AN INVESTIGATION INTO THE NEUROPROTECTIVE PROPERTIES OF CURCUMIN

Thesis

submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE (PHARMACY)

Of

RHODES UNIVERSITY

By

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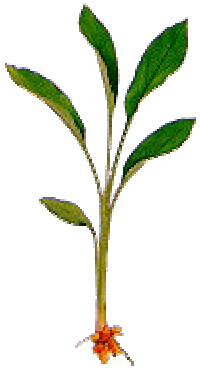
December 2002

Dedicated to my parents, Mary and George Daniel, for their unwavering belief in my capabilities, and for the sacrifices that they made to help me realise them.



'HISTORY' OF MEDICINE

'I have an earache:

2000 B.C.	*- Here eat this root.*
1000 A.D.	*- That root is heathen. Here, say this prayer.*
1850 A.D.	*- That prayer is superstition. Here, drink this potion.*
1940 A.D.	*- That potion is snake oil. Here, swallow this pill.*
1985 A.D.	*- That pill is ineffective. Here, take this antibiotic.*
2000 A.D.	*- That antibiotic is artificial. Here, eat this root."*

- Author Unknown

ABSTRACT

An increasing number of studies show that nutritional antioxidants such as vitamin E and polyphenols are capable of blocking neuronal death *in vitro* and may have therapeutic properties in animal models of neurodegenerative diseases including Alzheimer's and Parkinson's diseases. In the present study, the neuroprotective ability of one such polyphenolic antioxidant, curcumin, was investigated. Curcumin is the yellow curry spice derived from turmeric, and is widely used as a dietary component and herbal medicine in India.

Most neurological disorders are postulated to have an oxidative or excitototoxic basis. Thus the effects of curcumin on oxidative stress in the rat brain were investigated. Curcumin, administered to the rat *in vivo* and *in vitro*, was able to exert protective effects on oxidative damage in the brain, induced by cyanide, a mitochondrial inhibitor. Curcumin also offered protection against quinolinic acid induced lipid peroxidation, and this protection was extended to lipid peroxidation induced by metals such as lead and cadmium in the rat brain. Experiments conducted on the pineal gland revealed an increased production of the neuroprotective hormone melatonin in presence of curcumin *in vivo*.

The hippocampus is functionally related to vital behaviour and intellectual activities and is known to be a primary target for neuronal degeneration in the brains of patients with Alzheimer's disease. Histological studies were undertaken to assess the effects of

curcumin on lead induced toxicity on the rat hippocampus, the results of which show that curcumin affords significant protection to the hippocampus of the lead treated rats.

This study also sought to elucidate possible mechanisms by which curcumin exerts its neuroprotective capabilities. Curcumin was found to inhibit the action of cyanide on the mitochondrial electron transport chain, one of the most common sources of free radicals. Electrochemical, UV/VIS and Infrared spectroscopy were used to characterise interactions between curcumin and the metals lead, cadmium, iron (II) and iron (III). Curcumin was shown to directly chelate these metals with the formation and isolation of two new curcumin complexes with lead, and one complex each with cadmium and iron (III). These results suggest chelation of toxic metals as a mechanism of neuroprotection afforded by curcumin.

The need for neuroprotective agents is urgent considering the rapid rise in the elderly population and the proportionate increase in neurological disorders. The findings of this study indicate that curcumin, a well-established dietary antioxidant, is capable of playing a bigger role in neuroprotection, which needs to be further explored and exploited.

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LIST OF ABBREVIATIONS

µA	Microamperes
µg	Microgram
µl	Microlitre
µM	Micromolar
^{0}C	Degree Celsius
5-HIAA	5-Hydroxyindole acetic acid
5-HT	Serotonin
A	Amperes
AD	Alzheimer's disease
AFMK	N^{1}-acetyl-N^{2}-formyl-5-methoxykynuramine
Ag	Silver
AgCl	Silver chloride
aHT	N-acetylserotonin
AIF	Apoptosis inducing factor
aMT	Melatonin
ANOVA	One way analysis of variance
APP	Amyloid precursor protein
ASV	Adsorptive stripping voltammetry
ATP	Adenosine triphosphate
Aβ	β-amyloid
BBB	Blood brain barrier
BHT	Butylated hydroxytoluene
CA1	Cornu Ammonis *1*
CA2	Cornu Ammonis *2*
CAT	Catalase
Ca^{2+}	Calcium (II)
CA3	Cornu Ammonis 3
CA4	Cornu Ammonis 4
Camp	Cyclic adenosine mono phosphate
Cd^{2+}	Cadmium (II)
cm^{-1}	Per centimetre
CNS	Central nervous system
COX	Cyclooxygenase
CPM	Counts per minute
CSF	Cerebrospinal fluid
CuZnSOD	Copper-Zinc – catalysed Superoxide dismutase
CV	Cyclic voltammetry
DMBA	7,12-dimethylbenz(a)anthracene
DNA	Deoxyribonucleic acid
DPI	2,6-Diclorophenol-indophenol
e^{-}	Electron
EAA	Excitatory amino acid

EDTA	Ethylenediaminetetraacetic acid
ETC	Electron transport chain
Fe^{2+}	Iron (II)
Fe^{3+}	Iron (III)
g	Grams
GABA	Gamma-aminobutyric acid
GCE	Glassy carbon electrode
GSH	Reduced form of glutathione
H	Hydrogen
H_2O_2	Hydrogen peroxide
HA	5-Hydroxyindole acetic acid
HIOMT	Hydroxyindole – O – methyl transferase
HL	5-Hydroxytryptophol
$HO^{\bullet}_2$	Hydroperoxyl radical
i.p.	Intraperitoneal
i_{pa}	Anodic peak current
i_{pc}	Cathodic peak current
IR	Infrared
KCl	Potassium chloride
KCN	Potassium cyanide
K_f	Rate constant for forward reaction
K_r	Rate constant for reverse reaction
LMA	Limbic midbrain area
$LOO^{\bullet}$	Lipid peroxyl radical
LOOH	Lipid hydroperoxide
LOX	Lipoxygenase
LTP	Long term potentiation
M	Molar
MA	5-Methoxyindole acetic acid
MAO	Monoamine oxidase
MDA	Malondialdehyde
mg	Milligram
mg/kg	Milligram per kilogram
ML	5-Methoxytryptophol
ml	millilitre
mM	Millimole
MnSOD	Manganese – catalysed Superoxide dismutase
MPP^+	1-Methyl-4-phenyl-pyridinium ion
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
mV	Millivolts
n	Sample size
NA	Noradrenaline
Na^+	Sodium
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide

NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NAT	N-Acetyltransferase
NBD	Nitroblue diformazan
NBT	Nitroblue tetrazolium
NE	Norepinephrine
nm	Nanometre
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAID	Nonsteroidal anti-inflammatory drugs
O_2	Oxygen
$O_2^{-}\cdot$	Superoxide radical
$OH^{\cdot}$	Hydroxyl radical
ONOO-	Peroxynitrate
PARS	Poly-ADP ribose polymerase
Pb^{2+}	Lead (II)
PBS	Phosphate buffered saline
PD	Parkinson's disease
PhK	phosphorylase-kinase
PTP	Permeability transition pore
PUFA	Polyunsaturated fatty acid
QA	Quinolinic acid
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
$ROO^{\cdot}$	Peroxyl Radical
ROOH	Hydroperoxyl Radical
ROS	Reactive oxygen species
rpm	Revolutions per minute
s	Seconds
SD	Standard deviation
SEM	Standard error of mean
SOD	Superoxide dismutase
SP	Senile plaques
T	Transmission
TBA	2-Thiobarbituric acid
TBA-MDA	Thiobarbituric acid – Malondialdehyde
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
TPA	tetradecanoylphorbol-13-acetate
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
UV/VIS	Ultraviolet/Visible
V	Volts
v/v	Volume by volume

ACKNOWLEDGEMENTS

I would like to thank the following people for their contributions toward this thesis:

My supervisor, Professor Santy Daya for giving me the opportunity to be part of his research group. I am grateful for his guidance and supervision throughout the course.

Dr. Janice Limson, for the interest and enthusiasm she took in my work, which led to novel ideas and fruitful research. Furthermore I would also like to thank her for her help with the electrochemical part of this study and for her skillful editing of this thesis.

Dr. Bharathi Avula for inspiration, friendship, support and good advice.

Dr. Gary Watkins for his interest in my work and for lending his invaluable time and expertise in inorganic chemistry to this study.

The staff of the department of pharmacy and my lab mates for the warmth and cooperation extended towards me. Sally and Dave Morley for their competence in providing a well-organised work environment, as well as for their support and friendship. I also thank Leon Purdon for his kindness and willingness in assisting me whenever needed.

My friends, Jasmine, Anitha and Rubina for companionship and encouragement; Ami for his kind and enthusiastic help with many of my experiments, for proof-reading this thesis, and for his infectious cheer and encouragement; Nishal, for being “so sweet” and caring; Linda Emslie and Jeanette Coupe for always being ready to lend a listening ear or a helping hand; Linda Heideman for being the perfect “neuromum”; and all my dear friends at Lilian Britten House for understanding, encouraging and supporting me.

My parents for their unconditional love and support, and my sister, Shija, who has been a source of strength and friendship to me always.

The National Research Foundation for financial assistance towards this study.

Above all, I give thanks to God for opening doors for me, for His provision and care and for making all things possible.

CHAPTER ONE

LITERATURE REVIEW

1.1 AN INTRODUCTION TO NEUROSCIENCE

Two hundred years ago the brain was thought by our forefathers to consist of a concoction of spirits, which might be pure and tranquil, or even riotous and evil! The discovery that the central nervous system consists of an immense universe of highly complex units has been a slow and gradual one. In recent decades the brain and its mechanisms have become the focal point of much interest and research, and indeed many important and life changing discoveries and contributions have been made in the field of neuroscience.

The continued and increased growth of older population age groups in many countries, irrespective of whether they fall under the “developed” or “developing” status, is possibly one of the main driving forces fuelling neuroscience and the study of neurodegenerative diseases particularly Alzheimer’s disease, as well as age-related dementias in general. This is also a reflection of the growing appreciation of the enormous consequences of such disorders in terms of public health costs and shattered lives of the afflicted individuals and their families. The result is a virtual explosion in the understanding of how the brain functions, both at a cellular as well as molecular level.

1.2 THE BRAIN

1.2.1 NEUROANATOMY

The most common method of dividing the brain is into hindbrain, midbrain and forebrain as shown in fig. 1.1.

The hindbrain has three parts with functions in homeostasis, movement coordination, and signal conduction. These include the medulla oblongata, cerebellum and the pons.

The medulla oblongata and the pons are the lowest parts of the brain and appear as swellings at the top of the spinal cord (Campbell, 1990).

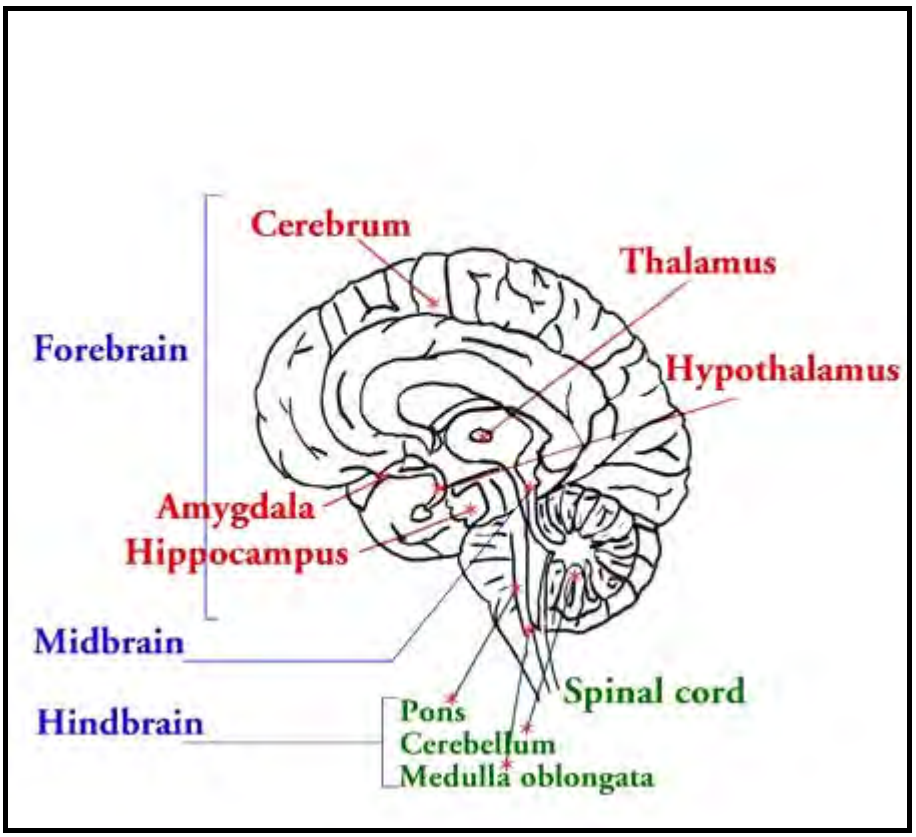


FIG. 1.1 *The main features of the brain depicted within its three main structural classifications (*http://www.educarer.com/brain.htm).

The medulla contains centres that perform various homeostatic and autonomic functions such as breathing, heart and blood vessel activity, swallowing and digestion. The pons also takes part in some of these activities such as regulating the breathing centres in the medulla. All the sensory and motor neurons which pass to and from higher brain regions pass through the hindbrain, making conduction of information one of the most important functions of the medulla and pons.

The cerebellum is attached to the brain stem by peduncles. Its primary function is the coordination of movement. This highly convoluted outgrowth on the dorsal surface of the hindbrain is tucked behind and partially beneath the cerebrum (Campbell, 1990). The

midbrain attaches the forebrain to the brain stem and it contains centres for the receipt and integration of several types of sensory information. The most prominent areas of the midbrain are two elevations known as the inferior and superior colliculi, which form part of the visual and auditory systems (Nauta & Feirtag, 1979).

The largest enlargement of the neuroaxis is the forebrain, which is attached to the brainstem by the midbrain and is the site where the most sophisticated neural processing in the brain occurs (Seminars in Neurosciences, 1993). It is divided into the lower diencephalon, which consists of two integrating areas, the thalamus and the hypothalamus, and the upper telencephalon, which comprises the cerebrum.

Most of the neural input to the cerebral cortex is relayed by neurons with cell bodies in the thalamus. It contains different nuclei that project information to specific anatomical areas of the cerebrum. The hypothalamus is one of the most important sites for regulation of homeostasis and is the source of the posterior pituitary hormones and the releasing factors for the anterior pituitary. It also regulates hunger, thirst and sexual response functions (Campbell, 1990).

The surface of the cerebral hemispheres, the largest and most complex part of the brain, is deeply folded into hemispheres, lobes and gyri. Such folds allow for a large area of cerebral cortex to be accommodated within the cranium. The longitudinal or saggital fissure divides the cerebrum into left and right hemispheres, which extend as far as the corpus callosum, white matter linking the two hemispheres of the cerebrum together (Seminars in Neurosciences, 1993). The cerebral hemisphere incorporates the hippocampus, the neocortex and the olfactory fields. Within the hemispheres are the amygdala and corpus striatum. The corpus striatum comprises the globus pallidus and the striatum, which includes the caudate nucleus and putamen (Nauta & Feirtag, 1979).

1.2.2 THE LIMBIC SYSTEM

The limbic system comprises the olfactory areas, hippocampus, amygdala, the septum, cingulate cortex, and regulates the hypothalamic area. Fig. 1.2 shows a general picture of

the limbic system. The significance of the limbic system lies in the role it plays in emotion, motivation and memory (Butler, 1993).

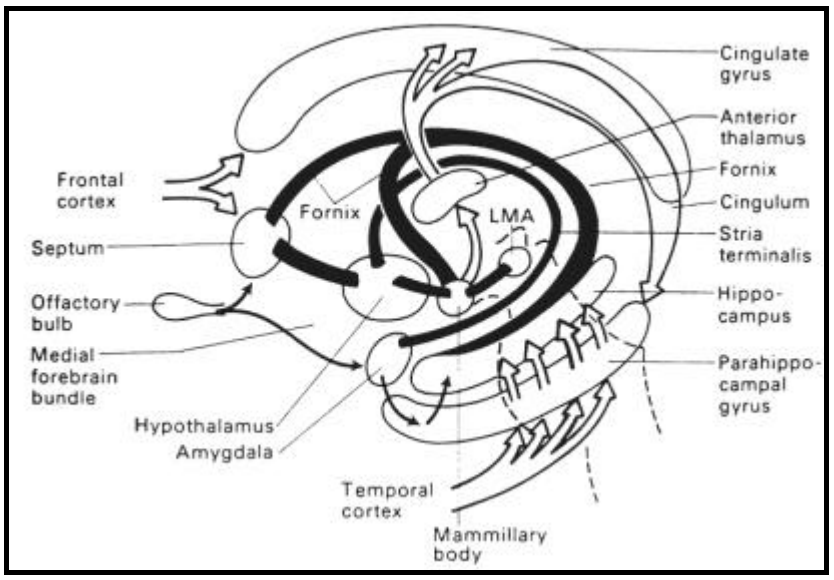


FIG. 1.2 *Diagram of the afferent and efferent connections of the limbic system. LMA: Limbic midbrain area (Banniser, 1992).*

1.2.3 THE HIPPOCAMPUS

The hippocampus is the most medial portion of the cerebral cortex and is developed from the stalk of the original cerebral vesicle (Smythies, 1970). Named for the seahorse shape, the hippocampus is the main relay station that determines whether a new memory should go into long-term storage or be deleted after its short-term usefulness is over.

The hippocampal formation is a bi-lateral limbic structure, which resembles two “C’s” that lean together at the top and spread apart at the base. The top portion is known as the “dorsal hippocampus”, and because of its proximity to the septum, the dorsal tip of the hippocampus is called the “septal pole” and the bottom tip is called the “temporal pole” as shown in Fig. 1.3 (Amaral & Witter,1989).

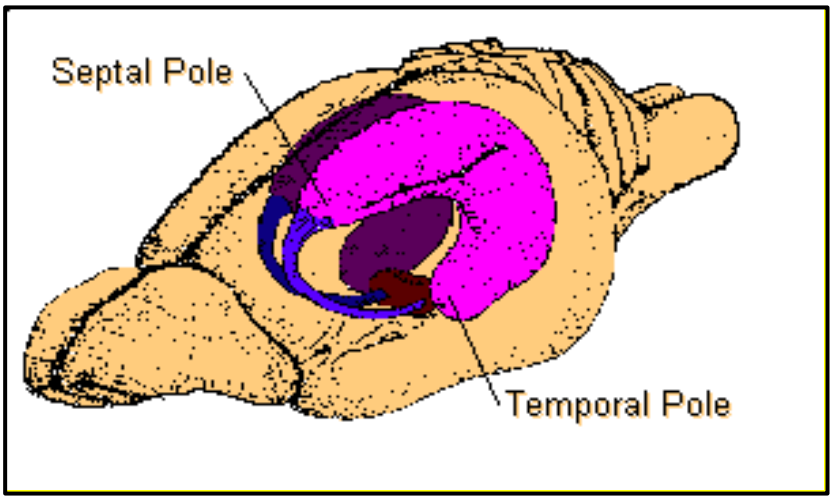


FIG. 1.3 *A depiction of a rat brain with hippocampus, fornix and mammillary bodies highlighted in mauve tones, and showing the septal and temporal poles of the hippocampus (Amaral & Witter, 1989).*

The internal structure of the hippocampus is the same throughout its length, and consists of an infolded convolution of the evolutionarily older and simpler archicortex or allocortex. A cross-section taken perpendicular to the long axis (septal-temporal) will reveal the internal structure as two interlocking "Cs", one reversed in relation to the other, each with its own principle cell layer. One "C" makes up Ammon's Horn or *Cornu Ammonis* (CA1-CA3), also known as the "Hippocampus proper".

The principle cell layer of Ammon's Horn is the *stratum pyramidale*, or the pyramidal cell layer. The other "C" is made up of the dentate gyrus, of which the *stratum granulosm*, or granule cell layer is the principle cell layer. Although the dentate gyrus is commonly included as part of the hippocampus, it is cytoarchitectonically distinct from the hippocampus proper (Amaral, 1978; Bayer, 1985; Amaral & Witter, 1989).

However, the hilus of the dentate gyrus, the area inside the C created by the granule cells is sometimes referred to as CA4. The hilar region is referred to as CA4 because the pyramidal cell layer of the CA1-CA3 regions begins to break down as a tightly packed

cell layer and becomes more spread out and sparse in this region, such that early neuroanatomists did not distinguish between these areas.

The intrinsic connections between the principle cell layers of the dentate gyrus and CA regions of the hippocampus are very clear. The hippocampus, when cut transverse to its longitudinal (septal-temporal) axis, exhibits a strong afferent set of three connected pathways known as the 'trisynaptic" circuit or loop (Swanson, 1978). The second and third layers, which are referred to as the "surface layers" of the entorhinal cortex, project to the granule cells of the dentate gyrus through the perforant-path. The granule cells of the dentate gyrus then project to the large pyramidal cells of CA3 region via the mossy fibers system. Finally, the CA3 pyramidal cells project to the pyramidal cells of the CA1 subfield, via the Schaffer collateral system (Lorente de No, 1934; Blackstad, 1956; Amaral, 1978).

The hippocampus is a primary target for neuronal degeneration in the brains of patients with Alzheimer's disease (Behl *et al.*, 1997).

1.3 MECHANISMS OF NEURODEGENERATION

1.3.1 FREE RADICALS AND OXIDATIVE STRESS

By definition, a free radical is a molecule or atom that possesses an unpaired electron in its outer orbit. They are generally unstable and very reactive. Free radicals are known to play critical roles in many biochemical reactions that maintain normal cell functions.

Oxygen radicals are known to exert critical actions such as signal transduction, gene transcription and regulation of soluble guanylate cyclase activity in cells (Zheng & Storz, 2000). However, as oxidants and inhibitors of enzymes containing an iron-sulfur center, free radicals and other reactive species cause the oxidation of biomolecules, leading to cell injury and death (McCord, 2000). Thus, free radicals demonstrate two "faces" in biology, in that they serve as signalling and regulatory molecules at physiologic levels

and highly deleterious and cytotoxic oxidants at pathologic levels (Freidovich, 1999). Any free radical involving oxygen can be referred to as reactive oxygen species (ROS).

1.3.1.1 Superoxide

When molecular oxygen accepts an electron from a reducing agent, the primary product generated is the superoxide anion (O_2^-). The superoxide anion and its conjugate acid, the hydroperoxyl radical ($HO^{\bullet}_2$) has been shown to be reactive to a number of compounds *in vitro*. Superoxide oxidises alpha-tocopherol to 8-alpha-tocopherone, which spontaneously forms alpha-tocopherol quinone (Nishikimi *et al.*, 1980). The reduction of cytochrome c by xanthine and xanthine oxidase is mediated by the superoxide anion (McCord & Fridovich, 1968) and this reduction is used as the basis of assays for both O_2^- and superoxide dismutase (SOD). The SOD enzymes are catalysts that have evolved a surface charge arrangement to facilitate the specific use of O_2^- as a substrate (Benovic *et al.*, 1983).

The superoxide free radical has come to occupy an amazingly central role in a wide variety of diseases. The toxicity of superoxide is seen in its ability to inhibit certain enzymes and thereby attenuate vital metabolic pathways, as well as in its effects on other major classes of biological molecules (McCord, 2000). *E. coli* deficient in SOD activity show increased rates of mutagenesis (Touati & Farr, 1990), illustrating the role of the radical, directly or indirectly, in DNA alterations. In conditions of ischaemia and reperfusion, the most acute problem resulting from the overproduction of superoxide appears to be greatly increased rates of lipid peroxidation. Here the superoxide radical plays paradoxical roles, in that it can both initiate and terminate lipid peroxidation chains (Nelson *et al.*, 1994).

1.3.1.2 Nitric Oxide

Nitric oxide (NO) is a free radical released by several cell types, especially vascular endothelial cells and phagocytes (Moncada *et al.*, 1991). Within the nervous system, NO is responsible for relaxation of the pyloric sphincter in the gastrointestinal tract (Yun *et*

al., 1996), mediation of penile erections (Melis *et al.*, 1994) and vasodilation (Holscher, 1997). Nitric oxide has been suggested to be involved in both the normal functioning of excitatory amino acids such as glutamate and in the damaging effects produced by their generation in excess (Dawson *et al.*, 1991; Forstermann *et al.*, 1991).Vascular NO is very unstable in oxygenated media with a half-life of about 6 seconds.The short lifetime of NO is generally attributed to the continuous presence of superoxide anions (Van Der Vleit & Bast, 1992).

1.3.1.3 Peroxynitrate

The interaction of NO with superoxide radical leads to the formation of peroxynitrite ($ONOO^-$), a reaction that occurs at a threefold faster rate than that of the dismutation of superoxide by SOD (Beckman *et al.*, 1993).

$$O_2^- + NO^{\cdot} \longrightarrow ONOO^- + H^+ \quad (1)$$

Therefore the formation of peroxynitrate depends on the concentrations of superoxide and NO in the cell. Peroxynitrate is a highly reactive oxidising agent that can cause tissue damage by an active intermediate that acts like an $OH^{\cdot}$ radical. At physiological pH, peroxynitrite may be able to diffuse over several cell diameters to produce cell damage by oxidising lipids, proteins and DNA (Beal, 1997).

1.3.1.4 Hydroperoxyl Radical

Protonation of O_2^- yields the hydroperoxyl radical ($HO_2^{\bullet}$). The pK_a of $HO_2^{\bullet}$ is 4.7 – 4.8 (Bielski & Allen, 1977)) so at physiological pH of around 7.4, not much $HO_2^{\bullet}$ exists. However, in close proximity to the membranes, the pH might be considerably lower than this, and more superoxide will exist as $HO_2^{\bullet}$. Although there is no clear evidence as to the cytotoxic effects of $HO_2^{\bullet}$ in any biological system, its potential importance arises from two factors. Firstly, it is less polar than O_2^- and is thus capable of crossing biological membranes as effectively as hydrogen peroxide (Halliwell & Gutteridge, 1986).

Secondly, $HO_2^{\bullet}$ is somewhat more reactive than O_2^{-}. It is capable of attacking fatty acids directly and has been shown to convert linolenic, linoleic and arachidonic acids to peroxides (Bielski *et al.*, 1983). $HO_2^{\bullet}$ has also been proven to initiate peroxidation of the lipid component of low density lipoproteins (Bedwell *et al.*, 1989).

1.3.1.5 Hydroxyl Radical

Apart from the damage it causes on its own, the superoxide anion secondarily forms further reactive oxidants, by dismutation to hydrogen peroxide which can be converted to the highly damaging hydroxyl radical or be catalysed and excreted harmlessly as water. Hydroxyl radicals are short-lived and can be formed from O_2^{-} and H_2O_2 through the Haber-Weiss reaction or through the interaction of metals such as iron or copper and H_2O_2, through the Fenton reaction (Halliwell & Gutteridge, 1985) as shown in the equations below.

$$O_2^{-} + H_2O_2 \longrightarrow H_2O + OH^{-} + OH^{\bullet}$$ Haber-Weiss Reaction (2)

$$Fe^{3+} + O_2^{-} \longrightarrow Fe^{2+} + O_2$$
$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$ Fenton Reaction (3)

The $OH^{\bullet}$ is capable of reacting with almost all molecules in living cells (Fridovich, 1986) and no enzyme system involving it as a substrate exists (Bird & Iversen, 1974). The hydroxyl radical has been implicated in damage to proteins, carbohydrates, DNA and lipids (Reiter *et al.*, 1995; Dawson & Dawson, 1996; Volterra *et al.*, 1994).

1.3.1.6 Lipid Peroxidation

Although all of the major classes of biomolecules may be attacked by free radicals, lipids are probably the most susceptible. Cell membranes are rich sources of polyunsaturated fatty acids (PUFAs), which are readily attacked by oxidising radicals (Cheeseman & Slater, 1993). Extensive lipid peroxidation in biological membranes results in alterations in fluidity, fall in membrane potential, increased permeability to H^{+} and other ions, and

eventual rupture leading to release of cell and organelle contents such as lysosomal hydrolytic enzymes (Fong *et al.*, 1973).

Although much attention is paid to malondialdehyde, various other toxic end products are also formed as a consequence of lipid peroxidation, such as 4,5-dihydroxydecenal and 4-hydroxynonenal (Benedetti *et al.*, 1984; Esterbauer *et al.*, 1988).

1.3.1.6.1 Mechanism of Lipid peroxidation

Fig.1.4 outlines the basic mechanism involved in lipid peroxidation. Any species which has enough reactivity to abstract a hydrogen atom from a methylene group provides the first step of a peroxidation sequence in a membrane. Abstraction of a hydrogen atom from the methylene group leaves behind an unpaired electron on the carbon to which it was originally attached. The presence of a double bond in the fatty acid weakens the C-H bonds on the carbon adjacent to the double bond, facilitating the removal of H.

The carbon radical stabilises itself by a molecular rearrangement as depicted in Fig.1.4, to form a conjugated diene, which, under aerobic conditions, can combine with oxygen to form a peroxyl radical, $ROO^{\cdot}$ or RO^{2}**.** The oxygen concentrations may alter the pathway of peroxidation since at very low oxygen concentrations the carbon-centered radicals may favour self-reaction or reactions with other membrane components such as proteins.

The peroxyl radicals formed can abstract H from another lipid molecule, possibly from an adjacent fatty acid side chain. This stage of the reaction is called the propagation stage. The carbon radical formed can react with oxygen to form another peroxyl radical and so the chain reaction of lipid peroxidation continues. The length of the propagation chain depends on factors such as the lipid/protein ratio in a membrane, the fatty acid composition, oxygen concentration as well as the presence of chain-breaking antioxidants, which are capable of interrupting the chain reaction by providing an easily donatable hydrogen for abstraction by the peroxyl radicals (Halliwell & Gutteridge, 1990).

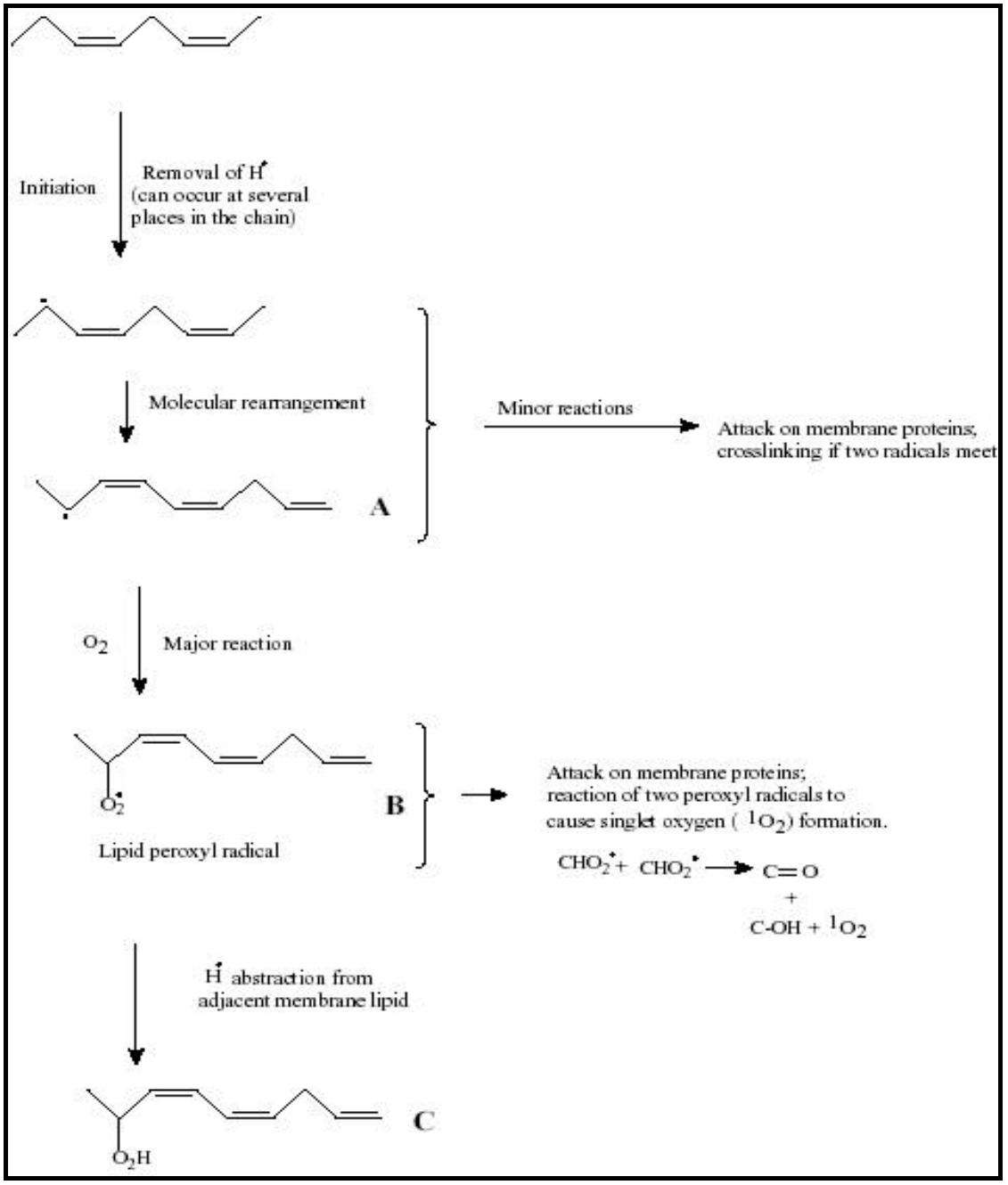


FIG. 1.4 *Mechanism of lipid peroxidation (Gutteridge &Halliwell; 1990).*

1.3.2 EXCITOTOXICITY

Excitotoxicity is the paradoxical property exhibited by excitatory amino acids (EAA) such as glutamate, of causing acute neuronal degeneration by excessive stimulation of postsynaptic EAA ionotropic receptors, i.e. receptors through which glutamate functions as a transmitter (Olney, 1995). Glutamate, the main excitatory neurotransmitter in the

mammalian brain, has important roles in several physiological and pathological events. Glutamatergic neurotransmission is achieved through ionotropic (ligand-gated ion channels) and metabotropic (G protein-coupled) receptors (Ozawa *et al.*, 1998).

Specifically, the NMDA ionotropic glutamate receptor subtype seems to be crucial in plasticity processes associated with normal brain function. However, overstimulation of the glutamatergic system, as observed when glutamate concentration in the synaptic cleft increases, may be neurotoxic (Izquierdo & Medina, 1997; Ozawa *et al.*, 1998).

There are two principle processes by which EAA causes neurotoxicity, based on the differences in time course and ionic dependence. The first process is termed the acute process and it is characterised by a rapid influx of sodium ions into the neuron, which causes a passive entry of chloride ions and water into the cell through osmotic pressure. This results in the rapid swelling of the neurons due to the excessive depolarisation, ion influx and water entry (Tilson & Mundy, 1995).

The second process, delayed toxicity, is one that involves Ca^{2+} influx, which is mediated by the NMDA receptor. The increased concentration of Ca^{2+} within the cells can trigger a number of enzymatic and metabolic processes which are neurotoxic. Among these processes is the activation of proteases such as calpain-1, which is capable of degrading major neuronal structural proteins and inducing cytoskeletal breakdown (Seubert *et al.*, 1988). Ca^{2+} can also bind to calmodulin to form Ca^{2+}-calmodulin complexes, which modulate the activity of a large number of enzymes. This complex can also disrupt the cytoskeleton, cause mitochondrial dysfunction and activate lipases (Scatton, 1994).

Ca^{2+} is also capable of activating the phospholipases, which can break down cell membranes and liberate arachidonic acid. This can lead to the breakdown of Ca^{2+} stores within the cells and thus further amplify the excitotoxic response. Moreover, arachidonic acid liberation leads to synthesis of prostaglandins, the mechanism of the anti-inflammatory response. Inflammatory cells have shown to produce ROS and reactive nitrogen species (RNS) as a mechanism for attacking opsonised targets (Akiyama *et al.*, 2000).

In 1989, Dawson and co-workers discovered a link between nitric oxide and excitatory transmission in the nervous system. These authors also discovered that by increasing the expression of neuronal nitric oxide synthase (NOS) in culture they could increase glutamate neurotoxicity. Thus, neuronally originated nitric oxide was discovered to play a critical role in behavioural function and can act as a trigger in the excitotoxic mechanism (Dawson *et al.*, 1993). As mentioned earlier, excess nitric oxide generation within the neuron can, in the presence of superoxide, lead to the production of the peroxynitrite radical (section 1.3.1.4).

The excitotoxic response is thus intimately related to the free radical generation in that excitotoxins increase free radical production, and free radicals, in turn, increase glutamate release extracellularly. Calcium entry into the neuron has been shown to be a trigger in activating NOS, thus completing the cycle (Blaylock, 1999).

1.3.3 THE ROLE OF THE MITOCHONDRIA IN OXIDATIVE STRESS AND EXCITOTOXICITY

Free radicals are sometimes formed as intermediates in mitochondrial and microsomal electron transport systems (Fantone & Ward, 1985). A deleterious consequence of energy production by the mitochondria is the production of free radicals such as the superoxide anion and hydroperoxides. This is because oxygen acts as the terminal electron acceptor within the electron transport chain (Fantone & Ward, 1985).

Impaired mitochondrial function leads to impaired cellular Ca^{2+} buffering and secondary activation of voltage-dependent NMDA receptors. This can, in turn, lead to further influxes of Ca^{2+}, which are buffered in mitochondria, leading to increased production of the superoxide anion. Moreover, the increase in Ca^{2+} results in an activation of neuronal nitric oxide synthase (NOS). Nitric oxide reacts with O_2^- to generate ONOO- (as discussed in section 1.3.1.3), which results in oxidative damage to DNA, proteins and lipids (Beal, 1997). This is shown in Fig. 1.5 below.

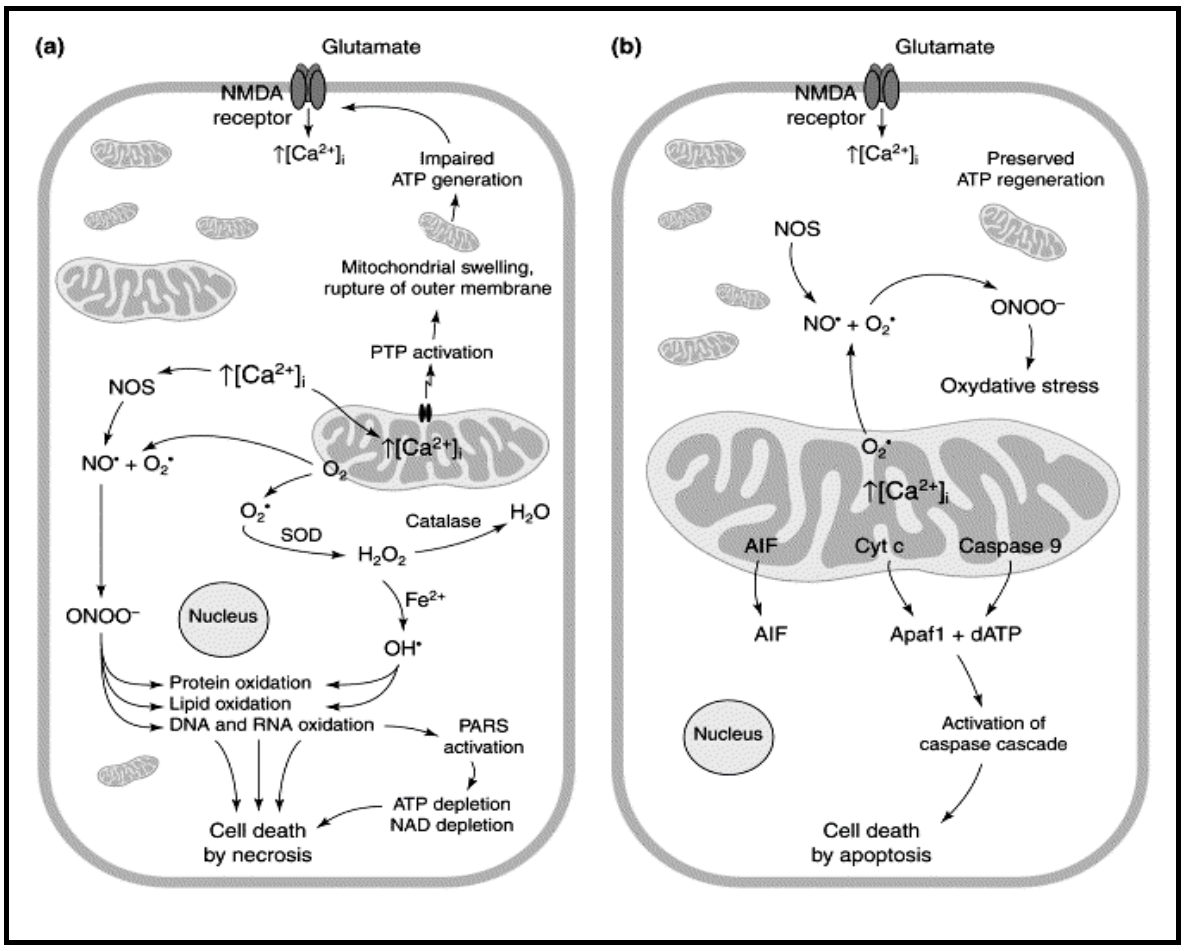


FIG. 1.5 *The mitochondrion: a major source of cellular free radicals. (a) depicts the effects of a severe excitotoxic insult that results in cell death by necrosis, whereas (b) depicts the results of a mild excitotoxic insult that results in apoptosis (Beal, 2000).*

As the diagram above depicts, the production of superoxide radicals can also lead to increased H_2O_2 production, which can react with transition metals like iron (II). This results in damage to cellular macromolecules like DNA, which leads to the activation of poly-ADP ribose polymerase (PARS). Activation of PARS leads to ATP and NAD depletion, which leads to death. Both mitochondrial accumulation of Ca^{2+} and oxidative damage lead to activation of the permeability transition pore (PTP) that is linked to excitotoxic cell death. The release of cytochrome c, caspase 9 and apoptosis inducing factor (AIF) by the mitochondria is also linked to increased mitochondrial Ca^{2+} and free radical production. These mediators lead to the activation of a caspase cascade, and finally apoptotic death (Beal, 2000).

1.3.4 NEUROTOXINS

Brain damage also results from chemical neurotoxicity, either from administered drugs or environmental toxins. Many neurotoxic agents are available which mimic human pathologies or are useful for the study of neurodegeneration because of their specificity in effect. Kainate, an excitotoxic agent, is useful as a model of epileptogenesis and seizures (Zaczek *et al*., 1980; Pennypacker *et al*., 1993). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration, resulting in the selective death of dopaminergic neurons in rodents, serves as a model for Parkinson's disease (Freyaldenhoven *et al.*, 1997).

1.3.4.1 Quinolinic Acid

Quinolinic acid (QA), an endogenous metabolite of tryptophan, is a potent endogenous neurotoxin, first demonstrated by Lapin (1978). It is normally present in low nanomolar concentrations in human brain and cerebrospinal fluid. However, substantial increase in QA levels to micromolar concentrations are found in the brain and CSF of patients with infectious and inflammatory neurological diseases (Heyes *et al.*, 1996; Stone, 2000).

In spite of its involvement in many diseases, the exact molecular mechanism linking QA and brain damage are far from being understood. However, some studies demonstrated that QA exerts its toxicity in the central nervous system (CNS) via glutamatergic receptors (Stone, 2001). Glutamate neurotoxicity is implicated in acute neurological disorders as well as in neurodegenerative diseases (Lipton & Rosenberg, 1994). Evidence suggests that QA is associated with neuronal damage through the overactivation of the postsynaptic NMDA subtype of glutamate receptors (Stone, 2001).

Striatal administration of QA is associated with neurotoxicity and development of neurological damage, which resembles that observed in Huntington’s disease, and probably results from excessive NMDA receptor activation (Beal *et al.*, 1991; Malcon *et al.*, 1997). Moreover, intracerebroventricular injection of QA has been shown to induce seizures (Lapin, 1978; Schmidt *et al.*, 2000; Lara *et al.*, 2001).

The neurotoxicity produced by QA may also depend at least partly on the formation of ROS, since its neurotoxic activity can be prevented by spin-trap reagents such as α-phenyl-*t*-butylnitrone (Nakao & Brundin, 1997) and free radical scavengers (Nakai *et al.*, 1999). QA has also been shown to induce lipid peroxidation (Rios & Santamaria, 1991) which can be prevented by nitroarginine and potentiated by L-arginine, suggesting that NO, a free radical itself and a precursor of potent toxic radicals such as peroxynitrite, may contribute to the activity of QA (Santamaria *et al.*, 1997).

1.3.4.2 Cyanide

Numerous biochemical, morphological and physiological studies have shown that cyanide is a potent and selective neurotoxin whose toxicity is mediated through histotoxic hypoxia, consequent to mitochondrial dysfunction (Bhattacharya & Lakshmana Rao, 2001). The cascading effects of cyanide-induced impairment of mitochondrial energy production include failure of ionic homeostasis, acidosis, elevated Ca^{2+} levels and lipid peroxidation, leading to activation of proteases, lipases, xanthine oxidases, etc. (Maduh, 1989). The elevation in brain calcium (Johnson *et al.*, 1986) and increase in free cytosolic calcium leads to increased oxidative stress and excitotoxcity.

The implication of oxidative stress and activation of endonucleases in oligonucleosomal cleavage of DNA following cyanide exposure is well documented (Trump & Berezesky, 1995). Subsequent to oxidative stress, widespread DNA fragmentation leading to an apoptotic type of cell death has been documented in cyanide poisoning (Mills *et al.*, 1996; Mills *et al.*, 1999), with Mills *et al.* (1996) reporting an apoptotic effect of cyanide in terminally differentiated PC12 cells. Cyanide also causes surface blebbing and cytoarchitectural defects of neuronal cells as a consequence of Ca^{2+}-activated phospholipases and proteases (Nicotera *et al.*, 1989).

1.3.4.3 Metals

Multivalent transition metals such as iron, copper, and manganese are essential in most biological reactions such as the synthesis of DNA, RNA, and proteins. These are also cofactors of numerous enzymes, particularly those involved in respiration. However, abnormal tissue accumulation of redox-active transition metals can be cytotoxic because

perturbations in metal homeostasis results in an array of cellular disturbances characterised by increased free radical production (Campbell, 2001).

The levels of copper and iron are increased in the rims of senile plaques (SP) (Lovell *et al.*, 1998) and the presence of these redox reactive metals in both SP and neurofibrillary tangles, has been shown to induce hydrogen peroxide dependent oxidation (Sayre *et al.*, 2001). This indicates that the metals may play a role in modulating oxidative events, which may eventually result in neurodegeneration.

Transition metal ions are effective catalysts in unsaturated lipid peroxidation and are therefore involved in membrane lipid peroxidation through free radical production. Hydrogen peroxide, a by-product of oxidases, has been considered a messenger for intracellular redox signaling pathways. In the presence of redox-active transition metal ions such as iron and copper, H_2O_2 is converted to reactive hydroxyl radicals via the Fenton reaction as shown below.

$$Mn^{+} + H_2O_2 \longrightarrow M^{(n+1)} + OH^{+} + OH^{-} \qquad (5)$$

Recently it has been discovered that transition metals bind to proteins involved in neurodegeneration and this association appears to preserve metal redox activity in a manner that is consistent with a pro-oxidant, free radical generating action (Campbell *et al.*, 2001). Whereas transition metal ions can initiate the formation of ROS *in vivo,* main group essential metal ions may limit oxidative damage through direct or indirect competition with these. This is especially the case with zinc (Favier, 1995) and magnesium (Rayssiguier & Mazur, 1995).

1.3.4.3.1 Iron

Free iron, more than any other transition metal, has been implicated in redox transitions and consequent generation of oxygen free radicals. The potential of iron to cause an increase in oxidative events leading to cellular damage is controlled by a series of iron-binding proteins (Campbell, 2001). Lactoferrin, which has been shown to protect against severe inflammation, is increased in the brain of patients with neurodegenerative diseases (Fillebeen *et al.*, 1999). The large amounts of polyunsaturated fatty acids present in the

brain are extremely sensitive to iron-induced oxidative stress and readily undergo lipid peroxidation in the presence of iron (Chiueh, 2001).

Haemoglobin has been postulated to be the major source for iron overload in the brain during the breakdown of the blood brain barrier (BBB) by promoting the generation of ROS, lipid peroxidation, axonal dystrophy and neurodegeneration. These deleterious effects of haemoglobin are blocked by desferrioxamine *in vitro* and *in vivo*, suggesting the involvement of free iron released from haemoglobin (Bergen *et al.*, 1999).

Microglia are the major sites of ferritin bound iron and are thought to be partly responsible for oxidative damage in Parkinson's disease and other neurodegenerative disorders. Abnormally high levels of iron as well as oxidative stress have been demonstrated in a number of neurodegenerative disorders including Alzheimer's disease and those characterised by nigral degeneration such as Parkinson's disease, multiple system atrophy, and progressive supranuclear palsy (Campbell, 2001).

Moreover, the interplay between oxidative stress and control of iron metabolism in the brain is exemplified by the finding of abnormal iron deposition associated with lipid peroxidation in a transgenic mouse model of a BBB defect linked to progressive neurodegeneration (Castelnau *et al.*, 1998).

1.3.4.3.2 Lead

Lead is one of the most widely used nonferrous metals that is present in the environment. Lead toxicity results from exposure to contaminated air, water, food, soil and other lead-contaminated non-food ingestants (Keogh, 1992). It interferes with haem metabolism, causes widespread tissue damage and antagonises many biological actions of Ca^{2+} ions (Dulka & Risby, 1976; Simons, 1986).

Several lines of evidence suggest that cellular damage mediated by oxidants is involved in some of the pathologies associated with lead intoxication. Lead is known to potentiate O_2 toxicity in rats (Kiesow, 1977), stimulate oxidative haemolysis of erythrocytes (Gelman *et al.*, 1978), decrease erythrocyte superoxide dismutase activity (Gelman *et al.*, 1978; Ito *et al.*, 1985) and accelerate the conversion of oxyhaemoglobin to

methaemoglobin, with the accompanying production of superoxide radicals and hydrogen peroxide (Ribarov & Bochev, 1982).

Lead has also been suggested to accelerate lipid peroxidation *in vivo* (Ribarov & Bochev, 1982) and it was observed to increase the rate of peroxidation of liposomes in the presence of haemoglobin (Ribarov *et al.*, 1981). Quinlan *et al.* (1988) reported an enhancement of Fe^{2+} initiated lipid oxidation in liposomes, erythrocytes, microsomal fractions and rat brain homogenates in the presence of lead.

A hypothesis that has been studied for many years is that lead is toxic by disrupting calcium-dependent mechanisms. Lead and calcium compete for the same binding sites on proteins that belong to a large family of ion binding proteins and lead often substitutes for calcium in such proteins (Habermann *et al.*, 1983). Members of this family include calmodulin, calretinin, calbindin and parvalbumin.

Furthermore, the dendritic branching observed in hippocampal neurons that were exposed to KN-62, an inhibitor of calmodulin – dependent protein kinase II, was blocked by lead (Kern & Audesirk, 1995). Dendritic branching is a mechanism essential in developing the learning process. Hence the effects of lead on calmodulin-dependent proteins may explain deficits observed in learning caused by lead exposure.

1.3.4.3.3 Cadmium

Cadmium is an abundant element of worldwide concern because it accumulates in the environment as a result of its manifold industrial uses. Discharges mainly from industries such as electroplating, plastic production, pigment and battery manufacturing, coal burning power plants, sludge from sewage treatment plants used as fertilizer, gas from the municipal incinerators, pesticides and cigarette smoking (Friberg, 1986) are the main sources of cadmium. Once absorbed, the extremely long biological half-life of cadmium (16 to 32 years) allows cadmium to slowly accumulate in the body (Nath *et al.*, 1984).
The picture of cadmium neurotoxicity is comprised of behavioural, neurochemical and histopathological effects (Murphy, 1996). Motor abnormalities, physiological dysfunctions in the nervous system and decreased intelligence are the main symptoms

observed in both children and adults exposed to cadmium (Taylor & Ennever, 1993; Thatcher & Lester, 1989).

Entry of cadmium into the brain is prevented by the BBB and the cerebrospinal fluid (CSF) barriers (Takeda *et al.*, 1999). However, Shukla *et al*. reported that BBB permeability is enhanced by chronic exposure to cadmium (Shukla *et al.*, 1996). In animal studies, chronic exposure to cadmium causes significant increases in cadmium concentration in the brain, especially in the olfactory bulb (Clark *et. al*, 1985; Evans & Hastings, 1992).

Cadmium accumulation in the brain causes behavioural alteration, which is exacerbated in rats fed with low protein diet (Ali *et al.*, 1991). Chronic exposure of rats to cadmium resulted in significant changes in the regional concentration of brain biogenic amines, namely a suppression of cortical acetylcholine and brain stem 5-hydroxytryptamine and an accumulation of striatal dopamine. Consequent to cadmium exposure, concentrations of lipid peroxidase are significantly elevated in the cerebellum, cerebral cortex, corpus striatum and midbrain (Shukla *et al.*, 1987). Based on the formation of thiobarbituric acid reactive substances (TBARS), cadmium has been shown to increase lipid peroxidation in various organs of adult rats, with the lungs and brain being the main targets (Manca *et al*., 1991).

Cadmium (II) is also known to decrease SOD activity as well as reduce the concentration of antioxidants such as vitamin E and C, in the liver and brain of treated rats (Sugiyama, 1994). It is suggested that cadmium (II) directly or indirectly through inhibition of superoxide dismutase, increases the lipid peroxidase levels of cell membranes and thus inhibits the associated physiological functions, leading to CNS dysfunction.

1.4 NEURODEGENERATIVE DISORDERS

In a structure as complex as the human brain, a multitude of things can go wrong. Disorders of the brain may occur in its structural architecture or in its electrical and chemical processes and the evidence for a role of oxidative damage in neurodegenerative diseases is becoming increasingly compelling. Pathological processes in the brain can be

brought about as a result of proximate and remote factors that may be classified as either genetic or environmental (Kety, 1979).

1.4.1 AGEING

The rate of ageing of an animal appears to be determined primarily by genetic factors. Thus the maximum lifespan of an animal is genetically determined, and hence it does not appear that significant manipulation of this maximum lifespan is possible utilising the techniques of free radical biology (Pryor, 1982; 1984). However, the lifespan of individuals in a population varies with stress, and free radicals play an important role in stress (Pryor, 1986). Free radicals increase ageing both by influencing the rate of development of specific chronic diseases and by causing general "wear and tear" on biopolymer molecules. The argument that free radicals cause general, non-specific damage to biopolymer molecules is called the "free radical theory of ageing" (Harman, 1982).

This theory is a popular hypothesis that has been used to explain the ageing process (Fantone & Ward, 1985). The intracellular generation of free radicals results in a progressive accumulation of defective molecules in the cells. This causes an alteration in the physiological function including the accumulation of the lipofuscins ("age-pigments"). Biochemical characterisation of these pigments indicates that they consist largely of oxidised lipids (Taubald *et al.*, 1975). Several other studies support a relationship between that of free-radical production within tissues and life span (Fantone & Ward, 1985).

It has been postulated that a reduced caloric intake could result in the decreased production of free radicals as by-products of normal biochemical reactions in cells, especially oxygen metabolic products of mitochondrial respiration (Harman, 1982). The correlation that exists between age and the onset of specific diseases in which free radical reactions are important, further supports the hypothesis that free radicals are intimately involved in the ageing process (Fantone & Ward; 1985).

Moreover, the aged brain has been shown to be more susceptible to ROS damage since many of the neuroprotective pathways are no longer as effective as they are in younger animals. The production of the neurohormone, melatonin, is also affected with age (Ludolph, 1995). This is probably because of the reduction in the number of β-adrenergic receptors (responsible for mediation of night time increase in melatonin production) with age (Saarela & Reiter, 1993). Another reason may be that the neuronal message to the pineal gland in older animals may be weakened by the gradual deterioration in the cells of the hypothalamic suprachiasmatic nuclei due to the action of EAA on the neurons at this level (Reiter *et al.*, 1996).

1.4.2 ALZHEIMER'S DISEASE

A case report was published by Alois Alzheimer in 1907, describing the cerebral cortex of a 55-year old woman with progressive dementia (Alzheimer, 1977). The famous "note" pointed out the presence of abnormal nerve cells that contained tangles of fibres (neurofibrillary tangles) and clusters of degenerative nerve endings (neuritic plaques). This presenile dementia later became known as Alzheimer's disease (AD).

AD involves the selective and progressive degeneration of the lower motor neurons in the spinal cord and the upper motor neurons in the cerebral cortex and is characterised by a progressive amnesic syndrome and loss of parietal and frontally based skills (Coyle & Puttfarcken, 1993). The characteristic pathology includes cerebral atrophy, neurofibrillary tangles, β-amyloid-containing plaques and gliosis. The cortex, hippocampus and amygdala are most affected (Barrett, 1993).

There is a marked dimunition of acetylcholine transferase, the biosynthetic enzyme for acetylcholine and this decrease is selective for the regions in which senile plaques and neurofibrillary tangles are found. Degeneration of cholinergic neurons is pronounced in these areas. Reports of loss of serotonin in the hippocampal region have also been reported (Bannister, 1992).

Substantial evidence for increased oxidative damage in AD exists from studies of postmortem brain tissue. There are consistent increases in lipid peroxidation as assessed by increases in malondialdehyde concentrations (Beal, 1997). Studies using novel spin-trapping techniques showed oxidative damage to both lipids and proteins (Hensley *et al.*, 1995). Amyloid β-protein which is derived from the amyloid precursor protein (APP) accumulates in plaques and has been implicated in neurotoxicity, where it is thought to induce the production of free radicals (Behl *et al.*, 1994, Goodman & Mattson, 1996) and disrupt calcium homeostasis (Mattson *et al.*, 1992).

It has been shown that patients with AD appear to suffer from diminished amplitude in the melatonin acrophase (Bevier *et al.*, 1992). This finding, in conjunction with other factors, has led to the conclusion that melatonin may be able to protect against AD. Rodent experimentation has demonstrated that pharmacological treatment with melatonin prevents premature ageing and delays the onset of various neurodegenerative diseases (Poeggeler *et al.*, 1993).

1.4.3 PARKINSON'S DISEASE

The possibility that oxidative damage may play a role in the pathogenesis of Parkinson's disease (PD) has aroused much interest. Mitochondrial complex I defects have been found in the substantia nigra, muscle and platelets of Parkinson's diseased patients (Beal, 1997). Direct evidence for oxidative damage comes from two studies, which show increased malondialdehyde and increased cholesterol hydroperoxides in the substantia nigra of Parkinson's diseased patients (Sanchez-Ramos *et al.*, 1994).

An indirect measure of oxidative stress is reduced glutathione concentration. Decreased glutathione concentrations in PD substantia nigra have been found in four separate studies (Perry *et al.*, 1982; Riederer *et al.*, 1989; Sofic *et al.*, 1992; Sian *et al.*, 1994). Furthermore, reduced glutathione concentrations are found in patients with incidental Lewy bodies in the substantia nigra, which may be a presymptomatic phase of PD (Dexter *et al.*, 1994).

Further evidence implicating oxidative damage in the pathogenesis of PD comes from studies of MPTP neurotoxicity. MPTP produces a Parkinsonian syndrome that closely mimics findings that occur in idiopathic PD. The pathogenesis of the lesions involves inhibition of mitochondrial complex I by 1-methyl-4-phenylpyridinium (MPP^+), the active metabolite of MPTP. MPP^+ results in increased free radical generation *in vivo*, and the toxicity of MPTP is attenuated in mice overexpressing SOD (Przedborski *et al.*, 1992). Schulz *et al.* (1995) showed that 7-nitroindazole, a relatively selective inhibitor of the neuronal isoenzyme of NOS, produces dose-dependent inhibition of MPTP neurotoxicity accompanied by reduced striatal concentrations of 3-nitrotyrosine. 7-nitroindazole produced complete protection against MPTP neurotoxicity in baboons (Hantraye *et al.*, 1996). These findings suggest that peroxynitrite plays a role in MPTP neurotoxicity and, by implication, in PD.

1.5 MECHANISMS OF NEUROPROTECTION

1.5.1 DEFENCES AGAINST FREE RADICALS

Due to the inevitable damages caused by free radical production in animal cells, defences within the body have evolved. These are known as antioxidant defences and may be categorised into those whose role is to prevent the generation of free radicals, and those that intercept any that are generated. These are present in both the aqueous and the membrane compartment of the cell and can be enzymes or non-enzymes (Cheeseman & Slater, 1993).

The principal defence systems against oxygen free radicals are SOD, GSH (reduced form of glutathione), GSH peroxidases, glutathione reductase, catalase (a haem enzyme), and antioxidant nutrients (Fig. 1.6).

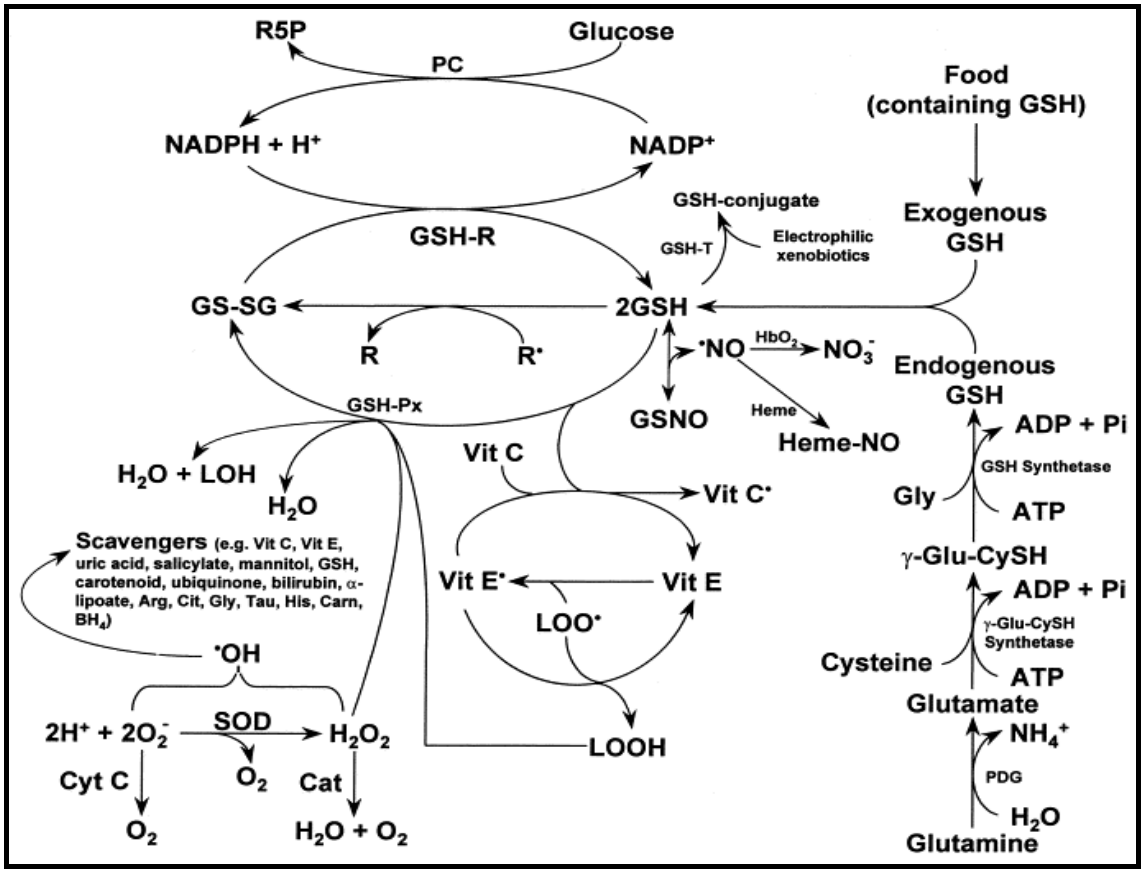


FIG. 1.6 *Removal of oxygen and nitrogen free radicals and other reactive species in mammalian cells (Fang et al., 2002).*

Vitamin E can transfer its phenolic hydrogen to a peroxyl free radical ($LOO^{\bullet}$) of a peroxidised PUFA, thereby breaking the radical chain reaction and preventing the peroxidation of PUFA in cellular and subcellular membrane phospholipids. As a reducing agent, vitamin C reacts with a vitamin E radical to yield a vitamin C radical while regenerating vitamin E. Like a vitamin E radical, a vitamin C radical is not a reactive species because its unpaired electron is energetically stable. A vitamin C radical is converted back to vitamin C by GSH (Fang *et al.*, 2002).

Cytochrome C and SOD catalyse the formation of O_2 from O_2^- as shown in Fig. 1.6. A co-product of SOD is H_2O_2, which is converted to H_2O by catalase (CAT) and the selenium-dependent GSH peroxidase. Lipid hydroperoxides (LOOH) are detoxified to alcohols by GSH peroxidase. Other ROS as well as RNS scavengers include uric acid (a metabolite of purines), salicylate, mannitol, carotenoid, ubiquinone, bilirubin (a product

of haemoglobin catabolism), α-lipoate, arginine, citrulline, glycine, taurine, histidine, creatine (a metabolite of arginine, glycine and methionine), carnosine (β-alanyl-L-histidine, which is abundant in skeletal muscle), tetrahydrobiopterin (a metabolite of guanosine triphosphate and thus glutamine), phytate, and tea polyphenols (Fridovich, 1999; Lawler, 2002; Machilin & Bandito, 1987;Wu & Meininger, 2000; Lass *et al.*, 2002; Akashi *et al.*, 2001; Redmond *et al.*, 1996).

Due to the continuous generation of superoxide anions in the mitochondria, the mitochondria possess an efficient antioxidant system composed of superoxide dismutase, glutathione peroxidase, glutathione reductase, glutatione, NAD(P) transhydrogenase, NADPH, vitamins E and C (Sutton & Winterbourn, 1989; Halliwell & Gutteridge, 1989), thiol peroxidases such as SSP-22 (Watabe *et al.*, 1997) and mitochondrial respiration itself (Guidot *et al.*, 1995).

Steroid hormones have been reported to have antioxidant neuroprotective properties, oestrogen being particularly important (Goodman *et al.*, 1996; Behl *et al.*, 1997). They have previously been reported to be a more potent inhibitor of iron-catalysed lipid peroxidation in brain tissue than vitamin E (Behl *et al.*, 1992; Hall *et al.*, 1991). With regard to AD, it has been demonstrated *in vitro* that 17-β-estradiol protects neurons from Aβ-mediated cytotoxicity (Behl *et al.*, 1995) and from oxidative insults attenuating membrane lipid peroxidation and stabilising neuronal calcium homeostasis (Goodman *et al.*, 1996; Keller *et al.*, 1997).

1.5.2 MELATONIN

Melatonin (5-methoxy-N-acetyl-tryptamine) is an indoleamine synthesised from tryptophan predominantly in the pineal gland and other organs in vertebrates (Reiter *et al.*, 2000). The synthesis of the hormone is under rhythmic control (Reiter, 1987) with peak levels produced at night in darkness. Melatonin has been demonstrated to be a powerful antioxidant and free radical scavenger (Tan *et al.*, 1993) and thus prevents oxidative damage to the cell membrane, cytosol organelles and nuclear and mitochondrial DNA .

Melatonin has been shown to scavenge reactants including the hydroxyl radical and hydrogen peroxide as shown in Fig. 1.7, as well as, nitric acid, peroxynitrite and peroxynitrous acid.

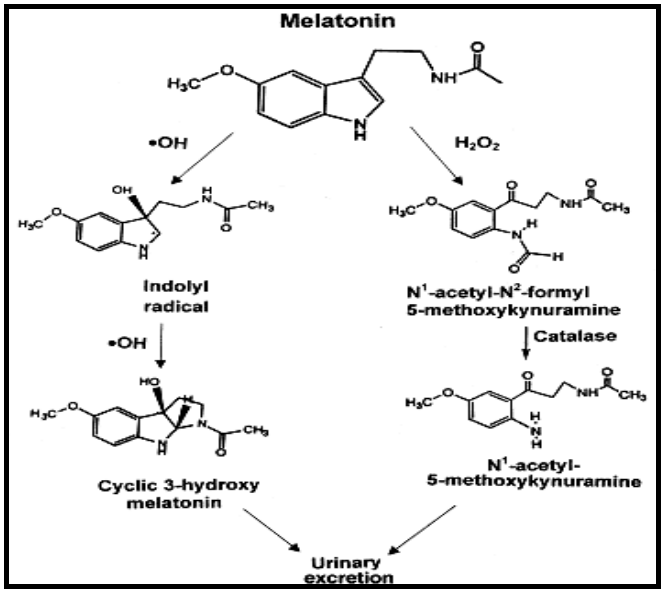


FIG. 1.7 *Melatonin: a scavenger of both $OH^{\cdot}$ and H_2O_2 as shown above, with the resulting products being cyclic 3-hydroxymelatonin and N^1-acetyl-N^2-formyl-5-methoxy-kynuramine, respectively (Reiter et al., 2002).*

One of the products of melatonin's interaction with hydrogen peroxide, N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), is also a highly efficient radical scavenger (shown in Fig. 1.7). The cascade of reactions where the secondary metabolites are also effective scavengers is believed to contribute to the high efficacy of melatonin in reducing oxidative damage (Reiter *et al.*, 2002).

In addition, melatonin stimulates the synthesis of antioxidant enzymes including superoxide dismutase, glutathione peroxidase and glutathione reductase (Pablos *et al.*, 1998; Albarran *et al.*, 2001) suggesting that melatonin may act not only directly against free radicals, but also indirectly as an enzyme stimulator, although the specific induction mechanism is unknown (Albarran *et al.*, 2001). Moreover, patients with AD have decreased levels of melatonin in the periphery and the administration of this indole induces improvement in circadian rhythms and insomnia in these patients (Monti & Cardinali, 2000).

1.6 PHYTOCHEMICALS

Animal studies have shown that dietary phytochemical antioxidants are capable of removing free radicals. Among them, phenolic and polyphenolic compounds, such as flavonoids and catechin in edible plants, exhibit potent antioxidant activities (Fang, 2002). A broad definition of the term, phytochemical, would be any compound found in plants. However phytochemical is more commonly used to define the biologically active molecules in plants that are not classified as vitamins or nutrients by more traditional definitions.

The number of phytochemicals in plants is staggering, and though research into their biological significance in humans is in its infancy, it has thus far yielded important observations. While phytochemicals have key functions in plants, they may also play a significant role in human health (Technical Bullein, 1998). As animal species evolved, many were able to "borrow" some of the protective phytochemicals from the plants composing their diets, saving these species the trouble of having to manufacture all their own chemical defence mechanisms.

Plants have developed extensive systems for managing reproduction, response to injury, protection from ultraviolet light, and resistance to disease. Attending to these important functions requires communication between plant cells and between the plant and its environment. Plants use phytochemicals, both as a means of communication and as a response to environmental stress. “Communication” also occurs between phytochemicals and the human organism when we eat plant-based foods. This communication involves a number of complex biological activities that influence health. While it is hard to quantify the exact amount of phytochemicals consumed by individuals, dietary consumption of flavonoids, one class of phytochemicals, is estimated to be between 23mg and 1 g/day (Hertog *et al.*, 1993; Kuhnau, 1976).

The process by which the plant produces the chemicals needed for communication, i.e., signalling molecules, is referred to as secondary metabolism (Technical bulletin, 1998).

The secondary metabolites produced from this process influence diverse communication activities. For example, carotenoids provide colour to signal pollinators such as birds, and polyphenols such as those present in grape skins, protect DNA from damage in dry and/or light intensive environments (Das *et al.*, 1994).

1.6.1 BIOLOGICAL ACTIVITIES INFLUENCED BY PHYTOCHEMICALS

The diversity of the phytochemical family is reflected in the number of biological activities which these molecules exhibit. Phytochemicals can act as potent antioxidants, can specifically inhibit or activate different enzyme systems in the body, and can effect gene expression and influence the types of molecules our cells synthesise. Different phytochemicals have been shown to possess anticarcinogenic, anti-inflammatory, and antiallergic properties (Caragay, 1992).

1.6.1.1 Phytochemicals as Antioxidants

The ability to act as antioxidants in some capacity is a common pathway shared by many phytochemicals. Interest in the antioxidative abilities of phytochemicals was generated in the mid 1930's when Szent-Gyorgi discovered a class of polyphenolic compounds that were more potent than vitamin C in treating scorbutic guinea pigs. This researcher termed this class of molecules "vitamin P" to reflect their ability to reduce capillary permeability (Liebovitz and Mueller, 1993). Since then it has been discovered that many phytochemicals have the ability to act as antioxidants, some even more potent than vitamins E or C due to their ability to donate single electrons (Technical Bulletin, 1998).

1.6.1.2 Phytochemicals as Modulators of Key Enzymes in Biological Pathways

Recent studies show that certain phytochemicals may have specific effects on enzyme pathways in the body. Much focus has been placed on one of the main pathways that contribute to chronic conditions such as inflammation: the arachidonic acid cascade.

Several enzymes in the arachidonic acid cascade have been shown to be modulated by different phytochemicals (Fig. 1.8.).

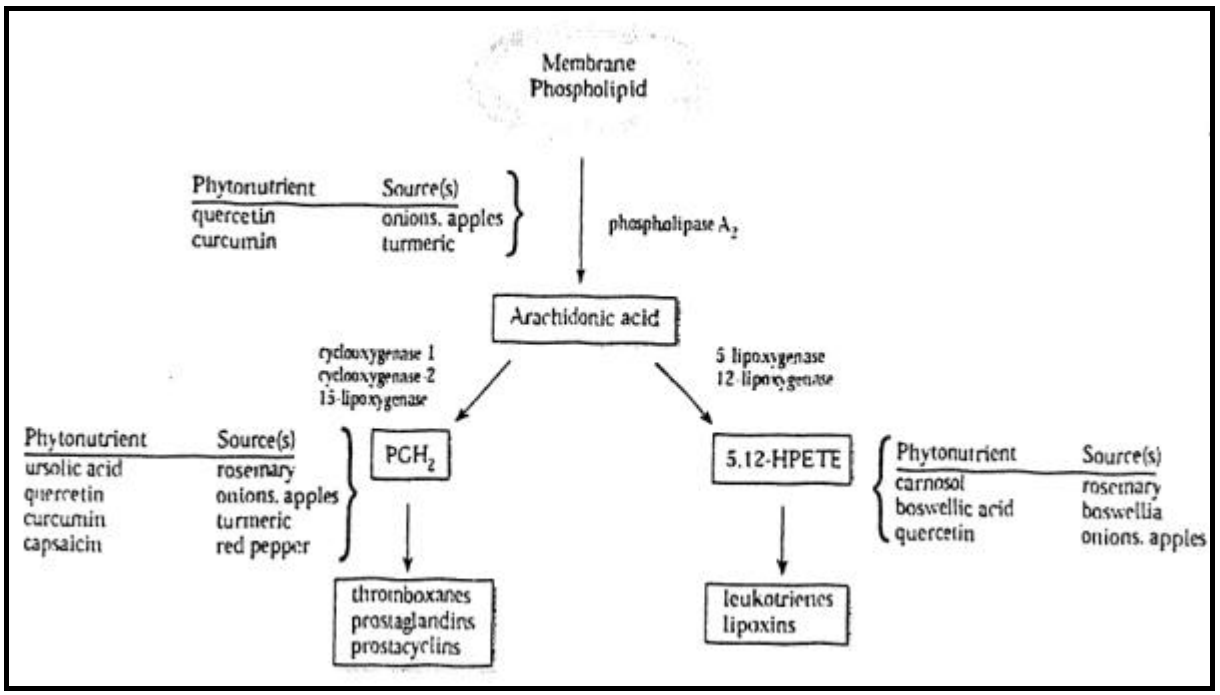


FIG. 1.8 *Modulation of key enzymes in the arachidonic acid cascade by phytochemicals (Technical Bulletin, 1998).*

1.6.1.3 Phytochemicals as Anti-inflammatory agents

The inflammatory process is a complex cascade, which involves the activation of the arachidonic acid cascade and release of oxidants as well as other chemical mediators that include eicosanoids (prostaglandins, prostacyclins, leukotrienes and thromboxanes), cytokines (e.g. tumour necrosis factor) and ROS. The controlled production and subsequent down-regulation of these inflammatory mediators produces acute inflammation, which is a process essential for maintaining health. When these inflammatory mediators are not down-regulated and continue to be produced, chronic inflammation results, and this leads to an excessive production of cytokines, eicosanoids and ROS.

Various phytochemicals have been shown to play important roles in combating chronic inflammation. Quercetin, found in onions and apples, has been shown to inhibit several

enzymes with pro-inflammatory activity in the arachidonic acid cascade, such as 5-lipoxygenase and cyclooxgenase (Carmen *et al.*, 1995). Hesperidin is a flavonoid found in citrus foods and is capable of inhibiting arachidonic acid and histamine release, actions that are thought to support its anti-inflammatory and analgesic properties (Emim *et al.*, 1994).

Phytochemicals are subdivided into different classes of compounds based on chemical structure and synthesis pathways, and are grouped into at least 14 general classes: carotenoids, coumarins, flavonoids, glucurates, indoles, isothiocyanates, lignans, mono-terpenes, di- and tri- terpenes, phenolic acids, phthalides, phytates, polyacetylenes, and sulfides. Of these, the flavonoids are the largest class, with over 4000 representatives (Hollman and Katan, 1998).

1.6.2 FLAVONOIDS

Flavonoids are brightly coloured chemical constituents found in most fresh fruit, vegetables and herbs. These are a ubiquitous group of polyphenolic substances occuring in high concentrations in the juice and peel of citrus fruits, and are also present in onions, grapes, wine and greens. Flavonoids have been shown in a number of studies to be potent antioxidants, capable of scavenging hydroxyl radicals, superoxide anions, peroxynitrite and lipid peroxyl radicals (Reuben, 1994). Several flavonoids are also known to be very effective metal chelators (Duthie *et al.*, 1997).

These phytochemicals can also inhibit free radical injury to neurons by inhibiting glutamate toxicity in a dose-dependent manner (Schubert *et al.*, 1992). These are also known to spare ascorbic acid and may spare the tocopherols as well, both of which significantly reduce excitoxicity (Hertog & Katan, 1998). Most flavonoids are present in plants as glycosides. In the intestines, this moiety is cleaved off leaving the aglycone form of the flavonoid (Griffiths, 1982). It is the aglycone form that is thought to have the highest antioxidant activity in biological systems.

1.6.3 TURMERIC

Turmeric is a member of the *Curcuma* species of the family Zingiberaceae (Fig. 1.9). The root and rhizome is crushed and powdered into ground turmeric. Turmeric is an ancient spice and a traditional remedy that has been used as a medicine, condiment and flavouring agent within both Chinese and Indian systems of medicine (Thaloor *et al.*, 1998).

FIG. 1.9 *Fresh rhizomes of turmeric* (www-ang.kfunigraz.ac.at/~katzer/engl/Curc_dom.html)

Turmeric has been used in Ayurvedic medicine for several thousand years in India for a number of medical conditions including treatment of common colds, coughs, jaundice, and upper respiratory disorders. In old Hindu texts it is ascribed for its aromatic, stimulant, and carminative properties. Turmeric mixed with slaked lime is known as a household remedy for the treatment of sprains and swellings cased by injury. For this purpose it is applied locally over the affected area. East Indian healers have also been reported to use turmeric as an antiviral agent and a stomach aid.

Besides its well-known medicinal history, turmeric is what gives curry dishes their distinctive colour and flavour. Ethnologically, *Curcuma longa* still occupies an important position, as every food should contain it in India. Religious ceremonies also, always make use of turmeric in any form. Raw turmeric contains 0.3 – 5.4% curcumin, 4 – 14% volatile oils including tumerone, atlantone, and zingiberone. Turmeric also contains

sugars (28% glucose, 12% arabinose), proteins and resins (Leung, 1980; Ammon & Wahl, 1991).

1.6.4 CURCUMIN

Turmeric is a rich source of a group of phenolic compounds called curcuminoids. Three main curcuminoids namely curcumin, demethoxycurcumin and bisdemethoxycurcumin occur naturally in these *Curcuma* species. Curcumin is the major yellow colouring pigment found in the *Curcuma* species, Zingiberaceae.

1.6.4.1 Chemistry

Although the chemical structure (Fig. 1.10) of curcumin was determined by Lampe in 1910 (Ammon & Wahl) it was only in the seventies and eighties that the potential uses of curcumin in medicine has been extensively studied.

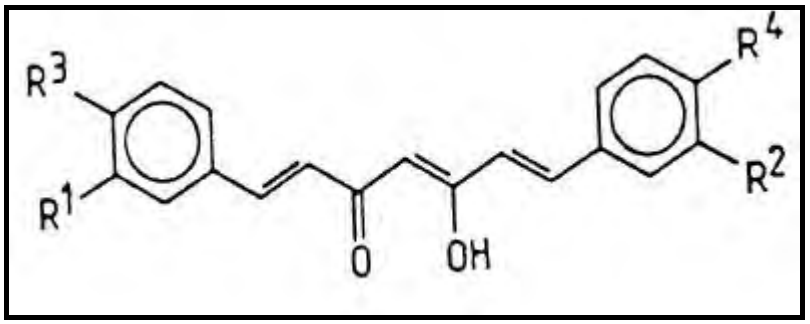


FIG. 1.10 *Structural representation of the three curcuminoids.*

Curcumin:	$R^1 = R^2 = OH$	$R^3 = R^4 = OCH_3$
Demethoxycurcumin:	$R^1 = R^2 = OH$	$R^3 = H; R^4 = OCH3$
Bisdemethoxycurcumin:	$R^1 = R^2 = OH$	$R^3 = R^4 = H$

As shown above, the primary structure of curcumin is a conjugated diene with an adjacent phenolic group that has general antioxidant property. It has been reported that in addition to the phenolic group, the methoxy group on the phenyl ring and the 1,3-diketone system are also important structural features that contribute to its antioxidant properties (Sreejayan *et al.*, 1996). It is insoluble in water but soluble in ethanol, alkalis,

ketone, acetic acid and chloroform. The main chain of curcumin is aliphatic and unsaturated and the aryl group can be substituted or not.

1.6.4.2 Biological Activities

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) has been shown to have a dual effect in oxygen radical reactions, it can act as a scavenger of hydroxyl radicals or catalyse the formation of hydroxyl radicals, depending on the experimental conditions (Tønnesen, 1989; Kunchandy & Rao, 1990).

Curcumin inhibits lipid peroxidation in rat liver microsomes, erythrocyte membranes and brain homogenates (Reddy & Lokesh, 1992; Salimath *et al.*, 1986; Sharma, 1976) and this has led to the postulation that curcumin could be useful as an anti-atherogenic agent in those processes which occur as a consequence of a marked increase in blood lipid peroxidation, such as myocardial infarction (Santos *et al.*, 1989) and diabetes (Yagi, 1989). Curcumin also inhibits the induction of NOS in activated macrophages and has been shown to be a potent scavenger of free radicals as well as nitric oxide (Sreejayan & Rao, 1997).

Pharmacological actions of curcumin as an anti-inflammatory agent have been examined by Srimal & Dhawan (1973) who showed that this compound was effective in acute as well as chronic models of inflammation. The same authors report curcumin to be less effective in adrenalectomized rats suggesting a participation of corticoidal steroids in the anti-inflammatory action of curcumin (Srimal & Dhawan, 1985).

Huang *et al.* (1991) reported an inhibitory effect of curcumin on arachidonic acid-induced inflammation and on arachidonic acid metabolism through the inhibition of cyclooxygenase (COX) and lipooxygenase (LOX) enzymes in mouse epidermis. COX is a bifunctional enzyme with cyclooxygenase and peroxidase activities (Zhang *et al.*, 1999; Campbell & Halushka, 1996). The cyclooxygenase activity of COX is important for the initial conversion of arachidonic acid to prostaglandin G_2, and the peroxidase activity of COX then converts it to prostaglandin H_2. Finally, prostaglandin H_2 is converted to other

prostaglandins by a variety of tissue specific enzymes. These prostaglandins then exert their biological actions on these tissues (Campbell & Halushka, 1996).

Conventional non-steroidal anti-inflammatory drugs (NSAIDS) do not inhibit the peroxidase function of the COX enzyme, which potentially limits their efficacy as anticancer agents whereas curcumin inhibits both the cyclooxygenase and peroxidase activities of this enzyme (Zhang *et al.*, 1999). The anti-cancer action of curcumin has been studied in a standard model of radiation-induced tumour in rat mammary gland and was reported to have decreased the incidence and proportion of tumours by 50% (Inano *et al.*, 2000). Likewise, Limtrakul *et al.* (1997) showed an inhibitory effect of curcumin on mouse skin carcinogenesis initiated by 7,12-dimethylbenz(a)anthracene (DMBA) and promoted by tetradecanoylphorbol-13-acetate (TPA).

As a result of its immune-modulating, anti-inflammatory and cycloxygenase inhibitory action curcumin shows a favourable effect on a mouse model of psoriasis (Miquel *et al.*, 2002). A study by Heng *et al.* (2000) on patients with active psoriasis showed that topical treatment with curcumin results in resolution of psoriatic activity as assessed by clinical, histological and immunological criteria and the authors postulate this anti-psoriatic activity of curcumin to be due to the modulation of phosphorylase-kinase (PhK) activity that integrates multiple calcium/calmodulin-dependent signaling pathways. The PhK activity is found to be high in untreated psoriasis and was decreased to near normal values by curcumin treatment. An overall view of the biochemical actions of curcumin is listed below:

Biochemical Action of curcumin	**Reference**
Scavenges superoxide anion and hydroxyl radical.	Kunchandy & Rao (1990).
Scavenges singlet oxygen.	Subramanian *et al.* (1994).
Inhibits lipid peroxidation.	Sreejayan (1994).
Inhibits TPA-induced ornithine decarboxy-lase mRNA and activity.	Huang *et al.* (1988).
Inhibits TPA-induced cellular	Shih & Linn (1993).

8-hydroxydeoxyguanosine.	
Inhibits TPA-induced skin inflammation.	Huang *et al.* (1997).
Inhibits lipoxygenase and cycloxygenase activities.	Huang *et al.* (1991).
Inhibits arachidonic acid metabolism.	Conney *et al.* (1991).
Inhibits the formation of carcinogen-DNA adducts.	Conney *et al.* (1991).
Inhibits skin tumor initiation and promotion	Huang *et al.* (1992).
Inhibits azoxymethane-induced colon tumorigenesis in mice and rats.	Rao *et al.* (1995).

1.6.4.3 Biotransformations of Curcumin

1.6.4.3.1 Chemical Degradation

Curcumin is affected by UV under solar light exposure and the degradation kinetics of curcumin under various pH conditions were investigated by Wang *et al.* (1997). The results of the study showed that decomposition of curcumin is pH-dependent and occurs faster under neutral-basic conditions. Based on mass and spectrophotometrical analysis, trans-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexenal was identified as the major degradation product, while vanillin, ferulic acid and feruloylmethane were identified as minor degradation products as depicted in Fig. 1.11 (Wang *et al.*, 1997).

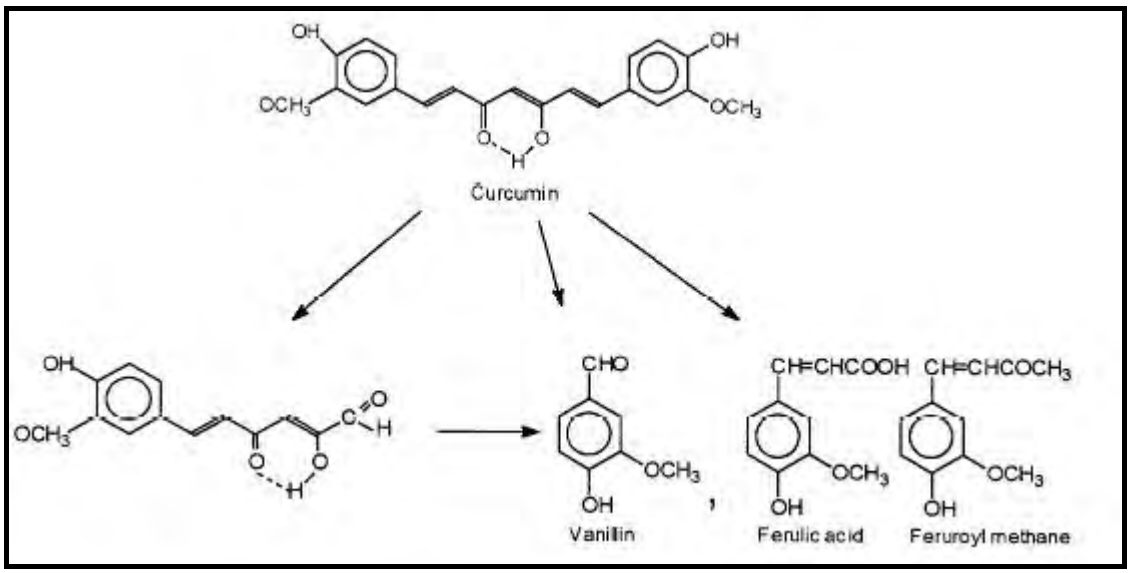


FIG. 1.11 *Chemical degradation of curcumin in aqueous solution (Wang et al., 1997).*

1.6.4.3.2 Biotransformations of Curcumin in Mice

Pan *et al.* (1999) investigated the nature of the metabolites of curcumin *in vivo* and showed that curcumin is first biotransformed into dihydrocurcumin and tetrahydrocurcumin. These compounds are then converted to monoglucuronide conjugates. The results of their experiments pointed at curcumin-glucuronide, dihydrocurcumin-glucuronide, tetrahydrocurcumin-glucuronide and tetrahydro-curcumin as being the major metabolites of curcumin *in vivo.*

1.7 OBJECTIVES OF THIS STUDY

This study was undertaken to investigate the possible role of the Eastern spice component, curcumin, as a neuroprotectant against oxidative stress in the brain, as well as damage induced by various neurotoxins. Previous research conducted on curcumin and Alzheimer's disease has brought to light statistics that confirm the reduced incidence of this disease among inhabitants of Indian villages in comparison to the western world (Ganguli *et al*., 2000; Lim *et al*., 2001). This has been attributed to the presence of several antioxidant and neuroprotective components in various spices commonly consumed in the daily diet of Indian cultures, one of them being curcumin.

The role of oxidative stress, endogenous toxins like quinolinic acid, mitochondrial inhibitors like cyanide, and metals like iron, lead and cadmium, in various neurodegenerative disorders are well established. The objectives of this study was thus to observe the effects of curcumin on damage induced by these factors through the employment of various biological assays as well as inorganic studies. Furthermore, this study also entailed the elucidation of possible mechanisms by which curcumin may exert any protective effects against oxidative stress and damage caused by the above mentioned neurotoxins in the rat brain.

CHAPTER TWO

SUPEROXIDE RADICAL GENERATION

2.1. INTRODUCTION

Reduction of molecular oxygen by the transfer to it of a single electron produces the superoxide free radical anion also referred to as superoxide (Cheeseman *et al.*, 1993).

$$O_2 + e^- \longrightarrow O_2^{\cdot -} \quad (6)$$

The mitochondrion is the organelle in eukaryotes responsible for aerobic respiration and is the most common source of ROS during apoptosis. The respiratory chain loses two to three percent of the electrons during their transfer to molecular oxygen, most of them participating in the production of superoxide anion (Boveris, 1977). Complex I, NADH–ubiquinone oxidoreductase and Complex III, ubiquinol-cytochrome *c* oxidoreductase are the two sites in the electron transport chain (ETC) where superoxide is produced and are diagrammatically represented in Fig. 2.1 (Beyer, 1992). Superoxide generated by the cycling of ubiquinol in the inner mitochondrial membrane, is produced primarily in the mitochondrial matrix (Raha *et al.*, 2000).

Superoxide toxicity appears to be through an indirect action on living cells by giving rise to more powerful oxidants such as the hydroxyl radical. As mentioned in section 1.3.1.6 the dismutation of O_2^- generates H_2O_2 and oxygen, and this reaction is a spontaneous reaction which can also be catalysed by the enzyme superoxide dismutase.

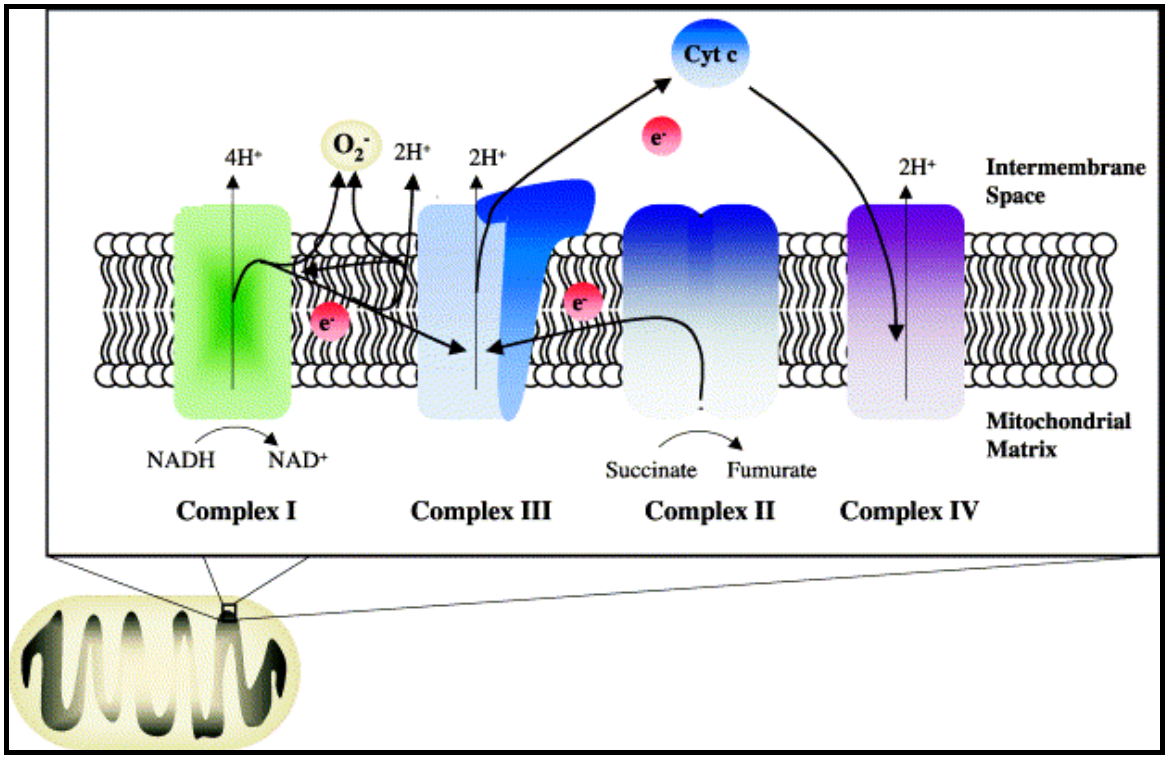


FIG. 2.1 *Schematic diagram illustrating the major subunits of the electron transport chain and sites of $O_2^{\cdot -}$ production. Electrons enter the ETC at either complex I (NADH-ubiquinone oxidoreductase) or complex II (succinate dehydrogenase) following the oxidation of NADH and succinate, respectively. Ubiquinone is a lipid-soluble electron carrier and carries the electrons from complex I and complex II to complex III (ubiquinol-cytochrome c oxidoreductase). Superoxide ($O_2^{\cdot -}$) can be produced at both complex I and complex III. It is believed that semiquinone formation at both complex I and complex III results in the production of $O_2^{\cdot -}$* (Curtin *et al.*, 2002).

The importance of hydrogen peroxide in free radical biochemistry lies in the fact that it breaks down easily in the presence of transition metal ions to produce the most reactive and damaging of the oxygen free radicals, the hydroxyl radical ($OH^{\cdot}$) and this is shown by equation 5 (section 1.3.2.3). Moreover, as shown in equation 2 (section 1.3.1.6) the superoxide anion is capable of producing the hydroxyl radical in presence of hydrogen peroxide (Haber-Weiss reaction). Since these reactions are reversible, there is a constant generation of superoxide and the promotion of free radical reactions is allowed for.

In pH environments of approximately 7.4 superoxide partially protonates to form the hydroperoxyl radical ($HO_2^{\cdot}$) a more reactive oxidising species (Rao & Hayon, 1973). In a study by Antunes *et al*. (1996) the perhydroxyl radical is estimated to inflict more than 5 times the damage that the hydroxyl radical is capable of. The protective measures that the cells employ in response to such damage are usually sufficient except when there is excessive ROS production, causing the biological defences to be overwhelmed thus leading to oxidative stress (DiFiglia, 1990).

2.2. EFFECT OF POTASSIUM CYANIDE ON SUPEROXIDE RADICAL GENERATION IN RAT BRAIN HOMOGENATE

2.2.1. INTRODUCTION

Cyanide is a well-established respiratory poison, which exerts its primary toxic effect by inhibiting cytochrome oxidase, the terminal enzyme of the mitochondrial electron transport chain (Isom & Way, 1984). The consequence is an impaired tissue utilisation of oxygen, and with time, oxidative metabolism is brought to a halt. Due to its high dependence on oxidative metabolism and limited anaerobic capacity, the CNS is particularly vulnerable to cyanide intoxication (Way, 1984).

The following experiments were conducted to determine the extent of toxicity that cyanide exerted in rat brain homogenate with respect to the generation of superoxide free radicals.

2.2.2 MATERIALS AND METHODS

2.2.2.1. Animals

Adult, male, Wistar rats purchased from the South African Institute for Medical Research (Johannesburg, South Africa) were used throughout the study. The animals weighed between 250 and 300g and were housed under artificial illumination with a daily photoperiod of 12 hours. The temperature in the animal house was maintained at 20^{o}C to 24^{o}C with four animals per cage and provided with food and water *ad libitum*. An extractor fan ensured the constant removal of stale air.

For the purpose of these experiments, the animals were sacrificed by cervical dislocation and rapidly decapitated. The brains were surgically removed and either used immediately or stored at –70 ^{0}C.

2.2.2.2 Chemicals and Reagents

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

2.2.2.3 Preparation of the Brain Homogenate

The brains were weighed and rapidly homogenised with 0.01M phosphate buffer saline (PBS), pH 7.4, so as to give a final concentration of 10% w/v. This is necessary to prevent lysosomal damage of the tissue.

2.2.2.4 Preparation of Standard Curve

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

2.2.2.5 Nitroblue Tetrazolium Assay

The NBT assay is generally accepted as a simple and reliable method for assaying the superoxide free radical (Ottino and Duncan, 1997). A modification of the assay used by Ottino and Duncan (1997) was used in the following set of experiments. Homogenate (1 ml) containing varying concentrations of KCN (0, 0.25, 0.5, 1mM) was incubated with 0.4 ml 0.1% NBT in an oscillating water bath for 1 hour at 37^{o}C. Termination of the assay and extraction of reduced NBT was carried out by centrifugation of the samples at 2000g and the resuspension of the pellet with 2 ml glacial acetic acid. The absorbance of the glacial acetic acid fraction was measured at 560 nm and converted to μmoles diformazan using a standard curve generated from NBD.

2.2.3 RESULTS

The final results were corrected for dilutions and expressed as µmoles of diformazan produced per mg tissue. Potassium cyanide showed a dose-dependent increase in superoxide radical generation in rat brain homogenate, when compared to the control (Fig. 2.2). The data represented in these experiments is the mean ± SD of five determinations. The results were statistically analysed using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test. The level of significance was accepted at $p<0.05$ (Zar, 1974).

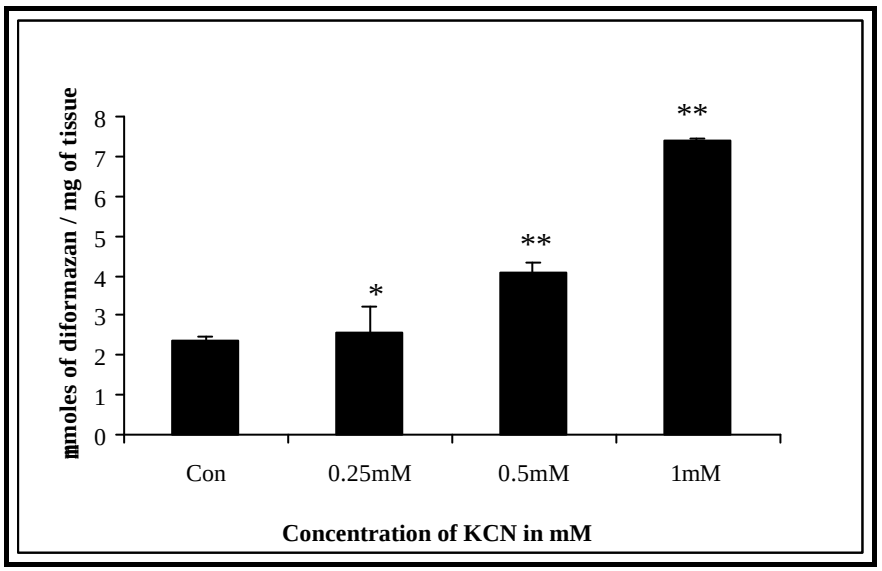


FIG. 2.2 *Concentration-dependent effect of KCN on superoxide generation in whole rat brain homogenate. Each bar represents the mean ± SD, n=5; * (p < 0.05). *** (P < 0.001 in comparison to Control); Student-Newman-Keuls Multiple Range Test.*

2.2.4 DISCUSSION

The results of this study show a concentration-dependent increase in superoxide formation by potassium cyanide in rat brain homogenate, as compared to the control.

Cyanide is a complex IV inhibitor of the mitochondria. ROS production occurs primarily within complex I and complex III. A free radical (ubisemiquinone) is normally generated during the electron transport process, which can potentially donate its unpaired electron to O_2 and generate superoxide. Distal inhibition of the ETC by cyanide augments ROS production in contrast to proximal site inhibitors such as rotenone or myxothiazol, which

allows for the ubiquinone pool to become fully oxidised and ROS generation at complex III is thus nullified (Gregory *et al.*, 2002).

Previous research has also shown cyanide to elevate brain calcium levels (Johnson *et al.* 1986) and produce an increase in free cytosolic calcium in an isolated neuronal cell model (Johnson *et al*. 1987). Elevated calcium levels can lead to activation of numerous neuronal calcium-dependent events and ultimately results in oxidative stress and free radical generation. Isolated cerebral and cerebellar mitochondria have been shown to produce free radicals when exposed to elevated levels of Ca^{2+} (Dykens 1994). Hence, the elevation of calcium levels by cyanide in the mitochondria could be another mechanism by which it induces oxidative stress.

2.3 EFFECT OF CURCUMIN ON CYANIDE INDUCED SUPEROXIDE RADICAL GENERATION IN RAT BRAIN HOMOGENATE

2.3.1 INTRODUCTION

In section 2.2, potassium cyanide showed potent induction of oxidative stress in the form of superoxide radical generation, in rat brain homogenate. Curcumin has been demonstrated to have potent antioxidant properties (Kunchandy & Rao, 1990; Subramanian *et al*., 1994; Sreejayan, 1994). In this study the ability of curcumin to effectively scavenge $O_2^{-\cdot}$ induced by cyanide in rat brain homogenate was investigated.

2.3.2 MATERIALS AND METHODS

2.3.2.1 *In vitro* Exposure of Rat Brain to Curcumin

Curcumin was purchased from Sigma Chemical Co., St. Louis, USA and dissolved in a mixture of ethanol and water in a ratio of 50: 50. The experiments were conducted as described in section 2.2.2. where brain homogenate was incubated at 37^0C for one hour, with the highest concentration of cyanide used previously, alone and in combination with increasing concentrations of curcumin. Following this, the NBT assay was performed.

2.3.2.2 *In vivo* Administration of Curcumin

Curcumin was dissolved in ethyl oleate for the purpose of *in vivo* administration of this agent to the rat. The rats were divided into two groups each containing six rats. One set of rats was injected intraperitoneally with 30mg/kg of curcumin twice daily (test group). The second group of rats was the control group and were injected i.p. with ethyl oleate at the same time and for the same duration as the test group. The animals were injected for three consecutive days and on the third day, were sacrificed and brains removed as described in section 2.2.2.3.

The experiments were conducted as described in section 2.2.2 and in this case, the homogenate was allowed to incubate with three concentrations of cyanide at 37^0C for one hour, after which the NBT assay was performed.

2.2.3 RESULTS

Curcumin showed a dose-dependent inhibition of superoxide generation induced by 1mM potassium cyanide in the rat brain homogenate. Curcumin at concentrations of 0.05 and 0.1mM reduced the levels of diformazan to below that of the control with formation after KCN treatment (Fig. 2.3).

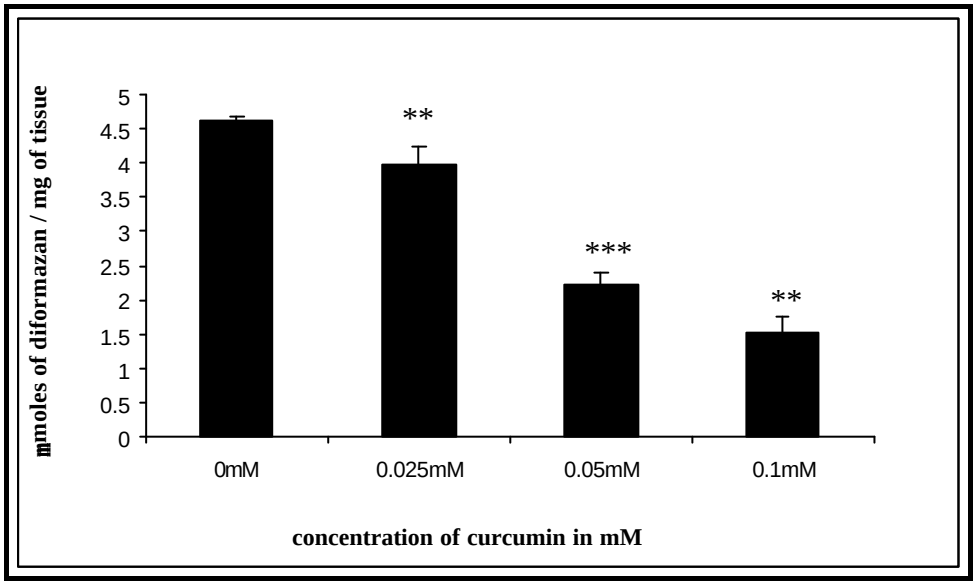


FIG. 2.3 *The effect of curcumin on KCN (1mM)-induced superoxide anion generation on whole rat brain homogenate. Each bar represents the mean ± SD; n=6. ** ($p < 0.01$), *** ($p < 0.001$); Student-Newman-Keuls Multiple Range Test.*

Brain homogenate obtained from rats that were pre-treated with curcumin 30mg/kg i.p. for three consecutive days, showed a significant reduction in superoxide generation by increasing concentrations of potassium cyanide, in comparison to the control (Fig. 2.4). The data represented in these experiments is the mean ± SD of six determinations. The results were statistically analysed as described in section 2.2.3.

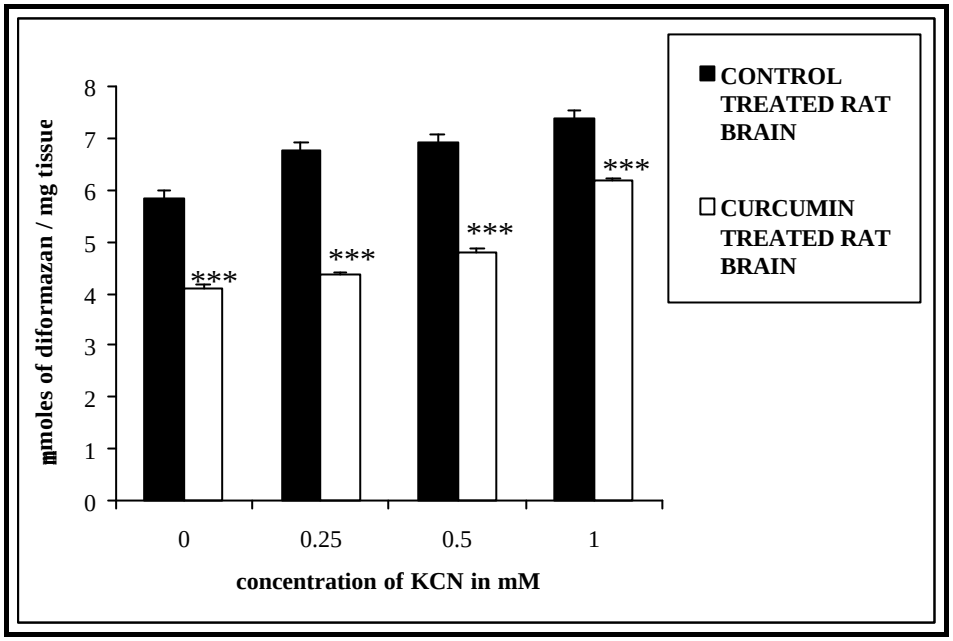


FIG. 2.4 *The effect of increasing concentrations of KCN on superoxide generation in rat brain homogenate obtained from rats pretreated with curcumin 30mg/kg i.p. for three consecutive days. Each bar represents the mean ± SD; n=6. *** ($p < 0.001$); Student-Newman-Keuls Multiple Range Test.*

2.2.4 DISCUSSION

Curcumin at all concentrations significantly reduced the concentration dependent induction of superoxide radicals by KCN. Brains obtained from rats that were pre-treated with curcumin also showed significant protection against cyanide toxicity. The results of this study clearly show that curcumin is a potent inhibitor of superoxide anions generated by cyanide in the brain.

Curcumin has gained much interest in current research circles due to its potent anti-inflammatory and anticancer activities (Srimal, 1987; Ammon *et al*., 1991). Petrone *et al*. (1980) suggests a chemotactic role for superoxide anions in inflammation. Neutrophil generated superoxide reacts with an extracellular precursor to generate a substance chemotactic for neutrophils and this appears to play a major role in communication in neutrophil-mediated inflammatory events. Superoxide dismutase inhibits the appearance of this chemotactic activity but catalase does not. Prevention of production of this factor appears to be the major anti-inflammatory action of superoxide dismutase (Petrone *et al*.,

1980). Hence it could be postulated that the anti-inflammatory activity of curcumin may be attributable to its superoxide scavenging properties. Further studies need to be conducted in this respect.

CHAPTER THREE

BIOLOGICAL OXIDATION ASSAY

3.1 INTRODUCTION

The primary function of the mitochondrion is to generate adenosine triphosphate (ATP), the energy currency of the cell. ATP is a ubiquitous store of energy needed for transport across membranes for all synthetic processes and for the mechanical work involved in motor activities of the cell (Hammans & Tipton, 1994). Hence energetically compromised mitochondria may have detrimental effects on the survival of the cell. Mitochondrial respiratory chain defects have been implicated in the pathogenesis of AD (Grunewald & Beal, 1999) and mitochondrial dysfunction has been associated with the neurodegeneration of PD (Berman & Hastings, 1999).

As mentioned earlier, the mitochondria is also an important site of ROS production particularly the superoxide anions. Oxygen free radicals are natural by-products of respiration. The electrons formed during the oxidation of glucose are passed along the electron transport chain and the protons created during electron transfer are used to drive the synthesis of ATP. A faulty electron transfer at any point in the ETC results in an electron being accepted by atomic oxygen thus resulting in the creation of a superoxide free radical ($O^{2}.^{-}$).

3.2 EFFECT OF CYANIDE ON BRAIN MITOCHONDRIAL RESPIRATORY FUNCTION

3.2.1 INTRODUCTION

Cyanide has already been described in chapter two to be a well established respiratory poison, exerting its toxic effects on the mitochondria by virtue of its inhibition of complex IV, the cytochrome oxidase enzyme, of the mitochondrial ETC (Isom & Way, 1984). The extent of damage that cyanide causes on rat brain mitochondrial function due to impaired respiration was investigated using a modification of the biological oxidation assay described by Plummer (1971).

3.2.2 MATERIALS AND METHODS

3.2.2.1 Animals

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

3.2.2.2 Chemicals and Reagents

All reagents were of the highest quality available. Potassium cyanide (KCN), curcumin, NAD, 2,6 – diclorophenolindophenol (DPI), and L-Malate were purchased from Sigma Chemical Corporation, St. Louis, MO, U.S.A. Sucrose was purchased from Saarchem (PTY) Ltd, Krugersdorp, South Africa. KCN was dissolved in Milli-Q water and curcumin in 50% ethanol for the purposes of the experiment. All other reagents were prepared in 0.1M potassium phosphate buffer, pH 7.4.

3.2.2.3 Isolation of Mitochondria from Rat Brain Homogenate

The rats were killed by cervical dislocation, and the brains were rapidly excised and homogenised with 0.1M potassium phosphate buffer, pH 7.4 to yield a 10% w/v homogenate.

Mitochondrial suspensions were prepared by differential centrifugation according to Plummer (1971). The brain homogenate was centrifuged at 600g for 10 minutes and then separated into supernatant and pellet. The pellet was resuspended in half the volume of 0.32M sucrose and once again centrifuged at 600g for 10 minutes and the supernatant obtained was combined with the previous supernatant. The combined supernatant was centrifuged at 8000g for 10 minutes and the pellet obtained (crude mitochondria) was washed twice in sucrose and then stored on ice until required.

3.2.2.4 Biological Oxidation Assay

A modification of the spectrophotometric method described by Plummer (1971) was employed. The rate of reduction of the synthetic electron acceptor dye, 2,6-dichlorophenol-indophenol (DPI) in presence of the substrate, L-Malate, is used as an indication of the extent of "activity" of the inner mitochondrial ETC. Homogenate containing KCN (1mM) was incubated at 37^0C in a water bath for incubation times of 0, 30 and 60 minutes, after which 1ml of the homogenate was removed and incorporated with NAD (0.5mM), L-Malate (90 μM), DPI (1.5mM) and potassium phosphate buffer. The decrease in absorbance over a 5-minute period was read at 1-minute intervals on a UV/VIS spectrophotometer at 600nm. All data is expressed as absorbance versus time in minutes and corrected for appropriate controls.

3.2.3 RESULTS

The results obtained demonstrate a progressive decline in mitochondrial ETC activity in presence of 1mM cyanide, with time. Fig. 3.1 demonstrates the *in vitro* effect of this agent on ETC activity in comparison to the control at zero incubation time. A significant decrease in absorbance is observed after the first two minutes of taking the readings.

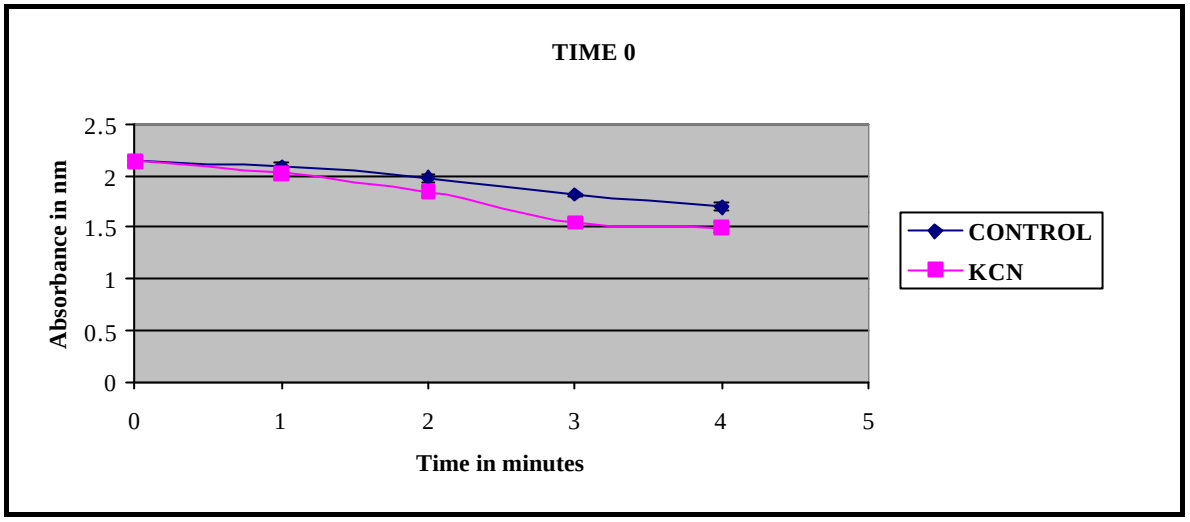


FIG. 3.1 *The in vitro effect of cyanide (1mM) on brain mitochondrial ETC utilising L-Malate as the substrate, after 0 minutes of incubation time.*

Fig. 3.2 shows the effect of cyanide on the ETC activity after 30 minutes of incubation. In comparison to zero time, there is a decrease in activity of both control and cyanide treated brain mitochondrial ETC. The decrease in cyanide treated mitochondria can be observed after the first minute of reading, in contrast to the zero time graph. This can be attributed to the increased time of contact between the mitochondria and the toxin.

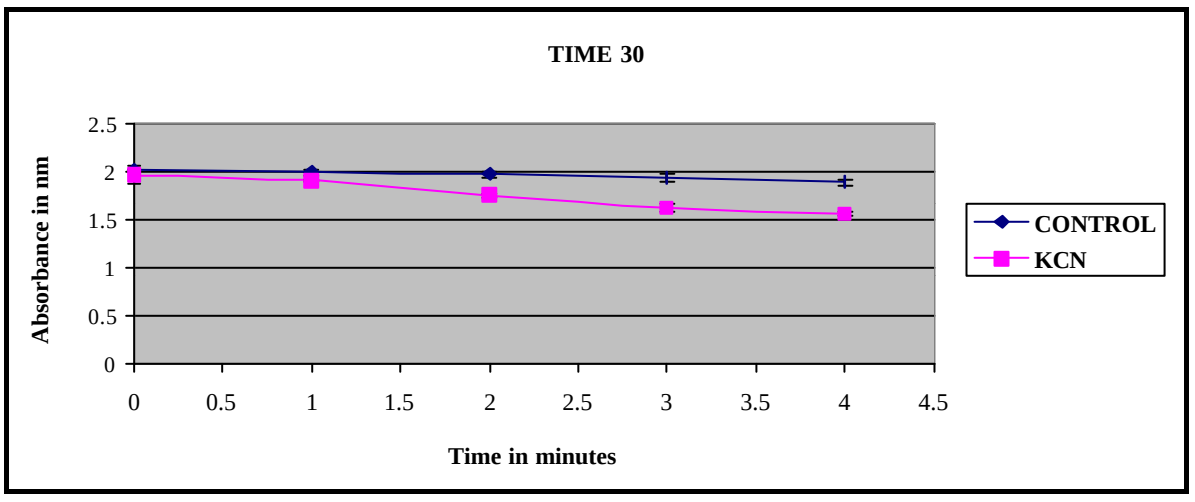


FIG. 3.2 *The in vitro effect of cyanide (1mM) on brain mitochondrial electron transport, using L-Malate as the substrate, after 30 minutes of incubation time.*

Fig. 3.3 depicts the *in vitro* effect of cyanide on brain mitochondrial ETC activity after 60 minutes of incubation. Here again, there is an overall decrease in absorbance for both the control and cyanide treated mitochondria due to the increased time of incubation. A significant decrease in the cyanide treated mitochondrial ETC activity, compared to the control, can be observed just before the 1-minute reading. This drastic change is again attributable to the increased time of contact between the mitochondria and cyanide.

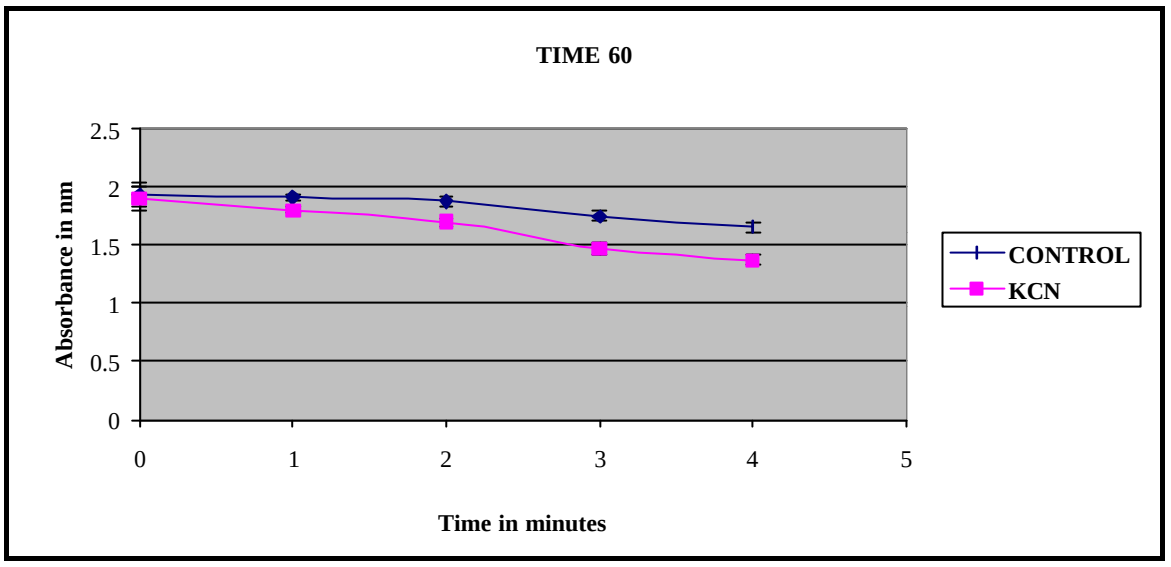


FIG. 3.3 *The in vitro effect of cyanide (1mM) on brain mitochondrial electron transport using L-Malate as the substrate, after 60 minutes of incubation time.*

3.2.4 DISCUSSION

The brain is acutely dependent on energy supplies for normal functioning. The mitochondrion is known to be the intracellular fount of the brain's energy supplies and subtle functional alterations in these essential energy dynamos may lead to insidious pathological changes within neurons (Beal *et al.*, 1993; Beal, 1995). Free radical reactions as well as mitochondrial inhibitors can decrease the activity of the ETC, which in turn leads to the obvious impairment in ATP production, and causes the diversion of electrons from their normal ETC recipients and further formation of damaging free radicals (Cassarino & Bennett, 1999).

The present study has shown that cyanide is capable of significantly reducing the activity of the ETC to levels below that of the control at 0, 30 and 60 minutes of incubation time. At all time intervals a significant decrease in ETC activity of cyanide treated mitochondria can be observed after the first 1-2 minutes depending on the time of contact allowed beween the mitochondria and cyanide. These findings are in accordance with the fact that cyanide interacts with the haem-a-3 portion of cytochrome oxidase (Slater; 1967), thus preventing oxygen utilisation by the mitochondria, and resulting in respiratory dysfunction and oxidative stress.

3.3 EFFECT OF CURCUMIN ON CYANIDE INDUCED IMPAIRMENT OF BRAIN MITOCHONDRIAL RESPIRATORY FUNCTION

3.3.1 INTRODUCTION

Chapter two has shown curcumin to be a potent scavenger of superoxide anions in the brain homogenate. Since much of the superoxide free radicals originate in the mitochondria, the following experiment was conducted to assess any effects of curcumin on the ETC, and whether it was able to offer protection against cyanide induced impairment of the mitochondrial respiratory functioning.

3.3.2 MATERIALS AND METHODS

3.3.2.1 Animals

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

3.3.2.2 Chemicals and Reagents

Curcumin was purchased from Sigma Chemical Corporation, St. Louis, MO, U.S.A. and dissolved in 50% ethanol for the experiment. All other reagents were of the highest quality available and were purchased and solubilised as described in 3.2.2.2.

3.3.2.3 Isolation of Mitochondria from Rat Brain Homogenate

The brain was excised and homogenised as described in 3.2.2.3. Mitochondrial suspensions were prepared by differential centrifugation according to Plummer (1971).

3.3.2.3 Biological Oxidation Assay

A modification of the spectrophotometric method described by Plummer (1971) was employed and was carried out as described in section 3.2.2.4. Homogenate containing

KCN (1mM) and curcumin (1mM), as well as curcumin (1mM) alone, was incubated at 37^0C in a water bath for incubation times of 0, 30 and 60 minutes, after which 1ml of the homogenate was removed and incorporated with NAD (0.5mM), L-Malate (90 μM), DPI (1.5mM) and potassium phosphate buffer. The decrease in absorbance over a 5-minute period was read at 1-minute intervals on a UV/VIS spectrophotometer at 600nm.

All data is expressed as absorbance versus time in minutes and corrected for appropriate controls.

3.3.3 RESULTS

The results obtained show significant protection afforded by curcumin against cyanide induced brain mitochondrial respiratory dysfunction, with respect to an increase in the activity of the ETC in presence of curcumin. Fig. 3.4 shows the *in vitro* effect of curcumin alone and curcumin in presence of cyanide, on the ETC of brain mitochondria, in comparison to the control and cyanide treated mitochondria, at 0 incubation time. The activity of the ETC of curcumin treated mitochondria is significantly higher than that of the control and cyanide treated mitochondria. The absorbance shows a proportionate decrease with time.

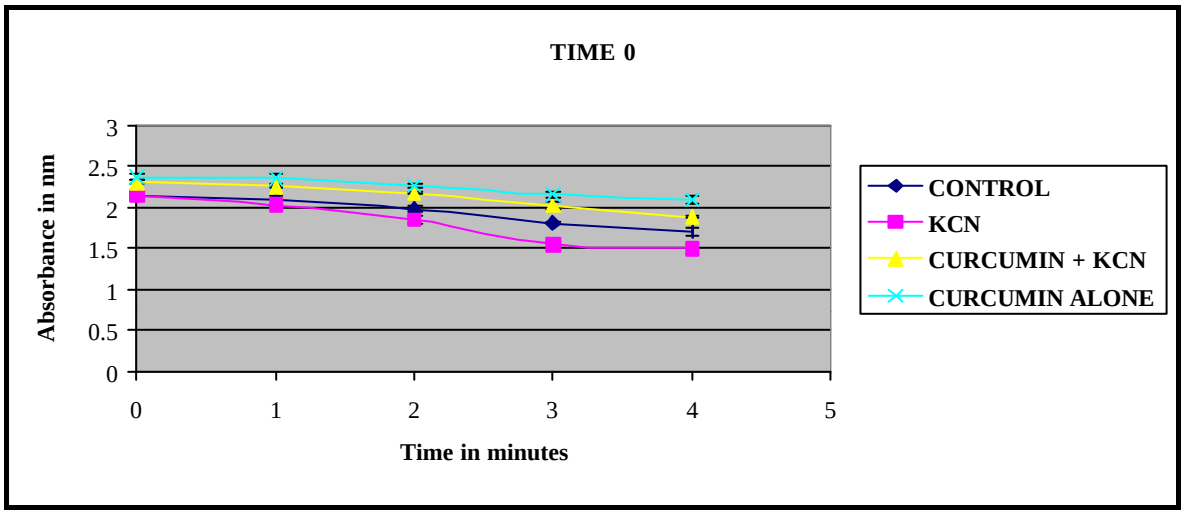


FIG. 3.4 *A comparison of the in vitro effect of curcumin (1mM) alone and in presence of cyanide (1mM) to that of cyanide (1mM) alone and control, on the brain mitochondrial ETC using L-Malate as the substrate, after 0 minutes of incubation.*

Fig. 3.5 shows the *in vitro* effect of curcumin alone, and curcumin in presence of cyanide on the ETC of brain mitochondria, in comparison to the control and cyanide treated mitochondria, after 30 minutes of incubation. Again curcumin shows significant protection against cyanide induced decline in ETC activity and this protection lowers down to the level of the control after the third minute of reading. Mitochondria treated with curcumin alone shows a significantly higher absorbance reading than all the other values, indicating that it is capable of increasing the basal levels of activity within the ETC.

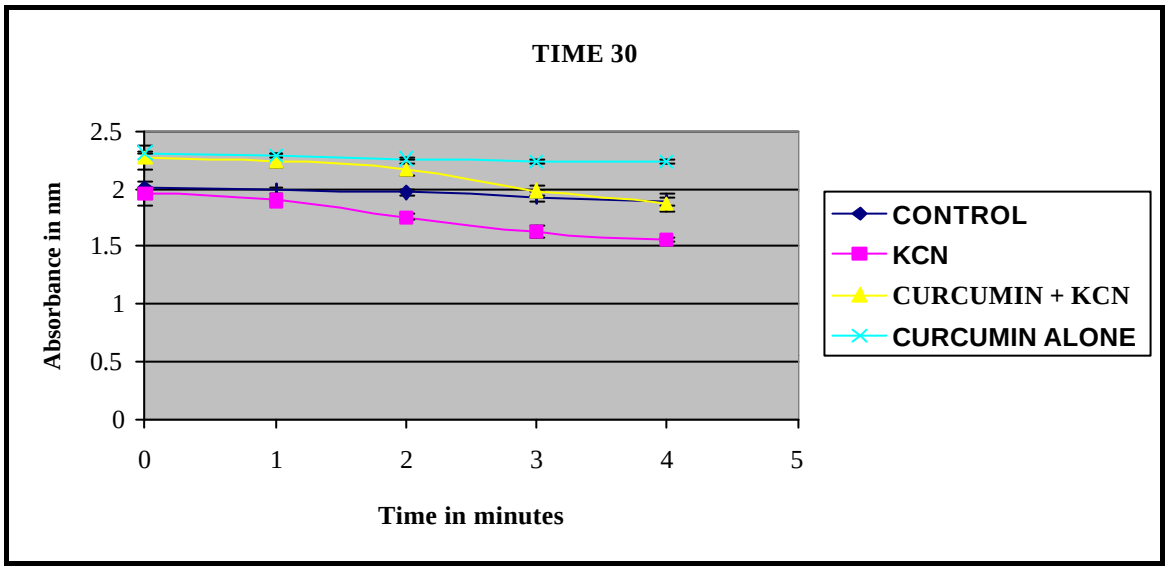


FIG. 3.5 *A comparison of the in vitro effect of curcumin (1mM) alone, in presence of cyanide (1mM), cyanide (1mM) alone and control, on the brain mitochondrial electron transport using L-Malate as the substrate, after 30 minutes of incubation.*

Fig. 3.6 depicts the *in vitro* effect of curcumin alone, and curcumin in presence of cyanide on the ETC of brain mitochondria, in comparison to the control and cyanide treated mitochondria, after 60 minutes of incubation. The overall absorbance is decreased in comparison to the previous two graphs possibly due to the increased time of incubation. Curcumin shows significant protection and the level of protection decreases to below levels of the control after around 2.5 minutes of reading. This could be due to the higher vulnerability of the mitochondria due to the increased time of incubation.

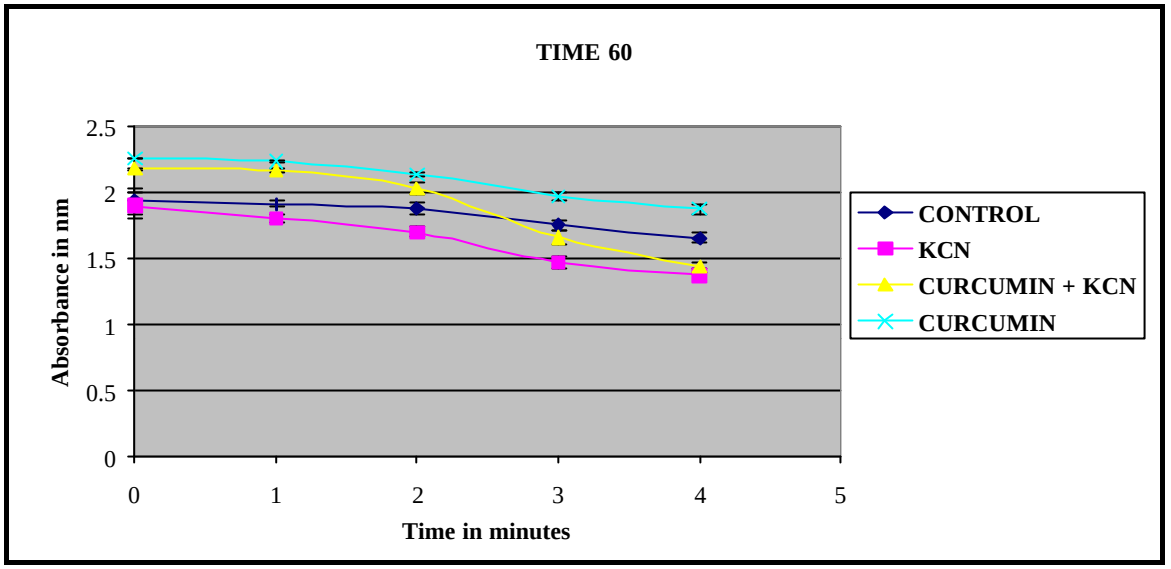


FIG. 3.6 *A comparison of the in vitro effect of curcumin (1mM) alone and in presence of cyanide (1mM) to that of cyanide (1mM) alone and control, on the brain mitochondrial electron transport using L-Malate as the substrate, after 60 minutes of incubation.*

3.3.4 DISCUSSION

The extent of intoxication by cyanide on the ETC of brain mitochondria has been demonstrated and discussed in sections 3.2.3 and 3.2.4. The present results show that curcumin affords significant protection to the mitochondria against cyanide insult with respect to the activity of ETC, and this protection has shown to decrease with time. This however would be expected considering the fact that this experiment is an *in vitro* one, and that the vulnerability of the mitochondria is bound to increase with an increase in time. Curcumin has also shown to increase the basal levels of respiration and ETC activity within the brain mitochondria.

Previous research has shown curcumin to produce an increase in basal oxygen consumption of the mitochondria when succinate was used as substrate and a very small or no effect when pyruvate plus malate was used (Morin *et al.*, 2001). However no data on the effect of curcumin on the respiratory activity of the mitochondria in presence of the substrate malate has been shown. The same authors have demonstrated the capability of curcumin in inducing the mitochondrial permeability transition pore by membrane

protein thiol oxidation under specified conditions and have postulated that this effect is the possible mechanism by which it induces apoptosis in tumour cells. This effect however, has been shown to be independent of the respiratory chain function of the mitochondria (Morin *et al.*, 2001).

Impaired ETC functioning in the brain leads to cell death via a decrease in ATP production or an increase in free radical or ROS production (Beal, 1995). The fact that the major neurodegenerative diseases are age-related may well be accounted for by the fact that the efficiency of the mitochondrial electron transport decreases with age, and the production of hydrogen peroxide and superoxide radicals increases with age (Beal, 1995; Hagen *et al.*, 1997). Curcumin has been shown to potently scavenge superoxide anions in the previous chapter. By increasing the basal level of respiration in the brain mitochondrial electron transport chain, and protecting against cyanide induced impairment of the same, curcumin could serve as a valuable defence system against mitochondria associated dysfunctions in the body.

CHAPTER FOUR

LIPID PEROXIDATION

4.1 INTRODUCTION

Biological membranes are known to contain an astonishing variety of lipids, which satisfy the demands related to membrane structure, fluidity, and permeability (Cullis, *et al.*, 1991). The oxidative destruction of polyunsaturated fatty acids is termed lipid peroxidation, and is known to be extremely damaging because of the self–perpetuating chain reactions they cause (Reiter, *et al., 1996*). Extensive lipid peroxidation in biological membranes results in alterations in fluidity, falls in membrane potential, increase in permeability to H^+ and other ions, and eventual rupture of the cell and its contents such as the lysosomal hydrolytic enzymes (Halliwell, 1990).

Oxidative stress can be reflected in lipid peroxidation to an extent and the brain and nervous system is especially prone to such oxidant damage. This is because of its high levels of unsaturated membrane lipids, which are rich in polyunsaturated fatty acid side chains, and the presence of transition metals such as iron and copper. These metals are capable of participating in the Fenton reaction and generating one of the most destructive of all free radical species, the hydroxyl radical, ultimately resulting in lipid peroxidation. Moreover the brain has shown high utilisation of oxygen and is relatively deficient in antioxidant defence mechanisms such as the protective enzymes superoxide dismutase and catalase (Halliwell, 1990).

4.1.1 Analysis of Lipid Peroxidation by Thiobarbituric Acid Assay

Aldehydes are always produced when lipid hydroperoxides break down in biological systems (Esterbauer, 1982). Malondialdehyde (MDA) is the most abundant individual aldehyde resulting from lipid peroxidation and *in vitro* it has been shown to be capable of altering proteins, DNA, RNA and other biomolecules (Schaunenstein, 1977). The

thiobarbituric assay measures the amount of malondialdehyde resulting from the break-down of lipid hydroperoxides and has served as the most commonly used method for measuring the extent of lipid peroxidation (Yamamoto, 1990).

In the TBA assay one molecule of MDA reacts with two molecules of TBA to produce a pink coloured complex having an absorption maximum at 532 – 535nm. The reaction takes place in acidic conditions (pH of 2 – 3) at 90 to 100^{o}C for one hour. Trichloroacetic acid (15% w/v) is added to the tissue sample to precipitate protein, which is pelleted by centrifugation. An aliquot of the supernatant is allowed to react with 0.66% w/v TBA in a boiling water bath for one hour. Butylated hydroxytoluene in ethanol is added prior to TCA precipitation to ensure that no lipid oxidation occurs during the assay and interferes with the results obtained. After cooling the absorbance is read at 532nm and the concentration of MDA determined from a standard curve generated from 1, 1, 3, 3-tetramethoxypropane.

4.2 EFFECT OF QUINOLINIC ACID ON LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE

4.2.1. INTRODUCTION

Quinolinic acid (2,3-pyridine dicarboxylic acid) is a neurotoxic metabolite of the tryptophan – kynurenine pathway (Heyes & Morrison, 1997) and is present in the human and rat brain in nanomolar concentrations (Stone, 1993). Substantial increases in the concentration of this neurotoxin occur in the body as a consequence of a broad spectrum of infections and inflammatory neurological diseases. The following experiment was conducted to investigate the effect of QA on lipid peroxidation in rat brain homogenate.

4.2.2. MATERIALS AND METHODS

4.2.2.1. Animals

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

4.2.2.2. Chemicals and Reagents

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

4.2.2.3. Preparation of the Brain Homogenate

The rats were sacrificed by cervical dislocation, and the brains were removed and homogenised in the same manner described in section 2.2.2.3.

4.2.2.4. Preparation of Standard Curve

A series of standards (0-20 nmoles/ml) was prepared using 1,1,3,3-tetramethoxypropane (MDA) and a standard curve generated by measuring the absorbance at 532nm using a GBC UV/VIS 916 spectrophotometer and plotted against the molar equivalent weight of MDA in the complex assayed (appendix 2).

4.2.2.5. The TBA Assay

A modification of the method of Ottino & Duncan (1997) was used in this experiment. Rat brain homogenate was prepared as described earlier and incubated in a shaking water bath for one hour at 37^0C with varying concentrations of quinolinic acid to make a final volume of 1ml. 0.5ml of BHT (0.5g/l ethanol) was added to each tube after incubation, followed by the addition of 1ml of TCA (10%w/v). The tubes were then centrifuged at 2000rpm for 20 minutes to remove insoluble proteins. 2ml of the supernatant was transferred to a clean set of tubes and 0.5ml TBA (0.33g/100ml Millipore water) was added. The tubes were boiled for one hour at 95^0C, and then cooled on ice. Thereafter the TBA-MDA complex was extracted with butanol and read at 532nm. MDA levels were determined from the standard curve generated from 1,1,3,3-tetramethoxypropane.

4.2.3. RESULTS

The final results are expressed as nanomoles of MDA produced per mg tissue. Statistical analysis was carried out using ANOVA followed by the Student Newman Keuls multiple range test. The level of significance was accepted at $p<0.05$. The data represented for these experiments is the mean ± SD of five determinations ($n = 5$). The results show a significant concentration dependent induction in lipid peroxidation by QA with respect to the control (Fig. 4.1).

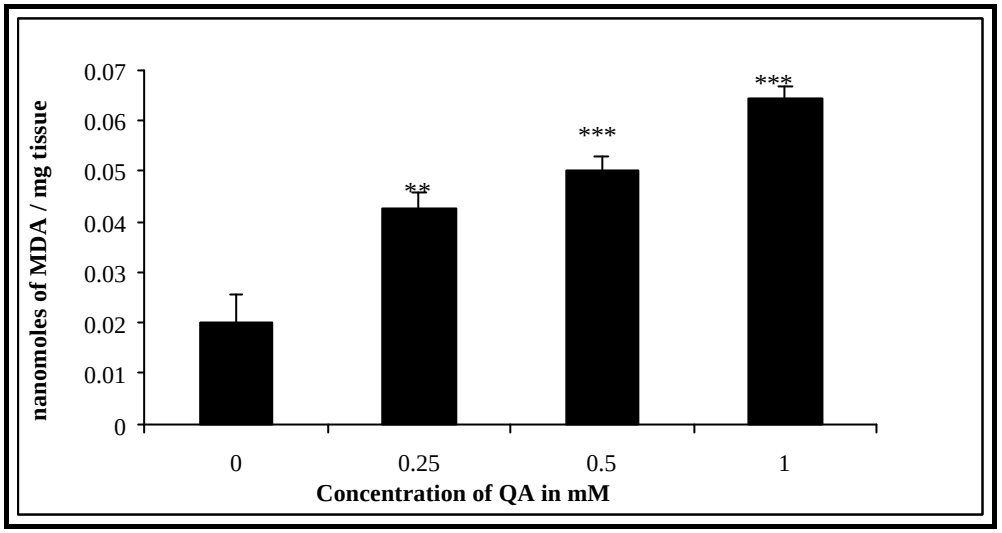


FIG. 4.1 *Concentration dependent effect of quinolinic acid on lipid peroxidation in whole rat brain homogenate. Each bar represents the mean ± SD; n=6. ** (p < 0.01) *** (p<0.001)(Student-Newman-Keuls Multiple Range Test).*

4.2.4. DISCUSSION

Quinolinic acid is an established agonist of the glutamate receptor and previous research has shown that among a number of glutamate receptor agonists including QA, Kainic acid, and NMDA, only QA potently induced lipid peroxidation in the rat brain homogenate (Rios *et al.*, 1991). These findings are consistent with the results obtained in the present experiment where QA in concentrations of 0.25, 0.5 and 1mM showed significant induction of lipid peroxidation in the rat brain homogenate in a dose-dependent manner.

Since QA is not readily metabolised in the synaptic cleft, it stimulates the NMDA receptor for prolonged periods. This sustained stimulation results in opening of calcium channels causing Ca^{2+} influx followed by Ca^{2+} dependent-enhancement of free radical production leading to molecular damage and often to cell death (Stone and Perkins, 1981). In the brain homogenate, however, the mechanism by which QA induces oxidative stress is not well understood. Studies performed by Stipêk *et al.* (1996) showed that QA did not induce lipid peroxidation in brain homogenate when co-incubated with an iron-chelator.

Spectral analysis revealed that QA was capable of chelating ferrous ions to form a complex and it was suggested that it is this complex that is responsible for stimulating lipid peroxidation in the rat brain homogenate. Thus the implication is that QA does not have a direct peroxidative effect *in vitro*, but modulates lipid peroxidation through its interaction with ferrous ions.

4.3. EFFECT OF CURCUMIN ON QUINOLINIC ACID INDUCED LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE

4.3.1. INTRODUCTION

The oxidative damage caused by QA in rat brain homogenate has been demonstrated and discussed in the section 4.2. The effect of curcumin, a known antioxidant (Kunchandy & Rao, 1990), on QA induced lipid peroxidation in the rat brain homogenate, was investigated.

4.3.2 MATERIALS AND METHODS

4.3.2.1. *In Vitro* Exposure of Rat Brain to Curcumin

The experiments were carried out as described in section 2.2.2. except that only the highest concentration (1mM) was used, and three sets of tubes containing QA in the presence of increasing concentrations of curcumin were employed. Curcumin was purchased from Sigma Chemical Co, St. Louis, USA and dissolved in a mixture of ethanol and water (50: 50) for the purposes of this experiment.

4.3.2.2. *In Vivo* Administration of Curcumin

Curcumin was dissolved in ethyl oleate for the purposes of these experiments. The rats were divided into two groups each containing six rats each. One set of rats was injected intraperitoneally with 30mg/kg curcumin, twice daily. The other set was injected with ethyl oleate at the same time and for the same duration as the test rats. The rats were injected for three days, and on the third day were sacrificed and the brains removed and homogenised as described in 2.2.2.3. Lipid peroxidation assays were carried out as described earlier, on these brains, in presence of three concentrations of QA.

4.3.3. RESULTS

The co-treatment of homogenate with increasing concentrations of curcumin showed a dose-dependent and significant decrease in lipid peroxidation induced by QA (Fig. 4.2). Curcumin in concentrations of 0.05 and 0.1mM significantly decreased MDA produced by the control treated samples.

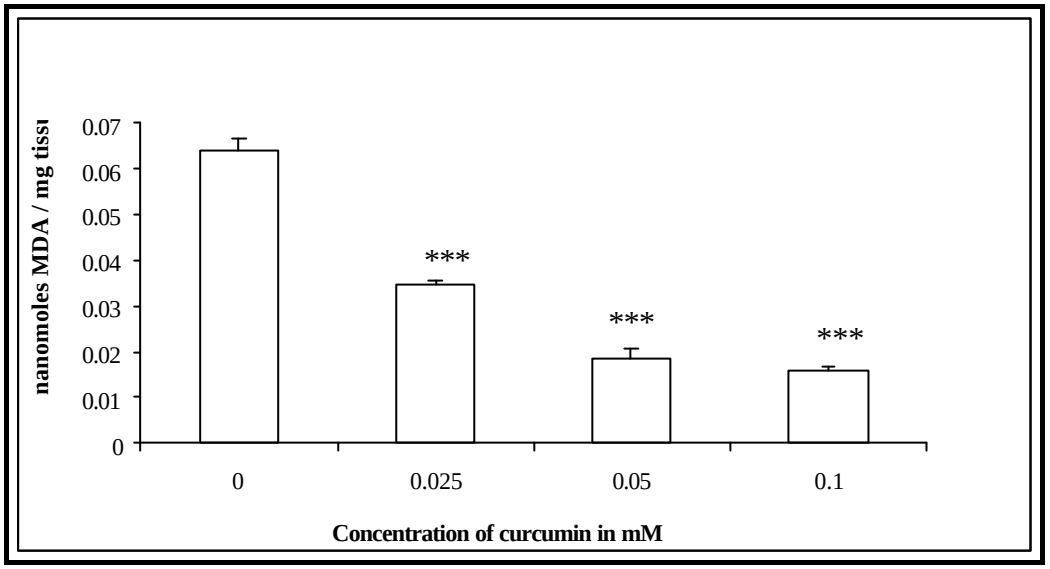


FIG. 4.2 *The effect of curcumin (in vitro administration) on QA (1mM)-induced lipid peroxidation in whole rat brain homogenate. Each bar represents the mean ± SD; n=6; *** (p < 0.001). Student-Newman-Keuls Multiple Range Test.*

With respect to the brains treated with curcumin 30mg/kg *in vivo*, the test showed a significant reduction in MDA produced by all three concentrations of QA (Fig. 4.3). Curcumin administered at this dose also showed a significant inhibition of lipid peroxidation produced by the control in absence of QA.

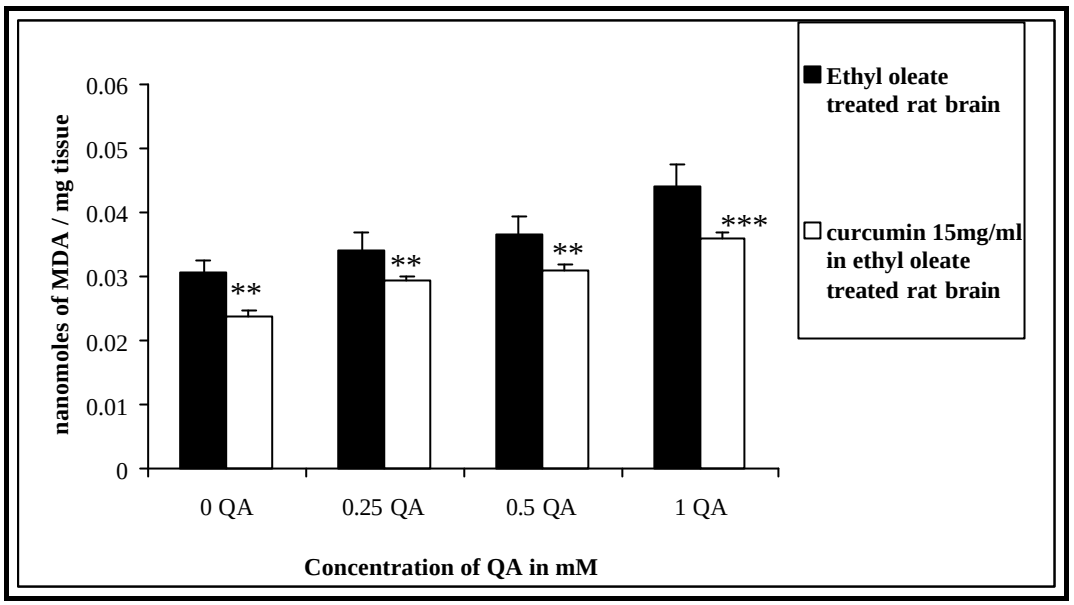


FIG. 4.3 *The effect of increasing concentrations of QA on MDA levels in rat brain homogenate obtained from rats treated with 30mg/kg curcumin (i.p.). Each bar represents the mean±SD (n=5). ** (p<0.01) ***(P<0.001) Student Newman Keuls Multiple Range Test.*

4.3.4. DISCUSSION

The results show clearly that curcumin, administered *in vivo* and *in vitro* to the rat brain, is capable of decreasing QA acid induced lipid peroxidation in a dose-dependent manner, as well as reducing the lipid peroxidation that occurred in the control samples. Curcumin is a known antioxidant and free radical scavenger and much of its protective abilities could be attributed to these properties.

Although the phenolic group and methoxy group are essential for its antioxidant effect, previous studies conclude that these groups are not essential for curcumin to exert its inhibiting action on lipid peroxidation in general. Tønnesen & Greenhill (1992) found that 5-hydroxy-1,7,diphenyl-1,4,6-heptatriene-3-one, a curcumin analog that lacks the phenolic and methoxy groups, was as active in scavenging hydroxyl radicals and in other redox properties as curcumin is. A report by Jovanovic *et al.* (1999) describes the H-atom donation from the β-diketone moiety to a lipid alkyl or a lipid peroxyl radical as a potentially more important antioxidant action of curcumin with respect to lipid peroxidation (Fig. 4.4).

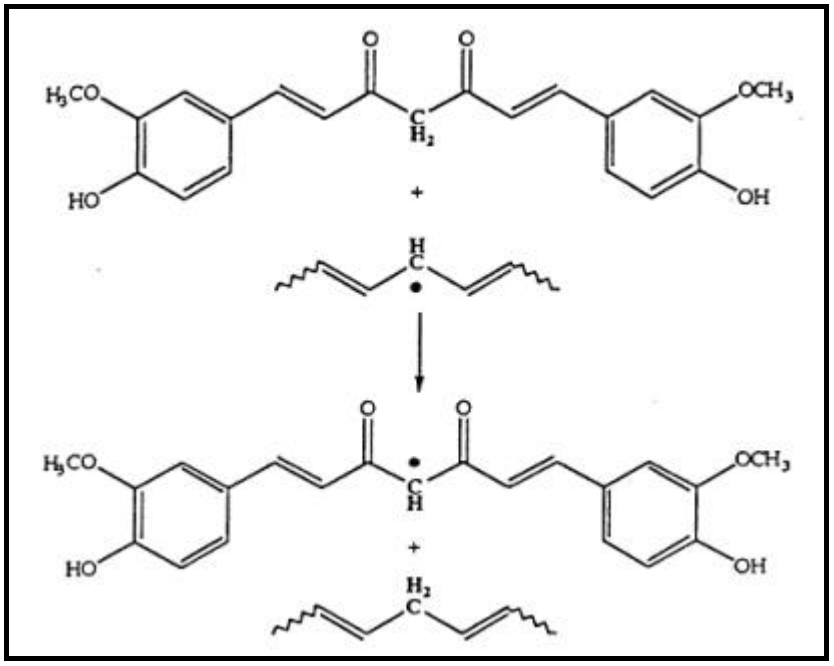


FIG. 4.4 *H-atom donation of curcumin to a bis-allylic radical (e.g. the linoleic acid radical (Jovanovic et al., 1999).*

The result is the formation of a curcumin radical, with unpaired electron density between three carbon and two oxygen atoms. If oxygen is added to the central carbon atom, a peroxyl radical is formed. This is an undesirable reaction because the peroxyl radicals propagate lipid peroxidation. However it is also possible that the curcumin radicals may react with each other or with other free radicals to yield stable products such as vanillin, ferrulic acid and curcumin dimers (Masuda *et al.*, 1999).

Jovanovic *et al.* (2001) showed that the predominant curcumin radical that is formed is the phenoxyl radical. Studies performed by these authors state that curcumin, being the potent lipid-soluble antioxidant that it is, positions itself within the cell membrane, where it is able to intercept lipid radicals and thereby converts itself into the phenoxyl radical. This radical is more polar than curcumin so it is capable of moving to the surface of the membrane, where it may be “repaired” by any water-soluble antioxidant (Fig. 4.5).

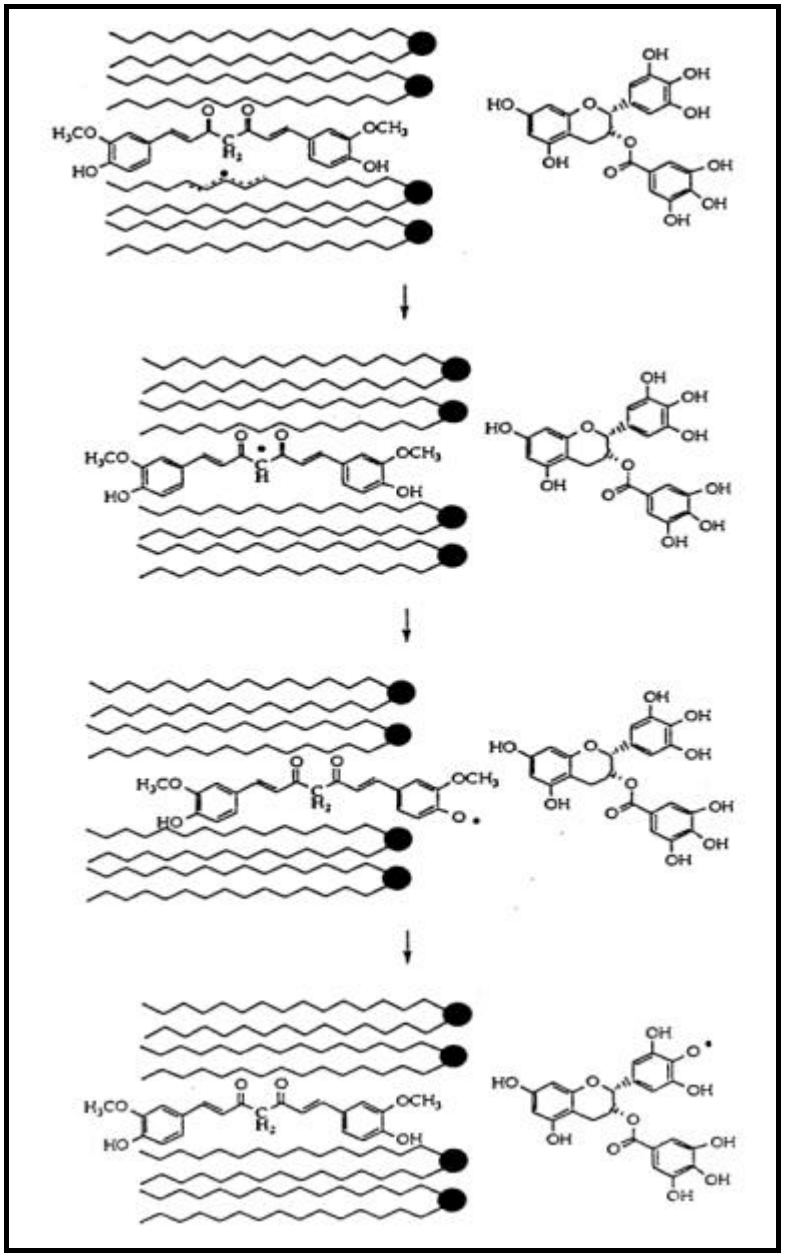


FIG. 4.5 *Diagramatic representation of the antioxidant action of curcumin within the cell membrane (Jovanovic et al.1999).*

The mode of action of QA in inducing lipid peroxidation in rat brain homogenate has been discussed as one involving a complex formation between QA and ferrous ions. The possibility that curcumin may be competing with QA for ferrous ion complexation has been investigated in chapter six and seven.

4.4. EFFECT OF TOXIC METALS, CADMIUM AND LEAD, ON LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE

4.4.1. INTRODUCTION

The brain is known to be a rich depot of metals, both essential and toxic. Lead and cadmium are two established neurotoxins and the following experiment was aimed at investigating whether induction of oxidative stress in the form of lipid peroxidation in the rat brain homogenate, could be a possible mechanism of neurotoxicity.

4.4.2. MATERIALS AND METHODS

Cadmium chloride was dissolved in Milli-Q water to give final concentrations of 0.1, 0.05 and 0.025mM in the brain homogenate. Lipid peroxidation assay was carried out with cadmium chloride on 10 % w/v rat brain homogenate in the same manner as described in section 4.2.2. Lead acetate was solubilised in Milli-Q water to give a final concentration of 0.01mM in the brain homogenate. Only one concentration of lead acetate was used and a time-dependent lipid peroxidation assay was carried out on the brain homogenate by assaying at time intervals of 0, 60, 120 and 180 minutes.

4.4.3. RESULTS

Cadmium chloride in increasing concentrations showed a proportionate and significant increase in lipid peroxidation when incubated with the rat brain homogenate for 1 hour as shown in Fig. 4.6. Lead acetate in a concentration of 0.01mM showed significant lipid peroxidation when compared to the control as shown in Fig. 4.7. As the time of incubation increased, there was a proportionate and significant increase in lipid peroxidation.

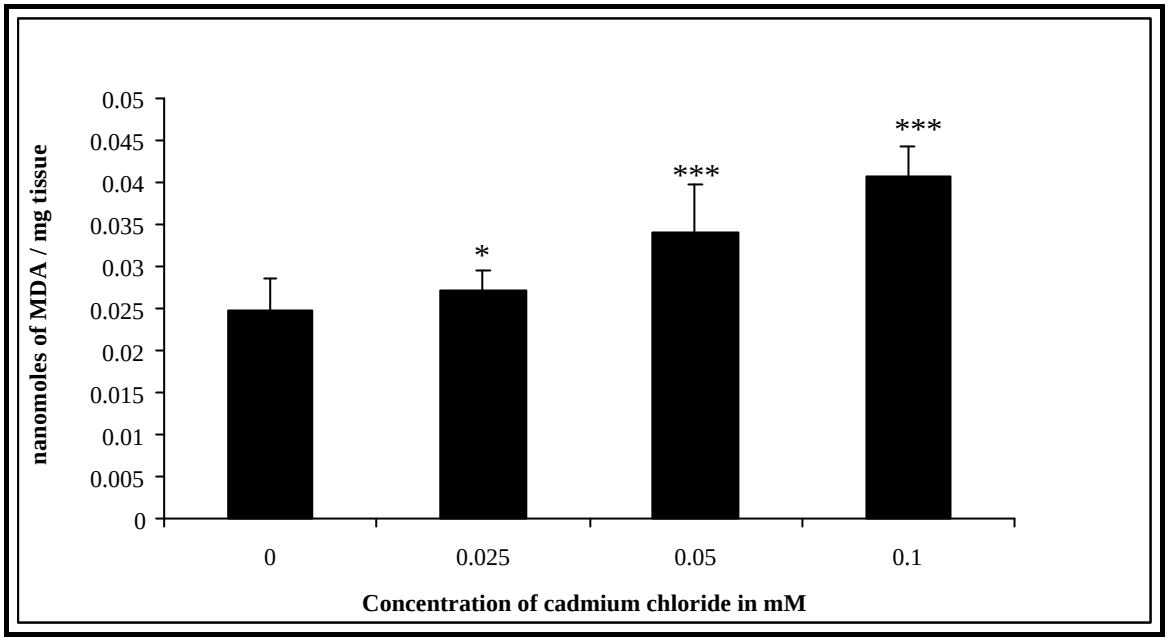


FIG. 4.6 *The effect of increasing concentrations of cadmium chloride on MDA production in rat brain homogenate after incubation for 1 hour. Each bar represents the mean ± SD; n=5. * (p<0.05) *** (p< 0.001). Student-Newman-Keuls Multiple Range Test.*

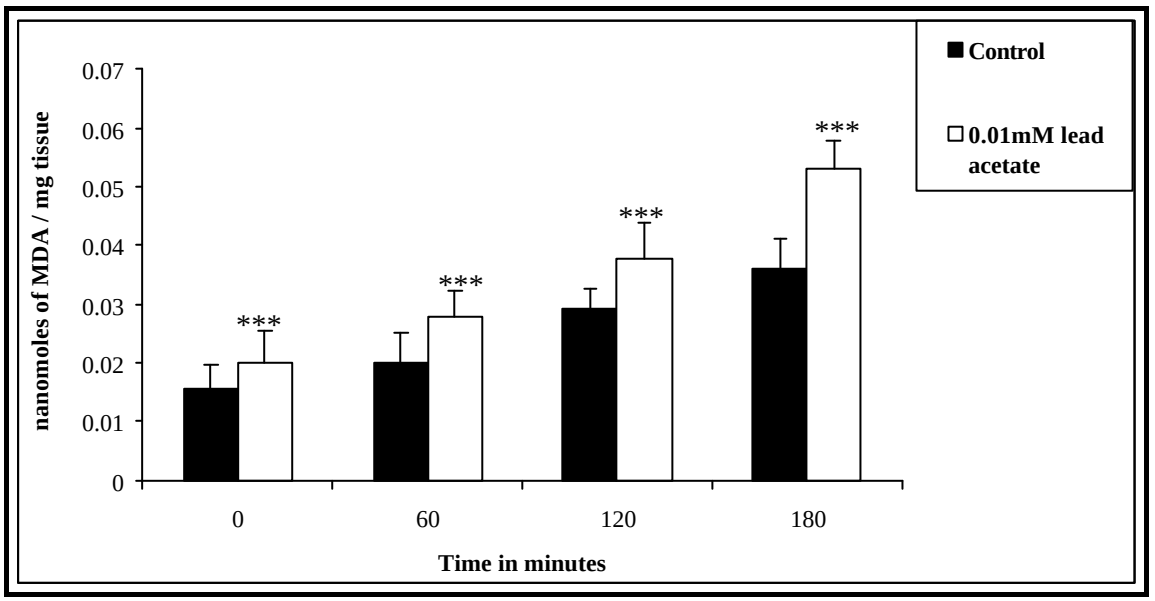


FIG. 4.7 *The effect of 0.01mM lead acetate on MDA production in rat brain homogenate. Each bar represents the mean±SD; n=5. *** (p< 0.001). Student-Newman-Keul's Multiple Range Test.*

4.4.4. DISCUSSION

A dose-dependent induction of MDA was obtained when homogenate was co-treated with cadmium. Cadmium-induced lipid peroxidation has been extensively studied in several laboratories but its peroxidative mechanism is a controversial matter, as cadmium does not undergo redox cycling and probably acts through indirect mechanisms (Casalino *et al.,* 1997; Yiin *et al.,* 2001).

Casalino suggested that cadmium displaces iron from its binding sites resulting in iron-induced lipid peroxidation (Casalino *et al.,* 1997). *In vitro* experiments showed that cadmium also displaced and substituted manganese and zinc, resulting in a strongly reduced MnSOD and CuZnSOD activity and thus proposing a non-specific interaction between cadmium and superoxide dismutase activity (Hussain *et al.,* 1987, 1999).

The experiments conducted also reveal a significant induction in lipid peroxidation by lead acetate in the rat brain homogenate, and this was proportionately increased as the time of incubation increased. In brain homogenate the mode by which lead causes lipid peroxidation is conjectural. Enhanced lipid peroxidation is associated with stimulation of arachidonic acid metabolism and prostaglandin formation (Takahashi *et al.,* 1991).
A close association among phospholipid methylation, arachidonic acid release from phospholipid and increased prostaglandin was found (Crew *et al.* 1980). Lead is postulated to be a facilitator of the conversion process of phosphatidylcholine (a major methylated phospholipid) into arachidonic acid and the metabolite prostaglandin.

Early work on lead toxicity has also established an interaction between iron, zinc and lead. Lead, zinc and iron are postulated to interact with identical proteins, possibly transport proteins, which mediate metal uptake (Bressler *et al.*1999). Lead ions may directly or indirectly alter the integrity of the membrane by reacting with sulphydryl groups and lipids and displace Cu^{2+} (Nakagawa *et al.,* 1980). Rivarov showed that lead is capable of accelerating lipid peroxidation in the presence of haemoglobin by interaction with it leading to the production of hydrogen peroxide (Rivarov *et al.,* 1982).

Lead has also shown to enhance ferrous ion initiated lipid peroxidation in liposomes, erythrocytes, microsomal fractions and rat brain homogenates (Quinlan *et al.*, 1988, Oeiza *et al.*, 1995). These observations indicate that lead does cause the degradation of phospholipids and possibly augments lipid peroxidation caused by other metals such as iron and copper. This corresponds to the findings of this study, which show a proportionate rise in lipid peroxidation in the rat brain homogenate with an increase in time of incubation.

4.5. EFFECT OF CURCUMIN ON CADMIUM AND LEAD INDUCED LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE

4.5.1. INTRODUCTION

Since curcumin was shown to protect the rat brain from lipid peroxidation induced by QA in section 4.3, the following experiments were conducted to determine whether curcumin could protect against lipid peroxidative damage induced by toxic metals cadmium and lead.

4.5.2 MATERIALS AND METHODS

The experimental protocol was carried out as described in section 4.2.2. and the homogenate was exposed directly to curcumin as described in section 4.2.2.1.

4.5.3 RESULTS

Three increasing concentrations of curcumin were able to significantly decrease MDA production in the cadmium treated brain homogenate to levels that were lower than the control. The decline in lipid peroxidation was dose-dependent, with maximum protection occurring at co-incubation of the homogenate with equimolar concentrations of curcumin and cadmium as shown in Fig. 4.8.

The steady increase in lipid peroxidation induced by 0.01mM of lead acetate over a time period of 0 till 180 minutes was significantly reduced in the presence of equimolar concentrations of curcumin to levels below that of the control (Fig. 4.9).

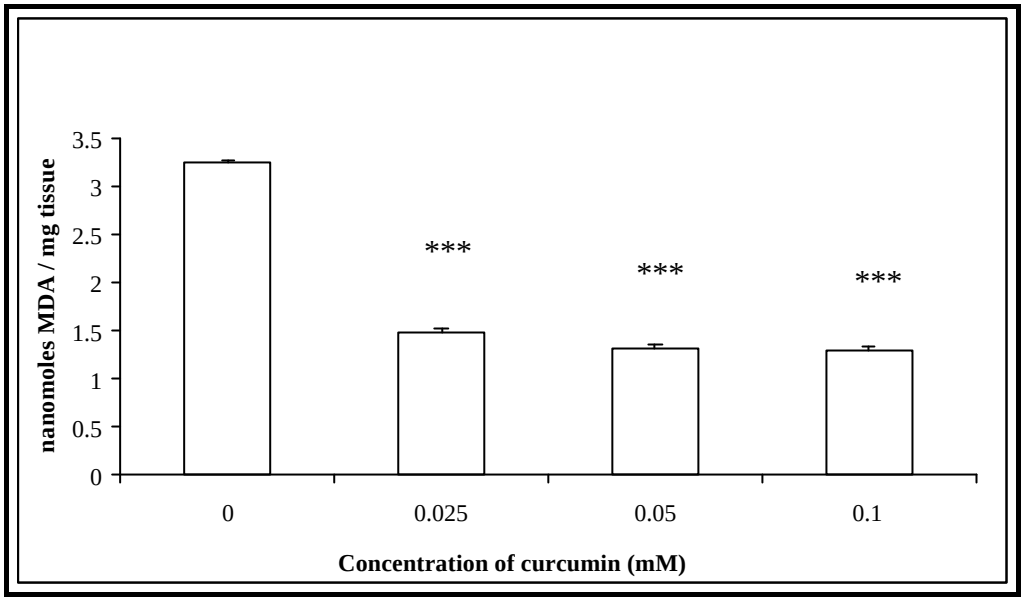


FIG. 4.8 *The effect of increasing concentrations of curcumin on MDA produced by 0.1mM cadmium in rat brain homogenate. Each bar represents the mean±SD; n=5. *** (p< 0.001). Student-Newman-Keuls Multiple Range Test.*

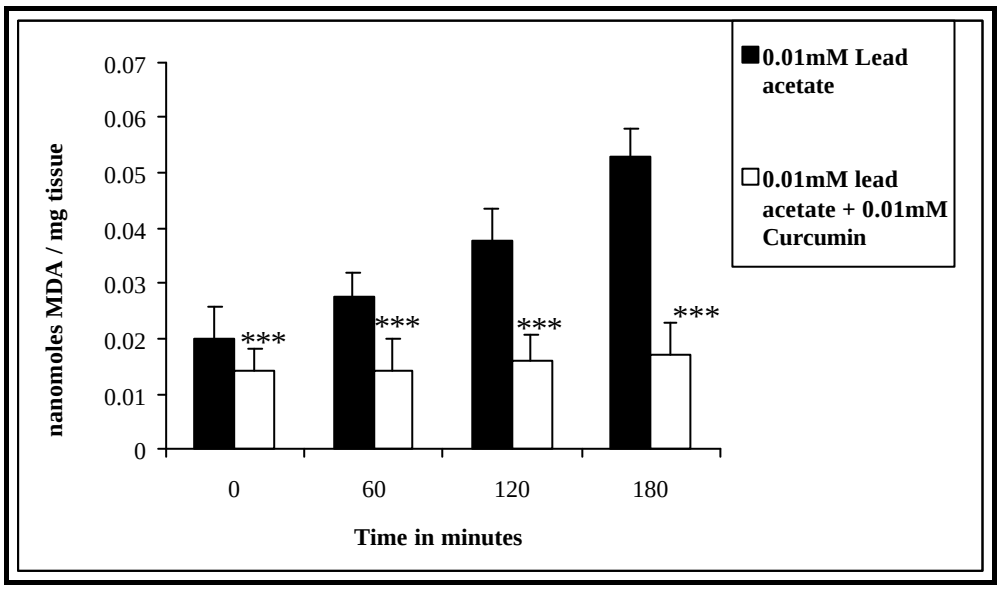


FIG. 4.9. *The effect of 0.01mM curcumin on MDA produced by equal concentrations of lead in rat brain homogenate. Each bar represents the mean±SD; n=5. *** (p< 0.001). Student-Newman-Keuls Multiple Range Test.*

4.5.4 DISCUSSION

Cadmium has been shown to produce a dose-dependent increase in lipid peroxidation in section 4.4.3. and it has been suggested that the mechanism by which this occurs is through the displacement of iron from its binding sites, resulting in iron-induced lipid peroxidation, as well as the displacement of the metals crucial to the optimal functioning of the protective enzyme, superoxide dismutase. Curcumin in increasing doses showed a proportionate decline in lipid peroxidation induced by 0.1mM cadmium (Fig. 4.8).

Curcumin has been shown to protect against iron-induced lipid peroxidation by forming a complex with ferric ions (Reddy *et al.*, 1994) and if cadmium is inducing toxicity through this manner, then it is likely that curcumin is preventing iron-induced lipid peroxidation. Curcumin being a free radical scavenger, could also be exerting a direct effect on the free radicals that are being produced. A direct effect of curcumin on cadmium is another possibility, i.e. the formation of a complex between curcumin and cadmium, thus preventing cadmium from displacing iron. This has been investigated in chapter six and seven.

The protective effects of curcumin on lead induced lipid peroxidation (Fig. 4.8) could be directly attributed to its antioxidant as well as its anti-inflammatory properties. Curcumin exerts its anti-inflammatory action through the inhibition of the cyclooxygenase pathway, thereby inhibiting arachidonic acid and prostaglandin synthesis. As mentioned earlier, lead has been postulated to facilitate the production of arachidonic acid, which is speculated to be a mode of contribution toward oxidative stress in the form of lipid peroxidation. Thus curcumin could be reducing lead-induced increase in arachidonic acid production. The possibility of chelation between lead and curcumin has also been investigated as another mode of protection by this agent against lead-induced toxicity, in chapter six and seven.

CHAPTER 5

HISTOLOGICAL ANALYSIS

5.1 INTRODUCTION

Histology is a term derived from the Greek word *histos*, meaning tissue, and *logia*, meaning "the study of" or knowledge and it involves the examination of preserved, sectioned and stained tissues of both plants and animals.

Nervous tissues have a wide distribution throughout the body, innervating most visceral and peripheral tissues. Within the nervous system, there are two basic types of cells, neurons and supportive cells. Neurons are highly specialised cells that easily conduct nerve impulses and are easily excited to produce them. Typical neurons as shown in Fig. 5.1 show a large cell body with large central nucleus and many cytoplasmic extensions of which there are two types: dendrites and axons. These features are also visible on a stained section of nervous tissue observed under the microscope depicted in Fig. 5.2).

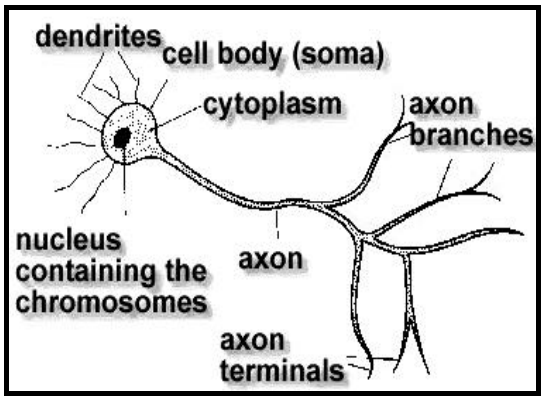
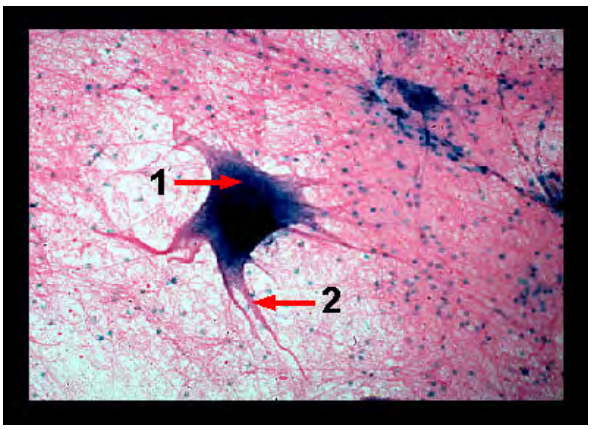


FIG. 5.1 *The basic structural features of a neuron* (www.wutsamada.com/alma/).

The dendrites are numerous and serve to collect incoming signals either from the periphery or other neurons, and send them to the cell body. The axon is a long process that conducts an electrical impulse away from the neuronal cell body to stimulate other

neurons or tissues. Most neurons have a single axon, although some can have more than one. Some axons are covered with a sheath of myelin.



1 Nerve Cell Body

2 Nerve Cell Processes

FIG. 5.2 *A high power magnified section of nervous tissue showing large star shaped neuronal cell body and the nerve cell processes (*bioweb.uwlax.edu/.../ nervous_tissue__spinal_neuron_.htm).

The supportive cells assist the neuron with its functioning and are found in conjunction with the neurons. There are various types of supportive cells also known as glial cells:

- **Astrocytes**: These cells in the brain have a small and round nucleus and many thin processes radiate from them. The cytoplasm contains intermediate filaments that are composed of glial fibrillary acidic protein which serves to identify these cells.

- **Oligodendroglia**: These are small, round cells that are most prominent in the white matter of the brain. These cells are responsible for myelinating the axons of the myelinated neurons of the CNS. Myelination of the nerve fibres allows for faster conduction of nerve impulses. A single oligodendrocyte can provide myelin for as many as 40 neurons.

- **Microglia**: These cells are part of the mononuclear phagocyte system of fixed macrophages in tissues. These are produced in numerous amounts during injury.

- **Schwann cells**: These cells are responsible for myelination of the nerve fibres in the peripheral nervous system.

5.2 HISTOLOGICAL ANALYSIS OF THE EFFECTS OF LEAD ON THE HIPPOCAMPUS

5.2.1 INTRODUCTION

The greatest amount of lead found in the brain is localised in the hippocampus, cerebellum, cerebral cortex, and medulla (Davidson, 1994). Since lead at low doses damages the functioning of the BBB, it is difficult to analyse whether the distribution of lead within the CNS reflects the relative vascularisation of different brain regions as opposed to a specific uptake and distribution process (Goldstein, 1994). The neuropathological effects of lead on cellular elements of the CNS include cell loss, haemorrhage and oedema (Goldstein, 1990).

In the present study, the structural effects that lead causes in the hippocampal CA1 and CA3 regions were examined using histological analysis.

5.2.2 MATERIALS AND METHODS

5.2.2.1 Animals

Adult, male, Wistar rats weighing between 250 and 300g were used for this study and were cared for as described in section 2.2.2.1. A test group of rats received 25mg/kg of lead acetate intraperitoneally (i.p), once daily for seven days and control rats received water in the same manner and for the same duration of time. On the seventh day the rats were sacrificed and brains removed as described in section 2.2.2.3.

5.2.2.2 Chemicals and Reagents

Lead (II) acetate and cresyl violet stain was purchased from BDH Laboratory Supplies, England. DPX was purchased from Philip Harris Ltd. England. Haupt's adhesive consisted of the following: 1g gelatine, 100ml water, 2g phenol and 15ml glycerol. All other chemicals used were of the highest quality available and were purchased from commercial distributors.

5.2.2.3 Histological Techniques

5.2.2.3.1 Fixation of the Brain

Neural tissues are extremely fragile and easily subjected to rapid anoxic and postmortem changes hence immediate fixation of these tissues is required (Chang, 1995). This process usually entails submerging the tissues in stabilising or cross – linking agents or perfusing them with these substances in order to preserve as much as possible of their morphological and molecular characteristics. Here, the brains were immediately and rapidly fixed in a mixture of formol (30%), glacial acetic acid and ethanol in a ratio of 2:1:7 v/v for two hours.

5.2.2.3.2 Tissue Processing

Moisture was extracted from the tissue fragments by bathing them successively in graded series of mixtures of ethanol, and this step was followed by the clearing process that involves the removal of ethanol by immersing the tissue in xylene twice for one hour each. The tissue was then submerged in molten paraffin wax at 57^0C twice for one hour each, which facilitated the removal of xylene and the thorough penetration of the tissue. This stage provides the hardness and support that the tissue requires for sectioning.

5.2.2.3.3 Blocking Out

This procedure was performed to form a support that would facilitate sectioning using the rotary microtome. This was done by fixing the brain material into a block using a mould. A plastic ice tray coated with ethanol- glycine was used as the mould. Molten wax was added to the mould and allowed to harden. The brain was then removed from the final molten wax stage and placed carefully onto the mould with warmed forceps. Molten wax was then poured onto the brain so that it was completely submerged. Air was then gently blown over the surface of the wax to allow the top to solidify. The entire mould was then immersed in cold water overnight to facilitate quicker solidification and to prevent the formation of crystals that might disrupt the tissue.

5.2.2.3.4 Sectioning

Sectioning is a technique performed using a microtome. This is an instrument, which consists of a sharp metal knife held in a fixed position, and a chuck in which a block of wax with the tissue is held. Depending on the type of microtome, a particular mechanism oscilates the chuck up and down and with each oscillation, the chuck is brought closer to the knife by a fixed distance. In this way sections are cut from the wax block (Hodgson, 1992).

The wax block was trimmed with a razor blade so that two of the sides were parallel to each other and the other two sides converged slightly. The sides were cut so as to leave about 2mm of wax around the tissue. The entire wax block was attached to a small wooden block with a small amount of wax. A rotary microtome was used to cut ribbons of sections of 10μm thickness. Once the sections were cut, they were floated on a warm water bath (40^0C), which smoothens out the wrinkles.

5.2.2.3.5 Transferring Sections to Slides

The sections were removed from the water bath and placed on a glass microscope slide using a thin paint brush. The glass slide was initially brushed with a thin layer of Haupt's adhesive before the sections were mounted. The slides were left overnight in an oven at 40^0C to enable the section to adhere to the slide.

5.2.2.3.6 Staining

The sections were Nissl stained using cresyl violet. This stains the Nissl substances intense purple and the nuclei purple. The background is left clear (Bauer, 1974). Fig. 5.1 shows a Nissl-stained coronal section through the caudal telencephalon of a rat brain depicting the hippocampus.

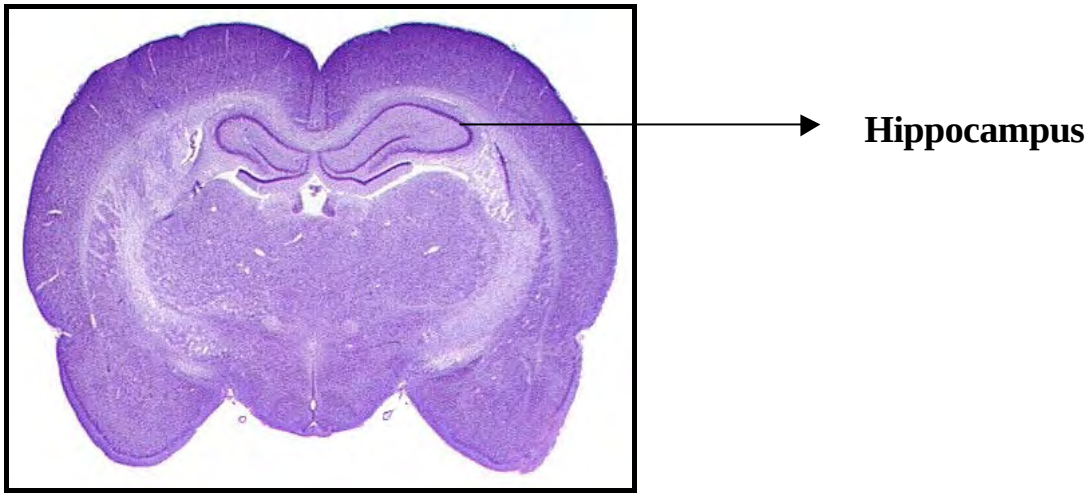


FIG. 5.3 *A Nissl-stained coronal section of the caudal telecephalon of a rat brain showing the hippocampus.*

Since this dye is water soluble, the entire embedding process had to be reversed in order to remove the paraffin wax from the tissue and allow penetration of the dye. The paraffin was removed by running the slides through xylene twice for five minutes each, followed by immersion in a mixture of xylene and absolute ethanol (1:1) for three minutes. This step was followed by immersion in absolute ethanol for five minutes, and then re - immersion in absolute ethanol overnight.

Sections were stained by placing the slides in 0.1% cresyl violet solution for two hours. These were then differentiated rapidly by rinsing them in 95% ethanol until the background was clear. The sections were then dehydrated by processing in absolute ethanol twice and xylene twice as described in section 5.2.2.3.2 for five minutes each.

5.2.2.3.7 Mounting of Slides

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

5.2.2.3.8 Photo – microscopy

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

5.2.3 RESULTS

Neurons in the CA1 and CA3 regions of the hippocampus were examined. Sections of the CA1 and CA3 regions of the control treated rats (Fig. 5.4 and 5.5 respectively) showed optimally sized, pyramidally shaped neuronal cells with a clearly observable cell nucleus and continuous cell membrane. The cells are grouped closely together to form a band – like appearance, which is characteristic of both the CA1 and CA3 regions.

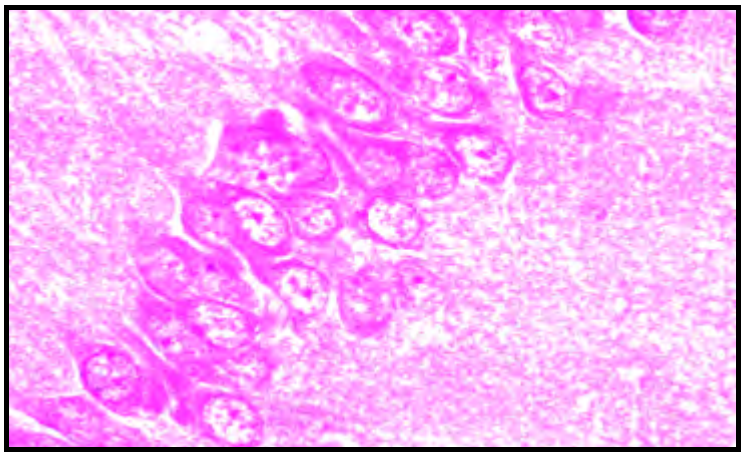


FIG. 5.4 *Cells of the CA1 region of the hippocampus from an animal of the control group. Magnification x 1000*

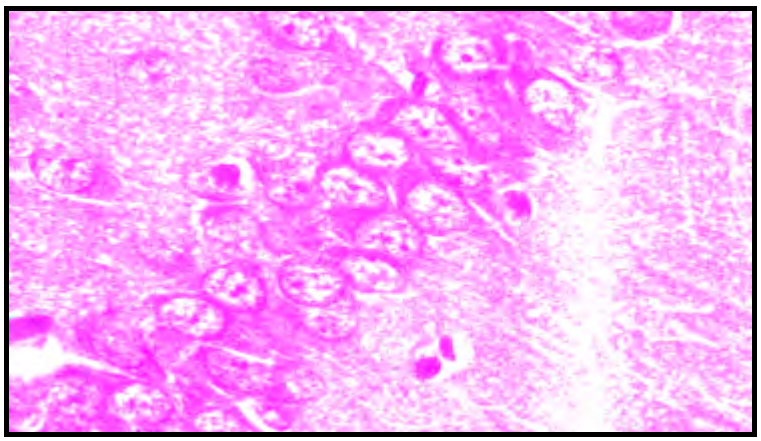


FIG. 5.5 *Cells of the CA3 region of the hippocampus from an animal of the control group. Magnification x 1000*

The cells of both regions of the lead treated animals (Fig. 5.6 and 5.7) show extensive violation by virtue of their roundness and swelling. The cells appear scattered with little integrity of cell membrane and appearance of dense nuclei. Necrosis of the cells in many areas also appears to have taken place.

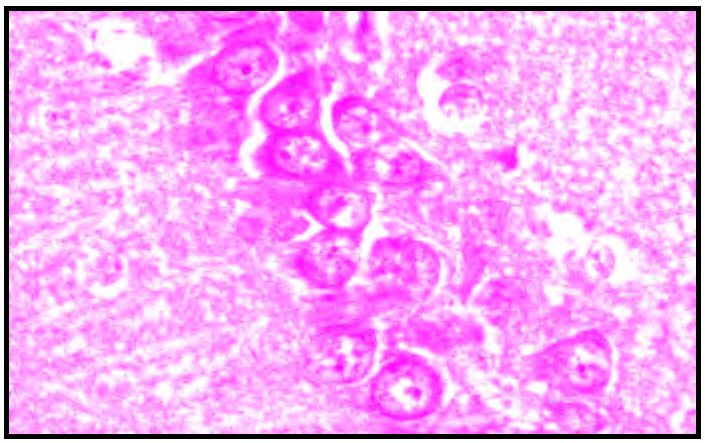


FIG. 5.6 *Cells of the CA1 region of the hippocampus from an animal treated with lead acetate (20mg/kg i.p.) for seven days. Magnification x 1000*

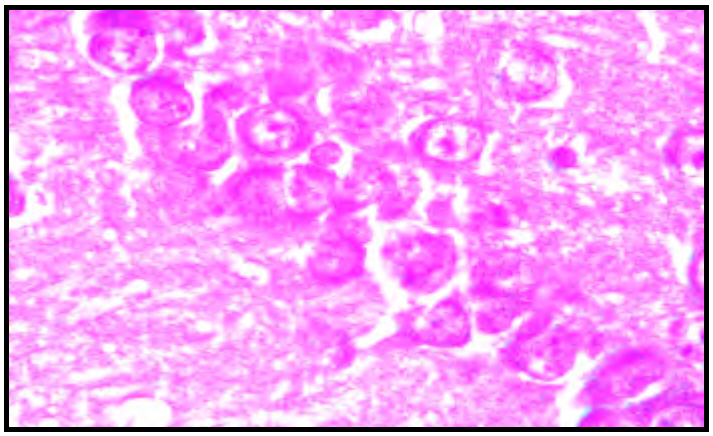


FIG. 5.7 *Cells of the CA3 region of the hippocampus from an animal treated with lead acetate (20mg/kg i.p.) for seven days. Magnification x 1000*

5.2.4 DISCUSSION

Traumatic injury of the brain is characterised by disruption of cell bodies and axons, followed by little or no axonal regeneration and virtually no recovery of function by the lesioned tissue. These results clearly demonstrate the structural damage that lead causes

in the CA1 and CA3 regions of the hippocampus, the part of the brain responsible for learning and memory processing.

A direct link has been established between low-level, long-term exposure to lead and deficits in cognitive performance and behaviour in childhood through adolescence. It has been concluded that no threshold level exists below which lead remains without effect on the central nervous system (Finkelstein *et al.,* 1998). After the third day of injecting the rats with lead acetate, visible observations of lead intoxications were demonstrated by slowness in movement and response as well as relative inactivity of the animals.

Chronic exposure of rats to lead is known to impair the induction of long term potential (LTP), a component of the hippocampal memory system, in the dentate and the CA1 region of the hippocampus (Gilbert *et al.*, 1996; Zaiser & Miletic, 1997).

One of the mechanisms by which lead induces damage to the brain appears to be through the inhibition of the myelination process as well as hampering the process of myelin membrane assembly (Konat, 1984). This explains the complete loss of axons of the cells of the CA1 and CA3 regions leading to a loss in their pyramidal shape, as shown in Fig. 5.6 and 5.7. In chapter four, it was demonstrated that lead acetate has induced lipid peroxidation of cell membranes, and this is further supported by the finding that many of the cells shown in Fig. 5.6 and 5.7 have little integrity of cell membrane or structure.

Reports on the effects of lead on the NMDA receptors have been contradictory, suggesting activation, inhibition or no change in NMDA receptor activity following lead exposure (Brook *et al.*, 1993; Guilarte *et al.*, 1994; Jett *et al.*, 1995; Schulte *et al.*, 1995). However, previous research has postulated lead-calcium interactions to be one of its molecular mechanisms of toxicity (Bressler *et al.*, 1999). The rise in intracellular levels of calcium following lead treatment may be due to either lead stimulating calcium entry or lead inhibiting calcium exit and storage (Zhao *et al.*, 1998). The increased swelling and necrosis of the cells in the CA1 and CA3 regions may be attributable to increased intracellular levels of calcium as a direct consequence of lead exposure.

The observations reported in this experiment demonstrate the hippocampus as an excellent model for examining the neurotoxic effects of lead and further strengthens the suggestion that the hippocampus may have a crucial role to play in mediating many of the behavioural and memory problems associated with lead toxicity.

5.3 HISTOLOGICAL ANALYSIS OF THE EFFECT OF CURCUMIN ON LEAD – INDUCED STRUCTURAL DAMAGE TO THE HIPPOCAMPUS

5.3.1 INTRODUCTION

Previous experiments show extensive damage caused by exposure to lead in the CA1 and CA3 regions of the hippocampus of rats (section 5.2). Curcumin has been shown to afford protection against lead mediated oxidative stress (chapter 4) and the following experiment was carried out to determine whether curcumin afforded protection against structural violation of the hippocampus by lead in rats.

5.3.2 MATERIALS AND METHODS

5.3.2.1 Animals

Adult, male, Wistar rats weighing between 250 and 300g were used and cared for as described in section 2.2.2.1. Test rats were injected with 30mg/kg curcumin intraperitoneally twice daily and 20mg/kg lead acetate once a day, for seven days. The animals were injected with curcumin in the morning and evening, and lead acetate at noon. Both agents were injected on the left and right side respectively, so as to avoid interaction at the site of administration. Sections obtained from the CA1 and CA3 regions of these animals were compared to those obtained from the control and lead alone treated animals

5.3.2.2 Chemicals and Reagents

Chemicals and reagents were obtained as mentioned in section 5.2.2.1. Curcumin was purchased from Sigma Chemical Co, St. Louis, USA and was solubilised in ethyl oleate for purposes of administration to the rats.

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5.3.2.3 Histological Techniques

These were carried out as described in section 5.2.2.3 and sections were viewed under the light microscope and photographed using a digital camera.

5.3.3 RESULTS

In comparison to the sections of the CA1 and CA3 regions of the rats treated with lead only (Fig. 5.6 and 5.7) the neurons of the CA1 and CA3 areas of the lead and curcumin treated rats (Fig. 5.8 and 5.9) showed significant protection.

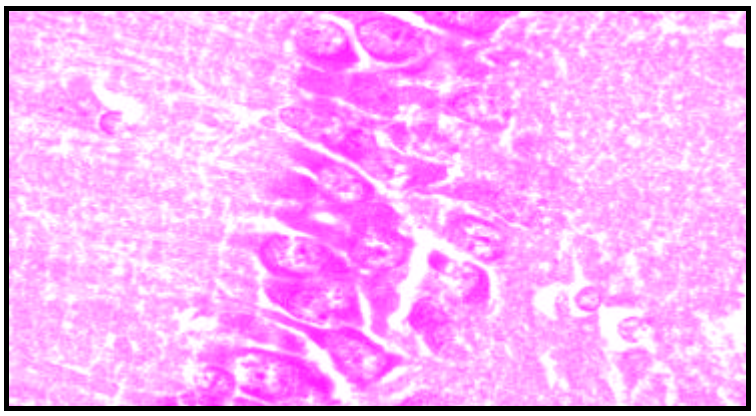

FIG. 5.8 *Cells of the CA1 region of the hippocampus from an animal treated with lead acetate (20mg/kg i.p.) and curcumin (30mg/kg i.p.) for seven days. Magnification x 1000*

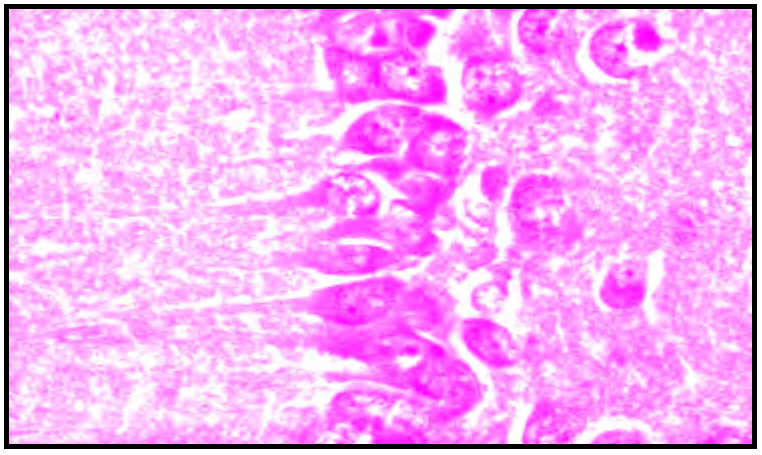

FIG. 5.9 *Cells of the CA3 region of the hippocampus from an animal treated with lead acetate (20mg/kg i.p.) and curcumin (30mg/kg i.p.) for seven days. Magnification x 1000*

Many of the cells appear to have retained their pyramidal appearance and are not as inflamed as those present in the hippocampus of the lead alone treated rats. The cells show better orientation, integrity of cell membrane and appear closer to each other as though in an attempt to reconstruct their typical “band-like” appearance.

5.3.4 DISCUSSION

The results of this experiment have shown curcumin to significantly protect against the structural damage that lead produced in the hippocampus. External observations in the rats treated with lead and curcumin showed a relatively greater activity and faster response when compared to those that were treated with lead alone.

The antioxidant activity of curcumin is already well established and its protective action against lead mediated oxidative stress has already been demonstrated in chapter 4. Since oxidative stress has been postulated as a possible mechanism of lead mediated toxicity, curcumin could be scavenging free radicals generated through lead induced calcium levels in the cell, thus accounting for the reduced swelling in the cells shown in fig. 5.9 and 5.10. Curcumin as an anti-inflammatory agent, could also be playing an important role in protection.

A third hypothesis is the possible binding of curcumin to this heavy metal, forming a complex and thus inactivating its toxic effects. This has been investigated in chapter 7.

CHAPTER SIX

ELECTROCHEMICAL ANALYSIS

6.1 INTRODUCTION

Previous biological work showed curcumin to be capable of protecting against lead and cadmium induced toxicity in the brain (chapter four and five). The direct interaction and possible complexation of these heavy metals by curcumin was suggested as a possible mechanism. Moreover, the protection that curcumin offered against QA and cadmium induced oxidative insult in the rat brain homogenate (chapter four) were postulated to be due to an interaction between curcumin and ferric as well as ferrous ions. Electrochemical analysis was one of the techniques used to probe any such interactions between curcumin and the metals, lead, cadmium, ferric and ferrous ions.

A chemical reaction in which one or more electrons are transferred from one species to another is the basis for electrochemical analysis (Pecsok *et al.*, 1968). Electrochemistry has been used with much success in metal-ligand studies (Limson *et al.*, 1998, Limson & Nyokong, 2001, Lack & Nyokong, 2001). Limson *et al.*, (1998) used electrochemistry to show metal-ligand interactions between the pineal hormone melatonin and a range of metals. In the following experiments two dynamic electroanalytical techniques, cyclic voltammetry and adsorptive stripping voltammetry were employed to determine whether curcumin was capable of interacting with lead, cadmium and iron.

The electrochemical cell used in these studies comprises of three electrodes, the working, reference, and auxiliary electrode as depicted in Fig. 6.1. The first electrode is the working electrode, where the analyte is oxidised or reduced. Working electrodes of metallic origin are mostly used. The main requirements of the material considered ideal for constitution of a working electrode are that it be durable under various solution conditions, it has a low electrical resistance, and that it has an easily reproducible surface.

In the studies conducted, the glassy carbon electrode (GCE) was used due to its ideal surface chemistry for adsorption of analytes. It also possesses excellent mechanical and electrical properties, is chemically inert and exhibits reproducible performance.

The reference electrode is the standard electrode against which the potential of the working electrode is referenced. It maintains a constant potential, and is also referred to as the primary electrode. Commonly used reference electrodes are the hydrogen electrode, smooth platinum electrode, saturated calomel electrode, silver/silver chloride electrode, and the glass electrode. The silver/silver chloride electrode was used due to its suitability in working with aqueous solutions. It consists of a silver wire anodised with silver chloride in a glass tube, the wire being in direct contact with saturated or concentrated solutions of AgCl and either KCl or NaCl. The electrode is protected from the solution by a semi-permeable salt bridge (Sole, 1995, Hawkridge, 1996).

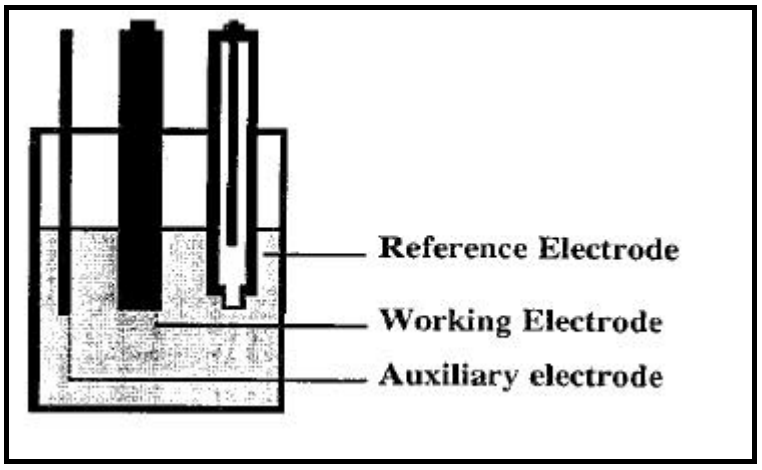

FIG. 6.1 *Most dynamic electrochemistry utilizes the classic three-electrode system as shown here (Limson, 1998).*

The third electrode used is called the auxiliary electrode, which is usually constructed of an inert material such as platinum, and it is used to prevent voltage drop across the working and reference electrodes. This electrode is not usually isolated from the working electrode while the reference electrode is (Sole, 1995, Hawkridge, 1996). Current passes between the working and auxiliary electrode.

Oxygen is electroactive at almost all electrodes, and seriously interferes with voltammeric and electrochemical measurements (Sawyer & Roberts, 1974). To remove the oxygen, nitrogen or any other inert gas is bubbled through the solution.

6.1.1 Cyclic Voltammetry

Cyclic voltammetry (CV) is often the technique of choice for characterising or providing a fingerprint of electro-active species in solution, mapping the current response produced at an electrode as a function of potential. During cyclic voltammetry, the electrode potential is rapidly scanned in search of redox couples. Once located, a couple is characterised from the potentials of peaks on the cyclic voltammogram and from the changes caused by variation of the scan rate (Kissinger *et al.*, 1996). The forward scan generates the oxidised species and the reverse scan, the reduced species (Limson, 1998). Changes in current response and potential are good indicators of alterations in the chemistry of a target compound, such as that which occurs during an exchange of electrons in a metal-ligand bond.

During the redox process the transfer of electrons can be represented by the following equation:

$$O + ne^- \underset{k_r}{\overset{k_f}{\longrightarrow}} R \qquad (7)$$

O refers to the oxidised species, e^- the electron and R, the species in reduced form on acceptance of the electron. K_f and K_r are the formal rate constants for the forward and reverse reactions, respectively. The conversion of O to R by reduction gives rise to a cathodic current i_c. Oxidation of R to O produces an anodic current, i_a. The observed current resulting in a change of oxidation state is the faradic current, which is a direct measure of the rate of redox reaction. The resultant current potential plot is the voltammogram.

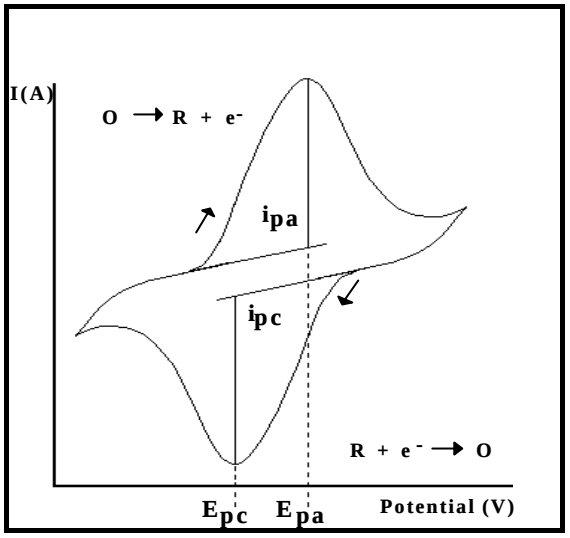

FIG. 6.2 *A typical cyclic voltammogram (Limson, 1998).*

A typical voltammorgram is shown in Fig. 6.2, depicting the i_{pa}, which is the anodic peak current formed when the electrode oxidises the analyte. The i_{pc} is the cathodic peak current formed during negative scanning which permits the reduction of the analyte (Limson, 1998). The cathodic peak potential, E_{pc}, and the anodic peak potential, E_{pa}, can be read from the voltammograms and the formal reduction potential, E^0 of a redox couple is determined according to the equation below (Wang, 1994; Hawkridge, 1996).

$$\mathbf{E^{0'} = (E_{pa} + E_{pc})/2} \qquad (8)$$

E0' = formal reduction potential
E_{pa} = anodic peak potential
E_{pc} = cathodic peak potential

6.1.1 Adsorptive Stripping Voltammetry

Adsorptive stripping voltammetry (ASV) exploits the natural tendency of analytes to pre-concentrate at an electrode and is a useful technique for gauging metal-ligand interactions. The electrode is polarised at a more negative potential than the reduction potential of the metal, and this serves to pre-concentrate the analyte at the electrode.

A metal species at an electrode will produce a characteristic response at a specific potential, with current being proportional to the amount of analyte at the electrode. When a suitable ligand is added to a metal solution, the ligand facilitates movement of the metal to the surface of the electrode. Theoretically then, a ligand which forms a bond with a metal will bring about a change in the current response observed, as well as a shift in potential resulting from the reduction of a new species, the metal-ligand complex at the electrode. The affinity of the ligand toward the metal is expressed by the extent of increase in current response of the metal on addition of the ligand. A lowering in current response indicates possible competition by the ligand for binding sites at the electrode at relatively high analyte concentrations, or formation of strong metal- ligand bonds where the metal is not easily reduced upon scanning. A negative potential shift indicates a strong metal-ligand interaction while a large positive shift is associated with a weaker metal-ligand interaction (Limson *et al.*, 1998; Lack & Nyokong, 2001).

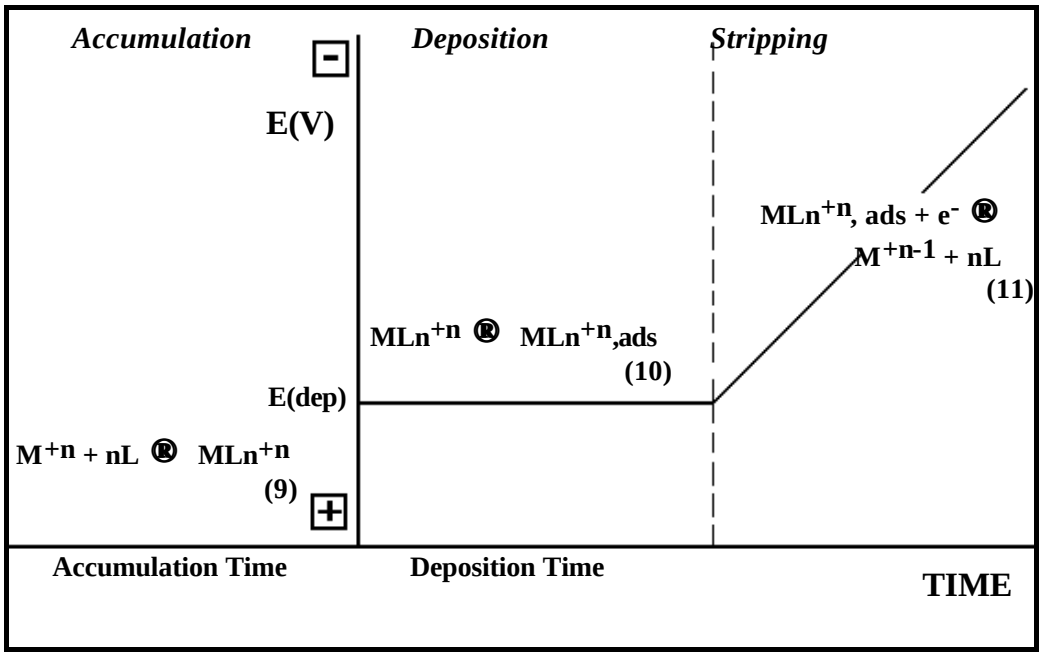

FIG. 6.3 *A diagrammatic representation of the adsorptive stripping voltammetric technique, showing the accumulation, deposition and stripping steps The equation numbers are shown in brackets (Limson, 1998).*

The steps involved in this technique is diagrammatically explained in Fig. 6.3. To a solution of metal analyte (M) and electrolyte, a suitable chelating ligand (L) is added and

metal-ligand complexation (ML) is facilitated. This is the accumulation time during which the metal-ligand complex has begun to adsorb at the electrode surface by diffusion from the solution. The electrode potential is held at a fixed deposition potential for a controlled time (deposition time), while the solution is stirred at a fixed convection rate. In the stripping step, the adsorbed complex is reduced during a negative going potential scan and both the metal and ligand are released back into solution.

6.1 MATERIALS AND METHODS

6.2.1 Chemicals and Reagents

Curcumin, lead acetate and cadmium sulphate were purchased from Sigma Chemical Corporation, St. Louis, U.S.A. Curcumin was dissolved in 50% ethanol-water and the heavy metals, in Milli-Q water for the electrochemical analysis. Fresh solutions of curcumin were prepared daily. Saarchem buffer tablets were used to prepare pH 4 buffer solutions. Ferric and ferrous solutions were prepared from ferric chloride and ferrous sulphate salts respectively, both were purchased from Merck, Midrand, South Africa.

6.2.2 Apparatus

Cyclic and stripping voltammograms were obtained with the Bio Analytical Systems (BAS, Lafayette, Indiana, U.S.A.) CV-50W voltammetric analyzer using a BAS C2 cell stand to maintain constant atmosphere. A 3mm diameter glassy carbon electrode (GCE) was employed as a working electrode for voltammetric experiments. A silver/silver chloride [KCl = 3 M] and a platinum wire were employed as reference and auxiliary electrodes, respectively, in all electrochemical work. Prior to use, the GCE was cleaned by polishing with alumina on a Buehler pad, followed by washing in nitric acid and rinsing in water followed by the buffer solution. Between scans, the GCE was cleaned by immersion in a dilute acid solution and rinsed with water.

6.2.3 Technique

6.2.3.1 Cyclic Voltammetry

For cyclic voltammetric experiments, appropriate concentrations of the metal and ligand (curcumin) in buffer were introduced into a glass cell and degassed for 5 min with nitrogen before scanning a potential window. All potential values quoted are referenced against the silver/silver chloride reference electrode.

6.2.3.2 Adsorptive Stripping Voltammetry

For adsorptive stripping experiments appropriate concentrations of the metal and of the ligand (curcumin) were introduced into an electrochemical cell. The solution was then de-oxygenated with nitrogen for 5 minutes, after which a flow of nitrogen was maintained over the solution throughout the measurement. Optimum deposition potential for each metal was identified and applied for 60 s to effect the formation and adsorption of the metal and ligand species onto the GCE.

The voltammograms were then scanned in the negative direction from the deposition potential to at least 0.50V beyond the reduction of the metal at the scan rate of 0.10V s^{-1} to strip the adsorbed metal-ligand species from the electrode. During the stripping step, current responses due to the reduction of the metal-ligand species were measured as a function of potential. All potential values quoted are referenced against the silver/silver chloride (Ag/AgCl) reference electrode.

6.3 RESULTS

6.3.1 CURCUMIN

Fig. 6.4 shows the adsorptive stripping voltammogram for 2.1 x 10^{-4} M curcumin in pH 4 buffer, with a peak potential of –0.92 V *vs* Ag/AgCl. The cyclic voltammogram for curcumin (Fig. 6.5) shows two quasi-reversible oxidation couples: 0.15V with a weak cathodic return peak [Fig. 6.5 (a)]. The second oxidation is observed at 0.69 V [Fig. 6.5 (b)].

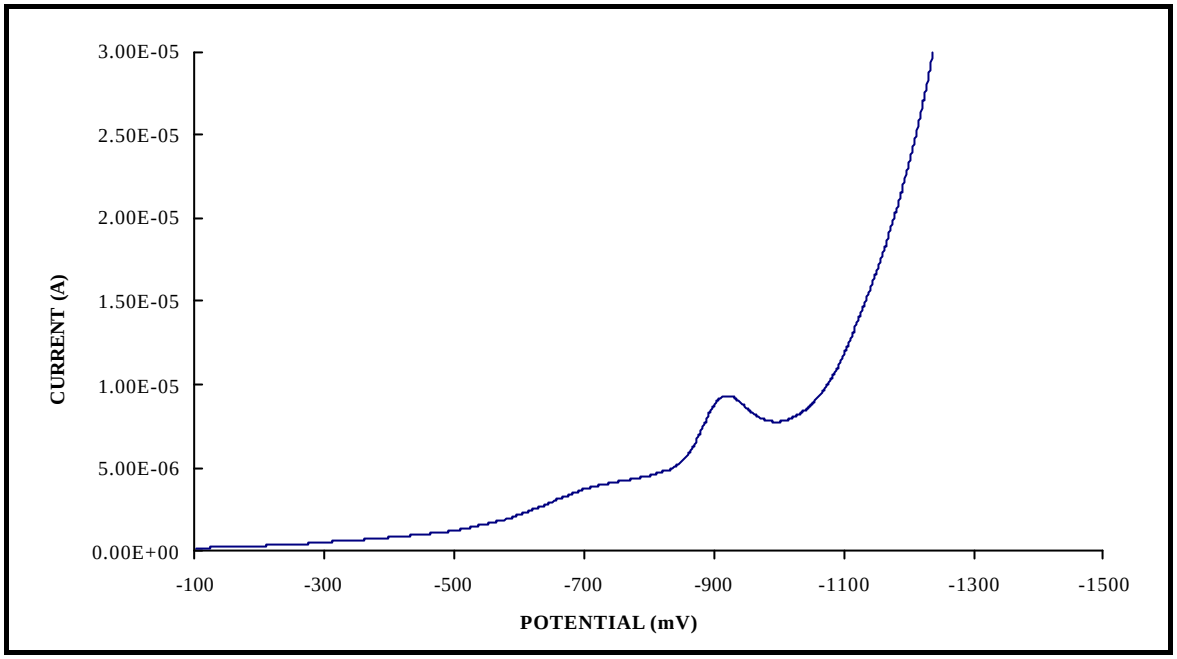

FIG. 6.4 *Adsorptive stripping voltammogram of curcumin in pH 4 buffer.*

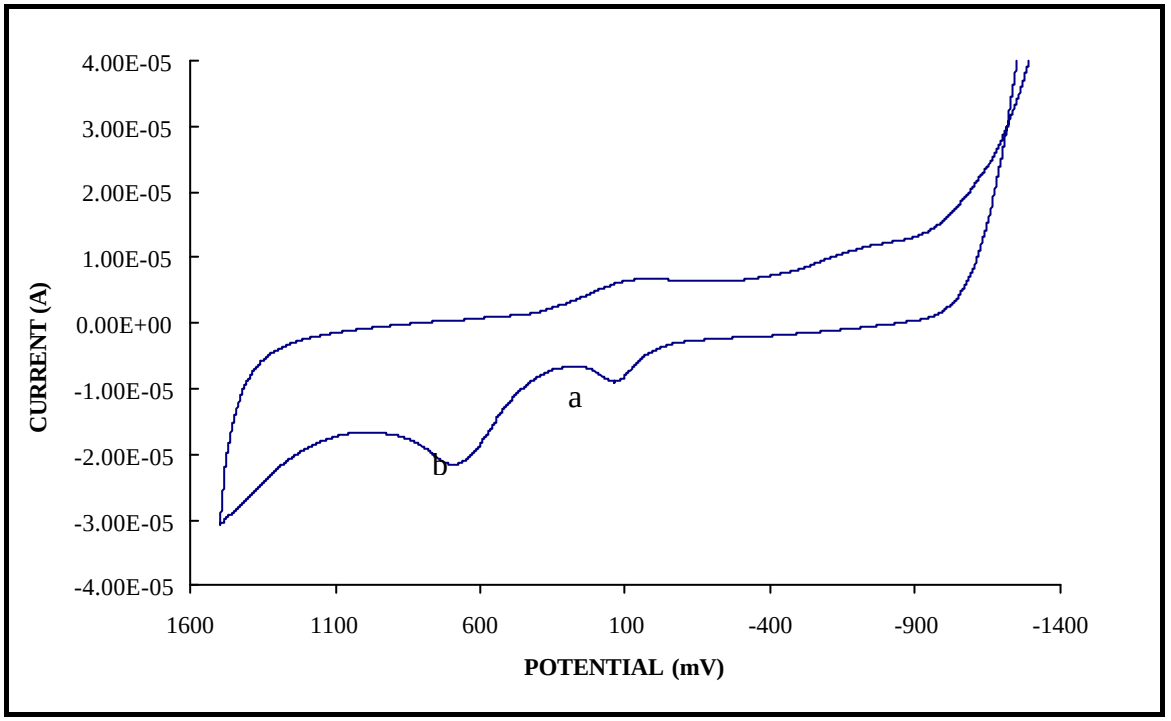

FIG. 6.5 *Cyclic voltammogram of curcumin in pH 4 buffer.*

6.3.2 CURCUMIN & LEAD

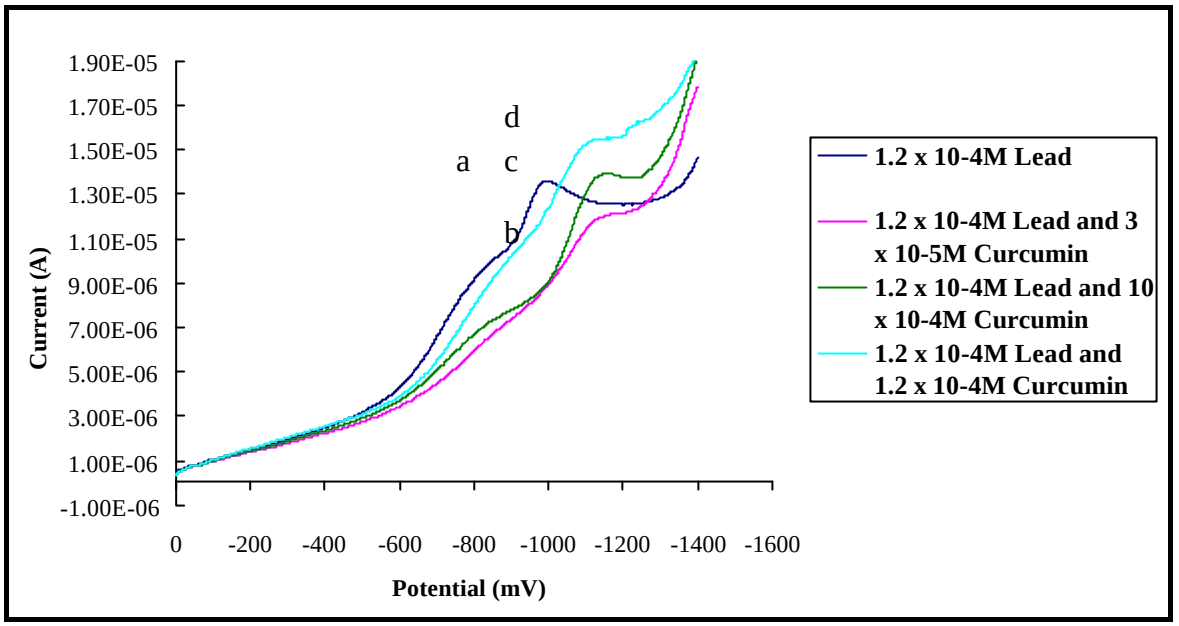

FIG. 6.6 *Adsorptive stripping voltammogram of lead in pH 4 buffer, and lead in presence of increasing concentrations of curcumin.*

Fig. 6.6 (a) shows the ASV of lead (1.2 x 10^{-4}M) in pH 4 buffer at –0.99V vs Ag/AgCl. In the presence of low concentrations of curcumin, this peak is shifted to negative potentials of –1.15V. This peak increases with increase in concentration of curcumin and is attributed to the reduction of the Pb^{2+}-curcumin complex. This is evident in Fig. 6.6 (b to d), which shows the shifted lead peak in response to increasing concentrations of curcumin.

At curcumin concentrations at and above (1.3 x 10^{-4}M) a peak at –1.09 V [Fig. 6.7 (a)] with a new peak observed at –1.38V (*) is observed. The peaks at –1.09V and –1.38V increased and shifted to more positive potentials with increase in concentration of curcumin.

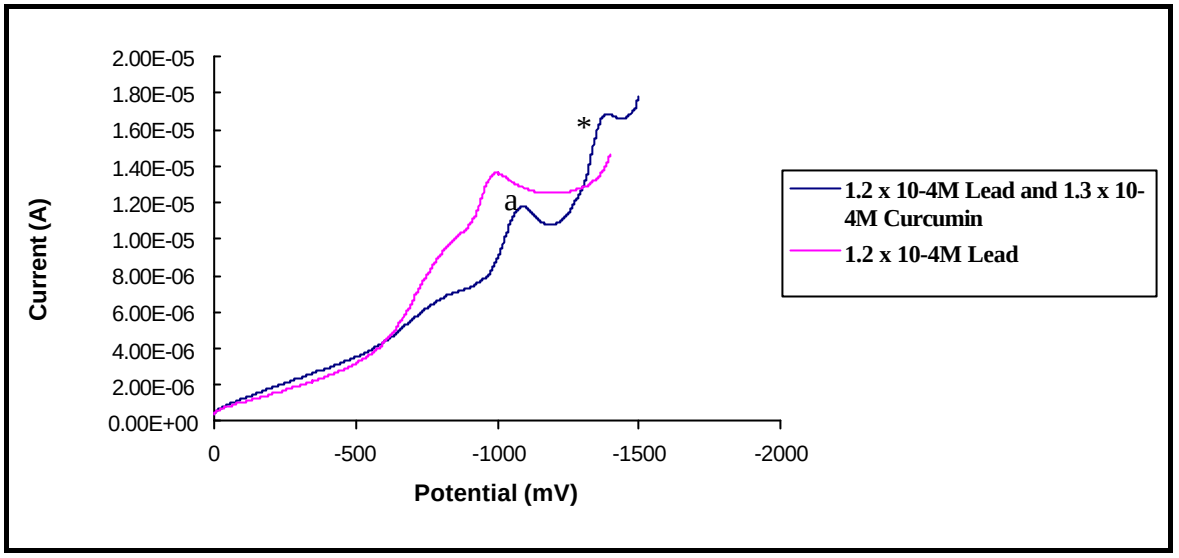

FIG. 6.7 *Adsorptive stripping voltammogram of lead alone and in presence of 1.3 x 10^{-4}M curcumin showing a peak at –1.09V as well as the formation of a new peak (*).*

It has been postulated that above equimolar concentrations of Pb^{2+}- curcumin, the Pb^{2+} peak is not observed while the reduction peak observed at –1.09V is that of a new species. The peak at –1.38V is also attributed to the reduction of a Pb^{2+}- curcumin complex, therefore, it is postulated that at these metal- ligand concentrations, two lead-curcumin species are formed in solution.

6.3.3 CURCUMIN & CADMIUM

Similar electrochemical behaviour was observed for studies of curcumin with Cd^{2+}. Fig. 6.8 (a) shows the ASV for Cd^{2+} (6 x 10^{-5}M) in pH 4 buffer at –0.82V. In the presence of curcumin, (3 x 10^{-5}M) a peak at –1.02V is observed [Fig. 6.8 (b)]. Increasing concentrations of curcumin increase this peak without any potential shifts [Fig. 6.9 (c – d)]. It is likely then that this peak is the reduction wave of curcumin. A new peak (*) as before is observed at –1.32V for Cd^{2+} studies in the presence of increasing concentrations of curcumin. This peak, which has not been observed either for the metal or the ligand alone and is similar to ASV for Pb^{2+} with curcumin, has been ascribed to the reduction of a Cd^{2+} -curcumin complex.

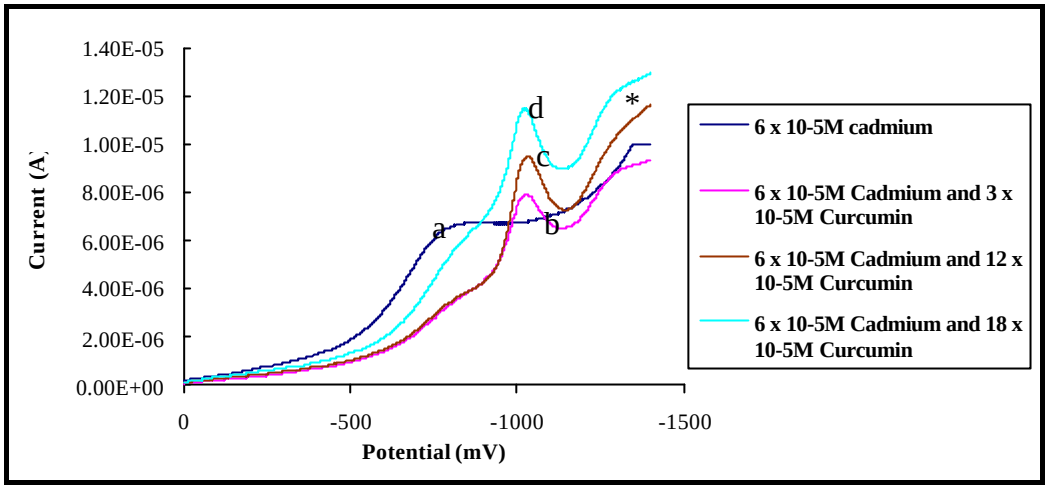

FIG. 6.8 *Adsorptive stripping voltammogram of cadmium alone (a) and in presence of increasing concentrations of curcumin (b, c and d) with the formation of a new peak at 10 x 10* $^{-5}$M curcumin (*).

6.3.4 CURCUMIN & IRON (II)

ASV of Fe^{2+} (4 x 10^{-5} M) produces a peak at a potential of –0.68V [Fig. 6.9 (a)]. In the presence of 2 x 10^{-5} M and 6 x 10^{-5} M curcumin, decreases in current response were observed with increase in concentration of curcumin. The second peak seen in presence of curcumin (*) was ascribed to curcumin. No potential shifts were observed.

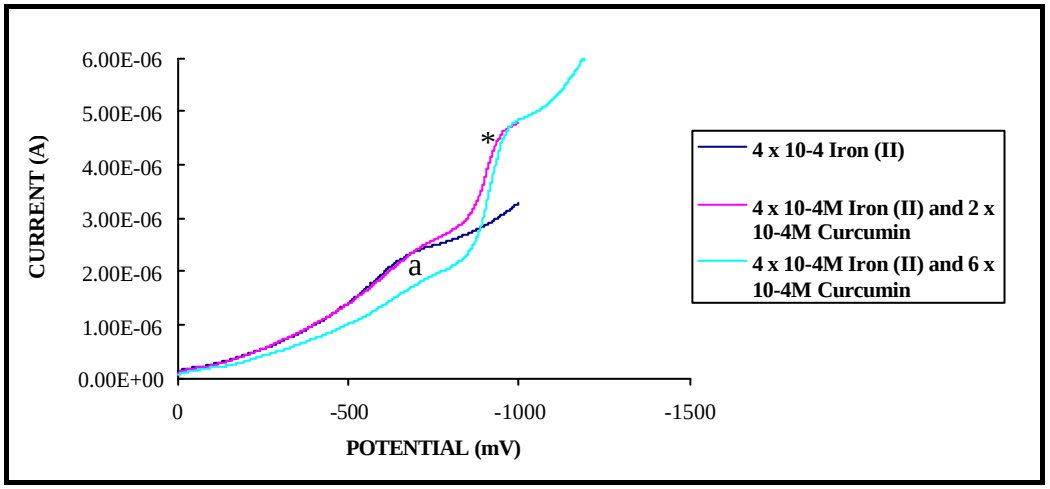

FIG. 6.9 *ASV of* Fe^{2+} *alone (a) and in presence of increasing concentrations of curcumin. The asterix indicates the curcumin peaks.*

6.3.5 CURCUMIN & IRON (III)

Fig. 6.10 (a) shows the ASV of Fe^{3+} (4 x 10^{-5} M) at –0.48V. In the presence of 2 x 10^{-5}M curcumin [Fig. 6.10 (b)] a decrease in the current response for Fe^{3+} as well as a slight shift to less negative potentials is observed. At higher curcumin concentrations (6 x 10^{-5} M) the Fe^{3+} peak disappears as shown in Fig. 6.10 (c) and a new broad peak is observed at –1.17V (*). This peak is not evident in ASV of curcumin, even at very high concentrations (2 x 10^{-4}M).

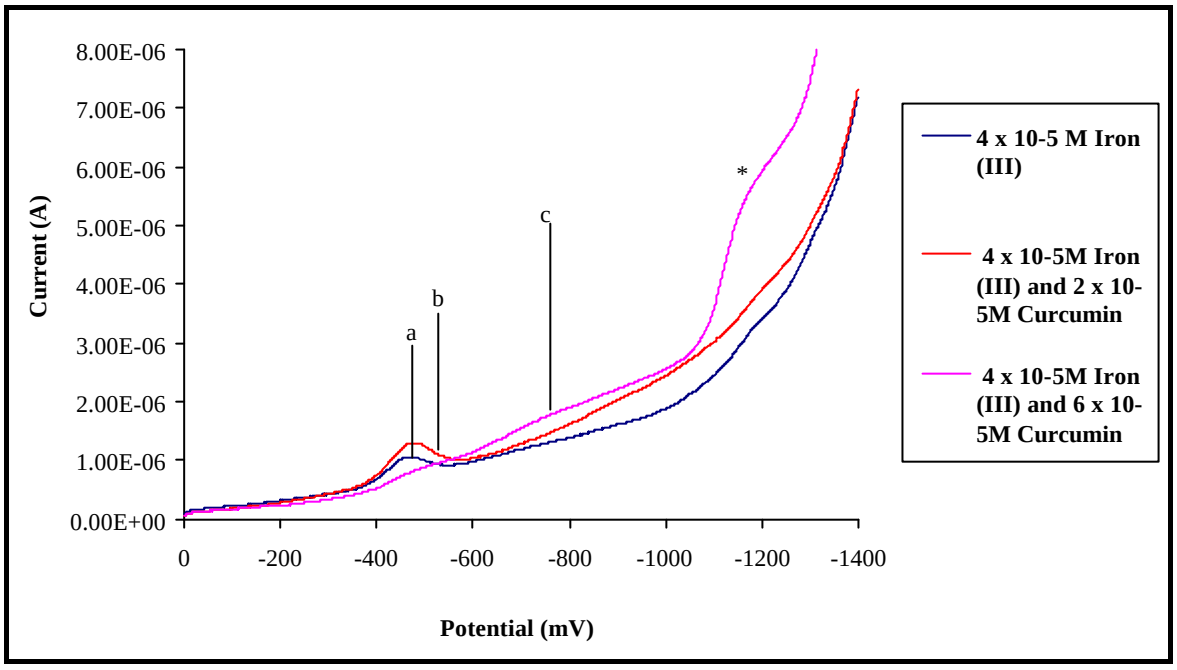

FIG. 6.10 *Adsorptive stripping voltammogram of Fe^{3+} alone (a) and in presence of increasing concentrations of curcumin (b and c) with a new peak formation at 6 x 10-5M curcumin (*).*

Curcumin has been shown to form complexes with Fe^{3+} by cyclic voltammetric methods (Borsari *et al.*, 2002). It is likely that the disappearance of the Fe^{3+} peak may be indicative of a very strong metal-ligand bond, such that the metal is not easily reduced during the stripping step. The new peak at –1.17V is thus likely to be the reduction of the Fe^{3+} - curcumin complex formed.

6.4 DISCUSSION

The electrochemical data reported above clearly supports the postulation that curcumin binds to lead and cadmium, forming new complexes and thereby inactivating their toxic effects. Curcumin has also been shown to strongly bind to Fe^{3+}, thus supporting earlier studies (Borsari *et al.*, 2002) showing a metal-ligand bond between this metal and curcumin. Thus it is likely that the chelation of ferric ions is the mechanism involved in reduction of iron – induced lipid peroxidation (Reddy *et al.*, 1994) and also one of the mechanisms involved in the reduction of cadmium induced lipid peroxidation, as discussed in section 3.4.4.

The above studies do not however, support the likelihood of an interaction between curcumin and Fe^{2+}. This finding does not favour the postulated curcumin-iron interaction being the mechanism involved in reducing QA-induced lipid peroxidation because QA binds with ferrous and not ferric ions. In this case it is more likely that curcumin is acting as a scavenger of free radicals induced by QA and thereby reducing oxidative stress induced by QA within the membranes.

The highly negative peak observed for curcumin and the metals Pb^{2+}, Cd^{2+} and Fe^{3+} at –1.38V, –1.32V and -1.17V respectively are indicative of strong metal-ligand interactions, as these reduction potentials are more negative than that of the metal alone. A high negative potential shift indicates that the species formed between the metal and the ligand is harder to reduce than the metal alone. Strong metal-ligand bonds are vital for the efficiency of metal chelators of toxic metals. For Fe^{3+} and Cd^{2+} only one species of metal – curcumin complex was observed in keeping with the smaller ionic radius of these metal species and thus their ability to bind curcumin in a 1:1 ratio. Pb^{2+} by comparison has a larger ionic radius. The two Pb^{2+} - curcumin species observed thus corresponds to this metal species' ability to bind curcumin at higher ratios.

These results are in keeping with earlier electrochemical studies of the known antioxidant and free radical scavenger, melatonin (Limson *et al.*, 1998), where weak interactions

were found between melatonin and essential metals and stronger metal-ligand interactions with toxic metals such as cadmium and lead.

CHAPTER SEVEN

METAL BINDING STUDIES - A SPECTROSCOPIC STUDY

7.1 INTRODUCTION

In chapter four it was established that curcumin is capable of protecting the rat brain against QA, lead and cadmium-induced lipid peroxidation. Curcumin has also been demonstrated in chapter five to prevent lead-induced structural damage to the rat hippocampus. The mechanism of action of curcumin has been postulated to involve metal-binding activity with lead, cadmium and iron, which has been supported by electrochemical analysis as shown in chapter six. In the following experiments, UV/VIS and infrared spectroscopic techniques were employed to further establish and characterise such an interaction between the metals mentioned above and curcumin.

The methods of spectrophotometry are based on the fact that all chemicals, to some extent, absorb electromagnetic radiations of specific wavelengths. Spectroscopy involves the technique of measuring the absorption of a beam of light after it passes through a sample or after reflection from a sample surface.

Ultraviolet and visible spectroscopy involves the energy absorbed by a transition from one electronic energy level to another. Any species with an extended system of alternating double and single bonds will absorb UV light, and anything with colour absorbs visible light, making UV-VIS spectroscopy applicable to a wide range of samples. Different chemicals may absorb light at different wavelengths so a qualitative analysis of an unknown chemical may be made by determining the absorption spectrum of that chemical. Any change in structure or composition of a compound will result in an electronic change, resulting in a change in the spectrum, either a shift in wavelength or a change in the extinction coefficient of the absorbance (Newman, 1969).

The principle involved in infrared IR analysis is similar. If absorption is accompanied by a transition from one vibrational energy level to another, the radiation is from the infrared portion of the electromagnetic spectrum, and the technique is known as infrared

spectroscopy, also known as vibrational spectroscopy (Willard *et al.*, 1958). An infrared (IR) spectrum is generally displayed as a plot of the energy of the IR radiation versus the percent of light transmitted by the compound. The IR spectrum of a compound is essentially the superimposition of the absorption bands of specific functional groups, and even subtle interactions with the surrounding atoms of the molecule affect the spectrum of the compound (Willard *et al.*, 1958).

7.2 MATERIALS AND METHODS

7.2.1 Chemicals and Reagents

Lead, cadmium, curcumin and potassium bromide were obtained from Sigma Chemical Corporation, St. Louis, U.S.A. and were prepared as described in section 6.2.2.1. Ferric and ferrous solutions were prepared from ferric chloride and ferrous sulphate salts respectively, both were purchased from Merck, Midrand, South Africa. All other chemicals and reagents were of the highest quality available.

7.2.2 UV/VIS Spectroscopy

The interaction between curcumin and the metal was studied by comparing the absorption spectra of curcumin alone in solution, and then upon addition of increasing concentrations of the metal solution. A range of concentrations of equimolar mixtures of curcumin and metal ($2.5 \times 10^{-4} - 3.9 \times 10^{-6}$ M) were investigated. All the samples were analysed on a GBC UV/VIS 916 spectrophotometric detector.

7.2.3 Infrared Spectroscopy

A range of solutions of curcumin and metal with metal:ligand ratios between 1:1 and 1:6 were allowed to react. Precipitates obtained were separated, washed, dried, and analysed by IR using KBr pellets between 4000 and 400cm^{-1}, using a Perkin Elmer 2000 FTIR spectrometer.

7.3 RESULTS

7.3.1 UV/VIS Spectroscopy

At 3 x 10^{-5} M curcumin shows a lamda max of 437nm and upon addition of equal concentrations of ferric ions, a shift is observed to 429nm with a considerable decrease in absorbance (Fig. 7.1). This is indicative of metal- ligand complexation.

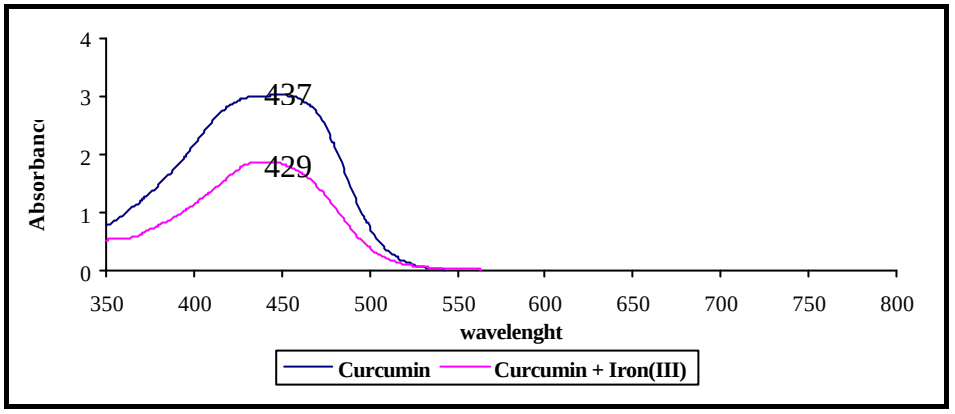

FIG. 7.1 *Spectroscopic analysis of curcumin and Fe^{3+} (3 x 10^{-5} M).*

For concentrations of 1.6 x 10^{-5} M, 7.8 x 10^{-6} M and 3.9 x 10^{-6} M, the lamda max of curcumin remained at 430nm and no shift in the band was observed upon addition of equal concentrations of ferric ions. However a considerable decrease in the absorbance was noted (Fig. 7.2). This is also supportive of iron (III)-curcumin chelate formation.

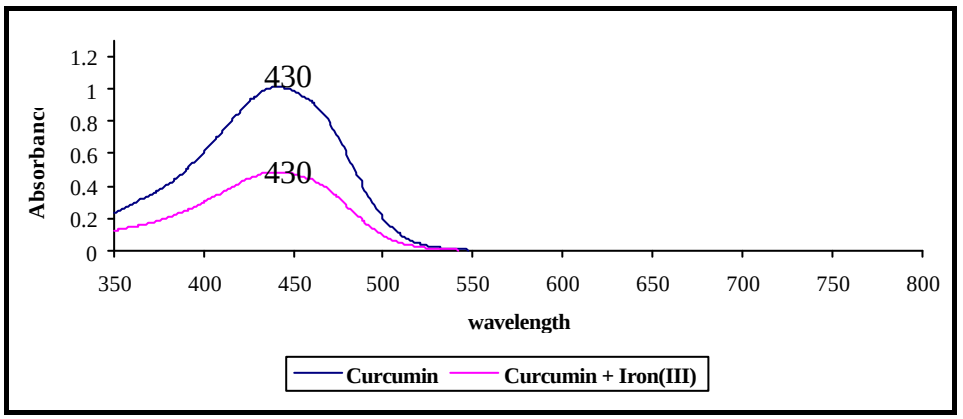

FIG. 7.2 *Spectroscopic analysis of curcumin and Fe^{3+} (7.8 x 10^{-6} M).*

Similar results were obtained when the interaction of curcumin with ferrous ions, lead and cadmium were studied spectrophotometrically. This is shown in Figs. 7.3, 7.4, 7.5, 7.6, 7.7, and 7.8.

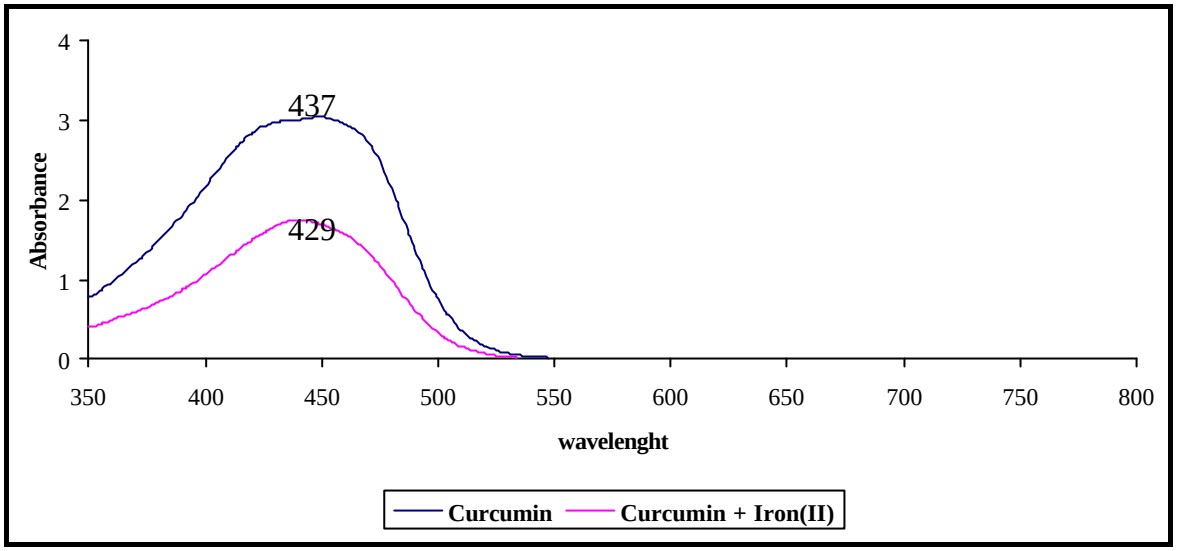

FIG. 7.3 *Spectroscopic analysis of curcumin and Fe^{2+} (3 x 10^{-5} M).*

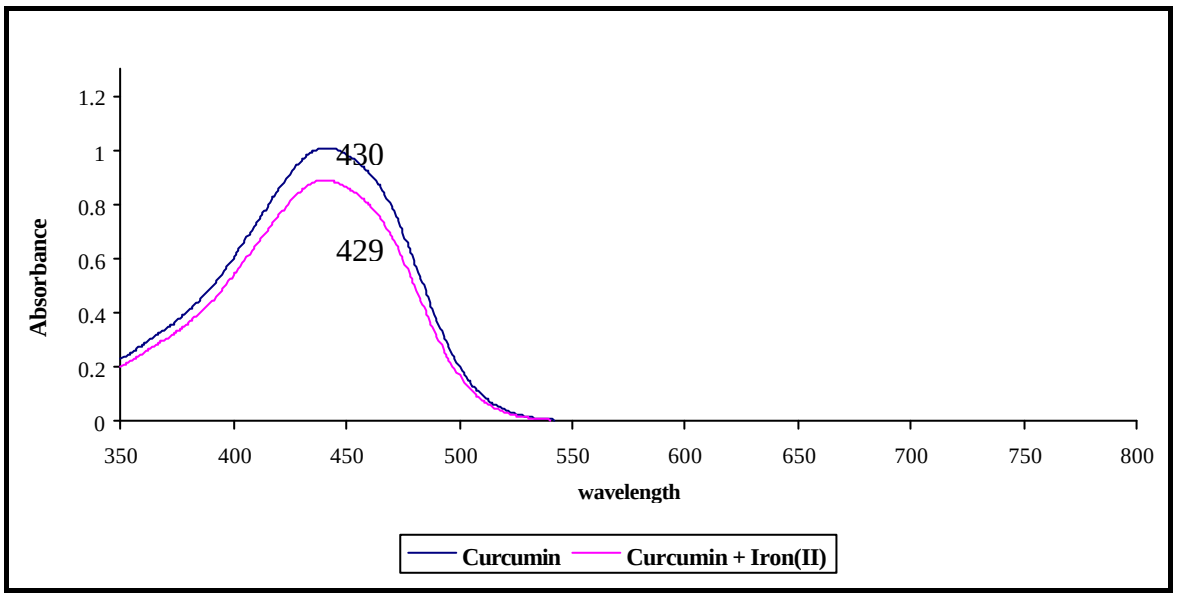

FIG. 7.4 Spectroscopic analysis of curcumin and Fe^{2+} (7.8 x 10^{-6} M).

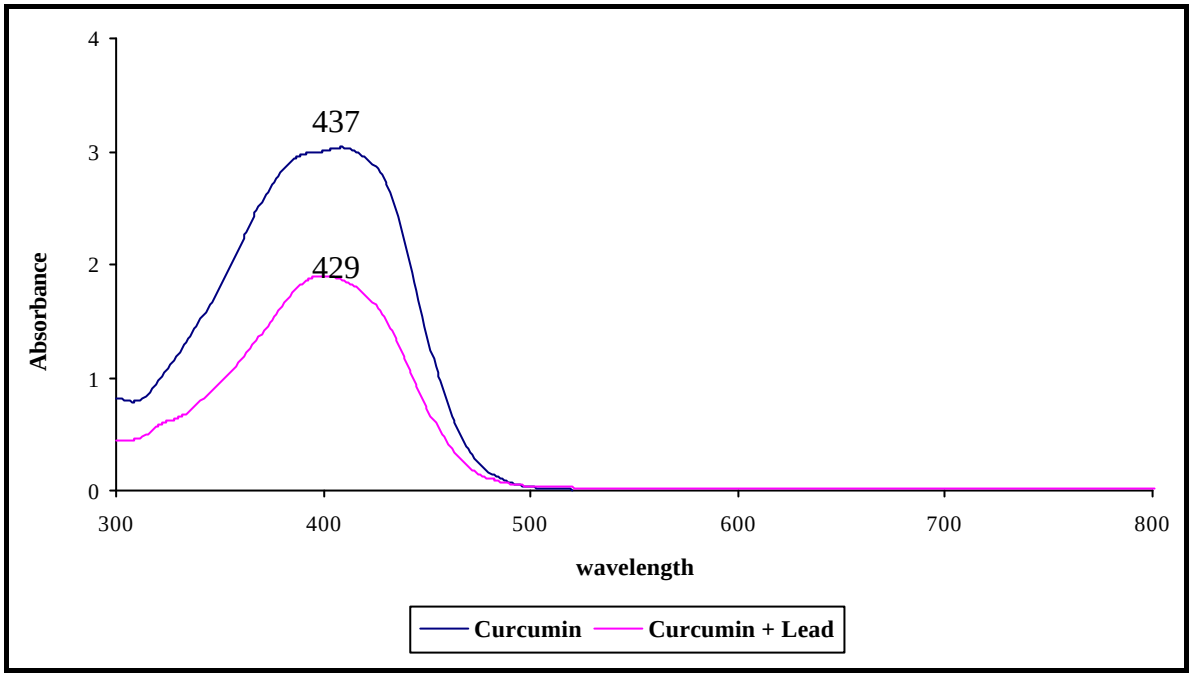

FIG. 7.5 *Spectroscopic analysis of curcumin and lead (3 x 10^{-5} M).*

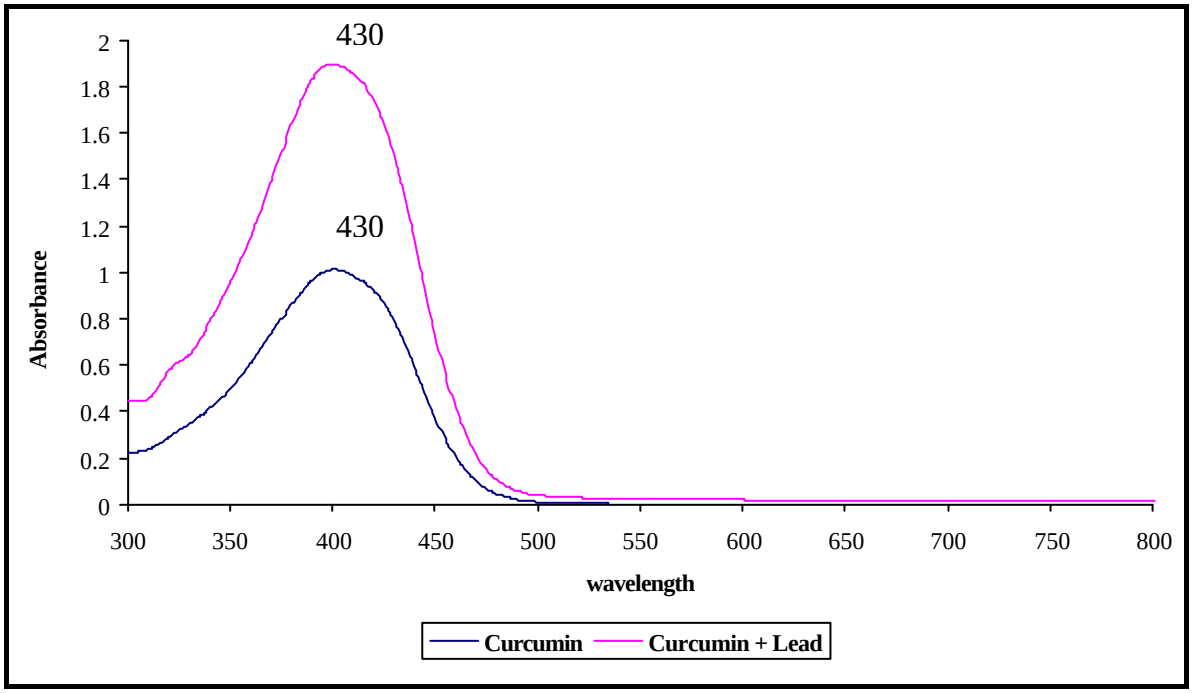

FIG. 7.6 *Spectroscopic analysis of curcumin and lead (7.8 x 10^{-6} M).*

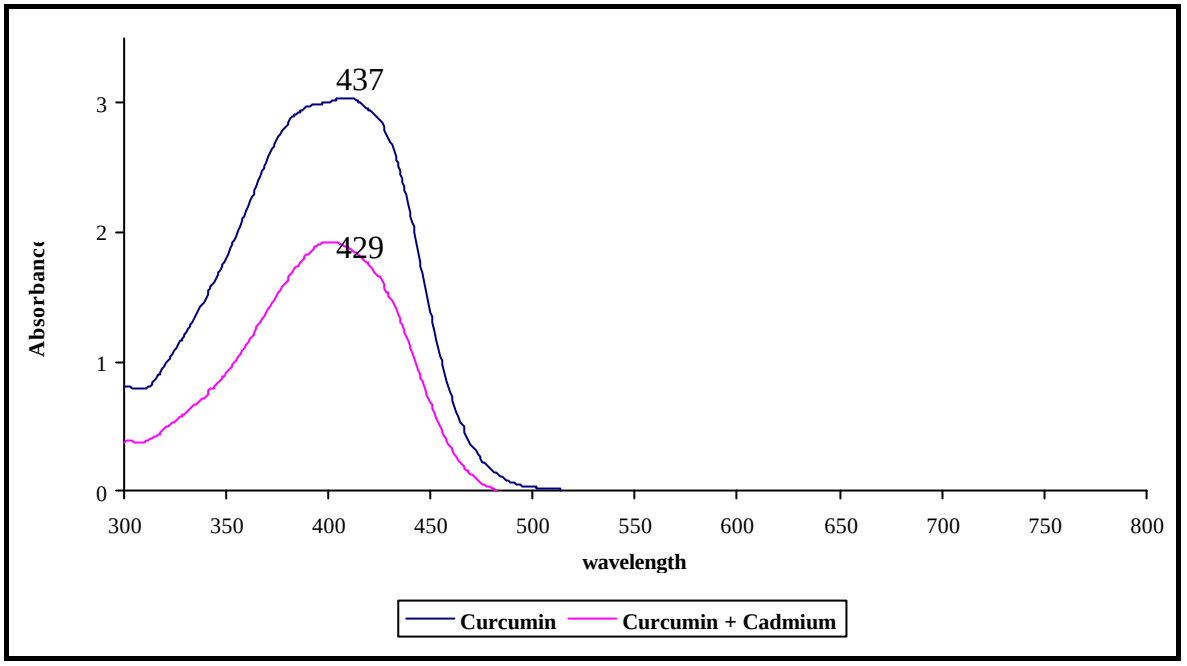

FIG. 7.7 *Spectroscopic analysis of curcumin and cadmium (3 x 10^{-5} M).*

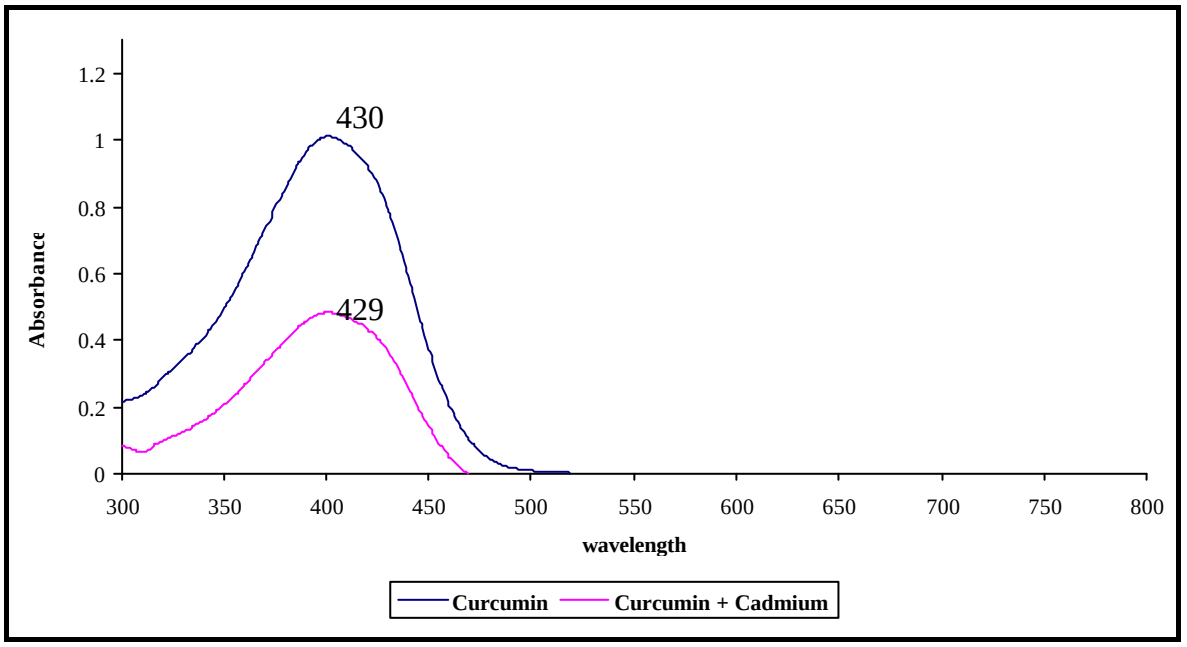

FIG. 7.8 *Spectroscopic analysis of curcumin and cadmium (7.8 x 10^{-6} M).*

At concentrations higher than 3 x 10^{-5} M, the absorption of curcumin was seen to develop shoulder bands at 354, 424, 438 and 452nm. Upon addition of equimolar concentrations of the metal to curcumin solution at a final concentration of 6 x 10^{-5} M, the split curcumin peaks collapses to a single peak at 452nm. The splitting of absorbance was retained however, when metal is added to curcumin at final concentrations of 1.25 x 10^{-4} M, with shifts of the bands to lower wavelengths. This behaviour was noted whether a 1cm or 0.2mm cuvette was employed, and is hence clearly a concentration effect rather than a detector response effect.

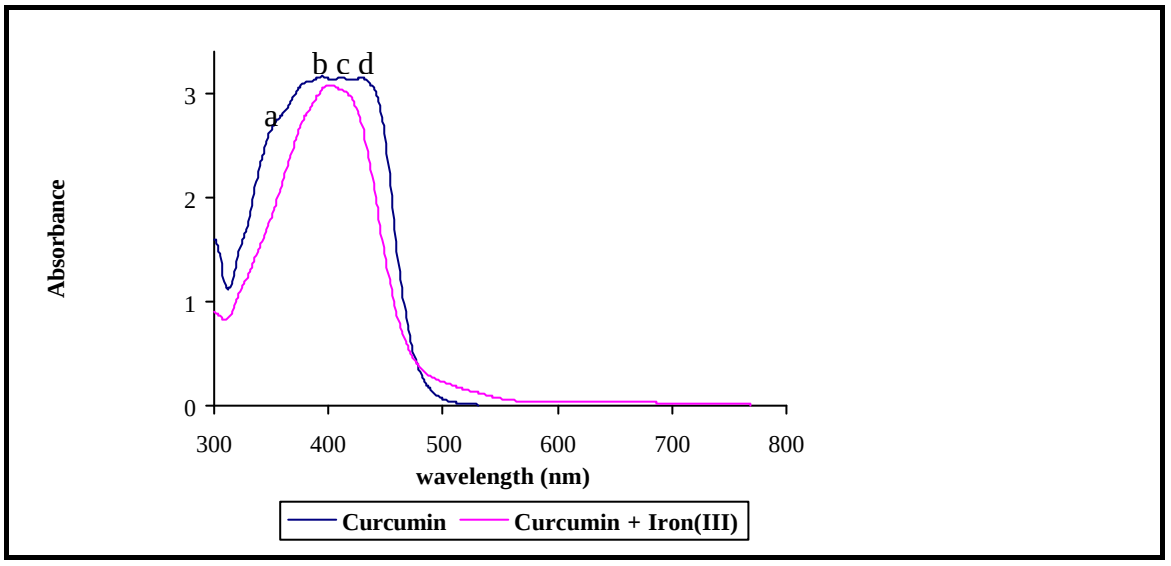

FIG. 7.9 *The development of absorption shoulders of curcumin (a, b, c and d) at concentrations higher than 3 x 10^{-5} M, and the collapsing thereof into a single peak upon addition of metal (Fe^{3+}).*

7.3.2 Infrared Spectroscopy

The IR spectra showed curcumin (appendix 3) to have a strong sharp O-H stretch at 3541 cm^{-1}, which may be ascribed to the hydroxyl group, and a medium, broad O-H stretch indicating strong hydrogen bonding at 3514 cm^{-1}, ascribed to the hydrogen-bonded enol form of the β-diketone moiety. The metal derivatives (appendix 4 and 5) showed two bands with shifts to lower wavelengths (3430 and 3280 cm^{-1}), both showing strong hydrogen bonding. This is indicative of metal complexation with both the hydroxyl groups attached to the two rings and the β-diketone moiety of curcumin.

Curcumin possesses two strong carbonyls at 1628 and 1603cm^{-1}, and a strong ν(C = C) at 1430cm^{-1} is observed in a rich spectrum between 1400 and 1100cm^{-1}. The infrared spectra for both the cadmium and lead complexes are identical, suggesting they are isostructural. Cadmium and lead showed the presence of two carbonyls (1585 and 1528cm^{-1} for cadmium, and 1586 and 1528 cm^{-1} for lead) and a shift of ν(C = C) 1264cm^{-1} for both complexes. In addition, several other bands in the infrared spectra are indicators for complexation.

There is a 10cm^{-1} shift of the strong curcumin band at 1022cm^{-1} to higher wave number, a shift of the medium intensity band of 854cm^{-1} to 845cm^{-1} and a collapse of a group of three weak bands at 574, 562 and 543cm^{-1} into a single band at 551cm^{-1} on complexation. These spectral changes are possibly due to symmetry charges associated with the benzene rings on complexation.

7.4 DISCUSSION

Curcumin is reported to have an absorption peak at 420 nm, which is ascribed to the presence of its conjugated diaryl heptanoid chromophore (Matsuda *et al*., 1999). However, the results obtained with this study show that at concentrations of 1.6 x 10^{-5} M and less, curcumin has a lamda max of 430nm and at concentrations of 3 x 10^{-5} M, a lamda max of 437nm. The splitting of the curcumin band at final concentrations higher than 3 x 10^{-5} M suggests that curcumin may be forming oligomers at higher concentrations.

The possibility of curcumin to dimerise has been noted by Matsuda *et al.* (1999) who have identified at least two dimeric species with slight shifts of the conjugated diaryl heptanoid chromophore due to changes in the conjugated system. They have also identified the radical reaction products vanillin (characteristic absorbance at 238nm) and ferulic acid (characteristic absorbance at 335nm). In the present study these were observed in solutions of concentrations greater than 6 x 10^{-5} M. These results point to the possibility that at concentrations of 6 x 10^{-5} M and higher, curcumin is susceptible to radical degradation under the given conditions.

At concentrations of 1.6 x 10^{-5} M and less, the addition of equimolar concentration of each metal studied caused a decrease in intensity of absorbance but no shift in the wavelength of the curcumin band. This is indicative of metal-ligand complexation, which occurs as long as the metal-ligand ratio is not greater than 1: 1 (Tønnesen & Greenhill, 1992). The shift in the curcumin band upon addition of metal (both at final concentrations of 3 x 10^{-5} M) accompanied by a decrease in the absorbance suggests that the interaction here is different to that of the dimeric species mentioned above.

The results of this study also showed the merging of the split bands of curcumin, upon addition of equimolar concentrations of metal at a final concentration of 6 x 10^{-5} M, which suggests the stabilisation of curcumin on complex formation. Oligomerisation of the complex appears to be retained at a concentration of 1.25 x 10^{-4} M, however, due to the presence of the split bands of curcumin with a shift to lower wavelengths, when metal was added.

The spectrophotometric data presented in this study clearly implies the direct interaction between curcumin and the metals, lead, cadmium, Fe^{2+} and Fe^{3+}. This is in support of the electrochemical work done with respect to curcumin and the metals, lead, cadmium and Fe^{3+} and in contrast to the electrochemical results obtained with curcumin and Fe^{2+}. Further studies need to be conducted in this respect. The present study also suggests that a suitable concentration for the therapeutic use of curcumin with regard to its metal – binding properties is limited to 1.25 x 10^{-4} M before its degradation products are formed.

The interaction between curcumin and lead, as well as curcumin and cadmium was further probed by examining the precipitates formed between metal and ligand using infrared spectroscopic techniques. Curcumin shows two possible sites of interaction with a metal- the hydroxyl group attached to the two rings and the β-diketone moiety bridging the two rings. Both sites have been identified as being important to biological activity (Tønnesen & Greenhill, 1992). The IR results obtained in this study suggest that both these sites are involved in metal-ligand complexation, either directly bonding to the metal, or in intermolecular hydrogen bonding.

Four major forms of metal interaction are known for the β-diketo moiety, which undergoes keto-enol tautomerism (Nakamoto, 1977). These are diagrammatically represented in Fig. 7.10.

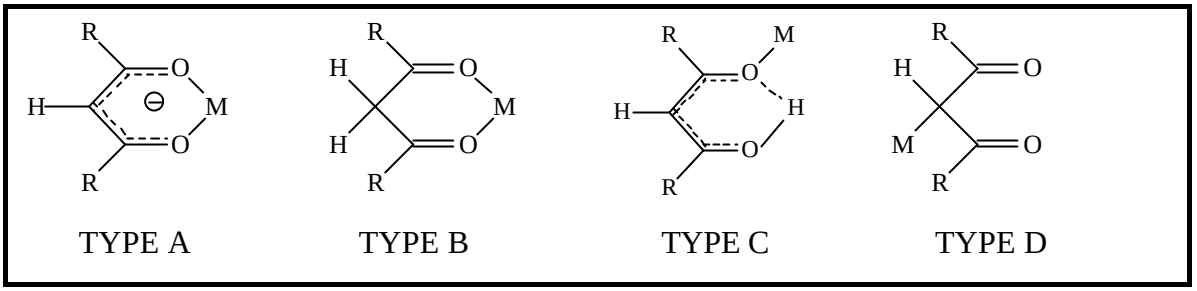

FIG. 7.10 *Four forms of metal interaction known for the b-diketo moiety (Nakamotoyo, 1977)*

Type A is associated with chelation in the ionic-enolic form. It is characterised by two strong bands between 1570 – 1525cm^{-1} and two between 1400 – 1280 cm^{-1} associated with the coupled ν(C = O) and ν(C =C) stretches respectively. Type B is associated with chelation in the neutral ketonic form. It is characterised by higher wavelengths of the carbonyls found between 1700 – 1650cm^{-1}. Type C is associated with the neutral enolic form with only monodentate coordination to the metal. It is characterised by 2 carbonyl bands at 1630cm^{-1} (between type A and B) and a lower band at 1560 cm^{-1}. Type D is associated with the metal binding to the central γ-carbon of the ionic ketonic form. It is characterised by two ν(C = O) stretches between 1650 and 1600 cm^{-1} and two ν(C = C) between 1350-1200 cm^{-1}, lower than that in type A.

The IR data presented above suggests that the metal-ligand binding is in accordance with type A in the solid state. This is consistent with the results observed for the gallium complex in solution (Borsai *et al.*, 2002).

CHAPTER EIGHT

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

8.1 INTRODUCTION

The pineal gland is recognized as an integral and important component of the neuroendocrine system (Reiter, 1989). The pattern of secretion of its major hormone, melatonin, conveys information regarding light-dark cycles to the body physiology for the organization of seasonal and circadian rhythms (Arendt, 1995). This is due to the fact that the cardinal feature of the melatonin synthesis pathway appears to be its rhythmicity.

Melatonin is a known free radical scavenger and has thus been promoted as an antioxidant agent with potential roles in protecting against a wide variety of neurological disorders that stem from ageing and age-related stress, such as AD and PD (Pappolla *et al*. 1998). Melatonin is synthesised within the pineal gland itself through the indoleamine metabolic pathway (Fig. 8.1) and this process can be divided into two parts. The first is the synthesis of serotonin from tryptophan and the second is the conversion of serotonin to melatonin and deaminated products.

8.1.1 Synthesis of Serotonin

Tryptophan is hydroxylated to 5-hydroxytryptophan by tryptophan hydroxylase, which is present in high concentrations in the pineal gland, and this appears to be the rate-limiting step in the synthesis of serotonin (Lovenberg *et al*, 1968). However it is the amount of substrate, tryptophan, available and not the enzyme activity, which influences the rate of this reaction. (Deguchi *et al*. 1972; Bensinger *et al*. 1974). 5-hydroxytryptophan is dehydroxylated to 5-hydroxytryptamine, also called serotonin, in presence of aromatic-L-amino acid decarboxylase, which is a nonspecific enzyme found in all tissues, but which

is present in the pineal organ at high concentrations. (Christenson *et al*. 1972). The activity of this enzyme is increased by exposure to constant lighting conditions, and this increase in activity in response to constant light is mimicked by the removal of the sympathetic nerves, implying that adrenergic stimulation depresses the activity of this enzyme. (Synder *et al*. 1965).

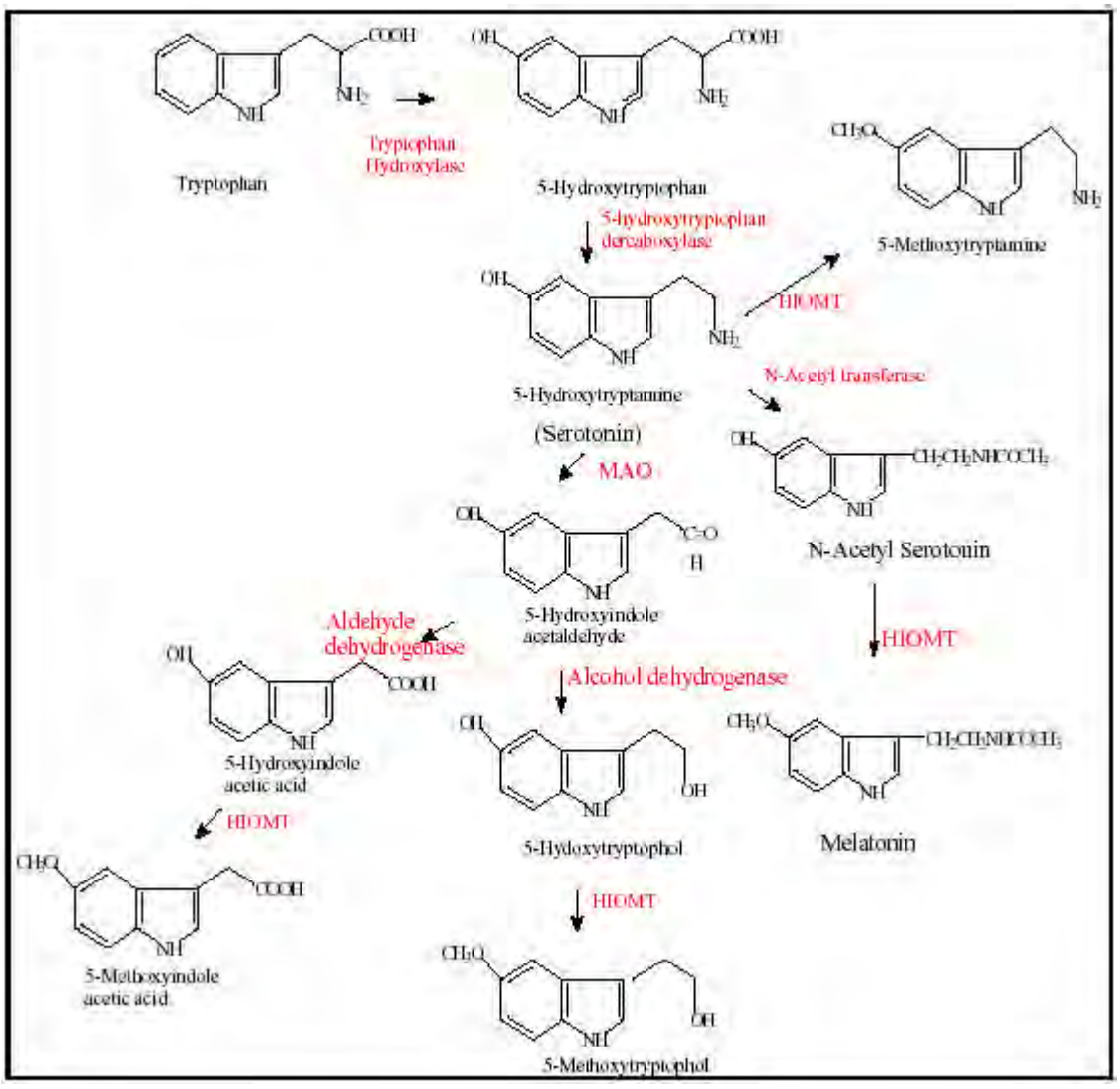

FIG. 8.1 *Pineal Indole Metabolism (Young & Silman, 1982).*

8.1.2 Conversion of Serotonin to its Metabolites

There are two pathways of serotonin metabolism resulting in either deaminated compounds or N-acetylated compounds. Serotonin may be deaminated by monoamine oxidase, an enzyme whose activity is known to be particularly high in the pineal gland, into 5-hydroxyindoleacetylaldehyde. This aldehyde is highly unstable and is immediately oxidized to the corresponding acid, 5-hydroxyindoleacetic acid, by the enzyme aldehyde dehydrogenase or reduced to the corresponding alcohol, 5-hydroxytryptophol by alcohol dehydrogenase (Winters *et al*., 1977; Morrissey *et al*., 1978). The 5-hydroxy group undergoes O-methylation to form corresponding 5-methoxyindoleacetic acid and 5-methoxytryptophol.

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

The quantification of pineal indoles and metabolism, and the influence of curcumin on the pineal indole metabolic pathway and melatonin synthesis were studied using organ culture. This is a valuable and sensitive technique, which enables the researcher to manipulate experimental conditions in such a way as to avoid complications of in vivo interactions. The pineal gland is ideal for organ culture due to its size, and remains viable for as long as six days under optimum conditions. It is capable of using exogenous radioactive (^{14}C) serotonin to produce various indoles including melatonin and serotonin (Daya *et al.*, 1989).

A bi-dimensional thin layer chromatographical system was used to isolate the pineal indoles (Klein & Notides, 1969). The first solvent comprises of three components including chloroform: methanol: glacial acetic acid (93:7:1) and it separates melatonin

(aMT) from N-acetylserotonin (aHT), and the 5-hydroxyindoles from the 5-methoxyindoles. The second solvent is ethyl acetate, and it separates 5-methoxyindole acetic acid (MA) and 5-methoxytryptophol (ML) from aMT, as well as 5-hydroxyindole acetic acid (HA) and 5-hydroxytryptophol (HL) from aHT. Serotonin and 5-methoxytryptamine are not affected by either of the solvents and remain at the origin (Anoopkumar-Dukie *et al*., 2001).

8.2 MATERIALS AND METHODS

8.2.1 Animals

Adult, male, Wistar rats weighing between 250 and 300g were used in this experiment and were obtained and cared for as described in section 2.2.2.1. The rats were randomly distributed into two groups, labelled control and test, each group containing five rats. The rats in the test group were administered with 0.25 ml of curcumin in ethyl oleate intraperitoneally in a dosage of 30mg/kg daily for three consecutive days, while the control group received 0.25 ml of ethyl oleate i.p. at the same time for three consecutive days. Thereafter all the rats were sacrificed by cervical dislocation and rapidly decapitated.

The brain was exposed in the manner described in section 2.2.2.1, and the pineal organ was carefully removed. The stalk, and any tissue adhering to the gland were also removed.

8.2.2 Chemicals and Reagents

^{14}C labelled serotonin was obtained from Amersham International, England. The culture medium, BGJb culture medium, was purchased from Gibco, Europe, and fortified with the antibiotics streptomycin and benzyl penicillin (Hoechst, South Africa). The composition of this medium is represented in the Table 8.1 below. The aluminium TLC plates coated with silica gel 60, Type F254 (0.25mm), were purchased from Merck, Darmstadt, Germany. Beckman Ready-Sol multipurpose liquid scintillation fluid was

purchased from Beckman RIIC Ltd, Scotland. The indole standards, MT, HA, HL, MA, ML, aMT, and aHT were purchased from Sigma Chemicals Co, St Louis, USA. All other reagents and chemicals were obtained locally and were of the highest purity available.

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

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

Table 8.1 *Composition of BGJb culture medium*

8.2.3 Organ culture of the Pineal Glands

The pineal glands were placed individually into sterile borosilicate (75 x 10 mm) Kimble tubes containing 52ul of BGJb culture medium. 8ul of (^{14}C) serotonin (specific activity 55mCi/mmol) was added. The tubes were then saturated with carbogen (95% Oxygen / 5% Carbon dioxide) and sealed. The tubes were incubated at 37 ^{0}C in the dark for 24 hours in a Forma Scientific model 3028 incubator. At the end of the 24-hour incubation period the reaction was terminated by the removal of the pineal glands from the culture medium.

8.2.4 Separation of Indoles by Thin Layer Chromatography

A modification of the technique employed by Klein and Notides (1969) was used to separate the radiolabelled indoles. The TLC plates were activated by placing them in an oven at 100 ^{0}C for 10 minutes. 10ul of the culture medium was spotted on a 10 x 10 cm thin layer chromatographic (TLC) plate. The spot was dried using a gentle stream of nitrogen. 10ul of the standard solution was spotted onto the culture medium. The standard solution was prepared as follows: 0.1mg of each standard indoleamine was dissolved together in a test tube containing 95% ethanol and 1% ascorbic acid. The ascorbic acid acts as an antioxidant. The second spot was also dried using a gentle stream of nitrogen.

The spotted plates were placed in a TLC tank containing chloroform: methanol: glacial acetic acid in a ratio of 93:7:1 and developed twice in this direction. The total front movement allowed during each development was 9cm and after each development the plates were dried under a gentle stream of nitrogen. After this, the plates were allowed to develop once in ethyl acetate at right angles to the first direction of development. The plates were once again dried under a gentle stream of nitrogen and the spots were observed and marked under UV light.

The spots were cut out and individually placed in plastic scintillation vials. 1ml of absolute ethanol was added to each vial, together with 3ml Beckman Ready-Sol scintillation fluid and then vortexed for 30s. The radioactivity of each metabolite was then measured in a Beckman LS 2800 scintillation counter.

8.3 RESULTS

A typical bi-dimensional thin layer chromatogram of the pineal indole metabolites was obtained which is shown in Fig. 8.2. Good separation of the indole metabolites and high counts were obtained. Since serotonin and methoxytryptamine did not migrate from the origin, the results expressed for the origin are those of methoxytryptamine and serotonin. The results were obtained as counts per minute (CPM) and each indole metabolite was expressed in terms of percentage of total counts per minute per 10ul /pineal gland.

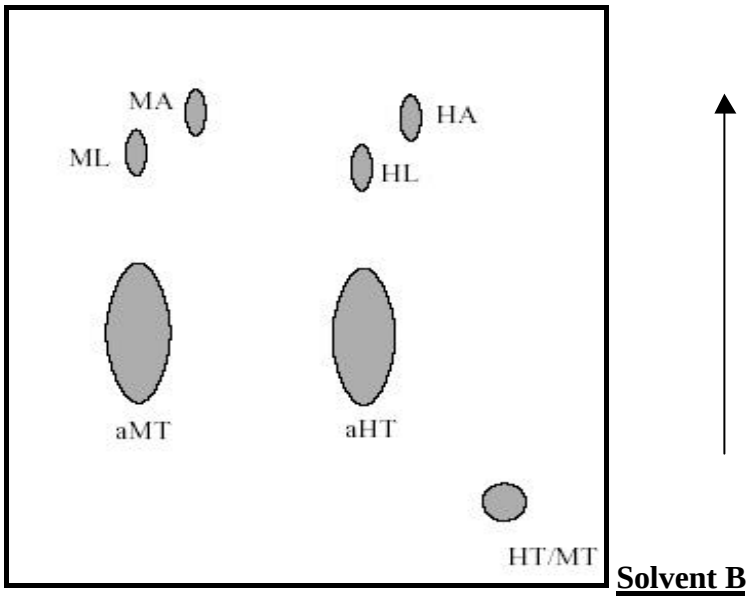

FIG. 8.2 *An illustration of the bi-dimensional thin layer chromatogram of the pineal indole metabolites showing the direction in which the plate was run and the location of the pineal indole metabolites (Klein & Notides, 1969).*

Following administration of curcumin (30mg/kg) intraperitoneally, there was an increase in combined serotonin and 5 – methoxytryptamine levels by 15.5%, N – acetylserotonin levels by 1.32% and melatonin levels by 0.8% in comparison to the control. A decrease in 5-hydroxyindole acetic acid (6.4%), 5 – hydroxytryptophol (3.9%), 5 – methoxyindole acetic acid (0.3%) and 5 – methoxytryptophol (0.2%) in comparison to the control, was also observed (Fig. 8.3).

All results were analysed using ANOVA followed by the Student-Newman-Keuls Multiple Range Test. The level of significance was accepted at $p<0.05$ (Zar, 1974).

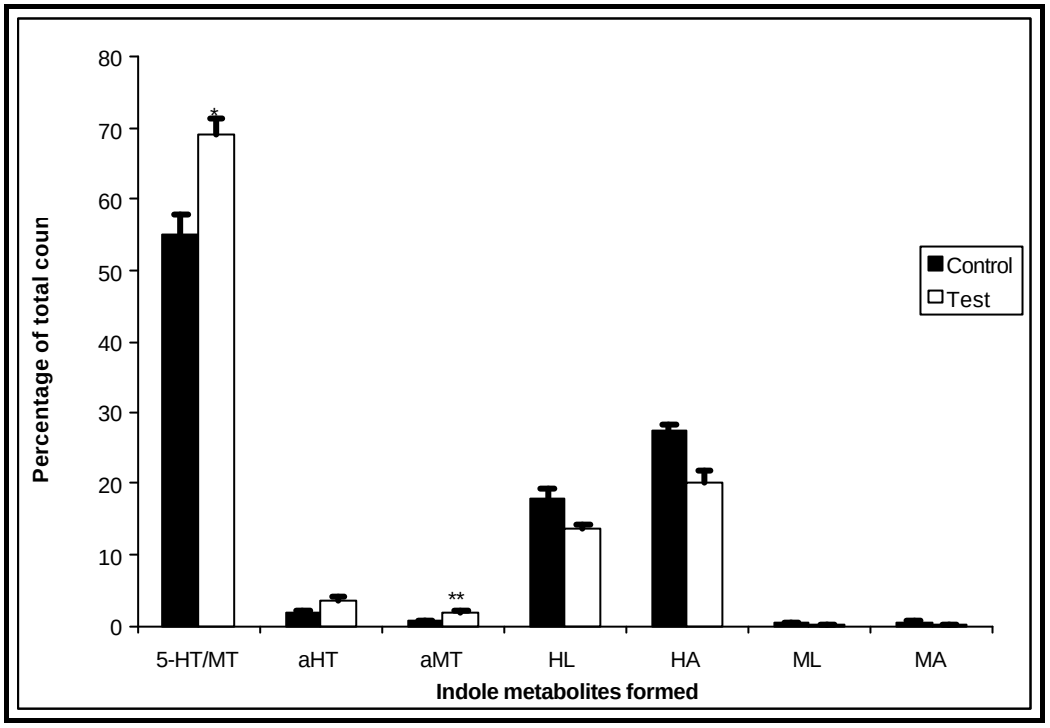

FIG. 8.3 *The effect of curcumin on pineal indole metabolism. Each bar represents the mean ± SD; n = 5. *(p = 0.0001) ** (p = 0.0076).*

8.4 DISCUSSION

The increase in radiolabelled - melatonin observed in curcumin treated rats could be possibly due to improved uptake of radiolabelled – serotonin by the pinealocytes. Another possibility for the increased levels of radiolabelled serotonin is that curcumin could be inhibiting monoamine oxidase (MAO) enzyme which is primarily involved in the conversion of serotonin to its oxidation products. It can be noted that all the deaminated products of serotonin was found to be decreased in curcumin – treated rats, with respect to the control. Hence more serotonin is available for the production of melatonin.

Mazzio *et al*. (1998) conducted studies on the effect of various dietary components on MAO activity in rat C6 glial cells and the results showed a dose – dependent inhibition of MAO-B activity by curcumin. MAO-B is the predominant form of MAO in human brain. However serotonin is a specific substrate for MAO-A and further research needs to be undertaken to determine whether curcumin is capable of specifically inhibiting MAO-A.

The conversion of serotonin to N – acetylserotonin is catalysed by the enzyme serotonin N – acetyl transferase that is present in the cytoplasm of the cell (Weissbach *et al*. 1961). The activity of this enzyme is regulated by an adrenergic cyclic AMP mechanism (Klein 1979). The possibility that curcumin could be increasing N – acetyl serotonin by inducing this enzyme does not seem likely because Curcumin is a cyclooxygenase – 2 (COX – 2) inhibitor (Reddy *et al*. 2002) and this is one of the mechanisms involved in its anti-inflammatory properties (Huang *et al* 1991). COX-2 is responsible for the synthesis of prostaglandin E_2 that, on binding to its receptor, induces intracellular cyclic adenosine monophosphate (cAMP) accumulation (O'Byrne *et al*. 2000). Inhibition of the COX – 2 pathway and subsequent inhibition of the prostaglandin synthesis results in an inhibition of cAMP and consequent decrease in N – acetyl transferase. Hence the increase in N – acetyl serotonin in curcumin treated rats could be more likely attributed to the increased level of serotonin available as substrate for the enzyme.

N – acetylserotonin is converted to melatonin by hydroxyindole – O – methyl transferase (HIOMT), which transfers a methyl group from the co – factor S – adenosyl methionine to the acceptor molecule (Axelrod *et al.,* 1961). The intracellular mechanisms involved in the regulation of HIOMT have not yet been clearly defined. However it can be postulated that since there is an increase in N – acetylserotonin, more substrate is available for HIOMT to produce more melatonin. The direct effect of curcumin on this enzyme is yet to be investigated.

The increase in melatonin synthesis by curcumin has a direct implication on neuroprotection based on the ability of melatonin to function as a free radical scavenger and an antioxidant in the brain. Melatonin has also been shown to protect against

neuronal cerebral damage induced by acute global cerebral ischemia (Letechipía-Vallejo, 2001) and glial cell survival increased in the presence of melatonin during cerebral ischaemia (Borlongan, 2000). Although the enhanced production of melatonin and its precursors by the pineal gland in the presence of curcumin suggests a valuable mechanism of neuroprotection by curcumin, the experiment conducted demonstrate only a slight increase of melatonin production (0.8 %) in presence of curcumin. It is thus debatable as to whether this increase can contribute significantly to neuroprotection or not.

CHAPTER NINE

GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

The experiments conducted in this study point conclusively to a new and emerging role of curcumin as a neuroprotective agent.

The extent of protection that curcumin as an antioxidant, could afford to the brain, against oxidative stress in the form of superoxide radicals was investigated in chapter two. The experimental results indicate that curcumin affords significant protection against cyanide induced superoxide radical generation in the rat brain homogenate. Brain homogenate that was obtained from rats pre-injected with curcumin (30mg/kg, i.p.) also showed significant protection. The superoxide scavenging abilities of curcumin were postulated to be due to its anti-inflammatory actions.

Since curcumin showed protection to the rat brain homogenate from cyanide induced superoxide radical generation, and considering the fact that cyanide is a respiratory poison which acts by blocking complex IV of the ETC, the study in chapter three was conducted to determine the effect that curcumin had on cyanide induced depression of the ETC within a mitochondrial fraction isolated from the rat brain. The results demonstrated an increase in ETC activity in curcumin treated samples compared to cyanide treated samples. Curcumin also demonstrated an increase in basal level of respiration within the brain mitochondria. Considering the fact that respiratory inhibition of mitochondria by cyanide is the basis for its superoxide radical generation, it can be concluded that the protection offered by curcumin against superoxide radical generation by cyanide was not only due to its antioxidant properties, but also due to the direct inhibition of cyanide insult within the ETC.

Chapter four investigated the neuroprotective effects of curcumin against oxidative stress in the form of lipid peroxidation induced by quinolinic acid, and the toxic metals, lead

and cadmium. Curcumin significantly reduced lipid peroxidation induced by all three neurotoxins. Previous work has postulated that the mechanism by which QA induces lipid peroxidation involves the binding of this toxin to ferrous ions to form a complex, which then induces peroxidation of the membranes. It is suggested that the protection curcumin offered to lipid peroxidation induced by QA, lead and cadmium was not only attributable to the antioxidant properties of this phytochemical, but also due to a possible chelation of metals, iron (II), iron (III), lead and cadmium.

Histological investigation (chapter five) of nervous tissue within the hippocampal CA1 and CA3 regions showed visible disruptions of the structural features of these regions in rats treated with lead acetate (25mg/kg i.p.) and significant protection in rats treated simultaneously with lead acetate (25mg/kg i.p.) and curcumin (30mg/kg i.p.). Moreover external observations in the rats treated with lead and curcumin showed a relatively greater activity and faster response as compared to those that were treated with lead alone. It is suggested that the ability of curcumin to prevent lipid peroxidation induced by lead could be one of the factors pertaining to the higher degree of integrity of cells within the hippocampal tissue of the curcumin treated rats. The anti-inflammatory property of curcumin could also have contributed to the reduced amount of swelling within neuronal cells in the brains of curcumin treated rats. The possibility that curcumin may be binding to lead and thus inactivating its neurotoxic effects was also investigated in later chapters.

Chapter six was devoted to the investigation of the metal-binding capabilities of curcumin with lead, cadmium iron (II) and iron (III), using electrochemical techniques. The results implied an interaction between curcumin and lead as well as cadmium to form two new species with lead and one new complex with cadmium. Curcumin also showed strong interaction with iron (III) with the possibility of a iron (III)-curcumin complex having been formed in solution. The electrochemical results did not, however, support the possibility of an interaction with iron (II).

Further analytical work was conducted to probe the interactions between curcumin and the metals mentioned above, in chapter seven. Solutions of appropriate mixtures of

curcumin and metal were analysed using UV/VIS spectrophotometric analysis, which suggested interactions between curcumin and all four metals in terms of a shift in the wavelength and an increase in absorbance of curcumin. The results of the study also indicated that the suitable concentration for the therapeutic use of curcumin with regard to its metal – binding properties is limited to 1.25×10^{-4} M before its degradation products are produced.

Precipitates formed upon interaction with curcumin and each metal, were also investigated by spectroscopy, and compared to curcumin alone. Results obtained with both cadmium and lead – curcumin precipitates, implied the binding of both these metals by curcumin to form new complexes. Both the hydroxyl groups, and the β-diketone moiety of curcumin are suggested to be involved in metal-ligand complexation, either directly bonding to the metal, or in intermolecular hydrogen bonding.

Thus the results of this study strengthens the postulation made earlier suggesting that one of the mechanisms by which curcumin protects against lead and cadmium toxicity in the body is by binding to it and forming new complex. This study has also suggested interaction between curcumin and iron (III) as well as iron (II) but the actual mode of interaction has not been established. The electrochemical analysis and UV/VIS analysis showed conflicting results with regards to iron (II)-curcumin interactions, and further studies need to be conducted in order to clear the ambiguity.

Chapter eight investigated the effect of curcumin on the production of melatonin, a natural antioxidant and neuroprotectant, produced by the pineal gland. The results obtained showed that curcumin stimulated an increase in melatonin production. This was attributed to an increased uptake of serotonin by the pineal gland in the presence of curcumin and the inhibition of the degradation pathway of serotonin by this phytochemical. However, the exact mode of action remains to be established. The increased production of melatonin produced in presence of curcumin appeared to be minute and hence the neuroprotective capabilities of curcumin may not necessarily be

attributable to increased production of melatonin, but rather to its antioxidant, anti-inflammatory and metal-binding capabilities.

No effective cure has so far been reported to treat neurological disorders associated with aging other than therapeutic interventions that could forestall or delay the normal and pathological ageing process. With respect to such a prophylactic approach, nutritional interventions may be viewed as a viable option (Deschamps *et al.*, 2001; Youdim & Joseph, 2001). Curcumin, the principle component of a daily dietary component of the Asian population, has been shown to afford protection to the brain against cyanide, quinolinic acid, lead and cadmium induced insult *in vitro* and *in vivo.* This study has also shown that the antioxidant and anti-inflammatory activities of curcumin contribute toward its neuroprotective abilities. Moreover the metal binding capabilities of curcumin with lead, cadmium and iron, have implications on neuroprotection because of the extent of involvement of metals with the brain with respect to oxidative stress and neurodegeneration.

Although the results of this study point to a possible role of curcumin as neuroprotectant, further research on this polyphenolic compound needs to be conducted in order to confirm the deductions made. The metal-chelating property of curcumin with lead and cadmium as demonstrated in this study, is an exciting prospect which also needs to be analysed in depth as to the characterisation of the new complexes formed, its biological and toxicological properties, as well as pharmacokinetic properties. The structure and properties of the flavonoids predict that they have a good chance of reaching and mobilising heavy metals within the body as well as form strong ligand complexes with heavy metal ions (Havsteen, 2002). Hence, the interactions of curcumin with a wider range of metals can be analysed, along with its effects on the biological damages that such metals produce in the body.

It would also be worthwhile investigating the consequences of the long-term use of curcumin especially with regard to its metal-chelating properties. This is of high significance with metals like iron and zinc, which are essential to various functions in the

body. Depletion of these metals lead to various disease conditions. Iron deficiency not only leads to anaemia, but can also lead to adverse effects on the cells of the CNS and on neurotransmitters. Iron-deficiency anaemia can also lead to reduced oxygen delivery to the brain (Gordon, 2003).

Another area for future study would be an investigation into the molecular mechanisms by which curcumin exerts its various neuroprotective abilities. The possibility that curcumin could be regulating a number of heat shock responses, a universally fundamental mechanism necessary for cell survival under a variety of unfavourable conditions, has already been raised, and such studies need to be facilitated in order to determine the exact mode of action of curcumin. The effects of curcumin on various receptors on the brain, such as those involved in excitotoxicity, also needs to be explored.

While pharmaceutical companies continue to invest huge resources into identifying agents which could be used to alleviate debilitating neurodegenerative diseases, a source of potentially beneficial agents, namely phytochemicals, appears to offer significant benefits that are yet to be fully exploited. Interventions at an early stage of neurological diseases is an important step in combating the illness, since by the time the symptoms of such disorders occur, a certain amount of neuronal death has already occurred. In this light, it is interesting to note that some epidemiological studies have shown that dietary habits can influence the incidence of neurodegenerative disorders (Esposito, 2002).

Thus, it is desirable and of benefit, that more studies on the potential benefits of curcumin as a neuroprotectant and metal-chelator be conducted, as well as investigations into the relationship between curcumin and the relative risk for the development of various neurodegenerative disorders such as AD and PD be facilitated.

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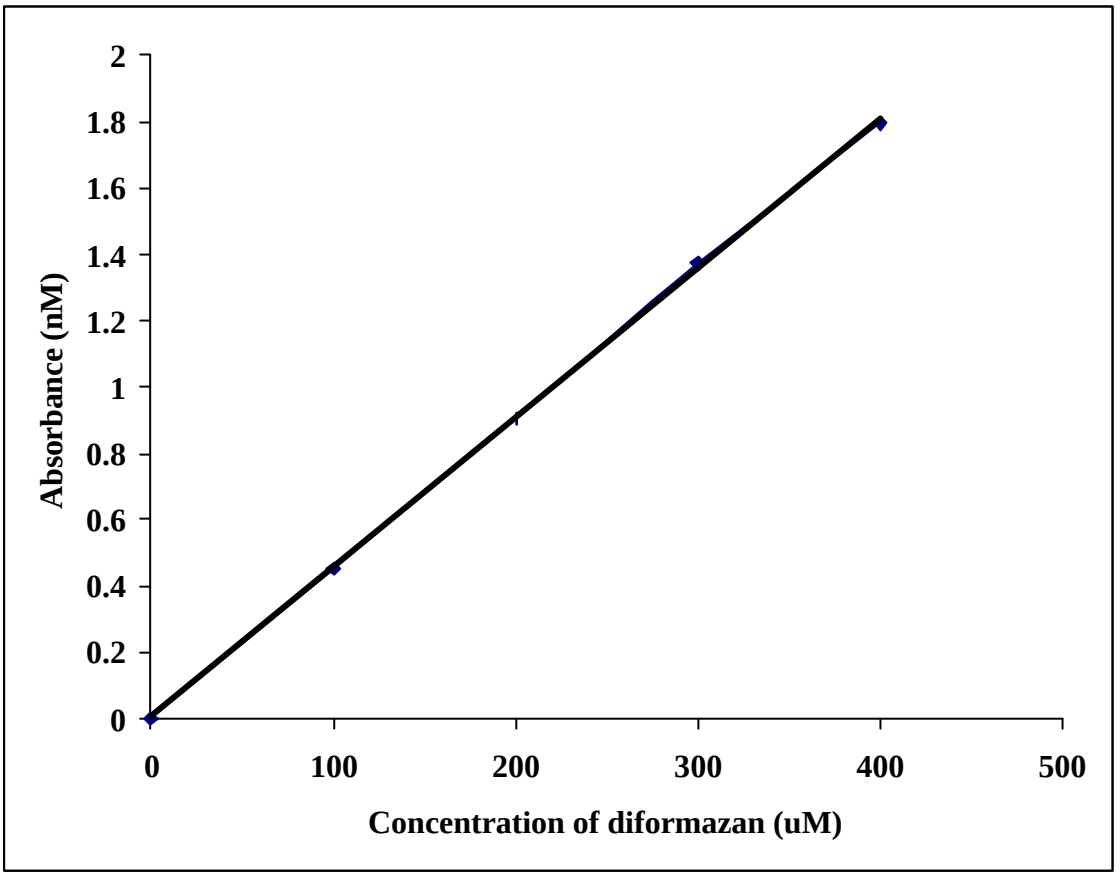
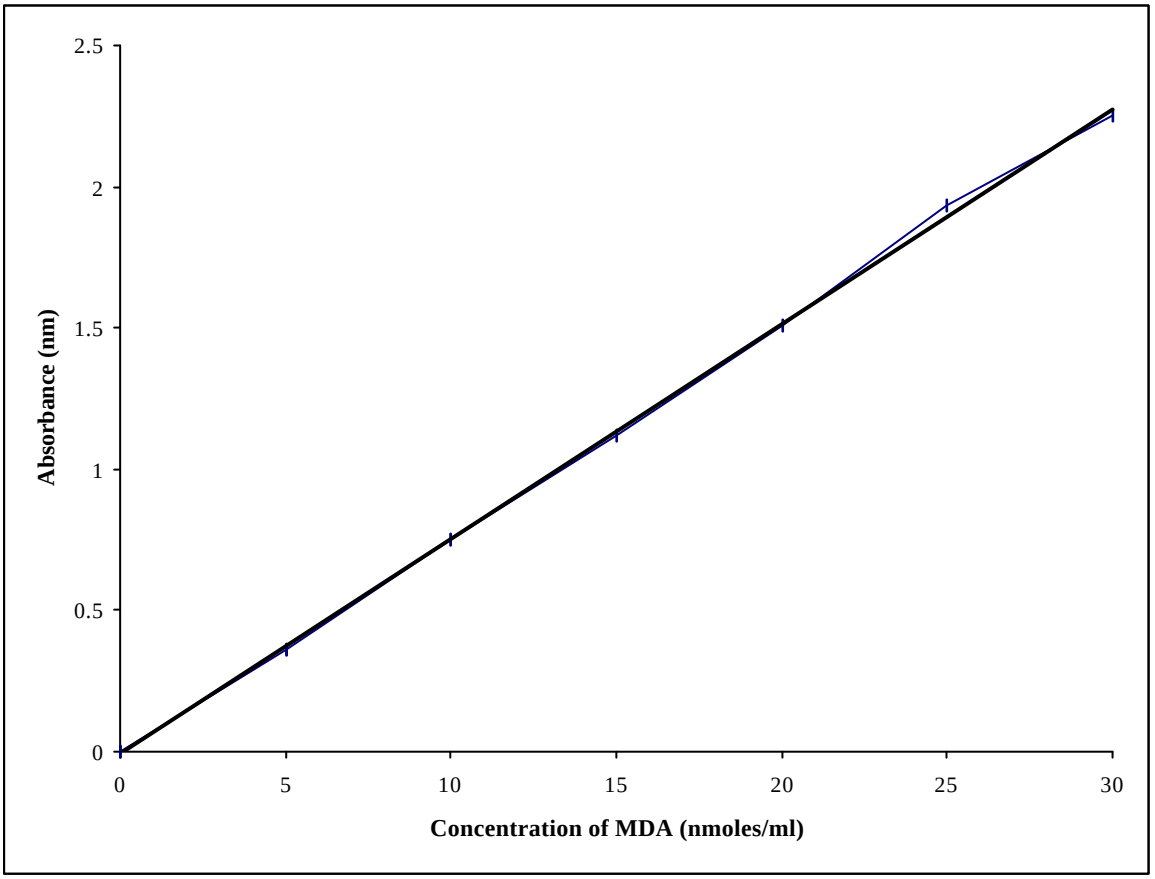
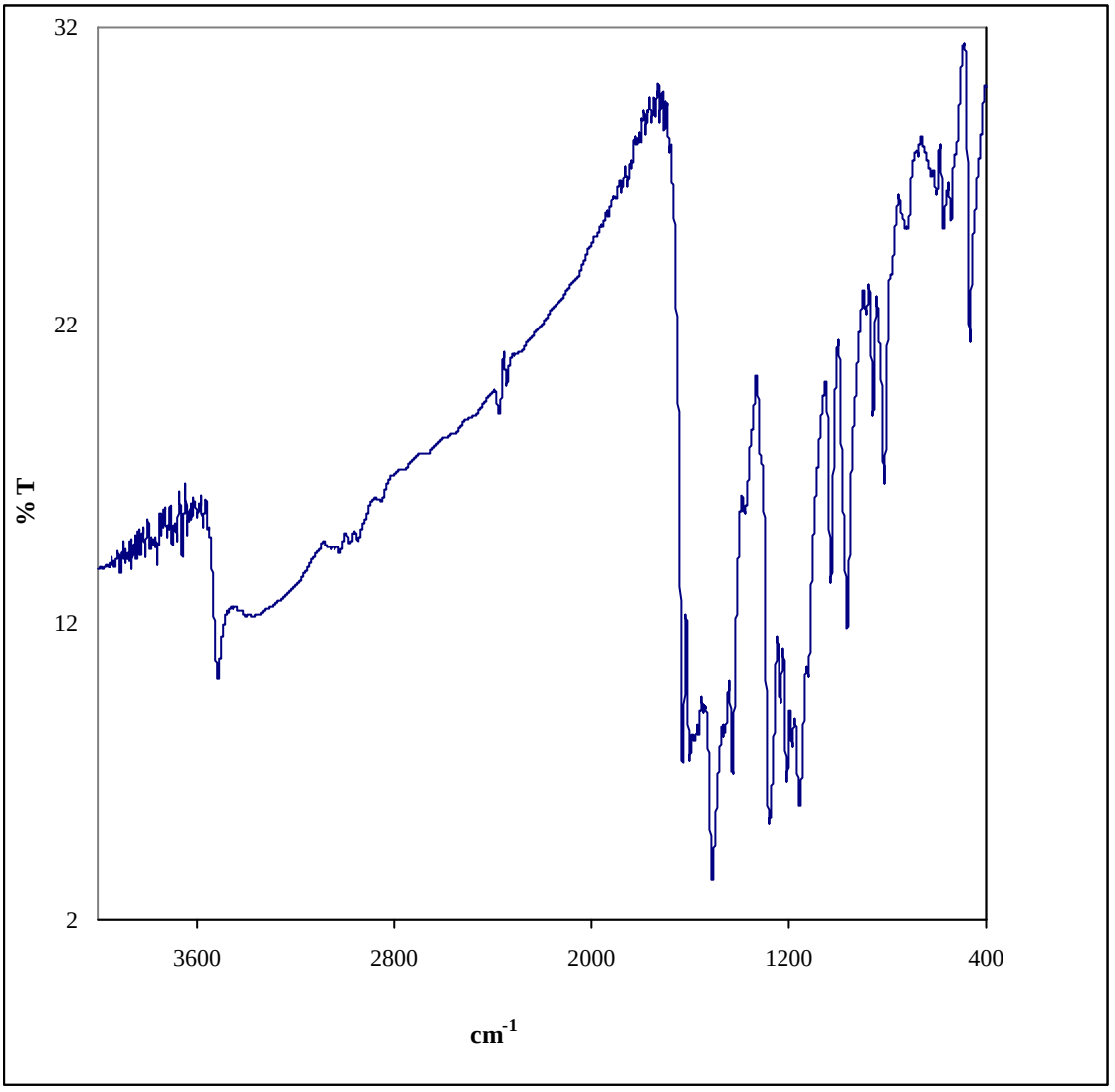
APPENDICES



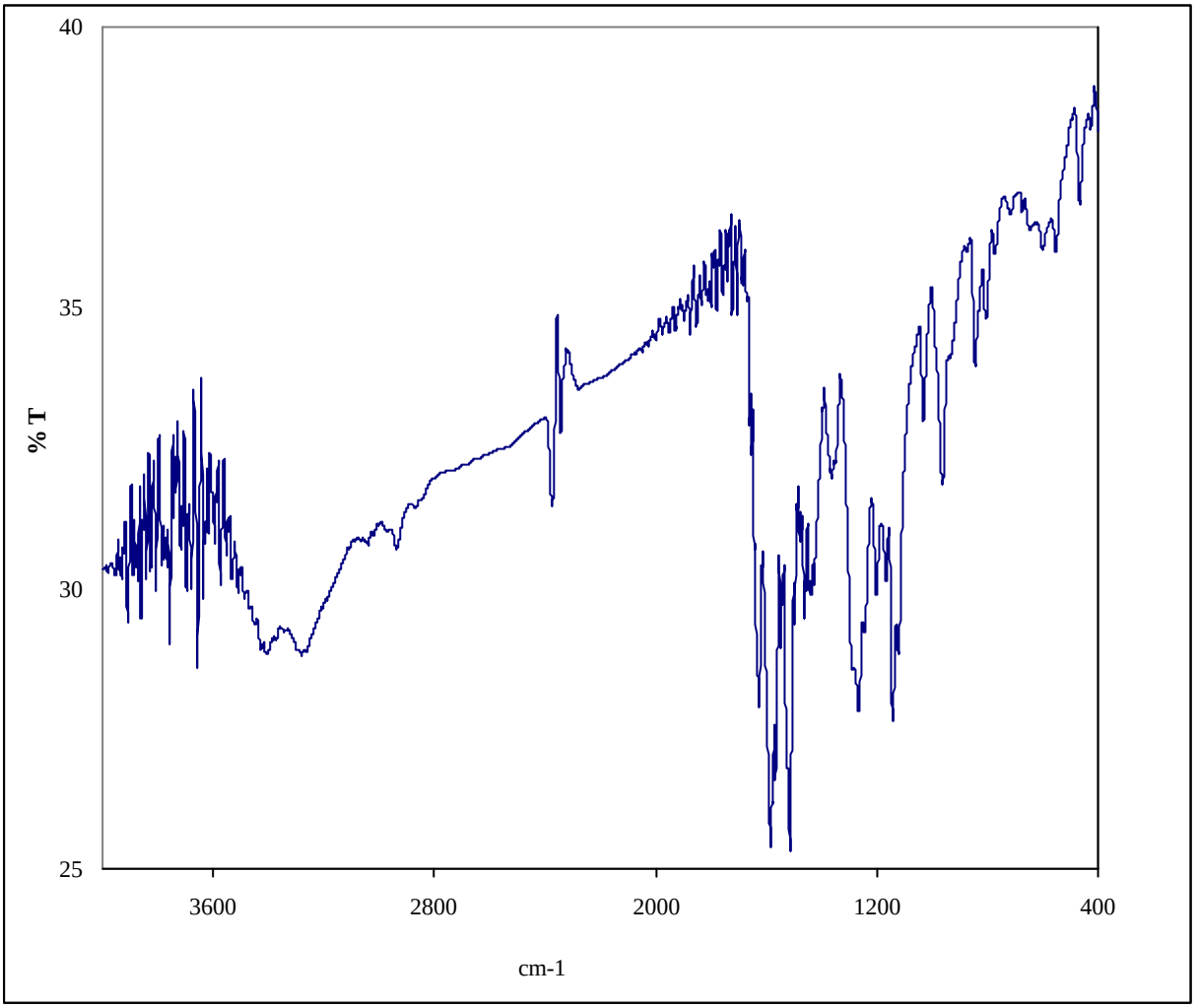
APPENDIX 1 CALIBRATION CURVE FOR DIFORMAZAN (R^2 = 0.999)



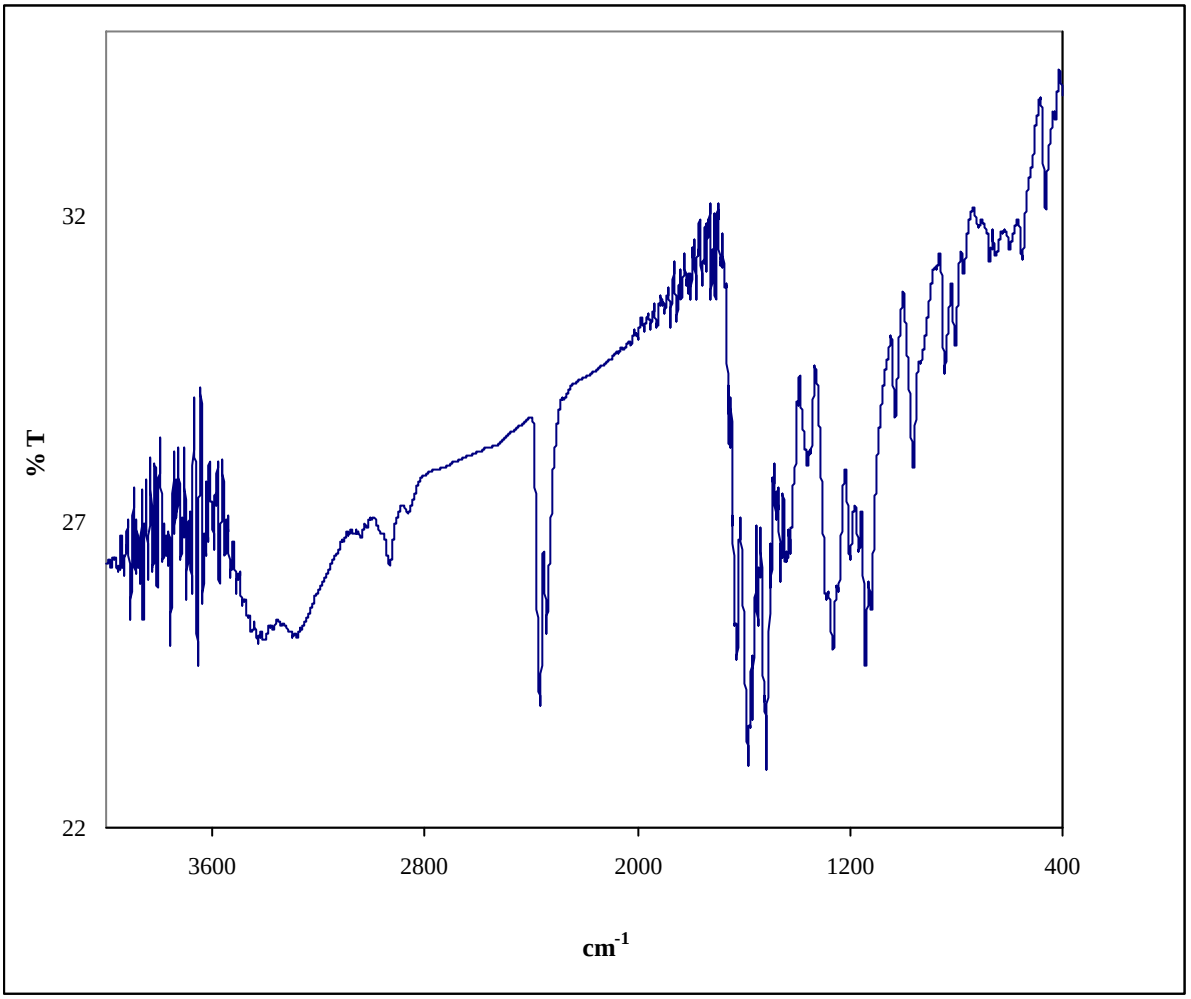
APPENDIX 2 MALONDIALDEHYDE STANDARD CURVE (R^2 = 0.999)



APPENDIX 3 INFRARED SPECTROSCOPY OF CURCUMIN



APPENDIX 4 **INFRARED SPECTROSCOPY OF ISOLATED CURCUMIN-LEAD COMPLEX**



APPENDIX 5 INFRARED SPECTROSCOPY OF THE ISOLATED CURCUiroMIN – CADMIUM COMPLEX