AN INVESTIGATION INTO THE POSSIBLE NEUROPROTECTIVE PROPERTIES OF PHENYTOIN

THESIS

Submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE (PHARMACY)

Of

Rhodes University

By

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December 2002

DEDICATED TO ALL THE ANIMALS SACRIFICED FOR THIS THESIS

Humankind has not woven the web of life.
We are but one thread within it.
Whatever we do to the web, we do to ourselves.
All things are bound together.
All things connect.

Chief Seattle, 1854

ABSTRACT

Cerebral ischaemia, traumatic injury to the brain, inflammatory neurological disorders and HIV infections are amongst the most prevalent causes of neurodegeneration. Neuroprotective strategies are usually to limit the progressive secondary injury that generally occurs, thus limiting overall tissue damage.

Neuroprotective strategies are usually to limit the progressive secondary injury that generally occurs, thus limiting overall tissue damage. Sodium channel blockers have been often used for this matter as they prevent the cascade of events culminating in free radical generation and eventually neuronal apoptosis. Newer compounds, such as antiperoxidants and free radical scavengers, show encouraging experimental results, but their clinical use is still very limited.

Phenytoin being a popular drug in the treatment of epilepsy has also been used as a neuroprotectant during certain neurological emergencies and in pharmacological prophylaxis of post-traumatic epilepsy. Furthermore this agent functions by prolonging inactivation of voltage gated sodium channels. In these sets of experiment the neuroprotective properties of phenytoin were examined.

The histological study revealed that phenytoin confers protection to the CA1 and CA3 regions of the hippocampus under the insult of QUIN. Cells maintain their characteristic shape and minimal tissue necrosis occurs in the presence of this agent.

The *in vitro* effect of this antiepileptic drug on free radicals generation shows that phenytoin does not reduce or prevent the formation of these reactive species. Lipid peroxidation was induced using QUIN and iron (II), two known neurotoxins. The study reveals that only lipid peroxidation induced using iron (II) is reduced by phenytoin. These experiments were carried out in whole rat brain homogenate. These studies show that phenytoin possesses poor free radical scavenging properties. However, the dose-related reduction of iron-induced lipid peroxidation allows for speculation that phenytoin interacts with iron in order to reduce neuronal damage.

Metal binding studies were performed using UV, IR and electrochemical analysis to examine the interaction of phenytoin with iron (II) and iron (III). Phenytoin, when added to iron (II) in solution, first oxidises the latter to iron (III) and maintains it in that form. A shift in the peak was observed in the UV spectrum when iron was added to phenytoin. Moreover, electrochemical studies indicate that the interaction between the metal and the ligand is very weak. The IR analysis it shows that phenytoin may be coordinating with iron through the Nitrogen atom on the phenytoin molecule. These studies show that phenytoin maintains iron in its oxidised form, which is a good property to possess as a neuroprotectants.

Pineal organ culture showed that phenytoin does not increase melatonin production but slightly and non-significantly reduces the levels of this pineal hormone. However there is a significant rise in precursor NAS levels. As melatonin is known to possess antioxidant and free radical scavenging properties, this could mean that this drug can cause the CNS to become more susceptible to attacks by reactive oxygen species.

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ABBREVIATIONS

3-HAO:	3-hydroxyanthranilic acid oxygenase
4-HAD:	4-hydroxyalkenals
5HT:	Serotonin
5MT:	5-Methoxytryptamine
Ag:	Silver
AgCl:	Silver chloride
AIDS:	Acquired immuno deficiency syndrome
ANOVA:	Analysis of variance
AP5:	2-amino-5-phosphonovalerate
ATP:	Adenosine triphosphate
ASV:	Adsorptive stripping voltammograms
BHT:	Butylated Hydroxytoluene
^{o}C:	Degrees celcius
Ca^{2+}:	Calcium
Cl^{-}:	Chloride
cm^{-1}:	per centimetre
CNS:	Central nervous system
CPM:	Counts per minute
CSF:	Cerebrospinal fluid
CV:	Cyclic Voltammograms
DMSO:	Dimethyl sulphoxide
DNA:	Deoxyribonucleic acid
EAA:	Excitatory amino acids
EDTA:	Ethylenediaminetetraacetic acid
Fe^{2+}:	Iron (II)
Fe^{3+}:	Iron (III)
FHS:	Foetal Hydantoin Syndrome
GABA:	γ-aminobutyric acid
GCE:	Glassy carbon electrode
GSH:	Glutathione

GSHPx:	Glutathione peroxidase
GSSG:	Reduced glutathione
H^+:	Proton
H_2O:	Water
H_2O_2:	Hydrogen peroxide
HIAA:	Hydroxyindoleacetic acid
HIOMT:	Hydroxyindole-O-methyltransferase
HIV:	Human immuno virus
HPPH:	5-(*p*-hydroxyphenyl)-5-phenylhydantoin
HTOH:	Hydroxytryptophol
IKr:	Delayed rectified potassium channel
IR:	Infrared
IV:	Intra-venous
K^+:	Potassium
KA:	Kainic acid
KBr:	Potassium bromide
KCN:	Potassium cyanide
kg:	Kilogram
mCi/ml:	millicurrie per millilitre
MDA:	Malondialdehyde
MEL:	Melatonin
MIAA:	Methoxyindoleacetic acid
μl:	microlitre
ml:	millilitre
mg:	milligram
mg/L:	milligrams per litre
Mg^{2+}:	Magnesium
mM:	millimolar
Mn:	Manganese
MTOH:	Methoxytryptophol
mV:	millivolt

Na^+:	Sodium
NAD:	Nicotinic acid dinucleotide
NADPH:	Nicotinamide adenine dinucleotide phosphate
NAS:	N-acetyl serotonin
NAT:	N-acetyltransferase
NBD:	Nitro-blue diformazan
NBT:	Nitro-blue tetrazolium
nm:	Nanometre
NMDA:	N-methyl-D-aspartate
NO:	Nitric oxide
NOS:	Nitric oxide synthase
O_2:	Oxygen
O^{2-}:	Superoxide anion radical
OH^-:	Hydroxyl radical
$ONOO^-$:	Peroxynitrite
P450:	Cytochrome P450
PBS:	Phosphate buffered saline
PCP:	Phencyclidine
PUFA:	polyunsaturated fatty acids
QPRTase:	Quinolinate phosphoribosyl transferase
QUIN:	Quinolinic acid
ROS:	Reactive oxygen species
SD:	Standard deviation
SCN:	Suprachiasmatic nucleus
SHE:	Standard hydrogen electrode
SOD:	Superoxide dismutase
TBA:	2-Thiobarbituric acid
TCA:	Trichloroacetic acid
TEAP:	Tetraethylammonium phosphate
TBSI:	Traumatic Brain and Spinal Cord Injury
TLC:	Thin layer chromatography

UV:	Ultraviolet
V:	Volts
V s^{-1}:	Volts per second
w/v:	Weight per volume
Zn:	Zinc

ACKNOWLEDGEMENTS

I would like to thank the following people:

Prof. Santy Daya, my supervisor, for his guidance and support.

Dr. Janice Limson, for her support and expertise with electrochemistry.

The Pharmacy department for all the assistance and facilities offered within the department.

Andy Soper, Dave and Sally Morley, for all their technical assistance.

The Biochemistry and Botany department of Rhodes University for allowing me to use their equipment, a special thanks to Prof. B Ripley from Botany and Prof J Duncan from Biochemistry.

Prudence, for maintaining the cleanliness of the laboratory and taking care of the animals

Mira for all her encouragements, support and love, thank you for being there for me☺.

My parents and family for all their encouragement. Without you this would not have been possible.

Shailendra “Shai” Annopkumar-Dukie, for all his help and guidance.

Amichand “Ami” for being a good motivator, thank you brother.

My fellow MSc colleagues, everyone from Worcester Mews, African lodge and all my friends.

The NRF and Rhodes University for the financial assistance towards this study.

Merci beaucoup
☺

CHAPTER 1
LITERATURE REVIEW

1. INTRODUCTION

1.1. PHENYTOIN

1.1.1. HISTORY OF PHENYTOIN

Phenytoin (5,5-diphenylhydantoin) was first synthesised in 1908 by the German chemist Heinrich Biltz. He sold phenytoin along with other compounds to the company Parke - Davis. The company did not patent it, nor did they find a use for it. As a result, phenytoin was forgotten for twenty-nine years.

In 1937, Putnam and Merritt, two doctors outside Parke - Davis, discovered phenytoin's first clinical use, in epilepsy. This discovery had an extremely important impact on the treatment of epilepsy; in fact phenytoin proved to be superior to phenobarbital in treating epilepsy and at the same time became the first anticonvulsive drug not to cause sedation. This therapeutic property of phenytoin is still used at present with its principle application being the cases of Grand Mal epilepsy and tonic-clonic seizures. Another major application of phenytoin is in its pronounced antiarrhythmic property and phenytoin is especially indicated for the treatment of ventricular arrhythmias, and more precisely of the arrhythmias caused by digitalis toxicity and by acute myocardial infarction.

Both above-mentioned applications are due to phenytoin’s ability to stabilize the cellular membranes by means of decreasing sodium conductivity through the sodium channels of these membranes. But these properties do not

represent the whole broad spectrum of its clinical usefulness. In fact, phenytoin is useful for over fifty symptoms and disorders. These consist of the following:

Thought, mood and behaviour disorders:

- Psychosis;
- Violent behaviour;
- Alcoholism and drug addiction;
- Sleep disorders;
- Enuresis;
- Anorexia, bulimia and binge Eating.

Cardiovascular disorders:

- Q-T interval syndrome;
- Torsade de Pointes;
- Tricyclic antidepressant overdose;
- Angina pectoris;
- Hypertension;
- Atherosclerosis.

Neuromuscular disorders:

- Chorea minor (Sydenham's Horea);
- Athetosis;
- Dystrophic myotony;
- Stiff-Man syndrome;
- Restless legs syndrome;
- Steroid myopathy;
- Intractable Hiccups;
- Respiratory myoclonus;

- Palatal myoclonus;
- Hypocalcemia;
- Tetanus.

Treatment of pain in:

- Trigeminal neuralgia;
- Glossopharyngeal neuralgia;
- Migraine;
- Diabetic neuropathy;
- Tabes dorsalis;
- Postherpetic neuralgia.

Healing in:

- Periodontitis;
- Ulcers;
- Burns;
- Epidermolysis bullosa;
- Pachyonychia.

Treatment of Other Disorders:

- Diabetic diarrhoea;
- Ulcerative colitis;
- SIADH;
- Transfusion reactions;
- Head injuries and surgery;
- Glaucomatous field loss.

The Dreyfus Health Foundation, a not-for-profit organisation, began thirty years ago as the Dreyfus Medical Foundation. Jack Dreyfus, founder of the Dreyfus Funds and Corporation, started the foundation after treatment with

phenytoin led to his recovery from endogenous depression. Finding that the uses of phenytoin were not widely known, he established the Dreyfus Medical Foundation sponsoring worldwide research and dissemination of information on this drug.

Since 1970, three bibliographies summarising and analysing the medical literature on the uses of phenytoin have been distributed to physicians worldwide. These bibliographies were derived from the Foundation's library, which now contains over 16,000 papers. In 1980, Jack Dreyfus wrote a book, *"A Remarkable Medicine Has Been Overlooked"*, for the government, the medical profession and the public. A new edition of this narrative, plus the latest phenytoin bibliography *"The Broad Range of Clinical Use of Phenytoin"*, were sent free of charge to 508,000 physicians in the United States, in 1987. These books have also been translated into Italian, French, Russian, Chinese, Spanish and German (www.dhf-bg.org/phenen.html).

Phenytoin is one of the oldest drugs used for the treatment of epilepsy. It has a very long history of effectiveness and safety. Because of this excellent track record, it is frequently the first drug used by many physicians to treat seizures.

Over the past 5 years, a handful of new agents have been approved for use as antiseizure medications. Fosphenytoin is an example. It is a pro-drug form of phenytoin, and is rapidly cleaved by phosphatases *in vivo* to form phenytoin, as discussed further in section 1.1.2.

1.1.2. STRUCTURE-ACTIVITY RELATIONSHIP AND PHYSICOCHEMICAL PROPERTIES

Phenytoin, 5,5-Diphenyl-2,4-imidazolidinedione; diphenylhydantoin (see Figure 1.1) exists as a white crystalline powder or granule. It is a diphenyl substituted hydantoin with much lower sedative properties than compounds with alkyl substituents at the 5 position and has a molecular formula $C_{15}H_{12}N_2O_2$. It is only minimally soluble in normal saline and must be dissolved in solution at a pH of 12.

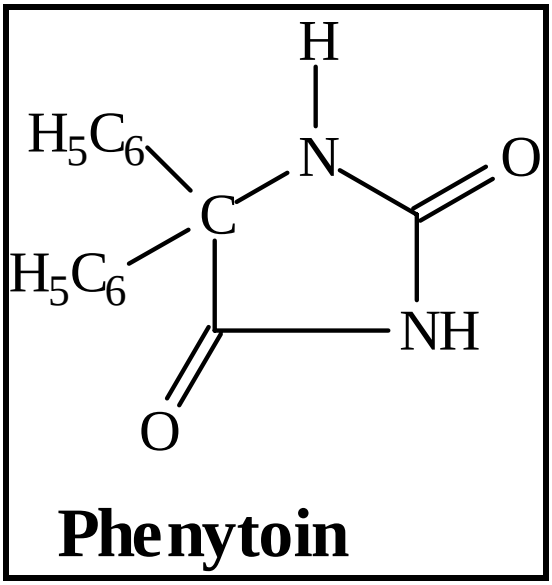


Figure 1.1. Structure of Phenytoin

The hydantoin [imidazoline-2,4-dione] ring containing two amide functionalities provide two chelatable electronegative atoms, N and O, in close proximity on the three faces of the heterocycle. This makes phenytoin a good candidate to interact with metallic ions (Milne, P. *et al.*, 1999).

Fosphenytoin, a new drug, is a phosphorylated inactive version of phenytoin that is rapidly dephosphorylated by endogenous phosphatases to active phenytoin (Browne TR, *et al.*, 1996) (see figure 1.2). It is quickly and completely converted to phenytoin and has no separate mechanism of

anticonvulsant activity. It is soluble in water and saline at pH 8.6 to 9.0. It is effective in animal models of Status Epilepticus (SE), and preliminary reports in humans are encouraging (Allen FH, *et al*, 1994).

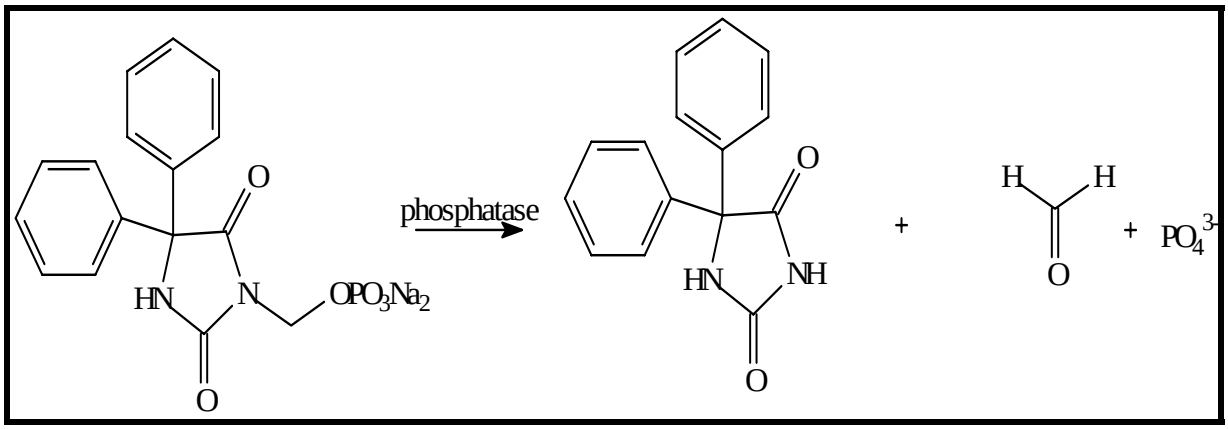


Figure 1.2.: Prodrug Fosphenytoin on the left going to Phenytoin *in vivo*

1.1.3. SYNTHESIS

Phenytoin can be synthesised in three steps from benzaldehyde. The first step in the synthesis is catalysed by thiamine (vitamin B_1). The mechanism is fairly straightforward. The second step in the mechanism is a simple oxidation. The third step is complicated and involves an interesting rearrangement. This can be seen in Figure 1.3.

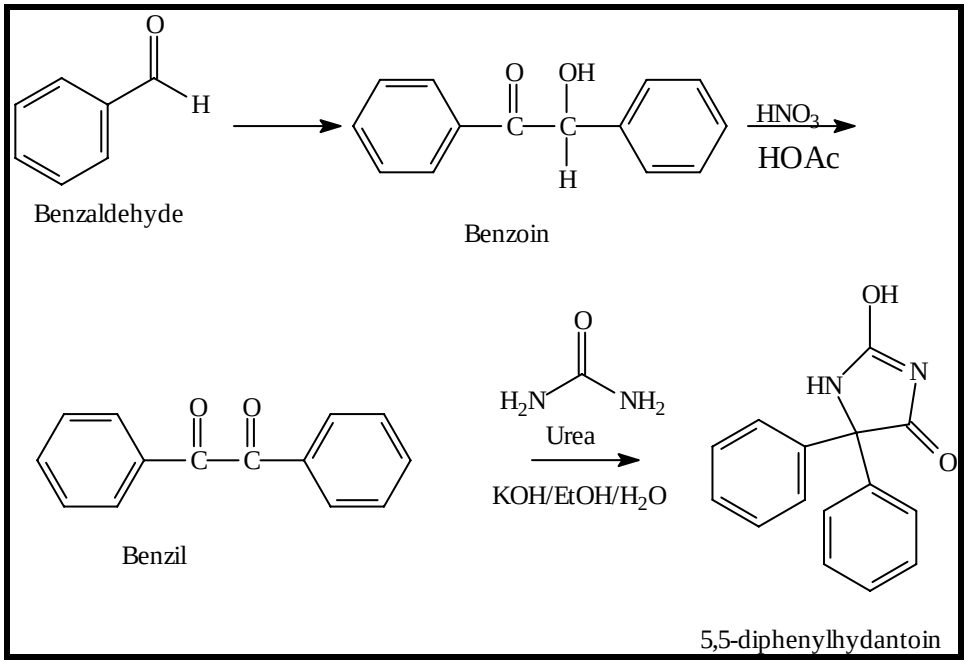


Figure 1.3. Synthesis of 5,5-diphenylhydantoin (Mohrig, J.R. *et al. 1997)*

1.1.4. MODE OF ACTION AND RECEPTOR INTERACTIONS

Phenytoin acts by promoting sodium efflux from neurons. The sodium ions tend to stabilize the threshold against hyperexcitability caused by excessive stimulation or environmental changes capable of reducing the sodium membrane gradient, depressing neuronal excitability.

Phenytoin has major effects on several physiological systems. It alters Na^+, K^+ and Ca^{2+} conductances, concentrations of amino acids and the neurotransmitters norepinephrine, acetylcholine and γ-aminobutyric acid (GABA). Phenytoin blocks post titanic potentiation in spinal cord preparations, but the role of this effect in suppressing seizure spread is not yet defined. This effect is observed at therapeutically relevant concentrations. It is a use-dependent effect on Na^+ conductance, arising from preferential binding to and prolongation of the inactivated state of the Na^+ channel (Macdonald *et al*., 1993)**.**

Phenytoin may act via several different mechanisms, but the most studied is based on its interference with voltage-gated sodium channels (Macdonald RL & Kelly KM, 1993).

In normal neurons, when the cell depolarises the action potential threshold, the voltage change transiently activates, or "opens," voltage gated Na^+ channels at the axon hillock, which then propagates a single action potential down the axon. This allows cells to communicate with each other. The structure of the axon hillock and axon collaterals as well as the direction of signal propagation can be seen in Figure 1.4. The normal direction of information flow is from the axon terminal to the target neuron. As a result of this, the axon terminal is termed presynaptic and the target neuron is referred to as postsynaptic (Bear, *et al.*, 2001). Figure 1.5. shows the typical synaptic transmission between the axon terminal of one neuron with the dendrite of the other.

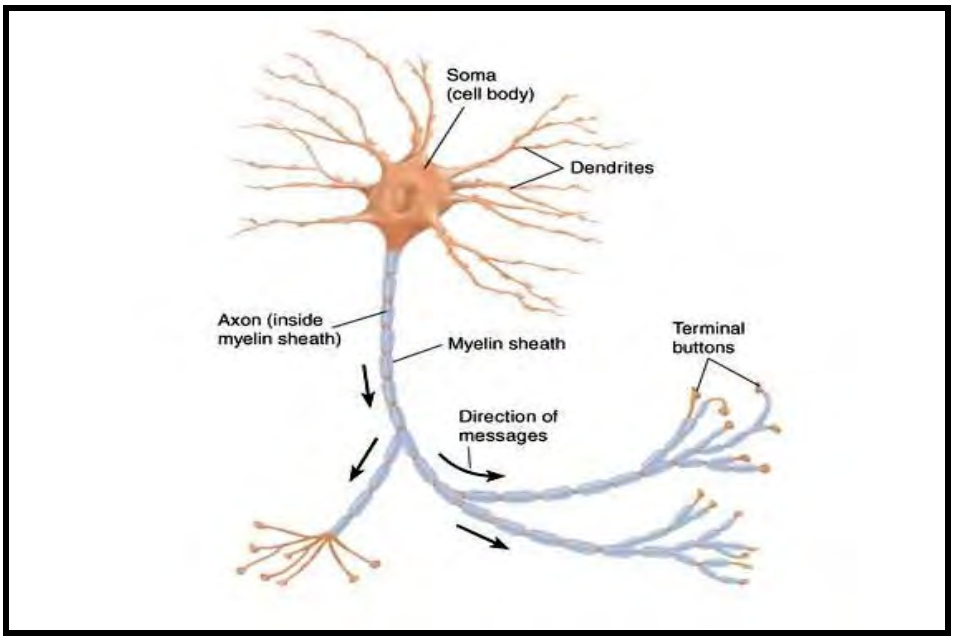


Figure 1.4. A diagram demonstrating the structure of the axon and cell body. The arrows indicate the direction and propagation of information flow (www.driesen.com/nervecell.jpg)

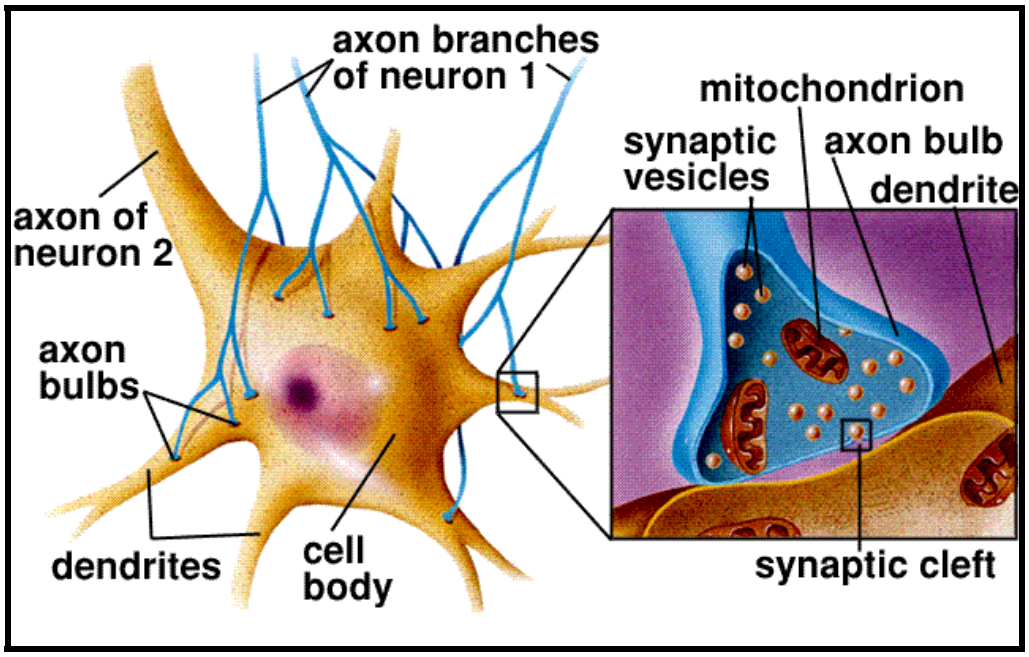


Figure 1.5. Synaptic transmission from the presynaptic neuron to the postsynaptic terminal (www.mhhe.com/biosci/ap/dynamichuman2/content/gifs/0191s.gif)

During prolonged depolarisation, for example during status epilepticus, the cell repetitively fires many action potentials and thus repetitively triggers other neurons. The same effect occurs in normal cells when they are artificially depolarised, resulting in sustained repetitive firing. Phenytoin prolongs inactivation of voltage gated Na^+ channels and prevents sustained repetitive firing *in vitro*, but allows at least a single action potential to pass. Therefore, it allows "normal" signals to pass, but blocks passage of repetitive action potentials.

There is also evidence that phenytoin prevents Ca^{2+} entry into neurons and thus may decrease excitability by this mechanism. Moreover, phenytoin, which decreases the excitability of neurons, has recently also been shown to block the rapid component of the delayed rectified potassium channel (IKr),

resulting in prolongation of the repolarisation phase of the action potential, in addition to its known blockade of sodium channels.

1.1.5. PHARMACOKINETICS

1.1.5.1. Absorption

Absorption of phenytoin is highly dependent on the formulation of the dosage form. Absorption of phenytoin sodium from the gastrointestinal tract is nearly complete in most patients, though the time to peak may range from 3 hours to 12 hours. Absorption from intramuscular injection is unpredictable and some drug precipitation in the muscle occurs.

1.1.5.2. Distribution

Phenytoin is highly bound to plasma proteins. It appears certain that the total plasma level decreases when the fraction that is bound decreases, as in uremia or hypoalbuminemia, but correlation of free levels with clinical states remain uncertain. Drug concentration in the cerebrospinal fluid is proportionate to the free plasma level and phenytoin accumulates in and becomes bound to the endoplasmic reticulum of cells in brain, liver, muscle and fat.

1.1.5.3. Metabolism and Elimination

Phenytoin is metabolised primarily by parahydroxylation to 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (HPPH), which is subsequently conjugated with glucuronic acid. The metabolites are clinically inactive and are excreted unchanged. At very low blood levels, phenytoin metabolism is proportionate to the rate at which the drug is presented to the liver, that is, first-order metabolism. However, as blood levels rise within the therapeutic range, the maximum capacity of the liver to metabolise phenytoin is

approached. Further increases in dose, even though relatively small, may produce very large changes in the drug's concentration and patients can develop toxicity. The half-life of phenytoin varies from 12 hours to 36 hours, with an average of 24 hours for most patients in the low- to mid-therapeutic range. At low blood levels, it takes 5-7 days to reach steady state blood levels after every dosage change; at higher levels it may be 4-6 weeks before blood levels are stable.

Phenytoin's metabolism has been well studied. Four oxidative metabolites, 49-HPPH, 39-HPPH, 39,49-diHPPH, and 39,49-dihydrodiol, are reported in humans (Figure 1.6.) (Maguire, 1988; Szabo et al., 1990).

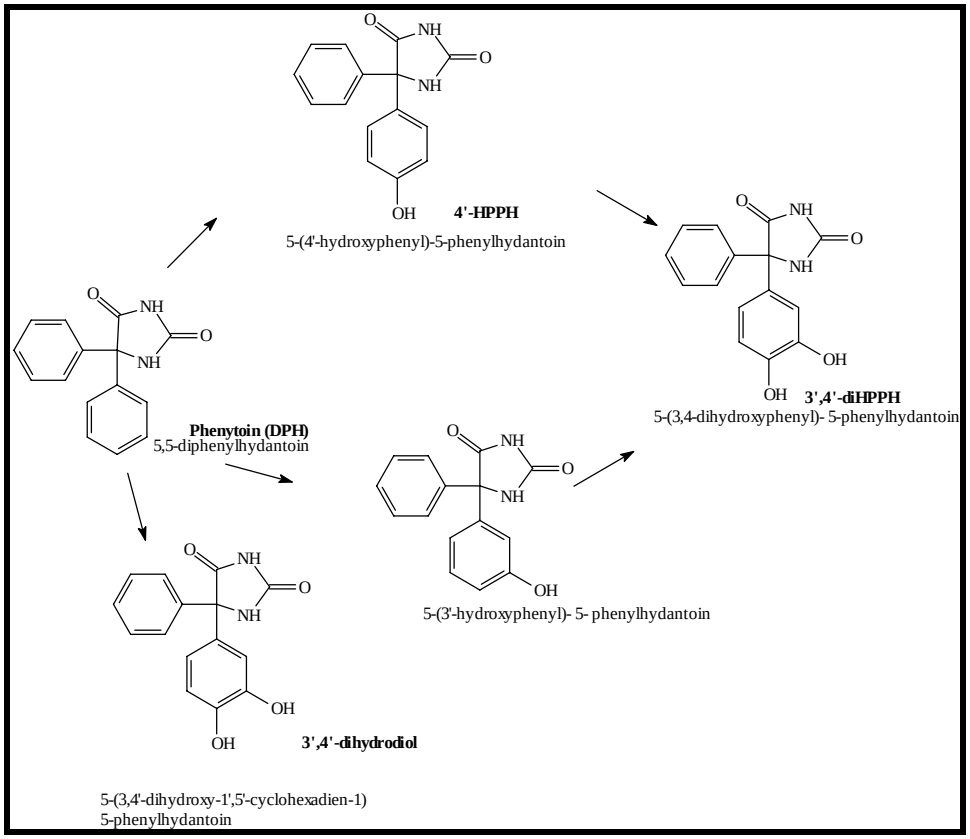


Figure 1.6. Proposed pathways of phenytoin metabolism in humans. (Maguire, 1988; Szabo et al., 1990)

It has been suggested that the anticonvulsant drug phenytoin is mainly metabolised to 49-HPPH by CYP2C9 (Bajpai et al., 1996). In addition Komatsu, T *et al.* (2000) discovered that CYP2C19, CYP2C9, and CYP3A4 contributes significantly to 39,49-diHPPH formation from primary hydroxylated metabolites. Roles of these P450s vary among different human liver samples having compositions of various P450 enzymes. As a result, this might indicate potential contributions of multiple P450 enzymes in 39,49-diHPPH formation. They also suggest that 49-HPPH formation and also other reactions (e.g., 39,49-diHPPH formation) can be involved in nonlinearity of phenytoin plasma concentrations or adverse phenytoin reactions in humans.

1.1.6. THERAPEUTIC LEVELS AND DOSAGE

The therapeutic level of phenytoin for most patients is between 10 to 20 μg/ml. A loading dose can be given either orally or intravenously; the latter is the method of choice for convulsive status epilepticus. When oral therapy is initiated, it is common for adults to begin at a dosage of 300 mg/day regardless of body weight. While this may be accepted in some patients, it frequently yields steady state blood levels of 10 μg/ml, the minimum therapeutic level for most patients. If seizures continue, higher doses are usually necessary to achieve plasma levels in the upper therapeutic range. This is normally done with only small increments in dose. In children, a dosage of 5 mg/kg/day should be followed by readjustment after steady state plasma levels are obtained.

1.1.7. SIDE EFFECTS AND TOXICITY

Nystagmus occurs early, as does loss of smooth extraocular pursuit movements, but neither is an indication for decreasing the dose. Diplopia and ataxia are the most common dose-related adverse effects requiring dosage adjustment; sedation occurs only at considerably higher levels. Gingival hyperplasia and hirutism occur to some degree in most patients; the latter can be especially unpleasant in females. Long-term chronic use is associated in some patients with the coarsening of facial features and mild peripheral neuropathy. Chronic use may also result in abnormalities of vitamin D metabolism, leading to osteomalacia. Low folate levels and megaloblastic anaemia have been reported, but clinical importance of this observation is unknown. Idiosyncratic reactions to phenytoin are relatively rare (Extra Pharmacopoeia, 1998)

1.1.8. INTERACTIONS OF PHENYTOIN

Phenytoin is known to interact with a number of other drugs. These interactions are summarised in the Extra Pharmacopoeia (1998) and have been well-documented by Martin *et al.* (1971), Hartshorn (1972), Buchanan & Sholiton (1972). Kutt (1975) reports that a range of interactions may occur between antiepileptic drugs or between antiepileptic drugs and other therapeutic or chemical agents. As a result of these interactions, pharmacokinetic parameters such as solubility, absorption, metabolism, elimination and tissue distribution of the drug in question will be altered.

From all the existing interactions, the specific interaction of phenytoin with metal ions will be dealt with.

1.1.8.1. Interactions of phenytoin with metal ions

Polyvalent cations such as aluminium, magnesium and calcium released from antacid preparations have been reported to interact with phenytoin, owing to a decreased in bioavailability of the drug (Garnett *et al.*, 1979). Compounds can normally form chelates or complexes with these cations, and this may be the underlying cause of reduced drug absorption.

Phenytoin forms a characteristic copper complex when treated with copper sulphate in pyridine solution. This reaction forms the basis for the B.P.C. identification test for this agent. In addition, studies carried out by Glazko and Chang (1972) reported the following:

- A copper chelate is formed by adding $CuCl_2$ to a solution of phenytoin dissolved in 0.1 M ammonium hydroxide and shaking continuously for 1 hour.
- Phenytoin also appeared to form a complex with cobalt salts.
- There was no indication of chelate formation with calcium, magnesium or ferrous salts.

Furthermore, it was important to note that this formation took place in ammonia solution, which has a much higher pH as compared to the physiological pH of 7.4.

Another study showed that zinc [Zn (II)] demonstrates affinity for each heteroatom (O and N) in phenytoin and that the Zn (II) cation coordinates with two phenytoin molecules at these respective sites. This suggests that phenytoin should be an excellent ligand for Zn (II) complexation (Milne, P. *et al.,* 1999).

1.1.9. TERATOGENICITY

The Foetal Hydantoin Syndrome (FHS) consists of craniofacial defects and any two of the following: pre/postnatal growth deficiency, limb defects, major malformations, and mental deficiency. Available data suggest a prevalence of FHS of 10-30% in infants of women ingesting 100-800 mg/kg of phenytoin during the first trimester or beyond.

Phenytoin produces multiple behavioural dysfunctions in rat offspring at subteratogenic and non-growth retarding doses. These behaviourally teratogenic doses produce maternal serum phenytoin concentrations in rats comparable to those found in humans. The dysfunctions in rats are dose-dependent and exposure-period-dependent, but independent of nutritional habits. Effects include vestibular dysfunction, hyperactivity and deficits in learning and memory (Adams, J., *et al.*, 1990)

Azarbayjani, F., *et al.* (2002) found evidence that drug-induced embryonic arrhythmia initiates phenytoin teratogenicity. The arrhythmia, which links to the potential of phenytoin to inhibit a specific potassium channel (IKr), may result in episodes of embryonic ischaemia and generation of reactive oxygen species (ROS) at reperfusion. Danielsson BR, *et al*. (2001), also reported the same findings.

Another important mechanism of teratogenicity of PHT involves the production of free radical reactive intermediary metabolite that lead to ROS formation, including hydroxyl radicals. In addition, ROS-initiated oxidation of DNA, proteins, glutathione (GSH), lipids and embryotoxicity have been shown to occur with phenytoin (Wells, P.G. *et al.*, 1997).

According to Miranda AF *et al.* (1994), the depletion of GSH due to its oxidation by the toxic reactive free radical intermediate also contributes to the teratogenicity, as the latter plays a critical role in detoxifying a phenytoin free radical or subsequent activated oxygen species, thereby reducing covalent binding, lipid peroxidation, and oxidative stress that may initiate embryotoxicity or death.

1.1.10. PHARMACOLOGICAL NEUROPROTECTION BY PHENYTOIN

Phenytoin is discussed here as a cerebroprotective premedication largely because it is cerebroprotective when given before global cerebral ischaemia but not when administered after the insult. It is a medication prescribed to patients dying from malignancies such as brain metastasis or primary brain tumour, HIV or other diseases, all of which involve the CNS.

It is not generally recommended that phenytoin be used as a primary cerebroprotective drug except when administered IV during the agonal period because of its unfavourable adverse reaction profile that many patients suffer from.

1.1.10.1. *In vivo* evidence

Voltage-dependent sodium channels have long been recognized targets for anti-arrhythmic and local anaesthetic drugs. Since the mid-1980s, Na^+ channels have become widely accepted as the primary target of anticonvulsants with pharmacological profiles similar to phenytoin, carbamazepine, and lamotrigine. Results from animal models and a few

preliminary clinical trials suggest that this class of drugs may also offer significant potential for reducing the neuronal damage caused by ischaemia, stroke, head trauma, and perhaps certain neurodegenerative diseases. Studies using site-directed mutations of Na^+ channels with electrophysiology have provided extensive insight into both the physiology and the interaction of drug molecules with ion channels.

In a rabbit model of spinal cord ischaemia, retrograde perfusion with the sodium channel antagonist, phenytoin, is reported to be safe and provides significant protection during prolonged spinal cord ischaemia (Gangemi, J.J. *et al.,* 2000)

In 1990, Boxer PA *et al.* showed that phenytoin, which is not a glutamate antagonist, reduces the infarct volume in the brain by 45% at 28 mg/kg. A single dose of phenytoin (28 mg/kg) administered 30 minutes after hypoxia, was neuroprotective, but delaying drug administration for more than 2 hours was ineffective. The neuroprotective activity of phenytoin suggests that this may be related to the common anticonvulsant action due to sodium channel blockade, thus reducing the hyperexcitablility of neuronal membranes. Without question, the blockade of the excitatory response by phenytoin is due to its non-NMDA rather than NMDA glutamate receptor antagonistic effect. This is in agreement with Kawano *et al.* (1994).

Moreover it is also suggested that non-NMDA glutamate receptor blockade results in considerable enhancement of the efficacy of antiepileptic drugs (Zarnowski *et al.,* 1993). As a result this might be an important feature in the anticonvulsant profile of phenytoin and might be contributing towards neuronal protection in the hippocampal area where there is a great number of glutaminergic neurons.

Vartanian MG *et al.* (1996) used a model of hypoxia-ischaemia with neonatal rats where they showed that pre-treatment with phenytoin is neuroprotective at a plasma phenytoin concentration of approximately 12 µg/ml. Phenytoin is also reported to be able to reduce lipid peroxide levels significantly in the rat brain, when given orally at a dose of 20 mg/kg as compared to a control group treated only with kainic acid (KA), an excitotoxic agent (Hsieh, C.L., *et al.*, 2001).

In the next section the aetiology via which damage can occur in neuronal cells by various excitatory amino acids is discussed.

1.2. EXCITOTOXICITY

1.2.1. INTRODUCTION

Glutamate though recognised as a beneficial excitatory neurotransmitter, also has the potential to be neurotoxic. Glutamate being an excitotoxin was recognised largely due to the work of Olney and his colleagues (1971 & 1974) who demonstrated an association between the excitatory properties of various glutamate analogues, and their ability to produce neurotoxic damage at elevated concentrations in the brain (Meldrum, B. & Garthwaite, J. 1990).

Excitotoxicity as described by Olney (Olney, J.W. 1995) refers to the contradictory property, shown by glutamate and certain particular EAA analogues, of causing severe degeneration to neuronal cells, by excessive stimulation of postsynaptic EAA ionotropic receptors through which glutamate functions as a transmitter.

Agonists acting at the N-methyl-D-aspartate (NMDA) and KA receptors are among the most potent agents able to produce a combination of neuronal excitation resulting in neurodegeneration. In most cases the factors that are believed to start the neurodegenerative process is the accumulation of intracellular Ca^{2+}. Agonists acting at the NMDA receptor induce increases in Ca^{2+} by activating ionic channels through which Na^{+}, K^{+} and Ca^{2+} can enter the cell (Stone, T.W. 1993). KA receptor agonists cause an increase in Ca^{2+} by triggering the release of Ca^{2+} from intracellular stores.

It is also possible that under certain pathological conditions such as hypoxia, hypoglycemia, seizure and epilepsy (MacDonald, J.F. & Nowak, L.M. 1990), the transport system, namely these ionic channels mentioned above,

can be caused to operate in reverse. This results in the release of glutamate into the synaptic cleft. High synaptic glutamate concentrations can prove toxic to neurons. This is because glutamate toxicity is thought to be mediated by an increase in intracellular Ca^{2+} as the Ca^{2+} channel blocker nifedipine is able to prevent damage to cultured neurons treated with quinolinic acid (QUIN) and other excitotoxins having similar actions like glutamate (Weiss, J.H., *et al.* 1990).

1.2.2. NMDA RECEPTORS

The NMDA receptor has a very complex molecular structure, containing binding sites for both glycine and glutamate (Jansen, K.L.R., *et al.* 1989). Both the glycine and glutamate sites must be occupied for activation to occur (Andjus, P.R., *et al.* 1996), and so they are referred to as "co-agonists" of the NMDA receptor (Blandini, F., *et al.* 1996). As discussed above, the NMDA receptors play a critical role in excitotoxicity and are classified as ionotropic glutamate receptors. Furthermore, the complex molecular entity has a number of distinct recognition sites for endogenous and exogenous ligands, each with discrete binding domains (Cooper, *et al.,* 1996). In addition the NMDA receptor contains six pharmacologically distinct sites through which compounds can alter the activity of this receptor. The structure of the NMDA receptor is shown in Figure 1.7.

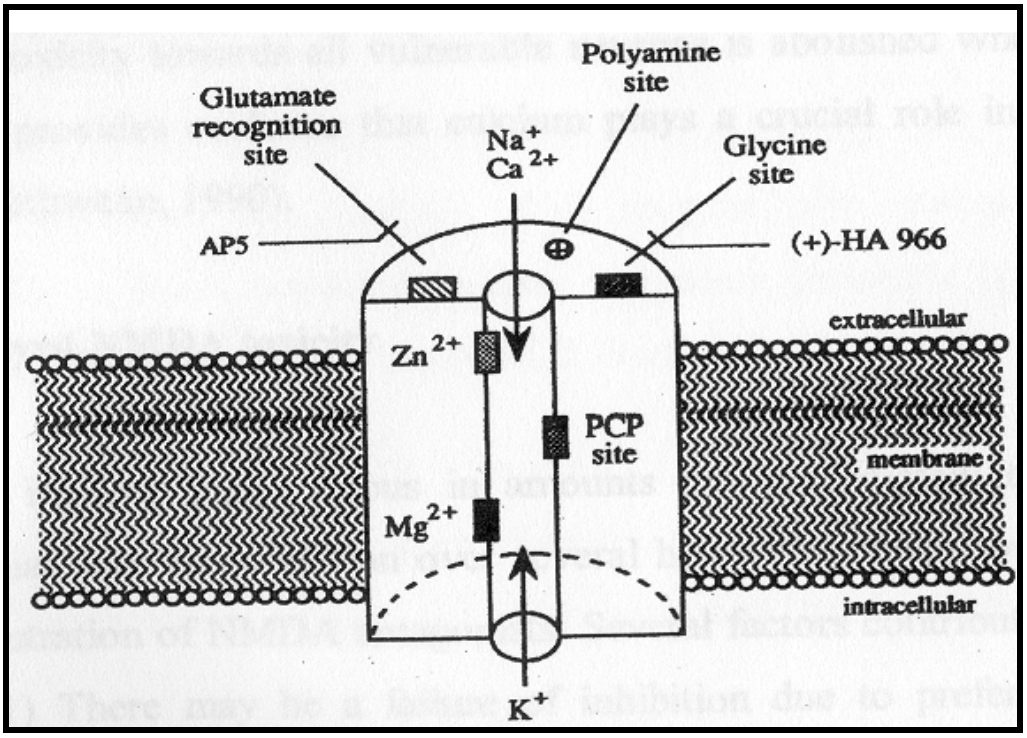


Figure 1.7. Diagrammatic representation of the NMDA receptor and sites of action of different agents on the receptor (Cooper, *et al.*, 1996)

Activation of the NMDA receptor is modulated by polyamines, such as spermine and spermidine (Williams, K., *et al.* 1991). Although the polyamine site need not be occupied for receptor activation, the presence of polyamines increases the ability of glutamate and glycine to open the NMDA-receptor ion channel (Yeneda, Y., *et al.* 1991). One of the most important factors of the NMDA receptor is the voltage-dependent blockade of the receptor ion channel by Mg^{2+} (Mayer, M.L., *et al.* 1984). At resting potential (about -70mV), normal extracellular concentrations of Mg^{2+} block the NMDA-receptor ion channel and prevent current flow, even when the glutamate and glycine sites are occupied. However, because the Mg^{2+} blockade is voltage-dependent, the degree of Mg^{2+} block is reduced as a neuron becomes depolarised (Dodd, P.R., *et al.* 1994) allowing more movement of ions. The NMDA receptor is ionotropic, permitting an

intracellular influx of Ca^{2+}, as well as an exchange of Na^{+} and K^{+} across the cell membrane (Tilson, H.A. *et al.,* 1995). Ca^{2+} permeability of the NMDA receptor is important as the increase in Ca^{2+} concentration activates a number of cellular and secondary messenger systems, and contributes to the initiation of long-term potentiation (Mayer, M.L. *et al.* 1990 & Madison, D.V. *et al.,* 1991). Extracellular Zn^{2+} appears to act as an inhibitory modulator of channel function at a site near the "mouth" of the ion channel (Tilson, H.A. *et al.* 1995).

A variety of competitive and non-competitive inhibitors exist for the NMDA receptor. Among the more common competitive inhibitors are 2-amino-5-phosphonovalerate (AP5) which competes directly with glutamate for the binding site (Tilson, H.A. *et al.* 1995), while the non-competitive inhibitors dibenzocycloheptеneimine (MK-801), phencyclidine (PCP) and remacemide act within the ion channels to block current flow (Greenamyre, J.T. *et al.* 1994, Tilson, H.A *et al.* 1995). High concentrations of the NMDA receptors are found in the cerebral cortex and CA1 regions of the hippocampus (Monaghan, D.T. *et al.* 1986).

1.2.3. MECHANISM OF EXCITOTOXICITY

Normally glutamate, being a neurotransmitter, acts as a chemical signal between two neurons. The release of this neurotransmitter triggers the short-lived opening of receptor-coupled channels, which allows ions to pass through the cell's biological membrane [Poeggeler, B., *et al.* 1993]. High levels of glutamate, however, cause these channels to remain open for extended periods of time allowing an unusual, prolonged and finally fatal influx of ions into the neuronal cells. There are two principle processes in

which excitatory amino acids induce neurotoxicity that can result in loss of membrane integrity and cell death (Meldrum, B. *et al.* 1990).

The first process, termed the acute process, involves the rapid swelling of neurons. This usually follows the excessive depolarisation, ion influx and water entry into the neurons. The second phase is slower. It is a delayed neurotoxic process that occurs secondary to Ca^{2+} influx (Tilson, H.A. *et al.* 1995). These two processes are discussed in more detail in the next section.

1.2.3.1. Acute Toxicity

Acute toxicity occurs following the rapid influx of mainly Na^{+} into the neuron. This causes passive Cl^{-} and water entry into the cell via changes in osmotic potentials. This toxic process occurs within minutes and depends on the presence of extracellular Na^{+} and Cl^{-} ions stores. It may be associated with abnormalities in membrane permeability and may lead to cell death via osmotic lysis. Under certain conditions, the neuron may be able to re-establish osmotic pressure and hence stay alive. This process can be mimicked by depolarising agents such as veratridine, an agent which prevents the inactivation of voltage-dependent Na^{+} channels, opening them for long periods of time (Churchwell, K.B., *et al.*1996) or by increasing extracellular K^{+} concentrations.

The process that takes place may be direct, by over-excitation of the neuronal cells resulting in a prolonged depolarisation and depletion of energy stores, or indirectly, for example by excessive Na^{+} ion influx resulting in Ca^{2+} build up via Na^{+} / Ca^{2+} exchange ion channels present within the cell membrane.

1.2.3.2. Delayed Toxicity

Delayed toxicity results from a Ca^{2+} influx which is mediated by the NMDA receptor. The increase in intracellular Ca^{2+} concentration can lead to a cascade of pathophysiological events. These can promote oxidative stress leading to neuronal damage and subsequent cell death (Coyle, J.T. *et al.* 1993). The cascade of events that results from the increase in intracellular Ca^{2+} and leads to neurotoxicity can be seen in Figure 1.7.

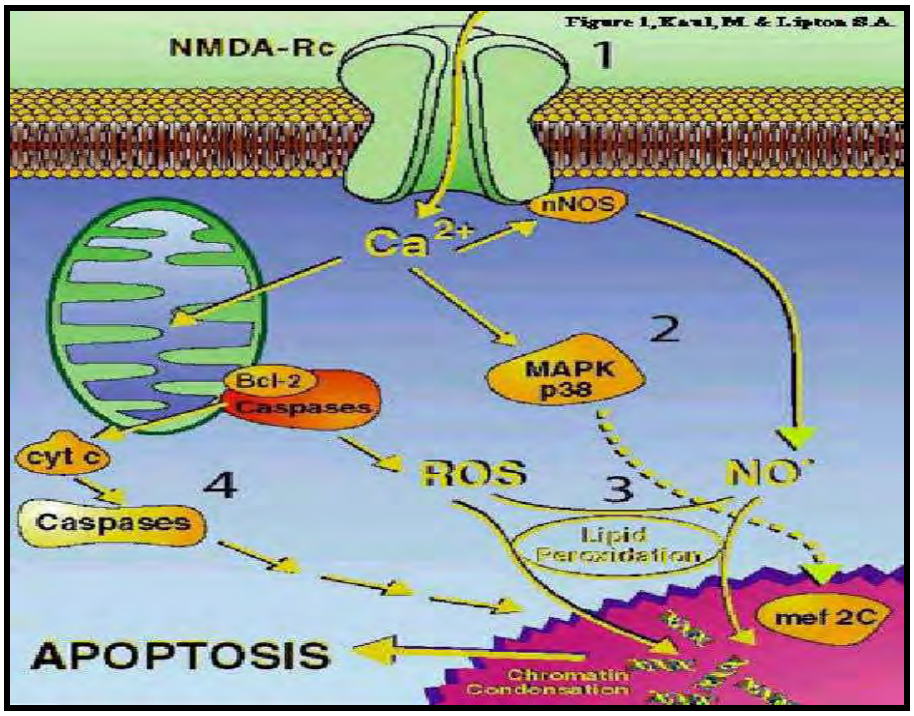


Figure 1.8. Schematic representation of the NMDA-receptor mediated processes (Kaul M & Lipton SA, 2000**)**

In the majority of instances Ca^{2+} influx does not actively initiate cell death, but rather activates a number of enzymatic and metabolic processes that set in motion a process that can prove to be neurotoxic. To a certain extent,

these alterations can be reversible. This can be deduced from the fact that glutamate receptor antagonists do confer an extent of neuroprotection several hours after an excitotoxic insult (Leigh, P.N. *et al.* 1996).

Nevertheless, in most cases, once the process has been initiated, neuronal cell death results. Among the processes triggered by increased Ca^{2+} concentrations, is the activation of proteases such as calpain-1. These enzymes can degrade major neuronal structural proteins, and induce cytoskeletal breakdown (Seubert, P *et al.* 1988). Calpain also cleaves xanthine dehydrogenase to convert it to xanthine oxidase (Meldrum, B. & Garthwaite, J. 1990, Atlante, A *et al.*1997). This enzyme is responsible for the conversion of hypoxanthine to uric acid and also the production of superoxide anions in the process (Coyle, J.T., *et al.* 1993, Wie, M.B., *et al.*1997).

Ca^{2+} can also bind to calmodulin to form Ca^{2+}-calmodulin complexes. The role of this complex in mammalian cells is to modulate the activity of a large number of enzymes (Ross, E.M. *et al.* 1980). Among the processes activated are Ca^{2+}-calmodulin-linked protein kinases, which disrupt the cytoskeleton (Wang, Y.T *et al.* 1996) and can cause mitochondrial dysfunction. Ca^{2+}-calmodulin complexes can also activate nitric oxide synthase (NOS) (Scatton, B. 1994), which produces nitric oxide (NO), a toxin that has been implicated in numerous neurological diseases (Dodd, P.R, *et al.* 1992).

Further more, free radical production has been linked to a loss of cellular Ca^{2+} homeostasis. Dykens (1994) reported that isolated cerebral and cerebellar mitochondria produce free radicals when exposed to elevated

Ca^{2+}. Mitochondria play an important role in the removal of Ca^{2+} from cells after a glutamate insult (White, R.J. *et al.* 1997, Khodorov, B., *et al.*1996).

Ca^{2+} is sequestered to the mitochondrial matrix, (Budd, S.L. *et al.* 1996) driven by the proton electron chemical gradient generated by the electron transport chain (Ankarcrona, M., *et al.* 1996). The reduction in the electrochemical gradient caused by the Ca^{2+} influx decreases ATP synthesis (Khodorov, B., *et al.*1996), at a time when there is enormous demand for ATP by the plasma membrane Ca^{2+} pump, and indirectly by the Na^{+}/Ca^{2+} exchanger (Schinder, A.F., *et al.* 1996). Mitochondrial Ca^{2+} accumulation and the subsequent permeability transition may therefore be a critical early event specific to the NMDA receptor mediated excitotoxic cascade (White, R.J. *et al.* 1996).

1.3. QUINOLINIC ACID

1.3.1. INTRODUCTION

Quinolinic acid (QUIN) is a naturally produced metabolite of tryptophan metabolism. QUIN is a structural analogue of NMDA and also a precursor for NAD (Foster, A.C *et al.* 1983, Heyes, M.P. *et al.* 1997, Heyes, M.P., *et al.* 1996, Wolfensberger, M., *et al.* 1984). QUIN has been shown to be present in normal post-mortem human brains at levels similar to those of other species (Wolfensberger, M., *et al.* 1984) and with concentrations not varying greatly among the different regions of the brain. Heyes and Morrison (1997) demonstrated that the brain naturally synthesises QUIN, and that the rate of QUIN formation increases in conditions of brain and systemic immune activation. QUIN concentrations have also been shown to

increase during the natural ageing process in rats (Moroni, F., *et al.* 1984). Foster *et al.,* 1984 showed that there is no active uptake of QUIN from the extracellular space, nor is there extracellular metabolism of QUIN. It is therefore unlikely that QUIN is a neurotransmitter in the classical sense. QUIN is however a selective agonist for the NMDA category of receptors.

Stone and Perkins (1981) demonstrated that QUIN is about 25% as active as NMDA, and approximately as active as glutamate and aspartate in stimulating NMDA receptors. Moreover, the latter compounds have a rapid, high-affinity uptake system for their removal from the synapse, while QUIN does not. Because QUIN is not removed from the synaptic cleft, QUIN will continue stimulating the NMDA receptor. As a result, this makes it a very potent neurotoxin.

The role of QUIN in physiology is not yet very clear, while in pathology an abnormal accumulation of QUIN in the brain has been proposed as one of the causes of convulsions (Lapin, 1981), Huntington's disease (Schwarcz *et al.* 1986), hepatic encephalopathy (Moroni *et al.*1986*b, c*) and recently also acquired immuno deficiency syndrome (AIDS)-related neurological disorders (Brown, 1990).

1.3.2. SYNTHESIS AND METABOLISM OF QUINOLINIC ACID

QUIN is synthesised according to the kynurenine pathway shown in Figure 1.9. The kynurenine pathway starts with oxidative cleavage of the amino acid, tryptophan by the haem dependent enzyme, tryptophan-2,3-dioxygenase. The product of this cleavage is N-formylkynurenine, which is

hydrolysed by a formamidase enzyme to give kynurenine, the amino acid responsible for this particular pathway's name. Kynurenine is situated at a branching point and metabolism can result in a number of secondary metabolites. Kynurenine could either be converted to anthranilic acid, utilised in the biosynthesis of aromatic amino acids; or transamination catalysed by kynurenine amino transferase yielding kynurenate or sometimes be employed in the biosynthesis of quinolines. However the third possibility and the most important route for the synthesis of QUIN, is the oxidation of the benzene ring catalysed by kynurenine-3-hydroxylase. At this point, kynureninase fragments the 3-hydroxykynurenine to give 3-hydroxyanthranilic acid and alanine (Botting, 1995).

The synthetic enzyme for QUIN in the kynurenine pathway, 3-hydroxyanthranilic acid oxygenase (3-HAO), catalyses the conversion of 3-hydroxyanthranilic acid to an unstable intermediate, α-amino-β-carboxymuconic semialdehyde also referred to as 3-acroleyl-3-amino-fumarate. This then spontaneously rearranges to form QUIN (Foster *et al.*, 1986). 3-hydroxyanthranilic acid oxygenase is an iron dependent enzyme requiring Fe^{2+} ions and sulfhydryl groups for its activity. It has become an important target for drug action as its inhibition provides the most direct method of reducing QUIN levels.

QUIN is eventually catabolised to NAD (nicotinic acid dinucleotide) and carbon dioxide after being acted upon by quinolinate phosphoribosyl transferase (QPRTase). This enzyme has been identified in rat and human brain tissue (Foster, *et al.,* 1985). Magnesium ions are required for QPRTase activity and there is evidence that an active site containing a

cysteine residue is necessary for catalysis, while lysine and histidine residues are also essential (Botting, 1995).

Usually, there exists a differential localization of the two enzymes, 3-HAO and QPRTase in the brain, with 3-HAO localised almost entirely in the soluble fraction of brain homogenate and QPRTase to the particulate component of P2 synaptosomal fractions (Stone, 1993). In view of this, QUIN must be synthesised in one category of glial cells and must exit those cells to be metabolised by QPRTase in a separate population of QPRT-containing glial cells and neurons (Heron, P.M., 2001)

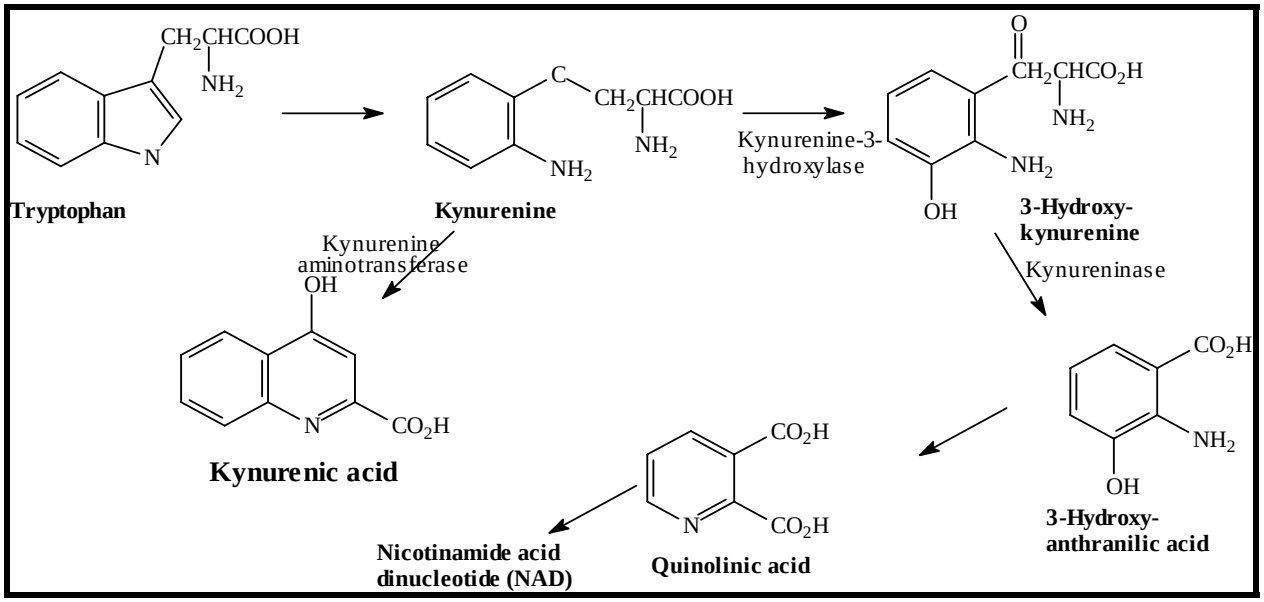


Figure 1.9. A schematic outline of the kynurenine pathway-yielding QUIN (Stone, 2000)

1.3.3. QUIN AND NEUROLOGICAL DISORDERS.

QUIN is a known neurotoxin and has been implicated in several neurological disorders. There are numerous factors that contribute towards

QUIN toxicity. One of the factors is the involvement of the enzymes in the metabolic and synthetic pathway of this neurotoxic agent. Previous studies have shown that two of the enzymes namely 3-HAO and QPRTase even though they have almost similar Km values exhibit different kinetic profiles. The kinetics of 3-HAO was 80 times higher than that of QPRTase (Foster, *et al.,* 1986). As a result, the production of QUIN occurs at a much faster rate within the CNS than the conversion to NAD. An imbalance in this equilibrium may play an important role towards the accumulation of the neurotoxin in the brain during certain neuro-pathophysiological diseases.

Moreover, it has been reported that 3-HAO is primarily found in areas of the frontal cortex, striatum and hippocampus, which possess little detectable QPRTases (Stone, 1993). Thus QUIN could reach high enough concentrations to induce excitotoxic damage in these regions. Furthermore, if the damage occurs in the hippocampal area, this can influence memory processes resulting in a cognitive decline and memory loss resembling Alzheimer's disease. Saito *et al.,* 1992, reported that the concentration of QUIN in the hippocampus of gerbils is normally 65 nM and this concentration was found to increase to 1466 nM after 4 days of ischaemia. Given the fact that Fe^{2+} ions are released during neuronal damage and considering that the activity of 3-HAO may normally be restrained by factors such as availability of Fe^{2+} ions (Stone, 1993). It is possible that this phenomenon might contribute towards the significant increase in levels of QUIN in the CNS during neurodegenerative diseases.

In addition, when radiolabelled QUIN was injected into the hippocampus, the radiolabelled compound was cleared from this CNS region with a half-life of 22 minutes and all residual radioactivity recovered after 2 hours was

still due to QUIN. This region thus does not appear to possess mechanisms neither for the rapid removal of QUIN nor for its metabolic degradation in the extracellular space by QPRTase (Foster, *et al.*, 1984). Consequently this lack of uptake systems and extracellular metabolism within these hippocampal neurons will promote the ability of QUIN to induce excitotoxic damage to these cells.

1.3.4. MECHANISMS BY WHICH QUIN INDUCES NEUROTOXICITY.

QUIN is an endogenous excitotoxic L-tryptophan and kynurenine pathway metabolite. It can function as a neurotoxin by acting as an agonist of the *N*-methyl-D-aspartate (NMDA) receptors (Stone, T.W. *et al.* 1981). In addition, neurotoxicity caused by this agent can also involve transcription factors, apoptosis, cytoskeletal disruption and lipid peroxidation. NMDA receptor agonists such as QUIN, glutamate and NMDA cause neuronal excitation. The chain of events culminating from this excitation and subsequent neurodegeneration can be referred to as neurotoxicity.

QUIN is able to produce neurotoxicity only in the presence of afferent fibres to the area of investigation. Thus the latter is ineffective as a neurotoxin in the hippocampus following transection of the perforant path as reported by Keiloff, *et al.* (1992). This suggests that presynaptic terminals may be an essential feature of the neurotoxic activity of QUIN. This further means that either QUIN may promote the release of secondary neurotoxic agents from nerve terminals, or that the postsynaptic effects of QUIN depend on the permissive or enhancing effects of factors released from presynaptic terminals (Stone, 1993).

Direct intra-hippocampal QUIN acid injections are reported not to be able to induce the disruption of DNA molecules characteristic of apoptosis (Ignatowicz, *et al.*, 1991). This suggests that QUIN is not likely to be involved in the initiation of such above-mentioned process.

Stone, 1993, has also suggested that the initial disruption of the cytoskeleton by QUIN through changes in the protein, fodrin, a major component of the neuronal cytoskeleton in the CNS, leads to the efflux of amino acids resulting in cell death.

Moreover, QUIN is able to increase lipid peroxidation in rat brain homogenate (Rios & Santamaria, 1991) and since lipid peroxidation is the damage related to free radical formation, this may suggest that this neurotoxic agent might also mediate its toxicity partly via this free radical mechanism.

1.3.5. NEUROPROTECTION AGAINST QUIN

Lees, 1987 reported that several agents, namely 2AP5, MK-801 and ketamine, all antagonists at the NMDA receptor, are protective against QUIN-induced toxicity. Certain metallic ions such as zinc and even systemically administered magnesium are effective (Wolf, *et al.*, 1990), however this may be related to their general depressant effect on neuronal excitability, or on the release of essential presynaptic factors, than to selective suppression of NMDA receptor function (Stone, 1993).

Moreover with respect to QUIN toxicity, there have been few reports of agents that can be applied systemically to confer protection. Most

antagonists mentioned above have very little activity in this respect, except MK-801 and more recently, mermantine. The latter, when administered orally can be protective against QUIN (Keiloff & Wolf, 1992). Moreover newer antioxidants compounds such as melatonin have also been proven effective *in vitro* against QUIN neurodegeneration (Southgate *et al.*, 1999) but their clinical uses are still limited.

1.4. OXIDATIVE STRESS AND FREE RADICALS

1.4.1. INTRODUCTION

Almost all of the processes described in the previous section lead either directly or indirectly to the elevated production of free radicals. The production of oxygen free radicals is a natural consequence of aerobic metabolism, with these molecules constantly being generated in the body by normal metabolic processes (Ames, B.N., *et al.* 1993). By definition, oxyradicals are molecular species capable of independent existence and contain one or more unpaired electrons (Halliwell, B. 1992). The loss of an electron leaves a molecule more reactive than its paired counterpart. If two radicals react, both radicals are quenched, while if a radical reacts with a nonradical, another free radical must be produced. This characteristic allows free radicals to participate in chain reactions, which may be thousands of events long (McCord, J.M. 1985). This chain of reaction is discussed further in section 1.4.5.

Although there are a variety of free radicals produced by molecules, those that are produced from molecular oxygen have received the most investigative interest (Reiter, R.J., *et al.* 1995). The oxygen species that are

typically linked to oxidative stress are superoxide anion radical ($O^{2-\cdot}$), the hydroxyl radical ($^{\cdot}OH^{-}$) (Cheesman, K.H. *et al.* 1993), hydrogen peroxide (H_2O_2) (Hoyt, K.R., *et al.* 1997), nitric oxide (NO) and peroxynitrite ($ONOO^{\cdot}$). The collective term for these chemicals is "reactive oxygen species" (ROS), but not all of these species are particularly active in aqueous biological solutions (Dawson, V.L. *et al.* 1996).

1.4.2 FREE RADICALS

1.4.2.1. The Superoxide Radical

The free radical $O^{2-\cdot}$ can be generated by multiple enzymatic and non-enzymatic pathways and is often found at the initial stages of the oxidative stress cascade. Mitochondria are one of the main producers of other $O^{2-\cdot}$ (McCord, J.M. 1985). In addition other organelles such as chloroplasts and the endoplasmic reticulum also produce these reactive species where electron transport chains occur too (Halliwell and Gutteridge, 1990). During the production of ATP via the electron transport chain (Dawson, V.L. and Dawson, T.M. 1996), the oxygen molecule can be reduced to $O^{2-\cdot}$. The body protects itself against this by kinetically restricting these reactions. Nevertheless, 1 to 3% of high-energy electrons "escape" in close proximity to oxygen resulting in the formation of $O^{2-\cdot}$ (Coyle, J.T. & Puttfarcken, P. 1993). As mentioned previously, cumulatively this minute amount of $O^{2-\cdot}$ leakage leads to the production of up to 2 kg of $O^{2-\cdot}$ during the year. The $O^{2-\cdot}$ radicals can also be generated chemically by auto-oxidative reactions with catecholamines, tetrahydrofolates and reduced flavins. This can lead to a free radical chain reaction as the production of $O^{2-\cdot}$ leads to the autocatalysis of further $O^{2-\cdot}$ species.

Most of the $O^{2-\cdot}$ generated in a cell is converted to H_2O_2 by superoxide dismutase (SOD)-catalysed dismutation (DiFiglia, M. 1990, Halliwell, B. 1992). This conversion can also occur by non-enzymatic processes. The H_2O_2 that results from dismutation is then metabolised by either catalases, or one of the peroxidases namely glutathione peroxidases to produce water and oxygen (Mathews, K. & Van Holde, K.E. 1990). Hence H_2O_2 is not itself a free radical and has limited reactivity. However it can cross biological membranes, which superoxide can only do at a much slower rate. The toxicity related to H_2O_2 to cells varies. This can be attributed to the activity of H_2O_2-removing enzymes and presence of metallic ions such as Fe^{2+}. Fe^{2+} can catalyse the conversion of H_2O_2 into more highly reactive radicals by the Fenton reaction. One such radical is the hyroxyl radical $^{\cdot}OH^{\cdot}$ (Halliwell & Gutteridge, 1990).

1.4.2.2. The Hydroxyl Radical

The $^{\cdot}OH$ radical is probably the most reactive of the ROS species (Poeggeler, B., *et al.*1993, Dawson, V.L. & Dawson, T.M. 1996). This is because it will react with almost all molecules in living cells (Fridovich, I. 1986). It is so reactive that no enzyme systems utilising it as a substrate exist (Bird, E.D. & Iversen, L.L. 1974). The estimated diffusion distance is 0.3 nm or one-hundredth the diameter of a typical protein (Beckman, J.S. 1994). This implies that reaction of the radical followed by damage will occur where the $^{\cdot}OH$ is formed. The $^{\cdot}OH$ radical has been implicated in damage to macromolecules such as proteins, carbohydrates, DNA and lipids (Reiter, R.J., *et al.* 1995). Most of the hydroxyl radicals generated in biological systems are formed when transition metals (Oliver, C.N., *et al.* 1990) or

biologically active chelators, such as porphyrins and flavins are present simultaneously with an oxidant, such as hydrogen peroxide (Poeggeler, B., *et al.*1993). The breakdown of hydrogen peroxide is described by the Fenton reaction in detail in section 1.4.4. (Coyle, J.T.*et al.* 1993, Poeggeler, B, *et al.*1993).

1.4.3. DEFENCE MECHANISMS AGAINST ROS NEUROTOXICITY

The body has many defence mechanisms to counteract oxidative stress (Machlin, L.J. *et al*. 1987). This can occur by enzymatic and non-enzymatic processes (see Table 1.1). These enzyme systems are normally distributed evenly inside cells (McCord, J.M. 1985), and under normal circumstances these defence mechanisms can deal with the production of ROS in the neuron. It is only when there is either an increase in oxygen radical production, or cellular ROS defence systems are compromised, that neurotoxicity occurs (DiFiglia, M. 1990).

Table 1.1: Cellular defence / anti-oxidant mechanisms accessible to neurons to protect against ROS species (Machlin, L.J. *et al*. 1987)

Enzymatic	*Non-Enzymatic*
Cu / Zn-Superoxide Dismutase Mn - Superoxide Dismutase Glutathione Peroxidase Glutathione-S-Transferase Glutathione Reductase Catalase	Ascorbic Acid (Vitamin C) **Tocopherol (Vitamin E)** Glutathione

The functioning of these enzymes depends to a large extent on scavenging of the reactive chemical entities. The non enzymatic-pathway mostly consists of agents that have good ability to accept electrons. These agents are found predominantly in the reduced state rather than their oxidised form and this optimises their electrophillic properties (Dawson & Dawson, 1996)

1.4.3.1 Chemical reactions of the defence mechanisms

$2O_2^{\cdot} + 2H^+ \rightarrow H_2O_2 + O_2$ (*SOD, Enzymatic*) [1]

$2H_2O_2 \rightarrow O_2 + 2H_2O$ (*Catalase, Enzymatic*) [2]

$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$ (*Enzymatic*) [3]

$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$ (*Enzymatic*) [4]

Reaction [1] involves the breakdown of $O_2^{\cdot}$ by SOD to produce non-toxic hydrogen peroxide and oxygen. This reaction can happen at the mitochondrial matrix and in the cytoplasm. This is due to the fact that two distinct SODs have been isolated in these regions in eukaryotes.

The route of H_2O_2 decomposition is concentration dependent. At low concentrations of H_2O_2, reaction [3] is favoured and glutathione peroxidase catalyses the conversion of hydrogen peroxide to water by the oxidation of glutathione. The reduced form of glutathione is regenerated in reaction [4] by the action of glutathione reductase. However, at high concentrations, H_2O_2 is removed by the enzyme catalase via reaction [2], (Fahn & Cohen, 1992)

1.4.4. THE INVOLVEMENT OF METAL IONS

As reported by Halliwell and Gutteridge, (1989), ions of transition metals such as copper and iron are involved in free radical generation. In the case of iron, only the unbound free iron is able to stimulate free radical generation. Iron in human plasma is normally bound to protein carriers such as transferrin. Free radicals, more precisely hydroxyl radicals, are generated via the Fenton reaction [5] in the presence of iron (II) as shown below.

$$H_2O_2 + Fe^{2+} \rightarrow OH^- + OH^{\cdot} + Fe^{3+} \quad [5]$$

$$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2 \quad [6]$$

$$O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + OH^- + OH^{\cdot} \quad [7]$$

The Fenton reaction can be further potentiated by the regeneration of the toxic iron (II) from the reaction of iron (III) generated in reaction [5] and superoxide anions as seen in reaction [6]. Consequently, reaction [5] and [6] give rise to the net reaction equation [7], the iron-catalysed Haber-Weiss type of reaction.

In summary, these reactive species produced have the capacity to cause severe damage to the neurons. In addition cells have the ability to reduce their toxicity by preventing their formation, metabolising hydrogen peroxide as stated previously and finally not allowing iron to take part in the reaction by keeping it in inactive sites or bound to carrier proteins (Fahn & Cohen, 1992).

1.4.4.1. Implication of iron in neurological disorders

There is considerable evidence to demonstrate that endogenous iron stores are partially available for participating in peroxidative damage in tissue (Halliwell & Gutteridge, 1986). Iron has been linked to the oxidative stress hypothesis for example, in Parkinson's disease. In this disease the substantia nigra is the main region of the brain where cell damage occurs. Moreover, the substantia nigra is rich in dopamine and iron (Youdim, 1988a). This is further discussed in section 1.4.6.2.

1.4.5. LIPID PEROXIDATION

Most of our current information concerning biological membranes comes from the fluid mosaic model, proposed by Singer and Nicholson in 1972. They proposed that a membrane consists of a lipid bi-layer, interspersed with proteins and carbohydrates. The membrane is a dynamic system, and the composition depends on where in the cell it is located. Membranes function to compartmentalise the cell, as well as providing a surface from which ATP can be produced, receptors can bind, and where the transport of substances can be controlled. The structure and integrity of membranes is therefore essential for the successful functioning of the cell.

Lipid peroxidation is a process whereby membranes are destroyed by oxidative attack from ROS. Of all the major classes of biomolecules attacked by ROS, membrane lipids are probably the most susceptible to oxidative attack because of the high levels of polyunsaturated fatty acids (PUFA) present in these macromolecules. Lipid peroxidation is also extremely damaging because self-perpetuating chain reactions are caused by ROS attack.

Table 1.2. illustrates the three features, which characterise the lipid peroxidation process. During the initiation step (reaction 1) PUFA are attacked by ROS ($In^{\cdot}$), resulting in the removal of a hydrogen atom from the PUFA (RH) and the formation of a lipid-derived radical ($R^{\cdot}$). The propagation step (reaction 2) normally begins with the rapid addition of molecular oxygen to $R^{\cdot}$, to form the lipid peroxyl radical ($ROO^{\cdot}$). This radical can attack other lipids, to generate a further lipid radical and a lipid hydroperoxide (ROOH). A self-perpetuating autocatalytic reaction then follows with reactions 2 and 3 undergoing a number of cycles (Krinsky, N.I. 1992). The final step is a termination step, which results when two $ROO^{\cdot}$ radicals react together to form a non-radical product (Burton, G.W. & Ingold, K.U. 1989). The removal of ROS by various antioxidant systems is therefore essential to limit lipid peroxidation in cells.

Table 1.2. Chain sequence for free radical auto-oxidation.

Initiations step	$In^{\cdot} + RH \rightarrow InH^{\cdot} + R^{\cdot}$	*Reaction 1*
Propagation Reactions	$\mathbf{R^{\cdot} + O_2 \rightarrow ROO^{\cdot}}$	**Reaction 2**
	$\mathbf{ROO^{\cdot} + RH \rightarrow R^{\cdot} + ROOH}$	**Reaction 3**
Termination	$\mathbf{2\ ROO^{\cdot} \rightarrow}$ **Non-radical products**	**Reaction 4**

1.4.6. FREE RADICALS AND NEUROPATHOLOGICAL CONDITIONS

1.4.6.1. Free radicals and Ageing

From the day an animal is born it begins to age. Ageing is therefore a natural phenomenon, and although the process cannot be stopped there are many physiological factors that can either advance or slow the ageing process. ROS are believed to be responsible for acceleration of the ageing process. These molecules affect the integrity of the macromolecules in the cell. Although most of the ROS produced are rapidly removed from the cell by the antioxidant defence system, there is a slow accumulation of damage that occurs during an animal's life. It is estimated that 10000 oxidative hits occur on DNA in the human per day (Ames, B.N., *et al.,* 1993). These damages are repaired by enzymes that excise the lesion, however there is a steady increase in the number of lesions as the animal ages. Viani *et al* (1991) compared young, adult and old rat brains. The group found that old brains were more susceptible to lipid peroxidation, demonstrating that peroxidation injury can have even more dramatic consequences when it takes place in the aged brain.

The reason why the aged brain is more susceptible to ROS damage, is due to the fact that many of the neuroprotective pathways are no longer as effective as they are in younger animals.

1.4.6.2. Free radicals in neurological disorders

Ischaemia, cardiac arrest, thrombotic stroke and asphyxia all involve very different pathophysiologies though they all are thought to contribute to the neuronal degradation that occurs following cerebral ischaemia (Meldrum, B. & Garthwaite, J. 1990). Phillis *et al* (1994) found that while ischaemia causes an increase in glutamate release into the extracellular space, glutamate levels tend to undergo a further, unexpected rise during the initial 30 to 40 minutes reperfusion. This could have resulted from the formation of toxic free radicals, including hydroxyl radicals (McIntosh, T.K., *et al.*, 1990). Following reperfusion there is also a burst of ROS production as xanthine oxidase metabolises hypoxanthine that has accumulated during the ischaemia (McCord, J.M. 1985).

Parkinson's disease is a chronic, progressive disorder of late life, which is characterized by rigidity, unintentional tremor and bradykinesia. Glutamate appears to play a very important role in the pathophysiology of Parkinson's disease, as abnormal patterns of glutamatergic neurotransmission are important symptoms of Parkinson's disease (Blandini, F., *et al.,* 1996). The metabolism of dopamine by monoamine oxidase can yield 6-hydroxydopamine, which is known to yield ROS (Cohen, G. & Spina, M.B. 1989). Although proof that ROS stress actively causes the loss of monoaminergic neurons in Parkinson's disease is lacking, there is considerable evidence in animals and humans to support the hypothesis (DiFiglia, M. 1990). Total iron is increased and ferritin is reduced in the substantia nigra pars compacta in patients with Parkinson's disease. When this occurs, the transition metal is in a low molecular weight form, capable

of catalysing non-enzymatic oxidative reactions, especially the conversion of H_2O_2 to $OH^{\cdot}$ (Simon, R.P., 1984).

Alzheimer's disease on the other hand involves selective and progressive degeneration of lower motor neurons in the spinal cord, and the upper motor neurons in the cerebral cortex. Symptoms normally become apparent in mid-life (Coyle, J.T. & Puttfarcken, P., 1993). The disease is characterised by an abundance of senile plaques and neurofibrillary tangles in certain areas of the brain (Dodd, P.R., 1994). Amyloid β-protein has been implicated in neurotoxicity, where it is thought to induce the production of free radicals (Goodman, Y., *et al.* 1996) and disrupt calcium homeostasis (Mattson, M.P., *et al.*, 1992). Mark *et al.* (1995) showed that amyloid β-peptides induce free radical production, which results in impairment of ion channels and calcium influx (Goodman, Y., *et al.* 1996).

1.4.7. MOLECULAR TARGETS OF OXIDATIVE STRESS

Damage resulting from oxidative stress does not always lead to the formation of lipid peroxides in damaged or injured neuronal cells. Damage to DNA and proteins are of equal or greater importance *in vivo* (Halliwell, 1987). Further evidence suggests that this process is instrumental in a variety of normal and pathological processes, such as ageing, neutrophil function, rheumatoid arthritis and oxygen toxicity (Oliver, *et al.*, 1990).

1.4.8. NEUROPROTECTIVE STRATEGIES

Much of the focus for neuroprotective strategies has been placed on specific targets elucidated through basic studies of neurotoxicity. It should be noted that each of the neuroprotective therapeutic targets identified in some way

contributes to the sparing of energy in nervous tissue (Obrenovitch & Urenjak, 1997). Thus, agents such as voltage dependent sodium and calcium channel blockers, glutamate receptor agonists, GABA and adenosine agonists all decrease membrane excitability and reduce the utilization of ATP for membrane repolarisation and calcium compartmentalization. Reduction of oxygen free radicals and nitric oxide may lower the utilization of ATP for membrane and macromolecular repair and may prevent the futile depletion of NAD and ATP (Azbill et al., 1997).

Other therapeutic strategies such as hypothermia probably also work by reducing demands on cellular ATP levels. Future clinical neuroprotective strategies in traumatic brain and spinal cord injury may benefit by focusing on the maintenance of brain spinal cord energy levels through a combination of pharmacological approaches (Verma, A., 1999)

1.4.8.1. Inhibition of Glutamate Release

Many neuroprotective strategies for limiting CNS injury in recent years have focused on reducing the release of glutamate from presynaptic endings and preventing its actions at postsynaptic receptors. While excessive extracellular glutamate levels undoubtedly promote secondary brain injury following trauma (Liu *et al.*, 1997), agents that block presynaptic sodium or calcium channels may potentially prevent the large increase in extracellular glutamate concentrations following brain injury. Several such agents including riluzole, lubelazole, BW619C89, phenytoin and fos-phenytoin are currently under investigation for this purpose.

1.4.8.2. Inhibition of Glutamate Receptors

Several compounds that inhibit NMDA receptors non-competitively or competitively with respect to the glutamate-binding site have been developed for potential use as neuroprotective agents. Non-competitive NMDA antagonists like ketamine, phencyclidine (PCP), dizolcipine (MK-801), dextrorphan and dextromethorphan bind to the phencyclidine recognition site in the NMDA-gated ion channel (Wong *et al*., 1986). Studies with dizolcipine and other NMDA receptor blockers indeed have demonstrated that such agents do improve functional outcome and histological morphology following experimental TBSI (Haghighi *et al.*, 1996; Rienert & Bullock, 1999).

1.4.8.3. Free Radical Scavengers

Several potential agents exist for neuroprotective strategies aimed at reducing free radical formation in the acute setting following TBSI. These include superoxide dismutase, catalase, vitamin E, iron chelators, lazaroids and phenyl-t-butyl-nitrone. Tirilazad, a lipid peroxidation inhibitor, which had been found to reduce infarct size in several stroke models (Xue *et al*., 1992), did not show improved functional outcome in patients with acute cerebral ischaemia (Peters *et al*., 1996), although a larger trial using higher doses is in progress. Phenyl-t-butyl-nitrone, a spin-trapping agent has been shown to reduce damage in animal stroke models, supporting the concept that free radicals contribute to brain injury (Floyd R.A *et al.* 2002).

Antioxidants are normally molecules or compounds that can attenuate the damage caused via oxidative stress, for example in lipid peroxidation. They

function by reacting to intermediate peroxy radicals (Halliwell, 1992). An important exogenous antioxidant in the brain is α-tocopherol. Although the levels of this agent take a considerable time to increase in the brain tissue after vitamin E supplementation, it has been found that severe and prolonged deprivation of this antioxidant produces pronounced neurological derangements (Muller & Goss-Sampson, 1990). Also considering that lipid peroxidation is not the sole end result of oxidative damage (Halliwell, 1992), the implication is that these anti-lipid peroxidant may not be as effective in limiting tissue damage.

N-acetyl-5-methoxytryptamine (melatonin) an example of an endogenous antioxidant that is produced in the pineal gland of all mammalian species, including humans (Reiter, 1991). This neuro-hormone has been implicated as a powerful quencher of free radical generation at high concentration and functions as a prooxidant at lower concentrations (Ianas, O., *et al.* 1991). The pineal gland and melatonin is discussed in more details in section 1.5.

This suggests an important role of antioxidants in limiting tissue damage mediated by oxidative stress.

1.5. THE PINEAL GLAND

1.5.1. INTRODUCTION

The pineal gland, in man, was first discovered by the famous anatomist, Herophilos (325-280 B.C.) at the University of Alexander in Egypt (Reiter RJ, 1980). Subsequently, in the late 19th century and the beginning of the 20th century, there was an explosion in pineal organ research.

In the last 50 years, scientists have established that the pineal gland is an endocrine organ. The neuronal connections linked to the pineal gland and the discovery of melatonin, the chief hormone produced by this gland stimulated worldwide research in this field.

1.5.2. STRUCTURE AND LOCATION OF THE PINEAL

The pineal gland is located within the brain. It varies in size, shape and location in different organisms. In humans, the gland is situated on the dorsal surface of the hypothalamus, occupying a central position between the two cerebral hemispheres (Ebadi, M, 1993).

It is a highly vascularised tissue consisting of two types of cells, pinealocytes and neuroglia. In humans, pinealocytes predominate and produce both melatonin and peptides e.g. arginine-vasotocin (Brzezinski, 1997).

In the rat, the pineal is located in the superior part of the *sulcus transverses cerebri* at the surface of the brain. It lies between the cerebral hemispheres, anteriorly, and posterior to the cerebellum. The pineal gland is connected to the commissural region by a thin filament-like stalk (Figure 1.10.). The pineal gland is attached to the brain by the pineal stalk, and it consists of pinealocytes, pinealoblasts and fibrocytes.

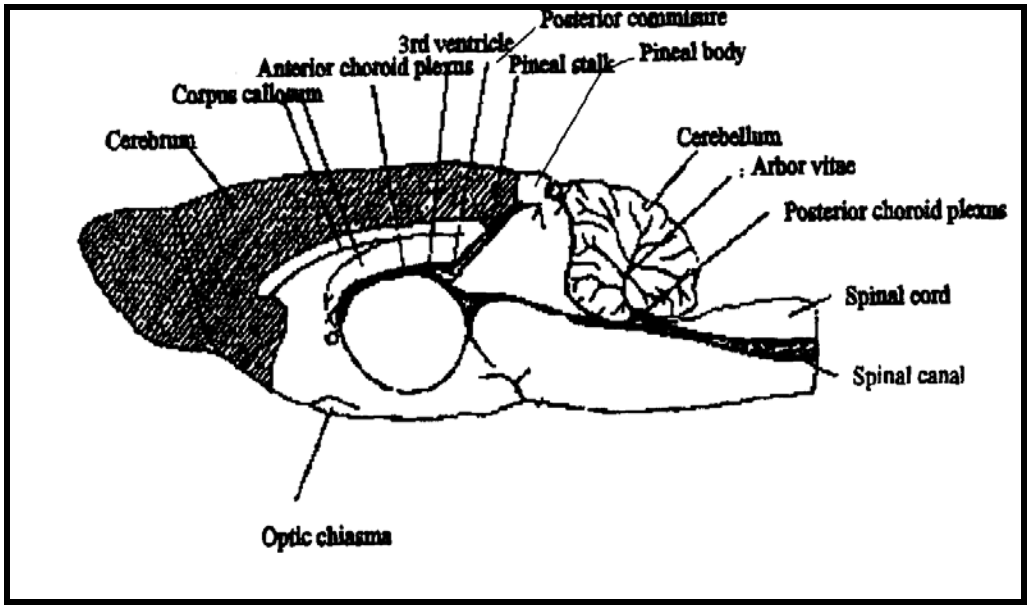


Figure 1.10. Transverse section of the rat brain showing the relative position of the pineal body and pineal stalk (Rowett, 1962).

1.5.3. PINEAL INNERVATION

The pineal gland in mammals is heavily innervated by norepinephrine containing sympathetic fibres (Guerillot C *et al*., 1979). It was also demonstrated by Wolfe *et al.* (1962) that the secretory functions of the gland was linked to its sympathetic connections. In addition, the gland is also termed as the "neuroendocrine transducer organ", due to its capability to synthesise and release melatonin in response to a neuronal stimulus. An external light stimulus that is converted to photic signals in the retina, pass through the suprachiasmatic nucleus (SCN), to the tubural hypothalamus, over the medial forebrain bundle, reticular formation and upper thoracic intermediolateral cell column, to the superior cervical ganglion. The latter conveys the signal via the postganglionic sympathetic fibres to the conarian nerve, which in turn passes the signal to the pineal gland. The pineal gland subsequently releases norepinephrine, which binds to the β-adrenergic

receptors present on the pineal cell surface. The result is a cascade of events leading to the production of melatonin (Ebadi *et al.* 1986).

1.5.4. PINEAL INDOLE METABOLISM

The pineal gland secretes melatonin, which is an indoleamine (Lerner *et al.*, 1958). Tryptophan is taken up from the blood stream into the pinealocytes and utilized in the synthesis of pineal proteins. Most of the tryptophan is converted to 5-hydroxytryptophan by the action of the enzyme tryptophan hydroxylase in the mitochondria (Hori *et al.,* 1976). 5-hydroxytryptophan is then converted by L-amino decarboxylase to serotonin (5HT), also referred to as 5-hydroxytryptamine (Snyder *et al.,* 1965).

The 5HT produced can in turn undergo three different metabolic pathways (Figure 1.11.) to produce the following metabolites by the action of specific enzymes:

1. **N-acetylserotonin** which is the precursor of **melatonin** (Klein *et al.*, 1971).
2. **5-hydroxyindole acetaldehyde** which can further be metabolised, due to its unstable nature and normally exists as an intermediate. It is converted to **5-hydroxyindoleacetic acid** (Wurtman & Larin, 1968) and **5-hydroxytryptophol** which is further methoxylated to form **5-methoxytryptophol** (Wurtman & Axelrod, 1967).
3. **5-methoxytryptamine.**

The detailed reaction schemes can be seen in Figure 1.11.

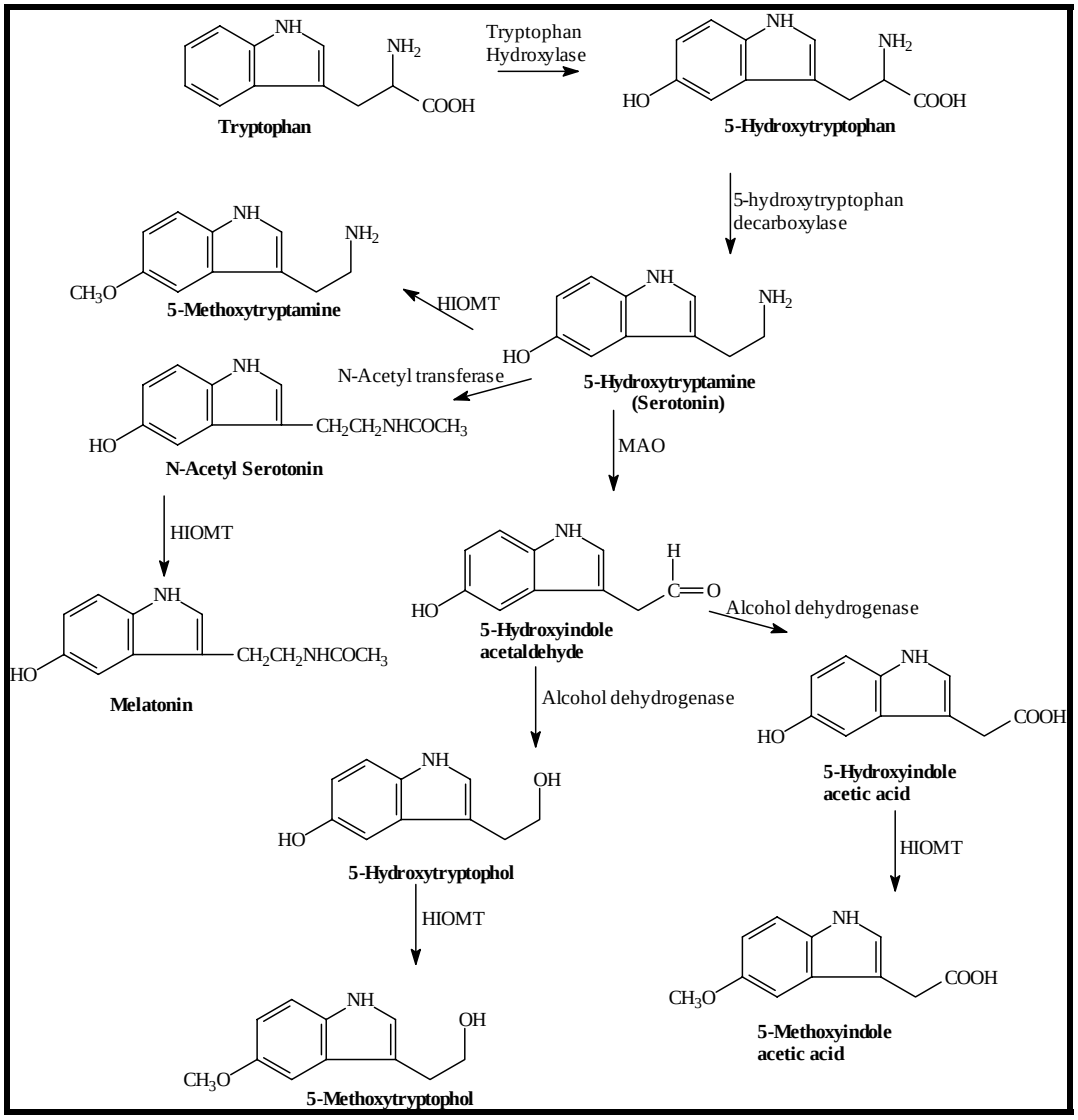


Figure 1.11. Shematic representation of pineal indole metabolism (modified from Young & Silman, 1982)

1.5.5. MELATONIN

1.5.5.1. History of melatonin

For quite a long time it was known that a substance is produced by the mammalian pineal gland that causes the blanching of melanophores in amphibian skin. In 1917, McCord and Allen demonstrated that extracts of the pineal gland cause the skin of tadpoles to lighten. A major breakthrough

in pineal studies occurred in 1958 when the dermatologist Aaron Lerner and co-workers (1958) extracted bovine pineal glands and were able to isolate a skin lightening compound, and determine its structure (1959) after 4 years of work and the use of more than 250 000 pineal glands (Lerner 1999). It was identified to be N-acetyl-5-methoxytryptamine, and due to its blanching effect on melanophores, it was named melatonin. It's chemical structure is shown in Figure 1.12.

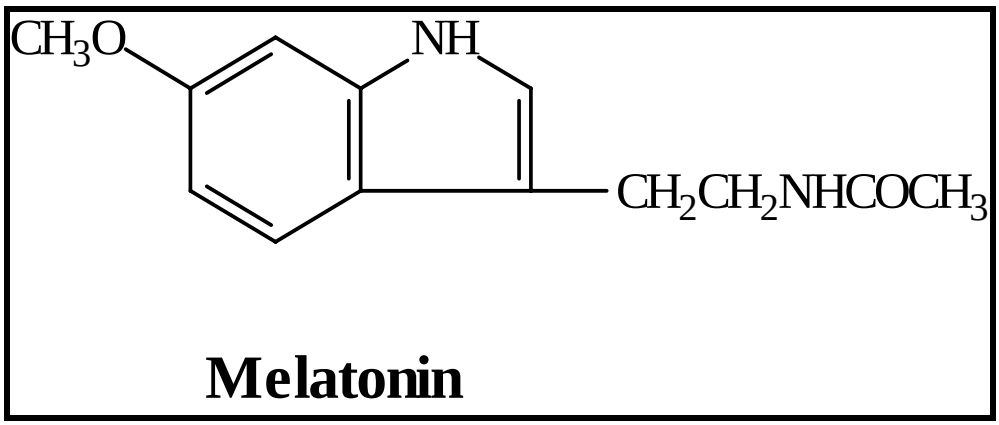


Figure 1.12. The chemical structure of melatonin (Feuer, 1990)

1.5.5.2. Functions of melatonin in the mammalian system

Ianas *et al.* (1991) were the first to report melatonin's antioxidant action. The agent exhibited antioxidant actions when its concentration exceeded 0.25 mM, and at concentrations below that, melatonin was reported to be prooxidative.

Tan *et al.* (1993) reported that melatonin was a more powerful free radical scavenger relative to known scavengers such as mannitol and GSH and in addition, melatonin was a more effective $OH^{\cdot}$ scavenger when compared to serotonin, N-acetylserotonin or 5-methoxytryptamine, all structurally related

to melatonin. These structure-activity studies showed that the 5-methoxy group on melatonin's indole nucleus and the N-acetyl group on the side chain, are both important for this property of melatonin. Melatonin is the most potent hydroxyl radical scavenger (Reiter *et al.,* 1994) and it has also been established that melatonin scavenges the peroxyl radical ($ROO^{\cdot}$) (Melchiorri *et al.,* 1995).

Melatonin's free radical scavenging action occurs in a two-step process (Figure 1.13.): In the first step, the melatonin molecule donates an electron to the $OH^{\cdot}$ radical, to produce an indolyl cation radical. The second step consists of the oxidation of the indolyl cation radical, by the $O_2^{\cdot -}$ to form 5-methoxy-N-acetyl-N-formyl-kynuramine and the proposed mechanism by which melatonin acts as a free radical scavenger (Hardeland *et al.,* 1993) can be seen in Figure 1.13. Melatonin is thus a suitable antioxidant due to its ability to terminate free radical chains and not to participate in redox cycling.

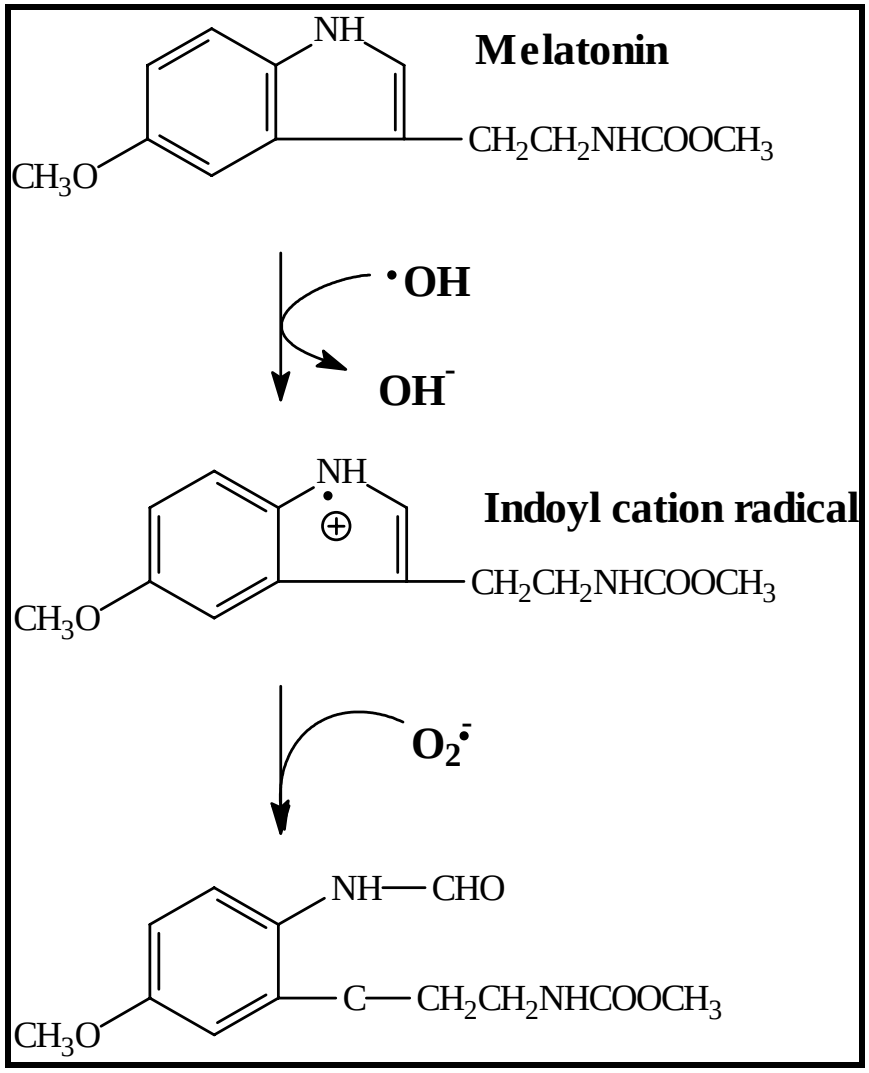


Figure 1.13. The proposed mechanism by which melatonin acts as a free radical scavenger (Hardeland *et al.*, 1993)

When Reiter *et al.* (1997) examined the antioxidant properties of melatonin in rats treated with Safrole. Safrole is a known carcinogen that causes DNA damage as a consequence of free radical generation. The results of the experiment showed that when melatonin was co-administered, it reduced the DNA adduct formation, confirming its antioxidant actions.

Melatonin was also found to stimulate the activity in the brain of glutathione peroxidase (GSHPx). GSHPx metabolises H_2O_2 to H_2O, thereby reducing the production of toxic $OH^{\cdot}$ (Reiter, 1997). Melatonin can also stimulate hepatic and cerebral glucose-6-phosphate dehydrogenase activity in mice, increasing NADPH levels. This promotes the enzymatic production of GSH via glutathione reductase. GSH is an important co-factor for GSHPx. (Pierrefiche & Laborit, 1995). This implies that melatonin possesses both direct and indirect antioxidant properties, thus making it a potent antioxidant in the mammalian system.

1.6. OBJECTIVES

The objectives of this study are to determine whether phenytoin could act as a neuroprotective agent and secondly to attempt to expose the mechanism of neuroprotection, should it occur.

The potential neuroprotective properties of phenytoin were investigated:

I. By determining whether this agent can protect the hippocampus under the insult of QUIN in an *in vivo* situation. This was done by histological examination of the hippocampal area of the brain under the insult of QUIN in phenytoin free and phenytoin pre-treated rat brains using stereotaxic techniques.

II. By assessing its free radical scavenging properties using the NBT assay with cyanide as the neurotoxin in whole rat brain homogenate.

III. The ability of the drug to prevent lipid peroxidation in whole rat brain homogenate incubated with the neurotoxins QUIN and Iron (II).

IV. The interaction of the drug with iron (II) and (III) ions using UV, IR analysis and electrochemical detection.

V. By determining its influence on the pineal gland on the release of the endogenous antioxidant, melatonin and the other indoleamines.

QUIN which acts at the NMDA receptor was chosen because it is an endogenous compound that is extremely neurotoxic, and is thought to play a role in the pathology of numerous neurodegenerative diseases such as Huntington's disease, AIDS related dementia. Its level is also found to increase during seizures which results in area specific neuronal damage.

In addition phenytoin protects the brain against a broad spectrum of neurological injuries resulting from strokes, ischaemic episodes (Vartanian M G *et al.* 1996) and has been used in an attempt to prevent post-traumatic epilepsy, which is thought to happen after neuronal degeneration (Iudice A. *et al*., 2000). This agent acts as a neuroprotectant due to its anti-seizure and sodium channel antagonistic properties in neurons. New compounds such as free radical scavengers and antiperoxidants, show encouraging experimental results, but their clinical use is still very limited.

It is hoped that this investigation will expose certain underlying mechanisms by which phenytoin offers neuroprotection and also help to better understand the factors that might contribute towards neurodegeneration caused by an increase in QUIN and free iron levels in the brain.

CHAPTER 2

EFFECT OF QUINOLINIC ACID AND PHENYTOIN ON HIPPOCAMPAL NEURONS

2.1. INTRODUCTION

In previous studies it had been demonstrated that QUIN induces neuronal cell death in primary neuronal cell cultures and also causes degeneration of neurons of the hippocampal areas after directly injecting the neurotoxin in that particular area (Southgate *et al.* 1999). It is also known that phenytoin, being a sodium channel blocker, is able to protect neurons to a certain degree after trauma and ischaemic episode (Boxer PA *et al.*, 1990).

Quinolinic acid is an agonist of the neuronal NMDA receptor (Stone, T.W. & Perkins, M.N. 1981), which has been detected in both the human and rat brain (Williams, K., *et al.*, 1991). Intrastriatal and intrahippocamal injections of QUIN into the rat brain have been shown to induce neuronal lesions (Schwarcz, R., *et al.*, 1983).

Moreover there is increasing evidence that during trauma, inflammation and HIV infections there is an increase in concentrations of EAAs more specifically QUIN, which induces neurotoxicity and is thought to cause certain types of dementia (Heyes & Morrison 1997).

The role of QUIN in physiology is not yet very clear, while in pathology an abnormal accumulation of QUIN in the brain has been proposed as one of the causes of convulsions (Lapin, 1981), Huntington's disease (Beal *et al.*

1986), hepatic encephalopathy (Moroni *et al.*1986*b*, *c*) and recently also AIDS-related neurological disorders (Brown, 1990).

2.1.1. STRUCTURE AND FUNCTION OF THE HIPPOCAMPUS

The hippocampus is a region of the brain, situated medial to the lateral ventricle in the medial temporal lobe. This region forms part of the cerebral cortex, which is folded onto itself in a peculiar shape (Bear *et al.*, 2001). After a Nissl-staining of the coronal section of the caudal telencephalon of a rat brain (Figure 2.1) this structure mentioned above can be clearly distinguished. The glutamatergic system, requiring glutamate as the main neurotransmitter, is the main pathway that is involved in the functioning of the hippocampus. This chemical messenger causes the activation of postsynaptic NMDA receptors involved in memory and learning.

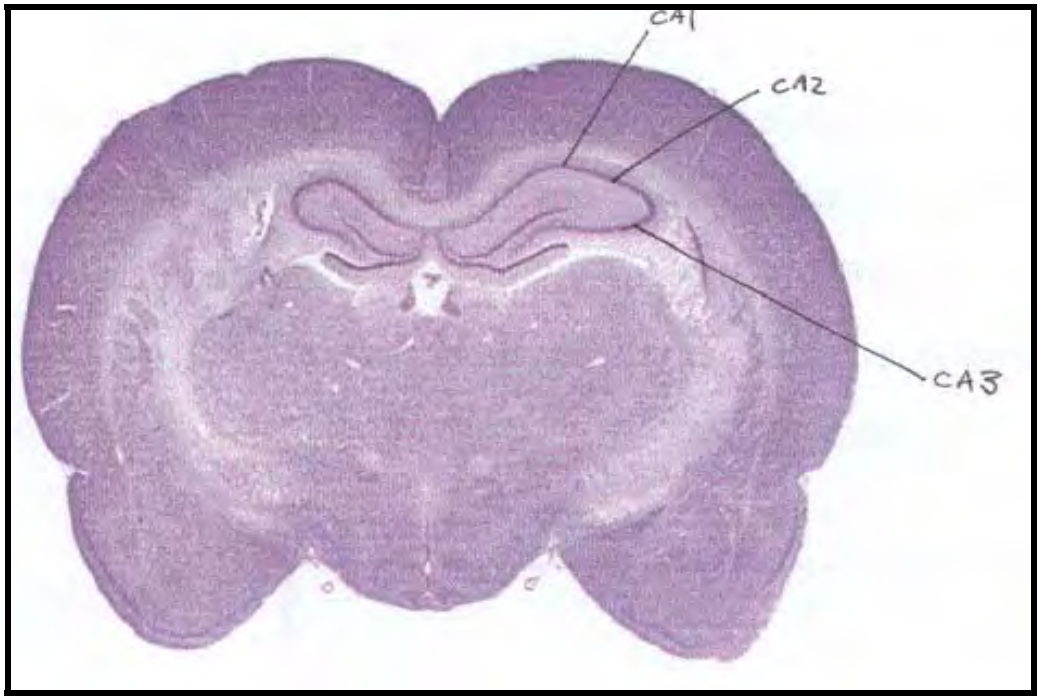


Figure 2.1. A coronal section through the caudal telencephalon of a rat brain displaying the hippocampal structure and the three subdivided regions. (www.bio.davidson.edu/courses/genomics/method/Brainparts.html)

The hippocampus consists of two thin sheets of neurons, namely the dentate gyrus and the Ammon's horn, folded onto each other. The Ammon's horn is divided into four areas, of which the most important are the CA1 and CA3 regions as shown in Figures 2.1. and 2.2. The major input to the hippocampus is the entorhinal cortex, which sends information to the hippocampus through a bundle of axons called the perforant path. These axons are connected to the neurons of the dentate gyrus via synapses. The dentate gyrus neurons give rise to axons referred to as mossy fibres which provide synaptic connection to the cells in the CA3 region. The CA3 cells in turn give rise to two axonal branches, namely the formix and Shaffer collateral, which synapses on the neurons of the CA1 region (Figure 2.2)

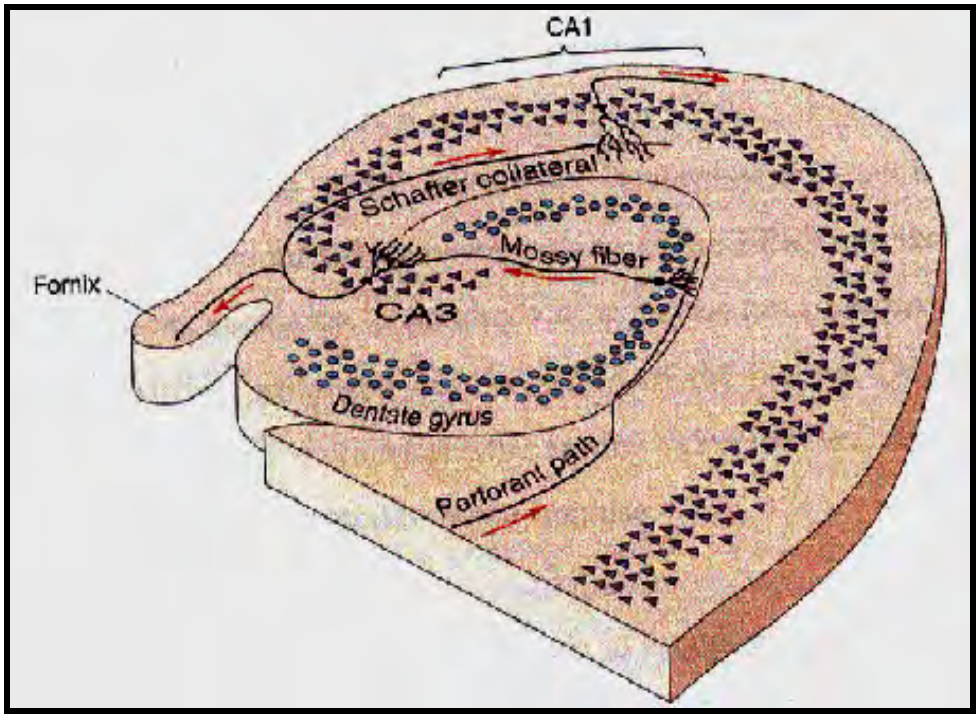


Figure 2.2. An illustration of some microcircuits on the hippocampus (Bear, *et al.*, 2001)

2.2. HISTOLOGICAL INVESTIGATION

2.2.1. INTRODUCTION

Histology is derived from the Greek word for web or tissue, and involves the examination of preserved, sectioned and stained tissues. Most of our knowledge of internal tissue structure has come from this branch of science (Hodgson, A.N. & Bernard, R.T.F. 1992).

In this experiment it was decided to investigate whether phenytoin offered neuroprotection against QUIN-induced intra-hippocampal lesions in the rat brain. After treatment, the hippocampus of the rats was sectioned and examined microscopically for evidence of any morphological changes. The

hippocampus was selected as it is very densely populated with glutamate receptors, and is also known to be susceptible to QUIN attack. Moreover, damage to this area correlates well with the symptoms observed in patients suffering from neurodegenerative diseases such as Huntington's disease (Beal, M.F., *et al.* 1986, Popoli, P *et al*, 1994).

2.2.2. MATERIALS AND METHODS

2.2.2.1. Chemicals and reagents

Quinolinic acid and phenytoin were purchased from Sigma Chemical Company (USA). Paraffin wax was obtained from Lasec (South Africa). Cresyl violet stain was purchased from BDH Chemicals Ltd (England), while DPX was purchased from Philip Harris Ltd (England). Haupt's adhesive consisted of the following; 1 g gelatine, 100 ml water, 2 g phenol and 15 ml glycerol. Sodium Phenobarbitone was purchased from Merck (Germany). All other chemicals were of the highest quality available and were purchased from commercial distributors.

2.2.2.2. Animals

Adult male Wistar rats, purchased from the South African Institute for Medical Research (Johannesburg, South Africa) were used throughout the study. The animals were housed under artificial illumination with a daily photoperiod of 12 hours (lights on at 06h00). The animal-house temperature was maintained at a constant 20^{o}C to 24^{o}C, while an extractor fan ensured the constant removal of stale air. The rats were housed four per cage with food and water provided *ad libitum*.

2.2.2.3. Surgical procedures

I. BILATERAL INTRAHIPPOCAMPAL INJECTIONS OF QUINOLINIC ACID

Male rats were anaesthetised with sodium phenobarbital (60 mg / kg i.p.) and placed in a stereotaxic frame. The rat skull was orientated according to the König and Klippel stereotaxic atlas. After a sagittal cut in the skin of the skull, the bregma suture was located and holes were drilled with an electrical trepan drill at the following co-ordinates; 4.4 mm anterior, 2 mm lateral of the sagittal suture. The bregma suture can be seen in Figure 2.3. Care was taken not to lesion the meninges. A Hamilton syringe, with a cannula of diameter 0.3 mm, was used to inject 250 nmol of quinolinic acid in 2 µl of PBS, 3 mm ventral of the dura. The injection was administered at a rate of 1 µl per minute and the cannula was left *in situ* for a further 3 minutes following the drug injection, to allow for passive diffusion away from the cannula tip and to minimise spread into the injection tract. The cannula was then slowly removed and the scalp was closed with sutures. Animals were kept in an incubator (37^{o}C) until they recovered from the anaesthesia.

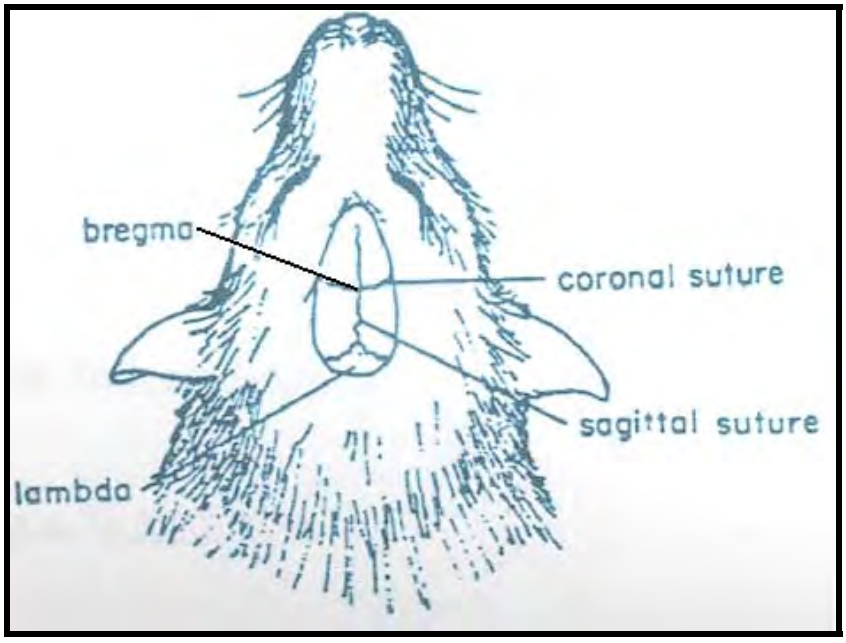


Figure 2.3. A view of the rat skull, exposing the reference point for the measurement of the coordinates, after the skin has been cut.

II. SHAM LESIONED RATS

Rats to be used as controls were subjected to the surgical procedures outlined in section 2.2.2.3(I). However, stereotaxic injections into the hippocampus were free of QUIN, and comprised only of PBS at a pH of 7.4.

2.2.2.4. Treatment regimens

Animals were divided into three groups as shown in Table 2.1. Ethyl Oleate was administered sub-cutaneously at the same dosage as that given to the rats treated with phenytoin. QUIN was dissolved in PBS made up to pH 7.4.

Table 2.1: Treatment regimen with phenytoin and QUIN for each group of animals

GROUP	**Received intra-peritoneally for 5 days prior to surgery**	**Intra-hippocampal injection consisted of:-**	**Received daily doses for 2 days after surgery**
CONTROL	Ethyl oleate and ethanol	PBS	Ethyl oleate and ethanol
PHENYTOIN	Phenytoin (20 mg/kg) in ethyl oleate and ethanol	500 nmol QUIN in 2 μl of PBS	Phenytoin (20 mg/kg) in ethyl oleate and ethanol
NO PHENYTOIN	Ethyl oleate and ethanol	500 nmol QUIN in 2 μl of PBS	Ethyl oleate and ethanol

Animals were treated for 2 days following surgery. Injections of ethyl oleate vehicle or phenytoin were administered at the same time (12h00) each day, so as to maintain a steady-state concentration in the CSF.

2.2.2.5. Brain removal

Rats were sacrificed by neck fracture and decapitated. The brain was exposed by making an incision through the bone on either side of the parietal suture from the foramen magnum to near the orbit. The calvarium was removed, exposing the brain. The brain was easily removed for use in experiments.

2.2.2.6. Histological techniques

I. Fixing the brain

Immediately after death, animal tissues begin to break down as a result of autolysis and bacterial attack. Fixation functions to chemically stabilise proteins, and thus preserve their structures. Brains were removed from the skull as in section 2.2.2.5, and rapidly fixed in a mixture of formaldehyde (30%), glacial acetic acid, and ethanol (2:1:7 v/v) for 2 hours. After fixation, the brains were stored in 70% ethanol and processed as mentioned in Table 2.2.

II. Specimen preparation and embedding

In order to be cut, the slices need to be supported. Embedding involves the infiltration and orientation of tissue in the paraffin wax support medium. The tissue was dehydrated using increasing concentrations of ethanol, followed by the removal of the ethanol using xylene. Finally the tissue was immersed in molten paraffin wax, which removed the xylene, while infiltrating the tissue without encountering water. The method used is shown in Table 4.2.

Table 2.2: Procedure for embedding brain in paraffin wax

STEP	PROCESSING AGENT	TIME
1	Ethanol	1 hour
2	Ethanol	1 hour
3	Absolute Ethanol I	1 hour
4	Absolute Ethanol II	1 hour
5	Xylene I	1 hour

6	Xylene II	1 hour
7	Melted Paraffin Wax I (57^0C)	1 hour
8	Melted Paraffin Wax II (57^0C)	1 hour

III. Blocking out

The brain material was fixed into a block so that it could be cut with a microtome. A mould was formed from two L-shaped metal bars. These were coated with ethanol: glycine to prevent the block sticking to the mould. A little molten wax was added to the mould and allowed to harden. The brain was removed from the final molten wax stage (previous section) and placed into the mould with warmed forceps. The brain was then completely covered in molten wax. Air was gently blown over the surface of the wax until the top solidified. The whole mould was then immersed in a large dish of cold water. This was to enable the wax to solidify quickly, and prevent the formation of crystals that might have disrupted the tissue. The blocks were left overnight in cold water to ensure that the wax had completely solidified.

IV. Sectioning

The wax block was trimmed with a razor blade so that two of the sides were parallel, while the other two converged slightly. The sides were cut so as to leave about 2 mm of wax around the tissue. The wax block was attached to a small wooden block with a small amount of molten wax.

Sectioning was done using a rotary microtome. The microtome was set to cut sections of 5 µm thickness. As sections were cut they would stick to one another, so as to form long ribbons. When the part of the brain containing

the hippocampus was reached, every second section was removed and placed in a water bath (25^{o}C) using a paintbrush.

V. Transferring sections to slides

Four to five sections at a time were removed from the water bath and placed onto microscope slides containing a thin layer of Haupt's adhesive. The slides were left overnight in an oven, which had a saturated atmosphere of formaldehyde and the temperature was maintained at 40^{o}C.

VI. Staining

The sections were Nissl stained using cresyl violet. This stains Nissl substances intense purple, the nuclei purple and leaves the background clear (Bauer, J.D., *et al.* 1974)

Before the section could be stained, it first had to be dewaxed and rehydrated as the stain is water-soluble. This was done as per Table 2.3.

Table 2.3: Procedure for dewaxing and rehydrating brain sections

STEP	PROCESSING AGENT	TIME
1	Xylene I (dewaxing)	5 minutes
2	Xylene II	5 minutes
3	Xylene / Absolute Ethanol (1:1)	3 minutes
4	Absolute Ethanol	Overnight at 30^{0}C

Sections were stained by placement in a 0.1% cresyl violet solution for 2 hours. The slides were differentiated rapidly in 95% ethanol by rinsing in a

flat dish until the background was clear. Sections were then dehydrated again as shown in Table 2.4.

Table 2.4: Procedure for dehydrating brain sections after staining

STEP	PROCESSING AGENT	TIME
1	**Absolute Ethanol I**	5 minutes
2	Absolute Ethanol II	5 minutes
3	Xylene I	5 minutes
4	Xylene II	5 minutes

VII. Mounting of the slides

While the slides were kept moist with xylene, enough DPX was added to just cover the tissue. A cover slip was placed over the tissue. The slides were then allowed to dry on a flat surface for 48 hours.

2.2.2.7. Photo-microscopy

The slides were photographed using a combination of an Olympus digital camera and light microscope at a magnification of 1000 times.

2.2.3. RESULTS

The area of the hippocampus that was investigated was adjacent to the site of injection of QUIN where no necrosis of neurons was visible. Samples that may have been physically damaged by the cannula were not selected. All damage that resulted was therefore of a neurochemical nature and not a result of physical damage caused by the cannula.

Neurons in the CA1 and CA3 regions of the hippocampus were examined. From the control group, injected intra-hippocampally with PBS, the neuronal cells from the CA1 region (Figure 2.4.) and CA3 region (Figure 2.6) appeared to be undamaged. Neurons from animals treated with QUIN showed signs of degeneration by virtue of their roundness and swelling in the CA1 region (Figure 2.5), while the CA3 region (Figure 2.7) showed signs of degeneration and necrosis. The neurons in the CA1 region (Figure 2.5) and those in the CA3 region (Figure 2.7) of those animals treated concomitantly with the neurotoxin, QUIN and phenytoin, appeared to be less damaged relative to the rats injected with QUIN only. In addition a higher degree of protection could be noted in the CA1 region as compared to the CA3 regions of the rat hippocampus.

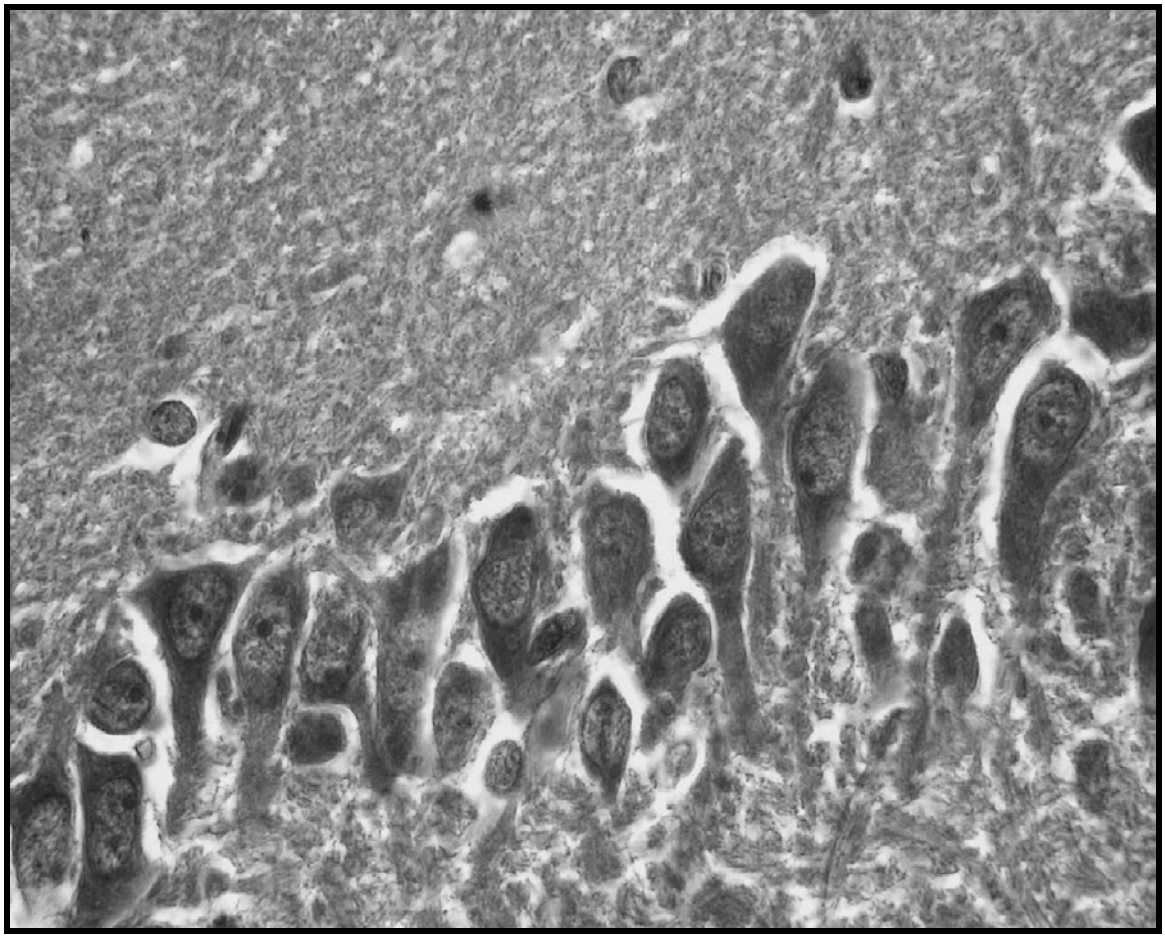


Figure 2.4. The CA1 region of rat brain, injected with PBS only, cells have distinct pyramidal shape and morphology (Magnification × 1000).

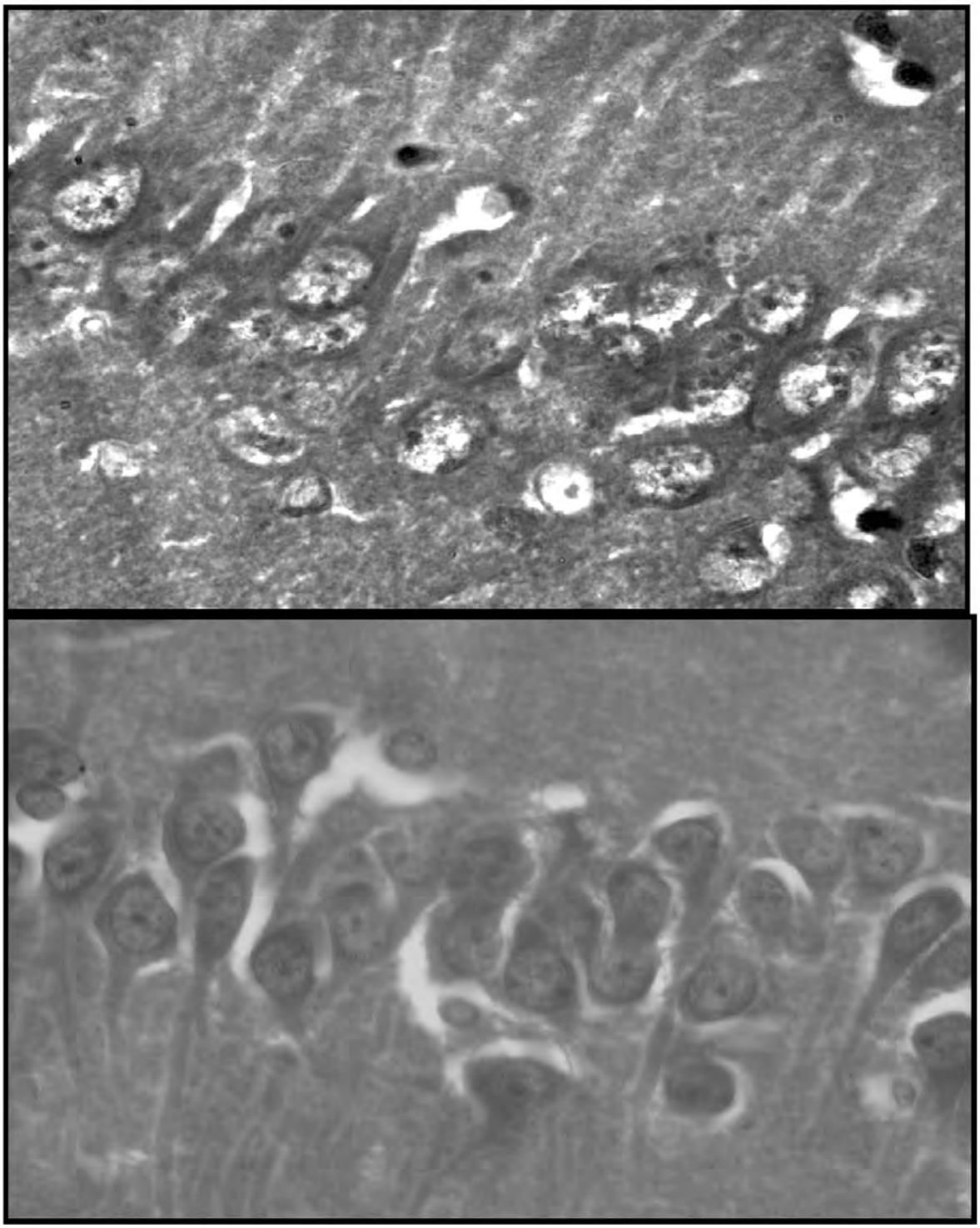


Figure 2.5. (Top) CA1 regions of rat brain injected with QUIN only. Swelling and necrosis of neurons are visible. (Bottom) CA1 regions of rat brain pretreated with phenytoin and injected with QUIN intra-hippocampally. Cells appear less swollen and more integrity was retained (Magnification × 1000)

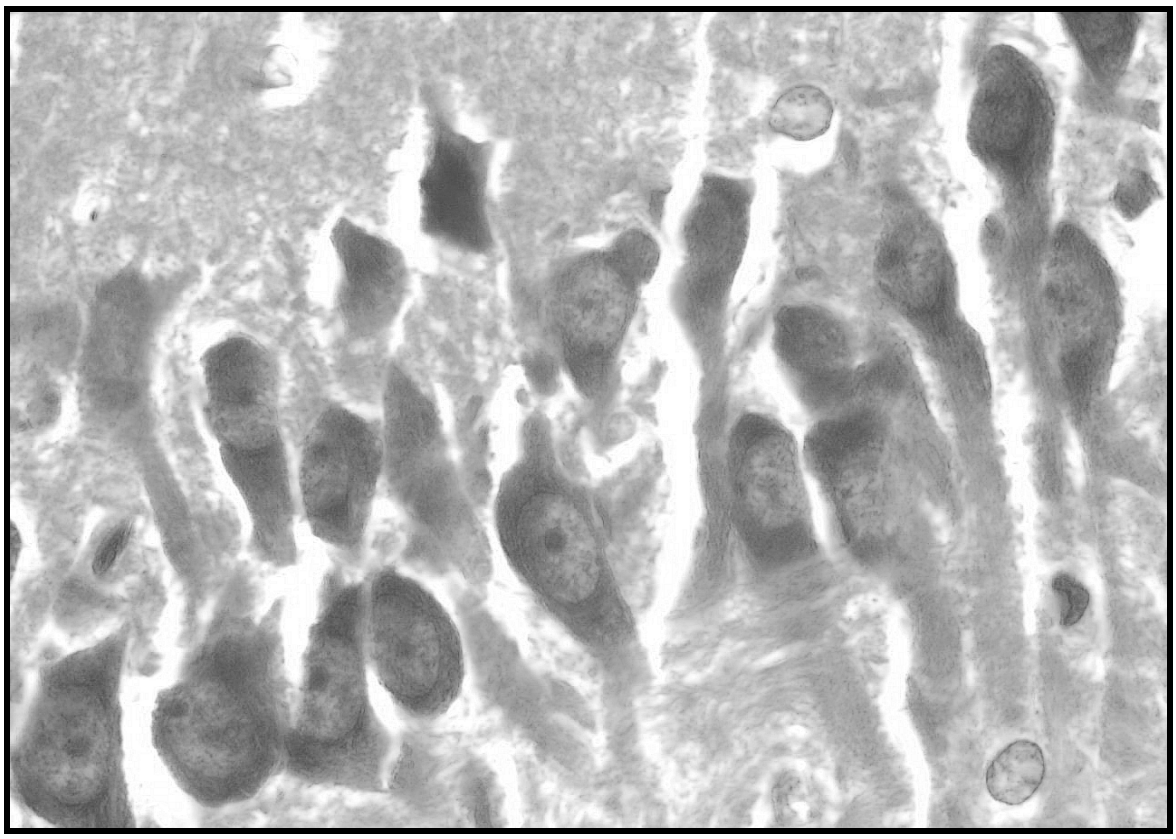


Figure 2.6. CA3 region of rat brain, injected with PBS only, cells have distinct pyramidal shape and morphology suggesting no major damage (Magnification × 1000).

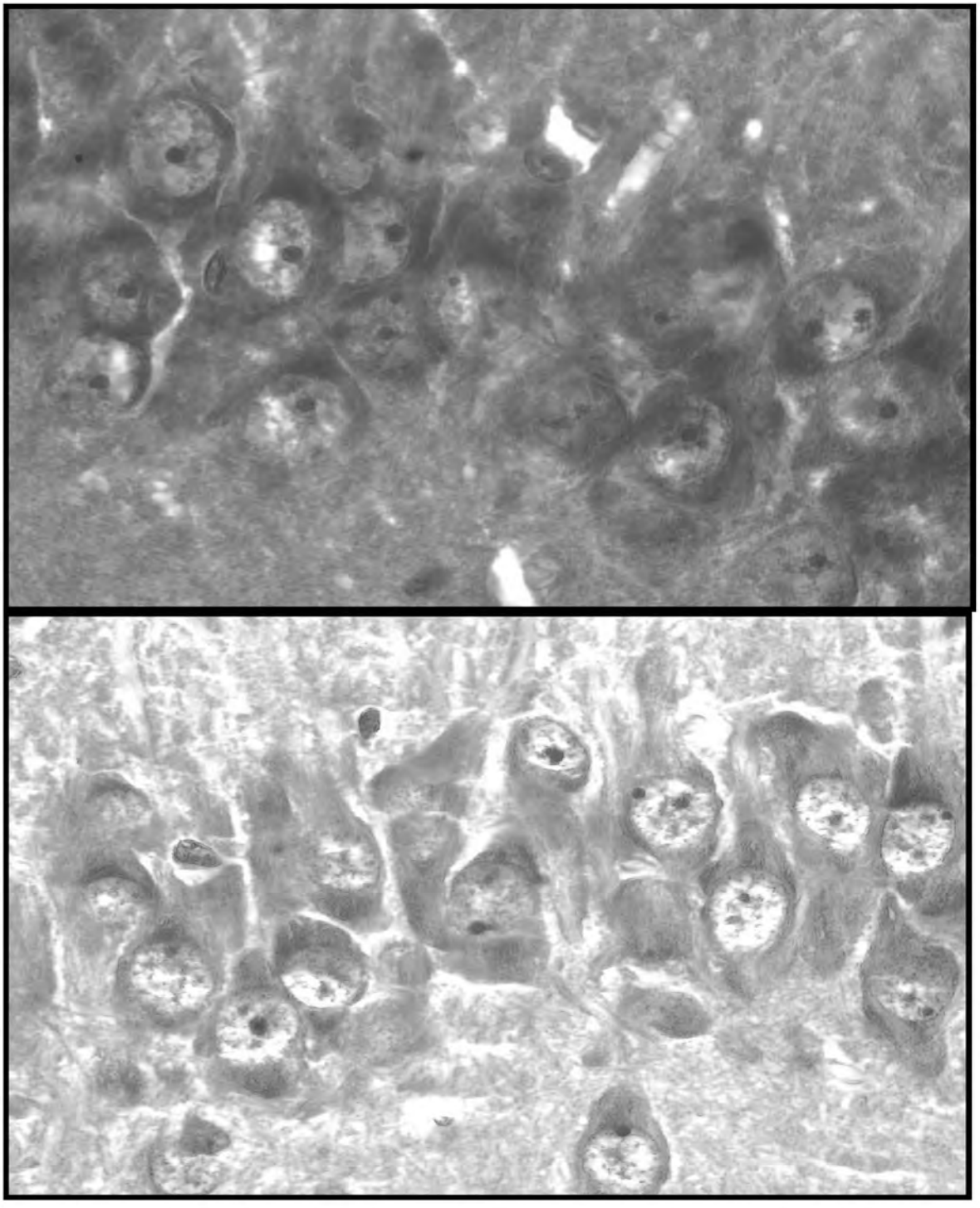


Figure 2.7. (Top) CA3 regions of rat brain injected with QUIN only. Swelling and necrosis of neurons are visible. (Bottom) CA3 regions of rat brain pretreated with phenytoin and injected with QUIN intra-hippocampally. Cells appear less swollen and more integrity was retained (Magnification × 1000).

2.2.4. DISCUSSION

QUIN induced considerable damage to the CA1 and CA3 regions of the hippocampus as seen in Figures 2.5. and 2.7. The damage by this neurotoxin is thought to be dependent on the NMDA receptors, an NMDA antagonist prevents this damage (as discussed in section 1.3.4). The activation of the NMDA receptors results in an influx of Na^+ ions into the cells causing a depolarisation of the membrane. This further results in the deactivation of the magnesium block, thus mediating the opening of the channel. In addition, this allows Ca^{2+} ions to move through into the neuronal cells.

As mentioned in section 1.2.3, acute toxicity results from excessive activation of the glutamate receptors. This type of toxicity can be attributed to the rapid influx of Na^+ ions into the neuron, which subsequently allows passive diffusion of Cl^- ions, both of the processes resulting in a change in osmotic pressure and allowing an inward movement of water molecules. This eventually results in osmotic lysis of the cell (Southgate, 1999). Thus two main processes contribute to the toxicity of QUIN. Firstly, over-stimulation of the neuron usually causes a prolonged depolarisation that results in depletion of energy reserves and secondly due to Ca^{2+} accumulation, which happens because of the Na^+ influx via the Na^+/Ca^{2+} exchanger. This increase in intracellular Ca^{2+} levels can also initiate a chain of events culminating in free radical generation. All of the above mentioned processes together with the osmotic water intake result in the swelling and cell damage observed in the photomicrographs in Figure 2.5 and 2.7. of the animals treated with QUIN only.

In the rats treated with both QUIN and phenytoin, the degree of damage to the areas of the hippocampus viewed, was lesser than the rats treated with QUIN only. This can be seen in Figures 2.5 and 2.7. This is believed to be mainly due to the Na^+ channel blocking properties of this antiepileptic drug. This property of phenytoin reduces the excitability of the neurons which, as stated above can lead to cell damage.

Furthermore, phenytoin might also act through other mechanisms such as scavenging free radicals or by interfering with the events leading to their formation. Phenytoin may also prevent lipid peroxidation by stabilization of the neuronal cell membranes. These processes could contribute to the maintenance of the cell integrity to a higher extent in the rats treated with both phenytoin and QUIN as compared to the ones treated with QUIN only.

The next two chapters examines the free radical scavenging properties of phenytoin and its effects on lipid peroxidation in whole rat brain homogenate.

CHAPTER 3

SUPEROXIDE ANION GENERATION

3.1. INTRODUCTION

Reactive oxygen species are continually generated intracellularly in aerobic organisms. If allowed to react uncontrollably, these are capable of causing extensive damage to DNA, carbohydrates, proteins and lipids within the cell (Hugon, J *et al.*, 1996). Cells possess highly effective antioxidant defence mechanisms to protect neurons against free radical species (Braughler, J.M. & Hall, E.D. 1989). These protective mechanisms can be classified into two main categories (Dawson, V.L. & Dawson, T.M. 1996), namely the enzymatic defence mechanisms, which include superoxide dismutase, and the non-enzymatic cellular antioxidants, which include Vitamins A and E. Under normal conditions these defence mechanisms are quite capable of protecting neurons, but under conditions of excessive ROS production, these mechanisms may not be able to cope and neurotoxicity may result (DiFiglia, M., 1990). ROS are generated by the addition of a single electron to $O^{2-\cdot}$. The radical is usually generated as a result of electron "leakage" from the electron transport chain located in the mitochondria (McCord, J.M., 1985), and by activation of certain enzymes. One of the by-products of the activities of these enzymes is the production of $O^{2-\cdot}$ superoxide anions, which can be very reactive and can lead to neurodegeneration (Patel, M., *et al.* 1996).

In this experiment cyanide was used to generate the superoxide radicals. Cyanide is a known neurotoxin and the primary mechanism of action of this neurotoxic agent involves the inhibition of cytochrome oxidase aa_3, the terminal oxidative enzyme of the electron transport chain (Albaum, H.G., *et*

al., 1946, Isom, G.E & Way, J.L., 1984). Studies have revealed that the sequence of reactions that follow cyanide-induced histotoxic hypoxia demonstrate that KCN produces elevated levels of brain calcium (Johnson, J.D., *et al.*, 1986). Moreover there is also an increase in free cytosolic calcium in an isolated neuronal cell model (Johnson, J.D., *et al.*, 1987). This elevation in calcium in the cytosol is associated with free radical generation and subsequent damage to cells. In addition, a number of antioxidant enzymes are also reported to be inhibited by cyanide, thus again it is believed that this further potentiates the oxidative stress and subsequent neurotoxicity of this agent (Ardelt *et al.*, 1989).

In this study, it was investigated whether the known antiepileptic agent phenytoin has the ability to either scavenge or reduce the production of these superoxide free radicals.

3.2. EXPERIMENT 1: The effect of phenytoin on cyanide induced superoxide anion formation in whole rat brain homogenate

3.2.1. INTRODUCTION

The nitro-blue tetrazolium (NBT) assay, which is generally accepted [Ottino, P. & Duncan, J.R. 1997] to be a reliable method for assaying for superoxide anions, was used. 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 560 nm.

3.2.2. MATERIALS AND METHODS

3.2.2.1. Chemical reagents

Nitro-blue tetrazolium (NBT), Nitro-blue diformazan (NBD), potassium cyanide (KCN) and phenytoin were purchased from the Sigma Chemical Company (USA). Glacial acetic acid was purchased from Holpro Chemical Company (South Africa). All other chemicals used were of the highest quality available from commercial sources.

3.2.2.2. Preparation of standards

NBD was used as a standard. A series of reaction tubes, each containing appropriate aliquots of NBD dissolved in acetic acid was prepared. A standard curve (see Appendix 1) was determined by measuring the absorbance at NBD concentration intervals of100 µM. The absorbance was

read in plastic cuvettes having a path length of 1 cm at 560 nm using a Varian Cary 500 Scan UV-VIS NIR Spectrophotometer (EL 99053199)

3.2.2.3. Preparation of whole Rat brain Homogenate

Adult, male Wistar rats were used for the experiment. These were cared for as explained in section 2.2.2.2. On the morning of the experiment, the brains were removed as described in section 2.2.2.5. The whole brains were homogenised in a Teflon® coated glass homogeniser for 60 seconds on ice. A 10% w/v homogenate was prepared with 0.1M PBS of pH 7.4.

3.2.2.4. The Nitro-blue tetrazolium assay

A modified method of Ottino and Duncan (1997) was used for this assay. A 0.1% NBT solution was made by dissolving the NBT in ethanol before making up to the required volume with Milli-Q water. All the other reagents used were dissolved only in Milli-Q water. The final ethanol concentration in the incubation flasks was less than 1.5%.

To 1 ml samples of brain homogenate, 0.4 ml of the 0.1% NBT solution and different concentrations of phenytoin (0, 0.25, 0.50, 0.75, 1.0 and 1.25 mM) and KCN (0, 0.25, 0.5, 1mM) being tested were added and allowed to incubate for 60 minutes at 37^{o}C in an oscillating water bath. On termination of the incubation, the suspensions were centrifuged at 2000 x g for 10 minutes. The supernatant was poured off and the pellet was dissolved in 2.0 ml glacial acetic acid.

The relative absorbance of the glacial acetic fraction was measured at 560 nm and converted to μmoles diformazan using a standard curve generated

from NBD. Final results were expressed as µM diformazan produced / mg wet tissue.

3.2.2.5. Statistical analysis

All data are represented as means ± SD from six determinations. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by the Student– Newman–Keuls multiple range test. Values were accepted as being statistically significantly different if a *P* value was < 0.05.

3.2.3. RESULTS

Exposure of the whole rat brain homogenate with various concentrations (0–1 mM) of KCN increased superoxide anion production in a concentration dependent manner (Figure 3.1). Furthermore, phenytoin did not cause a significant reduction in superoxide generation with 0.5 mM and 1.0 mM cyanide concentrations in the incubation vessels as seen in Figures 3.2. and 3.3, respectively.

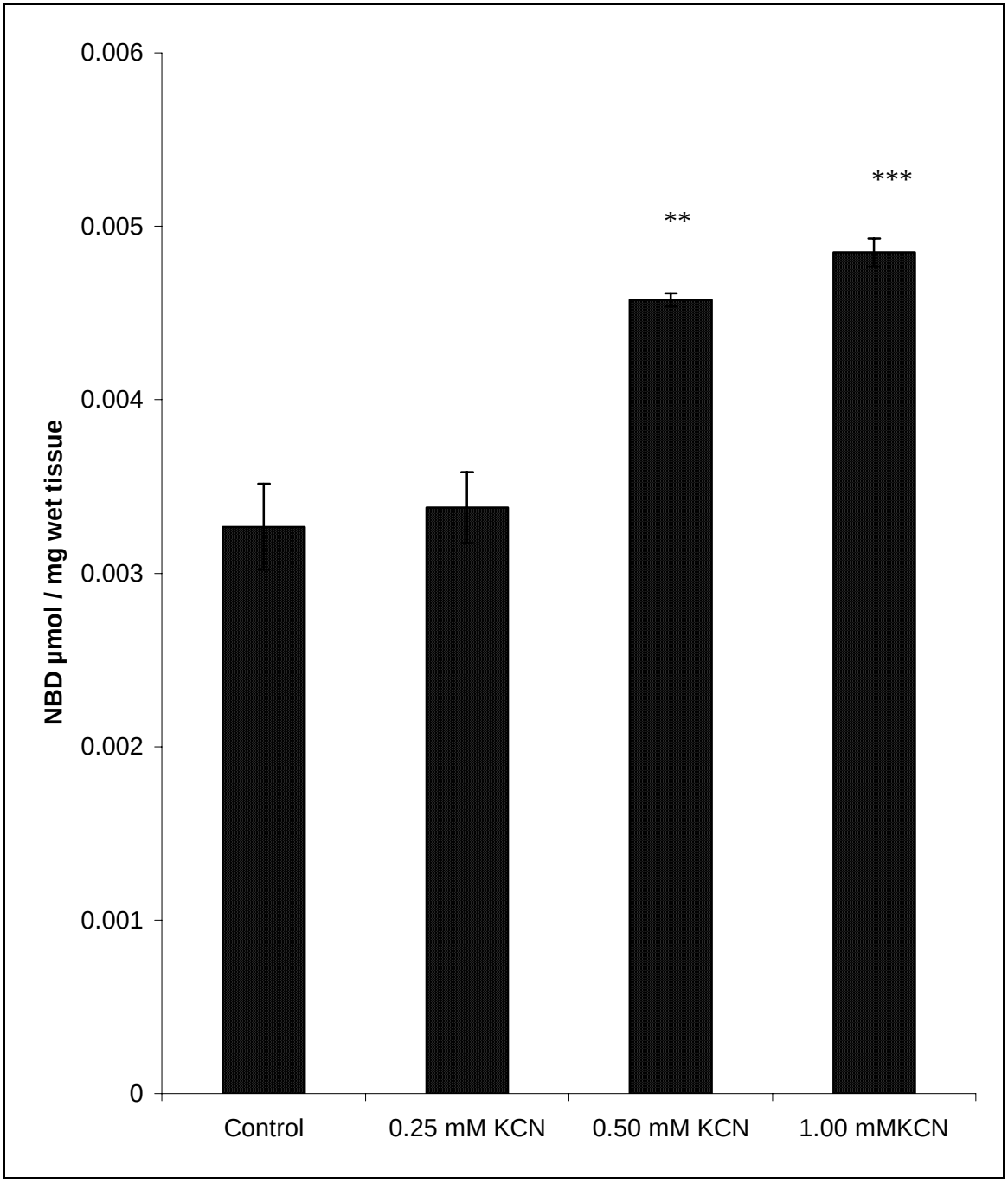


Figure 3.1 Concentration-dependent effect of KCN (mM) on superoxide anion generation in whole rat brain homogenate. Each bar represents the mean ± SD of six determinations. *($p < 0.05$); Student Newman Keuls multiple range test.

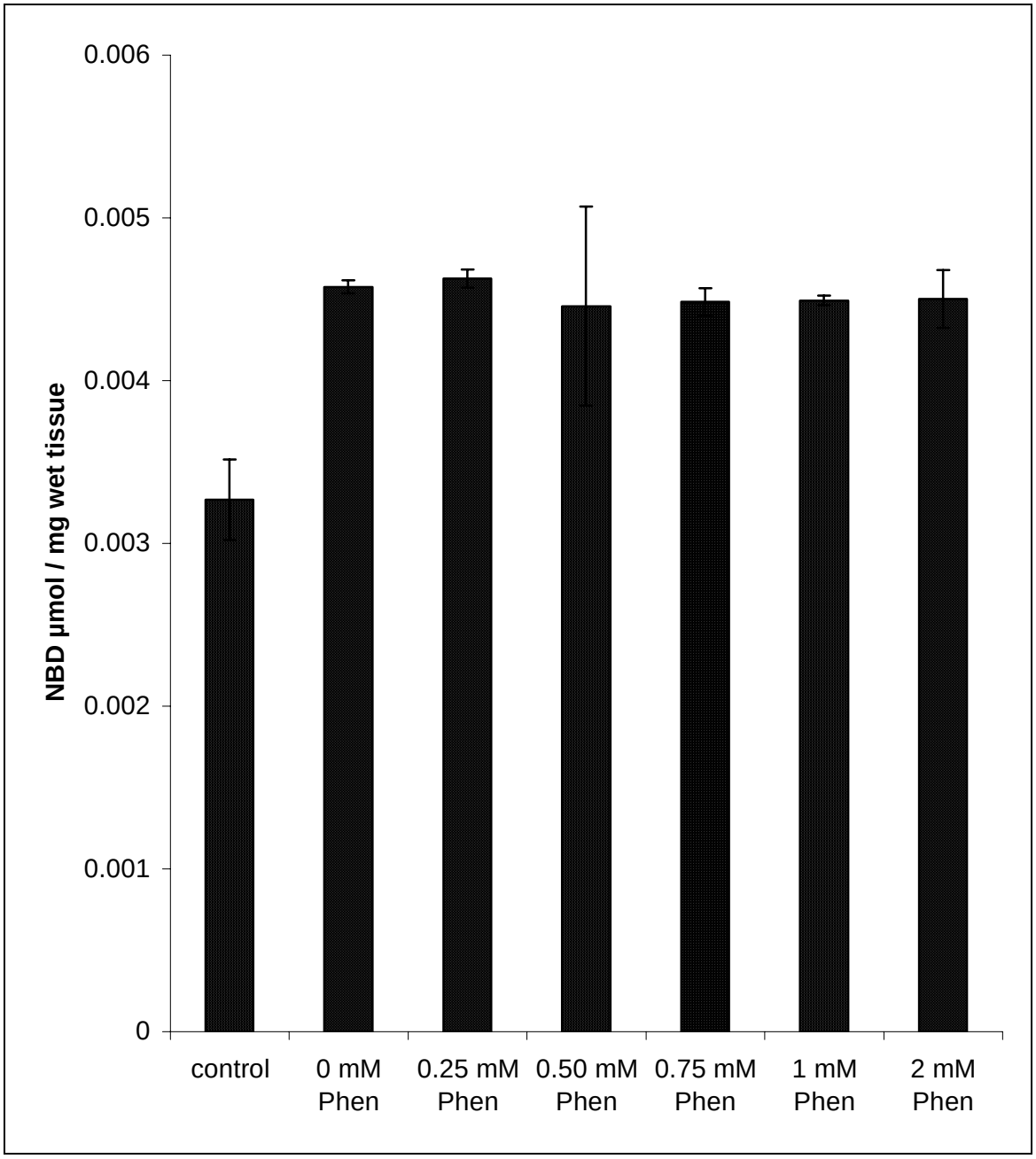


Figure 3.2. Effect of Phenytoin on 0.5 mM KCN- induced superoxide production in whole rat brain homogenate. Each bar represents the mean ± SD of six determinations. *($p<0.05$); Students Newman Keuls multiple range test.

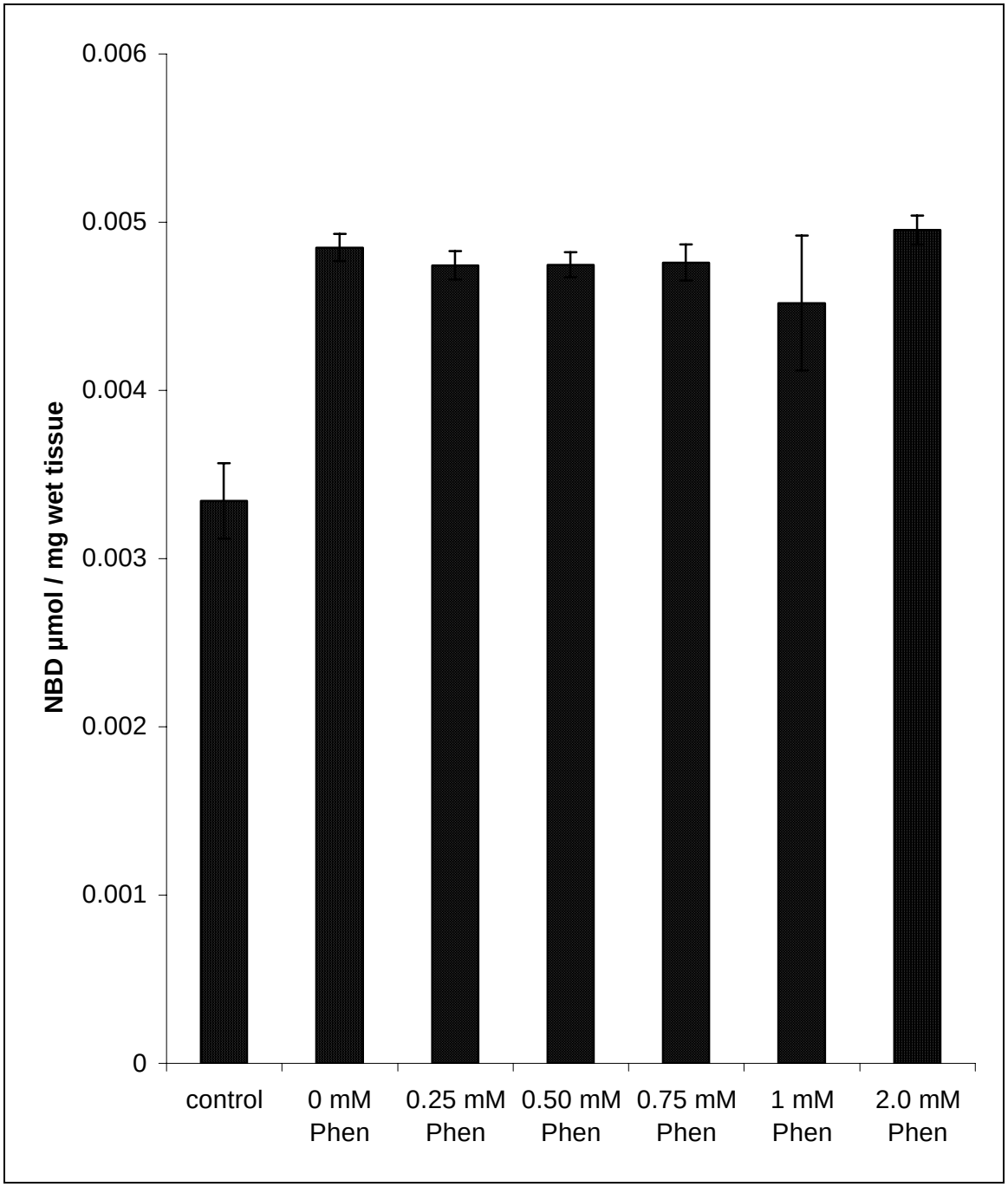


Figure 3.3. Effect of Phenytoin on 1.0 mM KCN- induced superoxide production in whole rat brain homogenate. Each bar represents the mean ± SD of six determinations. *($p<0.05$); Students Newman Keuls multiple range test.

3.2.4. DISCUSSION

The significance of free radical generation has been studied in the pathophysiology of many neurological disorders where there is massive neuronal loss. The production of superoxide in rat forebrain homogenate was significantly increased as expected by exposure to KCN and as reported by Lambat *et al.* (2000).

Moreover this dose-dependent increase in levels of NBD with KCN can be directly linked to the amount of superoxide radical produced as more NBT was reduced to produce the reduced form of the compound, NBD. However the increase in NBD levels was not significantly lowered by the antiepileptic drug phenytoin at concentrations of between 0 and 2.0 mM of this drug in the presence of 0.5 mM and 1.0 mM KCN.

These results therefore demonstrate that phenytoin was not able to act as a superoxide anion scavenger or chemically react with KCN to make cyanide unavailable to produce toxicity.

Another possibility is that phenytoin may react with superoxide anions formed and the product of this reaction could be a free radical of phenytoin. A free radical of phenytoin could thus have favoured the reduction of NBT accounting for the elevated levels of NBD observed in all the reaction tubes as compared to the control.

CHAPTER 4

LIPID PEROXIDATION

4.1. INTRODUCTION

In all living cells, the cell membrane separates the cell interior from the external environment, and in addition functions to hold and contain the internal structures of the cell. Membranes are not stagnant boundaries that segregate regions, but are dynamic systems responsible for among other things; the production of ATP, the discriminating transport of substances into and out of the cells, the binding of regulatory agents (such as hormones and growth factors), and the binding of neurotransmitters that mediate the conduction of nerve impulses (Dykens, J.A., *et al.* 1987).

Membranes are composed chiefly of protein and lipid molecules. The fluid mosaic model of membrane structure proposed by Singer and Nicholson (Singer, S.J. *et al.* 1972) is the accepted model of membrane structure. Membranes are understood to consist of a lipid bi-layer containing a mixture of proteins and carbohydrates.

Changes in membrane fluidity through physical and chemical disturbances, e.g. oxidative stress, can cause changes to neuronal characteristics, and the activities of the transport proteins. Neuronal membranes are especially vulnerable to free radical mediated oxidative stress, since the brain consumes 20% of the total body oxygen, is relatively deficient in protection mechanisms such as glutathione and vitamin E, and contains large amounts of polyunsaturated lipids (Reiter, R.J., *et al.* 1996). The oxidative destruction of polyunsaturated fatty acids is known as lipid peroxidation, and is

enormously damaging as a result of the self-perpetuating chain reactions they cause (Fahn, S. *et al.* 1992). Since some free radical production is inevitable in cells, several enzymatic and non-enzymatic defence mechanisms have evolved to protect cells. As discussed in section 1.4.3, any alterations either in the defence mechanisms or the production of free radicals, could upset this balance and lead to neurotoxicity.

This series of experiments were conducted to investigate the effect of phenytoin on QUIN and iron-induced lipid peroxidation in whole rat brain homogenate. The thiobarbaturic acid (TBA) assay was used to determine lipid peroxidation. The principle of the experiment involves the reaction of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA) with TBA to yield a pink complex, which can be extracted with butan-1-ol and read at 532 nm. MDA and 4-HDA are degraded lipid products from cell membranes, and are taken as reliable indicators of oxidative stress (Reiter, R.J., *et al.* 1995; Packer, L *et al.* 1990).

4.2. MATERIALS AND METHODS

4.2.1. CHEMICAL REAGENTS

Butylated Hydroxytoluene (BHT) and 2-Thiobarbaturic acid (TBA) were purchased from the Sigma Chemical Company (USA). 1,1,3,3-Tetramethoxypropane was obtained from Fluka AG (Switzerland). Butan-1-ol was supplied by Holpro Chemical Company (South Africa). Ascorbic acid, Fe_2SO_4, Trichloroacetic acid (TCA) and EDTA were purchased from Saarchem (Krugersdorp, South Africa). BHT was dissolved in absolute ethanol, while all the other chemicals were dissolved in Milli-Q water. Stock

solutions of known concentrations of QUIN, KCN and phenytoin were prepared in Milli-Q water. All solutions were prepared fresh on the day of the experiments.

4.2.2. ANIMALS

Animals were kept as described in section 2.2.2.2.

4.2.3. BRAIN REMOVAL

Rats were sacrificed by neck fracture and decapitated. The brain was handled as described in section 2.2.2.5.

4.2.3.1. Preparation of brain homogenate

On the morning of the experiment, following removal, the whole brains were weighed and homogenised in a Teflon® coated glass homogeniser for 60 seconds on ice. A 10% w/v homogenate was produced with 0.1 M PBS at a pH of 7.4.

4.2.4. PREPARATION OF STANDARD CURVE

1,1,3,3-Tetramethoxypropane was used as a standard. A series of reaction tubes, each containing appropriate aliquots of water and standard solution were prepared with Milli-Q water to a final volume of 1 ml. A calibration curve was generated by measuring the absorbance at concentration intervals of 2 nmol. The absorbance was read at 532 nm using a Varian Cary 500 Scan UV-VIS NIR Spectrophotometer (EL 99053199) and plotted against

the molar equivalent weight of MDA in the complex assayed (see appendix 2).

4.3. EXPERIMENT 1
In vitro incubation of rat brain homogenate with the Glutamate receptor agonist, QUIN, with varying concentrations of phenytoin.

4.3.1. METHODS

Whole rat brain homogenate was prepared as explained in section 4.2.3.1. A modified method of the TBA assay as described by Ottino (Ottino, P. 1996) was used to estimate lipid peroxidation.

Whole rat brain homogenate 10% w/v (0.5 ml) containing varying concentrations of phenytoin (0–1.25 mM) was incubated with 0.5mM QUIN, both dissolved in Milli-Q water, in a shaking water bath for 1 hour at 37^{o}C. At the end of the incubation, 0.5ml BHT (0.5 g/L in absolute ethanol) and 1 ml 25% TCA in Milli-Q water were added to the mixture to stop the reaction. The samples were centrifuged at 2000 x g for 20 minutes at 4^{o}C to remove insoluble proteins. Following centrifugation, 2 ml of protein-free supernatant was removed from each tube, and a 0.5 ml aliquot of 0.33% TBA in Milli-Q water was added to this fraction. The concentration range of phenytoin used in this experiment was 0–1.25 mM.

All tubes were heated for an hour at 95^{o}C in a water bath. After cooling, the TBA-MDA complexes were extracted by vortexing the contents with 2 ml of butan-1-ol, to optimise partitioning of the chromogen into the organic upper layer. The tubes were then centrifuged again at 2000 x g for 5 minutes to

allow complete separation of the two immiscible solvents. The absorbance of the top layer was then read at 532 nm in plastic cuvettes having a path length of 1 cm and MDA levels were determined from the standard curve generated from 1,1,3,3-tetramethoxypropane. Final results were expressed as nmoles MDA per mg wet tissue. The incubation mixtures did not contain any ethanol or other solvents.

4.3.2. STATISTICAL ANALYSIS

All data are represented as mean±SD. Statistical analysis was performed using a one way analysis of variance (ANOVA) followed by the Student–Newman–Keuls multiple range test. Values were accepted as being statistically significantly different if a *P* value was < 0.05.

4.3.3. RESULTS

The results clearly demonstrate that exposure of the whole rat brain homogenate to QUIN increased MDA production. This can be noted in Figure 4.1, QUIN (0.5 mM) significantly increased lipid peroxidation compared to the control. From Figure 4.1, phenytoin at any of the concentrations used did not result in an overall decline in MDA production.

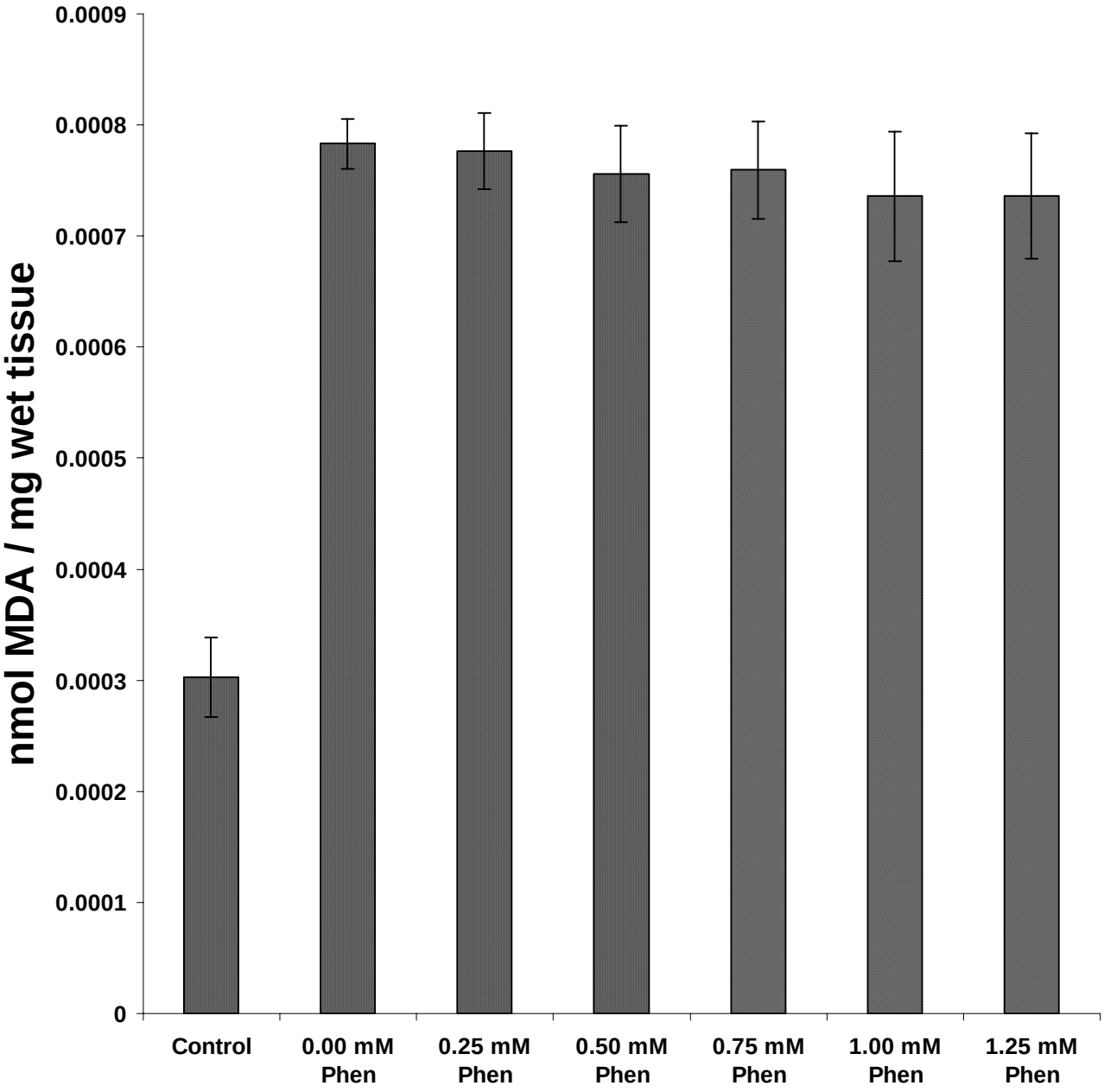


Figure 4.1. Effect of increasing levels of phenytoin (0 – 1.25 mM) on 0.5 mM QUIN induced lipid peroxidation in whole rat brain homogenate. The height of the columns shows the amount of MDA production per mg of wet tissue. The incubation time was 60 min. Each bar represents the mean ± SD (*n*=6); Student Newman Keuls multiple range test.

4.3.4. DISCUSSION

The significance of free radical generation is currently the subject of much research in the pathophysiology of many neurodegenerative disorders. Neurotoxins, including QUIN (Southgate and Daya. 1999) induce neurodegeneration by free radical mechanisms in live neurons. QUIN (0.5mM) has a significant outcome on MDA production. These results are parallel to those of Rios and Santamaria (1991) who found that QUIN is a potent lipid peroxidant in rat brain homogenate. QUIN causes lipid peroxidation in a dose-dependent manner. The reason for this is not clear, but it is possible that QUIN is acting as a pro-oxidant, thus initiating lipid peroxidation.

This study shows that the antiepileptic drug used, phenytoin, does not reduce QUIN induced lipid peroxidation. The protective effect of neuroprotectants is likely to be attributable, at least in part, to their direct free radical scavenging properties (Reiter, 1998). Moreover, other mechanisms such as stabilisation of cell membranes, (allowing them to resist free radical processes more efficiently) (Garcia *et al.* 1998) and the stimulation of antioxidative enzymes that renders toxic free radicals to non-toxic products (Barlow-Walden *et al.* 1995, Reiter. 1998) should play a role in decreasing QUIN induced lipid peroxidation. Phenytoin clearly does not possess these properties when used in whole rat brain homogenate. It is also possible that, because brain homogenate contains a big proportion of dead cells and that phenytoin can only protect from damage due to NMDA receptor mediated toxicity as discussed in chapter 1, the drug was not able to prevent damage.

4.4. EXPERIMENT 2

Effects of phenytoin on iron induced lipid peroxidation in whole rat brain homogenate

4.4.1. INTRODUCTION

The role of iron on free radical and lipid peroxidation reactions has been studied extensively and its role in this regard is widely accepted (Fahn *et al*, 1992). Transition metals promote lipid peroxidation in two ways. These can catalyse the formation of oxygen free radical species capable of initiating lipid peroxidation. In addition, they can also favour the decomposition of preformed lipid peroxides to propagate lipid peroxidation (Rikans *et al*, 1997).

In this study, iron was used to generate free radicals and the effects of phenytoin on iron-induced lipid peroxidation in whole rat brain homogenate were investigated.

4.4.1. MATERIALS AND METHODS

Rat whole brain homogenate was prepared as explained in section 4.2.3.1. A modified method of the TBA assay as described by Ottino (1996) was used to determine the extent of lipid peroxidation lipid peroxidation.

0.5 ml of a 10 % w/v whole rat brain homogenate containing the following reagents, iron sulphate (5 mM), EDTA (100 μM), H_2O_2 (2.8 mM), phenytoin (0.0-1.50 mM), and ascorbate (100 μM), were added in the sequence stated.

The reaction mixture was incubated in an oscillating water bath for 1 hour at 37°C. The experiment was continued as outlined earlier in Section 4.3.1. Final results are expressed as nmoles of MDA per mg wet tissue.

4.4.3. STATISTICAL ANALYSIS

Refer to section 4.3.2.

4.4.4. RESULTS

Exposure of whole rat brain homogenate to various concentrations (0–1.50 mM) of phenytoin decreased iron-induced lipid peroxidation significantly as compared to the control (Figure 4.2.). Iron II at a concentration of 5 mM produced a significant increase of about 700% in MDA production. Phenytoin caused a dose-related reduction in lipid peroxidation. A 500% reduction of iron-induced lipid peroxidation was achieved with a 1.50 mM phenytoin as shown in Figure 4.2.

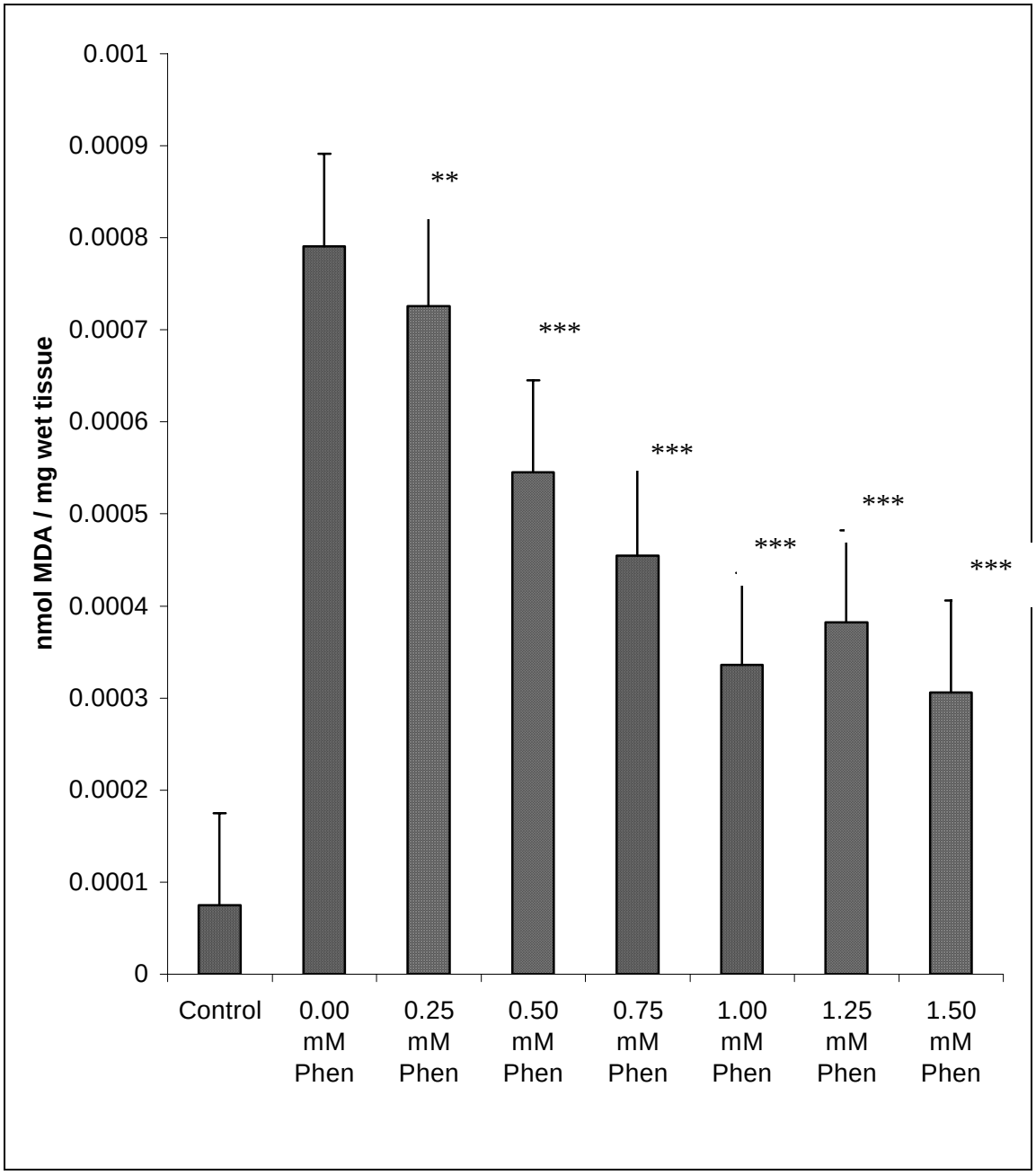


Figure 4.2. Effect of increasing levels of Phenytoin (0 – 1.50 mM) on iron (5 mM)-induced lipid peroxidation in whole rat brain homogenate. The height of the columns shows the amount of MDA production per mg of wet tissue. The incubation time was 60 min. Each bar represents the mean ± SD (n=6); *($P < 0.05$); Student Newman Keuls multiple range test.

4.4.5. DISCUSSION

A significant dose related reduction of iron II induced MDA production by the antiepileptic agent, phenytoin was observed whereas this behaviour was not seen for QUIN-induced lipid peroxidation. It is possible that Phenytoin acts by scavenging the hydroxyl radicals generated, chelating or complexing with the iron (II) or oxidising it to iron (III), such that iron (II) becomes unavailable hydroxyl radicals via the Fenton type reactions as mentioned earlier.

It had been suggested that phenytoin binds to metallic ions and forms complexes. Because phenytoin is not a good free radical quencher as found in the last chapter and with QUIN-induced lipid peroxidation, it can be inferred that a possible interaction between the drug and iron might be occurring and thereby preventing the generation of the potent damage causing hydroxyl radicals. Hence this interaction with iron might be an additional mechanism by which the drug confers protection when used during ischaemic episodes, strokes and trauma, during which it has been reported that an increase in free iron concentration occurs in the CNS.

This concept of phenytoin interacting with metals, especially iron (II) will be further investigated in the next chapter in an attempt to shed more light on this interaction.

CHAPTER 5

IRON BINDING STUDIES

5.1 INTRODUCTION

The iron contained in the body is absorbed from the gut, and originates from the diet that is consumed. Most body iron is contained in haemoglobin, with smaller amounts in myoglobin, co-factors of various enzymes and the transport protein transferrin. Excess iron is stored as ferritin, which can hold up to 45 000 ions of iron (Halliwell & Gutteridge, 1989). Transferrin enters the cytoplasm in a vacuole, which is acidified to release the iron. The transferrin is then ejected leaving behind non-protein-bound iron. This can be used in the synthesis of iron proteins. Cells use this process to minimize the intracellular iron concentration (Halliwell & Gutteridge, 1989). Oxidative processes can provide the iron required for Fenton chemistry by mobilizing iron from ferritin or by degradation of haem proteins. Studies have reported that the formation of the very reactive hydroxyl radical *in vivo* may increase during tissue injury.

This is significantly important in the brain since CSF has no considerable iron-binding capacity, and mechanical disruption of the brain releases iron that can stimulate radical reactions such as lipid peroxidation. Consequently, brain homogenate, for example, is known to undergo lipid peroxidation at a much faster rate than isolated intact brain (Halliwell & Gutteridge, 1989). Another important aspect to consider is that free radicals are able to initiate lipid-destroying chain reactions (Southgate & Daya, 1999), particularly in the brain, because it contains large amounts of polyunsaturated lipids. Many neurological disorders are associated with iron overload (Halliwell B.,

1992). These include asymptomatic deposition of iron in the basal ganglia (Berg D *et al.*, 2000), psychiatric diseases (Cutler P., 1994), dementia, ataxia and myoclonic jerks.

Moreover, Halliwell & Gutteridge (1989), have reported that iron (II) complexes react with lipid peroxide to generate iron (III) and an alkoxyl radical. They also noted that iron (II) complexes stimulate membrane peroxidation to a greater extent as compared to iron (III).

Iron is a grey metal with the outer electronic configuration $3d^6 4s^2$. The metal is found amongst the d-block elements of the periodic table. Iron exhibits several oxidation states in its compounds, of which the two most important ones are;

- Iron (II) electronic configuration $3d^6$
- Iron (III) electronic configuration $3d^5$

Iron (III) is more stable than iron (II) since each of its five 3d-orbitals is singly occupied by an electron. The iron (II) ion, Fe^{2+}, is hydrated in aqueous solution. It exists as the pale green hexa-aquairon (II) complex ion $[Fe(H_2O)_6]^{3+}$. The ion is stable in acidic conditions and readily forms hydrated iron (III) ion in neutral or alkaline solution.

One of the most important chemical properties of d-block elements is their ability to form complex ions. A complex ion is formed when one or more molecules or negatively charged ions become attached to a central atom. The molecule or negative ion that becomes attached is called a ligand. Some ligands are able to form ring structures with central atoms. This property is

referred to as chelation and the resulting compounds known as chelate compounds (Freemantle, M., 1987).

The additional protective mechanism conferred by phenytoin during iron-induced lipid peroxidation was investigated by gauging the interaction of phenytoin with iron using the following techniques listed below:

- UV/VIS spectrophotometry
- Electrochemistry
- IR analysis

5.2 MATERIALS AND METHODS

5.2.1. CHEMICAL REAGENTS

Phenytoin was purchased from Sigma Chemical Co., St. Louis, MO, USA. Ferrous sulphate ($FeSO_4 \cdot 7H_2O$) and Ferric chloride ($FeCl_3$) were obtained from Merck, Midrand, South Africa.

5.2.2. PREPARATION OF THE PHENYTOIN-IRON COMPLEX

An excess of 4 mM solution of phenytoin (ligand) was added to 1 mM solution of ferrous sulphate (central atom) and stirred continuously for 2 hours. Both compounds were dissolved in deaereated Milli-Q water so that iron (II) stays in its reduced form for a longer period as dissolved oxygen favours the conversion of iron (II) to the oxidised state, iron (III). The pH of the reacting solution was found to be 7.9. No buffer was used in the preparation. On addition of the ligand, the pale green colour of the metal ion solution changed to clear deeper green solution. Immediately afterwards, (approximately 2 seconds), a reddish brown precipitate was formed and this was believed to be the complex. The same procedure was used to synthesise the iron (III)- phenytoin complex using $FeCl_3$.

The precipitate formed was filtered under vacuum and rinsed with an excess of Milli-Q water. It was then dried in an oven at 40^0C for 12 hours to remove water.

A dry weight of 1.5 g of the phenytoin-metal complexes was obtained for both iron (II) and iron (III) ions.

5.2.3. UV/VIS analysis

Ultraviolet radiation normally excites electrons from one molecular orbital to another. An example is when an electron, such as a pi electron of a carbon-carbon double bond, after absorbing a particular quantity of energy, is promoted from the highest occupied molecular orbital to the lowest unoccupied molecular orbital. As a result, by irradiating UV light of continuously changing wavelength, a UV spectrum unique to an organic compound is produced. Moreover if that particular organic compound interacts chemically with any other compounds or metallic ions, a change in the absorption spectrum is expected.

The UV/Visible spectra were monitored using a Varian Cary 500 Scan UV-VIS NIR Spectrophotometer (EL 99053199). Concentrations of metal were kept constant (1 mM) and spectra measured on addition of excess phenytoin (2 mM) solution. Spectra were scanned at 30 second time intervals, time starting immediately after the addition of the phenytoin solution to the metal, iron (II), solution. This was carried out in a quartz cuvette of 1 cm path-length over a wavelength range of 190-800 nm. The experiment was carried out until no further shifting of peaks occurred and stopped 30 seconds after the precipitate became visible on the wall of the cuvette. All solutions were freshly prepared in deaerated Milli-Q water and glassware were treated with a dilute solution of nitric acid before usage. The experiment was repeated using iron (III) instead of iron (II).

5.2.4.. INFRARED SPECTROSCOPY

IR analysis simplistically determines the functional group of compounds. The full interpretation of an IR spectrum is difficult because most organic

molecules are so large that there are dozens or hundreds of different possible bond stretching and bending motions. Thus, an IR spectrum contains dozens or hundreds of absorptions. This complexity is valuable, since the spectrum serves as a unique fingerprint of a specific compound.

Essentially IR spectroscopy involves the interaction of a molecule with electromagnetic radiation. When an organic molecule is irradiated with IR light, the molecule absorbs particular frequencies of light. The frequencies absorbed correspond to the amounts of energy needed to increase the amplitude of specific molecular vibrations such as bond stretching and bending. Every functional group has a characteristic set of infrared absorptions.

In this experiment, the IR spectra of the phenytoin-metal complex formed and phenytoin were produced using potassium bromide pellets.

Care was taken to ensure that no air was trapped between the disks, before samples were scanned from 1000 cm^{-1} to 4000 cm^{-1}. IR spectra of phenytoin were also run for comparison. Blank IR spectra were run in air.

5.2.5. ELECTROCHEMISTRY

Electrochemistry offers a valuable tool in gauging metal ligand interactions. Cyclic voltammetry, used to characterise species in solution and adsorptive stripping Voltammetry, a tool to examine metal-ligand complex formation at an electrode, were used in this work. A three-electrode system was used comprising the working electrode, a reference electrode and an auxiliary electrode. These techniques are discussed in more detail below.

5.2.5.1. The Electrodes

The *working electrode* is the electrode where electron transfer reactions occur. The most utilised working electrodes are those of metallic origins such as mercury, platinum and gold, which offer fast electron transfer rates. A glassy carbon electrode (GCE) was used in this thesis. GCEs comprise of a material that has excellent mechanical and electrical properties, a wide potential window, is chemically inert and exhibit reproducible performance.

In this experiment the silver/silver chloride electrode was used as the *reference electrode* against which all potentials are referenced. It consists of a silver wire anodised with silver chloride.

Usually constructed of an inert metal such as platinum in the form of a wire, loop or foil, the *auxiliary electrode* completes the circuit.

5.2.5.2. Cyclic / Adsorptive Stripping Voltammetry

In Cyclic Voltammetry the underlying theory is that electroactive analytes in solution produce characteristic redox patterns when a potential range is scanned. Consequently, any changes in potentials or the intensity of current response are an indication of new species in solution. Cyclic Voltammograms (CV) were obtained with the Bio Analytical Systems (BAS) CV-50W voltammetric analyser using a BAS C2 cell stand to maintain constant atmosphere. A 3mm diameter glassy carbon electrode (GCE) was employed as a working electrode for voltammetric experiments.

A silver/silver chloride ([AgCl = 3 M]) and a platinum wire were employed as reference and auxiliary electrodes, respectively. Between scans, the GCE was cleaned by polishing with alumina on a Buehler pad, immersion in a dilute nitric acid and rinsed with deionised water and the buffer solution.

For cyclic voltammetric experiments, appropriate concentrations of phenytoin in buffer were introduced into a glass cell and degassed for 5 min with nitrogen before scanning a potential window. In contrast, in organic solutions, a silver wire was used instead of the silver/silver chloride reference electrode as a pseudo reference electrode. In these instances ferrocene was used as an internal standard. Potentials were referenced to ferrocene by subtracting $E_{1/2}$ ferrocene from potentials measured at the Ag wire. The $E_{1/2}$ of ferrocene has been determined in DMSO to be 0.50 V vs. a saturated calomel electrode. Potentials vs. ferrocene were thus referenced to the saturated calomel electrode by adding 0.05 V to these values. Potential values were then referenced to Ag/AgCl, 3M KCl by adding 0.05 V to these values. All potential values reported are vs. Ag/AgCl.

Adsorptive Stripping Voltammetry is a technique used to examine metal-ligand complex formation at an electrode. It is dependent on the tendency of analytes to preconcentrate at the surface of a working electrode. The introduction of a ligand into a metal solution causes an increase in the pre-concentration of the metal at the electrode if it forms a metal-ligand complex. The formation of this complex causes an increase in the current response due to metal reduction. A shift in the reduction potential indicates that a new species is being reduced.

For adsorptive stripping experiments appropriate concentrations of the metal (Fe^{2+} or Fe^{3+}) and of the ligand (phenytoin) were introduced into an electrochemical cell. The solution was then deaerated with nitrogen for 5 min, after which a flow of nitrogen was maintained over the solution throughout the experiment.

Optimum deposition potential for iron (II) and iron (III) were identified and applied for 60 s to effect the formation and adsorption of the metal and ligand species onto the glassy carbon electrode. The voltammograms were then scanned in the negative direction from the deposition potential to 500 mV beyond the reduction potential of the metal species at a scan rate of 0.1 V s^{-1} to strip the adsorbed metal-ligand species from the electrode. During the stripping step, current response due to the reduction of the metal–ligand species were measured as a function of potential.

5.3. RESULTS

5.3.1. UV/ VIS analysis

Table 5.1. The major peaks observed for Fe^{2+} and phenytoin

Peak	Wavelength (nm)
Phenytoin only	200.8
Fe^{2+} + Phenytoin (T= 0.0 mins)	210.1
Fe^{2+} + Phenytoin (T= 0.5 mins)	211.0
Fe^{2+} + Phenytoin (T= 1.0 mins)	211.0

The major band observed for phenytoin was at 200.8 nm. This band shifted to 210.1 nm immediately after the addition of the ligand to the metal ion solution without shift in peak height. No new bands were observed. The reaction was stopped after 1.0 minute when no further changes in absorbance values and in band shifting occurred and precipitation of the metal-ligand complex occurred.

In addition to the changes in peak wavelengths of phenytoin, a distinct colour change was observed from the pale green colour of the Fe (II) solution to a deeper green colour immediately after the addition of phenytoin. Following this, the colour quickly changed to a reddish brown clear solution and subsequently, precipitation of what was believed to be the end product of a ligand-metal interaction occurred.

When the UV/VIS analysis was repeated using $FeCl_3$ instead of $FeSO_4$ with phenytoin in the same aqueous environment the following results were

obtained and are shown in table 5.2. below. This experiment also gave a shift of the phenytoin peak at 200.8 nm to 210.9 nm after a 1 minute time period.

Table 5.2. The major peaks observed for Fe^{3+} and phenytoin

Peak	Wavelength (nm)
Phenytoin only	200.8
Fe^{3+} + Phenytoin (T= 0.0 mins)	209.9
Fe^{3+} + Phenytoin (T= 0.5 mins)	210.9
Fe^{3+} + Phenytoin (T= 1.0 mins)	210.9

5.3.3. ELECTROCHEMICAL STUDIES

All potentials are referenced against the Ag/AgCl reference electrode, unless otherwise stated.

Figure 5.1. shows the CV for phenytoin alone in solution in pH 4.25 sodium acetate buffer. One quasi-reversible couple with a cathodic potential of –0.65 V and an anodic potential of 0.75 V was observed. The halfwave potential ($E_{1/2}$) was equal to 0.05 V

Adsorptive stripping voltammograms (ASV) of studies of Fe^{2+} and phenytoin at pH 4 are shown in Figure 5.2. Figure 5.2 (a) shows the ASV for 3×10^{-5} M Fe^{2+}, with a reduction potential of –0.28 V. In the presence of increasing concentrations of phenytoin, this peak increases and shifts to less negative potentials of –0.27 V as shown for 3×10^{-5} M phenytoin in Figure 5.2 (b) and 9×10^{-5} M phenytoin in Figure 5.2 (c).

ASV for studies of Fe^{3+} and phenytoin at pH 4.25 (sodium acetate buffer), are shown in Figure 5.3. Figure 5.3 (a) shows the ASV for 3 x 10^{-5} M Fe^{3+}, with a reduction potential of –0.65V. Increasing concentrations of phenytoin reduces the current response observed for Fe^{2+} with no shifts in potential, as shown in Figure 5.3.(b) to 5.3.(d) for 3 x 10^{-5} M, 9 x 10^{-5} M and 1.2 x 10^{-4} M phenytoin respectively.

CV of the iron-phenytoin complexes isolated were conducted using dimethyl sulphoxide (DMSO) as a solvent and tetraethylammonium phosphate (TEAP) as the electrolyte and compared to the CV of phenytoin alone. Based on the quoted $E_{1/2}$ values of ferrocene in DMSO, all potentials were recalculated and final potentials reported against Ag/AgCl. Figure 5.4 (a) shows the CV for phenytoin alone in solution with a quasi-reversible couple. A cathodic peak potential of –0.57 V and anodic potential of –0.38 V was observed. Figure 5.4 (b) shows the CV for a phenytoin-Fe complex. Here, the cathodic peak potential is observed at –0.53 V with an anodic peak potential of –0.25 V. The cathodic return peak potential in Figure 5.4 (b) is slightly less negative than that of phenytoin alone, while the anodic peak potential is slightly more negative than that of phenytoin alone. These subtle shifts indicate an altered cyclic voltammetric profile for the phenytoin-iron complex.

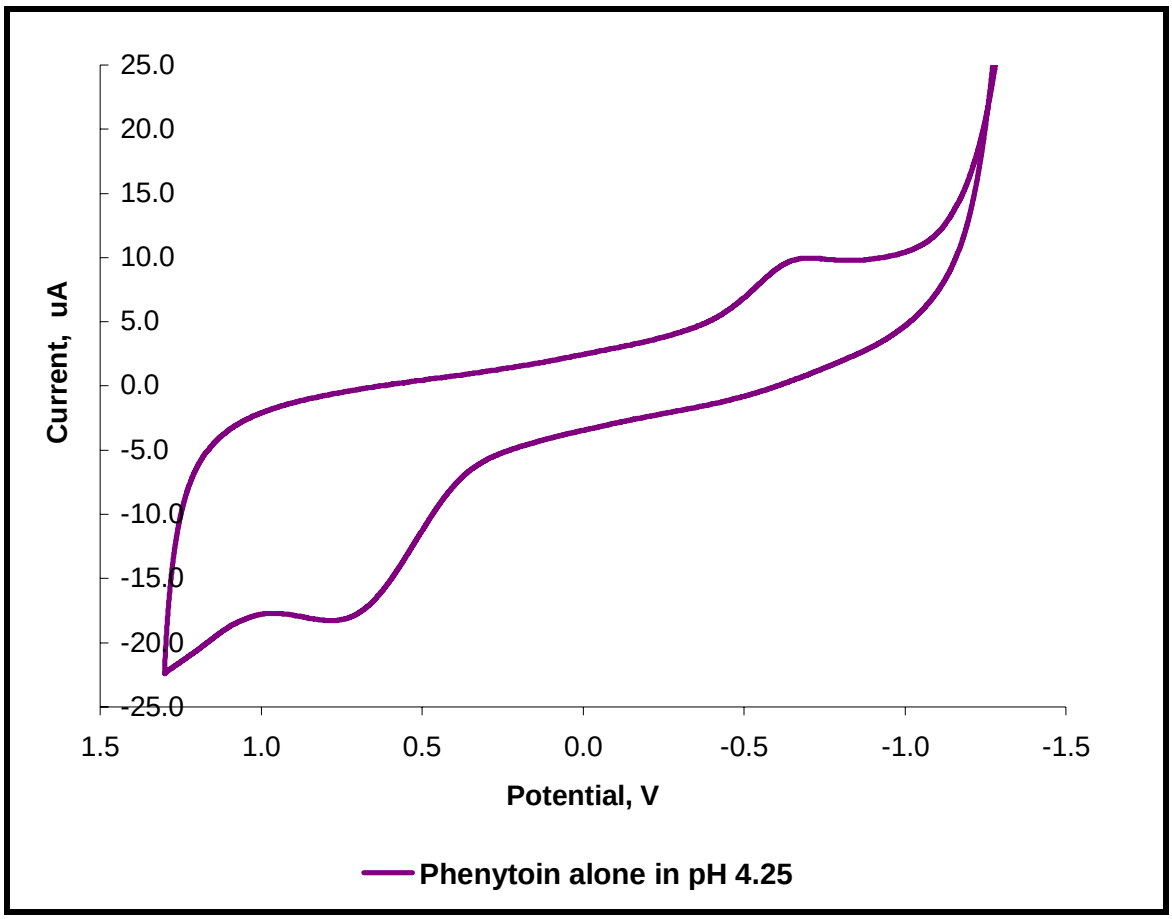


Figure 5.1. CV for phenytoin alone in solution in pH 4.25 sodium acetate buffer

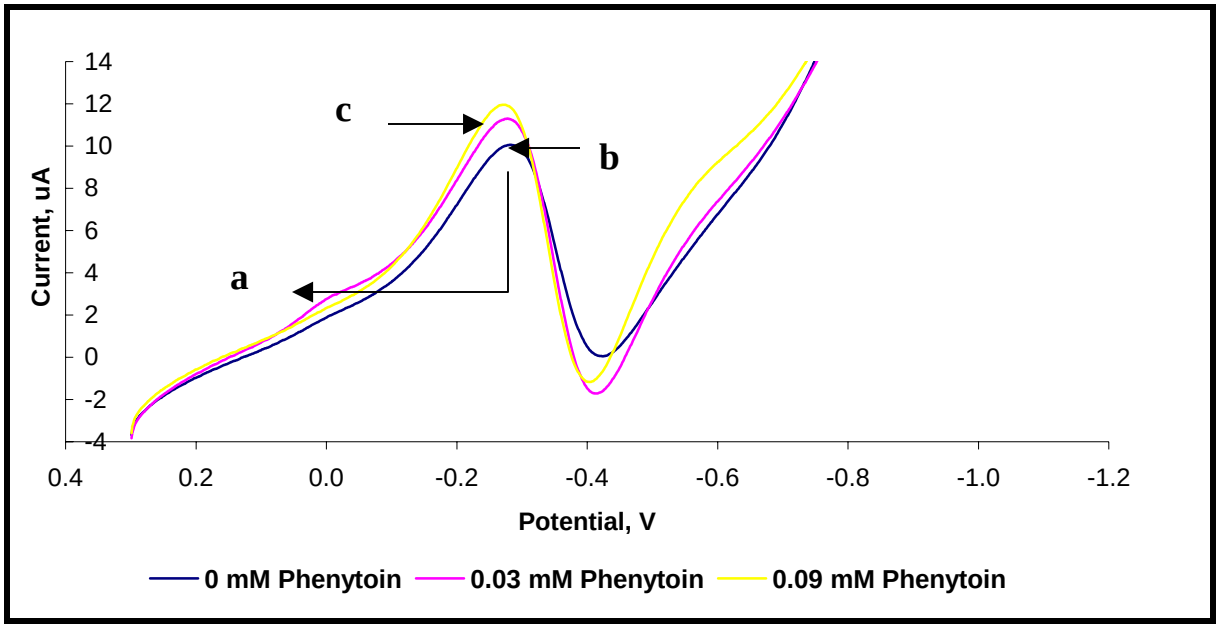


Figure 5.2. ASV for studies of Fe^{2+} and phenytoin at pH 4.

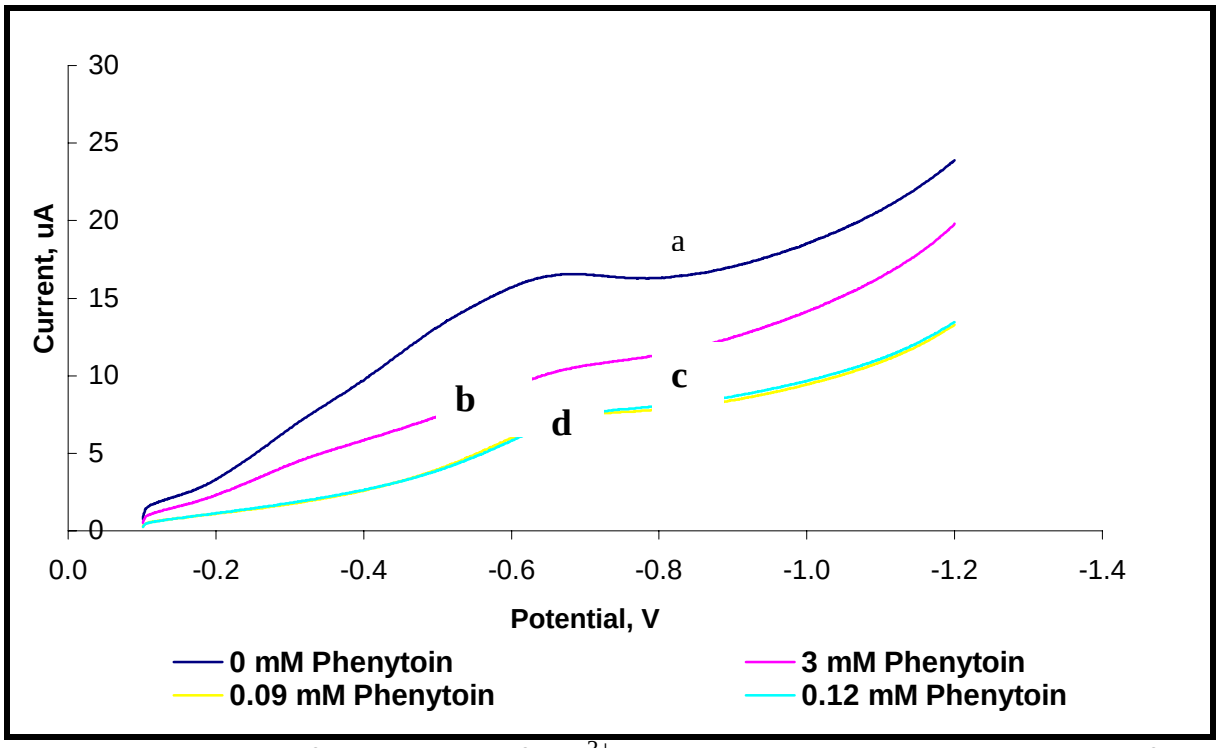


Figure 5.3. ASV for studies of Fe^{3+} and increasing concentrations of phenytoin at pH 4.25

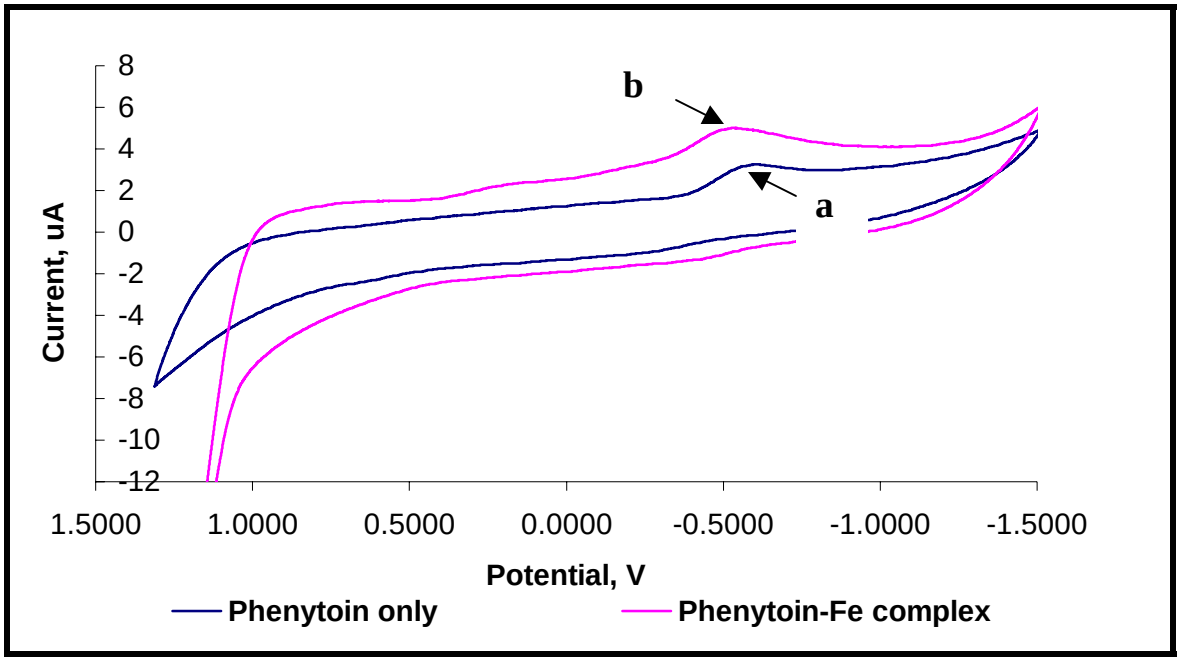


Figure 5.4. CV of phenytoin and the iron-phenytoin complexes in DMSO and TEAP as electrolyte.

5.3.2. INFRARED SPECTROSCOPY

The IR spectroscopic graphs for phenytoin, Fe^{2+}-phenytoin and Fe^{3+}-phenytoin (Figures 5.5, 5.6 and 5.7, respectively) indicate a shift in the major peak obtained at 3312.9 cm^{-1}. This peak represents the NH group present on the phenytoin molecule. In the Fe^{2+}-phenytoin and Fe^{3+}-phenytoin (Figures 5.6 and 5.7, respectively), the signal appearing due to this NH group occurring at 3312.9 cm^{-1} in the phenytoin molecule only, is split and shifted to 3198.4 cm^{-1} and 3267.1 cm^{-1}.

In addition, comparing the carbonyl peak in the infrared spectrums of phenytoin only and the metal-ligand product, a shift from 1596.1 cm^{-1} and 1687.6 cm^{-1} to 1714.2 and 1771.4 cm^{-1} respectively was noticed (Figures 5.5, 5.6 and 5.7). No important splitting was observed after interaction.

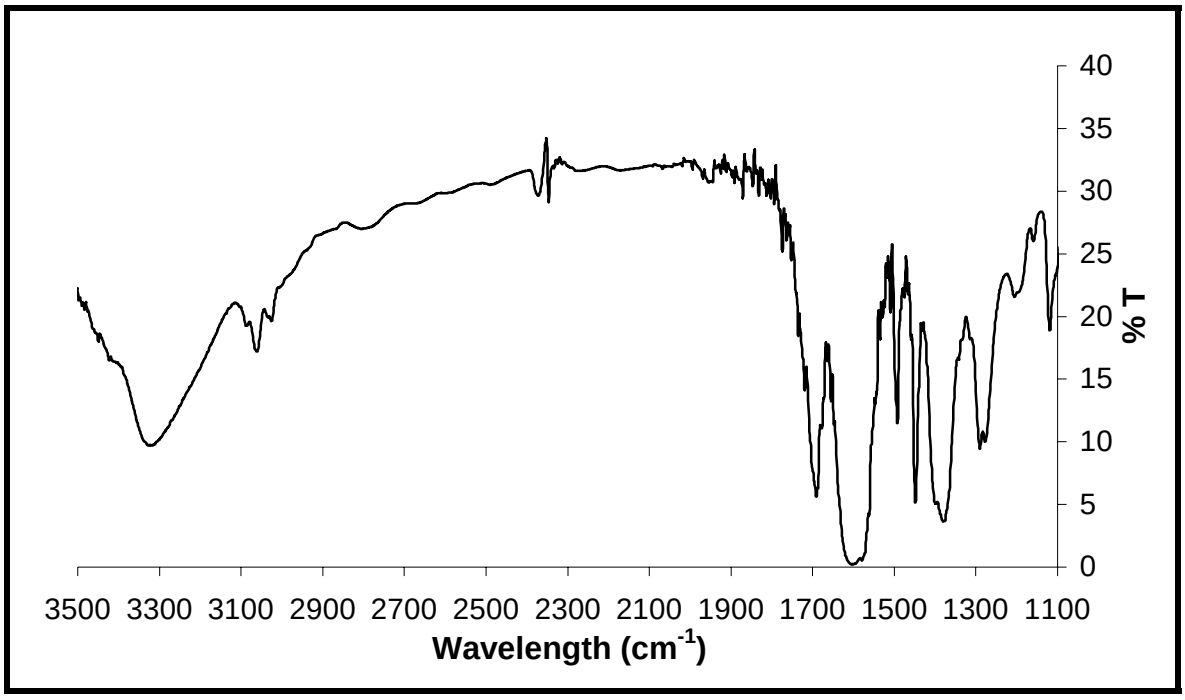


Figure 5.5. Infrared spectrum of phenytoin

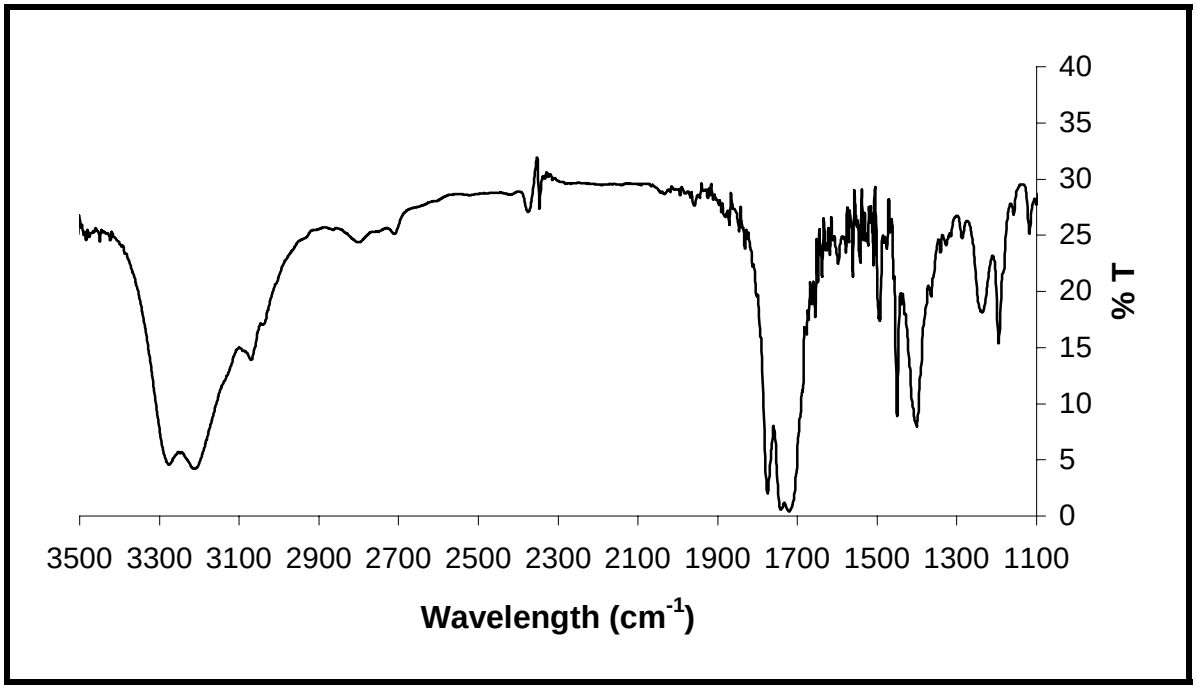


Figure 5.6. Infrared spectrum of iron (II) –phenytoin interaction product

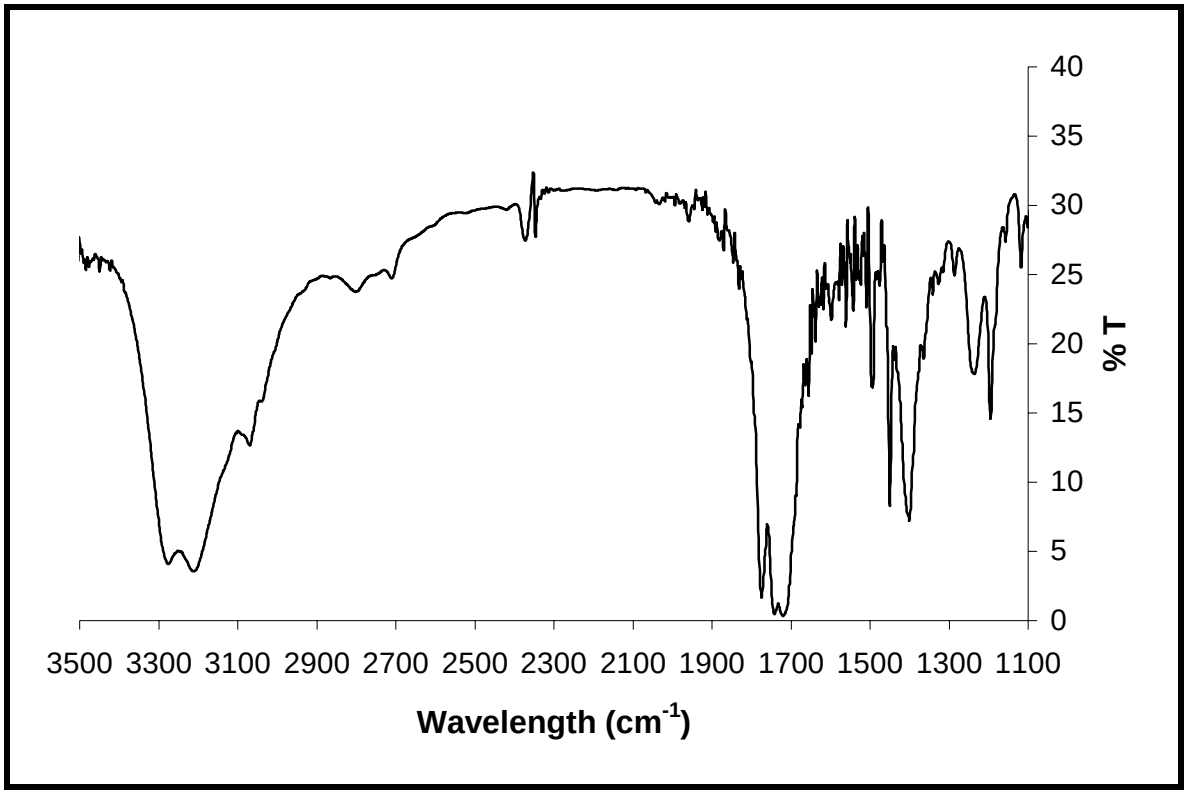


Figure 5.7. Infrared spectrum of iron (III) –phenytoin interaction product

5.4. DISCUSSION

5.4.1. UV/VIS analysis

Table 5.1.and 5.2. shows the shift in the major bands observed. The shift observed indicates that phenytoin is able to interfere with these ions to form a new entity that absorbs energy at these new wavelengths. This suggests a possible interaction between Fe^{2+} and Fe^{3+} with phenytoin in an aqueous environment.

In addition, the changes in colour observed in the cuvettes, permits speculation that phenytoin might be firstly reacting with the pale green iron (II) solution and oxidising it to the iron (III) state that has a characteristic

reddish brown colour followed by precipitation of the reaction product. In addition, apart from the colour changes that were not observed with iron (III) as the latter was already in its oxidised form, the UV/VIS spectrum had a very similar profile to that when Fe^{2+} was allowed to react with phenytoin. This allowed speculation that phenytoin maintains iron in its iron (III) state when bound.

From this experiment, it can be postulated that phenytoin interacts with Fe (II) and Fe (III) ions in a different manner but the resulting product of the reaction can be said to be identical. The next two sections will deal with the Infrared spectroscopy and electrochemical techniques to confirm and shed more light on this interaction.

5.4.2. Electrochemical studies

From the ASV technique used to study the interaction of Fe^{2+} and phenytoin at pH 4, the increase in current response observed for the reduction of Fe^{2+} in the presence of phenytoin indicates a metal-ligand interaction between these species in solution. The shift to less negative potentials indicates that the metal-ligand species is easier to reduce than the metal alone. This indicates a weak metal-ligand interaction between Fe^{2+} and phenytoin.

In addition, the decrease in current response with the studies for Fe^{3+} and phenytoin in Figure 5.3 may indicate that the electrode is saturated, but this is not likely at such low metal and ligand concentrations, or it may indicate that the Fe^{3+} is bound in a form which is not easily reduced by the ligand. The latter may be the case as this is the response observed for such studies with strong ligands. However, the lack of a potential shift does not support

this theory and therefore the interaction of Fe^{3+} and phenytoin in this study is not conclusive but warrants further examination through other solution phase techniques.

5.4.3. Infrared Spectroscopy

From the results obtained it can be noted that splitting and shifting of peaks occurred in the region representing the NH functional group. This suggests that coordination might be taking place at the NH functional group or that there has been a change in the chemical environment of the functional group after interacting with the metal ions.

Moreover the spectra obtained with the iron (II)–phenytoin and iron (III)-phenytoin were not different which suggests that phenytoin is bound to iron (III). This further suggests that phenytoin oxidises iron (II) to iron (III) before binding of the ligand to the metal occurs.

CHAPTER 6

ORGAN CULTURE STUDIES: THE EFFECT OF PHENYTOIN ADMINISTRATION ON RAT PINEAL INDOLE METABOLISM

6.1. INTRODUCTION

Organ culture originated in the Strangeways laboratory more than 60 years ago (Strangeways & Fell, 1926) and the method was used chiefly for the culture of organ rudiments. With various modifications of apparatus and techniques being used for organ culture, it is now possible to isolate and culture specific organs so as to keep a number of fully differentiated organs alive *in vitro* without growth or differentiation. Thus, the organ culture system allows pharmacological and biochemical manipulations of different organs without the complicating influences of *in vivo* milieu.

The choice of organ was the pineal gland located in the brain. The pineal gland is recognized as a fully functional organ that is responsible for the synthesis of indoleamines. Of particular interest is the pineal hormone, melatonin that has been shown to be an effective free radical scavenger (Reiter et al, 1995).

In this study, the effect of phenytoin on rat pineal indole metabolism was assessed.

incubation period, the pineal glands take up the radioactive serotonin and synthesize [^{14}C] indoles. Approximately 98% of the [^{14}C] indole products are secreted into the culture medium, apparently by passive diffusion (Klein & Rowe, 1970; Morton, 1990). The relative amount of medium is considered a good reflection of pineal indole biosynthesis. After the incubation period, an aliquot of the culture medium is analysed for its content of radioactive serotonin metabolites by using TLC and scintillometry.

6.2. MATERIALS AND METHODS

6.2.1. ANIMALS

Male Wistar rats weighing 250g -300g were used in this experiment. These were randomly assembled into 2 groups of 5 and maintained as described in section 2.2.2.2. 10 rats were used and from each the pineal gland was removed and cultured. The control group received the drug vehicle only and the test group was treated with phenytoin. The treatment regimen used can be viewed in Table 6.1 below.

Table 6.1. Treatment regimen for each group of animals

GROUP	Received intra- peritoneally for 7 days
CONTROL	PBS 0.1 mM
PHENYTOIN	Phenytoin (20 mg/kg) in PBS

6.2.2. MATERIALS

(^{14}C) serotonin (specific activity 57.0 mCi/ml) was obtained from Amersham International, England. The BGJb culture medium was purchased from Gibco, Europe and supplemented with the antibiotics streptomycin and benzyl penicillin (Hoechst, South Africa). The aluminium TLC plates coated with silica gel 60, Type F254 (0.25 mm), were purchased from Merck, Darmstadt, Germany. Beckman ready-Sol multipurpose liquid scintillation fluid was purchased from Beckman RIIC Ltd, Scotland. Phenytoin and the indole standards MT, HA, HL, MA, ML, aMT and aHT were purchased from Sigma Chemical Co, St. Louis, USA. All other materials used were obtained from local sources and were of the highest quality available.

6.2.2.1. Composition of BGJb medium used for organ culture studies (Fitton-Jackson modification)

Table 6.2. Composition of BGJb medium used for organ culture studies (Fitton-Jackson modification) Mean tonicity = 390 milliosmoles

COMPONENTS	**CONCENTRATION (mg/L)**
Inorganic Salts	
Dihydrogen sodium ortho phosphate	90.00
Magnesium sulphate $7H_2O$	200.00
Potassium chloride	400.00
Potassium dihydrogen phosphate	160.00
Sodium bicarbonate	3 500.00
Sodium chloride	5 300.00
Amino Acids	
L – Alanine	250.00

L – Arginine	175.00
L – Aspartic acid	150.00
L- Cysteine HCl	90.00
Glycine	800.00
L – Histidine	150.00
L – Isoleucine	30.00
L – Leucine	50.00
L –Lysine	240.00
L – Methionine	50.00
L – Phenylalanine	50.00
L – Proline	400.00
L – Serine	200.00
L – Threonine	75.00
L – Tryptophane	40.00
D, L – Valine	65.00
Vitamins	
Alpha tocopherol phosphate	1.00
Ascorbic acid	50.00
Biotin	0.20
Calcium pantothenate	0.20
Choline chloride	50.00
Folic acid	0.20
Inositol	0.20
Nicotinamide	20.00
Para aminobenzoic acid	2.00
Pyridoxal phosphate	0.20

Riboflavin	0.20
Thiamine hydrochloride	4.00
Vitamin B_{12}	0.04
Supplements	
L – Glutamine	200
Streptomycin sulphate	0.1
Amphotericin B	2.5 μg/ml
Benzyl Pennicillin	100 units/ml
Other components	
Calcium lactate	555.00
Glucose	10 000.00
Phenol red	20.00
Sodium acetate	50.00

6.2.2.2. Organ culture method

The pineal glands were placed individually into sterile 75 x 10mm Kimble tubes containing 52 μl of BGJ culture medium. 8 μl of (^{14}C) serotonin (specific activity 57.0 mCi/ml) was added to each tube. The culture vessels were placed in sterile tubes and before being sealed were gassed with carbogen. The tubes were then placed in the dark in a Forma scientific model 3028 incubator at 37°C for 24 hours.

After the incubation period of 24 hours the reaction was stopped by the removal of the pineal glands from the culture vessels. A 20 μl aliquot of the culture medium from each of the ten tubes was spotted on different TLC plates cut 10 x 10 cm. Following this 10 μl of standards of all the pineal

After the incubation period of 24 hours the reaction was stopped by the removal of the pineal glands from the culture vessels. A 20 μl aliquot of the culture medium from each of the ten tubes was spotted on different TLC plates cut 10 x 10 cm. Following this 10 μl of standards of all the pineal serotonin metabolites were spotted on top of the culture medium spot. All spotting was carried out under low intensity light to prevent photo-oxidation of the indoleamines.

The solution containing the standards was made up as follows:

1 mg of each of the 8 standards used were pooled and dissolved in a test tube containing 2.5ml of 95 % ethanol. 2.5ml of 1% ascorbic acid in 0.1M HCl was added. A gentle stream of nitrogen was used to dry the spots which were not greater them 0.5cm. The purpose of the nitrogen was to prevent atmospheric oxidation of the metabolites.

The plates were then placed in a TLC tank containing chloroform: methanol: glacial acetic acid (97:7:1) as mobile phase. The plates were developed twice in one direction, and allowed to develop until the solvent front had migrated approximately 9 cm. The plates were allowed to dry and then placed at right angles to the first direction of development, to develop once in ethyl acetate. The plates were allowed to dry and then placed under UV light to visualise the separation of the indoles. A typical trace of a TLC plate is seen in Figure 6.1. The spots were cut and individually placed into scintillation vials to which 1 ml of absolute ethanol was added, vortexed and

allowed to stand for 1 hour. After this time 3 ml of beckmans Ready-Sol scintillation fluid was added and vortexed again for 30 seconds.

Finally all the vials were left for 24 hours before the radioactivity of each metabolite was measured using a Beckman LS 2800 scintillation counter.

6.2.2.3. Statistical analysis

The results obtained were analysed using a One Way Analysis of Variance (ANOVA), followed by a Student Newman-Keuls Multiple Range test. * $P \leq 0.05$, ** $p \leq 0.01$, which indicates that the results obtained are significant.

6.3. RESULTS

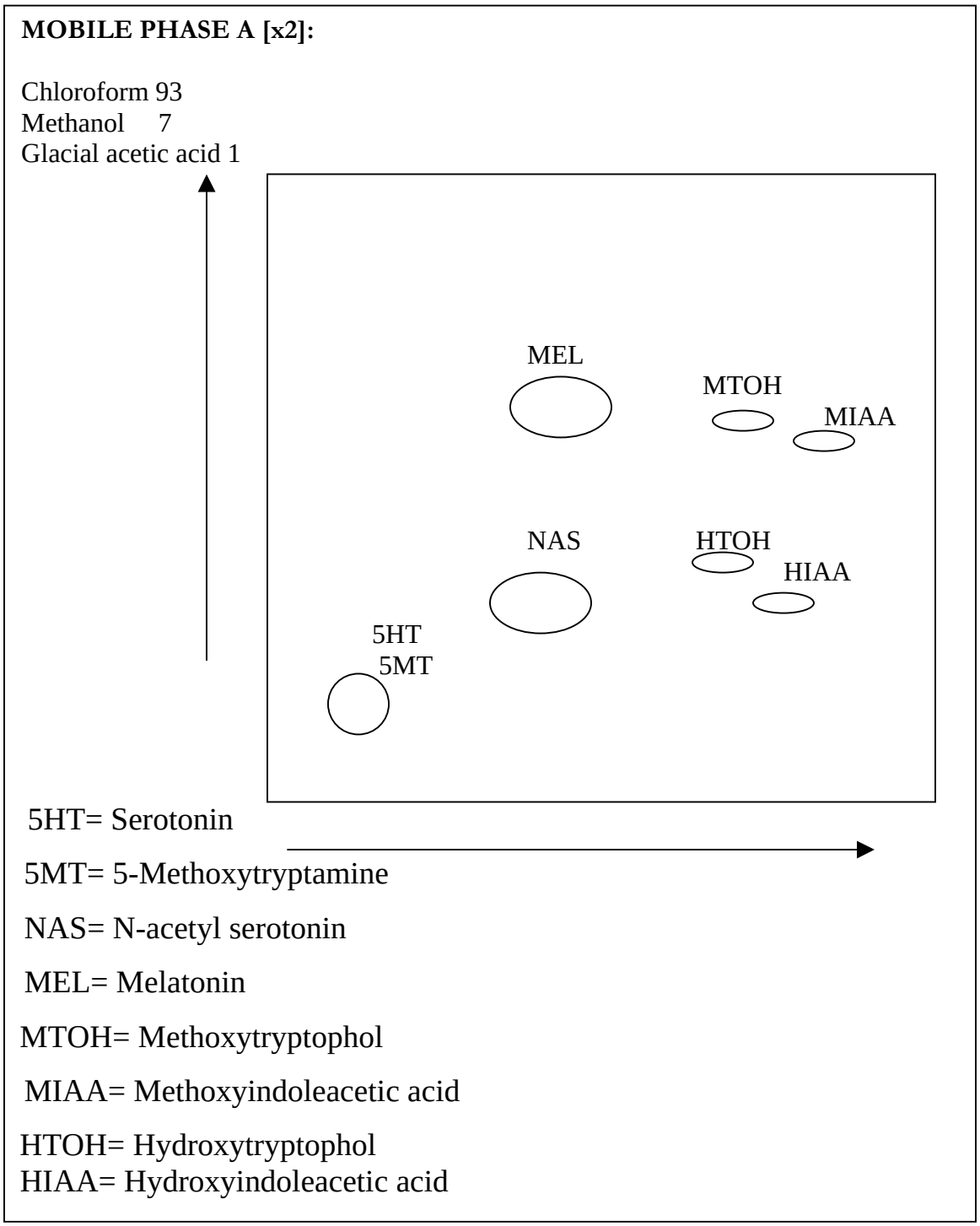


Figure 6.1. Tracing of a typical bi-dimensional thin layer chromatographic separation of pineal indole compounds

Excellent separation of the indoles was achieved and the different metabolites separated can be seen in the typical bi-dimensional thin layer chromatogram from Figure 6.1.

Results are an average of five estimations and expressed as CPM/10 μl/pineal gland (mean ± SD) for each indole. The background counts were negligible.

The rats treated with phenytoin (Table 6.3) showed a statistical difference only for the NAS, MIAA and the MTOH levels. In addition, a non-significant decrease in MEL was observed in the phenytoin treated rats.

Table 6.3. **Concentrations of indoles in CPM/10μl/pineal (n=5)**

Pineal Metabolites	**Control CPM/10 μl/pineal Mean ± SD**	**Phenytoin CPM/10 μl/pineal Mean ± SD**
N-acetyl serotonin (NAS)	1258 ±254	2055±636 **
5-Hydroxyindoleacetic acid (HIAA)	35984±5385	36378±3574
5-Hydroxytryptophol (HTOH)	9980±1697	10284±663
Melatonin (MEL)	705±94	532±160
5-Methoxyindole acetic acid (MIAA)	1840±272	1077±154**
5-Methoxytryptophol (MTOH)	1888±659	1692±658*

6.4. DISCUSSION

The serotonin taken up by the pineal gland further can undergo three different metabolic pathways to synthesise the pineal indoles. This can be seen in Figure 1.10. from section 1.5.4. There are numerous enzymes, co-enzymes and cofactors that take part in these reactions.

Serotonin that is taken up by the pineal is acetylated to N-acetylserotonin by the enzyme serotonin N-acetyltransferase (NAT), with acetyl coenzyme A being the acetyl donor (Weissbach *et al.,* 1960). N-actylserotonin can further be used by the pineal as a precursor of melatonin (Klein *et al.,* 1971). This conversion occurs by O-methylation in the 5-position by another enzyme hydroxyindole-O-methyltransferase (HIOMT). Moreover serotonin can also undergo other reactions such as deamination, oxidation and methoxylation to produce the other metabolites such as HIAA, HTOH, MTOH, and 5MT by the action of specific enzymes.

From this experiment it is observed that the rat pineal gland was able to metabolise (^{14}C) serotonin to the various pineal indoles. From Table 6.3, the 7 days administration of 20 mg/kg phenytoin did not result in significant changes in melatonin synthesis although a lower level of melatonin was obtained in the rats treated with phenytoin.

Phenytoin administration reduced MEL synthesis very insignificantly. This probably occurs because phenytoin may interfere with the rate of uptake of serotonin into the pineal gland. Moreover, in the group treated with phenytoin, the amount of NAS was significantly higher than that of the control. Because NAS is the precursor of MEL and taking into consideration

that the latter is the precursor of melatonin, theoretically more MEL should have been synthesised. However this was not the case. This is probably because phenytoin might be interacting with the enzyme HIOMT, which is responsible for the O-methylation of NAS to form MEL, in some manner that may be reducing its activity.

CHAPTER 7

SUMMARY OF RESULTS AND CONCLUSIONS

7.1. CHAPTER 2: Histochemical investigations of quinolinic acid induced damage in the hippocampal area of rat brain.

Light microscopy was employed to examine the effects of QUIN-induced neuronal damage in the hippocampus of rats after intra-hippocampal injections of 500 nmoles QUIN using stereotaxic techniques. The Nissl body of the neurons of the hippocampus were stained purple using cresyl violet. The micrographs of QUIN injected rats indicate increased neuronal damage in the CA1 and CA3 regions of the rat brain. The cells in these two above-mentioned regions appeared rounded and swollen and necrosis of tissue could also be noted. In contrast the control slides had cells that were well structured and maintained their characteristic pyramidal shapes and integrity with no signs of necrosis. At a systemic dose of 20 mg/kg of phenytoin administered intra-peritoneally, the damage caused by QUIN was reduced as the cells seem to closely resemble the ones of the control where no toxin was present. This shows that the sodium channel blocker, phenytoin confers some degree of protection in the hippocampus under the insult of QUIN *in vivo*. QUIN, an excitotoxin, acts on the NMDA receptor where it causes overstimulation of the receptor culminating in a cytotoxic effect that consequently leads to neuronal cell death. The protection offered by phenytoin is believed to occur because of the sodium ion channel blocking property of this drug, resulting in decreased excitability of the neurons, thereby preventing the events leading to cell death.

7.2. CHAPTER 3: Effects of phenytoin on superoxide generation in whole rat brain homogenate.

This study examined the effect of phenytoin on superoxide free radical generation in whole rat brain homogenate by using the NBT assay. KCN was used to generate superoxide radicals by incubating the neurotoxin with brain homogenate in the presence

and absence of phenytoin at concentrations ranging from 0 to 1.25 mM. The experiment showed that increasing concentrations of KCN (0, 0.25, 0.5, 1mM) increased superoxide generation significantly. This increase was not reduced by phenytoin at any of the concentrations used. This indicates that phenytoin was neither reducing the rate of production nor scavenging the superoxide anions generated by the neurotoxin KCN and thus it can be concluded that phenytoin will not be effective in preventing superoxide generation after episodes that lead to their generation in the CNS.

7.3. CHAPTER 4: Effects of phenytoin on quinolinic acid and iron (II) induced lipid peroxidation in whole rat brain homogenate.

This study was conducted to examine the effect of phenytoin on MDA levels after inducing lipid peroxidation with two known neurotoxins, namely QUIN and iron (II) in whole rat brain homogenate. Both neurotoxins induced damage, as a significant increase in lipid peroxidation was noted. When the brain homogenate was incubated with QUIN as the neurotoxin, phenytoin at any of the employed concentrations (0 to 1.25 mM) did not result in a decline in MDA production. QUIN is known to induce lipid peroxidation mostly via a free radical mechanism in whole rat brain homogenate, whilst phenytoin did not have any significant effect. Hence it can be said that the drug does not interact with free radicals to reduce their toxicities but somehow could enhance lipid peroxidation in a manner that could involve the formation of a reactive binary complex between phenytoin and the existing free radicals. However under the insult of iron (II), the increase in MDA levels after lipid peroxidation were significantly lowered by the drug in a dose related manner. Iron (II) functions as a lipid peroxidant by forming hydroxyl radicals via the Fenton reaction. Because phenytoin is not a good free radical quencher as found from chapter 3, it can be speculated that possible interaction between the drug and iron might be occurring and thereby preventing the generation of the potent damage causing hydroxyl radicals.

7.4. CHAPTER 5: Iron binding studies

Phenytoin's ability to bind/chelate iron (II) and iron (III) ions was examined using ultraviolet (UV), electrochemical analysis and infrared (IR) spectroscopy. The UV results indicate a small shift of the phenytoin major peaks from 200.8 nm to 211.0 nm and 210.9 nm with iron (II) and iron (III) respectively. Furthermore the colour changes observed while adding phenytoin to the iron (II) solution indicates that iron (II) is getting oxidised to iron (III) before the latter binds to phenytoin. Essentially, this experiment suggests that there exists an interaction between iron ions and phenytoin.

In the electrochemical study, the ASV of phenytoin and iron (II) from Figure 5.2. indicates a weak metal-ligand interaction between Fe^{2+} and phenytoin. This was because there was a shift to less negative potentials, indicating that the metal-ligand species is easier to reduce than the metal alone. When the experiment was repeated using iron (III) and phenytoin, the decrease in current response as shown in Figure 5.3 may indicate that the Fe^{3+} is bound in a form, which is not easily reduced by the ligand. However, the lack of a potential shift does not support this theory and therefore the interaction of Fe^{3+} and phenytoin in this study is inconclusive.

The infrared studies indicated splitting and shifting of peaks in the region representing the NH functional group. This suggests that coordination might be taking place at the NH functional group or that there has been a change in the chemical environment of the functional group on the phenytoin molecule after interacting with the metal ions. Moreover the graphs obtained with the Fe (II)–phenytoin and Fe (III)- phenytoin were not different and could be almost superimposed indicating that the product formed were similar. This suggests that iron (II) is being maintained in its oxidised form, that is its iron (III) form that is less toxic and that might have been the reason why there was a significant dose related reduction when iron (II) was used as a lipid peroxidant.

7.5. CHAPTER 6: Phenytoin and its interaction with the pineal gland.

This study was performed in order to assess if phenytoin can cause the pineal gland to augment the synthesis of melatonin, a powerful endogenous antioxidant. This was done using a combination of techniques consisting of organ culture, thin layer chromatography and scintillometry. It was found that phenytoin does not increase melatonin production but in fact shows a non-significant reduction in the levels of the pineal hormone even though the level of its precursor, NAS, was found to be higher in the phenytoin treated rats. This could be due to the fact that phenytoin might be interacting with the enzyme HIOMT, which is responsible for the O-methylation of NAS to form MEL, in some manner that may be reducing its activity.

7.6. FINAL CONCLUSION

In this thesis, some of the possible neuroprotective properties of phenytoin were investigated. The histological study of the CA1 and CA3 regions of the hippocampus under the insult of QUIN, with or without pre-treatment with phenytoin revealed that this agent confers protection in these regions. Because QUIN is believed to mediate neurotoxicity via a free radical mechanism, the effects of phenytoin on free radicals were tested. These studies were done using the NBT assay which assays for superoxide anions and lipid peroxidation assay which measures the amount of MDA produced. Phenytoin does not reduce KCN-induced superoxide generation and QUIN-induced lipid peroxidation, but however there was a dose-dependent decrease of lipid peroxidation when induced with iron (II) as neurotoxin because of a probable interaction between phenytoin and iron (II). In order to shed more light on this possible interaction, metal binding studies with iron (II) and iron (III) were performed using UV, IR and electrochemical analysis. These studies point out that this interaction between phenytoin and iron may be occurring through the Nitrogen atom on the phenytoin molecule and it is possible that this perceived interaction is related to the observed reduction in lipid

peroxidation in the presence of iron (II). The effect of phenytoin on levels of endogenous antioxidant, melatonin, was also established. Phenytoin does not increase melatonin production, but slightly and non-significantly reduces the levels of this pineal hormone.

7.7. FUTURE RECOMMENDATIONS

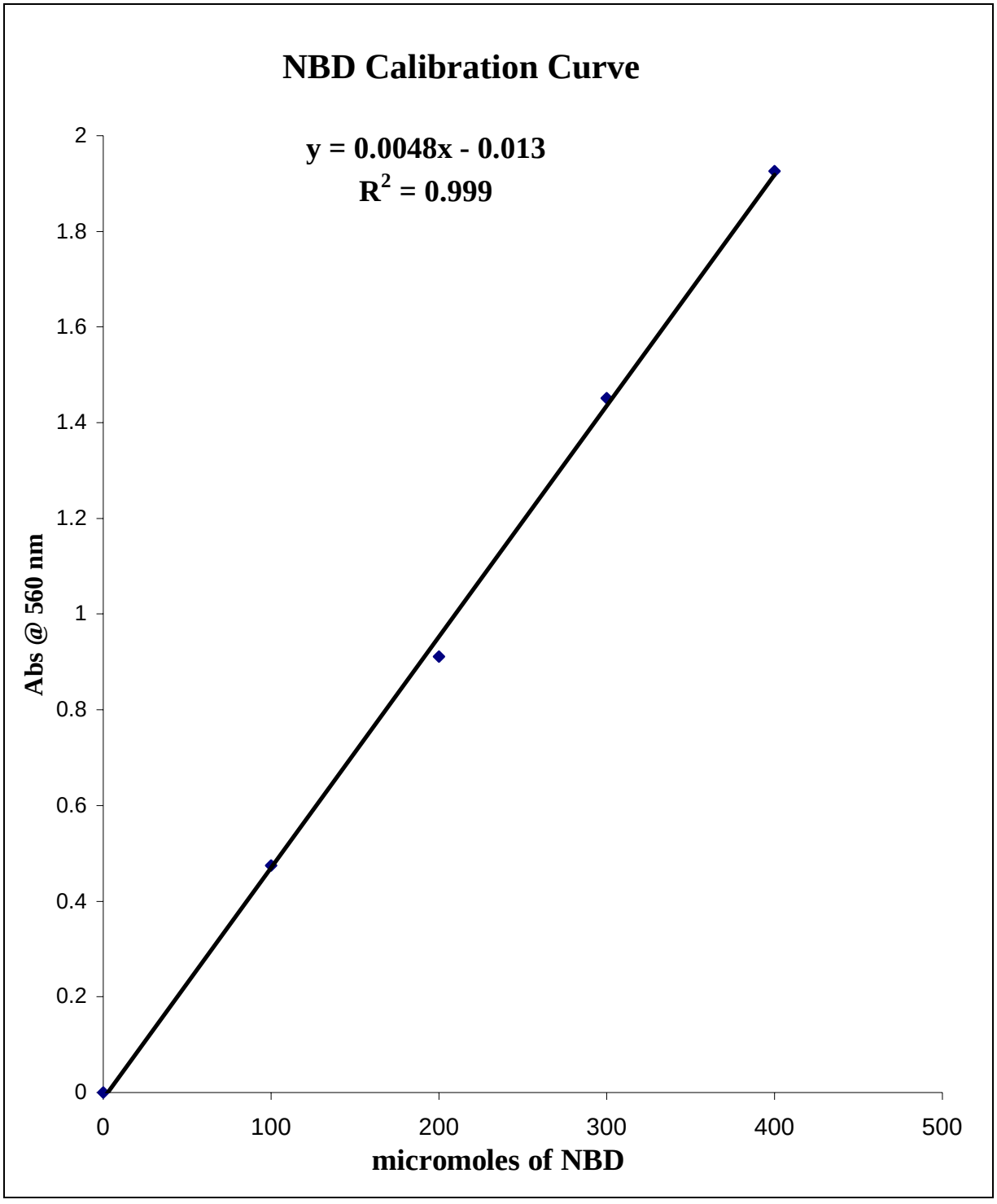
The effect of phenytoin on live cultured neurons should be performed in order to see whether it could reduce neuronal damage by lipid peroxidation with quinolinic acid. Moreover the effect of phenytoin on the different enzymes in the kynurenine pathway, which are responsible for the synthesis of QUIN, should be performed to see whether this drug could reduce the level of this neurotoxin.

Further studies using techniques such as nuclear magnetic resonance, computer modelling studies and elemental analysis can be performed to determine the number of phenytoin molecule binding to iron and confirm the exact site where coordination is occurring so that more can be learnt about this interaction which is essential as iron has been reported to contribute towards numerous neurodegenerative diseases.

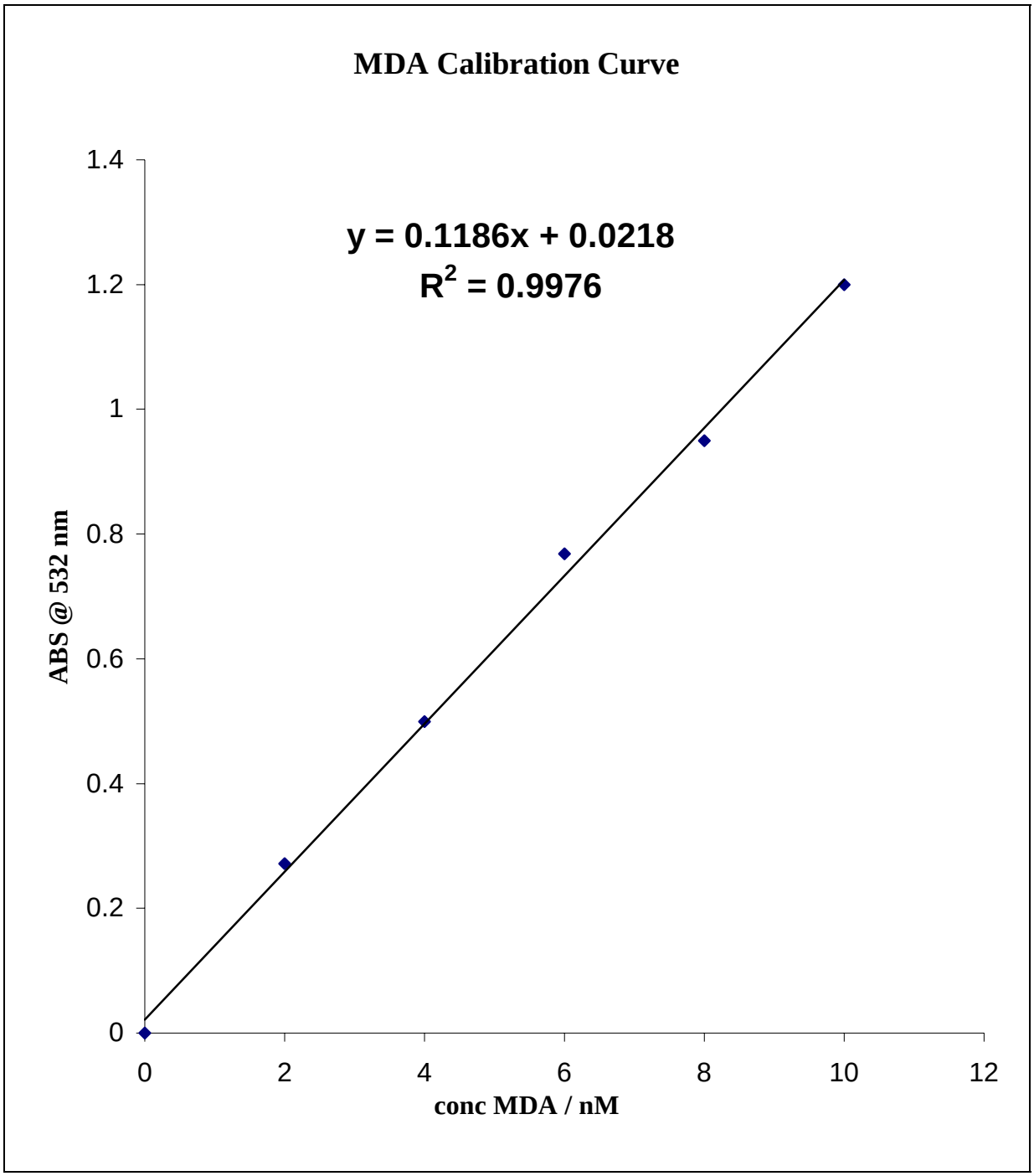
Considering the fact that phenytoin has been a popular drug, in the treatment of epilepsy, for decades, with moderate success and given the knowledge that high levels of free radicals are generated during such an attack, it is important that the effects of co administering with an antioxidant on neurodegenerative processes be examined.

APPENDICES

APPENDIX 1



APPENDIX 2


MDA Calibration Curve
y = 0.1186x + 0.0218
R2 = 0.9976
ABS @ 532 nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
conc MDA / nM

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