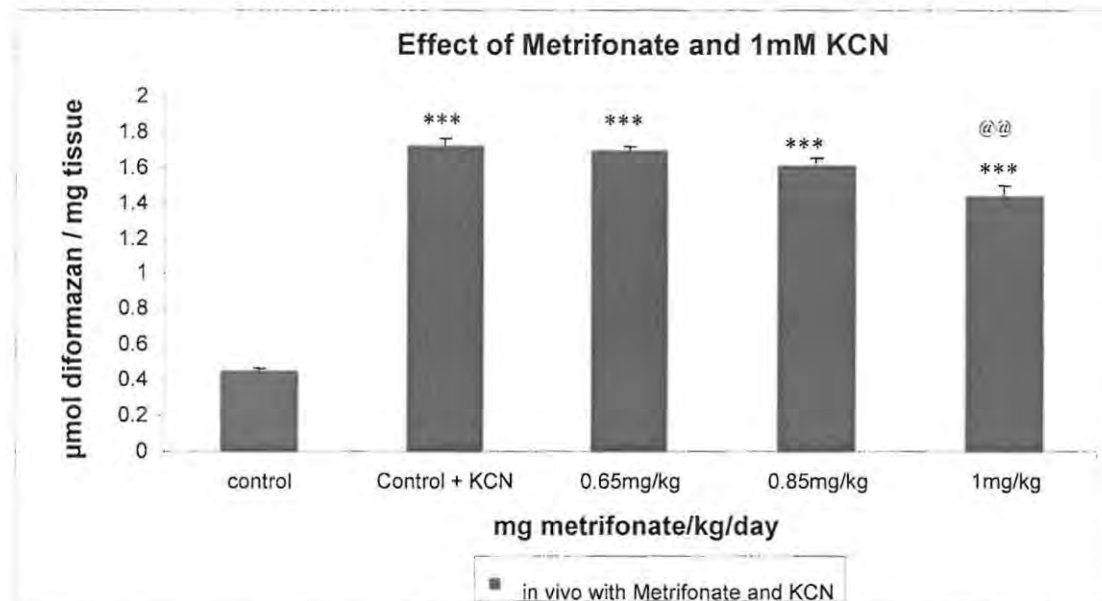


*Fig. 2.7 The effect of increasing doses of MET and 1mM KCN on levels of diformazan formed in rat brain homogenate obtained from rats pretreated with 0.65 and 1 mg/kg/day i.p for 7 consecutive days. Each bar represents the mean + SEM; n=5. Student-Newman-Keuls Multiple Range Test applied. (*** $p < 0.001$ in comparison to the control and @@$p^- < 0.01$ in comparison to the control group with toxin 1mM KCN).*

When 1mM KCN was added to the pre-treated rat brain homogenate, it significantly increased the levels of NBD formed in comparison to the control.

However, the 1mg/kg/day treatment of MET seemed to provide a degree of protection and thus reduction in NBD formed, to the addition of 1mM KCN, when compared to the control and 1mM KCN.

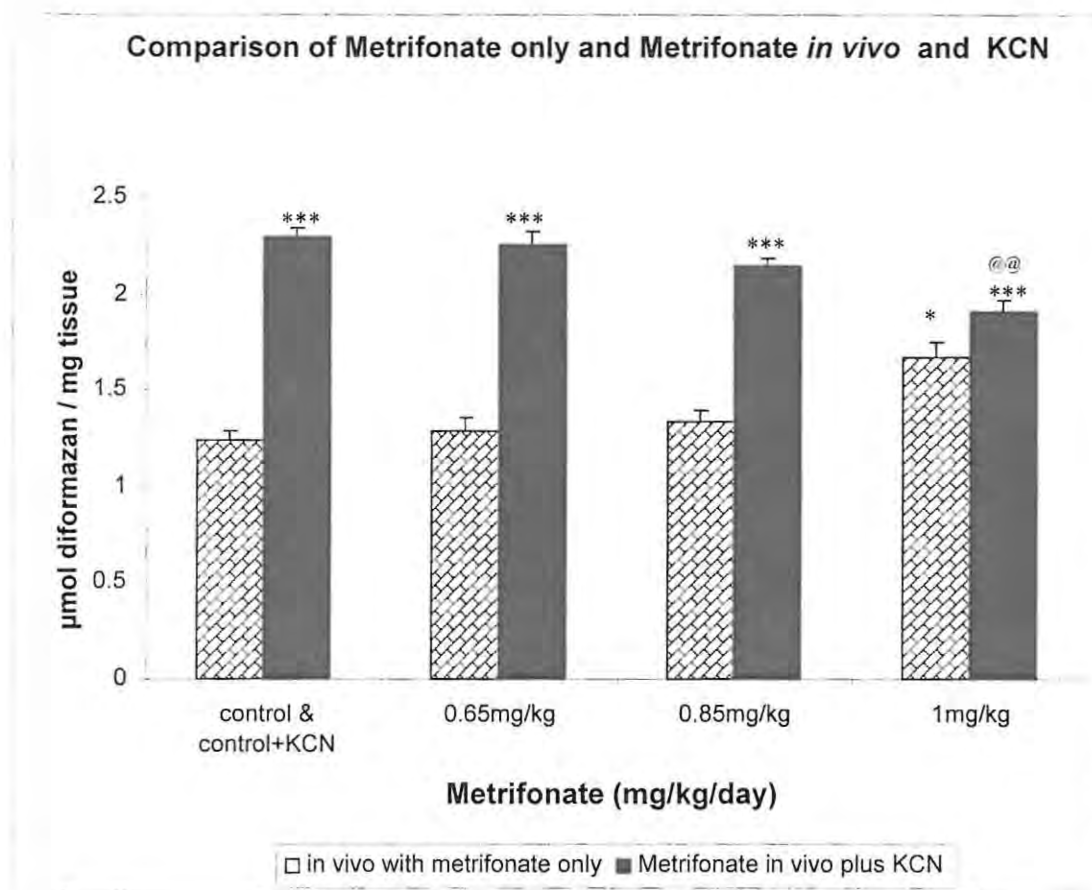


*Fig.2.8 Comparison of the effect of increasing doses of MET only and MET and 1mM KCN on levels of diformazan formed in rat brain homogenate obtained from rats pretreated with 0.65 0.85 and 1 mg/kg/day i.p for 7 consecutive days. Each bar represents the mean + SEM; n=5. Student-Newman-Keuls MultipleRange Test applied. (*** $p<0.001$ and * $p<0.05$ in comparison to control and @@ $p<0.01$, in comparison to control with KCN)*

There was a significant increase of superoxide generation between the control and the control with KCN, the 0.65, 0.85, and 1mg/kg/day MET with KCN and 1mg/kg/day without 1mM KCN.

This indicates that the KCN induced superoxide formation in the control and treated groups, and that the 1mg/kg/day without the KCN were also able to generate superoxides. However, in the 1mg/kg/day dose, there was a significant decrease in superoxide generation when compared to the control with KCN.

2.3.4 DISCUSSION

In the groups receiving MET alone\, *in vivo*, there was a significant increase in superoxide anion formation in the group treated with MET 1mg/kg/day. However, in comparison to the control group with 1mM KCN, the 1mg/kg/day group with KCN was able to reduce the superoxide formation. This slight degree of protection could be due to the increase in ACh because of the lowere anticholinesterase activity. ACh has been shown to be neuroprotective against KCN-induced neurotoxicity (Pillay et al. 2000).

Glutamate and GABA are two major fast neurotransmitters (excitatory and inhibitory, respectively) in the CNS, including the hypothalamus. These neurotransmitters play a key role in the control of excitation/inhibition balance and determine the activity and excitability of neurons in many neuronal circuits (Belousov *et al.*, 2001). A relative increase in glutamate excitation or decrease in GABA inhibition in different regions of the CNS lead to glutamate dependent neuronal hyper-excitability, which, if sustained, causes cell death (Mody *et al.*, 1992; Choi, 1994; Thompson *et al.*, 1996).

Cyanide is known to increase the levels of glutamic acid, an excitatory amino acid and decrease GABA levels. (Persson *et al.*, 1985). Inhibition of AChE with an organophosphate, in the striatum itself can increase extracellular striatal GABA concentration through activation of muscarinic cholinoceptors (Grasshoff *et al.*, 2003).

Thus, it is possible that MET, an organophosphate, stimulated enough GABA at that specific dose to overcome the KCN-induced glutamate excitotoxicity.

CHAPTER THREE – LIPID PEROXIDATION

3.1 INTRODUCTION

Cell membranes are important components of a cell because these are responsible for the integrity and functioning of the cell, as it provides protection from its surrounds and is a compartmentalizing structure. The membranes consist of irregular lipid mixtures, phospholipids, cholesterol and embedded globular proteins (Matthews and Van Holde, 1991). The variety of lipids is to ensure that membrane fluidity, permeability and structure requirements are met (Cullis, *et al.*, 1991).

Events inside and outside the cell seem to induce a fast response in the cell membrane and any change in the cell membrane structure is apparently connected with the activation of enzymes that react with polyunsaturated fatty acids (PUFAs) incorporated into the cell membrane to generate oxidation products (Spiteller, 2003) which is shown in fig. 3.1.

Thus physical or chemical disturbances could leave the cell vulnerable to oxidative damage. Peroxidation of membrane lipids bring about fluidity changes in the membranes, which in turn disrupt vital functions, such as signal transduction or selective permeability to ions. (Rikans and Hornbrook, 1997; Zs.-Nagy, 1978; Von Zglinicki, 1987; Yu *et al.*, 1992; Chen *et al.*, 1994; Sastre *et al.*, 1996). One example of such a disturbance would be the damage caused to membranes by ROS. Reactive oxygen species are products of partial reduction of molecular oxygen and comprehend both free radicals and neutral molecular species. The molecular oxygen is capable of accepting an additional electron to create a superoxide, a more reactive form of oxygen (Cheeseman and Slater, 1993). This free radical, due to the unpaired electron can cause cell damage or death.

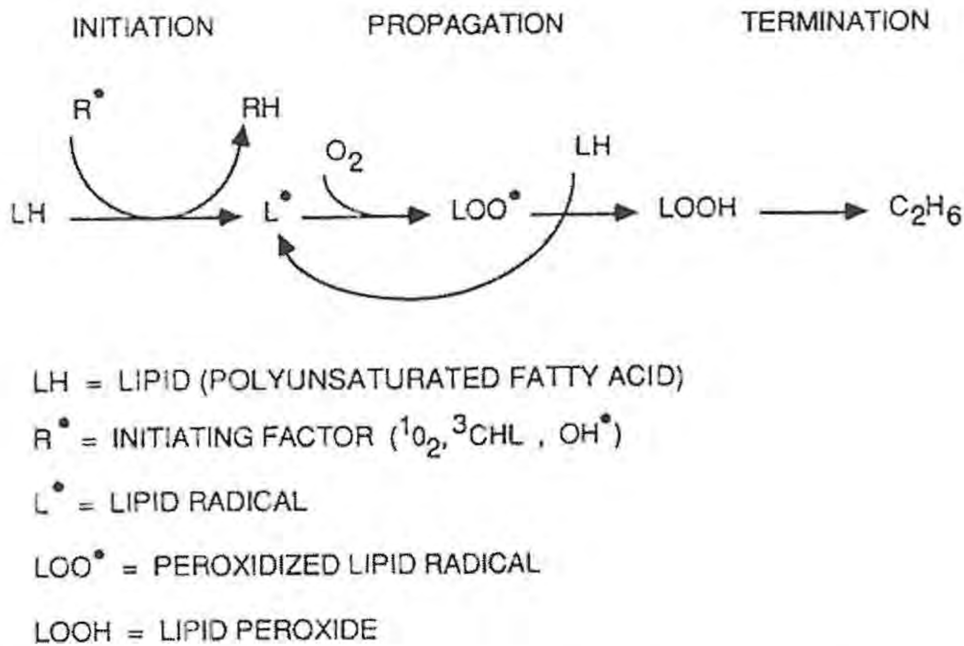


Fig.3.1. Free radical chain reaction where the free radical formed starts the self-perpetuating chain reaction. (billie.btny.purdue.edu/ btny504/lipidperox.html).

The brain is highly susceptible to oxidative stress because of its high use of oxygen and glucose consumption; high rate of energy consumption; large amount of peroxidisable fatty acids; relatively low antioxidant capacity e.g. superoxide dismutase and catalase and the ease with which brain membranes can undergo peroxidation (Floyd, 1999; Gupta et al., 2001). Also, the presence of transition metals e.g. iron and copper, which are capable of generating one of the most destructive of all free radical species, the hydroxyl radical which tends to react with all sites unselectively, due to the participation in the Fenton reaction (Halliwell, 1990).

Lipid peroxidation is the oxidative damage of PUFAs, and is known to be extremely damaging because of the self-perpetuating chain reactions these cause (see fig.3.1. above) (Reiter, *et al., 1996*). Propagation of free radical formation could continue for thousands of reactions and the damage caused will be extensive (Campbell and Abdullah, 1995; Halliwell, 1992). Lipid peroxidation is a well-known example of oxidative damage cell membranes, lipoproteins, and other lipid-containing structures (Girotti, 1998).

3.2 EFFECT OF METRIFONATE ON QUINOLINIC ACID INDUCED CHANGES IN RAT BRAIN HOMOGENATE

3.2.1 INTRODUCTION

The kynurenine pathway (KP) is a major route of L-tryptophan catabolism leading to production of a number of biologically active molecules. Quinolinic acid (2, 3-pyridine dicarboxylic acid) (QA) is a heterocyclic amino acid (Wolfensberger *et a*l., 1983) and is a neurotoxic metabolite of the tryptophan – kynurenine pathway (Heyes and Morrison, 1997). It is present in the human and rat brain in nanomolar concentrations (Stone, 1993).

This excitotoxin is synthesized in the liver and CNS (Flanagan *et al.*, 1995) and has been reported to be present in the brain in an uneven distribution pattern. Quinolinic acid is considered to be involved in the pathogenesis of a number of inflammatory neurological diseases. Quinolinic acid induced neurotoxicity closely mimics the patterns of selective nerve cell loss obseverd in Huntington's disease and temporal lobe epilepsy (Martin and Beal, 1992) A major aspect of QA toxicity is lipid peroxidation its markers in the brain, found in Alzheimer's disease, (Guilleman and Brew, 2002). The following experiment was conducted to investigate the effect of QA on lipid peroxidation in rat brain homogenate. QA induced neurotoxicity results from the activation of ion channels through which sodium, potassium and calcium flood into the cell (Stone, 1993). The increased intracellular calcium concentration sets of a cascade of events that culminate in the generation of free radicals. Free radicals are especially toxic because these initiate lipid destroying chain reactions (Halliwell and Chirico, 1993).

Metrifonate is an acetylcholinesterase inhibitor used in the symptomatic treatment of Alzheimer's disease. This experiment aimed to investigate whether metrifonate has any neuroprotective properties, i.e. if it can protect rat brain homogenate from QA induced neurotoxicity and free radical damage.

THE THIOBARBITURIC (TBA) – MALONDIALDEHYDE (MDA) ASSAY

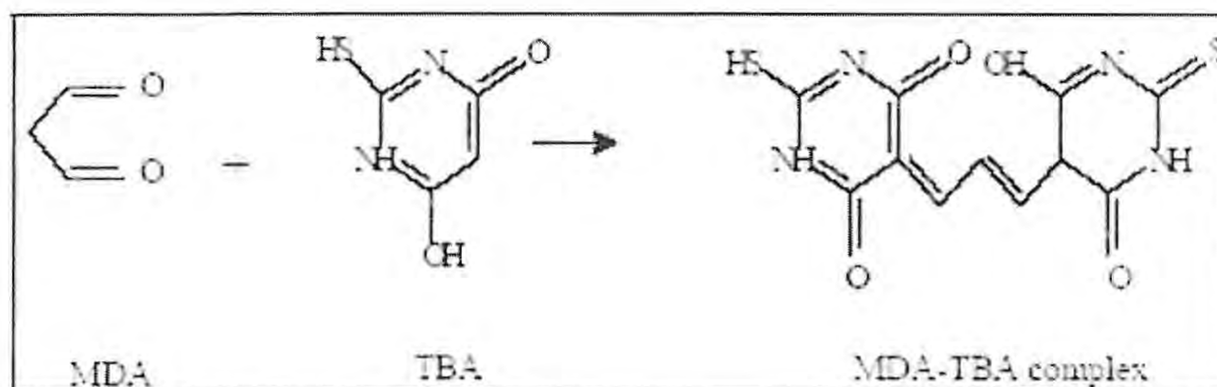


Fig.3.2. The reaction of MDA with TBA to yield a pink TBA-MDA complex (Mead *et al.*, *1*986).

Lipid peroxidation often occurs in response to oxidative stress, and a great diversity of aldehydes is formed when lipid hydroperoxides break down in biological systems. Some of these aldehydes are highly reactive and may be considered as second toxic messengers which disseminate and augment initial free radical events (Esterbaur, 1991). The aldehydes most intensively studied so far are 4-hydroxynonenal, 4-hydroxyhexenal, and malondialdehyde (Esterbaur, 1991). Malondialdehyde is a reliable indicator of free radical-induced damage (Reiter *et al*, 1995; Yamamoto, 1990; Wang *et al.*, 1996).

A widely used technique in the determination of peroxidation of tissue, is the thiobarbituric assay (TBA). Malondialdehyde (MDA), is an end product of lipid peroxidation (Wang *et al.*, 1996), and is the most abundant individual aldehyde resulting from lipid peroxidation and it has been shown to be capable of altering proteins, DNA, RNA and other biomolecules *in vitro* (Schaunenstein, 1977).

This test is based on the reaction of one molecule of MDA with two molecules of TBA, resulting in the formation of a pink chromogen (shown in fig. 3.2. above). This complex has an absorption maximum at 532nm. The reaction takes place in acidic conditions (pH of 2 – 3) at 90 – 100 °C for one hour.

Butylated hydroxytoluene (BHT) in methanol (0.5% w/v) is added to stop oxidative processes during the assay. Trichloroacetic acid (TCA) in Milli-Q water (10% w/v) is added to the tissue sample. TCA is also used to precipitate protein, which is pelleted by centrifugation. An aliquot of the supernatant is allowed to react with TBA in Milli-Q water (0.33% w/v) in a boiling water bath for one hour. After cooling, and extracting with butanol, the absorbance is read at 532nm and the concentration of MDA determined from a standard curve generated from 1, 1, 3, 3- tetramethoxypropane.

3.2.2 MATERIALS AND METHODS

3.2.2.1 Animals

Adult male Wistar rats weighing between 250 and 300 g were used in this experiment, and were housed and maintained under the conditions described in section 2.2.2.1.

3.2.2.2 Chemicals and Reagents

1,1,3,3-Tetramethoxypropane (MDA) was obtained from Fluka AG, Switzerland. Butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA) and QA and Metrifonate were purchased from Sigma Chemical Co, St. Louis, USA. Trichloroacetic acid and butanol were purchased from Saarchem (PTY) Ltd., Krugersdorp, South Africa.

3.2.2.3 Brain Removal

The brains were surgically removed and either used immediately or stored at –70 ° C as described earlier in section 2.2.2.3.

3.2.2.4 Preparation of the homogenate

The rats were sacrificed by cervical dislocation and the brains were surgically removed and homogenised in the same manner described in section 2.2.2.4.

3.2.2.5 Preparation of the standard curve

A series of standards (0-20 nmol/ml with 5nmol/ml intervals) was prepared using 1,1,3,3-tetramethoxypropane (MDA) and a standard curve generated by measuring the absorbance at 532nm using a GBC UV/VIS 916 spectrophotometer and plotted against the molar equivalent weight of MDA in the complex assayed (appendix 3).

3.2.2.6 Lipid Peroxidation

A modification of the method of Placer *et al*., (1966), was used in this experiment. Rat brain homogenate was prepared as described earlier and incubated in an oscillating water bath for one hour at 37^0 C with varying concentrations of metrifonate and 1mM QA, to make a final volume of 1ml after which 0.5ml of BHT (0.5% w/v in methanol) was added to each tube after incubation, followed by the addition of 1ml of TCA (10%w/v). The tubes were then centrifuged at 2000rpm for 20 minutes to remove insoluble proteins. Two millilitres of the supernatant was transferred to a clean set of tubes and 0.5ml TBA (0.33g/100ml Milli-Q water) was added. The tubes were boiled for one hour at 95^0C, and then cooled on ice. Thereafter the TBA-MDA complex was extracted with butanol and read at 532nm. MDA levels were determined from the standard curve generated from 1,1,3,3 -tetramethoxypropane.

3.2.2.7 *In vitro* exposure of rat brain homogenate to Quinolinic Acid and Metrifonate

Quinolinic acid and MET were dissolved in Milli-Q and to dilute to yield the following concentrations in the final volume of 1ml of the reaction mixture. For the *in vitro* assay of MET and QA, homogenate (1mL) containing varying concentrations of metrifonate (0, 0.25, 0.5, and 1mM) was incubated in an oscillating water bath for 1 hr at 37°C, to which 1mM QA was added and incubated for a further 1 hour. At the end of the incubation period, 0.5mL BHT (0.5mg/ml in methanol) and 1mL TCA (15% in water) were added to the mixture. The samples were centrifuged at 2000x g for 20min at 4°C to remove insoluble proteins. Following centrifugation, 2mL of protein free supernatant was removed from each tube and a 0.5mL aliquot of TBA (0.33% in water) was added to this fraction. The tubes were sealed and heated for an hour at 95°C in a water bath. After cooling, the TBA-MDA complexes were extracted with 2mL of butanol. The absorbance was read at 532nm and the MDA levels were determined from a standard curve generated from 1, 1, 3, 3-tetramethoxypropane. Final results were represented as nmol MDA/mg tissue.

3.2.3 RESULTS

The final results were expressed as nanomoles of MDA produced per mg tissue. The *in vitro* exposure of brain homogenate to 1mM QA alone produced a significant increase in lipid peroxidation when compared to the control. There was also a significant increase in lipid peroxidation of the varying concentrations of MET with 1mM QA compared to the control and 1mM QA alone.

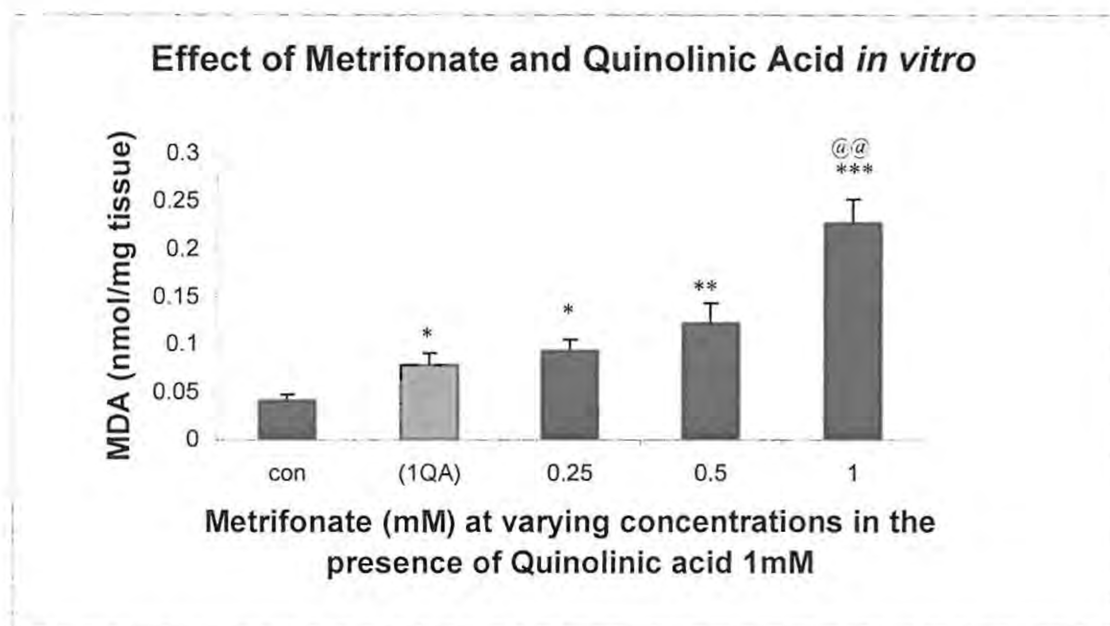


*Fig.3.3. Concentration-dependent effect of MET and QA on Lipid peroxidation in rat brain homogenate. Each Bar represents the mean ± SEM, n=5; (*** p<0.001 in comparison to control, ** p<0.01 in comparison to control, * p<0.05 in comparison to control and @@ P<0.01 in comparison to 1mM QA only).*

3.2.4 DISCUSSION

The graph and data imply that MET does not protect the brain homogenate from the lipid peroxidation damage induced by QA. In the presence of MET with QA, there is an increase in the levels of MDA produced. The level of MDA produced at 1mM MET with 1mM QA is higher than the MDA level produced in the presence of 1mM QA only. Therefore, the presence of MET increases lipid peroxidation. Thus MET does not protect the brain homogenate from QA-induced damage, but instead it causes additional peroxidation.

3.3 EFFECT OF METRIFONATE ON LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE

3.3.1 INTRODUCTION

From the results obtained in the previous experiment, it was observed that MET increases the production of lipid peroxidative products. Thus it was decided to determine the effect of MET alone. This assay was done *in vitro* and *in vivo*. The *in vivo* testing had two sets of dosing regimens. The reason for this is that at higher doses (80mg/kg and 120mg/kg twice daily orally) it was observed that MET could increase cerebral glucose utilization (Poindessous-Jazat *et al.*, 1998). The brain is especially susceptible to free radical formation and damage as a result of its high consumption of total body oxygen and the relatively low concentration of antioxidant enzymes (Coyle and Puttfarcken, 1993). Therefore this dosage regimen was used to determine whether these doses have an effect on lipid peroxidation. The lower dosage regimen in patients receiving orally administered 0.65mg/kg to 1mg/kg daily MET for 15 days with an initial loading dose of 4mg/kg daily for 6 days produce improvement on the Alzheimer's disease Assessment Scale. These are the dosages used in symptomatic treatment of Alzheimer's disease (Pettigrew *et al.*, 1998). Therefore a second dosing regimen was used as well, i.e. 0.65mg/kg/day and 1mg/kg/day MET. The drug was administered intraperitoneally (i.p.) in order to reduce the variation in absorption and which may occur if administered by an oral route.

3.3.2 MATERIALS AND METHODS

3.3.2.1 Animals

Adult male Wistar rats weighing between 250 and 300 g were used in this experiment, and were housed and maintained under the conditions described in section 2.2.2.1.

3.3.2.2 Chemicals and Reagents

1,1,3,3-Tetramethoxypropane (MDA) was obtained from Fluka AG, Switzerland. Butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA) and metrifonate were purchased from Sigma Chemical Co, St. Louis, USA. Trichloroacetic acid and butanol were purchased from Saarchem (PTY) Ltd., Krugersdorp, South Africa.

3.3.2.3 Preparation of the homogenate

The rats were sacrificed by cervical dislocation and the brains were surgically removed and homogenised in the same manner described in section 2.2.2.4.

3.3.2.4 Lipid peroxidation Assay.

Lipid peroxidation was determined using the TBA assay as described in section 3.2.2.6 in the following set of experiments i.e. :

(a) *In vitro* exposure to MET: 0, 0.125mM, 0.250mM, 0.5mM, 1mM and 2mM.

(b) *In vivo* exposure to MET at high doses: 20mg/kg/day, 50mg/kg/day and 80mg/kg/day i.p.

(c) *In vivo* exposure to MET at therapeutic doses: 0.65mg/kg/day and 1mg/kg/day i.p.

3.3.2.5 (a) *In vitro* exposure of homogenate to Metrifonate

Homogenate was prepared as described in section 2.2.2.4. For the *in vitro* assay, homogenate (1mL) containing varying concentrations of metrifonate (0, 0.125, 0.25, 0.5, 1 and 2mM) were incubated in an oscillating water bath for 1 hr at 37°C. The experiment was then carried out as detailed in 3.2.2.6.

3.3.2.6 (b) *In vivo* exposure to Metrifonate at high doses

For the *in vivo* study, animals were divided into four groups (I - IV), each group containing 4 animals:
Group I: Control – vehicle;
Group II: 20mg/kg/day MET;
Group III: 50mg/kg/day of MET;
Group IV: 80mg/kg/day MET.

Metrifonate was dissolved in PBS (pH 7.4) and administered by intraperitoneal injections daily. The animals were treated for a period of seven days after which the animals were sacrificed by decapitation on the eighth day. Brain homogenate of rats treated with metrifonate (control, 20mg/kg/day, 50mg/kg/day and 80mg/kg/day) was incubated at 95 °C for 15 min with the addition of the 0.5mL BHT (0.5mg/ml in methanol) and 1mL TCA (15% in water) to the mixture. The samples were centrifuged at 2000x g for 20min at 4°C to remove insoluble proteins. Following centrifugation, 2mL of protein-free supernatant was removed from each tube and a 0.5mL aliquot of TBA (0.33% in water) was added to this fraction. The tubes were sealed and heated for an hour at 95°C in a water bath. After cooling, the TBA-MDA complexes were extracted with 2mL of butanol. The absorbance was read at 532nm and the MDA levels were determined from a standard curve generated from 1,1,3,3-tetramethoxypropane. Final results were represented as nmol MDA/mg tissue.

3.3.2.7 *In vivo* exposure to Metrifonate at low doses

For the *in vivo* study, animals were divided into three groups (I - III), each group containing 4 animals:

Group I: Control - vehicle

Group II: 0.65 mg/kg/day MET;

Group III: 1mg/kg/day of MET.

Metrifonate was dissolved in PBS (pH 7.4) administered via intraperitoneal injections daily. The animals were treated for a period of seven days after which the animals were sacrificed by decapitation on the eighth day. Homogenate (1mL) was prepared as described in section 2.2.2.4 and the experiment was carried out as described in section 3.2.2.6. Final results were represented as nmol MDA/mg tissue.

3.3.3 RESULTS

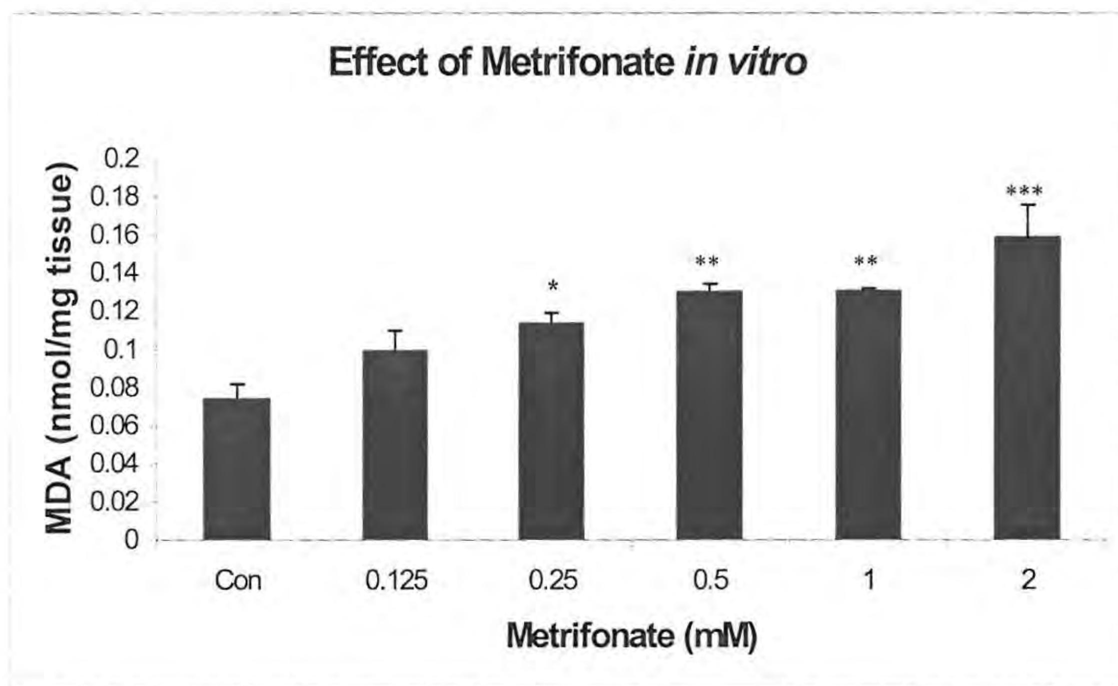


*Fig.3.4.The effect of increasing concentrations of MET in vitro on MDA levels in rat brain homogenate. Each bar represents the mean ± SEM; n= 5. (*** $p < 0.001$ in comparison to the control, ** $p < 0.01$ in comparison to the control and * $p < 0.05$ in comparison to the control.*

From the results of the *in vitro* study, shown in fig.3.4, it is evident that MET increases the production of lipid peroxide products in comparison to the control value. Increasing concentrations of MET (mM), induced a rise in lipid peroxidation levels as indicated by the increased levels of MDA in rat brain homogenate.

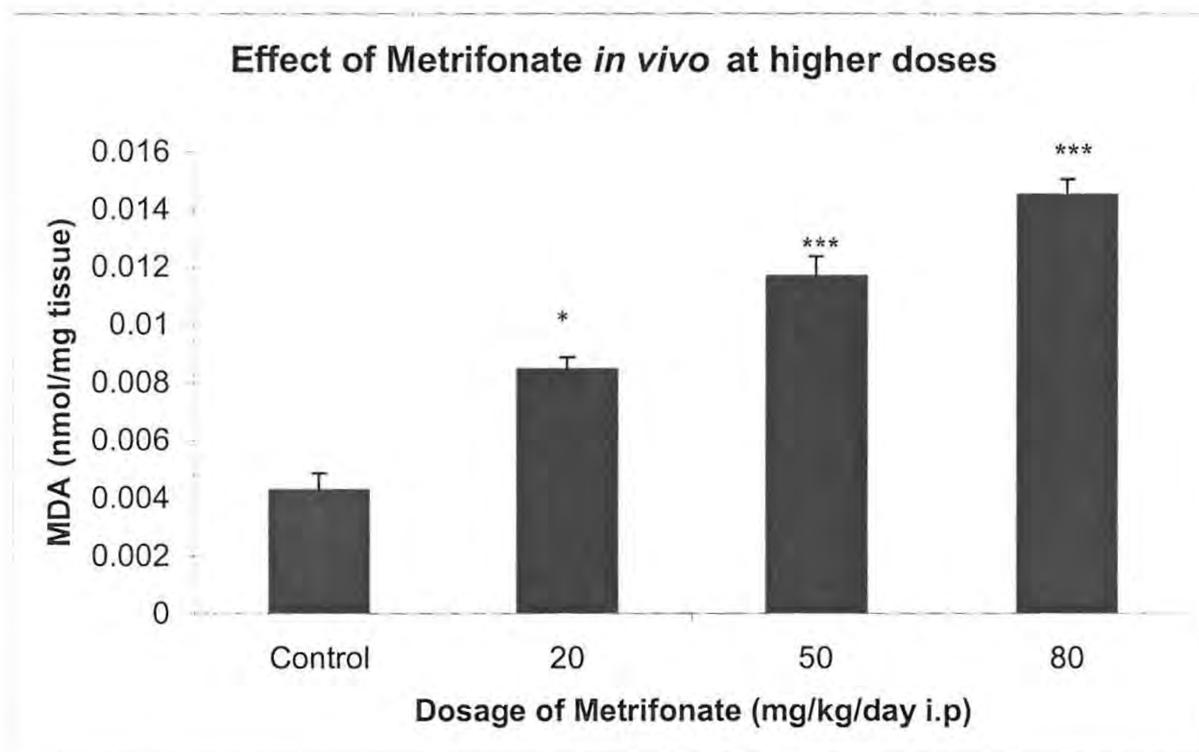


Fig.3.5. The effect of increasing doses of MET on MDA levels in rat brain homogenate obtained from rats pretreated with 20, 50 and 80 mg/kg/day i.p for 7 consecutive days. Each bar represents the mean $\pm$ *SEM; n=5. (*** p< 0.001 in comparison to the control and * p < 0.05 in comparison to the control).*

The results of the *in vivo* lipid peroxidation study, (figure 3.5) show that lipid peroxidation levels significantly increase in a dose-dependent manner in brain homogenates of those rats which had received metrifonate (20-80mg/kg/day) in comparison to the control.

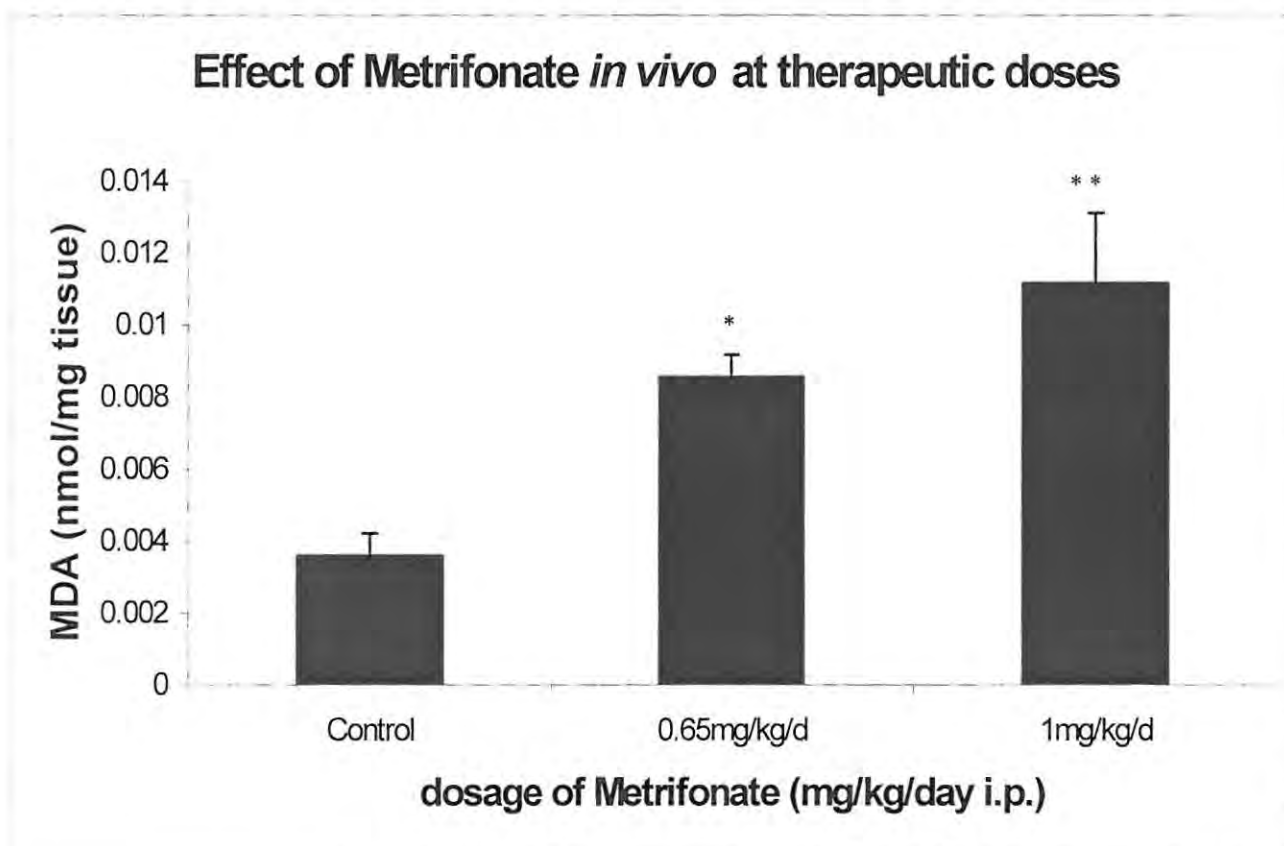


*Fig.3.6. The effect of increasing doses of MET on MDA levels in rat brain homogenate obtained from rats pretreated with 0.65 and 1 mg/kg/day i.p for 7 consecutive days. Each bar represents the mean ± SEM; n=5. (** $p < 0.01$ in comparison to the control and * $p < 0.05$ in comparison to the control).*

The results of the *in vivo* lipid peroxidation study, (figure 3.6) also demonstrates that lipid peroxidation levels significantly increase in a dose-dependent manner in brain homogenates of those rats which had received metrifonate (0.65-1mg/kg/day) in comparison to the control rats.

3.3.4 DISCUSSION

As can be gleaned from the results, it can be observed that metrifonate causes lipid peroxidative damage *in vitro*. These results are in agreement with Yamano, (1996) where the author showed that dichlorvos causes oxidative damage to isolated rat hepatocytes at the doses, 0.25mM, 0.5mM, 1.0mM. Furthermore, metrifonate and dichlorvos have been shown to produce toxic effects on hepatocytes as evidenced by the by MDA production and lactate dehydrogenase leakage in a dose-dependent manner up to the concentration of 2mM (Yamano and Morita, 1992).

There have also been studies which show that dichlorvos, at doses of 200mg/kg body weight, in rats, induces hyperphosphorylation of tubulin and microtubule associated protein-2 (MAP-2), which in turn destabilizes microtubule assembly and may ultimately result in axonal degeneration leading to dichlorvos induced delayed neurotoxicity (Choudhary *et al*., 2000).

In the present study, once-daily, intra-peritoneal doses of MET 0.65mg/kg/day and 1mg/kg/day and the subsequent effect on lipid peroxidation levels were investigated. The results show, that even at these doses, lipid peroxidation damage occurs in rat brain.

A twice daily dose of MET increases glucose utilization in the cerebral cortex and the hippocampus (Poindessous-Jazat *et al*., 1998). This action on energy metabolism probably reflects the activation of cholinergic transmission, which might explain, at least in part, the cognitive effects of metrifonate (Poindessous-Jazat *et al*., 1998). However the increased glucose utilization, results in an increase in activity in mitochondria. This may lead to an increase in the production of ROS in the cell, and therefore could explain the increase in lipid peroxidation levels for those animals receiving the higher doses.

Presently there is no evidence which suggests that AchEI's change the underlying neuropathology of AD (Bellantonio and Kuchel., 2002). Some authors have speculated that, AchEI's could accelerate the progression of AD (Soreq and Seidman., 2001).

Dichlorvos has been noted to have the potential to cause axonal degeneration leading to delayed dichlorvos induced neurotoxicity (Choudhary *et al.*, 2001). The present report is the first to provide evidence that metrifonate increases lipid peroxidation levels and thus causes oxidative stress in rat brain homogenate and that this agent can lead to further neurodegeneration.

3.4 EFFECT OF MELATONIN ON METRIFONATE-INDUCED LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE

3.4.1 INTRODUCTION

Melatonin (5-methoxy-N-acetyltryptamine) is a product of tryptophan metabolism by the pineal gland. It is both highly lipophilic as well as relatively water soluble (Shida *et al.*, 1994), thus it can easily cross the blood brain barrier, other morphophysiological barriers and is believed to have ready access to every cell in the organism (Menendez-Palaez and Reiter, 1993). Melatonin is a well known neuroprotectant and free radical scavenger (Tan *et al.*, 1993). It scavenges the most potent hydroxyl radical scavenger (Reiter *et al.*, 1994) and the peroxyl radical ($ROO^{\bullet}$) (Melchiorri *et al.*, 1995).

Metrifonate increases MDA levels in rat brain homogenate, in the presence of QA. Exposure of the homogenate to MET only increased MDA levels. This indicates that Metrifonate may have the potential to be neurotoxic by inducing lipid peroxidation. Since lipid peroxidation is one of the major consequences of free radical-mediated injury (Halliwell and Chirico, 1993), rat brain homogenate was exposed to MET in the presence of the free radical scavenger, melatonin. to determine whether melatonin could reduce or prevent metrifonate –induced lipid peroxidation.

3.4.2 MATERIALS AND METHODS

3.4.2.1 Animals

Adult male Wistar rats weighing between 250 and 300 g were used in this experiment, and were housed and maintained under the conditions described in section 2.2.2.1

3.4.2.2 Chemicals and Reagents

1, 1, 3, 3-Tetramethoxypropane (MDA) was obtained from Fluka AG, Switzerland. Butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA) metrifonate (MET) and Melatonin (MEL) were purchased from Sigma Chemical Co, St. Louis, USA. Trichloroacetic acid and butanol were purchased from Saarchem (PTY) Ltd., Krugersdorp, South Africa.

3.4.2.3 Preparation of the homogenate

The rats were sacrificed by cervical dislocation and the brains were removed and homogenised in the same manner described in section 2.2.2.4.

3.4.2.4 Lipid peroxidation Assay.

Lipid peroxidation was determined using the TBA assay as described in section 3.2.2.6. in the following set of experiments i.e.:

(a) *In vitro* exposure to MET and MEL: control; 1mM MET only; MEL (0.125, 0.25 0.5, 1 and 2mM) + 1mM MET.

(b) *In vivo* exposure to MET and MEL: control; 1mg/kg/day i.p. MET only; MEL (1mg/kg/day, 5mg/kg/day, 10mg/kg/day) + 1mg/kg/day MET i.p.

3.4.2.5 *In vitro* exposure to Metrifonate and Melatonin

Homogenate was prepared as described in section 2.3.2.3. For the *in vitro* assay, homogenate (1mL) containing varying concentrations of MEL (0, 0.125, 0.25, 0.5, 1 and 2mM) and 1mM MET were incubated in an oscillating water bath for 1 hr at 37°C. At the end of the incubation period, 0.5mL BHT (0.5mg/ml in methanol) and 1mL TCA (15% in water) were added to the mixture. The samples were centrifuged at 2000x g for 20min at 4°C to remove insoluble proteins. Following centrifugation, 2mL of protein free supernatant was removed from each tube and a 0.5mL aliquot of TBA (0.33% in water) was added to this fraction. The tubes were sealed and heated for an hour at 95°C in a water bath. After cooling, the TBA-MDA complexes were extracted with 2mL of butanol. The absorbance was read at 532nm and the MDA levels were determined from a standard curve generated from 1, 1, 3, 3-tetramethoxypropane. Final results were represented as nmol MDA/mg tissue.

3.4.2.6 *In vivo* exposure to Metrifonate and Melatonin

MET (1mg/kg/day i.p.) was dissolved in PBS and MEL (varying doses in mg/kg/day i.p) was dissolved in 30% ethanol in PBS for the purposes of these experiments. The rats were divided into five groups each containing four rats each i.e.

GROUP I: control - vehicle
GROUP II: 1mg/kg/day Metrifonate only and 30 % ethanol in PBS,
GROUP III: 1mg/kg/day Melatonin and 1mg/kg/day Metrifonate
GROUP IV: 1mg/kg/day Metrifonate, 5mg/kg/day Melatonin
GROUP V: 1mg/kg/day Metrifonate, and 10/kg/day Melatonin.

Animals were injected intraperitoneally for five days, and on the sixth day these were sacrificed and the brains removed and homogenized as described in 2.2.2.4. Lipid

peroxidation assays were carried out on these brains as described earlier iin section 3.2.2.6.

3.4.3 RESULTS

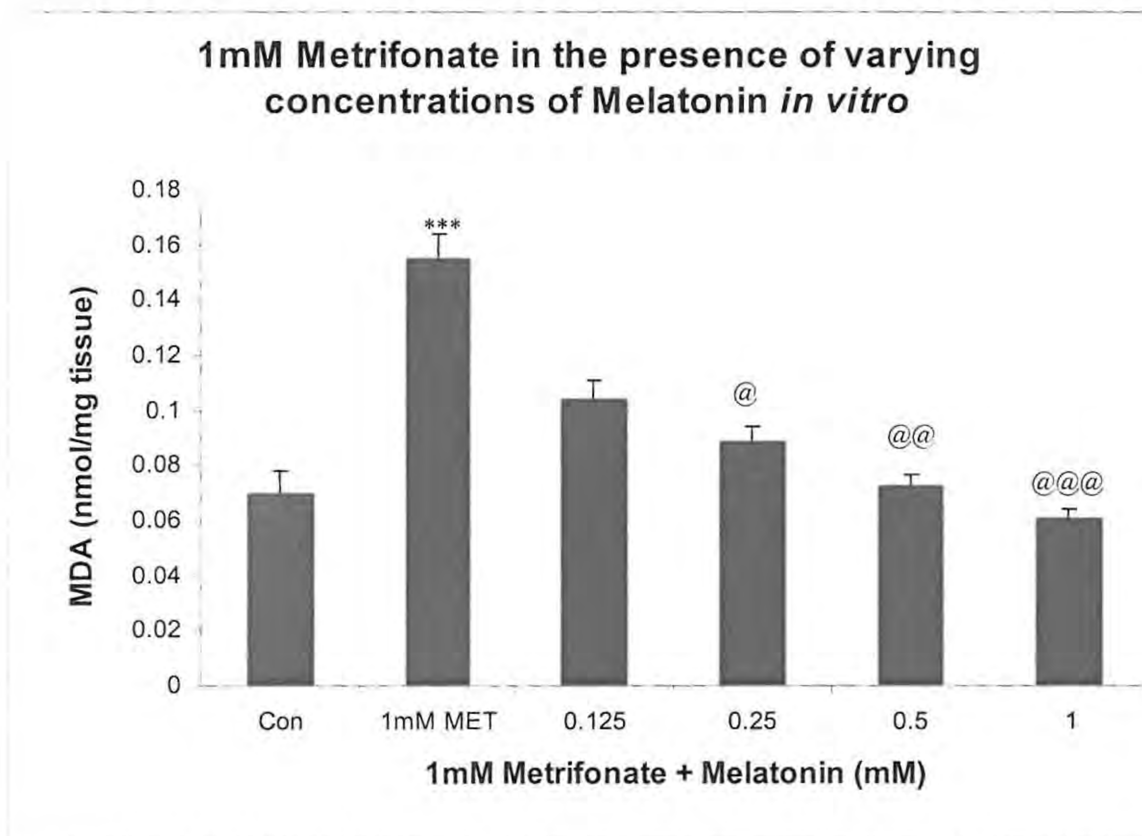


*Fig.3.7. The effect of increasing concentrations of MEL and 1mM MET on MDA level in rat brain homogenate. Each bar represents the mean ± SEM; n=5. (*** $p < 0.001$ in comparison to the control and @ $p < 0.05$ in comparison to 1mM MET only, @@ $p < 0.01$ in comparison to 1mM MET only and @@@ $p < 0.001$ in comparison to 1mM MET only)*

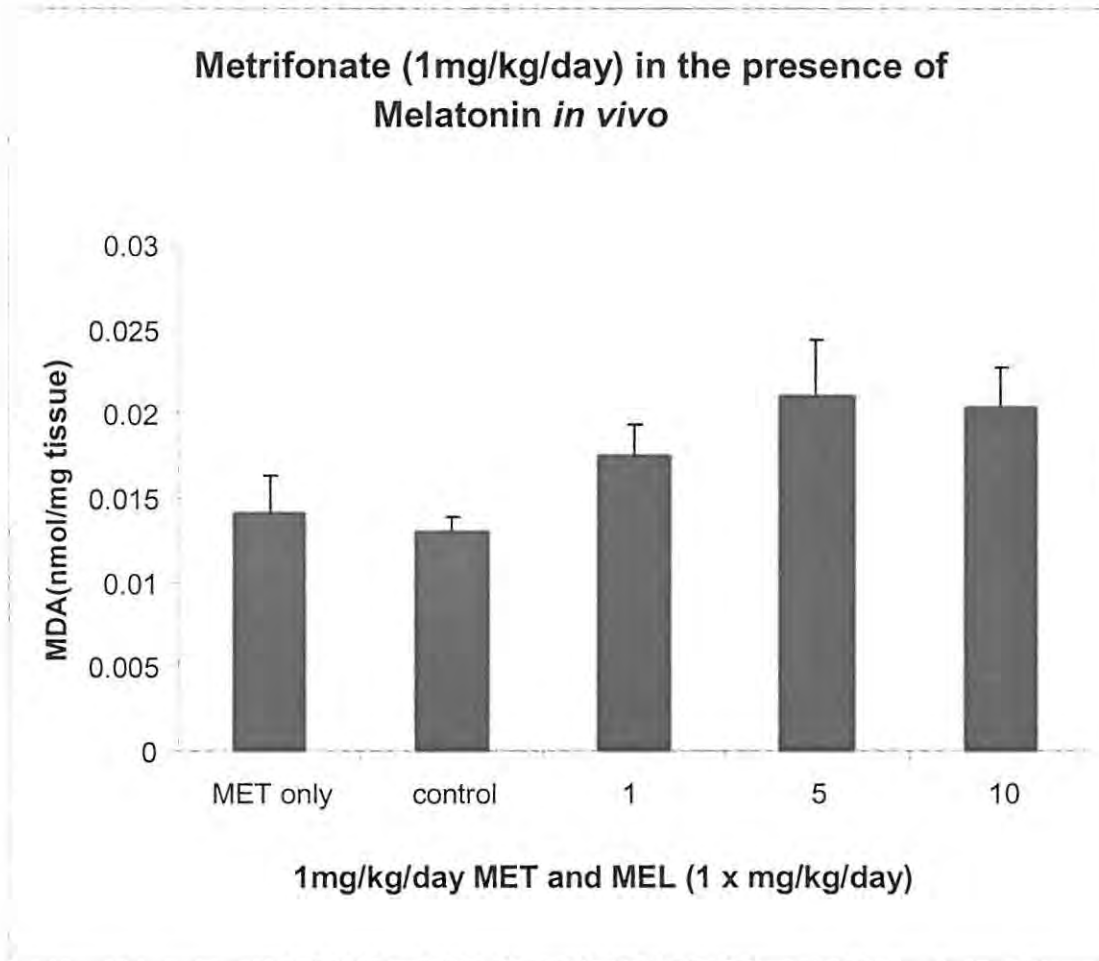


Fig.3.8 The effect of increasing concentrations of MEL and 1mg/kg/day MET i.p. on MDA levels in rat brain homogenate. Each bar represents the mean ± SEM; n=5. There is no significant difference between the concentrations.

3.4.4 DISCUSSION

As mentioned earlier lipids, proteins and DNA are all susceptible to attack by free radicals. The brain is particularly vulnerable as it contains large amounts of polyunsaturated fats, consumes 20% of the total body oxygen, has a low concentration of antioxidant enzymes and is rich in iron, which generates free radicals via the Fenton reaction (McCord, 1985).

Although Melatonin *in vitro* is able to produce a significant reduction in lipid peroxidation, it fails to do so *in vivo*. Melatonin is a free radical scavenger and powerful antioxidant (Tan *et al*., 1993), but is unable to significantly reduce the damage caused by MET *in vivo*. Melatonin is rapidly metabolized in the liver to its main metabolite, 6-hydroxymelatonin, by CYP1A2 (Sakano *et al*., 2004, Skene *et al*., 2001, Matuszak et *al*., 1997).

It has been noted that MEL in certain concentrations and its metabolite, 6-hydroxymelatonin under certain conditions also act as a pro-oxidant causing oxidative DNA damage via a unique non-o-quinone type of redox cycle (Sakano *et al*., 2004). It is therefore possible that the concurrent administration of MEL with MET is unable to protect the tissue from lipid peroxidation as it's metabolite produces oxidative damage. The reason for the absence of this occurrence *in vitro* is the absence of metabolism of MEL via the CYP1A2 in the liver (Sakano *et al*., 2004, Skene *et al*., 2001, Matuszak et *al*., 1997).

3.5 EFFECT OF METRIFONATE ON IRON (Fe^{2+}) INDUCED LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE

3.5.1 INTRODUCTION

Promotion of lipid peroxidation by transition metals can occur by one of two mechanisms i.e. catalyzing the formation of ROS (via the Fenton reaction) which are capable of initiating lipid peroxidation or by catalyzing the decomposition of preformed lipid peroxides which propagate lipid peroxidation (Rikans and Hornbrook, 1997).

Iron is required by Fenton reaction in order to allow free radical formation in the brain (Halliwell, 1992). Iron and the chelated forms thereof are involved in radical reactions at many different levels, e.g. the oxidation of Fe^{2+} in the presence of H_2O_2 to form $\cdot OH$ and Fe^{3+} - OH (the ferryl ion) (Braughler and Hall, 1989). Both species formed are powerful

oxidants which can react with a wide variety of of biological substrates (Ottino and Duncan, 1997; Dawson and Dawson, 1996).

Once these oxidants react, following lipid peroxidation, PUFAs form relatively unstable fatty acid hydroperoxides which are then converted via oxidation, consecutive scission and rearrangement reactions to form more stable carbonyls including malondialdehyde which is then measured as an indicator of the extent of lipid peroxidation occuring, by means of the TBA test.

This experiment, iron induced lipid peroxidation in rat brain homogenate, was performed in the presence of metrifonate to investigate whether or not metrifonate has an effect on the oxidative damage induced by iron.

3.5.2 MATERIALS AND METHODS

3.5.2.1 Animals

Adult male Wistar rats weighing between 250 and 300 g were used in this experiment, and were housed and maintained under the conditions described in section 2.2.2.1.

3.5.2.2 Chemicals and Reagents

Butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA) and metrifonate were purchased from Sigma Chemical Co, St. Louis, USA. Trichloroacetic acid, ascorbic acid and butanol were purchased from Saarchem (PTY) Ltd., Krugersdorp, South Africa. The ferrous sulphate was purchased from BDH Laboratory Supplies, UK and EDTA from Holpro Analytics (PTY) LTD, Johannesburg. All reagents were prepared in deaerated Milli-Q water.

3.5.2.3 Preparation of the homogenate

The rats were sacrificed by cervical dislocation and the brains were surgically removed and homogenised in the same manner described in section 2.2.2.4.

3.5.2.4 Lipid peroxidation Assay.

Lipid peroxidation was determined using the TBA assay. The following set of experiments were performed i.e. :

(a) *In vitro* exposure to MET: 0, 0.125mM, 0.250mM, 0.5mM, and 1mM

(b) *In vitro* exposure to Fe^{2+} and MET:0,0.125mM,0.250mM,0.5mM and 1mM

3.5.2.5 *In vitro* exposure of homogenate to metrifonate

Homogenate was prepared as described in section 2.2.2.4. For the *in vitro* assay, homogenate (0.5mL) containing varying concentrations of metrifonate (0, 0.125, 0.25, 0.5,and 1mM) 1mM ascorbate, 100μM EDTA, 0.5mM H_2O_2 and PBS buffer to yield a final volume of 1ml were incubated in an oscillating water bath for 1 hr at 37°C. Afterwhich 0.5ml BHT (0.5%) and 1ml TCA (25%) were added to the mixture and then heated at 95^0 C for 15minutes, cooled on ice and centrifuged at 2000g for 20 minutes. The supernatant (2ml) were removed and added to 1ml TBA (0.33%) and then incubated at 95^0C for 1 hour. The tubes were then removed, cooled, 2ml of butanol added and centrifuged at 2000g for 5minutes. The supernatant (1.5ml) was removed and the absorbance was read at 532nm. The MDA levels were determined from a standard curve generated from 1,1,3,3-tetramethoxypropane. Final results were represented as nmol MDA/mg tissue.

3.5.2.6 *In vitro* exposure of homogenate to metrifonate and Iron (Fe^{2+}) Sulphate

Homogenate was prepared as described in section 2.2.2.4. For the *in vitro* assay, homogenate (0.5mL) containing varying concentrations of metrifonate (0, 0.125, 0.25, 0.5, and 1mM) 1mM ascorbate, 100µM EDTA, 0.5mM H_2O_2 and 1mM Fe^{2+} to yield a final volume of 1ml were incubated in an oscillating water bath for 1 hr at 37°C. The experiment was performed and represented as detailed in 2.5.2.4.

3.5.3 RESULTS

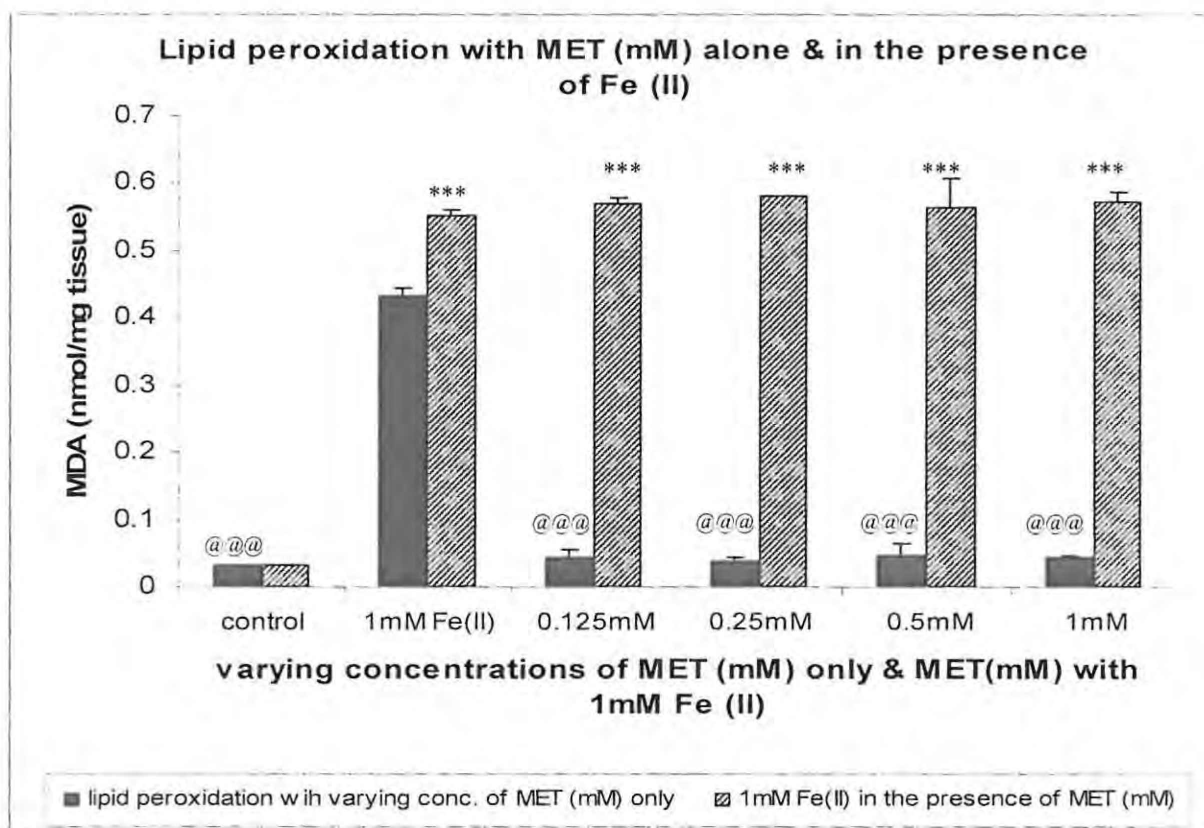


*Fig.3.9. The effect of increasing concentrations of MET and MET with Iron (II) in vitro on MDA levels in rat brain homogenate. Each bar represents the mean ± SEM ; n= 5. (*** $p < 0.001$ in comparison to the control, @@@ $p < 0.001$ in comparison to the toxin).*

The results of the *in vitro* study, (fig.3.9.), show that there is no significant difference with the homogenate containing varying concentrations of MET only in comparison to the control. However, there is a significant difference in the levels of MDA produced in the homogenate with iron (II) and MET in comparison to the control. This difference is due to the presence of iron (II). As there is no significant difference between the increasing doses of MET and iron (II) in comparison to the iron (II) only, this implies that MET does not effect iron (II) induced lipid peroxidation.

3.5.4 DISCUSSION

The lipid peroxidation induced by Fe^{2+} is the result of a different chain of events as compared to that induced by QA. Quinolinic acid induces lipid peroxidation as a result of activation of NMDA receptors, consequent influx of Ca^{2+} into the cell and the cascade of events triggered by calcium in the formation of ROS (Lipton and Rosenberg, 1994).

Iron (II) sulphate induces lipid peroxidation by catalysis of the formation of free radical species and decomposition of hydroperoxides to peroxyl and alkoxyl radicals (Halliwell and Gutteridge, 1990). The concentration of free Fe^{2+} available and levels of oxygen in the tissue determine the amount of ROS and resultant lipid peroxidation induced. In the presence of metrifonate, there was no significant increase in the levels of iron-induced lipid peroxidation with the increase in concentration of metrifonate. This indicates that metrifonate does not affect iron induced lipid peroxidation.

In comparison to earlier results with the increase in lipid peroxidation with QA in the presence of metrifonate, this indicates that metrifonate may be causing lipid peroxidation through a mechanism which is similar to QA rather than the Fenton reaction. However to rule out the possibility of the lack of interaction with iron, further investigation has to be carried out with metal (iron) binding studies and metrifonate.

CHAPTER FOUR – ELECTRON TRANSPORT CHAIN & COMPLEX I

[A] THE ELECTRON TRANSPORT CHAIN

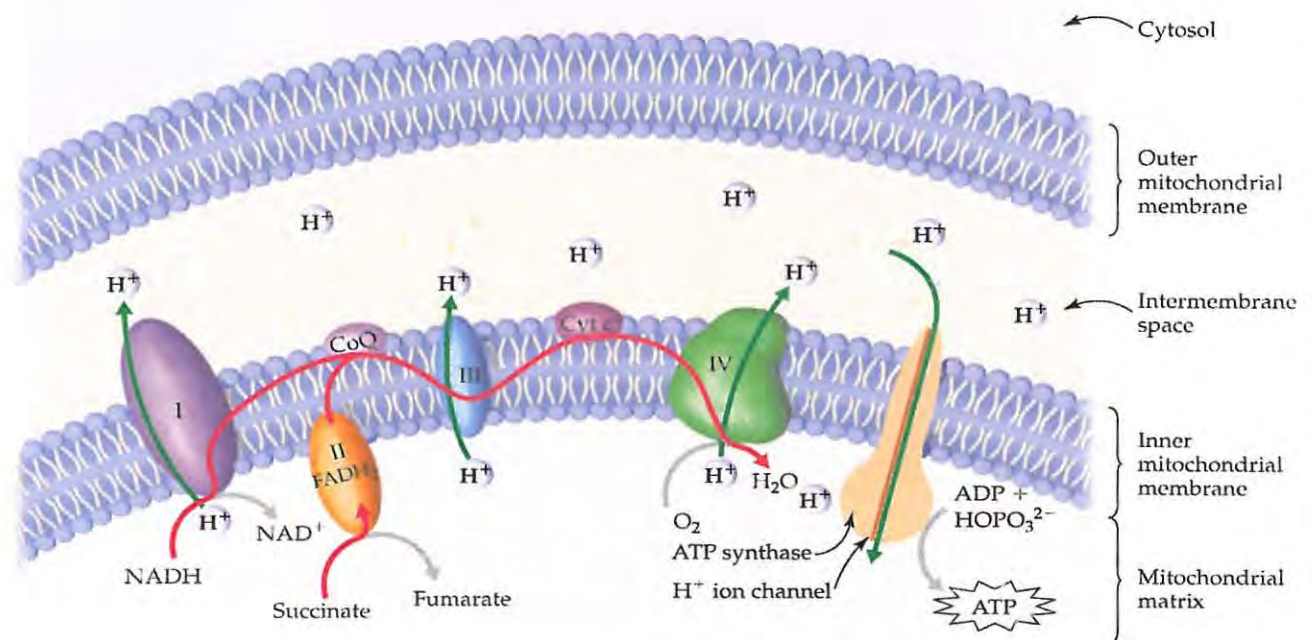


Fig.4.1. Represents the illustration of electron transport and ATP production in the membrane i.e. the mitochondrial electron-transport chain and ATP synthase. The red line shows the path of electrons, and the green lines show the paths of hydrogen ions. The movement of hydrogen ions across the inner membrane creates a higher concentration on the intermembrane side of the inner membrane than on the matrix side. The energy released by hydrogen ions returning through the membrane (through ATP synthase) provides the energy necessary to make ATP.
(wps.prenhall.com/.../ chapter_21/21.html)
(http://wps.prenhall.com/wps/media/objects/376/385232/Media-Portfolio/chapter_21/21.html)

4.1 INTRODUCTION

Over 120 human mitochondrial diseases have been discovered since the 1960's (Luft, 1997).
For normal functioning the brain relies on energy (Rossen and Kabat, 1943) with the mitochondria being the main source. Damage to mitochondria due to DNA mutations and pathological free radical reactions would impair the function of the electron transport chain (ETC) in the

mitochondria, thus impairing electron transport and therefore impaired ATP production. This would result in the diversion of electrons from their normal ETC recipients and further formation of damaging free radicals (Cassarino and Bennet, 1999). Thus decreased ATP production and increased ROS production is a possible mechanism whereby an impaired ETC would lead to cell death (Beal, 1995; Dykens, 1997; H. Mochizuki *et al.*, 1994; Wu *et al.*, 1990; Wolvetang *et al.*, 1994).

The complex I inhibitors rotenone and *N*-methylpyridinium ion (Zhang *et al.*, 2001; Smith and Bennett, 1997) generate free radicals *in vivo* and *in vitro* (Adams *et al.*, 1993; Mochizuki *et al.*, 1994; Wolvetang *et al.*, 1994) thus indicating that generation of free radicals via disruption of the ETC, have harmful effects if not neutralized by the cell. Damage to cells due to these free radicals, i.e. oxidation of cellular constituents including proteins, lipids, and DNA, can be detected through certain measurements for example, malonaldehyde formation, (Esterbaur *et al.*, 1991; Lovell *et al.*, 1997).

With age, there is oxidative membrane damage to membranes due to mitochondrial ROS production, which parallels the increase in mitochondrial lipid peroxides with aging (Zoratti and Szabo, 1995; Yu *et al.*, 1996; Harman, 1996). Progressive impairment of the ETC activity may also occur with aging due to the accumulation of oxidatively damaged proteins (Bowling and Beal, 1995; Dykens, 1997; Hagen *et al.*, 1997; Gutteridge and Halliwell, 1989; Sohal and Dubey, 1994; Symonyan and Nalbandyan 1972), leading to further ROS production and oxidative damage. The progressive loss of ATP may therefore result in the loss of Na/K-ATPase activity with consequent plasma membrane depolarization, loss of cellular homeostasis and activation of the apoptotic cascade (Beal, 1995; Bowling and Beal, 1995; Dykens, 1997).

Susceptibility to ROS-induced damage is higher in the CNS, than other organs, due to the relative lack of oxidative defenses (Marklund *et al.*, 1982; Martilla *et al.*, 1988). Therefore any symptomatic therapy for Alzheimer's disease, an affliction especially in the aged, should not further compromise the functioning of the ETC.

4.2 EFFECT OF METRIFONATE ON THE MITOCHONDRIAL CHAIN ACTIVITY

4.2.1 INTRODUCTION

Free radical-mediated cellular damage and impaired ETC have been exhibited in neurodegenerative diseases e.g. Alzheimer's disease and Parkinson's disease. (Beal, 1995; Bowling and Beal 1995; Harman, 1996; Kish *et al.*, 1985; Parker *et al.*, 1989, Parker *et al.*, 1994; Swerdlow *et al.*, 1996; Swerdlow *et al.*, 1997). Therefore it is important that the agents used in symptomatic treatment of neurodegenerative disorders e.g. Alzheimer's disease not cause further ROS generation or damage. However, as shown earlier, MET has the potential to induce lipid peroxidation and superoxide generation. Thus it is imperative that the source be determined, i.e. is the cause of increased superoxide generation, due to a disturbance of the ETC resulting in a decreased ETC activity ? This chapter aims to investigate the possibility of the disruption of the ETC as a source of free radical production.

4.2.2 MATERIALS AND METHODS

4.2.2.1 Animals

Adult, male, Wistar rats, weighing between 250 and 300g were used for the experiment, and were housed and maintained under the conditions described in section 2.2.2.1.

4.2.2.2 Chemicals and Reagents

All reagents were of the highest quality available. MET, NAD, 2,6 – diclorophenol-indophenol (DPI), and L-Malate were purchased from Sigma Chemical Corporation, St. Louis, MO, U.S.A. Sucrose was purchased from Saarchem (PTY) Ltd, Krugersdorp, South Africa. All reagents were prepared in 0.1M potassium phosphate buffer, pH 7.4.

4.2.2.3 Isolation of Mitochondria from rat brain

The rats were killed by cervical dislocation, and the brains were rapidly excised and homogenized with 0.1M potassium phosphate buffer, pH 7.4 to yield a 10% w/v homogenate. Mitochondrial suspensions were prepared by differential centrifugation according to

Plummer (1971). The brain homogenate was centrifuged at 600g for 10 minutes and separated into supernatant and pellet. The supernatant was collected and the pellet was re-suspended in half the volume of 0.32M buffered sucrose, and once again centrifuged at 600g for 10 minutes and the supernatant obtained was combined with the previous supernatant. The combined supernatant was centrifuged at 8000g for 10 minutes and the pellet obtained (crude mitochondria) was washed twice in the 0.32M buffered sucrose, and then stored on ice until required.

4.2.2.4 Biological Oxidation Assay

A modified method described by Plummer (1971) was used. This spectrophotometric technique was employed to determine the 'activity' of the inner mitochondrial membrane electron transport chain of the mitochondrial suspension. The rate of reduction of the synthetic electron acceptor dye, 2,6-dichlorophenol-indophenol in presence of the substrate, L-Malate, is used as an indication of the level of "activity" of the mitochondrial ETC. Homogenate (0.3ml), potassium phosphate buffer (1.6ml) and containing varying concentrations of MET (0.1ml) were incubated at 37^0C in a water bath for incubation times of 5 and 60 minutes, after which 1ml of the homogenate mixture was removed and incorporated with NAD (0.5mM), L-Malate (90 μM), DPI (1.5mM) and potassium phosphate buffer. This was inverted once to mix and the decrease in absorbance over a 5-minute period was read at 30 second intervals on a UV/VIS spectrophotometer at 600nm. All data is expressed as the rate of change in absorbance per minute at 600nm and corrected for appropriate controls.

4.2.3 RESULTS

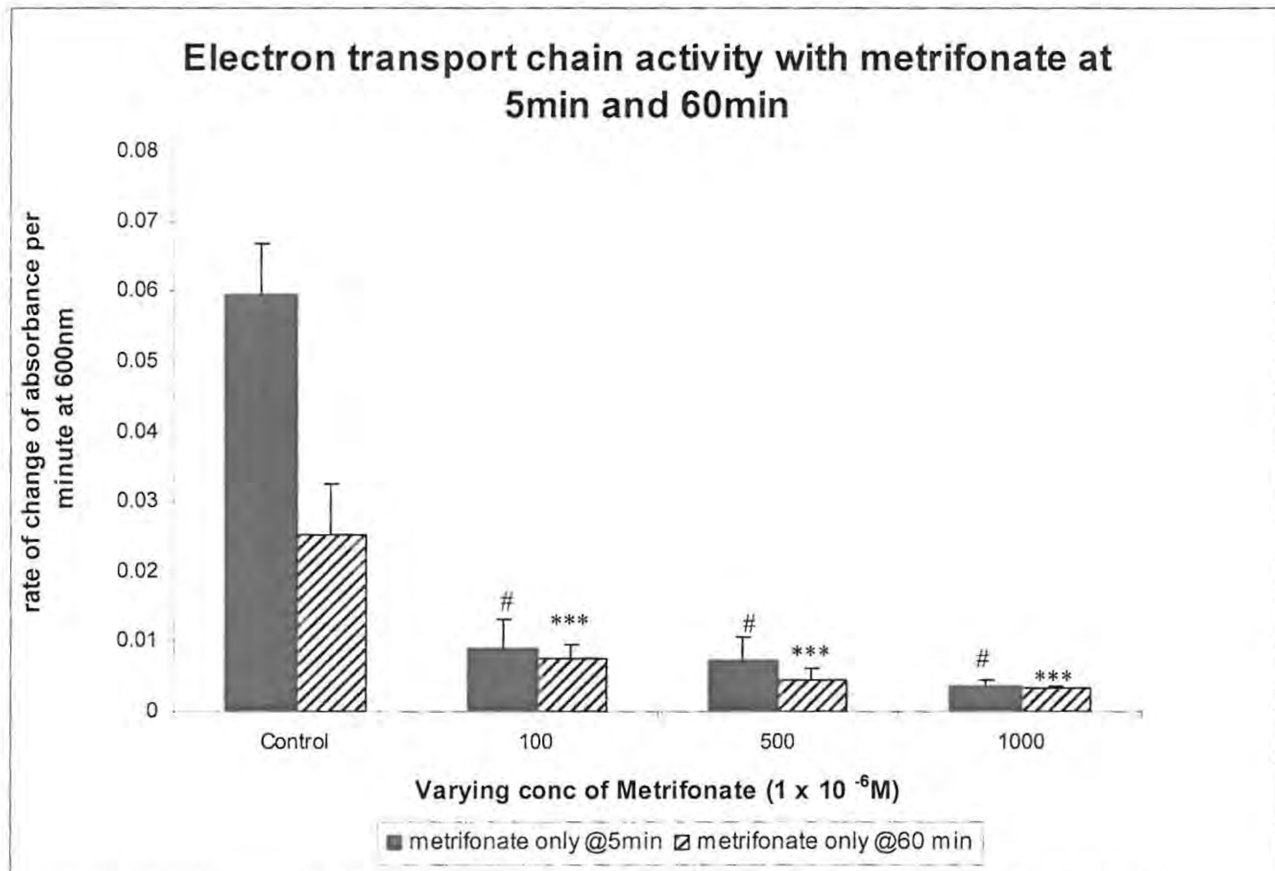


*Fig.4.2. The in vitro effect of metrifonate in varying concentrations (1 x 10 $^{-6}$M) on brain mitochondrial ETC utilizing L-Malate as the substrate, after 5 minutes and 60 minutes of incubation time. Data represents mean $\pm$ SEM and n=5.Student-Newman-Keuls Multiple Range Test applied. (*** $p<0.001$ vs. control for 5 min, $^{\#}p<0.05$ vs. control for 60 min)*

As shown above, as the concentration of MET increases with the 5 minutes of incubation there is a significant decrease in the ETC activity compared to the control which was incubated for 5 minutes. The same is apparent with the samples incubated for 60 minutes in comparison to the control incubated for 60 minutes. There is no significant difference between the varying concentrations at 5 minute and 60 minute incubation periods.

4.2.4 DISCUSSION

The significant decrease in ETC activity with the 5 and 60 minute incubation period, compared to the control, indicates that MET has a significant effect on the ETC activity. The lack of significance between the samples which had a 5 minute incubation and 60 minute incubation, could imply that the presence of MET reduces the ETC activity to a great degree within 5 minutes of incubation, that there is little ETC activity left.

Chemical substances which cause mitochondrial dysfunction and damage, lead to the depletion of ATP and increased production of ROS and therefore increased oxidative stress (Tsujimoto, 1997; Nicotera *et al.*, 1998; Delanty and Dichter, 1998; Taylor *et al.*, 1998; Murphy *et al.*, 1999). Certain organophosphates and carbamates possessing anticholinesterase activity prove to be neurotoxic (Wecker *et al.*, 1978; Gupta *et al.*, 1985; Misulis *et al.*, 1987; Gupta and Kadel, 1989; Yang *et al.*, 2000). This was supported by biochemical changes such as lactate dehydrogenase leakage and creatine kinase leakage. In a study in rats, when an organophosphate was administered, it induced status epilepticus and an impaired energy metabolism (a significant reduction in ATP) which lead to neuronal injury and neuronal loss, and ROS generation (Gupta *et al.*, 2000). This effect was reduced in the presence of an antioxidant, thus indicating that the production of ROS lead to the impaired energy metabolism.

In this experiment with MET, which is an organophosphate, a similar effect was observed, i.e. energy depletion or impairment of energy metabolism which may be due to the production of ROS. The production of ROS due to the presence of MET has been observed earlier in lipid peroxidation and superoxide generation.

4.3 EFFECT OF MELATONIN ON METRIFONATE-INDUCED REDUCTION OF ELECTRON TRANSPORT CHAIN ACTIVITY

4.3.1 INTRODUCTION

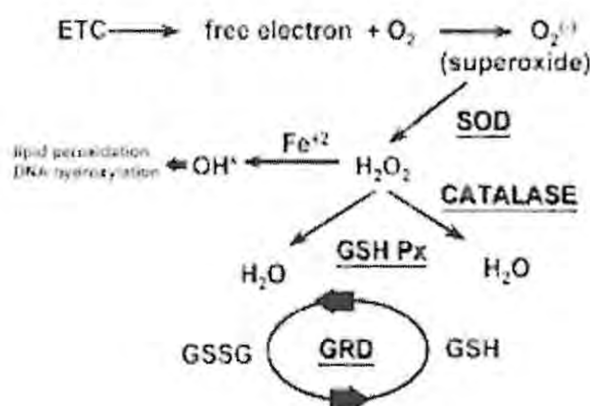


Fig.4.3. Production of reactive oxygen species by the mitochondrial electron transport chain and scavenging by nuclear-encoded antioxidant defenses. Abbreviations: ETC,electron transport chain; GRD, glutathione reductase; GSH Px, glutathione peroxidase. (Cassarino & Bennett, 1999)

Antioxidants exhibit promise in the prevention and treatment of those diseases which are associated with ETC disruption e.g. Alzheimer's disease and Parkinson's disease (Beal, 1995; Beal and Matthews, 1997; Harman 1996; Sano *et al.*, 1997; Schultz *et al.*, 1996). It has been generally accepted that superoxide anion generated by the mitochondrial respiratory transport chain are vectorially released into the mitochondrial matrix, where these are converted to hydrogen peroxide through the catalytic action of Mn-superoxide dismutase (Han *et al.*, 2001). Shown above in fig 4.3. are some of the superoxide scavenging mechanism that exist in the body.

The production of free radicals are counterbalanced by a complex, interdependent system of antioxidant defenses, including vitamins eg., vitamins A, C, and E , trace minerals Se, Zn, Mn, GSH, ubiquinone or coenzyme Q, NADPH, and melatonin (Ceballos-Picot *et al.*, 1996; Behl *et al.*, 1997; Lovell *et al.*, 1995; Southgate and Daya, 1999). Therefore, melatonin in the presence of MET and the subsequent effect of this combination on the ETC will be investigated, which may confirm that the damage occurring in the presence of MET may be due to generation of ROS.

4.3.2 MATERIALS AND METHODS

4.3.2.1 Animals

Adult male, Wistar rats, weighing between 250 and 300g were used for the experiment, and were housed and maintained under the conditions described in section 2.2.2.1.

4.3.2.2 Chemicals and Reagents

All reagents were of the highest quality available. NAD, 2, 6 – diclorophenol-indophenol (DPI), L-Malate, Metrifonate (MET) and Melatonin (MEL) were purchased from Sigma Chemical Corporation, St. Louis, MO, U.S.A. Sucrose was purchased from Saarchem (PTY) Ltd, Krugersdorp, South Africa. Melatonin was dissolved in 30% ethanol for the purposes of the experiment. All other reagents were prepared in 0.1M potassium phosphate buffer, pH 7.4.

4.3.2.3 Isolation of Mitochondria from rat brain

A mitochondrial suspension was prepared as described in section 4.2.2.3

4.3.2.4 Biological Oxidation Assay

A modification of the spectrophotometric method described by Plummer (1971) was employed. The rate of reduction of the synthetic electron acceptor dye, 2,6-dichlorophenol-indophenol (DPI) in presence of the substrate, L-Malate, is used as an indication of the extent of "activity" of the mitochondrial ETC. Homogenate (0.3ml) and potassium phosphate buffer (1.5ml) mixtures containing varying concentrations of MEL (0.1ml) and 1mM MET (0.1ml) were incubated at 37°C in a water bath for incubation times of 5 and 60 minutes, after which 1ml of the homogenate was removed and incorporated with NAD (0.5mM), L-Malate (90 μM), DPI (1.5mM) and potassium phosphate buffer. This was inverted once to mix solutions and the decrease in absorbance over a 5-minute period was read at 30 second intervals on a UV/VIS spectrophotometer at 600nm. All data is expressed as rate of change in absorbance per minute at 600nm and corrected for appropriate controls.

4.3.3 RESULTS

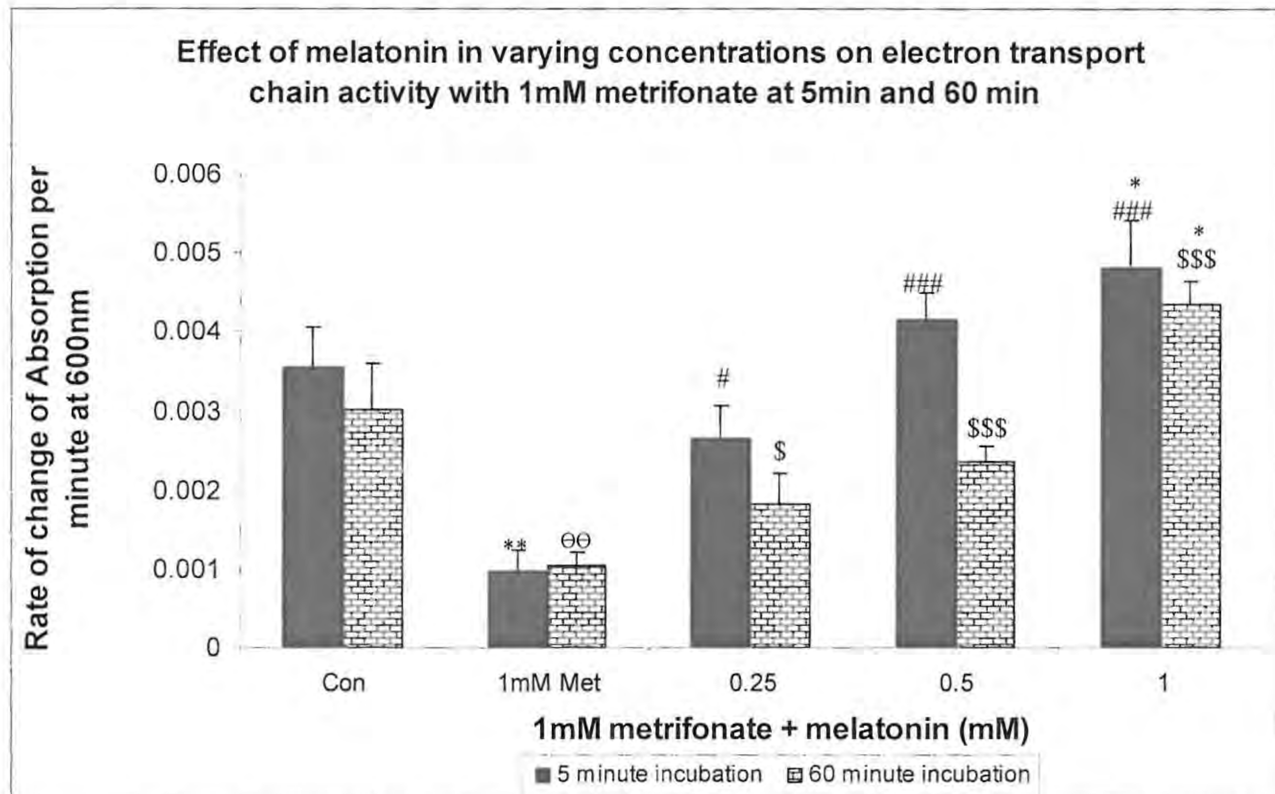


*Fig.4.4 The in vitro effect of Metrifonate(1mM) and Melatonin in varying concentrations (mM) on brain mitochondrial ETC utilizing L-Malate as the substrate, after 5 minutes and 60 minutes of incubation time. Data represents mean ± SD and n=5. Student-Newman-Keuls Multiple Range Test applied. (** $p<0.01$ vs. control for 5min; $^{\#}p<0.05$ vs. 1mM MET only at 5min.; $^{\#\#\#}p<0.001$ vs. 1mM MET only at 5min. $^{\Theta\Theta}p<0.01$ vs. Control at 60min. $^{\$}p<0.05$ vs. 1mM MET only at 60 min. $^{\$\$\$}p<0.001$ vs. 1mM MET only at 60min; *$p<0.05$ vs. control for 5min and 60min).*

These results show that there are significant differences between the activity of the ETC of the sample with 1mM MET only and those samples with 1mM MET and varying concentrations of MEL. The ETC activity in the 1mM MET decreases significantly in comparison to the control at both 5min and 60min. However in comparison to 1mM MET only, the samples incubated with 1mM MET and MEL increase in ETC activity. There were no significant differences between the samples with regard to the incubation times i.e. the results show that there is no influence of incubation times in the 1mM MET and varying concentrations of MEL and 1mM MET only when compared at 5min and 60min. There is a significant difference between the control and 1mM MEL and 1mM MET at 5min and 60min respectively.

4.3.3 DISCUSSION

Mitochondria are present in every cell and provide energy in the form of ATP (Orth and Schapira, 2002) via the ETC. In comparison to 1mM MET only, the samples incubated with 1mM MET and MEL showed an increase in ETC activity. Melatonin, a potent antioxidant, (Tan, *et al.*,1993) has been reported to improve the functioning of the ETC activity at the level of mitochondrial respiratory complexes I and IV, thus promoting electron flux through the ETC, with a resultant increase in ATP production (Martin *et al.*, 2002). The ETC activity decreased to below control in 1mM MET only, yet above this level in the varying concentration of MEL and 1mM MET, implying that MET decreases ETC activity. This is being counterbalanced with improved ETC functioning by the MEL. Since MEL affects complex I and IV (Martin *et al.*, 2002) and reducing the decrease of the ETC activity of MET, there may be a possibility that MET has an effect at complex I or IV level. However, complex I is the major site of superoxide anion generation (Hasegawa, 1995), which has been shown earlier with superoxide anion generation. Therefore, this in conjunction with the decrease in ETC activity without MEL is a stronger indication that MET may be exerting an effect at the level of complex I. Further investigation of the major site of ROS production (complex I), in the presence of MET needs to be explored.

[B] COMPLEX ONE

4.4 INTRODUCTION

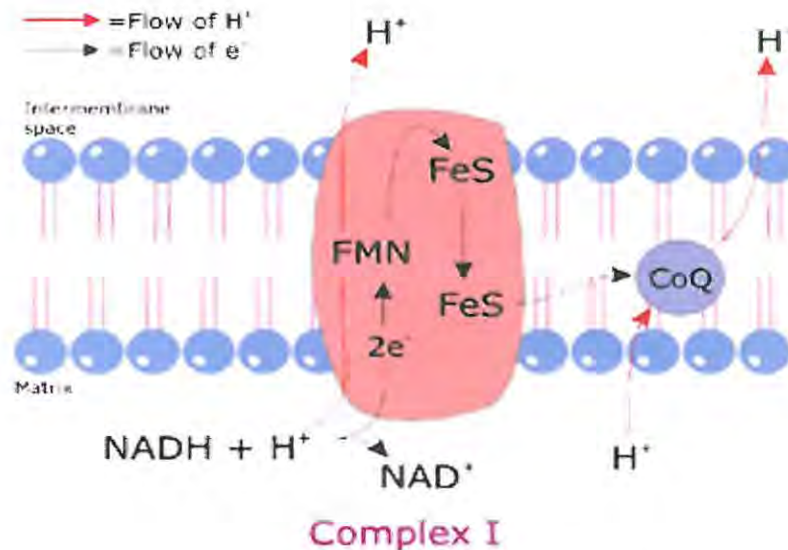


Fig.4.5. Represents the NADH dehydrogenase complex (also called NADH-Q reductase) is the largest of the respiratory enzyme complexes; it accepts electrons from NADH and passes them through a flavin and at least five iron-sulfur centers to coenzyme-Q. The binding of NADH and the transfer of its two high-potential electrons to the flavin mononucleotide (FMN) prosthetic group of this complex to give the reduced form, $FMNH_2$ is the initial step. Electrons are then transferred from $FMNH_2$ to a series of iron-sulfur clusters (abbreviated as Fe-S), the second type of prosthetic group in NADH-dehydrogenase. Iron atoms in these Fe-S complexes cycle between Fe^{2+} (reduced) and Fe^{3+} (oxidized) states. Electrons in the iron-sulfur clusters of NADH-dehydrogenase are then shuttled to ubiquinone, also known as coenzyme-Q (CoQ) . Ubiquinone is reduced to a free-radical semiquinone anion by the uptake of a single electron. Reduction of this enzyme-bound intermediate by a second electron yields ubiquinol (QH_2). In a nutshell, the NADH: Ubiquinone reductase activity of complex I catalyses the oxidation of NADH + H^+ to NAD^+ with the ultimate reduction of ubiquinone to ubiquinol with the help of the prosthetic groups.

(hem.passagen.se/hazard1/ projekt/komplex1.htm)

(http://hem.passagen.se/hazard1/projekt/komplex1.htm)

Shown above in figure 4.5., is the activity of complex one in the electron transport chain.

A variety of cellular reactions occurring in the mitochondria can lead to the formation of superoxide anions, hydroxyl radicals and hydrogen peroxide. These reactive oxygen species can cause oxidative stress which are consequently damaging to cells. Complexes I and III in particular are associated with superoxide production. Inhibition of these complexes results in increased free radical production (Orth and Schapira, 2002).

Late onset of neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, appear to be sporadic. However, biochemical evidence has accumulated that the mitochondrial ETC is defective in these diseases (Cassarino and Bennett, 1999). In particular, in PD, NADH: ubiquinone oxidoreductase, or complex I, of the ETC is defective (Beal *et al.*, 1993; Bindhoff *et al.*, 1991; Oblin *et al.*, 1994) and in AD, cytochrome C oxidase, or complex IV, is defective (Kish *et al.*, 1992; Mutisya *et al.*, 1994; Parker *et al.*, 1990).

Tissues with high energy demands, such as brain, skeletal and cardiac muscle contain the greatest number of mitochondria (Orth and Schapira, 2002). The superoxide anion radical formed during ubisemiquinone autoxidation and, secondarily, NADH dehydrogenase activity, is considered the stoichiometric precursor of mitochondrial H_2O_2 (Boveris *et al.*, 1976; Boveris and Cadenas, 1975; Turrens, and Boveris, 1980; Cadenas, *et al.*, 1977).

Mitochondria contain the only source of extranuclear DNA. Each mitochondrion harbours 2–10 molecules of mitochondrial DNA (mtDNA) which encodes 22 transfer RNAs (tRNAs) and 12S and 16S ribosomal RNA as well as 13 proteins, all part of the respiratory chain and oxidative phosphorylation system (OXPHOS). Of these 13 polypeptides, seven are sub-units of complex I, one is a sub-unit of complex III, three are sub-units of complex IV and two are sub-units of complex V. The remaining 70 sub-units of OXPHOS are encoded by the nucleus and imported into mitochondria. The OXPHOS complexes are located on the inner mitochondrial membrane. Of the five complexes, complex I (NADH CoQ_0 reductase) is the largest (Orth and Schapira, 2002).

Mitochondria are highly active metabolically, producing ATP from oxygen and generating free radicals in the form of superoxide ions. Reactive oxygen species are thought to be responsible for the high mutation rate of mtDNA (Shenkar et al., 1996) which have been associated with human diseases. There is now evidence indicating that superoxide anion formation is generated by the electron transport chain of mitochondria, probably at the coenzyme Q site (Hasegawa et al 1995).

4.5 EFFECT OF METRIFONATE ON COMPLEX ONE ACTIVITY OF THE ELECTRON TRANSPORT CHAIN

4.5.1 INTRODUCTION

The measurement of activity of individual respiratory chain complexes are important in the investigation of diseases with mitochondrial dysfunction especially since there is now evidence indicating that superoxide anion generation ($O_2^{\cdot-}$) is generated by the electron transport chain of mitochondria, probably at the coenzyme Q site (Hasegawa et al 1995).

Since MET is able to produce an increase in superoxide anions and a subsequent rise in lipid peroxidative products, the effect on the ETC was investigated. The ETC activity decreased in the presence of MET, and this decrease was counterbalanced by the presence of a ROS scavenger (MEL) which has been reported to improve ETC functioning (Martin *et al.*, 2000). This indicates that MET may be disrupting the ETC and producing ROS. The major site for ROS production is complex I. Therefore, the effect of MET on complex one was examined. The presence of an antioxidant (MEL) was not employed in this assay, as it is known to improve functioning of the ETC at the level of Complex I and the identification of the source of ROS in the presence of MET was to be elucidated.

4.5.2 MATERIALS AND METHOD

4.5.2.1 Animals

Adult male, Wistar rats, weighing between 250 and 300g were used for the experiment, and were housed and maintained under the conditions described in section 2.2.2.1.

4.5.2.2 Chemicals and Reagents

All reagents were of the highest quality available. MET, antimycin, 2,3-dimethoxy-5-methyl-1,4-benzoquinone (CoQ_0), NADH and Tris were purchased from the Sigma Chemical Corporation, St Louis, MO, USA. Potassium cyanide, magnesium chloride and sucrose were purchased from Saarchem, Merck Laboratory Supplies (Pty) Ltd. Gauteng, South Africa. Defatted bovine serum albumin was purchased from Boehringer Mannheim, Germany.

4.5.2.3 Isolation of Mitochondrial P^2 Fraction from rat brain

This mitochondrial fraction was prepared by differential centrifugation as described by Mitra (Mitra *et al.,* 1994). The rats were killed by cervical dislocation, and the brains were rapidly excised and homogenized in ice cold 0.32M sucrose in 10mM potassium phosphate buffer pH 7.2, in a manual glass-teflon homogenizer, yielding a 10% w/v homogenate. This homogenate was centrifuged at 1500xg for 10 minutes at 4^0C. The pellet was discarded and the supernatant centrifuged at 10000xg for 30minutes at 4^0C to separate and remove cell debris from the mitochondria. The supernatant was discarded, the pellet re-suspended ice cold 50mM Tris in potassium phosphate buffer, pH 7.2 (1:1 v/v) and re-centrifuged at 10000xg at 4^0C for 30minutes. The supernatant obtained was discarded and the pellet re-suspended in ice cold 10mM potassium phosphate buffer, pH 7.2. The suspension was then sonicated for uniform dispersion of the pellet and completion of mitochondrial lysis. This suspension was used in the determination of complex I activity, in the presence of MET.

4.5.2.4 Complex I (NADH: ubiquinone oxidoreductase) Assay

The complex I activity was measured, using a method described by Schults (Shults *et al.*, 1995) with minor modifications, by following the decrease in absorbance due to the oxidation of NADH at 340nm. The buffer utilised for complex I was 10mM potassium phosphate buffer, at pH 7.2. The assay buffer was prepared containing buffer, 5mM KCN, 5mM $MgCl_2$, 2.5mg/ml defatted bovine serum albumin, and 2ug/ml antimycin. An aliquot of the submitochondrial suspension (mitochondrial protein 30 to 50ug diluted in buffer) and 0.05mM co-enzyme Q_0 were added to the assay buffer. The total assay volume was maintained at 1ml. The reaction mixture was incubated at 30^0 C for 2 minutes. To this, the reaction was initiated by the addition of NADH (0.12mM final concentration) and the decrease in absorbance was monitored at 340nm using a spectrophotometer. The difference in absorbance between samples with NADH to those without was calculated. The assay was then performed with various concentrations of MET (uM). The complex I activity was expressed as nanomoles of NADH oxidised/min/mg protein at 340nM.

4.5.2.5 Protein determination

The protein concentration of the mitochondrial suspension was determined using the method described by Lowry et al., 1951. A standard curve was generated using BSA as a standard at concentration intervals of 60ug/ml (Appendix 2).

4.5.3 RESULTS

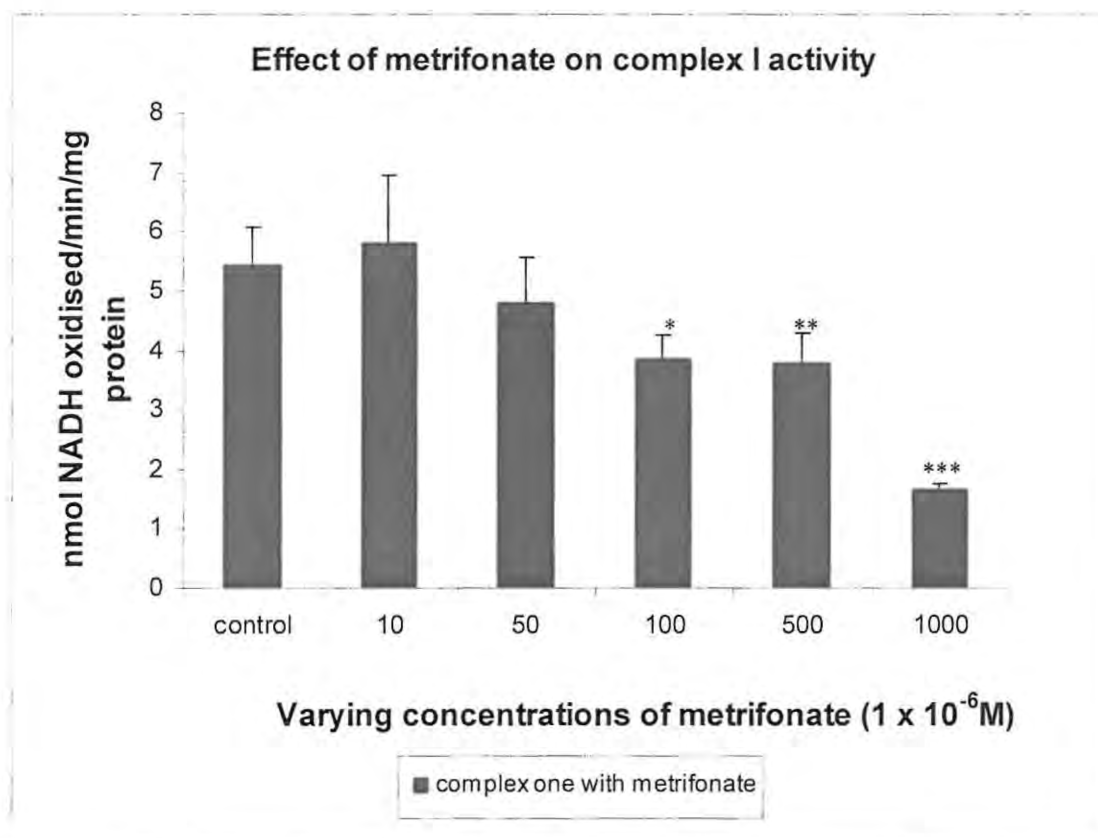


*Fig.4.6. The in vitro effect of Metrifonate in varying concentrations (uM) on complex I of rat brain homogenate. Data represents mean ± SEM and n=5. Student-Newman-Keuls Multiple Range Test applied. (***p<0.001 vs. control, **p<0.01 vs. control and *p<0.05 vs. control).*

From the above results, MET at lower concentrations (10μM and 50μM) have no significant effect on complex I activity. However at higher concentrations, 100, 500 and 1000μM respectively, there is a significant effect on complex I activity in comparison to the control. It is greatly reduced to less than fifty percent as compared to the control.

4.5.4 DISCUSSION

Almost all intracellular ATP is known to be generated in the mitochondria and about one third of cellular adenine nucleotides are found in this organelle (Pedersen, 1999). Therefore chemicals causing mitochondrial dysfunction or damage, leading to the depletion of ATP, excessive generation of ROS, resulting in oxidative stress (Delanty and Dichter, 1998; Tsujimoto, 1997; Murphy *et al.*, 1999; Nicotera *et al.*, 1998). Some organophosphates have been used as insecticides due to their action on complex I activity (Lummen, 1998).

The results imply that MET, in higher concentrations, has the ability to disrupt complex I activity, which ultimately results in cell death, due to impaired energy production. The results from the previous experiments show that the tissue exposed to MET undergoes superoxide generation, lipid peroxidation, and disruption of the ETC activities. From this experiment, it may be deduced that the root cause of the ETC disruption, ROS generation, and lipid peroxidation stems from the disruption of complex I activity. Fortunately, this disruption only occurs at a very high concentration, levels to which MET are not able reach in the brain. The levels reached may be sufficient to increase superoxide formation and lipid peroxidation, which can be offset due to the presence of antioxidant systems.

CHAPTER FIVE - ORGAN CULTURE STUDIES

THE EFFECT OF METRIFONATE ON RAT PINEAL INDOLE METABOLISM

5.1 INTRODUCTION

The pineal gland is no longer an insignificant phylogenic relic; it has been recognized as a functional organ which is an important and integral part of the neuroendocrine system (Reiter, 1989). The secretory products that the pineal produces are released into the systemic circulation and exert an effect on several endocrine organs (Kappers, 1976).

One of the most important indoleamines predominantly synthesized from tryptophan in the pineal gland is melatonin (Reiter, 2000). Serotonin N-acetyltransferase (NAT) catalyses the conversion of serotonin to N- acetylserotonin and is known as the rate-limiting enzyme in the biosynthetic pathway of melatonin (Ellison *et al.*, 1972). Pineal gland and retina are the primary sites of melatonin production and NAT expression. Therefore, regulation of NAT has been studied in these tissues i.e. the pineal gland and retina (Krause and Dubocovich, 1990; Yuwiler, 1989; Borjigin *et al.*, 1995). Melatonin synthesis is under rhythmic control (Reiter, 1987) which peaks at night. Apart from its potent free radical scavenging properties which directly protect the cell from ROS damage (Tan *et al.* 1993), it also stimulates the synthesis of antioxidant enzymes by an unknown mechanism (Albarran *et al.*, 2001), for example, superoxide dismutase, glutathione reductase and gluthione peroxidase (Pablos *et al.*, 1998; Albarran *et al.*, 2001) which also protect the cell from free radical damage, thus exerting a further indirect protective activity.

In patients suffering with Alzheimer's disease (AD), there are decreased levels of peripheral melatonin and improvement of insomnia and circadian rhythms is observed in these patients when administered melatonin. (Monti and Cardinali, 2000). If the levels of melatonin decrease, so does the anti-oxidizing neuroprotective effect. Therefore, any medication administered in the symptomatic treatment of AD should not decrease or inhibit the synthesis of melatonin.

A technique which allows the quantification of and monitoring of metabolites which are undergoing biochemical and pharmacological manipulations has to be sensitive, reproducible and replicate or imitate normal physiological conditions as much as possible.

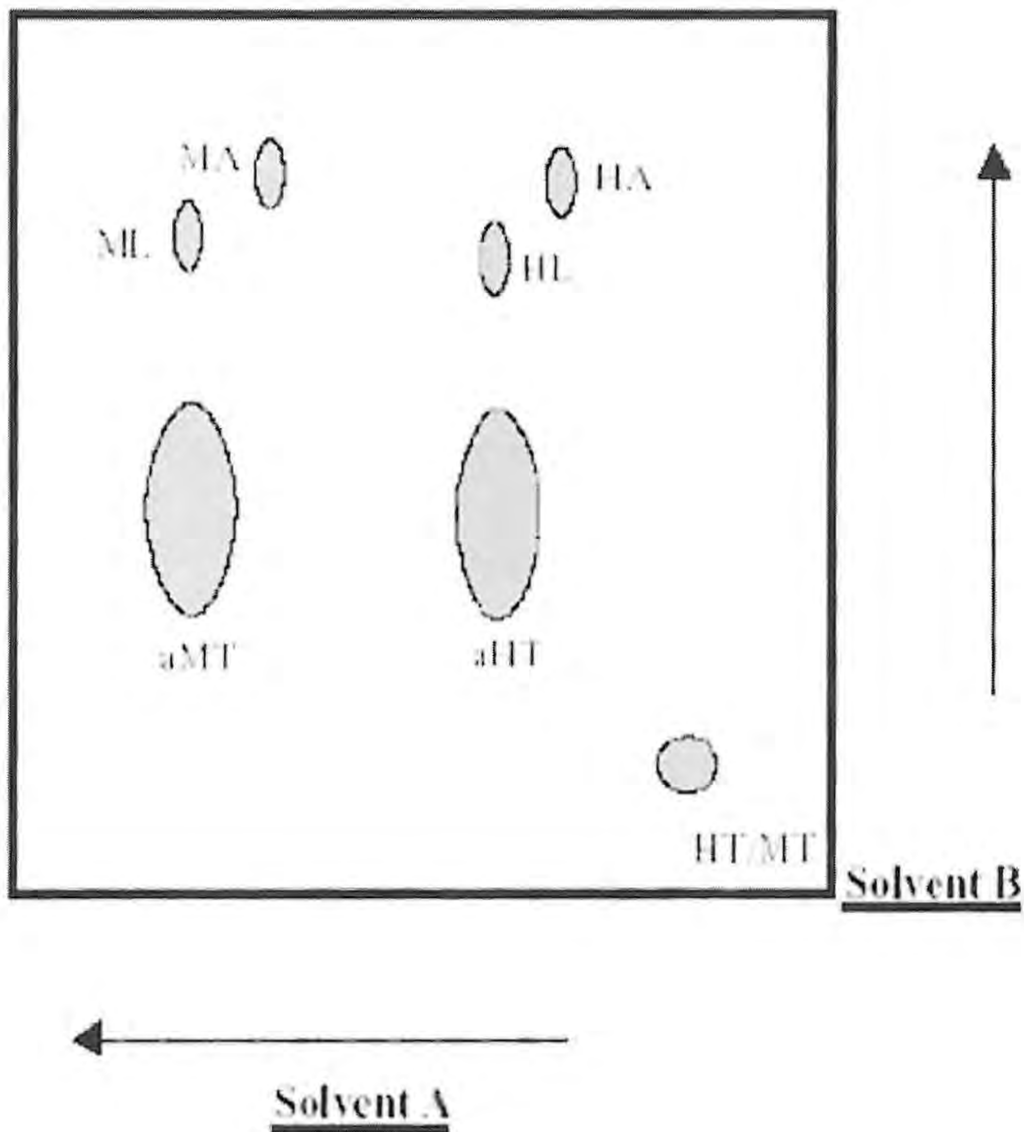
The size and accessibility of the rat pineal gland, makes it suitable for the intact organ culture technique. Previously, a number of the developed techniques had disadvantages in that these were not sensitive enough to allow the use of a single gland, or the results obtained were not representative of all metabolites (Morton, 1990). This technique is useful since it eliminates the complexities of organ or *in vivo* interaction, and allows for direct pharmacological manipulation Thus this technique has been utilized and optimized by a number of researchers (Klein and Rowe, 1970; Daya *et al*, 1989). The pineal gland in organ culture is able to remain viable for six days under optimal conditions and maintain its metabolic function thereby synthesizing various indole metabolites. It is able to utilize exogenous radioactive precursor (C^{14}) serotonin to produce the various indoles associated with pineal metabolism, including the neurohormone melatonin (Daya *et al.*, 1989). As much as 95% of the synthesized indoles are secreted into the culture medium which can then be isolated and quantified.

5.2 ORGAN CULTURE TECHNIQUE

5.2.1 THEORY OF ASSAY

The technique employed involves the separation of the radioactive indoles via a bi-dimensional thin layer chromatography (TLC) system, using organic solvents as the mobile phases, as can be seen in fig.... The first solvent system (A) which consists of consists of chloroform:methanol:glacial acetic acid in the ratio 97:3:1 and developed twice in this direction, separates Melatonin (MEL) from N-acetylserotonin (aHT), and the 5-hydroxyindoles from the 5-methoxyindoles. The second solvent system (B) consists of ethyl acetate and separates 5-methoxyindole acetic acid (MA) and 5-methoxytryptophol (ML) from MEL and 5-hydroxyindole acetic acid (HA) and 5-hydroxytryptophol (HL) from aHT. The serotonin (HT) and 5-methoxytryptamine (MT) are not affected by these solvents and therefore remain at the origin (Klein and Notides, 1969).

The radioactivity of the radioactive indoles is then quantitated by radiospectometry. This is a modified technique described by Klein and Notides (Klein and Notides, 1969).



aHT - N-acetylserotonin
MEL - Melatonin
HA- 5-hydroxyindole acetic acid
HL-5-hydroxytryptophol

HT - serotonin
MA-5-methoxyindole acetic acid
MT - 5-methoxytryptamine
ML-5-methoxytryptophol

Fig.5.1. An illustration of the bi-dimensional thin layer chromatogram of the pineal indole metabolites showing the direction in which the plate was run and the location of the pineal indole metabolites (Klein & Notides, 1969).

The trace of the TLC plate above (fig.5.1) indicates the directions in which the solvents were run and the subsequent location of the metabolites. The thin layer chromatographic technique is rapid, simple, and effectively separates trace quantities of the pineal indoles.

5.2.2 SYNTHESIS OF SEROTONIN (5- HYDROXYTRYPTAMINE)

Tryptophan is hydroxylated to 5-hydroxytryptophan via tryptophan hydroxylase, which is present in high concentrations in the pineal gland, and while this appears to be the rate-limiting step in the synthesis of serotonin (Lovenberg *et al*, 1968), it is the amount of substrate, tryptophan, available and not the enzyme activity, which influences the rate of this reaction (Deguchi and Barchas, 1972; Bensinger *et al.* 1974). The 5-hydroxytryptophan in the presence of an aromatic L-amino acid decarboxylase is dehydroxylated to 5-hydroxytryptamine viz. serotonin. Adrenergic stimulation may depress the activity of this enzyme since, exposure to constant lighting increases the activity of this enzyme and this increase in activity in response to the constant light is mimicked by the removal of the sympathetic nerves (Snyder *et al.*, 1965). This nonspecific enzyme is found in all tissues, but in high concentrations in the pineal gland (Christenson *et al.* 1972).

5.2.3 CONVERSION OF SEROTONIN TO ITS METABOLITES

Serotonin may be metabolised in one of two pathways which result in either the formation of deaminated compounds or N-acetylated compounds as can be seen in fig.5.2.

Deamination of serotonin via monoamine oxidize enzyme results in 5-hydroxyl-indoleacetylaldehyde, which is highly unstable and is undergoes immediate oxidation or reduction. It oxidizes to form the corresponding 5-hydroxyindoleacetic acid via the enzyme aldehyde dehydrogenase. It is reduced to 5-hydroxytryptophol by alcohol dehydrogenase (Winters *et al.*, 1977; Morrissey *et al.*, 1978). The 5-hydroxy groups then undergo O-methylation to form corresponding 5-methoxyindoleacetic acid and 5-methoxytryptophol.

Serotonin may also be N-acetylated to form N-acetyl serotonin via the N-acetyltransferase (NAT). The activity of the enzyme NAT increases from 30 fold to 70 fold at night, and is in most circumstances, the rate-limiting step in melatonin synthesis (Klein *et al.*, 1997). The 5-hydroxygroup of N-acetyl serotonin is O-methylated via hydroxy-O-methyltransferase to form melatonin. This enzyme transfers the methyl group from a co-factor, S-adenosyl methionine to N-acetyl serotonin.

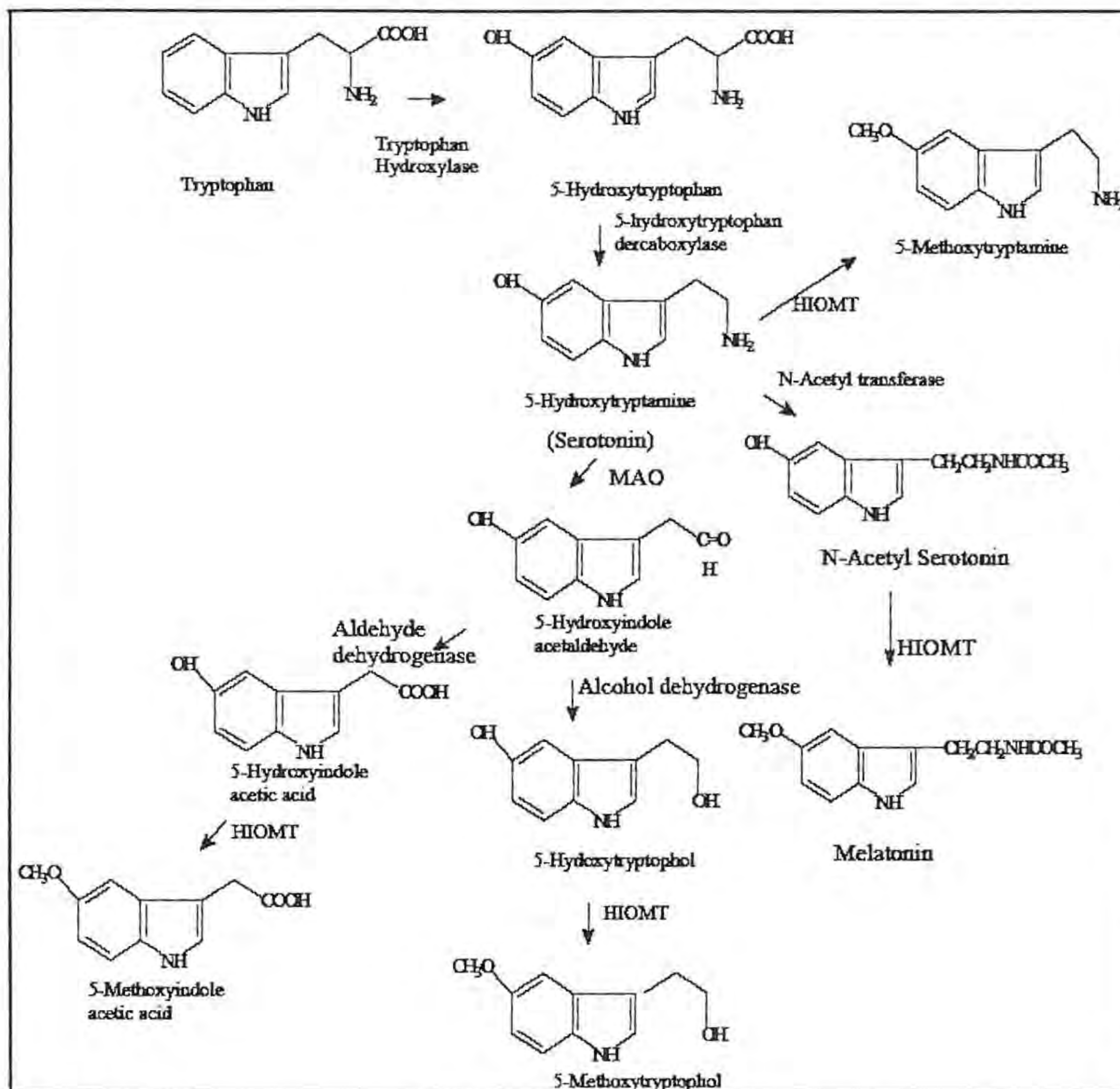


Fig.5.2. Represents a schematic diagram of the Pineal Indole Metabolism (Young & Silman, 1982). (The abbreviations used to represent metabolites shown in diagram above: aHT - N-acetylserotonin; HT – serotonin; MEL- Melatonin; MA-5-methoxyindole acetic acid; HA- 5-hydroxyindole acetic acid; MT - 5-methoxytryptamine; HL-5-hydroxytryptophol; ML-5-methoxytryptophol).

5.3 MATERIALS AND METHODS

5.3.1 Animals

Male Wistar rats weighing 250g-300g were used in the experiment. The animals were maintained as described in section 2.2.2.1. The animals were sacrificed by cervical dislocation and decapitation. Using a pair of scissors, an incision was made through the bone from the foramen magnum to the area near the orbit of the eyes. A clean sterile dissecting forceps was inserted into the incision and the skull and adhering meninges lifted and folded back. The brain was gently peeled away from the dura and the skull, exposing the pineal gland. The pineal gland and stalk was removed.

5.3.2 Chemicals and Reagents

Serotonin (with specific activity 55mCi/ml - C^{14}) was obtained from Amersham International, England. The culture medium, BGJb culture medium, was purchased from Gibco, Europe, and fortified with the antibiotics streptomycin and benzyl penicillin (Novartis, South Africa). The composition of this medium is represented in Table 5.1 . The aluminium (10cm x 10cm) TLC plates coated with silica gel 60, Type F254 (0.25mm), were purchased from Merck, Darmstadt, Germany. Beckman Ready-Sol multipurpose liquid scintillation fluid was purchased from Beckman RIIC Ltd, Scotland. The indole standards, MT, HA, HL, MA, ML, MEL, and aHT and Metrifonate were purchased from Sigma Chemical Co, St Louis, MO, USA. All other reagents and chemicals were obtained locally and were of the highest purity available.

5.3.3 Organ Culture of the Pineal Glands

The pineal glands were placed individually into sterile 75×10mm Kimble tubes containing 52µl BGJb culture medium (contents as seen in table 5.1) and 10µl of 7 x 10^{-5} M Metrifonate (MET) dissolved in medium. Then 8µl serotonin (specific activity 55mCi/ml C^{14}) was added to each tube. The final concentration of MET in the 70µL of culture medium was 1 x 10^{-5}M. The tubes were then saturated with carbogen and sealed. The tubes were the placed at 37^0 C in the dark for 24 hours in a Forma Scientific model 3028 incubator. At the end of the 24 hour incubation period, the reaction was terminated by the removal of the pineal glands from solution.

5.3.4 Separation of Indoles by Thin Layer Chromatography

Separation of the radio labelled indoles was carried out by modifying a technique employed by Klein and Notides (1969). Activation of the TLC plates was achieved by placing them in an oven at 70^0C for 60 minutes. The standard solution was prepared as follows: 0.1mg of each standard indoleamine was dissolved together in a test tube containing 95% ethanol and 1% ascorbic acid (which serves as an antioxidant). Ten µl of the culture medium was spotted on a 10×10cm TLC. Plate which was dried using a gentle stream of nitrogen. Spots of 10µl standard solution was superimposed onto the culture medium which was also dried using a gentle stream of nitrogen. It is important to note that the plates were spotted in subdued light to prevent photo-oxidation of the indoleamines. The spotted plates were then placed in a TLC tank containing chloroform: methanol: glacial acetic acid (93:7:1). The plates were developed twice in this direction, and were allowed to develop until the solvent front had migrated approximately 9cm. The plates were then allowed to dry, after which they were developed once in ethyl acetate. This was done at right angles to the first

direction of development. Following this the plates were dried. The spots were observed and marked under U.V light, then cut out and individually placed in into scintillation vials to which 1ml absolute ethanol and 3ml Beckman Ready-Sol scintillation fluid was added. The vials were vortexed on a Vortex Rotor-mixer for 30 seconds, and the radioactivity of each metabolite was then measured in a Beckman LS 2800 scintillation counter.

Table 5.1 : Composition of the BGJb culture medium

CONTENTS	CONCENTRATION (mg/litre)
1.6 AMINO ACIDS	
L-Alanine	250.0
L-Arginine	175.0
L-Aspartic acid	150.0
L-Cysteine (HCL)	90.0
L-Glutamine	200.0
Glycine	800.0
L-Histidine	150.0
L-Isoleucine	30.0
L-Leucine	50.0
L-Lysine (HCL)	240.0
L-Methionine	50.0
L-Phenylalanine	50.0
L-Proline	400.0
L-Serine	200.0
L-Threonine	75.0
L-Tryptophan	40.0
DL-Valine	65.0
1.7 VITAMINS	
α-Tocopherol phosphate	1.0
Ascorbic acid	50.0

Biotin	0.2
Calcium pantothenate	0.2
Choline chloride	50.0
Folic acid	0.2
Inositol	0.2
Para-aminobenzoic acid	2.0
Pyridoxal phosphate	0.2
Riboflavin	0.2
Thiamine HCl	4.0
Vitamin B12	0.04
1.8 INORGANIC SALTS	
Dihydrogen sodium ortho-phosphate	90.0
Magnesium sulphate	200.0
Potassium Chloride	400.0
Potassium dihydrogen phosphate	160.0
Sodium bicarbonate	3500.0
Sodium chloride	5300.0
1.9 OTHER COMPOUNDS	
Calcium lactate	555.0
Glucose	10000.0
Phenol red	20.0
Sodium acetate	50.0

5.4 RESULTS

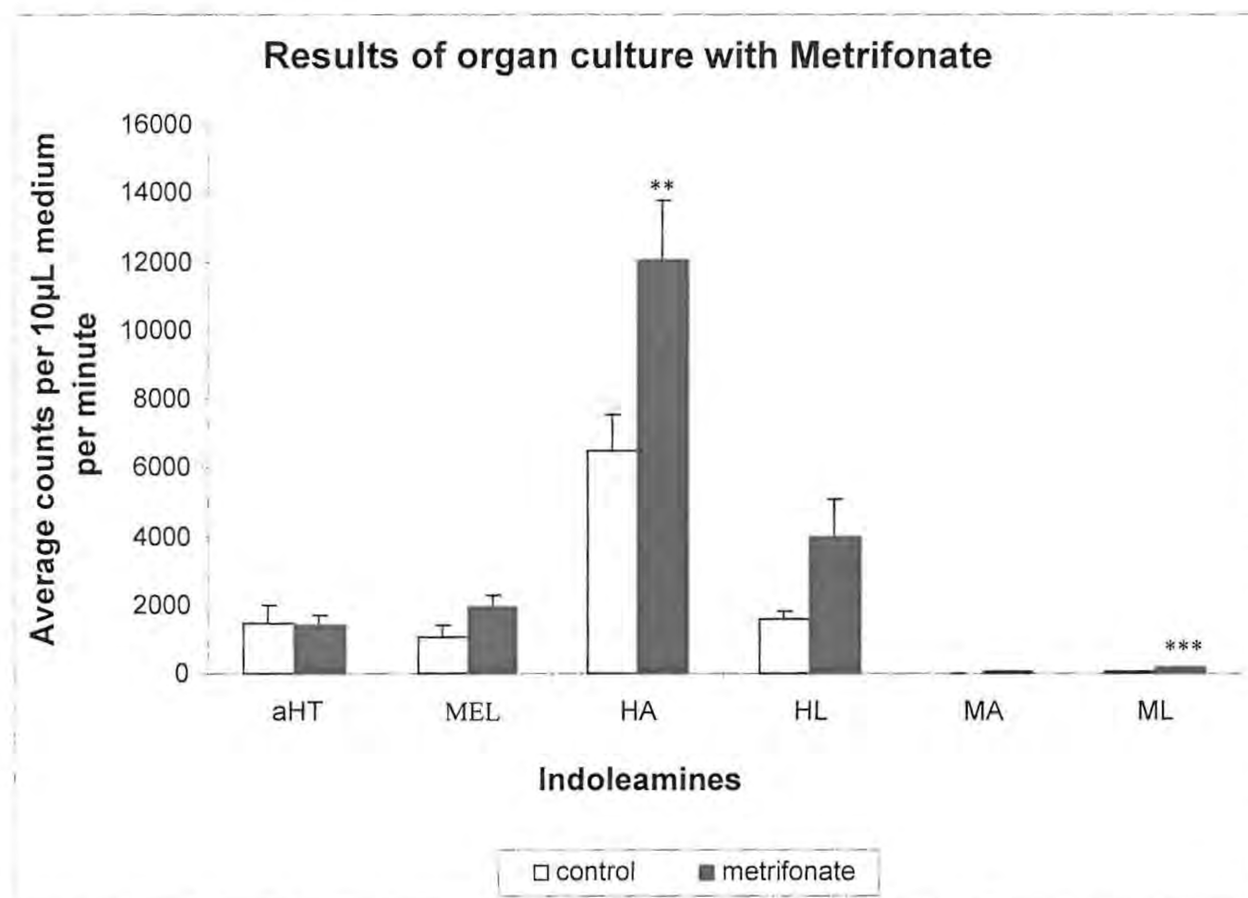


*Fig.5.3. The effect of metrifonate on pineal indole metabolism. Each bar represents the mean ± SEM; n = 5.(***p < 0.001 vs. control and ** p < 0.01 vs. control – Student Newman-Keuls test)*

A typical bi-dimensional thin layer chromatogram of the pineal indole metabolites was obtained which is shown in Fig.5.3. The results were obtained as counts per minute (CPM) and each indole metabolite was expressed in terms of the average of total counts per minute per 10µl medium. From the results obtained, there was only a significant increase in the deaminated products, HA and ML.

5.5 DISCUSSION

In the mammalian pineal gland, the driving force behind melatonin production is noradrenaline release from the sympathetic nerves originating in the superior cervical ganglia. Stimulation of α_1 and β_1 adrenoreceptors by noradrenaline results in an increased activity of NAT, the rate-limiting enzyme in the biosynthesis of melatonin. However, there is accumulating evidence for parasympathetic modulation of melatonin production as well (Drijfhout *et al.*, 1996; Laitinen, 1995). The presence of acetylcholinesterase in pineal nerve terminals has been reported rabbit (Romjin, 1973), gerbil (Moller and Korf, 1983), guinea pig (Luo *et al.*, 1990) and rat (Rodriguez de Lores Arnaiz and Pellegrino de Iraldi, 1972).

There have been reports whereby the presence of the parasympathetic tonus under physiological conditions was tested with the acetylcholinesterase inhibitor, neostigmine, which resulted in a gradual decrease of aHT and MEL and only a slight, non-significant increase in serotonin (Drijfhout *et al.*, 1996). Following the administration of cholinesterase inhibitors including carbamates (physostigmine) and organophosphate including soman, brain serotonin turnover increased which was gauged by the levels of the serotonin metabolite 5-hydroxyindoleacetic acid (Prioux-Guyonneau, Coudray-Lucas, *et al.*, 1982; Pscheidt, *et al.,* 1967; Kleinrok, *et al.*, 1975; Haubrich and Reid, 1972; Rausch and Janowsky, 1985)

However, other anticholinesterase inhibitors, namely the organophosphate insecticide, parathion, has been shown to increase night time NAT in the pineal gland and increase melatonin levels (Attia, *et al.*, 1995a) but this effect is speculated to occur via sympathetic innervations to the pineal gland as this effect was blocked by beta adrenergic receptor blockers (Attia, *et al.*, 1995b)

It would appear as though MET has a similar effect to physostigmine and soman in that there is an increase in 5-hydroxyindoleacetic acid. There is also an increase in 5-methoxytryptophol. Overall, there is an increase in the deaminated products rather that the N-acetylated products.

CHAPTER SIX - HISTOLOGICAL STUDIES

6.1 INTRODUCTION

Changes in enzymatic levels in neuronal tissue not only alter the functional capacity of the central nervous system but may also serve as sensitive markers for neurotoxicity. Neurotoxicity has been defined as any adverse effects on the structure and function of the nervous system. In the previous chapters, different markers for neurotoxicity were examined. Neuropathology is the "visual science" where cells and tissues are examined visually with the aid of optical instruments (e.g. microscopes) for the determination of the lesion. The use of chemical stains and dyes are used to bind to or react with the tissue components for the enhancement of the optical images. Histology involves the examination of preserved, sectioned and stained tissues of both plants and animals. Thus, histology is used as a technique to examine the neuropathological effect that metrifonate may exert on neuronal tissue.

Neurons are the basic cellular unit of a nervous system. A neuron is a single cell consisting at the largest level, a soma (the cell body), dendrites, and an axon as seen in Fig. 6.1 below. (people.cornell.edu/ pages/mlk24/boom/bio.html) Nervous tissues have a wide distribution throughout the body, innervating most visceral and peripheral tissues. Within the nervous system, there are two basic types of cells, neurons and supportive cells. Neurons are highly specialised cells that easily conduct nerve impulses and are easily excited to produce them. The basic structure and function of the neuron is as follows: the dendrite is a neuron branch which detects stimuli (changes in the environment), the cell body is body of the neuron has a central nucleus and is where normal metabolic activities occur and the axon is longest dendrite covered by a myelin sheath which provides electrical insulation carries nerve message or impulses to the axon terminals. Most neurons have a single axon, although some can have more than one.

Supportive cells assist the neuron with its functioning and are found in conjunction with the neurons. There are various types of supportive cells also known as glial cells: astrocytes; oligodendroglia; microglia and Schwann cells.

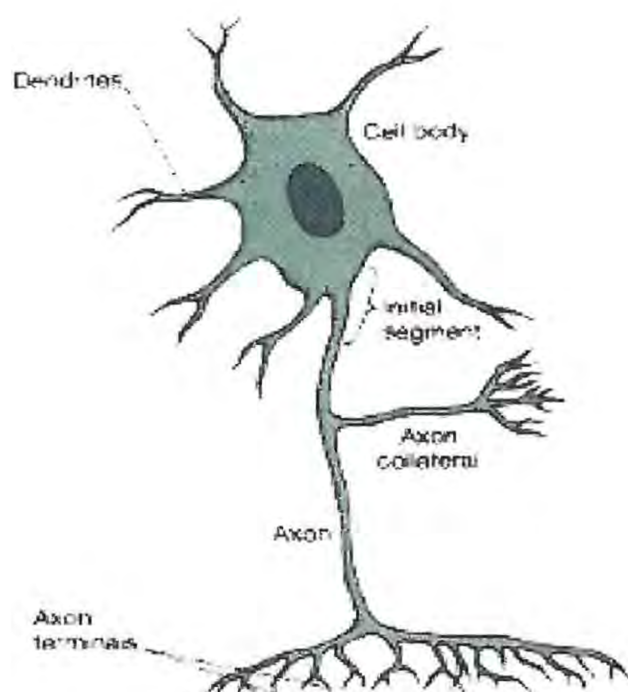


Fig.6.1 Illustration of the basic anatomy of a neuron
(http://www.bme.jhu.edu/~jsorger/LSD/neuron.html)

6.2 INVESTIGATION INTO THE HISTOLOGICAL EFFECT OF METRIFONATE IN RAT BRAIN TISSUE

6.2.1 INTRODUCTION

Metrifonate is used for the symptomatic treatment of Alzheimer's disease. MET is slowly and non-enzymatically converted to dichlorvos which competitively inhibits acetylcholinesterase, leading to an increase in brain acetylcholine within 1hour of oral metrifonate administration (Poindessous-Jazat *et a*l., 1998). The main metabolite, dichlorvos, has been reported to cause organophosphate induced delayed neurotoxicty through microtubule destabilization and axonal degeneration (Choudhary *et al.* 2001). It has also been noted to induce symptoms characteristic of oxidative stress (Hai *et al.*, 1995). The hippocampus is a primary target for neuronal degeneration in the brains of patients with Alzheimer's disease (Behl *et al.*, 1997). Therefore the effect of MET on neuronal tissue was investigated.

As observed in the previous chapters, MET produce superoxide damage, lipid peroxidation, reduced biological oxidation and decreased mitochondrial complex one activity. The present study was conducted in order to investigate whether MET induces neuronal leisons. After treatment with

MET the rat brain was sectioned and the hippocampal cells were examined for evidence of morphological changes.

6.2.2 MATERIALS AND METHODS

6.2.2.1 Animals

Adult, male, Wistar rats weighing between 250 and 300g were used for this study and were housed as described in section 2.2.2.1.

6.2.2.2 Chemicals and Reagents

Metrifonate, haemotoxylin and eosin Y were purchased from Sigma Chemical Co, St Louis, MO, USA. Paraffin wax was obtained from Lasec (South Africa). Cresyl violet stain was purchased from BDH Laboratory Supplies, England. DPX was purchased from Philip Harris Ltd. England. Haupt's adhesive consisted of the following: 1g gelatine, 100ml water, 2g phenol and 15ml glycerol. All other chemicals used were of the highest quality available and were purchased from commercial distributors. Metrifonate was dissolved in PBS and melatonin was dissolved in 30% ethanol in PBS.

6.2.2.3 Treatment Regime

The animals were divided into groups of three animals each, which received the varying doses of MET and MEL.

(a) GROUP I: control – vehicle ;
(b) GROUP II: 0.65mg/kg/day MET i.p. ;
(c) GROUP III: 1mg/kg/day MET i.p.;
(d) GROUP IV: 1mg/kg/day MET and 1mg/kg/day MEL i.p.
(e) GROUP V: 1mg/kg/day MET and 5mg/kg/day MEL i.p and
(f) GROUP VI: 1mg/kg/day MET and 10mg/kg/day MEL i.p.

The animals were injected once daily for seven days and were sacrificed on the eighth day. The whole brain was removed and immediately fixed.

6.2.2.4 Histological Techniques

The following techniques were used in order to fix, prepare, block and section the brain tissue. The paraffin wax method involves dehydration of the tissue, impregnation with molten wax and subsequently allowed to solidify by cooling. It is a commonly used embedding material in both

normal and pathological tissue. The cutting qualities are good, and the blocks are durable, and may be stored for a period of time (Drury and Wallington, 1967). The tissue was then transferred to slides and stained using two different stains and was then examined for neuronal damage.

6.2.2.5 Fixing the brain

Once the brain was removed, it was immediately fixed. Fixation of nervous tissue preserves cells in its native state in regard to its morphology and localization of chemical constituents. Fixation of neural tissues is required due to its fragility and susceptibility to rapid anoxia and post-mortem changes (Chang, 1995). The brains were immediately transferred into a fixing mixture containing of formol (30%), glacial acetic acid and ethanol in a ratio of 2:1:7 v/v for two hours. It was then transferred to 70% ethanol for preparation and embedding.

6.2.2.6 Specimen Preparation and Embedding

After fixation, tissues must be supported during the process of cutting. Embedding involves the infiltration and orientation of tissue in paraffin wax support medium. In order to do this, moisture has to be removed from the tissue. Therefore the tissue is subject to a dehydration process with a graded series of alcohol followed by immersion of tissue in xylene and finally submersion into the paraffin wax. The following table 6.1. outlines this process. This stage is required to provide hardness and support of tissue required during sectioning.

STEP	PROCESSING AGENT	TIME
1	70 % Ethanol	1 hour
2	90% Ethanol	1 hour
3	100% Ethanol	1 hour
4	100% Ethanol	1 hour
5	Xylene	1 hour
6	Xylene	1 hour
7	Melted Paraffin Wax at 57^0C	1 hour
8	Melted Paraffin Wax at 57^0C	1 hour

Table 6.1.: Illustrating the Specimen Preparation an Embedding Procedure

6.2.2.7 Blocking Out

Sectioning is performed using a rotary microtome. In order for the tissue to be sectioned, it had to be in a firm support; therefore the tissue was fixed into a paraffin wax mould. The mould is formed from 2 L-shaped metal bars coated with ethanol-glycine to prevent adherence to the mould. Molten wax was added to the mould and allowed to harden. The brain was then removed from the final molten wax stage and placed carefully onto the mould, using warmed forceps. Molten wax was then poured onto the brain until it was completely submerged. Air was then gently blown over the surface of the wax to allow the top to solidify. The entire mould was then immersed in cold water overnight to promote solidification and prevention of crystal formation that might disrupt the tissue.

6.2.2.8 Sectioning

Sectioning is a technique performed using a microtome. The wax block was removed from the mould and trimmed using a razor blade, so that two sides were parallel, while the other two converged slightly. The sides were cut so as to leave about 2mm of wax around the tissue. This was attached to a small wooden block with a small amount of molten wax. A rotary microtome was used for the sectioning. This instrument consists of a sharp metal blade held in a fixed position and a chuck in which the block of wax containing the tissue was held. Depending on the type of microtome, a particular mechanism oscillates the chuck up and down, and with each oscillation, the chuck is brought closer to the knife by a fixed distance (Hodgson, 1992). The microtome was set to cut sections of 10μm thickness. As sections were cut, it would stick to one another so as to form long ribbons. Once sections were cut, they were floated on a warm water bath at 40^0C to smoothen out wrinkles.

6.2.2.9 Transferring to Slides

Glass slides were coated with a thin layer of Haupt's adhesive before the sections were mounted. The section was gently removed from the water bath and placed onto the coated slide, using a thin paint brush. These slides were left overnight in an oven at 40^0C to enable the section to adhere to the slide.

6.2.2.10 Staining

Coronal sections of the midbrain were stained using two different stains:

(a) Nissl Staining

(b) Haemotoxylin and Eosin Staining (H & E staining)

In the H & E staining, the nuclei of the cells in the nervous tissue are stained blue (haematoxylin) against a pink (eosin) background. This method is useful for screening acute or extensive neurotoxicity (Chang, 1995). Certain neuronal tissue is rich in Nissl bodies (Chang, 1994). The presence or alterations of Nissl substance may therefore be used as an indicator or marker of these nerve cells. Disintegration of the Nissl substance (chromatolysis), signifies early degenerative change or injury. It can therefore be used as a stain to estimate cellular density changes in toxic conditions (Chang, 1995).

Before staining, due to the dyes being water soluble, the sections had to be dewaxed and rehydrated using xylene and ethanol as seen in table 6.2. below. The paraffin wax was removed by dipping the slides in xylene (for the dewaxing) twice for five minutes each, followed by immersion in a mixture of xylene and 100% ethanol (1:1) for three minutes, in absolute ethanol for five minutes and lastly overnight immersion in 100% ethanol at 30^0C.

STEP	PROCESSING AGENT	TIME
1	Xylene	5 minutes
2	Xylene	5 minutes
3	Xylene/100% Ethanol (1:1)	3 minutes
4	100% Ethanol	5 minutes
5	100% Ethanol	Overnight at 30^0C

Table 6.2 . Illustrating the procedure for Dewaxing and Rehydrating brain sections.

6.2.2.11 Nissl Staining

Sections were Nissl stained using cresyl violet. This stains Nissl substances intense purple and the nuclei purple while the background is left clear (Bauer, 1974), thus being extremely useful, as it distinguishes neurons and glia from one another. The 0.1% cresyl violet solution contains 0.25g cresyl violet, 250ml Milli-Q water, 0.75ml glacial acetic acid and 0.0512g sodium acetate. The pH was adjusted to 3.5 before use.

Sections were stained by placing them in the cresyl violet solution for two hours. The slides were then rapidly differentiated by rinsing in a flat dish with 95% ethanol until the background was clear. The sections were then dehydrated after staining as can be seen in table 6.3. below. The slides were processed in absolute ethanol twice and then xylene twice for five minutes each.

STEP	PROCESSING AGENT	TIME
1	100% Ethanol	5 minutes
2	100% Ethanol	5 minutes
3	Xylene	5 minutes
4	Xylene	5 minutes

Table 6.3.: Illustrating the procedure for Dehydrating of slides after staining.

6.2.2.12 Haemotoxylin and Eosin Staining (H & E)

This technique allows for differentiation between the nuclei and cytoplasm as it stains the nuclei blue and cytoplasm pink. It is useful in screening for neuronal oedema (cytoplasmic swelling) and neuronal losses.

The sections were stained in Harris haematoxylin solution for twenty minutes, rinsed in running tap water, followed by one to six dips in differentiating solution and then blued in Scott's tap water substitute for two minutes. These were then differentiated in 95% alcohol. The slides were then counterstained in alcoholic eosin Y solution for twenty minutes. The sections were then dehydrated as described in section 6.1.1.5.6 and seen in table above. (Please refer to appendix for H & E staining solution preparations).

6.2.2.13 Mounting of Slides

In order to protect the tissue from external damage, and to provide better optical quality for viewing under the microscope, the stained section on the slide was covered with a thin piece of glass (cover slips). While the slides were moist with xylene, sufficient DPX was added to just cover the tissue. A cover slip was then placed over the tissue. The slides were then allowed to dry over a flat surface for 48 hours.

6.2.2.14 Photo microscopy

All the slides were viewed under the light microscope and photographed using a digital camera.

6.2.3 RESULTS

6.2.3.1 Histological Analysis

The hippocampus is a primary target for neuronal degeneration in the brains of patients with Alzheimer's disease (Behl *et al.*, 1997). Thus the effect of MET, which is used for the symptomatic treatment of Alzheimer's disease was investigated. The neurons in the hippocampus (CA3 region) were carefully examined.

6.2.3.2 Nissl Staining

Neurons in the CA3 region of the hippocampus were examined. Sections of the CA3 regions of the control and 0.65mg/kg/day MET i.p. treated rats (fig.6.2. and fig.6.3. respectively) showed optimally sized, pyramidal shaped neuronal cells with a clearly observable cell nucleus and continuous cell membrane. The cells are grouped closely together to form a band – like appearance, which is characteristic of the CA3 region.

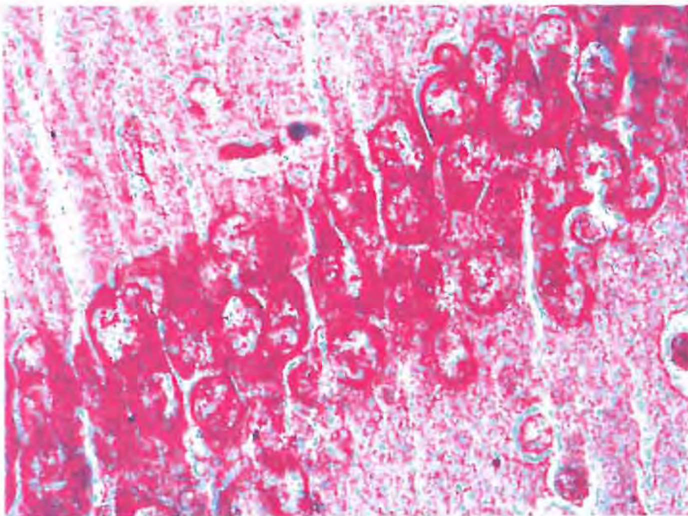


Fig. 6.2. Cells of the CA3 region of the hippocampus from an animal of the control group treated with PBS i.p. for 7 days. Magnification x 1000.

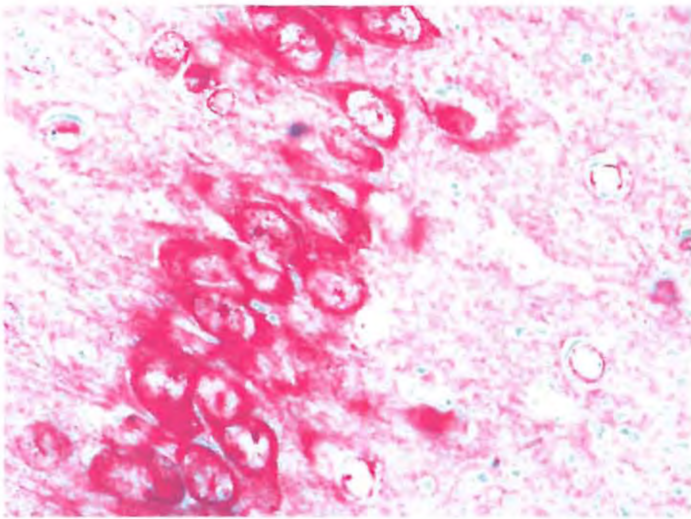


Fig.6.3. Cells of the CA3 region of the hippocampus from an animal treated with 0.65mg/kg/day MET i.p. for 7 days. Magnification x 1000.

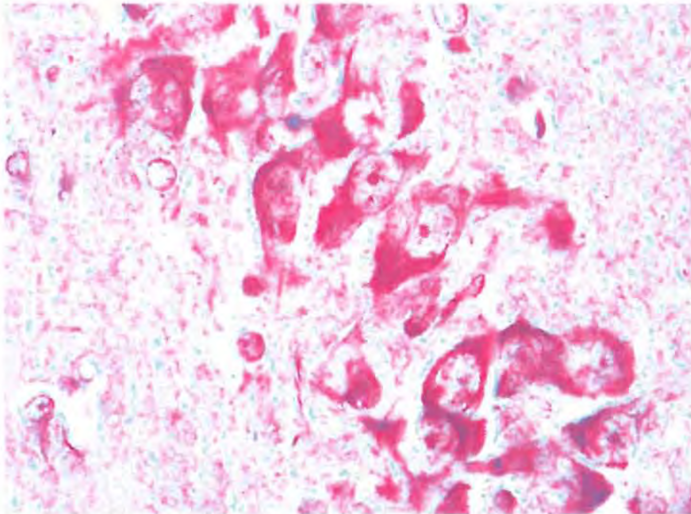


Fig.6.4 Cells of the CA3 region of the hippocampus from an animal treated with 1mg/kg/dayMET i.p. for 7 days Magnification x 1000.

The cells of the CA3 region of the 1mg/kg/day i.p. treated rats show cell damage by virtue of their roundness and swelling. The cells appear scattered, poor orientation with little integrity of cell membrane and appearance of dense nuclei.

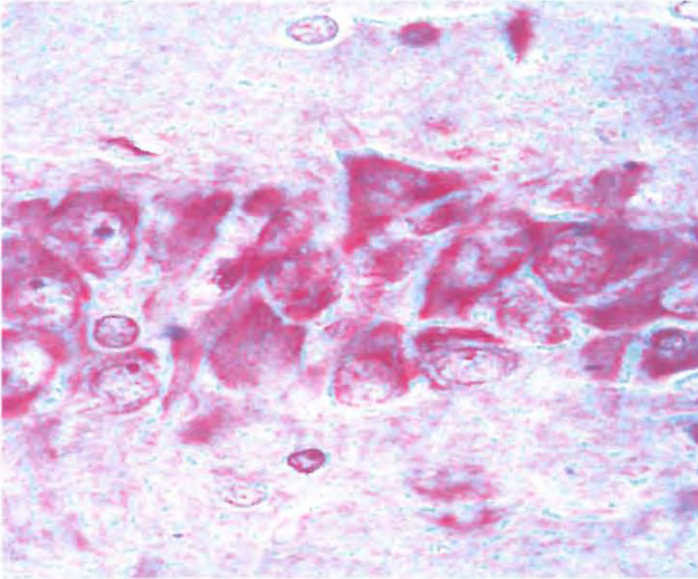


Fig.6.5. Cells of the CA3 region of the hippocampus from an animal treated with 1mg/kg/day MET i.p. and 1mg/kg/day MEL i.p for 7 days. Magnification x 1000.

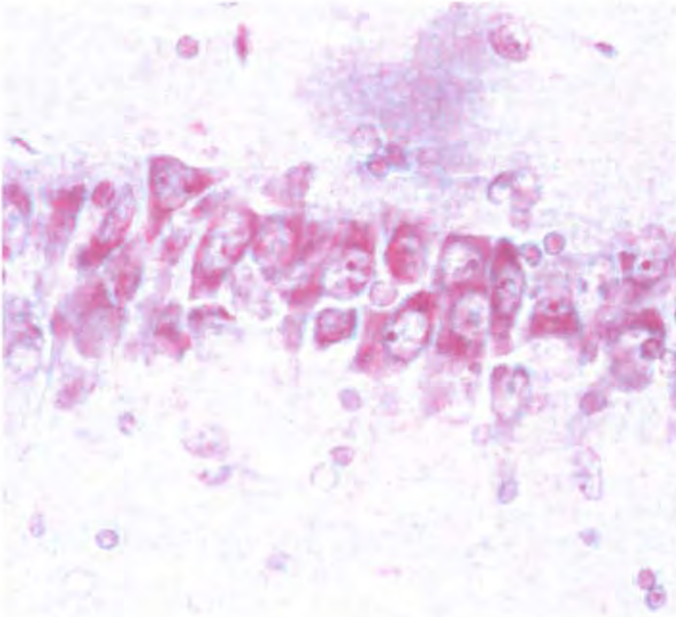


Fig.6.6. Cells of the CA3 region of the hippocampus from an animal treated with 1mg/kg/day MET i.p. and 5mg/kg/day MEL for 7 days Magnification x 1000

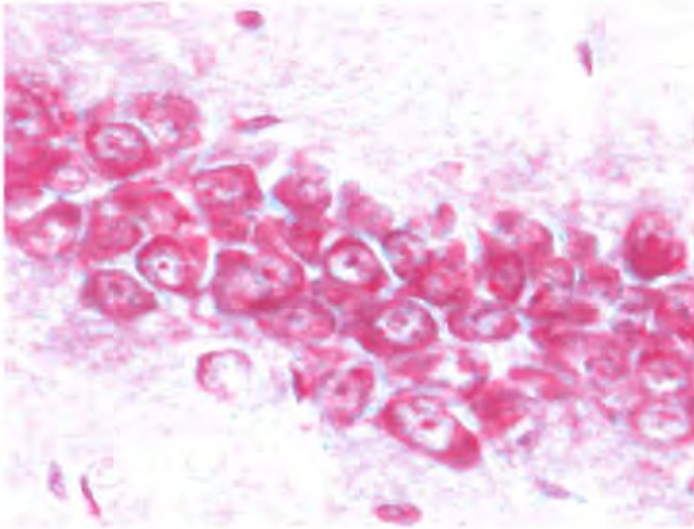


Fig.6.7. Cells of the CA3 region of the hippocampus from an animal treated with1mg/kg/day MET i.p. and 10mg/kg/day MEL for 7 days. Magnification x 1000.

The CA3 cells of the 1mg/kg/day MET with 5mg/kg/day MEL and 10mg MEL/kg/day i.p, treated animals (fig.6.6. and fig.6.7. respectively) appear to have retained their pyramidal appearance. The cells have good orientation, distinguishable nuclei, integrity of cell membrane and appear in the typical "band-like" appearance.

6.2.3.3 Haemotoxylin and Eosin Staining

The CA3 cells of the control and 0.65mg/kg/day MET i.p. treated animals (fig.6.8 and fig 6.9. respectively) appear to have retained their pyramidal appearance. The cells have good orientation, distinguishable nuclei, integrity of cell membrane and appear in the typical "band-like" appearance.

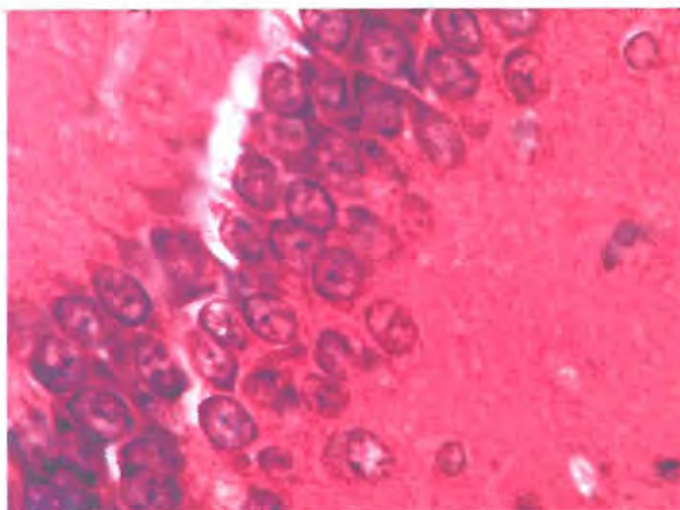


Fig.6.8.Cells of the CA3 region of the hippocampus from an animal of the control group treated with vehicle i.p. for 7days. Magnification x 1000.

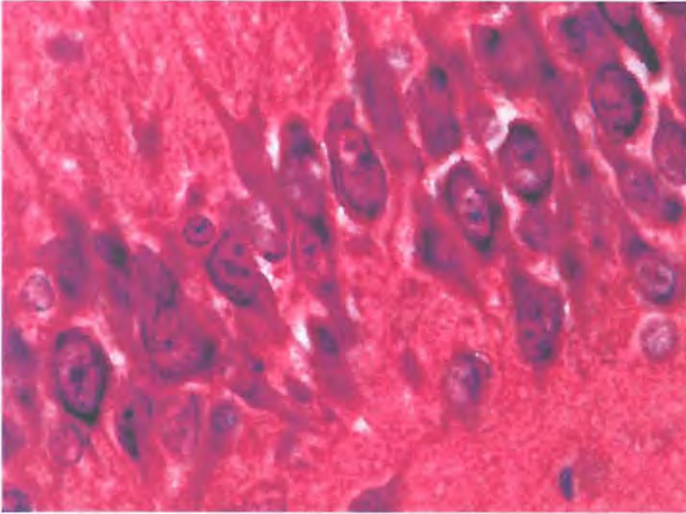


Fig.6.9. Cells of the CA3 region of the hippocampus from an animal treated with 0.65mg/kg/day MET i.p. for 7 days. Magnification x 1000.

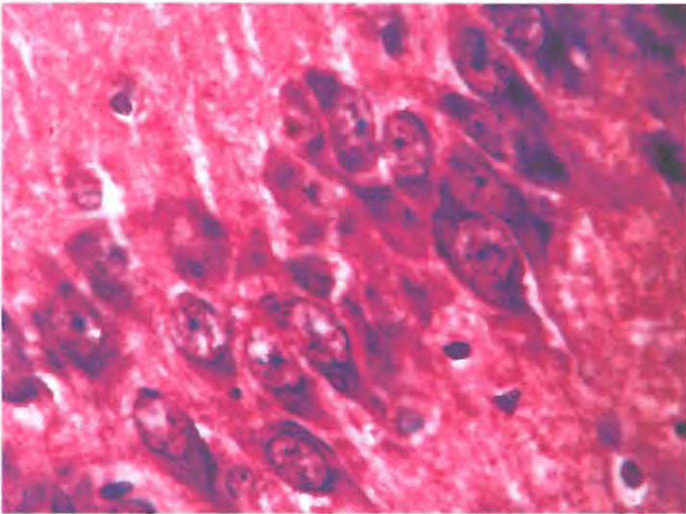


Fig.6.10. Cells of the CA3 region of the hippocampus from an animal treated with 1mg/kg/day i.p. for 7 days. Magnification x 1000.

The cells of the CA3 region of the animals treated with 1mg/kg/day MET i.p. (as seen in fig 6.10.) display signs of damage by virtue of their roundness and swelling. The cells appear slightly cattered with poor orientation and the lack of the tight band-like appearance in comparison to the control.

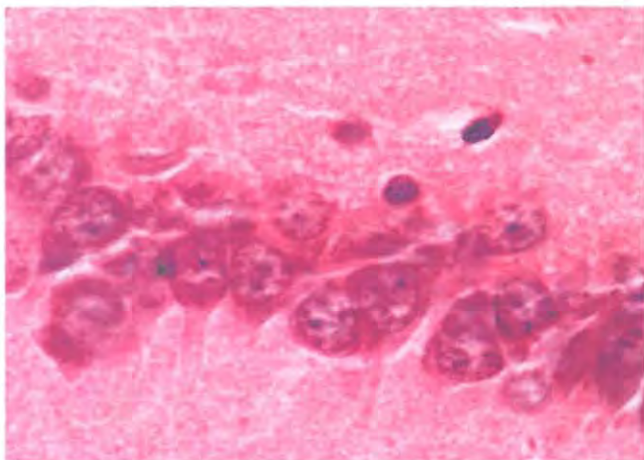


Fig.6.11. Cells of the CA3 region of the hippocampus from an animal treated with 1mg/kg/day MET i.p. and 1mg/kg/day MEL for 7 days. Magnification x 1000.

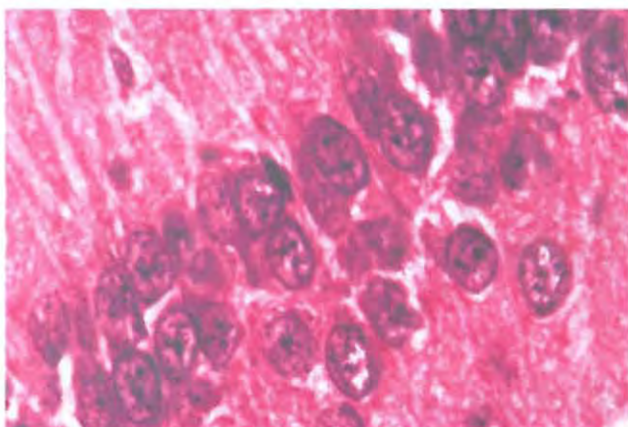


Fig.6.12. Cells of the CA3 region of the hippocampus from an animal treated with 1mg/kg/dayMET i.p. and 5mg/kg/day MEL for 7 days. Magnification x 1000.

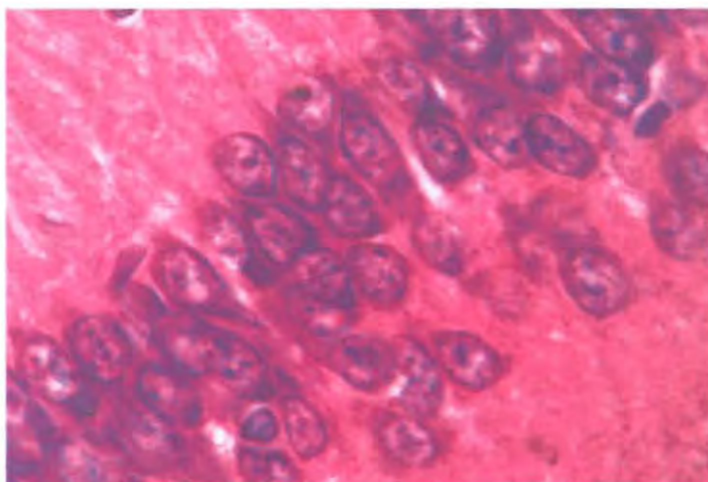


Fig.6.13. Cells of the CA3 region of the hippocampus from an animal treated with 1mg/kg/day MET i.p. and 10mg/kg/day MEL for 7 days. Magnification x 1000.

The CA3 cells of the 1mg/kg/day MET with 5mg and 10mg/kg/day MEL i.p, treated animals (fig.6.12 and fig.6.13 respectively) appear to have retained their pyramidal appearance. The cells have good orientation, distinguishable nuclei, integrity of cell membrane and appear in the typical "band-like" appearance.

6.3 DISCUSSION

Many organophosphate compounds have been shown to induce a delayed neurotoxic effect which is characterized by the appearance of muscular incoordination, prolonged motor ataxia and eventually paralysis, two to three weeks after a single exposure to the organophosphate compound (Abou-Donia., 1981). Histopathological lesions include axonal degeneration with secondary breakdown of myelin, muscle denervation and degeneration (Jortner and Ehrich, 1987). Metrifonate, also known as trichlorfon, is an organophosphorous cholinesterase inhibitor used in the treatment of AD. It is transformed, non-enzymatically, *in vivo*, to it's active metabolite, dichlorvos (O,O-dimethyl 2,2 dichlorovinyl phosphate), (Poindessous-Jazat *et al.*, 1998). The first case of human exposure to dichlorvos and subsequent development of delayed neurotoxicity was reported in 1985 (Wadia *et al.*, 1985). Later, dichlorvos was reported (Lotti *et al.*, 1993) to display delayed neurotoxic potential whereas the same was denied (Johnson, 1978). An earlier report (Sarin and Gill, 2000) also showed dichlorvos to be capable of inducing delayed neurotoxicity in rats. Metrifonate and dichlorvos have been shown to produce toxic effects on hepatocytes as evidenced by the by MDA production and lactate dehydrogenase leakage in a dose-dependent manner up to the concentration of 2mM (Yamano and Morita, 1992). Previous experiments showed oxidative damage caused by the exposure of rat brain to metrifonate. This experiment was conducted in order to determine whether metrifonate induced morphological changes in the hippocampus. As gleaned from the results, the neuronal cells of the CA3 region from the hippocampus of animals which received MET 1mg/kg/day i.p. lacked good orientation, the tight band-like packing (which is characteristic of cells in that region), and appear round and swollen. Even though these changes were very slight in comparison to the control, this provides histological evidence as to the neurotoxic potential of metrifonate and its active metabolite dichlorvos. The neuronal cells of the CA3 region from the hippocampus of animals which received treatment with 5mg and 10mg/kg/day MEL i.p and 1mg/kg/day MET maintained its shape and orientation, which are indicative of the neuroprotective properties, characteristic of melatonin (Tan *et al.*, 1993; Pablos *et al.*, 1998; Reiter *et al.*, 2002). These results indicate that melatonin is able to protect the tissue against the damage induced by metrifonate.

CHAPTER SEVEN - APOPTOSIS

7.1 INTRODUCTION

Apoptosis or programmed cell death, important in both health and disease, is a common physiological and pathological process in which cell death is mediated by activation and synthesis of a series of gene products (Green and Reed, 1998). Many factors can induce apoptosis, however it is generally believed that reactive oxygen species serves as the common initiator of apoptosis (Jabs, 1999). Shown below in fig. 7.1. is a representation of the sequence of the events involved in apoptosis.

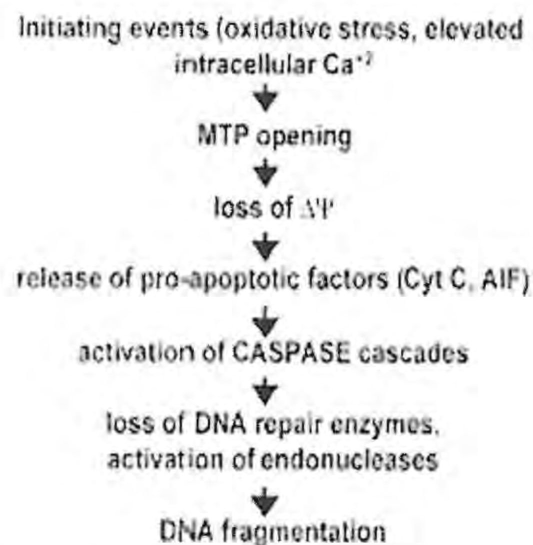


Fig.7.1. Proposed sequence of events for mitochondrial damage leading to apoptosis through the mitochondrial transition pore. Abbreviations: AIF,apoptosis inducing factor; Cyt C; cytochrome c; D c, mitochondrial membrane potential; MTP, mitochondrial transition pore (Cassarino and Bennett Jr., 1999).

The brain is an organ which has high oxygen consumption and is constantly exposed to oxidative stress. The brain also has a relatively low antioxidative capacity, which increases susceptibility to oxidative damage (Facchinetti *et al.*, 1998). It is increasingly apparent that reactive oxygen species play a key role in neuronal apoptotic cell death (Greenlund *et al.*, 1995; Hockenberry *et al.*, 1993; Li *et al.*, 2001).

Depending on the cell model and level of the oxidative insult (Bonfoco *et al.,* 1995; Nicotera *et al.,* 1997) i.e. severity and duration of the oxidative injury, cell death may manifest as apoptosis or necrosis. Apoptosis is now recognized as a critically important mechanism of neuronal cell death. It occurs in a variety of acute neurological insults and chronic neurodegenerative disorders (Mattson and Furukawa, 1996). In animals, apoptosis has been detected in the brain after ischemia (Li *et al.,* 1995; Paradanian *et al.,* 1998), seizures (Keller *et al.,* 1998; Pollard *et al.,* 1994), or traumatic injury (Yakovlev *et al.,* 1997). It has also been proposed that apoptotic cell death contributes to chronic neurodegenerative conditions, including Parkinson's disease, amyotrophic lateral sclerosis, and Alzheimer's disease (Laferla *et al.,* 1995; Mattson and Furukawa, 1996; Mochizuki *et al.,* 1996). Apoptosis has been described following exposure to a number of chemicals associated with delayed, progressive degeneration of the brain (Wertz and Hanley, 1996).

A reduced cell volume, condensations of chromatin and fragmented nuclei with preservation of cellular membranes are characteristic of apoptosis and exhibit apoptotic bodies containing cellular material which are recognized and removed by phagocytes. Cascade activation of proapoptotic proteins (caspases) precedes the morphological changes. Eventually, activated caspase-3 cleaves an inhibitory protein of the caspase-activated DNAase (ICAD) which can then act as an endonuclease to digest DNA (Enari *et al.*, 1998; Sakahira *et al.*, 1998). Mitochondria are involved in the regulation of apoptosis. Opening of the mitochondrial transition pore (MTP) and mitochondrial depolarization are the initial step of apoptosis, as shown in fig.7.2. below. (Green and Reed, 1998; Marchetti *et al.*, 1996; Zamzami *et al.*, 1995). Consequently, cytochrome *c* and the apoptosis inducing factors (AIFs) can exit mitochondria and activate proapoptotic proteins close to the outer mitochondrial membrane (Kroemer *et al.*, 1997; Reed, 1997). Antiapoptotic proteins, such as bcl-2, are also localized close to the outer mitochondrial membrane which makes mitochondria the location for pro and antiapoptotic events (Rossé *et al.*, 1998; Zhivotovsky *et al.*, 1998).

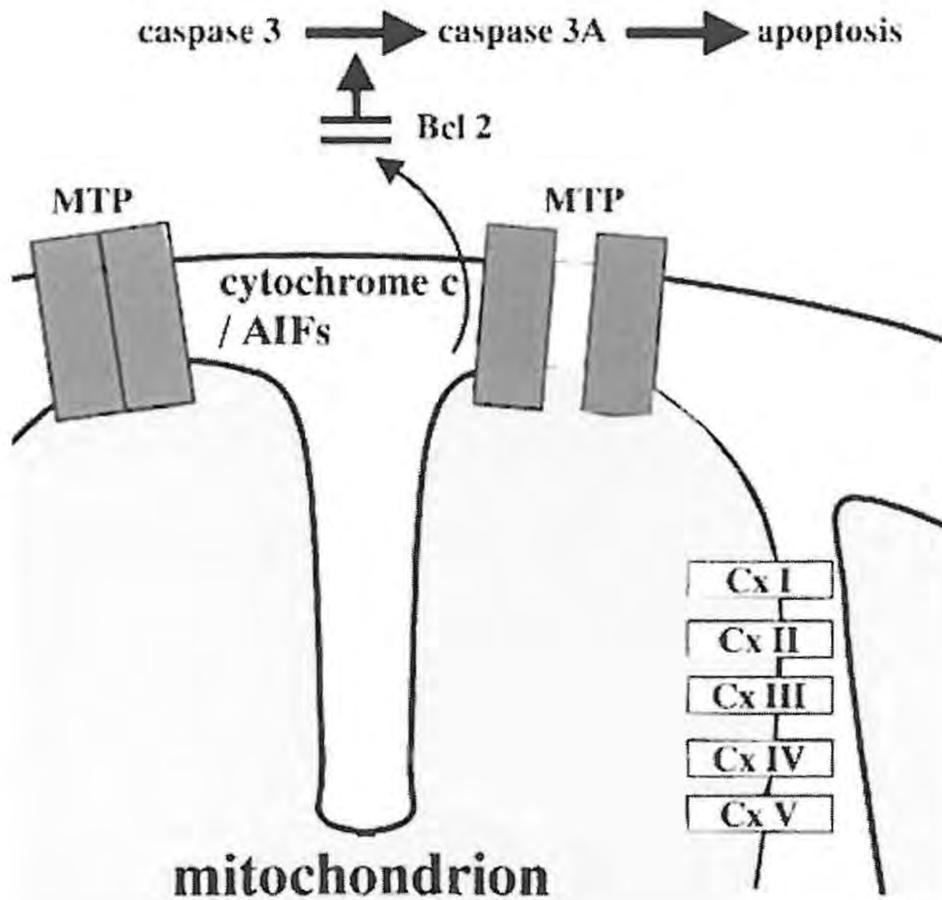


Fig.7.2. Cytochrome c and AIFs can be released by an incompletely understood mechanism when the MTP opens. These can activate proapoptotic proteins at the outer membrane initiating apoptosis which is inhibited by Bcl 2 (Orth and Schapira, 2002).

Whether or not the cell ultimately capitulates to the death program is probably dependent on the balance of pro- and anti-apoptotic factors shown in fig.7.2. above. Reactive oxygen species and perturbed Ca^{2+} dynamics arising from ETC defects may stimulate pathological MTP opening, inducing activation of apoptosis, anti-apoptotic factors such as the antioxidant enzymes. The endogenous MTP inhibitors bcl-2 and bcl-X favor the closed state of the pore and inhibit the death process. In neurodegeneration, the balance between pro and anti-apoptotic factors is eventually shifted towards death in particular neuronal populations, but the mechanisms by which this occurs remain to be fully elucidated (Cassarino and Bennett Jr., 1999).

7.2 THE EFFECT OF METRIFONATE ON APOPTOSIS IN RAT BRAIN

7.2.1 INTRODUCTION

In the earlier chapters, it is possible that reactive oxygen species play a role in the underlying mechanisms of metrifonate-induced neurotoxicity. Studies have shown that acetylcholinesterase inhibitor-induced damage may be associated with excessive generation of intracellular reactive oxygen species (Steevens and Benson, 1999; Yang *et al.*, 1996b, Li *et al.*, 2001). Reactive oxygen species are intiators of neuronal injury through various mechanisms including oxidative damage to cellular lipids, proteins and DNA, and gene expression associated with apoptosis. Earlier studies have shown pyridostigmine, an organophosphate acetylcholine esterase inhibitor used in the treatment of myesthenia gravis, to induce apoptotic cell death in the rat brain (Li *et al.*, 2000). This study investigated the involvement of metrifonate in apoptosis. In the current experiment, the *in situ* cell death detection kit was used for the detection of apoptosis at a single cell level based on labelling of DNA strand breaks (TUNEL method i.e. TdT-mediated dUTP nick end labelling method). The assay uses an optimized terminal transferase (TdT) to label free 3' OH ends in genomic DNA with fluorescein d-UTP. The assay procedure is as follows in table 7.1. :

Tissue fixing
Paraffin embedding
Blocking out
Sectioning
Transference to slides
Dewaxing
Rehydrate
Protease treatment
Permeabilization of tissue
Addition of TUNEL mixture
Analysis of samples by microscopy

Table 7.1.: The assay procedure for detection of apoptosis.

7.2.2 MATERIALS AND METHODS

7.2.2.1 Animals

Adult male Wistar rats, weighing between 250g and 300g were used for the experiments, and were housed and maintained under the conditions described in section 2.2.2.1.

7.2.2.2 Chemicals and Reagents

The *in situ* cell death detection kit, POD, DNase and Protein K was purchased from Roche diagnostics, Mannhein, Germany. Metrifonate, melatonin and aminopropyl triethoxysilane (APES) were purchased from Sigma Chemical Co, St. Louis, MO, USA. Paraffin wax was obtained from Lasec (South Africa). All other chemicals were of the highest quality available and purchased from commercial distributors. Metrifonate was dissolved in PBS and melatonin was dissolved in 30% ethanol in PBS.

7.2.2.3 Treatment Regime

The animals were divided into six groups of five animals each which received varying doses of metrifonate and melatonin.

GROUP I: Control group – vehicle only i.p. daily;
GROUP II: 0.65mg/kg/day MET i.p. daily;
GROUP III: 1mg/kg/day MET i.p. daily;
GROUP IV: 1mg/kg/day MET i.p and 1mg/kg/day MEL i.p. daily;
GROUP V: 1mg/kg/day MET i.p and 5mg/kg/day MEL i.p. daily and
GROUP VI: 1mg/kg/day MET and 10mg/kg/day MEL i.p. daily.

The animals received treatment as above for seven days. On the eighth day the animals were sacrificed and the brains removed as described in section 2.2.2.3. The brain tissue was rapidly fixed in Davidson's Alcohol Formalin Acetic Acid Fixative (AFA) (see appendix 5 for formula)

7.2.2.4 Techniques involved in Apoptosis

The method used for the immunohistochemical detection and quantification of apoptosis (programmed cell death) at single cell level, based on the labelling of DNA strand breaks using TUNEL technology and analysis by microscopy, as described by the instruction manual. This technique is sensitive and therefore some of the protocols involved for the processing the tissue are different from that mentioned in the previous chapter.

7.2.2.4.1 Fixing the brain

The role of the fixative is to maintain the morphology of the tissues as close to the *in vivo* morphology as possible and to prevent post- sampling necrosis. Fixation of brain tissue, should therefore be performed rapidly. The brain tissue, once removed, was immediately fixed in Davidson's AFA solution for forty-eight hours.

7.2.2.4.2 Specimen Preparation and Embedding

The embedding of samples in paraffin requires several steps during which the water contained in the tissues is progressively replaced, initially by alcohol, then xylene and lastly paraffin. A modification of the protocol outlined by (Judes 1997) was used. After fixing the brain tissue in Davidson's AFA for 48 hours the tissue was prepared for embedding as outlined in table 7.2. :

STEP	PROCESSING AGENT	TIME
1 – Dehydration	50% Absolute Ethanol	1 x 2 hours
2	70% Absolute Ethanol	1 x 2 hours
3	80% Absolute Ethanol	1 x 2 hours
4	90% Absolute Ethanol	1 x 2 hours
5	96% Absolute Ethanol	2 x 2 hours
6	100% Absolute Ethanol	3 x 2 hours
7 – Clearing	Absolute Ethanol: Chloroform (1:1)	1 x 2 hours
8	Chloroform	1 x 2 hours
9	Xylene: Chloroform (1:1)	1 x 1 hour
10 – Wax Immersion	Melted Paraffin Wax at 60^0C	1 x 1 hour
11	Vacuum at 60^0C	15 minutes
12	Melted Paraffin Wax at 60^0C	2 x 1 hour
13 - Embedding	Molten Wax	Overnight

Table 7.2.: The Procedure for processing the tissues for paraffin embedding

Once the tissue was embedded, it was then prepared for blocking.

7.2.2.4.3 Blocking Out

This procedure was carried out as described in section 6.2.2.7.

7.2.2.4.4 Sectioning

The coronal sections of the mid-brain were chosen and sections of 5μm thickness were cut for use. This procedure was carried out as described earlier in section 6.2.2.8.

7.2.2.4.5 Transferring Sections to Slides

Before transferring the sections to slides, the slides had to be treated with appropriate adhesive. Use of the appropriate adhesive to mount paraffin sections onto a slide is very important in order to avoid loss of sections during the subsequent washing procedures during staining. For TUNEL staining, the sections were mounted on slides that were coated two days prior to use with APES as it has been shown to be superior to poly-L-lysine in preventing tissue detachment from the glass (Ben-Sasson *et al.*, 1995). The slides were placed in a rack and cleaned by immersion for 30 minutes in 2% Decon 90 made in warmed distilled water (60^0C). This was followed by rinsing in distilled water. This was followed by rinsing in the distilled water and then in acetone and air drying. The slides were then immersed into 2% APES solution in acetone, for 30 minutes. Finally the slides were rinsed in acetone, washed in distilled water and air dried at 37^0C. When dried, these slides were stored at room temperature in a dry place, for at least two days prior to use.

Once the slides were treated, the paraffin wax blocks containing the treated brain tissue were sectioned. These sections were removed from the water bath and placed onto the treated slides using a thin paint brush. The slides were then stored at room temperature to allow the section to adhere to the slide. It was then used for the TUNEL staining.

7.2.2.5 The TUNEL Staining Procedure

Apoptotic cells were detected by immunohistochemistry, using the procedure outlined in the manufacturers' recommendations.

7.2.2.5.1 Pre-treatment of paraffin embedded tissue

Before the commencement of the TUNEL staining, the paraffin embedded tissue had to be dewaxed and rehydrated. This was achieved by heating the slides for 20minutes at 60^{0}C. Hydration was achieved by transferring the slides through xylene twice for 5 minutes each, followed by immersion in absolute ethanol twice for 3 minutes and then through a graded series of ethanol (95-70%) and finally rinsed in PBS (pH 7.4).
The process was followed as outlined in table .

STEP	PROCESSING AGENT	TIME
1	Heat at 60^{0}C	20minutes
2	Xylene	2 x 5 minutes
3	100% Absolute Ethanol	2 x 3 minutes
4	95% Absolute Ethanol	1 x 3 minutes
5	90% Absolute Ethanol	1 x 3 minutes
6	80% Absolute Ethanol	1 x 3minutes
7	70% Absolute Ethanol	1 x 3minutes

Table 7.3: The Procedure for dewaxing and rehydrating the brain tissue sections.

The slides were then rinsed twice in PBS and the tissue sections were incubated with Proteinase K (20µg/ml in 10mM Tris/HCl, pH 7.4-8) for 20 minutes at 37^{0}C in a humidified chamber. The Proteinase K treatment digests cross-linked proteins and thereby increases cell permeability and access to the nucleic acid targets i.e. DNA. The slides were then rinsed twice with PBS.

The experimental slides were kept in PBS while the positive control slide was removed for Dnase treatment. Two negative control slides and one positive control slide was

included with each experimental run, due to the variation in positive staining when using the TUNEL method.

For the positive control slide, since DNA fragmentation is characteristic of apoptosis, application of DNase I to the control slides is ideal, as it introduces breaks by hydrolysis of double stranded or single stranded DNA and therefore results in intensive labelling of all nuclei. The Dnase I was used as 3000U/ml and prepared in 50mM Tris-HCl, pH 7.4 containing 1mg/ml BSA. This DNase I mixture was applied to the control slides and incubated for ten minutes at 25^0C. This mixture was only prepared until required as thawing of the DNase I would cause its inactivation. After treating for ten minutes, the slides were washed with PBS.

During the ten minute incubation time of the positive control slides, the TUNEL reaction mixture was prepared. In the *in situ* cell death detection kit, are vials containing the following:

(a) vial 1 - an enzyme solution (Terminal deoxynucleotidyl transferase (Tdt) from calf thymus recombinant in E.coli, in storage buffer;

(b) vial 2 - a labelling solution (nucleotide mixture in reaction buffer)

For the negative controls, 100µl of the labelling solution, vial 2, is removed and kept aside. The TUNEL reaction mixture is prepared by combining the enzyme solution from vial 1 with the balance of the labelling solution from vial 2, which yields 500µl of the TUNEL reaction mixture. The mixture is mixed well and prepared in the dark due to sensitivity to light.

7.2.2.5.2 Labelling

Once the positive control slide had been treated with Dnase I, labelling solution for the negative control set aside, and the TUNEL reaction mixture prepared, labelling began. This was done by adding 50µl of the TUNEL reaction mixture to each section, as well as the positive Dnase I control slides, while 50µl of the labelling solution only was added to the negative control slides. All the slides were then covered with Parafilm to prevent the

slides from drying out. The slides were then incubated for one hour at 37^0C in a darkened humidified chamber. This reaction was then terminated by immersing slides in PBS and washing three times in PBS.

7.2.2.5.3 Microscopy

Whilst the slides were still wet, SHUR/MOUNT ™ which is an aqueous mountant, was added to the slides. The tissue sections were then covered with coverslips and analysed under a microscope and photographed using a digital camera.

7.2.3 RESULTS

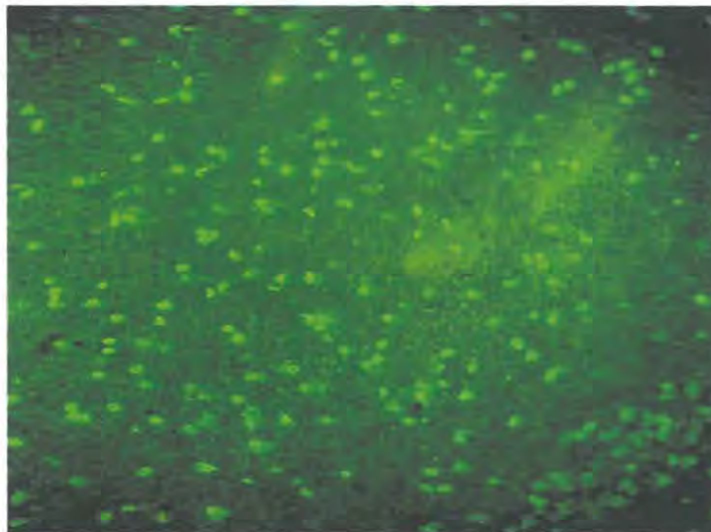


Fig.7.3. The positive control slide at x 200 magnification.

Fig.7.3. above illustrates the positive control. The TUNEL positive cells are indicative of DNA fragmentation.

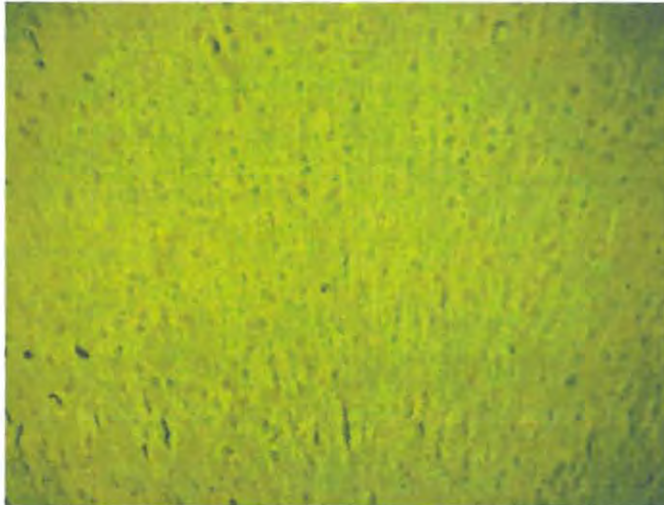


Fig. 7.4. Represents the TUNEL stained neurons from the midbrain of a rat treated with vehicle only, i.p. x 200 magnification.

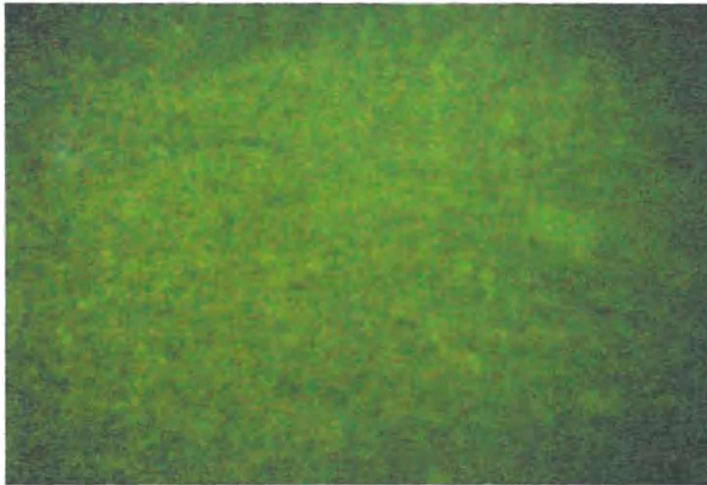


Fig. 7.5. Represents the TUNEL stained neurons from the midbrain of a rat treated with 0.65mg/kg/day MET only i.p. x 200 magnification.

The section the midbrain of a rat which received 0.65mg/kg/day MET i.p. indicates only a light staining with no distinct apoptotic bodies observed.

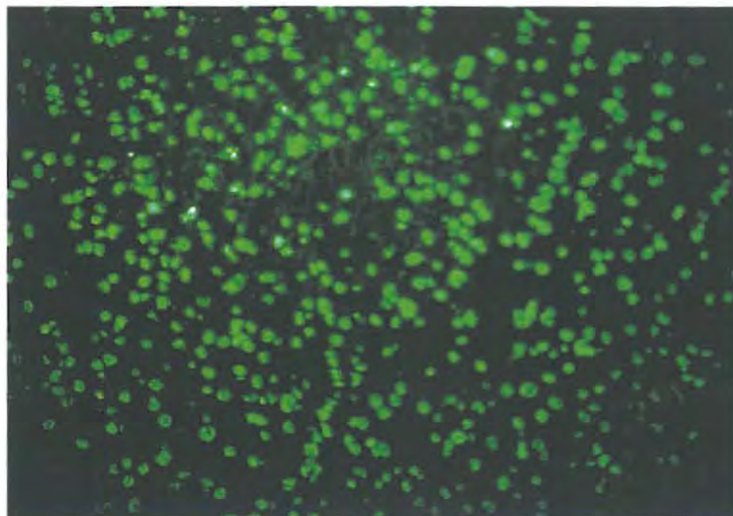


Fig. 7.6. Represents the TUNEL stained neurons from the midbrain of a rat treated with 1mg/kg/day MET only i.p. x 200 magnification.

Intense TUNEL staining in the section of midbrain of the rat receiving 1mg/kg/day MET only i.p. can be observed, indicating the presence of apoptotic bodies.

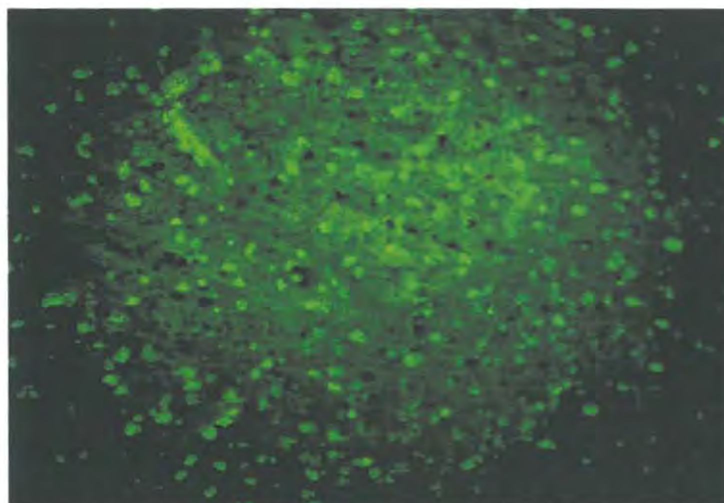


Fig. 7.7. Represents the TUNEL stained neurons from the midbrain of a rat treated with 1mg/kg/day MET and 1mg/kg/day MEL i.p. x 200 magnification.

Fig.7.7. above shows the TUNEL staining of the group receiving 1mg/kg/day MET and 1mg/kg/day MEL indicate the presence of apoptotic bodies.

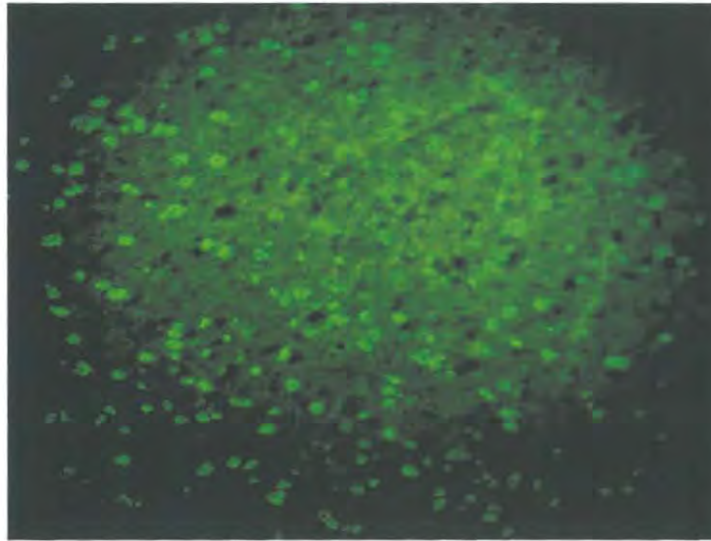


Fig.7.8. Represents the TUNEL stained neurons from the midbrain of a rat treated with 1mg/kg/day MET and 5mg/kg/day MEL i.p. x 200 magnification.

The TUNEL staining the midbrain section of the rat receiving 1mg/kg/day MET and 5mg/kg/day MEL i.p. as seen in fig.7.8. above indicate the presence of apoptotic bodies but has less fluorescence than the midbrain section of the rat receiving 1mg/kg/day MET i.p. only.

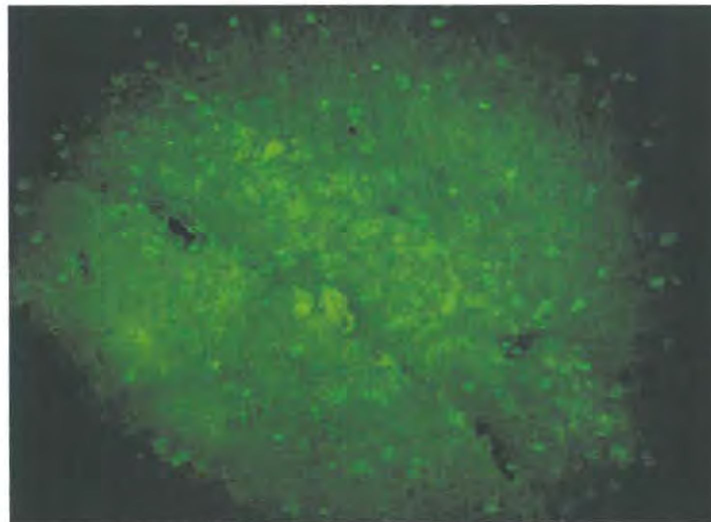


Fig.7.9. Represents the TUNEL stained neurons from the midbrain of rats treated with 1mg/kg/day MET and 10mg/kg/day MEL i.p. x 200 magnification.

The TUNEL staining of the group receiving 1mg/kg/day MET and 10mg/kg/day MEL i.p. also indicate the presence of apoptotic bodies, but greatly decreased in the intensity in comparison to the midbrain section of the rat receiving 1mg/kg/day metrifonate only.

7.2.4 DISCUSSION

Extreme generation of intracellular reactive oxygen species produces neuronal damage by the initiation of damage of the cellular macromolecules and perhaps by influencing gene expression and post-translational modification of key elements of the apoptotic cascade (Sakaguchi *et al.*, 1998). Several studies have shown that anticholinesterases cause tissue damage associated with reactive oxygen species (Bagchi *et al.*, 1995; Yang *et al.*, 1996a, b; Milatovic *et al.*, 2000; Li *et al.*, 2000; Li *et al.*, 2001). In the previous chapters, metrifonate demonstrated the ability to induce damage characteristic of reactive oxygen species. In this chapter, metrifonate induced extensive apoptosis, which was reduced in the presence of free radical scavenger, melatonin. This indicates that the damage induced by metrifonate may be due to reactive oxygen species generation.

In another study, carbachol, a muscarinic agonist, increased production of reactive oxygen species (Naarala *et al.*, 1997), thus indicating that extreme activation of cholinergic receptors increased the production of reactive oxygen species. Over stimulation of muscarinic receptors leads to excitotoxicity-like cell damage in which intracellular calcium increases, followed by enhanced generation of reactive oxygen species (Jansson *et al.*, 1991; Naarala *et al.*, 1997; Mangelus *et al.*, 2001).

Furthermore, organophosphate-induced delayed apoptosis mediated by nitric oxide (NO), in rat brain has also been reported (Kim *et al.*, 1999). Activation of muscarinic receptors can mediate NO release in rat cortical primary cultures (Castoldi *et al.*, 1993), mouse neuroblastoma (Hu and El-Fakahany, 1993) and ganglionic neurons (Quinson *et al.*, 2000). Nitric oxide has been shown to trigger apoptosis by mechanisms such as excitotoxicity, inhibition of the mitochondrial respiratory chain and the subsequent

decrease in cellular ATP. (Wei *et al.*, 2000). Therefore metrifonate may cause organophosphate-induced delayed apoptosis mediated by NO and reactive oxygen species, via over stimualtion of the muscarinic receptors due to the build-up of acetylcholine due to it's mechanism of action.

Melatonin, is a free radical scavenger, inhibits NO production in the rat brain (Pozo *et al.* 1997; Noda *et al.*, 1999), and has the ability to stimulate brain glutathione peroxidase (antioxidant) activity (Barlow-Walden *et al.*, 1995), thus suggesting three mechanisms by which it can provide a protective role against apoptosis and explain the protection provided against the metrifonate-induced apoptosis.

CHAPTER EIGHT- ELECTROCHEMICAL AND UV/VISIBLE *IN VITRO* INVESTIGATION

8.1 INTRODUCTION

Iron is a redox-active metal that is able to promote the formation of reactive oxygen species within humans. Along with the increase in oxidant stress due to imbalances in free radical generation and various enzymatic and non-enzymatic antioxidant defense mechanisms (Ames et al., 1993), the presence of metals such as iron are able to catalyze and promote the further formation of ROS within humans (Bush, 2000), thus increasing oxidative damage caused by ROS. There is increasing evidence that during disease states, reactive iron becomes available (Gutteridge and Halliwell, 1984). In the brain of patients suffering from neurodegenerative disorders, lactoferrin levels, which has been shown to protect against severe inflammation, is increased (Fillebeen *et al.*, 1999). Abnormally high levels of iron as well as oxidative stress have been demonstrated in a number of neurodegenerative disorders including Alzheimer's disease and those characterised by nigral degeneration such as Parkinson's disease, multiple system atrophy, and progressive supranuclear palsy (Campbell, 2001). The antioxidant defense system in the body is also affected with age e.g. of the neurohormone, melatonin, is reduced (Ludolph, 1995), thus leaving the brain more susceptible to oxidative damage caused by iron and free radicals.

In chapter three it was noted that although metrifonate can potentiate the lipid peroxidative damage induced by quinolinic acid, it fails to do so in the presence of iron. This may be due to OPIDN and interference with calcium influx regulation and not due to metal binding. In order to confirm the postulated lack of interaction with Fe^{2+}, metal binding studies were performed, using two methods:

(a) UV/VIS Spectroscopic technique and
(b) Electrochemical Analysis

8.1.1 UV/VIS Spectroscopic technique

An UV/Visible study was undertaken in order to elucidate whether an electronic interaction occurs between MET and iron (II) sulphate *in vitro*. All chemicals, to an extent, absorb electromagnetic radiation of specific wavelength. By using spectroscopy, the extent absorption of a beam of light after the light is passed through the sample or after reflection from the sample surface, is measured. By using ultraviolet and visible spectroscopy, the energy absorbed by a transition from one electronic energy level to another is measured. Light absorption occurs at different wavelengths, which are different for different substances, thus allowing a qualitative analysis of a chemical substance. A change in structure or composition of a compound will result in an electronic change, thus resulting in a change in the spectrum, either a shift in wavelength or a change in the extinction coefficient of the absorbance (Newman, 1969).

8.1.2 Electrochemistry using Adsorptive Stripping

The transfer of electrons during a chemical reaction is the basis of electrochemical analysis (Pescok et al., 1968). This technique is used to examine the metal-ligand complex formation at an electrode. It relies on the tendency of an analyte to preconcentrate at the surface of an electrode (the working electrode) due to the working electrode being more polarized at greater negative potential than the reduction potential of the metal. In theory, the introduction of a successful ligand to a metal solution will increase the preconcentration of the metal at the electrode and due to the resulting metal reduction, and an increase in current will result. The reason for this is that applying a voltage in a negative direction against the electrode in order to strip the adsorbed metal complex from the electrode, scans a voltammogram. In this stripping step, the current response due to the reduction of the metal complex is measured as a function of potential. Therefore, to a solution of metal analyte (e.g. a solution of ferrous (II) sulphate) and electrolyte (0.1 M citric acid buffer at pH 3.4), a suitable chelating ligand (e.g. the 1mM

solution of MET) is added. The metal-ligand complexation is facilitated by allowing time for it to accumulate at the electrode. This is the accumulation time (e.g. 2 minutes) during which the metal-ligand complex has begun to adsorb at the electrode surface by diffusion from the solution. The electrode potential is held at a fixed deposition potential for a controlled time (deposition time), while the solution is stirred at a fixed convection rate. A metal species at an electrode will produce a characteristic response at a specific potential, with current being proportional to the amount of analyte at the electrode. This is followed by the above-mentioned stripping step, in which the adsorbed complex is reduced during a negative going potential scan and both the metal and ligand are released back into solution. A shift in the reduction in potential is indicative of the new metal-ligand species being reduced or the strength of the metal-ligand complex. A lowering in current indicates possible competition by the ligand for binding sites at the electrode at relatively high analyte concentrations, or formation of strong metal-ligand bonds where the metal is not easily reduced upon scanning. A negative potential shift indicates a strong metal-ligand interaction while a large positive shift is associated with a weaker metal-ligand interaction (Limson *et al.*, 1998; Lack & Nyokong, 2001).

Electrochemistry has been used with much success in metal-ligand studies (Limson *et al.*, 1998, Limson & Nyokong, 2001, Lack & Nyokong, 2001). Limson *et al.*, (1998) used electrochemistry to show metal-ligand interactions between the pineal hormone melatonin and a range of metals. Thus electrochemical analysis was one of the techniques used to probe any such interactions between metrifonate and ferric and ferrous ions.

8.2 MATERIALS AND METHODS

8.2.1 Chemicals and reagents

8.2.1.1 UV/VIS Spectroscopy

Metrifonate was purchased from Sigma Chemical Co, St. Louis, USA. The ferrous sulphate was purchased from BDH Laboratory Supplies. All reagents were prepared in deaerated Milli-Q water.

8.2.1.2 Electrochemistry

Citric acid and trisodium citrate used in preparing the citric acid buffer at pH 3.4 and TRIS used in preparation of the tris-HCl buffer at pH 7.4 were purchased from Merck, Midrand, South Africa. Ferrous (II) sulphate and ferric (III) chloride (the ferrous and ferric solutions were prepared from respectively) were purchased from BDH Laboratory Supplies. All other chemicals and reagents were obtained locally and were of the highest chemical purity available.

8.2.2 Apparatus

8.2.2.1 UV/VIS Spectroscopy

The interaction between MET and the metal was studied by comparing the absorption spectra of MET alone in solution, and then upon addition of the metal solution. The final concentrations of MET used were 6.25mM; 12.5mM; and 50mM. The final concentration of ferrous (II) sulphate used was 1mM. All the samples were analyzed using a Cary 500 UV/VIS/NIR over a wavelength of 190-800 nm.

8.2.2.2 Electrochemistry

The electrochemical cell used in these studies comprised of three electrodes, viz. the working, reference and auxiliary electrode as depicted in fig.8.1. below. The first electrode is the working electrode, where the analyte is oxidized or reduced. Working electrodes of metallic origin are mostly used in the studies conducted. The glassy carbon electrode (GCE) was used due to its ideal surface chemistry for adsorption of analytes and possesses excellent mechanical and electrical properties, and is thus chemically inert and exhibits reproducible performance. The reference electrode may also be referred to as the primary electrode and is the standard electrode against which the potential of the working electrode is referenced as it maintains a constant potential and is isolated from the other electrodes. Commonly used reference electrodes are the hydrogen electrode, smooth platinum electrode, saturated calomel electrode, silver/silver chloride electrode, and the glass electrode. Here, the silver/silver chloride electrode was used due to its

suitability in working with aqueous solutions. It consists of a silver wire anodized with silver chloride in a glass tube, the wire being in direct contact with saturated or concentrated solutions of AgCl and either KCl or NaCl. The electrode is protected from the solution by a semi-permeable salt bridge (Sole, 1995, Hawkridge, 1996).

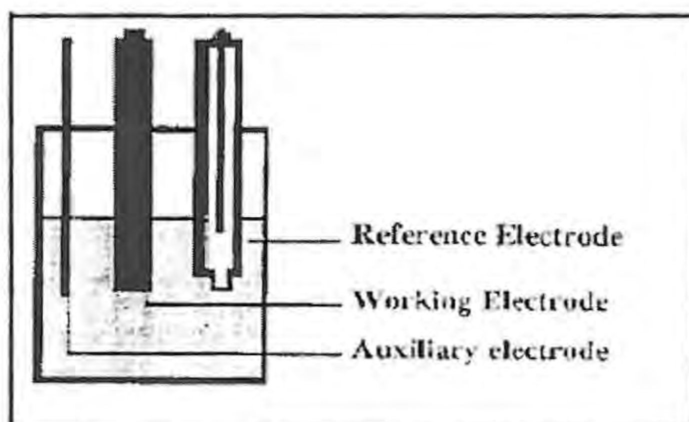


Fig.8.1. Illustrates the three-electrode system used (Limson, 1998).

The third electrode used is named the auxiliary electrode, which is usually constructed of an inert material such as platinum, and is used to prevent voltage drop across the working and reference electrodes. This electrode is not usually isolated from the working electrode while the reference electrode is (Sole, 1995, Hawkridge, 1996). Current passes between the working and auxiliary electrode. Due to oxygen being electroactive at almost all electrodes, and the interference with voltammetric and electrochemical measurements (Sawyer & Roberts, 1974), removal of the oxygen, is performed by bubbling nitrogen or any other inert gas through the solution.

Adsorptive stripping voltammograms (ASVs) were obtained with the Bio Analytical Systems (BAS, Lafayette, Indiana, U.S.A.) CV-50W voltammetric analyzer using a BAS C2 cell stand to maintain a constant atmosphere. A 3mm diameter glassy carbon electrode (GCE) was employed as a working electrode for voltammetric experiments. A silver/silver chloride [KCl = 3 M] and a platinum wire were employed as reference and auxiliary electrodes, respectively, in all electrochemical work. Prior to use and between

scans, the GCE was cleaned by polishing with alumina on a Buehler pad, followed by washing in nitric acid and rinsing in Milli-Q water.

For adsorptive stripping experiments appropriate concentrations of the metal and of the ligand (MET) was introduced into an electrochemical cell. The solution was then de-oxygenated with nitrogen for 2 minutes, after which a flow of nitrogen was maintained over the solution throughout the measurement. Optimum deposition potential for each metal was identified and applied for 2 minutes to effect the formation and adsorption of the metal and ligand species onto the GCE. The voltammograms were then scanned in the negative direction from the deposition potential to at least 0.50V beyond the reduction of the metal at the scan rate of 0.10V s^{-1} to strip the adsorbed metal-ligand species from the electrode. During the stripping step, current responses due to the reduction of the metal-ligand species were measured as a function of potential. All potential values quoted are referenced against the silver/silver chloride (Ag/AgCl) reference electrode.

Ferrous (II) sulphate and drug were dissolved in citric acid buffer at pH 3.4, in order to prevent oxidation of this compound into the ferric (III) ion. The drug was dissolved and tested at both pHs 3.4 and 7.4 in the presence of ferrous (II) sulphate. When working with ferric (III) chloride, both the drug and ferric (III) chloride was tested at pH 7.4.

8.3 RESULTS AND DISCUSSION

8.3.1 UV/VIS Spectroscopy

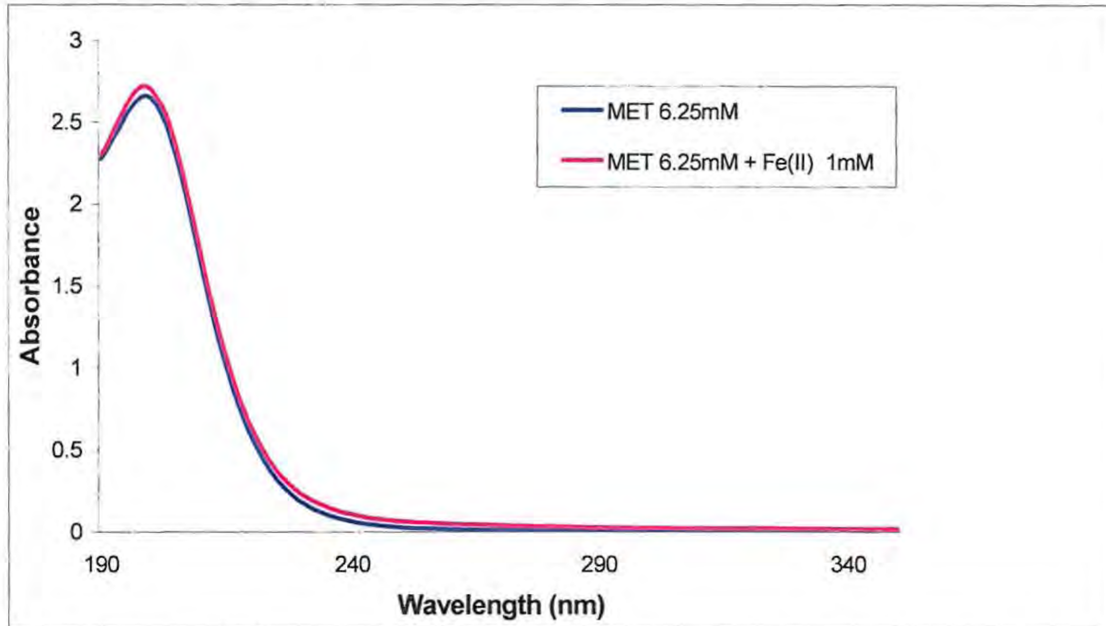


Fig.8.2. Illustrates the results of the spectroscopic analysis of metrifonate (6.25mM) only and in the presence of 1mM Fe^{2+}.

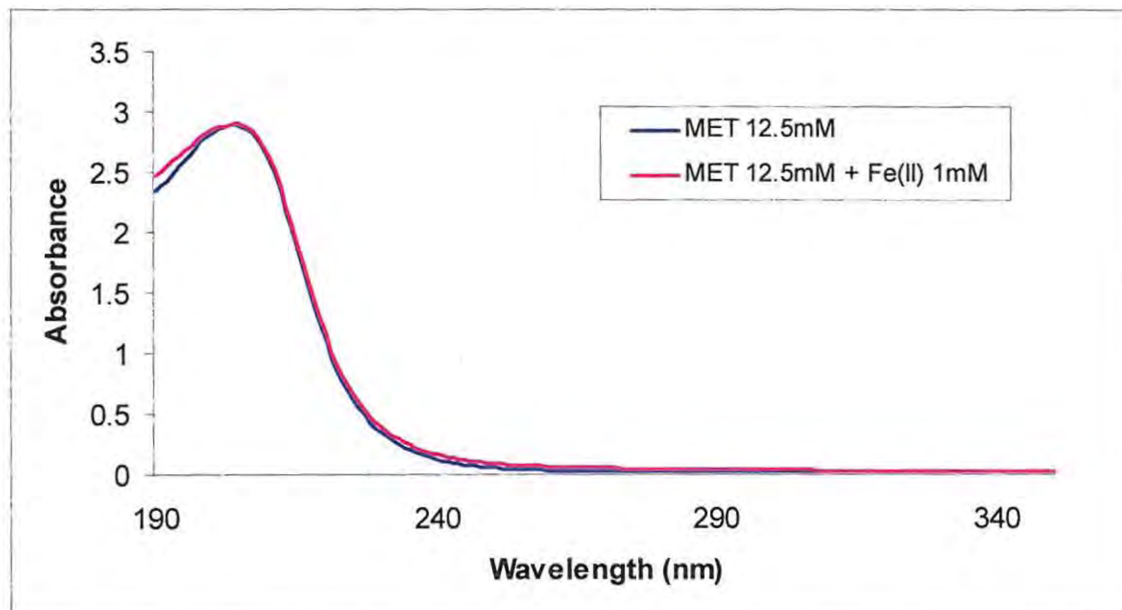


Fig.8.3. Illustrates the results of the spectroscopic analysis of metrifonate (12.5mM) only and in the presence of 1mM Fe^{2+}.

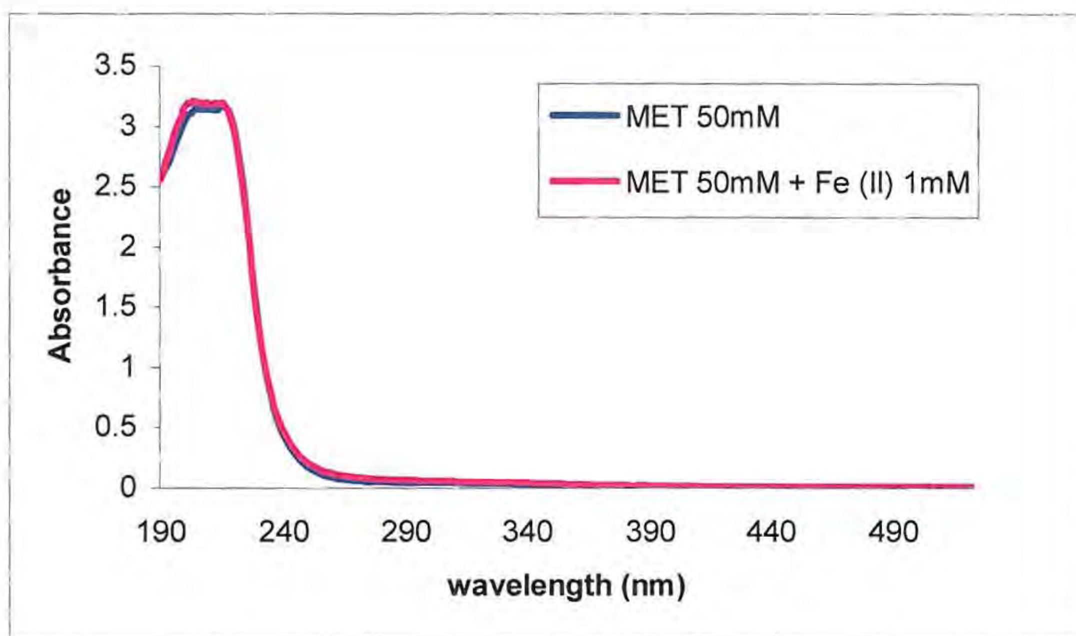


Fig.8.4. Illustrates the results of the spectroscopic analysis of metrifonate (50mM) only and in the presence of 1mM Fe^{2+}.

UV /VIS Spec

Metrifonate shows a lambda max of 210 nm and upon addition of ferrous ions to the various concentrations of MET, no shift or decrease in absorbance occurred. This is indicative of no metal- ligand complexation.

8.3.2 Adsorptive Stripping Voltammetry

Metrifonate & Iron (II) – at pH 3.4

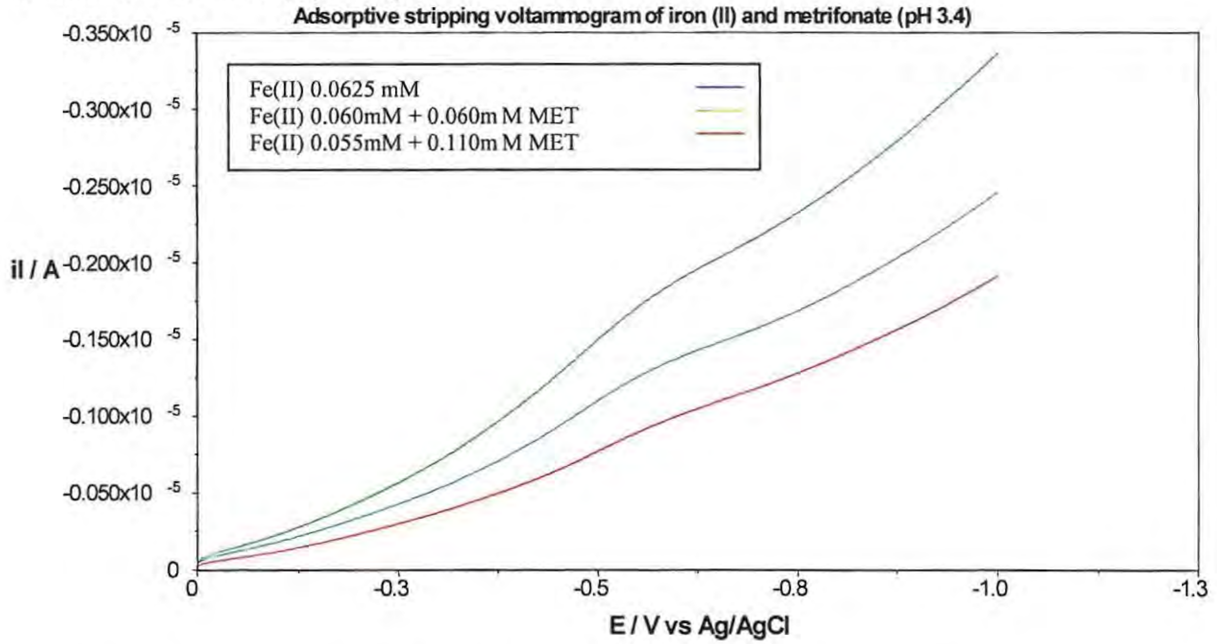


Fig.8.5. Illustrates the result of ASV of Fe^{2+} alone at pH 3.4 and in presence of increasing concentrations of Metrifonate at pH 3.4.

Metrifonate at pH 7.4 & Iron (II) at pH 3.4

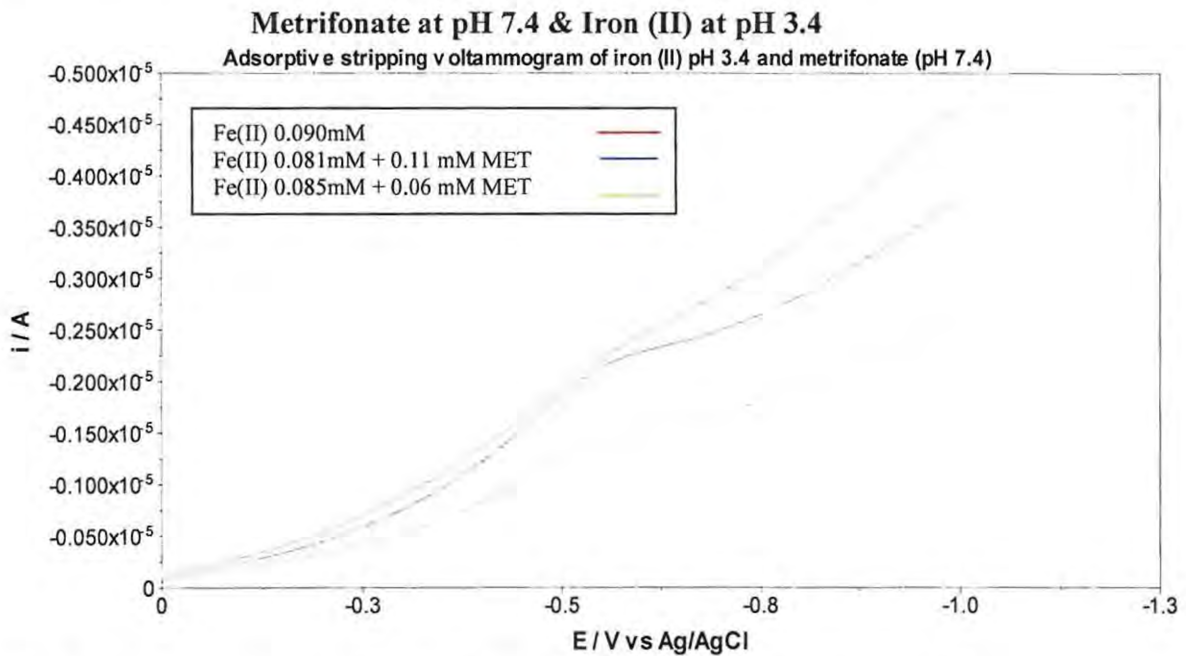


Fig.8.6. Illustrates the results of ASV of Fe^{2+} alone at pH 3.4 and in presence of increasing concentrations of Metrifonate at pH 7.4.

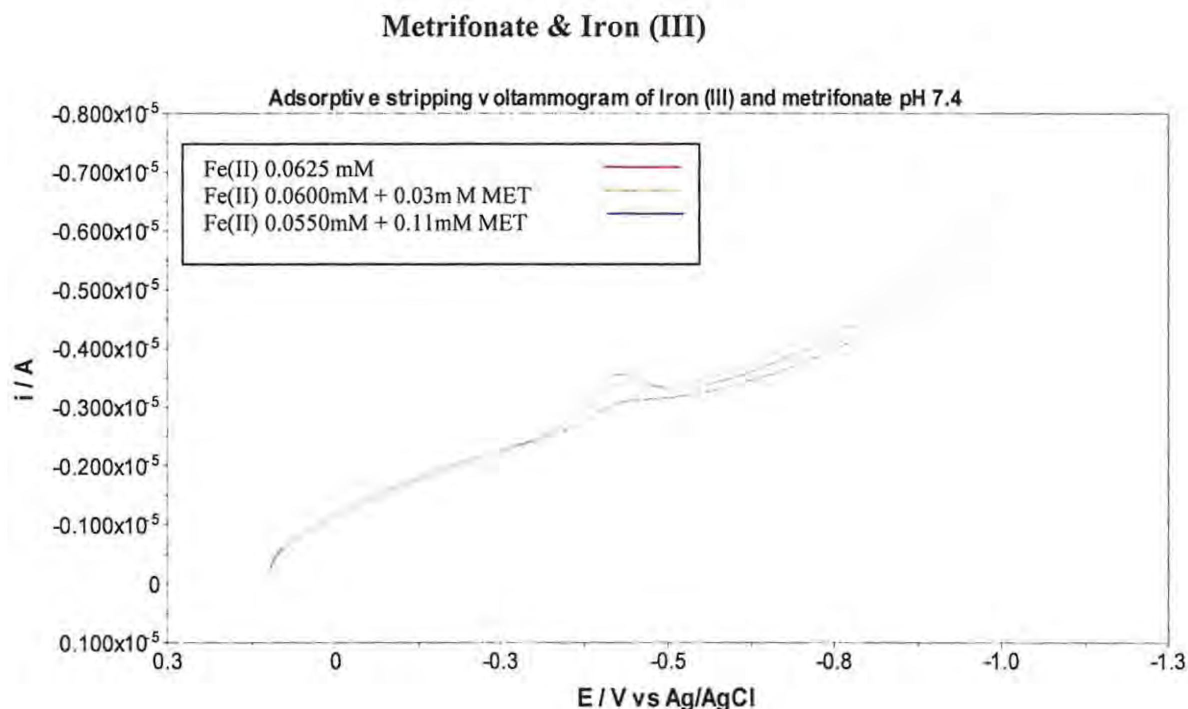


Fig.8.7. Illustrates the results of ADS voltammogram of Fe^{3+} alone at pH 7.4 and in presence of increasing concentrations of Metrifonate at pH 7.4.

Electrochemistry

From electrochemical studies of MET in the presence of both ferrous and ferric irons, no shift in potential was seen, indicating that no metal-ligand or very weak metal ligand complexes were formed. As mentioned earlier, a lowering in current indicates possible competition by the ligand for binding sites at the electrode at relatively high analyte concentrations (i.e. due to overloading of ions at the electrode), or formation of strong metal-ligand bonds where the metal is not easily reduced upon scanning. Since there was no change in potential, and the current both increased and decreased upon further addition of MET, which may have been caused due to the changes in volumes, competition at the electrodes or the formation of strong metal-ligand bonds which are not easily reducible, this study therefore is inconclusive.

In chapter three it was noted that there is no protection but rather a significant increase in lipid peroxidation with MET in the presence of QA which did not occur in the presence

of iron (II) sulphate. Therefore this was postulated to be due to a lack of interaction between metrifonate and ferric as well as ferrous ions. From the results of this *in vitro* study, even though the results of the UV/VIS spectroscopy offered no interaction, the ASV offered an inconclusive result, making it unlikely that there is any interaction between metrifonate and iron.

CHAPTER NINE – SUMMARY, CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

The experiments conducted in this study show that metrifonate has the potential to cause neurotoxicity. In chapter two, metrifonate increased the formation of superoxide anions, and therefore the level of oxidative stress in the cell. Chapter three investigated the neuroprotective effects of metrifonate against oxidative stress in the form of lipid peroxidation induced by quinolinic acid, and the toxic metal iron. Metrifonate significantly increased lipid peroxidation in the presence of quinolinic acid yet not in the presence of iron. Metrifonate alone induced lipid peroxidation. However, levels of lipid peroxidation were reduced *in vitro* in the presence of melatonin. This indicated that metrifonate induces lipid peroxidation by the generation of free radicals, but does not influence lipid peroxidation via the Fenton reaction.

In chapter four, due to the increase in oxidative stress seen thus far, the study in this chapter was conducted to determine the effect that metrifonate had on the electron transport chain within a mitochondrial fraction isolated from the rat brain. The results demonstrated a decrease in electron transport activity in metrifonate treated samples, whereas those samples treated with both melatonin and metrifonate, had sustained the electron transport chain activity. Since melatonin is a free radical scavenger and known to affect the mitochondria at the level of complex I, (Martin *et al.*, 2002), these results indicated that metrifonate was disrupting the electron transport chain, possibly at the level of complex I. Thus, the effect of metrifonate on complex I activity was determined. The results from this study show that metrifonate does affect the functioning of complex I, and this may be the reason for the increase in superoxide generation and oxidative damage.

In chapter five, pineal organ culture investigated the effect of metrifonate on the production of melatonin, a natural antioxidant and neuroprotectant, produced by the pineal gland. The results obtained show that metrifonate does not stimulate an increase in

melatonin production, yet an increase in deamination products, hydroxyindole acetic acid and methoxytryptophol. However, the exact mode of action remains to be established. Further studies may be undertaken to establish the effect of metrifonate on the enzymes responsible for the production of hydroxyindole acetic acid and methoxytrptophol, as the cause of the increase in these products. Metrifonate therefore, does not have indirect neuroprotective properties by the simulation of melatonin production.

In chapter six histological investigation of nervous tissue within the hippocampal CA3 regions show visible disruptions of the structural features of these regions in rats treated with metrifonate only 1mg/kg/day i.p. and significant protection in rats treated simultaneously with melatonin (1, 5 and 10 mg/kg/day i.p.). It is suggested that the ability of metrifonate induces lipid peroxidation and this could be one of the factors pertaining to the higher degree of swelling within neuronal cells in the brains of metrifonate treated rats. The antioxidant action of melatonin prevented this swelling thereby inactivating the neurotoxic effects.

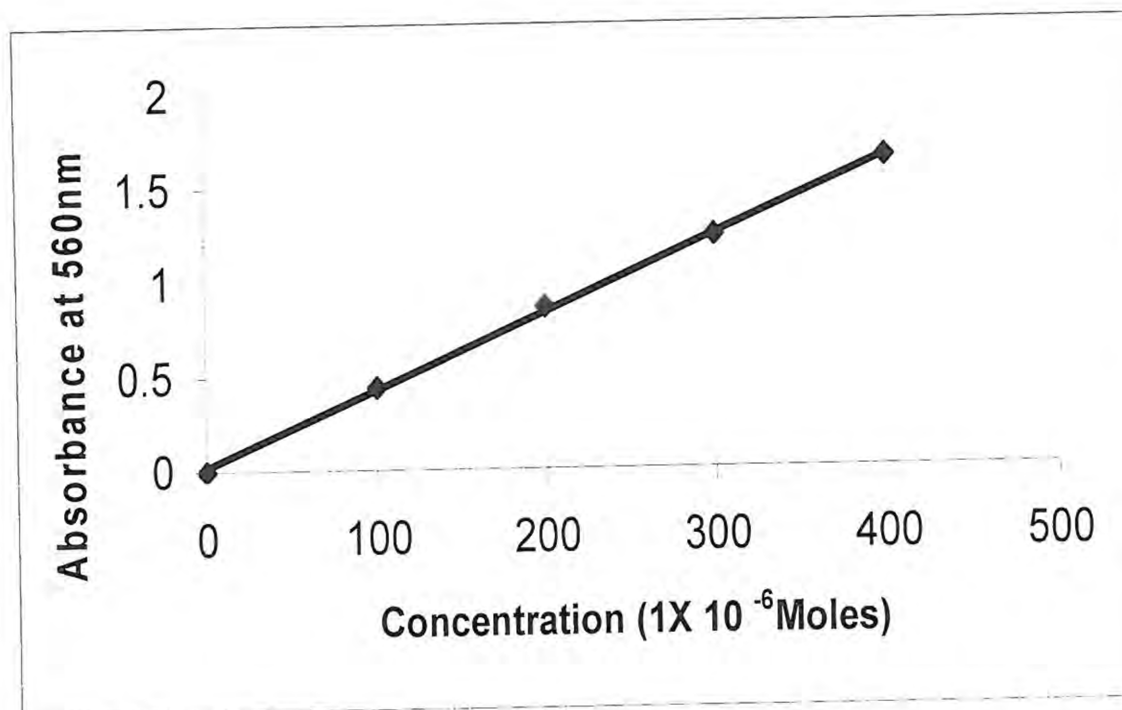
Chapter seven investigated the effect of metrifonate on the apoptotic cell processes of the neuronal tissue. It causes an increase in apoptosis at higher dose of 1mg/kg/day i.p. which was reduced in the presence of melatonin (1, 5 and 10mg/kg/day i.p). This indicates that metrifonate does affect levels of oxidative stress which lead to apoptotic cell death, which was further strengthened by the protection of the cell by an anti-oxidant, melatonin.

Chapter eight was devoted to the investigation of the metal-binding capabilities of metrifonate with iron (II) and iron (III), using spectrophotometry and electrochemical techniques. Metrifonate and metal ion were analysed using UV/VIS spectrophotometric analysis, which did not suggest interactions between metrifonate and iron (II) or iron (III) in terms of a shift in the wavelength and an increase in absorbance of metrifonate. The results imply a lack interaction between with iron (II) and metrifonate spectrophotometrically. The electrochemical results do not support the possibility of an interaction with iron (II) and iron (III).

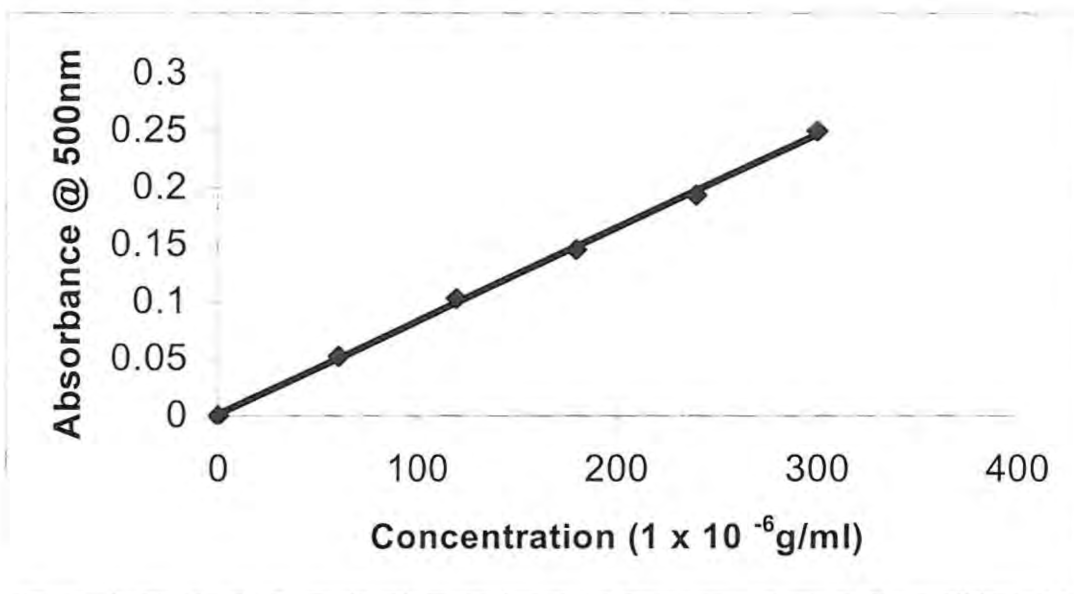
Metrifonate, is essentially an organophosphate, which has been noted to cause, apart from acute toxicity viz. over-stimulation of the muscarinic system, OPIDN due to alterations $Ca^{2+}$and calmodulin levels in cells, as mentioned earlier. Therefore the affect of metrifonate on intracellular calcium levels require further investigation as the potential source of the increase in oxidative stress and damage.

Due to the potential neurotoxic effects of metrifonate, it is suggested that it should not be used in neurodegenerative conditions such as Alzheimer's disease.

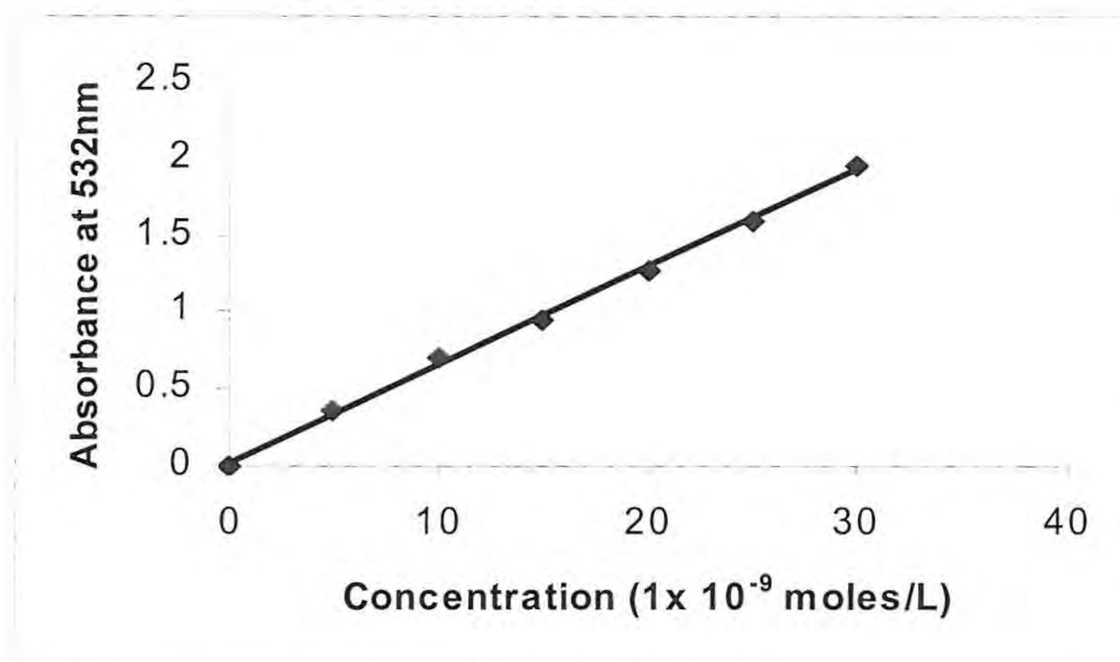
APPENDICES



APPENDIX 1 : THE CALIBRATION CURVE FOR DIFORMAZAN ($R^2 = 0.999$)



APPENDIX 2: PROTEIN STANDARD CURVE (R^2 = 0.998)



APPENDIX 3 : MALONDIALDEHYDE STANDARD CURVE (R^2 = 0.998)

APPENDIX 4: HARRIS HAEMOTOXYLIN AND EOSIN STAINING

Preparation of Harris Haemotoxylin

Haemotoxylin crystals	5.0 grams
Alcohol (100%)	50.0 ml
Ammonium or Potassium Alum	100.0 grams
Distilled water	1000.0 ml
Mercuric oxide (red)	2.5 grams

Dissolve the haemotoxylin crystals in the alcohol, and alum in water, aided by heating. Remove from heat and combine the two solutions. Bring to boil as rapidly as possible. Remove from heat and add the mercuric oxide slowly. Reheat to simmer, until mixture becomes dark purple in colour, remove from heat and immediatley plunge the mixture containing vessel into a basin of cold water until mixture is cool. Once cooled, the mixture is ready for use. Addition of 2-4ml glacial acetic acid per 100ml of solution increases the precision of the nuclear stain. Filter before use.

1% Stock Alcoholic Eosin

Eosin Y, water soluble	1.0 grams
Distilled water	20.0 mls
Alcohol (95%)	80.0 mls

Dissolve the eosin Y in distilled water. Add the 95% alcohol.

Scott's Tap Water Substitute

Tap Water	1000.0 mls
Magnesium Sulphate (anhydrous)	10.0 grams
Sodium Bicarbonate	2.0 grams

To the tap water, add in the magnesium sulphate and sodium bicarbonate. Stir until dissolved.

APPENDIX 5 : DAVIDSON'S ALCOHOL FORMALIN ACETIC ACID FIXATIVE

Fixation with Davidson's Alcohol Formalin Acetic Acid Fixative (Davidsons AFA)

Ethyl alcohol (95%)	330.0 ml
Formalin (37-39% solution)	220.0 ml
Glacial Acetic Acid	115.0 ml
Distilled Water	335.0 ml

Combine liquids. Store at room temperatue.

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