AN INVESTIGATION INTO THE NEUROPROTECTIVE PROPERTIES OF ACYCLOVIR

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ABSTRACT

Accumulating evidence suggests that quinolinic acid has a role to play in disorders involving impairment of learning and memory. In the present study, the effect of the guanosine analogue antiherpetic, acyclovir, on quinolinic acid-induced spatial memory deficits was investigated, as well as some of the mechanisms which underlie this effect.

Behavioural studies using a Morris water maze show that post-treatment of rats with acyclovir significantly improves spatial memory deficits induced by intrahippocampal injections of quinolinic acid. Histological analysis of the hippocampi show that the effect of acyclovir is related to its ability to alleviate quinolinic acid-induced necrotic cell death, through interference with some of the mechanisms of neurodegeneration. However, acyclovir is unable to alter a quinolinic acid-induced increase in glutamate release in the rat hippocampus, even though it alleviates quinolinic acid induced oxidative stress by scavenging the superoxide anion *in vitro* and *in vivo* in whole rat brain and hippocampus respectively. Due to the inverse relationship which exists between superoxide anion and glutathione levels, acyclovir also curtails the quinolinic acid-induced decrease in hippocampal glutathione levels.

Acyclovir suppresses quinolinic acid-induced lipid peroxidation *in vitro* and *in vivo*, in whole rat brain and hippocampus respectively, through its alleviation of oxidative stress and possibly through the binding of iron (II) and / or iron (III), preventing the participation and redox recycling of iron (II) in the Fenton reaction, which quinolinic acid is thought to enhance by weak binding of ferrous ions. This argument is further

strengthened by the ability of the drug to suppress iron (II)-induced lipid peroxidation *in vitro* directly. Inorganic studies including ultraviolet and visible spectroscopy, electrochemistry and the ferrozine assay show that acyclovir binds to iron (II) and iron (III) and that quinolinic acid forms an easily oxidisable association with iron (II).

Acyclovir inhibits the endogenous biosynthesis of quinolinic acid by inhibiting the activity of liver tryptophan-2,3-dioxygenase, intestinal indoleamine-2,3-dioxygenase and rat liver 3-hydroxyanthranillic acid oxygenase *in vitro and in vivo*, possibly through competitive inhibition of haeme, scavenging of superoxide anion and binding of iron (II) respectively. An inverse relationship exists between tryptophan-2,3-dioxygenase activity and brain serotonin levels. Acyclovir administration in rats induces a rise in forebrain serotonin and 5-hydroxyindole acetic acid and reduces the turnover of forebrain serotonin to 5-hydroxyindole acetic acid. Furthermore, it shows that acyclovir does not alter forebrain norepinephrine levels. The results of the pineal indole metabolism study show that acyclovir increases 5-hydroxytryptophol, N-acetylserotonin and the neurohormone melatonin, but decreases 5-hydroxyindole acetic acid.

The results of this study show that acyclovir has some neuroprotective properties which may make it useful in the alleviation of the anomalous neurobiology in neurodegenerative disorders.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	percent
ΔE_P	change in electrical potential
ΔI_A	change in current
°C	degrees Celsius
µg	microgram
µl	microlitre
µm	micrometres
3-HA	3-hydroxyanthranillic acid
3-HAO	3-hydroxyanthranillic acid oxygenase
3-HK	3-hydroxykynurenine
5-HIAA	5-hydroxyindole acetic acid
5-HT	serotonin
5-HTOH	5-hydroxytryptophol
5-MIAA	5-methoxyindole acetic acid
5-MT	5-methoxytryptamine
5-MTOH	5-methoxytryptophol
7-nACh-R	7-nicotinic acetylcholine receptor
A	amperes
AC	acyclovir
ACN	acetonitrile
AD	Alzheimer's disease
AdCSV	adsorptive cathodic stripping voltammogram
ADP	adenosine diphosphate
AGE	advanced glycation endproducts
AMPA	α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid
aMT	melatonin
ANOVA	analysis of variance
AP	beta amyloid plaques
ASV	adsorptive stripping voltammatery
ATP	adenosine triphosphate

BHT	butylated hydroxytuolene
BSA	bovine serum albumin
CA	cornu ammonis
$CaCl_2$	calcium chloride
CAT	catalase
cGMP	cyclic guanylate monophosphate
-CH(NH2)COOH	secondary amine
Ci	curies
cm	centimetre
CNS	central nervous system
CPM	counts per minute
CR	conditioned response
CS	conditioned stimulus
DA	dopamine
dATP	deoxy adenosine triphosphate
DG	dentate gyrus
DNA	deoxyribonucleic acid
DNA-PK	deoxyribonucleic acid-dependent protein kinases
DPM	disintegrations per minute
DPX	distrene plasticiser in xylene
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EC	entorhinal cortex
EDTA	ethylene diamine tetra acetic acid
EGTA	ethylene glycol bis (2-ainoethylether)-N,N,N',N'-tetra acetic acid
E_P	electrical potential
ETC	electron transport chain
FAD	flavin adenine dinucleotide
$FADH_2$	dihydroflavin adenine dinucleotide
Fe^{2+}	ferrous ion
Fe^{3+}	ferric ion
$FeCl_3$	anhydrous ferric chloride
$FeSO_4.7H_2O$	hydrated ferrous sulphate
FMN	flavin mononucleotide

$FMNH_2$	dihydroflavin mononucleotide
fmoles	faromoles
g	gram
GCE	glassy carbon electrode
GDP	guanosine diphosphate
GMP	guanosine monophosphate
GPx	glutathione peroxidase
GR	glutathione reductase
G_s	guanine nucleotide-binding regulatory protein
$GS^{\cdot}$	glutathione radical
GSH	reduced glutathione
GSSG	oxidised glutathione
GTP	guanosine triphosphate
$H^{\cdot}$	hydrogen radical
H_2O	water
H_2O_2	hydrogen peroxide
HBSS	Hepes/Cl buffered salt solution
HCl	hydrochloric acid
$HClO_4$	perchloric acid
HEPES	4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid
HIOMT	hydroxyindole-O-methyl transferase
HNRNPs	heteronuclear ribonucleoproteins
$HO^{\cdot}$	hydroxyl radical
$HOO^{\cdot}$	hydroperoxyl radical
HSA	heptane sulphonic acid
HSE	herpes simplex encephalitis
HSV-1	herpes simplex virus type-1
H-W	Haber-Weiss
i.p.	intraperitoneal
i.v.	intravenous
I_A	current
IDO	indoleamine-2,3-dioxygenase
IFN-γ	interferon gamma
IL	interleukin

-indole-NH	indole amine
Iz	imidazolone
KCl	potassium chloride
kg	kilograms
KH_2PO_4	potasssium dihydrogen phosphate
K_m	Michaelis Menton equilibrium constant
KP	kynurenine pathway
LHPA	limbic-hypothalamic-pituitary-adrenal
L-KYN	L-kynurenine
LP	lipid peroxidation
LTP	long term potentiation
M	molar (moles per litre)
MAO	monoamine oxidase
MDA	malondialdehyde
MEM	minimum essential medium
mg	milligram
$MgSO_4$	magnesium sulphate
min.	minute
ml	millilitre
mm	millimetre
mM	millimolar (millimoles per litre)
mmoles	millimoles
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger ribonucleic acid
mV	millivolt
Na^+/K^+ ATPases	sodium/potassium ATPases
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide hydrogen
NAS	N-acetyl serotonin
NAT	N-acetyl transferase
NBD	nitroblue diformazan
NBT	nitroblue tetrazolium
NE	norepinephrine

NFT	neurofibrillary tangles
NFκB	nuclear factor kappa B
NHLS	National Health Laboratory Services
nm	nanometres
NMDA	N-methyl-D-aspartate
nmoles	nanomoles
NO	nitric oxide
NOS	nitric oxide synthase
NP-SH	non-protein thiol
O_2	molecular oxygen
$O_2^{\cdot-}$	superoxide anion radical
$ONOO^-$	peroxynitrite radical
PA	phosphoric acid
PARP	polyADPribose polymerase
PBS	phosphate buffered saline
PLA_2	phospholipase A_2
pmoles	picomoles
PPP	pentose phosphate pathway
PT	permeability transition
PUFA	polyunsaturated fatty acids
QA	quinolinic acid
RAGE	receptor for advanced glycation endproducts
redox	reduction-oxidation
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
$RO^{\cdot}$	alkoxyl radical
$ROO^{\cdot}$	peroxyl radical
ROOH	lipid peroxide
ROS	reactive oxygen species
R-SH	thiol-containing compounds
s	seconds
SD	standard deviation
SOD	superoxide dismutase
TBA	thiobarbituric acid

TCA	trichloroacetic acid
TDO	tryptophan-2,3-dioxygenase
TEA	triethylamine
TK	thymidine kinase
TLC	thin layer chromatography
TNF-α	tumour necrosis factor alpha
TRP	tryptophan
UR	unconditioned response
US	unconditioned stimulus
UV	ultraviolet
UV/VIS	ultraviolet and visible
V	volt
v/v	volume by volume
w/v	weight by volume
x g	relative centrifugal field
Z	oxazolone
ε	extinction coefficient
λ_{max}	lambda max

In memory of my dear brother, Simeon Aelred Müller

(25.10.1978 – 24.05.2004)

who inspired my love for learning

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PUBLICATIONS

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Müller, A.C., Dairam, A., Limson, J.L. and Daya, S. (2006). Mechanisms by which acyclovir reduces the oxidative neurotoxicity and biosynthesis of quinolinic acid. *Life Sciences* (in press).

CHAPTER ONE

LITERATURE REVIEW

1.1 LEARNING AND MEMORY

1.1.1. Introduction

"Learning has always been a crucial activity in human culture. From a young age, as members of society we are expected to learn beliefs, norms of behaviour, skills and facts. We even have educational institutions devoted to learning!" (Anderson, 2000). Learning can be formally defined as the ability to acquire new knowledge or skills through instruction or experience. However, learning would be a pointless activity unless we have the ability to recapture or recall the information, thus memory is an important associated process, in which the knowledge or skill learnt is retained over time. Research on learning and memory has been part of psychology since it began as a science in the 1800's (Anderson, 2000). Later in that same century, scientists began the search for an explanation of learning and memory at the molecular level. Eventually, the neural basis of learning and memory emerged from the assumption that information is stored in the brain as changes in synaptic efficiency (Bliss and Collingridge, 1993). This was suggested in the 1890's upon the discovery that networks of neurons are not in cytoplasmic continuity but communicate with each other at junctions called synapses (Bliss and Collingridge, 1993). The ability of the brain to re-organise neural pathways based on experience is termed, neuroplasticity.

1.1.2. The Hippocampus and Learning and Memory

The hippocampal formation is a relatively small bilateral structure (Amaral and Witter, 1989) in the brain surrounded by the temporal cortex (Anderson, 2000). It forms part of

the limbic system which has a general role to play in emotion, motivation and memory (Butler, 1993).

Each hippocampus consists of two thin sheets of neurons which are shaped like two inter-locking C's folded onto each other; one sheet is the dentate gyrus (DG) and the other is the Ammon's horn. The Ammon's horn has four divisions, of which the most important are the CA1 and CA3 (figure 1.1.). CA stands for *Cornu Ammonis* and means Ammon's Horn in Latin (Amaral, 1978; Amaral and Witter, 1989; Bear *et al*., 2001). It is also known as the hippocampus proper. Figure 1.1. shows that the structures are curved and subfields or regions are defined along the curves (only CA3, CA1 and DG are labelled). The regions are also structured depthwise in clearly defined strata. The *stratum pyramidale* is the main cell layer of Ammon's horn and contains the cell bodies of pyramidal cells, whilst the *stratum granulosum* is the main cell layer of the DG, consisting of the cell bodies of granule cells (Amaral, 1978; Amaral and Witter, 1989).

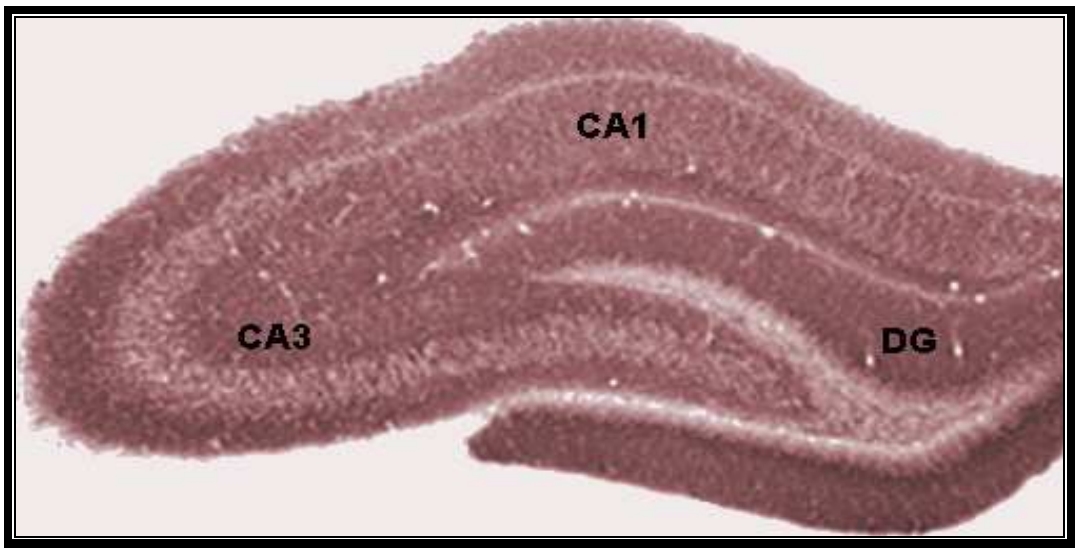


Figure 1.1. Photomicrograph of the major regions of the hippocampus. (http://en.wikipedia.org/wiki/Hippocampal_subfields)

Most neural input to the hippocampus occurs via the entorhinal cortex (EC), which is connected to the granule cells of the dentate gyrus through the perforant-path of axons.

The granule cells of the dentate gyrus then project their axons, called the mossy fibre system to the large pyramidal cells of CA3 region (Amaral, 1978). Finally, the CA3 pyramidal cells synapse on pyramidal cells of the CA1 subfield, via their axons, known as the Schaffer collateral system (Amaral, 1978).

The importance of the hippocampus in memory was brought to the attention of researchers by the patient, HM. HM suffered from a number of anterograde and temporally-graded retrograde memory impairments following the bilateral removal of various medial-temporal lobe structures (including bilateral ablation of his hippocampi) to relieve frequent epileptic seizures (Terry, 2005). In later years, it was discovered that a few seconds of high-frequency electrical stimulation of a glutamatergic fibre pathway in the hippocampus enhances synaptic neurotransmission between stimulated axons and post-synaptic cells and that this change persists for weeks (Bliss and Lomo, 1973). This effect, called long term potentiation (LTP) was proposed to be the neural process which underlies learning and memory. Thus, this further defined the role of the hippocampus in learning and memory.

1.1.3. Long Term Potentiation

1.1.3.1. Induction of Long Term Potentiation

The best understood form of LTP is by activation of N-methyl-D-aspartate (NMDA) receptors, which allows for the transduction of electrical signals to chemical signals at the post-synaptic membrane, which, in turn, activates both pre- and post-synaptic mechanisms to generate a persistent increase in synaptic strength (Bliss and Collingridge, 1993).

In the hippocampus, LTP is initiated by stimulation of the presynaptic neuron (Danysz and Parsons, 2003). This results in the release of glutamate, which binds to ionotropic glutamatergic receptors on the postsynaptic neuron. The α-amino-5-hydroxy-3- methyl-4-isoxazole propionic acid (AMPA) receptors contain ligand-gated channels only, thus the binding of glutamate immediately results in an influx of sodium ions into the

postsynaptic neuron. On the other hand, the ion channels of NMDA receptors are both ligand-gated and voltage-gated, because of the voltage-dependent block of these channels by a magnesium ion. Thus, on NMDA receptors, glutamate binding alone is insufficient to bring about ion channel opening. It is the influx of sodium ions (due to AMPA stimulation) across the postsynaptic membrane which causes the depolarisation required to remove the magnesium ion. Since glutamate is already bound, once the magnesium ion has dissociated from the receptor, the ion channel is open to allow the passage of sodium and calcium ions (Bliss and Collingridge, 1993; Lodish *et al*., 1995). The induction of LTP could be blocked by the intracellular injection of ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid (EGTA), a calcium ion chelator (Lynch *et al.*, 1983). This has implicated the post-synaptic cell, and in particular calcium signalling, in the induction process.

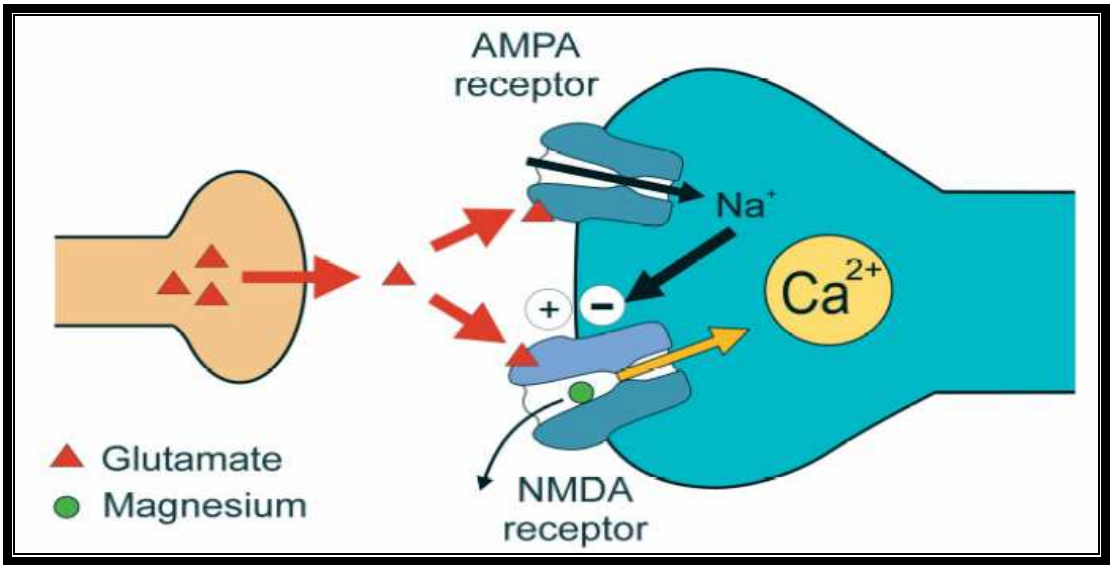


Figure 1.2. Diagram showing the involvement of AMPA and NMDA receptors in the induction of LTP (Danysz and Parsons, 2003).

During the induction of LTP, the NMDA receptor functions as a co-incidence detector, integrating activity of the post-synaptic cell (depolarised membrane) with the release of glutamate from the pre-synaptic cell, generating a greater response by the postsynaptic neuron than that caused by the neurotransmitter release alone (Lodish *et al.*, 1995) The

increase in calcium ions also causes activation of nitric oxide synthase (NOS) to form nitric oxide (NO), which diffuses out of the postsynaptic cell to the presynaptic cell where it activates guanylate cyclase to synthesise cyclic guanylate monophosphate (cGMP), which increases the amount of glutamate released at each subsequent action potential (Lodish *et al.*, 1995). Thus, it can be argued that both sides of the synapse are strengthened during induction of LTP.

1.1.3.2. Expression of Long Term Potentiation

Several different calcium-sensitive enzymes have been proposed to play a part in converting the induction signal into persistent modifications of synaptic strength. These include the protease calpain (Oliver *et al*., 1989), phosphatases such as calcineurin (Halpain and Greengard, 1990), phospholipases and protein kinases. In addition to post-translational modification of existing proteins there is evidence that protein synthesis itself is also necessary for LTP (Bliss and Collingridge, 1993). Proteins synthesised from pre-existing messenger ribonucleic acid (mRNA) are required for the maintenance of LTP during the first few hours. This suggests that LTP does not depend on gene transcription during this time. It does not preclude, however, the possibility that genes are normally transcribed at or shortly after the time of induction but exert their effects at later times (Bliss and Collingridge, 1993). Thus, it appears that the increase in synaptic response generated at potentiated synapses could be due to:

1. Presynaptic modifications which result in an increase in the amount of L-glutamate released per impulse,
2. Postsynaptic modifications, such as an increase in the number of receptors or a change in their functional characteristics,
3. An extrasynaptic change, such as reduction in uptake of L-glutamate by glial cells leading to increased neurotransmitter availability at the receptors, or
4. Morphological modifications.

(Bliss and Collingridge, 1993).

1.1.4. Disorders Involving Impairment of Learning and Memory

Irrespective of socio-economic class, most spheres of our lives depend on our ability to acquire and retain information. Conceivably then, impairment of learning and memory is likely to have devastating effects on the quality of life of such affected patients. It may even become psychologically debilitating for family and friends as they watch their loved one deteriorate in cognitive function. Based on the important role the hippocampus plays in learning and memory, any disease which results in damage or death of hippocampal neurons is likely to cause impairment of these two cognitive processes. The neuropathology of these disorders is often related to one or more of the following:

1. The presence of foreign bodies;
2. An immune response to foreign bodies;
3. An immune response to physical or psychological stress.

1.1.4.1. The Immune Mechanism of the Central Nervous System

Microglia are non-neuronal cells in the central nervous system (CNS) which serve as the first line of defense as antigen-presenting cells (Kreutzberg, 1996). During this process, these become activated and thereafter follow a stereotyped pattern of events irrespective of the antigen (Mori and Kimura, 2001). In the first stage of activation, if the injury to the neurons is sublethal, microglia become activated but do not become phagocytic. Microglia at this stage likely provide neurons with trophic support by releasing a battery of molecular factors such as neurotrophins, basic fibroblast growth factor, and annexin V (Mori and Kimura, 2001). If neuronal death is induced by the injury, activated microglia are further transformed into phagocytic, and thus cytotoxic cells, known as microglia-derived brain macrophages (Mori and Kimura, 2001). These microglia kill injured cells by releasing neurotoxic molecules such as free oxygen intermediates, NO, proteases, arachidonic acid derivatives, excitatory amino acids and cytokines like tumor necrosis factor α (TNFα) (Kreutzberg, 1996). TNFα is one of the many pro-inflammatory cytokines, including some interleukins (IL) and interferon-γ (IFN-γ) which have the ability to stimulate the activity of indoleamine-2,3-dioxygenase (IDO), possibly resulting in an increased production of another neurotoxin, quinolinic acid (QA), via the

kynurenine pathway (KP) (figure 1.10.) Within the brain, IL-1 has been found in several brain regions including the hippocampus (Fagan and Gage, 1990).

1.1.4.2. Alzheimer's Disease

Alzheimer's disease (AD) is a chronic, progressive neurodegenerative disorder which is characterised by a decline in cognitive abilities, including learning and memory. Two of the hallmark morphological features of AD are ß-amyloid plaques (AP) and neurofibrillary tangles (NFT) (Mirra *et al.*, 1993; Gandy and Petanceska, 2000) present in specific subcortical and cortical areas (Mirra *et al.*, 1993). These are formed by the extracellular deposition of ß-amyloid peptide and the intra-neuronal accumulation of abnormal tau protein respectively (Selkoe, 2004). These abnormal bodies directly compromise the function and viability of neurons (Trojanowski and Lee, 2005), possibly leading to neuronal and synaptic loss. The most vulnerable circuit appears to be the perforant path (Hyman *et al.*, 1984; Hyman *et al.*, 1986) which, as described in section 1.1.2., originates in the EC and terminates in the DG of the hippocampal formation. This circuit is progressively affected by NFT formation prior to the appearance of substantial cognitive decline (Gomez-Isla *et al.*, 1996). Several studies have shown that the CA1 subfield of Ammon's horn is also severely affected by NFT (Braak and Braak, 1985; Squire and Zola-Morgan, 1988). AP are associated with evidence of neuroinflammation, especially activated microglia. Guillemin *et al.*, (2003) discovered that ß-amyloid 1-42, one of the ß-amyloid peptides found in AP, is able to induce the production of the potent neurotoxin, QA, in neurotoxic concentrations, by human macrophages and microglia. Brain regions involved in learning and memory demonstrate the greatest degree of microglia cell activation early in the disease, ultimately showing the highest rate of neurodegeneration (Cagnin *et al.*, 2001). In AD, neurons within the EC are particularly vulnerable to the consequences of chronic neuroinflammation (Hauss-Wegrzyniak *et al.*, 1998; Hauss-Wegrzyniak *et al.*, 2002). This includes the loss of NMDA receptors (Rosi *et al.*, 2004), which have a pivotal role to play in LTP (section 1.1.3.1).

1.1.4.3. Herpes Simplex Encephalitis

Herpes viruses are ubiquitous and usually cause asymptomatic or mild infections (Cinque and Lazzarin, 2000). However, following primary infection, these remain latent in the body and may subsequently be reactivated under particular circumstances such as immunodeficiency. During primary infection or reactivation, the virus may enter the CNS by nerve pathways or by crossing the blood brain barrier (Cinque and Lazzarin, 2000). Neuronal destruction is initially sustained by the direct cytotoxic effect of the virus and thereafter continued by immune mechanisms (Esiri, 1982). The relative importance of either mechanism will most likely depend on the balance between viral replication and the host inflammatory response (Price *et al*., 1973). For example, direct virus cytotoxicity is likely to be the most relevant pathogenic mechanism in an immunocompromised patient, in whom there is a limited inflammatory response. Although herpes simplex virus type-1 (HSV-1) may induce diffuse infection of the neonatal brain, adult herpes simplex encephalitis (HSE) is pathologically characterised by necrotising lesions commonly found in the CNS structures implicated in learning and memory, including the amygdala, hippocampus, and EC (Gordon *et al*., 1990) Consequently, the most frequently observed clinical finding in survivors of HSE is a deficit in intellectual function, in particular, a profound inability to store and recall information.

1.1.4.4. Mental Depression

The monoamine hypothesis of depression proposes that low levels of one or more of the brain monoamine neurotransmitters; serotonin (5-HT), norepinephrine (NE) and dopamine (DA) could produce depression (Hirschfeld, 2000). This hypothesis has been further refined, emphasising that mental depression may arise, specifically, from decreased brain 5-HT function (Coppen, 1967). During psychological stress, more 5-HT is needed to cope with demands and up-regulation of 5-HT_2 receptors (Hrdina, 1996) is a compensatory response. Ultimately, however, 5-HT turnover dysfunction occurs and major depression will develop (Stokes, 1995).

It has been hypothesised that cytokine hypersecretion may also be involved in the pathophysiology of depressive disorders (Leonard, 2001). A cytokine-5-HT interaction, linking the above two hypotheses has been identified through the discovery that pro-inflammatory cytokines have profound effects on the metabolism of brain 5-HT, NE and DA in mice and rats (Dunn *et al.*, 1999). In clinical studies, significant decreases in serum tryptophan (TRP) concentrations have been noted in patients receiving IL-2 or IFN-γ (Brown *et al.*, 1989; Brown *et al*., 1991). There are two major mechanisms involved in cytokine-induced TRP depletion. The first mechanism is that cytokines may induce TRP depletion directly by reducing food intake since TRP levels are strongly modulated by dietary intake (Plata-Salaman, 1998). The second mechanism is that cytokines induce TRP depletion by enhancing the activity of IDO. TRP is a precursor of 5-HT, thus depletion of TRP means that there is less of this amino acid available for the production of the brain monoamine. Moreover, as described in section 1.1.4.1, enhanced activity of IDO increases the production of the neurotoxin QA. It is this action which has given rise to the neurodegenerative hypothesis of depression (figure 1.3.), proposed by Myint and Kim, (2003).

This hypothesis proposes that psychological stress induces TRP depletion due to the increased demand for 5-HT and mood changes develop. This is balanced by modification of 5-HT synthesis and expression of different 5-HT receptors. However, when the psychological stress continues over a period of time, an increase in pro-inflammatory cytokines occurs. The adverse effects of pro-inflammatory cytokines may be balanced by an increased production of anti-inflammatory cytokines. The pro-inflammatory cytokines activate IDO further depleting TRP through the KP. As a result of this pathway, neurodegenerative QA and neuroprotective kynurenic acid are produced in the brain, creating a challenge between neurodegeneration and neuroprotection. If the balance between pro- and anti-inflammatory cytokines is disturbed or stress continues further, the TRP degradation through the KP becomes more pronounced and the neurodegeneration–neuroprotection challenge becomes extensive. When this challenge shifts to the degenerative side, neurodegeneration of areas of brain involved in neuroendocrinology of stress and memory such as the hippocampus occurs. This neurodegeneration may further

perturb serotonergic function. This neurodegeneration hypothesis is supported by the findings of Bremner *et al.,* (2000) and Sheline (1996; *et al.,* 1999) who showed that atrophy of the hippocampus occurs in major depression and that this effect increases with longer duration of depression.

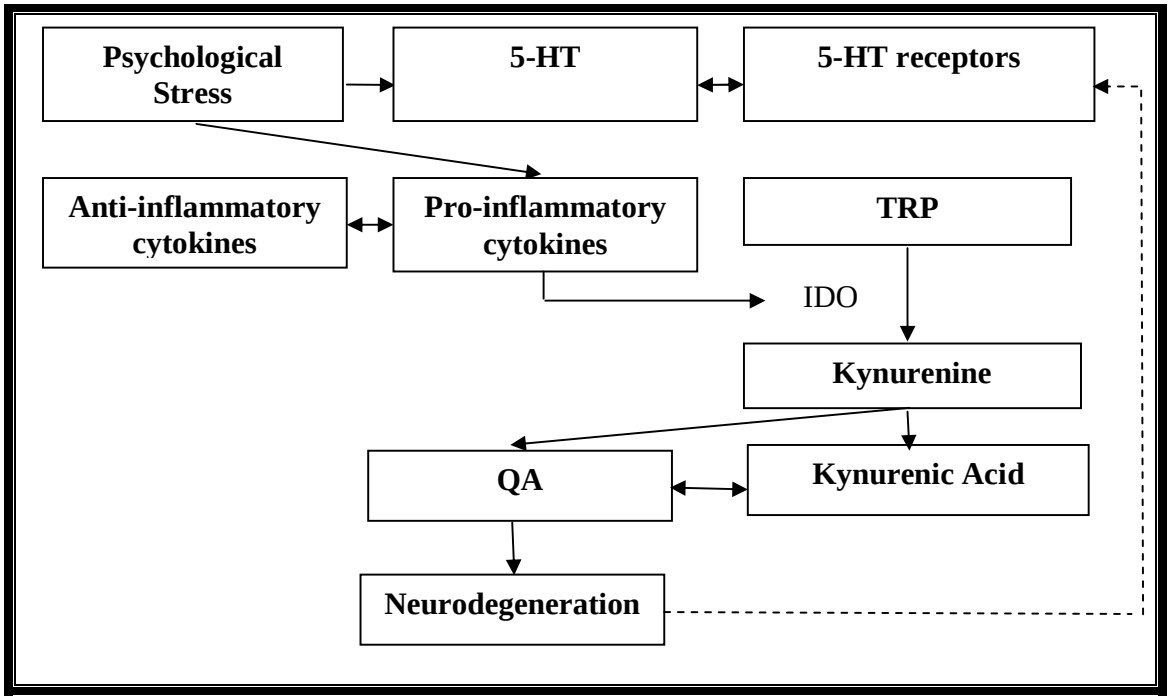


Figure 1.3. Schematic diagram outlining the neurodegenerative hypothesis of depression, modified from Myint and Kim (2003).

1.2. NEURODEGENERATION

1.2.1. Neuronal Cell Death

1.2.1.1 Introduction

Neuronal death is normal during nervous system development but becomes potentially pathological (Farber, 1994) in brain and spinal cord disease and injury (Martin, 2001). There are two types of cell death, namely, necrosis and apoptosis.

1.2.1.2. Necrosis

Necrosis is a passive process (Ankarcrona *et al.,* 1995), ultimately resulting in the lysis of cells. It is characterised by a catastrophic deregulation of cellular metabolism, primarily due to adenosine triphosphate (ATP) depletion and electrolyte homeostasis (Toescu, 1998). This imbalance results in typical morphological changes which include organelle and cell swelling (Ankarcrona *et al.*, 1995), followed by the loss of integrity (disintegration) of the cell membrane. The latter may result in the release of cytoplasmic content into the extracellular medium (Toescu, 1998). This triggers an important inflammatory response, which includes local cellular infiltration, vascular damage and oedema (Ankarcrona *et al.,* 1995), which initiates injury to the surrounding tissue, and, eventually leads to fibrosis.

1.2.1.3. Apoptosis

Apoptosis is a genetically controlled, energy-dependent process which removes unwanted cells from the body (Blankenberg *et al.,* 2000). Thus, the nuclei and mitochondria of cells are likely to play an important role in the process; in this respect, it is a disturbance of the function of one or both of these organelles, which ultimately initiates apoptosis by the activation of a cascade of cysteine aspartate-specific proteases (caspases) (van Loo *et al.,* 2002). Cytochrome C is a necessary component in caspase activation, suggesting that mitochondria participate in apoptosis by releasing cytochrome C into the cytosol (Liu *et al*., 1996; Li *et al.,* 1997). Furthermore, cytosolic deoxy-ATP (dATP), a product of mitochondrial activity is a required cofactor for cytochrome C-induced caspase activation. (Liu *et al*., 1996, Li *et al.,* 1997). The mitochondria also release caspase 9, apoptosis inducing factor (AIF) and perhaps other mediators that cause apoptosis (Beal, 2000). Cytochrome C and dATP are involved in the conversion of pro-caspase 9 to an active caspase, which initiates a caspase cascade that leads to apoptotic cell death (Beal, 2000).

1.2.1.3.1 The Role of Caspases in Apoptosis

Caspases are constitutively expressed proenzymes that undergo proteolytic cleavage and rearrangement of subunits to produce active enzymes (Schwartz and Milligan, 1996). The substrates of these enzymes are primarily nuclear proteins, including, polyADPribose polymerase (PARP), deoxyribonucleic acid-dependent protein kinases (DNA-PK), heteronuclear ribonucleoproteins (HNRNPs), lamins, and cytoskeletal proteins such as actin and fodrin (Schwartz and Milligan, 1996). It is also possible that these proteases could contribute to deoxyribonucleic acid (DNA) fragmentation by activation of endonucleases (Wright *et al.,* 1994).

1.2.1.3.1.1. Cleavage of PARP

Under normal circumstances, PARP is an important molecule, which has a role to play in DNA repair, in response to DNA damage. Its precise role in DNA repair or replication remains to be defined (Herceg and Wang, 2001). The cleavage of PARP inhibits most of its DNA repair activity (Kaufmann *et al.,* 1993), and this may contribute to the apoptotic demise of the cell.

PARP cleaves nicotinamide adenine dinucleotide (NAD) into nicotinamide and adenine dinucleotide-ribose and uses the latter to synthesise long branching polyADPribose polymers covalently attached to acceptor proteins including histones, DNA repair enzymes, transcription factors and PARP (Virág, 2005). Depletion of NAD pools reduces the cells' ability to generate ATP and maintain energy-dependent processes (Sims *et al.,* 1983). Therefore, it has been postulated that PARP cleavage also occurs in order to prevent depletion of energy pools, and in this way, steer the neuron towards apoptotic, rather than necrotic death, which would occur due to overactivation of PARP (Eliasson *et al*., 1997; Endres *et al*., 1997).

1.2.1.3.1.2. Cleavage of DNA-PK

DNA-PK is essential in the repair of DNA double-strand breaks (Hartley *et al.,* 1995, Jackson and Jeggo, 1995). The DNA-PK catalytic site is the 470-kDa catalytic subunit of DNA-PK. DNAPK. Catalytic site activity is induced by binding to the 70- and 80-kDa Ku heterodimer (Lees-Miller *et al.,* 1990; Anderson and Lees-Miller, 1992), which in turn, binds to DNA in double-strand break repair reactions and thereby recruits DNA-PK catalytic sites to sites of DNA damage (Lees-Miller *et al.,* 1995). The available evidence indicates that the DNA-PK catalytic site is cleaved into 240-, 150-, and 120-kDa fragments and that cleavage is associated with loss of DNA-PK activity (Song *et al.,* 1996) and thus an impaired ability of the cell to repair DNA double strand breaks.

1.2.1.3.1.3. Cleavage of HNRNPs

The HNRPs belong to a class of proteins that bind heterogeneous nuclear ribonucleic acids (RNA) or pre-mRNA (Waterhouse *et al.,* 1996). These are thought to play a role in processing (splicing) pre-mRNA; however, the full range of functions and mechanisms of action are not yet known. There is evidence of cleavage of the HNRNPs C1 and C2 in apoptotic cells (Waterhouse *et al.*, 1996). This interferes with the synthesis of mRNA. Without mRNA, the genetic code cannot be translated for the production of proteins, which undoubtedly constitute an integral part of normal cell function.

1.2.1.2.1.4. Cleavage of Actin and Fodrin

The cytoskeleton has an important role to play in different types of cellular motility, such as, intracellular transport, locomotion, maintenance of cell shape and cell division (Jokusch *et al*., 1986). Thus, proteolytic cleavage of cytoskeletal actin (Kayalar *et al*., 1996) and the actin-associated fodrin (Martin *et al.*, 1995) is likely to cause disassembly of the cytoskeleton. This contributes to the morphological changes of the cell membrane observed in apoptosis.

1.2.1.3.1.5. Cleavage of Lamins and Endonucleases

Lamin B1 degradation during apoptosis could lead to collapse of the chromatin due to the loss of attachment points on the nuclear matrix (Neamati *et al.,* 1995). The cleavage of endonuclease activates the enzyme, which fragments the chromatin into nucleosomal units, of about 180-base pair oligomers (Matassov *et al.*, 2004). This is an important early feature of apoptosis.

1.2.1.3.2. Morphological Features of Apoptosis

The early morphological changes in apoptosis include cell shrinkage, organelle relocalisation and compaction, and nuclear chromatin condensation (Kerr *et al*., 1972, Arends and Wyllie, 1991). The condensed chromatin network then moves towards the nuclear envelope (Wyllie, 1987). The plasma membrane starts to convolute, giving the cell a blebbed surface. The cytoplasmic blebs pinch off as apoptotic bodies, which are usually rapidly phagocytised by macrophages, (Toescu, 1998) preventing inflammation and damage to the surrounding tissue (Duvall *et al*., 1985; Savill *et al*., 1993). Preventing the release of intracellular molecules is particularly important in the nervous system, where liberation of excitotoxic mediators, such as glutamate from dying cells can cause severe injury to neighbouring neurons (Leist and Nicotera, 1998). However, if local phagocytic capacity is limited, the apoptotic bodies can undergo secondary necrosis, in which the membrane breaks and the contents are released into the extracellular space (Toescu, 1998). These morphological features of apoptosis are illustrated in figure 1.4., and compared to those of necrosis.

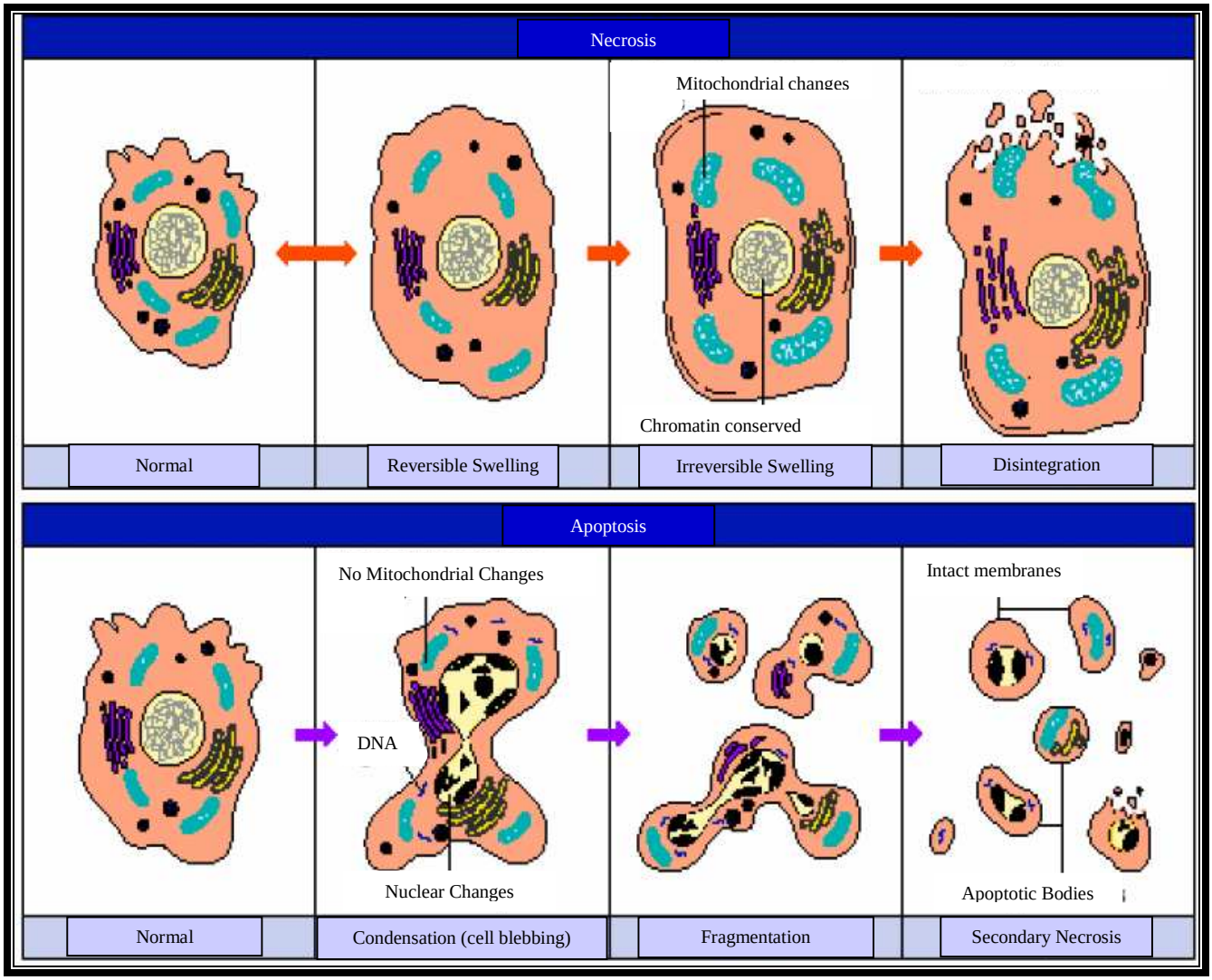


Figure 1.4. Illustration of the morphological features of necrosis and apoptosis. (Wyllie *et al*., 1998).

1.2.2. Mechanisms of Neurodegeneration

1.2.2.1. Introduction

Necrosis and apoptosis are initiated by three inter-related mechanisms, namely, oxidative stress, excitotoxicity and mitochondrial dysfunction (Greene and Greenamyre, 1996, Simonian and Coyle, 1996; Alexi *et al*., 1998; Cassarino and Bennett, 1999). This relationship is depicted in figure 1.5.

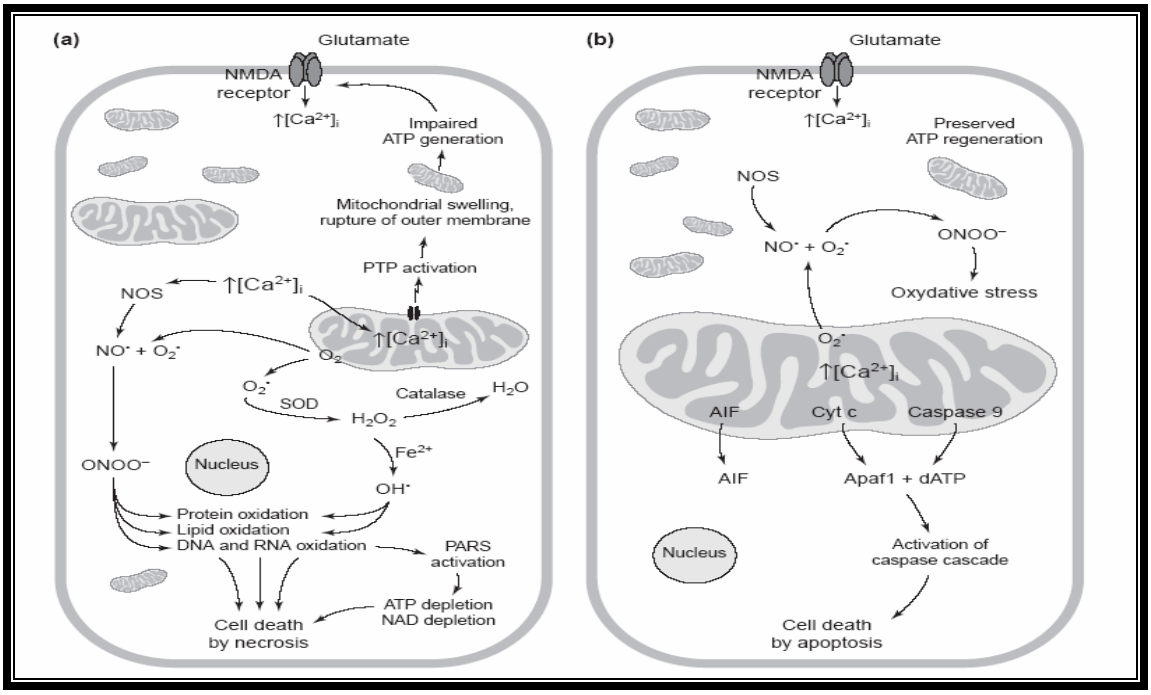


Figure 1.5. The inter-relationship between excitotoxicity, free radicals and oxidative stress, and mitochondrial dysfunction in causing necrosis and / or apoptosis. (a) depicts the effects of a severe excitotoxic insult which results in cell death by necrosis. (b) depicts the effects of a mild excitotoxic insult that results in apoptosis (Beal, 2000).

1.2.2.2. Free Radicals and Oxidative Stress

Free radicals are chemical species which are unstable because these possess an unpaired electron in their outer orbit. Free radicals are generally very reactive (Cheeseman and Slater, 1993) due to the attempt to achieve stability by gaining an electron from molecules in close proximity. Thus, in biological systems, free radicals may do so by oxidising biomolecules, including nucleic acids, amino acids, proteins and fatty acids and lipids, which lead to cell injury and possibly cell death (Fridovich, 1999; McCord, 2000).

1.2.2.2.1. Superoxide Anion

The superoxide anion ($O_2^{\cdot-}$) is most commonly formed when molecular oxygen (O_2) accepts an electron from a reducing agent. This occurs via cellular electron transport chains located in mitochondria and the endoplasmic reticulum (Halliwell and Gutteridge,

1990) where some electrons passing through the chain "leak" directly from the intermediate electron carriers onto O_2. There are a number of other biochemical reduction-oxidation (redox) reactions which generate $O_2^{\cdot-}$. Examples of these include, the auto-oxidation of catecholamines and oxidation of xanthine by the enzyme xanthine oxidase (Colton and Gilbert, 1987). The calcium-dependent activation of phospholipase A_2 (PLA_2) also yields $O_2^{\cdot-}$ through the metabolism of arachidonic acid by the lipoxygenases and cyclooxygenases to form eiconasoids (Chan and Fishman, 1980).

The direct toxicity of $O_2^{\cdot-}$ is two fold. It has the ability to inhibit certain enzymes, including calcineurin, thereby attenuating vital metabolic pathways and the expression of LTP (section 1.1.3.2), and it has been shown to have a role to play in DNA damage (Touati and Farr, 1990).

1.2.2.2.2. Nitric Oxide

NO is one of the most widespread signalling molecules and plays a vital role in the functioning of many different types of cells (Ignarro *et al.,* 1999). Physiologic levels of NO produced by endothelial cells are essential for regulating the relaxation and proliferation of vascular smooth muscle cells, leukocyte adhesion, platelet aggregation, angiogenesis, thrombosis, vascular tone, and hemodynamics (Ignarro *et al.,* 1999) In addition, NO produced by neurons serves as a neurotransmitter, and NO generated by activated macrophages is an important mediator of the immune response (Fridovich, 1999).

It is known that in the course of ischaemia, AD and other degenerative conditions, NO concentrations increase in the brain (Torreilles *et al.*, 1999). This excess NO is able to promote apoptosis in primary neuronal cultures, due to the formation of a far more potent free radical, peroxynitrite (Tomaszewicz *et al*., 1997; Brorson *et al.*, 1999; Kim *et al.*, 1999).

1.2.2.2.3 Peroxynitrite

The short lived, potent oxidant peroxynitrite ($ONOO^-$) is the biological reaction product of $O_2^{\cdot-}$ and NO (Radi *et al.*, 1991). The broad spectrum of toxic effects produced by $ONOO^-$ in biological systems includes lipid peroxidation (LP), protein oxidation and nitration, DNA cleavage, a reduction in cellular antioxidant defenses and inhibition of mitochondrial respiration (Misko *et al*., 1998; Szabó, 2003; Virág *et al*., 2003). These events are known to trigger apoptotic cell death at low concentrations of $ONOO^-$, and necrosis at higher concentrations (Virág *et al.,* 2003).

1.2.2.2.4. Hydroperoxyl Radical

Hydroperoxyl ($HOO^{\cdot}$) is produced in biological systems by the reaction of $O_2^{\cdot-}$ with any substrate that contains acidic protons (Collins *et al.,* 2001). The pH at sites very close to a membrane surface is very acidic, so the formation of $HOO^{\cdot}$ there is favoured (Halliwell and Gutteridge, 1989). This is particularly relevant because the intracellular generation of its precursor, $O_2^{\cdot-}$ is formed in membrane bound systems, such as the mitochondria. Furthermore, $HOO^{\cdot}$, being uncharged, crosses membranes readily and appears to be somewhat more reactive than $O_2^{\cdot-}$. For example, it, unlike $O_2^{\cdot-}$, has the ability to initiate LP (Halliwell and Gutteridge, 1989).

1.2.2.2.5. Hydroxyl Radical

In many systems in which $O_2^{\cdot-}$-generation has been shown to do damage, there is protection by known scavengers of the hydroxyl radical ($HO^{\cdot}$), such as mannitol and thiourea (Halliwell and Gutteridge, 1989). It was thus suggested by Fridovich, (1983) that $O_2^{\cdot-}$ and hydrogen peroxide (H_2O_2) react in the Haber-Weiss reaction to form the highly reactive $HO^{\cdot}$. However, the second-order rate constant for the reaction was found to be virtually zero and therefore not likely to occur at the low steady state concentrations of $O_2^{\cdot-}$ and H_2O_2 that exist *in vivo*. It was later shown (Halliwell and Gutteridge, 1986) that the $HO^{\cdot}$ formation can be accounted for if the Haber-Weiss (H-W) reaction is catalysed

by traces of transition metal ions, for example, Fe^{2+} and Fe^{3+} in the Fenton reaction (Halliwell and Gutteridge, 1986), as shown in equations 1.1, 1.2 and 1.3.

$$Fe^{3+} + O_2^{\cdot -} \longrightarrow Fe^{2+} + O_2 \quad \text{.....equation 1.1}$$

$$Fe^{2+} + H_2O_2 \longrightarrow HO^{\cdot} + {}^{-}OH + Fe^{3+} \quad \text{....equation 1.2}$$

Fenton Reaction

$$O_2^{\cdot -} + H_2O_2 \xrightarrow{\text{metal catalyst}} O_2 + HO^{\cdot} + {}^{-}OH \quad \text{...equation 1.3}$$

Net reaction:H-W

The potency of $HO^{\cdot}$ lies in its ability to react with almost all molecules in living cells (Fridovich, 1986). Furthermore, no $HO^{\cdot}$-detoxifying enzyme exists in biological systems (Bird and Iversen, 1974).

During normal cell functioning, free radicals are produced primarily during mitochondrial respiration, where oxygen is the last acceptor of the electron transport chain. However, the levels of these radicals are controlled by the deactivation mechanisms of the antioxidant defense system (Alexi *et al.*, 2000). Oxidative stress occurs when generation of free radicals exceeds the capacity of antioxidant defense mechanisms (Poston and Raijmakers, 2004). Thus, excess free radicals have the ability to oxidise important cell components, including membranes, proteins and DNA and can be detrimental to cells if they accumulate in high levels or the antioxidant defense system is defective or saturated (Alexi *et al.*, 2000). Mitochondrial dysfunction and excitotoxicity, although mechanisms of neurodegeneration in their own right, may contribute to one or both of these criteria. For years now, oxidative stress has been associated with the pathology of AD (Martins *et al.,* 1986; Franceschi *et al.,* 1990; Butterfield *et al.,* 1999a;, 1999b; Ansari *et al.*, 2006; Onyango and Khan, 2006), whilst in HSE (Valyi-Nagy and Dermody, 2005) and depression (Khanzode *et al.,* 2003), the notion and evidence is very recent and not well known.

1.2.2.3. Mitochondrial Dysfunction

Mitochondrial dysfunction may occur as a consequence of excitotoxicity (section 1.2.2.4.) or due to the presence of respiratory poisons such as cyanide and 1-methyl-4-

phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Alexi *et al.*, 2000). Dysfunctioning of the mitochondria results in depression of oxidative phosphorylation (Zhang *et al.*, 1990), the consequences of which are leakage of excess electrons and possible ATP depletion through the reduced production of ATP (Alexi *et al.*, 2000). The electrons combine with O_2 to form $O_2^{\cdot-}$ leading to the production of other free radicals, and ultimately, oxidative stress (Beal, 2000). Oxidative stress also has the potential to cause permeability transition (PT) of mitochondria (Gunter and Pfeiffer, 1990; Beal, 2000). This is associated with the opening of a pore in the inner mitochondrial membrane, which makes it permeable to ions and small molecules (Gunter and Pfeiffer, 1990, Beal, 2000). PT may be a key switch responsible for the induction of apoptosis by release of the caspase activator cytochrome C through the pore (Hengartner, 1998). However, if PT causes a dramatic change in ion homeostasis to occur, a breakdown of mitochondrial membrane potential and swelling of the mitochondria results (Hengartner, 1998) potentially leading to necrotic cell death. Since neurons rely on ATP for all energy-dependent processes, ATP depletion results in ATP-independent necrotic cell death. More specifically, it causes failure of ATP-dependent ion pumps. This may cause uncontrolled ion influx and thus depolarisation of the membrane (Alexi *et al.*, 2000). Together with another consequence of mitochondrial dysfunction, which is the loss of intracellular calcium buffering capacity (Alexi *et al.*, 2000); this may lead to calcium-dependent toxic processes as described in section 1.2.2.4. Experimental evidence has shown that mitochondrial dysfunction is very likely involved in the pathology of AD (Hauptmann *et al.*, 2006; Onyango *et al.*, 2006) and depression (Modica-Napolitano and Renshaw, 2004).

1.2.2.4. Excitotoxicity

Excitotoxicity is a mechanism of neuronal cell death mediated by overstimulation of ionotropic glutamate receptor sub-types, such as NMDA and AMPA (Griffiths *et al.*, 2000). Thus, in addition to modulation of LTP (section 1.1.3.1) these receptors are potential agents of neurodegeneration. The resulting massive depolaristion of neuronal membranes opens the ion pores of the receptors, allowing an influx of large amounts of

sodium and calcium ions (Cotman *et al*., 1989; Farooqui and Horrocks, 1991). It is this disturbance in ion homeostasis that can ultimately result in necrosis and apoptosis.

It has been suggested that two separate processes occur in the excitotoxic mechanism. The influx of sodium ions is accompanied by influx of chloride ions as well, creating an osmotic imbalance between the neuron and the extracellular environment (Rothman and Olney, 1987). Water flows into the cell, causing acute cell swelling, damage and possible lysis, that is, necrosis (Olney, 1969; Rothman, 1985; Choi, 1987; Tilson and Mundy, 1995).

The massive calcium ion influx triggers the activation of intracellular calcium-dependent enzymes (Meldrum and Garthwaite, 1990). This causes a delayed toxicity in cells that survive the osmotic crisis and accounts for most cell deaths due to glutamatergic hyperstimulation (Choi, 1992).

High concentrations of calcium ions activate various hydrolytic enzymes including proteases, nucleases and lipases (Choi, 1992). The general homeostatic disturbance of the nucleus and / or mitochondria signals the mobilisation of caspases in their zymogen form for activation by calcium-dependent proteases, ultimately resulting in the initiation of the apoptotic cascade.

Among lipases, the calcium-dependent PLA_2 has been implicated in neurotoxicity. Its activation by calcium ions results in the release of arachidonic acid and related polyunsaturated fatty acids (PUFA) from lipid membranes, which are further metabolised by lipoxygenases or cyclooxygenases with concomitant generation of free radicals (Traystman *et al.*, 1991). The cleavage of PUFA may cause disruption of the structure and integrity of lipid membranes and, as discussed in section 1.2.2.2., the free radicals contribute to oxidative stress, which causes cell damage and may facilitate cell death. Furthermore, free radicals promote elevations in calcium ion concentrations, (Mattson and Mark, 1996), precipitating a vicious cycle. The release of arachidonic acid following activation of PLA_2, can also inhibit glutamate uptake into both neurons and glial cells and

may, therefore, prolong the excitotoxic action of this amino acid on its receptors (Volterra *et al.*, 1992).

There is also evidence which suggests that intracellular calcium overload could result in the activation of NOS, which catalyses the production of NO (Kiedrowski *et al.*, 1992), the potential toxicity of which was discussed in section 1.2.2.2.2. Moreover, increases in intracellular calcium may cause mitochondrial dysfunction by depression of oxidative phosphorylation (Zhang *et al.*, 1990) and PT, the consequences of which were mentioned in section 1.2.2.3. Metabolic and neurochemical perturbations, which give rise to excitotoxicity, are prevalent in AD (Olney *et al.*, 1995; Hynd *et al.*, 2004) and depression (Hayley *et al.*, 2005; Yao and Reddy, 2005).

1.2.3. Oxidation of Macromolecules

1.2.3.1. Introduction

The three mechanisms of neurodegeneration, whether causes or effects of neurodegeneration, ultimately result in oxidation of biomolecules and thus damage to cells. The role of oxidative damage in the pathogeneses of AD (Sultana *et al.*, 2006), HSE (Valyi-Nagy and Dermody, 2005) and depression (Bilici *et al.*, 2001) have all been explored.

1.2.3.2. Oxidation of Nucleic Acids

The presence of oxidised DNA bases is often used as a marker for free radical-mediated DNA damage (Helbock *et al.*, 1999). The guanine base is particularly sensitive to oxidation making this a reasonable biomarker for oxidative injury. Oxidised DNA bases always exist at some basal level, although cells have numerous repair systems to remove such species (Lindahl and Wood, 1999). However, if these occur at critical sites, or are not quickly repaired, oxidised purines or pyrimidines can cause functional problems. As a result, oxidised DNA bases are considered an important event in chemical carcinogenesis (Klaunig *et al.*, 1998). The extreme reactivity of the $HO^{\cdot}$ makes it a particularly important player in free radical-mediated toxic processes. The reaction of $HO^{\cdot}$ with aromatic ring

structures can proceed by addition, and similar reactions occur with the purine and pyrimidine bases present in DNA and RNA (Halliwell and Gutteridge, 1989). For example $HO^{\cdot}$ can add on across a double bond in the pyrimidine base thymine to form a thymine radical, as shown in figure 1.6.

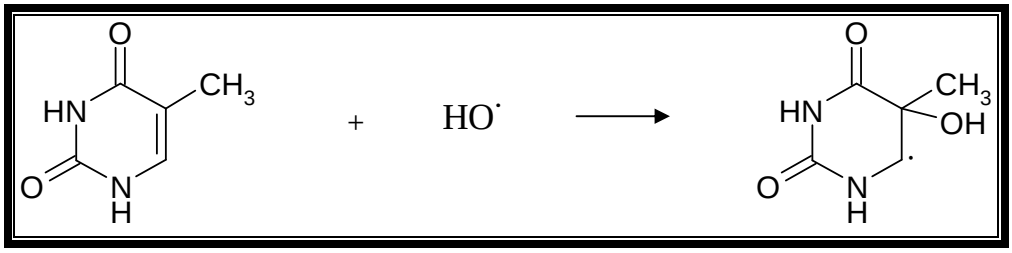


Figure 1.6. The oxidation of thymine by HO· to form a thymine radical (Halliwell and Gutteridge, 1989).

The thymine radical then undergoes a series of further reactions. For example, with oxygen it forms the fairly reactive thymine peroxyl radical ($ROO^{\cdot}$). Thus, $HO^{\cdot}$ has the potential to severely damage DNA, even inducing strand breakage, which could lead to overactivation of PARP (section 1.2.1.3.1.1.) and thus, necrotic cell death.

1.2.3.3. Oxidation of Proteins

Radical-mediated damage to proteins may be initiated by electron leakage, metal-ion-dependent reactions and autoxidation of lipids and sugars (Dean *et al.*, 1997). The consequent protein oxidation is O_2-dependent, and results in reactive compounds, such as protein hydroperoxides and propagating alkoxyl radicals (Dean *et al.*, 1997) which undergo further reactions, usually until the protein carbonyl is isolated.

This whole process may alter protein functions, but this is not often quantitatively significant because of the sheer number of each type of protein (Grune *et al.*, 1997). Except for enzymatic repair of oxidised thiols from *S*-alkyl derivatives, protein repair systems do not appear to exist, since it is apparently more efficient to either prevent the formation of oxidised proteins through the action of various cellular antioxidant systems, or to simply destroy the modified species and start over (Grune *et al.*, 1997). Although

most oxidised proteins that are functionally inactive are rapidly removed, some can gradually accumulate with time and thereby contribute to the damage associated with ageing as well as various diseases including diabetes, atherosclerosis and neurodegenerative diseases (Kehrer, 2000).

1.2.3.4. Lipid Peroxidation

The brain is predisposed to LP, more so than most other organs, because it is rich in PUFA (Schafer *et al.*, 2000). Peroxidation is initiated by the attack of a free radical that has sufficient reactivity to abstract a hydrogen atom from a methylene carbon in the side chain of an unsaturated fatty acid (Cheeseman and Slater, 1993). The removal of the hydrogen atom leaves behind an unpaired electron on the carbon atom to which it was originally attached (Gutteridge and Halliwell, 1990). The resulting carbon-centred radical undergoes molecular rearrangement (resonance stabilisation of the radical) followed by reaction with O_2 to form a lipid $ROO^{\cdot}$. These radicals are capable of abstracting hydrogen from adjacent fatty acid side chains in a membrane and so cause propagation of LP. The resulting product is a lipid hydroperoxide (**C** in figure 1.7.) (Gutteridge and Halliwell, 1990). The carbon-centred radical (**A** in figure 1.7.) formed during the initiation of LP may undergo minor reactions which include radical attack of membrane proteins and cross-linking of the side chain of the fatty acids should two of these radicals combine. The $ROO^{\cdot}$ (**B** in figure 1.7.) could also attack membrane proteins and when they combine with each other, they form singlet oxygen, which is itself a highly reactive species capable of causing oxidative damage to macromolecules within a cell (Gutteridge and Halliwell, 1990).

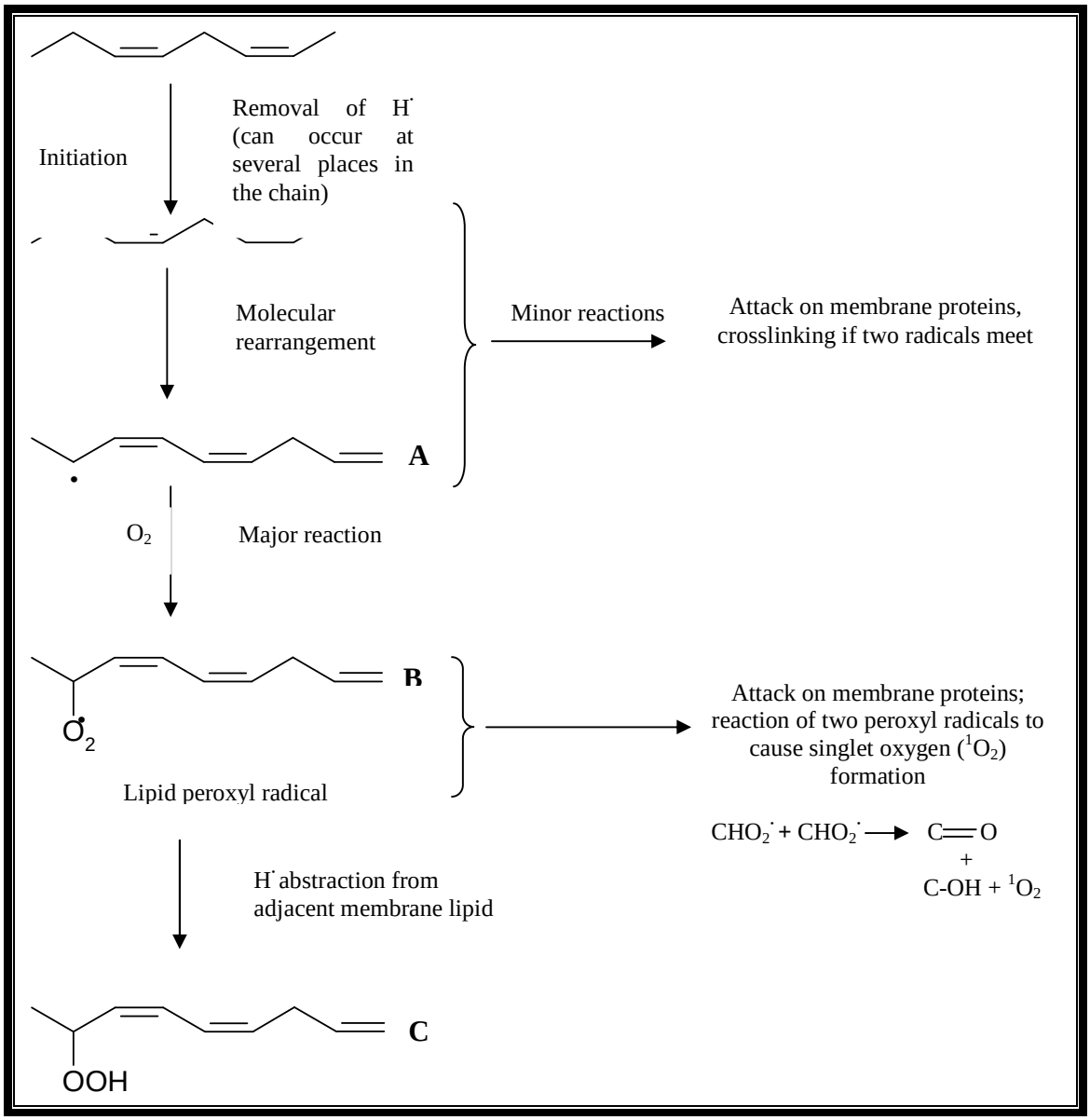


Figure 1.7. Outline of the mechanism of LP (Gutteridge and Halliwell, 1990).

The occurrence of LP in biological membranes causes impairment of membrane function in the following ways: (1) decreased fluidity and increased non-specific permeability to ions. (2) inactivation of membrane-bound receptors and enzymes (Gutteridge and Halliwell, 1990).

Lipid peroxides (ROOH) within the membrane have a devastating effect on membrane integrity because they alter membrane fluidity and permeability, thereby allowing ions such as calcium to leak into the cell (Halliwell, 1994). These ions then have a role to play in the cell death processes as discussed in section 1.2.2.4., creating a vicious cycle of deleterious events in and around neurons.

In rat brain, LP decreases the specific binding of [^{3}H] 5-HT and [^{3}H] spiperone, indicating the loss of serotonergic binding sites of receptors (Muakkassah-Kelly *et al*., 1982). Furthermore, the presence of phospholipids influences the binding of ligands to ß-adrenergic receptors, suggesting that the interaction of the receptor with phospholipids is essential (Thang *et al*., 1980). NMDA and insulin receptors are known to have sulfhydryl groups that are damaged during LP (van der Vliet and Bast, 1992).

LP is also known to affect the activities of a number of enzymes (Chan *et al*., 1985; Yeagle, 1989). Some of these enzymes are membrane-bound; others are found in the cytosol and become associated with the neural membrane during the activation process, whilst others such as sodium-potassium ATPase, protein kinase C, PLA_2, guanylate cyclase, and 5′-nucleotidase require specific membrane phospholipids (Chan *et al*., 1985; Yeagle, 1989) for normal functioning.

1.2.4. Quinolinic Acid

1.2.4.1. Introduction

QA is a neurotoxic TRP metabolite, normally present at low concentrations in the CNS of man (Wolfensberger *et al.,* 1983). However, in pathological states with signs of inflammation, these levels are markedly elevated (Heyes *et al.,* 1992) because it is produced by immune activated macrophages (Pláteník *et al.,* 2001). Increased CNS levels of QA have been found in patients with mental depression (Mangoni, 1974) and AD (Guillemin *et al.,* 2003) and in mice with HSE (Reinhard, 1997).

1.2.4.2. Biosynthesis of Quinolinic Acid

1.2.4.2.1. Introduction

In mammals, the vast majority of dietary TRP is metabolised via the KP (figure 1.10.), which is initiated by the oxidative opening of the indole ring and eventually produces the ubiquitous enzyme cofactor NAD. This pathway contains neuroactive intermediates, all of which derive from L-kynurenine (L-KYN), the primary degradation product of TRP. Most of the neuroactive metabolites including QA can produce neuronal damage via the overstimulation of NMDA receptors, whilst kynurenic acid, which has proved to be an antagonist at these same receptors, is neuroprotective (Schwarcz and Pellicciari, 2002). 3-hydroxykynurenine (3-HK) and 3-hydroxyanthranilic acid (3-HA) can also produce neuronal damage, primarily by the induction of free radicals (Stone, 2000a).

1.2.4.2.2. Enzymes Involved in the Degradation of Tryptophan

1.2.4.2.2.1. Tryptophan-2,3-Dioxygenase

Tryptophan-2,3-dioxygenase (TDO) is one of the enzymes responsible for the degradation of TRP to L-KYN. It is a haeme-dependent enzyme, present in the livers of most mammals, including humans and rats. There is a considerable proportion of the enzyme that is present as the apoenzyme form, and is therefore inactive until the additional haeme is made available, either by the administration of haeme precursors *in vivo* or by the addition of haematin during incubation *in vitro* (Bender, 1983). Haeme is essential for activity and binds to the inactive apoenzyme in a reversible manner (step 1 in figure 1.8.) (Feigelson *et al*., 1964a), to form the oxidised, inactive holoenzyme, containing ferrihaeme (Knox and Piras, 1966). TRP binds to the inactive holoenzyme (step 2 in figure 1.8.) and reduces it to the active holoenzyme, which contains haeme in the ferrous form. This results in TRP becoming a free radical. The new complex reacts with O_2 (step 3 in figure 1.8.), during which O_2 is reduced to $O_2^{\bullet -}$ and haeme iron is reoxidised to the ferric form. Still on the enzyme surface, the TRP radical and $O_2^{\bullet -}$ combine (Feigelson *et al*., 1964b), yielding indole peroxide as an intermediate (Hayaishi

et al., 1957). This intermediate then undergoes further reactions independent of TDO to form L-formylkynurenine, which hydrolyses to L-KYN.

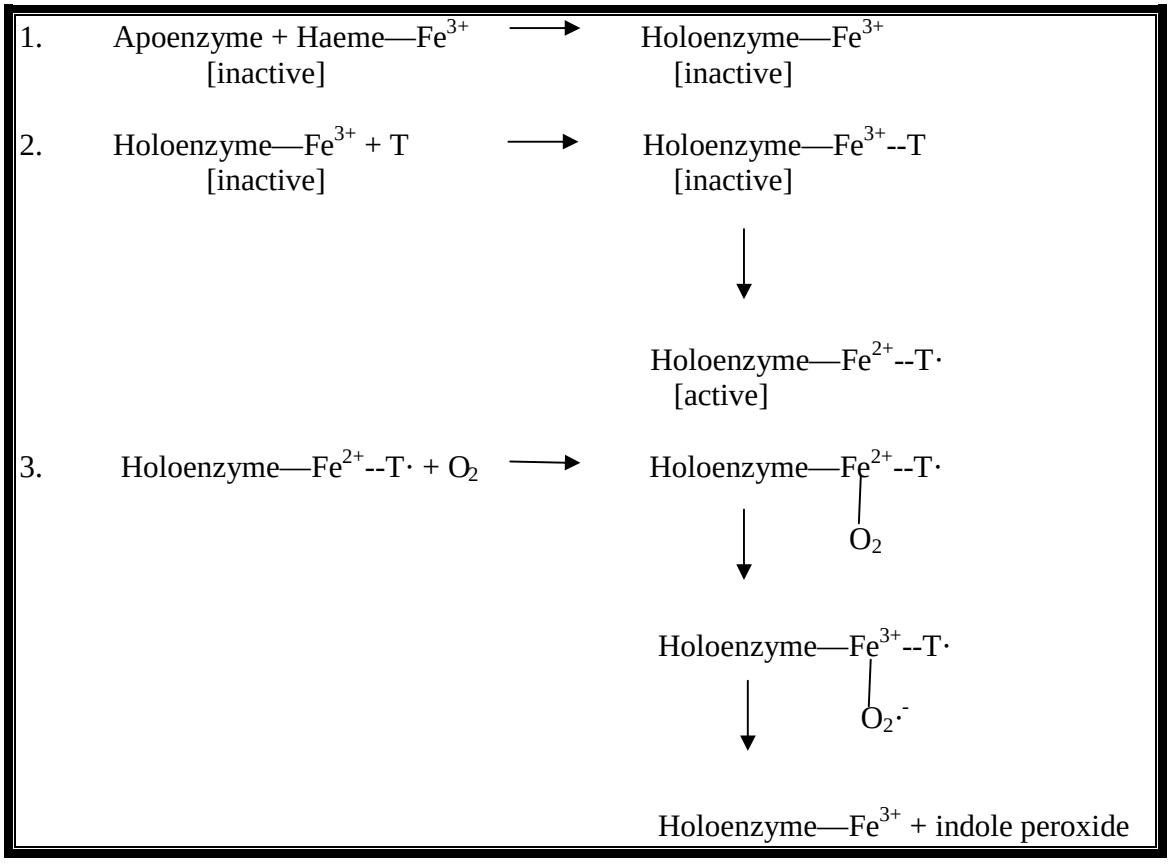


Figure 1.8. Activation and catalytic mechanism of TDO (modified from Feigelson *et al.*, 1964b).

1.2.4.2.2.2. Indoleamine-2,3-Dioxygenase

The second enzyme which catalyses the conversion of TRP to L-KYN in mammals is IDO. IDO has a broader specificity than TDO, as it can catalyse the oxidative cleavage of other indoleamines such as, 5-hydroxytryptophan, tryptamine, 5-HT and melatonin as well (Hirata and Hayaishi, 1972).

IDO contains protohaeme IX as its prosthetic group (Hirata *et al.*, 1977).The enzyme is only catalytically active in an oxygenated form. This active form of the IDO can be

formed by the interaction of either $O_2^{-\cdot}$ with the ferric form of the enzyme or O_2 with the ferrous form of the enzyme (Hirata *et al.*, 1977). The enzyme may then react with substrate to produce the product and the ferrous form of the enzyme. The ferrous form either undergoes oxidation to the ferric form or it can be oxygenated to regenerate the enzyme-O_2 complex (figure 1.9.) (Hirata *et al.*, 1977).

The specific cofactor for IDO has not been identified. Dihydroflavin mononucleotide ($FMNH_2$) has the capacity to activate O_2 via a single electron donation to produce $O_2^{-\cdot}$ (Ozaki *et al.*, 1987). Secondly, since it has another electron to donate, it may also function as a reducing agent converting [Fe^{3+}-haeme] to a [Fe^{2+}-haeme] state to which O_2 can bind to form a [Fe^{2+}-O_2-haeme] complex (Ozaki *et al.*, 1987). These authors, after extensive work using $FMNH_2$ on murine epididymal IDO, suggest that $FMNH_2$ may therefore be the biological cofactor of IDO *in vivo.*

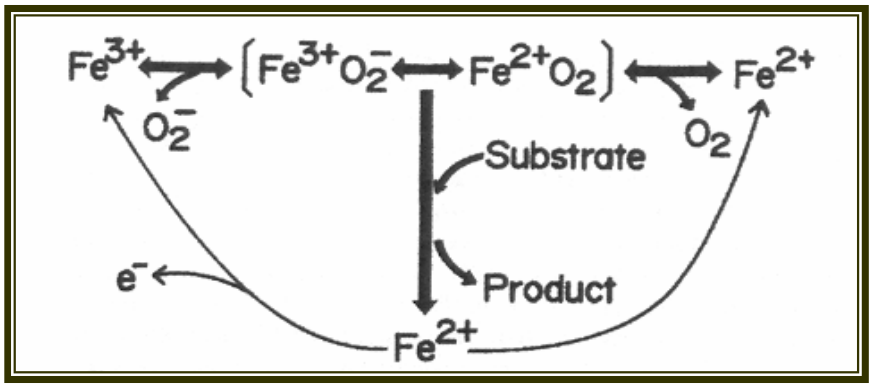


Figure 1.9. Proposed mechanism of action of IDO (Hirata *et al.*, 1977).

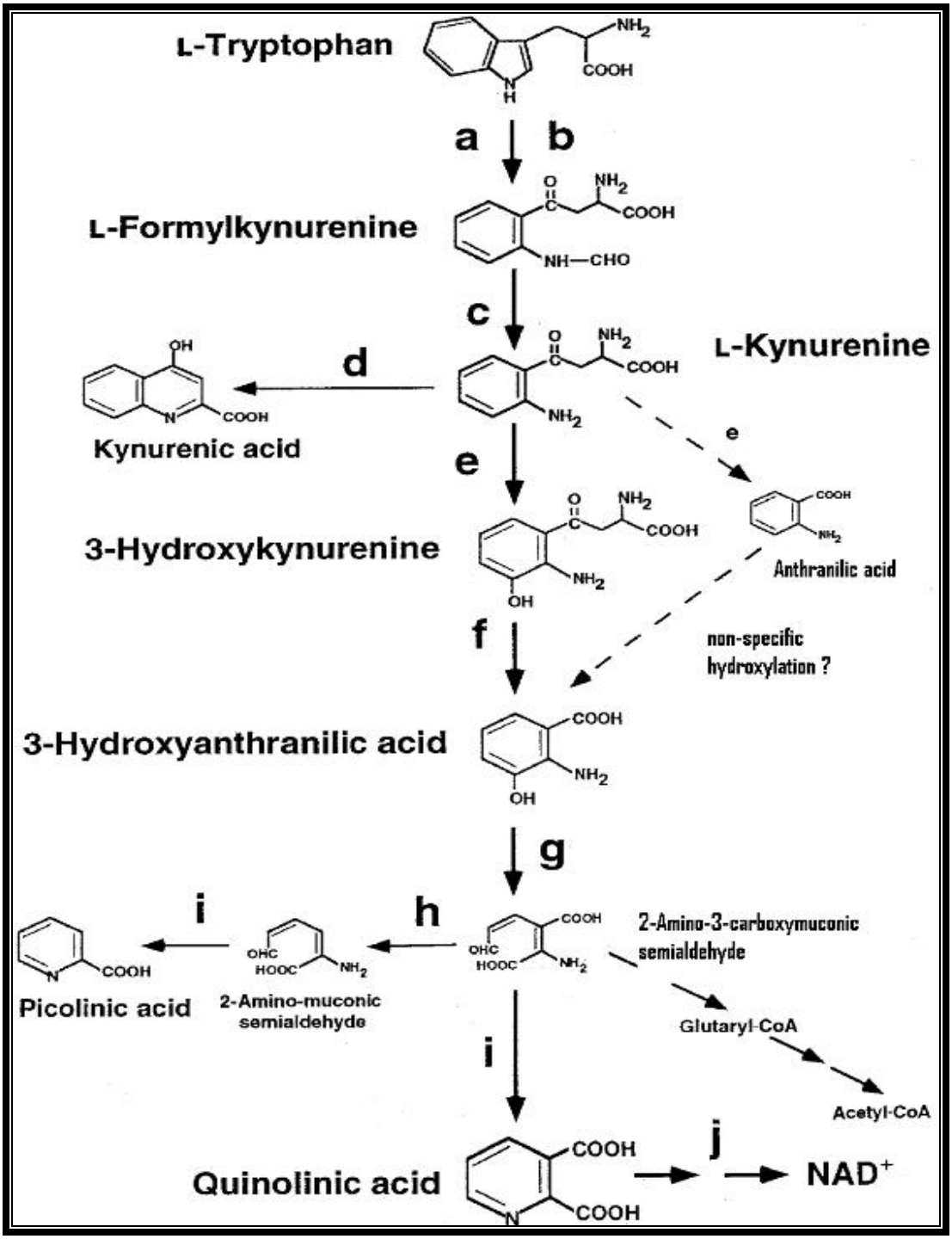


Figure 1.10. Graphical representation of the KP. (a) IDO; (b) TDO; (c) kynurenine formamidase; (d) kynurenine aminotransferase; (e) kynurenine-3-hydroxylase; (f) kynureninase; (g) 3-HAO; (h) picolinic carboxylase; (i) non-enzymatic cyclisation (spontaneous); (j) QA phosphoribosyltransferase (Heyes *et al.*, 1997).

1.2.4.2.3. Enzymes Regulating the QA / Kynurenic Acid Balance

Interference with the degradative enzymes of L-KYN, especially kynureninase and kynurenine-3-hydroxylase might favourably alter the kynurenic acid / QA ratio and thus correct pathophysiologically relevant chemical impairments in the brain (Schwarcz and Pellicciari, 2002).

1.2.4.2.3.1. Kynurenine-3-Hydroxylase

Kynurenine-3-hydroxylase is a flavin adenine dinucleotide (FAD)-dependent monooxygenase, which catalyses the hydroxylation of L-KYN to 3-HK (Schwarcz and Pellicciari, 2002). It catalyses the incorporation of one atom of O_2 into kynurenine in the presence of nicotinamide adenine dinucleotide hydrogen (NADPH) as an electron donor (Breton *et al*., 2000). During the reaction, the prosthetic group FAD is reduced to dihydroflavin adenine dinucleotide ($FADH_2$) by NADPH and is subsequently oxidised by O_2 to FAD (Breton *et al*., 2000).

1.2.4.2.3.2. Kynureninase

Kynureninase is a pyridoxal phosphate-dependent enzyme that catalyses the hydrolysis of both L-KYN and 3-HK into alanine and anthranilic acid or 3-HA respectively (Schwarcz and Pellicciari, 2002).

A Schiff base (**I** in figure 1.11.) forms between the pyridoxal phosphate enzyme and kynurenine. A proton from the α-carbon is eliminated. This results in the shifting of electrons and cleavage to form the o-aminobenzoyl radical (**II** in figure 1.11.) and the Schiff base of α-amino acrylic acid (**III** in figure 1.11.). Intermediate II (as its hydrate), either before or after stabilisation as o-aminobenzaldehyde, then undergoes a redox reaction with III to yield anthranilic acid and the Schiff base of alanine, which hydrolyses to alanine with regeneration of the phosphopyridoxal enzyme (Longenecker and Snell, 1955).

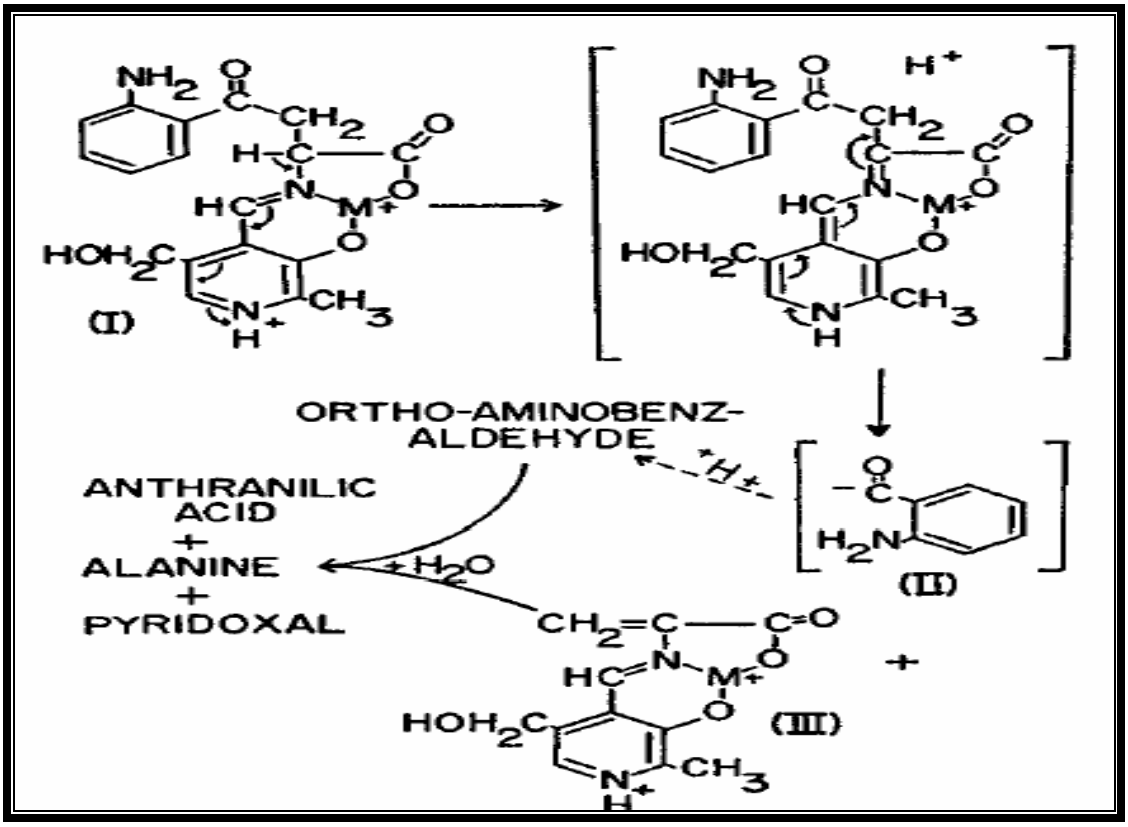


Figure 1.11. Possible mechanism for the cleavage of kynurenine by kynureninase. For simplicity, the reactions are formulated with "pyridoxal" instead of "pyridoxal phosphate" (Longenecker and Snell, 1954).

1.2.4.2.3.3. 3-Hydroxyanthranilic Acid Oxygenase

3-Hydroxyanthranillic acid oxygenase (3-HAO) catalyses the conversion of 3-HA to an unstable intermediate, 2-amino-3-carboxymuconic semialdehyde, which in turn can spontaneously rearrange by non-enzymatic cyclisation and dehydration (Schwarcz *et al.*, 1984) to QA or serve as a substrate for picolinic acid carboxylase to form picolinic acid.

3-HAO is a non-haeme ferrous extradiol dioxygenase enzyme (Li *et al*., 2006). Key enzymes in the pathways of aromatic compounds are the metal-dependent ring cleavage dioxygenases, which act on the corresponding catechol-type derivatives, cleaving these at the intradiol position (*ortho* cleavage) or the extradiol position (*meta* cleavage)

(Harayama *et al.*, 1992). While intradiol dioxygenases typically depend on ferric ions (Fe^{3+}), most extradiol dioxygenases depend on ferrous ion (Fe^{2+}) (Andújar *et al.*, 2000). A complete active site contains an Fe^{2+} ion (Zhang *et al.*, 2005). The coordination ligands include two histidine residues (His51 and His95), a bidentate glutamate residue (Glu57), and a water molecule (Zhang *et al.*, 2005). In this mechanism, the substrate binds to the active site Fe^{2+} by displacing one water molecule and the carbonyl oxygen of Glu57. This is followed by O_2 binding and an electron transfer from Fe^{2+} to O_2 to form an Fe^{3+}-containing product, **5** (figure 1.12.). Electron transfer from the electron rich aminophenol to the Fe^{3+} then produces the diradical, **6**. Radical recombination would give a peroxide, **7,** followed by a Criegee rearrangement to form lactone, **8**. Hydrolysis of **8** would then produce **10**. The enzyme dissociates from the product, **2**, which is 2-amino-3-carboxymuconic semialdehyde (Zhang *et al.*, 2005). It spontaneously rearranges to form QA (product **3**).

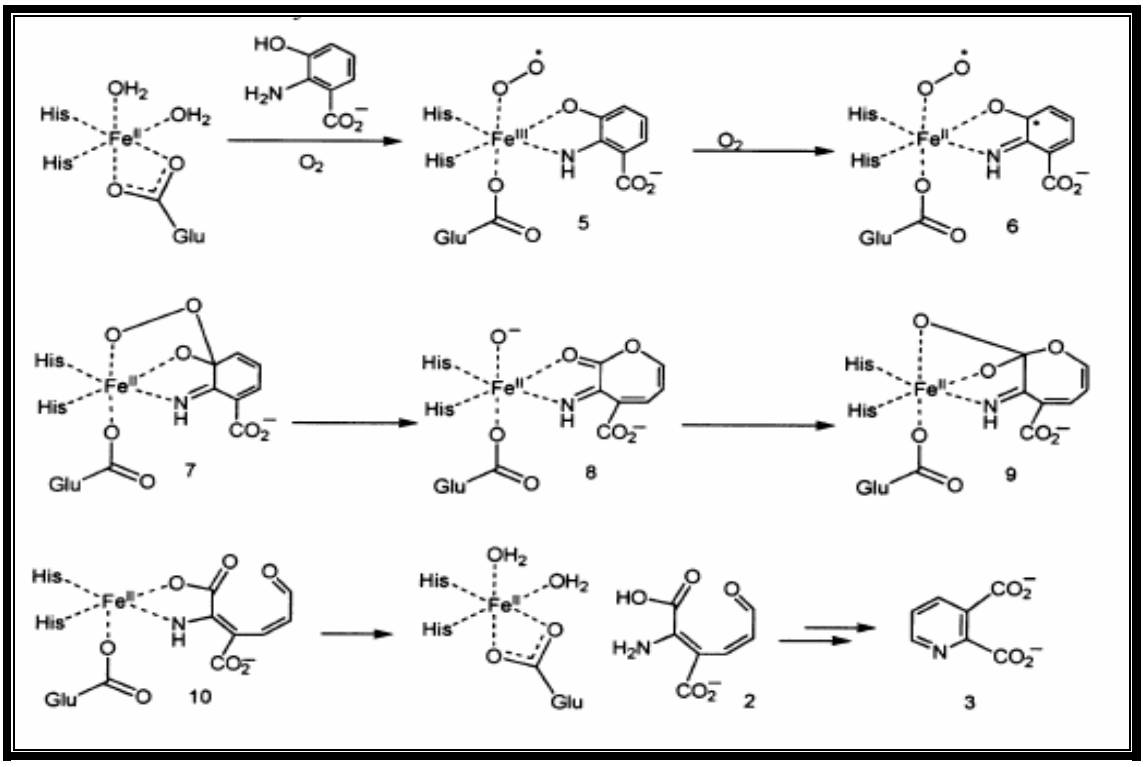


Figure 1.12. Proposed mechanism of action of 3-HAO (Zhang *et al.*, 2005).

1.2.4.2.4. The Effect of QA Biosynthesis on Brain Indoleamine Metabolism

In indoleamine metabolism (figure 1.13.), TRP is converted to 5-hydroxytryptophan by hydroxylation (Lovenberg *et al*., 1967). Decarboxylation of this indole derivative leads to the formation of 5-HT (Lovenberg, *et al*., 1962). Thus, the degradation of TRP by TDO and IDO, in the KP, during the biosynthesis of QA, reduces the amount of TRP available in the brain for conversion to 5-HT. Correlations between reduced levels of brain 5-HT and mental depression were discovered over twenty years ago (van Praag, 1982a). Depressed patients have a reduced level of plasma TRP (Delgado *et al*., 1990) and post-mortem studies on brains of depressed or suicidal patients have shown reduced concentrations 5-HT (Gibbons and Davis, 1986). However, research findings thus far point to aberrant 5-HT synthesis and / or metabolism increasing the risk of depression (Jimerson *et al*., 1990), rather than being a cause of the disease. Postmortem brain studies on AD patients have reported loss of serotonergic neurons (Mann and Yates, 1983; Yamamoto and Hirano, 1985) and nerve terminals (Bowen *et al*., 1983; Palmer *et al*., 1987) as well as reduced levels of 5-HT, its major metabolite 5-hydroxyindoleacetic acid (5-HIAA), and serotonergic receptor density (Adolfsson *et al*., 1979; Cross *et al*., 1984). In rabbits with experimental HSE, 5-HT levels are significantly reduced in the raphe nuclei (Päivärinta *et al*., 1993).

5-HT may be acetylated to N-acetylserotonin (Weissbach *et al.*, 1960), or oxidised by monoamine oxidase (MAO) to form 5-hydroxyindole acetaldehyde (figure 1.13.). Normally this is oxidised further to 5-HIAA by acetaldehyde dehydrogenase (Lerner and Case, 1960). Under some metabolic conditions, when the redox potential of the brain has shifted to a more reducing state than normal, a significant proportion of the aldehyde may be reduced to 5-hydroxytryptophol (Bender, 1983). All the 5-hydroxyindoles, namely, 5-hydroxytryptophan, 5-hydroxytryptamine, 5-HIAA, 5-hydroxytryptophol and N-acetylserotonin can be methylated by hydroxyindole-O-methyl transferase (HIOMT) (Axelrod and Weissbach, 1960; Axelrod and Weissbach, 1961) to form 5-methoxytryptophan, 5-methoxytrytamine, 5-methoxyindoleacetic acid, 5-methoxytryptophol and melatonin, respectively (Klein and Notides, 1969). Melatonin is

an important neurohormone known to act against free radicals both directly and indirectly (Albarran *et al.*, 2001). Patients with certain neurodegenerative disorders, such as AD have decreased levels of melatonin in the periphery (Monti and Cardinali, 2000). Ohashi *et al*., (1999) have suggested that this may be due to the neurodegenerative process of the disease. Moreover, previous studies have shown a reduction in melatonin secretion in patients with depression (Wetterberg *et al*., 1979, Miles and Philbrick, 1988), which may make these patients more susceptible to neurodegeneration.

1.2.4.3. The Neurotoxicity of QA

QA induced neurotoxicity has been associated with the overstimulation of NMDA receptors (Stone and Perkins, 1981). Thus, it would be expected to evoke a degenerative sequence of events within neurons, as shown in figure 1.5. *In vivo*, QA has been shown to impair learning and memory (He *et al.*, 2002), induce apoptosis and necrosis (Ferrer *et al.*, 1995), LP (Santamaria and Rios, 1993), DNA damage (Bordelon *et al.*, 1999), and generate free radicals (Pérez-Severianoa *et al.*, 2004). Furthermore, the fact that QA also has a lipid peroxidative potential *in vitro* (Rios and Santamaria, 1991) indicates a mechanism of generating free radicals independent of NMDA receptor activation.

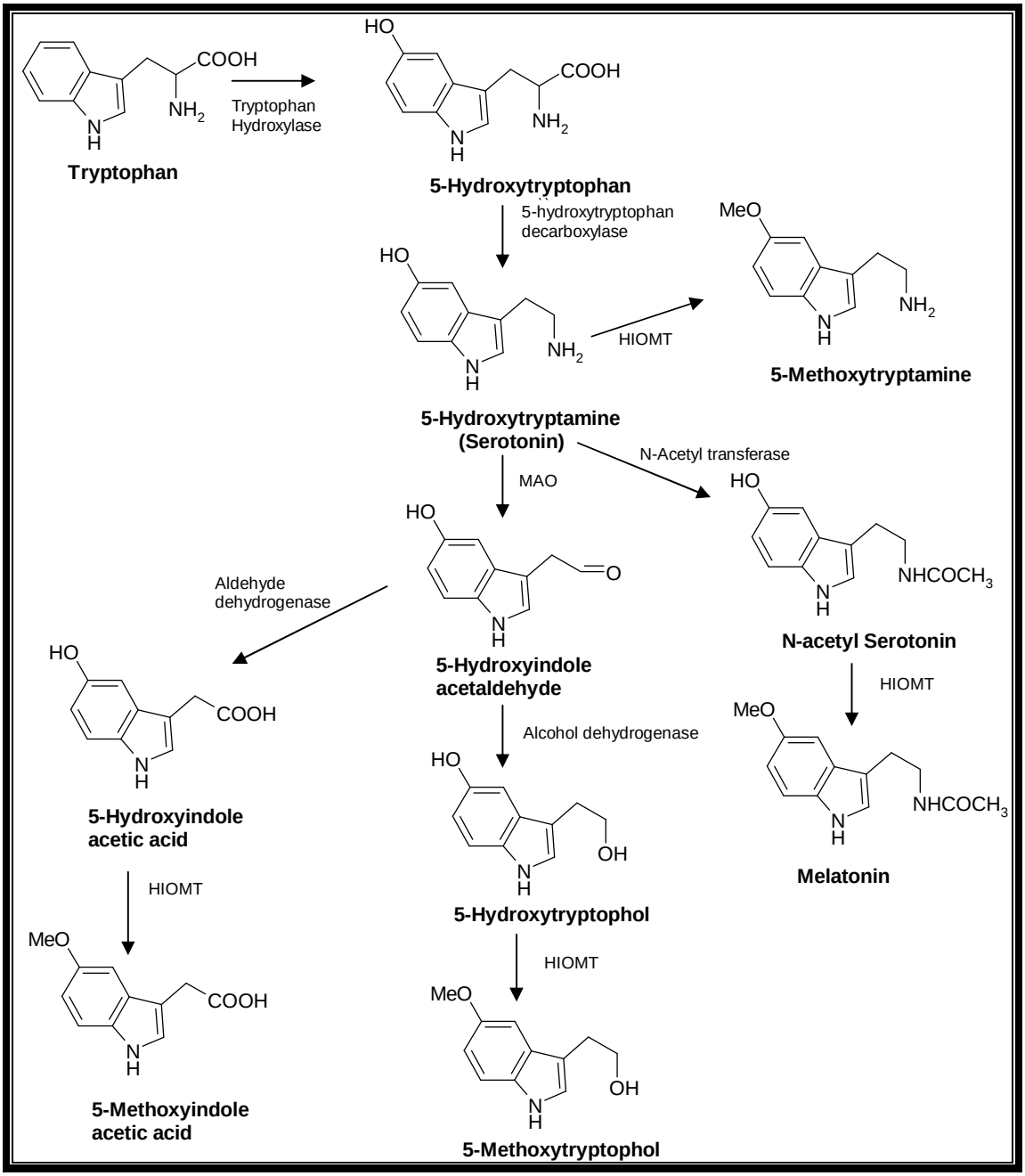


Figure 1.13. Pineal Indole Metabolism (Young and Silman, 1982).

1.3. NEUROPROTECTION

1.3.1. Introduction

Neuroprotectants have a critical role to play in the therapeutic intervention of neurodegenerative disorders to partially reverse or slow their progression. Neuroprotective strategies should either aid neuronal regeneration or, ideally disrupt the cascade of events which eventually lead to apoptosis or necrosis at one or more points

1.3.2. Neuronal Regeneration

1.3.2.1 Cellular Transplantation Therapy

Cellular transplantation was one of the first neuroprotective approaches employed clinically and it has been tried extensively in human patients as an experimental treatment (Alexi *et al.*, 2000). There are studies which have reported that foetal cortical tissue which has been transplanted 24 h after traumatic brain injury, survives and integrates into the injured brain. This has been shown to attenuate behavioural deficits and histological damage including hippocampal cell loss (Soares and McIntosh, 1991; Soares *et al.*, 1995; Sinson *et al.*, 1996).

1.3.2.2. Neurotrophic Factors

One of the most promising neuronal rescue strategies is treatment with neurotrophic factors. Neurotrophic factors influence cell survival and proliferation, differentiation, bio-chemical function, and morphological plasticity (Loughlin and Fallon, 1993; Hefti, 1997). Indeed, endogenous levels of neurotrophic factors are often found to increase in response to neuronal injury, suggesting a physiological regenerative response (Connor and Dragunow, 1998; Hughes *et al.*, 1999). Pharmacological application of many neurotrophic factors has been found to rescue injured neurons in animal models of Parkinson's disease and Huntington's disease (Alexi *et al.*, 2000). Furthermore, intracerebroventricular administration of glial derived neurotrophic factor following brain

injury in rats decreased the post-traumatic neuronal loss in the hippocampus at one week postinjury (Kim *et al.,* 2001).

1.3.3. Anti-Apoptotic Therapy

Another strategy to prevent acute cell death may be to inhibit the caspase enzymes (Hara *et al.,* 1997; Raghupathi, 2004) Several caspase inhibitors of varying specificity have been successfully evaluated in models of experimental traumatic brain injury including ketones such as the pan-caspase inhibitor z-VAD-fmk, the caspase-1 specific inhibitor acetyl-Tyr-Val-Ala-Asp-chloromethyl ketone and the caspase-3-specific z-DEVD-fmk (Raghupathi, 2004). In NMDA lesioned rats, intraventricular administration of the caspase inhibitor zVAD-fmk just prior to and 3 h after lesioning partially reduces the lesion volume (Hara *et al.,* 1997).

1.3.4. Antioxidant Therapy

1.3.4.1. Introduction

Halliwell and Gutteridge (1989) define an antioxidant as “any substance that, when present at low concentrations compared to those of an oxidisable substrate, significantly delays or inhibits oxidation of that substrate”. According to this definition, antioxidants can act at various levels in the oxidative sequence.

1.3.4.2. Enzymes

The antioxidant enzymes form an important part of the adaptation of organisms to protect themselves against free radical injury. These enzymes, as illustrated in figure 1.14., include catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and superoxide dismutase (SOD) (Hung and Lee, 1998). SOD catalyses the dismutation of $O_2^{\bullet-}$ to H_2O_2 and in this way reduces the concentration of $O_2^{\bullet-}$ available i) to exert the deleterious effects described in section 1.2.2.2.1. ii) for the conversion to other toxic free

radicals such as $OONO^-$ and $HOO^\cdot$ (Hung and Lee, 1998). CAT and GPx catalyse the conversion of H_2O_2 to water (H_2O) thus slowing the progression of the Fenton reaction and the generation of the potent $HO^\cdot$ (section 1.2.2.2.5.). GPx is the major enzyme for the detoxification of H_2O_2 in the brain since the brain has very low CAT activity (Bharath *et al*., 2002). Glutathione (GSH) serves as the reducing agent in this reaction (Hung and Lee, 1998). Antioxidant enzymes were found to be reduced in an animal model of AD (Marcus *et al*., 1998; Montilla-López *et al.*, 2002).

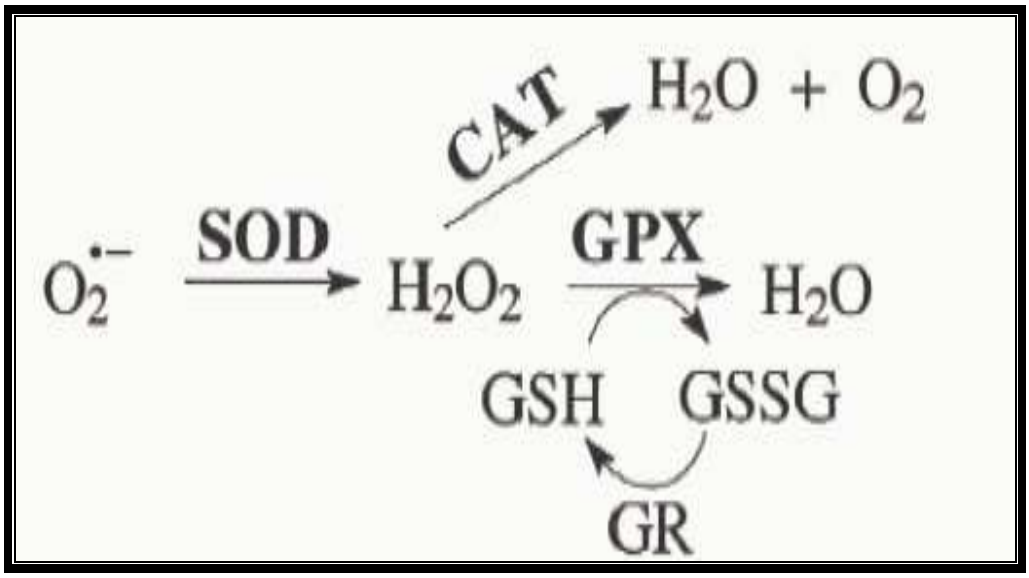


Figure 1.14. Schematic diagram of the antioxidant enzyme system, modified from Hung and Lee (1998).

1.3.4.3. Iron Chelators

Free or weakly bound ferrous iron is very sensitive to O_2 and leads to the formation of ferric iron and $O_2^{\cdot-}$ which rapidly dismutates to H_2O_2. Ferrous iron can also react with H_2O_2, leading to $HO^\cdot$ by Fenton chemistry. Moreover, in the presence of physiological reductants, iron can redox cycle between the two oxidation states, thereby generating the production of free radicals continuously (Galey, 1997). Increased iron content has been found in the substantia nigra of postmortem parkinsonian brains (Gerlach *et al.*, 1994) and the toxicity of AP in AD toward some neural cells is mediated by H_2O_2 (Behl *et al.*,

1994) Thus, agents with the ability to chelate iron and thus decrease its content in the brain may prove to be useful in oxidative stress-induced disorders of the CNS. By definition, chelation requires the presence of two or more electron donating groups, such as carboxylate and tertiary amine to form a coordinate bond with Fe^{2+} or Fe^{3+} (Galey, 1997). Intramuscular injection of the well established iron chelator desferrioxamine was found to significantly reduce the brain iron content in a model of iron-loaded rats (Ward *et al.*, 1995).

1.3.4.4. Scavengers of Free Radicals

1.3.4.4.1. Introduction

Scavengers of free radicals are antioxidants which act by being oxidised by free radicals in place of biomolecules (Halliwell and Gutteridge, 1989).The specific mechanism of action involves free radicals reacting with the antioxidant via hydrogen abstraction or addition to form an antioxidant radical (Halliwell and Gutteridge, 1989). Since most antioxidants have aromatic ring structures, the antioxidant radical so produced is itself insufficiently reactive to cause hydrogen abstraction or addition of biomolecules, because of delocalisation of the unpaired electron into the aromatic ring (Halliwell and Gutteridge, 1989). The antioxidant radical can however either undergo radical terminating reactions with other free radicals, further reducing the availability of these to initiate oxidative damage, or be regenerated by reducing agents.

1.3.4.4.2. Endogenous Scavengers of Free Radicals

Vitamin E and vitamin C are both endogenous scavengers of free radicals that work synergistically (Fang *et al.*, 2002). Vitamin E can transfer its phenolic hydrogen to a $ROO^{\cdot}$ of a peroxidised PUFA, thereby breaking the radical chain reaction and preventing the peroxidation of PUFA in membrane phospholipids (Fang *et al.*, 2002). As a reducing agent, vitamin C reacts with a vitamin E radical to yield a vitamin C radical while regenerating vitamin E. The vitamin C radical is converted back to vitamin C by GSH (Fang *et al.*, 2002). Peripheral levels of these vitamins have been found to be depleted in

patients with AD (Rinaldi *et al.*, 2003) and have shown promise in the prevention of this disease (Bradford and Gupta, 2005). Serum levels of vitamin E have also been found to be lower in patients suffering from major depression (Maes *et al.*, 2000).

1.3.4.5. Melatonin as an Antioxidant

Melatonin is a neurohormone produced in the pineal gland of all mammalian species. This production is regulated by the photoperiodic environment, such that the daily period of light is associated with minimal release whereas during darkness, the synthesis and secretion is at a maximum (Reiter, 1991c).

The functions of melatonin as an antioxidant are broad and thus it cannot be categorised as one specific type of antioxidant. Melatonin is able to detoxify free radicals via electron donation (Reiter *et al.*, 1996). The indoyl cation that is formed is able to scavenge both the $O_2^{\cdot-}$ and $ONOO^-$ forming N-acetyl-N-formyl-5-methoxykynuramine. Limson *et al.*, (1998) showed that melatonin can chelate iron. In addition, melatonin stimulates the synthesis of antioxidant enzymes including SOD, GPx and GR (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).

1.3.5. Anti-excitotoxin Therapy

As described in section 1.2.2.4., excitotoxicity has a critical role to play in inducing neurodegeneration. Thus, it stands to reason that opposing or preventing excitotoxicity may have therapeutic potential in diseases, such as Parkinson's (Alexi *et al.*, 2000) AD and Huntington's (Hynd *et al.*, 2004), where the pathology is thought or known to include this phenomenon. There are various ways in which excitotoxicity can be curtailed. Memantine and zonampanel are antagonists at the NMDA and AMPA / kainate receptors respectively. Both these agents have been successful in treating experimental traumatic brain injury (Pitkanen *et al.*, 2005), whilst the former has also been used in AD (Grossberg *et al.*, 2006). There are also agents which modulate the release and uptake of

glutamate. Riluzole, which enhances glutamate uptake (Frizzo *et al.*, 2004) and is an inhibitor of glutamate release has shown neuroprotective properties against hypoxic brain damage, MPTP-induced neurotoxicity, and both global and focal cerebral ischaemia in rodents (Mufson *et al.*, 1999). In a clinical study, riluzole appears to slow the progression of amyotrophic lateral sclerosis (Pitkänen and McIntosh, 2006).

1.3.6. Bioenergetic Supplements

Metabolic supplements such as nicotinamide diminish the neurotoxicity of metabolic poisons such as 3-nitropropionic acid and malonic acid in rodents. Nicotinamide is the precursor of NAD, an electron acceptor which releases energy when it is oxidised during phosphorylation of ADP to ATP. Thus it has the ability to possibly reverse ATP depletion during mitochondrial dysfunction. Nicotinamide provides neuroprotection from malonic acid lesioning when administered as four systemic injections beginning 30 min prior to malonic acid (Beal *et al*., 1994; Jenkins *et al*., 1996; Schulz *et al*., 1996).

1.3.7. Purine Nucleotides / Nucleosides

Purine nucleosides and nucleotides are extensively released from degenerating cells, particularly under hypoxic/ischaemic conditions, thus it was suggested that these may have a role to play in regulating the response of nervous tissue to injury (Ciccarelli *et al*., 2001). The interaction of guanine nucleotides with excitatory amino acid receptors, at the extracellular level, is already a well-established fact: both the common native nucleotides, guanosine triphosphate (GTP), guanosine diphosphate (GDP) and guanosine monophosphate (GMP), and the non-hydrolysable, synthetic analogues have been shown to displace agonists from both ionotropic receptors, including NMDA (Sharif and Roberts, 1981; Monahan *et al.*, 1988) and non-NMDA (Souza and Ramirez, 1991; Gorodinsy *et al*., 1993) types, and metabotropic receptors (Tasca *et al*., 1995; Tasca *et al*., 1998). This is particularly relevant in the CNS, where this action may depress glutamatergic neurotransmission, thus reducing excitotoxic neuronal damage (Rudolphi, 1991; Rudolphi *et al*., 1992). Moreover, it has been shown that guanosine is able to

induce neurite outgrowth of neuronal pheochromocytoma cells (Gysbers and Rathbone, 1996) and to preserve glial cell viability (Jurkowitz *et al*., 1998). Recent data gave evidence that purines protect against oxidant-induced cell injury by inhibiting the activation of PARP (Virág and Szabo, 2001).

These studies have encouraged the use of purines as therapeutic agents in neurological disorders. Unfortunately, the clinical use of naturally occurring molecules in humans is undermined by pharmacokinetic and pharmacodymanic problems (Ciccarelli *et al*., 2001). The ubiquitous distribution of purine receptors results in numerous side effects when purines are administered systemically. Moreover, the widespread distribution of enzymes that metabolise purines implies that the half-life, and hence duration of action of these is very short. In contrast, synthetic purine derivatives that penetrate the blood brain barrier and enter the CNS are good candidates for clinical use, particularly if these are metabolised slowly.

1.3.7.1. Acyclovir

1.3.7.1.1. Introduction

A screening programme for antiviral drugs which began in the 1960s resulted in the discovery of acyclovir (AC) in 1974. Preclinical investigation brought the drug to clinical trials in 1977 and the first form of this antiviral drug was available to physicians in 1982 (King, 1988).

1.3.7.1.2. Pharmacology

1.3.7.1.2.1. Indications

AC is indicated for the treatment of infections due to HSV (types I and II) and varicella-zoster viruses.

1.3.7.1.2.2. Adult Dosing

Topical AC is used to treat the symptoms of HSV infections of the skin and mucous membranes by applying it to the affected area four to six times a day for up to 10 days (Gibbon, 2003).

Oral treatment is 400 mg eight hourly or 200 mg five times daily, for ten days (primary episode) or 5 days (recurrent episode) (Gibbon, 2003).

Parenteral AC is used in the treatment of severe primary herpes infections, disseminated herpes infections and in HSE. Intravenous (i.v.) infusion over one hour, 5mg/kg eight hourly for 5-7 days; 10mg/kg is recommended in HSE and in varicella-zoster (Gibbon, 2003).

1.3.7.1.2.3. Drug Classification and Activation

AC (figure 1.15.) is a deoxyguanosine analogue with an acyclic side chain that lacks the 3-hydroxyl group of natural nucleosides (Wagstaff *et al.*, 1994). After preferential uptake by infected cells, AC is monophosphorylated by virus-encoded thymidine kinase (TK); host cell TK is approximately 1 millionfold less capable of converting AC to its monophosphate derivative (Elion, 1982). Subsequent diphosphorylation and triphosphorylation are catalysed by host cell enzymes, resulting in the active form, AC triphosphate, the concentrations of which are 40 to 100 times higher in viral-infected cells than in uninfected cells (Elion, 1982).

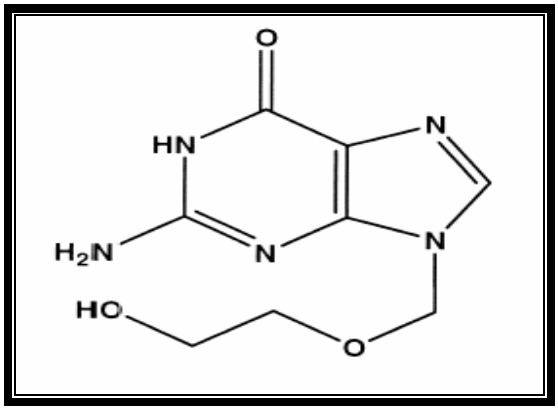


Figure 1.15. Structure of acyclovir (Herrero *et al*., 2001).

1.3.7.1.2.4. Mechanism of Action

AC triphosphate prevents viral DNA synthesis by inhibiting the viral DNA polymerase (Reardon and Spector, 1989; Kimberlin, 2001). *In vitro*, AC triphosphate competes with deoxyguanosine triphosphate as a substrate for viral DNA polymerase (Reardon and Spector, 1989). Because AC triphosphate lacks the 3-hydroxyl group required to elongate the DNA chain, the growing chain of DNA is terminated. In the presence of the deoxynucleoside triphosphate complementary to the next template position, the viral DNA polymerase is functionally inactivated (Reardon and Spector, 1989). In addition, AC triphosphate is a more suitable substrate for the viral polymerase than for cellular DNA polymerase, resulting in little incorporation of AC into cellular DNA (Kimberlin, 2001).

1.3.7.1.2.5. Adverse Effects

AC is generally well tolerated (Gibbon, 2003). However, when administered i.v., it may cause inflammation and phlebitis at the injection site (Gibbon, 2003). Renal impairment may occur, although this appears to be reversible as it has reported to respond to hydration and / or dosage reduction or withdrawal (Harrington *et al*., 1981) If not controlled, it may progress to acute renal failure (Giustina *et al.*, 1988; Eck *et al.*, 1991). Occasionally, i.v. AC has caused neurological effects including lethargy, confusion, agitation, tremors and convulsions (Wade and Meyers, 1983).

1.3.7.1.2.6. Drug Interactions

Patients being treated with both zidovudine and AC together can develop severe somnolence and lethargy (Kimberlin, 2001). The likelihood of renal toxicity is increased when AC is administered with nephrotoxic drugs such as cyclosporine and amphotericin B (Kimberlin, 2001; Gibbon, 2003). Concomitant administration of probenicid decreases renal clearance of AC and prolongs its half-life; conversely, AC can decrease the clearance of drugs such as methotrexate that are eliminated by active renal secretion (Kimberlin, 2001).

1.3.7.1.3. Pharmacokinetics

1.3.7.1.3.1. Absorption

AC is slowly and poorly absorbed, as the time to reach peak concentrations is 1.5–2 hrs and the estimated bioavailability is 13–21 % (de Miranda and Blum, 1983). With multi-dose administration, steady-state plasma concentrations are achieved by the second day. The mean plasma half-life is approximately 3.32 hrs and 3.03 hrs after oral and i.v. administration respectively (Blum *et al*, 1982).

1.3.7.1.3.2. Distribution

AC is widely distributed in tissues and body fluids including the brain, kidney, lung, liver, muscle, spleen, uterus, vaginal mucosa and secretions, and the cerebrospinal fluid (CSF) (Blum *et al.*, 1982). After multiple dose therapy, concentrations in the brain, spinal chord and CSF were 25–70 % of the plasma level (Blum *et al.*, 1982).

1.3.7.1.3.3. Metabolism

Radiolabelled disposition studies in individuals with normal renal function show that almost all of an administered i.v. dose is recovered from the urine. About 80 % is unchanged and a further 8.5–14 % appears as the oxidised metabolite, carboxymethoxymethyl guanine (de Miranda *et al.*, 1981). Another minor metabolite, 8-hydroxy-9-(2-hydroxyethoxymethyl guanine can be detected in urine at very low concentrations. Neither of these metabolites is known to be pharmacologically active (de Miranda *et al.*, 1981).

1.3.7.1.3.4. Excretion

Renal excretion is the major route of elimination in individuals with normal renal function (de Miranda *et al.*, 1981). The renal clearance of AC is approximately three

times greater than that of creatinine, thus indicating that the drug is removed by tubular secretion as well as glomerular filtration (de Miranda *et al.*, 1981).

1.4. RESEARCH OBJECTIVES

Due to the activation of the immune response in the CNS during diseases such as AD, HSE and depression, the neurotoxin QA is produced. These diseases have the potential to impair learning and memory and hippocampal lesioning has been reported. Although QA may not be the sole cause of this neurodegeneration, it is likely to have some role to play, particularly if neurotoxic concentrations in the hippocampus are achieved. Numerous studies point to a neuroprotective potential for guanine, guanosine and their derivatives and analogues. AC, a guanosine analogue is of particular relevance in this regard because it is the current drug of choice in the treatment of one of these debilitating diseases, namely, HSE.

The objective of this study is to determine whether AC has the ability to reduce QA-induced spatial memory deficits and the neurodegeneration associated therewith. In addition, it also seeks to establish some of the mechanisms by which AC affords neuroprotection, if any, through the employment of various biological assays and inorganic studies.

Since an increase in QA could enhance neuronal damage produced by a primary brain insult, the reduction in QA synthesis could limit the extent of CNS injury (Stone, 2000a). Thus, the effect of AC on the activity of some of the enzymes in the biosynthetic pathway of QA will be assessed to see if this drug could favourably regulate the KP. Due to the interplay between the cytokine activated KP and brain 5-HT, any profound effect exhibited by AC on the KP is expected to reflect in brain 5-HT and related neurotransmitter levels, thus changes in these levels will be investigated. Furthermore, preserving 5-HT levels within the brain may aid in preventing or alleviating mental depression, therefore the effect of AC on 5-HT metabolism will also be determined.

It is hoped that this study will provide more information on the neuroprotective potential of AC, which may contribute to the effectiveness of this antiviral in HSE. Furthermore, this work may provide a foundation for further research on structure-activity relationships of neuroprotective guanosine analogues to effectively harness maximal neuroprotective activity in one or some of these properties investigated.

CHAPTER 2

LEARNING AND MEMORY

2.1. INTRODUCTION

The majority of studies on learning (Tsilovosky *et al.,* 2004) include classical and instrumental conditioning. Classical conditioning is characterised by the contingent reinforcement of a specific signal, called a conditioned stimulus (CS) (Tsitolovsky *et al.,* 2004) with an unconditioned stimulus (US) to produce an unconditioned response (UR), resulting in the CS acquiring the ability to evoke a conditioned response (CR). This type of learning was first discovered by Pavlov who experimentally found that food as a US reflexively evokes salivation, the UR in dogs (Anderson, 2000). After repetitively giving the dogs food with the concomitant ringing of a bell (CS), the bell ringing could eventually evoke the salivation by itself (Anderson, 2000). On the other hand, the basis of instrumental conditioning is the contingent reinforcement of a specific type of behaviour (Tsitolovsky *et al.,* 2004) with a stimulus. In 1898, Thorndike studied this type of learning by placing a hungry cat in a box with food outside (Anderson, 2000). If the cat hit an unlatching device, the door would open and the cat would escape and eat the food. After repeated trials, Thorndike found that the random behaviour of the cat diminished (Anderson, 2000) and when placed in the box, unlatching the door became instinct. Thus, in instrumental conditioning, the response (in this case, unlatching the door) to a stimulus (being placed in the box) is performed to obtain a reinforcer (escape and food).

One of the most widely accepted ideas about memory is the distinction between short-term memory and long-term memory (Atkinson and Shiffrin, 1968; Baddeley, 1976). More recently, it was suggested that long-term memory can be divided into two major components referred to as declarative or explicit and non-declarative or procedural memory (Schacter, 1987; Soliveri *et al.,* 1992; Squire *et al.,* 1993). While declarative memory relates to the ability to store and recall or recognise specific images, events, or facts, procedural memory is based on information which is accessible only by

improvement in performance and includes, for example, the cognitive and motor skill acquisition (Rammsayer *et al.,* 2000) required to remember how to ride a bike. Declarative memory is generally conceived as a combination of episodic and semantic memory (Schacter and Tulving, 1994). Episodic memory involves the capacity to remember specific personal experiences (Eichenbaum, 2003). These memories contain the detailed sequence of events that constitute an experience and the spatial and temporal context in which the experience occurred (Eichenbaum, 2003). Semantic memory involves the acquisition of world knowledge, an organisation of information about the physical features of the world, about the content and meaning of language, social structure, geography and a multitude of other spheres of knowledge (Eichenbaum, 2003).

Memory may further be classified according to purpose. Reference memory refers to the memory of information that remains constant over a relatively long period of time, whereas working memory refers to memory of information which is rapidly changing (Olton *et al.,* 1979).

Memory processing consists of an encoding phase, a consolidation phase and a retrieval phase. In the encoding phase, the stimulus material has to be learned (intentional) or processed deeply (incidental) (van der Veen *et al.,* 2006). In the consolidation phase, the previously learned or encoded stimulus material is transformed into a permanent memory trace. In the retrieval phase, the stored information has to be retrieved from memory by using either recognition or recall (van der Veen *et al.,* 2006).

There is a well-established tradition of studying the effects of drugs using behavioural methods (Cook and Kelleher, 1963; Gollub and Brady, 1965). Defining behaviour in terms of underlying biological events is not a new concept (Bondy, 1985). Such correlations have been attempted in several areas ranging from learning and memory to neurological disease. Neurological disorders can formally be described as disturbances in structure or function of the nervous system resulting from a developmental abnormality, disease, injury, or toxin (http://www.alz.org/Resources/Glossary.asp#n). Increased information concerning the relationship between behaviour and neurobiological

mechanisms is important because it is often abnormal behaviour that is a first sign of exposure to a neurological disease and such changes may give clues as to the anatomical or chemical sites of attack on the nervous system (Bondy, 1985). Unfortunately, there appears to be a shortfall of literature reporting on both behavioural and biological disturbances caused by an agent of neurological disorders (developmental abnormality, disease, injury, or toxin) in the same experimental animal (Bondy, 1985).

2.2. THE EFFECT OF QA ALONE AND IN COMBINATION WITH AC ON RAT SPATIAL REFERENCE MEMORY

2.2.1. Introduction

Declarative memory, in general, is highly dependent on the integrity of the hippocampus and anatomically related structures in the medial temporal lobe and diencephalon (Zola-Morgan and Squire, 1993; Nelson, 1995). The hippocampal formation is particularly important in the development of episodic declarative memory (Squire *et al.,* 1993; Schacter *et al.,* 1996; Vargha-Khadem *et al.,* 1997) and the working memory (Olton *et al.,* 1979), necessary for the processing of cognitive mapping (O'Keefe and Nadel, 1978), or relational association (Eichenbaum *et al.,* 1990; Bunsey and Eichenbaum, 1996). The hippocampus encodes the association or relation of numerous factors by representing sequences of multiple events or factors that compose the spatial and temporal context of an episode (Furusawa *et al.,* 2006). Thus, many studies have suggested that the hippocampus is imperative for spatial memory.

Rolls (1987) produced a theory of the hippocampus in which the dentate granule cells encode episodic memory input from the cortex by performing pattern separation so that the mossy fibres can act to set up different representations for each memory to be stored in the CA3 cells. The CA3 neurons then facilitate autoassociation. Autoassociation refers to the ability of a network of neurons to store information, after constant exposure to the same pattern of information (Hertz *et al.,* 1991). When a distorted image of the same pattern (partial cue) is detected, the task is to retrieve the pattern. Rolls (1987) further suggested that the CA1 cells operate as a recoder for the information recalled from the CA3 cells so that the recalled information would be represented more efficiently to enable recall, via backprojection synapses to the neocortical areas similar to that which had been present during the original episode. As described in section 1.1.2., the CA3 pyramidal neurons synapse on the CA1 pyramidal neurons via their axons known as the Schaffer collateral system. It is these synapses which undergo synaptic modification in

the form of LTP during learning (Morris, 1989), thus making it structurally possible for autoassociation to occur. By recording the firing patterns of hippocampal neurons of rats, Wood *et al*. (2000) showed that certain hippocampal neurons fire selectively and, therefore, likely undergo autoassociation when a rat is in a particular locus in its environment. This finding has been described as evidence that the hippocampus is dedicated to forming spatial maps (O'Keefe, 1979).

Since the hippocampus plays such an important role in episodic memory, lesioning of this limbic structure after learning a spatial orientated task would induce spatial memory deficits (Winocur *et al.,* 2005). QA is known to have the ability to cause lesioning in the hippocampus (Schwarcz *et al.,* 1984 Speciale *et al.,* 1987; Haberny *et al.,* 1992), thus the aim of the experiment in this study is to investigate the effect of QA on spatial reference memory in male Wistar rats. Furthermore, since lesioning of the hippocampus commonly occurs during adult HSE and there is a link between HSE and QA (section 1.2.4.1.), it was also decided to assess whether AC, the current treatment of HSE could alter the effect, if any, of QA on spatial reference memory. This is deemed particularly relevant because AC belongs to the nucleoside class of guanosine analogues which have known neuroprotective effects (section 1.3.7.). The results of the study may also provide a foundation for further research into guanosine analogues and other potential memory impairing disorders, associated with QA, such as AD and depression (section 1.2.4.1.).

Morris, (1981) first demonstrated that rats can rapidly learn to locate an object that they cannot see, hear, or smell provided it remains in a fixed spatial location relative to distal room cues. Instrumental associative learning is employed by training rats to escape from an opaque tank of water by swimming onto a platform, which is in a fixed location and submerged below the level of the water. Room cues are available to the rats to use for spatial orientation. After training, the hippocampi of the rats are lesioned and the rodents undergo a treatment regime. The effects thereof on the spatial memory of the water maze task experienced before the lesioning is determined by reintroducing the rats to the same Morris water maze and assessing the latency (time in seconds) to escape to the platform.

Since the location of the platform is not varied, this standard water maze task measures predominantly spatial reference memory, rather than spatial working memory.

2.2.2. Materials and Methods

2.2.2.1. Animals

Adult male Wistar rats were purchased from the National Health Laboratory Service (NHLS), Johannesburg, South Africa. The rats were randomly assembled into groups of five and housed in separate cages under artificial illumination with a daily photoperiod of 12 hours (lights on at 06h00). The temperature of the animal house was maintained at 20–24 ˚C, while an extractor fan ensured the constant removal of stale air. The rats (200–250 g) were provided with food and water *ad libitum.* All protocols for the experiments were approved by the Rhodes University Animal Ethics Committee.

2.2.2.2. Chemicals and Reagents

AC sodium (Zovirax®, powder for injection) was obtained from Glaxo Wellcome, Midrand, South Africa. QA was purchased from Sigma Chemical Co, St. Louis, USA. Sodium phenobarbitone was purchased from Merck, Germany and was at a working concentration of 60 mg/ml.

2.2.2.3. Animal Training in the Morris Water Maze Task

A black (opaque) platform, 12.5 cm in diameter and 31 cm in height, was placed on a marked position in a black circular water tank, 150 cm in diameter and 40 cm in height. Water at a temperature of 23 ˚C was filled in the tank to a height of 33 cm, thus the platform was submerged 2 cm below the level of the water. The tank was located in a room with colourful pictures (cues) external to the tank, which could be used by the rats for spatial orientation. The positioning of the cues remained unchanged throughout the study. During the training sessions, each rat was put into the water at the same starting point, and left to swim in the tank until they found the platform. If they failed to find the

platform within 90 s, they were gently guided to it. Training was conducted twice a day for 7 days, with each session consisting of two trials.

2.2.2.4. Surgical Procedures

Each rat was anaesthetised by an intraperitoneal (i.p.) injection of sodium phenobarbitone (60 mg/kg) and placed in a stereotaxic frame (Stoelting, IL, USA). The rat skull was orientated according to the stereotaxic atlas of König and Klippel (1963). After a sagittal cut in the skin of the skull, the bregma suture was located and bilateral holes were manually formed with a 21 gauge needle, at the following co-ordinates; 4.0 mm anterior, 2.5 mm lateral of the suture. Care was taken not to damage the meninges. A Hamilton syringe with a 26 s gauge cannula with an outer diameter of 0.47 mm was used to inject 120 nmoles of QA in 2 µl phosphate buffered saline (PBS), pH 7.4., 3 mm ventral of the dura. The rats in the control group were sham-lesioned with injections containing PBS only. Each injection was administered at a rate of 1 µl per minute and the cannula was left *in situ* for a further 2 minutes to allow for passive diffusion away from the cannula tip and to minimise spread into the injection tract. The cannula was then slowly removed and the scalp was closed with sutures. The rats were kept warm until recovery from the anaesthesia.

2.2.2.5. Drug Treatment

The rats were treated as described in table 2.1. The rats in the control group, which were sham-lesioned received an i.p. injection of saline (vehicle) twice a day for 5 days. The rats in the QA only group received this same treatment, whilst those in the QA + AC group received an i.p. injection of 5 mg/kg AC (dissolved in saline) twice a day for 5 days.

Table 2.1. Treatment regime for each group of rats

Treatment Group	Intrahippocampal Injection	Intraperitoneal Injection (Twice a day for 5 days)
Control	PBS	Saline
QA alone	120 nmoles QA	Saline
QA + AC	120 nmoles QA	AC 5mg/kg

2.2.2.6. Morris Water Maze Test of Spatial Reference Memory

During these test runs, the rats were placed into the tank at the same starting point as during training, with their heads facing the wall of the tank. The time taken (latency to escape) for each rat to find the platform from the starting point was measured, using a stopwatch, by a person unaware of the experimental conditions. The test runs were performed once a day for 7 days.

2.2.2.7. Brain and Hippocampi Removal

On the 8^{th} day after drug treatment, the rats were killed by cervical dislocation and decapitated. The brain was exposed by making an incision on either side of the parietal structure, from the foramen magnum to near the orbit. The calvarium was removed, exposing the brain, which was easily removed. The hippocampus was then dissected out from each hemisphere of the whole brain, rapidly frozen in liquid nitrogen and stored at -70 ˚C for use in chapter 5.

2.2.2.8. Statistical Analysis

All results were analysed using repeated measures analysis of variance (ANOVA) with group as a factor and latency to escape measured over 7 days. Student–Newman–Keuls multiple range test was used for post hoc analysis. The level of significance was accepted at $p < 0.05$ (Zar, 1974).

2.2.3. Results

The statistical analysis shows a significant difference in latency to escape between the groups ($F = 20.39$, $p < 0.001$). Post hoc tests (Student–Newman–Keuls' Multiple Range tests) show that the latency to escape (over 7 days) of rats in the QA only group is significantly longer in comparison to the control group ($p < 0.001$). The QA + AC group has significantly faster escape times in comparison to the QA alone group ($p < 0.001$). There is no difference between the latencies to escape of the control group in comparison to the QA + AC group ($p > 0.05$).

As shown in figure 2.1., it is evident that the latency to escape of the QA group decreases sharply between day 5 and day 7 on which the mean escape time was equal to that of the QA + AC.

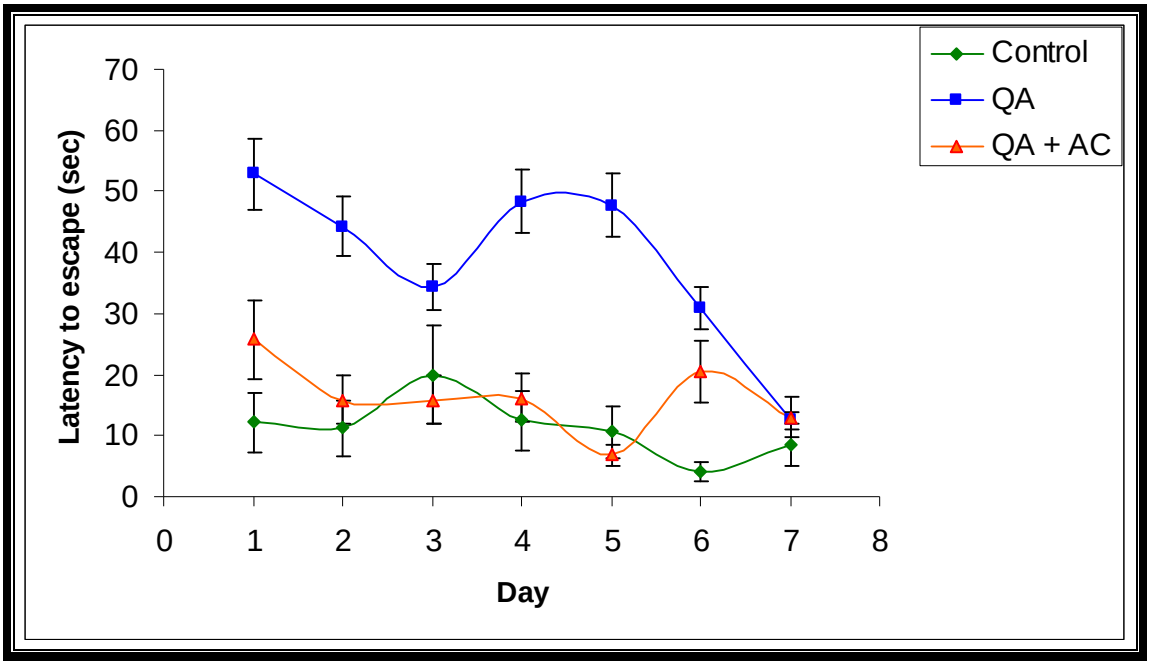


Figure 2.1. Performance of the rats in the Morris water maze task. Each point represents the mean latency ± SD to escape of 10 rats.

2.2.4. Discussion

A previous study has shown that intrahippocampal injections of QA have the ability to impair memory (He *et al.,* 2002). This is likely through the formation of lesions, which prevent the hippocampi from engaging in normal functioning. Lesioning by QA infusion occurred after memory encoding and consolidation (during training), but before memory retrieval (test runs), thus it is likely to be the last phase (memory retrieval) in the memory process which QA impairs. QA is an agonist at NMDA receptors (Stone and Perkins, 1981; Pérez-Navarro *et al.,* 2005), which are present in the glutamatergic synapses of the hippocampus and which are involved in LTP, the neuroplastic process which makes autoassociation possible (section 2.2.1.). It is this property of QA which may be involved in altering the ability of the rats to retrieve spatial reference memory.

A signal-to-noise hypothesis has been proposed to be a consequence of enhanced activity of the glutamatergic system (Danysz and Parsons, 2003). Since QA is not metabolised in the synaptic cleft, it remains there for prolonged periods (Choi, 1988) and may thus cause continuous overstimulation of the NMDA receptors on the postsynaptic neuron. These receptors are usually only stimulated by glutamate released from the presynaptic neuron when it (the presynaptic neuron) receives an impulse (Danysz and Parsons, 2003). This normal physiological stimulation of the NMDA receptor evokes an influx of calcium ions, known as the calcium signal, required to induce LTP (section 1.1.3.1.). In the presence of QA, temporally uncoordinated, continuous pathological stimulation of NMDA receptors may occur, producing enhanced noise, decreasing the probability of detecting the relevant calcium signal (Danysz and Parsons, 2003) produced in response to firing of the presynaptic neuron.

During the test trials (after the lesioning), the firing of hippocampal neurons occurs when partial cues are detected and the retrieval process of autoassociation is put in motion. All groups of rats have potentiated glutamatergic synapses between CA1 and CA3 subfields of the hippocampus, where the spatial information is stored and from which it needs to be retrieved. These potentiated synapses are formed by the induction and subsequent expression of LTP (section 1.1.3.1. and section 1.1.3.2.) in the encoding and

consolidation process during the 7 days of training. However, in these synapses in the hippocampi of rats lesioned with QA, the calcium signal generated as a result of an impulse is not detected. Therefore it is likely that the CA1 cells are unable to operate as recoders for the information recalled from the CA3 cells. This in turn suggests that the recalled information would not be represented more efficiently to enable recall, via backprojection synapses to the neocortical areas (section 2.2.1.).

Overstimulation of ionotropic glutamate receptor sub-types, such as NMDA and AMPA may also lead to neurodegeneration via an excitotoxic mechanism described in section 1.2.2.4. This implies that concentrations of QA which are high enough may cause degeneration of the postsynaptic neurons of the potentiated glutamatergic synapses. This poses a second mechanism by which QA impairs memory. If these neurons are destroyed then the information stored within them cannot be retrieved.

It has been reported that intravenous AC treatment is able to improve the memory deficits in adult patients with HSE (Ishida *et al.*, 2002; MacLean and Douen, 2002). This finding is coincident with a decrease in serum HSV DNA to undetectable levels (MacLean and Douen, 2002), suggesting that AC brings about this effect through its antiviral mechanism. However, in the present study AC appears to employ a mechanism independent of its antiviral activity, because it improves spatial reference memory deficits induced by QA, a product of the viral-induced immune response (Reinhard, 1997). Thus AC either has the ability to reduce the noise-to-signal ratio or to protect the neurons against excitotoxicity-induced neurodegeneration, the two proposed mechanisms by which QA causes spatial memory deficits. AC appears to be very effective as it improves the memory to the extent that there is no significant difference in comparison to the control group ($p>0.05$). The ability of 120 nmoles of QA injected intrahippocampally, in the absence and presence of subsequent AC treatment, to cause neurodegeneration will be investigated in chapter 3. This will be the first step towards attempting to elucidate the neurobiological mechanisms which underlie this hitherto unreported effect of AC.

The improvement in memory of the QA alone group between days 5 and 7 shown in figure 2.1. may indicate that parts of the hippocampi were still able to function normally and that 120 nmoles QA did not lesion the entire hippocampus. This suggests that during the first few days of the test trials, the rats may undergo relearning, by encoding and consolidating the spatial information again, further strengthening the glutamatergic synapses through LTP in the unlesioned parts of the hippocampus. The autoassociative retrieval process and recall is therefore more easily achieved in the last few days (day 5–day 7) of the test trials from these parts of the hippocampus than previously because the synapses are stronger. This increase in synaptic strength further implies that a more distorted (vague) partial cue may be detected, causing normal firing of the presynaptic neuron, which releases normal physiological levels of glutamate. The glutamate stimulates the NMDA receptors subsequently producing a signal great enough to activate the retrieval process. This concept of relearning shows the adaptability of neuroplastic processes in the brain.

CHAPTER 3

NEURODEGENERATION

3.1. INTRODUCTION

Necrosis and apoptosis cannot be taken as two completely distinct modes of cell death. Since these can be encountered in the same tissue and initiated by the same type of stimuli, it would appear possible that, at least some of the early events are common to both types of cell death, and that downstream controllers may decide the direction towards one outcome or another (Toescu, 1998). Necrosis, associated with extreme energy failure in mitochondria, may simply reflect the failure of neurons to carry out the "default" apoptotic death program used to dispose efficiently of aged or otherwise unwanted cells (Ankarcrona *et al.*, 1995). The preservation of mitochondrial function may therefore be a decisive factor in determining the degree and progression of neuronal injury caused by excitotoxins (Ankarcrona *et al.*, 1995) such as glutamate or QA. Both forms of cell death occur after exposure of brain regions which contain high concentrations of NMDA receptors for glutamate. These regions include the cerebral cortex and CA1 subfield of the hippocampus (Monaghan and Cotman, 1986). First, a rapid necrotic cell killing occurs during and immediately after glutamate exposure and is associated with loss of mitochondrial function. Second, a delayed apoptotic type of neuronal death occurs in the surviving neurons that recover their mitochondrial function and cellular energy levels (Ankarcrona *et al.*, 1995).

Histology is the anatomical study of the microscopic structure of animal and plant tissues. Histologic sections may be used to define the normal or abnormal appearance of brain tissue (Schmitz and Hof, 2005). Such descriptions, although subjective, are very useful to describe features of sections when there are distinct differences between pathologic and physiologic conditions (Schmitz and Hof, 2005).

Brain tissue consists of neurons, which are the impulse conducting cells of the nervous system and non-conducting, supporting glial cells, including astrocytes, oligodendrocytes and microglia, which nourish and protect the neurons. Glial cells are far more numerous than neurons and, unlike neurons, are capable of mitosis.
(http://en.wikipedia.org/wiki/Neuron)

A typical neuron is shown in figure 3.1. It consists of a cell body, containing the nucleus and the surrounding cytoplasm, several short radiating processes (dendrites) and one long process (the axon), which terminates in twig-like branches.
(http://en.wikipedia.org/wiki/Neuron)

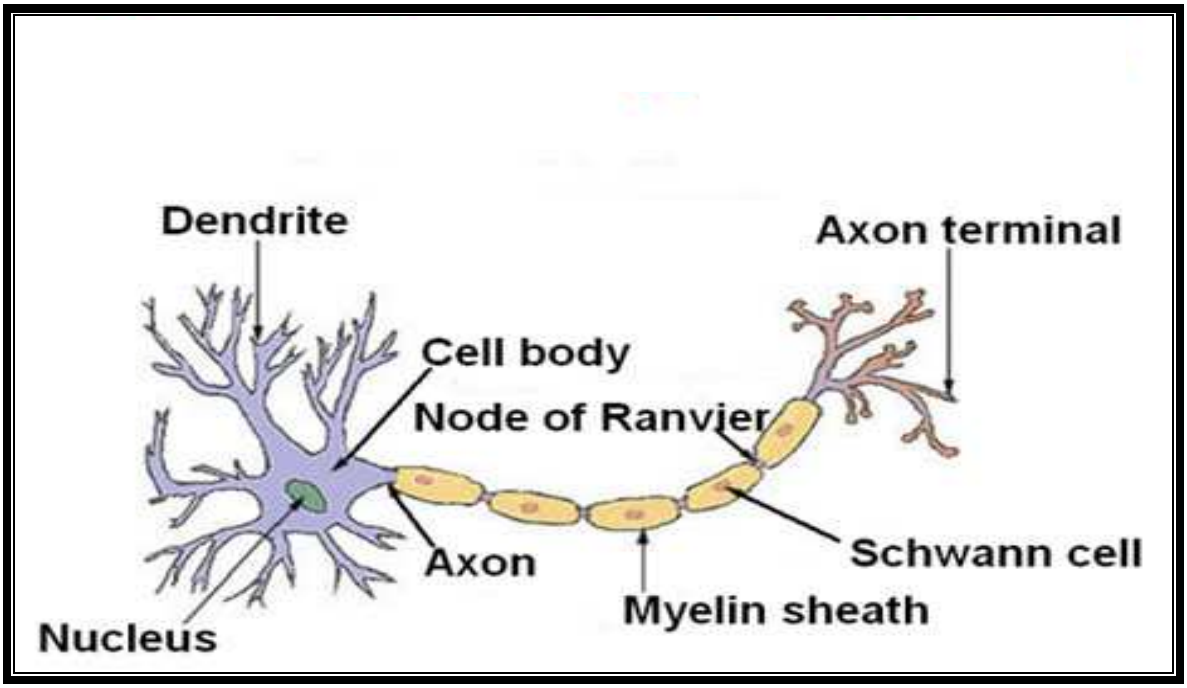


Figure 3.1. Structure of a typical neuron. (http://en.wikipedia.org/wiki/Neuron).

The dendrites serve to collect incoming electrochemical signals either from the periphery or other neurons, to send them to the cell body. The axon is a long process that conducts an electrical impulse away from the neuronal cell body. At the other end of the axon, the axon terminals transmit the electrochemical signal across the synapse. Myelin,

manufactured by Schwann cells coats and insulates the axon (except for periodic breaks called nodes of Ranvier), increasing transmission speed along the axon. (www.med.mun.ca/anatomyts/nerve/neuron.htm).

Neurons are metabolically very active and are thus involved in large amounts of protein synthesis. It is not surprising therefore that the most prominent organelle in the cell body is the rough endoplasmic reticulum (RER). RER is formed into large Nissl bodies and is responsible for protein synthesis (www.med.mun.ca/anatomyts/nerve/neuron.htm). The cell bodies of neurons also contain extensive neurofilaments and microtubules. These organelles are involved in the transport of materials synthesised in the cell body out into the processes. Neurons also generally have clearly observable nuclei. The Nissl bodies, neurofilaments and microtubules and clearly observable nuclei are the unique features of neurons in comparison to other cells. Other organelles present are common to most cells. (http://www.med.mun.ca/anatomyts/nerve/neuron.htm).

Astrocytes are the largest and most abundant of all glial cells. These appear star shaped due to their large number of cytoplasmic processes that extend outwards from the cell body. The cytoplasm contains intermediate filaments that are composed of glial fibrillary acidic protein which serves to identify these cells (Björkland *et al.*, 1984)

Another salient feature of neurons in contrast to other cells of the body is that the nuclei of these do not possess centrioles. This is consistent with the amitotic nature of neurons in a developed brain and implies that degenerated neurons cannot be restored or replaced (http://training.seer.cancer.gov/module_anatomy/unit5_2_nerve_tissue.html).This is the primary challenge which neuroscientists are faced with in attempting to find treatments for neurodegenerative disorders.

AD is a well established chronic progressive neurodegenerative disorder, in which brain regions involved in learning and memory, such as the hippocampus, are severely affected by QA produced in response to microglial activation, ultimately showing the highest rate of neurodegeneration (section 1.1.4.2.). The neurodegeneration in HSE is not as well

known and usually acute in nature. Increases in QA have been found in mice with experimental HSE (section 1.2.4.1.) and adult human HSE is pathologically characterised by necrotising lesions commonly found in the CNS structures implicated in learning and memory, including the amygdala, hippocampus, and entorhinal cortex (section 1.1.4.3.). QA production in the neurodegenerative hypothesis of depression is initiated through IDO activation by pro-inflammatory cytokines. The hypothesis has been supported by findings that hippocampal atrophy occurs in major depression (section 1.1.4.4.).

Degeneration of the supporting glial cells may indirectly cause neurodegeneration as neurons are no longer supplied with the sustenance required to survive. This however is not likely to be a long term or major mechanism of neurodegeneration as the glial cells have the ability to undergo mitosis and therefore degenerated glial cells are rapidly replaced.

3.2. THE EFFECT OF QA ALONE AND IN COMBINATION WITH AC ON RAT HIPPOCAMPAL NEUROMORPHOLOGY AND CYTOARCHITECTURE

3.2.1. Introduction

In section 1.1.2., the structure of the hippocampus was described. The important features of this description with respect to hippocampal neuromorphology and cytoarchitecture are aptly depicted in figure 3.2.

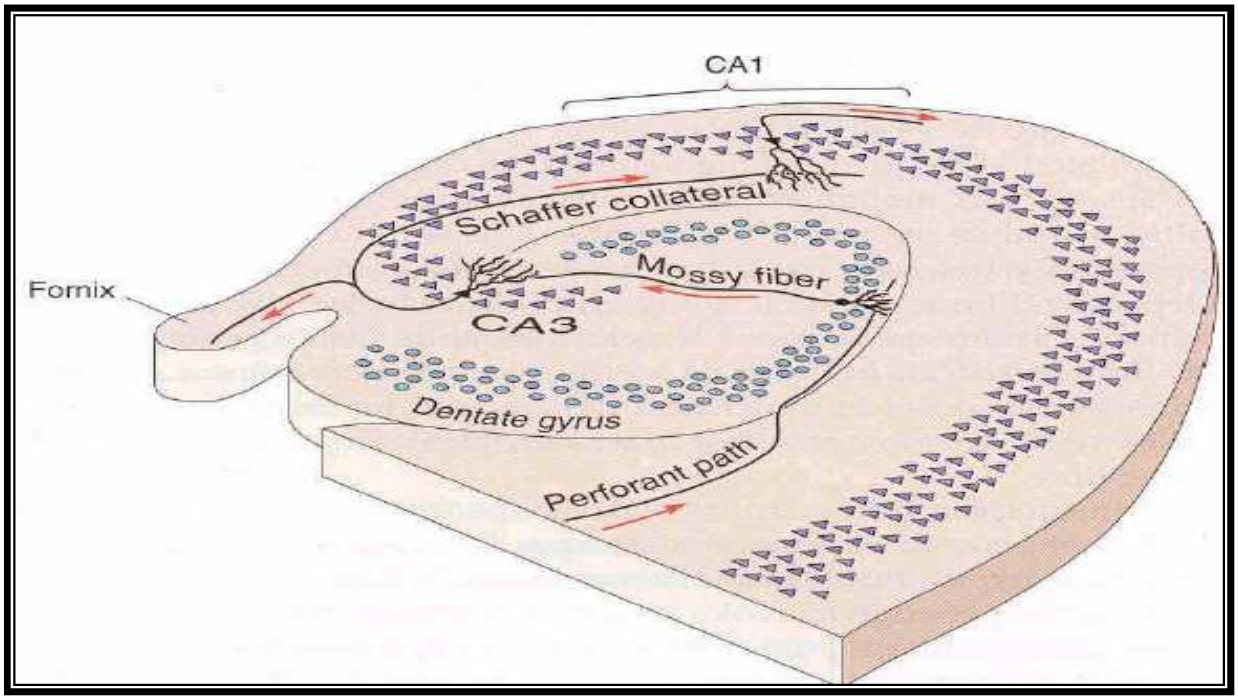


Figure 3.2. Schematic diagram illustrating some aspects of the neuromorphology and cytoarchitecture of the rat hippocampus (Bear *et al.*, 2001).

Figure 3.2 shows the dentate gyrus and Ammon's horn (only CA1 and CA3 are labelled) as two interlocking "C" shaped structures. It further indicates that the cell bodies of the neurons making up the primary cell layer of Ammon's horn are pyramidally shaped, whilst those of the dentate gyrus are round and granular. Moreover, these primary strata

of Ammon's horn and the dentate gyrus both take on a characteristic band–like appearance.

QA has been shown to cause dose–dependent selective pyramidal cell death in the hippocampal CA1, CA3 and CA4 regions (Behan *et al*., 1999) and results in the infiltration of microglial cells into the pyramidal cell layers and surrounding tissue (Stone *et al*., 2000). Thus, the present study aims to determine whether 120 nmoles QA has the ability to elicit such an effect in the CA1 and CA3 regions of the hippocampus, using histological analyses; and moreover, to assess the ability of AC to protect against pyramidal cell death, if any. This investigation will provide useful information on the degree to which the extent of neurodegeneration dictates the efficiency of spatial reference memory and therefore determine whether a correlation exists between the two processes.

Histology as a technique relies on subjective visual assessment. Therefore, to improve the visibility of histologic sections, dyes and stains which react with tissue components are employed before examination under a microscope. The Nissl stain, introduced by the German neurologist Franz Nissl in the late nineteenth century, is commonly used to study neurons under the light microscope (Bear *et al*., 2001). This stain is extremely useful since it distinguishes neurons and glia from one another (Bear *et al*., 2001). This is because of the unique high Nissl body content of neurons which was described in section 3.1. The elevated RNA content makes Nissl bodies highly basophilic. The bodies are thus sharply stained with basic aniline dyes, such as cresyl violet (figure 3.3.).

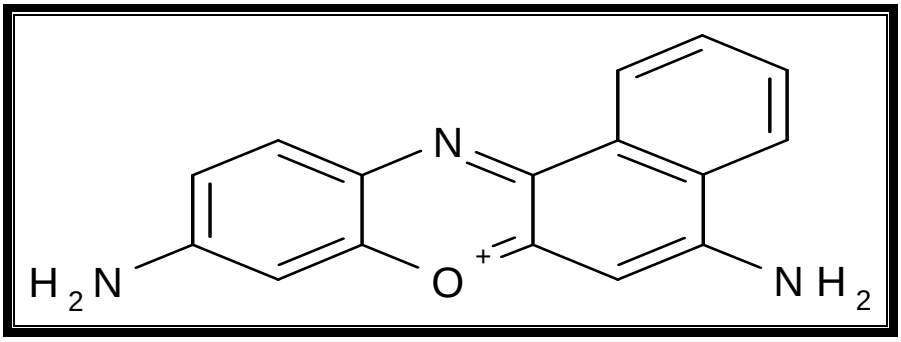


Figure 3.3. Structure of cresyl violet, showing that it is an aniline (http://www.coleparmer.com/catalog/large_image.asp?img=AGROS22963.jpg)

3.2.2. Materials and Methods

3.2.2.1. Animals

Adult male Wistar rats were purchased from the NHLS, Johannesburg, South Africa and cared for as described in 2.2.2.1.

3.2.2.2. Chemicals and Reagents

AC sodium (Zovirax®, powder for injection) was obtained from Glaxo Wellcome, Midrand, South Africa. QA and Cresyl violet acetate were purchased from Sigma Chemical Co, St. Louis, USA. Sodium phenobarbitone was purchased from Merck, Germany and was at a working concentration of 60 mg/ml. Paraffin wax was purchased from Lasec, South Africa and Distrene Plasticiser in xylene (DPX) was a donation from the Rhodes University Botany Department. Haupt's Adhesive was prepared as follows: 1 g gelatine, 100 ml water, 2 g phenol and 15 ml glycerol. Ethanol and xylene were purchased from Saarchem, Krugersdorp, South Africa.

3.2.2.3. Surgical Procedures

These procedures were conducted as described in section 2.2.2.4.

3.2.2.4. Drug Treatment

This was performed as described in section 2.2.2.5.

3.2.2.5. Brain Removal

The rats were killed and the brains removed in the manner described in section 2.2.2.7.

3.2.2.6. Histological Techniques

3.2.2.6.1. Fixation of the Brain

The brains were immediately stored in a fixative medium, which consisted of formaldehyde (30 %), glacial acetic acid and ethanol (2: 1: 7 v/v), for 48 hours. This process is necessary to preserve, as much as possible, the morphological and molecular structure of the tissue. Coronal sections were then cut from the forebrain.

3.2.2.6.2. Tissue Processing

Moisture was extracted from the tissue fragments by bathing these successively in graded series of ethanol for one hour each. 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 ˚C twice for one hour each, which facilitated the removal of xylene and the thorough penetration of the tissue. This last step provides the hardness and support that the tissue requires for sectioning. An outline of the tissue processing is summarised in table 3.1.

Table 3.1. Outline of the steps in tissue processing.

Step	Processing Agent	Time
1	70 % ethanol	1 hour
2	90 % ethanol	1 hour
3	Absolute ethanol I	1 hour
4	Absolute ethanol II	1 hour
5	Xylene I	1 hour
6	Xylene II	1 hour
7	Molten wax (57 ˚C)	1 hour

3.2.2.6.2. Blocking Out

Blocking out was performed to form a support that would facilitate sectioning using a rotary microtome. This was done by fixing the brain material into a block using a mould. A plastic ice tray coated with lubricating gel was used as the mould. Molten wax (10 ml) was added to the mould, the surface of which was allowed to harden. The brains were then removed from the final molten wax stage (step 7 in table 3.1) 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 layer 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.

3.2.2.6.3. Sectioning

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 2 mm of wax around the tissue. The entire wax block was attached to a small wooden block with a small amount of wax. Sectioning was done using a rotary microtome, set to cut sections with a thickness of 10 µm. As the sections were cut, these stuck together, forming long ribbons. When the part of the brain containing the

hippocampus was reached, the long ribbons were removed and placed in a water bath (40 ˚C) using warm forceps.

3.2.2.6.4. Transferring Sections to Slides

Each glass microscope slide was brushed with a thin layer of Haupt's adhesive, using a thin paint brush, before being dipped into the water bath to allow 4–5 sections (1 long ribbon) to be mounted. The slides were left overnight in an oven set at 40 ˚C and saturated with formalin, to enable the section to adhere to the slide.

3.2.2.6.5. Staining

The sections were Nissl stained using cresyl violet. This stain stains Nissl substances intense purple, the nuclei purple, and leaves the background clear (Bauer *et al.*, 1974). 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, at 30 ˚C. The procedure is outlined in table 3.2.

Table 3.2. Outline of the procedure for dewaxing and rehydrating brain sections.

Step	Processing Agent	Time
1	Xylene I	5 minutes
2	Xylene II	5 minutes
3	Xylene: Absolute ethanol (1:1)	5 minutes
4	Absolute ethanol I	5 minutes
5	Absolute ethanol II	Overnight at 30 ˚C

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 slides were then dehydrated again by placing them in absolute ethanol twice and xylene twice for five minutes each, as shown in table 3.3.

Table 3.3. Procedure for dehydrating brain sections after staining.

Step	Processing Agent	Time
1	Absolute Ethanol I	5 minutes
2	Absolute Ethanol II	5 minutes
3	Xylene I	5 minutes
4	Xylene II	5 minutes

3.2.2.6.5. Mounting the 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 such as scratching, and to provide better optical quality for viewing under the microscope. The slides were removed from xylene (step 4 in table 3.3.) and enough DPX was added to cover the stained tissue. A coverslip was placed over the stained tissue and pressed to remove air bubbles. The slides were allowed to dry on a flat surface for 48 hours.

3.2.2.6.6. Photomicroscopy

Slides were viewed using a bright light microscope. The CA1 and CA3 regions of the hippocampus were located and photographed with a digital Olympus camera.

3.2.3. Results

Sections of the CA1 and CA3 regions of the control treated rats (figure 3.4 (a) and (b) respectively) show optimally sized, pyramidal shaped neurons which are grouped closely together to form the characteristic band-like appearance (section 3.2.1). The cells also

have clearly observable cell nuclei and continuous cell membranes. Thus these neurons in the CA1 and CA3 region appear to be undamaged.

The hippocampal neuronal cells of both regions of the QA treated animals (figure 3.4 (c) and (d), respectively) have lost the pyramidal shape and are instead, round and swollen. These cells have also lost the band-like arrangement, appearing scattered, and furthermore, have little integrity of the cell membranes. There also appear to be fewer neurons indicating that lysis of some of the neurons has occurred.

The majority of the hippocampal neuronal cells in the QA + AC treated rats (figure 3.4 (e) and (f)) appear to have retained the pyramidal appearance, with better orientation, appearing closer to each other as though in an attempt to reconstruct the typical band-like appearance. These cells also show greater integrity of cell membranes in comparison to those of the QA only treated rats. There are also fewer neurons which have undergone lysis.

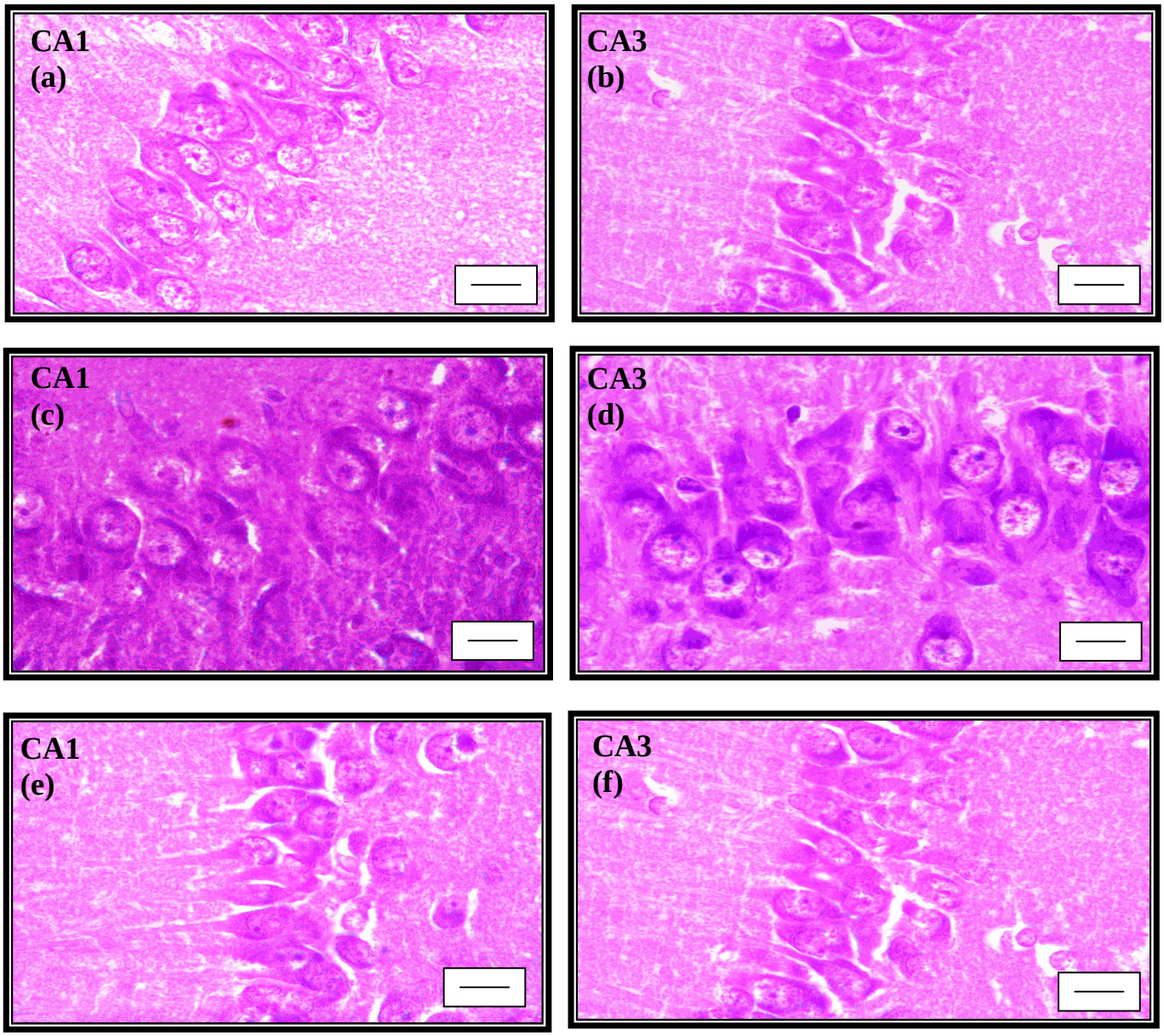


Figure 3.4. Effect of QA alone and in combination with AC on rat hippocampal neuromorphology and cytoarchitecture. Figure 3.4 (a and b) show the CA1 and CA3 regions of the control treated rats, respectively. The QA alone treated CA1 and CA3 regions are evident as 3.4 (c and d), respectively while 3.4 (e and f) show the photomicrographs of the CA1 and CA3 regions of the QA + AC treated rats. Bar = 10 μm.

3.2.4. Discussion

The cell damage and neurodegeneration occurring in the presence of an intrahippocampal injection of 120 nmoles QA without AC treatment, portrayed in figure 3.4 (c and d), fits the profile and characteristics of the various stages of necrotic cell death, described in section 1.2.1.2. The hypothesis that neurodegeneration is responsible for the spatial reference memory deficits induced by 120 nmoles QA (section 2.2.4) is therefore justified. Similarly, AC appears to protect against the QA-induced cell damage and neurodegeneration, which provides an explanation for the improvement in spatial reference memory (section 2.2.4). This is the first study to report on the neuroprotective effect of AC or any other guanosine analogue using a histological analysis.

QA-induced necrosis has been associated with each of the three main mechanisms of neurodegeneration, namely, excitotoxicity (Stone and Perkins, 1981), mitochondrial dysfunction (Bordelon *et al.,* 1997) and oxidative stress (Vega-Naredo *et al.,* 2005), primarily through overstimulation of the NMDA receptor.

Mitochondrial dysfunction may cause ATP depletion and therefore inevitably direct the neurons toward necrotic cell death rather than the more regulated apoptotic death, which is ATP-dependent.

Excitotoxicity is a mechanism of neuronal cell death mediated by overstimulation of ionotropic glutamate receptor sub-types, such as NMDA and AMPA (Griffiths *et al.,* 2000). As described in section 1.1.3.1, in order for NMDA receptors to be activated, non-NMDA glutamate receptors such as AMPA must be activated. This activation results in an influx of sodium ions into the cell causing a depolarisation of the membrane. The depolarisation results in the removal of the magnesium block on the NMDA receptor, allowing the opening of the calcium ion channel once QA has bound. Once opened, calcium ions move across the postsynaptic membrane and into the neuron. It has been suggested that two separate processes occur in the excitotoxic mechanism (Cotman *et al*., 1989; Farooqui and Horrocks, 1991), namely acute toxicity and delayed toxicity.

The influx of sodium ions is accompanied by influx of chloride ions as well, creating an osmotic imbalance between the neuron and the extracellular environment (Rothman and Olney, 1987). Water flows into the cell, causing acute cell swelling, damage and possible lysis (Olney, 1969; Rothman, 1985; Choi, 1987, Tilson and Mundy, 1995), which is clearly observed in figure 3.4 (c and d).

The massive calcium ion influx triggers the activation of intracellular calcium-dependent enzymes (Meldrum and Garthwaite, 1990). This causes a delayed toxicity in the round swollen cells that survive the osmotic crisis. The calcium ions activate various hydrolytic enzymes including PLA_2 which cleave PUFA from lipid membranes. This may explain the loss of integrity of the cell membranes of the swollen round cells left after the acute QA insult (figure 3.4 c and d). The disintegration of the cell membranes may, in turn, result in the release of cytoplasmic content into the extracellular medium (Toescu, 1998). This triggers an important inflammatory response, which includes local cellular infiltration, vascular damage and oedema (Ankarcrona *et al.,* 1995), which may account for the scattered arrangement of the cells and loss of the band-like appearance.

QA in the hippocampus is known to elicit microglial cell infiltration (section 3.2.1) in response to the CNS injury. If neuronal death is induced by the injury, activated microglia are further transformed into phagocytic, and thus cytotoxic, cells known as microglia-derived brain macrophages (Mori and Kimura, 2001). These microglia kill injured (swollen) cells by releasing neurotoxic molecules, including free oxygen intermediates (section 1.1.4.1.) resulting in oxidative stress and therefore a rise in oxidative damage to DNA, proteins and lipids. The disintegration of the cell membranes may also be evidence of LP in conjunction with the action of PLA_2. This will be investigated further in chapter 6.

The neuroprotection offered by AC against the gross adverse changes in hippocampal neuromorphology and cytoarchitecture caused by QA (figure 3.4 e and f), may be related to an ability of this antiviral to interfere with one or more of the three mechanisms of neurodegeneration and the oxidative damage associated therewith. The ensuing chapters

will attempt to elucidate the neurobiological mechanisms which underlie the effect of AC on neurodegeneration. This includes direct effects on the mechanisms of neurodegeneration and the oxidative damage which correlates to it, as well as indirect effects. The latter includes the consequences of AC treatment on QA biosynthesis and the biosynthesis and metabolism of 5-HT, to assess whether AC could enhance an endogenous environment which challenges the neuroprotective-neurodegenerative balance such that the neuroprotective status of the cells is favoured.

CHAPTER 4

EXCITOTOXICITY

4.1. INTRODUCTION

An increase in the concentration of a NMDA receptor agonist in the synaptic cleft of glutamatergic synapses may lead to excitotoxicity as a mechanism of neurodegeneration (section 3.2.4.). Figure 4.1., shows that this is a particularly important mechanism because the sequence of events it evokes effectively contributes to the other mechanisms of neurodegeneration as well, namely, oxidative stress and mitochondrial dysfunction.

The arrival of the action potential to the presynaptic terminal (1) depolarises the plasma membrane and activates voltage-dependent calcium channels (2). Calcium influx induces the fusion of presynaptic vesicles with the presynaptic neuronal plasma membrane (synaptic exocytosis), (3) promoting glutamate release and increasing its concentration in the synaptic cleft (4). Glutamate will activate AMPA receptors on postsynaptic neurons (5), inducing sodium influx and depolarisation of the plasma membrane. In this condition, the magnesium ion that normally blocks the NMDA receptors is extruded (6), and the receptor activated by the binding of glutamate and its co-agonist glycine (7). The activation of the NMDA receptor induces sodium and calcium influx (8). Voltage-dependent calcium channels (CCDV) also become activated (9) and in some circumstances calcium can also enter the cell through AMPA receptors (10). Calcium influx will induce cytoplasmic calcium overload and activation of diverse enzymes such as phospholipases (11), proteases (12) and nucleases, which drive the breakdown of phospholipids, proteins and nucleic acids (13). In addition, cytoplasmic calcium overload will lead to free radical production (14), thus oxidative stress, and impairment of mitochondrial function, which leads to energy failure (15) (Camacho and Massieu, 2006). As a result, neuronal death is likely to take place.

The detailed description of excitotoxicity above clearly illustrates that an understanding of the complexities of this process is crucial to finding ways to nullify it. The need for this is urgent since excitotoxicity is known to contribute towards the pathophysiology of several neurodegenerative disorders, such as AD (Olney, 1995; Hynd *et al.,* 2004) and depression (Hayley *et al.*, 2005; Yao and Reddy, 2005).

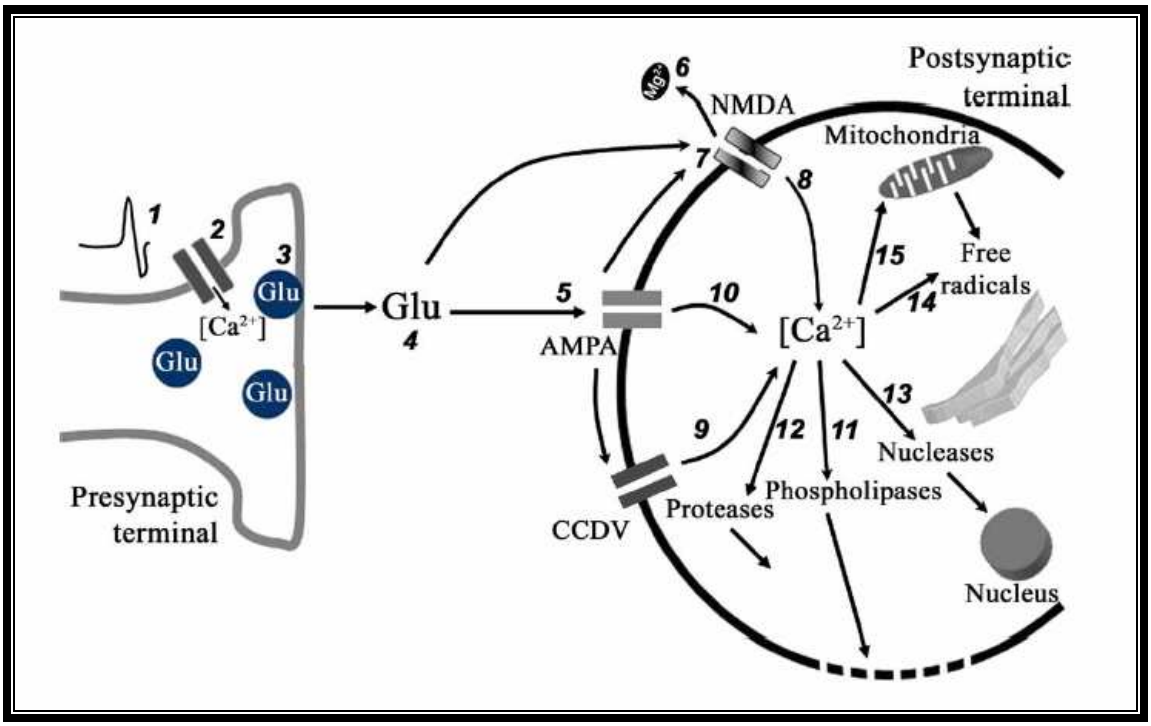


Figure 4.1. Excitotoxic neuronal death induced by glutamate (Camacho and Massieu, 2006).

The extracellular concentration of glutamate after its synaptic release is highly regulated by Na^+-dependent high affinity transporters located both in neurons and astrocytes (Storck *et al.,* 1992; Kanai and Hediger, 2004; Maragakis and Rothstein, 2004). Glutamate transporter 1 is an astroglial transporter highly distributed in the entire forebrain (Danbolt *et al.*, 1992; Pines *et al.*, 1992; Furuta *et al.*, 1997), whilst excitatory amino acid carrier 1 is a neuronal transporter abundantly expressed in the hippocampus, striatum and cerebellum (Olney *et al.*, 1989; Schmitt *et al.*, 2002).

As shown in figure 4.2., the activity of glutamate transporters depends on the sodium electrochemical gradient generated by the membrane Na^+/K^+ ATPases. One glutamate molecule is transported from the synaptic cleft across the neuronal cell membrane to the cytoplasm together with two sodium ions, while one potassium ion is extruded to the extracellular medium (Szatkowski *et al.*, 1990; Attwell *et al.*, 1993). Recovery of the sodium electrochemical gradient after the uptake of extracellular amino acids occurs at the expense of the ATP consumed by the membrane Na^+/K^+ ATPases (Bonvento *et al.*, 2002). Therefore, the maintenance of energy levels is essential for the control of glutamatergic transmission.

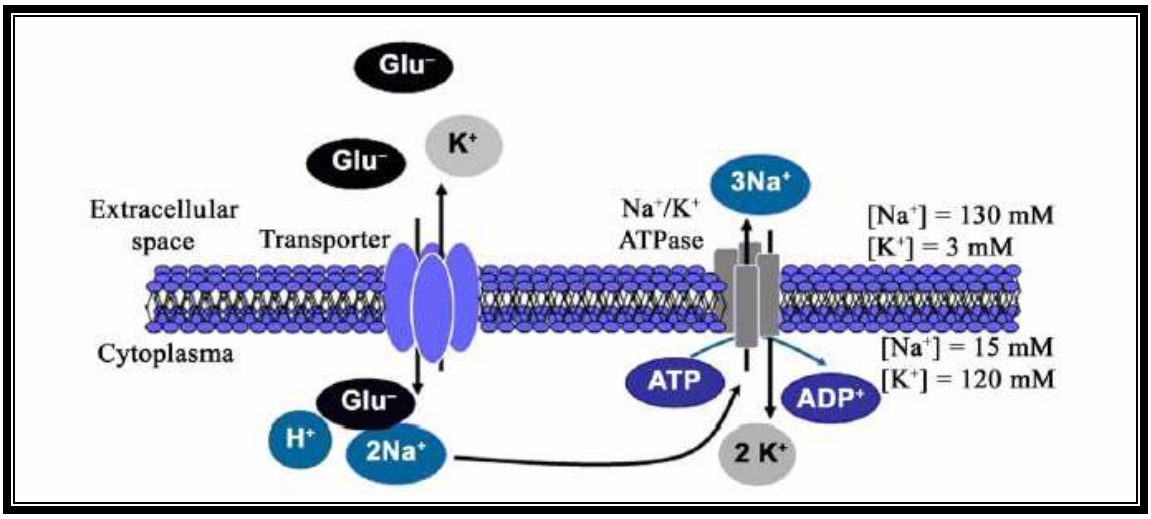


Figure 4.2. Mechanism of extracellular glutamate uptake (Camacho and Massieu, 2006).

Once glutamate is taken up into the presynaptic neuron, a low affinity sodium-independent carrier located at the membrane of synaptic vesicles is responsible for vesicular glutamate uptake (Fykse and Fonnum, 1996; Wolosker *et al.*, 1996). The coordinated actions of both systems (for neuronal and vesicular uptake) effectively maintain the glutamate concentration in the synaptic cleft at micromolar levels (Tavares *et al.*, 2005). From the mechanisms of the systems described above, it is evident that these rely on certain ionic and energy requirements, which if not met, may culminate not only in failure, but even possible reversal of the highly regulated processes through attempts by the neurons to maintain homeostasis.

4.2. THE EFFECT OF QA ALONE AND IN COMBINATION WITH AC ON GLUTAMATE RELEASE FROM RAT HIPPOCAMPAL SYNAPTOSOMES *IN VIVO*

4.2.1. Introduction

Although microglia, astrocytes and neurons all express IDO, only microglia produce detectable amounts of QA. Conversely, astrocytes and neurons have the ability to catabolise this neurotoxin (Guillemin *et al.*, 2005a). Astrocytes alone can thus be considered neuroprotective because these cells minimise QA production and maximise QA metabolism (Guillemin *et al.*, 2001). However, QA selectively induces apoptosis of human astrocytes (Guillemin *et al.*, 2005b) and hence effectively prolongs its own life and presence in the synaptic cleft. The availability of QA for perpetual NMDA stimulation is extended, thus aggravating excitotoxicity.

In addition to eliciting a direct excitotoxic effect through NMDA overactivation, it has more recently been shown that *in vivo*, QA has the ability to inhibit vesicular and astrocytic glutamate uptake and to enhance synaptosomal glutamate release (Tavares *et al.*, 2005). In this manner, QA indirectly enhances excitotoxicity through the elevation of glutamate levels in the synaptic cleft. Interestingly though, QA appears to have no effect on synaptosomal glutamate uptake (Tavares *et al.*, 2005). Judging from the biological systems in place to regulate glutamate levels in the synaptic cleft and the inter-relationship between these, it seems plausible to predict that QA may at least in part bring about these trends through exploitation of the above mentioned pathways and that any drug which counteracts this effect may have the ability to negate the QA-induced manipulation. Therefore, with the knowledge that QA has the ability to enhance synaptosomal glutamate release, and that this is reversed by guanosine (Tavares *et al.*, 2005), the effect of QA alone and in combination with AC, a guanosine analogue, on synaptosomal glutamate release was investigated.

4.2.2. Materials and Methods

4.2.2.1. Animals

Adult male Wistar rats were purchased from the NHLS (Johannesburg, South Africa) and were cared for as described in section 2.2.2.1.

4.2.2.2. Chemicals and Reagents

AC sodium (Zovirax®, powder for injection) was obtained from GlaxoWellcome, Midrand, South Africa. [^{3}H]-glutamate (specific activity of 42 Ci/mmole) was purchased from Amersham Biosciences, Buckinghamshire, United Kingdom. Sodium phenobarbitone was purchased from Merck, Germany and was at a working concentration of 60 mg/ml QA, Folin's reagent, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and bovine serum albumin (BSA) were purchased from Sigma Chemical Corporation, St. Louis, MO, USA. All other bench reagents were of the highest quality and were purchased from Saarchem (PTY) Ltd, Krugersdorp, South Africa.

4.2.2.3. Surgical Procedures

These procedures were conducted as described in section 2.2.2.4.

4.2.2.4. Drug Treatment

This was performed as described in section 2.2.2.5.

4.2.2.5. Brain and Hippocampi Removal

The rats were killed and the brains and hippocampi removed in the manner described in section 2.2.2.7. However, the rats were killed the day after the last dose of the drug treatment.

4.2.2.6. Preparation of Synaptosomes

Synaptosomes were prepared from each rat as previously described by Bonnet and Costentin (1989), with minor modifications. The hippocampi were homogenised in 20 volumes of 0.32 M ice-cold sucrose in a pre-chilled manual glass Teflon homogeniser. The homogenates were then centrifuged at 1000 x g for 10 minutes at 4 ˚C to form a nuclear pellet. Supernatants were stored at 4 ˚C for 10 minutes and the pellet was resuspended in 20 volumes of 0.32 M sucrose and centrifuged for 10 minutes at 1000 x g. The two supernatants were then pooled and centrifuged at 17 500 x g for 30 minutes at 4 ˚C, after which the supernatant was discarded and the pellets resuspended in Hepes/Cl buffered salt solution (HBSS), pH 7.4 (27 mM HEPES, 133 mM NaCl, 2.4 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.2 mM glucose, 1 mM $CaCl_2$), so as to obtain a protein concentration of 1.4 mg/ml.

4.2.2.7. Protein Assay

Protein estimation was done using the method previously described by Lowry *et al*., (1951). A calibration curve was generated using BSA which was prepared in Milli-Q water (1 ml) at concentration intervals of 60 µg/ml. The aliquots of BSA were incubated with copper reagent (6 ml) for 10 minutes at room temperature. Folin's reagent (0.3 ml) was added and samples were left to stand for 30 minutes in the dark, at room temperature. The absorbance was then read at 500 nm on a GBC 916 UV/VIS spectrophotometer and the curve generated by plotting concentration against absorbance (Appendix I).

4.2.2.8. L-[^{3}H] Glutamate Release from Synaptosomes

L-[^{3}H] Glutamate release was measured according to Migues *et al.,* (1999), with minor modifications. Synaptosomal preparations (1 ml) were incubated in the presence of L-[^{3}H] glutamate (50 µl) at a final concentration of 500 nM. Samples were then centrifuged at 13 000 x g for 1 minute at 4 ˚C. The supernatants were discarded and the pellets washed three times with HBSS by centrifugation at 13 000 x g for 1 min at 4 ˚C. In order to measure the release of L-[^{3}H] glutamate, the final pellet was resuspended in

HBSS and incubated for 60 s at 37 ˚C. Incubation was terminated by immediate centrifugation at 16 000 x g for 1 min at 4 ˚C. Radioactivity present in supernatants and pellets was determined separately in a Beckman, LS 2800 Scintillation Counter with a counting efficiency of 65 %. The results were initially expressed as L-[^{3}H] glutamate (fmoles/mg protein). L-[^{3}H] glutamate released (supernatant) was then calculated and expressed as a percentage of the total amount of L-[^{3}H] glutamate (supernatant + pellet) in the synaptosomal preparations.

4.2.2.9. Statistical Analysis

The differences in the means were analysed using a one-way analysis of variance (ANOVA) for statistical significance. If the F values were significant, the Student–Newman–Keuls test was used to compare the groups. The level of significance was accepted at $p < 0.05$ (Zar, 1974).

4.2.3. Results

Figure 4.3. illustrates that intrahippocampal QA significantly enhances the percentage of [^{3}H] glutamate released ($p < 0.05$). Administration of AC does not alter the effect induced by QA.

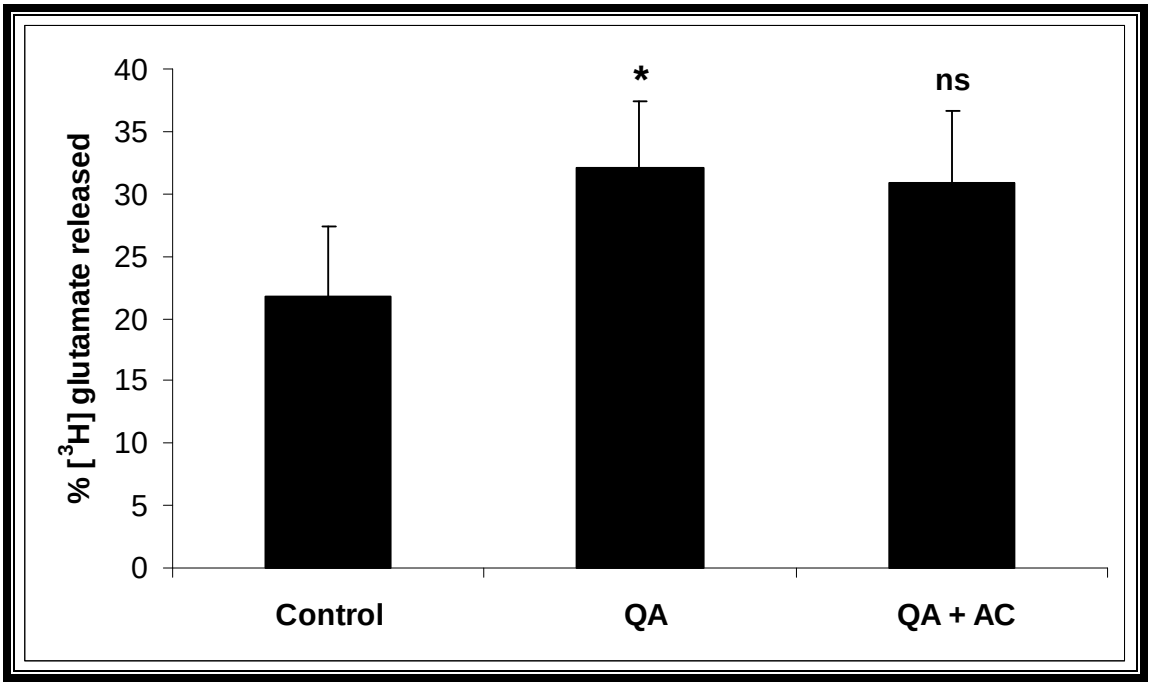


Figure 4.3. Effect of QA alone and in combination with AC on glutamate release from rat hippocampal synaptosomes *in vivo*. Each bar represents the mean ± SD; n = 5. * ($p < 0.05$) in comparison to control; ns ($p > 0.05$) in comparison to QA (ANOVA and Student–Newman–Keuls Multiple Range Test).

4.2.4. Discussion

Bordelon *et al.*, (1997) have shown that intrastriatal injections of QA *in vivo* produce progressive mitochondrial dysfunction, which may be a common and critical event in the cell death cascade. Mitochondrial dysfunction is likely to cause energy failure, during which ionic gradients are disrupted and the regulation of glutamate uptake is lost, leading to increased excitatory amino acid levels (Benveniste *et al.*, 1984; Massieu *et al.*, 2000). As shown in figure 4.2., recovery of the sodium electrochemical gradient occurs at the expense of ATP, thus a deficiency of this energy-bearing molecule will result in accumulation of intracellular sodium, which, in turn will drive the extrusion of osmotically active compounds, such as glutamate and aspartate for cell volume regulation (Phillis *et al.*, 1997; Estevez *et al.*, 1999). This process may also facilitate the reverse action of the sodium-dependent excitatory amino acid transporters, extruding glutamate

to the synaptic cleft (Longuemare *et al.,* 1999; Phillis *et al.,* 2000). This means that the mitochondrial dysfunction which occurs during excitotoxicity (figure 4.1.) may perpetuate itself and a cycle of deleterious effects predominates within the synapse. The finding that AC has no effect on the QA-induced increase in glutamate release could possibly indicate that AC is not able to salvage mitochondrial respiratory function after a QA-induced excitotoxic insult.

Tavares *et al.,* (2002) suggested that the increase in glutamate release induced by QA occurs through the activation of presynaptic NMDA receptors since MK-801 completely abolishes the effect. This hypothesis may explain further observations by these authors that this effect could be reversed by guanosine (Tavares *et al.,* 2005), as this nucleoside is known to inhibit the binding of glutamate or its analogues to the NMDA receptor (Monahan *et al.,* 1988; Souza and Ramirez, 1991). A study has demonstrated that activation of presynaptic NMDA autoreceptors may provide a positive rather than the usual negative feedback on presynaptic glutamate release in synaptosomal preparations (Breukel *et al.,* 1998). These authors speculate that stimulation of these receptors causes depolarisation of the presynaptic membrane by sodium entry, inducing the same kind of homeostatic imbalance as previously described, which may induce reversal of amino acid transporters thereby releasing amino acids from a cytoplasmic pool. It was anticipated that AC, a guanosine analogue would have a parallel effect. A possible reason for this discrepancy is that Tavares and coworkers (2005) administered guanosine i.p. prior to the infusion of QA, thus, guanosine was already present to prevent the binding of QA, whereas in the present study, QA was infused 24 hours before initiation of the AC treatment and may therefore have more time to bind to presynaptic NMDA autoreceptors to elicit the response (glutamate release) before sufficient AC is present to inhibit the QA effect to a sufficiently substantial extent so as to pose a challenge.

CHAPTER 5

OXIDATIVE STRESS

5.1. INTRODUCTION

Oxidative stress is a state within cells where the generation of free radicals exceeds the capacity of the endogenous antioxidant defence system (Poston and Raijmakers, 2004). The brain, in comparison to the other organs of the body, is especially susceptible to this phenomenon as a result of its high consumption of total body O_2, the primary source of free radicals, and its relatively low concentration of antioxidant enzymes (Coyle and Puttfarcken, 1993).

The major reactions for the normal, regulated production of oxygen and nitrogen free radicals in the body are depicted in figure 5.1. NO is formed from L-arginine by one of the three NOS isoforms, which all require oxygen, tetrahydrobiopterin, NADPH, calmodulin, FAD, flavin mononucleotide (FMN), and haeme for catalytic activity, whilst, in addition, nNOS found in neuronal tissue requires calcium (Wu and Morris, 1998).

$O_2^{\cdot-}$ is generated from O_2 by various metabolic pathways: 1) NADPH oxidation by NADPH oxidase; 2) oxidation of xanthine or hypoxanthine by xanthine oxidase; 3) oxidation of reducing equivalents (e.g., NADH, NADPH, and $FADH_2$) via the mitochondrial electron transport chain; 4) auto-oxidation of monoamines (e.g., DA, epinephrine, and NE), flavins, and haemoglobin in the presence of trace amounts of transition metals; 5) one-electron reduction of O_2 by cytochrome P-450 (Fridovich, 1999; Evans and Halliwell, 2001).

The short lived, potent oxidant $ONOO^-$, as described in section 1.2.2.2.3., is the biological reaction product of $O_2^{\cdot-}$ and NO. In addition, the H_2O_2 produced as a result of the auto-oxidation of monoamines or the dismutation of $O_2^{\cdot-}$ contributes to the formation

of ˙OH**,** in the presence of reducing agents such as Fe^{2+} (Fenton reaction) or ascorbic acid (vitamin C) (Fang *et al.*, 2002).

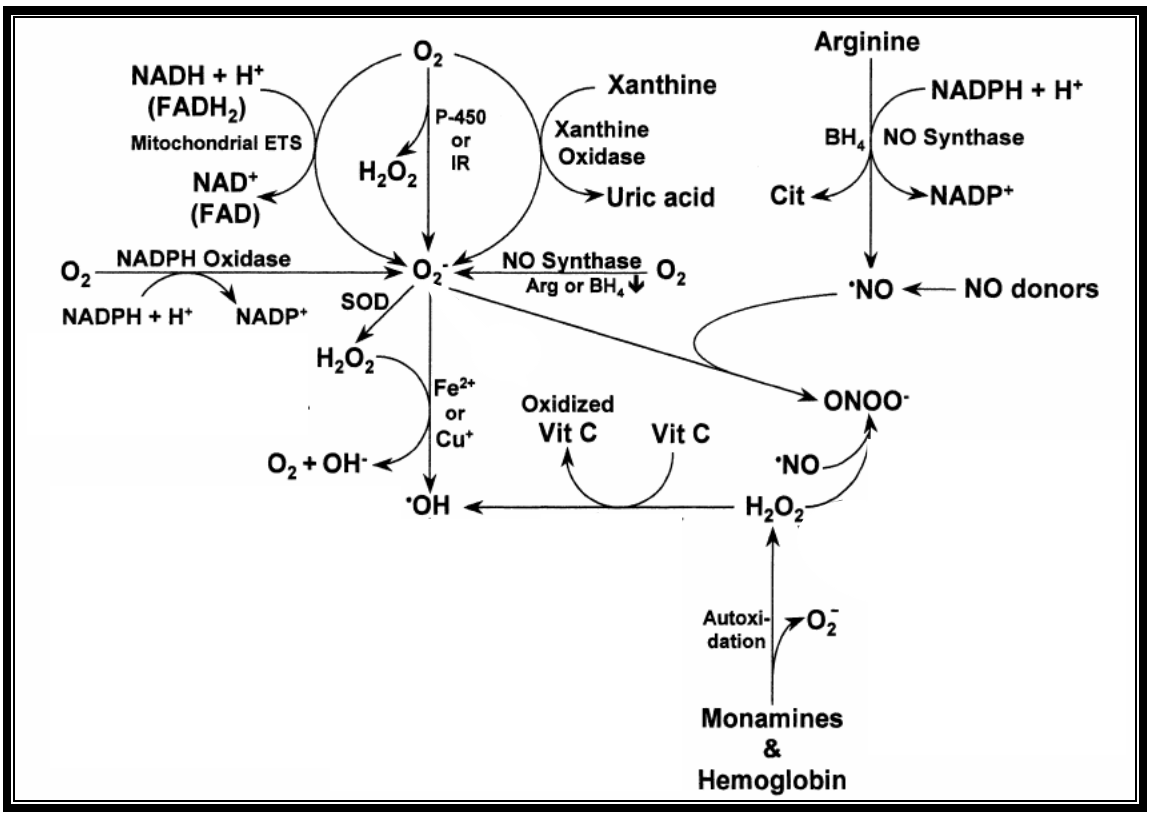


Figure 5.1. Production of oxygen and nitrogen free radicals and other reactive species in mammalian cells (modified from Fang *et al.*, 2002).

The antioxidant defense system, outlined in figure 5.2. consists of enzymes, namely, SOD, CAT, GSH-Px, and GSH-R and antioxidant molecules such as reduced GSH, α-tocopherol (vitamin E) and vitamin C (Fang *et al.*, 2002).

SOD enzymes catalyse the removal of $O_2^{\cdot-}$ in a dismutation reaction where one $O_2^{\cdot-}$ radical is oxidised and another is reduced to form H_2O_2 and O_2 (Fridovich, 1975). The H_2O_2 that is produced can be metabolised to H_2O and O_2 by CAT or GSH-Px. This latter enzyme uses GSH as a co-factor which is oxidised by H_2O_2 (Chance *et al.*, 1979). The oxidised glutathione (GSSG) is recycled to its reduced form by GR (Barber *et al.*, 2006).

Furthermore, GPx also detoxifies lipid hydroperoxides formed during lipid peroxidation to alcohols (Fang *et al.*, 2002). These hydroperoxides are initially produced by the transfer of the phenolic hydrogen of vitamin E to a $ROO^{\cdot}$ free radical, thereby breaking the radical chain reaction and preventing propagation of LP. A vitamin E radical is generated in this process, however it is not a highly reactive species because the unpaired electron is resonance stabilised (Fang, 2002). Vitamin C then reacts with the vitamin E radical to yield a vitamin C radical while regenerating vitamin E. The vitamin C radical is also not reactive and is converted back to vitamin C by GSH (Fang, 2002; Fang *et al.*, 2002).

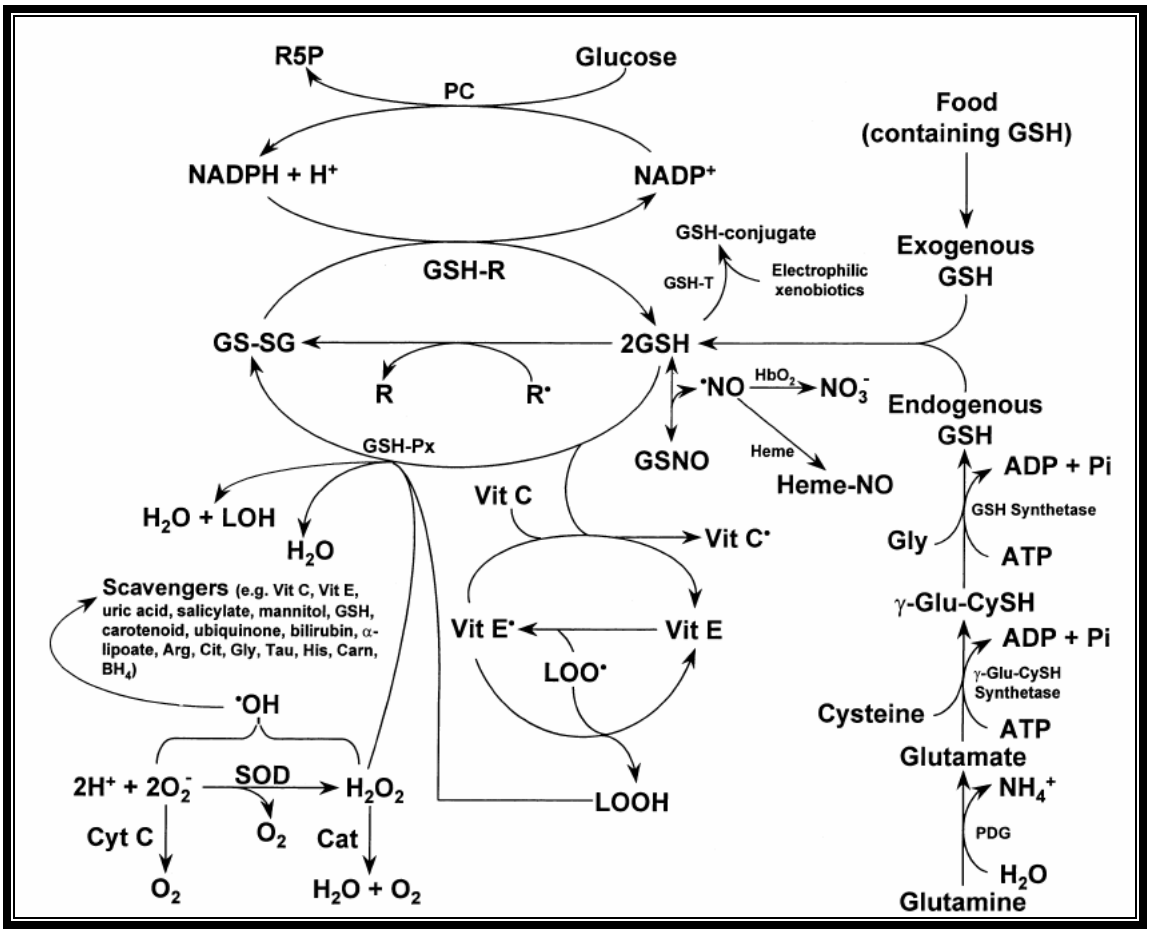


Figure 5.2. Removal of oxygen and nitrogen free radicals and other reactive species in mammalian cells (Fang *et al.*, 2002).

The pathophysiology of neurodegenerative disorders further predisposes the brain to free radical attack as these either allow additional sources of free radicals to predominate, or alternately, exploit the usual sources of these reactive species mentioned above. Activated microglia and exaggerated calcium signalling in excitotoxicity (figure 4.1.) both stimulate NOS activity to enhance synthesis of NO (Kreutzberg, 1996). Figure 4.1. also shows that excitotoxicity may lead to mitochondrial dysfunction, another mechanism of neurodegeneration, the initial stages of which include depression of oxidative phosphorylation and thus the leakage of excess electrons which drive the production of the various free radicals.

In AD, AP and NFT accumulate redox-active iron which, as described in section 1.2.2.2.5., catalyses the formation of $OH^{\cdot}$ via the Fenton reaction. Advanced glycation endproducts (AGE), a less well known morphological feature of AD (Steele *et al.,* 2006), can also undergo redox recycling in the presence of redox active iron, thereby enhancing free radical production (Perry *et al.,* 2002). Activated microglia, such as those surrounding senile plaques, can produce NO and $O_2^{\cdot -}$ (Kreutzberg, 1996) which can subsequently react to form $ONOO^-$ (Radi *et al.,* 1991). AGE can further increase free radical formation by activation of specific receptors, such as the receptor for AGE (RAGE) and the class A scavenger-receptor (Gasic-Milenkovic *et al.,* 2003). AP are also capable of this receptor activation. Mitochondrial metabolic abnormalities, such as changes in the mitochondrial genome or deficiencies in key metabolic enzymes, may also be a major initiating source of reactive oxygen species (ROS) in AD (Perry *et al.,* 1998; Perry *et al.,* 2002).

The notion that oxidative stress is involved in the pathogenesis and pathology of AD now appears to be well established and unrefuted (Martins *et al.,* 1986; Franceschi *et al.,* 1990; Butterfield *et al.,* 1999a; Butterfield *et al.,* 1999b; Ansari *et al.,* 2006; Onyango and Khan, 2006). On the other hand it is a relatively new concept with respect to major depression, in which evidence of an imbalance in oxidant and antioxidant defence systems in favour of the pro-oxidant status has recently arisen (Khanzode *et al.,* 2003). Similarly, oxidative stress has lately become recognised as a key feature of viral

infections including acute encephalitis caused by HSV-1 (Valyi-Nagy and Dermody, 2005).

The anomalous role of oxidative stress in any disease is that it is likely to lead to oxidative injury to the lipid, nucleic acid and protein biomolecules (Azbill *et al.,* 1997; Sun and Chen, 1998; Bergamini *et al.,* 2004), structures vital in the facilitation of normal functioning of the cell. The cells are thus steered towards degeneration (Bus and Gibson, 1982), and moreover, the damage to some of these biomolecules may in turn further predispose the cells toward fatality. For example, the antioxidant enzymes and glutamate transporters (described in chapter 4) are protein molecules which may be structurally damaged during oxidative stress, thus rendering them dysfunctional (Mo *et al.,* 1995; Camacho and Massieu, 2006) effectively enhancing oxidative stress and excitotoxicity respectively. Damage to DNA may cause mutations of genes and thus reduced and/or defective translation of proteins, including the antioxidant enzymes and glutamate transporters (Wei *et al.,* 1998; Wei and Lee, 2002).

5.2. THE EFFECT OF QA ALONE AND IN COMBINATION WITH AC ON SUPEROXIDE ANION GENERATION IN RAT BRAIN HOMOGENATE *IN VITRO*

5.2.1. Introduction

O_2 is required for the generation of most free radicals (Fridovich, 1999), and since these are defined as molecules having an unpaired electron in the outer orbit (Gilbert, 2000), the simplest free radical to form is the $O_2^{\cdot-}$, which is then the precursor of a diverse group of highly reactive species, including $HO^{\cdot}$ (Fridovich, 1983), $ONOO^{-}$ (Radi *et al.,* 1991) and $HOO^{\cdot}$ (Halliwell and Gutteridge, 1989).

Mitochondria are the major sites of the generation of free radicals (Curtin *et al.,* 2002). In particular, it is the electron transport chain (ETC) which is a source of $O_2^{\cdot-}$. Figure 5.3. shows that complex I, NADH ubiquinone oxidoreductase and Complex III, ubiquinol–cytochrome C oxidoreductase are the two sites where $O_2^{\cdot-}$ is produced (Beyer, 1992).

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) (Beyer, 1992). In this process, a semiquinone anion, an intermediate in the oxidation of ubiquinol to ubiquinone is formed. This intermediate is responsible for the production of $O_2^{\cdot-}$ (Curtin *et al.,* 2002) in the ETC because the electron which it surrenders upon oxidation back to ubiquinone is taken up by O_2 to form $O_2^{\cdot-}$. This free radical generated by the cycling of ubiquinol in the inner mitochondrial membrane, is produced primarily in the mitochondrial matrix (Raha *et al.,* 2000). It is therefore plausible to predict that any agent capable of enhancing the activity of the ETC at complex I, II and at a point in complex III which is proximal to the formation of the semiquinone, will enhance $O_2^{\cdot-}$ production. Similarly, agents which inhibit the activity of complex III distal to the semiquinone formation and complex IV will probably also have such an effect.

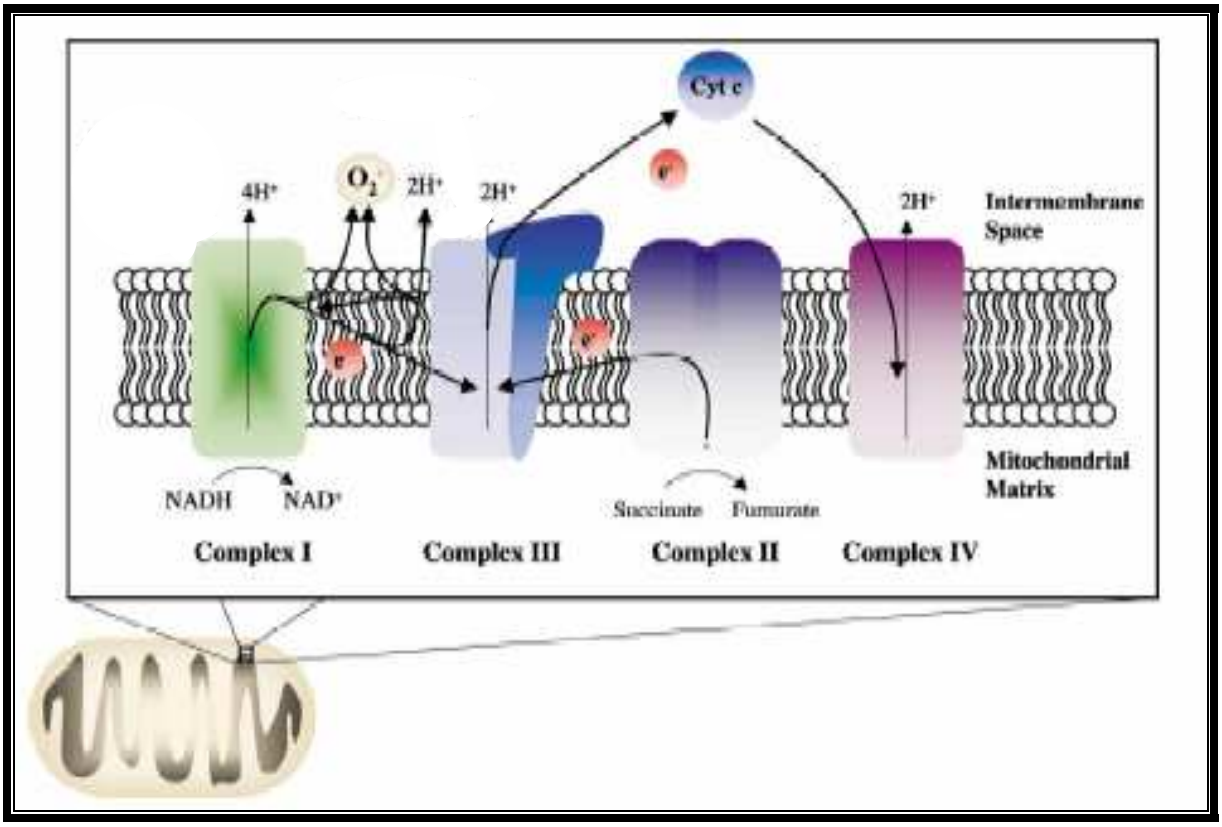


Figure 5.3. Schematic diagram illustrating the major subunits of the ETC and sites of $O2^{\cdot-}$ production (Curtin *et al.*, 2002).

Bordelon *et al*., (1997) have shown that intrastriatal injections of QA produce progressive mitochondrial dysfunction, thus the possibility of such an effect, specifically involving the ETC, on rat brain homogenate was investigated *in vitro*. The consequences, if any, of AC in combination with QA was also studied.

The nitroblue tetrazolium (NBT) assay, generally accepted (Das *et al.,* 1990; Sagar *et al.,* 1992) as a reliable method to assay for $O_2^{\cdot-}$**,** was used. The principle behind the assay is that $O_2^{\cdot-}$ reduces NBT to the water insoluble nitroblue diformazan (NBD), which can be extracted using glacial acetic acid and quantified by spectrophotometric analysis.

5.2.2. Materials and Methods

5.2.2.1. Animals

Adult male Wistar rats were purchased from the NHLS (Johannesburg, South Africa) and cared for as described in section 2.2.2.1.

5.2.2.2. Chemicals and Reagents

AC sodium (Zovirax®, powder for injection) was obtained from Glaxo Wellcome, Midrand, South Africa. NBD, QA and NBT were purchased from Sigma Chemical Co, St. Louis, USA. Ethanol and glacial acetic acid were purchased from Saarchem, Krugersdorp, South Africa.

5.2.2.3. Removal of the brain

Rats were killed and the brains removed as described in section 2.2.2.7.

5.2.2.4. Preparation of Tissue

Each brain was weighed and rapidly homogenised in a glass-teflon hand held homogeniser in ice cold 0.1 M PBS at pH 7.4 to obtain a homogenate concentration of 10 % w/v.

5.2.2.5. Preparation of the NBD Standard Curve

A series of NBD standards (0–400 nmoles/ml) were prepared in triplicate using glacial acetic acid as the diluent to give a total volume of 1 ml. An NBD standard curve was generated by measuring the absorbances at 560 nm on a GBC UV/VIS 916 spectrophotometer and plotting these against the molar equivalent weight of NBD (Appendix II).

5.2.2.6. Nitroblue Tetrazolium Assay

A modification of the NBT Assay as described by Das *et al*., (1990) was used.

Samples of rat brain homogenate (1 ml), in triplicate, contained 1 mM QA (250 µl) in the absence and presence of increasing concentrations (0–2 mM) of AC (250 µl) and 0.4 ml NBT (0.1 % dissolved in ethanol and then made up to the required volume with milli-Q water). The samples were incubated in an oscillating water bath for an hour at 37 ˚C. Control samples did not contain QA or AC.

The reaction was terminated and the NBD formed extracted by centrifuging the samples for 10 minutes at 2000 x g and resuspending the pellets with 2 ml glacial acetic acid. To remove insoluble debris, the samples were centrifuged for 5 minutes at 2000 x g. An aliquot of the supernatant (extracted NBD in glacial acetic acid) was read at 560 nm, using a GBC UV/VIS spectrophotomter. NBD levels were then determined from a standard curve generated as described in section 5.2.2.5. Final results were expressed as NBD (nmoles/mg tissue).

5.2.2.7. Statistical Analysis

This was performed as described in section 4.2.2.9.

5.2.3. Results

Figure 5.4. shows that 1 mM QA has no significant effect on $O_2^{\bullet-}$ generation *in vitro*. Co-incubation with AC at low concentrations ($\leq$ 0.5 mM) also produces no effect on the formation of this free radical. However, 1 mM AC ($p < 0.05$) and 2 mM AC ($p < 0.01$) are able to significantly reduce the amount of NBD that is already present.

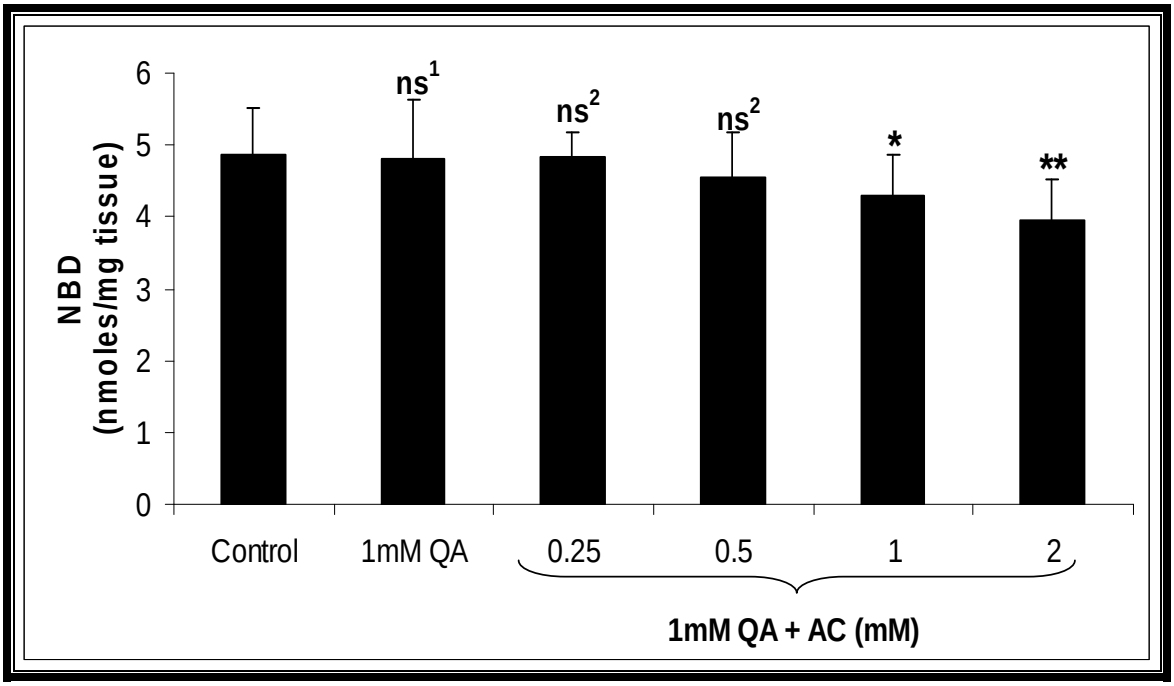


Figure 5.4. Effect of QA alone and in combination with AC on $O_2^{\bullet-}$ generation in rat brain homogenate *in vitro*. Each bar represents the mean ± SD (n = 5). ns^1 ($p > 0.05$) in comparison to control; ns^2 ($p > 0.05$), * ($p < 0.05$) and ** ($p < 0.01$) in comparison to 1 mM QA (ANOVA and Student–Newman–Keuls Multiple Range Test).

5.2.4. Discussion

The results (figure 5.4.) show that QA has no direct effect on ETC activity as there was no difference in the generation of $O_2^{\bullet-}$ in comparison to control. This suggests that the ability of QA to cause mitochondrial dysfunction reported by Bordelon and coworkers (1997) is mediated by NMDA receptor overactivation (excitotoxicity). As described, in the previous chapter, it is the rise in intracellular calcium levels subsequent to NMDA stimulation in particular, which initiates the sequence of events. In the present experiment, QA is exposed to homogenised tissue in which the mitochondria remain intact, but the integrity of the outer neuronal cell membranes is destroyed. This may not have an adverse effect on receptor positioning and functioning within the cell membranes. However a compartmentalised intracellular environment to facilitate the cellular response (in this instance, increased intracellular calcium levels) to binding at the

receptor no longer exists. Thus the sequence of events leading up to mitochondrial dysfunction and a rise in $O_2^{\cdot-}$ does not occur.

Even though QA does not enhance the formation of $O_2^{\cdot-}$, it is important to note that at the higher end of the concentration range, AC is able to reduce the amount of $O_2^{\cdot-}$ present under those conditions. The presence of this $O_2^{\cdot-}$ is not influenced by the NMDA stimulation associated with QA, which implies that the reduction in $O_2^{\cdot-}$ brought about by AC must be through its ability to scavenge this free radical, rather than through prevention of its formation.

Guanine is the most easily oxidisable nucleic acid base in DNA and is therefore a primary target of oxidative modification (Cadet *et al.*, 2002). The ease with which free radicals react with guanine could thus possibly be exploited by the production of analogues, such as AC, as suitable free radical scavengers to alleviate oxidative stress.

The interaction of $O_2^{\cdot-}$ with a guanine base is shown in figure 5.5. The G (-H)$^{\cdot}$ radical can be formed by H abstraction of the hydroxyl group at C-6. This abstraction could be carried out by the potent OH$^{\cdot}$ radical which is known to be formed by QA activity *in vitro* (Pláteník *et al.*, 2001) The G (-H)$^{\cdot}$ radicals are usually considered to be O-centered radicals with the unpaired electron positioned on the O-6 atom (Hildenbrand and Schulte-Frohlinde, 1990), which explains the low reactivity of this radical with O_2 (Al-Sheikhly, 1994). However, resonance stabilisation allows for the placement of the unpaired electron at C-5. The $O_2^{\cdot-}$ can react via an addition reaction at C-5 and at C-8 of both resonance structures (i.e. both forms of the G (-H)$^{\cdot}$ radical) (Cadet *et al.*, 1994). However, the former reaction is the major reaction (Misiaszek *et al.*, 2003), which is depicted in figure 5.5. Cadet and co-workers (1994) proposed that the addition of $O_2^{\cdot-}$ to the C-5 position of the G (-H)$^{\cdot}$ radical is followed by a rapid protonation of the peroxide anion to form 5-HOO-G-(H) Typically, hydroperoxides generated in the course of oxidative degradation of guanine bases are very unstable (Kang and Foote, 2002). Thus, according to the mechanism proposed by Cadet and co-workers (1994), the cleavage and decarboxylation of 5-HOO-G-(H) occurs via the opening of the pyrimidine ring at the C-

5—C-6 bond in 5-HOO-G(-H) leading to an unstable intermediate that is easily hydrated at the 7,8-C=N double bond. Ring chain tautomerisation and cleavage of the carbinolamine results in the opening of the imidazole ring with a subsequent intramolecular cyclisation of the guanidine residue forming imidazolone (Iz) which can be hydrated to oxazolone (Z).

AC also has the purine ring involved in the mechanism of $O_2^{\cdot -}$ scavenging proposed for guanine, thus it probably acts in the same manner. The salient feature of this mechanism is that from unstable reactive radicals, namely $^{\cdot}OH$, $O_2^{\cdot -}$ and G (-H)$^{\cdot}$, stable, non-reactive products, namely Iz and Z are formed, thus the propagation of free radicals is terminated and the progression of oxidative stress restrained.

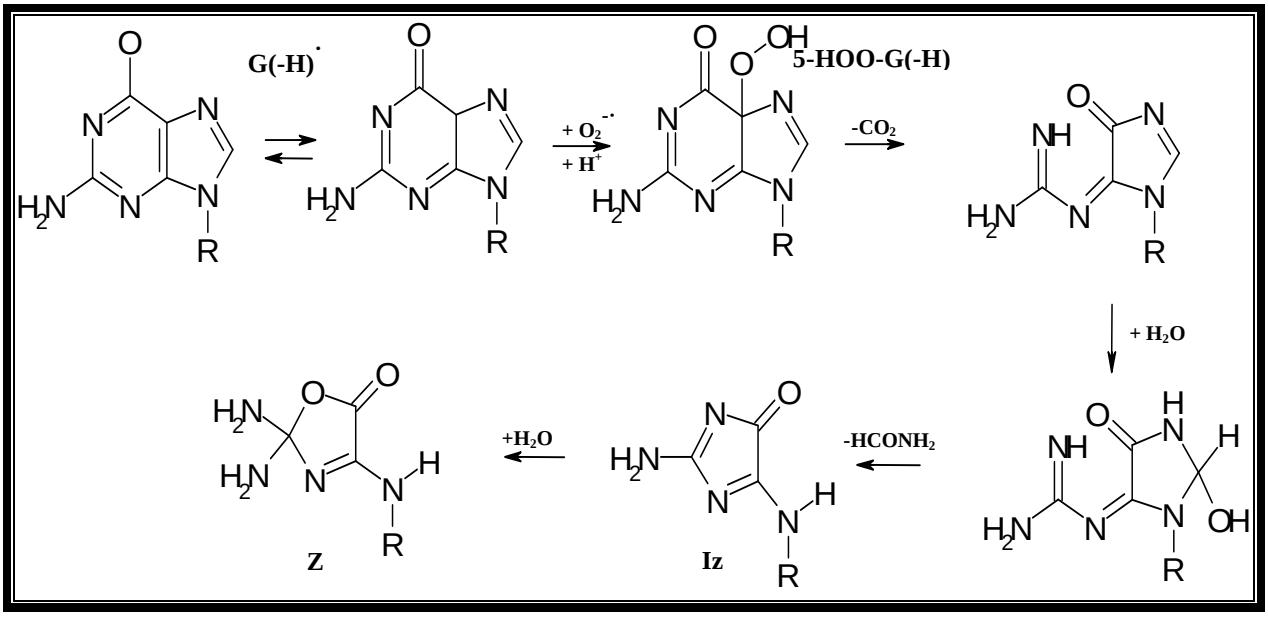


Figure 5.5. Interaction of O2·- with a guanine base to form imidazolone (Iz) and oxazolone (Z) products (Misiaszek *et al.*, 2003).

5.3. THE EFFECT OF QA ALONE AND IN COMBINATION WITH AC ON SUPEROXIDE ANION GENERATION IN RAT HIPPOCAMPAL HOMOGENATE *IN VIVO*

5.3.1. Introduction

In the previous experiment, QA was found to not induce $O_2^{\cdot-}$ generation *in vitro*. The present study was therefore conducted to assess whether QA administered *in vivo* to intact cells in the hippocampus could generate increases in $O_2^{\cdot-}$, through an insult on mitochondrial function, reported by Bordelon *et al.* (1994) through its long known, well established excitotoxic, NMDA receptor mediated, effect (Stone and Perkins, 1981). Furthermore, the investigation also sought to determine whether AC treatment in combination with QA could bring about any alterations to the effect of QA alone despite it not being successful at modifying glutamate release (chapter 4), an important aggravating factor of excitotoxicity.

5.3.2. Materials and Methods

5.3.2.1. Animals

Adult male Wistar rats were purchased from the NHLS (Johannesburg, South Africa) and cared for as described in section 2.2.2.1.

5.3.2.2. Chemicals and Reagents

As per section 5.2.2.2. In addition, sodium phenobarbitone was purchased from Merck, Germany and was at a working concentration of 60 mg/ml.

5.3.2.3. Surgical Procedures

These procedures were conducted as described in section 2.2.2.4.

5.3.2.4. Drug Treatment

This was performed as described in section 2.2.2.5.

5.3.2.5. Brain and Hippocampi Removal

The hippocampi from the brains of the rats which were killed a day after their test trials (8 days after the last dose of the drug treatment) in the Morris Water Maze (chapter 2) were removed as described in section 2.2.2.6.

5.3.2.6. Preparation of Tissue

The preparation of each portion of the hippocampal tissue was performed as described for each whole brain in section 5.2.2.4. However, the homogenate concentration was 5 % w/v instead of 10 % w/v.

5.3.2.7. Preparation of the NBD Standard Curve

This was performed as described in section 5.2.2.5.

5.3.2.8. Nitroblue Tetrazolium Assay

The NBT assay was performed as described in section 5.2.2.6. However, no exogenous QA and AC was added to the triplicate samples of rat hippocampal homogenate (0.5 ml).

5.3.2.9. Statistical Analysis

This was performed as described in section 4.2.2.9.

5.3.3. Results

Figure 5.6. illustrates that the intrahippocampal injection of QA (120 nmoles) has the ability to significantly enhance ($p < 0.001$) the generation of $O_2^{\cdot-}$, in comparison to control, and that AC treatment significantly reduces ($p < 0.001$) QA-induced $O_2^{\cdot-}$.

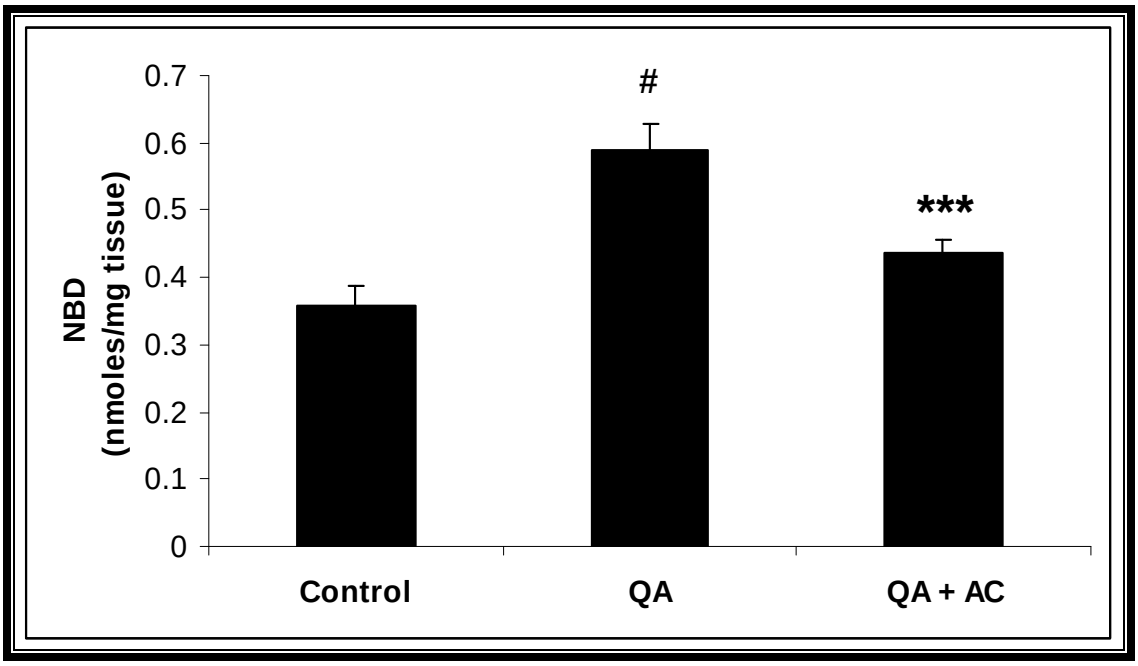


Figure 5.6. Effect of QA alone and in combination with AC on $O_2^{\cdot-}$ generation in rat hippocampal homogenate *in vivo*. Each bar represents the mean ± SD; n = 5. # ($p < 0.001$) in comparison to control; *** ($p < 0.001$) in comparison to QA (ANOVA and Student–Newman–Keuls Multiple Range Test).

5.3.4. Discussion

In cultured neurons, QA stimulation of the NMDA receptors is known to generate ROS, such as $O_2^{\cdot-}$, H_2O_2 and $NO^{\cdot}$ (Lysko *et al.*, 1992; Lafon-Cazal *et al.*, 1993; Gunasekar *et al.*, 1995; Reynolds and Hastings, 1995; Küçükkaya *et al.*, 1996). As in cultured neurons, QA injected intrastriatally (*in vivo*) causes NMDA stimulation (Giordano *et al.*, 1990), thus it is expected that QA injected intrahippocampally is also able to induce a significant increase in $O_2^{\cdot-}$.

The ability of QA to cause mitochondrial dysfunction (Bordelon *et al.*, 1994), via its perpetuating excitotoxic mechanism (chapter 4) may account for the QA effect on $O_2^{\bullet-}$, although this needs to be investigated further. The dampening of the QA-induced increase in $O_2^{\bullet-}$ by AC is most likely primarily or even solely related to its capacity to scavenge this free radical, which was described in the previous experiment. This is argued in the light of the doubt cast on AC being able to restore mitochondrial function and to have a significant role to play as an anti-excitotoxin (discussed in chapter 4). Since QA impairs the activity of the $O_2^{\bullet-}$ detoxifying enzyme, SOD (Rodríguez-Martínez *et al.*, 2000), this ROS becomes longer-lived and therefore more readily available for conversion to other noxious free radicals (as described previously), ultimately augmenting oxidative stress. Furthermore, the direct toxicity of $O_2^{\bullet-}$ includes the inhibition of the calcineurin enzymes, which attenuates the expression of LTP (section 1.2.2.2.1. Thus the enhanced generation of $O_2^{\bullet-}$ may be an additional mechanism by which QA induces spatial memory deficits (chapter 2). Through its capacity to scavenge $O_2^{\bullet-}$, AC is therefore improving spatial memory deficits directly as well as indirectly by curbing oxidative stress.

5.4. THE EFFECT OF QA ALONE AND IN COMBINATION WITH AC ON TOTAL GLUTATHIONE LEVELS IN RAT HIPPOCAMPAL HOMOGENATE *IN VIVO*

5.4.1. Introduction

The antioxidant properties of GSH, the most abundant thiol-containing peptide in the CNS (Hermann *et al.,* 2002), are vast and include direct and indirect mechanisms. (1) GSH can react with a variety of xenobiotic electrophilic compounds in the catalytic reaction of glutathione-S-transferase (Baars and Breimer, 1980). (2) It effectively scavenges ROS (e.g., $LOO^{\cdot}$, $ONOO^{-}$, and H_2O_2) directly and indirectly through enzymatic reaction with GPx (Fang *et al.,* 2002). (3) GSH can conjugate with NO, resulting in the formation of an S-nitrosoglutathione, an adduct which has exhibited neuroprotective effects against iron-induced oxidative stress in the substantia nigra (Rauhala *et al.,* 1998a). Although a pro-oxidant, GSH radical (GS•) is formed when GSH reacts directly with free radicals; it can be rapidly detoxified by reaction with another GS• to yield a non-radical GSSG (Fang *et al.*, 2002).

The additional versatility of GSH lies in its ability to regenerate from GSSG in the presence of GR and the reducing equivalent, NADPH (figure 5.2.), which is produced during glucose metabolism via the pentose phosphate pathway (PPP) (Puskas *et al.,* 2000). When the intracellular concentration of GSH decreases and that of GSSG increases, the cellular demand for NADPH increases markedly (Sies, 1999) and glucose utilisation is shunted towards the PPP.

GSH is synthesised from glutamate, cysteine, and glycine (Sies, 1999), as shown in figure 5.2. Almost all dietary glutamate is subjected to first pass metabolism by the small intestinal mucosa (Wu, 1998). Thus, the hydrolysis of glutamine to glutamate by glutaminase and the production of glutamate from α-ketoglutarate and branched-chain amino acids via transamination is necessary for GSH synthesis, which is an energy-

dependent process. GSH in the diet can be partly absorbed from the small intestine, thus it can be classified as an endogenous and exogenous antioxidant (Fang *et al.,* 2002).

The levels of GSH in the cerebral cortex of stress-induced depressed mice is appreciably depleted and different antidepressants were able to correct this to varying degrees, suggesting a close link between stress-induced depression, increased monoaminergic utilisation, oxidative stress and brain GSH (Pal and Dandiya, 1994). Adams and coworkers (1991) found that GSH levels were increased in the hippocampi, whereas Gu and coworkers (1998) found that these levels were reduced in the cingulate cortex of the brains of AD patients postmortem. The discrepancy lies in the unexpected increase in the former, which may suggest that after an initial decrease in GSH levels in the brain in AD, a compensatory response (Adams *et al.,* 1991) increases the *de novo* synthesis and regeneration of GSH. Thus, whether a decrease or increase in the GSH levels in the brains of AD patients postmortem in comparison to controls is reflected depends on the progression of AD at the time of death. Intracellular endogenous GSH levels dramatically decreased in the first 24 h after virus adsorption after *in vitro* HSV-1 infection of cells with inhibition of viral replication upon addition of exogenous GSH (Palarama *et al.,* 1995).

Rodríguez-Martinez *et al.,* (2000) showed that striatal levels of GSH are reduced after intrastriatal injections of QA. This has raised the possibility that QA could have an analogous effect in the hippocampus. Thus the aim of this study was to assess the effect of QA in this regard and furthermore to probe whether AC could influence it.

GSH is the most abundant intracellular low molecular weight, non-protein thiol (NP-SH) compound in mammalian cells (Sies, 1999), thus it is expected that it will account for most NP-SH in tissues. A rapid, simple spectrophotometric method for the determination of sulfhydryl groups in NP-SH in various tissues would be an appropriate technique to use to measure GSH levels in rat hippocampal homogenate. The technique is based on the principle that 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) is reduced by SH groups to

form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of SH (Sedlak and Lindsay, 1968). This reaction is illustrated in figure 5.7.

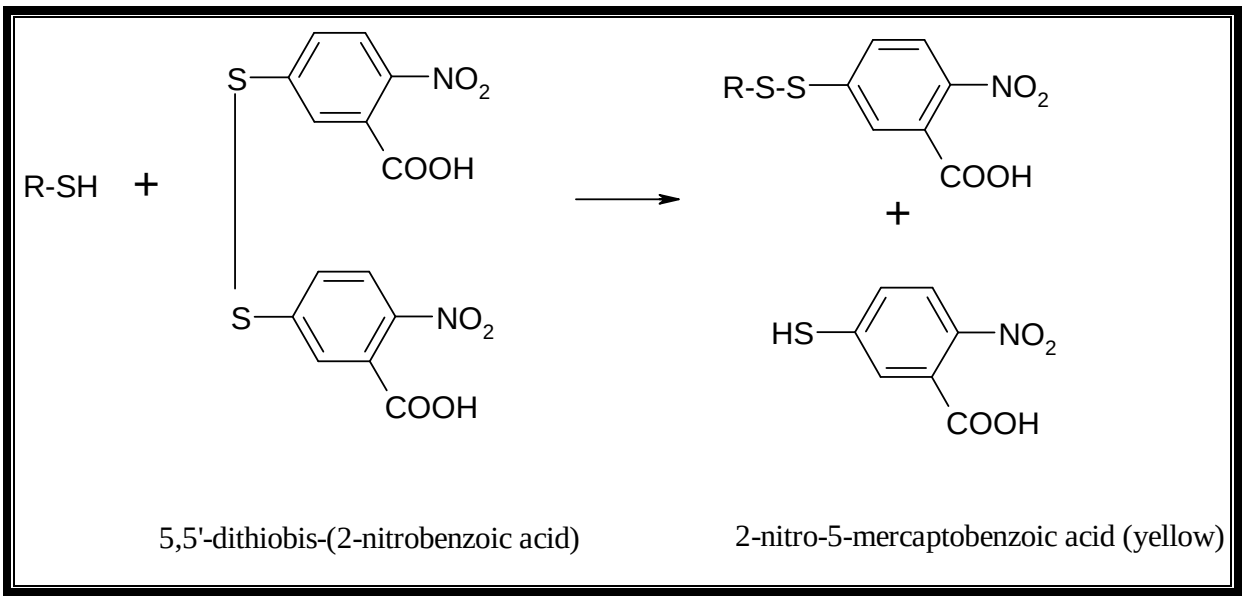


Figure 5.7. Reaction of thiol-containing compounds (R-SH) with DTNB to produce 2-nitro-5-mercaptobenzoic acid (Sedlak and Lindsay, 1968).

5.4.2. Materials and Methods

5.4.2.1. Animals

Adult male Wistar rats were purchased from the NHLS (Johannesburg, South Africa) and cared for as described in section 2.2.2.1.

5.4.2.2. Chemicals and Reagents

AC sodium (Zovirax®, powder for injection) was obtained from Glaxo Wellcome, Midrand, South Africa. QA (2, 3-pyridinedicarboxylic acid), GSH and DTNB were purchased from Sigma Chemical Co, St. Louis, USA. Methanol was purchased from Saarchem, Krugersdorp, South Africa. Sodium phenobarbitone was purchased from Merck, Germany and was at a working concentration of 60 mg/ml.

5.4.2.3. Surgical Procedures

These procedures were conducted as described in section 2.2.2.4.

5.4.2.4. Drug Treatment

This was performed as described in section 2.2.2.5.

5.4.2.5. Brain and Hippocampi Removal

The hippocampi from the brains of the rats which were killed a day after their test trials (8 days after the last dose of the drug treatment) in the Morris Water Maze (chapter 2) were removed as described in section 2.2.2.6.

5.4.2.6. Preparation of Tissue

The preparation of each portion of the hippocampal tissue was performed as described for the whole brains in section 5.2.2.4., except that the homogenate concentration was 5 % w/v instead of 10 % w/v.

5.4.2.7. Preparation of the GSH Standard Curve

A series of GSH standards (0–10 nmoles/ml) were prepared in triplicate using PBS as the diluent to give a total volume of 0.5 ml. To these tubes, 1.5 ml 0.2 M tris HCl (pH 8.2), 0.1 ml 0.01 M DTNB and 7.9 ml methanol were added and thereafter left to stand in the dark for 30 minutes. A standard curve was generated by measuring the absorbances at 412 nm on a GBC UV/VIS 916 spectrophotometer and plotting these against the molar equivalent weight of GSH (Appendix 3).

5.4.2.8. Glutathione Assay

GSH levels were estimated by measuring NP-SH using the method previously described by Sedlak and Lindsay (1968).

To 1 ml of rat hippocampal homogenate, 0.5 ml TCA (25 % in milli-Q water) was added to precipitate proteins and thus separate protein bound thiol containing compounds from non-protein-bound thiol-containing compounds (Sedlak and Lindsay, 1968), the predominant one being GSH (Sies, 1999; Hermann *et al.*, 2002). These samples were centrifuged at 2000 x g for 15 minutes. Aliquots of the supernatant (0.5 ml) were transferred to a clean set of test tubes and 1.5 ml 0.2 M tris-HCl (pH 8.2), 0.1 ml DTNB (0.01 M in methanol) and 7.9 ml methanol were added. All the samples were incubated for 30 minutes at room temperature. The tris-HCl buffer (pH 8.2) was necessary to maintain a final pH of the complete reaction mixture above 8.0 since the intensity of the colour of 2-nitro-5-mercaptobenzoic acid varies with pH below pH 8 (Sedlak and Lindsay, 1968). The incubation mixture was then centrifuged at 2000 x g for 15 minutes and the supernatant was read at 412 nm. The GSH levels were determined from a standard curve generated from GSH as described in section 5.4.2.7. Final results were expressed as GSH (nmoles/mg tissue).

5.4.2.9. Statistical Analysis

This was performed as described in section 4.2.2.9.

5.4.3. Results

Figure 5.8. illustrates that the intrahippocampal injection of QA (120 nmoles) has the ability to significantly reduce ($p < 0.001$) GSH levels in the rat hippocampus, in comparison to control, and this effect is blunted by treatment of the rats with AC ($p < 0.01$).

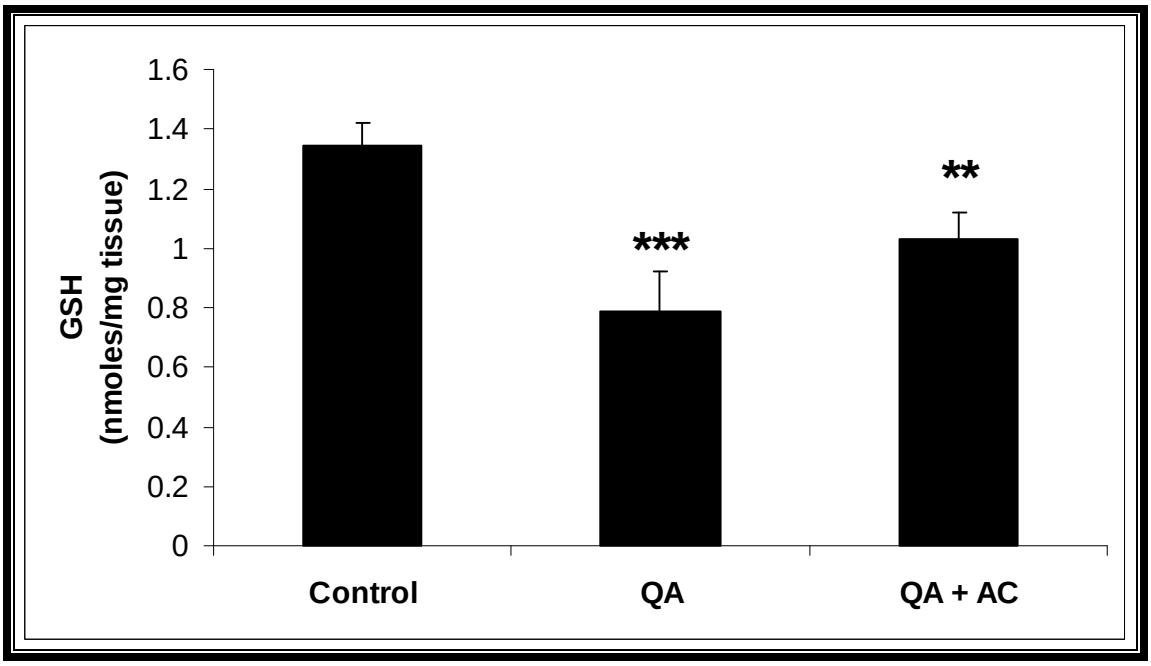


Figure 5.8. Effect of QA alone and in combination with AC on GSH levels in rat hippocampal homogenate *in vivo*. Each bar represents the mean ± SD; n = 5. *** ($p < 0.001$) in comparison to control; ** ($p < 0.01$) in comparison to QA (ANOVA and Student–Newman–Keuls Multiple Range Test).

5.4.4. Discussion

From the nature of the antioxidant defense system, it appears that it is possible that an inverse relationship exists between $O_2^{\bullet-}$ levels and GSH levels. After the dismutation of $O_2^{\bullet-}$ to H_2O_2, this substrate of the Fenton reaction and thus $^{\bullet}OH$ formation, is reduced to H_2O by GPx with GSH as a reducing agent (figure 1.14 and figure 5.2). Increased $O_2^{\bullet-}$ generation exhibited by QA *in vivo* in the rat hippocampus (section 5.3.) should then drive increased production of H_2O_2 and this in turn should enhance GPx activity and thus GSH consumption. This at least may explain in part the diminished GSH levels present in the rat hippocampus after an intrahippocampal injection of QA. Furthermore, GSH is known to have its own direct free radical scavenging properties (Kavanagh *et al.,* 1993) and may thus also become depleted by the free radicals generated during an excitotoxic insult by QA (Ganzella *et al.,* 2006).

The ability of QA to cause mitochondrial dysfunction has been referred to previously. This may reduce the production of ATP which is the energy-bearing molecule required for GSH synthesis (figure 5.2), thus reducing GSH levels in comparison to control rat hippocampi where ATP production is not adversely affected.

A potential $O_2^{\bullet-}$scavenging property of AC has already been discussed and implies that AC treatment could swing the inverse relationship between $O_2^{\bullet-}$ and GSH in the opposite direction to that brought about by QA in the absence of such treatment. The $O_2^{\bullet-}$ levels are reduced by AC, thus less H_2O_2 is formed, thereby decreasing the activity of GPx and the requirement for GSH associated therewith. Moreover, the loss of $^{\bullet}OH$ by hydrogen abstraction from AC means that less GSH is required for its direct free radical scavenging activity. The unlikelihood of AC restoring mitochondrial function was articulated in the previous chapter, indicating that it cannot aid the increased generation of ATP needed to overcome the deficit of *de novo* production of GSH induced by QA.

The significance of the conservation of GSH levels by AC is largely related to the alleviation of oxidative stress in the potential neurodegenerative disorders, AD, HSE and depression. In HSE, this, in turn, may not only limit oxidation of biomolecules and neurodegeneration directly, but also reduce activation of nuclear factor kappa B (NFκB), a nuclear transcription factor known to be activated in response to oxidative stress and thus suppress HSV-1 replication (Palamara *et al.,* 1995). Moreover, GSH appears to have direct anti-HSV-1 activity by inhibition of viral glycoprotein B synthesis which is necessary for the production of enveloped (i.e. infectious) virus particles (Palarama *et al.,* 1995). These antiviral effects brought about by GSH may aid in reducing CNS injury during HSE, by dampening the direct viral invasion of neurons and the subsequent viral induced immune response (section 1.1.4.1.).

CHAPTER 6

LIPID PEROXIDATION

6.1. INTRODUCTION

PUFAs, such as arachidonic, docosatetraenoic, and docosahexaenoic acids are essential for normal cell functioning because many membrane properties such as fluidity and permeability are closely related to their presence. LP results in a loss of membrane PUFA, which increases membrane rigidity (Choe *et al*., 1995). Furthermore, ROOH within the membrane have a devastating effect on membrane integrity because these too alter membrane fluidity and permeability (Halliwell, 1994). This alteration in membrane integrity due to LP, in turn, markedly affects the functioning of membrane bound enzymes and receptors (Farooqui and Horrocks, 1994).

Oxidative damage to lipids rather than the other biomolecules is of particular significance in neuroscience, because the brain is rich in peroxidisable PUFAs (Floyd, 1999). The products of LP are increased in the plasma of depressed patients (Bilici *et al.,* 2001) and in the brains of AD patients post-mortem (Sultana *et al.,* 2006; Williams *et al.,* 2006). In HSE, LP is thought to be part of the phagocytic function of macrophage, through the release of neurotoxic oxidative agents such as QA (Reinhard, 1997). However, the extent to which oxidative damage plays a beneficial role for the host by limiting viral replication is largely unknown (Valyi-Nagy and Dermody, 2005), since inevitably, surrounding healthy tissue is also damaged.

It has not been determined unequivocally whether LP is a cause of cell death or merely a consequence of cell damage by some other mechanism (Smith *et al.,* 1983). It was established many years ago that disrupted tissues undergo peroxidation more rapidly than healthy ones due to the inactivation of some antioxidants and the release of metal ions, especially iron from storage sites within cells (Barber, 1963, Gutteridge and Stocks, 1976). This finding provides supporting evidence that increased LP often occurs as a

result of cell damage or death and not vice versa (Halliwell and Gutteridge, 1984a). However, whether LP occurs before or after cell death, it remains a biomarker for oxidative cell damage, which may result in neurodegeneration.

LP can be assayed by quantifying malondialdehyde (MDA), which is a product of LP. The test material is heated at low pH with thiobarbituric acid (TBA) and the resulting pink chromogen, formed by a MDA-TBA complex (figure 6.1.) is extracted into butanol and measured by absorbance at 532 nm or by fluorescence at 553 nm (Gutteridge and Halliwell, 1990). Some of the MDA detected in the TBA test is formed from the hydroperoxides during the peroxidation process itself, but most is generated during the acid-heating stage of the test (Gutteridge and Halliwell, 1990).

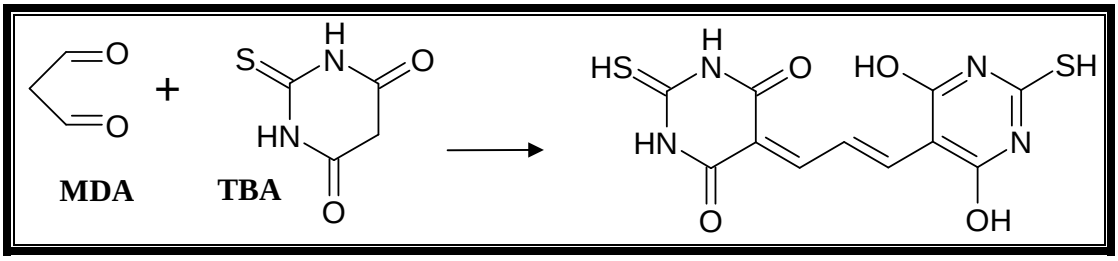


Figure 6.1. Reaction between 1 molecule of MDA and 2 molecules of TBA to form an MDA-TBA complex (modified from Mead *et al.*, 1986).

The value of the TBA test is that the peroxidation process beginning in the reaction mixture is effectively amplified in the assay itself, hence making it fairly sensitive (Gutteridge and Halliwell, 1990). A problem with the assay is that added metal ions, antioxidants and chelating agents may interfere with peroxide decomposition (Gutteridge and Quinlan, 1983).

MDA is produced from ring LOOH, as shown in figure 6.2. These peroxides can only be formed during the oxidation of methylene-interrupted polyunsaturated systems with three or more double bonds (Dahle *et al.*, 1962). The reason for this is that lipid $ROO^{\cdot}$ with an unsaturation β, γ with respect to the $ROO^{\cdot}$ function are required (Dahle *et al.*, 1962). This

is illustrated in figure 6.3., using the fatty acid linolenate as an example. Hydrogen abstraction of linolenate can give rise to four different isomeric lipid ROO˙. Of the four radicals, only two possess an unsaturation β, γ with respect to the ROO˙ function (Dahle *et al*., 1962). The β, γ unsaturated lipid ROO˙ undergo cyclisation to form five-membered ring peroxides (Dahle *et al*., 1962). If the β, γ-unsaturated free radical is stabilised by addition of a hydrogen atom to become a hydroperoxide, it could again yield a peroxide radical upon loss of hydrogen to either RO˙ or HO˙ .The latter radicals could be formed from ROOH under the acidic conditions of the TBA assay (Dahle *et al*., 1962). In contrast, figure 6.4. shows that ring peroxides cannot be formed from linoleate, which only has two double bonds.

The TBA test is particularly useful in neuroscience because neural membranes contain large amounts of phospholipids that are characterised by their high content of arachidonic, docosatetraenoic, and docosahexaenoic acids (Farooqui and Horrocks, 1994). All these fatty acids contain three or more double bonds, thus MDA can be formed and used as a marker for LP. The amount of MDA present is directly proportional to the incidence of LP.

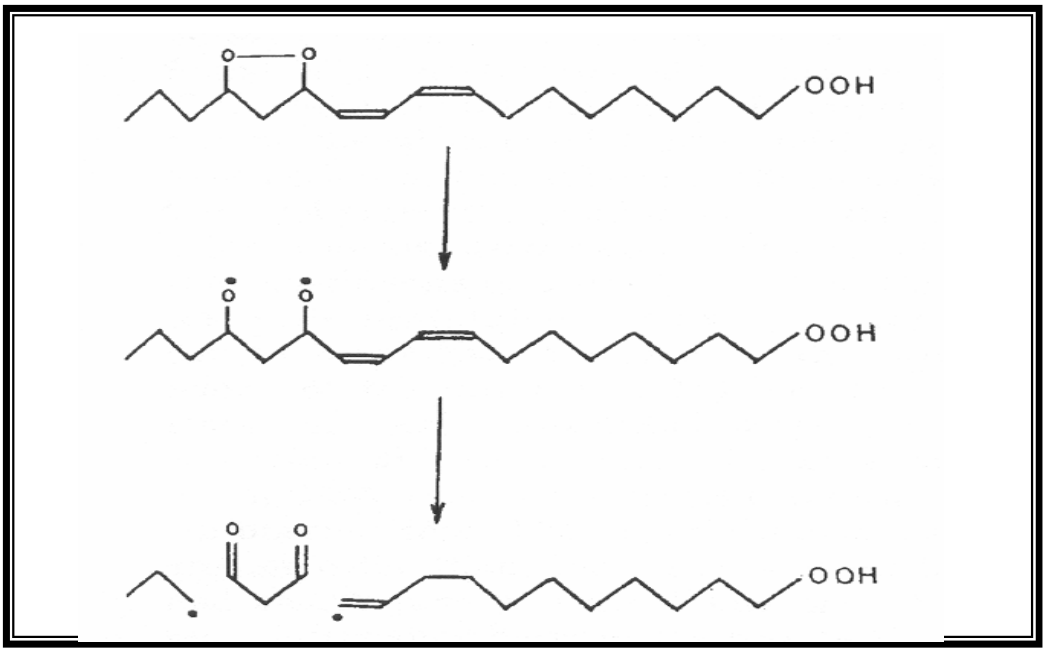


Figure 6.2. Mechanism of formation of MDA from cyclic ring peroxides (Dahle *et al.*, 1962).

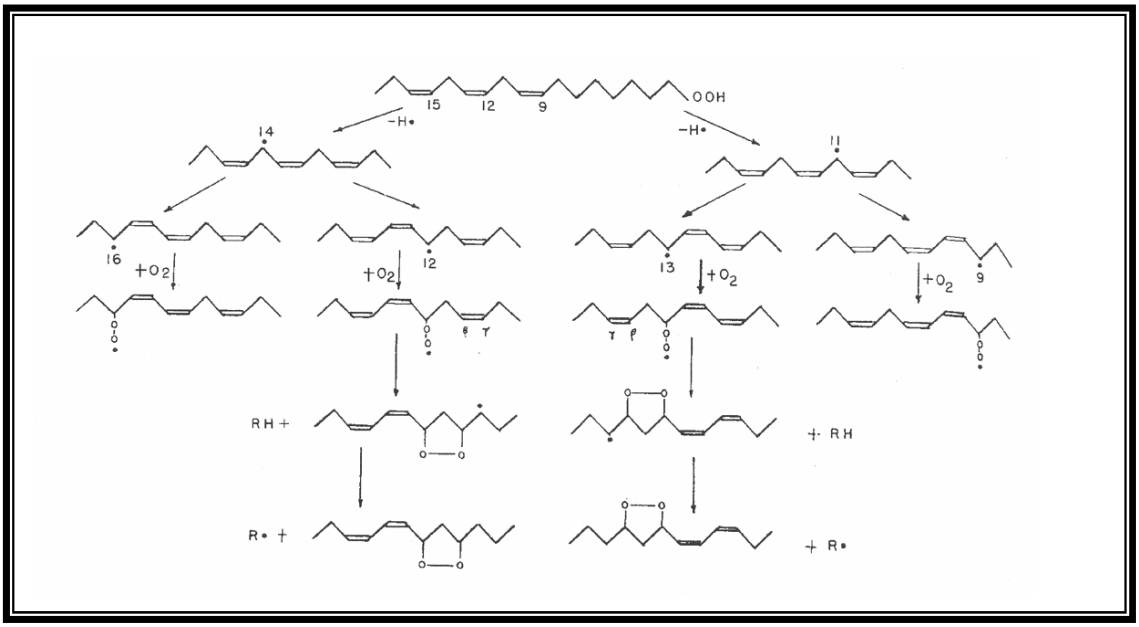


Figure 6.3. The formation of ring peroxides during LP of linolenate (Dahle *et al.*, 1962).

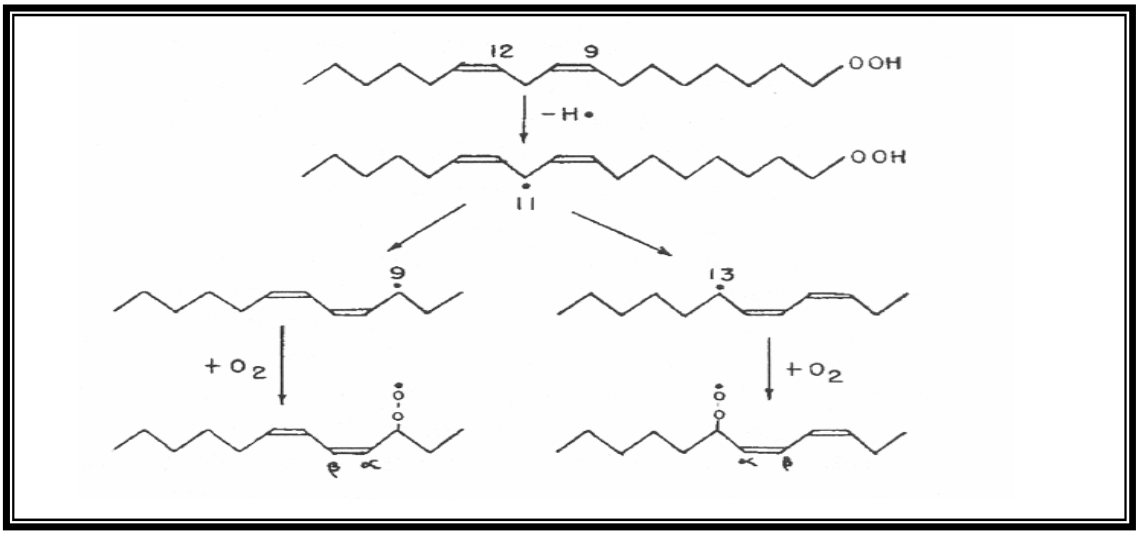


Figure 6.4. No ring peroxides formed during LP of linoleate (Dahle *et al.*, 1962).

6.2. THE EFFECT OF QA ALONE AND IN COMBINATION WITH AC ON LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE *IN VITRO*

6.2.1. Introduction

Rios and Santamaria, 1991 first described the lipoperoxidative effect of QA *in vitro* in rat brain homogenates and concluded that lipid peroxidation may be a damaging process involved in the neurotoxicity of QA.

AC was able to protect against QA-induced necrosis of hippocampal cells and the loss of integrity of hippocampal cell membranes (chapter 3). In the previous chapter, AC was found to have the ability to attenuate oxidative stress. Theoretically then, attenuation of oxidative stress should diminish oxidative damage to lipids (LP). Hence, the effect of QA on LP in rat brain homogenate *in vitro,* in the absence and presence of AC was investigated.

6.2.2. Materials and Methods

6.2.2.1. Animals

Adult male Wistar rats were purchased from the NHLS (Johannesburg, South Africa) were cared for as described in section 2.2.2.1.

6.2.2.2. Chemicals and Reagents

AC sodium (Zovirax®, powder for injection) was obtained from Glaxo Wellcome, Midrand, South Africa. 1, 1, 3, 3-tetramethoxypropane (98%), QA, Butylated Hydroxytuolene (BHT) and TBA and trichloroacetic acid (TCA) were purchased from

Sigma Chemical Co, St. Louis, USA. Ascorbic Acid, ethanol and butanol were purchased from Saarchem, Krugersdorp, South Africa.

6.2.2.3. Removal of the brain

This was performed as described in section 5.2.2.3.

6.2.2.4. Preparation of Tissue

This was performed as described in section 5.2.2.4.

6.2.2.5. Preparation of the MDA Standard Curve

Preparation of the calibration curve is complicated by the fact that MDA is unstable. Thus, MDA must be prepared immediately before use, by hydrolysing one of its derivatives, either 1,1,3,3-tetramethoxypropane or 1,1,3,3-tetraethoxypropane (Halliwell and Gutteridge, 1989). A series of 1, 1, 3, 3-tetramethoxypropane standards (0–20 nmoles/ml) were prepared in triplicate using PBS as the diluent to give a total volume of 1 ml. To these tubes 0.5 ml BHT (0.5 % in ethanol) and 1 ml TCA (15 % in milli Q water) were added. An aliquot of 2 ml of this solution was added to 0.5 ml TBA (0.33 %) and incubated for 1 hour. A standard curve was generated by measuring the absorbances at 532 nm on a GBC UV/VIS 916 spectrophotometer and plotting these against the molar equivalent weight of MDA in the complexes formed (Appendix IV).

6.2.2.6. Lipid Peroxidation Assay

A modification of the TBA Assay as described by Placer *et al*. (1966) was used.

Triplicate samples of rat brain homogenate (1 ml) contained 1 mM QA (100 µl) in the absence and presence of increasing concentrations (0–2 mM) of AC (100 µl). The samples were incubated in an oscillating water bath for an hour at 37 ˚C. Control samples did not contain QA or AC.

The reaction was terminated at the end of the incubation period by the addition of 1 ml TCA (15 % in milli-Q water) and 0.5 ml BHT (0.5 % in ethanol) to each sample. The samples were heated at 95 ˚C for 15 minutes in a water bath to release protein-bound MDA. Following this, samples were cooled and centrifuged at 2000 x g for 20 minutes to yield a protein-free supernatant. This supernatant (2 ml) was then transferred to a clean set of test tubes and 0.5 ml TBA (0.33 % in milli-Q water) was added. All samples were heated at 95 ˚C for an hour in a water bath to allow for the formation of the MDA-TBA complex. After rapidly cooling the test tubes on ice, 2 ml butanol was added to extract the pink complex. The samples were then centrifuged at 2000 x g for 15 minutes. An aliquot of the top layer (extracted complex in butanol) was read at 532 nm, using a GBC UV/VIS spectrophotomter. MDA levels were then determined from a standard curve generated from 1, 1, 3, 3-tetramethoxypropane as described in section 6.2.2.5. Final results were expressed as MDA (nmoles/mg tissue).

6.2.2.7. Statistical Analysis

This was performed as described in section 4.2.2.9.

6.2.3. Results

Figure 6.5. illustrates that 1 mM QA increases the amount of MDA in comparison to control approximately six-fold, and that AC blunts the 1 mM QA-induced LP. The latter response proceeds in a concentration-dependent manner and the suppression of MDA is highly significant for each concentration used ($p < 0.001$) in comparison to samples containing QA alone.

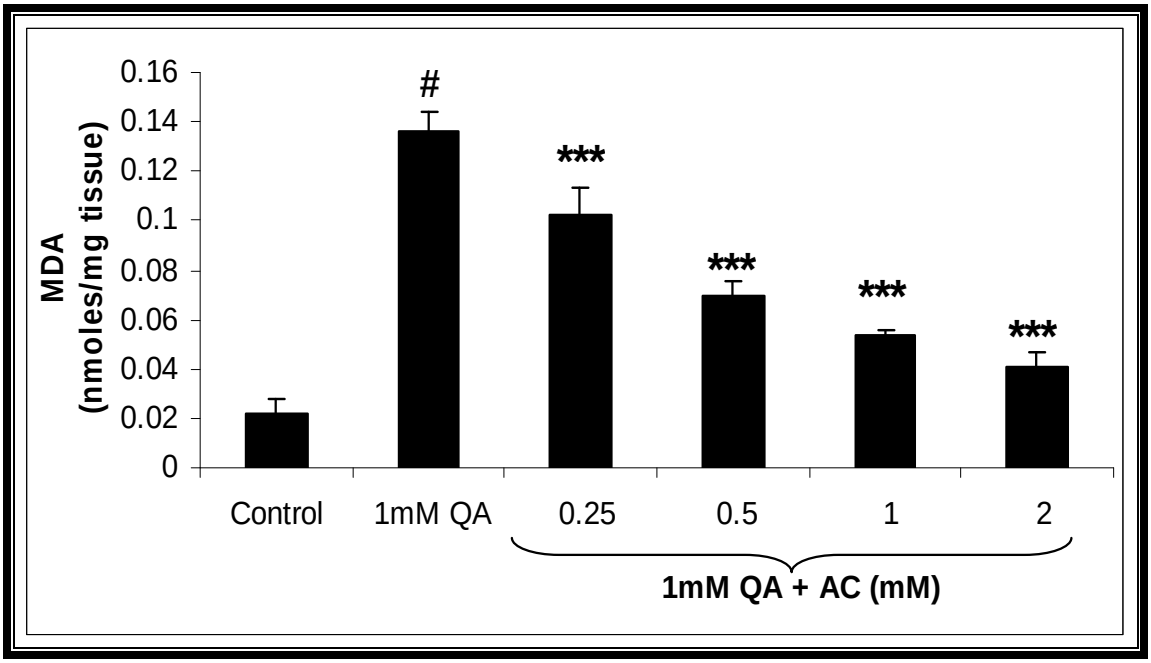


Figure 6.5. Effect of 1 mM QA alone and in combination with AC on LP in rat brain homogenate *in vitro*. Each bar represents the mean ± SD (n = 5). # ($p < 0.001$) in comparison to control and *** ($p < 0.001$) in comparison to 1mM QA (ANOVA and Student–Newman–Keuls Multiple Range Test).

6.2.4. Discussion

Štípek *et al.,* 1997 found that the effect of QA on LP in the rat brain *in vitro,* reported by Rios and Santamaria (1991) depends on Fe^{2+} and that this mechanism is likely to involve Fe^{2+}-chelation by QA. In this experiment, QA was added to homogenised and therefore disrupted tissues. The homogenising action results in the release of metal ions especially iron from storage sites within cells (Barber, 1963, Gutteridge and Stocks, 1976). Ultra-violet (UV) absorption spectra have shown that QA has the ability to chelate the Fe^{2+} ion. This was also observed with 2,6-pyridinedicarboxylic acid, which like QA, contains a 2-pyridinecarboxylic acid moiety (figure 6.6) (Iwahashi *et al.,* 1999).

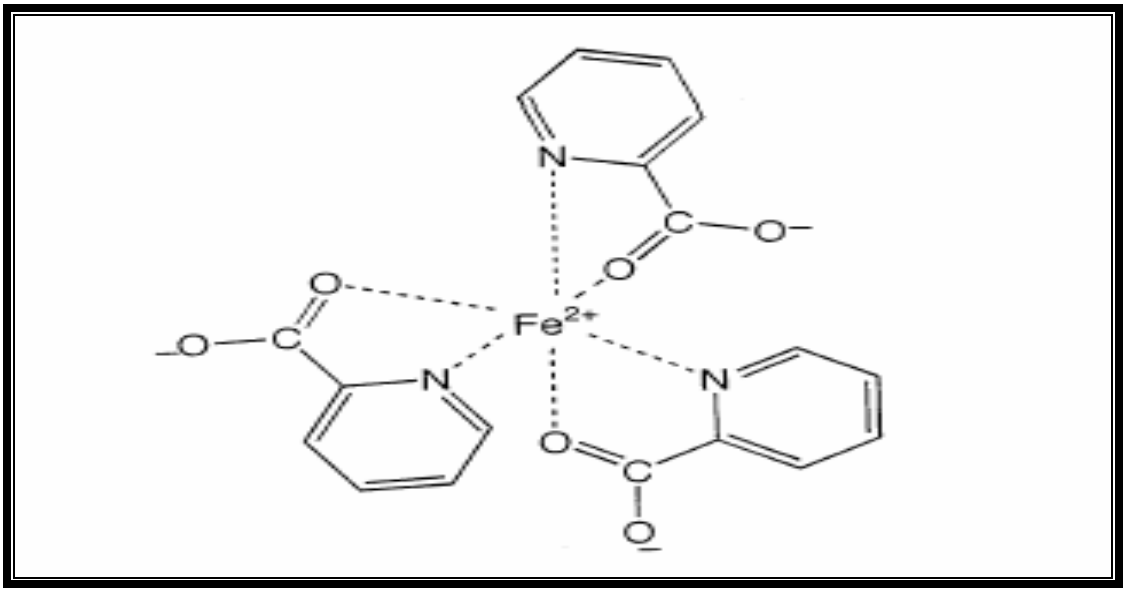


Figure 6.6. Binding of Fe^{2+} by the 2-pyridine carboxylic acid moiety (Iwahashi *et al.*, 1999)

The UV spectral wave so generated disappeared upon addition of H_2O_2, indicating that the Fe^{2+} ions in the complex may have been oxidised by H_2O_2 to Fe^{3+} (Iwahashi *et al.*, 1999), with the subsequent formation of the potent $HO^{\cdot}$ radical, shown in Equation 6.1. This free radical is potent enough to initiate LP.

Fe^{2+}-2,6-pyridinedicarboxylic acid + H_2O_2 ⟶

Fe^{3+} + OH^- + $HO^{\cdot}$ + 2,6-pyridinedicarboxylic acid **Equation 6.1.**

It has also been reported that the QA-Fe^{2+} complex is able to inhibit the auto-oxidation of Fe^{2+} (Murakami, *et al.* 1998), implying that iron is kept in the ferrous form which participates in the Fenton reaction. Thus, it appears that, through weak chelation of Fe^{2+}, QA may enhance the Fenton reaction. Furthermore, the ability of Fe^{3+} to undergo rapid redox recycling to Fe^{2+} (Gutteridge, 1994) in the presence of $O_2^{\cdot-}$ provides a constant supply of Fe^{2+} for the Fenton reaction.

Štípek, *et al.,* (1997) further found that upon addition of the iron chelator, desferoxamine, the increase in QA-induced LP is abolished. It is thus evident that the ability of AC to ameliorate QA-induced LP is possibly related to an ability to strongly bind Fe^{2+} and/or Fe^{3+}. If AC binds strongly to endogenous Fe^{2+} in rat brain homogenate, reduction of these ions will not occur even in the presence of H_2O_2 and fewer of these ions will be available to bind to QA to form the QA-Fe complex. Similarly, strong binding of Fe^{3+} by AC would prevent redox recycling of this ion and thus the perpetual formation of Fe^{2+}. AC has been reported to bind various other 3d metal ions, including nickel, copper and platinum (Herrero *et al.,* 2001). The ability of AC to bind the 3d metal ions Fe^{2+} and Fe^{3+} shall be investigated in chapter 7.

6.3. THE EFFECT OF IRON (II) ALONE AND IN COMBINATION WITH AC ON LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE *IN VITRO*

6.3.1. Introduction

In addition to having a high content of peroxidisable PUFAs, the brain also contains high levels of transition metals (Floyd, 1999). In biological systems, the formation of the LP initiating $HO^{\cdot}$ radical, is catalysed by the Fenton reaction, which occurs between H_2O_2 and the transition metals, iron or copper in a low valence state (Halliwell and Gutteridge, 1986), as shown in the equation 6.2. below:

$$Metal^{n+} + H_2O_2 \rightarrow Metal^{n+1} + HO^{\cdot} + OH^{-} \qquad \textbf{Equation 6.2}$$

The Fenton reaction has gained such prominence in neuroscience that the two transition metals have been classified as mediators of oxidative stress in a number of neurodegenerative disorders (Sayre *et al.,* 1999; Thompson *et al.,* 2001).

This reaction may be accelerated by a reducing agent such as ascorbic acid, which promotes the redox cycling of soluble iron complexes (Rauhala *et al.,* 1998b). Ethylenediamine tetraacetate (EDTA) is an anionic hexadentate ligand which has the ability to bind Fe^{3+}. Upon chelation with ferric ions, a soluble complex ion is formed. This prevents the formation of neutral insoluble complexes which cannot be reduced even in the presence of a reducing agent such as ascorbic acid. It has been shown that ferric complexes with EDTA can generate $HO^{\cdot}$ radicals and initiate LP in the presence of reducing agents such as ascorbate (Rauhala *et al.,* 1998b).

In section 6.2.4., evidence that the Fenton reaction is implicated in the lipoperoxidative effect of QA *in vitro* was explored. Thus, in this experiment, the direct effect of the

Fenton reaction on LP was investigated by simulating, as accurately as possible the conditions under which this reaction occurs.

6.3.2. Materials and Methods

6.3.2.1. Animals

Adult male Wistar rats were purchased from the NHLS (Johannesburg, South Africa) and cared for as described in section 2.2.2.1.

6.3.2.2. Chemicals and Reagents

Chemicals and reagents used were as described in section 6.2.2.2., with the exception of QA. In addition, ferrous sulphate ($FeSO_4.7H_2O$) was obtained from Merck, Darmstadt, Germany. EDTA was obtained from Holpro Chemical Corp., Johannesburg, South Africa. H_2O_2 was obtained from BDH Laboratory Supplies, Poole, England and ascorbic acid was obtained from Saarchem, Krugersdorp, South Africa.

6.3.2.3. Brain Removal

This was performed as described in section 5.2.2.3.

6.3.2.4. Preparation of Tissue

The tissue was performed as described in section 5.2.2.4.

6.3.2.5. Preparation of the MDA Standard Curve

This was performed as described in section 6.2.2.5.

6.3.2.6. Lipid Peroxidation Assay

The assay was performed as described in section 6.2.2.6. However, triplicate samples of rat brain homogenate (1 ml) containing 1 mM Fe^{2+} (100 µl) in the absence and presence of increasing concentrations (0–2 mM) of AC (100 µl), together with 100 µM H_2O_2 (100 µl), 100 µM EDTA 100 µl and 10 µM (100 µl) as**o**rbic acid were incubated at 37 ˚C for an hour. Control samples contained no $Fe^{2+}$and no AC.

6.3.2.7. Statistical Analysis

This was performed as described in section 4.2.2.9.

6.3.3. Results

Figure 6.7. illustrates that 1 mM Fe^{2+} increases the amount of MDA produced in comparison to the control by approximately seventy five times. AC has the ability to significantly reduce 1 mM Fe^{2+}-induced LP. This effect does not appear to be entirely concentration dependent as the difference between the amount of MDA formed by co-incubation of 1 mM QA and 1 mM AC (0.97 nmoles/mg tissue) and that formed by co-incubation of 1 mM QA and 2 mM AC (0.95 nmoles/mg tissue) is insignificant.

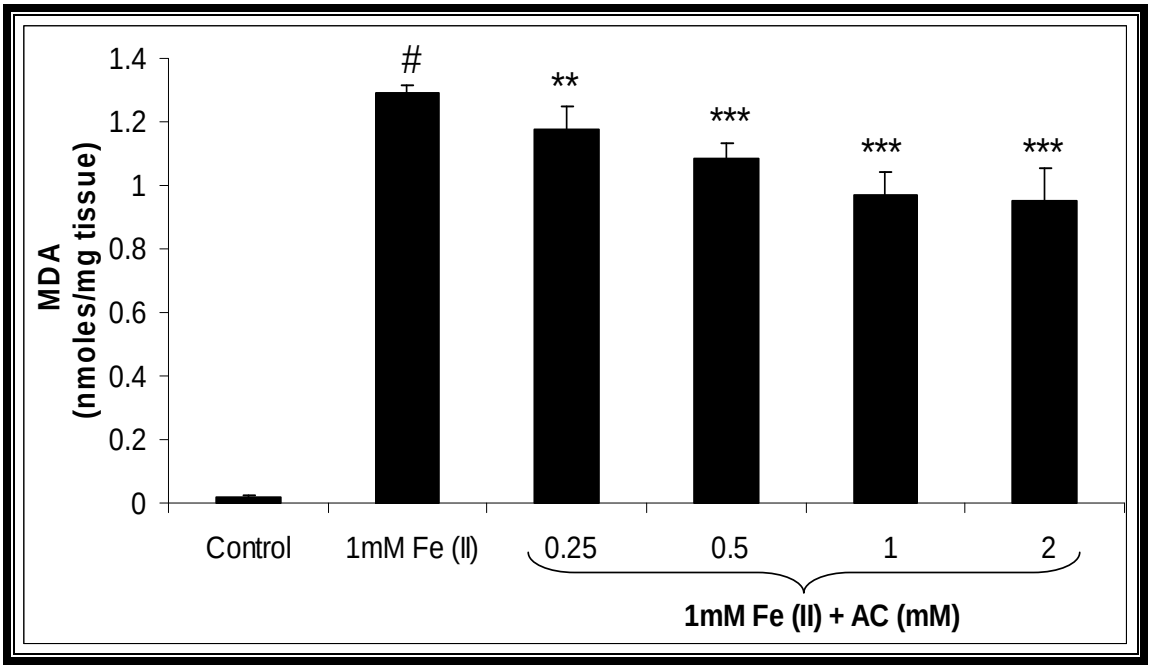


Figure 6.7. Effect of 1 mM Fe^{2+} alone and in combination with AC on LP in rat brain homogenate *in vitro*. Each bar represents the mean ± SD; n = 5. # ($p < 0.001$) in comparison to control; ** ($p < 0.01$) and *** ($p < 0.001$) in comparison to 1 mM Fe^{2+} alone (ANOVA and Student–Newman–Keuls Multiple Range Test).

6.3.4. Discussion

The finding that 1 mM Fe^{2+} produced a significantly higher amount of MDA compared to control indicates that the Fenton reaction has the potential to play a major role in LP. Fe^{2+} ions catalyse the formation of $HO^{\cdot}$ from H_2O_2 (Halliwell and Gutteridge, 1989). These radicals are sufficiently reactive to abstract $H^{\cdot}$ (Halliwell and Gutteridge, 1989) from membrane PUFA to initiate LP. Furthermore, ferrous and ferric ions also react with lipid hydroperoxides already formed as by-products of LP, decomposing them to $ROO^{\cdot}$ and alkoxyl radicals that can reinitiate LP (Halliwell, 1994). A reduced iron complex (e.g. EDTA- Fe^{3+} or QA- Fe^{2+}) reacts with these lipid hydroperoxides in a similar way to its reaction with H_2O_2, i.e. it causes fission of an O-O bond. This forms an alkoxyl radical (Halliwell and Gutteridge, 1989), as shown in equation 6.3.

$ROOH + Fe^{2+}\text{-complex} \longrightarrow Fe^{3+}\text{-complex} + OH^- + R\text{-}O^\cdot$ lipid peroxide alkoxyl radical	**Equation 6.3.**

A Fe^{3+} complex can form both $ROO^\cdot$ and alkoxyl radicals (Halliwell and Gutteridge, 1989) according to the overall equation 6.4.

$R\text{-}OOH + Fe^{3+}\text{-complex} \longrightarrow R\text{-}O\text{-}O^\cdot + H^+ + Fe^{2+}\text{-complex}$ peroxyl radical further reaction to alkoxyl radical	**Equation 6.4.**

The reaction of Fe^{2+} with R-OOH is faster than the reaction with H_2O_2 (Halliwell and Gutteridge, 1989). Thus, if both substrates are available, the reaction with R-OOH will be favoured. When cells are injured, LP is more likely to occur and traces of R-OOH are formed enzymatically in tissues by cyclo-oxygenase and lipoxygenase enzymes (Halliwell and Gutteridge, 1989). When these are decomposed, in the presence of ferrous and ferric ions, alkoxy and peroxy radicals are generated which can abstract $H^\cdot$ and stimulate LP further.

Since AC is able to reduce Fe^{2+}-induced LP, the argument that AC may be binding Fe^{2+} and/or Fe^{3+} is strengthened. However, it is possible that a maximum effect is achieved when the ratio of AC to Fe^{2+} is 1 to 1 because LP was not suppressed by much more when the ratio of AC to Fe^{2+} was greater than 1. This may suggest that the concentration-dependent decrease in QA-induced LP (section 6.3) may be attributed to other factors aside from iron binding, such as the scavenging of free radicals, particularly those associated with Fe^{2+}-induced LP, such as $HO^\cdot$, $O_2^{\cdot-}$ and alkoxyl radicals. The ability of AC to scavenge $O_2^{\cdot-}$ has been elucidated in chapter 5.

In general, of all the various biomolecule monomers, the guanine base is particularly sensitive to oxidation and these products are often used as a biomarker for oxidative injury (Halliwell and Gutteridge, 1989). Guanine reacts with the $HO^{\cdot}$ as illustrated in figure 6.8., to form 8-hydroxyguanine.

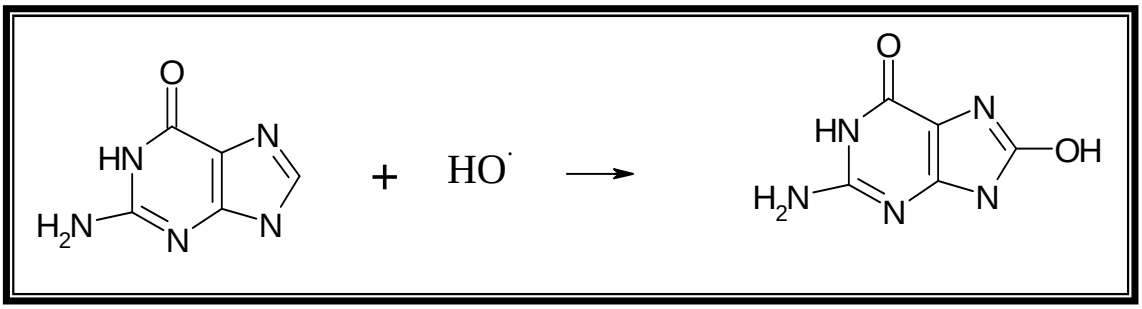


Figure 6.8. Reaction of guanine base with $HO^{\cdot}$ to form 8-hydroxyguanine (Halliwell and Gutteridge, 1989).

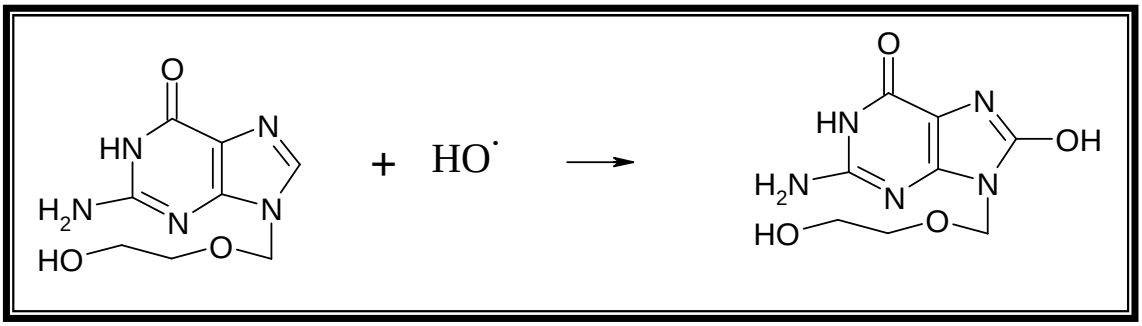


Figure 6.9. Proposed reaction of AC with $HO^{\cdot}$ to form 8-hydroxyAC.

Analogous to guanine, AC, a guanosine analogue may also be sensitive to oxidation and react with $HO^{\cdot}$ in a similar manner as proposed in figure 6.9., and therefore reduce the availability of this radical for reaction with lipid membranes, effectively diminishing the occurrence of LP in rat brain homogenate.

$HO^{\cdot}$ may also be inactivated by AC through the abstraction of $H^{\cdot}$ from the hydroxyl group at position 5 of AC, to form a guanine radical which scavenges $O_2^{\cdot -}$. This is described in section 5.2.4., as part of the $O_2^{\cdot -}$ scavenging of AC.

6.4. THE EFFECT OF QA ALONE AND IN COMBINATION WITH AC ON LIPID PEROXIDATION IN THE RAT HIPPOCAMPUS *IN VIVO*

6.4.1. Introduction

The disruption of the structure and integrity of the lipid membranes of the cell bodies in the CA1 and CA3 regions of the hippocampi of QA only treated rats (chapter 3) may occur due to the cleavage of arachidonic acid and related PUFA by calcium-dependent PLA_2 release after an excitotoxic insult (section 3.2.4.), or otherwise LP which may occur as a result of QA-induced oxidative stress (chapter 5). Therefore, the improvement in this structure and integrity observed in the hippocampi of QA + AC treated rats may be through protection of AC against QA-induced LP in rat hippocampal homogenate *in vivo*.

Ultimately, this section aims to assess the deleterious effect, if any, of the mechanisms of neurodegeneration, such as excitotoxicity (chapter 4) and oxidative stress (chapter 5), initiated by QA *in vivo,* on lipid biomolecules and to determine if AC could alter this. To reiterate what was mentioned in section 1.2.3.4. and 6.1., the importance of maintaining the structure and integrity of lipid membranes lies in its role in normal cell functioning. Impairment of this arrangement may (1) decrease fluidity and increase non-specific permeability to ions. (2) inactivate membrane-bound receptors and enzymes (Gutteridge and Halliwell, 1990).

6.4.2. Materials and Methods

6.4.2.1. Animals

Adult male Wistar rats were purchased from the NHLS (Johannesburg, South Africa) and cared for as described in section 2.2.2.1.

6.4.2.2. Chemicals and Reagents

Chemicals and reagents used were as described in section 6.3.2.2. Sodium phenobarbitone was purchased from Merck, Germany and was at a working concentration of 60 mg/ml.

6.4.2.3. Surgical Procedures

These procedures were conducted as described in section 2.2.2.4.

6.4.2.4. Drug Treatment

This was performed as described in section 2.2.2.5.

6.4.2.5. Brain and Hippocampi Removal

The rats were killed and the brains and hippocampi removed in the manner described in section 2.2.2.7. However, the rats were killed the day after the last dose of the drug treatment.

6.4.2.6. Preparation of Tissue

The preparation of each portion of the hippocampal tissue was performed as described for each whole brain in section 5.2.2.4. However, the homogenate concentration was 5 % w/v instead of 10 % w/v.

6.4.2.7. Preparation of the MDA Standard Curve

This was performed as described in section 6.2.2.5.

6.4.2.8. Lipid Peroxidation Assay

The LP assay was performed as described in section 6.2.2.6. However, no exogenous QA or AC was added and the first incubation at 37 ˚C for one hour was omitted. The assay resumed with the termination of LP by addition of TCA and BHT to the triplicate samples of rat hippocampal homogenate (1 ml).

6.4.2.9. Statistical Analysis

This was performed as described in section 4.2.2.9.

6.4.3. Results

As shown in figure 6.10, it appears that the intrahippocampal injection of QA (120 nmoles) causes approximately a three-fold increase ($p < 0.001$) in MDA levels in comparison to the control treated rats. However, the twice daily i.p. injections of AC (5mg/kg) for five days are able to significantly reduce the QA-induced MDA production ($p < 0.001$).

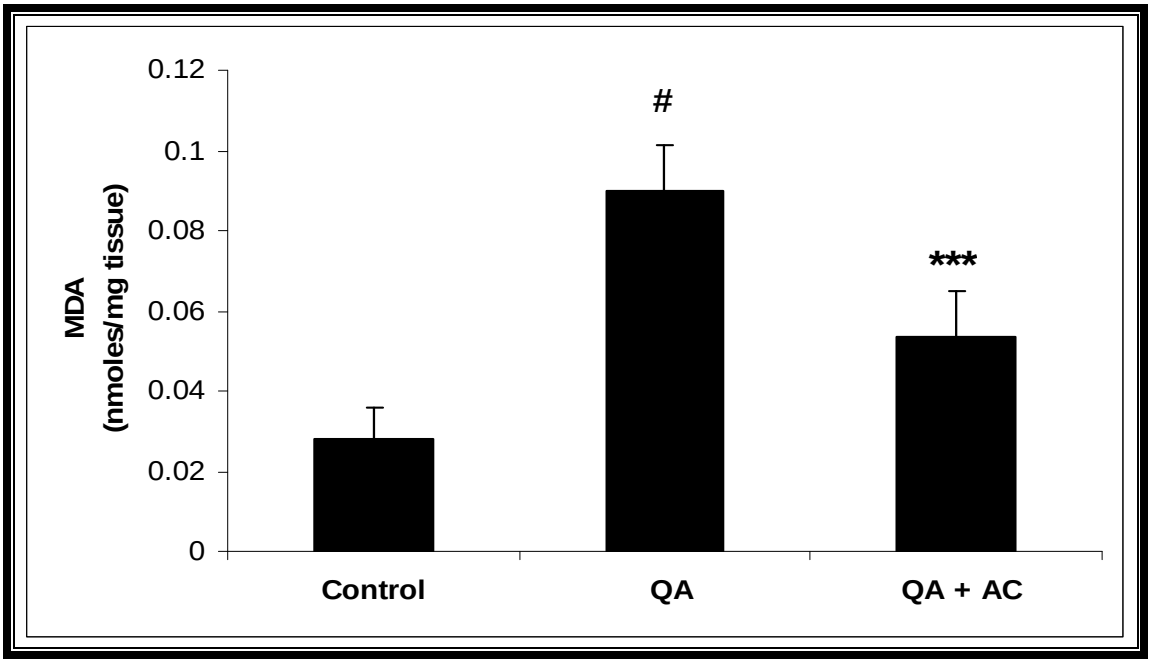


Figure 6.10. Effect of QA alone and in combination with AC on LP in rat hippocampal homogenate *in vivo*. Each bar represents the mean ± SD; n = 5. # ($p < 0.001$) in comparison to control; *** ($p < 0.001$) in comparison to QA (ANOVA and Student–Newman–Keuls Multiple Range Test).

6.4.4. Discussion

The effect of QA on the formation of LP products from hippocampal tissue in this study is in agreement with the results of Behan *et al*., (1999). These authors found that melatonin, a potent free radical scavenger and a stimulant of antioxidant enzyme synthesis (section 1.3.4.5.), is able to reduce 120 nmoles QA-induced LP in rat hippocampus *in vivo* and thus suggest that free radical formation contributes significantly to QA-induced damage. In Chapter 5, the ability of AC to reduce QA-induced oxidative stress in rat hippocampus *in vivo* was demonstrated. Therefore, through this mechanism, there will be fewer free radicals available to attack lipids and initiate LP, which may explain the amelioration of QA-induced LP by AC in rat hippocampus *in vivo.*

As expected, these results exhibit an inverse relationship to the degree of integrity of the cell membranes of the cell bodies of the hippocampal neurons in section 3.2. QA alone produced a decrease in the integrity of the cell membranes which was coupled with an increase in LP, in comparison to the control. In contrast, QA + AC treatment had an improved integrity of cell membranes and reduced LP in comparison to QA alone. It can therefore be concluded that the integrity of neural membranes in general is highly dependent on the degree of LP. As explained in section 6.1., LP may often occur as a result of cell damage or death, thus a correlation between the extent of cell damage and / or death and the extent of LP exists and these results may serve to confirm the findings of chapter 3.

NMDA and insulin receptors are known to have sulfhydryl groups which are damaged during LP (van der Vliet and Bast, 1992). Thus, the LP induced by the intrahippocampal injection of QA may inactivate the NMDA receptors of the glutamatergic system in the hippocampus, reducing the incidence of LTP at the glutamatergic synapses which have survived the excitotoxic insult. The amelioration of LP may therefore be an additional mechanism by which AC treatment improves the spatial memory deficits (Chapter 2) after intrahippocampal injections of QA.

It may be argued that if the NMDA receptors are inactivated during LP, excitotoxicity should also be dampened and the further effects thereof, such as LP should diminish over time. However, instead, hippocampal LP was enhanced 5 days after an intrahippocampal injection of QA. This points to the possibility that other mechanisms are involved in QA-induced LP *in vivo*. Shoham *et al.*, (1992) have shown that after single unilateral injections of QA into the rat ventral-striatal region, iron accumulates in high concentrations in the basal ganglia area such as globus pallidus and substantia nigra pars reticulate. Hence, it is possible that the intrahippocampal injection of QA has a similar effect on iron accumulation in the hippocampus, consequently forming the QA-iron complex which may also have a role to play in enhancing the production of the $HO^{\cdot}$ *in vivo* and in this manner initiate LP independent of excitotoxicity (NMDA stimulation).

CHAPTER 7

IRON CHELATION STUDIES

7.1. INTRODUCTION

Iron is an essential constituent of a number of proteins involved in oxygen transport or metabolism (Winterbourn, 1995). It is transported in the body, stored and made available for synthesis of these iron proteins (Brock, 1994; De Jong *et al.,* 1990). The ability of iron to undergo cyclic oxidation and reduction is an important aspect of its function. However, as described in sections 1.2.2.2.5., 6.2. and 6.3., such redox activity can generate free radicals and other strongly oxidising species capable of causing a wide range of biological injuries (Winterbourn, 1995), particularly via the Haber-Weiss and Fenton reactions (Halliwell and Gutteridge, 1984b).

Iron has also been associated with the pathological lesions of AD (Smith *et al.,* 1997). Accumulation of iron has been shown to be associated with AP and NFT, indicating alterations of iron homeostasis in AD, which is further supported by the elevated serum levels of the iron binding protein p97 found in patients with AD (Kennard *et al.,* 1996). Moreover, iron greatly increases the vulnerability of neurons to amyloid toxicity, which involves membrane LP resulting in the production of the aldehyde 4-hydroxynonenal, which, in turn, impairs the function of membrane ATPases and glucose and glutamate transporters (Goodman and Mattson, 1994; Mark *et al.,* 1997a,b).

Iron is also essential for the biosynthesis of the deoxyribonucleotides required for DNA synthesis and repair (Aisen *et al.,* 1999). DNA viruses, such as HSV-1 are directly dependent on iron for their proliferation since iron plays a role in the catalytic centre of ribonucleotide reductase (Cooper *et al.,* 1996; Lamarche *et al.,* 1996; Chabes *et al.,* 2000). Therefore, the presence of free iron may enhance HSV-1 replication, thereby facilitating increased viral infectivity in HSE.

Depression may be accompanied by the induction of an immune response which includes the production of pro-inflammatory cytokines (section 1.1.4.4.). Other features of this inflammatory process include alterations in iron metabolism (Fairbanks and Beutler, 1988; Brock, 1994), such as decreased serum iron and transferrin (Fairbanks and Beutler, 1988; Brock, 1994), the iron protein transporter, which delivers an iron supply to the tissues (Brock, 1994; De Jong *et al.,* 1990). Thus, in such patients, the provision of iron for the synthesis of iron proteins is reduced and may contribute to "sickness behaviour" consisting of psychomotor retardation, anorexia, weight loss, lethargy and sleep disorders (Kleesiek and Greiling, 1984; Kent *et al.,* 1992). These signs and symptoms are common in patients with major depression (Maes *et al.,* 1993).

Iron deprivation and/or the removal of free iron from plasma and cells may be beneficial in neurodegenerative disorders which are associated with iron-catalysed oxidative stress, particularly if the accumulation of iron is prevalent, as in AD. In HSE, reduction in the replication of HSV-1 is an additional advantage. However, such therapy cannot afford to be drastic as it may aggravate iron-related "sickness behaviour", in neurodegenerative disorders, such as HSE or depression where serum iron levels are low or normal.

By definition, chelation requires the presence of two or more atoms on the same molecule capable of forming a coordinate bond, an interaction between the electron donor (chelating ligand) and an electron acceptor (metal) to form a complex metal ion (Galey, 1997). Depending on the number of donor groups associated with the chelating ligand, varying ligand to metal ratios may occur to satisfy the coordination requirements of the metal ion. Both ferrous and ferric ions have a coordination number of 6 (Galey, 1997). Bidentate ligands therefore form 3 to 1 iron complexes, whereas tridentate ligands form 2 to 1 complexes.

There exists an ambiguous relationship between iron and its interaction with compounds with which it forms co-ordinate bonds. For example, many ferrous chelates, such as the QA-iron complex referred to in chapter 6 react with hydrogen peroxide to produce an

oxidising species, namely $HO^{\cdot}$ (Winterbourn, 1995). On the other hand, other chelates, such as the ATP-iron complex are not effective at driving the Fenton reaction (Winterbourn, 1995). The former category of chelates would enhance oxidative stress and oxidation of biomolecules whilst the latter would prevent the participation of iron in redox reactions, thus limiting its physiological and pathological activity.

Using qualitative analytical techniques, this chapter aims to firstly determine whether an interaction exists between AC and iron, in both the ferrous and ferric oxidation states. Secondly, an attempt will be made to characterise the interactions, if any, particularly with respect to the strength of the AC-Fe^{2+} complex in relation to the QA-Fe^{2+}. The results of these studies will predict whether the hypothesis that AC binds iron in the ferrous and ferric forms holds true or not. Such an effect would diminish the redox activity of iron and therefore its ability to catalyse the formation of $HO^{\cdot}$ and induce LP (chapter 6).

7.2. ULTRAVIOLET AND VISIBLE SPECTROSCOPIC ANALYSIS OF AC ALONE AND IN COMBINATION WITH IRON (II) AND IRON (III)

7.2.1. Introduction

The fundamental principle underlying spectrophotometric techniques is that all chemicals to some extent absorb electromagnetic radiation of specific wavelengths (Newman, 1964). These techniques measure the extent to which electromagnetic radiation is absorbed by any chemical substance. Ultraviolet and visible (UV/VIS) spectroscopy specifically measures the absorption of electromagnetic radiation in the UV/VIS range of the electromagnetic spectrum, which extends from 200 nm to 800 nm.

Electromagnetic radiation absorbed in the UV region produces changes in the electronic energy of the molecule resulting from transitions of valence electrons in the molecule (Silverstein *et al.*, 1981). These transitions consist of the excitation of an electron from an occupied molecular orbital, usually a non-bonding p or bonding π orbital to the next higher energy orbital, an antibonding π^* or σ^* orbital (Silverstein *et al.*, 1981). As a result, unsaturated organic compounds in particular absorb UV light (Newman, 1964).

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 (Newman, 1964). The principle characteristics of any absorption band in an absorption spectrum are its position and intensity (Silverstein *et al.*, 1981). The position of absorption corresponds to the wavelength of radiation whose energy is equal to that required for an electronic transition. The intensity of absorption is dependent on two factors: (a) the probability of interaction between the radiation energy and the electronic system and (b) the difference between the ground and excited state (Silverstein *et al.*, 1981). Therefore, any change in structure or composition of a compound will result in a

change in the electronic system, thereby introducing alterations in the spectrum; either a shift in wavelength or a change in the intensity of the absorbance (Newman, 1964).

7.2.2. Materials and Methods

7.2.2.1. Chemicals and Reagents

AC sodium (Zovirax®, powder for injection) was obtained from Glaxo Wellcome, Midrand, South Africa. Hydrated ferrous sulphate ($FeSO_4.7H_2O$) and anhydrous ferric chloride ($FeCl_3$) were purchased from BDH Laboratory Supplies, Poole, England. All solutions were prepared in deaerated Milli-Q water to prevent autooxidation.

7.2.2.2. UV/VIS Spectroscopy

The interaction between AC and Fe^{2+} and Fe^{3+} was studied by comparing the absorption spectrum of AC alone in solution to those of AC in equimolar combinations with each metal ion (0.125 mM-0.5 mM). All the samples were analysed on a GBC UV/VIS 916 spectrophotometer, using quartz cuvettes with a pathlength of 0.1 cm.

7.2.3. Results

Figures 7.1 and 7.2 show that free AC has an absorption band from 227.2 nm to 298.4 nm, with a λ_{max} of 252.8 nm and a shoulder extending from 262.4 nm to 298.4 nm. Upon the addition of Fe^{2+} (figure 7.1), there is an increase in the absorption intensity and a blue shift of λ_{max} by 2.4 nm from 252.8 nm to 250.4 nm. In the presence of Fe^{3+} (figure 7.2.), a blue shift of the band at 252.8 nm to 250.4 nm is also observed with a greater increase in absorption intensity. The observations are also noted at the other concentrations (0.25–0.5 mM).

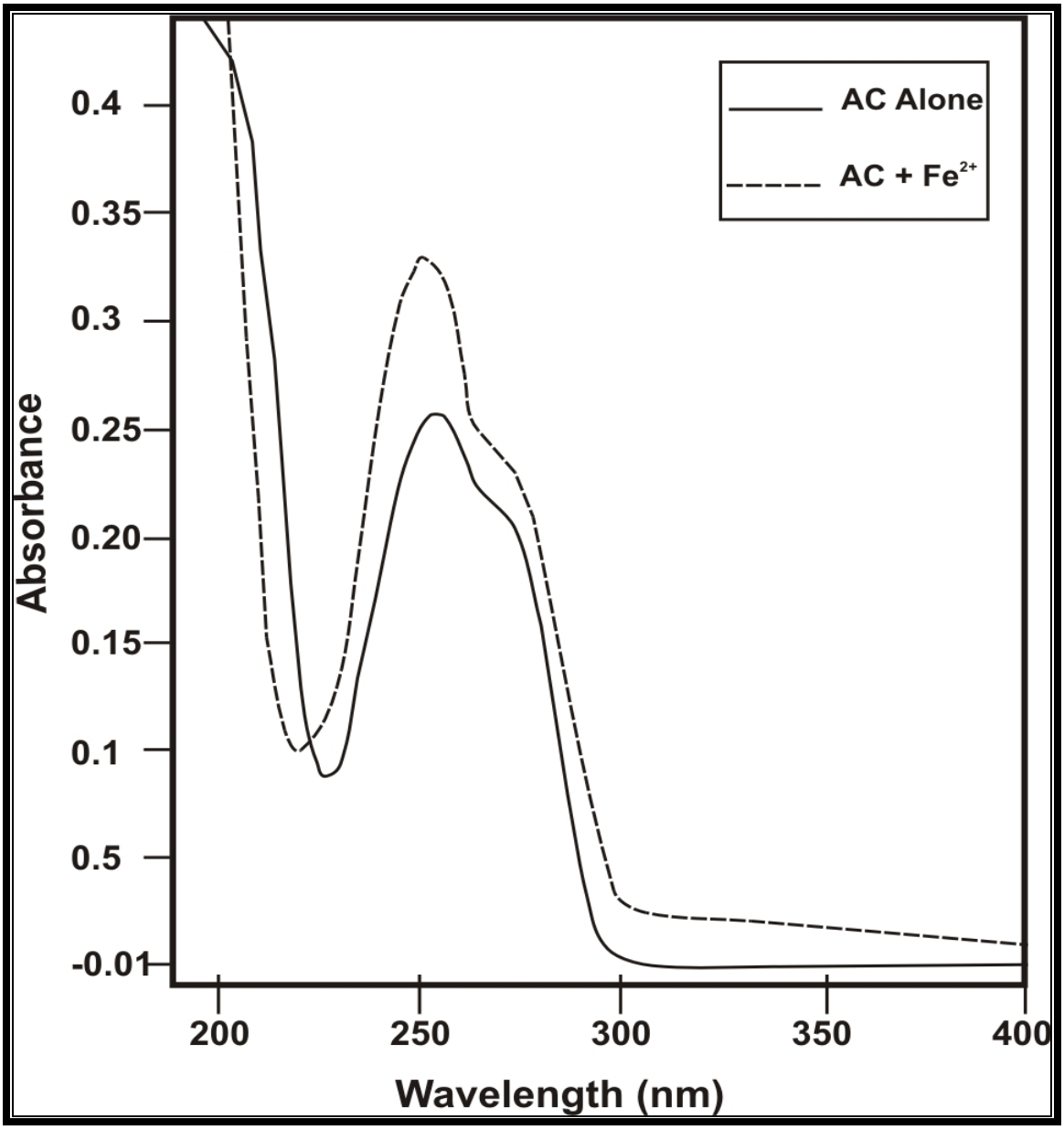


Figure 7.1. UV spectroscopic analysis of AC alone and in the presence of an equimolar concentration (0.125 mM) of Fe^{2+}.

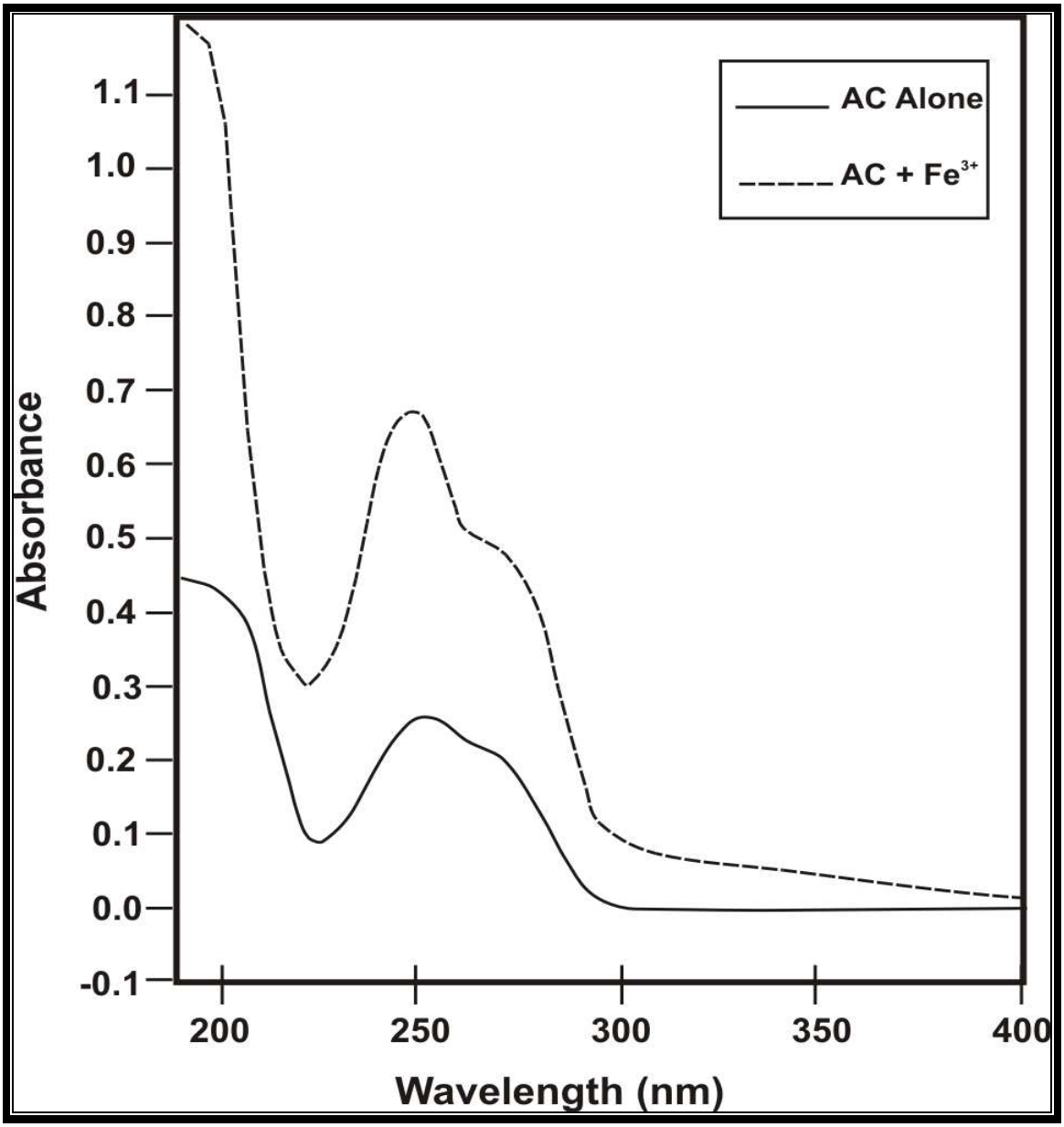


Figure 7.2. UV spectroscopic analysis of AC alone and in the presence of an equimolar concentration (0.125 mM) of Fe^{3+}.

7.2.4. Discussion

The λ_{max} absorption band of free AC at 252.8 nm is due to an aromatic, $\pi \rightarrow \pi^*$ electronic transition (Silverstein *et al.*, 1981). The absorption of sulphides, disulphides, thiols, amines, bromides and iodides frequently appear only as a shoulder or an inflection (Silverstein, 1981), thus the shoulder in the spectra (figures 7.1 and 7.2) most likely represents the amine group in AC (figure 1.15).

Possible complexation on addition of both Fe^{2+} and Fe^{3+} is evidenced by the increase in the intensity of the absorbance and by the blue shift of λ_{max} by 2.4 nm, which indicate that a change in the electronic system of AC has occurred (section 7.2.1). In co-ordinate bonding with metals, it is the ligand which provides both electrons for each bond. Thus, a good ligand is likely to be a compound which has a number of non-bonding electrons. AC (figure 1.15) has auxochromic substituents such as N and O-containing functional groups which have non-bonding electrons. The blue shift to a wavelength of higher energy may result from an increase in the energy required for an electronic transition to occur because of the reduced availability of non-bonded electrons in a possible AC-iron complex.

As mentioned previously, the intensity of absorption is largely dependent on two factors: (a) the probability of interaction between the radiation energy and the electronic system and (b) the difference between the ground and excited state (section 7.2.1.). The probability of transition [(a) above] is proportional to the change in the electronic charge distribution occurring during excitation, therefore a greater intensity of absorption is reflected on the absorption spectrum when the electronic transition during excitation is accompanied by a larger change in the electronic charge distribution (Silverstein *et al.*, 1981). During co-ordinate bonding, the electrons of the ligand move towards the metal ion, therefore any charge on the free ligand or metal ion is no longer isolated but spread over the entire complex, known as a complex ion. This change in the electronic structure may effect a larger change in the electronic charge distribution during the electronic transition and may thus explain the increase in absorption intensity on addition of the ferrous or ferric ions. The larger intensity of absorption of AC in combination with Fe^{3+}

than with Fe^{2+} may indicate that the interaction between AC and Fe^{3+} is stronger than that of AC and Fe^{2+}.

It has been suggested that purines act as chelating ligands of metals through the N(7) and the exocyclic atom attached to C(6) (Turel *et al.,* 1998). However, in aqueous systems, true chelation has only been observed in 6-thiopurines. Acyclovir is a 6-oxopurine (figure 7.3.), in which the typical bonding pattern in the metal complexes can be best described as indirect chelation where a water molecule in the coordination sphere forms interligand hydrogen bonds to the exocyclic substituent at C(6) (Turel *et al.,* 1998). In some cases, metal-AC bonds may also form through the hydroxylic oxygen atoms of a bridging AC molecule bound via the N (7), as in the cadmium complex (Garcia-Raso *et al.,* 1999). The proposed metal-ligand bonding of AC is shown in figure 7.4.

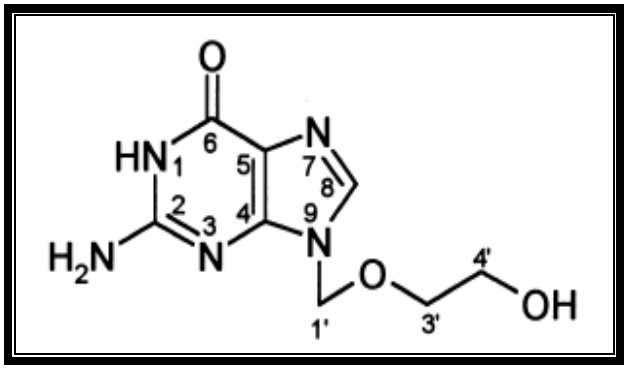


Figure 7.3. Structure of AC with atoms numbered (Turel *et al.,* 1998).

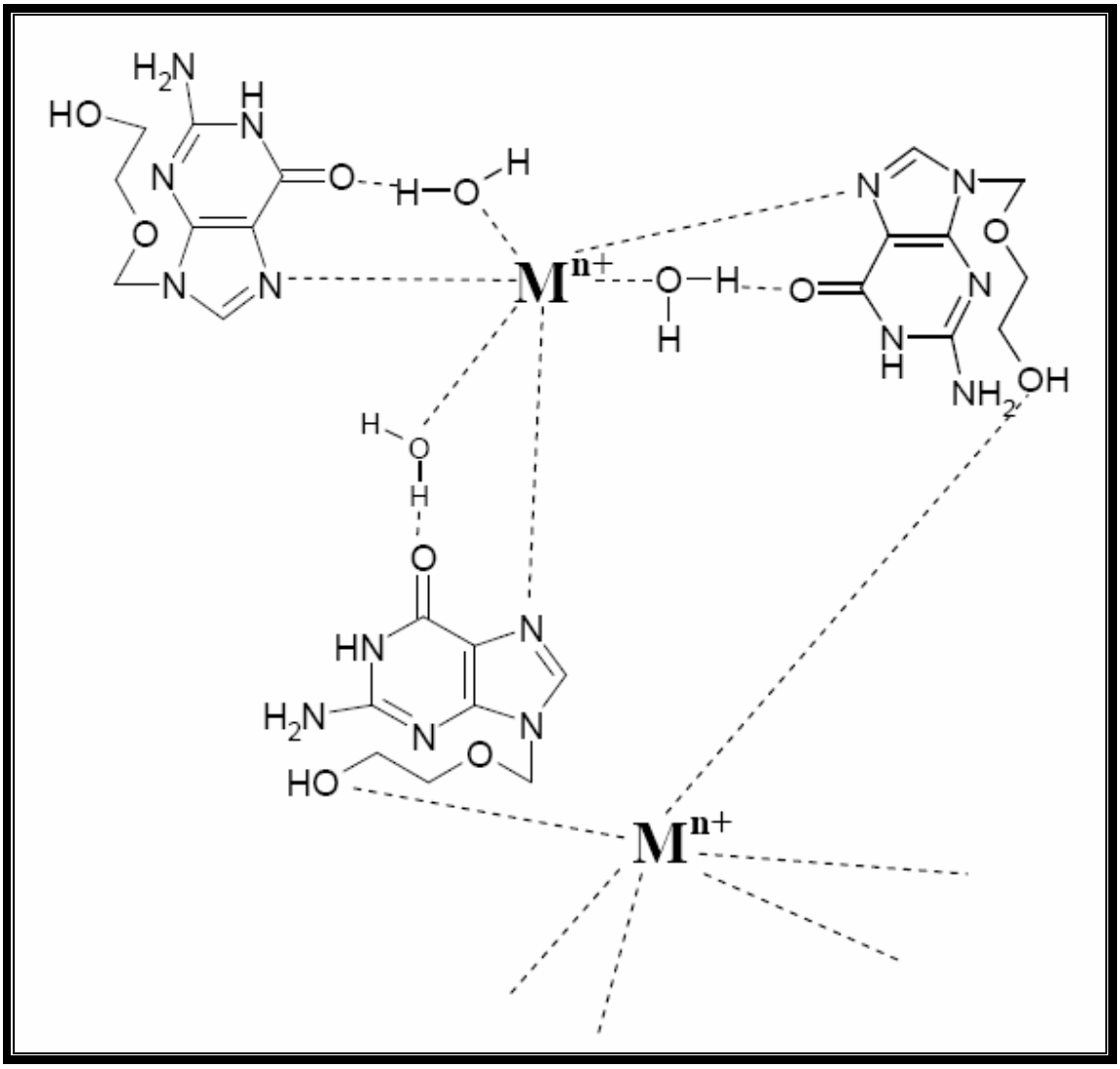


Figure 7.4. Proposed metal-AC chelation, showing the functional groups involved in the coordinate bonding.

7.3. AN ELECTROCHEMICAL ANALYSIS OF IRON (III) AND IRON (II) ALONE AND IN THE PRESENCE OF AC OR QA

7.3.1. Introduction

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). Electrochemical cells usually consist of three electrodes, namely, a working, a reference and an auxilliary electrode (Bard and Faulkner, 1980). The electron transfer reactions occur at the surface of the working electrode. These reactions are monitored in relation to a reference electrode, which maintains a constant potential. The current passes between the working electrode and the auxilliary electrode, the latter serving to complete the circuit (Bard and Faulkner, 1980).

Metal ions in solution are able to accept and donate electrons. This electroactive nature makes both qualitative and quantitative analysis possible. For a trace metals in solution it is necessary to employ a preconcentration step to isolate it at the working electrode where the reactions take place (Limson, 1998). This may be performed electrolytically or spontaneously. Adsorptive stripping voltammetry (ASV) is an electrochemical technique in which natural tendency of analytes to preconcentrate at the electrode is exploited (Limson, 1998). This extends the use of stripping voltammetry to organic compounds which cannot be electrolytically deposited (Wang, 1989). Furthermore, in metal analysis, ASV allows for the preconcentration of any oxidation state and not only the metallic state (Limson, 1998).

The first step in ASV is the formation of a metal-ligand complex (equation 7.1), followed by controlled interfacial accumulation (adsorption) of the complex onto the electrode at a fixed deposition potential during the deposition step (equation 7.2) (Limson *et al.*, 1998). The third phase is the electrochemically active stripping step (Limson *et al.*, 1998). It involves the application of a potential in the negative direction, during which reduction of

the adsorbed metal complex occurs, releasing the metal and ligand back into solution (equation 7.3) (Limson *et al.,* 1998). Thus, the third step further specifies the technique as adsorptive cathodic stripping voltammetry. The flow of electrons during the last step produces a cathodic current which peaks at the reduction potential of the complex. This current is a direct measure of the rate at which reduction occurs (Limson, 1998). The resulting current potential plot is a voltammogram.

$M^{n+} + x(Ligand)_{aq} \rightarrow M(Ligand)_{x,aq} + xnH^{+}$	Equation 7.1.
$M(Ligand)_{x,aq} \rightarrow M(Ligand)_{x,ads}$	Equation 7.2.
$M\,(Ligand)_{x,ads} + xnH^{+} + ne^{-} \rightarrow M^{n+} + x(Ligand)_{aq}$	Equation 7.3.

A metal species will produce a characteristic voltammogram, with current being proportional to the concentration of the species, due to the linear relationship between current, the amount of species adsorbed onto the electrode and the bulk concentration of the species in the electrolyte (Limson, 1998). When a suitable ligand is added to a metal solution, the ligand facilitates movement of the metal to the surface of the electrode, possibly due to the field dipole interactions between the electrode double layer and functional groups of the ligand. (Limson, 1998). Theoretically then, a ligand which forms a bond with a metal should bring about an increase in current response observed. There may also be 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 an increase in current response of the metal on addition of the ligand, while the extent of a shift in the reduction potential is a measure of the stability of the complex (Limson *et al.,* 1998). A lowering in current response at relatively high ligand concentrations indicates possible competition by free ligand with the metal-ligand complex for binding sites at the electrode, thereby decreasing the space available for adsorption of the metal-ligand complex (Limson, 1998). At low ligand concentrations, this is more likely due to the formation of strong metal-ligand bonds where the metal is not easily reduced (Limson,

1998) thus impeding cathodic current flow and decreasing the rate at which reduction occurs. A shift in potential may also be indicative of metal-ligand bond strength (Limson, 1998). Since reduction potential is the tendency of a chemical species to acquire electrons and become reduced, a negative potential shift indicates that the metal-ligand complex has a lower tendency to become reduced. A large shift to more negative potentials may occur as a result of a strong metal-ligand interaction. The converse may be ascribed to a large positive shift, which is associated with a weaker metal-ligand interaction.

7.3.2. Materials and Methods

7.3.2.1. Chemicals and Reagents

As described in section 7.2.2.1. QA was purchased from Sigma Chemical Co., St. Louis, USA.

7.3.2.2. Apparatus

Adsorptive cathodic stripping voltammograms (AdCSVs) were recorded on an Autolab PGSTAT 30 voltammeter equipped with a Metrohm VGA cell stand. A 3 mm 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 auxilliary electrodes respectively. Prior to use and between scans, the GCE was cleaned by polishing with alumina on a Buehler pad, followed by washing in nitric acid and rinsing in Milli-Q water.

7.3.2.3. Adsorptive Stripping Voltammetry

An appropriate concentration of either $FeSO_4.7H_2O$ or $FeCl_3$ was introduced into the electrochemical cell containing the electrolyte, 0.2 M tris-HCl buffer, pH 7.4 which was then deaerated for 5 minutes. Thereafter, an optimum deposition potential for each metal was identified and applied for 60 s to effect the formation and adsorption of the metal ion onto the GCE. A potential scan in the negative direction from the deposition potential to

at least 0.50 V beyond the reduction potential of the metal was applied, at a scan rate of 0.1 $V.s^{-1}$, to strip the adsorbed metal species from the electrode. During the stripping step, current responses due to the reduction of the metal species were measured as a function of potential to generate voltammograms. The GCE was then cleaned as described in section 7.3.2.2. The procedure was repeated between successive additions of an appropriate concentration of the ligand (0–0.06 mM) to the electrolyte containing the metal ion in the electrochemical cell.

Current vs concentration and potential vs concentration plots were constructed to measure the extent of shifts in current response and reduction potential of the metal species with increasing concentrations of the ligand.

7.3.3. Results

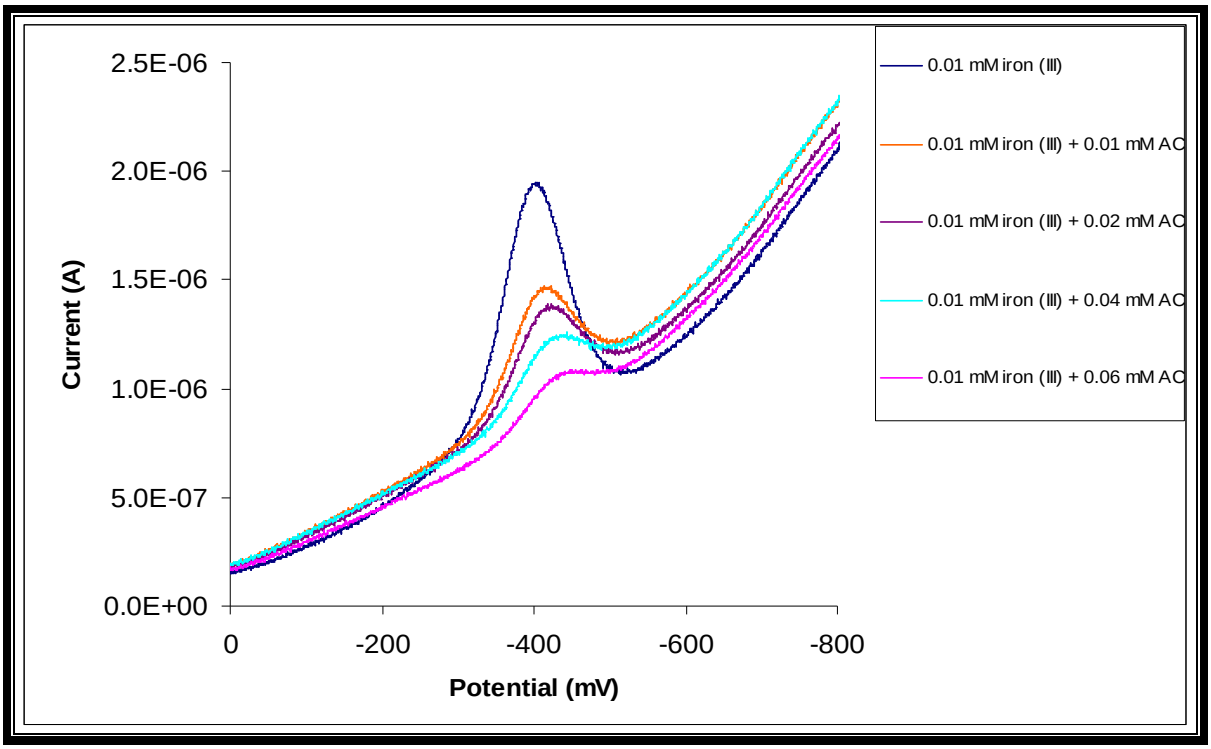


Figure 7.5. AdCSVs of Fe^{3+} (0.01 mM) alone and in the presence of increasing (0.01–0.06 mM) concentrations of AC.

In figure 7.5., the AdCSV for 0.01 mM Fe^{3+} alone in 0.2 M tris-HCl, pH 7.4 buffer shows a peak reduction potential of 1.952 x 10^{-6} A at -401.8 mV. The addition of increasing concentrations of AC causes a significant concentration-dependent shift in the reduction potential and decrease in current response (figure 7.5).

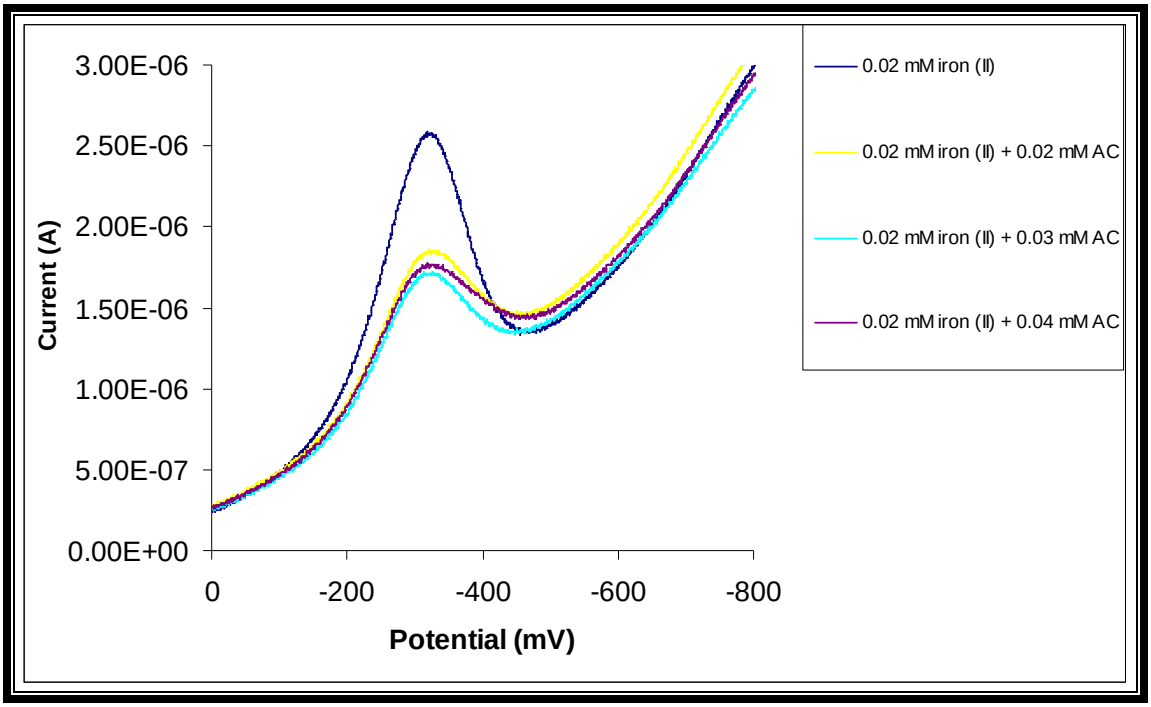


Figure 7.6. AdCSVs of Fe^{2+} (0.02 mM) alone and in the presence of increasing (0.02–0.04 mM) concentrations of AC.

In figure 7.6., the AdCSV for 0.02 mM Fe^{2+} alone in the electrolyte shows a peak reduction potential of 2.582 x 10^{-6} A at -322.4 mV. In the presence of 0.02 mM and 0.03 mM AC, there is a concentration-dependent decrease in the current response with a slight shift in the reduction potential of 1.2 mV to a more positive potential, which remains constant at both concentrations (figure 7.6). However, upon addition of 0.04 mM AC, where the metal to ligand ratio is 1 to 2, there is a slight increase in current response of 0.027 x 10^{-6} A and a very slight shift of 0.2 mV in the reduction potential to a more negative potential, in comparison to 0.03 mM AC.

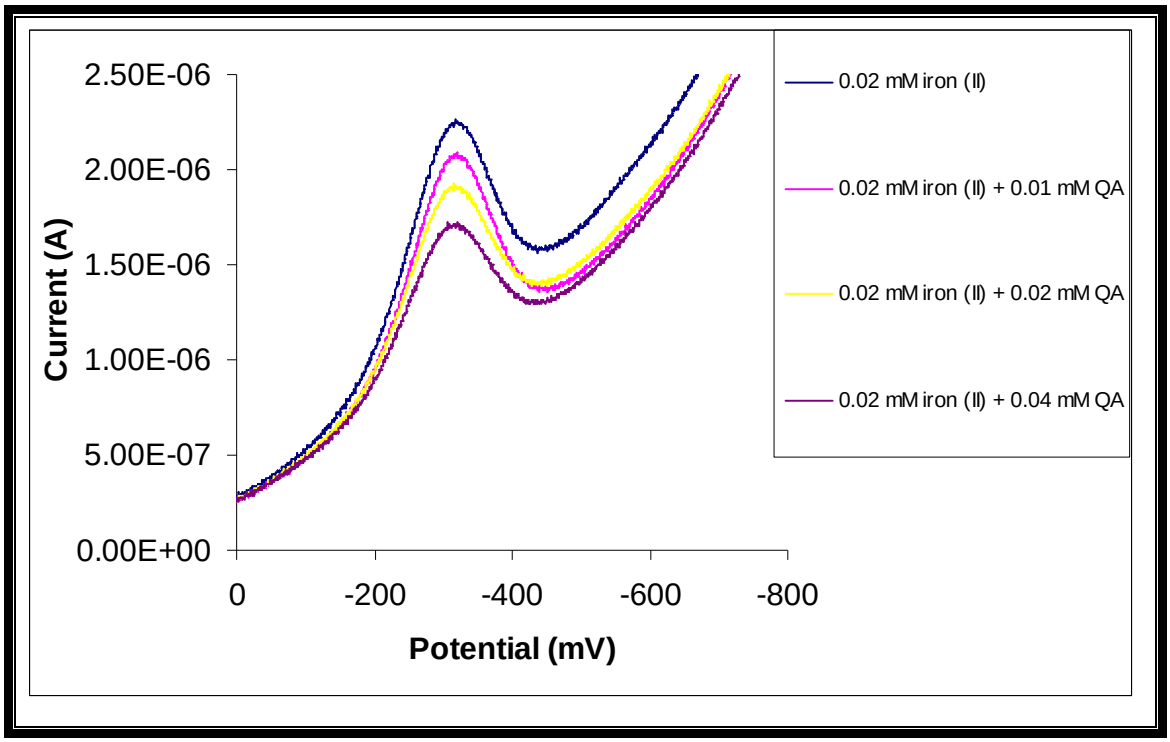


Figure 7.7. AdCSVs of Fe^{2+} (0.02 mM) alone and in the presence of increasing (0.01–0.04 mM) concentrations of QA.

Figure 7.7. shows that QA elicits a concentration-dependent decrease in the current response of 0.02 mM Fe^{2+}. At a metal to ligand ratio of 2 to 1, there is a slight shift of 0.6 mV in reduction potential to more negative potentials. A positive shift back to that of Fe^{2+} alone occurs at a metal to ligand ratio of 1 to 1. Finally, at a metal to ligand ratio of 1 to 2, there is a comparatively large shift of 3 mV to more negative potentials.

Figures 7.8. and 7.9. aptly illustrate the concentration-dependent decrease in reduction potential and peak current response respectively of 0.01 mM Fe^{3+}, exhibited by AC. The gradients of the curves are -6.638 (ΔE_p) and -0.1227 (ΔI_A) respectively.

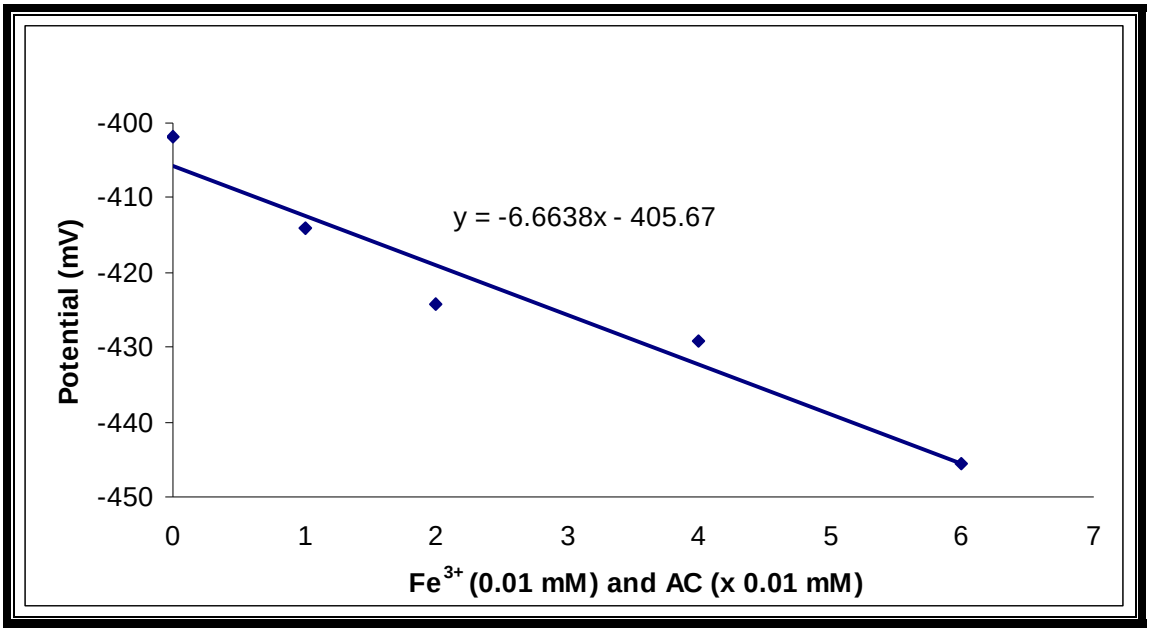


Figure 7.8. Effect of increasing concentrations of AC on the reduction potential of 0.01 mM Fe^{3+}.

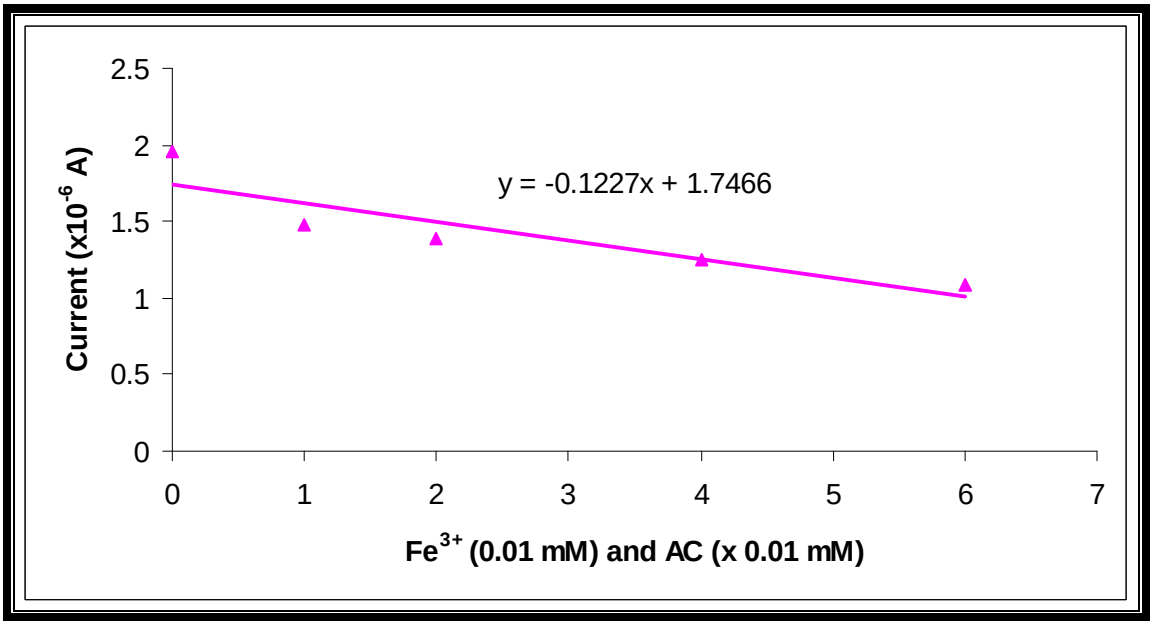


Figure 7.9. Effect of increasing concentrations of AC on the peak current response of 0.01 mM Fe^{3+}

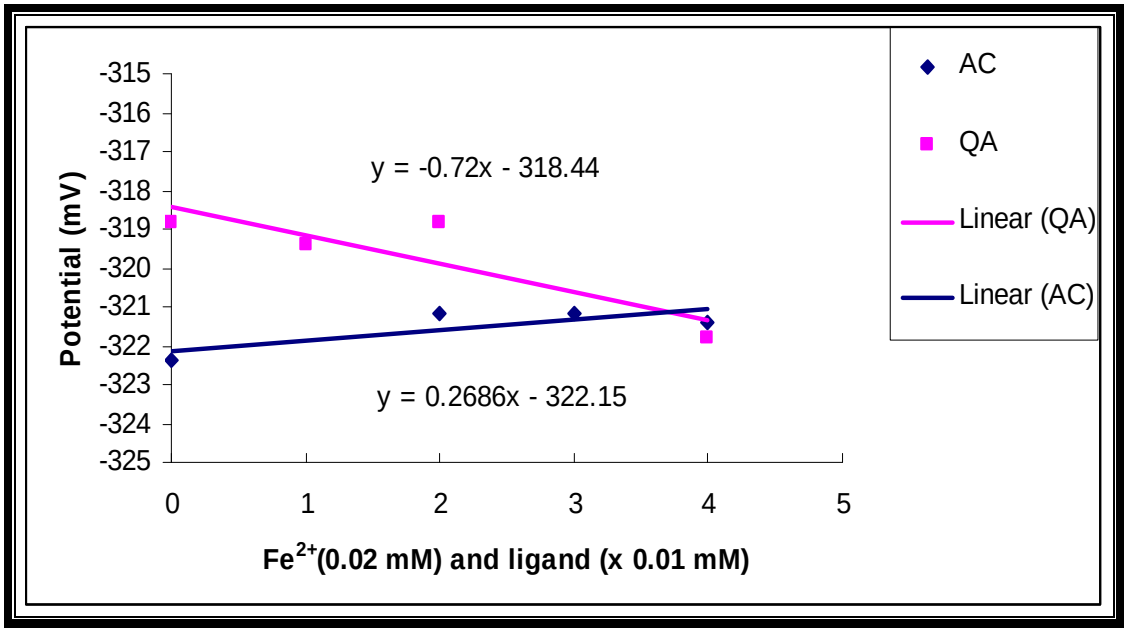


Figure 7.10. Effect of increasing concentrations of AC and QA on the reduction potential of 0.02 mM Fe^{2+}

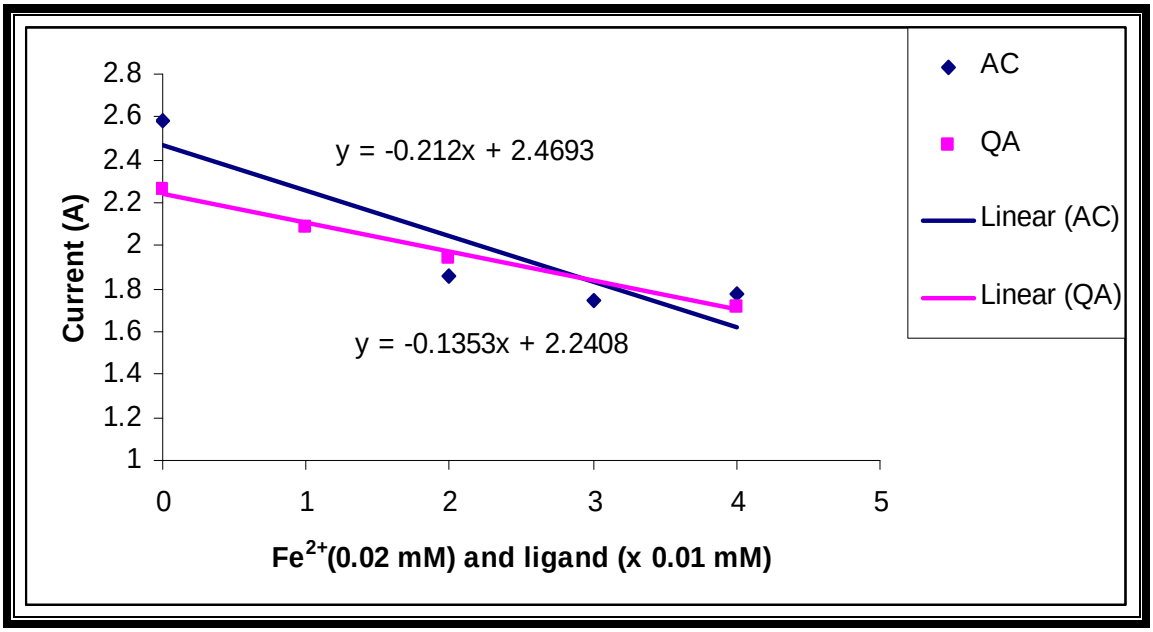


Figure 7.11. Effect of increasing concentrations of AC and QA on the peak current response of 0.02 mM Fe^{2+}

Figure 7.10. depicts an overall decrease in reduction potential of Fe^{2+} with a gradient (ΔE_p) of -0.72 in the presence of increasing concentrations of QA. A converse effect with ΔE_p of 0.2686 is observed with AC. Figure 7.11. shows that both ligands produce an overall reduction in peak current response of Fe^{2+} with concomitant increases in concentration. This effect is greater for AC (ΔI_A = -0.212), than for QA (ΔI_A = 0.1353).

7.3.4. Discussion

Chelators affect the reduction potential of iron, and therefore its effectiveness as a catalyst (Winterbourn, 1995). Equation 1.1 of the Fenton reaction (section 1.2.2.2.5.) involves the reduction of Fe^{3+} to Fe^{2+} and drives equation 1.2 (section 1.2.2.2.5.) in which H_2O_2 is reduced to $HO^{\cdot}$ with a concomitant oxidation of Fe^{2+} back to Fe^{3+}. Therefore, chelators which alter the reduction potential of Fe^{2+} and/or Fe^{3+}, such that it favours the reduction of Fe^{3+} or the oxidation of Fe^{2+}, will likely enhance the production of the potent $HO^{\cdot}$. Similarly, chelators which have the opposite effect will curb its formation.

The reduction potential is an indication of the ease with which a species is reduced. AC has the ability to form a complex with Fe^{3+} which has a more negative reduction potential than Fe^{3+} alone (figure 7.5.). Therefore, AC forms a strongly bonded complex with Fe^{3+} which is more difficult to reduce, but easier to oxidise than Fe^{3+} alone and is likely to curtail the reduction of Fe^{3+} to Fe^{2+}. Figure 7.8. emphasises that AC has a concentration dependent effect on the decrease in reduction potential. The gradient of figure 7.8. is an estimation of the magnitude of the reduction potential shift over the AC concentration range. This in turn is a measure of the stability of the AC-Fe^{3+} complex. The gradient of -6.6638 is relatively large and thus indicates that a stable complex has been formed. Through this strong binding of Fe^{3+}, AC prevents the reduction of Fe^{3+} to Fe^{2+} in Fe^{2+}-induced and QA-enhanced Fe^{2+}-induced LP, thereby diminishing this detrimental effect (Chapter 6).

Štípek *et al.,* 1997 found that the effect of QA on LP in the rat brain *in vitro,* reported by Rios and Santamaria (1991) depends on Fe^{2+} and that this mechanism is likely to involve

Fe^{2+}-chelation by QA. In section 6.2.4., it was suggested that through weak chelation of Fe^{2+}, QA enhances equation 1.2 of the Fenton reaction. QA has the ability to form a complex with Fe^{2+} which has a more negative reduction potential than Fe^{2+} alone except at a metal-ligand ratio of 1 to 1 (figure 7.7.). However, figure 7.10. shows that despite this deviation, overall there is a shift to more negative potentials. Thus, the QA-Fe^{2+} complex is more difficult to reduce, but easier to oxidise than Fe^{2+} alone. This result confirms and explains the enhancement of the oxidation of Fe^{2+} by QA in the Fenton reaction. The gradient of -0.72 of the QA graph in figure 7.10., which is very small, indicates that the QA-Fe^{2+} complex is unstable.

In contrast, AC forms a complex with Fe^{2+} which has more positive reduction potentials than Fe^{2+} alone except at a metal to ligand ratio of 1 to 2 (figure 7.6.). The AC graph of figure 7.10. shows that overall, there is a shift to more positive potentials. The AC-Fe^{2+} complex is easier to reduce, but more difficult to oxidise than Fe^{2+} alone, thus, in the presence of AC, the oxidation of Fe^{2+} in the presence of H_2O_2 to produce LP initiating $HO^{\cdot}$ is likely to be suppressed. This may explain why AC is efficient at ameliorating Fe^{2+}-induced LP (chapter 6). However, the gradient of the AC graph in figure 7.10. of 0.2686 is small indicating that the complex, like that of QA-Fe^{2+} is not stable. The instability of both complexes suggests that the results discussed so far are insufficient to determine which of the two ligands, QA and AC has the greater chelating activity of Fe^{2+}. This information is crucial to determine whether equation 1.2 of the Fenton reaction is enhanced by QA or inhibited by AC in the presence of both ligands.

Since low ligand concentrations were used, the decrease in the peak current response of the free metal after the formation of metal-ligand complex, observed in figures 7.5., 7.6. and 7.7., most likely indicates that the rate of reduction of the complexes is lower than the rate of reduction of the metal species. Figure 7.9. and 7.11. show that this was a concentration dependent effect for the AC-Fe^{3+} and QA-Fe^{2+} complexes respectively. This implies that the complexes are strong enough to hinder the reduction reaction and thus slow the cathodic current flow. This is expected for the AC-Fe^{3+} and QA-Fe^{2+}, which have negative ΔE_p's and are consequently more difficult to reduce than the

respective free metal ion. However the effect appears to be contradictory with respect to the AC-Fe^{2+} complex, which has a positive ΔE_p. As mentioned previously, a lowering in current response may be a result of competition by free ligand with the metal-ligand complex for binding sites at the electrode at relatively high ligand concentrations, thereby decreasing the space available for adsorption of the metal-ligand complex. Despite the overall decrease in peak current response of the free Fe^{2+}, in the presence of increasing concentrations of AC (AC graph of figure 7.11) this argument should be ruled out because figure 7.6. shows that upon addition of the final concentration of AC (0.04 mM), a slight increase in peak current response was observed. It can therefore be concluded that the electrode is not saturated with free ligand at the metal to ligand concentrations achieved in this experiment. This may indicate that the affinity of AC for Fe^{2+} is only enhanced once a metal to ligand ratio of 1 to 2 or greater is achieved in the electrochemical cell. At lower metal to ligand ratios, it may be that although the complex requires less energy to be reduced than free Fe^{2+}, the complex is so unstable that the rate of reduction is lower.

The results of the electrochemical study are in agreement with that of the UV/VIS study in that it appears that AC forms a stronger bond with Fe^{3+} than with Fe^{2+}. The electrochemical study has shown that the oxidation of the QA-Fe^{2+} complex is easier, whilst that of the AC-Fe^{2+} complex is more difficult than free Fe^{2+}. The former would therefore enhance the Fenton reaction (equation 1.1) and the latter would ameliorate it. However, the results were insufficient to predict the nature of, if any, the competition between AC and QA for binding of Fe^{2+}. This would provide the necessary evidence to determine whether Fe^{2+} is more likely to be bound by QA or AC and therefore, whether the Fenton reaction (equation 1.2) is enhanced or inhibited in the presence of both ligands. This needs to be investigated further.

7.4. A COMPARISON OF THE EFFECT OF AC, QA AND POSITIVE CONTROL EDTA ON THE INHIBITION OF THE FERROZINE-IRON (II) COMPLEX

7.4.1. Introduction

Ferrozine (figure 7.12.) can form purple complexes with Fe^{2+} (figure 7.13.) in a quantitative manner (Decker and Welch, 1990) with a metal to ligand ratio of 1 to 3. (figure 7.13.). It is the lone pairs of electrons in the nitrogen atoms adjacent to each other, but on different aromatic rings which are involved in the co-ordinate bonding to Fe^{2+} (figure 7.13.)

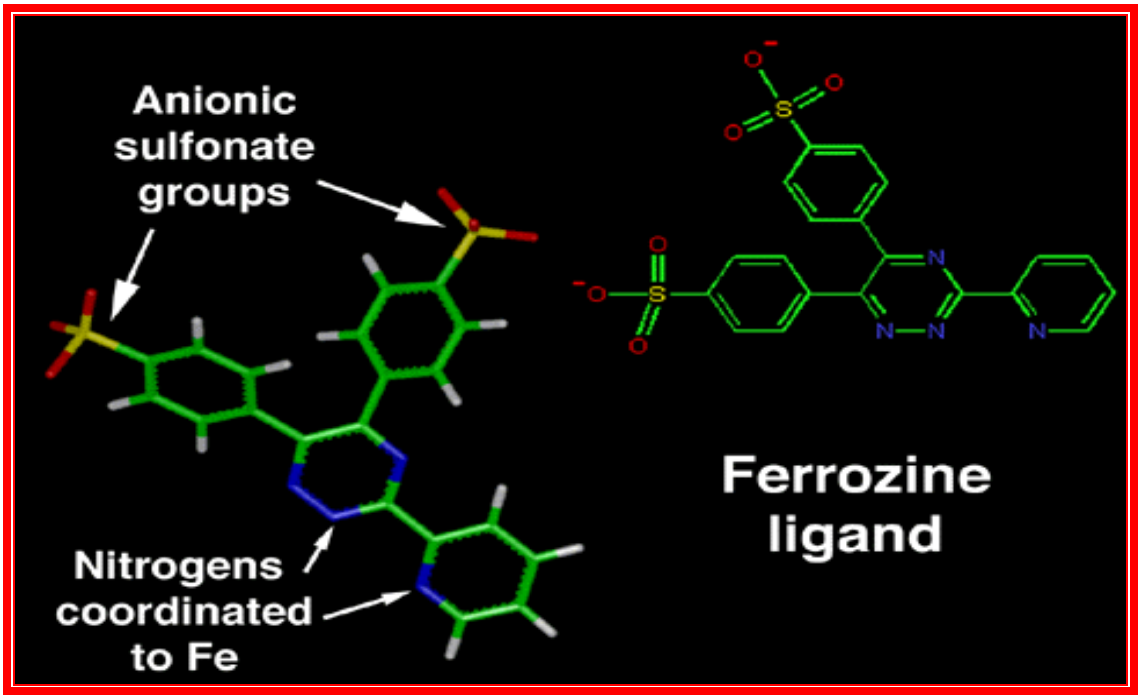


Figure 7.12. Representations of the ligand ferrozine. The figure on the left is a stick representation and the figure on the right is a ChemDraw® representation of the ligand. The carbon atoms are green, the hydrogens are white, the nitrogen atoms are blue, the oxygen atoms are red, and the sulphur atoms are yellow. (http://www.chemistry.wustl.edu/~edudev/LabTutorials/Ferritin/ferrozine.html).

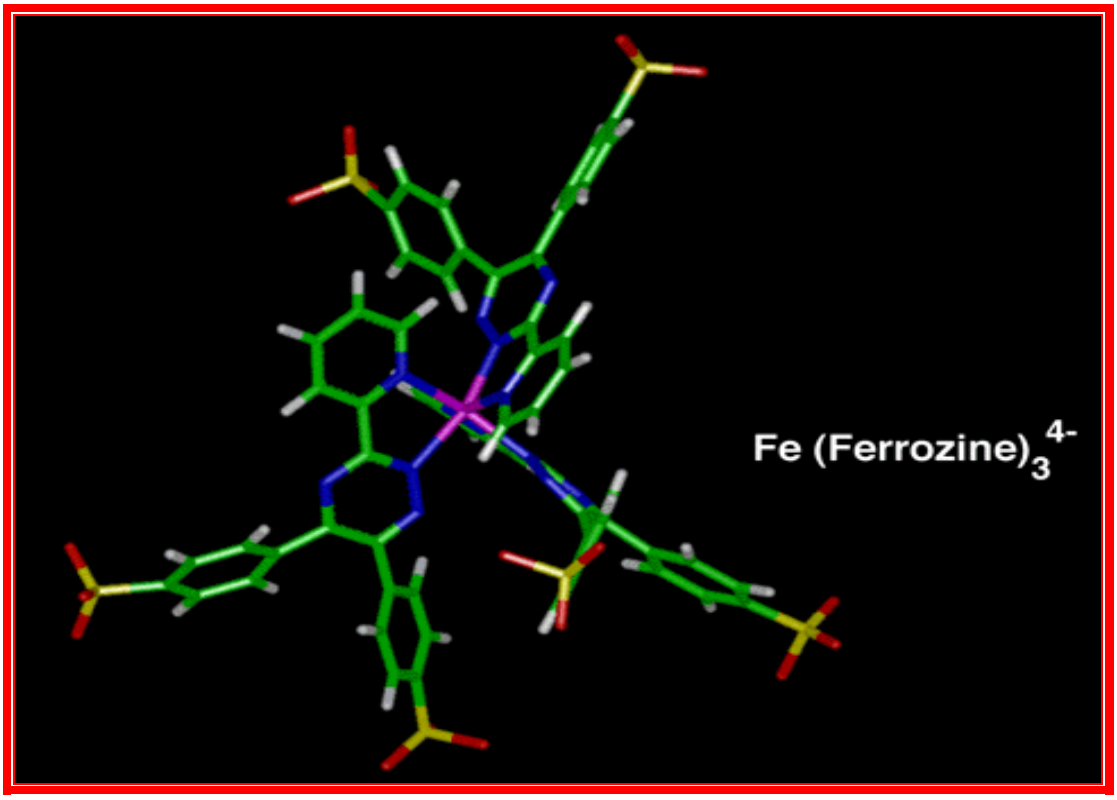


Figure 7.13. Molecular stick representation of Fe^{2+}-ferrozine complex. The carbon atoms are green, the hydrogens are white, the nitrogen atoms are blue, the oxygen atoms are red, the sulphur atoms are yellow and the Fe^{2+} is purple. (http://www.chemistry.wustl.edu/~edudev/LabTutorials/Ferritin/feferr.html).

In the presence of agents, which also have the ability to chelate Fe^{2+}, the formation of the Fe^{2+}-ferrozine complex is inhibited or reduced resulting in a decrease in the colour of the complex. EDTA is a well established metal chelator known to inhibit the Fe^{2+}-ferrozine complex (Que *et al.*, 2006). The greater the Fe^{2+} chelating activity of a chelator, the greater the inhibition of the complex. Therefore, measurement of the inhibition of the Fe^{2+}-ferrozine complex may be an ideal approach to elucidate which ligand, AC or QA, has a stronger affinity for Fe^{2+}. To reiterate, this is important because it would determine whether Fe^{2+} is more likely to be bound by QA or AC and therefore, whether the Fenton reaction (equation 1.2) is enhanced or inhibited in the presence of both ligands.

7.4.2. Materials and Methods

7.4.2.1. Chemicals and Reagents

AC sodium (Zovirax®, powder for injection) was supplied by Glaxo Wellcome, Midrand, South Africa. $FeSO_4.7H_2O$ and EDTA were purchased from Merck, Darmstadt, Germany. QA and ferrozine, was purchased from Sigma Chemical Co., St. Louis, USA.

7.4.2.2. Chelating Activity of AC and QA

The chelating activity of AC and QA on Fe^{2+} was assessed by measuring the percentage (%) inhibition of the Fe^{2+}-ferrozine complex using the method of Decker and Welch (1990). A 1 ml solution containing increasing concentrations (0, 1.56, 3.13, 6.25, 12.5, 25, 50, 100 µg/ml) of the test compound (QA or AC) was added to 3.7 ml of Milli-Q water. This mixture was reacted with 0.1 ml of 2 mM $FeSO_4.7H_2O$ and 0.2 ml of 5 mM ferrozine for 20 minutes. The absorbance was read at 562 nm in a GBC 916 UV/VIS spectrophotometer. Lower absorbance of the reaction mixture indicated higher chelating activity. The percentage inhibition of ferrozine–Fe^{2+} complex formation was calculated by the following formula: % inhibition of the ferrozine–Fe^{2+} complex = $\{(A_1-A_0)/A1\}$ x 100, where A_1 was the absorbance of 0 µg/ml test compound and A_0 was the absorbance in the presence of each of the other concentrations of the test compound. EDTA, at the same concentrations as the test compounds, was used as the positive control.

7.4.3. Results

Figure 7.14. shows that EDTA, AC and QA inhibit the Fe^{2+}-ferrozine complex in a concentration-dependent manner. However this concentration-dependent effect is negligible for QA. At 100 µg/ml, EDTA, AC and QA inhibited 99.98 %, 99.08 % and 11.98 % of the Fe^{2+}-ferrozine complex respectively. At 100 µg/ml, EDTA and AC have similar % inhibitions of the Fe^{2+}-ferrozine complex, but EDTA is still more potent than AC at lower concentrations. At concentrations between 1.5625 µg/ml and 6.25 µg/ml,

QA has greater % inhibitions of the Fe^{2+}-ferrozine complex than AC, whilst at higher concentrations, the opposite is observed.

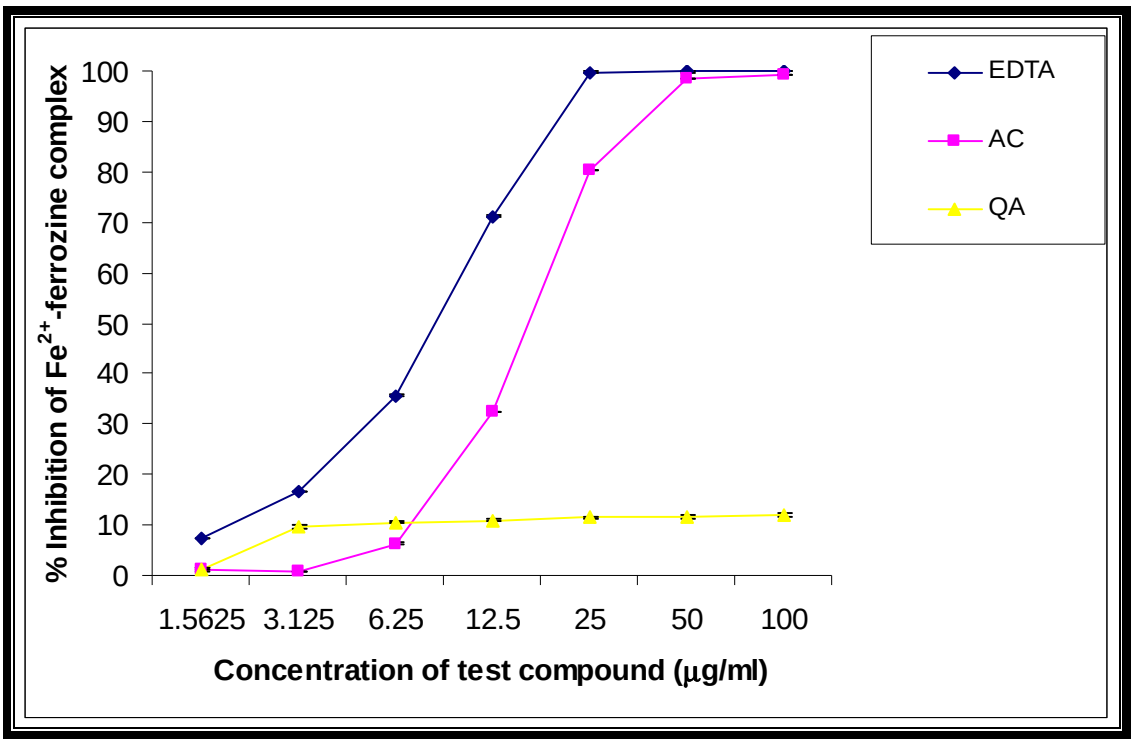


Figure 7.14. Percentage inhibition of Fe^{2+}-ferrozine complex by EDTA, AC and QA. Each point represents the mean ± SD (n = 10).

7.4.4. Discussion

The knowledge that EDTA is a well established metal chelator known to inhibit the Fe^{2+}-ferrozine complex (Que *et al.*, 2006) is confirmed and AC was found to be nearly as potent. The results clearly indicate that overall, AC has a greater ability to inhibit the Fe^{2+}-ferrozine complex and therefore a greater affinity and chelating activity of Fe^{2+} than QA. It may thus be concluded that Fe^{2+} is more likely to be bound by AC than by QA and therefore, the Fenton reaction (equation 7.2) is more likely to be impeded rather than enhanced in the presence of both ligands. This provides an adequate explanation for the inhibition of QA-induced LP by AC (Chapter 6).

This study provides supporting evidence of a metal-ligand interaction between the metal ion, Fe^{2+} and the ligands, QA and AC alluded to by the UV/VIS spectroscopic and electrochemical analyses. Moreover, it provides some substantiation on the nature of the interplay between these interactions.

CHAPTER 8

THE BIOSYNTHESIS OF QUINOLINIC ACID

8.1. INTRODUCTION

The KP contains neuroactive intermediates, all of which are derived from L-KYN, the primary degradation product of TRP (Schwarcz and Pellicciari, 2002). However, the KP does not only occur in the CNS, but in the periphery as well and as shown in figure 8.1., the dynamics of the KP is such that there is interplay between KP metabolism in the periphery and the brain (Schwarcz and Pellicciari, 2002). Although L-KYN can be produced in the brain to a moderate degree, under normal circumstances, the cerebral KP is driven mainly by blood-borne L-KYN, which enters from the circulation using the large neutral amino acid transporter (Fukui *et al.*, 1991). 3-HK can also penetrate the brain, using the same uptake mechanism (Reinhard *et al.*, 1994). The subsequent intracellular degradation of these substrates appears to be dictated primarily by the distribution of individual enzymes in astrocytes and microglial cells (Schwarcz and Pellicciari, 2002). For example, because of the efficiency of 3-HAO, which converts 3-HA to QA, as opposed to the low activity of the degradative enzyme of QA, quinolinic acid phosphoribosyltransferase, the extracellular CNS levels of QA are governed largely by the bioavailability of 3-HA (Schwarcz and Pellicciari, 2002).

In severe regional CNS inflammatory disease, the contribution of systemic L-KYN to QA pools is negligible (Kita *et al.*, 2002). However, in less severe regional CNS inflammatory disease or when local CNS inflammation is accompanied by systemic disease as well, the possibility remains that circulating L-KYN may make consequential contributions to the CNS QA pools (Kita *et al.*, 2002).

An increase in QA levels in the CNS could enhance the amount of neuronal damage produced by a primary brain insult; therefore the reduction of QA synthesis could limit

the amount of brain damage (Stone, 2000b). Chapters 2–6 have shown the deleterious actions of QA on the rat hippocampus and the neuroprotective effect of AC associated therewith. Thus, the aim of this chapter is to assess the ability of AC to ameliorate the activity of the enzymes which catalyse the first and last steps in the conversion of tryptophan to QA. As shown in figure 1.10., these enzymes are TDO, IDO and 3-HAO.

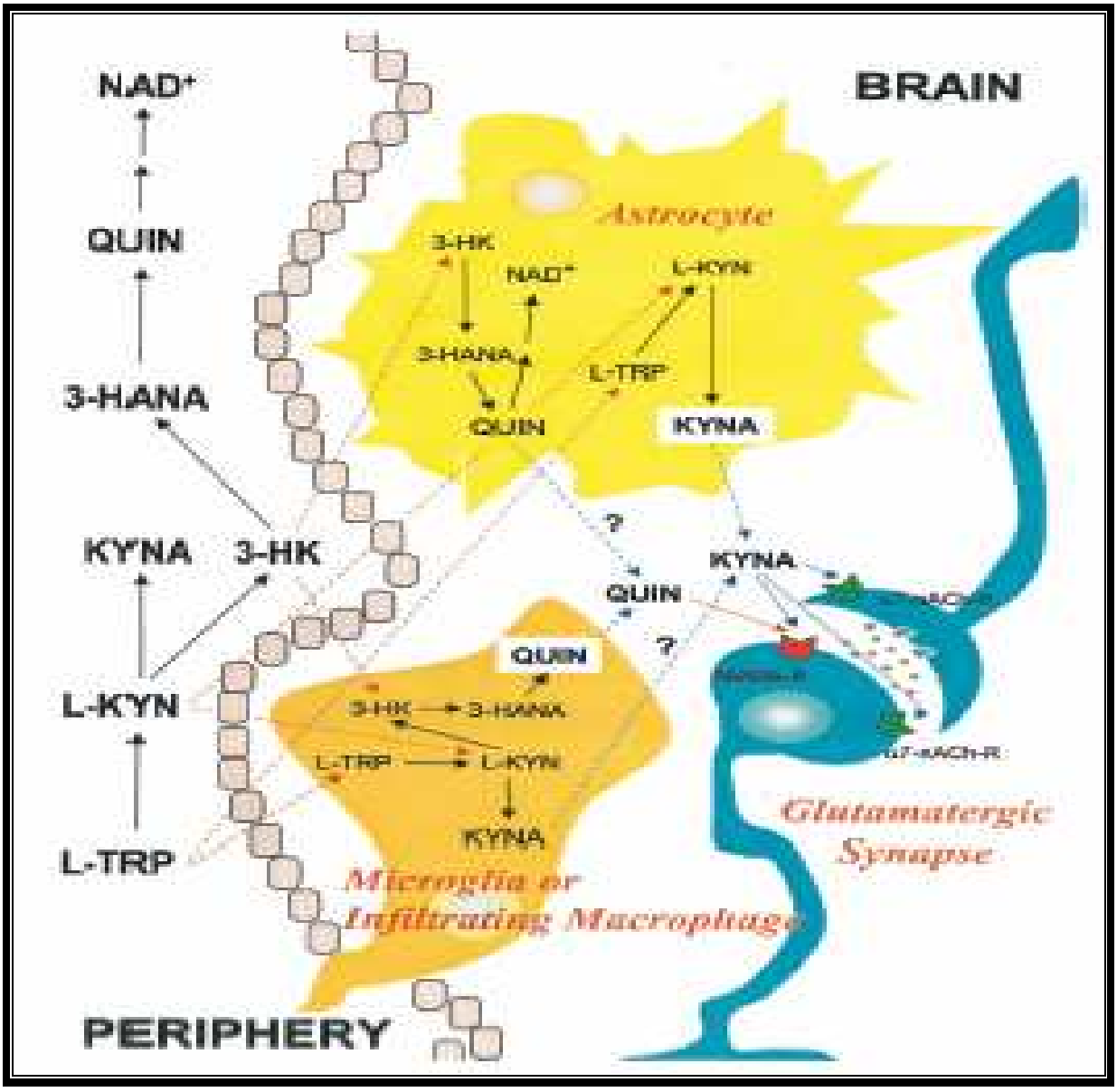


Figure 8.1. Dynamics of cerebral and extracerebral kynurenine pathway metabolism. L-TRP, L-tryptophan; L-KYN, L-KYN; 3-HK, 3-HK; 3-HANA, 3-hydroxyanthranilic acid; QUIN, quinolinic acid; NMDA-R, NMDA receptor; 7-nACh-R, 7 nicotinic acetylcholine receptor. Broken arrows: brain entry/cellular uptake (red), release (blue). Solid arrows: enzymatic conversion (black), receptor agonist (red), receptor antagonist (blue) (Schwarcz and Pellicciari, 2002).

8.2. THE EFFECT OF AC ON TDO ACTIVITY IN RAT LIVER HOMOGENATE *IN VITRO* AND *IN VIVO*

8.2.1. Introduction

The major site of TRP catabolism is the liver (Salter *et al.*, 1986). The liver cytosolic enzyme, TDO is the major determinant of the levels of peripheral, circulating TRP (Badawy and Evans 1981). Thus, the untoward effect of enhanced activity of TDO is not only an increase in blood-borne L-KYN for the biosynthesis of the neurotoxin QA, but it also reduces the availability of TRP for uptake into the brain. As shown in figure 1.13., TRP is required for 5-HT synthesis, the rate-limiting step of which is the conversion of TRP to 5-hydroxytryptophan by TRP hydroxylase (Knowles and Pogson, 1984). Therefore the cerebral concentration of TRP is an important factor in brain 5-HT synthesis (Fernstrom and Wurtman, 1971). 5-HT appears to be an important mood-enhancing neurotransmitter (section 1.1.4.3.), with decreased levels reported in patients with, and in animal models of, depression, AD and HSE (section 1.2.4.2.4.).

Glucocorticoids are potent inducers of TDO activity (Salter and Pogson, 1985). Most patients suffering from depression have elevated cortisol levels (Curzon, 1988). Because TRP hydroxylase is approximately 50% saturated with TRP *in vivo*, 5-HT levels will be more sensitive to decreases in TRP than increases in TRP (Pogson *et al.,* 1989). Elevated cortisol levels in depressed patients may, therefore, contribute to mood status by producing a TDO-induction dependent decrease in brain 5-HT (Salter *et al.*, 1994).

The hippocampal formation forms part of the limbic-hypothalamic-pituitary-adrenal (LHPA) axis; a negative feedback loop where increased glucocorticoid (mainly cortisol in humans) levels are “shut-off” by activation of receptors at one or several levels of this axis. Activation of these receptors also influences neurochemistry, neuronal excitability and structural plasticity (McEwen 2000). Notably, excessive glucocorticoid exposure to hippocampal neurons impairs neuronal plasticity and may contribute to or worsen

neurodegeneration in AD. This has been suggested to result in a vicious circle, where loss of neurons in the hippocampus leads to further loss of feedback inhibition leading to hypercortisolism with progressive hippocampal damage (Sapolsky *et al.,* 1986; Seckl and Olsson 1995). Part of this progressive hippocampal damage may be due the induction of liver TDO by the glucocorticoids. This enhances blood-borne L-KYN which is taken up into the brain where it can be converted to the neurotoxic QA.

There is much controversy surrounding the use of corticosteroids in combination with AC in the treatment of HSE. Corticosteroids could have an important role in the management of this disease because the anti-inflammatory action reduces cerebral oedema (Thompson *et al*., 2000). However the use of these anti-inflammatory drugs has been limited by concerns that their immunosuppressive actions could increase viral replication and spread (Thompson *et al*., 2000). Data published by Thompson *et al*., (2000) suggest that corticosteroids are not detrimental in this regard when combined with AC in the management of HSE. However, by virtue of the fact that glucocorticoids have the ability to induce the activity of TDO, it is reasonable to argue that these agents may indirectly contribute to neuronal damage through increased biosynthesis of QA, an action which is totally independent of its effect on viral replication.

Thus, due to the potential of enhanced TDO activity and the detrimental role of this phenomenon in neurodegeneration and the alteration of mood in AD, depression and HSE, the effect of AC on the activity of the Holo- and Apoenzyme of TDO was determined.

8.2.2 Materials and Methods

8.2.2.1. Animals

Adult male Wistar rats were purchased from the NHLS (Johannesburg, South Africa) and cared for as described in section 2.2.2.1.

8.2.2.2. Chemicals and Reagents

AC (Zovirax®, powder for injection) was obtained from Glaxo Wellcome, Midrand, South Africa. Haeme (porcine), BSA, TCA and Folin's reagent were purchased from Sigma Chemical Co, St. Louis, U.S.A. L-TRP was obtained from Merck, Darmstadt, Germany. Sodium hydroxide was obtained from Saarchem, Krugersdorp, South Africa.

8.2.2.3. *In vitro* Exposure of Rat Liver to AC

8.2.2.3.1. Removal of the Liver

Rats were killed by cervical dislocation and rapidly decapitated. To remove the liver, a mid-ventral incision was made through the abdominal musculature from the pelvic region to the posterior edge of the sternum. A transverse cut was made anteriorly to expose the liver, which was removed carefully. The liver was immediately perfused with cold 0.9 % saline to remove all endogenous haeme, frozen in liquid nitrogen and stored at -70 ˚C.

8.2.2.3.2. Preparation of the Tissue

The livers were thawed, weighed and chopped into fine pieces using a blade. The pieces of liver were then rapidly homogenised with a hand-held homogeniser in 0.01 M PBS pH 7.4, to give a final concentration of 10 % w/v. The homogenate was then sonicated for a period of two minutes at thirty second intervals for complete removal of the enzyme from cells. The entire procedure, where possible, was conducted on ice.

8.2.2.3.3. Determination of TDO Activity

A modification of the method described by Badawy and Evans, (1975) was used.

Homogenate (15 ml) and water (12.5 ml) was added to each of two flasks. To one flask, haematin (100 µl) was added, to make a final concentration of 2 µM. This flask was

stirred and allowed to stand for one minute to allow for activation of the enzyme by haematin. An aliquot of 2.5 ml 0.03 M L-TRP was then added to each flask. The flasks were stirred before aliquots of 2 ml were transferred to test tubes in triplicate. Thereafter, AC (1 ml) was added such that incubation concentrations of 100 µM and 10 µM were achieved. No drug was added to control samples. The test tubes were incubated for 60 minutes at 37 ˚C in an oscillating water bath. To stop the reaction, 0.9 M TCA (2 ml), was added to each test tube. The resulting precipitate was then centrifuged at 4500 x g for 15 minutes. The supernatant (2.5 ml) was transferred to a new set of test tubes and 0.6 M sodium hydroxide (1.5 ml) was added to each. An aliquot from each test tube was removed for analysis. The absorbance was read at 365 nm, which is the λ_{max} of N-formylkynurenine. The extinction co-efficient of N-formylkynurenine, $\varepsilon = 4540\ M^{-1}.cm^{-1}$ was used to determine the concentration of N-formylkynurenine. The activity of TDO was expressed as pmoles N-formylkynurenine/mg protein/min. The results from test tubes which contained haematin represent total activity, whilst the results from those which did not contain haematin represent holoenzyme activity. Apoenzyme activity is calculated as the difference between the total activity and holoenzyme activity.

8.2.2.3.4. Protein Assay

The determination of protein was performed as described in section 4.2.2.7.

8.2.2.4. *In vivo* Administration of AC

8.2.2.4.1. Drug Treatment

Ten rats were randomly divided into two groups. The animals in the first group (control) received i.p. injections of saline (0.25 ml) twice a day (at 0830 and 1630 hours) for five days. The animals in group two (treatment) received injections of AC (5 mg/kg) twice a day for five days, in the same manner and at the same times.

8.2.2.4.2. Removal of the Liver

On the morning of the sixth day, the rats were killed and the livers removed as described in section 8.2.2.3.1.

8.2.2.4.3. Preparation of the Tissue

This was performed as described in 8.2.2.3.2.

8.2.2.4.4. Determination of TDO Activity

This was conducted as described in section 8.2.2.3.3. However, after addition of 0.03 M L-TRP (2.5 ml) to each flask, aliquots of 3ml instead of 2ml were transferred in triplicate to test tubes. AC not added to any of the test tubes.

8.2.2.4.5. Protein Assay

The determination of protein was performed as described in section 4.2.2.7.

8.2.2.5. Statistical Analysis

This was performed as described in section 4.2.2.9.

8.2.3. Results

Figure 8.2. shows that 100 µM AC significantly reduces the total activity and apoenzyme activity of TDO ($p < 0.05$), whilst 10 µM AC produces no effect on either form of the enzyme. AC at a concentration of 100 µM has no significant effect on holoenzyme activity.

As shown in figure 8.3., it is evident that AC, administered 5 mg/kg twice a day for five days induces a significant ($p < 0.001$) decrease in the total apoenzyme and holoenzyme activity of TDO.

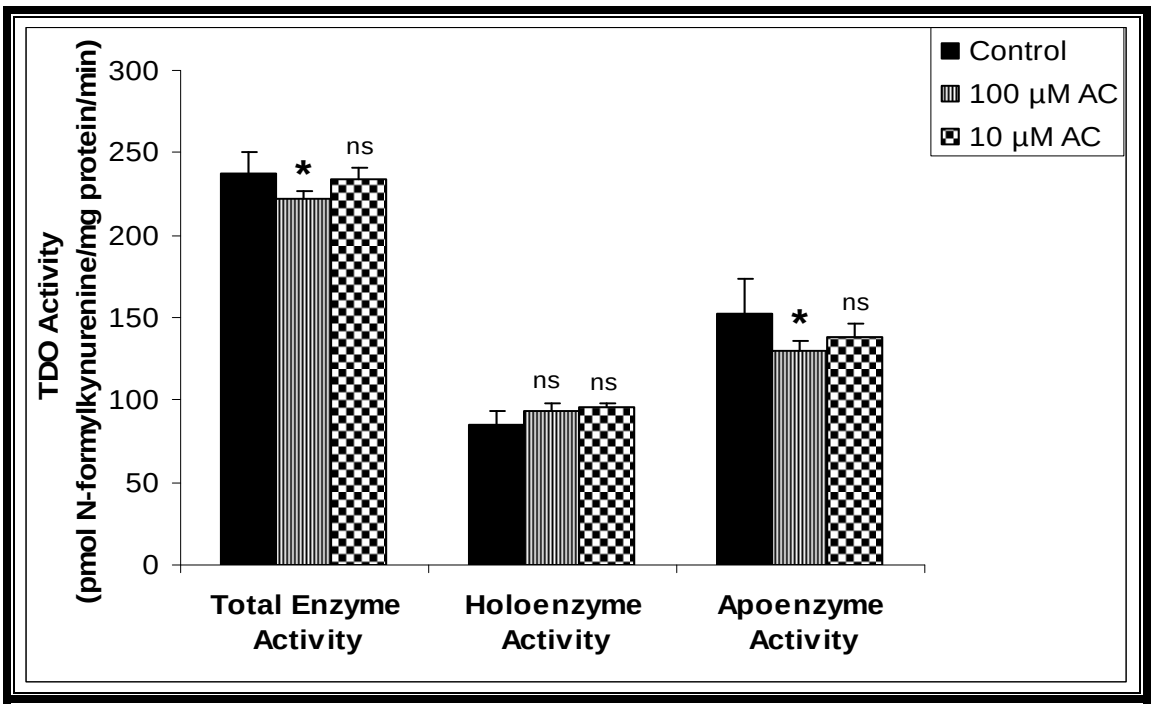


Figure 8.2. Effect of AC on TDO enzyme activity in rat liver homogenate *in vitro*. Each bar represents the mean ± SD ($n = 5$). * ($p < 0.05$) and ns ($p > 0.05$) in comparison to controls (ANOVA and Student–Newman–Keuls Multiple Range Test).

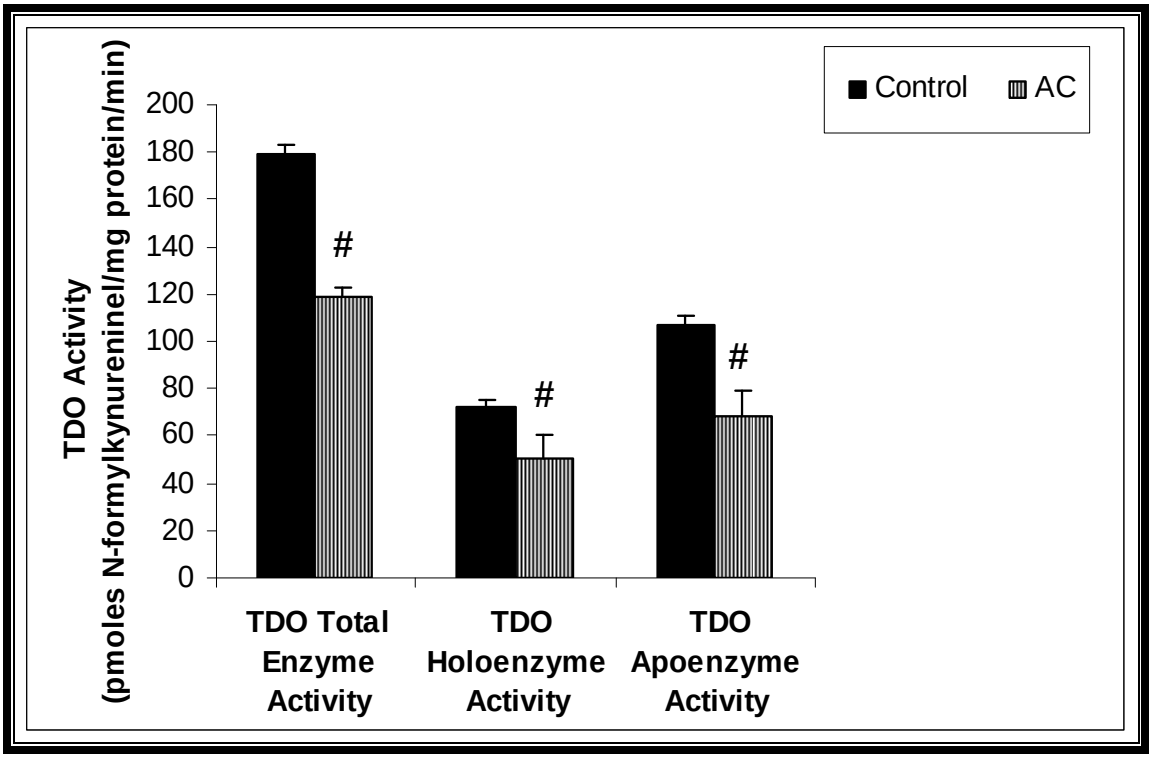


Figure 8.3. Effect of AC on TDO enzyme activity in rat liver homogenate *in vivo*. Each bar represents the mean ± SD (n = 5). # ($p < 0.001$) in comparison to control (ANOVA and Student–Newman–Keuls Multiple Range Test).

8.2.4. Discussion

The already active holoenzyme does not require the addition of exogenous haeme for demonstration of its activity *in vitro*, whereas the haeme-free predominant apoenzyme does (Badawy and Evans, 1975). Therefore, apoenzyme activity represents the activity of TDO which is in the apoenzyme form at the time of removal of the liver. This implies that it first has to be activated by exogenous haeme to become the active holoenzyme. Holoenzyme activity represents the activity of TDO which is already in the active holoenzyme form at the time of removal of the liver.

There are two mechanisms by which compounds may inhibit TDO:

(a) Some agents may interfere with the conjugation of the apoenzyme and its co-factor, haeme (Badawy and Evans, 1981), thus inhibiting the formation of new holoenzyme from the apoenzyme (step 1 of figure 1.8). Thus, effectively, apoenzyme activity is inhibited.

(b) An indole amine (indole–NH) group is required for substrate binding to the catalytic site of TDO, whilst a secondary amine [$CH(NH_2)COOH$] group ensures that the substrate takes up the correct configuration appropriate to the catalytic reaction (Uchida *et al.*, 1992). Thus, compounds which possess at least an indole–NH should competitively inhibit the binding of TRP to the inactive holoenzyme (step 2 of figure 1.8.), thereby inhibiting holoenzyme activity. Uchida *et al.*, (1992) further suggest that compounds without the indole -NH may inhibit TDO at this level by non-competitive inhibition, that is, interference with TRP binding through interaction at a site other than that designated for TRP.

In vitro, 100 µM AC could only inhibit apoenzyme activity and not holoenzyme activity, which implies that AC does not interfere with TDO when it is already in its active form. Instead, it is probably interfering with the conjugation of haeme to the apoenzyme. The relationship between this hypothesis and the finding that 10 µM AC does not have an effect on apoenzyme activity may be that this concentration is not sufficiently high to inhibit the haeme added exogenously. The type of inhibition displayed by AC may be competitive rather than non-competitive because it consists of functional groups and rings found in the structure of haeme. However, this needs to be investigated further.

Since *in vivo* AC was administered before removal of the liver, competition between endogenous haeme and AC may occur up to the time of liver removal. Holoenzyme activity represents the specific activity of TDO which is already in the active holoenzyme form at the time of removal of the liver. Thus, such competitive inhibition of TDO by AC might lead to decreased active holoenzyme and therefore decreased holoenzyme activity

in the livers of rats which received *in vivo* administration of AC. Any free AC still present in the liver at the time of its removal and which is not taking part in the competition with endogenous haeme may be available to compete with exogenous haeme and thus also cause competitive inhibition, resulting in decreased apoenzyme activity.

The inhibition of TDO by AC should decrease the catabolism of systemic TRP, raising its concentration not only in the blood but also in the brain. An increased availability of TRP will increase the activity of TRP hydroxylase, and therefore increase the synthesis and vesicular stores of 5-HT (Schaetchter and Wurtman, 1989). Furthermore, inhibition of TDO by AC will reduce the peripheral L-KYN levels thereby decreasing the consequential contributions to CNS QA levels, particularly as a result of TDO induction by endogenous and administered glucocorticoids implicated as described in section 8.2.1., in AD, HSE and depression.

8.3. THE EFFECT OF AC ON IDO ACTIVITY IN RAT SMALL INTESTINAL HOMOGENATE *IN VITRO* AND *IN VIVO*

8.3.1. Introduction

IDO is ubiquitously distributed in various organs including the brain, lung, spleen and alimentary tract (Hayaishi *et al*., 1975).

The pro-inflammatory cytokine IFN-γ induces the enzyme IDO in a variety of cells, both *in vitro* and *in vivo* (Wirleitner *et al.*, 2003). IDO activation limits availability of TRP. Because TRP is required for protein synthesis, withdrawal of this essential amino acid from the micro-environment arrests protein biosynthesis and subsequent growth of pathogens and proliferating cells (Schröcksnadel, *et al.*, 2006). Consequently, TRP depletion is regarded as a defence mechanism induced by IFN-γ in immunocompetent cells during an immune response (Schröcksnadel, *et al.*, 2006).

In addition however, the activation of IDO results in an increase in the biosynthesis of the intermediates of the kynurenine pathway, including the neurotoxic 3-HK, 3-HA and QA. As described in section 1.1.4.1., these TRP catabolites may also contribute to the immune mechanism by killing already injured neurons. However, at the same time, surrounding healthy tissue is compromised by oxidative damage. For example, in HSE, QA could be part of the macrophage's phagocytic function, by degrading lipid membranes, but it is also possible that the increased QA could augment the toxicity of HSV-1 by degrading the lipid membranes of cells in proximity to the virus (Reinhard, 1997). A similar and likely analogy may be drawn to AD and depression as well, where IDO activation and increased QA levels are also prevalent (sections 1.1.4.2., 1.1.4.4., 1.2.4.1.). Thus, the aim of this section is to investigate the effect of AC, an antiviral used in HSE, on the activity of IDO.

It has been shown that IDO utilises $O_2^{\cdot-}$ in the catalytic process *in vivo* (Taniguchi *et al.*, 1977) and *in vitro* (Hirata and Hayaishi, 1971, Hayaishi *et al.*, 1977). $O_2^{\cdot-}$ can be generated *in situ* enzymatically by xanthine oxidase or GR with their respective substrates or chemically by ascorbic acid and methylene blue (Hirata and Hayaishi, 1975).

8.3.2. Materials and Methods

8.3.2.1. Animals

Adult male Wistar rats were purchased from the NHLS (Johannesburg, South Africa) and cared for as described in section 2.2.2.1.

8.3.2.2. Chemicals and Reagents

AC sodium (Zovirax® powder for injection) was obtained from Glaxo Wellcome, Midrand, South Africa. CAT (specific activity of 2.250 units/mg protein), BSA, TCA and Folin's reagent were purchased from Sigma Chemical Co, St. Louis, U.S.A. L-TRP was purchased from Merck, Darmstadt, Germany. Ascorbic acid and methylene blue were purchased from Saarchem, Krugersdorp, South Africa.

8.3.2.3. *In vitro* Exposure of Rat Small Intestine to AC

8.3.2.3.1. Removal of the Small Intestine

Rats were killed by cervical dislocation and rapidly decapitated. To remove the small intestines, a mid-ventral incision was made through the abdominal musculature from the pelvic region to the posterior edge of the sternum. A transverse cut was made anteriorly to expose the small intestine, which was removed carefully. Food and faeces were removed, using PBS pH 6.0. The small intestines were frozen in liquid nitrogen and stored at -70˚C.

8.3.2.3.2. Preparation of the Tissue

The small intestines were thawed, weighed and chopped into fine pieces using a blade. The pieces of small intestine were then rapidly homogenised with a hand-held homogeniser in 0.01M PBS pH 6.0, so as to give a final concentration of 10 % w/v. A pH of 6.0 was found to be the optimal pH for stability of IDO when assayed with either L- or D-TRP as substrate (Shimizu *et al.*, 1978). The homogenate was then sonicated for a period of two minutes at thirty second intervals for complete removal of the enzyme from cells. This procedure was, where possible, performed on ice.

8.3.2.3.3. Determination of IDO Activity

A modification of the method described by Shimizu *et al.*, 1978 was used.

Reaction mixtures containing 0.01 M PBS pH 6.0 (0.75 ml), 10 µg catalase (0.15 ml), 10 µM or 100 µM AC (0.15 ml), 25 µM methylene blue(0.15 ml), 10 mM ascorbic acid (0.15 ml) and 5 mM L-TRP (0.15 ml), in that order, were prepared. IDO is easily inactivated by H_2O_2 formed by ascorbic acid, in the presence of methylene blue in the reaction mixture (Hirata *et al.*, 1977), hence CAT is included in the reaction mixture (Hirata and Hayaishi, 1975), to degrade any H_2O_2 produced. Four groups were prepared in triplicate. The first group (control) did not contain AC, methylene blue or ascorbic acid. The second group did not contain AC, whilst the third and fourth group contained 10 µM and 100 µM AC respectively. Homogenate (1.5 ml) was then added to the reaction mixture in each test tube. The test tubes were incubated for 60 minutes at 37 ˚C in an oscillating water bath. After incubation, 2ml TCA (0.9 M) was added to each test tube to terminate the reaction. The test tubes were then shaken for a further 30 minutes at 50˚C to hydrolyse N-formylkynurenine to L-KYN. The resulting precipitate was then centrifuged at 4500 x g for 10 minutes. An aliquot of the supernatant from each test tube was then removed for analysis. The absorbance was read at 335 nm, the isosbestic point of N-formylkynurenine and L-KYN. An extinction co-efficient, ε of 3500 $M^{-1}cm^{-1}$ was used to determine the concentration of the two kynurenines. The activity of IDO was expressed as pmoles kynurenines/mg protein/min.

8.3.2.3.4. Protein Assay

The determination of protein was performed as described in section 4.2.2.7.

8.3.2.4. *In vivo* Administration of AC

8.3.2.4.1. Drug Treatment

Ten rats were randomly divided into two groups and treated as described in section 8.2.2.4.1.

8.3.2.4.2. Removal of the Small Intestine

On the morning of the sixth day, the rats were killed and the small intestines removed as described in section 8.3.2.3.1.

8.3.2.4.3. Preparation of the Tissue

The tissue was prepared as described in section 8.3.2.3.2.

8.3.2.4.4. Determination of IDO Activity

This assay was performed as described in section 8.3.2.3.3. However, there were two groups each for the small intestines from control and AC treated rats. AC was excluded from all reaction mixtures. Group one contained no methylene blue or ascorbic acid, whilst group two did.

8.3.2.4.5. Protein Assay

This was performed as described in section 4.2.2.7.

8.3.2.5. Statistical Analysis

This was performed as described in section 4.2.2.9.

8.3.3. Results

Figures 8.4. and 8.5. show that the activity of IDO is significantly greater ($p < 0.001$) in the presence of *in situ* generation of $O_2^{\cdot-}$. Furthermore, 10 µM and 100 µM AC (figure 8.4.) significantly reduce the specific activity in comparison to 0 µM AC ($p < 0.01$ and $p < 0.001$ respectively), in a concentration dependent manner.

Figure 8.5. shows that AC, administered 5 mg/kg twice a day for five days induces a significant decrease in the specific activity of IDO in both the absence and presence of *in situ* generation of $O_2^{\cdot-}$ (both $p < 0.001$).

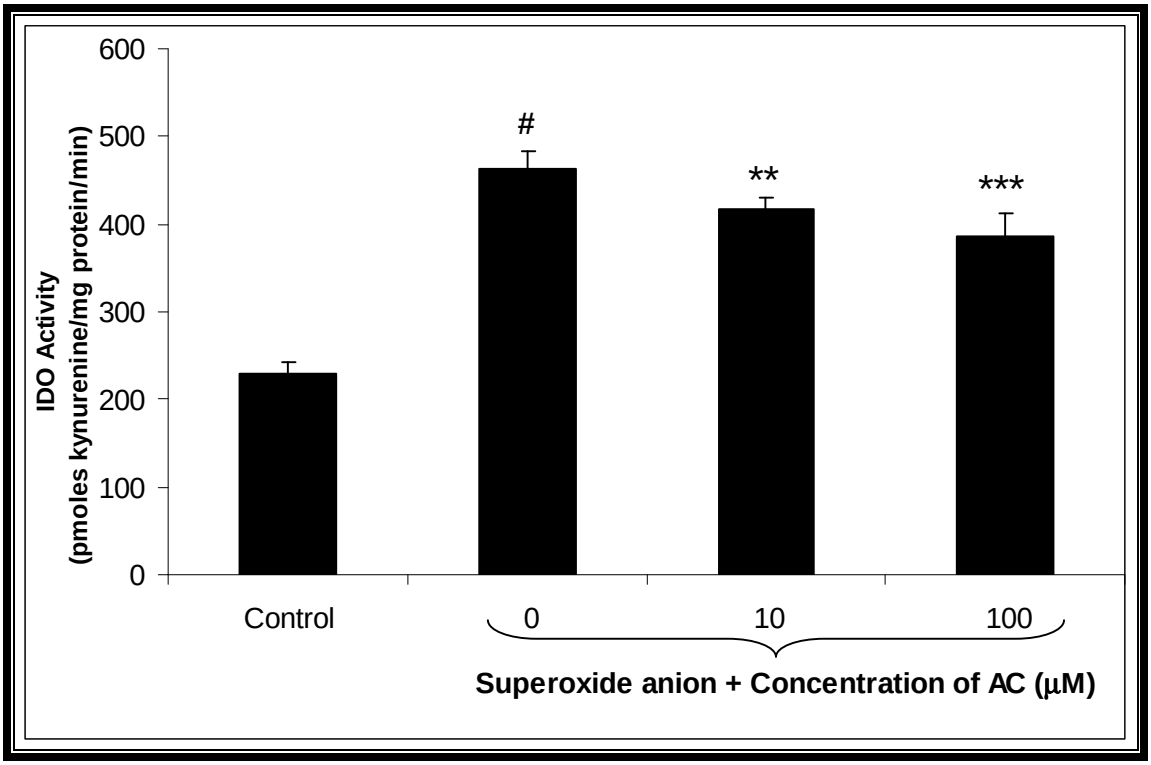


Figure 8.4. Effect of AC on IDO enzyme activity in rat small intestinal homogenate *in vitro*. Each bar represents the mean ± SD (n = 5). # ($p < 0.001$) in comparison to control; ** ($p < 0.01$) in comparison to 0 µM AC; *** ($p < 0.001$) in comparison to 0 µM AC (ANOVA and Student–Newman–Keuls Multiple Range Test).

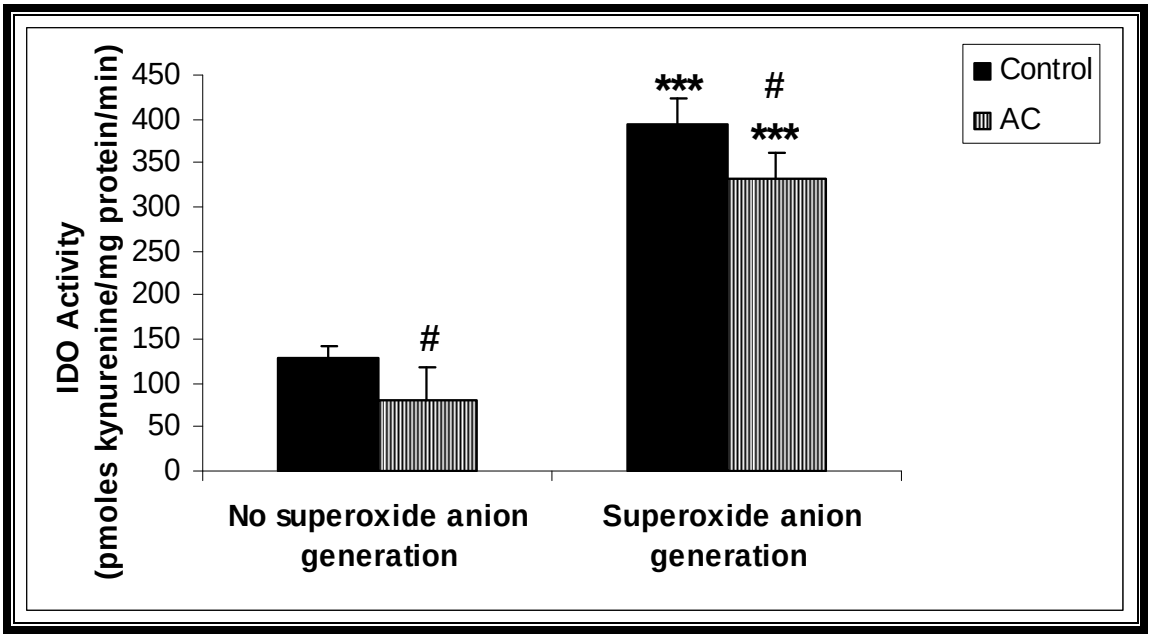


Figure 8.5. Effect of AC on IDO enzyme activity in rat small intestine homogenate *in vivo*. Each bar represents the mean ± SD (n = 4). *** ($p < 0.001$) in comparison to no *in situ* $O_2^{\cdot-}$ generation (control and AC treated animals); # ($p < 0.001$) in comparison to control (no *in situ* $O_2^{\cdot-}$ generation and *in situ* $O_2^{\cdot-}$ generation) (ANOVA and Student–Newman–Keuls Multiple Range Test).

8.3.4. Discussion

IDO contains haeme as a prosthetic group and it is the oxygenated form of the enzyme which is catalytically active (Hirata *et al.*, 1977; section 1.2.4.2.2.2.). Unlike TDO, the active form of the enzyme can be produced not only by the interaction of O_2 with the ferrous form of the enzyme, but also by the interaction of $O_2^{\cdot-}$ with the ferric form of enzyme (Hirata *et al.*, 1977; figure 1.9). This explains why, as shown in both figures 8.4 and 8.5, the presence of *in situ* $O_2^{\cdot-}$ generated by methylene blue and ascorbic acid increases the activity of IDO. Both interactions mentioned could occur under these conditions, therefore producing more IDO in the active form. In contrast, only the interaction of O_2 with the ferrous form of the enzyme could occur in the absence of methylene blue and ascorbic acid.

The success of AC at inhibiting the activity of IDO *in vitro* (figure 8.4.) is likely to be related to its ability to scavenge $O_2^{\bullet-}$, a property of AC which was demonstrated in Chapter 5. By scavenging $O_2^{\bullet-}$, there are fewer of these free radicals available to bind to the ferric form of the enzyme, thus reducing the formation of active IDO. This effect of AC is concentration dependent because a higher concentration of AC is able to scavenge more $O_2^{\bullet-}$ in comparison to lower concentrations.

The effect of AC on IDO activity *in vivo* may be two-fold. In the presence of *in situ* $O_2^{\bullet-}$ generation, the role of AC is likely to be the scavenging of this free radical, as described for the *in vitro* inhibition of IDO. In the absence of *in situ* $O_2^{\bullet-}$ generation, AC may have scavenged endogenous $O_2^{\bullet-}$, thus reducing active IDO. In addition, in the absence or presence of *in situ* $O_2^{\bullet-}$ generation, AC may be preventing the binding of endogenous haeme, the prosthetic group of IDO, reflecting a potential property of AC which was proposed for the inhibition of TDO in section 8.3.4. Thus, *in vivo,* there are two possible mechanisms by which AC may suppress the activation of the IDO enzyme.

Through inhibition of IDO activity in the small intestine, AC may counter the rise in peripheral L-KYN brought about by the induction of peripheral IDO by pro-inflammatory cytokines in diseases such as HSE where local CNS inflammation is accompanied by systemic disease. Furthermore, qualitatively, the characteristics of enzymes along the QA branch of the KP do not differ between the brain and the periphery (Schwarcz and Pellicciari, 2002), therefore AC is likely to inhibit CNS IDO as well, and thus may also be effective in diseases, such as AD and depression, which are primarily associated with neuroinflammation. Inherently then, AC has the potential to reduce the biosynthesis of QA.

8.4. THE EFFECT OF AC ON 3-HAO ACTIVITY RAT LIVER HOMOGENATE *IN VITRO* AND *IN VIVO*

8.4.1. Introduction

Since QA, in contrast to its bioprecursors tryptophan and L-KYN, does not cross the blood brain barrier, it can reasonably be assumed that in the brain it is synthesised locally by its anabolic enzyme, 3-HAO (Foster *et al.*, 1986). In the frontal cortex, striatum and hippocampus, 3-HAO activity is relatively high, whilst the activity of QA phosphoribosyltransferase, which catabolises QA, is low in these same areas (Perkins and Stone, 1983; Schwarcz and Köhler, 1983) This is particularly undesirable, as these cerebral structures are highly susceptible to the excitotoxic effects of QA (Perkins and Stone, 1983; Schwarcz and Köhler, 1983). These effects have been shown to impair spatial reference memory (Chapter 2), induce neurodegeneration (Chapter 3), enhance excitotoxicity (Chapter 4), oxidative stress (Chapter 5) and LP (Chapter 6) in the rat hippocampus. Thus, QA released during neuroinflammation in diseases such as AD, HSE and depression (sections 1.1.4.2., 1.1.4.3., 1.1.4.4.) has the potential to initiate a plethora of deleterious CNS effects.

The administration of kynurenine hydroxylase inhibitors appear to be the most logical way to decrease the amount of QA beyond TDO and IDO in the cerebral KP because the production of the neurotoxic precursors of QA, namely 3-HK and 3HA are also inhibited, with a simultaneous rise in the neuroprotectant, kynurenic acid (Stone, 2000b). However, being an NMDA receptor antagonist at the glycine binding site, kynurenic acid may block the induction of LTP, which would impair learning and memory. This has been illustrated in the CA1 region of the hippocampus, employing 7-chlorokynurenic acid, the potent analogue of kynurenic acid, (Izumi *et al.*, 1990). An alternative approach to preventing the synthesis of QA is to inhibit 3-HAO (Stone, 2000b). Since 3-HAO is distant from the branching point of the KP, enzyme inhibition provides a method to selectively attenuate QA formation without a concomitant rise in kynurenic acid.

(Schwarcz and Pellicciari, 2002). Thus, there being a good rationale for manipulation of the KP at this point, the effect of AC on 3-HAO activity was investigated.

There appears to be general accordance between 3-HAO in the brain and from peripheral sources with regard to dependency on the cofactor Fe^{2+}, substrate specificity, the K_m for 3-HA and cytosolic localisation (Foster *et al.*, 1986). 3-HAO has been studied primarily in the liver and kidney as these organs contain large amounts of the enzyme (Foster *et al.*, 1986). Therefore, rat liver 3-HAO activity was measured as a model of rat brain 3-HAO activity.

8.4.2. Materials and Methods

8.4.2.1. Animals

Adult male Wistar rats were purchased from the NHLS (Johannesburg, South Africa) and cared for as described in section 2.2.2.1.

8.4.2.2. Chemicals and Reagents

AC sodium (Zovirax®, powder for injection) was obtained from Glaxo Wellcome, Midrand, South Africa. 3-HA, BSA and Folin's reagent were purchased from Sigma Chemical Co, St. Louis, U.S.A. $FeSO4.7H_2O$ was obtained from Merck, Darmstadt, Germany. Ascorbic acid, hydrochloric acid (HCl) and sodium hydroxide were obtained from Saarchem, Krugersdorp, South Africa.

8.4.2.3. *In vitro* Exposure of Rat Liver to AC

8.4.2.3.1. Removal of the Liver

This was performed as described in section 8.2.2.3.1.

8.4.2.3.2. Preparation of the Tissue

This was performed as described in section 8.2.2.3.2.

8.4.2.3.3. Determination of 3-HAO Activity

A modification of the method described by Mehler, 1956 was used.

Reaction mixtures were prepared containing 0.01 M PBS pH 7.4 (0.5 ml), 10µM 3-HA (0.25 ml), 100µM ascorbic acid (0.25 ml), 100 µM $FeSO_4.7H_2O$ (0.25 ml) and 10 µM or 100 µM AC (0.25 ml), in that order. Four groups were prepared in triplicate. The first group (control) did not contain AC, $FeSO_4.7H_2O$ or ascorbic acid. The second group did not contain AC, whilst the third and fourth groups contained 10 µM and 100 µM AC respectively. Homogenate (1.5 ml) was then added to the reaction mixture in each test tube. The test tubes were incubated for 30 minutes at 37˚C in an oscillating water bath. After incubation, 2 ml HCl (3 M) was added to each test tube to terminate the reaction. The resulting precipitate was then centrifuged at 4500 x g for 10 minutes. An aliquot of the supernatant from each test tube was then removed for analysis. The absorbance was read at 360 nm, which is the λ_{max} of 2-amino-3-carboxymuconic semialdehyde, the unstable intermediate which spontaneously rearranges to form QA. An extinction co-efficient, ε of 47500 $M^{-1}.cm^{-1}$ was used to determine the concentration of 2-amino-3-carboxymuconic semialdehyde. The activity of 3-HAO was expressed as pmoles of 2-amino-3-carboxymuconic semialdehyde /mg protein/min.

8.4.2.3.4. Protein Assay

This was performed as described in section 4.2.2.7.

8.4.2.4. *In vivo* Administration of AC

8.4.2.4.1. Drug Treatment

This was performed as described in section 8.2.2.4.1.

8.4.2.4.2. Removal of the Liver

On the morning of the sixth day, the rats were killed and the livers removed as described in section 8.2.2.4.2.

8.4.2.4.3. Preparation of the Tissue

This was performed as described in section 8.2.2.4.3.

8.4.2.4.4. Determination of 3-HAO Activity

This was performed as described in section 8.4.2.3.3. However, there were two groups each for the livers from control and treated rats. AC was excluded from all reaction mixtures. Group one contained no $FeSO_4.7H_2O$, whilst group two did.

8.4.2.4.5. Protein Assay

This was performed as described in section 4.2.2.7.

8.4.2.5. Statistical Analysis

This was performed as described in section 4.2.2.9.

8.4.3. Results

Figures 8.6. and 8.7. show that the activity of 3-HAO is greater in the presence of Fe^{2+} ($p < 0.001$). Furthermore, 10 µM and 100 µM AC significantly reduce the activity of

3-HAO (figure 8.6.), in comparison to 0 µM AC ($p < 0.05$ and $p < 0.01$ respectively), in a concentration dependent manner. Figure 8.7. shows that AC, administered 5 mg/kg twice a day for five days induces a significant decrease in the activity of 3-HAO in both the absence and presence of Fe^{2+} ($p < 0.001$).

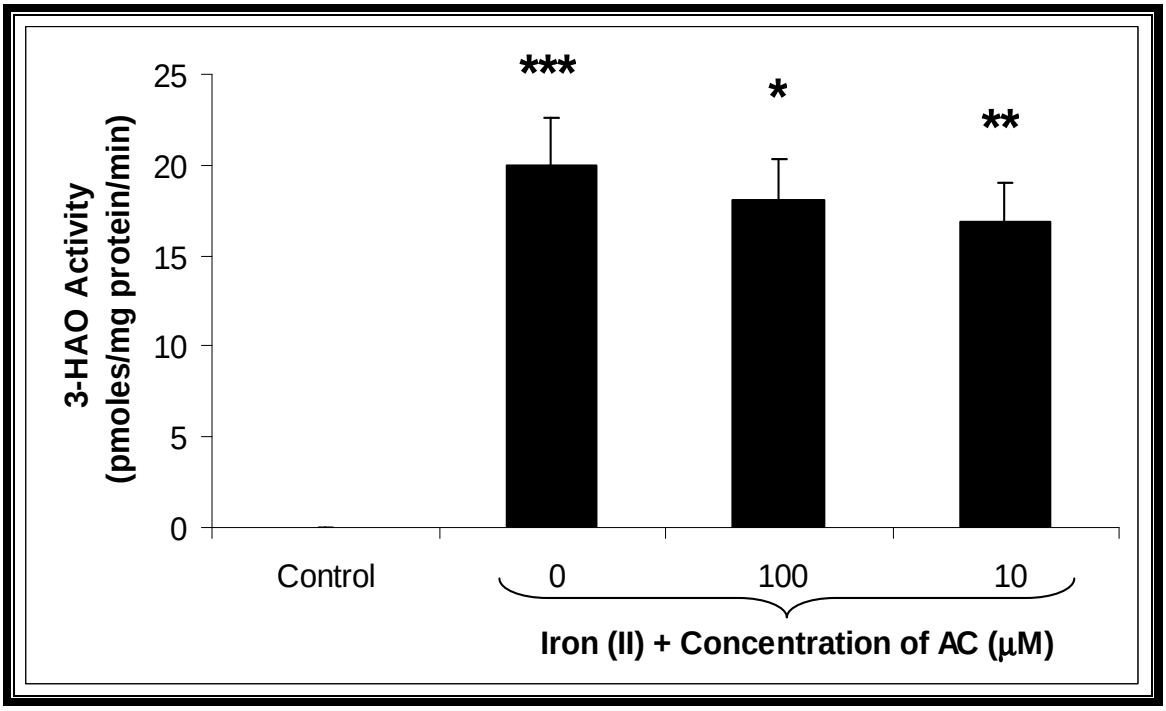


Figure 8.6. Effect of AC on 3-HAO enzyme activity in rat liver homogenate *in vitro*. Each bar represents the mean ± SD (n = 5). *** ($p < 0.001$) in comparison to control; ** ($p < 0.01$) in comparison to 0 µM AC; * ($p < 0.05$) in comparison to 0 µM AC (ANOVA and Student–Newman–Keuls Multiple Range Test).

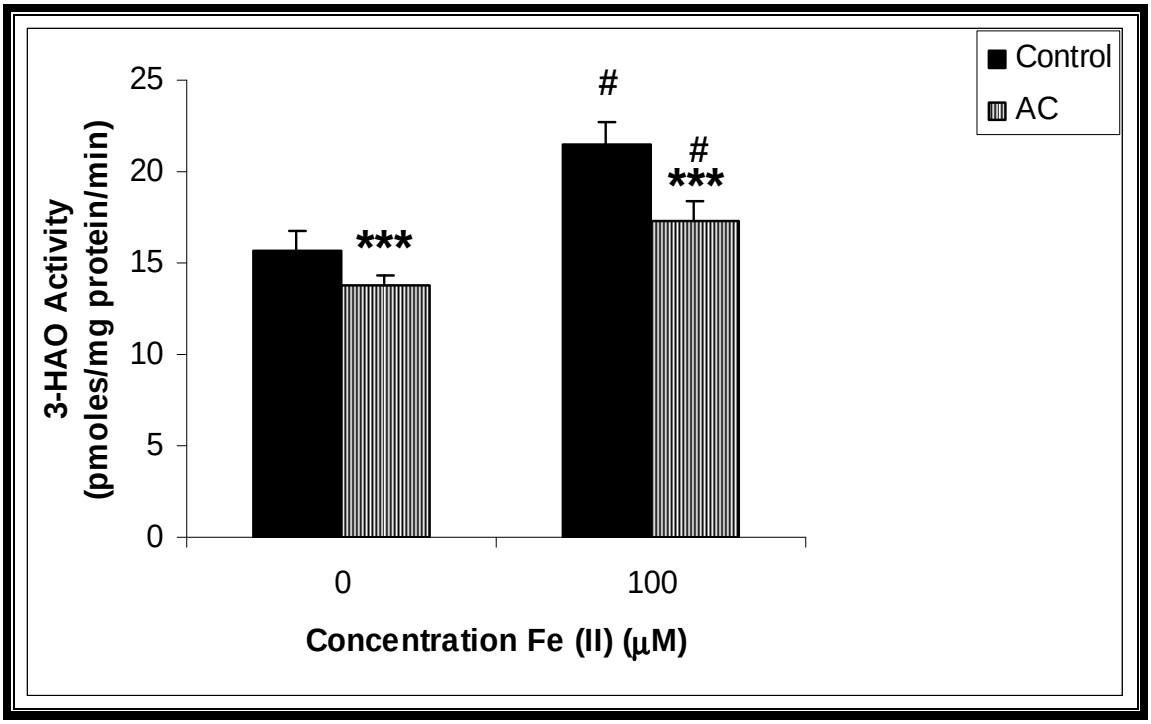


Figure 8.7. Effect of AC on 3-HAO enzyme activity in rat liver homogenate *in vivo*. Each bar represents the mean ± SD (n = 5). *** ($p < 0.001$) in comparison to controls (0 and 100 μM Fe^{2+}) # ($p < 0.001$) in comparison to 0 μM Fe^{2+} (control and AC treated rats) (ANOVA and Student–Newman–Keuls Multiple Range Test).

8.4.4. Discussion

3-HAO is a non-haeme ferrous extradiol dioxygenase enzyme (Schwarcz *et al.*, 1984). Key enzymes in the pathways of aromatic compounds are the metal-dependent ring cleavage dioxygenases, which act on the corresponding catechol-type derivatives, cleaving them at the intradiol position (*ortho* cleavage) or the extradiol position (*meta* cleavage) (Harayama *et al.*, 1992). While intradiol dioxygenases typically depend on Fe^{3+}, most extradiol dioxygenases depend on Fe^{2+} (Andújar *et al.*, 2000). In section 1.2.4.2.3.3., the role of Fe^{2+} in the transfer of electrons in the reaction which 3-HAO catalyses, was explained. In fact, the active site of 3-HAO is only complete if it contains a Fe^{2+} ion (Zhang *et al.*, 2005). Thus, it is expected that the presence of Fe^{2+} would enhance the activity of 3-HAO (figures 8.6. and 8.7.).

In Chapter 7, it was discovered that AC has the ability to bind Fe^{2+}; AC could be binding free Fe^{2+} ions, such that there are fewer of these ions available for interaction with the active site of 3-HAO. *In vitro,* this occurs in a concentration-dependent manner because a higher concentration of AC contains a greater number of AC molecules which can thus bind more Fe^{2+} ions, in comparison to lower concentrations of AC. As was the case for TDO, and IDO, *in vivo* AC inhibited 3-HAO activity in the absence and presence of exogenously added coenzyme or cofactor, implying that AC is binding endogenous and not only exogenous Fe^{2+}.

Similar to the inhibition of kynurenine-3-hydroxylase, inhibition of 3-HAO comes with great risk, as it too challenges the balance between neuroprotection and neurodegeneration. In the KP (figure 1.10), QA is the precursor of nicotinic acid which is used in the production of nicotinamide and ultimately NAD. As described in section 1.3.6., this molecule has a role to play in reversing ATP depletion in mitochondrial dysfunction. Thus, it can be concluded that inhibition of 3-HAO may adversely affect the ability of the CNS to replenish NAD in a cellular environment which may already be metabolically compromised, through mitochondrial dysfunction, one of the major mechanisms of neurodegeneration (section 1.2.2.3.). Thus it may be necessary to use agents such as AC which inhibit the production of QA together with bioenergetic supplementation to curtail or prevent this effect. This needs to be investigated further.

CHAPTER 9

THE BIOSYNTHESIS AND METABOLISM OF SEROTONIN

9.1. INTRODUCTION

The effect of viral infections on brain neurotransmitters is important since these are regarded as potential aetiological factors for the development of chronic degenerative diseases. Furthermore, certain clinical symptoms of encephalitis may reflect dysfunctions in monoamine metabolism induced by the infection (Lycke and Roos, 1972). Anatomical studies suggest that HSV-1 has a particular affinity not only for neurons located in or associated with limbic structures such as the hippocampus, as previously described (section 1.1.4.3.), but also for neurons in or near several brain regions known to contain monoaminergic cell bodies, for example, the substantia nigra pars compacta (DA cell-containing area), the dorsal raphe nucleus (5-HT cell-containing area) and the locus coeruleus (Norepinephrine-containing area) (Neeley *et al.,* 1985). Experimentally induced HSE in mice was observed to cause marked increases in brain concentrations of 5-HIAA, in comparison to uninfected mice. Work by Lycke and Roos, (1972) showed no evidence that impaired elimination of this metabolite from brain to blood could account for the phenomenon. Neither did the observed increase depend on a reduced capacity of nerve cell granules to take up 5-HT. However, after administration of substances inhibitory or stimulative to the synthesis of 5-HT, the HSV infection initiated increased neuronal activity characterised by a greater synthesis, release and ultimately turnover of 5-HT.

Interest in correlations between monoamines and depression was stimulated by the finding that first-generation antidepressant drugs enhance the availability of 5-HT and norephinephrine (NE) at central receptors (Kopin, 1981). Tricyclic antidepressants achieve this via reuptake inhibition (van Praag, 1982b) and inhibition of TDO in the liver

which, via increased TRP concentration in blood and then brain, increase central 5-HT synthesis (Badawy and Evans, 1981); and MAO inhibitors act via inhibition of 5-HT degradation. This observation led to the idea that antidepressants enhance serotonergic and noradrenergic activity and this effect is in part responsible for their therapeutic activity (van Praag, 1982b).

Postmortem brain studies of AD patients have reported loss of serotonergic neurons (Mann and Yates, 1983; Yamamoto and Hirano, 1985) and nerve terminals (Bowen *et al.*, 1983; Palmer *et al.,* 1987) as well as reduced levels of 5-HT, its major metabolite 5-HIAA, and serotonergic receptor density (Adolfsson *et al.*, 1979; Cross *et al.,* 1984). An intact hippocampal function necessitates both the cholinergic and the serotonergic systems (Vizi and Kiss, 1998; Gulyas *et al.,* 1999), as well as interaction between these two systems. Kornum and coworkers (2006) have recently discovered: (1) that an increase in acetylcholine tonus induces neuronal activation in the hippocampus, and (2) that 5-HT depletion results in a decrease of this acetylcholine-induced neuronal activation (Kornum *et al.,* 2006). Thus, the observations in the postmortem brain studies of AD patients may manifest as reduced neuronal activity in the hippocampus, possibly contributing to the depression of LTP and thus the impairment of learning and memory.

5-HT has thus emerged as a dynamic, important brain neurotransmitter in normal brain function. In view of the role of alterations in normal 5-HT synthesis, utilisation and metabolism in HSE, depression and AD, illustrated above, the effect of AC on these aspects of the monoamine was investigated, to assess whether this agent could favourably challenge or oppose these consequences of the disorders.

9.2. THE EFFECT OF AC ON 5-HT AND RELATED NEUROTRANSMITTERS LEVELS IN RAT FOREBRAIN HOMOGENATE *IN VIVO*

9.2.1. Introduction

An inverse relationship exists between liver TDO activity and brain 5-HT levels (Daya *et al.*, 1989). Thus, the inhibition of liver TDO by AC may lead to a rise in brain 5-HT and its major metabolite, 5-HIAA. NE accelerates the synthesis of [^{14}C] 5-HT from [^{14}C] TRP by cultured rat pineals (Shein and Wurtman, 1971). Since the enzymes which catalyse 5-HT biosynthesis in brain and pineal appear to be similar, this raises the possibility that noradrenergic neurons in brain which make functional synaptic contact with serotonergic neurons might stimulate 5-HT synthesis in these neurons (Shein and Wurtman, 1971). In turn, 5-HT is known to play a role in NE release in the brain (Xi-Ming Li *et al*., 2002). The precursors of 5-HT and NE are TRP and tyrosine respectively. Both these molecules are large neutral amino acids and may thus compete with one another for transport into the brain, by the neutral amino acid transporter. The increase in peripheral TRP through the inhibition of liver TDO by AC may thus reduce the uptake of peripheral tyrosine into the brain, possibly increasing 5-HT, but decreasing NE.

In general, there appears to be a distinct interdependency between the two monoaminergic systems, therefore, the effect of AC on 5-HT, 5-HIAA and NE levels in rat brain was determined. In the forebrain of saline-treated wild-type mice, the two most abundant monoamines are 5-HT and NE and of the two indoleamine metabolites, 5-HIAA is the most abundant, while 5-hydroxytryptophol represents only about 5% of total metabolite contents (Reader *et al.*, 2000), thus this region of the rat brain was chosen for assessment of these monoamines and metabolites.

9.2.2. Materials and Methods

9.2.2.1. Animals

Adult male Wistar rats were purchased from the NHLS (Johannesburg, South Africa) and cared for as described in section 2.2.2.1.

9.2.2.2. Chemicals and Reagents

AC sodium (Zovirax®, powder for injection) was obtained from Glaxo Wellcome, Midrand, South Africa. Heptane sulphonic acid (HSA), EDTA, triethylamine (TEA), phosphoric acid (PA), perchloric acid ($HCLO_4$), 5-HT, NE and 5-HIAA were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. HPLC grade acetonitrile (ACN) was purchased from Saarchem, Johannesburg, South Africa.

9.2.2.3. Drug Treatment

This was performed as described in section 8.2.2.4.1.

9.2.2.4. Brain Removal

On the morning of the sixth day, the rats were killed by cervical dislocation. The brain was removed as described in section 2.2.2.7. A portion of the forebrain was dissected, frozen in liquid nitrogen and stored at -70 ˚C.

9.2.2.5. Preparation of the Tissue

The forebrains were thawed on ice, weighed out and individually homogenised (1 mg/10 µl) in ice-cold $HCLO_4$ (0.1 M) containing 0.01 % EDTA, by sonication at 50 Hertz for 30 seconds using an ultrasonic cell disruptor. The homogenate was kept on ice for 20 minutes before being centrifuged at 10 000 x g for 10 minutes. The supernatant (10 µl) obtained was injected directly into a High Performance Liquid Chromotography-

Electrochemical Detector (HPLC-ECD) system for analysis (Muralikrishnan and Mohanakumar, 1998).

9.2.2.6. Instrumentation

Samples were analysed on an isocratic HPLC system coupled to an electrochemical detector. The chromatographic system consisted of a Waters Millipore Model 510 pump, Waters electrochemical detector and a Rikadenki chart recorder. The chart speed was set at 15 cm/hr. Samples were introduced into the system using a Rheodyne 772Si fixed loop injector, fitted with a 20 µl loop.

9.2.2.7. Chromatographic Conditions

Separation was achieved using a C_{18} (Waters Spherisorb, 5 µm, 250 x 4.6 mm n-octadecylsilane column). The mobile phase composition was 8.65 mM HSA, 0.27 mM EDTA, 13 % ACN, 0.4-0.45 % TEA and 0.22% PA (v/v) and was degassed using a 0.45µm membrane filter prior to use. It was recycled but changed every 3 days. The flow rate was set at 0.7ml/min and the electrochemical detection was performed at + 0.74 V. The results were expressed as pmoles/mg tissue.

9.2.2.8. Statistical Analysis

This was performed as described in section 4.2.2.9.

9.2.3. Results

Table 9.1. illustrates that the forebrains of rats treated with AC show a significant increase in the levels of 5-HT ($p < 0.001$) and 5-HIAA ($p < 0.001$) and a decrease in 5-HT turnover ($p < 0.05$) in the forebrain in comparison to controls. However, no significant changes are observed in the NE levels after treatment.

Table 9.1. Effect of AC on rat forebrain NE, 5-HT and 5-HIAA levels

Treatment Group	NE (pmoles/mg tissue)	5-HT (pmoles/mg tissue)	5-HIAA (pmoles/mg tissue)	5-HT turnover (5-HIAA: 5-HT)
Control	1.93 ± 0.44	2.00 ± 0.43	2.70 ± 0.31	1.37 ± 0.25
AC	2.01 ± 0.40 ^{ns}	3.76 ± 0.20 ***	3.91 ± 0.38 ***	1.11 ± 0.11 *

Each value represents the mean ± SD (n = 4). *** ($p < 0.001$), * ($p < 0.05$) and ns ($p > 0.05$) in comparison to controls (ANOVA and Student–Newman–Keuls Multiple Range Test).

9.2.4. Discussion

It has been shown that the inhibition of liver TDO has the potential to increase brain 5-HT levels (Hardeland and Rensing, 1968; Daya *et al.*, 1989). The results of this chapter show that AC increases the 5-HT level which implies that it may be effective in HSE, depression and AD where reduced 5-HT levels have been reported or suspected. In the previous chapter, AC has been shown to inhibit liver TDO, therefore this is likely to be the mechanism of action in this regard. 5-HT synthesis may also possibly be enhanced by NE (Shein and Wurtman, 1971), however, since AC had no effect on NE levels, the inhibition of TDO and thus increased brain TRP levels is confirmed as the only and most likely mechanism of increased brain 5-HT levels by AC.

By enhancing NE release 5-HT would be shifting vesicular NE to synaptic NE, thus the increase in 5-HT has no influence in NE levels in this experiment which is non-discriminatory in terms of vesicular and synaptic pools of neurotransmitters.

The proposed competition for transport into the brain between TRP and tyrosine did not decrease NE levels as expected. It appears that this hypothesis does not hold true because, unlike the direct relationship between TRP and 5-HT, levels of tyrosine in the brain do not directly correlate with levels of the neurotransmitter DA, from which NE is produced

(Groff and Gropper, 1999). The effect of increased dietary sources of tyrosine on levels of tyrosine in the brain appears to vary with region of the brain, and the effect of increased levels in the brain on neural activity is influenced by the firing rate of the neuron (McTavish *et al.*, 1999: Groff and Gropper, 1999). For example, when the firing rate is markedly increased as in Parkinson's disease or DA-depleting lesions, tyrosine can increase levels of DA (Groff and Gropper, 1999).

Most 5-HT is oxidised to 5-HIAA by the enzymes MAO and aldehyde dehydrogenase (Wurtman *et al.*, 1968). Thus, the rise in forebrain 5-HIAA levels after AC treatment may be a result of increased forebrain 5-HT levels being available as substrate for MAO. However, despite this, 5-HT turnover was reduced, which implies that AC may have the potential to slow the metabolism of 5-HT, thus maintaining the increased levels for longer. This would once again be useful in AD, depression and in particular, HSE, where 5-HT turnover is increased.

The elevation of 5-HT in diencephalic/telencephalic areas of serotonergic presynaptic terminal innervation and at the soma in the raphe (Fuller, 1994) decreases both cell firing and release of 5-HT by activating the somatodendritic 5-HT_{1A} and terminal 5-$HT_{1B/1D}$ autoreceptors respectively (Blier and de Montigny, 1994). This consequently dampens the increase in synaptic 5-HT. However, after chronic (14 days) administration of an agent which enhances 5-HT levels in these brain regions, for example, a selective 5-HT reuptake inhibitor, these receptors and the 5-HT transporter itself become desensitised allowing a larger increase in synaptic 5-HT, particularly in terminal areas (Bel and Artigas, 1993; Blier and de Montigny, 1994). The combined action of AC through the inhibition of TDO and a decreased rate of metabolism of 5-HT may produce 5-HT levels greater than either effect alone, which may lead to a faster downregulation of 5-$HT_{1A/1D/1B}$ receptors (and therefore a faster return to normal firing and release), as well as maintaining synaptic 5-HT at a higher level prior to autoreceptor downregulation. A rapid attainment of high synaptic 5-HT may enhance the onset of postsynaptic changes in emotive processing and hasten the therapeutic onset (Bel and Artigas, 1993; Blier and de Montigny, 1994). This hypothesis is supported by clinical data which shows a faster

onset of antidepressant action with combinations of a reuptake inhibitor and the 5-HT precursor, tryptophan (Walinder *et al.*, 1976; Thomson *et al.*, 1982). To assess the extent to which this hypothesis may be applicable to AC, its effect on 5-HT metabolism will be investigated in section 9.3.

9.3. THE EFFECT OF AC ON INDOLE METABOLSM IN RAT PINEAL GLANDS

9.3.1. Introduction

The 5-HT concentration is very high in the pineal gland and levels exceed any other organ in the body (Reiter, 1981; Reiter, 1989; Reiter, 1991a;). The pineal concentration of this indoleamine may be decreased due to three main pathways: (1) oxidative deamination by monoamine oxidase (MAO), (2) N-acetylation and (3) release to the extracellular spaces (Reiter, 1989; Reiter, 1981; Cardinali, 1981). The fate of 5-HT in the first two pathways is outlined in figure 1.13. Availability of 5-HT for these pathways depends on the granular 5-HT in equilibrium with "free" 5-HT (Cardinali, 1981). It is in the latter state in which 5-HT is metabolised. For these reasons, the pineal gland was the brain organ/region of choice to assess 5-HT metabolism.

After years of disregard, the pineal gland has finally taken its place in mainstream biology and medicine. It is an organ of particular fascination in that it serves as an interface between the environment and the body. Its primary functions co-ordinate the effects of light/dark cycles on physiology, via the secretion of one of the 5-HT metabolites, the neurohormone, melatonin (Arendt, 1988). Melatonin (aMT) synthesis in the mammalian pineal gland is regulated by endogenous oscillators and photoreceptors in the retina of the eye, which is functionally and anatomically connected to the pineal gland by a neural network (Hagan and Oakley, 1995). Neural pathways from the suprachiasmatic nucleus, where glutamate is the neurotransmitter, relay episodic sympathetic nerve impulses via the superior cervical ganglion, terminating at the postganglionic sympathetic fibres within the pineal gland (Petterborg *et al*, 1991). NE is released from the nerve terminals. This leads to a stimulation of postsynaptic ß-adrenergic receptors of the pinealocytes which activates the second messenger, adenylate cyclase via a stimulatory guanine nucleotide-binding regulatory protein, G_s (Reiter, 1991b). Adenylate cyclase catalyses the conversion of ATP to cAMP (Strada *et al.*, 1972). The increase in intracellular levels of cAMP (up to 60-fold in the rat pineal)

results in elevated N-acetyl transferase (NAT) activity. Through the cAMP dependent protein kinase, cAMP increases the transcription of mRNA (Reiter, 1991b), and thus translation to increase NAT synthesis. This enzyme then converts 5-HT to N-acetylserotonin (NAS) which is converted to aMT. Light has an inhibitory effect on the sympathetic nerves to the pineal gland (Klein, 1973), thus more aMT is synthesised nocturnally.

Collectively, data indicate a role for melatonin in the antioxidant defence system of organisms. Most importantly, there is evidence which suggests that these actions of aMT are not merely pharmacological, but physiologically relevant too (Reiter, 1997). For example, aMT is protective against free radical damage, even at physiological concentrations (Reiter, 1997).

Thus, the aim of this experiment is to investigate whether AC could manipulate 5-HT pineal indole metabolism to increase 5-HT and/or aMT. The value of the former was emphasised in section 9.2.4., whilst that of the latter has been highlighted above. These actions would confer on AC a novel role in neuroprotection with respect to learning and memory impairing disorders, HSE, AD and depression.

To carry out this investigation, organ culture, a valuable and sensitive technique was used. It 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 small size, and its ability to remain viable for as long as six days under optimum conditions. It is capable of using exogenous radioactive (^{14}C) 5-HT to produce the various indoles (Daya *et al.*, 1989), therefore the direct effect of AC on 5-HT metabolism could easily be established.

Bidimensional Thin Layer Chromatography (TLC) was used to separate the pineal indole metabolites. This method provides a remarkably simple and inexpensive means for separating and identifying components of small samples of complex, inorganic, organic and biochemical substances. Furthermore, it permits reasonably accurate quantitative

determination of the components of such mixtures (Skoog and West, 1980). In TLC, separation is achieved on a planar surface, rather than with a column, which offers the unique advantage of two-dimensional operation. In this manner, samples which could be only partially separated with either solvent alone may be completely separated by the combination of solvents. Thus the selective properties of two different solvents can be utilised in developing a single chromatogram (Ewing, 1960).

9.3.2. Materials and Methods

9.3.2.1. Animals

Adult male Wistar rats were purchased from the NHLS (Johannesburg, South Africa) and cared for as described in section 2.2.2.1.

9.3.2.2. Chemicals and Reagents

AC sodium (Zovirax®, powder for injection) was obtained from Glaxo Wellcome, Midrand, South Africa. ^{14}C labelled 5-HT was obtained from Amersham International, England. The culture medium, Minimun Essential Medium (MEM), was purchased from Highveld Biological (PTY) LTD, Lyndhurst, South Africa and fortified with the antibiotics, amphoteracin B, streptomycin and benzyl penicillin (Hoechst, South Africa). The aluminium TLC plates coated with silica gel 60, Type F254 (0.25 mm), were purchased from Merck, Darmstadt, Germany. Beckman Ready-Sol multipurpose liquid scintillation fluid was purchased from Beckman RIIC Ltd, Scotland. The indole standards, 5-HT, NAS, aMT, 5-hydroxytryptophol (5-HTOH), 5-methoxytryptophol (5-MTOH), 5-methoxytryptomaine (5-MT), 5-HIAA and 5-methoxyindoleacetic acid (5-MIAA) were purchased from Sigma Chemicals Co, St Louis, USA. Ascorbic acid, ethanol, chloroform and glacial acetic acid were purchased from Saarchem, Krugersdorp, South Africa.

9.3.2.3. Drug Treatment

This was performed as described in section 8.2.2.4.1.

9.3.2.4. Pineal Gland Removal

On the morning of the sixth day, the rats were killed by cervical dislocation. 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.

9.3.2.5. Organ Culture of the Pineal Glands

The pineal glands were placed individually into sterile borosilicate (75 x 10 mm) Kimble tubes containing 52 µl of MEM culture medium, supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (2.5 µg/ml). (^{14}C) 5-HT (8 µl) with a specific activity 55 mCi/mmoles was added. The tubes were then saturated with carbogen (95 % Oxygen/5 % Carbon dioxide) and sealed. The tubes were incubated at 37 ˚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.

9.3.2.6. 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 ˚C for 10 minutes. 10 µl 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. The indole standard solution was prepared using 1 mg of each of the pineal metabolites: 5-MT, aMT, 5-HT, NAS, 5-MIAA, 5-HIAA, 5-MTOH and 5-HTOH. The pineal indoles were dissolved in 2.5 ml absolute ethanol. The solution was then vortexed after the addition of 2.5ml 1% ascorbic acid (an antioxidant) in 0.1 M HCl. The standard

solution (10 µl) was spotted onto the culture medium and 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 addition of 1 part glacial acetic acid results in the complete separation of 5-MIAA from 5-HIAA. Solvent B separates 5-HTOH from NAS and improves separation of aMT from 5-MIAA and 5-MTOH. The small amount of acid which remains on the gel from the first solvent development enhances the effective separation by solvent B. 5-HT and 5-MT remain at the origin (Klein and Notides, 1969).

The spots were excised and individually placed in plastic scintillation vials. Absolute ethanol (1 ml) was added to each vial, together with 3 ml Beckman Ready-Sol scintillation fluid and then vortexed for 30 s. The radioactivity of each metabolite was then measured in a Beckman LS 2800 scintillation counter. The results were obtained as counts per minute (CPM) and corrected for the counting efficiency of the scintillation counter to disintegrations per minute (DPM). The results were expressed as DPM/10µl/ pineal.

9.3.2.7. Statistical Analysis

This was performed as described in section 4.2.2.9.

9.3.3. Results

A typical bi-dimensional thin layer chromatogram of the pineal indole metabolites obtained in this experiment is shown in Fig. 9.1. An acceptable extent of separation is

observed. As discussed in section 9.2.2.6., 5-HT and 5-MT do not migrate from the origin; therefore the results expressed for the origin are those of 5-MT and 5-HT.

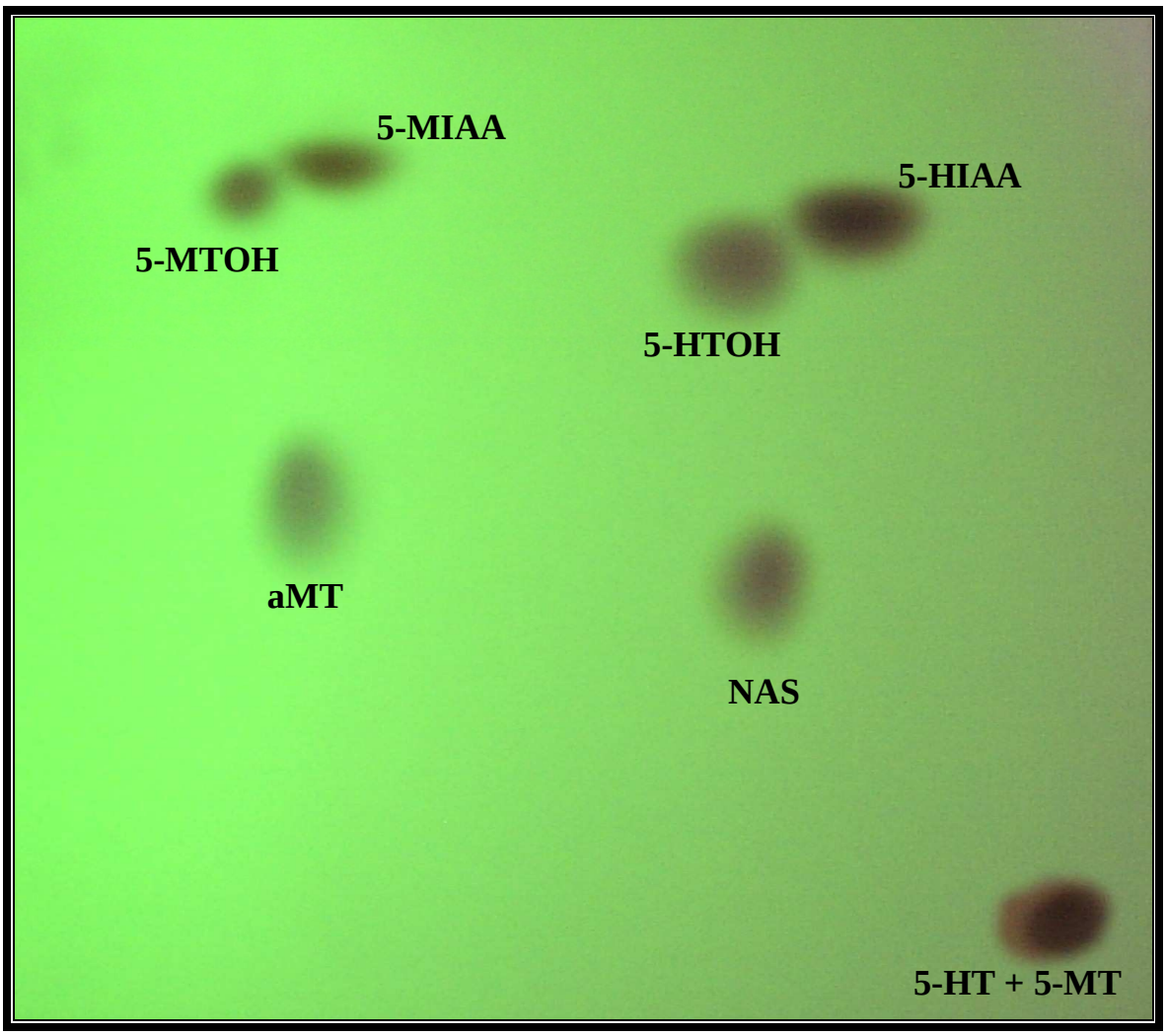


Figure 9.1. Typical bi-dimensional thin layer chromatogram illustrating the location of the pineal indole metabolites.

Figure 9.2. shows that there is a significant decrease in the DPM/10µl/pineal for the combination of 5-HT and 5-MT ($p < 0.001$) and for 5-HIAA ($p < 0.01$) of the AC treatment group in comparison to the control group, whilst a moderate increase in the DPM/10µl/ pineal is observed for 5-HTOH, NAS and aMT (all $p < 0.05$). The effect of AC on 5-MIAA and 5-MTOH is insignificant.

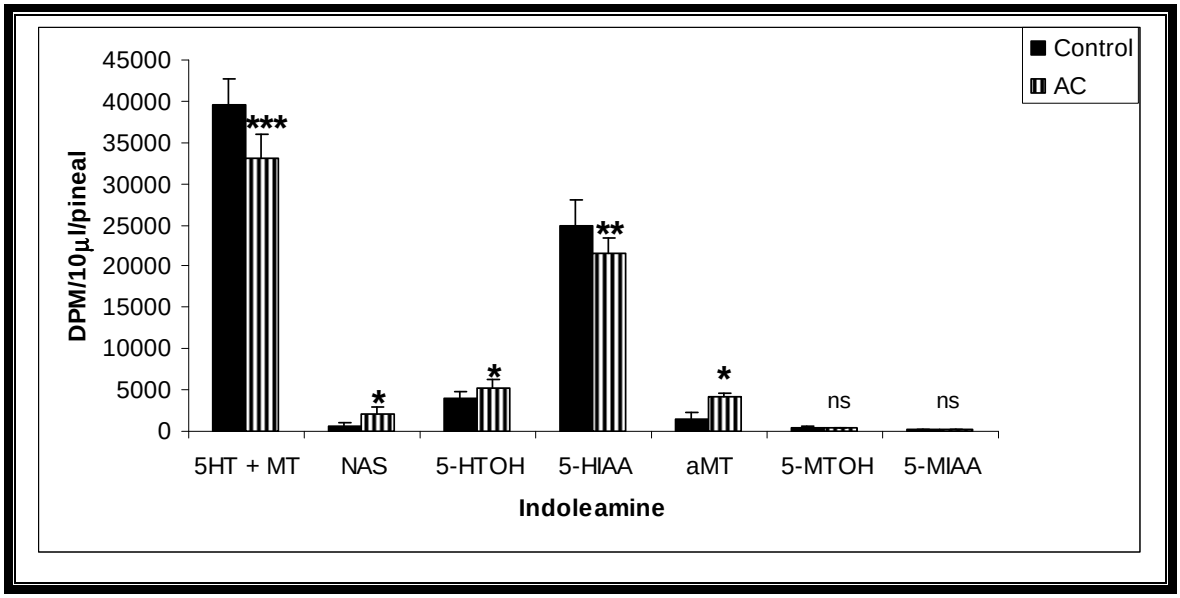


Figure 9.2. Effect of AC on indole metabolism in the rat pineal gland *in vivo*. Each bar represents the mean ± SD; n = 4. *** ($p < 0.001$), ** ($p < 0.01$), * ($p < 0.05$) and ns ($p > 0.05$) in comparison to controls (ANOVA and Student–Newman–Keuls Multiple Range Test).

9.3.4. Discussion

The results show that under normal circumstances (control conditions), 5-HT in the pineal gland is either taken up by the sympathetic nerve terminals and subjected to MAO degradation (Olivieri *et al.*, 1990) to form 5-HIAA and 5-HTOH or it is taken up by the pinealocytes for conversion to NAS and then aMT. The decrease in 5-HT + 5-MT after AC treatment is thus related to increased uptake and utilisation of 5-HT at one or both of these two sites. It is not likely due to a decrease in 5-MT, as this would generate a concomitant increase in 5-HT, because less of it is converted to 5-MT. It therefore appears that AC has a stimulatory rather than the expected inhibitory effect on 5-HT metabolism. Since there is a significant increase in both metabolites of 5-HT formed in the pinealocytes, namely,NAS and aMT, the increased uptake and utilisation of 5-HT after AC treatment most likely occurs there and not at the sympathetic nerve terminals..

The effect of AC on 5-HTOH and 5-HIAA is possibly through one of two mechanisms. AC may have the ability to inhibit the activity of aldehyde dehydrogenase or to enhance the activity of alcohol dehydrogenase. The effects of either of these postulated mechanisms would be an increase in 5-HTOH and a decrease in 5-HIAA, which may occur under some metabolic conditions, when the redox potential of the brain has shifted to a more reducing state than normal (Bender, 1983). AC may contribute to such conditions since many of the previous chapters have alluded to an antioxidant property of this agent. The shunt in this pathway of 5-HT metabolism may also account for the reduced turnover of forebrain 5-HT to 5-HIAA (section 9.2.) observed after AC treatment. More of the 5-HT acted upon by the MAO enzyme under control conditions was converted to 5-HTOH rather than 5-HIAA after treatment.

From section 9.3.1., it is evident that when provided with the same concentration of radioactive 5-HT, NAT activity and therefore the production of NAS in the pineal gland may be enhanced by i) increased levels of NE, ii) a ß-adrenoreceptor agonist, iii) increased levels of ATP, iv) increased activity of adenylate cyclase or v) increased levels of cAMP. Section 9.2. demonstrated that AC has no effect on rat forebrain NE levels, thus it is unlikely that AC enhances NAT activity in the pineal through increasing levels of this catecholamine.

The activation of adenylate cyclase in the pineal gland occurs via a stimulatory guanine nucleotide-binding regulatory protein, (G_s), (Reiter, 1991b). This activation, through the action of a hormone (in this case the catecholamine, NE) and a GTP coincides with a release of GDP bound to the G_s. This indicates that a GTPase reaction, which hydrolyses GTP to GDP at the regulatory site, G_s generates an inactive adenylate cyclase possessing tightly bound GDP, and that the hormone increases adenylate cyclase activity by inducing a repetitive introduction of the activator GTP to replace the hydrolysed nucleotide, GDP (Reiter, 1991b). Thus elevated GTPase activity in the presence of hormone results from the enhanced displacement of GDP by the substrate GTP (Cassel and Selinger, 1978). Triphosphorylated AC, as a GTP analogue may thus be substrate and AC treatment over 5 days may provide a constant supply of the analogue and therefore

contribute to increased NAT activity and consequently increased NAS. However, as described in section 1.3.7.1.3.3., host cell TK is approximately 1 millionfold less capable of converting AC to its monophosphate derivative than virus-encoded TK, which would slow the formation of the triphosphorylated AC and perhaps explain the modest increase ($p < 0.05$) in NAS. An increase in aMT follows an increase in NAS as there is more of the latter available for conversion to the neurohormone by the enzyme HIOMT.

As described in section 1.2.4.2.4., reduced levels of aMT are secreted from the pineal gland of patients with AD and depression. Whether or not the increase in aMT induced by AC is sufficient to regain the normal physiological concentrations of aMT is debatable. Furthermore, although aMT is protective against free radical damage, at physiological concentrations (Reiter, 1997), such low concentrations may not be successful at alleviating or eliminating oxidative stress caused by neurotoxins implicated in HSE, depression and AD, such as QA.

CHAPTER 10

SUMMARY OF RESULTS, GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

Irrespective of socio-economic class, most spheres of our lives depend on our ability to store and recall information. The neuropathology of CNS disorders in which learning and / or memory is impaired is often associated with an immune response to one or more stressors. In HSE, this is the HSV-1 virus, whereas in AD, it may be the AP and NFT, whilst in major depression, it is a psychological stressor. During the immune response, microglia are activated, producing and releasing many neurotoxic products including QA, which have the ability to cause neurodegeneration. Although QA may not be the sole cause of neurodegeneration, it is likely to have some role to play, particularly if neurotoxic concentrations in the hippocampus are achieved. Numerous studies point to a neuroprotective potential for guanine, guanosine and their derivatives and analogues. AC, a guanosine analogue, is of particular relevance in this regard because it is the current drug of choice in the treatment of one of these debilitating diseases, namely, HSE.

This research project has aptly demonstrated that the antiherpetic guanosine analogue drug, AC, has the ability to improve QA-induced spatial memory deficits in a rat model. Furthermore, it has proved successful in elucidating some of the mechanisms, both direct and indirect, by which this effect is elicited.

The extent to which i.p. post-treatment of AC improves spatial memory deficits induced by intrahippocampal injections of QA was investigated in Chapter 2 using the Morris water maze task. The results of the behavioural study show that AC significantly improves QA-induced spatial memory deficits by virtue of the shorter latencies to escape to the platform of QA-infused rats which received i.p. post-treatment in comparison to those which did not. It was speculated that this effect is related to the ability of AC to protect against QA-induced hippocampal neurodegeneration which prevents the retrieval

of the spatial information stored in potentiated synapses through LTP and autosassociation at the NMDA receptors.

It is well established that QA induces neurodegeneration via the two cell death processes, namely, necrosis and apoptosis. The aim of Chapter 3 was therefore to assess the effect of intrahippocampal QA alone and in combination with i.p. post-treatment of AC on hippocampal neuromorphology and cytoarchitecture. The Nissl stained cells in the CA1 and CA3 regions show visible destruction of the structural features of these regions in rats which received intrahippocampal QA. The nature of the damage fits the profile and characteristics of the various stages of necrotic cell death. The majority of the hippocampal neuronal cells in QA-infused rats, post-treated with AC, appear to maintain structural integrity, thus the QA-induced necrotic death is alleviated. It was suggested that AC exhibits this action via interference with the three mechanisms of neurodegeneration, namely, excitotoxicity, oxidative stress and mitochondrial dysfunction which QA is known to induce. Further studies are warranted to determine the effect of QA alone and in combination with AC on apoptotic cell death. Moreover, NMDA receptor binding and cell viability studies in which a reduction in the number of NMDA receptors correlates to a reduction in the number of viable neurons, could be performed. This would provide supporting, statistically significant evidence of an increased loss of neurons after intrahippocampal QA alone and an amelioration of this effect after i.p. post-treatment with AC.

Excitotoxicity is a mechanism of neuronal cell death mediated by overstimulation of ionotropic glutamate receptor sub-types, such as NMDA and AMPA. In addition to eliciting a direct excitotoxic effect through NMDA overactivation, it has more recently been shown that *in vivo*, QA has the ability to inhibit vesicular and astrocytic glutamate uptake and to enhance synaptosomal glutamate release. In this manner, QA indirectly enhances excitotoxicity through the elevation of glutamate levels in the synaptic cleft. Therefore, with the knowledge that QA has the ability to enhance synaptosomal glutamate release, and that this is reversed by guanosine (Tavares *et al.*, 2005), the effect of AC (a guanosine analogue) on QA alone and in combination with AC on synaptosomal

glutamate release was investigated in Chapter 4. The experimental results show that although QA enhances synaptosomal glutamate release, AC is not able to alter this. Interestingly, one of the effects of excitotoxicity is mitochondrial dysfunction. It was postulated that QA therefore causes mitochondrial dysfunction which results in energy failure. This in turn disrupts ionic gradients and the regulation of glutamate uptake is lost, leading to increased excitatory amino acid levels. The finding that AC has no effect on the QA-induced increase in glutamate release could possibly indicate that AC is not able to salvage mitochondrial respiratory function after a QA-induced excitotoxic insult. The effect of AC on mitochondrial function needs to be investigated further.

A previous study demonstrated that activation of presynaptic NMDA autoreceptors may provide a positive rather than the usual negative feedback on presynaptic glutamate release in synaptosomal preparations (Breukel *et al.,* 1998). This stimulating activity of QA at these specific receptors is thought to be another mechanism by which this neurotoxin elicits an increase in glutamate release. Despite AC belonging to the class of guanosine analogues, which are known to have antagonistic activity at the NMDA receptors (Tavares *et al.,* 2005), it failed to reduce QA-induced glutamate release. It was suggested that this discrepancy occurred because Tavares and coworkers (2005) administered guanosine i.p. prior to the infusion of QA and thus, guanosine was already present to prevent the binding of QA. In this study, however, QA was infused 24 hours before initiation of the AC treatment and may have had more time to bind to presynaptic NMDA autoreceptors to elicit the response (glutamate release) before sufficient AC is present to inhibit the QA effect. To confirm this hypothesis, this experiment needs to be repeated with a change in the treatment regime, in which pre-treatment rather than post-treatment of AC is administered. Even if the results of such a study do show that pre-treatment of AC reduces glutamate release, it would not mean that pre-treatment is necessarily superior to post-treatment, since the former would likely prevent LTP from occurring at the postsynaptic NMDA receptor and therefore aggravate rather than improve spatial memory deficits.

Further studies also need to be conducted to determine the effect of AC on QA-induced inhibition of vesicular and astrocytic glutamate uptake. Since it is the increase in intracellular calcium levels which initiates the various cell responses to NMDA overstimulation, it may also prove useful to investigate the effect of AC on intracellular calcium levels and the binding of calcium ions.

Oxidative stress is a state within cells where the generation of free radicals exceeds the capacity of the endogenous antioxidant defence system. Chapter 5 explores one such free radical, namely $O_2^{\bullet-}$ and one such endogenous antioxidant molecule, namely GSH. QA has the ability to induce oxidative stress primarily via the Ca^{2+}-dependent events during excitotoxicity but may also act independently of NMDA stimulation.

It was demonstrated that QA does not induce $O_2^{\bullet-}$ generation in rat brain homogenate *in vitro* which indicates that QA has no direct effect on the electron transport chain activity of the mitochondria from which most $O_2^{\bullet-}$ originates. This suggests that the ability of QA to cause mitochondrial dysfunction reported by Bordelon and coworkers (1997) is mediated by NMDA receptor overactivation (excitotoxicity). QA was added to homogenised tissue, in which the integrity of the cells is destroyed and thus unable to evoke a response to the NMDA overstimulation. Even though QA does not enhance the formation of $O_2^{\bullet-}$, it is important to note that at the higher end of the concentration range, AC is able to reduce the amount of $O_2^{\bullet-}$ present under those conditions. The presence of this $O_2^{\bullet-}$ is not influenced by the NMDA stimulation associated with QA, which implies that the reduction in $O_2^{\bullet-}$ brought about by AC must be through its ability to scavenge this free radical, rather than prevention of its formation. The mechanism by which AC scavenges $O_2^{\bullet-}$ was proposed in section 5.2.4.

QA injected intrahippocampally (*in vivo*) is able to induce a significant increase in $O_2^{\bullet-}$. The ability of QA to cause mitochondrial dysfunction, via its perpetuating excitotoxic mechanism (chapter 4) may account for the QA effect on $O_2^{\bullet-}$, although this needs to be investigated further. The dampening of the QA-induced increase in $O_2^{\bullet-}$ by AC is most likely primarily or even solely related to its capacity to scavenge this free radical, which

was described in the previous experiment. This is argued in the light of the doubt cast on AC being able to restore mitochondrial function or to act as an anti-excitotoxin when administered as post-treatment.

GSH levels are diminished in the rat hippocampus after an intrahippocampal injection of QA. After the dismutation of $O_2^{\cdot-}$ to H_2O_2, it is reduced to H_2O by GPx with GSH as a reducing agent. Therefore the effect of QA on GSH levels suggests that an inverse relationship exists between $O_2^{\cdot-}$ levels and GSH levels. GSH is known to have its own free radical scavenging properties. Therefore it may also become depleted through reaction with the various other free radicals produced during a QA-induced excitotoxic insult. AC treatment blunts the QA effect on GSH levels likely through its ability to scavenge $O_2^{\cdot-}$**.** It may also be able to scavenge various other free radicals, such as $HO^{\cdot}$ and thus reduce GSH depletion; this needs to be explored. Further investigations into the effect of AC, in the absence and presence of intrahippocampal QA on the activity of the antioxidant enzymes SOD, GPx and GR are also warranted.

The mechanisms of neurodegeneration, particularly oxidative stress ultimately cause oxidative damage to lipid, protein and nucleic acid biomolecules of the cells, which directly or indirectly leads to neurodegeneration. Chapter 6 illustrates that QA significantly induces LP in rat brain homogenates *in vitro* and that AC suppresses this effect. It is thought that *in vitro*, QA enhances the $HO^{\cdot}$-producing Fenton reaction by forming a complex with Fe^{2+} which is more easily oxidisable than free Fe^{2+} is. The ability of AC to inhibit the Fenton reaction directly was assessed by conducting Fe^{2+}-induced LP. AC once again has an inhibitory effect and therefore the ability of AC to reduce QA-induced LP was speculated to be through binding of Fe^{2+} and / or Fe^{3+}. AC also reduces QA-induced LP *in vivo*, which may occur in the presence of the free radicals generated from the QA-Fe complex or via NMDA-dependent excitotoxicity. The efficacy of AC in the *in vivo* experiment is thus possibly related to its ability to scavenge free radicals or to bind ferrous and / or ferric ions. AC has been shown to bind $O_2^{\cdot-}$ in Chapter 5. It was also argued that the *in vivo* results exhibited an inverse relationship to the degree of integrity of the cell membranes of the cell bodies of the hippocampal neurons in the histological

analyses (Chapter 3), which implied that the integrity of neural membranes in general is highly dependent on the degree of LP. To establish the nature of protection AC offers to biomolecules in its entirety, further studies should be conduced to determine its effects on the oxidation of proteins and nucleic acids.

In view of the possibility that AC alleviates Fe^{2+}-induced and QA enhanced Fe^{2+}-induced LP through the chelation of ferrous and/or ferric iron, the purpose of Chapter 7 was to conduct iron chelation studies. The UV/VIS data demonstrates that AC interacts with both Fe^{2+} and Fe^{3+} and that the interaction with the latter is stronger. A mechanism of AC-metal complex formation was proposed. The AdCSVs from an electrochemical analysis confirm that AC interacts with Fe^{2+} and Fe^{3+} and that the latter interaction is stronger, forming a more stable complex. The results also indicated that QA, like AC interacts with Fe^{2+} to form comparatively weak, unstable complexes. The electrochemical analysis further characterised the redox potentials of the complexes in comparison to the free ferrous or ferric ions. It was concluded that the AC-Fe^{3+} and the QA-Fe^{2+} complexes are more difficult to reduce, but easier to oxidise than the respective free metal ion, whilst the opposite is observed for the AC-Fe^{2+} complex. These results thus provided an explanation for the inhibition of redox cycling of iron by AC complexation to ferrous and ferric ions and the enhancement of the oxidation of ferrous iron by QA complexation to Fe^{2+}. In the last part of the chapter, the ferrozine assay was used to determine which ligand, QA or AC, has greater Fe^{2+} chelating activity. The results of the assay demonstrate that overall AC has a greater chelating activity, indicating that it is more likely to bind Fe^{2+} in the presence of both ligands and therefore provides an adequate explanation for the inhibition of QA-induced LP by AC (Chapter 6).

The KP, in which the neurotoxin QA is produced endogenously, contains neuroactive intermediates derived from L-KYN, the primary degradation product of TRP. However, the KP does not only occur in the CNS, but in the periphery as well and the dynamics of the KP are such that there is interplay between KP metabolism in the periphery and the brain. The aim of Chapter 8 was to assess the effect of AC on the biosynthesis of QA by measurement of the activity of the enzymes which catalyse the first and last steps in the

conversion of tryptophan to QA, namely, TDO, IDO and 3-HAO. Since it is the blood-borne L-KYN which drives the cerebral KP, TDO and IDO, which catalyse the conversion of TRP to L-KYN, were measured peripherally in the liver and small intestine respectively. Although cerebral QA can only be formed by 3-HAO from 3-HA in the brain, liver 3-HAO activity was used as a model of brain 3-HAO activity since the enzyme is present in greater abundance in the liver which simplifies the method of quantification required. There was merit in doing this because the there appears to be general accordance between 3-HAO in the brain and from peripheral sources.

AC inhibits apoenzyme and total enzyme activity of TDO *in vitro* at 100 µM, but not at 10 µM. The treatment of the animals with AC (5 mg/kg) decreases the apo-, holo- and total enzyme activity of TDO. It was proposed that AC most likely interferes with the conjugation of haeme to the apoenzyme by competitive inhibition, which explained the results of the *in vitro* studies. This argument was extended to the *in vivo* results where there is inhibition of endogenous and exogenous haeme and therefore blunting of the activity of both forms of the enzyme by AC. The competition between AC and haeme for binding to the apoenzyme needs to be explored further. Since stressors activate the HPA axis, causing the release of glucocorticoids which induce TDO, it may be interesting to assess the effect of AC on this phenomenon. This is particularly relevant because glucocorticoids regulate the LHPA axis by interacting with glucocorticoid receptors in the brain, including in the hippocampus.

The cofactors, $O_2^{\cdot-}$ and Fe^{2+} are required for the activity of IDO and 3-HAO respectively, thus the activity of both enzymes is enhanced in the presence of the respective cofactors, *in vitro* and *in vivo*. AC inhibited the activity of both enzymes in the presence of the cofactors *in vitro* and in the absence and presence of the cofactors *in vivo.* The results were expected due to the $O_2^{\cdot-}$ scavenging and Fe^{2+} binding properties of AC demonstrated in previous chapters of this thesis. In a similar pattern to that of TDO inhibition, it was speculated that *in vivo*, AC exhibited these properties obliterating exogenous and endogenous cofactor. To confirm the findings of the studies in Chapter 8, the effect of AC directly on QA levels in the different regions of the brain need to be investigated.

The liver cytosolic enzyme, TDO is the major determinant of the levels of peripheral circulating TRP, therefore, the untoward effect of enhanced activity of TDO is not only an increase in blood-borne L-KYN for the biosynthesis of the neurotoxin QA, but it also reduces the availability of TRP for uptake into the brain, where it is utilised for the synthesis of the neurotransmitter serotonin. 5-HT has thus emerged as a dynamic, important brain neurotransmitter in normal brain function and the consequences of its abnormal synthesis, utilisation and metabolism are dire. Therefore, Chapter 9 investigated the effect of AC on these aspects of the monoamine.

AC (5mg/kg for 5 days) increases the 5-HT forebrain levels, but has no effect on the NE level which may have a role to play in stimulating 5-HT synthesis from TRP. It was therefore concluded that the inhibition of TDO and thus increased brain TRP levels is confirmed as the only and most likely mechanism of increased brain 5-HT levels by AC. AC also induces a rise in the forebrain 5-HIAA level which was attributed to the fact that there is increased forebrain 5-HT levels available as substrate for MAO and aldehyde dehydrogenase which oxidise 5-HT to 5-HIAA. However, despite this, 5-HT turnover was reduced, which implies that AC may have the potential to slow the metabolism of 5-HT, thus maintaining the increased levels for longer. Increased 5-HT levels are known to have a role to play in aggression, therefore it would be interesting to assess whether AC could aggravate aggressive behaviour, using a rat model of aggression.

The results of the pineal organ culture showed that AC treatment decreases the pineal level of 5-HT + 5-MT. It was hypothesised that this effect is related to an increased uptake and utilisation of 5-HT by the pinealocytes to form NAS and subsequently, aMT, which are both increased after AC treatment. It therefore appears that AC has a stimulatory rather than the expected inhibitory effect on 5-HT metabolism. Further investigation is required using a liquid-phase extraction method rather than TLC as a method of separation, to allow for total separation of the two indoleamines, 5-HT and 5-MT, which could then be quantified individually. This would provide more conclusive results on the effect of AC on 5-HT uptake and utilisation by the pineal gland. An increase in the pineal level of 5-HTOH and a decrease in 5-HIAA is observed and is

thought to occur via one of two mechanisms; AC may have the ability to inhibit the activity of aldehyde dehydrogenase or to enhance the activity of alcohol dehydrogenase. Therefore, the direct effect of AC on the activity of these two enzymes needs to be investigated It was also concluded that the shunt in the serotonin metabolism pathway, such an effect on aldehyde dehydrogenase or alcohol dehydrogenase would cause, may also account for the reduced turnover of forebrain 5-HT to 5-HIAA.

It was speculated that AC, in its triphosphorylated form may provide a constant supply of substrate for GTPase, thereby enhancing adenylate cyclase activity, which in turn increases cAMP levels, enhancing NAT activity and therefore increasing the NAS level. This postulate may be explored further by assessing the effect of AC on adenylate cyclase activity, cAMP levels and NAT activity in the pineal gland. The increase in NAS was however modest, possibly due to the reduced capability of AC to be phosphorylated in non-virulent cells. An increase in aMT follows an increase in NAS as there is more of the latter available for conversion to the neurohormone by the enzyme HIOMT. The effect of AC on NAS and aMT levels should be investigated in models of experimental HSE to determine whether this increase is more pronounced in the presence of virulent cells. An effect of AC on endogenous aMT levels formed from endogenous 5-HT is also warranted.

This thesis points towards a possible neuroprotective role for AC and other guanosine derivatives and analogues in known memory impairing neurodegenerative disorders, such as AD or potential neurodegenerative disorders, such as HSE and depression. Excitotoxicity, oxidative stress, LP, accumulation of iron, the enhanced biosynthesis of QA and reduced biosynthesis of 5HT and aMT are reported to be commonly encountered in AD, HSE and depression. Therefore, these triggers and/or markers of neurodegeneration form the central themes around which this thesis is assembled. Each has ability to either directly or indirectly shift the neurodegenerative-neuroprotective balance towards neurodegeneration and the question which threaded these themes together was; to what extent, if any, could AC counter the one-sided effect and swing it towards neuroprotection?

It has emerged that AC has the potential to convincingly reduce oxidative stress, LP, iron levels and the biosynthesis of QA and to enhance the production of 5HT. Therefore, AC has direct neuroprotective effects and may be effective in AD, depression and HSE. The inhibition of TDO by AC indicates that the drug may prove very useful in negating the induction of this enzyme by glucocorticoids, released in response to activation of the LHPA axis by stressors in AD, depression and HSE, or by possible corticosteroid anti-inflammatory therapy used in HSE. Thus the increased biosynthesis of QA and decreased 5-HT, which may occur due to the action of glucocorticoids, is reduced. Through the amelioration of GSH levels, the binding of free iron and reducing the activity of IDO, AC may reduce proliferation of HSV-type 1 independent of its antiviral action, and in this manner accomplish an indirect neuroprotective effect in which reduced CNS viral load implies a reduced immune response and therefore reduced release of neurotoxic substances. These actions endow on AC additional mechanisms of action, other than its antiviral action which may contribute to its effectiveness in the treatment of HSE.

The inhibition of 3-HAO comes with great risk, as it challenges the balance between neuroprotection and neurodegeneration. QA is the precursor of nicotinic acid which is used in the production of nicotinamide and ultimately NAD, which has a role to play in reversing ATP depletion in mitochondrial dysfunction. Thus, it can be concluded that inhibition of 3-HAO may adversely affect the ability of the CNS to replenish NAD in a cellular environment which may already be metabolically compromised, through mitochondrial dysfunction. Thus it may be necessary to take agents such as AC which inhibit the production of QA together with bioenergetic supplementation to curtail or prevent this effect. This needs to be investigated further.

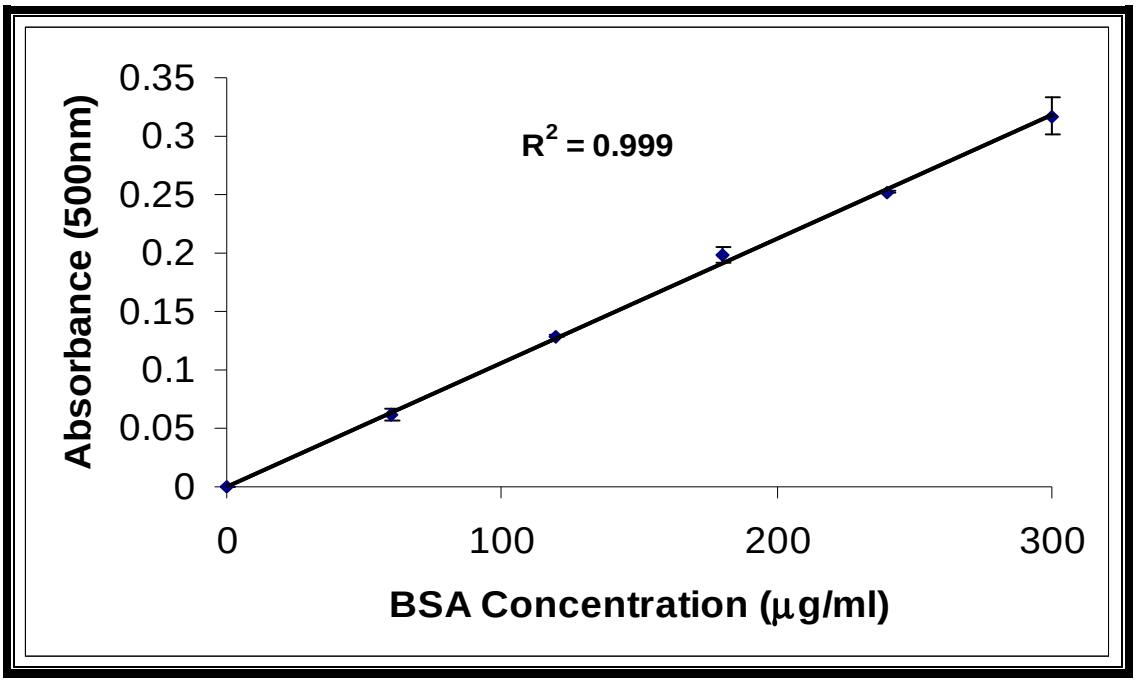
The effect of AC on reducing excitotoxicity and enhancing aMT was arguable and therefore inconclusive. It has emerged that the iron binding potential of AC may be a double-edged sword. Further investigations are warranted to assess the extent to which such iron deprivation may interfere with physiologically required levels of iron. This may contribute to “sickness behaviour” and anaemia particularly in patients already

predisposed to or exhibiting such signs and symptoms. If these speculations are confirmed, AC may not be the safest effective drug in such patients.

The next step forward may well be to continue preliminary animal studies, with the same or similar research objectives set out in this research project together with the recommended studies using more specific models of experimental HSE, AD and depression. Furthermore, it may also be worth comparing the effects of AC and other guanosine analogues and derivatives, so that structure-activity relationships with respect to neuroprotective properties can be generated. For example, it would be interesting to compare the neuroprotective effects of AC and guanine to determine the role that the acyclic chain plays in imparting these properties to AC.

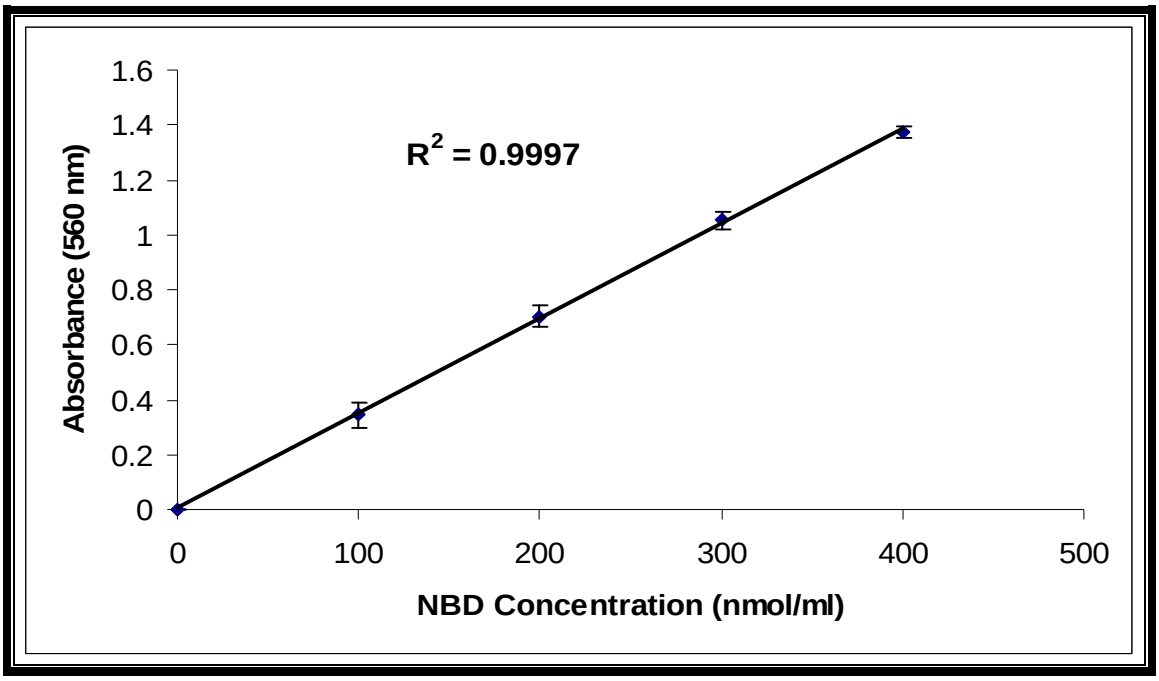
Pharmaceutical companies invest millions every year bringing novel drug molecules to phase I clinical trials for neurodegenerative disorders, whilst before them may lie a plethora of effective, registered drugs like the prototype, AC. After success in further preliminary animal studies, the advancement of such drugs directly to phase II or III clinical trials may be advocated since after years of use, the pharmacokinetic and safety profiles of these are already well established. This may assist in making the search for treatment of these debilitating disorders far more cost-effective.

APPENDIX I



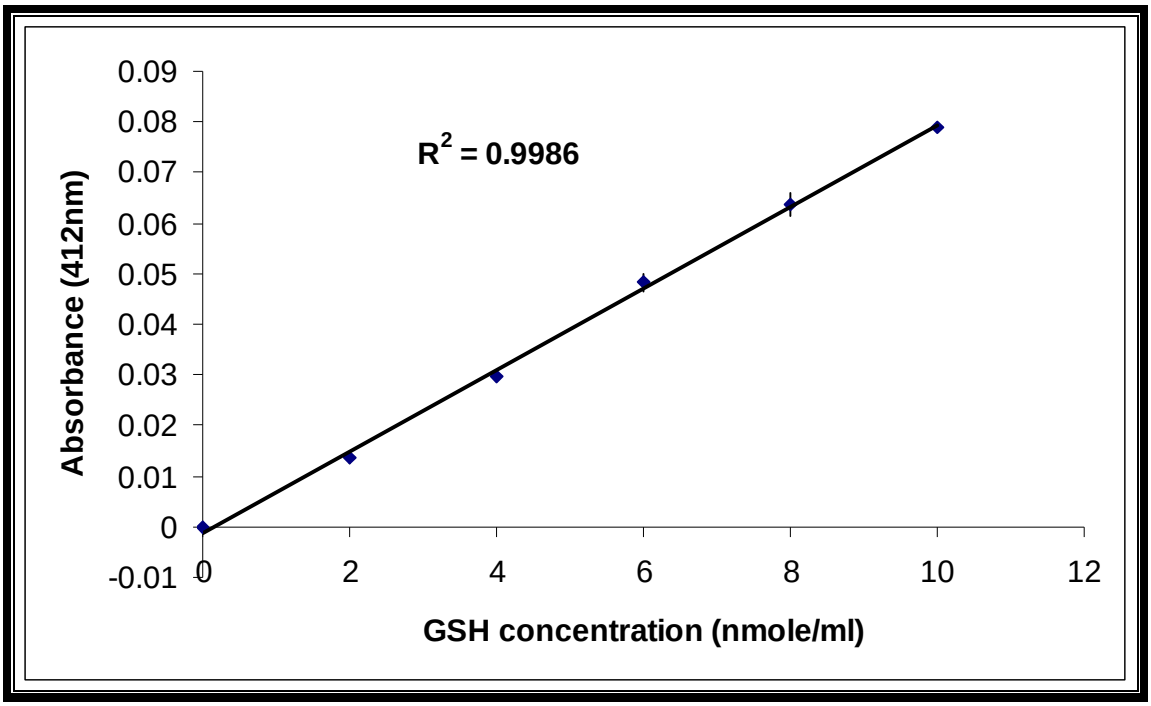
Protein Standard Curve

APPENDIX II



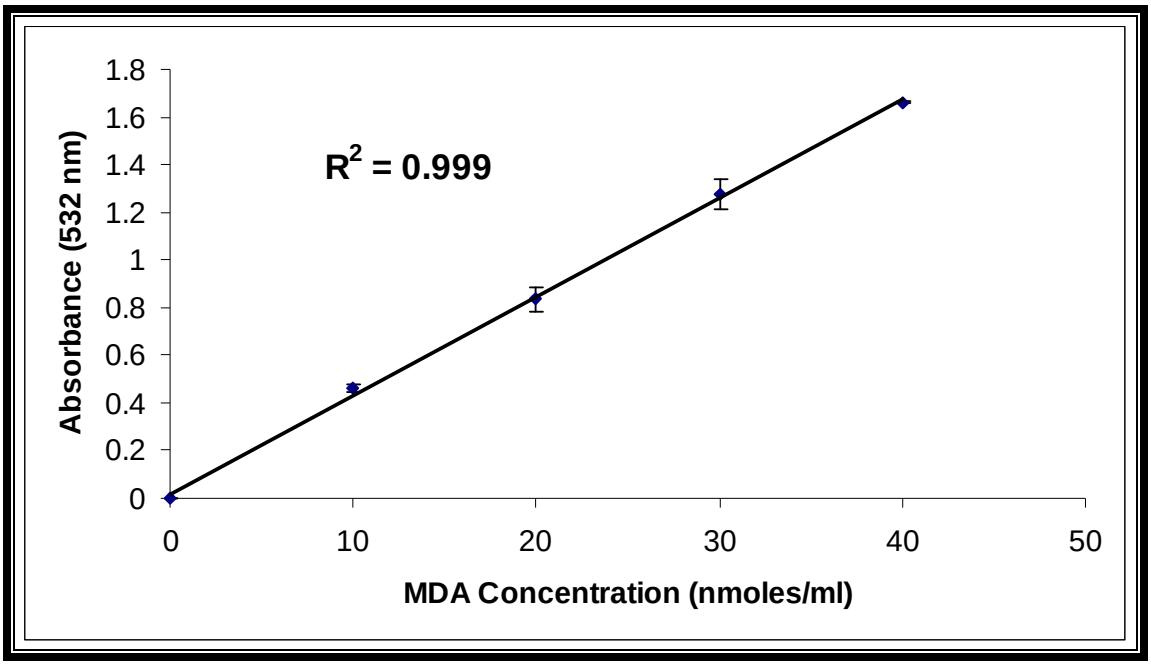
NBD Standard Curve

APPENDIX III



GSH Standard Curve

APPENDIX IV



MDA Standard Curve

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