AN INVESTIGATION INTO THE NEUROPROTECTIVE PROPERTIES OF ACETYLSALICYLIC ACID AND ACETAMINOPHEN

THESIS

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By

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Dedicated to my parents, Joey and Gita Maharaj for their encouragement, love and support…

"Vision is seeing what everyone else has seen and thinking what no-one else has thought."

Albert Szent-Gyorgi

ABSTRACT

The potent analgesic property of acetylsalicylic acid and acetaminophen makes these the most commonly used analgesics in the world. Easy accessibility and cost effectiveness of these agents are attractive to patients seeking pain relief. However, the abuse of non-narcotic analgesics such as acetaminophen and acetylsalicylic acid by alcoholics and patients seeking to relieve dysphoric moods is well documented. These agents therefore impact on the brain neurotransmitter levels and therefore all processes involved in the synthesis and metabolism of neurotransmitters may be affected. The use of non-narcotic analgesics has been reported to reduce the incidence of neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD). The mode of action by which acetylsalicylic acid and acetaminophen elicit neuroprotection is however unclear as many mechanisms of action have been inconclusively postulated.

The first part of this study aims to elucidate the various mechanisms by which acetylsalicylic acid and acetaminophen affect the enzymes responsible for the catabolism of tryptophan, which is a precursor for the mood elevating neurotransmitter serotonin, as well as to investigate whether these agents alter the interplay between serotonin and pineal indole metabolism. The second part of this study focuses on the neuroprotective properties of acetylsalicylic acid and acetaminophen utilizing the neurotoxic metabolite of the kynurenine pathway, quinolinic acid and the potent Parkinsonian neurotoxin, 1-methyl-4-phenylpyridinium (MPP^+).

The ability of acetylsalicylic acid and acetaminophen to alter TRP metabolism was determined by investigating the effects of these agents on the primary enzymes of the kynurenine pathway i.e. tryptophan 2, 3-dioxygenase and indoleamine 2,3-dioxygenase as well as to investigate whether these agents would have any effects on 3-hydroxyanthranilic acid oxygenase. 3-Hydroxyanthranilic acid oxygenase is the enzyme responsible for the synthesis of quinolinic acid. Acetylsalicylic acid and acetaminophen alter tryptophan metabolism by inhibiting tryptophan 2, 3-dioxygenase and indoleamine 2,3-dioxygenase thus increasing the availability of tryptophan for the production of serotonin. Acetylsalicylic acid and acetaminophen also inhibit 3-hydroxyanthranilic acid oxygenase thus implying that these agents could reduce quinolinic acid production.

Acetaminophen administration in rats induces a rise in serotonin and norepinephrine in the forebrain. Acetylsalicylic acid curtails the acetaminophen-induced rise in brain norepinephrine levels as well as enhances serotonin metabolism, indicating that analgesic preparations containing both agents would be advantageous, as this would prevent acetaminophen-induced mood elevation. The results from the pineal indole metabolism study show that acetylsalicylic acid enhances pineal metabolism of serotonin whereas acetaminophen induces an increase in melatonin levels in the pineal gland.

Neuronal damage due to oxidative stress has been implicated in several neurodegenerative disorders such as AD and PD. The second part of the study aims to elucidate and characterize the mechanism by which acetylsalicylic acid and acetaminophen afford neuroprotection. The hippocampus is an important region of the brain responsible for memory. Agents such as quinolinic acid that are known to induce stress in this area have detrimental effects and could lead to various types of dementia. The striatum is also a vulnerable region to oxidative stress and hence (MPP^+), which is toxic for this particular region of the brain, was also used as a neurotoxin. The results show that ASA and acetaminophen alone and in combination, are potent superoxide anion scavengers. In addition, the results imply that these agents offer protection against oxidative stress and lipid peroxidation induced by several neurotoxins in rat brain particularly, the hippocampus and striatum.

Histological studies, using Nissl staining and Acid fuchsin, show that acetylsalicylic acid and acetaminophen are able to protect hippocampal neurons against quinolinic acid-induced necrotic cell death. Immunohistochemical investigations show that QA induces apoptotic cell death in the hippocampus, which is inhibited by ASA and acetaminophen. In addition, ASA and acetaminophen inhibited MPP^+ induced apoptotic cell death in the rat striatum.

The study also sought to elucidate possible mechanisms by which ASA and acetaminophen exert neuroprotective effects in the presence of MPP^+ as these agents are shown to prevent the MPP^+-induced reduction in dopamine levels. The results show that acetylsalicylic acid and acetaminophen inhibit the action of this neurotoxin on the mitochondrial electron transport chain, a common source of free radicals in the cell. In addition, these agents were shown to block the neurotoxic effects of MPP^+ on the

enzymatic defence system of the brain i.e. superoxide dismutase, glutathione peroxidase and catalase. The reduction in glutathione levels induced by MPP^+ is significantly inhibited by acetylsalicylic acid and acetaminophen. The results imply that these agents are capable of not only scavenging free radicals but also enhance the cell defence mechanism against toxicity in the presence of MPP^+. These agents also block the MPP^+-induced inhibition of dopamine uptake into the cell. This would therefore reduce auto-oxidation of dopamine thus implying another mechanism by which these agents exert a neuroprotective role in MPP^+-induced neurotoxicity.

The discovery of neuroprotective properties of acetylsalicylic acid and acetaminophen is important considering the high usage of these agents and the increased incidence in neurological disorders. The findings of this thesis point to the need for clinical studies to be conducted as the results show acetylsalicylic acid and acetaminophen to have a definite role to play as antioxidants. This study therefore provides novel information regarding the neuroprotective effects of these agents and favours the use of these agents in the treatment of neurodegenerative disorders, such as AD and PD, in which oxidative stress is implicated.

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2. **H. Maharaj,** D. S. Maharaj**,** K.S. Saravanan, K.P. Mohanakumar, and S. Daya. Aspirin curtails the acetaminophen-induced rise in brain norepinephrine levels. Metabolic Brain Disease. 19 (1-2): 71-77 (2004).

3. D.S. Maharaj, H. Mollel, E. Antunes, **H. Maharaj**, D. Maree, T. Nyokong, B.D. Glass, and S. Daya. Melatonin generates singlet oxygen on laser irradiation but acts as a quencher when irradiated by lamp photolysis. J. Pineal Res. Online publication date 19-Oct-2004.

4. A. Muller, **H. Maharaj**, D.S. Maharaj, and S. Daya. Acyclovir protects against quinolinic acid induced oxidative neurotoxicity. J. Pharm. Pharmacol. In Press.

5. D.S. Maharaj, **H. Maharaj**, E.M. Antunnes, D. Maree, T. Nyokong, B.D. Glass, and S. Daya. Melatonin 6-hydroxymelatonin protects against oxidative neurotoxicity. J. Pharm. Pharmacol. In Press.

LIST OF SYMBOLS AND ABBREVIATIONS

AC	Adenylate cyclase
AD	Alzheimer's Disease
ADHD	Attention deficit hyperactivity disorder
AIF	Apoptosis inducing factor
ALA	Aminolaevulinic acid
aMT	Melatonin
ANOVA	One way analysis of variance
AP	Anterior posterior
ASA	Acetylsalicylic acid
ATP	Adenosine Triphosphate
BBB	Blood brain barrier
BHT	Butylated hydroxytoluene
BSA	Bovine Serum Albumin
CA1	Cornu Ammonis *1*
CA3	Cornu Ammonis *3*
CAD	caspase-activated deoxyribonuclease
CNS	Central nervous system
CO_2	Carbon dioxide
COMT	Catechol-*O*-methyltransferase
CSF	Cerebrospinal fluid
DA	Dopamine
DAT	Dopamine transporter
DNA	Deoxyribonucleic Acid
DOPAC	3, 4-dihydroxyphenylacetic acid
DPI	2, 6-dichlorophenolindophenol
DTNB	5,5'-Dithio-bis(2-nitrobenzoic acid)
DV	Dorsal ventral
EAA	Excitatory amino acid
EDTA	Ethylenediaminetetraacetic acid
Em	Emission
ETC	Electron transport chain
Ex	Excitation
Fe^{2+}	Iron (II)
Fe^{3+}	Iron (III)
Fmoles	Fentamoles
GPx	Glutathione peroxidase
GRd	Glutatione reductase
GSH	Glutathione
GSSG	Oxidized glutathione
HAD	Hydroxyalkenals
HAO	Hydroxyanthranilic acid oxygenase
HCl	Hydrochloric acid
$HClO_4$	Perchlorate
HD	Huntington's Disease
HE	Hepatic encephalopathy
HIAA	Hydroxyindole acetic acid
HIOMT	Hydroxyindole-O-methyltransferase
HK	Hydroxykynurenine
H_2O_2	Hydrogen peroxide

HPLC	High performance liquid chromatography
5-HT	Serotonin
HTOH	Hydroxytryptophol
HTP	Hydroxytryptophan
HVA	Homovanillic acid
ICAD	Caspase-activated deoxyribonuclease inhibitor
IDO	Indoleamine 2, 3-dioxygenase
i.p.	Intraperitoneal
ISEL	*In situ* end labelling
ISNT	*In situ* nick translation
KAT	Kynurenine aminotransferase
KCN	Potassium cyanide
KYN	Kynurenine
KYNA	Kainic acid
L	Lateral
LB	Lewy Bodies
LNAA	Large neutral amino acid
LOD	Limit of detection
LOQ	Limit of quantitation
$LOO^{\bullet}$	Peroxyl radical
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitors
MDA	Malondialdehyde
MIAA	Methoxyindole acetic acid
min	Minute
ml	Millilitre
mM	Millimolar
MOPS	3-[*N*-morpholino]propanesulfonic acid
MPTP	1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
MPP^{+}	1-methyl-4-phenylpyridinium
mRNA	Messenger ribonucleic acid
MTOH	Methoxytryptophol
NAAC	Neutral amino acid carrier
NAD	Nicotinic acid dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADP	Nicotinamide Adenine dinucleotide phosphate
$NaHCO_3$	Sodium Hydrogen Carbonate
NAL	Nicotinylalanine
NAS	N-acetylserotonin
NAT	Serotonin-N-acetyltransferase
NBD	Nitroblue Diformazan
NBT	Nitroblue Tetrazolium
NE	Norepinephrine
NK	Natural killer
NMDA	N-methyl-D-aspartate
$NO^{\bullet}$	Nitric oxide
NOCD	Naturally occurring cell death
NOS	Nitric oxide synthase
ns	Not significant
$O_2^{-\bullet}$	Superoxide anion radical

$^{\bullet}OH$	Hydroxyl radical
6-OHD	Hydroxydopamine
o-MBA	o-methoxybenzoylalanine
OXPHOS	Oxidative phosphorylation system
p	Probability
PARP	poly(ADP-ribose) polymerase
PARS	Poly-ADP ribose polymerase
PBS	Phosphate buffered saline
PCD	Programmed cell death
PDT	Photodynamic therapy
Pmoles	Picamoles
POD	Horse-radish peroxidase
Ppm	Parts Per Million
QA	Quinolinic acid
R_T	Retention Time
ROS	Reactive oxygen species
s	seconds
SAM	S-adenolsyl-L-methionine
s.c.	Subcutaneous
SCG	Superior cervical ganglia
SCN	Suprachiasmatic nucleus
SEM	Standard error of the mean
SN	Substantia niagra
SNpc	Substantia niagra pars compacta
SNS	Sympathetic nervous system
SOD	Superoxide dismutase
SPE	Solid-phase extraction
TBA	2-Thiobarbituric acid
TCA	Trichloroacetic acid
TDO	Tryptophan 2, 3-dioxygenase
TdT	Terminal deoxynucleotidyl transferase
TEM	Transmission electron microscopy
TH	Tyrosine hydroxylase
Tris	Tris (hydroxymethyl)-aminomethane
TLC	Thin layer chromatography
TRP	Tryptophan
TUNEL	TdT-mediated dUTP nick end labeling
UK	United Kingdom
USA	United States of America
USP	United States Pharmacopoeia
V	Volts
VMAT	Vesicular associated monoamine transporters
v/v	Volume by volume
$\propto$	Alpha
µCi	Microcurie
µg	Micrograms
µl	Microlitre
µM	Micromolar
^{o}C	Degrees celcius

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CHAPTER ONE

LITERATURE REVIEW

1.1. INTRODUCTION

Manipulation of the kynurenine pathway of tryptophan (TRP) metabolism has yielded a plethora of agents that are being developed as neuroprotectants and antidepressants. TRP is the essential amino acid that plays an integral role in the synthesis of the neurotransmitter serotonin (5-HT), which is implicated in depression. The interest in the neurobiology of the metabolites of this pathway was aroused with the discovery that integral components of this pathway such as quinolinic acid (QA), could be implicated in the neuronal damage that characterizes neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Stone, 2000).

Alzheimer's disease and PD are late-onset neurodegenerative diseases that have tremendous impact on the lives of affected individuals, their families and society as a whole. Remarkable efforts are being made to elucidate the dominant factors that result in the pathogenesis of these disorders. Extensive post-mortem studies of PD and AD patients suggest that oxidative stress is a prominent feature of these neurodegenerative disorders. The frequent presentation of intracytoplasmic inclusions in the patients of both disorders suggests the involvement of a common underlying aberrant process. It can therefore be surmised that oxidative stress, which is cooperatively influenced by environmental factors, genetic predisposition, and senescence, may be the link between these disorders (Trojanowski *et al.*, 2002).

Alzheimer's disease is the major cause of dementia in the United States with a typical age of onset as early as 60 years of age for sporadic cases and a prevalence that can reach as high as 30% by the age of 80 (Zabar and Kawas, 2000). Typical sporadic PD has a

prevalence of 0.6% at 65 years of age, but the risk of developing PD increases with age with a prevalence of 4-5% by the age of 85 (Zabar and Kawas, 2000).

Therefore there is a dire need to investigate the use of novel, cost effective agents in the treatment of these prevalent neurodegenerative disorders. Hence this study focused on using common, non-narcotic analgesics namely acetylsalicylic acid (ASA) and acetaminophen to investigate any possible neuroprotective effects that they may possess.

1.2. TRYPTOPHAN (TRP)

Tryptophan is a dietary essential amino acid (Bender, 1982). The chemical structure of TRP is shown in figure 1.1. It has the greatest molecular weight of the twenty amino acids and is encoded by messenger ribonucleic acid (mRNA) to be incorporated into protein synthesis.

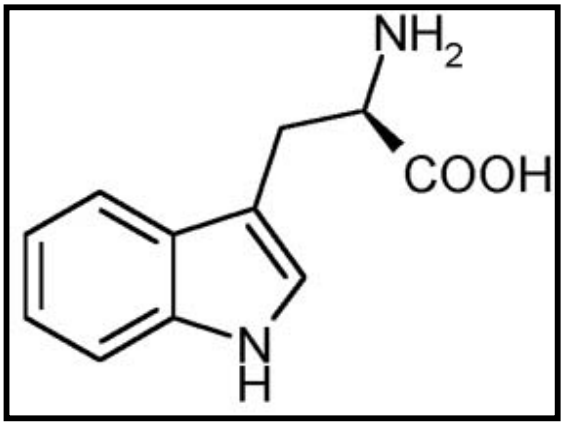

Figure 1.1. Chemical structure of the essential amino acid tryptophan (TRP)

Tryptophan derivatives constitute a number of physiologically important molecules such as the neurotransmitter 5-HT, the neurohormone melatonin (aMT), the vitamin niacin, proteins, and the pyridine nucleotides (Maurizi, 1990). There are essentially two sources of plasma TRP in the body: (1) increased hepatic portal output after a protein rich meal and (2) secretion by the tissues as a consequence of alterations in the concentration of bound and free TRP pools in the body.

Following protein ingestion and the efflux of TRP from bound and "free" pools in the tissues, TRP enters the circulation as the overflow from the portal circulation. TRP leaves the plasma via:

(1) Metabolism in the liver by TRP-2,3-dioxygenase and most significantly
(2) uptake into the tissues,
(3) excretion into the urine (Fernstrom and Wurtman, 1971).

In individuals, the metabolism of the dietary amino acid exhibits a rhythm depending on the food consumed. Peak levels are found to occur during the latter part of the dark period or the early light period (Sudgen, 1979).

1.2.1. TRP Uptake System

Green and Curzon (1968) demonstrated that following TRP induction of TDO in the liver by the administration of glucocorticoids, there was a significant reduction in the rate of 5-HT turnover, and in the steady state concentration of 5-HT in the central nervous system (CNS). Conversely a number of studies have shown that the administration of loading doses of TRP leads to an increase in the rate of 5-HT turnover (Eccleston *et al.*, 1965).

Little is known about the enzymology of the transport of amino acids across membrane systems. However there has been greater knowledge on the specificity of the amino acid transport systems, regardless of the enzymology of the process. The mechanism of uptake in the intestinal mucosa, renal tubule and the BBB have similar specificity. A common carrier, transports not only TRP but also phenylalanine, tyrosine, threonine, leucine, isoleucine and valine, the so-called large group of neutral amino acids. In the brain this common carrier system was first described in brain slices (Blasberg and Lajtha, 1966), and has since been demonstrated at the blood-brain barrier by single perfusion studies (Olendorf, 1971).

The rate of amino acid uptake into the brain from the bloodstream is extremely high. Thirty-fifty percent of a labeled amino acid present in an infusion bolus may be extracted during a single pass through the brain capillary bed (Olendorf, 1971). There are at least ten different groups of amino acids in the brain tissue, concerned with uptake from the bloodstream, efflux from the CNS, exchange across the BBB (counter transport rather than active transport) and uptake into specific cells and regions of the brain. These systems can be differentiated by their sensitivities to inhibition by substrates and analogues (Sershen and Lajtha, 1979).

The uptake of TRP by the brain is essential due to the fact that TRP is a precursor to the synthesis of useful indoleamines in the body such as 5-HT and aMT. The passage of TRP and other amino acids between the blood and the brain is facilitated by a carrier system. Tryptophan is a water-soluble molecule hence it is transported between tissues and organs via a diffusion process. Tryptophan transportation is dependent on a carrier system to cross the blood brain barrier. The neutral amino acids compete with each other for attachment to this carrier molecule and hence transport to the brain (Wurtman, 1982). The affinities of the BBB amino acid transport system for the various large neutral amino acids, their potencies as inhibitors of the transport of other amino acids and their potencies as inhibitors of transport in the bloodstream are all closely similar. This suggests that competition between amino acids will be an important factor in determining the rate of TRP uptake into the brain.

In individuals, the metabolism of the dietary amino acid exhibits a rhythm depending on the type of food consumed. Peak levels of TRP have been found to occur during the latter part of the dark period or the early light period (Sudgen, 1979). A high protein meal reduces the plasma ratio of TRP to the competing amino acids and would therefore decrease the amount of TRP crossing the BBB and subsequently its availability to the brain (Wurtman, 1982; Fernstrom *et al.*, 1971). A high carbohydrate intake would cause a rise in insulin secretion which reduces the plasma levels of competing amino acids and the ratio of TRP to competing amino acids is higher resulting in a an increased amount of TRP crossing the BBB. Tryptophan is bound to the plasma protein, albumin, isolated in

the albumin reservoir and is immune to the effects of insulin (Wurtman, 1982). The flux of amino acids traversing tissues is influenced by a variety of hormones such as glucocorticoids and by antidepressants (Fernstrom *et al.*, 1971).

1.2.2. The Binding of TRP to Serum Albumin

Tryptophan is unique among the amino acids in that it is bound non-covalently to serum albumin, a phenomenon that was first observed by McMenamy and Oncley (1958). The binding is specific, and only a limited number of analogues and metabolites of TRP will compete. Albumin binding is specific for the L-enantiomer; D-TRP is not bound. Non-esterified fatty acids displace TRP form albumin binding. The binding sites for TRP and fatty acids are separate, but there seems to be some degree of overlap or interaction between them (McMenamy, 1965).

This may be important under physiological conditions. The percentage of TRP that is free increases with the concentration of non-esterified fatty acids in response to fasting and exercise, and falls following the intake of food (Bender, 1982). A number of drugs have been shown to displace TRP from albumin binding. Compounds such as indomethacin, benzoate and clofibrate have been shown to displace TRP both *in vivo* and when added to albumin or serum *in vitro* (Spano *et al.*, 1974; Smith and Lakatos, 1971). The equilibrium of TRP binding to the albumin and is very rapid, and it has been suggested that because of this the binding of TRP to albumin will not be an important factor in controlling the uptake of TRP into the brain. Much of the bound TRP would be stripped off albumin during a single pass through the brain capillary bed (Yuwiler *et al.*, 1977). This therefore suggests that the factor that will be important in determining the rate of uptake into the brain will be total concentration of TRP in the bloodstream, rather than that which is freely diffusible, and not bound to albumin.

The albumin–bound TRP represents a considerable buffer pool that can replenish the free pool of the amino acid available and is considerably greater than the free pool of amino acid, which is immediately available to the transport system. TRP competes with other

large neutral amino acids for the transport system and approximately only 10% of serum TRP is free. The role of the albumin-bound pool of TRP is to maintain this pool of free TRP at a more or less constant level despite uptake (Bender, 1982). Further evidence of the importance of albumin binding in controlling the uptake of TRP into the nervous system has come from the perfusion studies of Etienne and coworkers (1976). Inclusion of albumin in the infusion bolus used in single perfusion studies of amino acid uptake leads to a considerable decrease in the rate of TRP uptake.

1.2.3. TRP and Mental Depression

Evidence indicates that TRP deficiencies are common and have a role to play in human disease (Vaughan, 1984). Tryptophan depletion appears to have a negative effect on the learning ability of individuals (Park *et al.*, 1994). A negative correlation exists between free plasma TRP levels and depression during acute TRP depletion (McDougle *et al.*, 1993), where a lack of the amino acid has a pronounced effect on the depressive symptoms (Curzon and Bridges, 1970). There is considerable evidence that TRP metabolism is disturbed during depression, namely due to:

(1) The therapeutic activity of MAO inhibitors (MAOI) which is enhanced by TRP (this implies a lack of 5-HT). TRP treatment has also been found to be efficacious in some depressives.

(2) Low levels of 5-hydroxyindoleacetic acid, the excretory product of TRP metabolism, implies a decreased level of 5-HT, and is also indicative of dysregulated TRP metabolism.

(3) There is increased secretion of cortisol with a consequent rise in TDO, 3-OH-kynurenine and 3-HAO concentrations.

Abnormal 5-HT levels in the brain and the cerebrospinal fluid could be due to a number of biochemical variables e.g. high tryptophan 2,3-dioxygenase (TDO) activity. Curzon

and Bridges (1970) demonstrated that there is elevated TDO activity even after recovery from depressive patients. Most antidepressives involve either 5-HT or noradrenaline (NA), or both neurotransmitters. It is believed that the therapeutic effect of certain drugs could be dependent on the availability of 5-HT (Delgado *et al*., 1990).

1.2.4. TRP Catabolism via the kynurenine pathway

Tryptophan is primarily metabolized via the kynurenine pathway which is illustrated in figure 1.2. This pathway is responsible for the formation of various kynurenines including the potent neurotoxin, QA.

1.2.4.1. TDO (EC 1.13.11.11)

Tryptophan 2, 3-dioxygenase is a heme-dependent enzyme, in the human liver, as in a number of species, including the rat. There is a considerable proportion of the enzyme that is present as the apoenzyme form, and is therefore inactive until the additional heme is made available, either by the administration of heme precursors *in vivo* or by the addition of hematin during incubation *in vitro* (Bender, 1982).

1.2.4.1.1. TDO Regulation

1.2.4.1.1.1. TDO Induction

Tryptophan 2, 3-dioxygenase is subject to hormonal and substrate induction as well as a number of other effectors (Knox and Auerbach, 1955). Tryptophan 2, 3-dioxygenase activity can be increased by the administration of cortisone or TRP. Adrenal glucocorticoids also increase synthesis in the rat liver (Civen and Knox, 1959) but via direct interaction with the genome which results in raised levels of mRNA leading to increased apoenzyme synthesis (Fiegelson *et al.*, 1961; Greengard *et al.*, 1963; Greengard *et al*., 1966).

Tryptophan administration prevents the rapid degradation of the pre-existing apoenzyme (Badaway *et al*., 1975; Schimke *et al*., 1965) and enhances the conjugation of hematin with the apoenzyme while preserving the active reduced holoenzyme (in the absence of TRP the reduced holoenzyme is inactivated reversibly to the oxidized form of the enzyme).

The similarity of the effect that adrenal glucocorticoids and TRP have on the regulation of TDO has been thought to be the result of different mechanisms. Puromycin, an inhibitor of protein synthesis, can inhibit cortisone-induced elevation of TDO. Actinomcyin D, which inhibits RNA synthesis, abolishes the cortisone-induced rise in apoenzyme and holoenzyme but does not influence the TRP-mediated increase in the level of TDO. This suggests that there is a difference between the hormonal- and substrate- induced stimulation of enzyme synthesis *in vivo* (Greengard *et al*., 1963; Greengard *et al*., 1966).

The administration of heme or its precursor 5-aminolaevulinate (5-ALA) also stimulates the enzyme activity (Badaway *et al*., 1987). The mode of action of these compounds is to increase the saturation of the endogenous apoenzyme and heme. Activation of TDO by exogenous heme is not prevented by actinomycin D or by puromycin. They block pyrollase activity only when 5–ALA is administered. This suggests that the step in heme synthesis beyond 5-ALA synthetase requires the continuation of mRNA and protein synthesis for activation to occur.

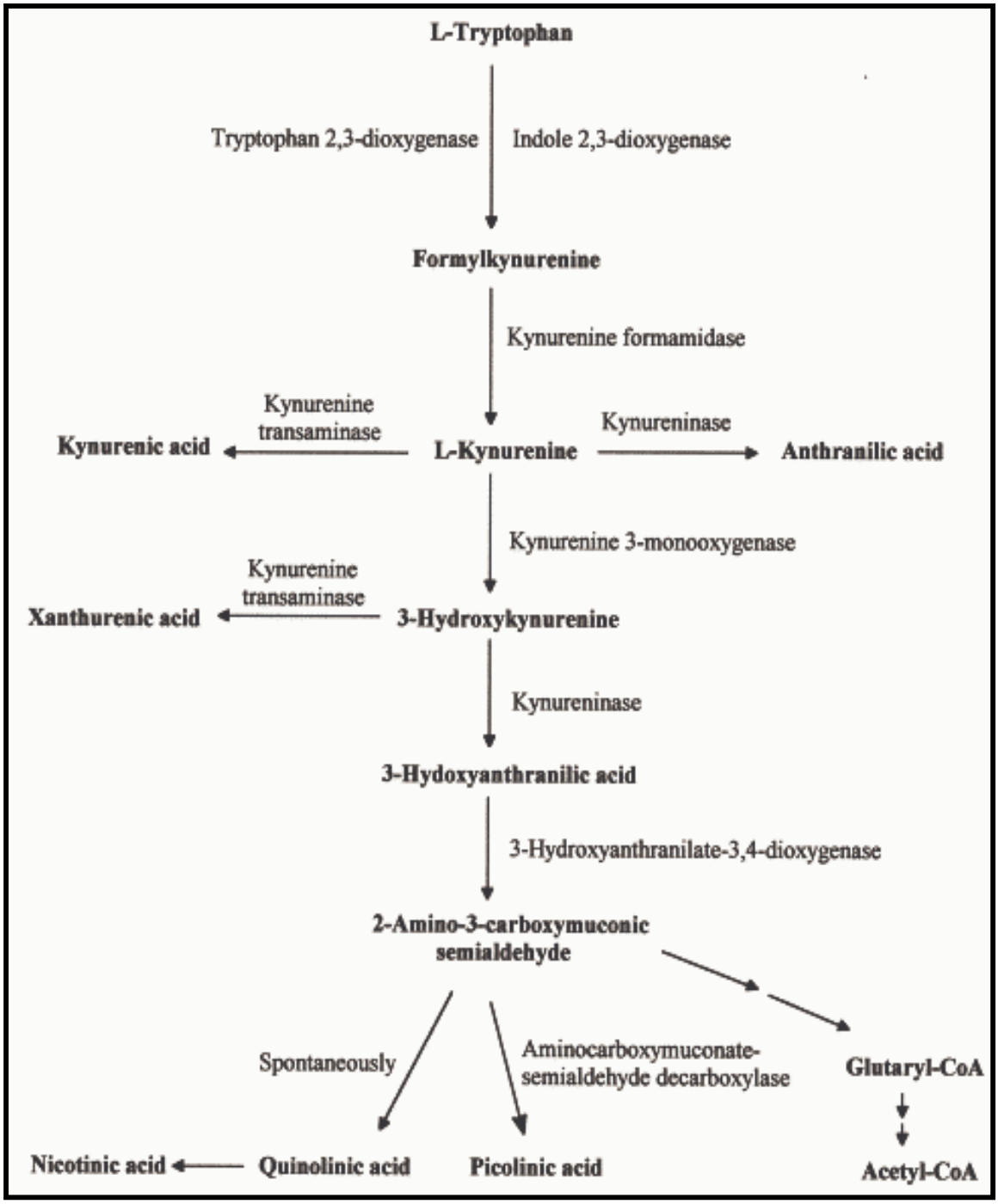

Figure 1.2. Representation of the kynurenine pathway which is responsible for the catabolism of tryptophan with the resultant production of kynurenic acid (KYNA) and quinolinic acid (QA), antagonist and agonist of the N-methyl-D-aspartate receptor respectively (Martin *et al*., 1992).

1.2.4.1.1.2. TDO Inhibition

Novel indoles have inhibitory effects on TDO activity with some members having 5-HT reuptake inhibitory activity (Madge *et al*., 1996). These compounds thus lead to increased levels of L-TRP and 5-HT in cerebrospinal fluid (CSF), which could be vitally important for antidepressant therapy. In support of this Walsh and Daya (1997) showed that TDO activity is regulated differentially by the indoles, aMT and 5-HT. Melatonin is a competitive inhibitor of TDO and 5-HT is predominantly an allosteric inhibitor. Badaway and Evans (1981) showed that MAO inhibitors are weak inhibitors of TDO.

1.2.4.1.2. Mechanism of Action of TDO

The mode of action of TDO is dependent on three factors:

1) TRP alone can activate the enzyme,
2) hemin is essential for the activity and binds to the enzyme in a reversible manner, and
3) the heme iron, which is indispensable for activity.

The inactive form can be converted to the fully active form by the substrate TRP (Fiegelson *et al*., 1961), and the prosthetic group hemin can activate the enzyme in the presence of TRP. Tryptophan binds directly to the enzyme and not to the prosthetic heme. During the catalytic process, the heme iron is subject to a cyclical chain of events due to TRP and oxygen. The binding of L-TRP to the enzyme induces a fundamental change in the ligand binding affinity of the catalyst in addition to increasing the reactivity of the heme iron towards the substrate (Makino *et al*., 1980). The sequential events in enzymatic catalysis due to substrate induction are:

1) enzyme saturation by activator heme, and
2) increases in levels of total enzyme concentrations (Frieden *et al*., 1961).

1.2.4.2. Indoleamine 2,3-Dioxygenase (IDO) (EC 1.13.11.17)

This enzyme was first discovered in the rabbit intestine (Higuchi and Hayaishi 1967). Hayaishi (1976) described that the enzyme IDO appears superficially to catalyse the same reaction as TDO, but with a broader specificity (figure 1.2). Tryptophan 2, 3-dioxygenase is specific for L-TRP, and is found only in the liver in mammals whereas IDO will act on D- or L-TRP. This enzyme is widely distributed in a number of tissues, including brain, lung, gastric and intestinal mucosa, kidney, heart and adrenal gland. Although like TDO, it is a heme enzyme, IDO uses the superoxide anion radical ($O_2^{-\bullet}$) as a source of oxygen, rather than molecular oxygen and is not induced by either TRP or glucocorticoids. Indoleamine 2, 3-dioxygenase is induced by IFN-γ, virus infections or the administration of bacterial endotoxins and tumor cells.

The induction of IDO causes a marked increase in TRP catabolism in the body with the production of kynurenine and total depletion of TRP in the cells as shown in figure 1.2 (Antonella *et al*., 2001). The activity of intestinal IDO is of the same magnitude as that of TDO in the liver (Bender, 1982). Indoleamine 2, 3-dioxygenase is distributed in a variety of tissues, unlike TDO, which is found only in the liver and may provide a pathway for extra-hepatic tissues. Since tissues such as kidney have the other enzymes of the TRP oxidative pathway, this suggests that IDO may be important in the overall metabolism of TRP in the body. Intestinal IDO possesses a requirement for a reducing agent e.g. sodium ascorbate in the presence of methylene blue. Indoleamine 2, 3-dioxygenase provides a pathway for the conversion of any ingested D-TRP to D-kynurenine. Ligands such as cyanide, azide, and CO inhibit catalysis by the intestinal enzyme (Yamamoto and Hayaishi, 1967).

1.2.4.3. Kynurenine Pathway

One percent of dietary TRP in the peripheral tissues is converted to 5-hydroxytryptophan, while 95% is metabolized to kynurenines (Stone, 1993; Schwarcz, 1993). The term kynurenine refers to a specific metabolite of the kynurenine pathway of TRP degradation

(Stone, 1993; Schwarcz, 1993). Tryptophan is oxidatively cleaved to form L-kynurenine via n-formylkynurenine by TDO and IDO in the peripheral tissues and brain as evident in figure 1.2 (Antunes, 1998).

1.2.4.4. Formamidase (EC 3.5.1.9)

The immediate product of TDO and IDO is formylkynurenine (figure 1.2). However, the activity of the hydroxylase, which removes the formyl group, formyl kynurenine formidase is extremely high. This means that under all conditions except when highly purified enzyme preparations are used, the apparent product of TDO or IDO is kynurenine rather than formylkynurenine (Bender, 1982). The enzyme has low substrate specificity and is able to release formate from a variety of aryl-formylkynurenines although its greatest activity is towards N-formylkynurenine (Bender, 1975; Stone, 1993).

1.2.4.5. Kynurenine

L-Kynurenine (L-KYN) is an intermediary formed in the KYN pathway of TRP metabolism as evident in figure 1.2. Approximately 5% of the TRP that enters the brain from the plasma is metabolized to KYN and is non-uniformly distributed ranging from 0.03 pmol/mg to 1.05 pmol/mg tissue in the cerebellum and putamen pool respectively (Speciale *et al*., 1989). Kynurenine uses a large neutral amino acid (LNAA) transporter, which can readily penetrate the BBB (Schwarcz, 1993). Kynurenine is competitive with other substrates for the high affinity, sodium independent transporter of LNAA's. Kynurenine is transported with high affinity and stereospecificity into the astrocytes by a sodium-independent process that prefers branched chain and aromatic neutral acids such as TRP, phenylalanine and leucine (Schwarcz, 1993: Speciale *et al*., 1989). Kynurenine is then stored or converted to kynurenic acid (KYNA) by the enzyme kynurenine aminotransferase (KAT) localized in the glial cells and then rapidly exits the cell. Uptake of KYN into glial cells is likely to be mediated by a neutral amino acid carrier (NAAC) (Stone, 1993).

1.2.4.6. Kynurenine transaminase (EC 2.6.1.7)

Kynurenine transaminase (KAT) acts both on KYN and 3-hydroxykynurenine in the presence of pyridoxal phosphate to give rise to both kynurenic acid and xanthurenic acid (figure 1.2). This enzyme is present in mammalian liver and kidney (De Castro *et al*., 1957). In humans KAT is present in 2 isoforms: KAT I and KAT II (Jauch *et al*., 1995). The enzyme is preferentially localized in the glial compartment, which suggests that astrocytes are in close proximity to the excitatory synapses making contact with the dendritic synapses (Antunes, 1998).

1.2.4.7. Kynurenic acid

Kynurenic acid (KYNA) is a broad-spectrum antagonist and is able to interact with ionotrophic excitatory amino acid (EAA) receptors (Schwarcz, 1993; Chiarugi *et al*., 1995). Kynurenic acid has been identified as a natural brain constituent with its concentration varying within the brain of several animal species. It has also been shown to possess anticonvulsant and neuroprotective properties and therefore could act as a possible endogenous anti-excitotoxin. The polar structure of KYNA prevents it from penetrating the BBB. Kynurenine aminotransferase is preferentially located in the glial cells, surrounds the synapses that may allow KYNA to be synthesized and released at the sites where it may reduce excitatory amino acids-mediated neurotransmission (Russi *et al*., 1992).

1.2.4.8. Kynurenine 3-Hydroxylase (EC 1.14.13.19)

Kynurenine 3-hydroxylase (3-HK) governs the conversion of KYN to 3-hydroxykynurenine as shown in figure 1.2. This enzyme was first identified by De Castro *et al*., (1956) in rat and cat liver and kidney. It is localized in the outer mitochondrial membrane and is NADPH-dependent monoxygenase. The enzyme present in the brain has low activity (Antunes, 1998).

1.2.4.9. 3-Hydroxykynurenine

3-Hydroxykynurenine (3-HK) is a metabolite of KYN in the production of QA in the periphery (figure 1.2). This is a neurotoxic metabolite of KYN with the ability to produce convulsions and neuronal damage (Stone, 1993; Schwarcz, 1993). This metabolite has been proven to exist in high concentration in neurological diseases such as Huntington's disease (HD) (Nakagami *et al*., 1996). Schwarcz (1993) and Nakagami (1996) showed that the damage caused by 3-HK is due to the generation of hydrogen peroxide (H_2O_2) rather than the direct action on the EAA receptors. The interaction of 3-HK with H_2O_2 results in 3-HK toxicity, which may be enhanced by the presence of iron and prevented by the presence of the antioxidant catalase (Eastmen *et al*., 1990; Nakagami *et al*., 1996).

1.2.4.10. Kynureninase (EC 3.7.13)

Kynureninase catalyses the hydrolysis of both L-KYN and L-3-HK to form anthranillic acid and 3-hydroxyanthranillic acid, respectively as shown in figure 1.2. Kynureninase has pyridoxal phosphate as co-enzyme (Braunstein *et al*., 1949) and is present in the liver and kidney of mammals (de Castro *et al*., 1957). The enzyme shows the highest activity towards 3-HK (Antunes, 1998). Oestrogenic compounds, nicotinylalanine (NAL) (KYN analogue) and o-methoxybenzoylalanine (o-MBA) are able to inhibit kynureninase which results in an increase in urinary and plasma KYN and 3-HK with the resultant modification of the cerebral concentration of the metabolites (Stone, 1993). Inhibition of this enzyme results in the enhancement of the KYNA levels and prevents the metabolism of QA.

1.2.4.11. 3-Hydroxyanthranilic acid oxygenase (EC 1.13.11.6)

3-Hydroxyanthranilic acid oxygenase (3-HAO) is present in the mammalian liver and kidney (Stevens, 1959). 3-Hydroxyanthranilic acid oxygenase is an anabolic enzyme and is responsible for the synthesis of QA via an unstable intermediate 2-acroleylaminofumurate QA as evidenced in figure 1.2 (Schwarcz, 1993). The enzyme is

present in the outer and inner surfaces of the mitochondrial membrane (Stone, 1993). The enzyme was shown to be located in the thin and highly ramified astrocytic processes, which engulf excitatory synapses (Antunes, 1998). Hence QA synthesis is in the position to act on the N-methyl-D-aspartate (NMDA) receptors (Stone, 1993; Schwarcz, 1993). There is an increase in activity of 3-HAO in response to lesions, which could result from an increase in QA production. Several excitatory amino acids, TRP and KYNA have no influence on the enzyme with regard to its activity (Stone, 1993).

1.2.4.12. Quinolinic Acid

Quinolinic acid (QA) is an endogenous metabolite of TRP, which is neurotoxic when injected into the rat striatum (Farmer *et al*., 1984; Stone *et al*., 1984). Quinolinic acid acts preferentially on the NMDA receptors as shown in figure 1.3 (Stone *et al*., 1984; Birley *et al*., 1982) and is synthesized in the liver and the CNS (due to activation by human macrophages). Quinolinic acid is a rigidly planar molecule due to its aromatic ring while NMDA is a flexible non-planar molecule (Stone, 1984). The action of QA on the NMDA receptors results in neuronal lesions after hippocampal or striatal injections by activation of the EAA receptors (Blight *et al*., 1995).

Activation of the NMDA receptor is accompanied with an influx of calcium and delayed calcium neurotoxicity (Rios and Santamaria, 1991) as shown in figure 1.3. The high concentrations of excitatory receptors in the cortex correlate with the high concentration of QA in the cortex, therefore increasing the susceptibility of the cortex to the excitotoxic effects of QA (Schwarcz *et al*., 1983; Moroni *et al*., 1984).

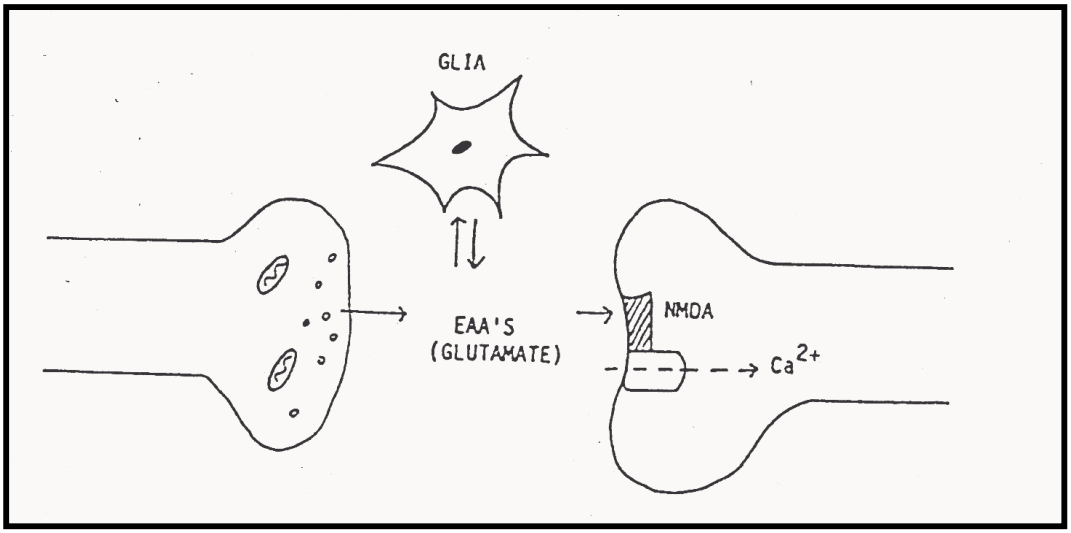

Figure 1.3. Calcium entry due to stress induction by the excitatory amino acid, glutamate. Abbreviations: **Ca^{2+}** (calcium), **EAA** (excitatiory amino acids), and **NMDA** (N-methyl-D-aspartate) (Daya, 1994).

Lipid peroxidation is enhanced through calcium entry hence it may be through this mechanism that QA induces lipid peroxidation. Quinolinic acid is found to be a potent convulsant. Quinolinic acid could possibly play a role in the etiology of various diseases such as HD, temporal lobe epilepsy, hepatic encaphalopathy and AD due to its ability to reproduce the various histological and neurochemical features of the various diseases especially in the case of HD (Misztal *et al*., 1996; Blight *et al*., 1995; Basile *et al*., 1995; Beal, 1992).

Huntington's disease was first described by George Huntington in 1872. It is an inherited neurodegenerative disorder in which the clinical features are divided into two areas: (1) progressively worsening choreoathetotic movement and (2) neuropsychiatric problems (Antunes, 1998). Long term lesions caused by QA resemble the neurochemical features of HD which are increases in: (1) 5-hydroxyindoleacetic acid, (2) 5-hydroxytryptamine, somatostatin and neuropeptide Y concentrations, hence increasing possibility that an NMDA receptor mediated process is involved in the pathogenesis of HD (Beal *et al*., 1991). Various disease states are accompanied by an increase in QA, such as hepatic encephalopathy (HE) (Saito *et al*., 1996).

Dexter *et al*., (1991) reported increased concentrations of copper and iron in the caudate nucleus, putamen, substantia nigra and cerebral cortex of post mortem HD human brains. This pattern of increased copper in HD patients is not shared by other neurodegenerative diseases of the basal ganglia, such as PD and progressive supranuclear palsy, suggesting that specific changes in transition metals are linked to the different neuronal populations affected. In the same report, however no difference in manganese content was found in HD brain regions as compared against control brains.

Increased release of glucocorticoids results in induction of TDO activity and hence an increase of QA production. Due to the poor integrity of the BBB, QA is able to penetrate it during the last stage (IV) of HE. Induction of the enzymes: IDO, 3-HK, kynureninase, and 3-HAO may result in an increase in synthesis of QA (Blight *et al*., 1995).

1.3. PINEAL GLAND

1.3.1. The History of The Pineal Gland

The human pineal gland was discovered by the famous anatomist Herophilis (325-280 BC). Rene Descartes proclaimed that the pineal gland was the “seat of the soul”. The word “pineal” is specified from the Latin *pinealis, pinea* which means pine cone, as this is the shape resembled by the human pineal gland. The term epiphysis means, “what is grown on something” (Erlich and Apuzzo, 1985). By the end of the 19th century there was a suggestion that the pineal gland had an endocrine role. It wasn’t, however until the 1950’s that scientists conducted serious research on the pineal gland. The subsequent research confirmed the endocrine role and also uncovered the neural connection between the pineal gland and the hypothalamus.

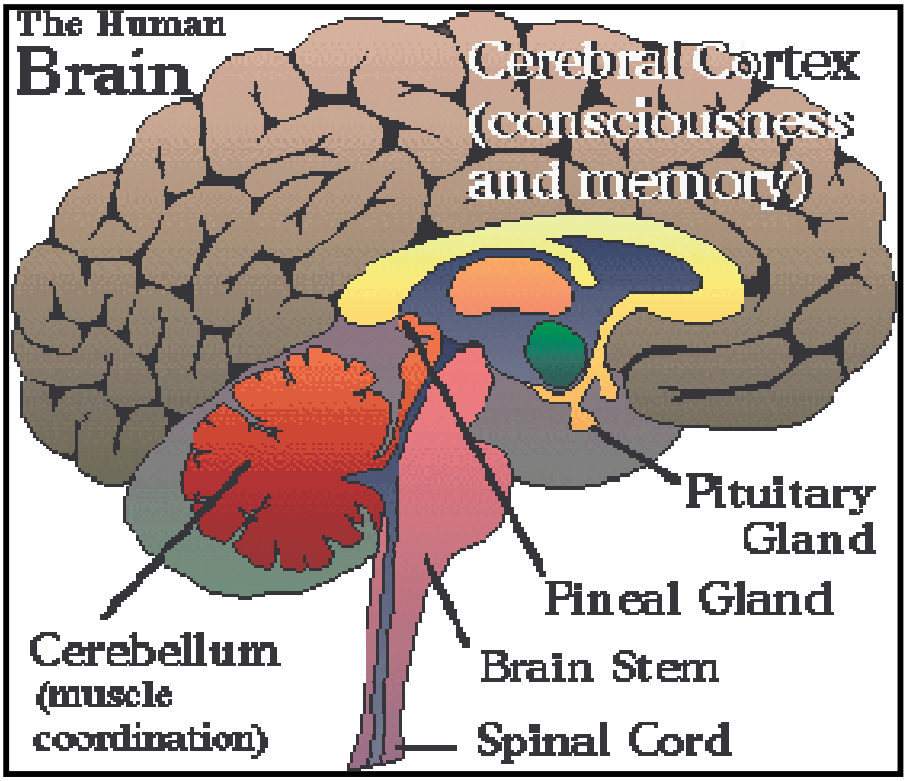

Figure 1.4. View of the brain showing location of the pineal gland (Rowett, 1968).

The pineal (figure 1.4.) is an endocrine gland ideally situated anatomically to receive, integrate and compare information from both the external environment and the internal physiological milieu. By transducing photoperiodic information into a hormonal signal, the pineal plays an integral role in the temporal organization of numerous metabolic, physiological and behavioural processes. For this reason the pineal has been defined as a "neuroendocrine transducer" (Axelrod, 1974) with the indoleamine aMT as the principal hormone secretion (Erlich and Apuzzo, 1985).

1.3.2. The Anatomy of the Pineal Gland

The mammalian pineal gland is a glandular structure (figure 1.5) derived as an evagination of the neural tubule (Kappers, 1965). In the rat it appears as a neuroepithelial protrusion from the roof of the diencephalons, the area between the habenular and posterior commissures. It's connection with the commisural region is through the pineal

stalk. The human pineal gland is attached to the posterodorsal aspect of the diencephalons and occupies a depression between the superior colliculi of the mesencephalon. Proximally the pineal gland, in humans, is closely associated to the third cerebral ventrical. The pineal gland is composed of two parenchymal cell types, the pinealocytes and interstitial cells (Wartenberg and Gusek, 1965), and are both of neuroectodermal origin. The chief cellular component of the pineal gland is the pinealocyte (pineal parenchyma cells) (Young *et al*, 1982). The rat pineal is highly vascularised, its major blood supply being provided by branches of the posterior choroidal and posterior cerebral (Hodde, 1979) arteries. Venous drainage occurs via the distal end of the cerebral vein into the superior saggital sinus (Hodde, 1979).

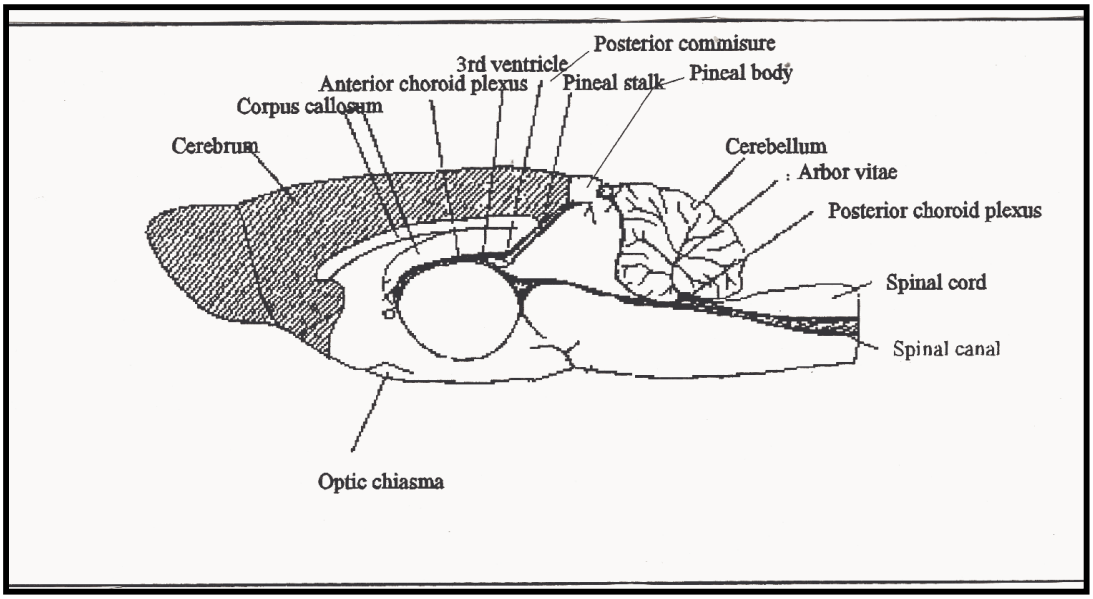

Figure 1.5. Median sagittal view of the rat brain (Rowett, 1968).

The pineal gland plays an important role in photoperiodism (figure 1.6.). Within the pineal, the neural (photic) information is transduced into a hormonal input (Reiter, 1980). The gland responds to the photic information via the lateral eyes. Mammals depend on neural connection between the eyes and the gland that assimilates the photic information. The pineal is connected to the phototransducing ganglion cells of both retinas through a specific polyneuronal pathway. An independent retinohypothalmic tract connects these ganglion cells to the paraventricular nucleus and suprachiasmatic nucleus (SCN) of the

anterior hypothalamus. From here, fibres run through the brain stem and the medial forebrain bundle, terminating on preganglionic sympathetic neurons in the intermediolateral cell columns of upper thoracic cord. Efferent axons pass in the cephalid direction via the sympathetic trunk to synapse in the superior cervical ganglia (SCG). Post-ganglionic fibres of the sympathetic nervous system (SNS) enter the cranial cavity via the internal carotid plexus and terminate primarily on the perivascular processes of the pinealocytes. In this way, the parenchymal cells of mammalian pineals are unusual in that they receive direct sympathetic innervation, mediating pineal responses to environmental lighting (Erlich and Apuzzo, 1985).

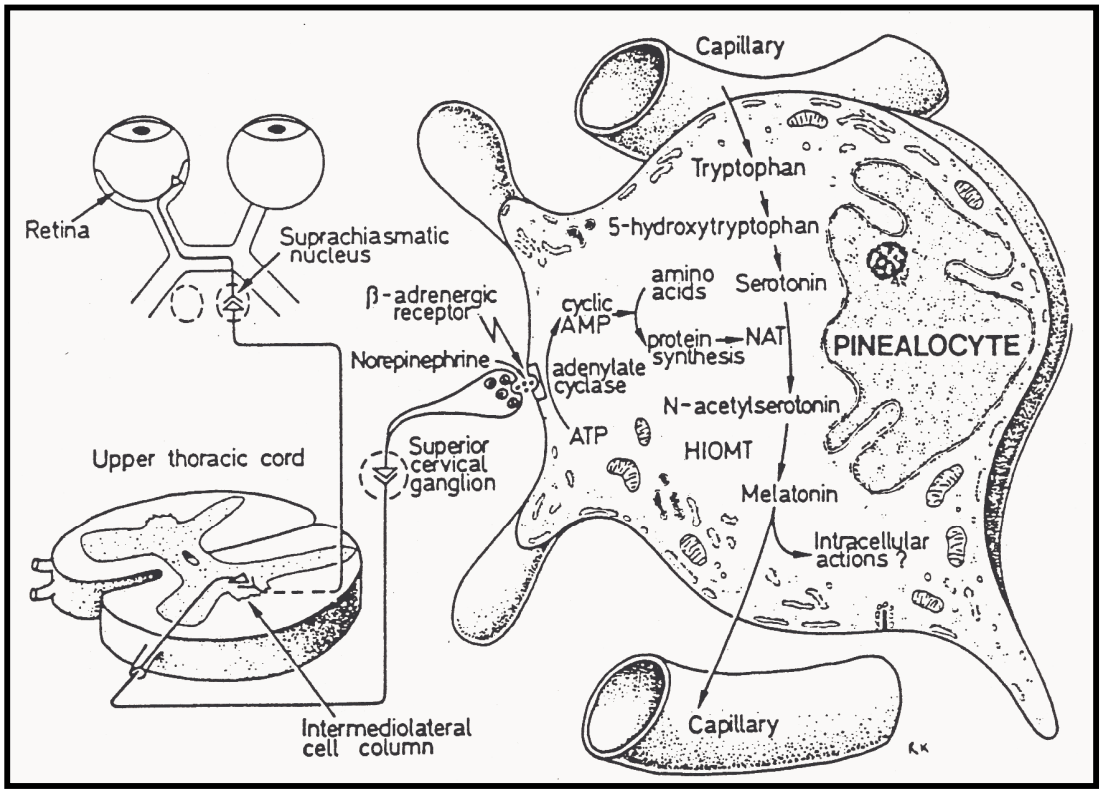

Figure 1.6. Schematic representation of the sensory input pathway and the effect of light and dark on the synthesis of the various indoleamines in the pineal gland (Reiter, 1989).

Hypothalamic SCN represents the site of the endogenous "biological clock" or the internal "zeitgeber". Light acts as an entraining agent to readjust the clock to a period of 24 hrs (i.e. a circadian rhythm) in response to daily and seasonal shifts in the photoperiod.

The onset of darkness (i.e. the absence of light) results in the disinhibition of the SCN triggering a surge in the synthesis and release of aMT at night (Armstrong, 1989; Reiter, 1989; Moore, 1993). Hence aMT has been called the "chemical expression of darkness" (Reiter, 1991).

1.3.3. Serotonin

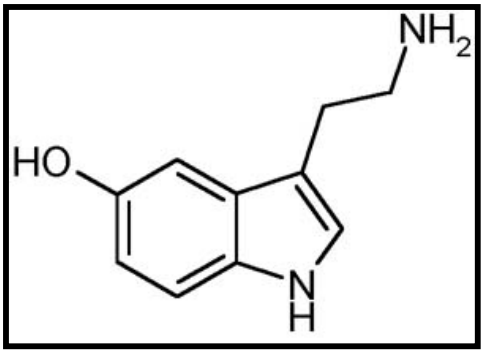

Figure 1.7. Chemical structure of serotonin.

1.3.3.1. Introduction

Serotonin was discovered approximately a century ago by physiologists as a vasoconstrictor in serum after blood clotting. In 1948 it was isolated as a crystalline complex and was named '5-HT'. The active moiety of 5-HT is 5-hydroxytryptamine. The chemical structure of 5-HT is shown in figure 1.7. Synthetically manufactured 5-HT possesses all the properties of naturally produced 5-HT. The 5-HT concentration is very high in the pineal gland and levels exceed any other organ in the body (Reiter, 1989; Reiter, 1991a; Reiter, 1981). Thirty percent of the total 5-HT stored in the rat pineal is located in the sympathetic nerve endings; only the pinealocytes, in the pineal gland, have the ability to produce 5-HT which is taken up into the adjoining nerve endings.

Serotonin, a precursor to several indoles may be acted upon by several enzymes and its pineal concentration at night may be decreased due to three pathways: (1) oxidative deamination by monoamine oxidase (MAO), (2) N-acetylation and (3) release to the extracellular spaces (Reiter, 1989; Reiter, 1981; Cardinali, 1981). 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 broken or metabolized.

1.3.3.2. Serotonin Receptors

There are several subtypes of the 5-HT receptor that are believed to exist, namely 5-$HT1_A$, 5$HT1_B$, 5$HT2_B$, 5-$HT1_D$, 5-$HT1_P$, 5-HT2, and 5-HT3 (Zemlan *et al.*, 1988). These subtypes are classified pharmacologically according to the drug that blocks the normal expression of the signal transducers and second messengers. The action of 5-HT at the receptors is shown in figure 1.8.

As is the case with other biogenic receptors (cholinergic, dopamanergic and adrenergic), the various 5-HT receptor subtypes are linked to a number of different second messengers (Frazer *et al.*, 1990). Three clearly defined 5-HT receptor subtypes have been found linked to adenyl cyclase (AC) in the vertebrate brain – namely, 5-$HT1_A$, 5-$HT1_B$ and 5-$HT1_D$. Even though these receptors are known to activate or stimulate AC via G-protein couplings, it is not yet possible to discern whether the individual receptor subtypes are linked to inhibitory (Gi) and stimulatory G-protein (Gs). Thus far, in the rat hippocampus, the 5-HT receptor has been found to be linked to both inhibitory and stimulatory G-protein. Two receptor subtypes 5-$HT1_C$ and 5-HT2 are believed to be linked to phosphoinositide hydrolysis via phospholipase C activity (Hanson *et al.*, 1987).

1.3.3.3. Serotonin and Depression: Indoleamine Hypothesis

Interest in the possible role of 5-HT receptor operation in depression was stimulated by a number of observations:

a) The requirement of an intact 5-HT system is needed for an antidepressant-induced effect to occur in adrenergic receptors (Sulser, 1984).

b) The effects of antidepressants due to sensitization of postsynaptic 5-HT_3 receptors (De Montigny, 1981).

c) Animal models revealed post-synaptic 5-HT receptor sensitization, as well a certain pre-synaptic effects on the 5-HT system using lithium (Price, 1989).

A substantial body of evidence has thus far been collected to support the 30-year-old indoleamine hypothesis, which asserts that modifications in the 5-HT neuronal function are a core feature of depression. It is hypothesized that there is a monoaminergic hyperfunction in depression, and antidepressants operate so as to downregulate post-synaptic monoamine receptors (Van Praag, 1982) as compared to the traditional hypothesis that asserts that there is a metabolic and functional aberration in depression, and drugs which curtail the availability of 5-HT and NA precipitate while those which enhance their abundance are therapeutic (Coppen, 1967).

Some major findings are:

a) The prime metabolite of 5-HT, 5-HIAA depicts reduced levels in the CSF of drug free patients (Davis *et al*., 1981).

b) Post-mortem studies on brains of depressed or suicidal patients have shown reduced concentrations 5-HT and 5-HIAA (Gibbons and Davis, 1986)

c) Chronic efficacious antidepressant treatment enhances 5-HT neuronal transmission in the laboratory rat (De Motigny and Aghajanian, 1978).

d) Depressed patients have a reduced level of plasma TRP, and serotonergic antidepressant-dependent remittance depicts a clear dependence on an adequate supply of TRP, given that a lack of this precursor results in an immediate relapse (Delgado *et al.*, 1990).

e) Post-mortem brain tissue of depressed suicide victims (Mann *et al.*, 1986) display definite increases in the levels of 5-HT2_A receptor-binding sites (Arora and Meltzer, 1989).

The question that arises from such conclusions is whether these abnormalities in 5-HT levels are necessary for depression to occur or whether they increase the susceptibility of the particular individual to depression. Research findings thus far point to aberrant 5-HT metabolism increasing the risk of depression. Hence by implication, increasing 5-HT levels should be prophylactic, because if 5-HT plays an important role in the pathogenesis of depression then factors that affect 5-HT synthesis should be effective in treating depression. The substances that are generally used increase 5-HT availability at the receptors. The effectiveness of anti-depressant therapy depends to a certain extent on dietary factors involved in 5-HT synthesis, as well as other changes in the implicated monoaminergic neurobiological system (Jimerson *et al*., 1990).

1.3.3.4. Antidepressant Drugs Targeting Serotonin

Monoamine oxidase inhibitors, such as isocarboxazid and phenelzine block the degradation of 5-HT by MAO. This permits more 5-HT accumulation in the presynaptic stores and therefore more 5-HT can be released. Understandably these drugs are more specific for MAO-A as MAO-B is responsible for DA catabolism (Hollister, 1995).

Another class of drug is the selective 5-HT reuptake inhibitors (SSRI's) and examples of this group are fluoxetine and paroxetine (Hollister, 1995). These drugs as the class name suggests prevent the reuptake of 5-HT, which is illustrated in figure below, thereby preventing 5-HT inactivation (Sanders-Bush and Meyer, 1996). Antidepressant drugs such as mianserin and mirtazepine, inhibit the 5-HT neurons (heteroreceptors) in the CNS thereby resulting in enhanced 5-HT release (Harvey, 1997).

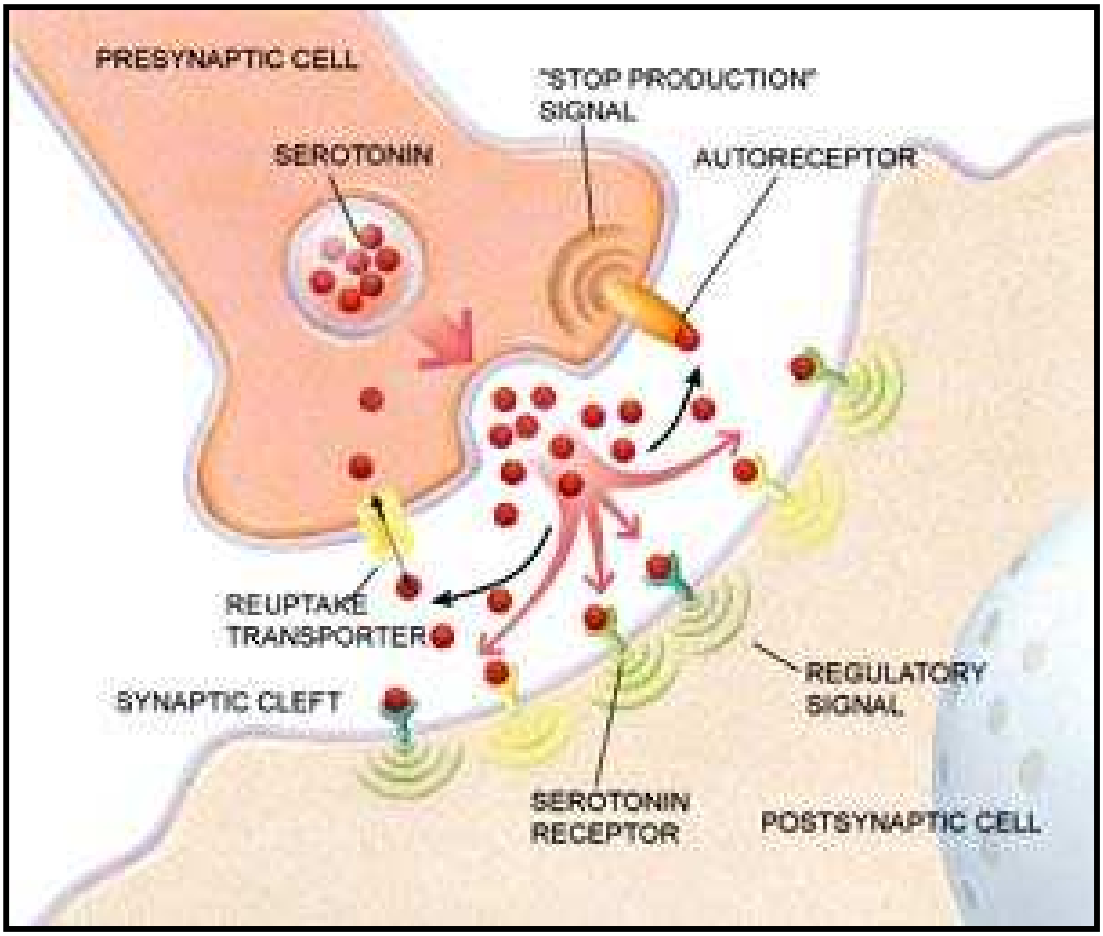

Figure 1.8. Action of serotonin (red spheres) at the receptors (shades of green) on a postsynaptic cell (Nemeroff, 1998).

1.3.3.5. Serotonin Pathway

The synthesis of 5-HT and the other indoleamines is shown in figure 1.9.

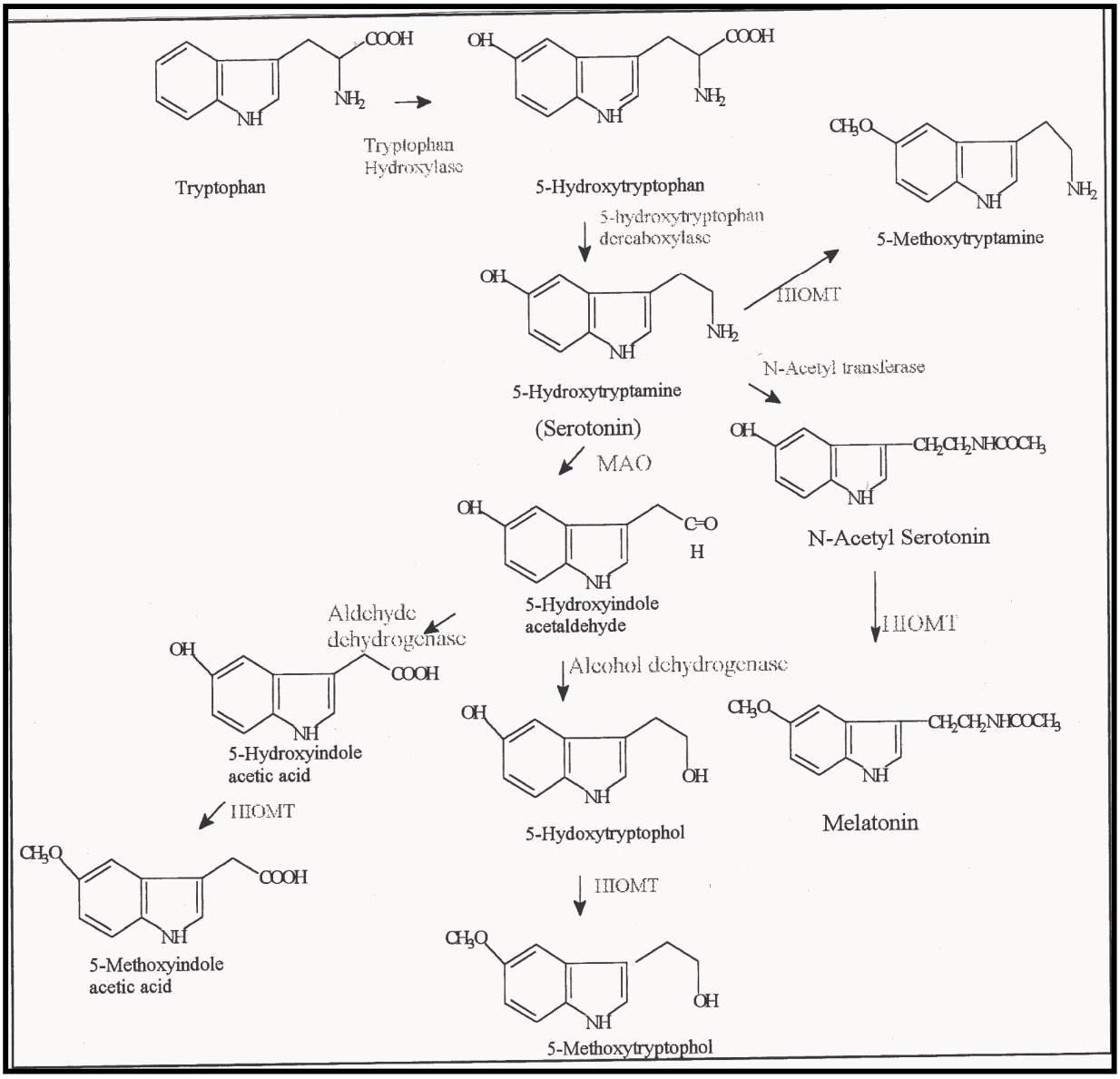

Figure 1.9. The pathway of indole metabolism (modified from Young and Silman, 1982).

1.3.3.5.1. Tryptophan-5-Hydroxylase (EC1.14.16.4)

The first step in 5-HT synthesis is 5-hydroxylation of TRP to yield 5-hydroxytryptophan (5-HTP) as shown in figure 1.9. Friedman and coworkers (1972) demonstrated that the TRP hydroxylase is a biopterin-dependent enzyme. It was noted that the stimulation by Fe^{2+} ions was not the result of the enzyme but the removal of H_2O_2, which accumulates as the result of side reactions of the enzyme. In the presence of catalase to remove this

peroxide, there is no stimulation by Fe^{2+} ions. The hydroxylation of TRP requires molecular oxygen, and in the reaction the co-factor, tetrahydrobiopterin, is oxidized to dihydrobiopterin. This is reduced to the active co-factor for further hydroxylation by a second enzyme, dihydrobiopterin reductase (EC1.6.99.7), which uses NADPH as a reductant. Friedman and coworkers (1972) noted that 5-HTP was inhibited by excess of TRP. The activity of TRP hydroxylase could therefore be the rate-limiting step in 5-HT synthesis.

1.3.3.5.2. Aromatic Amino Acid Decarboxylase (EC 4.1.1.28)

Aromatic amino acid decarboxylase (5-hydroxytryptophan decarboxylase) causes the conversion of 5-hydroxytryptophan to 5-hydroxytryptamine (figure 1.9). The enzyme requires the presence of pyridoxal phosphate (cofactor). Although the decarboxylation of 5-hydroxytryptophan is not normally the rate-limiting step of 5-HT synthesis, changes in the activity of the decarboxylase may be physiologically important (Bender, 1982). Bowen and coworkers (1974) showed that there is a significant loss of aromatic amino acid decarboxylase activity in patients with presenile dementia. In view of the importance of both 5-HT and catecholamines in affective disorders, and possibly in psychosis this may be an important finding.

1.3.3.6. Serotonin Metabolism

1.3.3.6.1 Monoamine Oxidase (MAO) (EC 1.4.3.4)

The principal metabolism of 5-HT in the CNS is oxidation, catalysed by MAO and aldehyde dehydrogenase, to yield 5-hydroxyindoleacetic acid and 5-hydroxytrytophol as shown in figure 1.10. Unlike the catecholamines, methylation of the hydroxyl group does not seem to be an important part of the inactivation of 5-HT.

There are 2 isoenzymes of MAO in the CNS. MAO-A acts preferentially on 5-HT, while MAO-B acts on phenylalanines and the catecholamines. The increase in CNS amines after the inhibition of MAO has been central to the development of the amine hypothesis of affective disorders. The development of inhibitors that are specific for one or both of the isozymes (Clorgyline for MAO-A and Deprenyl for MAO-B) has aided the dissection *in vivo* of serotonergic and catecholaminergic mechanisms, and the parts they play both in the affective disorders and in normal patterns of behaviour. MAO is distributed throughout the CNS and periphery. The ratio of MAO-A and MAO-B expression is distinctly cell-, tissue-, and species specific and shows ontogenic development. This differential expression of A and B isoforms in CNS and peripheral tissues may be regulated independently by tissue-specific factors (Zhu *et al.*, 1992).

The product of MAO action on 5-HT is 5-hydroxyindole acetaldehyde. Normally this is oxidised further to 5-hydroxyindoleacetic acid (5-HIAA) by acetaldehyde dehydrogenase. Measurement of the excretion of 5-HIAA is a useful indicator of serotonergic activity. Under some metabolic conditions, when the redox potential of the brain is shifted to a more reducing state than normal, as, for example after the ingestion of alcohol, a significant proportion of the aldehyde may be reduced to 5-hydroxytryptophol, which is excreted in the urine (Bender, 1982).

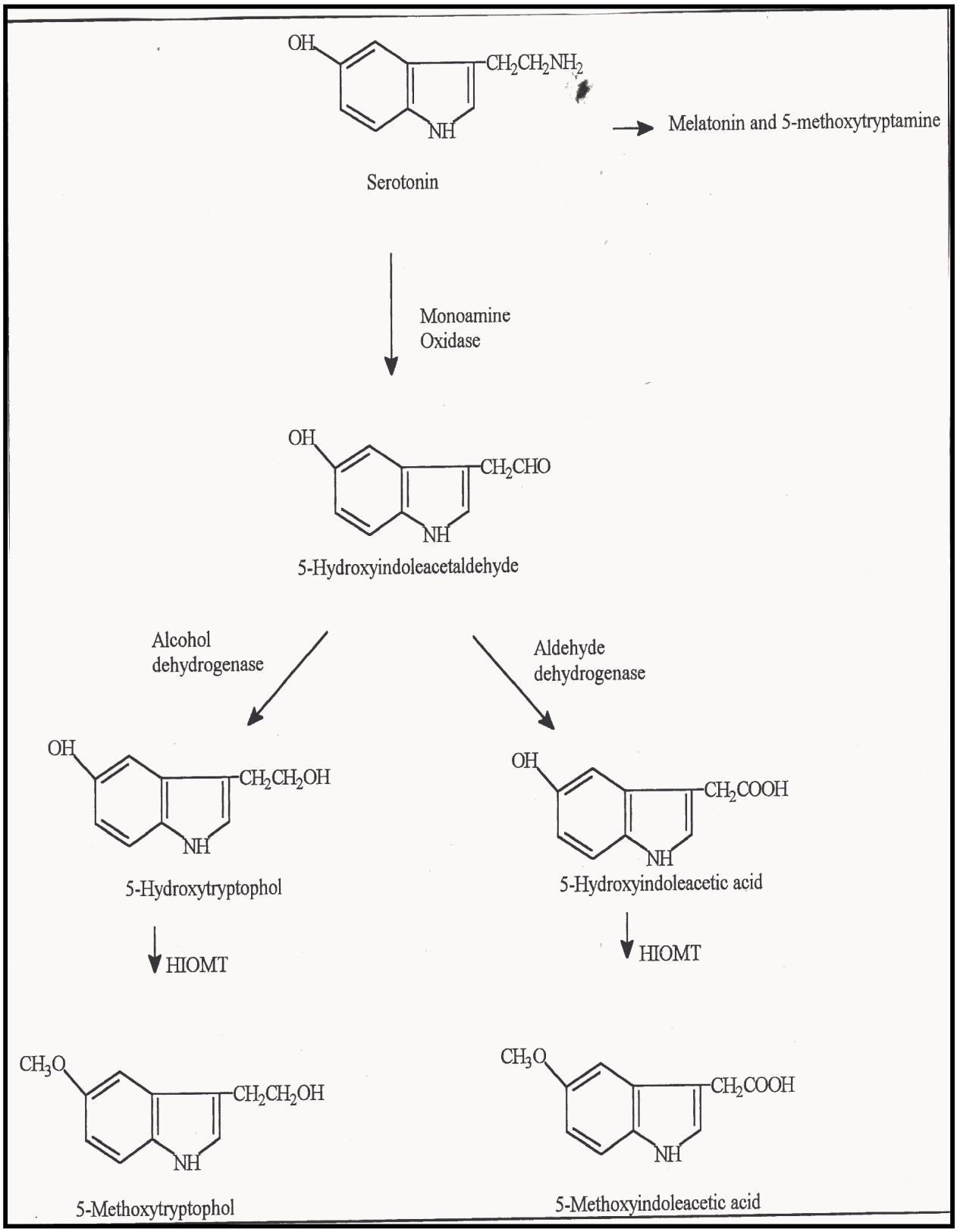

Figure 1.10. The catabolism of 5-HT (modified from Young and Silman 1982)

1.3.3.6.1.1. Clinical Significance of MAO

Monoamine oxidase has been implicated in numerous brain diseases and psychiatric disorders. Although human platelets contain exclusively MAO-A and MAO-B is of primary importance for the metabolism of central 5-HT, there is a strong positive correlation between central serotonergic turnover (e.g. 5-HIAA levels in CSF) and platelet MAO-B activity. For this reason, platelet MAO activity has been suggested as a trait-dependent indicator of vulnerability to psychopathology, which implies a stability of the enzyme in an individual irrespective of changes in mood, season and symptoms (Schalling *et al*., 1987). Low platelet MAO activity and low 5-HT turnover have been associated with eating disorders, impulsivity, novelty seeking, substance abuse, aggression, borderline personality, antisocial and recurrent suicidal behaviour (Sullivan *et al*., 1979; Moss *et al*., 1990). Transgenic mice lack the MAO-A gene, show abnormal levels of 5-HT and NA and aggressive behaviour (Cases *et al*., 1995). PD is also associated with low platelet MAO-B, whereas there is evidence for very high levels of MAO-B in plaque-associated astrocytes in the brains of Alzheimer's patients (Williams *et al*., 1991; Saura *et al*., 1994). Studies have failed to find an association between the intronic MaeIII polymorphism of MAO-B and PD (Kurth *et al*., 1993; Ho *et al*., 1994).

1.3.3.6.2. Melatonin (aMT)

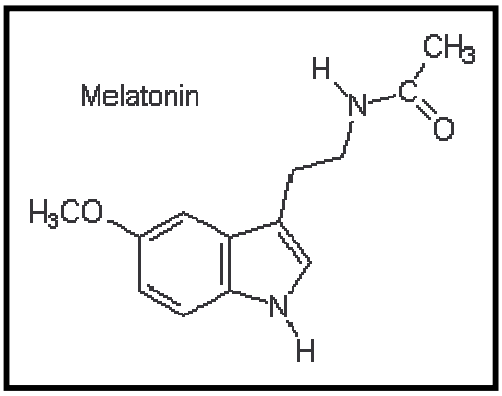

Figure 1.11. The chemical structure of melatonin.

1.3.3.6.2.1. Introduction

The neurohormone aMT is a ubiquitously acting biological substance within the mammalian body (Reiter, 1991b), and can be found in the pineal and extra-pineal tissue. The nocturnal patterns of aMT production in the pineal gland vary between species and its significance is not clear. Melatonin is not stored in any significant amount and is released into the blood vascular system and into other fluids such as the cerebrospinal fluid (CSF) (Reiter, 1981; Tricore *et al*., 2002). Blood and CSF levels of aMT closely relate to those in the pineal gland, with the highest concentration in the night. The chemical structure of aMT is illustrated in figure 1.11.

Melatonin is rapidly broken down in the blood or hydroxylated and conjugated with sulfate (70-80 %) and glucoronide (5%) by hepatic microsomes. These metabolites are excreted in the urine (Reiter, 1981; Kopin *et al*., 1961;Walhauser *et al*., 1993). Rats appear to have extra-pineal aMT synthesizing machinery due to the fact that large TRP doses increase serum aMT levels, but not pineal N-acetyl transferase (NAT) or hydroxyindole-O-methyltransferase (HIOMT) activity (Yaga *et al*., 1993). In humans no such effects are evident and no extra-pineal plasma aMT producing centers have been demonstrated as yet (Lewy *et al*., 1979). Melatonin secretion by the rat pineal gland provides a realistic assessment of noradrenergic neurotransmission within this species (Thompson *et al*., 1985). Denervation of the pineal gland prevents the nocturnal elevation in both brain and plasma aMT levels, which clearly demonstrates that the rise in the neurohormone is a consequence of pineal secretion (Walsh, 1996).

1.3.3.6.2.2. Functions of Melatonin

Studies have shown aMT to bind to complex metals thereby reducing metal-induced toxicity (Limson *et al*., 1998). These authors demonstrated aMT to form complexes with toxic metals, such as aluminium and copper. Considering that aluminium is implicated in AD, aMT may have serious implications in the treatment of AD.

Melatonin is also able to detoxify free radicals via electron donation (Reiter *et al*., 1996). The indoyl cation that is formed is able to scavenge both the $O_2^{-\bullet}$ and peroxynitrous forming N-acetyl-N-formyl-5-methoxykynuramine as illustrated in figure 1.12.

Glutathione peroxidase (GPx), an enzyme which is able to metabolize H_2O_2 to water, is known to be stimulated by aMT (Reiter, 1997), thereby stimulating a major antioxidative defense system. Melatonin has also been demonstrated to suppress the activity of nitric oxide synthase (NOS). Nitric oxide interacts with the oxygen molecule to generate the peroxynitrite anion, which degrades to produce the $^{\bullet}OH$.

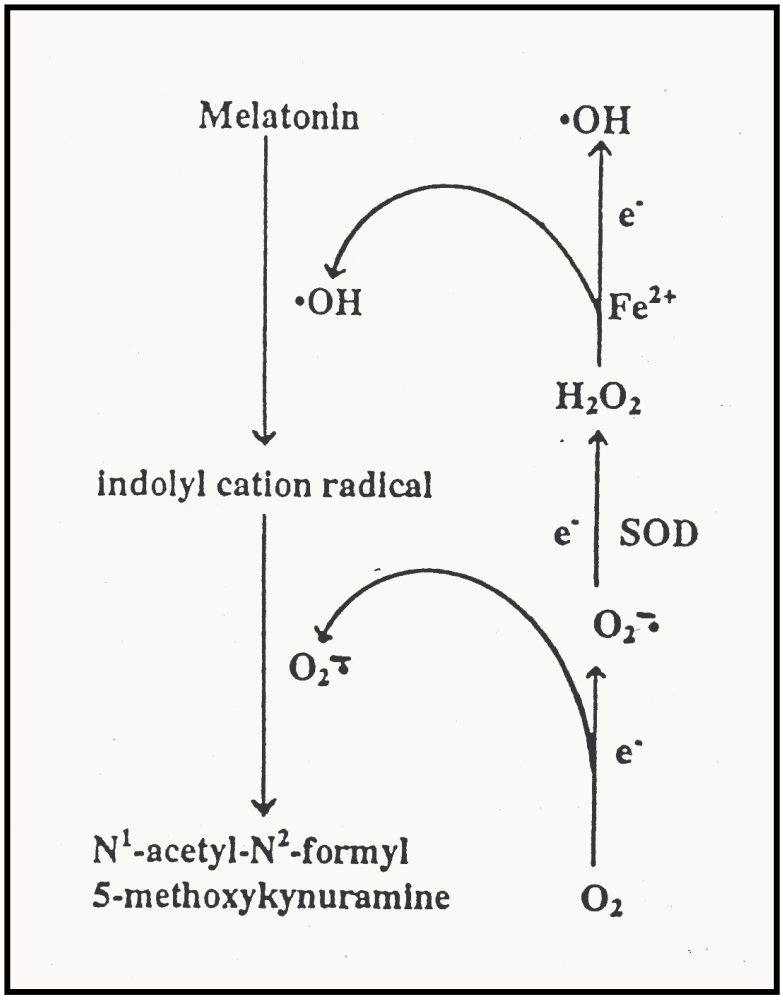

Figure 1.12. The mechanism thought to be responsible for the antioxidant effects of aMT (Reiter *et al*., 1996).

1.3.3.6.3. Serotonin N-acetyltransferase (NAT) (EC 2.3.1.5)

It is generally assumed that the first step, the N-acetylation of 5-HT during the scotophase, is rate limiting. The vesicular 5-HT is preferentially utilized (Racké *et al.*, 1991) in the N-acetylation. The activity of 5-HT N-acetyltransferase (figure 1.9) shows a diurnal variation in the pineal that corresponds to the variation in the concentrations in aMT in the CNS and the circulation in the light dark cycle (Deguchi, 1975). Daytime 5-HT content is crucial for determining the amount of NAS synthesized. The N-acetylation of 5-HT to yield N-acetylserotonin (NAS) is achieved by the provision of the acetyl group from the acetyl co-enzyme (figure 1.9) (Reiter, 1989).

Two forms of NAT are present in the pineal gland, one of which is highly specific for the arylalkylamines and exhibits the 24 hour rhythm. The other form shows weak activity towards arylamines and remains stable over a 24 hour period. The synthesis of aMT is the greatest during the hours of darkness. If adult rats are maintained in constant darkness, the cyclic variations in serotonin-N-acetyl-transferase activity persist (Deguchi, 1975). The activity of the circadian variation in serotonin N-acetyltransferase activity differs considerably in different species. In the rat there is as much as a 60-fold increase during the hours of darkness, while in the hamster and gerbil (which are nocturnal animals) it is only 3-fold, and in the (diurnal) guinea pig it is only 1.5 fold (Rudeen, 1978).

1.3.3.6.4. Hydroxyindole O-methyltransferase (EC 2.1.1.4)

Hydroxyindole-*O*-methyltransferase (HIOMT) (figure 1.10) consists of two 38 kilodalton subunits and is found in high concentrations in the pineal gland where it represents 2-4% of the total soluble protein (Reiter, 1981). Hydroxyindole-*O*-methyltransferase levels decrease by 70% in chronically sympathetically denervated glands, suggesting that NE is responsible for maintaining basal levels of the enzyme (Reiter, 1991). Hydroxyindole-*O*-methyltransferase is a cytosolic enzyme which catalyses the O-methylation of 5-hydroxyindoles by the methyl donor S-adenolsyl-L-methionine (SAM). The hyroxyindoles formed are 5-methoxyindole acetic acid (5-MIAA) from 5-HIAA, 5-

methoxytryptophol from 5-HTOH and aMT from NAS (Reiter, 1989). In addition to the pineal, the retinas, harderian glands, extra-orbital lacrimal glands, erythrocytes, mononuclear leucocytes and various areas of the gastrointestinal tract also contain HIOMT and are therefore capable of synthesizing melatonin (Reiter, 1989)

1.4. PARKINSONS DISEASE

1.4.1. History of Parkinson's Disease

James Parkinson became famous for *An assay on the shaking palsy* published in 1817 (Parkinson, 1817). He described six cases that formed the basis for his observations. Some observations are still associated with modern definitions of PD. "Involuntary tremulous motion, …in parts not in action and even when not supported; with propensity to bend the trunk forward, and to pass from walking to a running pace ….insidious onset…..The patient's infirmity gradually increased: the hand failed to answer with exactness to the dictates of the will". He hoped his description would excite others to extend researchers to this disease so they might point out the most appropriate means of relieving a tedious and most distressing malady. In a historical chapter Tyler describes how such prominent figures in medicines as Trousseau, Romberg and Charcot displayed interest in the disease but were at a loss to define its cause (Tyler, 1992). Charcot regarded palsy as less relevant in a disease where a muscle strength was kept until late stages, shaking was not quite adequate as even advanced disease could occur without tremor. In 1892 Charcot stated "everything or almost everything has been tried against the disease". Charcot was the first to use hyoscyamine, of which he wrote "..from which some patients have obtained relief; its action, however, is simply palliative."

1.4.2. Epidemiology

Parkinson's disease is a slowly neurodegenerative disease of which there is no single identifiable cause. Pathologically, PD is characterized by loss of pigmented neurons and gliosis, most prominently in the substantia niagra pars compacta (SNpc) and locus ceruleus (LC) and by the presence of ubiquitin-positive eosinophilic cytoplasmic inclusions in degenerating neurons (Forno, 1987). The Lewy bodies (LB) are concentric eosinophilic cytoplamic intraneuronal inclusions with peripheral halos and dense cores, and their presence is essential for the pathological confirmation of PD. Unfortunately, the LB is not specific to PD (Fearnley and Lees, 1991).

The diagnosis is clinical, as there is no test, which is specific to PD. The diagnosis of PD is based solely on clinical history and examination, which implies an obvious risk of misclassification. A clinico-pathological study showed clinical over-diagnosis in 18-24%, depending on which clinical diagnosis criteria wee applied (Hughes *et al.*, 1992). Thus, some cases of atypical Parkinsonism may be erroneously diagnosed as PD. The clinical manifestation of PD may be preceded by a long "latent" stage (Koller *et al*., 1991). The disease process is slow, as shown by the usually long time period from first symptoms to diagnosis. The finding of LB in the brains of people not known to have clinical evidence of PD during life is also suggestive of a pre-symptomatic period. Incidental Lewy bodies and clinical PD are both age-related phenomena (Tanner *et al*., 1997).

There are many factors associated with altered risk of PD. Demographic factors such as age, male gender and race (caucasian) appear to increase risk of developing PD. All studies find age associated to an increased risk, and this could be interpreted as an age-related neuronal vulnerability. Men appear to have a slightly (1.5 times) higher risk of acquiring PD than women (Fall *et al*., 1996; Marttila and Rinne, 1991). Some studies suggest that African blacks and Asians may have a lower occurrence of PD than Caucasians (Kessler, 1972). However, the differences may reflect different socio-economic factors that influence e.g. life expectancy and thus the risk of acquiring PD.

The tragedy with pyridine 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) which induced Parkinson's like disease in young people, created an interest in an environmental cause of PD (Langston *et al.*, 1983). Studies seeking an association between environmental exposure and PD have suggested an increased risk associated with farming, rural residence, herbicide/pesticide exposure, iron or mining, steel manufacture and employment in industries manufacturing chemicals, wood, pulp and paper (Aquilonius and Hartvig, 1986). Besides the increasing age, the strongest risk factor associated with PD is the presence of the disease in a family member. Most reports either suggest an autosomal-dominant inheritance or a multifactorial causation. Three different genes have been mapped out for rare familial Parkinsonian syndromes. At present there is no evidence that any of these genes for familial Parkinsonian syndromes have a direct role in the aetiology of the common sporadic form of PD (Gasser, 1998). The manifold putative causative factors are often supposed to act on genetically susceptible individuals.

1.4.3. Biochemical basis for Parkinsons Disease

It was not until almost half a century later that the biochemical basis for PD was clarified by Arvid Carlsson as a depletion of dopamine (DA) from the basal ganglia (Carlsson and Lindqvist, 1978). His investigation also led to the identification of the DA precursor L-DOPA, which thirty years later is still the principal medication used to alleviate the clinical manifestation of PD. Since these early studies, DA has been found to play a role in a number of other physiological processes, including cognition, emotion, reward, memory, and endocrine function. Moreover, DA neuronal dysfunction has been associated with several prevalent neurobehavioral disorders, including drug addiction, schizophrenia, and attention deficit hyperactivity disorder (ADHD).

But despite almost half a century of intense investigations into DA neurotransmission and its clear role in neurological disorders, much remains to be understood regarding the regulation of DA signaling during normal physiological states as well as what confers the unique sensitivity of DA neurons to damage in PD. The complexity of the human brain, which contains over a 100 billion neurons and tens of thousands of DA-containing cells

(Smith and Kieval, 2000), each capable of forming many thousands of synaptic connections, has greatly limited the ability to dissect the regulatory machinery involved in DA neurotransmission. Hence it is deduced that the loss of neurotransmitter DA from the basal ganglia, a consequence of the neurodegeneration in the substantia nigra, triggers postural instability and PD.

1.4.4. Dopaminergic Systems

Dopaminergic innervation in the brain is widespread and diffuse and is illustrated in figure 1.13. This is consistent with the modulatory nature of DA, behaving more like a hormone rather than a neurotransmitter. Dopamine represents more than 50% of the total catecholamine content of the CNS of most mammals with the highest levels being found in the caudoputamen, nucleus accumbens and tuberculum olfactorium. The main dopaminergic pathways are found in the basal ganglia (Moore and Bloom, 1978). Of particular importance is high expression of D1 and D2 DA receptors in the striatum, with a predominance of the D1 subtype (Camps *et al.*, 1990). GABAergic interneurons in the striatum and globus pallidus descending projections with nerve terminals located in both the pars compacta and pars reticula zones of the substantia nigra. This is known as the striato-nigral pathway.

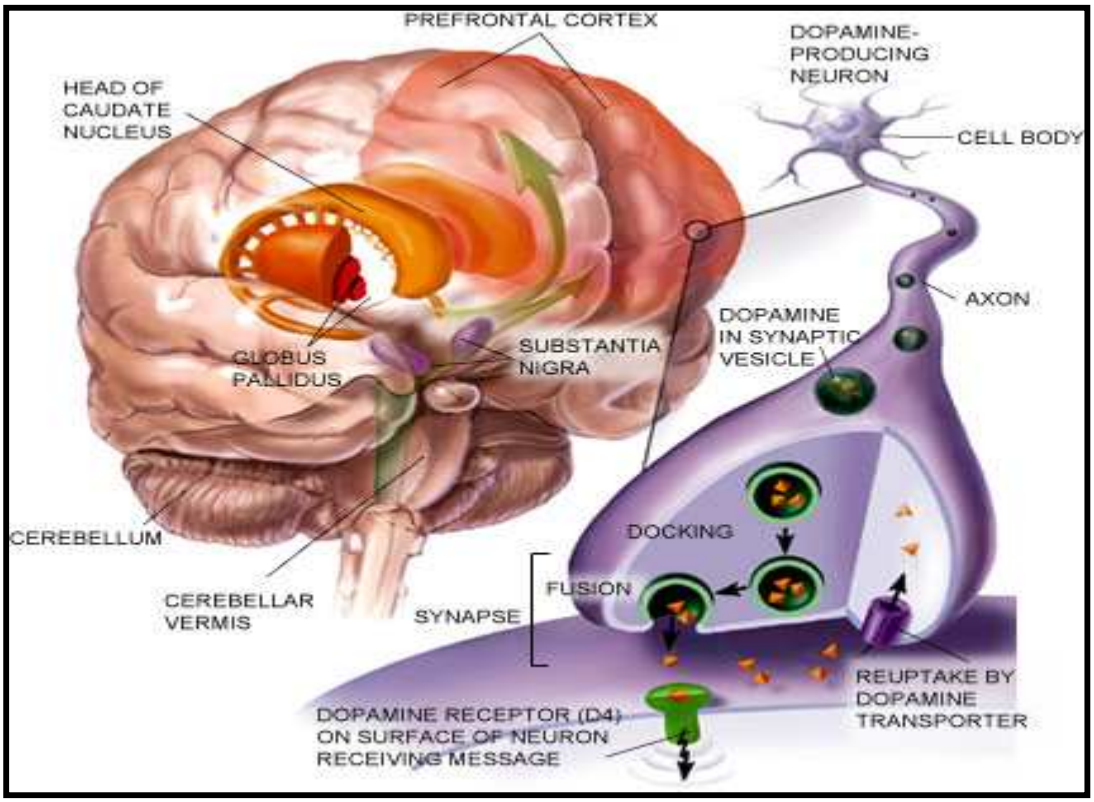

Figure 1.13. Illustration of the dopaminergic system in the brain and the interaction of DA with DA receptors and the DA transport system (www.hypsos.ch/presse/0998barkley_brain_large.jpg).

1.4.5. Dopamine Biosynthesis

Tyrosine hydroxylase (TH) is the key regulatory enzyme in the catecholamine synthesis (Ribeiro *et al*., 1992). It catalyses the hydroxylation of tyrosine to DOPA, the rate limiting step as shown in figure 1.14. The extent of L-tyrosine bioavailability is a crucial determinant of the synthetic rate and is dependent on the physiological rate of the neuron. Pyridoxal phosphokinase catalyses the formation of pyridoxal-5-phosphate, which is an essential co-factor of aromatic decarboxylase. The nonspecific enzyme aromatic L-amino acid decarboxylae is necessary for catecholamine and indoleamine biosynthesis. The final step in DA synthesis is the conversion of L-DOPA to dopamine via decarboxylation by aromatic-L-amino acid decarboxylase.

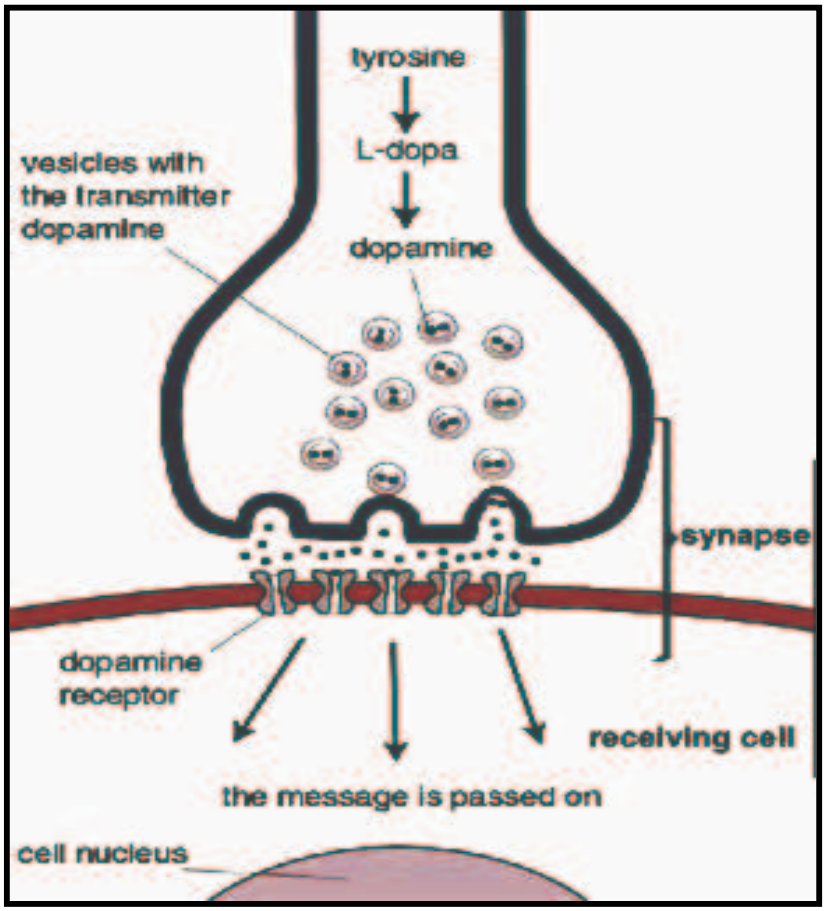

Figure 1.14. The biosynthesis and release of dopamine in the synapse. (www.sane.org.uk/images/ Research/dopamine%20si...).

1.4.6. Dopamine Catabolism

In mammals DA is metabolized by three distinct enzymes: catechol-*O*-methyltransferase (COMT), MAO and phenolsulphotransferase and these enzymes are shown in figure 1.15. MAO and COMT are the two main catabolic enzymes influencing dopaminergic neurotransmission (Napolitano *et al.*, 1995). Dopamine is broken down to form 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine.

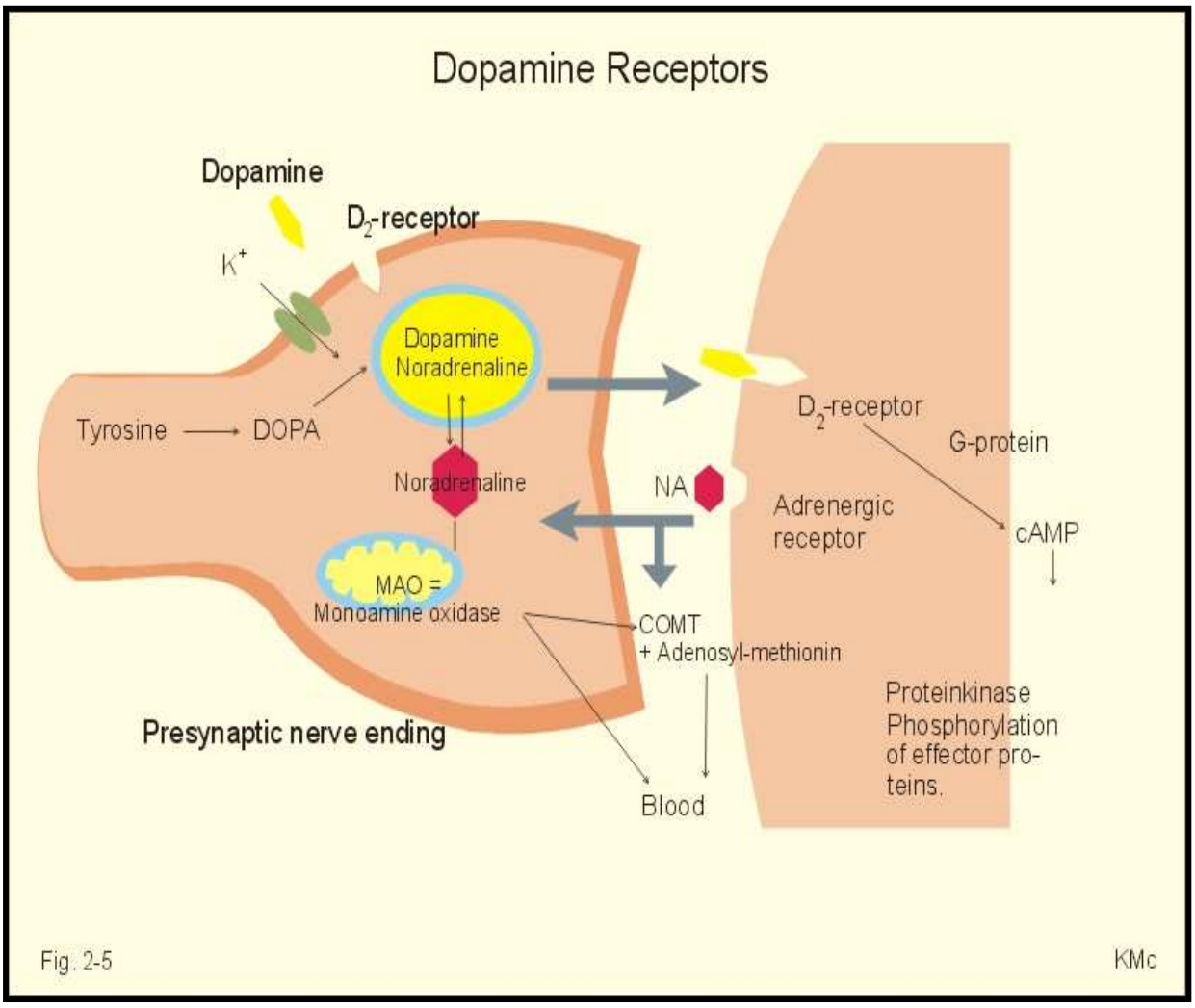

Figure 1.15. The anabolism and fate of DA in the neuron (www.mfi.ku.dk/ppaulev/chapter2/images/n2-5.jpg).

1.4.7. Dopamine and Parkinsons Disease

Parkinson's disease results from the loss of greater than 80% of the DA neurons within the SNpc. Although the specific etiology of PD is unknown, abundant pathological data suggests that oxidative stress and mitochondrial dysfunction plays a role in the DA neurodegeneration (Mitchell *et al.*, 1996). Dopamine itself may be an endogenous neurotoxin as shown in figure 1.16, clarifying the specificity of DA neuron vulnerability seen in PD.

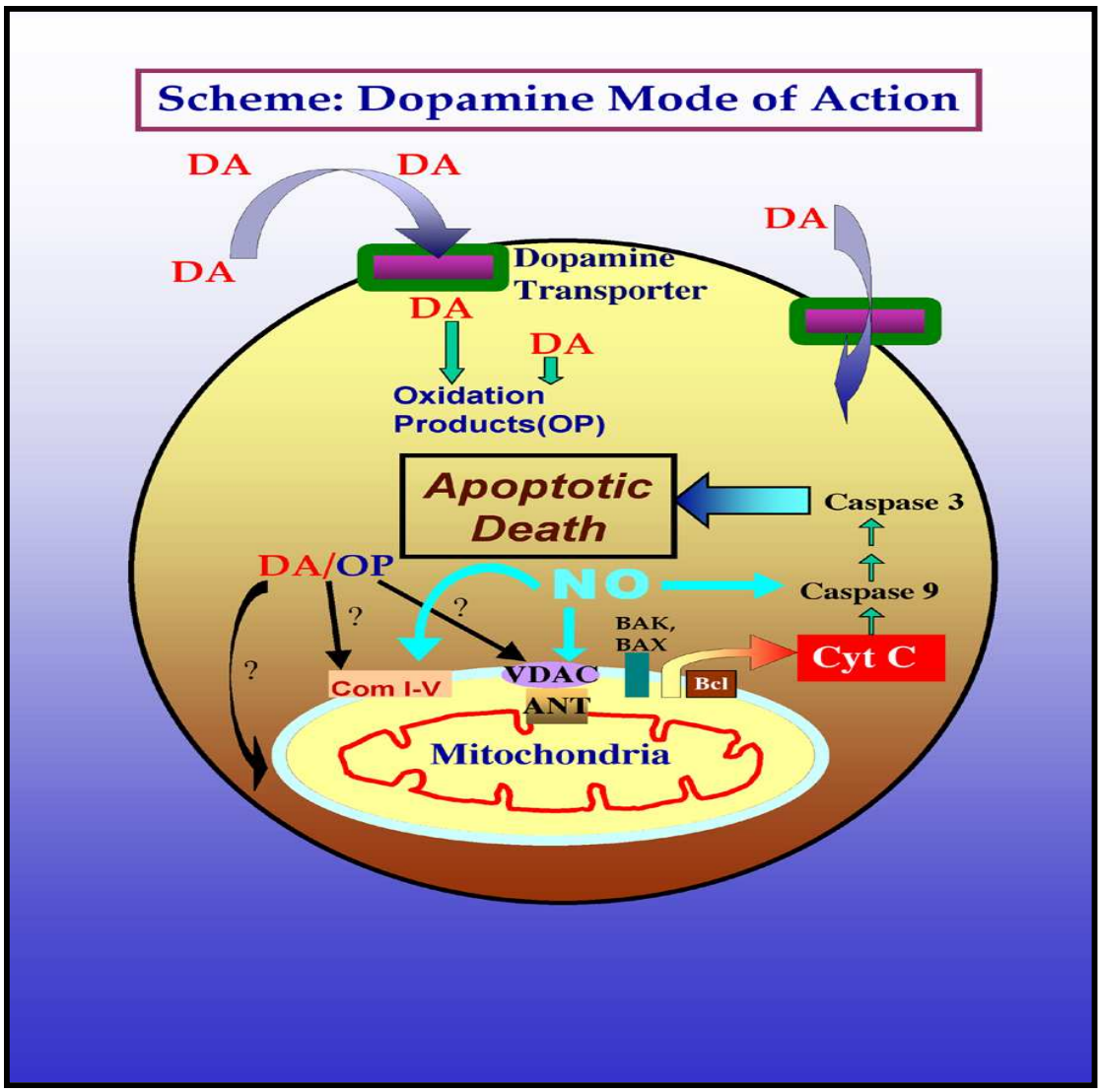

Figure 1.16. The role of DA in the production of oxidative products and apoptotic cell death (www.weizmann.ac.il/ molgen/images/schem.jpg).

Several studies have shown that a highly reactive DA molecule can cause striatal neuronal death both *in vitro* and *in vivo* in a variety of organisms (Yahr *et al*., 1986; Jenner, 1998). Dopamine can be oxidized by at least two independent pathways. It can be oxidized enzymatically by MAO and it can be nonenzymatically auto-oxidized to produce highly reactive quinones and the $O_2^{-\bullet}$. Both pathways produce H_2O_2, which in the presence of transition metals, such as iron (which is found in higher abundance in PD

substantia nigra, possibly due to abnormal ferritin matabolism), can be futher oxidized to form the highly reactive reactive $^{\bullet}OH$ (Yahr *et al*., 1986; Jenner, 1998). Several studies suggest these reactive oxygen species (ROS) and quinones could be major contributors to DA neuron cell death because of their ability to cause protein denaturation, lipid peroxidation, and DNA damage (Yahr *et al*., 1986; Jenner, 1998). DA has also been proposed to cause cell death via calcium channel activation in the DA receptor-dependent mechanism (Yahr *et al*, 1986). Figure 1.16 indicates the various mechanisms by which excess DA can produce oxidative products that induce apoptotic cell death.

Finally DA can confer toxicity to the DA neurons via the catechol hydroxylation to form 6-hydroxydopamine (6-OHDA) (Blum *et al*, 2001). Exposing the SNpc in rodents and mammals to neurotoxins that cause DA neurodegeneration is most commonly utilized animal model for PD. Vertebrates exposure to the neurotoxins, 6-OHDA, MPTP (MPP^+ is the active metabolite of MPTP), or the insecticide rotenone causes SNpc DA neuronal death within several weeks, and the animals mimic symptoms as PD.

1.4.8. MPTP model for Parkinson's Disease

Between 1979 and 1982, a population of young Californians addicted to a new synthetic fentanyl derivative developed an irreversible L-dopa responsive PD (Davies *et al*., 1979). The analysis of this synthetic compound showed that it contained around 3% MPTP (Langston *et al*., 1983). Patients exhibited symptoms very similar to PD with bradykinesia, rigidty, postural instability and resting tremors. Additionally, post-mortem investigations clearly confirmed the lesion in the substantia nigra (Davies *et al*., 1979). MPTP was thus considered a powerful drug to induce PD-like symptoms in several species including rat, mouse, dog, cat and monkey.

One of the most acceptable animal models in Parkinsons disease research is the MPTP model (Ghorayeb, 2002). MPTP needs to be metabolized by MAO-B to produce an active neurotoxic metabolite MPP^+. Species of animals that have inherent low activity of this enzyme are shown to be resistant to MPTP (Mitra *et al*, 1994). However MPP^+ can

be intracranially made available at the terminal region, the striatum or cell body region, the substantia nigra to make such animals parkinsonian (Wu *et al*., 1994; Mohanakumar *et al*., 2002).

1.4.8.1. Neurotoxicity of MPTP

In the monkey, MPTP induces the loss of pigmented neurons of the SNpc (Hantraye *et al*., 1993) but rarely causes the appearance of eosinophilic inclusions resembling Lewy Bodies (Forno *et al*., 1988). Injections of MPTP into the SN and the medial forebrain bundle as well as intrastriatal perfusion lead to a massive loss of DA in the striatum (Chiueh *et al*., 1992 and 1993) and, more generally, to the depletion of the dopaminergic markers in the nigrostriatal tract (Heikkila *et al*., 1985). The effects of MPTP on animals depend on several parameters such as the administration mode, dosage and animal age (Gerlach and Reiderer, 1996).

Interestingly, studies on mice support the notion that older animals are more susceptible to MPTP (Jarvis and Wagner, 1985). When the MPTP crosses the BBB and is converted, mainly in the glial cells, into its effective form, MPP^+, by MAO B explaining the effectiveness of MAO B inhibitors against MPTP neurotoxicity (Chiba *et al*., 1984). MPP^+ then accumulates in the dopaminergic cells after selective uptake by energy-dependent DA uptake sites (Chiba *et al*., 1985). Besides this uptake, intracytoplasmic accumulation of MPP^+ also depends on two intracellular trapping systems: (1) the neuromelanin that forms the complex with MPP^+ and delays it cytoplasmic release (D'Amato *et al*., 1986) and the vesicular monoamine transporters that confine the neurotoxin to synaptic vesicles. Free cytosolic MPP^+ finally enters mitochondria by an energy-dependent mechanism (Ramsay and Singer, 1986) inhibiting the activity of this organelle and leading to a drop in cellular ATP levels and subsequent cell death as illustrated in figure 1.17. Kalivendi *et al*. (2003) demonstrated that Fe^{2+} plays an important role in MPP^+-induced $^{\bullet}OH$ formation which ultimately results in lipid peroxidation as shown in figure 1.17. Blum *et al* (2001) reported that MPTP/MPP^+

administration results in enhanced Fe^{2+} levels in the brain which propogates the formation of ROS resulting in neurodegeneration.

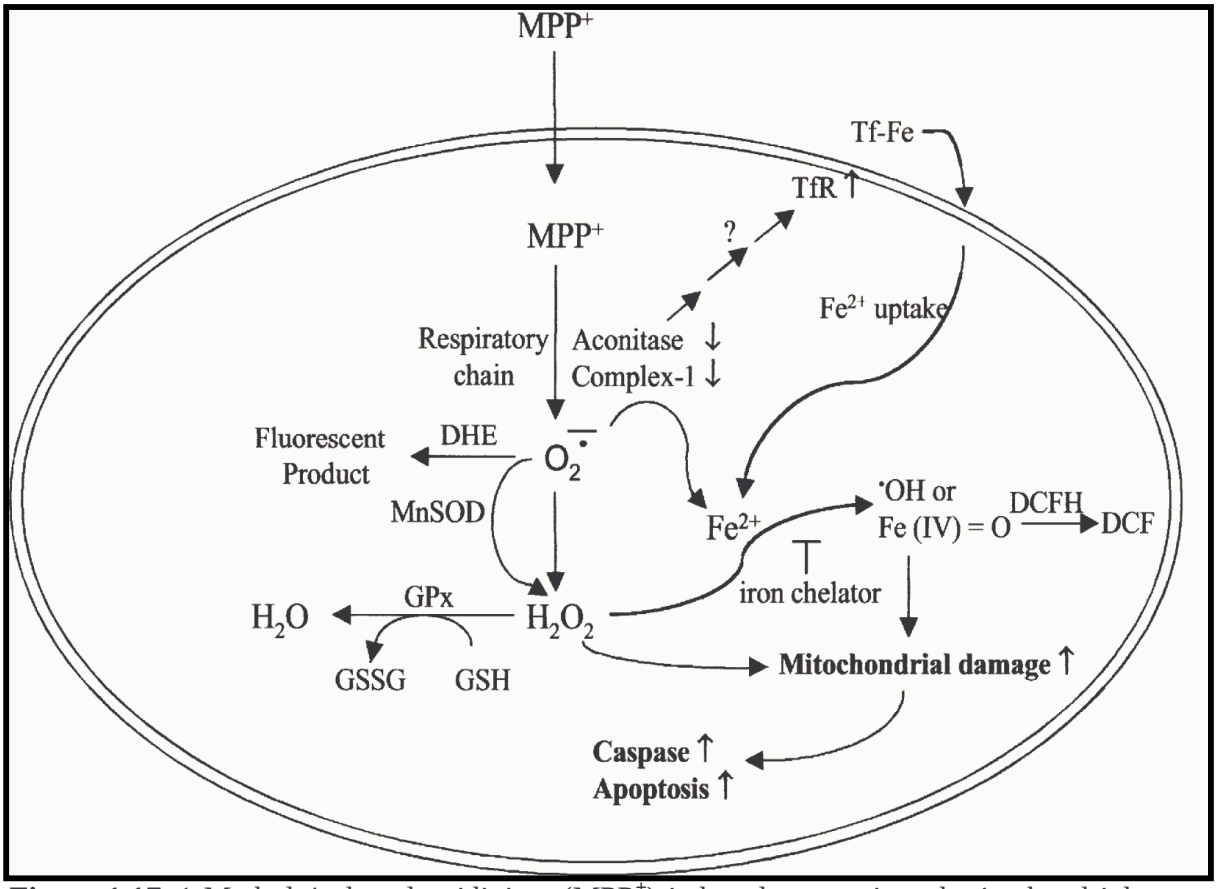

Figure 1.17. 1-Methyl-4-phenylpyridinium (MPP^+)-induced apoptosis and mitochondrial oxidant generation (Kalivendi *et al*., 2003).

1.4.9. Treatment Possibilities of Parkinsons Disease

Aims of treatment are to:

- restore dopaminergic neurotransmission in the nigrostriatal pathway
- adjust GABA-mediated effects on the ventrolateral nucleus of the thalamus.
- inhibit the relative excess of cholinergic neurotanmission within the striatum
- reduce the relatively excessive glutaminergic neurotransmission in the neural circuits.

GABA receptor agonists display a dual action on DA-mediated events. One includes a decrease in DA release, reduction in DA receptor density, and decreased response of postsynaptic cells to dopaminergic stimulation; it results in antidopaminergic effects. The other consists of a reduction of striatal cholinergic activity resulting in a facilitation of dopaminergic effects. These two effects could be dissociated depending on the dose of GABA receptor agonists. This dual action probably explains the results of clinical trials showing either amelioration of parkinsonian symptoms with aggravation of L-DOPA-induced dyskinesia or improvement of dyskinesia without or with aggravation of parkinsonian symptoms (Bartholini *et al*., 1987). In animal models of Parkinson's disease, NMDA and AMPA receptor antagonists were found to reverse Parkinsonian signs or potentiate the ability of L-DOPA to reverse akinesia and to alleviate muscular rigidity. Accordingly, the clinical use of NMDA antagonists has been considered for the symptomatic treatment of Parkinson's disease, based also on the observation that low doses of NMDA antagonists potentiate the therapeutic effects of DA agonists and on the hypothesis that even the beneficial effects of anticholinergic drugs may be mediated in part by NMDA receptor blockade (Greenamyre and O'Brien, 1991). Polypharmacy with L-DOPA and a glutamate antagonist as adjuvant may be a realistic prospect in the pharmacological management of Parkinsonian symptoms. This is based on the pathophysiological hint that Parkinson's disease is a glutamate hyperactivity disorder (Starr, 1995).

Unfortunately the drugs that have anticholinergic effects in the striatum will decrease cholinergic neurotransmission in the degenerating pathway from the nucleus basilis to the cerebral cortex. This may worsen memory failure in Parkinsonism. The best therapeutic approach is to enhance striatal dopaminergic neurotransmission. This relieves Parkinsonian rigidity, bradykinesia and loss of associated movements more effectively it helps tremor.

The agents currently being used in the treatment of PD are:

1) Dopamine agonists.

2) The dopamine precursor L-DOPA

3) Agents which delay dopamine degradation, e.g. MAO type B inhibitors

4) Centrally-acting anticholinergic agents.

5) Gene therapy e.g. genetically engineered cells, stems cells.

1.5. LIPID PEROXIDATION

1.5.1. Introduction

Biological membranes have an important function with regards to compartmentalization of structures and are essential for cell functioning. According to the 'Fluid Mosaic Model', biological membranes are dynamic, irregular lipid mixtures of phospholipids and cholestrol, with globular proteins embedded within the membrane (Matthews and van Holde, 1991). The membranes are equipped to transport molecules across them and also form part of metabolic activities such as electron transport. Membranes are also the sites of cell-cell interactions, such as hormone-cell interactions (Clark and Switzer, 1977). Physical or chemical disturbance lead to a change in integrity of cell membrane, which results in the influx of ions such as Ca^{2+}, which causes destruction of the cell. Lipid peroxidation is the ability of free radicals to alter the integrity of cell membranes.

1.5.2. The Role of Molecular Oxygen

Molecular oxygen is required by living organisms and biological systems to survive and is depended upon heavily. It has a very high oxidizing potential and has the ability to form non-toxic agents. It is therefore a very important part of metabolism in many organisms. However oxygen has the potential to be poisonous at higher concentrations than in air (Buechter, 1988). Single electron reductions of oxygen by compounds and enzymatic reactions occur *in vivo* and these reduced intermediates or oxygen species that are formed are reactive with toxic implications (Gerschman *et al.*, 1954; Green and Hill, 1984). These species are collectively known as ROS and include the following: superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$) as shown in figure 1.18 (Green and Hill, 1984).

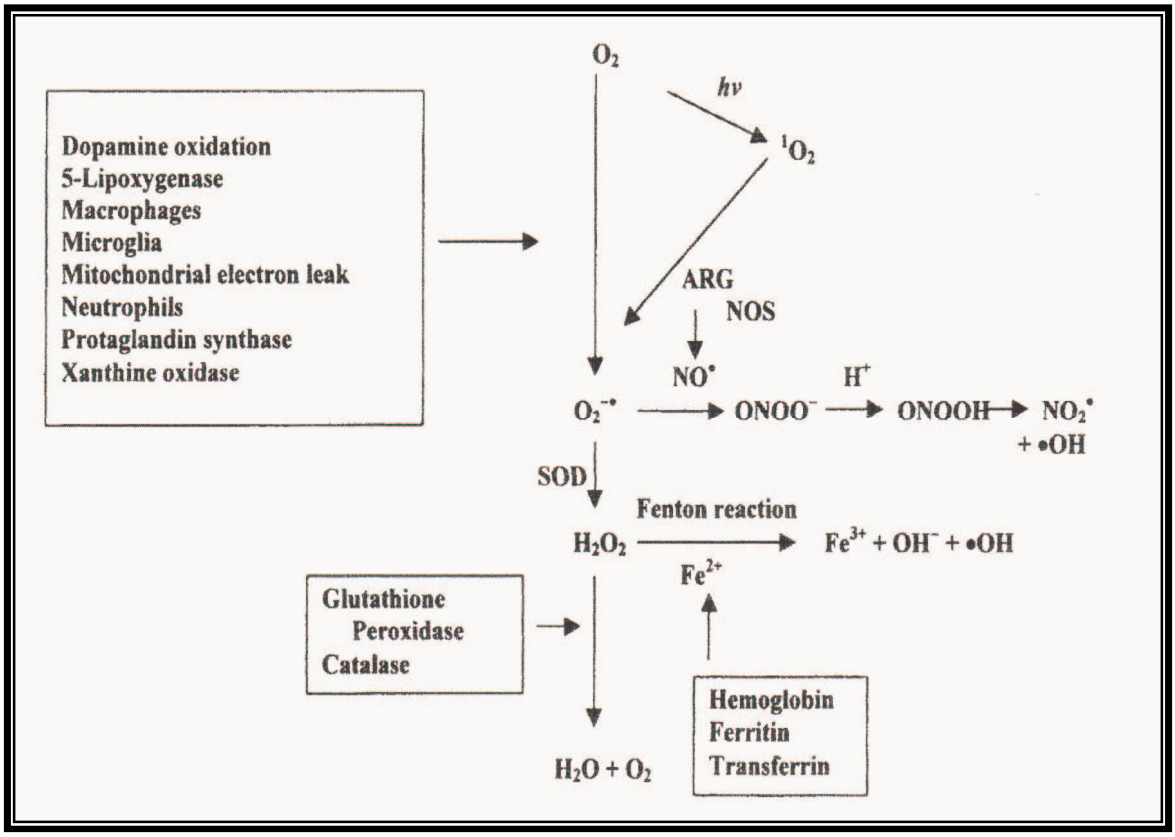

Figure 1.18. A summary of the multiple by-products generated by the partial reduction of O_2 (Reiter, 1998).

1.5.2.1. Reduction of Molecular Oxygen

The utilization of molecular oxygen (dioxygen or O_2) by the organisms comes with a high price. Under normal physiological circumstances, aerobic organisms utilize approximately 98% of cellular O_2 at cytochrome a; this is the terminal cytochrome in the respiratory chain in the mitochondria (Chance *et al*., 1979). In so doing, O_2 is reduced by 4 electrons without the release of partially reduced oxygen intermediates (Malmstrom, 1982). However, a small percentage (1-2%) of the O_2 is not consumed at the cytochrome a_3; rather, it is mono- and divalently reduced to either the $O_2^{-\bullet}$ or to reactive oxygen intermediate H_2O_2 as illustrated in figure 1.18.

Various physiological perturbations of cellular homeostasis lead to a dramatic enhancement in the production of the superoxide anion and H_2O_2 as well as related species such as the $^{\bullet}OH$ (Freeman *et al*, 1981). All these species, but especially the $^{\bullet}OH$, are potentially cytotoxic. These molecules interact with and frequently irreversibly damage a wide variety of biomolecules including: proteins, phospholipids, nucleic acids and sugars, i.e., virtually any molecule in the cell. Cellular damage produced by these reactive oxygen intermediates, often referred to as oxidative stress, seriously jeopardize cellular functions and eventually may kill cells. Oxidative damage may be prominent in pathophysiological damage such as hypoxia (Freeman and Crapo, 1980), tissue ischemia and reperfusion (Flohe, 1988), and inflammation (Zweier *et al*., 1988). The $^{\bullet}OH$ is the most reactive and therefore causes the most damage to the cell (Halliwell, 1992) (figure 1.18). Reactions of the $^{\bullet}OH$ with other molecules are usually classified into three main types: (1) hydrogen abstraction, (2) addition and (3) electron transfer (Halliwell and Gutteridge, 1991). The three reactions mentioned highlights the important principle of free radical chemistry, namely, the reaction of a different free radical, which may be more or less reactive in the formation of a different free radical, which may be more or less reactive than the original radical species.

$O_2^{-\bullet} + H_2O_2 \rightarrow H_2O + OH^- + {}^{\bullet}OH$ Haber-Weiss Reaction (equation 1)

$Fe^{3+} + O_2^{-\bullet} \rightarrow Fe^{2+} + O_2$

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$

} Fenton Reaction (equation 2)

Hydrogen peroxide itself is not especially toxic unless it is in high concentrations within the cells. There are features of the molecule, however, which make it hazardous. Hydrogen peroxide readily diffuses through cellular membranes and can thereby distribute to sites distant from where it was generated. Also, in the presence of transition metals, most often Fe^{2+} (figure 1.18.) but also Cu^{1+}, H_2O_2 is reduced to the $^{\bullet}OH$ via either Haber-Weiss or Fenton reactions (Halliwell & Gutteridge, 1990). The ultimate fate of H_2O_2 is not always the $^{\bullet}OH$. In most cells H_2O_2 is converted to innocuous products by

the actions of two important antioxidative enzymes, that is catalase and selenium dependent GPx (figure 1.18). In the brain GPx are considerably more important than catalase because of the low activity of the latter enzyme in most parts of the CNS (Jain *et al*., 1991). Glutathione peroxidase utilizes H_2O_2 and hydroperoxides as substrates during the conversion of reduced glutathione (GSH) to its disulfide oxidized glutathione (GSSG) (Griffith and Meister, 1985).

The $^{\bullet}OH$ is perhaps not the only destructive species that is formed during the Fenton reaction (Bielski, 1991) but its formation is well documented as, is its ability to oxidize adjacent molecules (Halliwell & Gutteridge, 1989). The $^{\bullet}OH$ is thus, probably the most reactive of the ROS species (Poeggeler *et al*., 1993; Dawson & Dawson, 1996) as it will react with almost all molecules in living cells (Fridovich, 1987). The $^{\bullet}OH$ is formed from $O_2^{-\bullet}$ and H_2O_2 through the Haber-Weiss reaction or through the interaction of metals such as Fe^{2+} or Cu^{1+} and H_2O_2 through the Fenton reaction (figure 1.18) as shown in equation 1 and 2 above. An intermediate in the reaction of H_2O_2 with Fe^{2+} may be the iron-oxygen complex referred to as ferryl which is highly oxidizing and which degrades to form the $^{\bullet}OH$.

Superoxide anions, H_2O_2 and the $^{\bullet}OH$ are all interrelated in the following way (figure 1.18). Superoxide anions are produced as a consequence of the $O_2^{-\bullet}$ metabolism and are metabolized in the presence of an antioxidant enzyme known as superoxide dismutase (SOD) (Zimmerman *et al*., 1990); this results in the formation of H_2O_2. However in the presence of transition metals especially Fe^{2+}, H_2O_2 is rapidly converted to $^{\bullet}OH$ (Finkelstein *et al*., 1980). This step is known as the Fenton reaction. Because of the high cytotoxicity of the $^{\bullet}OH$ it is especially devastating (figure 1.18).

1.5.2.1.1. Non-Enzymatic Lipid Peroxidation

Species that possess an unpaired electron or a fragment of molecule are called free radicals, and may be generated by three different mechanisms (Cheeseman and Slater, 1993):

a) haemolytic cleavage of a covalent bond, whereby each fragment retains one of the paired electrons, $X:Y \rightarrow X^{\cdot} + Y^{\cdot}$

b) loss of a single electron (oxidation) $A \rightarrow A^{+\cdot} + e^{-}$

c) addition of a electron $A + e^{-} \rightarrow A^{-\cdot}$ (where A is a normal molecule)

The detection and measurement of lipid peroxidation is the evidence most frequently cited to support the involvement of free radical reactions in toxicology and in human disease. Lipid peroxidation has been defined as the oxidative deterioration of polyunsaturated lipids, i.e. those lipids containing more than two carbon-carbon double covalent bonds (Halliwell, 1992). Cell membranes are rich in polyunsaturated lipids, and saturated fatty acids which give rise to membrane fluidity. Damage to these polyunsaturated fatty acid side chains reduces membrane fluidity and as a result the biological membrane is not able to function properly.

Schematic diagram of the sequence of events in lipid peroxidation:

- Cell damage $\rightarrow OH^{\cdot} + CH_2 \rightarrow C^{\cdot}H \rightarrow$ conjugated diene $+ O_2 \rightarrow CHO_2^{\cdot} + CH_2 \rightarrow C^{\cdot}H$ + lipid peroxide
- Fe^{2+}-complex + lipid peroxide $\rightarrow CHO^{\cdot}$
- Fe^{3+}-complex + lipid peroxide $\rightarrow CHO_2^{\cdot} + H^{+} + Fe^{2+}$-complex

Lipid peroxidation is initiated by the attack of any species that has sufficient reactivity to abstract a hydrogen atom from a methylene group ($-CH_2-$). An $^{\bullet}OH$ can do this, as well as

various iron-oxygen complexes. Abstraction from a methylene group leaves a carbon radical, which then stabilizes by molecular rearrangement to form a conjugated diene. This then combines with oxygen (which is hydrophobic and thus concentrates into the interior of membranes) to give a peroxyl or "peroxy" radical ($CHO_2^{\bullet}$). This radical can then abstract hydrogen from another lipid molecule or it may attack membrane proteins. Once the peroxyl radical is formed it can abstract another hydrogen atom from a methylene group and combines with this hydrogen to form a carbon radical and lipid peroxide. This carbon radical may then react with oxygen to form a peroxyl radical and so the chain of lipid peroxidation can continue as shown in figure 1.19.

Iron plays an important role in lipid peroxidation. Not only can it generate $^{\bullet}OH$ via the Fenton reaction, which initiates the chain of events leading to the formation of the alkoxyl radical, but it also plays a second important role in lipid peroxidation. Lipid peroxides are fairly stable at physiological temperatures, but in the presence of iron, their decomposition is greatly accelerated. Thus a reduced iron complex can react with lipid peroxides in a way similar to its reaction with H_2O_2; it causes fission of O-O bonds to form alkoxyl radicals. A Fe^{2+} complex can form peroxyl radicals and by further reaction with the Fe^{2+}–complex can form alkoxyl radicals (Halliwell & Gutteridge, 1990).

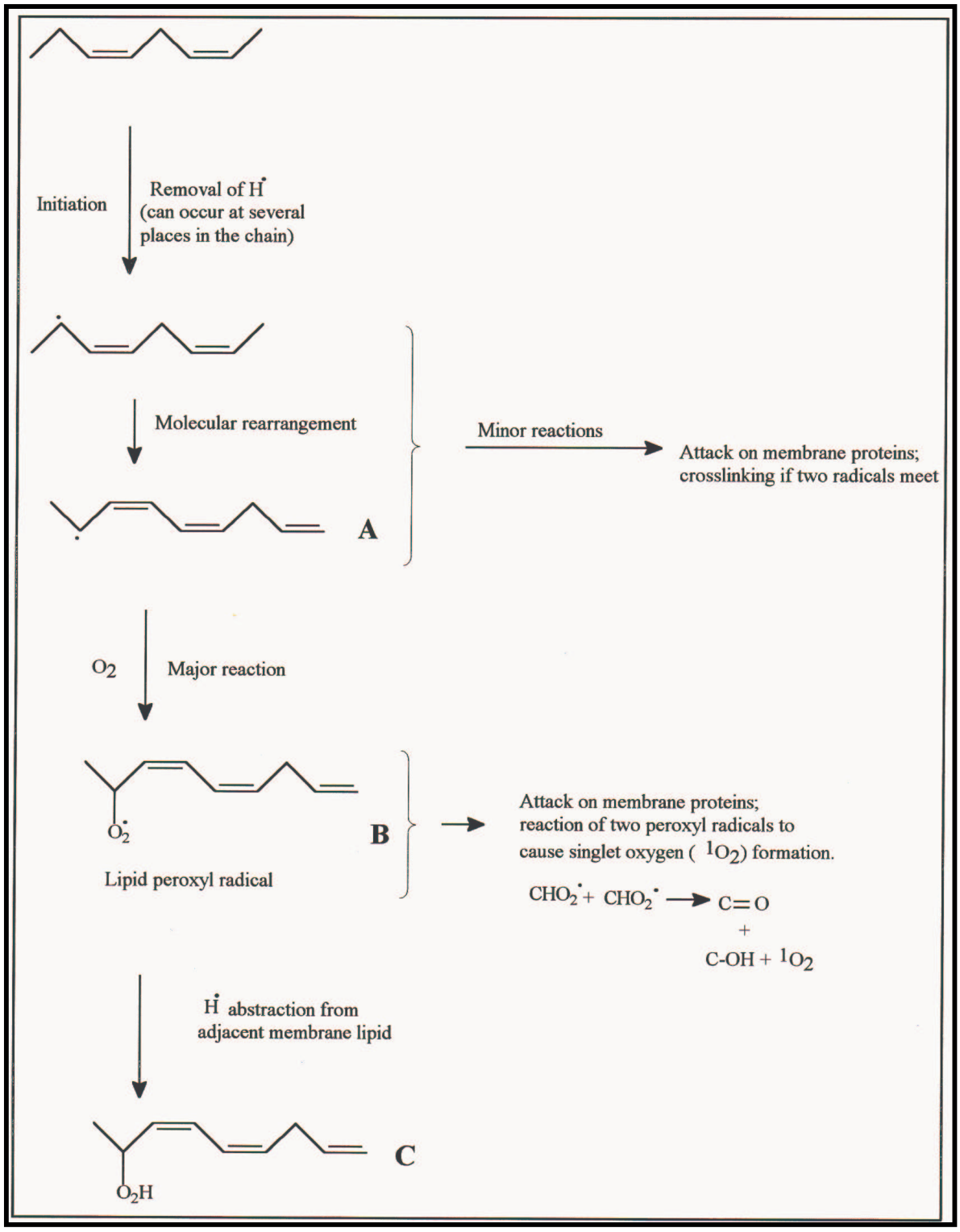

Figure 1.19. An outline mechanism of lipid peroxidation (Gutteridge & Halliwell, 1990).

1.5.2.1.2. Enzymatic Lipid Peroxidation

Superoxide anion production was conclusively demonstrated in a biological system with the oxidation of the xanthine oxidase system (Buechter, 1988). The $O_2^{-\bullet}$ is not considered to be directly responsible for the toxic effects of O_2. Superoxide anions are able to reduce transition metals and their complexes (Buechter, 1988).

Hydroxyl radicals may be formed in two ways: 1) iron catalysis and 2) by iron complexes. The addition of an iron complex to commercial and unsaturated fatty acids will stimulate peroxidation through radicals because in biological systems there is a surplus of lipid hydroperoxide (Gutteridge, 1987). Ferric irons precipitate rapidly to form ferric hydroxides in neutral solutions. However chelators such as ethylenediaminetetracetic acid (EDTA) alter the redox potential of iron thereby preventing this problem (Gutteridge, 1987). Hydrogen peroxide is not a free radical; however it has a status as a ROS in that it is non-radical oxygen that is involved in oxygen radical formation (Cheeseman and Slater, 1993). Hydrogen peroxide has the ability to cross the biological membrane but may diffuse a long distance from the site of production due to its limited activity (Fisher, 1987).

1.5.2.2. Antioxidant Defense System

The selectivity of damage to the membrane is increased by the efforts of $O_2^{-\bullet}$ which exerts its effect directly *in vivo* by producing more potent oxidants, by protonation or by metal salt-catalysed interaction with H_2O_2 (Fridovich, 1987). An increase in the availability of oxygen or the decrease in activity in the oxygen utilizing enzymes such as, SOD, catalase and glutathione peroxidase (GPx) as shown in table 1.1, may lead to an increase in the levels of $O_2^{-\bullet}$. Hydrogen peroxide is also produced with the electron oxidation of phenols, thiols and catecholamines (Turrens *et al*., 1982; Cohen and Heikkila, 1974). Induction of the cytochrome P-450 system may result in an increase in the production of H_2O_2, thus contributing to lipid peroxidation (Van Ginkel and Sevanian, 1994). Microsomes, red blood cells and liposome are sources of hydroxyl

radicals and therefore affect peroxidation profoundly. Sickle cell anaemia and glucose-6-phosphate dehydrogenase deficiency are disease which illustrate that red blood cells are susceptible to oxidative stress (Gutteridge, 1987).

Table 1.1. Cellular defense/anti-oxidant mechanisms accessible to neurons to protect against ROS species (Dawson & Dawson, 1996).

Enzymatic	**Non-Enzymatic**
Cu / Zn – $O_2^{\cdot}$ Dismutase	Ascorbic Acid (Vitamin C)
Mn – $O_2^{\cdot}$ Dismutase	α-tocopherol (Vitamin E)
Glutathione Peroxidase	Glutathione
Glutathione-S-Transferase	Melatonin
Glutathione Reductase	
Catalase	

As mentioned earlier, the presence of scavenging enzymes such as SOD and GPx enable the cells to remain resistant to oxidative stress (Gutteridge, 1987). Reactive oxygen species result in DNA, protein, and lipid damage. All components of DNA may be attacked by the $^{\bullet}OH$ and H_2O_2 (Aruoma, 1994), via chemical damage or ionizing radiation. Reactive oxygen species may attack the sulphydral (SH) groups in proteins. The protein often binds transition metals which makes them the target of attack by site-specific hydroxyl generation (Aruoma, 1994). The presence of polyunsaturated fatty acids makes the membrane phospholipids more susceptible to peroxidation (Van Ginkel

and Sevanian, 1994). Membranal functions, such as GABA uptake, are also altered by lipid peroxidation (Rios and Santamaria, 1991).

The principal cellular defence systems against oxygen free radicals are SOD, GSH, GPx, glutathione reductase (GRd), catalase (a heme enzyme), and antioxidant nutrients (figure 1.18). These enzymes scavenge reactive chemical species and help to maintain cells in a reduced state. Cellular reducing agents such as glutathione and α-tocopherol appear predominantly in the reduced state rather than their oxidized form to enable them to gain electrons (Fahn & Cohen, 1992). The breakdown of $O_2^{-\bullet}$ by SOD yields H_2O_2 and oxygen (reaction 1). There are two distinct SOD's in eukaryotes; the manganese-containing SOD localized in the mitochondrial matrix and the copper- zinc-containing SOD found in the cytoplasm.

$$2O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O \quad (2)$$

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+ \quad (3)$$

$$2H_2O_2 \rightarrow O_2 + 2H_2O \quad (4)$$

Hydrogen peroxide is decomposed by two reactions. At low concentrations, H_2O_2 is removed by reacting with GSH to form GSSG and water, catalysed by GPx (reaction 2). GSH is regenerated by the action of GRd (reaction 3). At high concentrations, however, H_2O_2 is removed by the enzyme catalase (reaction 4), (Fahn and Cohen, 1992).

An important antioxidant in the brain is α-tocopherol (figure 1.20). This is the most potent antioxidant that can break the propagation of the free radical chain reaction in the lipid part of the biological membrane. In rats, it was shown that in the long term, low levels of antioxidants, such as vitamin E, ascorbic acid (vitamin C) as shown in figure 1.20 and figure 1.21, and GSH in all tissues could lead to tissue peroxidizability. Vitamin E deficiency also influences the activities of SOD, catalase and GPx (Buechter, 1988). In addition, severe and prolonged deprivation of this antioxidant produces severe neurological derangements (Muller and Goss-Sampson, 1990).

1.5.3. Protection against Lipid Peroxidation by Antioxidants

The term 'antioxidant' is frequently used in biomedical literature. However Halliwell and Gutteridge (1989) refer to an antioxidant as 'any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate'. Antioxidants can act at different levels in the oxidative sequence. As far as lipid peroxidation is concerned, antioxidants could act by:

1. Decreasing localized O_2 concentrations (e.g. sealing of foodstuffs under nitrogen)
2. Preventing first-chain initiation radicals such as $^{\bullet}OH$.
3. Binding metal ions in forms that will not generate such initiating species as $^{\bullet}OH$, ferryl, or Fe^{2+} / Fe^{3+} /O_2 and /or will not decompose lipid peroxides to peroxy or alkoxy radicals.
4. Decomposing peroxides by converting them to non-radical products, such as alcohols.
5. Chain breaking, i.e. scavenging intermediate radicals such as peroxy and alkoxy radicals to prevent continued hydrogen abstraction. Chain-breaking antioxidants are often phenols or aromatic amines.

Antioxidants acting by mechanisms 1, 2, and 3, can be called preventative antioxidants. Those acting by mechanism 3 are not usually consumed during the course of reactions. Antioxidants of the fourth type are also preventative antioxidants, but they may not be consumed during the reaction, depending on their chemical behaviour (e.g. glutathione peroxidase acts by this mechanism and being and enzyme, is a catalyst and is not consumed). Chain-breaking antioxidants, act by combining with the intermediate radicals.

It should be stressed that many antioxidants have multiple mechanisms of action. In addition, rapid repair of oxidative damage (e.g. cleavage of peroxidized fatty acids from membrane lipids) will tend to diminish the rate of lipid peroxidation; repair mechanisms exist for damage to DNA and proteins.

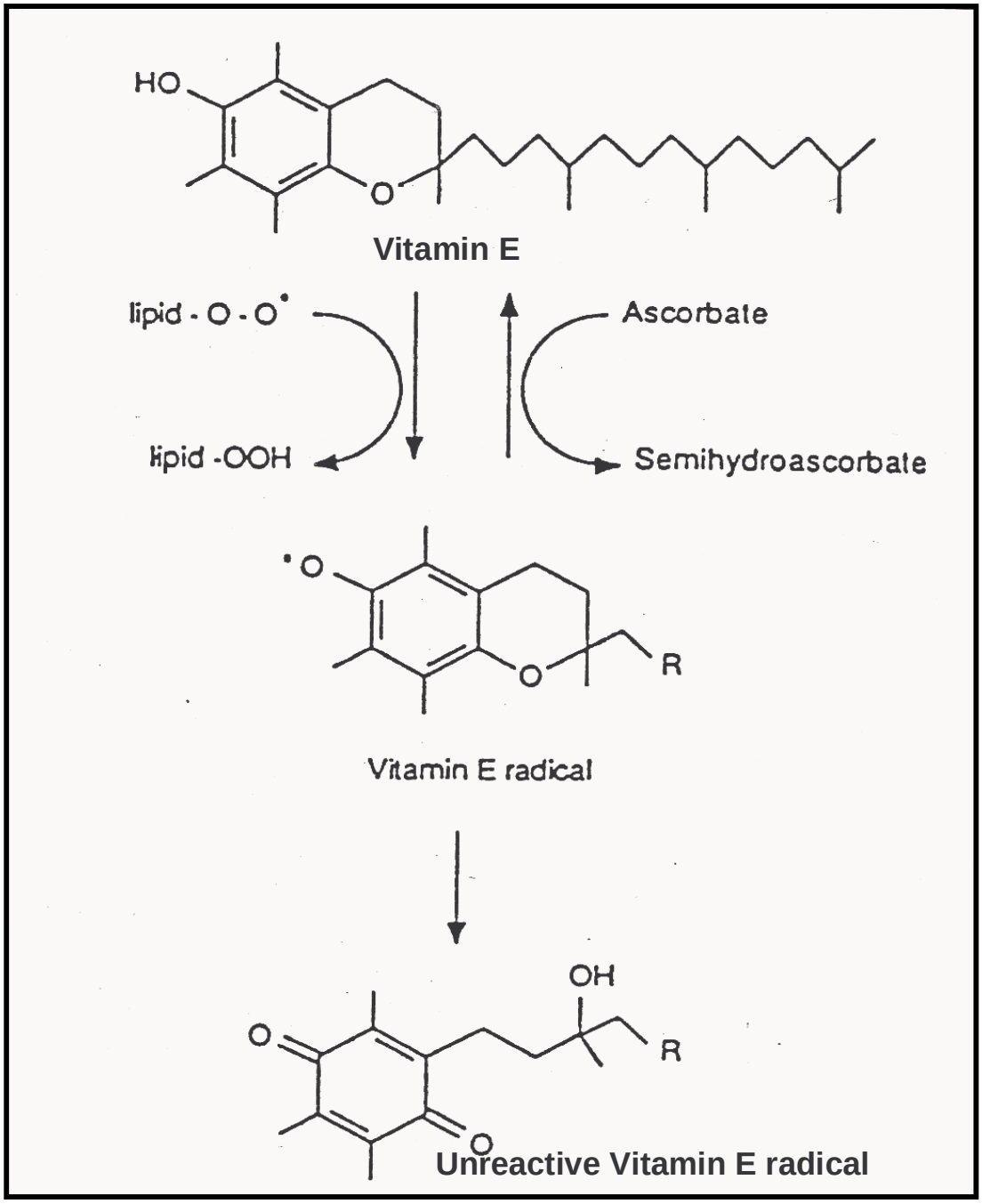

Figure 1.20. Vitamin E acting as a chain-breaking antioxidant, reacting with lipid peroxy radicals to give an unreactive vitamin E radical (Buechter, 1988).

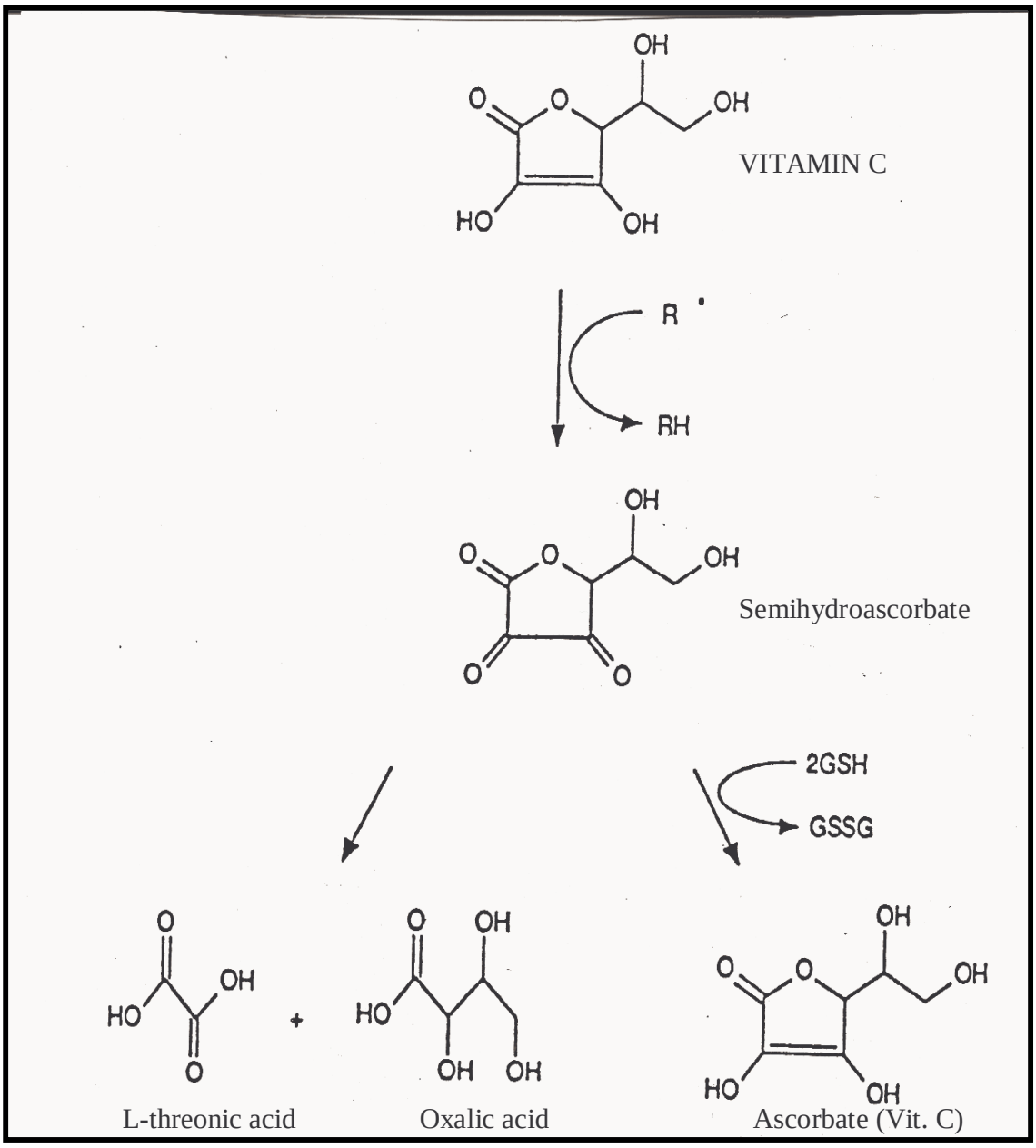

Figure 1.21. Vitamin C (ascorbic acid) plays a protective role by reacting with free radicals including the Vitamin E radical to give semihydroascorbate, which is then able to go on to form L-threonic acid and oxalic acid or be reduced back to ascorbate (Buechter, 1988).

1.5.4. Oxidative stress in Parkinsons Disease

The role of oxidative stress in neuronal degeneration in PD is substantiated by pathological findings and animal models that have provided experimental paradigms to delineate the possible mechanisms. Perhaps the main factor in the vulnerability of dopaminergic neurons are their intrinsic predisposition to generate reactive species. The normal enzymatic metabolism of dopamine results in the generation of H_2O_2 by MAO-B. The nonenzymatic autoxidation of dopamine at neutral pH results in the formation of reactive quinones and semiquinones, this process is enhanced in the presence of iron, leading to the further formation of H_2O_2, $O_2^{-\bullet}$, and $^{\bullet}OH$ radicals (Hastings *et al*., 1996). It also has been suggested that the oxidation of dopamine results in the formation of 6-OHDA, which readily undergoes rapid autoxidation with molecular oxygen to generate reactive free radical species (Baumgarten and Grozganovic, 2000). Although the importance of the formation of 6-OHDA under normal physiological conditions is still unknown, it has been widely used as a model of dopaminergic neuronal injuries. These findings have provided further credence to the proposal that dopamine metabolism results in oxidative stress (Ungerstedt, 1976).

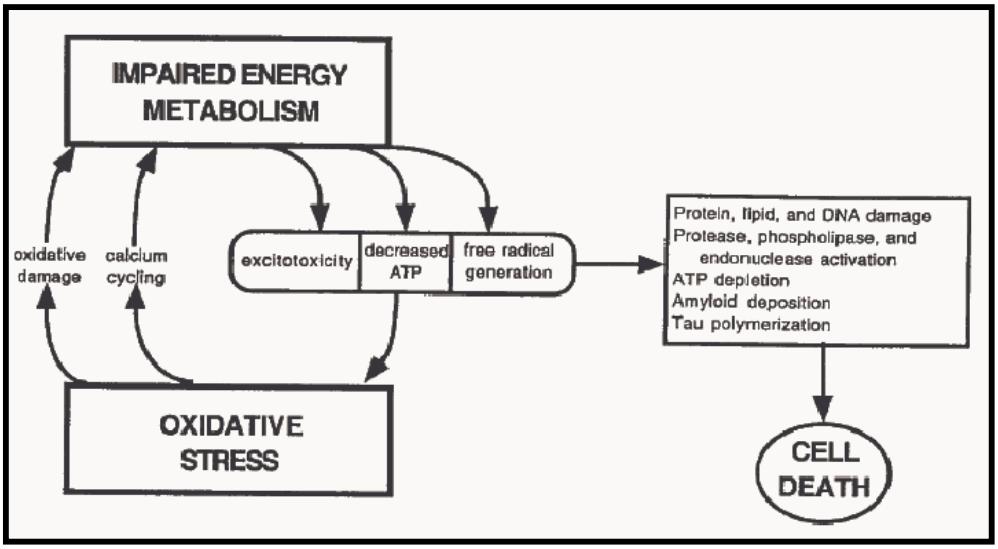

Figure 1.22. Possible cycling mechanism between impaired energy metabolism and oxidative stress (Bowling and Beal, 1995).

1.5.4.1. Mitochondrial dysfunction

It has been hypothesized that mitochondrial dysfunction and consequent production of ROS may cause neuronal death evolving in the process of PD (Halliwell, 1987).

1.5.4.1.1. The mitochondrial electron transport chain

Each mitochondrion consists of two phospholipid bilayers, the outer membrane and the inter membrane. The mitochondrial electron transport chain (ETC) is located in the inner mitochondrial membrane. Between the two bilayers is intermembrane space. The primary function of ETC is ATP synthesis. It comprises a series of electron carriers grouped into four enzyme complexes: (i) complex I (NADH ubiquinone reductase), (ii) complex II (succinate ubiquinone reductase), (iii) complex III (ubiquinol cytochrome *c* reductase), and (iv) complex IV (cytochrome *c* oxidase). In brief, flow of electrons along the ETC from NADH or $FADH_2$ to molecular oxygen is coupled to the pumping of protons across the inner mitochondrial membrane, resulting in the formation of a proton gradient. Dissipation of this proton gradient drives ATP synthesis (Mandavilli *et al*., 2002). The brain is dependent on mitochondrial energy supply to maintain normal brain function. Therefore, damage to one or more of the respiratory chain complexes may lead to impairment of cellular ATP synthesis. However, each of the complexes exerts varying degrees of control over respiration, and substantial loss of activity of an individual respiratory chain complex may be required before ATP synthesis is compromised (Schapira, 1998).

1.5.4.1.2. Mitochondrial DNA and oxidative phosphorylation system

Mitochondrial DNA is a circular double stranded molecule comprising a heavy (H) and a light (L) chain but without any histone coat. Mitochondrial DNA encodes a full complement of 22 transfer RNAs (tRNAs) and 12S and 16S ribosomal RNAs in addition to 13 proteins, all of which are part of the respiratory chain and oxidative phosphorylation system (OXPHOS). Five different complexes involve in OXPHOS

system: complex I, II, III, IV and ATP systhase. MtDNA is dependent upon the cell nucleus for encoding its replication, transcription, translation, repair and regulatory factors (Schapira, 1998).

1.5.4.1.3. Interaction between oxidative phosphorylation and oxidative damage

There are significant interactions between oxidative damage and mitochondrial energy metabolism, especially the OXPHOS. Oxidative phosphorylation results in generation of free radicals in cells. Inhibition of the ETC induces free radical generation. In addition to producing free radicals, OXPHOS is vulnerable to damage by free radicals. There are two mechanisms involved in this process: can be affected through damage to mtDNA. This susceptibility is probably due to lack of protective histones, limited repair ability, and proximity to ECT (Halliwell, 1987); ECT can also be affected directly by free radicals. Complex I is particularly sensitive to $^{\bullet}OH$ and $O_2^{-\bullet}$. The vulnerability of ECT may due to the damage that occurs in the protein and phospholipids (Zhang *et al*., 1990). A cycling process may occur between oxidative damage and oxidative phosporylation, due to the fact that free radicals attack this system that also generate them (Figure 1.22). When oxidative phosphorylation generates free radials, these radicals damage mtDNA, proteins, lipids and induce the greater level of free radicals that may result in addition oxidative damage. This process may also reduce ATP levels, excessive release and reuptake of mitochondrial calcium (Schlegel *et al.*, 1992).

1.5.4.1.4. Mitochondrial dysfunction in Parkinsons Disease

A consequence of mitochondrial dysfunction is increased by generation of free radicals and oxidative damage, which is strongly implicated in the pathogenesis of neurodegenerative diseases including PD. Substantial evidence, has shown a mitochondrial defect in PD. It has been demonstrated that MPP^+ inhibits complex I in SN and is not altered in other brain regions (Mann *et al*., 1992). Increased oxidative stress in PD also induces increases lipid peroxidation in SN in patients (Dexter *et al.*, 1989). It has been hypothesized that increased iron levels cause oxidative stress in substantia nigra. By

the Fenton reaction, iron can react with H_2O_2 to produce $^{\bullet}OH$. Total iron levels are elevated in PD substantia nigra. Iron is also found increased in MPTP-treated-primate (Mochizuki *et al*., 1994). Mitochondrial dysfunction in PD also might be a consequence of either nuclear DNA or mtDNA (Beal, 2000).

1.5.4.2. Antioxidant enzymes Systems in Parkinsons Disease

Free radicals are constitutively produced under normal physiological conditions therefore; organisms develop various defense mechanisms to protect themselves against free radical injury. Such defense mechanisms include the antioxidant enzymes, free radical scavengers, and metal chelating agents. The antioxidant enzymes, as illustrated in figure 1.23, include CAT, GPx, and SOD. SOD catalyses the dismutation of $O_2^{-\bullet}$ to H_2O_2, while catalase and GPx convert H_2O_2 to H_2O. The scavengers include ascorbate, α-tocopherol and GSH. GSH not only acts as a scavenger, but also regenerates other scavengers and serves as a substrate in the GPx reaction (Hung and Lee, 1998).

Compared to other organs in the body, the brain has lower lever activities of detoxifying enzymes like SOD, catalase and GR. It also contains excess unsaturated fatty acids which are targets for lipid peroxidation. Mitochondria, nitric oxide synthase, arachidonic acid metabolism, xanthine oxidase, MAO and p450 enzymes are all sources of ROS in the brain. GPx is the major enzyme for the detoxification of H_2O_2 in the brain since the brain has very low catalase activity (Bharath *et al*., 2002).

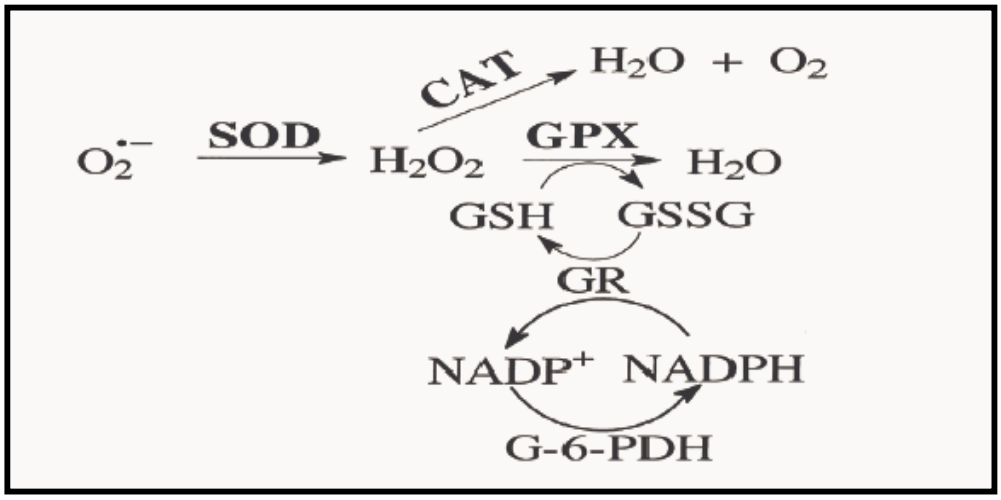

Figure 1.23. Schematic diagram of the antioxidant system. Abbreviations: **$O_2^{-\bullet}$** (superoxide anion), **SOD** (superoxide dismutase), **CAT** (catalase), **H_2O_2** (hydrogen peroxide), **GPx** (glutathione peroxidase), **GSH** (glutathione), **GSSG** (oxidised glutathione), **GR** (glutathione reductase), **$NADP^+$** (Nicotinamide adenine dinucleotide phosphate), **NADPH** (Nicotinamide adenine dinucleotide phosphate, reduced) and **G-6-PDH** (glucose-6-phosphate dehydrogenase)(Hung and Lee, 1998).

1.5.4.2.1. SOD

Several different SOD have evolved to inactivate both intracellular and extracellular $O_2^{-\bullet}$ as shown in figure 1.23. There are two types of intracellular SODs: the manganese SOD (MnSOD) and the copper/zinc SOD (CuZnSOD). MnSOD is localized within the mitochondrial matrix, while CuZnSOD is confined predominantly in the cytoplasm or the nuclear (Weisiger and Fridovich, 1973). Klivenyi *et al* (1998) found that overexpressing the human MnSOD gene in mice showed significant neuroprotection against MPTP-induced depletion of dopamine levels, as well as peroxynitrite-mediated oxidative damage. In a previous study, transgenic mice with increased Cu/ZnSOD activity were also found resistant to MPTP-induced neurotoxicity. Further, inhibition of SOD activity was reported to enhance MPTP toxicity *in vivo* (Bharath *et al*., 2002). .

1.5.4.2.2. GPx

The role of the key H_2O_2 converting enzyme, GPx, is controversial. Early studies report decreased GPx activity in the substantia nigra, caudate and putamen in PD patient (Ambani *et al*., 1975), however, are not supported by subsequent investigations (Chen and Yu, 1994). Importantly, *in vitro* assessment of GPx levels in post-mortem tissues may not reflect events occurring *in vivo*.

1.5.4.3. GSH

Glutathione plays an important role in the adult brain by removing H_2O_2 formed during normal cellular metabolism. In general, SN has lower levels of GSH compared to other regions in the brain. It has been observed that during PD, there is a further reduction in GSH levels within the SNpc. In fact, GSH depletion is the first indicator of oxidative stress during PD progression suggesting a concomitant increase in ROS. The magnitude of GSH depletion occurs prior to other hallmarks of the disease including decreased activity of mitochondrial complex I, decreased enzyme activities in the mitochondria as well as losses in ATP production. GSH may protect neurons against the build-up of protein aggregation, which form Lewy bodies within the cell, the deleterious effects of the lipid peroxidation by-product 4-hydroxynonenal (4-HNE) (Chen and Yu, 1994), and protein oxidation (Ziegler, 1985).

1.6. ACETYLSALICYLIC ACID

1.6.1. Introduction

Quinine from Cinchona bark is one of the oldest remedies for relief of mild pain and fever. Willow bark was used in folk medicine for years for similar indications. In 1763, Edmund Stone, in a letter to the president of the Royal Society, described his success in treating fever with a powdered form of the bark of the willow. He had noted that the bitterness of the willow bark was reminiscent of the taste of the chinchona bark, a source of Quinine. The active ingredient of willow bark, salicin, which on hydrolysis yields salicylic acid, was later found in other natural sources. Acetylsalicylic acid (ASA) was synthesized in 1853, but the drug was not used until 1899, when it was later found to be effective in arthritis and well tolerated. The name ASA was coined from the German word for the compound, acetylspirsauer (Spirea, the genus of the plant from which it as obtained and Saure, the German word for acid). Because of its greater efficacy and lower cost, ASA rapidly replaced the natural products then in use and has remained one of the most widely employed remedies for the last century.

1.6.2. Chemistry and Pharmacokinetics

Acetylsalicylic acid has a pK_a of 3.5. The salicylates are rapidly absorbed from the stomach and upper small intestine, yielding a peak plasma salicylate level within 1-2 hours. The acid medium in the stomach keeps a large fraction of the salicylate in the nonionized form, promoting absorption. However, when high concentrations of salicylate enter the mucosal cell, the drug may damage the mucosal barrier. If the gastric pH is raised to 3.5 by a suitable buffer then gastric irritation is decreased.

Acetylsalicylic acid is absorbed as such and is rapidly hydrolyzed to acetic acid and salicylate by esterases in tissue and blood. Salicylate is bound to albumin, but, as the serum concentration of salicylate increases then a greater fraction remains unbound and available to the tissues. Ingested salicylate and that generated by hydrolysis of ASA may

be excreted unchanged, but most is converted to water soluble conjugates that are rapidly cleared by the kidney. Alkalinization of the urine increases the rate of excretion of free salicylate. When ASA is used in lower doses (600mg), salicylate elimination is in accordance with first-order kinetics and serum half-life is 3-5 hours. With higher dosage, a mix of capacity-limited and first-order kinetics prevails. Figure 1.24 shows the chemical structures of ASA and salicylic acid.

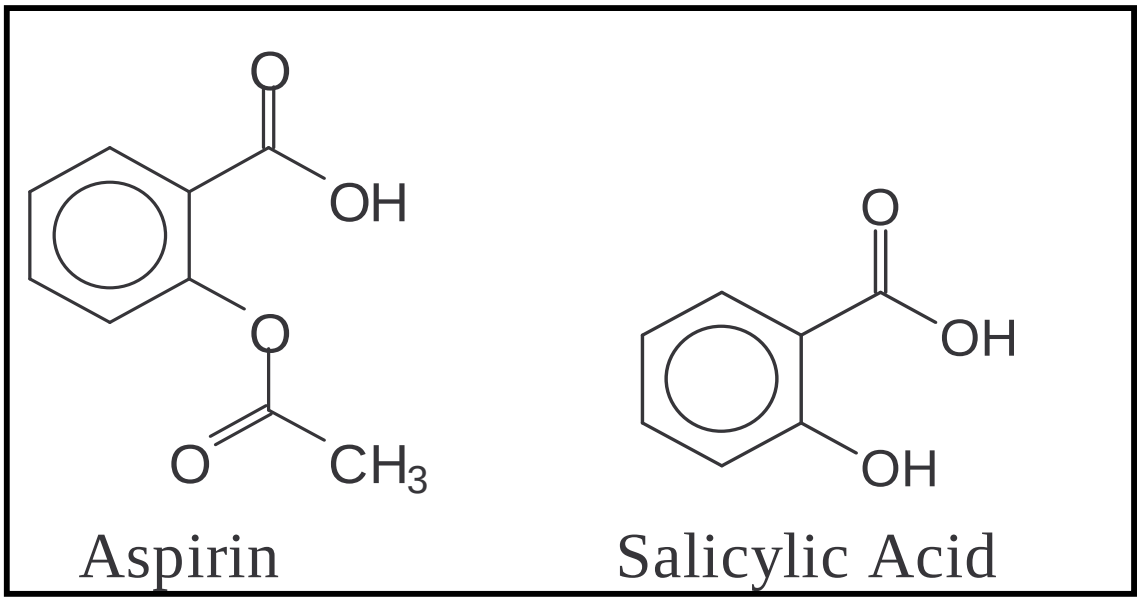

Figure 1.24. The chemical structures of acetylsalicylic acid and salicylic acid.

1.6.3. Principal Effects

Analgesic effects: is mild (less than that of codeine) because prostaglandin G/H synthase is readily regenerated. It seems to be due to both central and peripheral action. Acetylsalicylic acid is most effective against mild pain of somatic as opposed to visceral origin.

Antiplatelet effects: Acetylsalicylic acid appears to offer some protection against thrombotic and embolic events in various parts of arterial circulation, including transient ischaemic attacks, where it reduces the risk of stroke (Ramirez-Lassepas, 1984). Its

antiplatelet action depends on acetylation, and thus irreversibly inhibiting, the enzyme cyclo-oxygenase, which catalyses thromboxane A_2 formation in platelets, and prostocyclin formation in endothelial cells. Cyclo-oxygenase inhibition prevents thromboxane thromboxane-mediated platelet aggregation, but simultaneously prevents the anti-aggregatory effect of prostacyclin formed in endothelial cells. Platelet thromboxane A_2 formation may be more sensitive to inhibition by ASA than endothelial cell prostacyclin synthesis (De Gaetano *et al*., 1982).

Antipyresis: acetylsalicylic acid acts in the hypothalamus to place at a lower level the set point of temperature regulation which is controlled by prostaglandin synthesis. It does affect temperature raised by exercise or heat and does not lower normal temperature.

Respiratory Stimulation: is a characteristic of ASA intoxication and occurs directly both by stimulation of the respiratory centre and indirectly through increased CO_2 production.

Metabolic effects: including increased O_2 consumption and CO_2 production.

1.6.4. Uses

Acetylsalicylic acid relieves mild to moderate pain of nonvisceral origin e.g. headache, dysmonorrhoea, osteoarthritis, myalgia and painful bony metasteases. The anti-inflammatory reaction is prominent, e.g. rheumatoid disease, Still's disease and acute rheumatic fever.

1.6.5. Acetylsalicylic Acid in Neuroprotection.

Acetylsalicylic acid is the most widely used drug for the prevention of secondary ischemic cerebrovascular events (Antithrombotic Trialists Collaboration, 2002). It has been postulated that the ability of ASA to prevent cerebrovascular incidents is

complimented by a possible neuroprotective effect via a direct action on brain tissue (Hissin and Hill, 1976; Grilli *et al.*, 1996).

Several mechanisms have been invoked to explain the neuroprotective effects of ASA in brain tissue (Asanuma *et al.*, 2001; Moro *et al.***,** 2002). It has been postulated that ASA and its metabolite SA inhibit oxidative stress (Sagone and Husney, 1987). One of the principal mechanisms of brain damage during ischemia is the formation of free radicals, which, together with the impairment of the enzymatic antioxidant mechanisms, give rise to tissue oxidative stress (Kontos, 2001). Although ASA inhibits prostaglandin biosynthesis, a property that appears to explain part of the anti-inflammatory activity, this mechanism does not appear to be the only mode of action by which this drug exerts its activity. Sagone and Husney (1987) demonstrated that salicylates react with •OH radicals in granulocytes which might directly relate to their anti-inflammatory effects. Figure 1.25 illustrates the mechanism by which salicylates react with •OH radicals to form stable metabolites.

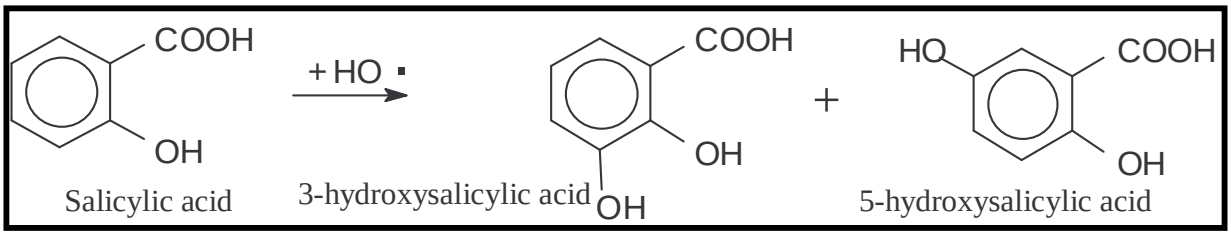

Figure 1.25. Mechanism by which metabolite of acetylsalicylic acid, salicylic acid, acts as a hydroxyl free radical scavenger **(**Aruoma and Halliwell, 1988).

Furthermore it has been shown that ASA rectifies calcium (Ca^{2+}) homeostasis via a mechanism involving the reduction of the $O_2^{\cdot}$ levels possible by acting on protein kinase C-induced NADPH activity and therefore decreases ROS in human endothelial cells, *in vitro* (Dragomir *et al.*, 2004). As mentioned in section 1.5.2.1.1, iron plays an important role in lipid peroxidation. Not only can it generate hydroxyl radicals via the Fenton reaction, which initiates the chain of events leading to the formation of the alkoxyl radical, but Fe^{2+} can also form peroxy radicals and by further reaction with the Fe^{2+}–

complex, can form alkoxyl radicals (Halliwell & Gutteridge, 1990). The various roles of Fe in oxidative stress are illustrated in figure 1.26.

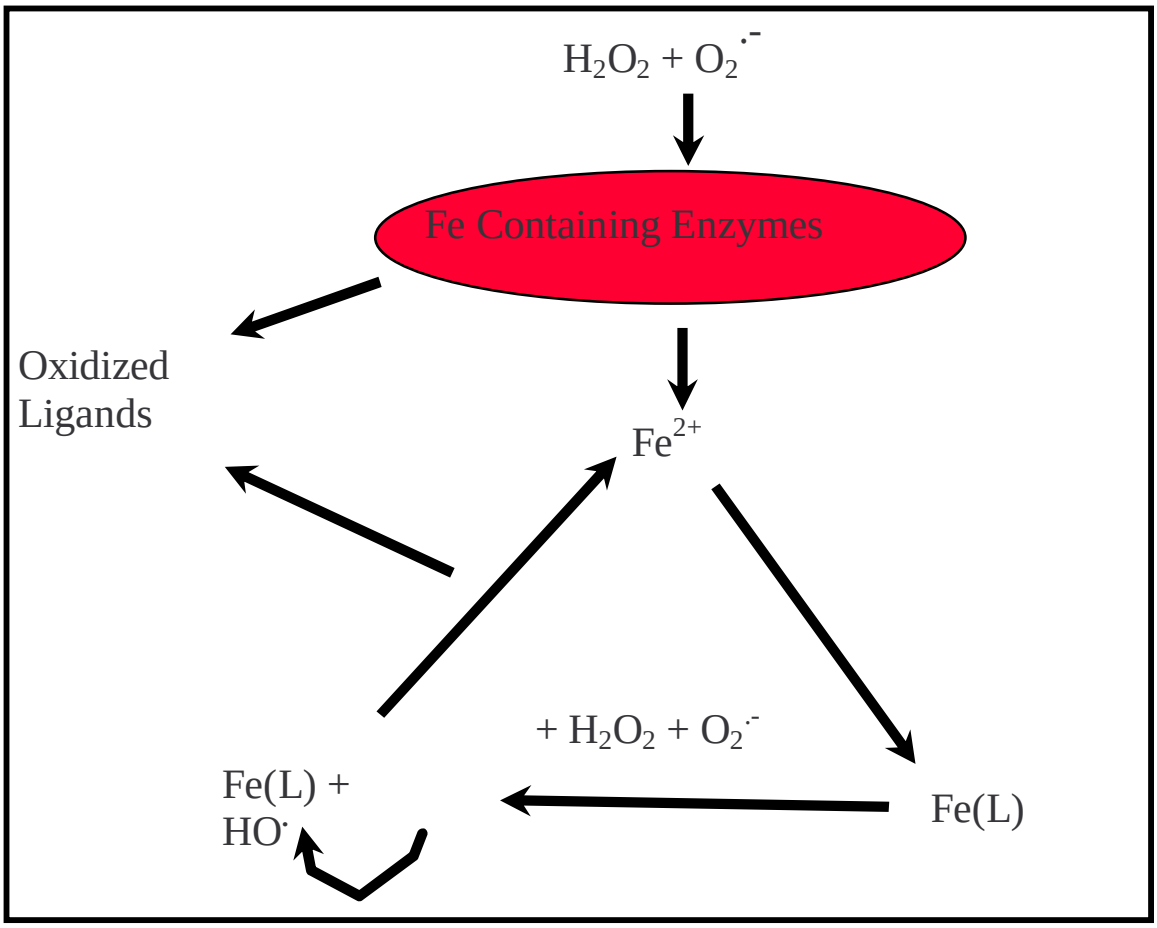

Figure 1.26. Migration of Iron (Fe) Under Conditions of Oxidative Stress (Kotrly and Sucha, 1985).

Acetylsalicylic acid has been shown to complex with Fe^{2+} (Kotrly and Sucha, 1985). Figures 1.27 and 1.28 illustrate the mechanism by which salicylates bind to Fe^{2+} and Fe^{3+}. Salicylates compete with oxidized EDTA for Fe^{2+} chelation and the mechanism is illustrated in figure 1.28.

Acetylsalicylic acid may have neuroprotective properties, and their chronic use is correlated with reduced incidence of AD (McGeer and McGeer, 2001). Acetylsalicylic acid has been demonstrated, *in vitro*, to attenuate the effects of 6-OHDA and MPP^+ in

dopaminergic cells (Carrasco and Werner, 2002). *In vivo* studies have shown that ASA can protect dopaminergic neurons against systemically administered MPTP and have attributed this protection to free radical scavenging (Aubin *et al*., 1998).

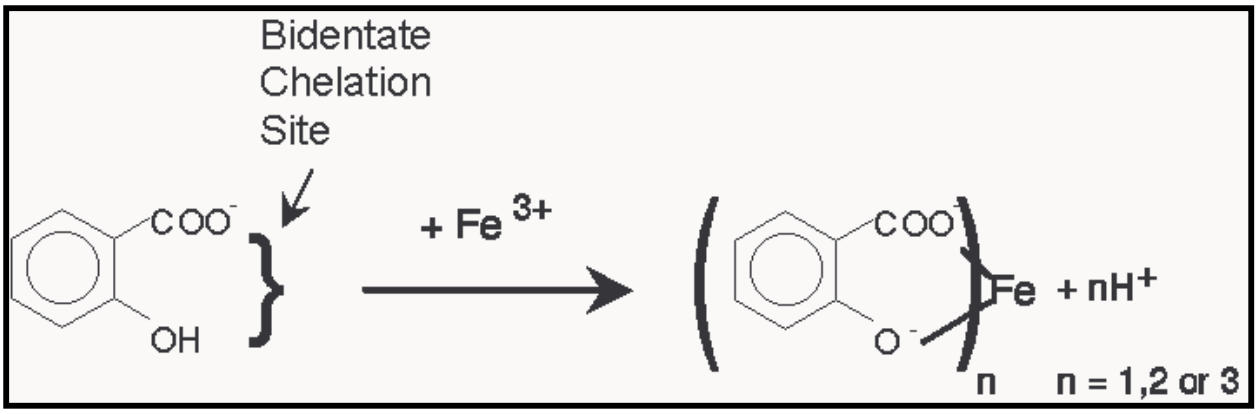

Figure 1.27. Mechanism by which salicylates bind to Fe^{3+} (Kotrly and Sucha, 1985).

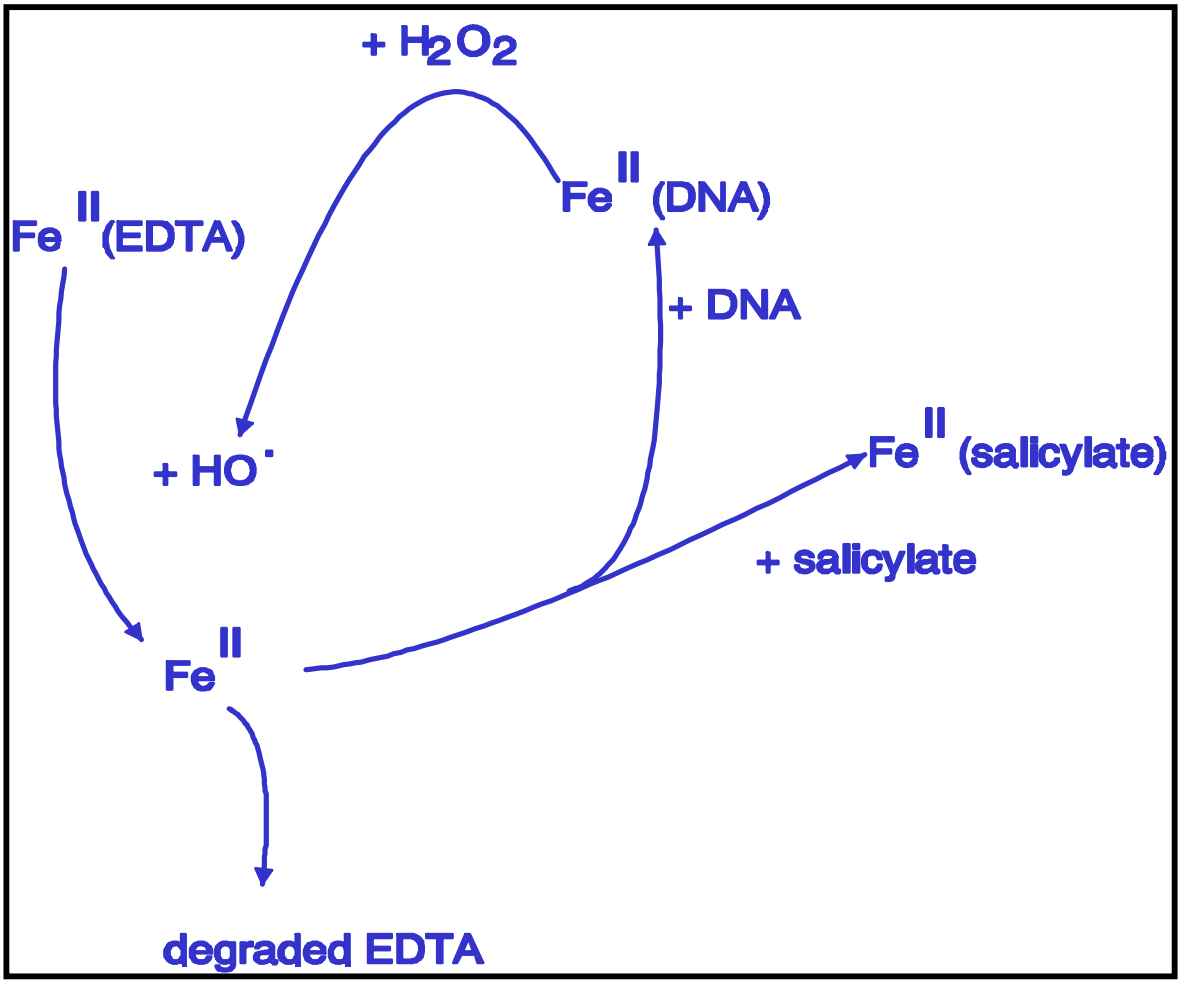

Figure 1.28. Mechanism by which salicylates compete with oxidized EDTA for Fe^{2+} chelation (Kotrly and Sucha, 1985).

1.7. ACETAMINOPHEN

1.7.1. Introduction

This is a popular domestic analgesic and antipyretic for adults and children. It is a major metabolite of the now obsolete Phenacetin. Its analgesic efficacy is equal to that of Acetylsalicylic acid but in therapeutic doses has very mild anti-inflammatory effects. Acetaminophen inhibits prostaglandin synthesis in the brain but hardly at all in the periphery; it does affect platelet function. Figure 1.29 shows the chemical structure of acetaminophen.

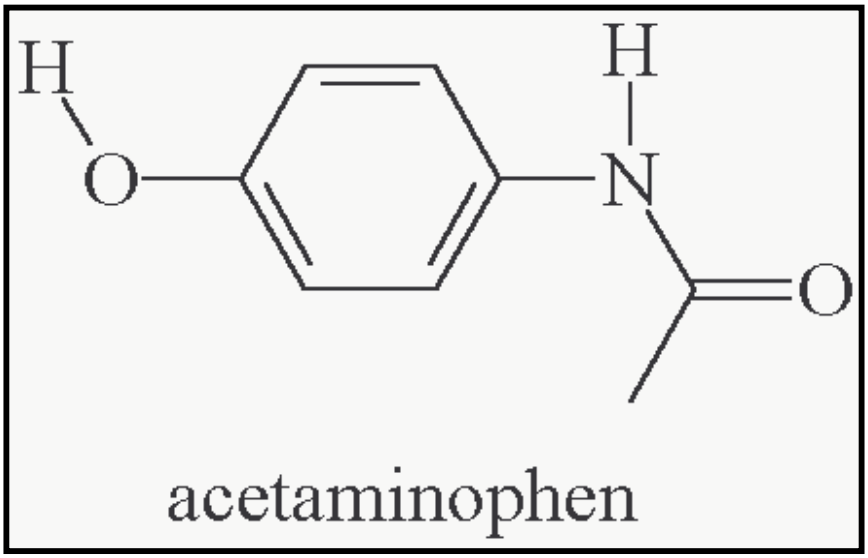

Figure 1.29. The chemical structure of acetaminophen

1.7.2. Principle Uses

Acetaminophen is effective in mild to moderate pain such as that of headache or dysmenorrhoea and it is also useful in patients who should avoid ASA because of gastric intolerance, a bleeding tendency or allergy, or because they are aged less than 12 years.

1.7.3. Pharmacokinetics

Acetaminophen is well absorbed from the alimentary tract and is inactivated in the liver principally by conjugation as glucoronide and sulphate. Minor metabolites of acetaminophen are also formed of which one oxidation product, N-acetyl-p-benzpoquinoneimine (NABQI), is highly reactive chemically. This substance is normally rendered harmless by conjugation with glutathione. But the supply of hepatic glutathione is limited and if the amount of NABQI formed is greater than the glutathione available, then the metabolite oxidizes thiol (SH-) groups of key enzymes, which causes cell death.

1.7.4. Adverse Effects

There are few adverse effects. The drug may rarely cause skin rash and allergy. It is well tolerated by the stomach because its inhibition of prostaglandin synthesis in the periphery is weak. But heavy long-term use may predispose to chronic renal failure.

1.7.5. Acetaminophen in Neuroprotection

Therapeutic interventions aimed at reducing the inflammatory response in AD recently suggested the application of non-steroidal anti-inflammatory drugs (NSAID's). Although the anti-inflammatory properties of acetaminophen are controversial, it emerged that in the amyloid-driven astrocytoma cell degeneration model acetaminophen proved to be effective (Bisaglia *et al.*, 2002). It has been demonstrated that acetaminophen rescues neuronal cells from mitochondrial redox impairment, apoptotic DNA fragmentation, lipoperoxidative products and the generation of malondialdehyde (MDA) (Bisaglia *et al.*, 2002). Acetaminophen has been shown to also reduce the cytoplasmic accumulation of peroxides and inhibition of NF-κB activation.

It has been reported, by numerous authors, that the importance of an anti-inflammatory therapy reduces the occurrence of AD (Stewart *et al.*, 1997; Bour *et al.*, 2000; Ogawa *et*

al., 2000). Recent data show that in the human glial cell line T98G, acetaminophen is able to reduce prostaglandin E_2 (PGE_2) production following Aβ stimulation (Landolfi *et al.*, 1998) thus providing evidence of inhibition of Aβ-triggered glial activation.

1.8. RESEARCH OBJECTIVES

The first objective of this study was to investigate the effect of ASA and acetaminophen on the enzymes involved in the regulation of TRP catabolism including the effects of these agents on the enzyme responsible for QA production to determine whether these agents are capable of favourably altering the kynurenine pathway which is implicated in a number of neurodegenerative disorders such as AD and HD. Furthermore, any effects that these agents may have on TRP catabolism will be investigated by measuring brain neurotransmitter levels such as 5-HT, which is affected by TRP levels in the brain. The rationale behind this study was due to the high usage and abuse of non-narcotic analgesics as it is hypothesized that this could result from an elevation of mood enhancing neurotransmitters in the brain. Due to a number of pharmaceutical preparations containing the combination of ASA and acetaminophen, it was decided to also investigate the effects of the combination of these agents. In addition, it was decided to investigate the effects of these non-narcotic analgesics on indoleamine metabolism in the pineal gland. This study would serve as confirmation of any changes that these agents may induce in brain neurotransmitter levels and their metabolites. These studies mentioned above would provide important information on the possible antidepressant properties of these drugs as well as any effects that they may possess in inhibiting the enzyme responsible for the synthesis of endogenous, QA, which is a potent neurotoxin.

The second objective was to determine whether ASA and acetaminophen could act as neuroprotective agents under a number of neuropathological conditions and to attempt to elucidate the mechanism of neuroprotection, should it be occuring. Thus, this part of the study was undertaken to observe the neuroprotective properties of ASA and acetaminophen against damage induced by various neurotoxins such as KCN, QA and MPP^+. This was investigated by measuring $O_2^{-\bullet}$ generation, lipid peroxidation histological and immunohistochemistry studies to elucidate whether these agents are effective against the neurotoxins employed. Due to the controversy surrounding the mechanism by which these agents are neuroprotective, it was decided that these studies

would provide information as to whether ASA and acetaminophen possess antioxidant properties.

Due to the implication of oxidative stress in MPP^+-induced neurotoxicity, it therefore would be beneficial to investigate any effects that these potential antioxidant agents may have in preventing MPP^+-induced neurodegeneration. It was therefore decided to investigate whether these non-narcotic analgesics are capable of attenuating MPP^+-induced striatal DA depletion in rats. Should these agents attenuate the MPP^+-induced striatal DA depletion, it would be beneficial to determine the mechanism by which this occurs. Therefore, it would be essential to investigate if these agents altered any of the mechanisms by which MPP^+-induces neurotoxicty in the striatum ie. Complex I inhibition, antioxidant enzymes such as SOD, CAT and GPx, glutathione levels and DA uptake.

It was hoped that following this investigation, this would provide scientists with a better understanding of the mechanisms by which ASA and acetaminophen affect indoleamine synthesis and catabolism as well as alter brain neurotransmitter levels. In addition, the potential neuroprotective properties and the implications of these non-narcotic analgesics in MPP^+-induced Parkinsonism would be better understood, and that knowledge gained could ultimately be used by future researchers in the treatment of a number of neurodegenerative diseases.

CHAPTER 2

REGULATION OF THE KYNURENINE PATHWAY

2.1. INTRODUCTION

In mammalian cells, the essential amino acid, (TRP) is degraded primarily by the kynurenine pathway via a cascade of enzymatic steps containing several biologically active compounds. This pathway is the major route of L-TRP catabolism (Heyes *et al*., 1997). Figure 2.1 is a schematic representation of TRP catabolism during inflammatory neurological diseases in the brain such as HD, PD and AD with the consequent production of QA, is a known agonist of the NMDA receptor and neurotoxin.

The liver is a major site of systemic kynurenine pathway metabolism, and substrate flux is predominantly regulated by tryptophan 2, 3-dioxygenase (TDO) (Bender, 1982). This enzyme has high substrate specificity for L-TRP and is induced by corticosteroids. Saturation of this enzyme with heme, whether by administration of hematin (Badaway and Evans, 1975) or with the heme precursor, 5-ALA results in an increased activity of the enzyme, resulting in enhanced catabolism of L-TRP.

Indoleamine 2, 3-dioxygenase (IDO) was first described in rat intestine in 1963 but only recently has the biological significance of this enzyme been examined in a broader context (Haiyaishi, 1963; Taylor and Feng, 1991; Mellor and Munn; 1999). Indoleamine 2, 3-dioxygenase degrades the indole moiety of TRP, serotonin and melatonin, and initiates the production of neuroactive and immunoregulatory metabolites, collectively known as kynurenines (Grohmann *et al*., 2003). Indoleamine 2, 3-dioxygenase is induced by interferon-γ in most human cells, and TRP, which is an indispensable constituent of all proteins as shown in figure 2.1. In parallel this amino acid represents the source for two pivotal biochemical pathways: the generation of 5-HT and the IDO-catalysed formation of a series of biologically active metabolites referred to as kynurenines.

Tryptophan is also utilized in the formation of QA. The product of the enzymatic reaction involving 3-hydroxyanthranilic acid was identified ultimately as α-amino-β-carboxymuconic ϵ-semialdehyde, which is unstable and spontaneously rearranges to QA (Mason, 1957). The enzyme responsible for the conversion of 3-hydroxyanthranillic acid to QA is 3-hydroxyanthranilic acid oxygenase (3-HAO). There is an increase in activity of 3-HAO in response to inflammation, which results in an increase in QA production (Heyes and Morrison, 1997) as evident in figure 2.1.

There is considerable evidence that the metabolism of biogenic amines is disturbed in depressive illness, and no doubt there exist mechanisms by which the various biogenic amines and other neurotransmitters are mutually regulated. Evidence suggests that 5-HT may play important role in the mood changes characteristic of the illness (Badaway and Evans, 1981). Hence increased TRP catabolism could lead to the disruption of serotonergic functions leading to neuronal disorders evoking neurological or psychiatric symptoms (Widner *et al*., 2002).

Hence for all the above-mentioned reasons it was investigated to determine if these enzymes; TDO, IDO and 3-HAO could be regulated by acetylsalicylic acid (ASA) and acetaminophen. Therefore any effect that these agents would have on the kynurenine pathway would therefore be consequential in disease states such as depression and QA-induced diseases such as HD.

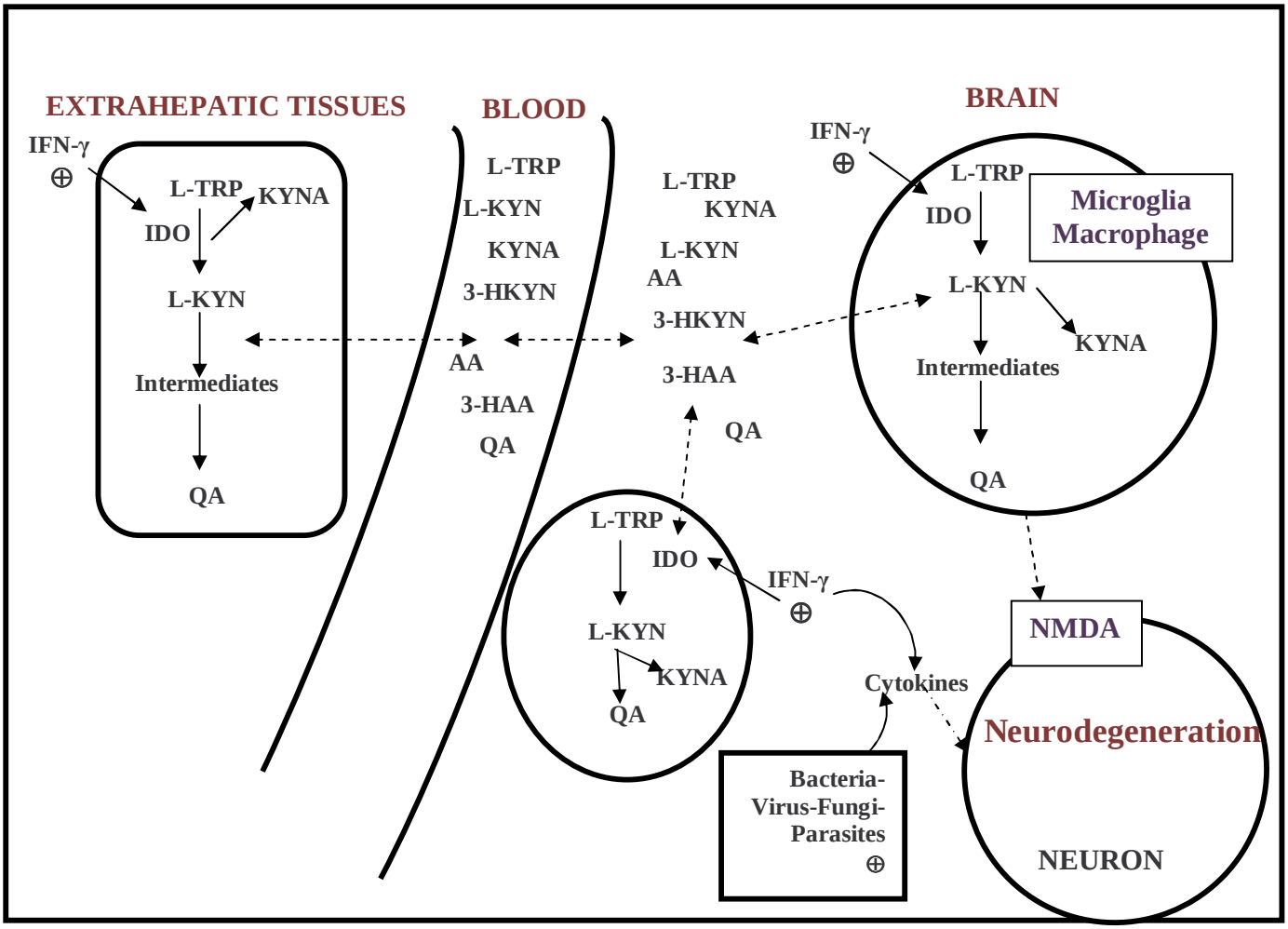

Figure 2.1. A schematic representation of TRP catabolism in inflammatory neurological diseases in the brain with the consequent production of kynurenic acid and QA. Macrophages play an important role in the cerebral synthesis of kynurenines by inducing enzymes responsible for the synthesis of kynurenines. The following abbreviations are used: **3-HIAA**: 3-hydroxyanthranilic acid, **IFN**: interferon, **QA**: quinolinic acid, **KYNA**: kynurenic acid, **AA**: anthranilic acid, **3-HKYN**: 3-hydroxykynurenine and **IDO**: indoleamine 2, 3-dioxygenase (modified from Heyes *et al*., 1993).

2.2. THE EFFECT OF ACETYLSALICYLIC ACID AND ACETAMINOPHEN ON TRYPTOPHAN 2,3-DIOXYGENASE ACTIVITY *IN VIVO*.

2.2.1. INTRODUCTION

The abuse of non-narcotic analgesics such as acetaminophen and ASA by alcoholics (Seifert *et al*., 1993) and patients seeking to relieve dysphoric moods (Abbot and Fraser, 1998; Courade *et al*., 2000) is well documented. It was previously reported that acetaminophen administered in high doses results in increased serotonin levels in rat forebrains (Daya and Anoopkumar-Dukie, 2000). This rise in serototin is accompanied by a concomitant inhibition of the heme-dependent liver cytosolic enzyme, TDO (EC 1.13.11.11) (Badaway *et al*., 1981). This liver cytosolic enzyme is the major peripheral determinant of circulating TRP levels. This enzyme specifically catalyses the oxidative cleavage of the pyrrole ring of L-TRP to form N-formylkynurenine which is metabolized further in the kynurenine pathway of TRP degradation (Badaway, 1979).

Thus, increased activity of this enzyme results in reduced amounts of TRP becoming available for uptake into the brain. The TRP taken up by the brain is then available for conversion to 5-HT (Fernstrom and Wurtman, 1971; Carlsson and Lindqvist, 1978). As a result, there exists an inverse relationship between liver TDO and brain 5-HT levels (Daya *et al*., 1989). Tryptophan 2, 3-dioxygenase is comprised of a holoenzyme as well as an apoenzyme. The apoenzyme is dependent on heme or hematin for its activation (Badaway and Evans, 1975) as shown in figure 2.2. Both the holoenzyme and apoenyme activities can be manipulated by drugs which in turn has the potential to alter brain serotonin levels accordingly (Hardeland and Rensing, 1968; Walsh and Daya, 1998).

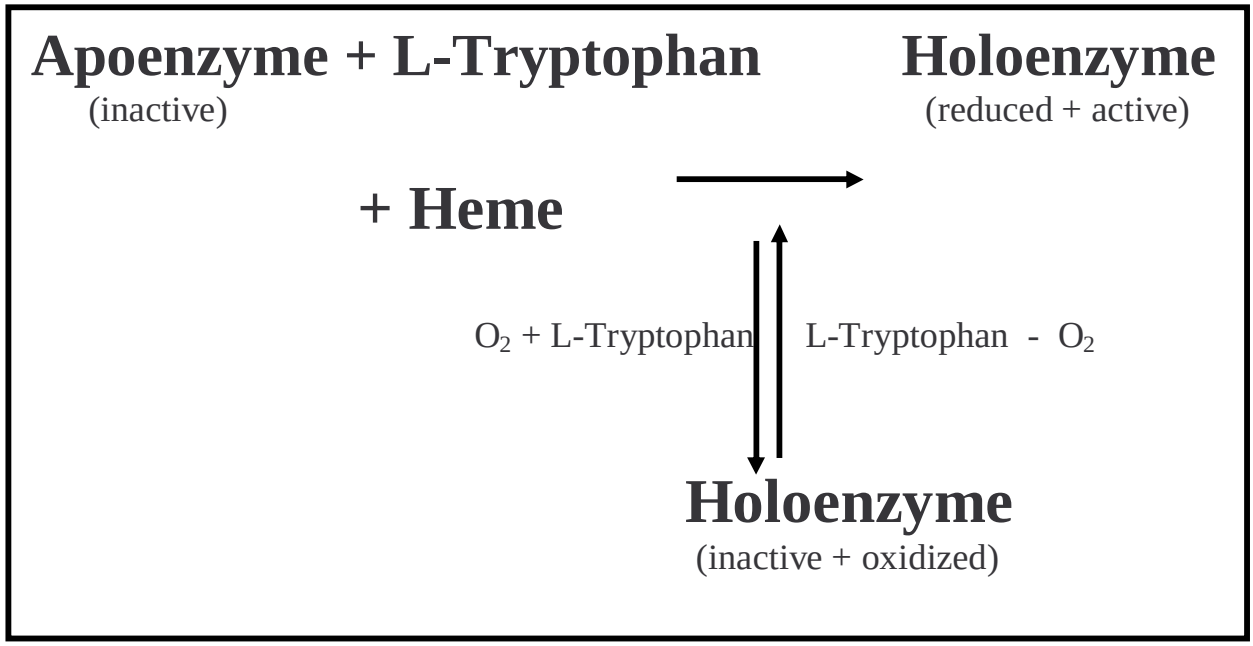

Figure 2.2. The activation of TDO (modified from Walsh, 1996)

2.2.2. MATERIALS AND METHODS

2.2.2.1. Chemicals and Reagents

All chemicals used were of the highest analytical grade available. Acetylsalicylic acid, acetaminophen, L-Tryptophan, hematin chloride, bovine serum albumin (BSA) and methylene blue were all purchased from Sigma Chemical Co., St. Louis, USA. Trichloroacetic acid (TCA), phosphate buffer, sodium hydroxide (NaOH), ethanol (Abs), potassium chloride (KCl), copper sulphate ($CuSO_4.5H_2O$), sodium tartrate, sodium carbonate (Na_2CO_3), sodium chloride (NaCl), Folin-Ciocalteu (F.C) and ferrous sulphate ($FeSO_4$) were all purchased from Saarchem Limited, Krugersdorp, South Africa.

2.2.2.2. Animals

Adult male rats of the Wistar strain, weighing between 200-250g were used in this experiment. The rats were randomly assembled into groups of five, and housed in

separate cages, in a controlled environment as described in appendix I. All protocols for the experiments were approved by the Rhodes University Animal Ethics Committee.

2.2.2.3. Drug Treatment

The animals were divided into four groups (n=5) and treated according to the protocol described in table 2.1. The animals were injected intraperitoneally with 100mg/kg/day, every hour for three hours and killed on the fourth hour. The dose was chosen from previous literature sources where these agents where shown to be protective (Daya *et al.*, 2000).

The animals were killed swiftly by cervical dislocation and rapidly decapitated between 12h00 and 13h00 as described in appendix II. The liver was removed and immediately perfused with 0.9% NaCl. The liver especially needed to be perfused so as to remove the blood and interfering heme, which could affect the conjugation of the exogenous heme with the apoenzyme. The liver was rapidly frozen in liquid nitrogen and stored at –70^{o}C. When needed the liver was allowed to thaw on ice and then weighed.

Table 2.1. Treatment protocol of animal groups used in TDO.

	Treatment Group	**Dosage**
Group 1	Control	Ethanol/Saline (40/60)
Group 2	ASA	100 mg/kg/day in Ethanol/Saline (40/60)
Group 3	Acetaminophen	100 mg/kg/day in Ethanol/Saline (40/60)
Group 4	ASA and Acetaminophen	100 mg/kg/day in Ethanol/Saline (40/60)

2.2.2.4. Sample Preparation

All chemical solutions were prepared in deionized water (Milli R/Q System, Millipore). L-TRP (0.03M and 0.4mM) was prepared in 4mM NaOH. Hematin chloride, final concentration 2μM, was dissolved in 0.1M NaOH and prepared prior to the start of the experiment.

2.2.2.5. Tissue Preparation

The liver was initially homogenized with 60 ml 140mM KCl - 2.5mM NaOH with a Junke and Kunkel Ultra Turrex warring blender for a period of 1 minute at 1000 rev/minute. During the homogenization of the liver, care as taken not to allow excessive foaming so as to prevent protein denaturation. The resultant suspension was further homogenized with a hand held glass homogenizer until a complete homogenous solution was obtained. Thereafter, sonication for a period of 2 minutes at 30s intervals proceeded for complete removal of enzymes from the cells. Finally, 0.2M sodium phosphate buffer, pH 7.0 was used to make up a volume required for a final 20% w/v before being gently stirred. The procedure was carried out on ice.

2.2.2.6. Determination of TDO Activity

Badaway and Evans, 1975, previously described the assay method for TDO employed in this study as described in table 2.2. An aliquot of 15ml of homogenate was added to a beaker containing 12.5 ml water and stirred. Where necessary 100 μl hematin (final concentration of 2μM) was added and a period of 1 minute as allowed for activation of the enzyme. Finally, 2.5 ml of 0.03M L-TRP was added to the overall mixture and was gently stirred. The rest of the assay was carried out in triplicate. Three milliliter aliquots of the reaction mixture was transferred to test tubes and stoppered in an atmosphere of CO_2/O_2 (5%: 95%) and incubated for a period of one hour. The reaction was terminated by the addition of 2ml of TCA to the test tubes. The mixture was then shaken for a period of 4 minutes. The resulting precipitate was filtered into another test tube using a

Whatman No.1 filter paper. To a volume of 2.5 ml of filtrate, 1.5 ml of 0.6M NaOH was added and vortexed. The final pH of the reaction mixture was tested to be 7. The kynurenine present was measured at 365 nm with a Shimadzu UV 160A UV-visible recording spectrophotometer and using an extinction coefficient of kynurenine: $\varepsilon = 4540$ $L.mol^{-1}.cm^{-1}$ the concentration of kynurenine was determined as nmoles/mg protein/minute. The blank consisted of 2 ml TCA and 1.5 ml 0.6M NaOH. Holoenzyme activity was measured in the absence of hematin while the total activity was measured in the presence of hematin. Apoenzyme activity was calculated as the difference between holoenzyme and total activity.

2.2.2.7. Protein Determination

All protein determinations were performed using the method described by Lowry *et al*., (1952). A standard curve was generated using bovine serum albumin (BSA) as a standard at concentration intervals of 60 μg/ml, described in appendix III.

2.2.2.8. Statistical Analysis

The results were analyzed using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range test. The level of significance was accepted at $p < 0.05$.

2.2.3. RESULTS

As shown in figure 2.3, acetaminophen significantly inhibits apoenzyme activity as well as total enzyme activity without altering holoenzyme activity. In contrast, ASA inhibits the apoenzyme, holoenzyme and total enzyme activity. The combination of ASA and acetaminophen potentiates the inhibition of apoenzyme activity compared to either agent alone ($p<0.001$). This is not observed with holoenzyme and total enzyme activity.

Table 2.2. Scheme representing the method of the assay for TDO.

20 % w/v HOMOGENATE		
Holoenzyme	**REAGANTS**	**Total Activity**
12,5 ml	Water	12.5 ml
15.0 ml	Homogenate	15.0 ml
0 ml	Hematin 2μM	100 μl
2.5 ml	0.03 L-TRP	2.5 ml
Continuation in triplicate		
3 ml	Sample Transfer	3 ml
Incubation for a period of 1 hour		
2 ml	TCA	2 ml
Shake for a period of 4 minutes and filter through Whatman No.1 filter paper. Remove 2.5 ml of filtrate into another test tube		
1.5 ml	0.6M NaOH	1.5 ml
Absorbance read at 365 nm		

2.2.4. DISCUSSION

Liver TDO plays an important role in determining the levels of circulating TRP in the blood (Badaway *et al*., 1981). An increase in activity of this enzyme enhances the conversion of TRP to N-formylkynurenine, thus reducing the amount of TRP available for uptake into the brain (Badaway, 1979). Thus agents, which inhibit these enzymes, increase plasma levels of TRP and subsequently induce a rise in brain TRP and serotonin levels (Daya *et al*., 1989). Tricyclic anti-depressants inhibit TDO and this could be part of their mode of action in attenuating depression (Badaway and Evans, 1981).

The inhibition of TDO has been shown to occur via two mechanisms: (a) interference of the test compound with the conjugation of the apoenzyme and its co-factor, heme (Badawy *et al*., 1981), (b) the structure-activity relationship of the test compound at the active site of the co-enzyme. The presence of an -NH group has been reported to be

essential for the active compound to bind to the catalytic site of the enzyme (Uchida *et al*., 1992). The structure of acetaminophen possesses an -NH group, however ASA does not possess a -NH group. This therefore explains the mode of action by which acetaminophen inhibits TDO. The results exhibited in figure 2.3 show that acetaminophen significantly inhibit all forms of TDO except the holenzyme. Acetylsalicylic acid significantly inhibited all forms of TDO. The combination of ASA and acetaminophen potentiated the inhibition of apoenzyme activity compared to either agent alone ($p<0.001$). This is, however, not observed with holoenzyme and total enzyme activity.

The inhibition of TDO by acetaminophen appears to occur at the apoenzyme level (Anoopkumar-Dukie and Daya, 2000). This implies that ASA individually exerts its inhibitory action on the enzyme by inhibiting the binding of the enzyme to its co-factor, hematin. The results imply that ASA and acetaminophen individually and in combination could cause a rise in circulating TRP levels and in turn could result in elevated brain serotonin levels.

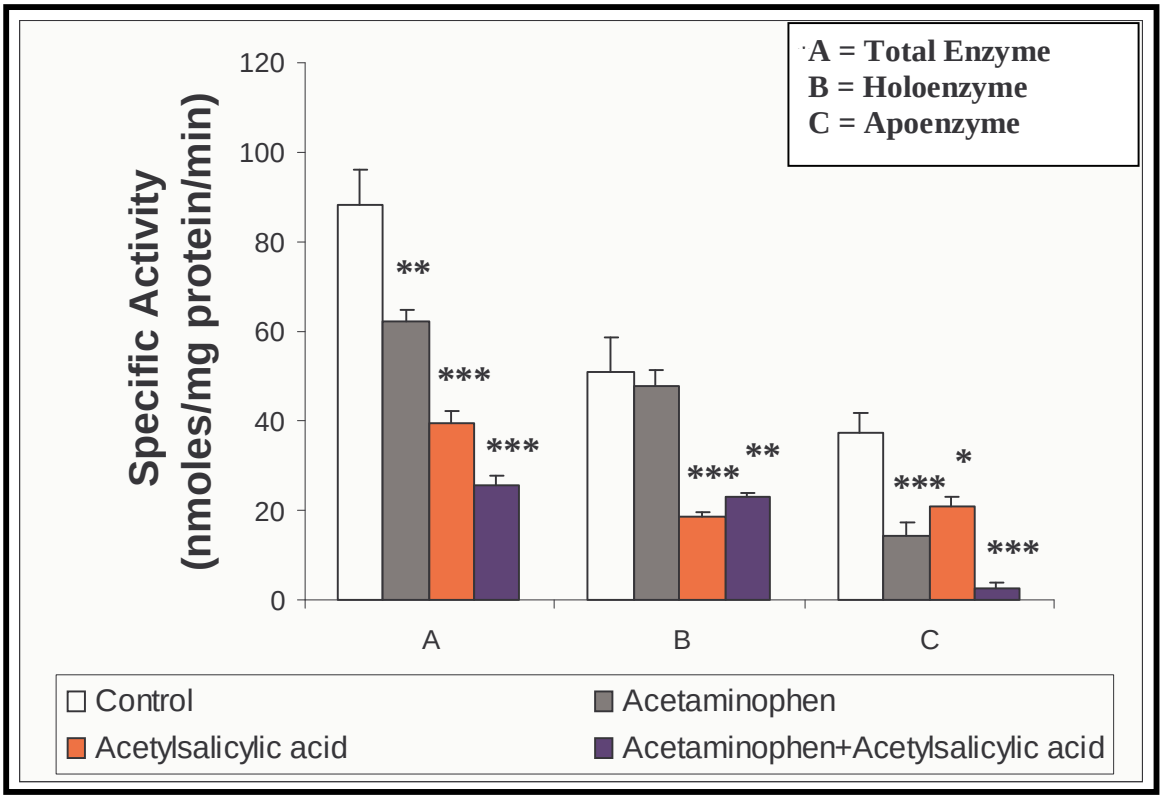

Figure 2.3. The effect of non-narcotic analgesic administration on rat liver tryptophan-2, 3-dioxygenase activity. The specific enzyme activity is expressed as nmols of L-Kynurenine formed/mg protein/min. Each bar represents the mean ± SEM; n=6. *(p<0.05), **(p<0.01), ***(p<0.001). Student-Newman-Keuls Multiple Range Test.

2.3. THE EFFECT OF ACETYLSALICYLIC ACID AND ACETAMINOPHEN ON INDOLEAMINE 2,3-DIOXYGENASE *IN VIVO*.

2.3.1. INTRODUCTION

In extra-hepatic tissues, the first enzyme of the kynurenine pathway is IDO rather than TDO. This enzyme has a much broader substrate profile for indoleamine containing compounds (Carlin *et al*., 1989). In order to elicit catalytic activity, *in vitro*, a requirement for ascorbate and methylene blue has been demonstrated (Higuchi and Haiyaishi, 1967). These enzymes, TDO and IDO, both catalyse the oxidative cleavage of the pyrrole ring of L-TRP and to produce N –formylkynurenine during the first and rate limiting step of the kynurenine pathway of TRP degradation as evident in figure 2.1 (Badaway, 1979).

The high efficiency of degradation of the TRP indole moiety in the periphery contrasts with the relatively poor efficiency of the corresponding reaction in the brain. Although L-kynurenine can be produced in the brain to a moderate degree, the cerebral pathway is driven mainly by the blood-borne L-kynurenine, which enters the circulation and is taken up by astrocytes and microglial cells through transporter-mediated mechanisms. It is reasonable to assume that the level of IDO activity in the periphery greatly affects the dynamics of kynurenine pathway in a normal or diseased brain. In particular, decreased delivery of TRP to the brain, due to increased IDO activity, might result in impaired serotonergic functions and enhanced production of neurotoxins, with far-reaching consequences for patients with conditions apparently as diverse as depression, Acquired Immunodeficiency Syndrome (AIDS)-associated dementia, epilepsy, neurodegenerative diseases, cognitive impairment and schizophrenia (Schwarcz and Pellicciari, 2002).

2.3.2. MATERIALS AND METHOD

2.3.2.1. Chemicals and Reagents

All chemicals were of the highest purity available. Ascorbic acid, catalase, and methylene blue were all purchased from Sigma Chemical Co., St. Louis, USA. All other bench reagents were purchased from Merck, Darmstadt, Germany, and were of the highest chemical purity.

2.3.2.2. Animals

As described in chapter 2, section 2.2.2.2.

2.3.2.3. Drug Treatment

As described in chapter 2, section 2.2.2.3.

2.3.2.4. Tissue Preparation

The small intestine was cut up and then homogenized in 50 mM sodium phosphate buffer pH 7.0 with a hand held homogenizer (20% homogenate) at 4^{o}C.

2.3.2.5. Determination of IDO Activity

The activity of IDO was assayed according to Shimizu *et al*., (1978). The homogenate was centrifuged at 10 000 x g for 20 minutes. The supernatant was removed and placed in test tubes. The experiment was carried out in triplicate. An aliquot of 1.5 ml of supernatant was added to 1.5 ml of reaction mixture. The reaction mixture consisted of 100mM phosphate buffer, pH 6.5, containing 50mM methylene blue, 10 µg of catalase, 50mM of ascorbate and 0.4 mM L-TRP. The buffer extracts were then shaken at 37^{o}C for

60 minutes. After incubation the reaction was terminated with the addition of 2 ml of 0.9M TCA. The mixture was then, further shaken at 50^{o}C for a further 30 minutes to hydrolyse N-formylkynurenine to kynurenine. The incubation mixture was then centrifuged at 12 000 x g for 10 minutes. An aliquot of 3 ml supernatant was used to measure the product formed. The product formed was measured at 335nm, the isosbestic point of formylkynurenine, with a Shimadzu UV 160A UV-visible recording spectrophotometer and an extinction coefficient of 3500 $M^{-1}cm^{-1}$.

2.3.2.6. Protein Determination

As described in Appendix III.

2.3.2.7. Statistical Analysis

The results were analyzed as described in section 2.2.2.8.

2.3.3. RESULTS

As shown in figure 2.4, ASA, acetaminophen and the combination of ASA and acetaminophen significantly inhibited IDO activity (p<0.001).

The administration of the combination of these drugs had a potentiating effect as compared to either agent used alone. Acetaminophen caused a more significant inhibition compared to ASA (p<0.05).

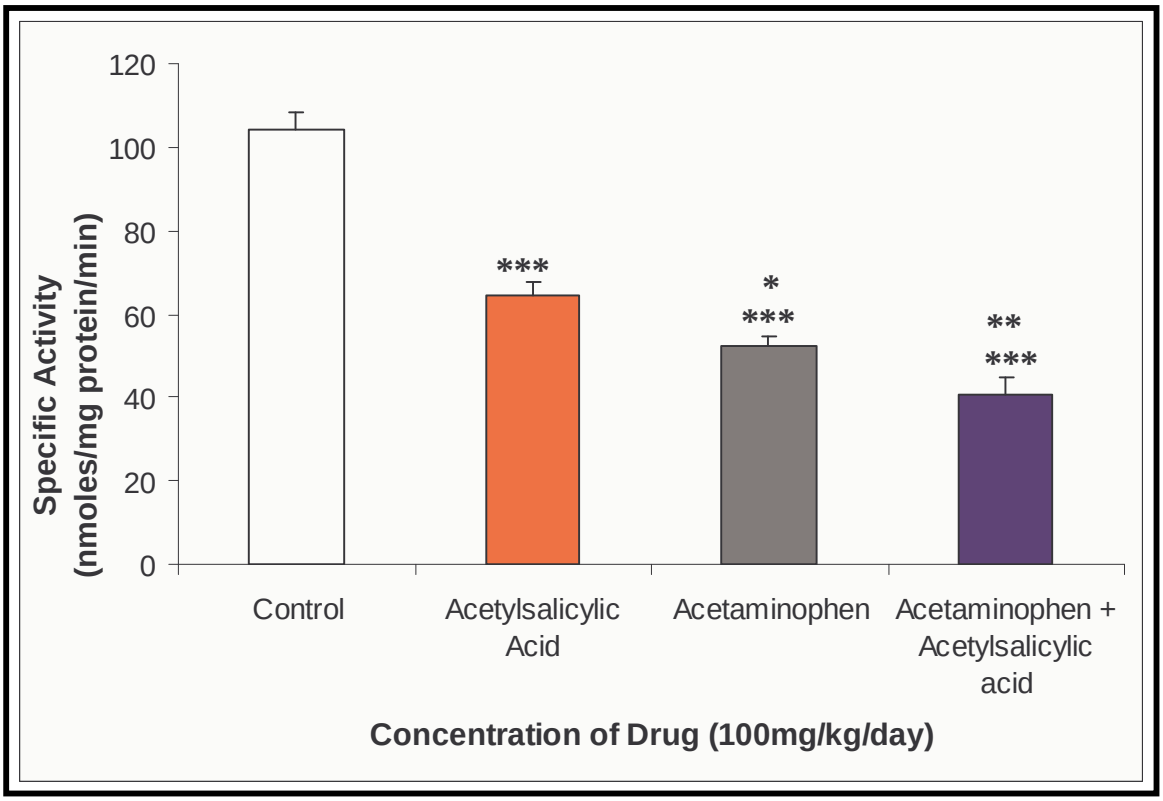

Figure 2.4. The effect of non-narcotic analgesic administration on rat intestine indoleamine-2, 3- dioxygenase activity. The specific enzyme activity is expressed as nmoles of L-Kynurenine formed/mg protein/min. Each bar represents the mean ± SEM; n=5. *(p<0.05) Acetaminophen group vs. Acetylsalicylic acid group, **(p<0.01) Acetaminophen + Acetylsalicylic acid group vs. Acetylsalicylic acid group and ***(p<0.001) vs. control . Student-Newman-Keuls Multiple Range Test.

2.3.4. DISCUSSION

Indoleamine 2, 3-dioxygenase uses a $O_2^{-\bullet}$ as a cofactor in its catalytic process (Hirata and Hayaishi, 1975) and is not induced by TRP and glucocorticoids (Shimizu *et al*., 1978). When immune activation is restricted to the CNS compartment, such as ischaemic brain damage (Saito *et al*., 1993), large increases in brain IDO are accompanied by a substantial increase in brain QA levels as evidenced by the pathway shown in figure 2.1. The induction of IDO causes a marked increase in TRP catabolism in the body (Takikawa

et al., 1986) with enhanced production of kynurenine, which is a precursor to the production of QA.

As is evident from figure 2.4, ASA, acetaminophen and a combination of these two agents caused a significant reduction of IDO activity. The combination of these agents results in further inhibition of IDO.

Maharaj *et al*. (2004) showed that ASA and acetaminophen are potent scavengers of $O_2^{-\bullet}$ in the presence of potassium cyanide, and that the combination of these agents has the greatest effect. This has also been confirmed in chapter five. It is therefore logical to assume that the mode of inhibition of these drugs on IDO activity could be due to the ability of these agents to scavenge the $O_2^{-\bullet}$ free radical, which is the essential cofactor for IDO activity. Hence the combination of these drugs has the greatest inhibitory effect.

Thus there are significant implications for the use of ASA and acetaminophen as novel agents in manipulating IDO activity. These agents could therefore be useful in the treatment of IDO-related serotonin depletion, in which high activity of IDO is reported, and therefore represents an important link in immunological network and the pathogenesis of depression in specific conditions, such as pregnancy or neoplasia (Widner *et al*., 2002).

2.4. EFFECT OF ACETYLSALICYLIC ACID AND ACETAMINOPHEN ON 3-HAO ACTIVITY *IN VIVO*.

2.4.1. INTRODUCTION

Tryptophan is also utilized in the formation of QA. The product of the enzymatic reaction involving 3-hydroxyanthranilic acid was identified ultimately as α-amino-β-carboxymuconic ϵ-semialdehyde, which is unstable and spontaneously rearranges to QA as shown in figure 2.1 (Mason, 1957).

The role of endogenous QA in the brain remains obscure and controversial, but there is increasing evidence that glia can synthesize and release relatively high concentrations of QA after activation by insults to the brain or inflammatory stimuli such as bacterial infection. The increase of QA could produce or enhance the amount of neuronal damage produced by primary brain insult. The reduction of QA synthesis could, therefore, limit the amount of brain damage. A novel approach to preventing the synthesis of QA is to inhibit 3-HAO (Schwarcz, 2004).

2.4.2. MATERIALS AND METHOD

2.4.2.1. Chemicals and Reagents

All chemicals were of the highest purity available. 3-hydroxyanthranilic acid was purchased from Sigma Chemical Co., St. Louis, USA. All other bench reagents were purchased from Merck, Darmstadt, Germany, and were of the highest chemical purity.

2.4.2.2. Animals

Animals were housed as described in section 2.2.2.2.

2.4.2.3. Drug Treatment

Animals were treated as described in table 2.1.

2.4.2.4. Tissue Preparation

The liver was initially homogenized with 60ml, 50mM Sodium phosphate buffer (pH 7.0) with a Junke and Kunkel Ultra Turrex Warring blender for a period of 1minute at 1000 rev/minute. During the homogenization, care was taken not to allow excessive foaming so as to prevent protein denaturation. The resulting suspension was further homogenized with a hand held glass homogenizer until a complete homogenous solution was obtained. Finally the weight was adjusted with 50mM Sodium phosphate buffer (pH 7.0) to give a 20% homogenate. Process was performed at 4^{o}C.

2.4.2.5. Determination of 3-HAO Activity

The activity was assayed spectrophotometrically in liver homogenate as described by Mehler (1956). The homogenate was centrifuged at 12 000 x g for 10 minutes at 4^{o}C. The supernatant was removed and placed in test tubes. The experiment was now carried out in triplicate. An aliquot of 1.5 ml of the cell homogenate supernatant was added to the reaction mixture. The reaction mixture consisted of 1.5 ml of phosphate buffer 0.1M (pH 7.5) containing 10 μM ascorbate, 6mM $FeSO_4$, and 10μM 3-hydroxyanthranillic acid. The buffer extracts were then incubated for 30 minutes at 37^{o}C. After the incubation the reaction was immediately terminated by the addition of 2 ml, 3M HCl. The mixture was then centrifuged at 12000 g for 10 minutes at 4^{o}C. An aliquot of 3ml of supernatant was removed and used to measure enzymatic activity. Enzymatic activity was calculated from

the difference between product content before (blanks) and after incubation. The absorbance was read at 360 nm with a Shimadzu UV 160A UV-visible recording spectrophotometer and an extinction coefficient of $47{,}500 M^{-1}cm^{-1}$ for the reaction product (α-amino-β-carboxymuconic ϵ-semialdehyde) was used to calculate the concentration of α-amino-β-carboxymuconic ϵ-semialdehyde.

2.4.2.6. Protein Determination

Protein determination was performed as described in 2.2.2.7.

2.4.2.7. Statistical Analysis

The results for the liver 3-hydroxyanthranilate 2,4-dioxygenase assay was analyzed using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range test. The level of significance was accepted at $p < 0.05$.

2.4.3. RESULTS

As seen in Figure 2.5, all of the treatments used in adult male rats significantly inhibited 3-HAO as compared to the control. Acetaminophen administration in rats results in a more significant inhibition ($p<0.001$) of 3-HAO than ASA ($p<0.01$) when compared to the control group. The combination of ASA and acetaminophen had a significant ($p<0.05$) inhibitory effect as compared to either agent alone.

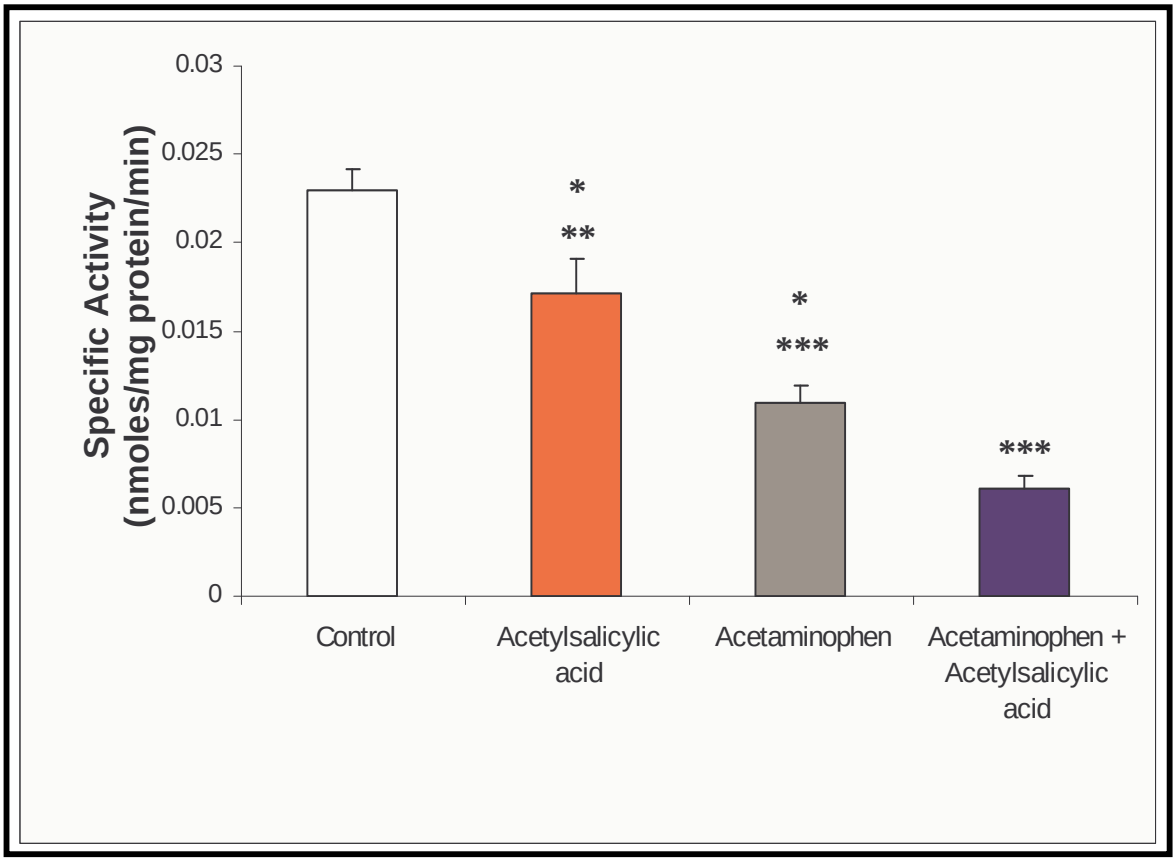

Figure 2.5. The effect of non-narcotic analgesic administration on rat liver 3-HAO activity. The specific enzyme activity is expressed as nmoles of α-amino-β-carboxymuconic ϵ-semialdehyde/mg protein/min. Each bar represents the mean ± SEM; n=5. * (p<0.05) vs. Acetaminophen + Acetylsalicylic acid and *** (p<0.001) vs. control. Student-Newman-Keuls Multiple Range Test.

2.4.4. DISCUSSION

Quinolinic acid is a neurotoxic metabolite of 3-HAO and is an endogenous agonist of NMDA glutamate receptor subtype. Quinolinic acid is also able to reproduce the pathological features of HD. Quinolinic acid also plays an important role in neurodegenerative inflammatory and infectious diseases (Heyes and Saito, 1992). Ischemic brain injury results in increased QA production in the hippocampal region resulting in major neuronal death and neurodegeneration, with increased activities of IDO and 3-HAO (Heyes and Morrison, 1997).

In an attempt to develop therapies for the treatment of neurodegeneration, the kynurenine pathway has been manipulated in several ways. The original approach involved the use of analogues of kynurenic acid as antagonists at glutamate receptors. A second approach uses pro-drugs of kynurenic acid or its analogues, which can be hydrolyzed within the CNS, whereas a third, and the most recent approach, is inhibition of the activity of the enzymes responsible for synthesizing the NMDA receptor agonist, QA. This diverts kynurenine metabolism away from the production of the potent excitotoxin, QA (Stone, 2000).

The administration of these non-narcotic analgesics are effective at inhibiting 3-HAO activity and the combination of these agents induce an inhibitory effect. As mentioned earlier, a novel approach to reducing QA synthesis is to inhibit 3-HAO activity. The inhibition of this enzyme was aptly achieved with the use of ASA and acetaminophen either individually or in combination thus promotes further research in the possible therapeutic use of these agents in diseases in which QA is implicated such as AD and HD.

2.5. CONCLUSION

The potent inhibitory effects of these non-narcotic analgesics, on IDO and 3-HAO, noted in the results (sections 2.3.3. and 2.4.3.) is significant in the treatment of inflammatory and infective disorders of the CNS and could help in the prevention of QA induced neurodegeneration which would further aid in explaining the anti-inflammatory effects of these agents. Thus indicating the possible use of these agents in HD and AD states.

The actions of these agents in regulating the kynurenine pathway suggest that use of ASA and acetaminophen could prove beneficial in the attenuation of various disease states. The action of these drugs in reducing the activity of TDO and IDO could result in an increase in brain serotonin levels, which could aid in the treatment of depression, which was further investigated in chapter 3.

CHAPTER 3

BRAIN NEUROTRANSMITTER LEVELS

3.1. INTRODUCTION

An inverse relationship exists between liver TDO and brain 5-HT levels (Daya *et al*., 1989). The inhibition of TDO by ASA and acetaminophen has lead to an investigation into the effects of these agents on brain 5-HT levels. Serotonin is known to play a role in norephinephrine (NE) release in the brain (Xi-Ming Li *et al*., 2002). It was also decided to determine the effects of this combination on brain NE levels. It has recently been shown that acetaminophen administration in rats induces a rise in brain 5-HT and NE levels (Courade *et al*., 2000). Since dopamine (DA) is a precursor of norepinephrine it stands to reason that acetaminophen has the potential to induce a rise in DA levels in the brain. Dopamine plays an important role in the biochemical basis of Parkinsons disease as well as schizophrenia. Figure 3.1, illustrates the synthesis of the neurotransmitters DA, NE, and EN from tyrosine in the brain.

Therefore in order to understand the effects of ASA and acetaminophen on normal brain function, it is especially important to study the *in vivo* changes in the regional extracellular neurotransmitter release and metabolism. HPLC and electrochemical detection were used for the examination of small changes if any in the brain tissue. Rather than investigate individual neurotransmitters as single extracellular biochemical events, a deeper understanding of the effect on the brain would result from the study of several neuronal systems simultaneously. Consequently the important interplay and interdependency between distinct monoaminergic systems could be better defined. Accordingly, the effect of these drugs on normal brain activity could be more accurately assessed as the understanding of normal brain function is further defined.

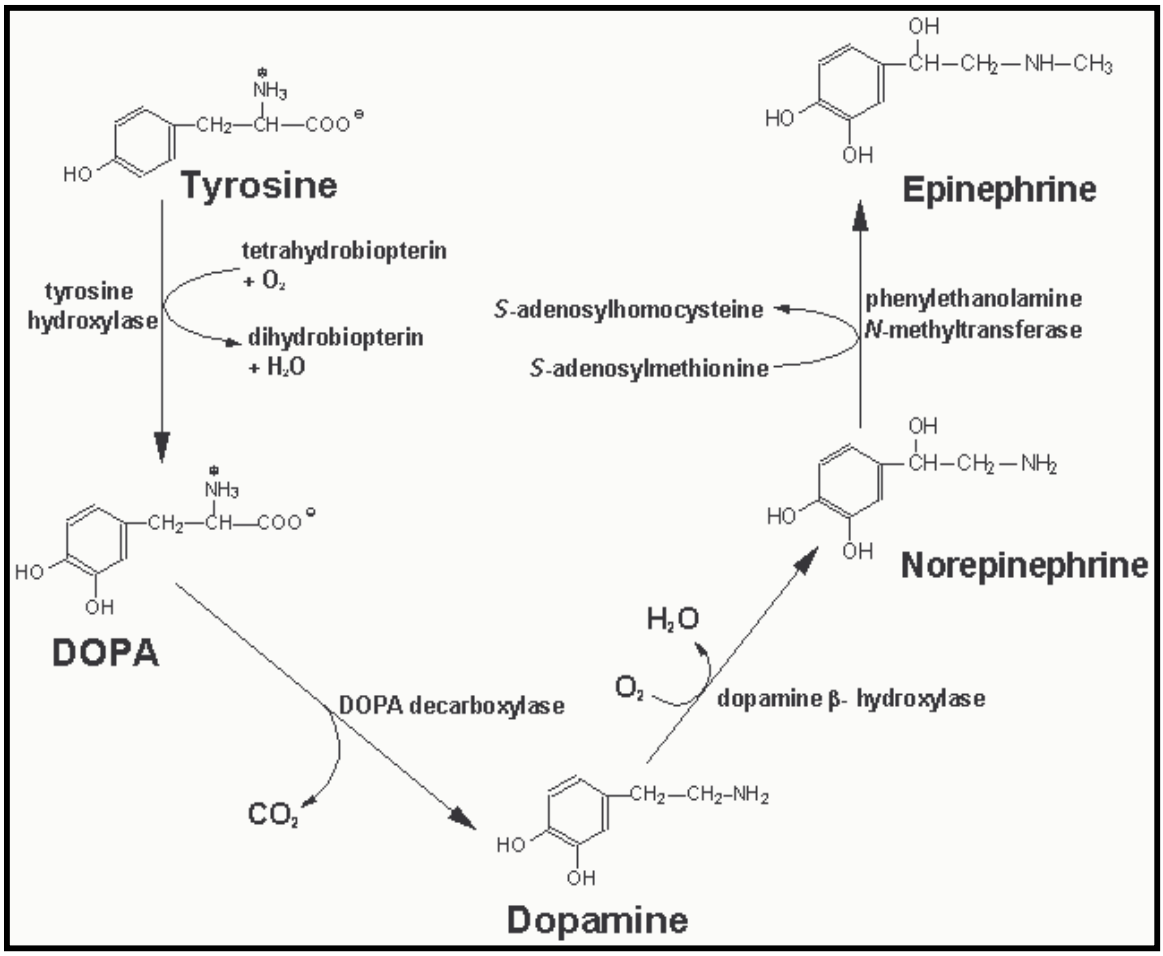

Figure 3.1. Illustration of the synthesis of the neurotransmitters DA, NE, and EN from tyrosine in the brain (Mohanakumar, 2004).

3.2. MATERIALS AND METHODS

3.2.1 Chemicals and Reagents

Serotonin, 5-hydroxyindole acetic acid (5-HIAA), norepinephrine (NE), dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC) standards were purchased from Sigma Chemical Co., St. Louis, USA. Perchlorate, Ethylenediaminetetraacetic acid (EDTA), heptane sulphonic acid, acetonitrile, triethylamine and phosphoric acid was purchased from Merck, Darmstadt, Germany. All other reagents used in this experiment were of the highest chemical purity.

3.2.2. Animals

Adult male Sprague-Dawley rats were used in this study. The experimental protocol met the National Guidelines on the "Proper Care and Use of Animals in Laboratory Research" (Indian National Science Academy, New Delhi, 2000) and was approved by the Animal Ethics Committee of the Institute as well as that of Rhodes University.

3.2.3. Drug Treatment

Animals were treated as described in chapter 2, section 2.2.2.3 and killed by cervical dislocation. The brain was removed, dissected and stored as described in appendix II.

3.2.4 Homogenate Preparation

The whole brains were dissected and rinsed in chilled normal saline blotted dry on ash-free filter paper. The forebrain was dissected from the occipital pole extending 4-5mm caudally. The forebrain was deproteinized for the analysis of the neurotransmitters and metabolites, following sonication (50Hz for 60s) in ice-cold 0.1M $HClO_4$ containing EDTA (0.01%) (1mg tissue in 10µl). The samples were kept in ice for 15 min and centrifuged at 10 000 x g for 10 min and the supernatant (10µl) was injected into the HPLC system for the analyses of the neurotransmitters, and their metabolites (Muralikrishnan & Mohanakumar, 1998).

3.2.5. Instrumentation

The forebrain samples were analyzed for 5-HT, 5-HIAA, NE, DA and DOPAC on a modular, isocratic high performance liquid chromatographic (HPLC) system (Merck/Hitachi; Germany). The chromatographic system consisted of a LaChrome pump L-7110, a LaChrome L 3500A amperometric detector and a Merck-Hitachi integrator D-

7500. Samples were introduced into the system using a Rheodyne 7725i injector, fitted with a 20µL loop.

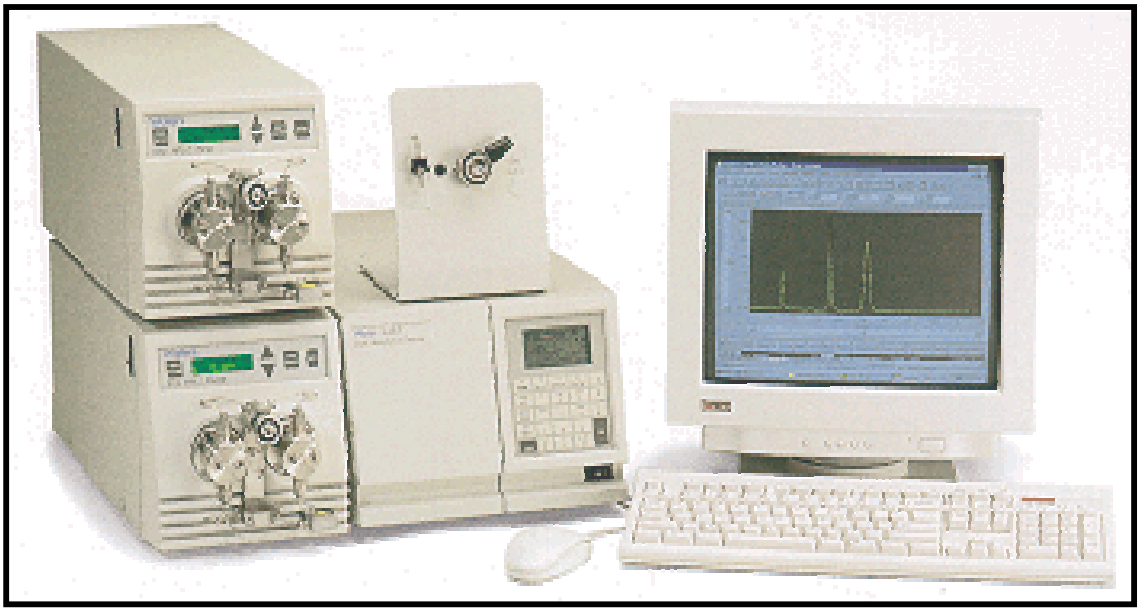

Figure 3.2. Photograph of a modular, isocratic HPLC system (Merck/Hitachi; Germany), the LaChrome pump L-7110, a LaChrome L 3500A amperometric detector and a Merck-Hitachi integrator D-7500 that was used to measure brain neurotransmitter levels (Mohanakumar, 2004).

3.2.6 Chromatographic Conditions

Separation was achieved using a C_{18}, ion pairing, analytical column (4.6 mm x 250 mm; Ultrasphere IP; Beckman, USA), with a particle size of 5µm and a pore size of 80Å. The mobile phase composition was 8.65mM heptane sulphonic acid, 0.27mM EDTA, 13% acetonitrile, 0.4-0.45% triethylamine and 0.22% phosphoric acid (v/v) and was degassed using a 0.45µm membrane filter prior to use. The mobile phase flow rate was 0.7ml/min and the electrodetection was performed at 0.74V.

3.2.7. Protein Determination

Protein determination was carried out according to the protocol described in appendix III.

3.2.8. Statistical Analysis

All results were analyzed using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test. A $p<0.05$ was considered as significant.

3.3. RESULTS

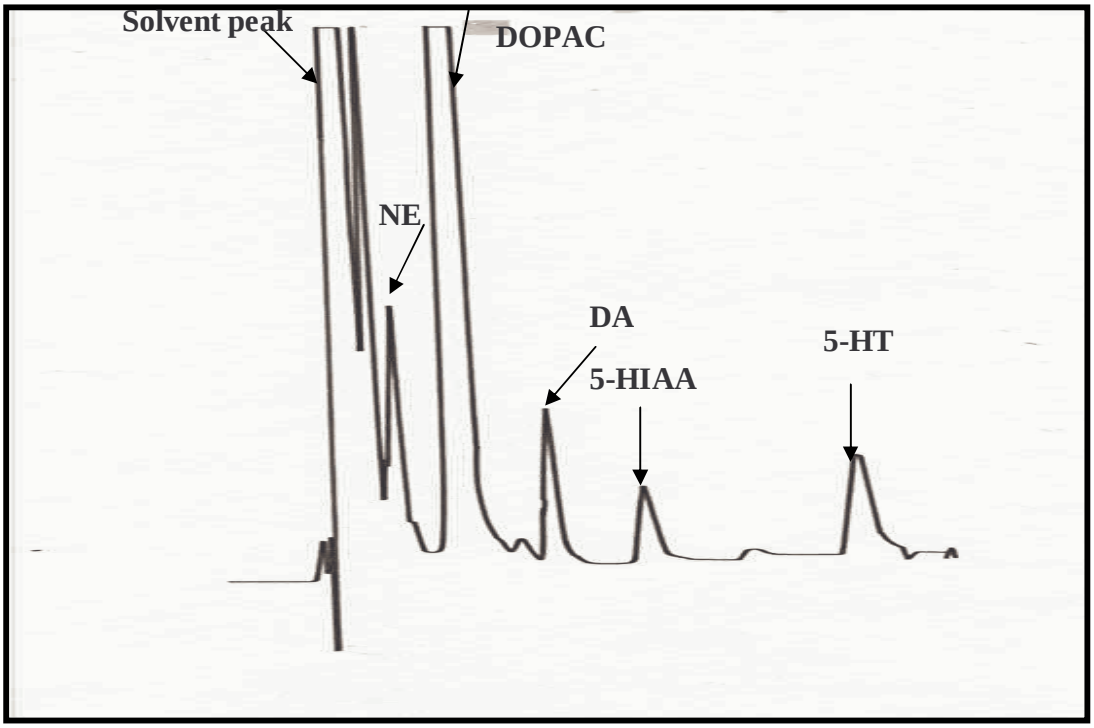

Figure 3.3. Illustration of a typical chromatogram trace of the neurotransmitters NE, DA, 5-HT and their metabolites obtained from rat forebrain homogenate.

The chromatogram obtained showing the separation of the forebrain neurotransmitter analysis is shown in Figure 3.3. The chromatograms that were obtained following homogenate injection showed consistent, sharp, and symmetrical peaks for each

neurotransmitter analyzed. Each neurotransmitter peak was well-resolved from the solvent front (Figure 3.3).

Table 3.1. Effect of non-narcotic analgesics on rat forebrain 5-HT, 5-HIAA, and NE levels.

Concentration (pmol/mg tissue) n=6				
Treatments	**NE**	**5-HIAA**	**5-HT**	**5-HT turnover (5-HIAA:5-HT)**
Control	5.53 ± 0.32	1.96 ± 0.36	2.40 ± 0.1	0.79 ± 0.02
Acetaminophen	6.44 ± 0.10**	2.40 ± 0.08**	3.50 ± 011**	0.69 ± 0.01***
ASA	5.52 ± 0.20	3.69 ± 0.21****	2.70 ± 0.12	1.31 ± 0.05****
Acetaminophen + ASA	5.49 ± 0.11	2.97 ± 0.08***	3.26 ± 0.06**	0.91 ± 0.02***

Each value represents the Mean ± SEM; n = 6. *(p<0.05), **(p<0.01), ***(p<0.001), ****(p<0.0001). All results were analyzed using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

As is evident in table 3.1, acetaminophen causes a significant rise in rat forebrain NE levels, with a significance level of p<0.01. However, ASA and the combination of ASA and acetaminophen did not show any significant effect on rat forebrain NE levels.

Administration of acetaminophen proved to induce a significant rise in 5-HIAA levels (table 3.1). The administration of ASA and the combination of ASA and acetaminophen also resulted in a significant rise in 5-HIAA levels (p<0.001 and p<0.0001 respectively) as is evident in table 3.1.

Acetaminophen induced a significant rise in forebrain 5-HT levels (p<0.01) with a concomitant rise in 5-HIAA levels as shown in table 3.1. Acetylsalicylic acid on the other hand did not change forebrain 5-HT levels but did result in a significant rise in 5-HIAA levels. The combination of ASA with acetaminophen did not alter the

acetaminophen induced increase in forebrain 5-HT levels or 5-HIAA levels. The turnover of 5-HT, as revealed by the metabolite to the neurotransmitter ratio was significantly inhibited by acetaminophen, but increased by ASA. Combined treatment of ASA and acetaminophen in rats caused a significant increase in turnover of 5-HT (p<0.0001).

From table 3.2 it is evident that neither acetaminophen nor ASA affected the forebrain levels of DA. A combination of acetaminophen and ASA caused a significant inhibition in the DOPAC levels (p<0.05).

Table 3.2. The effect of non-narcotic analgesics on rat forebrain DA and DOPAC levels.

	Catecholamines (pmol/mg tissue)	
TREATMENTS	**DOPAMINE**	**DOPAC**
Control	8.51 ± 0.21	1.00 ± 0.01
Acetaminophen	8.84 ± 0.46	1.35 ± 0.07
ASA	8.15 ± 0.30	1.47 ± 0.13
Acetaminophen + ASA	7.66 ± 0.17	0.72 ± 0.06*

Each value represents the Mean ± SEM; n = 6; (*p<0.05). All results were analyzed using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

3.4. DISCUSSION

Salient features of the present study are: (i) the significant and hitherto unknown influence of acetaminophen on brain NE levels following its systemic administration; (ii) overwhelming effects of ASA on 5-HT turnover over the acetaminophen effect, and (iii) reversal of acetaminophen-induced rise in the levels of NE by ASA. The study also confirms the earlier findings in chapter two that acetaminophen has a profound inhibitory effect on liver TDO activity, which culminates in a rise in central 5-HT levels (Anoopkumar-Dukie and Daya, 2000).

Changes in central concentrations of neurotransmitters by non-narcotic analgesics have not been properly delineated. Thus far, there are only two reports, which show that acetaminophen administration in rats increases brain 5-HT levels (Courade *et al*., 2000; Anoopkumar-Dukie and Daya, 2000). Such changes could account for non-narcotic analgesic abuse since these agents have the potential in high doses to increase the levels of mood enhancing neurotransmitters. The increase in brain 5-HT and 5-HIAA induced by acetaminophen is consistent with the inhibition of apoenzyme and total enzyme activity induced by this agent, since an inverse relationship is known to exist (Daya *et al*., 1989) between liver TDO and brain 5-HT levels.

The rise in NE induced by acetaminophen further demonstrates that this agent can bring about the elevation of mood enhancing neurotransmitters. One possible reason for the rise in NE levels is that 5-HT is known to play a role in NE release (Xi-Ming Li *et al*., 2002). In contrast, ASA did not alter brain 5-HT or NE levels but did cause a significant rise in 5-HIAA levels, despite this finding, as shown in chapter two, figure 2.1, that ASA inhibits all forms of TDO measured in this study. A possible reason for this could be that ASA enhances 5-HT metabolism thus masking a rise in 5-HT.

In comparison to ASA alone, the combination of ASA with acetaminophen did not cause a further suppression of total enzyme and holoenzyme activity but did cause a significant suppression of apoenzyme activity. However, this did not reflect a further rise in brain 5-HT and 5-HIAA in comparison to acetaminophen alone but did result in a blunting of the acetaminophen-induced rise in NE. A combination of ASA with acetaminophen did not alter the acetaminophen induced rise in 5-HT and 5-HIAA but did however curtail the acetaminophen induced rise in NE levels. A possible explanation for this phenomenon could be that if ASA does indeed enhance 5-HT metabolism, thus less 5-HT will be available to induce NE release.

The ASA-induced increase in the turnover of 5-HT is interesting and important in view of the indoleamine's role in analgesia (Mohanakumar *et al*., 1995) and depression (Walsh and Daya, 1998; Charney, 1998; Elkind, 1991). Increases in the turnover of 5-HT as

observed in the present study indicate enhanced oxidative metabolism of the mood influencing neurotransmitter, 5-HT, which is also known to mediate pain. On the other hand, acetaminophen causes a decrease in its turnover, indicating shutting down of the oxidative deamination, resulting in increased 5-HT levels, and thus compounding the influence of the drug on TDO. Interestingly ASA when administered along with acetaminophen could override the influence of the latter. This may have a direct relationship to acetaminophen abuse, and proved correct; ASA could be the drug of choice.

The individual administration of ASA and acetaminophen did not have any effect on DA or DOPAC levels. However the use of these agents in combination significantly reduced the production of DOPAC, which indicates that these agents do affect the dopaminergic system as well.

3.5. CONCLUSION

Both non-narcotic analgesics tested herein have analgesic, antipyretic and to some extent anti-inflammatory properties. Abuse of non-narcotic analgesics has been an area, which is generally underestimated. These results demonstrate the potential of these agents to alter neurotransmitter levels in the brain. This in turn can influence mood and behaviour leading to the abuse of these commonly used agents.

CHAPTER 4

PINEAL INDOLE METABOLISM.

4.1. INTRODUCTION

The pineal gland is recognized as a fully functional organ of the brain that is responsible for the synthesis of indoleamines. The pineal gland is an integral and important component of the neuroendocrine system (Reiter, 1989). The pineal metabolites are synthesized from the precursor amino acid TRP. Melatonin (aMT) is the characteristic neurohormone that is synthesized from its precursor 5-HT. The bulk of 5-HT synthesized in the pineal is metabolized by the enzyme MAO from TRP. The enzyme N-acetyltransferase (NAT) acetylates a minor portion of 5-HT, followed by methylation by hydroxyindole-O-methyltransferase (HIOMT) to synthesize aMT. Methoxyindoles are responsible for the mediation of pineal function and these are exhibited in ascending order of importance: 5-methoxyindoleacetic acid (5-MIAA), 5-methoxytryptophol (5-MTOH) and aMT.

The quantification of pineal indole metabolism requires a sensitive technique that is able to mimic normal physiological processes and conditions as closely as possible. The organ culture technique is an invaluable tool, which enables the researcher to finely control experimental conditions and avoid the complications of *in vivo* interactions. The pineal organ culture technique is well used to mimic normal physiological processes that occur in the pineal organ and is utilized by a number of researchers (Daya *et al*., 1989). The pineal gland is able to utilize exogenous radioactive (^{14}C) serotonin to produce the various indoles including aMT and 5-HT (Daya *et al*., 1989). As much as 95% of the various indoles synthesized are secreted into the culture medium. These can then be isolated and quantified. The pineal gland in the rat is small and easily accessible for intact organ culture.

Numerous researchers use organ and tissue culture techniques since it is convenient and not time consuming (Klein and Notides, 1969; Daya *et al*., 1989). The isolation of indoles can be achieved by organic extractions and quantification using paper thin layer adsorbents and finally different solvents. The bi-dimensional thin layer chromatography system was first employed by Klein *et al*., (1969) for separation of indoles. The pineal indoles are separated by two solvent systems; the first utilizes chloroform, methanol and glacial acetic acid [93: 7: 1] and the second solvent system uses ethyl acetate only. The primary solvent system separates aMT and N-acetylserotonin (NAS) and the 5-hydroxyindoles from the 5-methoxyindoles. The glacial acetic acid effectively separates 5-MIAA and 5-MTOH from aMT and the separation of 5-hydroxytryptophol (HTOH) and 5-HIAA from NAS.

The aim of this experiment is to assess the direct effects of ASA and acetaminophen on pineal indole metabolism. It is important to determine why acetylsalicylic acid, which inhibits TDO significantly, does not result in an increase in 5-HT levels. A possible reason for this is that ASA increases 5-HT metabolism thus masking any increase in 5-HT that may occur. The effect of acetaminophen on pineal indole metabolism was investigated to determine its effect on 5-HT metabolism in the pineal organ. The pineal gland contains high levels of 5-HT compared to other brain regions (Daya *et al*., 1989). It is possible that the acetaminophen induced increase in brain 5-HT levels could lead to changes in pineal 5-HT and aMT levels.

4.2. MATERIALS AND METHODS

4.2.1. Chemicals and Reagents

5-Hydroxy (side-chain-2-^{14}C) tryptamine creatinine sulphate was purchased from Amersham international, United Kingdom. The concentration of the radioactivity was 50microcurie (μCi) and the specific activity was 55mCi/mmol. BGJb culture medium (Fitton Jackson modification) was purchased from Gibco, Europe and aseptically fortified with antibiotics such as streptomycin and penicillin. The composition of the media is

schematically represented in table 4.1. The thin layer chromatography (TLC) plates, Kieselgel 60 F_{254}, (20 x 20cm) aluminium sheets coated with silica gel were obtained from Merck, Germany. The liquid scintillation cocktail, Packard® Scinillator 299™, was purchased from Packard Instrument Company, Inc., Netherlands.

The indole standards: melatonin (aMT), serotonin (5-HT), N-acetylserotonin (NAS), 5-methoxyindoleacetic acid (5-MIAA), 5-hydroxyindoleacetic acid (5-HIAA), 5-methoxytryptophol (5-MTOH), 5-hydroyxtryptophol (HTOH), were obtained from Sigma Chemical Co., St. Louis, USA. Ethanol, glacial acetic acid, chloroform, methanol and ethyl acetate were obtained from Saarchem Limited, Krugersdorp, South Africa.

4.2.2. Animals

Male Wistar rats of the albino strain were assigned into groups of 4 (n=5). The animals were maintained as described in Appendix I. Animals were killed by cervical dislocation and the brain was removed as described in Appendix II.

4.2.3. Sample Preparation

The indole standard solution was prepared using 1 mg of each of the pineal metabolites: aMT, 5-HT, NAS, 5-MIAA, 5-HIAA, 5-MTOH and 5-HTOH. The pineal indoles were dissolved in 2.5ml absolute ethanol. The solution as then vortexed after the addition of 2.5ml 1% ascorbic acid (an antioxidant) in 0.1N HCl. The resultant solution was stored in darkness at –20^{o}C until needed.

Table 4.1. Compostion of the BGJb culture medium (Fitton Jackson modification).

CONTENTS	CONCENTRATION (mg/ml)
AMINO ACIDS	
L-Alanine	250.00
L-Arginine	175.00
L-Aspartic Acid	150.00
L-Cysteine HCl	90.00
L-Glutamine	200.00
Glycine	800.00
L-Histidine	150.00
L-Isoleucine	30.00
L-Leucine	50.00
L-Lysine HCl	240.00
L-Methionine	50.00
L-Phenylalanine	50.00
L-Proline	400.00
L-Serine	200.00
L-Threonine	75.00
L-Tryptophan	40.00
L-Tyrosine	40.00
DL-Valine	65.00
INORGANIC SALTS	
Dihydrogen sodium orthophosphate	90.00
Magnesium sulphate ($7H_2O$)	200.00
Potassium Chloride	400.00
Potassium dihydrogen phosphate	160.00

CONTENTS	CONCENTRATION
Sodium bicarbonate	3500.00
Sodium chloride	5300.00
OTHER COMPONENTS	
Calcium Lactate	555.00
Glucose	10 000.00
Phenol Red	20.00
Sodium acetate	50.00
VITAMINS	
A-Tocopherol phosphate	1.00
Ascorbic acid	50.00
Biotin	0.20
Calcium Pantothenate	0.20
Choline chloride	50.00
Folic acid	0.20
Inositol	0.20
Nicotinamide	20.00
P-Aminobenzoic acid	2.00
Pyridoxal phosphate	0.20
Riboflavin	0.20
Thiamine HCl	4.00
Vitamin B_{12}	0.40

4.2.4. Assay Procedure

4.2.4.1. Pineal Organ Culture

The pineal gland was removed and individually placed into sterile (borosclicate 10x 75mm) Kimble glass tubes. Each test tube contained 52μl of BGJb by culture medium. In those experiments that involved the addition of test compounds, a volume of 10μl was added to the incubation medium to give the required final concentration in a total volume of 70μl. Finally, 8μl (^{14}C) serotonin (specific activity 55mCi/mmolar) was added. In the control test tubes, the test compounds were replaced with the vehicle test compound (10μl). All the test tubes were then saturated with carbogen (95% oxygen: 5 % carbon dioxide) and immediately sealed. The vials were incubated for a period of 24 hours at 37^{o}C in the dark. The incubation was terminated after 24 hours by he removal of the pineal glands from the culture medium. The culture medium was then analyzed by TLC.

4.2.4.2. Separation of Indoles by Thin Layer Chromatography

Aliquots of 10μl of the culture medium was applied to a 10 x 10cm chromatography plate, to form a spot no larger than 4–5mm. The spotting took place under a gentle stream of nitrogen to aid in the drying of the spotted-media. Drying with nitrogen prevented the atmospheric oxidation of the indoles. Thereafter, 10μl of the standard solution containing all the indoles, was spotted on top of the already spotted culture medium, the standard was dried under nitrogen.

The TLC plate was placed in a TLC tank which contained the following solvent system: chloroform: methanol: glacial acetic acid (93:7:1). The plate was allowed to develop until the solvent front had reached 9 cm from the starting point. The plate was removed from the tank and dried under a stream of nitrogen. Once the required distance was reached, the plate was dried under a stream of nitrogen and placed in a second solvent system. Once the required distance was reached, the plate was dried under nitrogen and placed in

the second solvent system (ethyl acetate) at right angles to the first direction. The movement of the second solvent front was approximately 6cm.

Once the plate was dried under nitrogen once again, the plates were placed under a UV-Visible light to detect the spots of indoles. The spots were cut out and placed in scintillation vials containing 3ml of scintillation liquid. The vials were tightly sealed and shaken for a period of 30 minutes. The radioactivity was quantified by a Beckman LS 2800 scintillation counter.

4.2.4.3. Statistical Analysis

The results were analyzed using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range test. The level of significance was accepted at $p < 0.05$.

4.3. RESULTS

Figure 4.1 shows a typical bi-dimensional thin layer chromatogram of the pineal indole metabolites. Clear separation of the six spots was achieved and the positions of the metabolites were identified by following the schematic representations of the chromatogram in figure 4.1.

The results were expressed as disintegrations per minute (DPM)/10μl spotted for each of the indoles spotted. The data was statistically analyzed and the difference between control and drug groups was determined using the Student's t-test. A p value of <0.05 was accepted to be statistically significant. The radioactivity corresponding to each of the metabolites isolated from the culture medium is represented in Figures 4.2, 4.3, and 4.4.

As evident in figure 4.2 and figure 4.3, ASA at a concentration of 10μM was able to increase 5-HIAA synthesis significantly (p<0.05) while acetaminophen significantly reduced 5-HIAA synthesis (p<0.01). However, the combination had no effect on 5-HIAA synthesis as seen in Figure 4.4.

5-Hydroxytryptophol synthesis was significantly increased by ASA (p<0.001) (Figure 4.2) and the combination of ASA and acetaminophen (p<0.05) (Figure 4.3.) while acetaminophen had no effect on HTOH synthesis (Figure 4.4).

From figure 4.3, it is clear that acetaminophen caused a significant rise in aMT levels (p<0.01). However, ASA and its combination with acetaminophen had no effect on aMT synthesis as evident in figure 4.2 and 4.4, respectively.

Acetylsalicylic acid caused a significant rise in NAS synthesis (p<0.05) as shown in figure 4.2, while acetaminophen and the combination of ASA and acetaminophen caused a significant reduction in NAS synthesis (p<0.05) as evident in figure 4.3 and 4.4 respectively.

From figure 4.2 and figure 4.4, ASA individually and the combination of ASA with acetaminophen caused a significant increase in MIAA synthesis ($p<0.05$ and $p<0.001$ respectively).

It is evident from figure 4.3 that acetaminophen had no significant effect on MTOH synthesis. However acetylsalicylic acid and the combination of ASA and acetaminophen enhanced MTOH production, $p<0.05$.

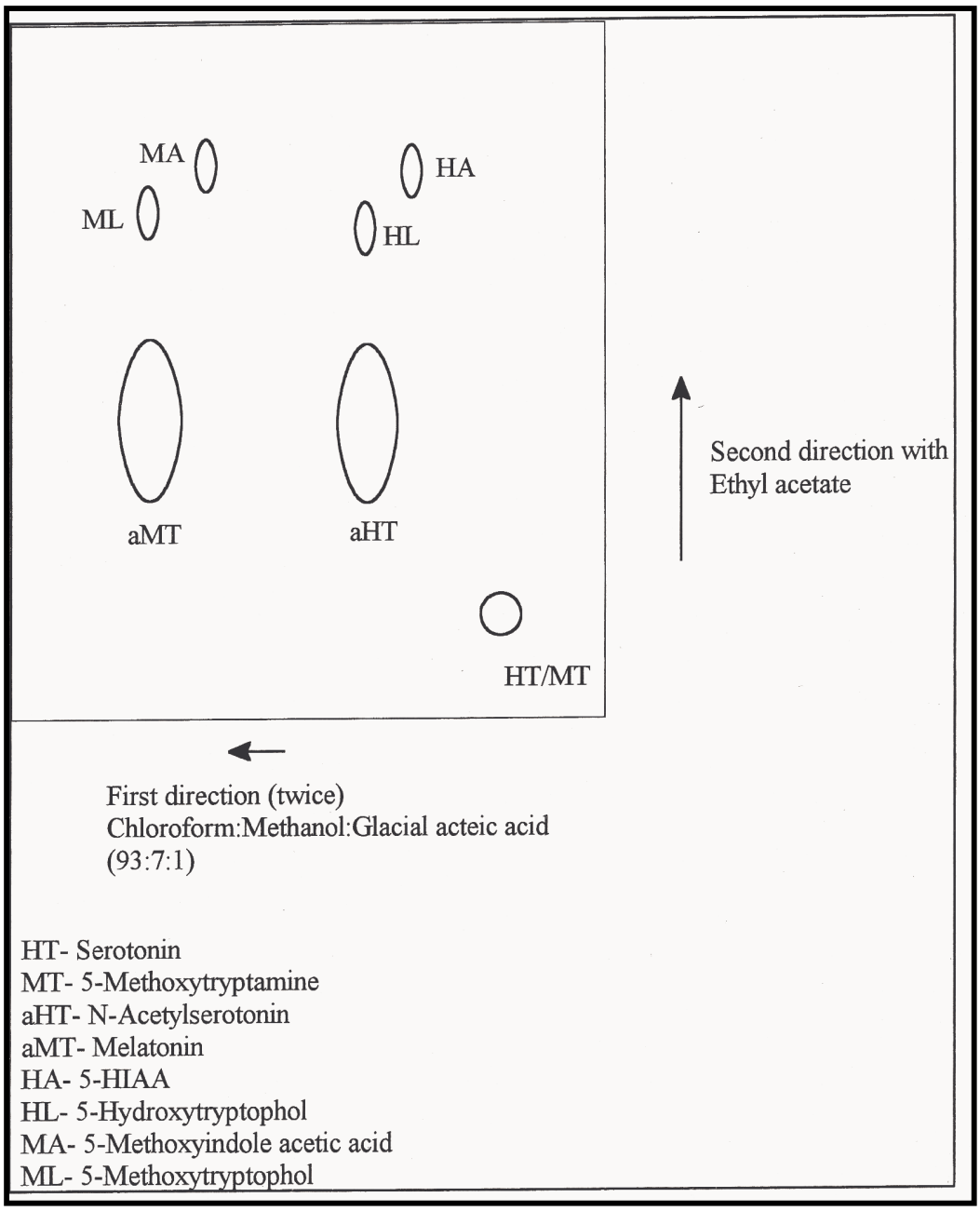

Figure 4.1. A typical bi-dimensional thin layer chromatogram trace illustrating the direction in which the plate was run and the location of the pineal indole metabolites (Klein and Notides, 1969).

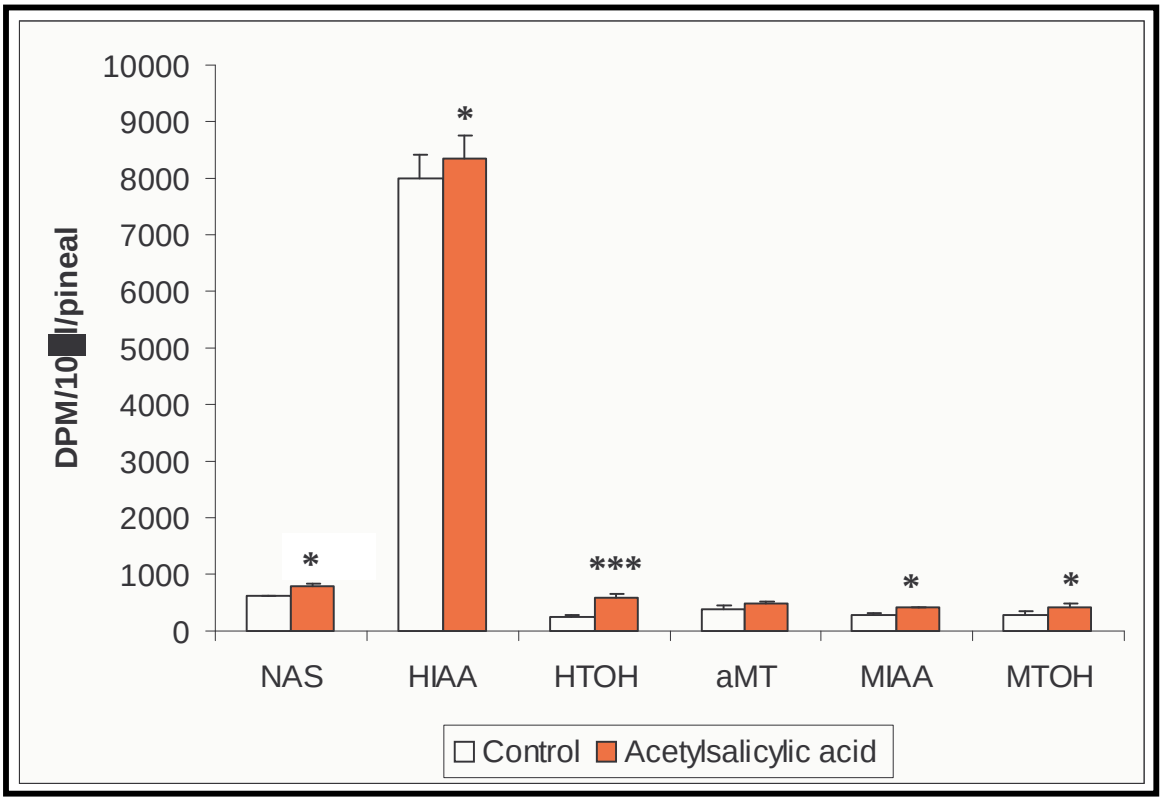

Figure 4.2. The effect of intraperitoneal administration of acetylsalicylic acid in rats on pineal indole metabolism. Each bar represents the ±SEM, n=5. *(p<0.05) and ***(p<0.001) vs. control. Students t test.

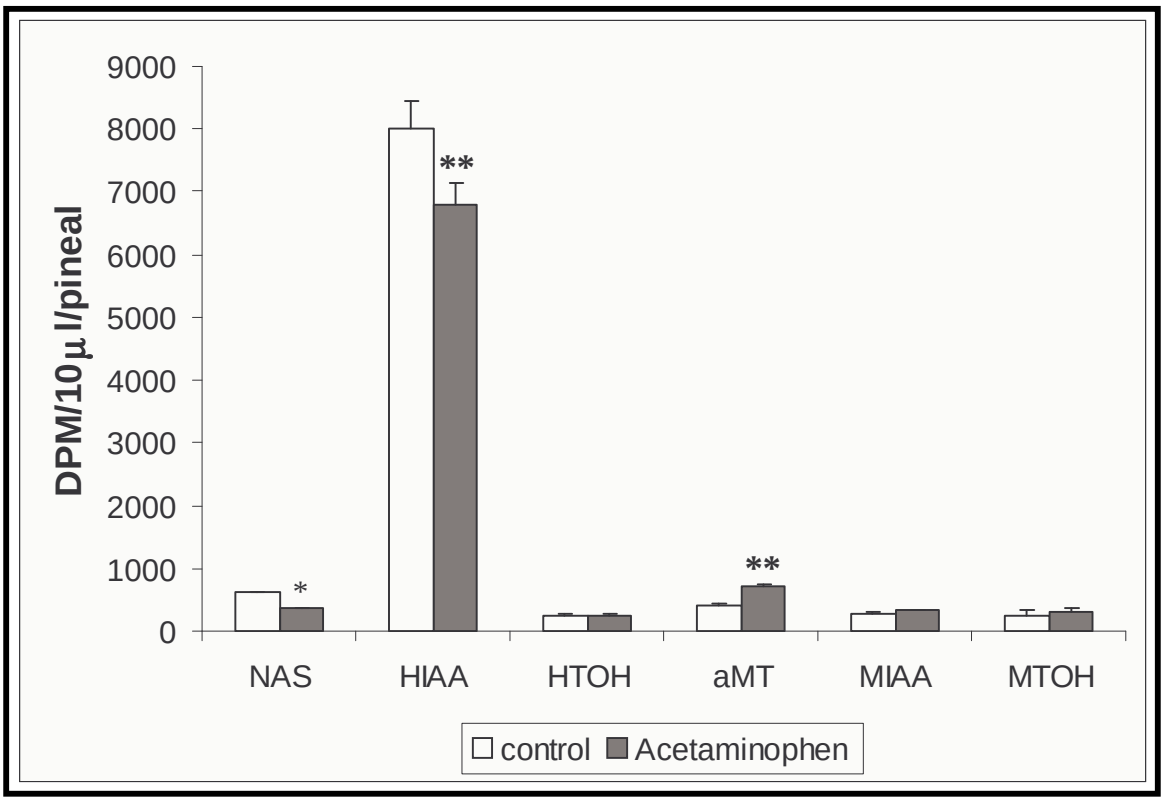

Figure 4.3. The effect of intraperitoneal administration of acetaminophen in rats on pineal indole metabolism. Each bar represents the ±SEM, n=5. *(p<0.05) and **(p<0.01) vs. control. Students t test.

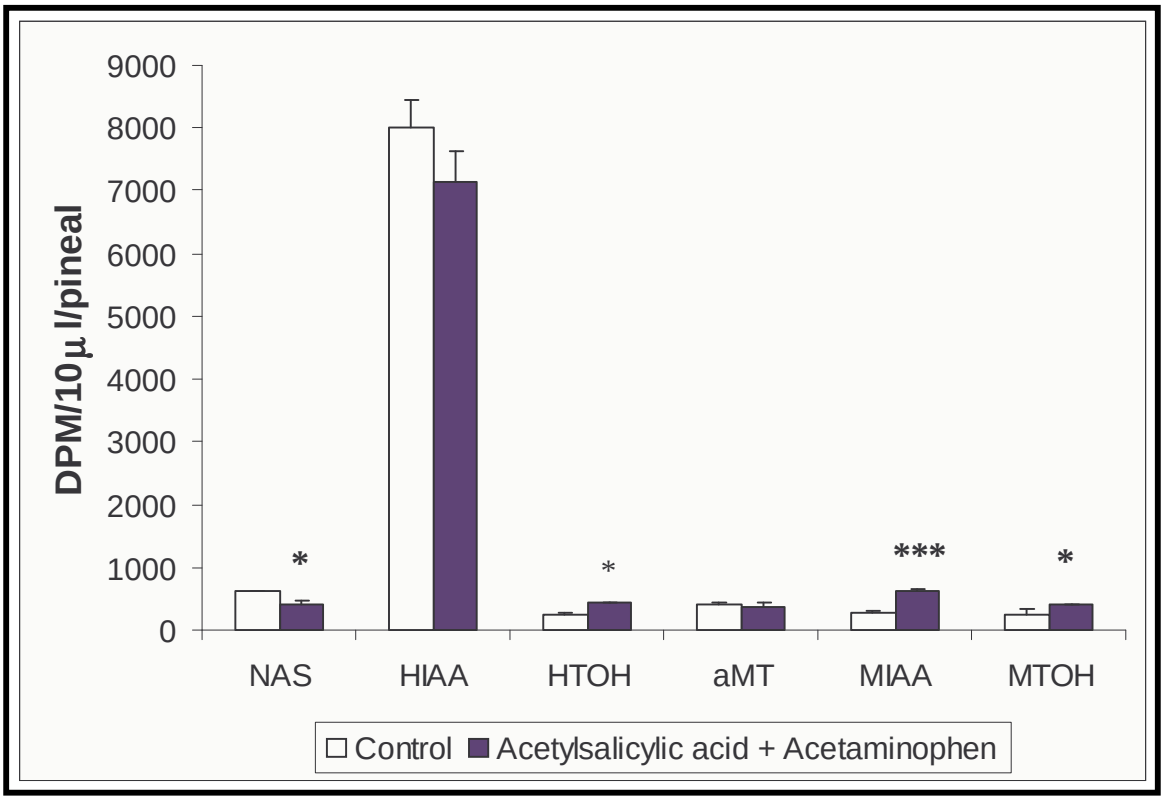

Figure 4.4. The effect of intraperitoneal administeration of the combination of acetaminophen and acetylsalicylic acid in rats on pineal indole metabolism. Each bar represents the ±SEM, n=5. *(p<0.05) and *** (p<0.001) vs. control. Students t test.

4.4. DISCUSSION

Indole metabolism in the pineal gland occurs in the pinealocytes. Pineal indole metabolism commences with the uptake of TRP from the bloodstream and TRP is converted to 5-hydroxytryptophan by the enzyme tryptophan hydroxylase. A decarboxylase enzyme then converts 5-hydroxytryptophan to serotonin. Serotonin is then N-acetylated by NAT to form N-acetylserotonin as described in chapter one, section 1.3.3.6.3.

The results of this experiment shown in Table 4.2, indicates that acetaminophen administration significantly increases melatonin levels ($p<0.01$). The acetaminophen-induced increase in pineal melatonin biosynthesis could be not be due to an increase in N-acetylation of [^{14}C] serotonin by NAT which would result in elevated N-acetylserotonin (NAS) as acetaminophen administration results in a decrease in [^{14}C] NAS in the rat pineal. N-acetylserotonin is a substrate for conversion to [^{14}C] aMT.

Acetaminophen has been shown to be a potent inhibitor of TDO activity and therefore reduces the primary metabolism of TRP thereby increasing serotonin levels. This confirms the results from chapter three where acetaminophen administration significantly increased forebrain serotonin levels. This increases the levels of serotonin available for melatonin production, and subsequently enhances melatonin levels. Studies have demonstrated that selective serotonin reuptake inhibitors increase melatonin synthesis in rats as well as in healthy human volunteers (Wirz-Justice and Arendt, 1980). It is also a possibility that the presynaptic inhibition of serotonin, possibly by acetaminophen, may increase the availability of serotonin within the pinealocyte as substrate for conversion to melatonin although pineal serotonin and pineal melatonin are unlikely to be affected by changes in brain serotonin because the levels of brain serotonin are 100-fold lower than in the pineal gland (Daya *et al*., 1989). Thus, the relationship between brain serotonin levels, and pineal serotonin and melatonin levels need to be examined in greater detail.

The results obtained confirmed earlier suspicions that although ASA blocked the TDO apo- and holoenzyme effectively (chapter two), it did not cause a rise in 5-HT levels (chapter three) in the rat brain. This is due to a rapid increase in 5-HT turnover in the rat pineal. The results obtained indicate that ASA caused a significant rise in 5-HIAA and 5-HTOH levels in the pineal. Therefore ASA is unlikely to play a role as a mood elevator as compared to acetaminophen, which did cause an increase in brain 5-HT levels.

4.5. CONCLUSION

This study provide novel information that these agents affect pineal indole metabolism. The rise in melatonin induced by acetaminophen in rats indicates that this agent could play a significant role in neuroprotection as melatonin has been shown to be a potent antioxidant (Maharaj *et al*., 2002; 2004). The study also indicated that acetylsalicylic acid enhances the catabolism of 5-HT in the pineal organ and therefore plays an important role in the monoaminergic system.

CHAPTER 5

SUPEROXIDE ANIONS

5.1. INTRODUCTION

Reactive oxygen species are continually generated under normal conditions as a consequence of aerobic metabolism. Reactive oxygen species are particularly transient species due to their high chemical reactivity and can react with DNA, proteins, carbohydrates and lipids in a destructive manner (DiFiglia, 1990).

The reduction of oxygen by its acceptance of a single electron produces the superoxide free radical also referred to as superoxide anion ($O_2^{-\bullet}$) (Reiter *et al*., 2002). The major source of $O_2^{-\bullet}$, under physiological conditions is the mitochondrion and these radicals are usually generated as a result of electron "leakage" from the electron transport chain located in the mitochondria (McCord, 1985), and by activation of certain enzymes. Other sources of $O_2^{-\bullet}$ include enzymes such as cytochrome p450 in the endoplasmic reticulum, lipoxygenases, cyclooxygenases, xanthine oxidase and NADPH oxidase (Curtin *et al*., 2002). Figure 5.1 diagrammatically illustrates the different sources of $O_2^{-\bullet}$.

During the production of ATP via the electron transport chain (Dawson and Dawson, 1996), the oxygen molecule can be reduced to $O_2^{-\bullet}$. Mitochondrial dysfunction and free-radical induced oxidative damage have been implicated in the pathogenesis of several neurodegenerative diseases such as HD (Reiter, 1997).

Superoxide toxicity appears to be through an indirect action on living cells as $O_2^{-\bullet}$ is capable of producing the more powerful and damaging hydroxyl radical ($^{\bullet}OH$) in the presence of hydrogen peroxide (Haber-Weiss reaction). Since these reactions are

reversible, there is a constant generation of $O_2^{-\bullet}$ and the promotion of free radical reactions is allowed for (Curtin *et al*., 2002).

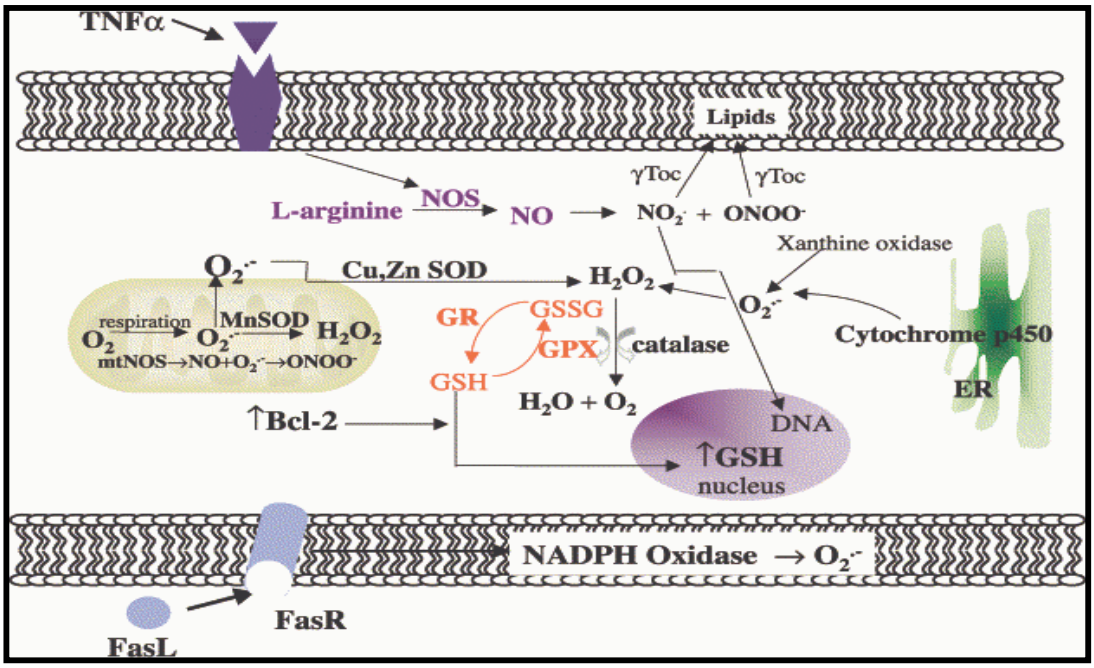

Figure 5.1. Diagrammatic representation of the intracellular sources of ROS and principle defense mechanisms. Major sources of ROS production include the mitochondria, endoplasmic reticulum, plasma membrane and cytosol. The mitochondria generate **$O_2^{-\bullet}$** (superoxide anion) during respiration, which is converted to **H_2O_2** (hydrogen peroxide) by **Mn-SOD** (Manganese-Superoxide Dismutase). In the cytosol, $O_2^{-\bullet}$ is converted to H_2O_2 by **Cu,Zn-SOD** (Copper,Zinc-Superoxide Dismutase). The two major defense systems against H_2O_2 are the GSH (glutathione) redox cycle present in both the cytosol and mitochondria and catalase present only in the peroxisome fraction. Other sources of $O_2^{-\bullet}$ include the enzymes xanthine oxidase in the cytosol, **NADPH** (nicotinamide adenine dinucleotide phosphate, reduced form) oxidase in the membrane and cytochrome p450 in the **ER** (endoplasmic reticulum). Abbreviations: TNFα (tumor necrosis factor-alpha), **NOS** (nitric oxide synthase), $ONOO^-$ (peroxynitrite), GR (glutathione reductase), **GSSG** (gluthatione, oxidized), **Bcl-2** (B-cell leukemia), **Toc** (tocopherol), and **GPx** (glutathione peroxidase) (Curtin *et al*., 2002).

The aim of the present chapter is to investigate the effect of the neurotoxin, cyanide, on the production of $O_2^{-\bullet}$ in rat brain homogenate *in vitro* and to determine the effect of the non-narcotic analgesics, ASA and acetaminophen to reduce cyanide-induced $O_2^{-\bullet}$ generation. Secondly, a comparative study was done to determine whether ASA and acetaminophen could prevent or reduce quinolinic acid and MPP^+-induced $O_2^{-\bullet}$ generation, *in vivo*, in rat hippocampal and striatal regions, respectively.

5.2. EFFECT OF CYANIDE ON SUPEROXIDE ANION FORMATION IN RAT BRAIN HOMOGENATE *IN VITRO*.

5.2.1. INTRODUCTION

Cyanide is a well-established respiratory poison, which exerts its primary toxic effect by inhibiting Complex IV (cytochrome *c* oxidase) which is the terminal electron acceptor enzyme of the mitochondrial electron transport chain (Isom and Way, 1984) and it must give up its reducing equivalents to allow continued electron transport and ATP production (Cadenas and Davies, 2000) as demonstrated in figure 5.2. Figure 5.2 further shows the different inhibitors of electron transport chain activity and the points where they act. As can be seen cyanide inhibits cytochrome *c* oxidase that is also known as Complex IV.

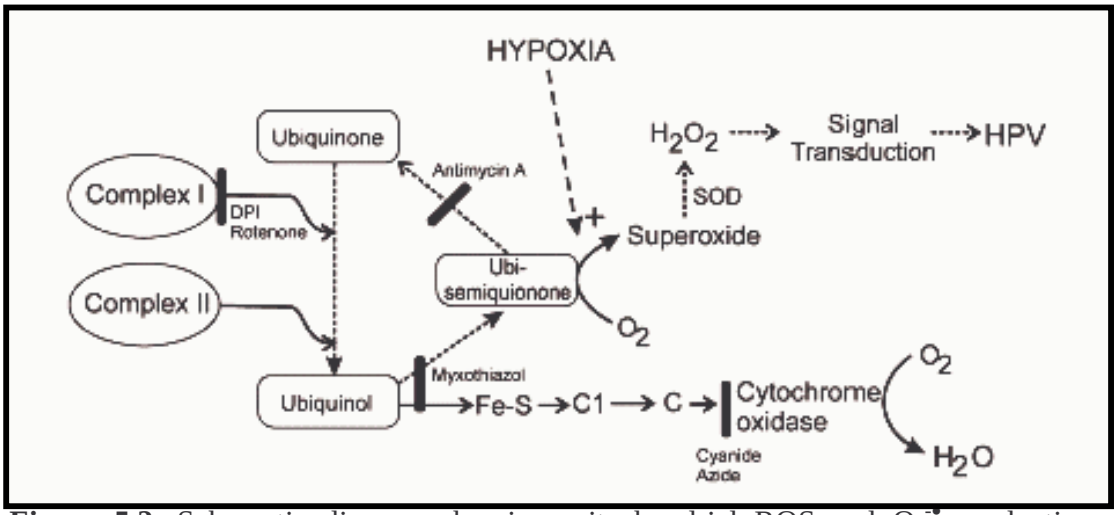

Figure 5.2. Schematic diagram showing mitochondrial ROS and $O_2^{-\bullet}$ production at Complex III. Solid arrows show electron transfer steps; solid bars show sites of electron transport inhibition. The Q cycle converts the dual electron transfer in Complex I and II into single electron transfer steps in Complex IV. Ubisemiquinone (a free radical) created in this process can generate $O_2^{-\bullet}$ (Waypa and Schumacker, 2002).

These ROS are known to exert destructive effects on cellular components with neurodegenerative diseases being one such consequence. Thus, the aim of this experiment was to demonstrate that cyanide induces $O_2^{-\bullet}$ generation in rat brain homogenate and to determine the concentration of cyanide that causes the most significant $O_2^{-\bullet}$ production.

5.2.2. MATERIALS AND METHODS

5.2.2.1. Chemicals and Reagents

Potassium cyanide (KCN), nitro-blue tetrazolium and nitro-blue diformazan (NBD) were purchased from the Sigma Chemical Company, St. Louis, MO (USA). Glacial acetic acid was purchased from Saarchem (PTY) Ltd, Krugersdorp (South Africa). All other chemicals used were of the highest quality available from commercial sources.

5.2.2.2. Animals

Adult male rats of the Wistar strain, weighing between 200-250g were used in this experiment. The rats were randomly assembled into groups of five, and housed in separate cages, in a controlled environment as described in appendix I. Protocols for the experiments were approved by the Rhodes University Animal Ethics Committee.

5.2.2.3. Sample Preparation

A 0.1% NBT solution was made by dissolving the NBT in ethanol before making up to the required volume with Milli-Q water. The final ethanol concentration in the incubation flasks was less than 0.5%.

Stock solutions were prepared so that on addition of 250µl of the toxin, the stock solution would be diluted to the correct incubation concentration. KCN was tested at the following concentrations; 0, 0.25, 0.5, 1, 1.5mM. The KCN was dissolved in Milli-Q water.

5.2.2.4. Preparation of Standards

Nitroblue diformazan was used as a standard. A series of reaction tubes, each containing appropriate aliquots of NBD dissolved in glacial acetic acid was prepared to a final volume of 1ml. A calibration curve (appendix IV) was generated by measuring the absorbance at 20µmoles/ml intervals. The absorbance was read at 560nm using a Shimadzu UV-160A UV-visible recording spectrophotometer.

5.2.2.5. Brain Removal

Rats were sacrificed swiftly by cervical dislocation and rapidly decapitated. The brains were removed for use in experiments as described in appendix II. The brains were either used immediately or stored at -70^{o}C until needed.

5.2.2.6. Tissue Preparation

Rat brain homogenate is a useful model for determining the efficacy of agents to reduce or potentiate $O_2^{-\bullet}$. Each brain was homogenized in a glass Teflon homogenizer with 0.1M phosphate-buffered saline buffer (PBS), pH 7.4 so as to give a final concentration of 10% w/v. This is necessary to prevent lysosomal damage of the tissue. PBS buffer was used as it has been shown not to scavenge free radicals (Anoopkumar-Dukie *et al*., 2001) unlike Tris-HCl buffer, which is an $^{\bullet}OH$ scavenger (Yamamoto and Tang, 1996). The homogenate was either used immediately or frozen in liquid nitrogen and stored at -70°C until use. All samples were used within 7 days of homogenate preparation. Test samples revealed that storage of the homogenate did not alter the $O_2^{-\bullet}$ levels compared to fresh brains.

5.2.2.7. Nitroblue Tetrazolium Assay (NBT)

A modified method of Sagar *et al.*, (1992) and Das *et al.*, (1990) was used for this assay. The lipid source viz. rat brain homogenate (1ml) containing varying concentrations of KCN (0, 0.25, 0.5, 1, 1.5mM) was incubated with 0.4ml of a 0.1%NBT solution in an oscillating water bath for 60 min at 37^{o}C. Termination of the assay and extraction of reduced NBT was carried out by centrifugation of the suspensions at 2000 x g for 10mins. The supernatant was decanted and the pellet was resuspended with 2ml glacial acetic acid. The relative absorbance of the glacial acetic acid fraction was measured at 560nm and converted to μmoles Diformazan using a standard curve generated from NBD. Final results are expressed as μmoles Diformazan/mg protein.

5.2.2.8. Protein Determination

All protein determinations were performed using the method described by Lowry *et al.*, (1952). A standard curve was generated using bovine serum albumin (BSA) as a standard at concentration intervals of 60μg/ml, described in appendix III.

5.2.2.9 Statistical Analysis

The results were analyzed using a one-way analysis of variance (ANOVA). If the F values were significant, the Student Newman-Keuls test was used to compare the treated and control groups. The level of significance was accepted at $p<0.05$ (Zar, 1974).

5.2.3. RESULTS

The final results are expressed as μmoles of diformazan produced/mg protein. This data represents the mean ±SEM of five determinations. It is clearly evident from figure 5.3, that the *in vitro* exposure of rat brain homogenate to the different concentrations of KCN caused a significant increase in $O_2^{-\bullet}$ generation in a dose-dependent manner.

As is evident from figure 5.3, 1.5mM KCN induced the significant $O_2^{-\bullet}$ generation. However there was no significant difference between the 1mM and 1.5mM KCN induced $O_2^{-\bullet}$ generation. Hence it was decided to use the 1mM KCN in the preceding experiment.

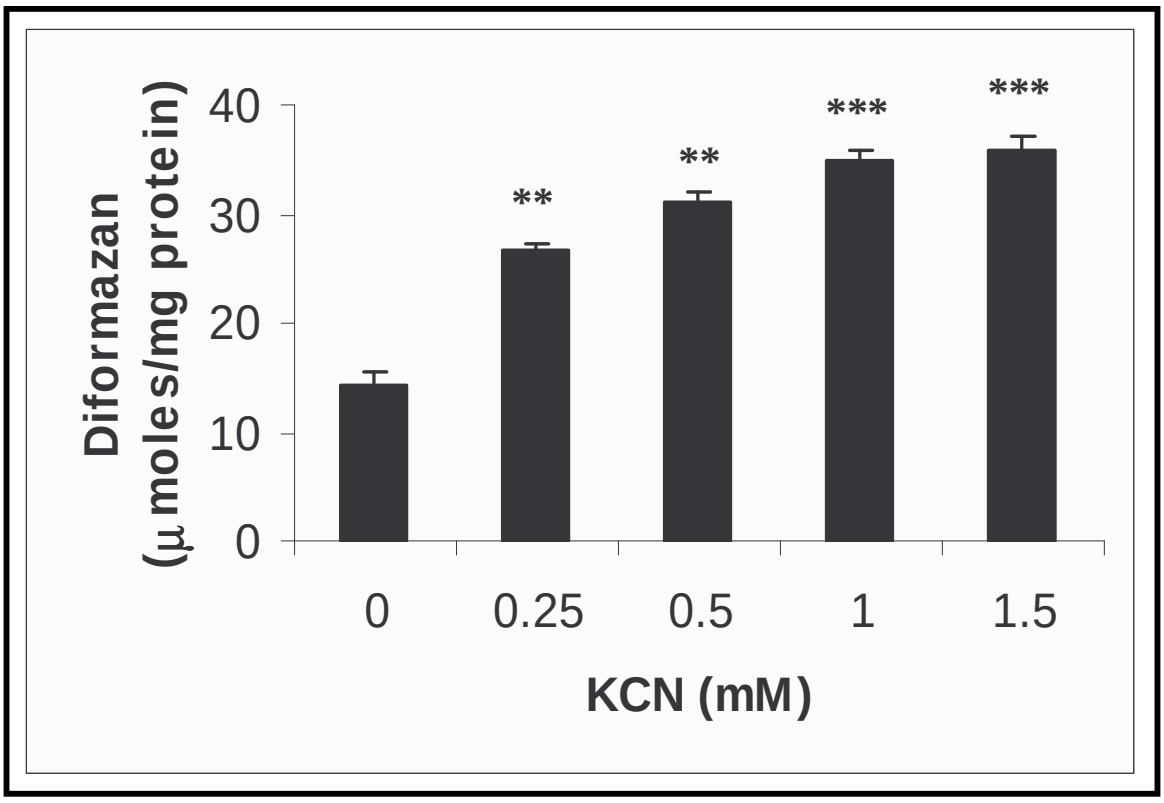

Figure 5.3. Concentration-dependent effect of KCN on $O_2^{-\bullet}$ generation in whole rat brain homogenates *in vitro*. Each bar represents the mean ± SEM; n=5; *p<0.05 ; **p<0.01 ; ***p<0.001 in comparison to control. Student-Newman-Keuls Multiple Range Test.

5.2.4. DISCUSSION

The mitochondria possess a mechanism known as mild uncoupling and this prevents a marked increase in $O_2^{-\bullet}$ formation. Mild uncoupling is the first line of mitochondrial antioxidant defense since it reduces $O_2^{-\bullet}$ generation (Skulachev, 1999). If, nevertheless, some $O_2^{-\bullet}$ is still formed, the next line of defense is activated. This role is carried out by the cytochrome *c* oxidase (cyt c) that oxidizes $O_2^{-\bullet}$ back to O_2**,** thus the $O_2^{-\bullet}$ is merely being converted back to O_2 (Skulachev, 1999).

The results from this chapter aptly demonstrate that KCN is a potent neurotoxin which results in the generation of $O_2^{-\bullet}$ as is evident from figure 5.3. Cyanide interacts with the hem-a-3 portion of cytochrome *c* oxidase in the electron transport chain, in the mitochondria (Slater, 1967) as illustrated in figure 5.2, resulting in the disruption of the homeostatic ATP-dependent Na^{2+}/K^{+} and Ca^{2+} pumps, causing an increase in intracellular calcium. This initiates a cascade of events, culminating free radical generation that will affect the lipids, proteins, and DNA (Southgate and Daya, 1999).

Elevated calcium levels can lead to activation of numerous neuronal calcium-dependent events and ultimately results in oxidative stress and free radical generation. Therefore the elevation of calcium levels by cyanide in the mitochondria could be another mechanism by which it induces oxidative stress.

5.3. EFFECT OF ACETYSALICYLIC ACID AND ACETAMINOPHEN ON CYANIDE INDUCED SUPEROXIDE ANION GENERATION IN RAT BRAIN HOMOGENATE *IN VITRO*.

5.3.1. INTRODUCTION

Cyanide has been shown to induce oxidative stress in rat brain homogenate (section 5.2.). The brain is susceptible to free radical formation and damage due to it its high oxygen consumption and relatively low concentration of antioxidant enzymes (Colye and Puttfarcken, 1993).

Cyanide induces inhibitory effects on SOD and catalase. Superoxide dismutase and catalase, as well as reduced glutathione and the antioxidant vitamins A, C and E are involved in the protection of biological membranes from the harmful effects of free radicals (Halliwell and Gutteridge, 1989; Bunce and Hess, 1988; Tessier *et al*., 1999; Paolisso *et al*., 1998).

These free radicals, which are generated in all biological systems, can cause tissue damage. Thus, if indeed the tissue damage caused by cyanide is due to inhibition of the free radical scavenging enzymes, the administration of antioxidants to cyanide toxified animals should exert an ameliorating influence on the severity of resultant tissue injury.

It is of vital importance that novel agents are found in order to prevent the toxic effects of free radicals in the brain. The present study was therefore performed to investigate whether ASA and acetaminophen possess any protective effects against cyanide induced $O_2^{-\bullet}$ generation in rat brain homogenate.

5.3.2. MATERIALS AND METHODS

5.3.2.1. Chemicals and Reagents

ASA and acetaminophen were purchased from the Sigma Chemical Company, St. Louis, MO (USA). All other chemicals used were of the highest quality available from commercial sources.

5.3.2.2. Sample Preparation

Samples for the NBT assay were prepared according to the method described in section 5.2.2.3. Acetylsalicylic acid, acetaminophen and the combination of ASA and acetaminophen were prepared by dissolving in absolute ethanol, and subsequently diluting it with Milli-Q water so that the final concentration in brain homogenate as 0.5%. Fresh solutions were prepared daily.

5.3.2.3. *In vitro* Exposure of Rat Brain to ASA and Acetaminophen

The experiments were conducted as described in section 5.2.2.7. The brain homogenate (1ml) was incubated at 37^0C for one hour, with the highest concentration of cyanide (1mM) used previously, alone and in combination with increasing concentrations (0, 0.25, 0.5, 1mM) of ASA, acetaminophen and the combination of ASA and acetaminophen. Following this, the NBT assay was performed.

5.3.3. RESULTS

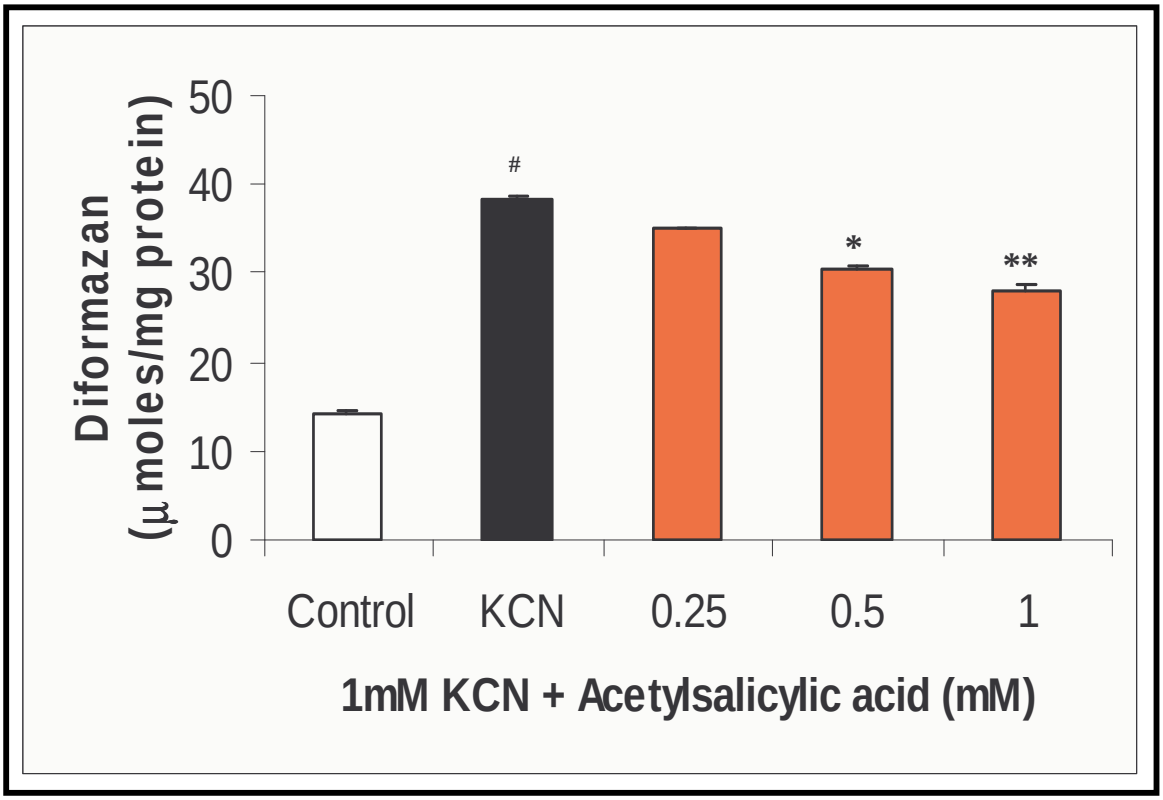

Figure 5.4. The effect of different concentrations of Acetylsalicylic acid on 1mM KCN-induced $O_2^{-\bullet}$ generation in rat whole brain homogenate *in vitro*. Each bar represents the mean ±SEM; n=5. $^{\#}$(p<0.001) vs. control, **(p<0.01) vs. KCN and *(p<0.05) vs. KCN. Student-Newmans-Keuls Multiple Range Test.

Figure 5.4, illustrates that the 0.5 and 1mM ASA is effective at reducing the concentration of diformazan produced by 1mM KCN. However the 1mM ASA together with an equivocal concentration of toxin was effective at reducing the $O_2^{-\bullet}$ product.

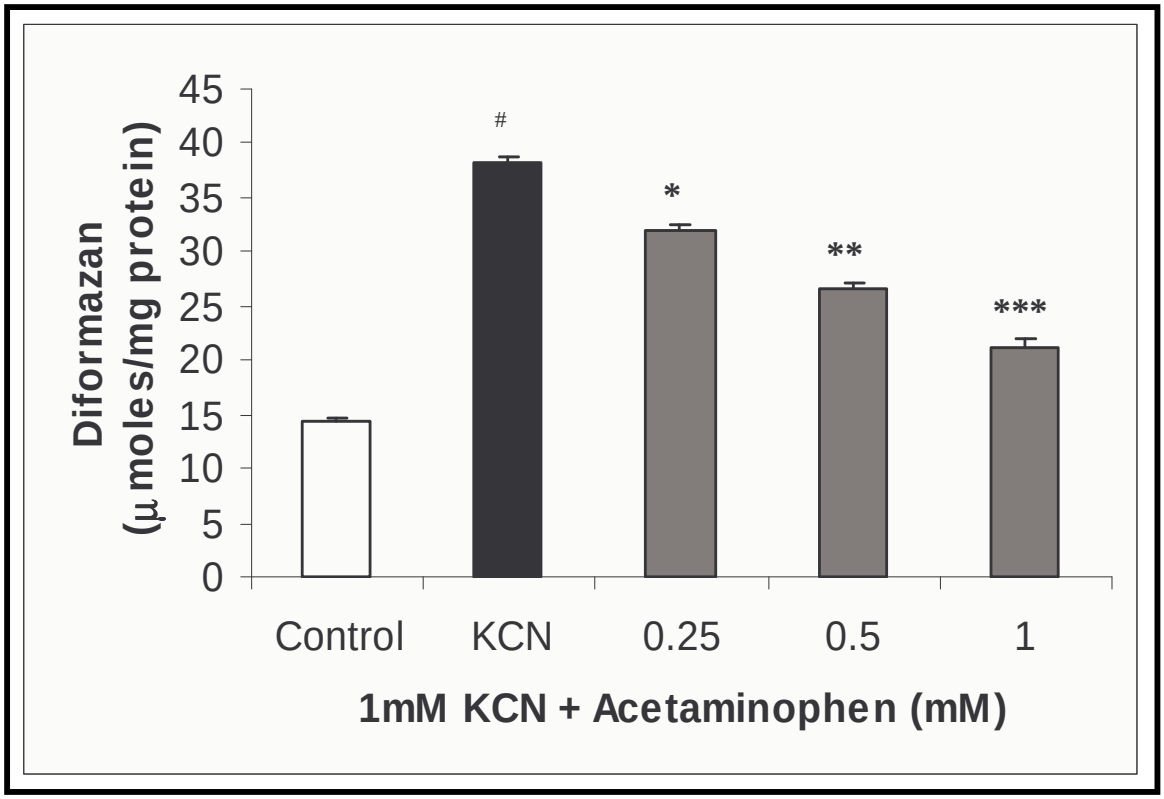

Figure 5.5. The effect of different concentrations of Acetaminophen on 1mM KCN-induced $O_2^{-\bullet}$ generation in rat whole brain homogenate *in vitro*. Each bar represents the mean ±SEM; n=5. $^{\#}$(p<0.001) vs. control, ***(p<0.001) vs. KCN; **(p<0.01) vs. KCN and *(p<0.05) vs. control. Student Newman-Keuls Multiple Range Test.

The treatment of the 1mM KCN-exposed rat brain homogenate with three varying concentrations (0.25, 0.5 and 1mM) of acetaminophen significantly inhibited $O_2^{-\bullet}$ generation as shown in figure 5.5. The 1mM acetaminophen was approximately 30% and 20% more effective at reducing $O_2^{-\bullet}$ generation than when compared to the 0.25mM and 0.5mM acetaminophen, respectively.

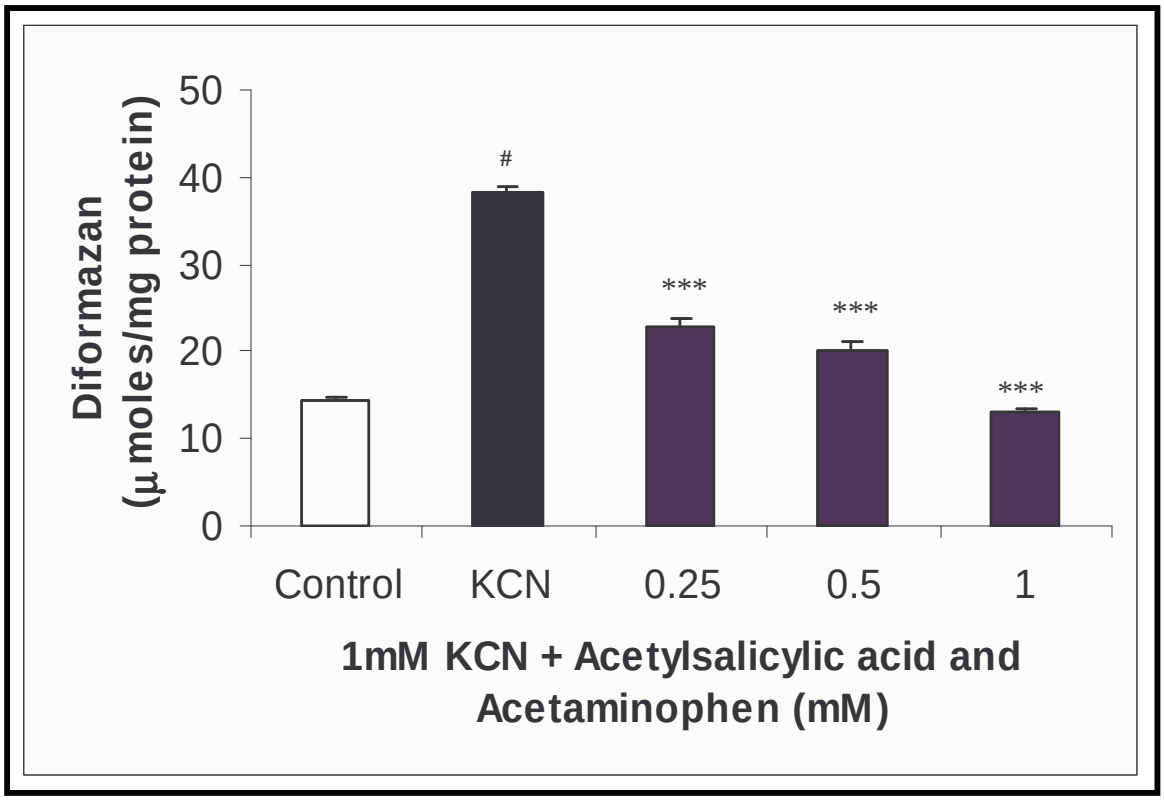

Figure 5.6. The effect of different concentrations of Acetaminophen and Acetylsalicylic acid on 1mM KCN-induced $O_2^{-\bullet}$ generation in rat whole brain homogenate *in vitro*. Each bar represents the mean ±SEM; n=5. $^{\#}(p<0.001)$ vs. control, and ***($p<0.001$) vs. KCN. Student Newman-Keuls Multiple Range Test.

All three concentrations (0.25, 0.5, 1mM) of the combination of ASA and acetaminophen were significantly effective at reducing the neurotoxic effects of KCN in rat brain homogenate, $p<0.001$ (figure 5.6). The 1mM combination of ASA and acetaminophen was effective as there was a low concentration of diformazan produced.

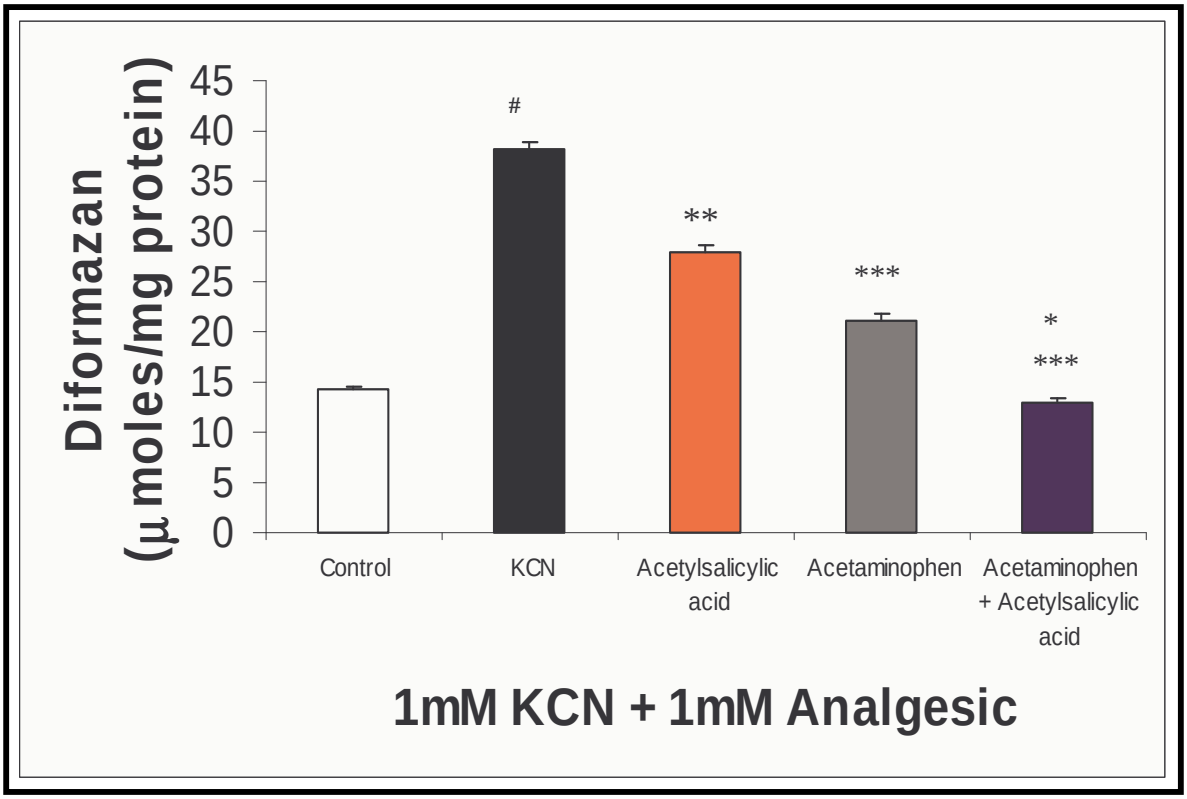

Figure 5.7. The effect of different concentrations of Acetaminophen and Acetylsalicylic acid on 1mM KCN-induced $O_2^{-\bullet}$ generation in rat whole brain homogenate *in vitro*. Each bar represents the mean ±SEM; n=5. $^{\#}(p<0.001)$ vs. control, **($p<0.01$) vs. KCN, ***($p<0.001$) vs. KCN and *($p<0.05$) vs. Acetylsalicylic acid alone and Acetaminophen alone . Student Newman-Keuls Multiple Range Test.

Figure 5.7 provides a comparative evaluation of the effects of the most effective concentration, 1mM, of the three treatments used in the study in rat brain homogenate in the presence of KCN. 1mM KCN induced a rapid increase in $O_2^{-\bullet}$ generation in the rat brain homogenates. However, 1mM aspirin, acetaminophen or a combination of the two significantly curbed this increase. It is also evident that the 1mM combination of ASA with acetaminophen significantly reduces $O_2^{-\bullet}$ generation by 50% in comparison to the 1mM ASA and 30% in comparison to 1mM acetaminophen alone.

5.3.4. DISCUSSION

Cyanide is a toxin that induces neurotoxicity through a series of steps culminating in the production of ROS that are responsible for cellular damage (Pillay *et al*., 2002). These ROS are known to exert destructive effects on cellular components with neurodegenerative diseases being one such consequence.

Free radical scavengers are becoming increasingly popular as a means of reducing or preventing the hazardous effects of free radicals and their inducers. The inhibition of antioxidant enzymes by cyanide is believed to produce oxidative stress and induce neurotoxicity (Ardelt *et al*., 1989).

Results in from figures 5.4-5.7 clearly indicate that these agents are also effective at lower concentrations but are more effective when an equivalent concentration of these agents with toxin was used. The results of the present study indicate that 1mM acetaminophen and 1mM ASA, individually and in combination, significantly reduce 1mM KCN-induced $O_2^{-\bullet}$ generation as illustrated in figure 5.7. Acetaminophen and ASA, either alone or in combination, have the potential to limit the production of $O_2^{-\bullet}$ thus limiting their undesirable effects, qualities that satisfy their use as neuroprotective agents in neurodegenerative disorders.

This study confirms that these agents possess antioxidant properties in the presence of the neurotoxin, KCN, in rat brain homogenate. However the antioxidant properties of these non-narcotic analgesics was investigated further to ascertain antioxidant effects *in vivo*. Since it is not viable to use KCN *in vivo* it was therefore decided to investigate the antioxidant effects *in vivo* using two different potent $O_2^{-\bullet}$ generating neurotoxins, QA and MPP^+.

5.4. EFFECT OF ACETYLSALICYLIC ACID AND ACETAMINOPHEN IN QA INDUCED SUPEROXIDE ANION GENERATION IN THE RAT HIPPOCAMPUS *IN VIVO*.

5.4.1. INTRODUCTION

Quinolinic acid has been shown to be present in normal post-mortem human brains at levels similar to those of other species (Wolfensberger *et al*., 1983), and with concentrations not varying greatly among the different regions of the brain. Heyes and Morrison (1997) demonstrated that the brain naturally synthesizes QA, and that the rate of QA formation increases in conditions of brain and systemic immune activation. Quinolinic acid concentrations have also been shown to increase during the natural aging process in rats (Moroni *et al*., 1984). The administration of quinolate is known to induce seizures in various species of mammals (Lapin, 1981; Lapin *et al*., 1998). The most vulnerable brain structures appear to be the striatum, globus pallidus and hippocampus (Schwarcz and Kohler, 1983).

The results obtained, from section 5.3, encouraged further investigation into the effects of ASA and acetaminophen on *in vivo* induced $O_2^{-\bullet}$ generation. The toxin used was QA as it was not viable to test KCN neurotoxicity, in rats, *in vivo*.

5.4.2. MATERIALS AND METHODS

5.4.2.1. Chemicals and Reagents

Quinolinic acid (2, 3-pyridinedicarboxylic acid) and diethylether were purchased from the Sigma Chemical Company, St. Louis, MO (USA). All other chemicals used were of the highest quality available from commercial sources.

5.4.2.2. Dosing of the Animals

Adult male rats of the Wistar strain were housed in separate cages, in a controlled environment as described in appendix I. The animals were separated into five groups of five animals each. The dose and treatment period was chosen according to Southgate (1999).

The control group received sweet oil (s.c.) while the animals in-group 3 received a dose of 100 mg/kg/d of ASA in 100µl sweet oil, injected subcutaneously, 20 min prior to intrahippocampal QA injection. Similarly the animals in-group 4 and 5 received a dose of 100mg/kg/d of acetaminophen and a combination of ASA and acetaminophen in 100µl of sweet oil respectively, administered in the same way.

The injections were administered at the same time (14H00) each day. The animals in groups 1 and 2 received the vehicle for these drugs, viz. sweet oil. On day one, 20 mins after dosing the animals with the respective drug or vehicle, the animals were injected with QA directly into the hippocampal region. Quinolinic acid was dissolved in PBS made up to pH 7.4. A dose of QA (120nmol) was used to induce neurotoxicity as this concentration of QA is known to cause severe behavioural disturbances and total loss of hippocampal neurons in rats (Lekieffre *et al*., 1990; Schwarcz *et al*., 1984).

Following the intrahippocampal injections of QA, the animals in groups 3, 4 and 5 received subsequent daily doses of the drugs respectively, each day for seven days, while as before, the animals in groups 1 and 2 received daily doses of sweet oil for seven days.

Table 5.1: Treatment regime for each group of animals

Treatment Group	Administered 20 mins prior to surgery (s.c.)	Intrahippocampal injection	Daily treatment for 7 days after surgery (s.c.)
1. Control	100µl Sweet oil	PBS	100µl Sweet oil
2. QA	100µl Sweet oil	120 nmol QA in PBS	100µl Sweet oil
3. ASA	100µl ASA in sweet oil	120 nmol QA in PBS	100µl ASA in sweet oil
4. Acetaminophen	100µl acetaminophen in sweet oil	120 nmol QA in PBS	100µl acetaminophen in sweet oil
5. ASA + Acetaminophen	100µl ASA + acetaminophen in sweet oil	120 nmol QA in PBS	100µl ASA+ acetaminophen in sweet oil

5.4.2.3. Surgical Procedures

5.4.2.3.1. Anesthesia

Diethyl ether anesthesia was employed for all surgical procedures carried out. Animals were placed, one at a time, in a dessicator containing cotton wool soaked in diethyl ether. Once the animals were sedated, and were removed and placed on the operating surface as shown in figure 5.8. A small conical flask containing cotton wool soaked in ether was placed approximately 3cm from the rats' nose. This flask remained in this position throughout surgery, except in cases where respiration became too weak. A good indication of the depth of anesthesia was monitored by the colour of the limbs and tail, which displayed a faint, almost pale pinkness. This was indicative of the optimum level of anesthesia, meaning a satisfactory rate and depth of respiration with good narcosis. A purple colour of the limbs was an indication of cyanosis.

Figure 5.8. A view of the stereotaxic apparatus and Hamilton syringe used for the bilateral intrahippocampal injection of QA (Stoelting, IL, USA).

5.4.2.3.2. Bilateral Intrahippocampal QA Injection

Quinolinic acid was injected intrahippocampally using stereotaxic surgical techniques. Each animal was anaesthetized as described above in section 5.4.2.3.1. Quinolinic acid was dissolved in phosphate buffered saline (PBS), pH= 7.4, and (120nmol in 2µl) was infused bilaterally into the hippocampii employing rat brain stereotaxic apparatus (Stoelting, IL, USA) (figure 5.9). The skull was orientated according to the König and Klippel stereotaxic atlas (1963). After a saggital cut in the skin of the skull, the bregma and lambda suture were located and holes were drilled with a Bosch electrical drill fitted with a drill bit of 0.5mm in diameter at the following coordinates; 4.0mm caudal to the bregma, 2.5mm lateral to the saggital suture, and 3.2mm ventral of the dura. Care was taken not to lesion the meninges. A Hamilton syringe, with a cannula of diameter 0.3mm

held rigidly in the stereotaxic micromanipulator, was used to inject 120 nmol of quinolinic acid in 2µl of PBS, 3.2mm ventral of the dura.

The injection was administered at a rate of 1µl per minute and the cannula was left *in situ* for a further 3 minutes following the drug injection, to allow for passive diffusion away from the cannula tip and to minimize spread into the injection tract. The cannula was then slowly removed and the scalp was closed with sutures. Animals recovered from the anesthesia after approximately 3-4 hours.

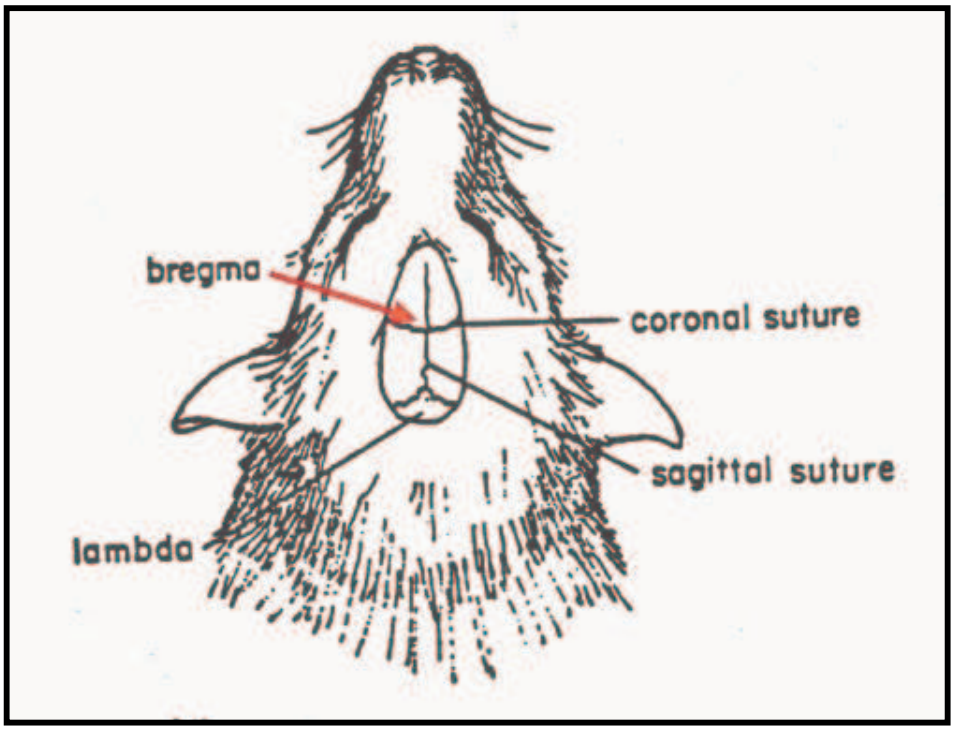

Figure 5.9. A view of the rat skull after the skin has been cut. The sutures shown are used as a reference point for the measurement of the coordinates for the intrahippocampal injection (König and Klippel stereotaxic atlas, 1963).

5.4.2.3.3. Sham Lesioned Rats

The rats used as controls were subjected to the same surgical procedures described in section 5.5.2.3 (i). However, stereotaxic injections into the hippocampus were free of QA and comprised solely of PBS.

5.4.2.4. Dissection of the Hippocampus

On the eighth day following the intrahippocampal injection of QA, the brains were removed as described in appendix II and the hippocampii rapidly dissected according to a modified method of Glowinski and Iversen, (1966) as shown in Figure 5.10. Briefly, the rhombencephalon is separated by a transverse section from the rest of the brain. A transverse section is then made at the level of the optic chiasma, which delimits the anterior part of the hypothalamus and passes through the anterior commissure (section 2). This section separates the cerebrum into two parts, B and C. Part B is divided into five fractions. The easiest way to reach the hippocampus is to first dissect the hypothalamus and the striatum from section B. The midbrain is then gently separated from the remaining part of the brain. The hippocampus is then dissected.

5.4.2.5. Nitroblue Tetrazolium Assay (NBT)

The rat hippocampii were homogenized in PBS, pH 7.4, to yield a 10% w/v homogenate as described in section 5.2.2.6. The nitro-blue tetrazolium (NBT) assay was performed as described in section 5.2.2.7. The only difference was that the lipid source viz. rat hippocampii homogenate (1ml) was incubated with 0.4ml of a 0.1% NBT solution in an oscillating water bath for 1 hr at 37^{o}C.

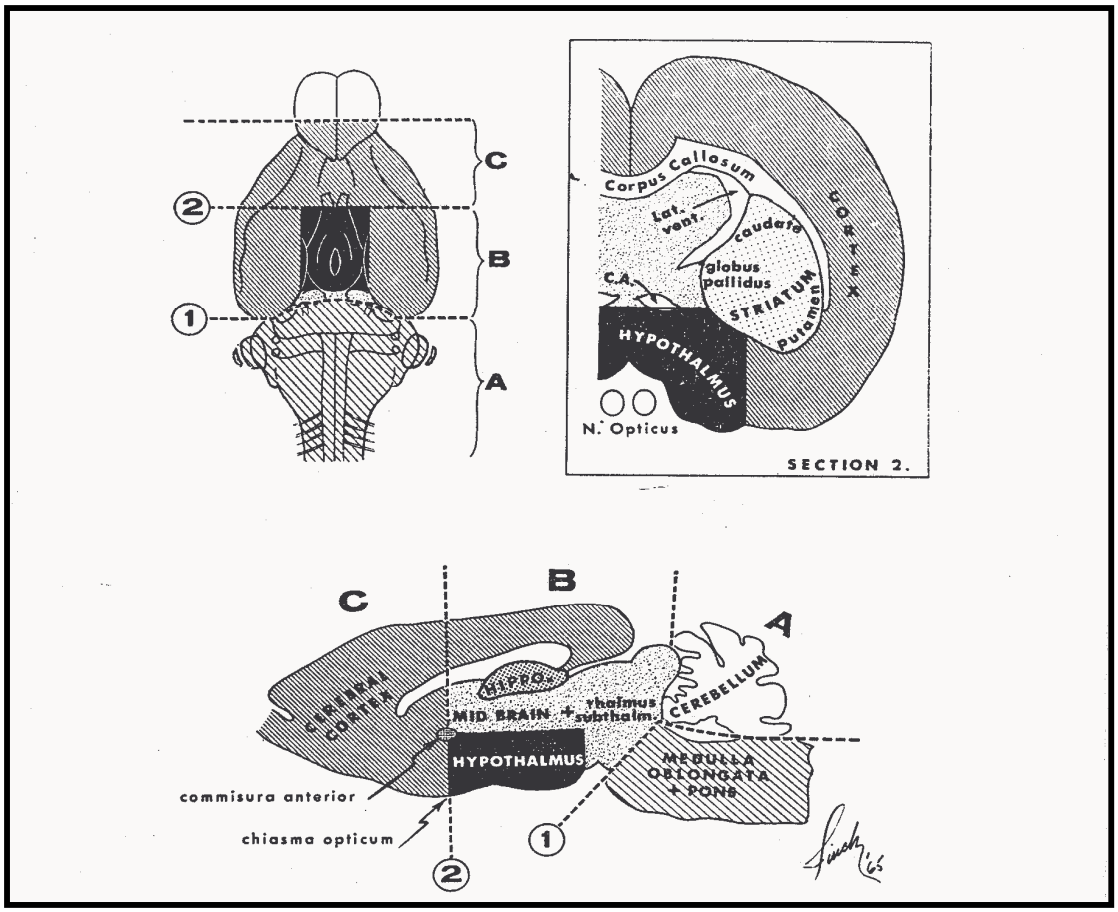

Figure 5.10. Diagrammatic representation of the dissection procedure for rat brain as described in section 5.4.2.4 (Glowinski and Iversen, 1966).

5.4.3. RESULTS

As is evident from figure 5.11, QA caused a significant increase in $O_2^{-\bullet}$ generation as compared to the control (p<0.001). However ASA and acetaminophen alone and in combination were all equally effective at significantly inhibiting the rise in $O_2^{-\bullet}$ generation (p<0.001) with no significant difference between the three non-narcotic analgesic drug treatments that were administered to the rats post operatively. The combination of acetaminophen and ASA was however able to significantly reduce the QA-induced $O_2^{-\bullet}$ generation below that of basal control value (p<0.05).

5.4.4. DISCUSSION

The rats treated with QA only showed severe behavioural disturbances and this in itself provided preliminary evidence that neurological damage had occurred.

The results show that intra-hippocampal injections of QA caused a significant induction of $O_2^{-\bullet}$ generation in the rat hippocampus. This is a result of the activation of the NMDA receptors in the hippocampus by QA, which results in Ca^{2+} dependent increase in oxidative stress (Stone and Perkins, 1981).

The results of the present study show that acetaminophen and ASA significantly reduce QA-induced $O_2^{-\bullet}$ generation. When these drugs are used in combination this effect is potentiated with a resultant effect significantly below that of basal control levels (Figure 5.11). $O_2^{-\bullet}$ is known to exert destructive effects on cellular components with lipid peroxidation being one such consequence. The results obtained from this study therefore indicate that acetaminophen and ASA have the potential to limit these undesirable effects.

The results obtained from the *in vivo* study, using QA as the neurotoxin, provided confirmation, of the antioxidant properties demonstrated by ASA and acetaminophen, in the *in vitro* study (section 5.3). Acetylsalicylic acid and acetaminophen either alone or in combination have demonstrated their ability to prevent $O_2^{-\bullet}$ generation *in vivo*. These agents therefore possess neuroprotective properties strengthening the argument for their use in neurodegenerative disorders.

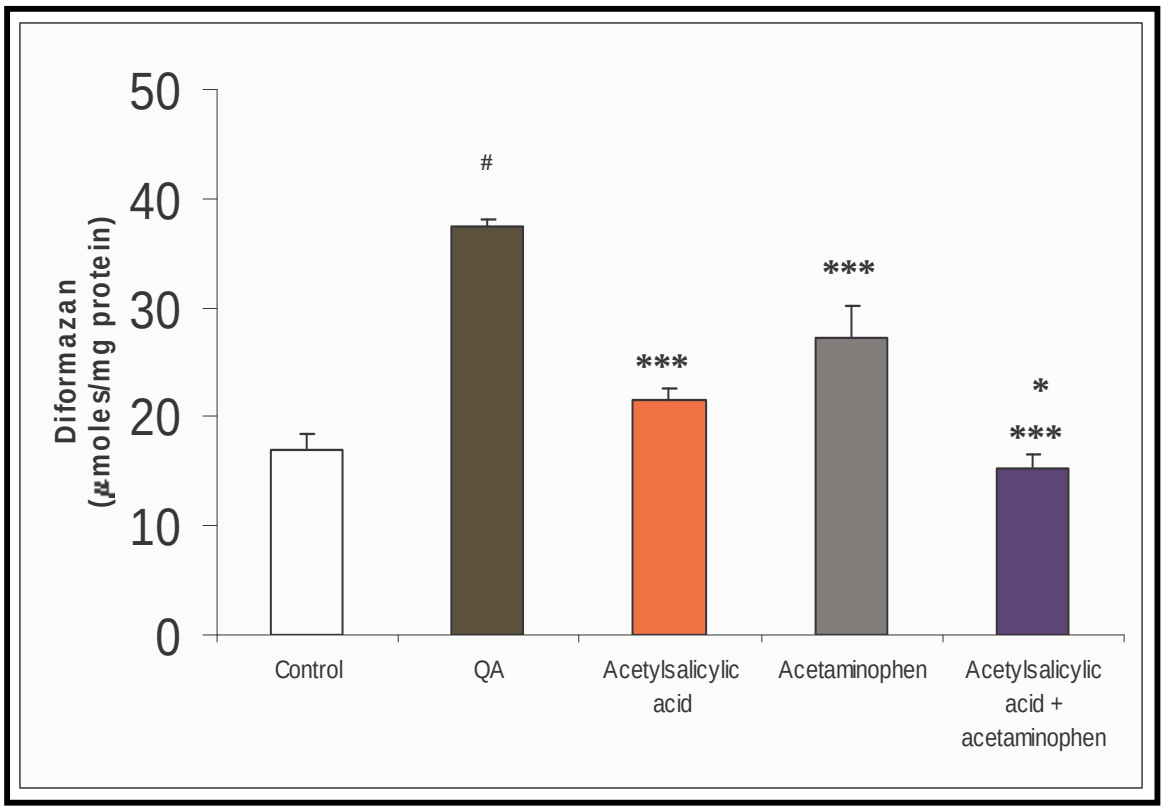

Figure 5.11. The effect of the *in vivo* administration of acetaminophen and acetylsalicylic acid alone or in combination on intrahippocampally injected QA-induced $O_2^{-\bullet}$ generation in rat hippocampal homogenate. Each bar represents the mean ± SEM; n=6; #(p<0.001) in comparison to control, ***(p<0.001) in comparison to QA and ***(**p<0.05) in comparison to the control value. Student-Newman-Keuls Multiple Range Test.

5.5. EFFECT OF ACETYLSALICYLIC ACID AND ACETAMINOPHEN IN MPP^+ INDUCED SUPEROXIDE ANION GENERATION IN RAT STRIATUM *IN VIVO*.

5.5.1. INTRODUCTION

The results obtained from the previous studies mentioned in sections 5.3 and 5.4 fostered an investigation into the possible effects of these agents in PD, in this disease state the generation of free radicals has been known to play an important role in the progression of the disease by contributing to the neurodegeneration in the substantia nigra (Lotharius and O' Malley, 2000).

The discovery of the selective neurotoxicity for nigral dopamine containing cells by 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) has provided possible clues as to the probable causes of Parkinson's disease (Langston *et al*., 1984). Thus it has been suggested that this disease may be induced by (a) compound(s) similar to MPTP (Kinmachi *et al*., 1987). 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) is a good substrate for MAO-B and is metabolized to the active neurotoxin, MPP^+. The primary effect of MPP^+ is to inhibit complex I in the mitochondria (Ramsay *et al*., 1986, Ramsay *et al*., 1991). It has been shown that the potent inhibition of complex I by MPP^+ results in the generation of $O_2^{-\bullet}$ (Hasegawa *et al*., 1990).

Several lines of evidence suggest ROS involvement in MPP^+–induced neurotoxicity. Increases in ROS have been detected by using, for example a ROS-dependent fluorescence probe *in vitro* (Kitamura *et al*., 1998) or electron spin resonance *in vivo* (Rossetti *et al*., 1988). The involvement of ROS in MPP^+ induced neurotoxicity was also confirmed by the cellular protection provided by SOD expression in transgenic mice (Przedborsky *et al*., 1992).

Several factors contribute to MPTP-induced ROS generation. Iron may be of great importance in MPTP toxicity and triggers the Fenton reaction in dopaminerigic cells that results in the generation of free radicals in the brain. MPTP has been shown to increase free iron levels in the SNpc of the brain (Mochizuki *et al*., 1994; Temlett *et al*., 1994). MPP^+, which is a substrate for xanthine oxidase, may lead to the formation of the MPP radical compound in the metabolism of MPTP, and can itself induce $O_2^{-\bullet}$ formation during auto-oxidation (Zang and Misra, 1992).

The aim of this study was to demonstrate the potent induction of $O_2^{-\bullet}$ in the rat striatum by MPP^+ *in vivo* and to investigate any possible effects that ASA and acetaminophen may have in MPP^+ -induced $O_2^{-\bullet}$ generation *in vivo*.

5.5.2. METHODS AND MATERIALS

5.5.2.1. Chemicals and Reagents

1-methyl-4-phenylpyridinium ion (MPP^+) was purchased from the Sigma Chemical Company, St. Louis, MO (USA). All other chemicals used were of the highest quality available from commercial sources.

5.5.2.2 Dosing of the Animals

Adult male rats of the Wistar strain were housed in separate cages, in a controlled environment as described in appendix I. The animals were separated into five groups of five animals each. The control group received ethanol/water (40/60); while the animals in group 3 received a dose of 100mg/kg/day of ASA in ethanol/water (40/60), injected intraperitoneal post intrastriatal MPP^+ injection. Similarly the animals in group 4 and 5 received a dose of 100mg/kg/d of acetaminophen and a combination of ASA and acetaminophen in ethanol/water (40/60), administered in the same way. The injections

were administered initially twice in four hour intervals, and followed by two injections in eight hour intervals. The animals were sacrificed on fourth day.

5.5.2.3. Surgical Procedures

5.5.2.3.1. Anesthesia

Diethyl ether is a desirable anesthetic to use because the mortality rate of the animals is lower than with halothane or phenobarbitone. Diethyl ether is also easy to administer and it is easy to monitor the depth of anesthesia.

5.5.2.3.2. Unilateral Intrastriatal Injections

The neurotoxin, MPP^+ was injected intrastriatal using stereotaxic surgical techniques. Each animal was anaesthetized as described above in section 5.4.2.3.1. MPP^+ was dissolved in saline, and (32nmol in 1µl) was infused unilaterally into the striatum employing rat brain stereotaxic apparatus (Stoelting, IL, USA). The skull was orientated according to the König and Klippel stereotaxic atlas (1963). After a saggital cut in the skin of the skull, the lambda suture were located and holes were drilled with a Bosch electrical drill fitted with a drill bit of 0.5mm in diameter at the following coordinates: L = 0.24; AP = 0.30; and DV = 0.78, from Lambda point (Paxinos and Watson, 1998). Care was taken not to lesion the meninges. A Hamilton syringe, with a cannula of diameter 0.3mm held rigidly in the stereotaxic micromanipulator, was used to inject 32nmol of MPP^+ in 1µl of saline.

The injection was administered at a rate of 1µl per minute and the cannula was left *in situ* for a further 3 minutes following the drug injection, to allow for passive diffusion away from the cannula tip and to minimize spread into the injection tract. The cannula was then slowly removed and the scalp was closed with sutures. Animals recovered completely from the anesthesia after approximately 3-4 hours.

5.5.2.3.3. Sham Lesioned Rats

The rats used as controls were subjected to the same surgical procedures described in section 5.5.2.3. However, stereotaxic injections into the striatum were free of MPP^+ and comprised solely of saline.

5.5.2.4. Dissection of Striatum

On the fourth day following the intrastriatal injection of MPP^+, the brains were removed as described in appendix II and the striata rapidly dissected according to a modified method of Glowinski and Iversen, (1966) as shown in Figure 5.10. Briefly, the rhombencephalon is separated by a transverse section from the rest of the brain. A transverse section is then made at the level of the optic chiasma, which delimits the anterior part of the hypothalamus and passes through the anterior commissure (section 2). This section separates the cerebrum into two parts, B and C. Part B is divided into five fractions. The easiest way to reach the striatum is to first dissect the cortex, which reveals the striatum as shown in Figure 5.10, section B. The striatum is then gently separated from the remaining part of the brain.

5.5.2.5. Nitroblue Tetrazolium Assay

The rat hippocampii were homogenized in PBS, pH 7.4, to yield a 10% w/v homogenate as described in section 5.2.2.6. The nitro-blue tetrazolium (NBT) assay was performed as described in section 5.4.2.5.

5.5.3. RESULTS

The results illustrated in figure 5.12, clearly indicate that MPP^+ is a potent neurotoxin that enhances the levels of $O_2^{-\bullet}$ in rat striatal homogenate as compared to that observed in the control group.

The use of ASA, acetaminophen and the combination of these agents significantly reduced MPP^+ $O_2^{-\bullet}$ generation with a *p* value of <0.001 in comparison to the MPP^+ treated group.

Postoperative treatment with ASA resulted in the $O_2^{-\bullet}$ generation being below basal control levels.

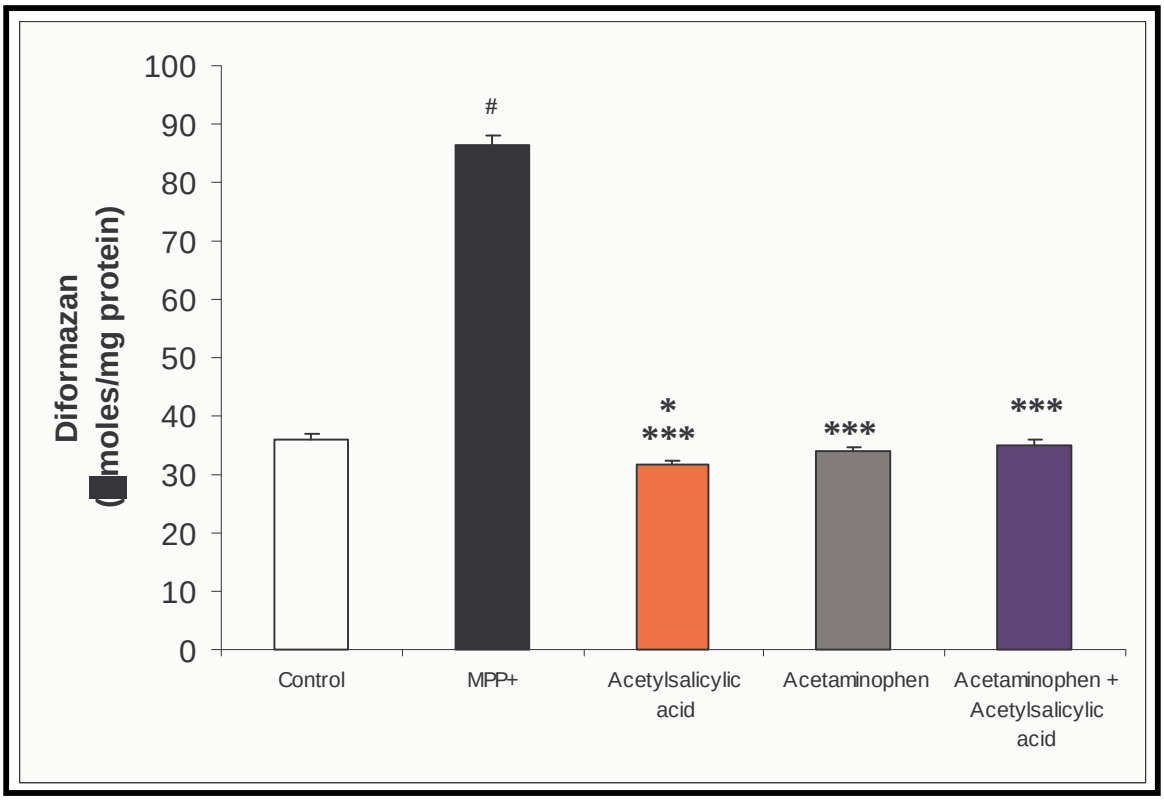

Figure 5.12-. The effect of the *in vivo* administration of acetaminophen and acetylsalicylic acid alone or in combination on intrastriatal injected MPP^+-induced $O_2^{-\bullet}$ generation in rat striatal homogenate. Each bar represents the mean ± SEM; n=5; #(*p*<0.001) in comparison to control, ***(*p*<0.001) in comparison to MPP^+ and ***(*p*<0.05) in comparison to the control value. Student-Newman-Keuls Multiple Range Test.

5.5.4. DISCUSSION

Reactive oxygen species formation due to MPP^+ may be dependent on indirect excitotoxicity resulting from neuronal impairment of energy metabolism and the subsequent increase in cytoplasmic calcium (Storey *et al*., 1992; Chen *et al*., 1995). This is due to the potent inhibition of complex I by MPP^+, which leads to a decrease of cellular ATP levels (Di Monte *et al*., 1986), loss of mitochondrial membrane potential, alterations of calcium homeostasis and free radical formation. N-methyl-D-aspartate receptor antagonists (Turski *et al*., 1991) and calcium channel blockers (Kupsch *et al*., 1995, 1996) were shown to protect the SNpc efficiently against MPP^+.

These results, figure 5.12, implore that ASA and acetaminophen, either individually or in combination, have the potential to combat MPP^+-induced $O_2^{-\bullet}$ generation. Acetylsalicylic acid and acetaminophen have been shown to be potent $O_2^{-\bullet}$ scavengers in section 5.3 and 5.4 counteracting capable inducers such as KCN and QA. Sairam *et al* (2003) demonstrated that the non-steroidal anti-inflammatory drug, such as sodium salicylate, has antioxidant properties, which eventually led to these agents having neuroprotective effects in MPP^+ treated rats. The present study establishes the potential and novel neuroprotective activity of ASA and acetaminophen in MPP^+ treated rats.

5.6. CONCLUSION

The search for novel neuroprotective agents in neurodegenerative diseases involves a number of screening methods to detect their ability to quench free radicals and prevent neuronal death.

The results of the present chapter illustrate that acetaminophen and ASA significantly reduce KCN (*in vitro*), QA and MPP^+-induced (*in vivo*) $O_2^{-\bullet}$ generation in rat brain tissue. These results are also significant and support the assumption (section 2.3.) that ASA and acetaminophen inhibit the enzyme IDO by scavenging $O_2^{-\bullet}$.

It is evident that acetaminophen and ASA, either alone or in combination, have the potential to limit the undesirable effects of $O_2^{-\bullet}$, indicating qualities that would satisfy the use of these agents as novel antioxidant agents in the treatment of neurodegenerative disorders. The generation of free radicals affects lipids, proteins and DNA (Southgate and Daya, 1999). The findings of this chapter encouraged further investigation into the ability of these non-narcotic analgesics to protect against neurotoxin induced lipid peroxidation, which is explored in chapter six.

CHAPTER 6

LIPID PEROXIDATION

6.1. INTRODUCTION

Lipid peroxidation is a deleterious process that may participate in the induction of various pathologies of man. Lipid peroxidation has been shown to occur in brain tissue *in vitro* and *in vivo*, and it has been associated with neuronal damage after trauma and increased membrane permeability (Rios and Santamaria, 1991). Membranal functions, such as GABA uptake, are altered by lipid peroxidation, and lipoperoxidative agents such Fe^{2+} can induce epilileptiform discharges in the rat.

The brain is most vulnerable to oxidative stress such as free radicals because: (1) the activity of the antioxidant enzymes which are responsible for the conversion of the ROS to less reactive components, is weak, (2) the brain possesses a high lipid content, and (3) the consumption of O_2 in the brain is high compared to the rest of the body (Choi and Rothman, 1990). One of the tests used for lipid peroxidation detection is the thiobarbituric acid (TBA) test, which is widely employed in the measurement of lipid peroxidation. The test measures the levels of malondialdehyde (MDA), which is a product of lipid peroxidation and is taken to be a reliable indicator for oxidative stress (Reiter *et al*., 1995) as shown in figure 6.1.

Membranes of living cells are remarkable in molecular architecture, displaying a variety of different functions. The plasma membrane surrounds all animal cells, including neurons, and separates the cell interior from the extracellular environment, and also compartmentalizes the internal structures of the cell, which is essential for cell functioning (Campbell, 1996). Membranes are not static boundaries that segregate regions but are dynamic systems responsible for among other things; to function as permeable barriers for the selective transport of molecules into and out of the cell,

membranes are also responsible for the production of ATP and the binding of regulatory molecules such as hormones, growth factors and binding of neurotransmitters that mediate neurotransmission (Bohinski, 1987). Therefore, biological membranes function as important barriers, protecting cells from possible harmful compounds in the surrounding medium.

The TBA test is widely used as an indicator of $^{\bullet}OH$ generation (Gutteridge & Halliwell, 1990). The TBA test was introduced to biological systems for the first time by Kohn & Liversedge in 1944 (Kohn & Liversedge, 1944) as a measurement for lipid rancidity in the food industry (Gutteridge and Quinlan, 1983). This test is now the most widely employed technique used in the determination of peroxidation in biological materials (Yamamoto *et al.*, 1990). Malondialdehyde is a degraded lipid product from cell membranes, and is taken as a reliable indicator of oxidative stress (Reiter *et al.*, 1995). The foundation of this test is based on the reaction of one molecule of MDA with two molecules of TBA to yield a pink coloured chromagen. Figure 6.1 outlines the reaction that occurs during the TBA test.

A simple and accurate ultraviolet absorbance spectrophotometric method was used to measure the TBA-MDA complex. This assay involves the reaction of malondialdehyde equivalents with TBA to yield a pink complex. A modified method of Sagar *et al.*, (1992) and Das *et al.*, (1990) was used in this assay. The MDA-TBA complex has an absorption maximum at 532nm. Materials of animal origin usually contain large amounts of protein, to which MDA may be bound (Draper & Hadley, 1990). It is therefore imperative to release the protein-bound MDA, which is achieved by hot acid hydrolysis (pH of 2-3) using TCA, and is required for the formation of the complex and the release of protein bound MDA as evident from figure 6.1. Butylated hydroxytoluene (BHT) in methanol is added prior to TCA precipitation to ensure that no lipid oxidation occurs during the assay and interferes with the results obtained.

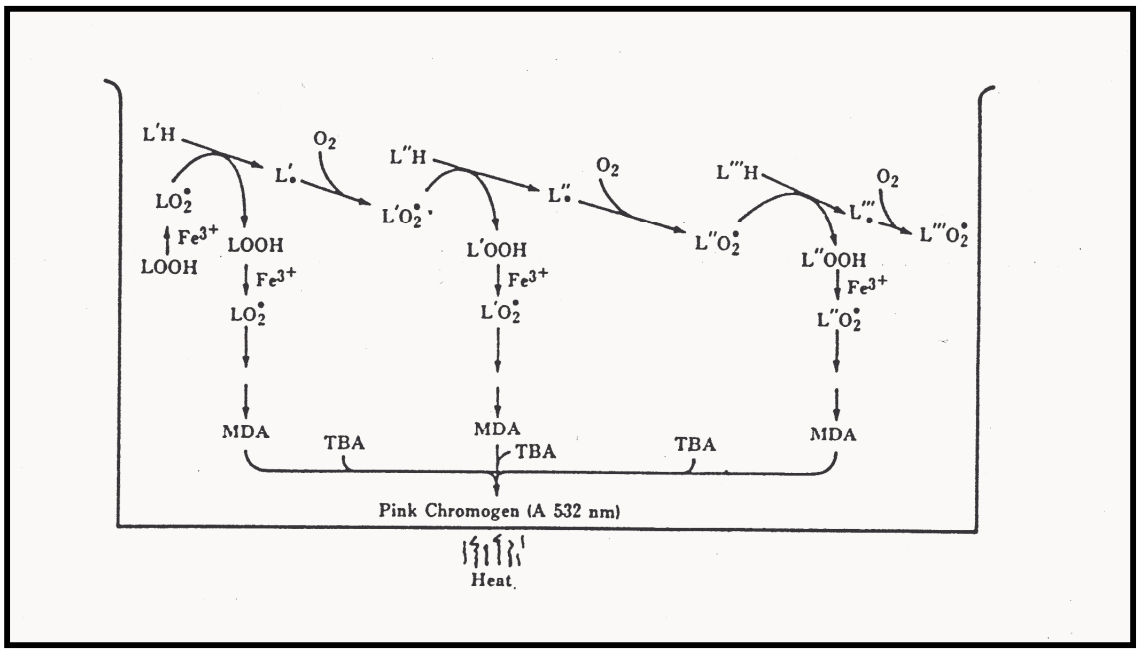

Figure 6.1. The formation of MDA by lipid peroxidation during incubation and to a much greater extent during the acid-heating stage (Gutteridge, 1987). Malondialdehyde (**MDA**) which is a degraded lipid product (**L'H**) from cell membranes reacts with two molecules of thiobarbituric acid (**TBA**) to yield a pink coloured chromagen. The MDA-TBA complex has an absorption maximum at 532nm. Materials of animal origin usually contain large amounts of protein, to which MDA may be bound thus it is therefore imperative to release the protein-bound MDA and this is achieved by hot acid hydrolysis (pH of 2-3) using TCA.

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6.2. EFFECT OF CYANIDE AND QA ON LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE *IN VITRO*.

6.2.1. INTRODUCTION

The brain is a primary target organ in cyanide toxicity (Gunasekar *et al*., 1996). Cyanide-induced neurotoxicity is mainly attributed to its production of cellular anoxia in the brain (Yamamoto & Tang, 1996). Acute toxicity of cyanide produces tonic and clonic seizures, convulsions (Way, 1984) and in some individuals a Parkinson-like condition may develop as a post toxicity sequel (Utti *et al*., 1985). Cyanide produces dopaminergic toxicity, characterized by loss of dopaminergic neurons in the basal ganglia, which is accompanied by impaired motor function (Gunasekar *et al*., 1996). Due to the number of antioxidant enzymes being inhibited by cyanide, it is also believed that oxidative stress plays an important role in cyanide induced neurotoxicity (Ardelt, 1989). Johnson *et al* (1987) proposed that increased intracellular calcium following cyanide treatment in the brain generates reactive oxygen species leading to peroxidation of lipids and subsequent neuronal damage.

Quinolinic acid induced neurotoxicity results from the activation of ionic channels through which sodium, potassium and calcium flood into the cell (Stone, 1993). The increased intracellular calcium sets off a cascade of events that culminate in the generation of free radicals.

The aim of this study is to determine the effect of the neurotoxins cyanide and QA on lipid peroxidation in rat brain homogenate *in vitro*. These neurotoxins have been previously reported to increase lipid peroxidation levels in brain homogenate (Southgate, 1999; Daya *et al*., 2000; Anoopkumar-Dukie *et al*., 2003; Maharaj *et al*., 2004).

6.2.2. MATERIALS AND METHODS

6.2.2.1. Chemicals and Reagents

All reagents used were of analytical grade. Cyanide, QA (2, 3-pyridinedicarboxylic acid), 2-thiobarbituric acid (98%) (TBA), 1, 1, 3, 3-tetramethoxypropane (98%) and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Corporation, St. Louis, MO, U.S.A. Methanol (HPLC grade) was purchased from BDH Laboratory Supplies, Poole, England. Trichloroacetic acid (TCA) was obtained from Saarchem, Johannesburg, South Africa.

6.2.2.2. Animals

Adult male rats of the Wistar strain, weighing between 200-250g were used in this experiment. The rats were randomly assembled into groups of five, and housed in separate cages, in a controlled environment as described in appendix I. All protocols for the experiments were approved by the Rhodes University Animal Ethics Committee.

6.2.2.3. Sample Preparation

Butylated hydroxytoluene (0.5mg/ml) was dissolved in methanol; TCA (15%) and TBA (0.33%) were prepared in Milli-Q water. All reagents were made up under de-aerated conditions. Stock solutions were prepared so that on addition of 100µl of the toxin, the stock solution would be diluted to the correct incubation concentration. Potassium cyanide and QA were tested at the following concentrations: 0, 0.25, 0.5, 1, and 1.5mM.

6.2.2.4. Brain Removal

Rats were sacrificed and the brains removed as described in appendix II.

6.2.2.5. Tissue Preparation

Rat brain homogenate is a useful model for determining the efficacy of agents to reduce or potentiate lipid peroxidation (Maharaj *et al*., 2002). Each brain was homogenized according to the method described in chapter five, section 5.2.2.6. The homogenate was either used immediately or frozen in liquid nitrogen and stored at -70°C until use. All samples were used within 7 days of homogenate preparation. Test samples revealed that storage of the homogenate did not alter the lipid peroxidation levels compared to fresh brains.

6.2.2.6. Preparation of the Standard Curve

1, 1, 3, 3-tetramethoxypropane was used as a standard. A series of reaction tubes each containing appropriate aliquots of water and standard solution were prepared with Milli-Q water to a final volume of 1ml. A calibration curve was generated by measuring the absorbance at 5nmoles/ml intervals as shown in appendix V. The procedure described in section 6.2.2.6 was followed. The absorbance was read at 532nm. The absorbance of MDA-TBA was plotted against the concentration of MDA.

6.2.2.7. The Thiobarbituric Acid (TBA) Test

Lipid peroxidation was determined using the thiobarbituric acid (TBA) assay. This assay involves the reaction of malionaldehyde equivalents with TBA to yield a pink complex. A modified method of Sagar *et al*., (1992) and Das *et al*., (1990) was used in this assay. In the *in vitro* study to determine the effective concentration, homogenate (1ml) containing varying concentrations of KCN (0, 0.05, 0.25, 0.5, 1, 1.5mM) or QA (0, 0.05, 0.25, 0.5, 1, 1.5mM) were incubated in a shaking water bath for 1h at 37^{o}C. At the end of the incubation 0.5 ml BHT (0.5 g/l in absolute methanol) and 1ml 25% TCA were added to the mixture. The samples were centrifuged at 2000 x g for 20min at 4^{o}C to remove insoluble proteins. Following centrifugation, 2ml of protein free supernatant was

removed from each tube and a 0.5ml aliquot of 0.33% TBA was added to this fraction. All tubes were heated for 1h at 95^{o}C in a water bath. After cooling, the TBA-MDA complexes were extracted with 2ml of butanol. The absorbance was read at 532 nm and MDA levels were determined from a standard curve generated from 1, 1, 3, 3-tetramethoxypropane. The final results were expressed as nmol MDA/mg tissue.

6.2.2.8. Statistical Analysis

The results were analyzed using a one-way analysis of variance (ANOVA). If the F values were significant, the Student's Newmans-Keuls Multiple Range test was used to compare the treated and control groups. The level of significance was accepted at $p<0.05$ (Zar, 1974).

6.2.3. RESULTS

As is evident from figure 6.2, all concentrations of QA induced a significant rise in MDA concentration. However it was the 1mM and 1.5mM QA that induced the significant rise in lipid peroxidation i.e. $p<0.001$. Therefore a concentration of 1mM QA acid was used to induce lipid peroxidation, *in vitro*, in the rat brain homogenate.

Cyanide induced lipid peroxidation was significant, $p<0.001$, at a concentration of 1.5mM as compared to the control (figure 6.3). Therefore a concentration of 1.5mM KCN was used to induce *in vitro* lipid peroxidation.

6.2.4. DISCUSSION

Quinolinic acid plays an important role in the etiology of various diseases such as HD, temporal lobe epilepsy, hepatic encaphalopathy and AD due to its ability to reproduce the various histological and neurochemical features of the various diseases especially in the case of HD (Misztal *et al*., 1996; Blight *et al*., 1995; Basile *et al*., 1995; Beal, 1992).

Quinolinic acid is an established agonist of the glutamate receptor and previous research has shown that among a number of glutamate receptor agonists including QA, kainic acid and NMDA however only QA potentially induces lipid peroxidation in rat brain homogenate (Rios & Santamaria, 1991). The results of the present study are consistent with this finding and show that QA causes a significant induction of lipid peroxidation products in rat brain homogenate *in vitro* in a concentration dependent manner.

Figure 6.2, clearly demonstrates that 1mM and 1.5mM QA are the most potent concentrations of QA with regard to the degree of lipid peroxidation that was produced in rat brain homogenate. This therefore provides evidence that these concentrations are extremely effective to induce lipid peroxidation *in vitro* in rat brain homogenate.

Figure 6.3 illustrated that KCN treatment in rat brain homogenate results in the peroxidation of lipids in the rat brain. The results from figure 6.3 indicate that 1mM and 1.5mM KCN were most effective at enhancing the MDA concentration levels.

Since the, 1mM and 1.5mM concentrations of both neurotoxins have no significant difference in the amount of lipid peroxidation being produced, it would therefore be ideal to use either concentration in the assessment of possible therapeutic agents in attenuating the deleterious effect produced by KCN and QA.

Johnson *et al*, (1987) proposed that increased intracellular calcium after cyanide treatment generates reactive oxygen species leading to peroxidation of lipids and subsequent neuronal damage. The result of the present study is consistent with this finding. It has also been proposed that nitric oxide, a promoter of $^{\bullet}OH$ formation, is a mediator of convulsions associated with cyanide toxicity (Yamamoto & Tang, 1996a). Studies have also shown the activation of NMDA receptors during cyanide toxicity (Gunasekar *et al*., 1996). Cyanide induced seizures are inhibited by MK-801, an antagonist of the NMDA receptor (Yamamoto & Tang, 1996b). MK-801 is also known to inhibit cyanide-induced cerebellar granule cell death (Gunasekar *et al*., 1996).

The concentrations of KCN and QA used are suprapharmacological and above the concentration that is normally present *in vivo*, in aging and in neurodegenerative diseases. However this study aptly demonstrates that these agents are capable of inducing lipid peroxidation in rat homogenate and the concentration that was most effective provides a benchmark to assess whether ASA and acetaminophen possess antioxidant capabilities in the presence of such toxins, in further lipid peroxidation studies.

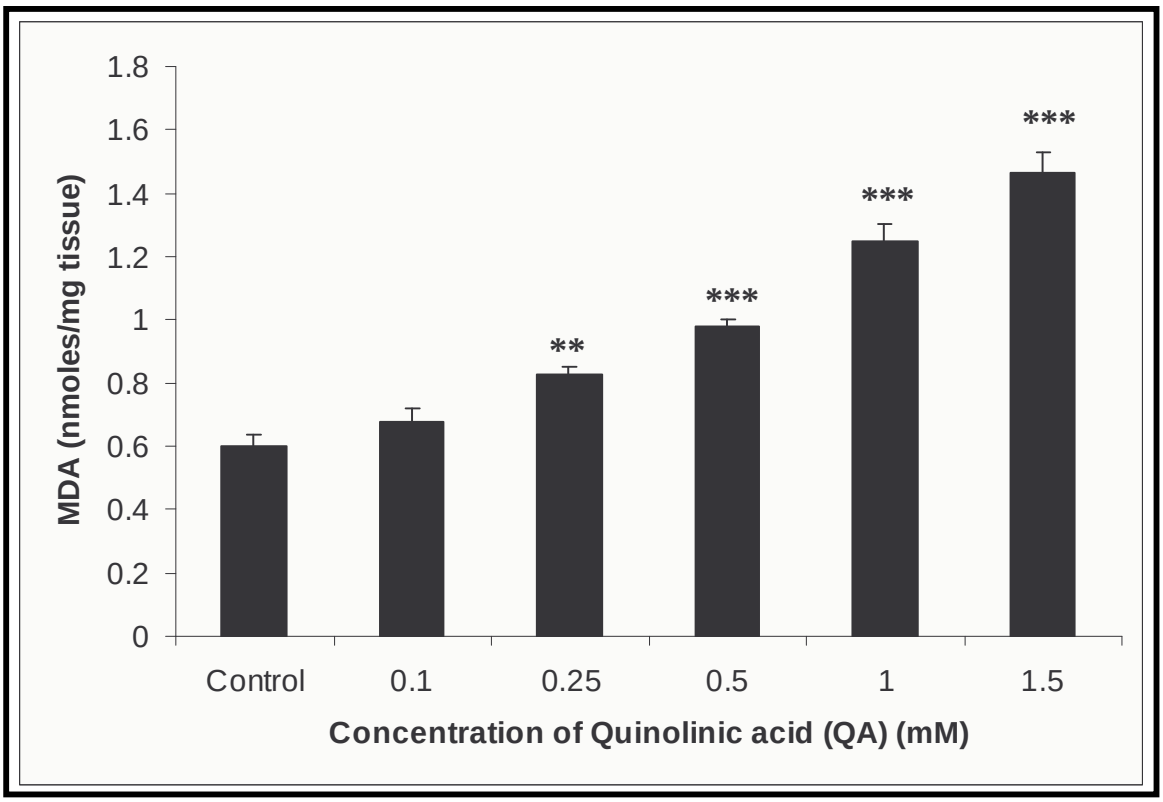


Figure 6.2. Concentration-dependent effect of QA of lipid peroxidation generation in whole rat brain homogenate *in vitro*. Each bar represents the mean ±SEM; n=5. **(p<0.01) vs. control and ***(p<0.001) vs. control. Student Newman-Keuls Multiple Range Test.

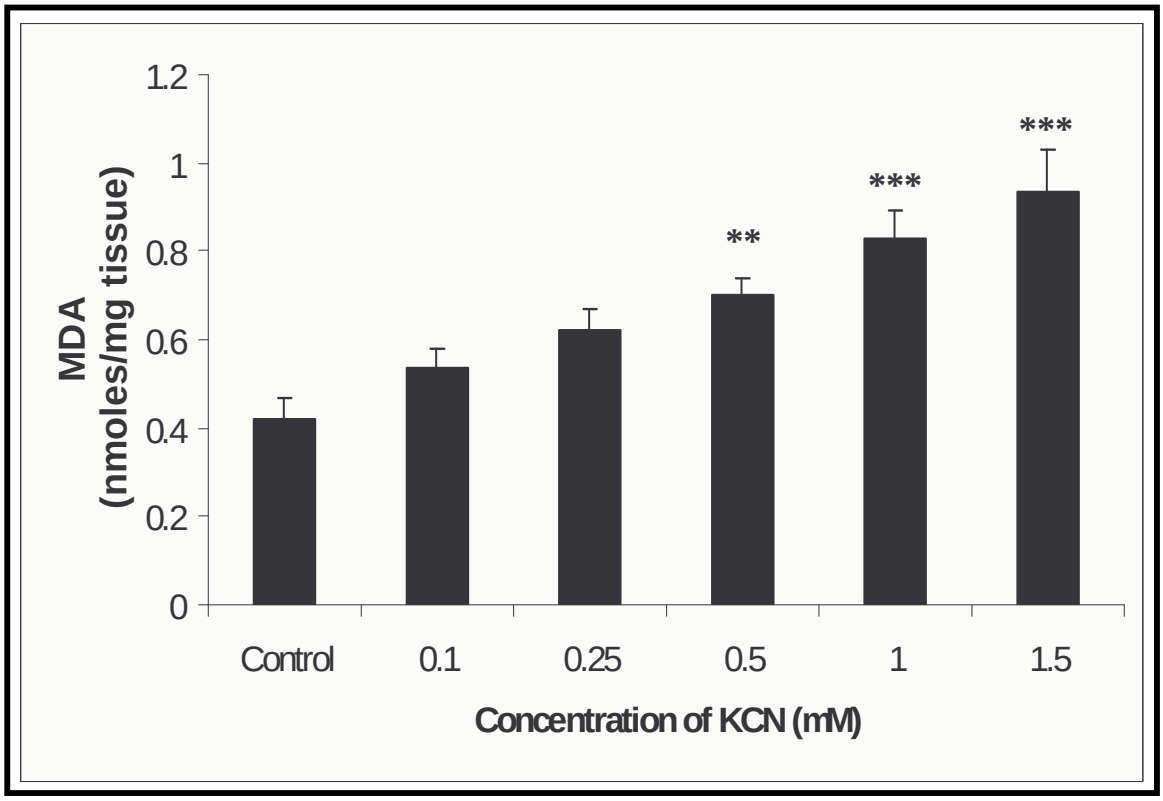


Figure 6.3. Concentration-dependent effect of QA of lipid peroxidation generation in whole rat brain homogenate *in vitro*. Each bar represents the mean ±SEM; n=5. **(p<0.01) vs. control and ***(p<0.001) vs. control. Student Newman-Keuls Multiple Range Test.

6.3. EFFECT OF ACETYLSALICYLIC ACID AND ACETAMINOPHEN ON KCN AND QA INDUCED LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE *IN VITRO*.

6.3.1. INTRODUCTION

The brain is particularly susceptible to oxidative attack by free radicals because of its high utilization of oxygen, its relatively low concentration of antioxidative enzymes and free radical scavengers (Reiter, 1995).

Lipid peroxidation is able to cause extensive damage and is known to play a major role in the deterioration of the brain and spinal cord that occurs after traumatic, excitotoxic or ischemic injury. Radicals have been postulated to be important mediators of tissue injury in several neurodegenerative models (Bautista & Spitzer, 1990; Shuter *et al*., 1990; Yoshikawa *et al*., 1994).

The results from the study in section 6.2 show that QA and KCN are potent inducers of lipid peroxidation in rat brain homogenate *in vitro*. Research has indicated that non-narcotic analgesics possess antioxidant activity (Kataoka *et al*., 1997; Maffei *et al*., 1993). Therefore the aim of the present study is to determine whether ASA and acetaminophen are capable of inhibiting QA and KCN–induced lipid peroxidation *in vitro* in rat brain homogenate.

6.3.2. MATERIALS AND METHODS

6.3.2.1. Chemicals and Reagents

ASA and acetaminophen were purchased from the Sigma Chemical Company, St. Louis, MO (USA). All other chemicals used were of the highest quality available from commercial sources.

6.3.2.2. Sample Preparation

Samples for the lipid peroxidation assay were prepared according to the method described in section 6.2.2.3. ASA, acetaminophen and a combination of ASA and acetaminophen were prepared by dissolving in absolute ethanol, and subsequently diluting with Milli-Q water so that the final ethanol concentration in the brain homogenate was 0.5%. The following concentrations of ASA, acetaminophen and combination of ASA and acetaminophen were used 0, 0.25, 0.5, 1, 1.5mM.

6.3.2.3. Lipid Peroxidation Assay

Adult male rats of the Wistar strain were used for this experiment and were maintained as described in appendix I. The rats were sacrificed and the brains were rapidly removed and placed on ice as described in appendix II. Each brain was homogenized as described in section 6.2.2.5.

Lipid peroxidation was determined using a modification of the method described in section 6.2.2.7. Briefly, homogenate (1ml) containing 1mM QA or 1mM KCN alone or in combination with varying concentrations of ASA, acetaminophen and a combination of ASA and acetaminophen were incubated on an oscillating water bath at 37^{o}C for 60min. The procedure as described in section 6.2.2.7 was then followed. Final results were

expressed as nmoles MDA/mg tissue and analyzed for statistical significance as demonstrated in section 6.2.2.8.

6.3.3. RESULTS

The exposure of rat brain homogenate with 1mM KCN at 37^{o}C for 1hr caused a significant increase in lipid peroxidation when compared to the control (figure 6.4). The 1mM and 1.5mM concentrations of ASA significantly inhibit KCN induced lipid peroxidation as shown in figure 6.4. However the 1.5mM ASA is the most effective concentration at inhibiting KCN induced lipid peroxidation in rat brain homogenate.

The 0.5, 1 and 1.5mM concentrations of acetaminophen are effective at significantly reducing the concentration of MDA in the presence of KCN (figure 6.5). The 1.5mM acetaminophen is the most effective concentration in providing protection against the KCN-induced lipid peroxidation with significance of $p<0.001$. However there is no significant difference when compared to the 1mM acetaminophen in the presence of KCN. In addition there was no significant difference between the protection offered by the 1.5mM acetaminophen concentration and that of the control value.

Figure 6.6 illustrates that the 1.5mM combination of ASA and acetaminophen is the most effective concentration used in attenuating KCN-induced lipid peroxidation, $p<0.001$.

Figure 6.7, demonstrates that all concentrations of ASA are effective at reducing the QA-induced lipid peroxidation, $p<0.001$. The concentration of MDA obtained, in ASA treated homogenate, is below basal control levels for all concentrations of ASA used indicating that ASA was effective in reversing QA-induced lipid peroxidation in the rat brain homogenate *in vitro* and that ASA is possible acting as an agonist at the NMDA receptor competing with QA.

The 1mM and 1.5mM acetaminophen are the most effective concentrations in inhibiting QA-induced rise in lipid peroxidation in the rat brain homogenate. In addition, the MDA

concentrations obtained for the 1 and 1.5mM acetaminophen are below basal control levels as illustrated in figure 6.8.

All concentrations of the combination of ASA and acetaminophen are effective at reducing lipid peroxidation in the presence of 1mM QA as shown in figure 6.9.

6.3.4. DISCUSSION

The results obtained from this study demonstrate that these non-narcotic analgesics agents either alone or in combination offer protection against cyanide and QA-induced lipid peroxidation in rat brain homogenate *in vitro*. Previous research shows that ASA is an antioxidant in rat brain homogenate (Daya *et al*., 2000) and the results of this study are therefore in agreement.

It has been shown previously that acetaminophen can act as a pro-oxidant (Anoopkumar-Dukie, 1999). However at lower doses than those reported to be pro-oxidant, as was used in this study, it was clearly demonstrated that acetaminophen acts as an anti-oxidant. This is confirmed in previous reports wherein acetaminophen has been shown to possess both these abilities (Mason and Fischer, 1986; Nakamato *et al*., 1997). These agents have a potentiating effect when used in combination, further motivating that the combination of these drugs could result in the birth of novel and potent neuroprotective agents.

This study provides novel and substantial evidence that proves ASA and acetaminophen are potent antioxidants in the presence of potent neurotoxins such as QA and KCN. The use of these agents resulted in the attenuation of the potent induction of lipid peroxidation by the neurotoxins QA and KCN. This therefore indicates the promising antioxidant activity of these agents *in vitro*, in rat brain homogenate and promotes further investigations in the antioxidant ability of these agents to reduce lipid peroxidation *in vivo*.

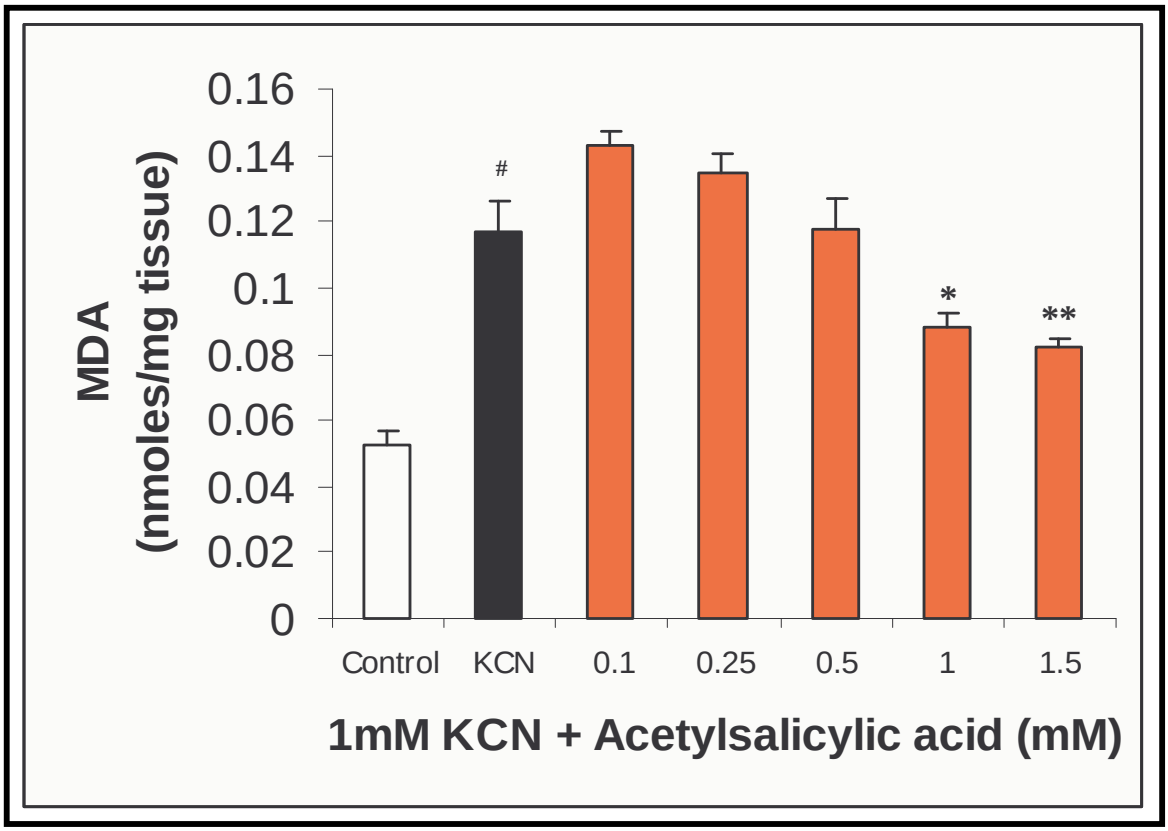


Figure 6.4. The effect of varying concentrations of Acetylsalicylic acid on 1mM KCN-induced lipid peroxidation in rat whole brain homogenate *in vitro*. Each bar represents the mean ±SEM; n=5. $^{\#}(p<0.001)$ vs. control, *($p<0.05$) vs. KCN and **($p<0.01$) vs. KCN. Student Newmans-Keuls Multiple Range Test.

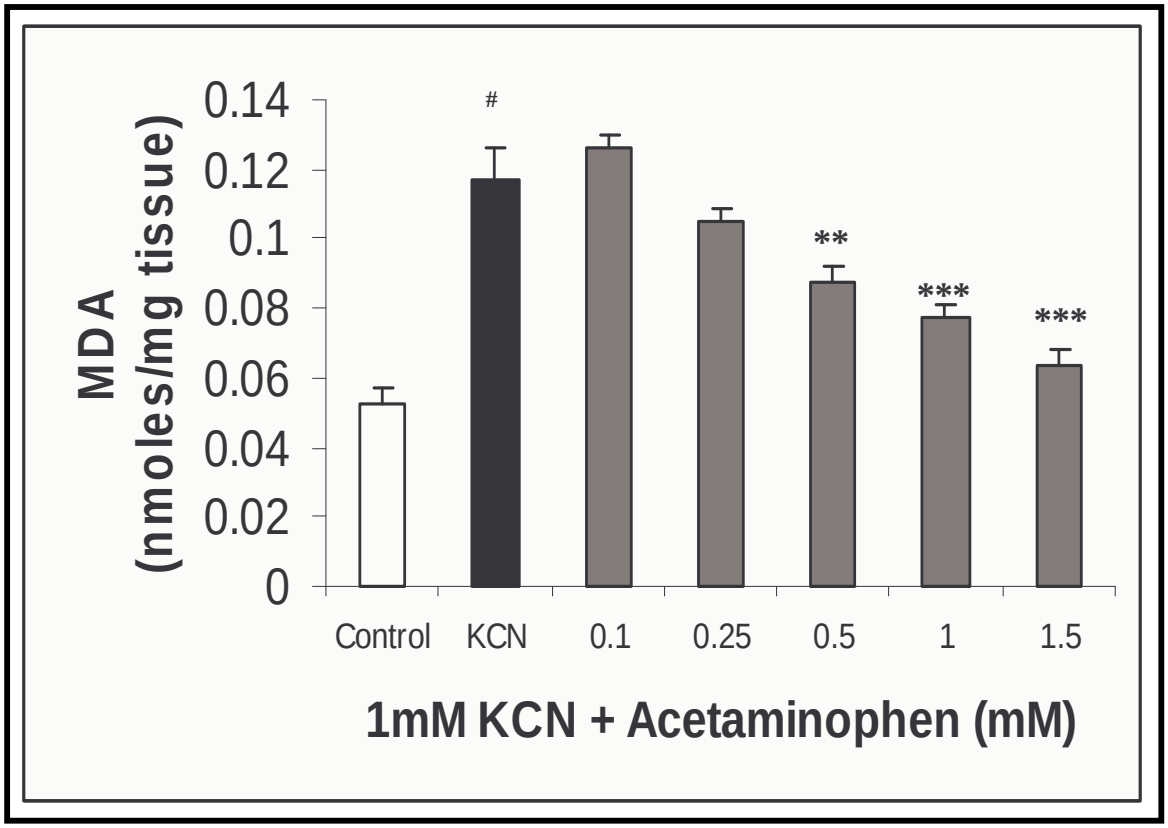


Figure 6.5. The effect of varying concentrations of Acetaminophen on 1mM KCN-induced lipid peroxidation in rat whole brain homogenate *in vitro*. Each bar represents the mean ±SEM; n=5. $^{\#}$(p<0.001) vs. control, ***(p<0.001) vs. KCN and **(p<0.01) vs. KCN. Student Newman-Keuls Multiple Range Test.

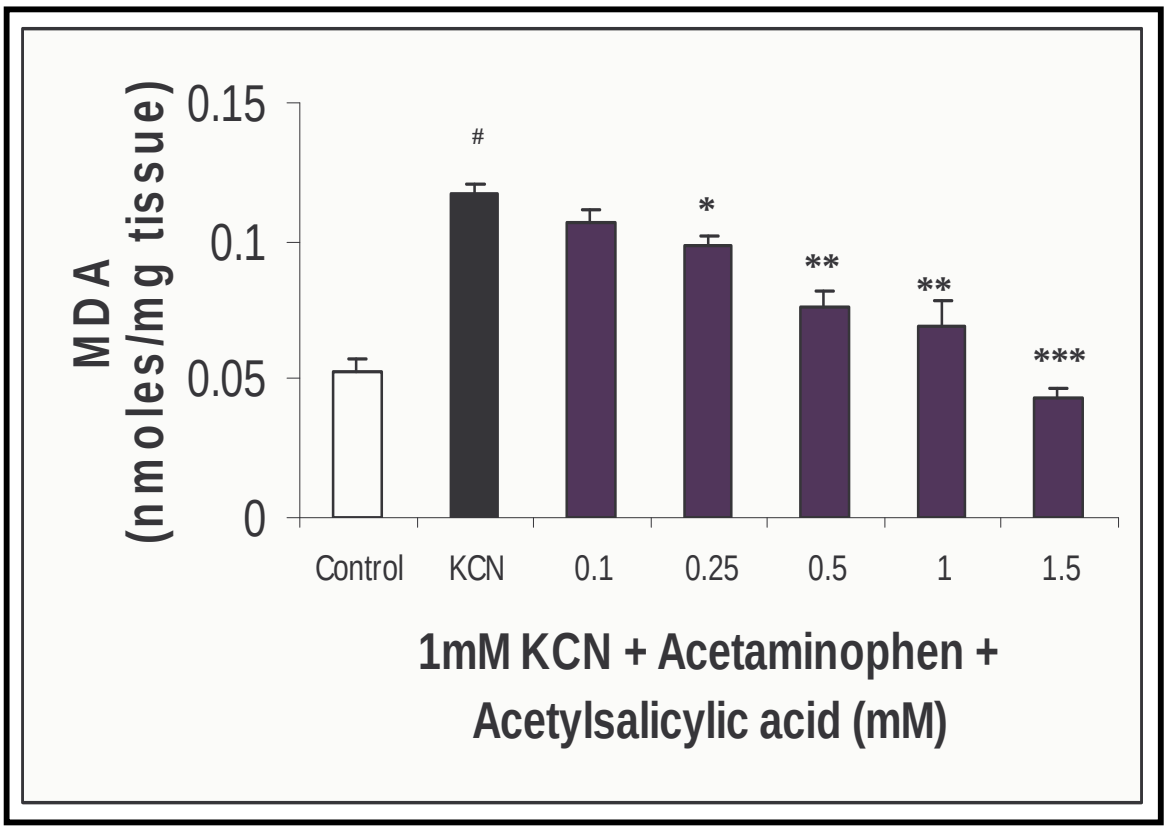


Figure 6.6. The effect of varying concentrations of Acetaminophen and Acetylsalicylic acid on 1mM KCN-induced lipid peroxidation in rat whole brain homogenate *in vitro*. Each bar represents the mean ±SEM; n=5. $^{\#}$(p<0.001) vs. control, ***(p<0.001) vs. KCN, **(p<0.01) vs. control and *(p<0.05) vs. control. Student Newman-Keuls Multiple Range Test.

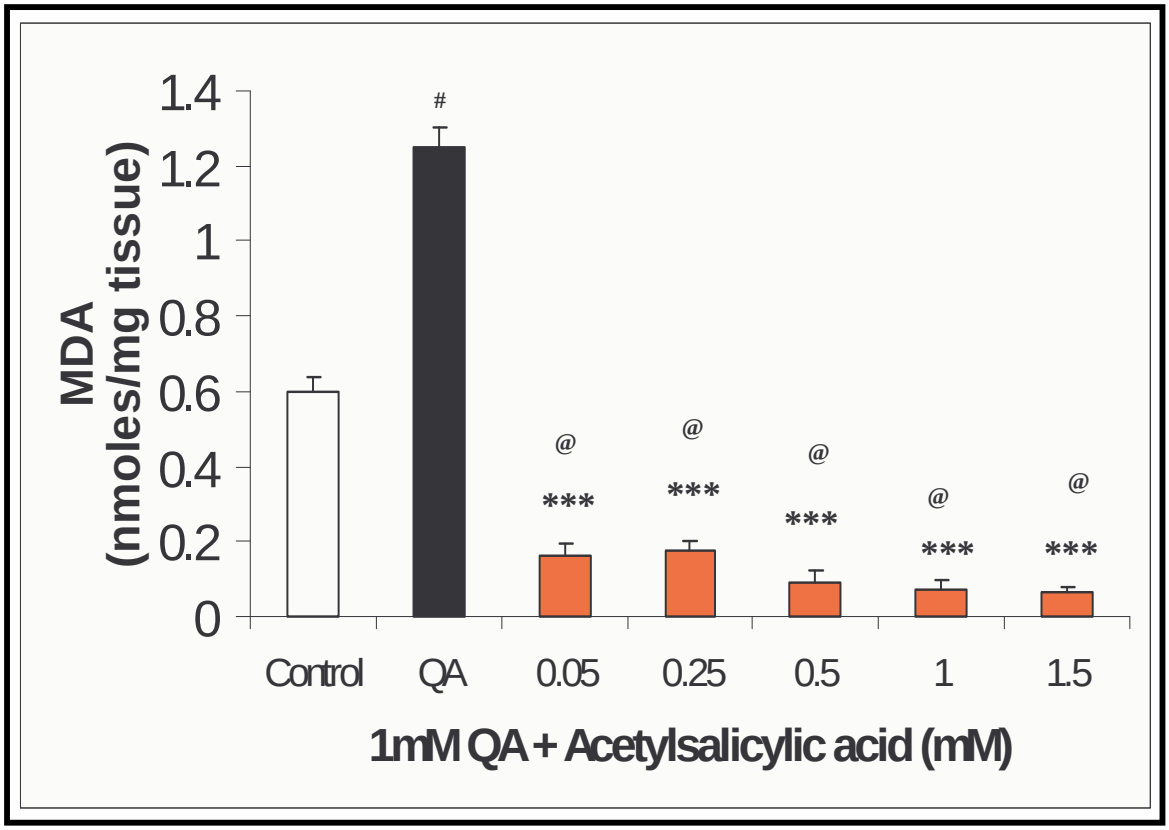


Figure 6.7. The effect of varying concentrations of Acetylsalicylic acid on 1mM QA-induced lipid peroxidation in rat whole brain homogenate *in vitro*. Each bar represents the mean ±SEM; n=5. $^{\#}(p<0.001)$ vs. control, ***($p<0.001$) vs. QA and $^{@}(p<0.001)$ vs. control. Student Newmans-Keuls Multiple Range Test.

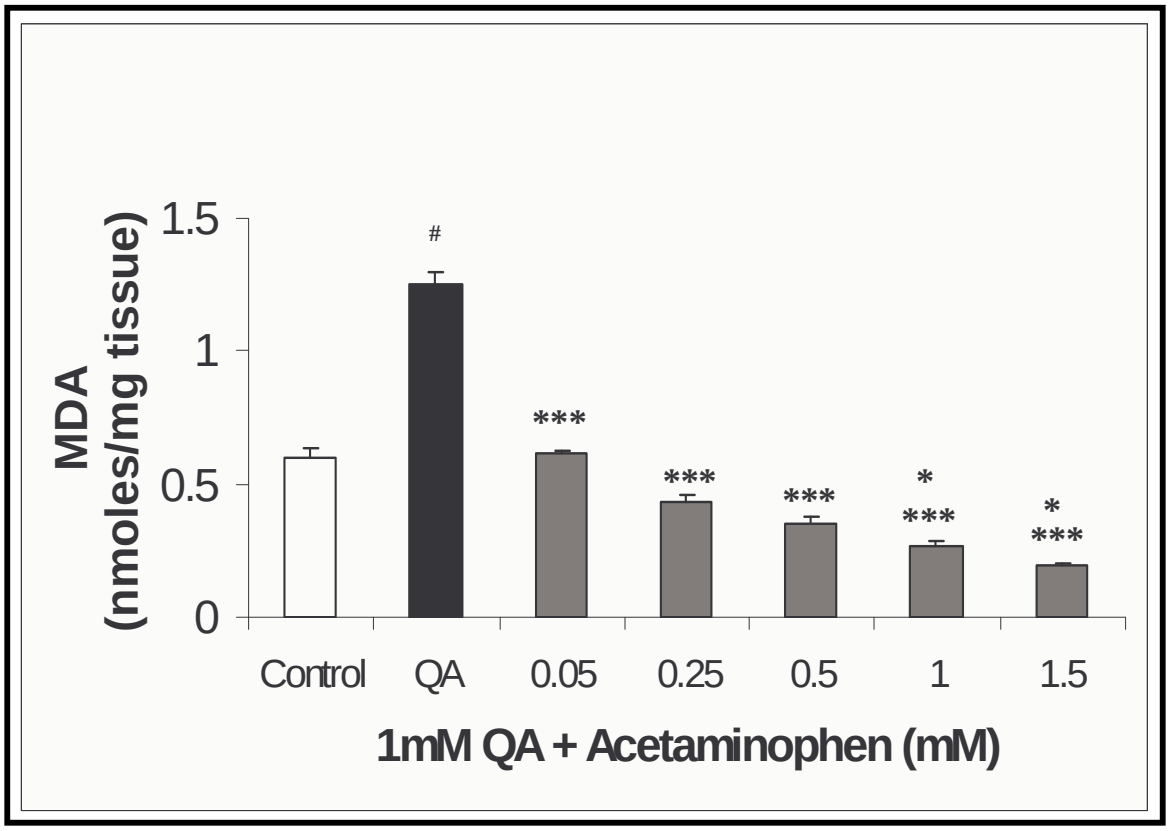


Figure 6.8. The effect of varying concentrations of Acetaminophen on 1mM QA-induced lipid peroxidation in rat whole brain homogenate *in vitro*. Each bar represents the mean ±SEM; n=5. $^{\#}$(p<0.001) vs. control, ***(p<0.001) vs. QA and *(p<0.05) vs. control. Student Newman-Keuls Multiple Range Test.

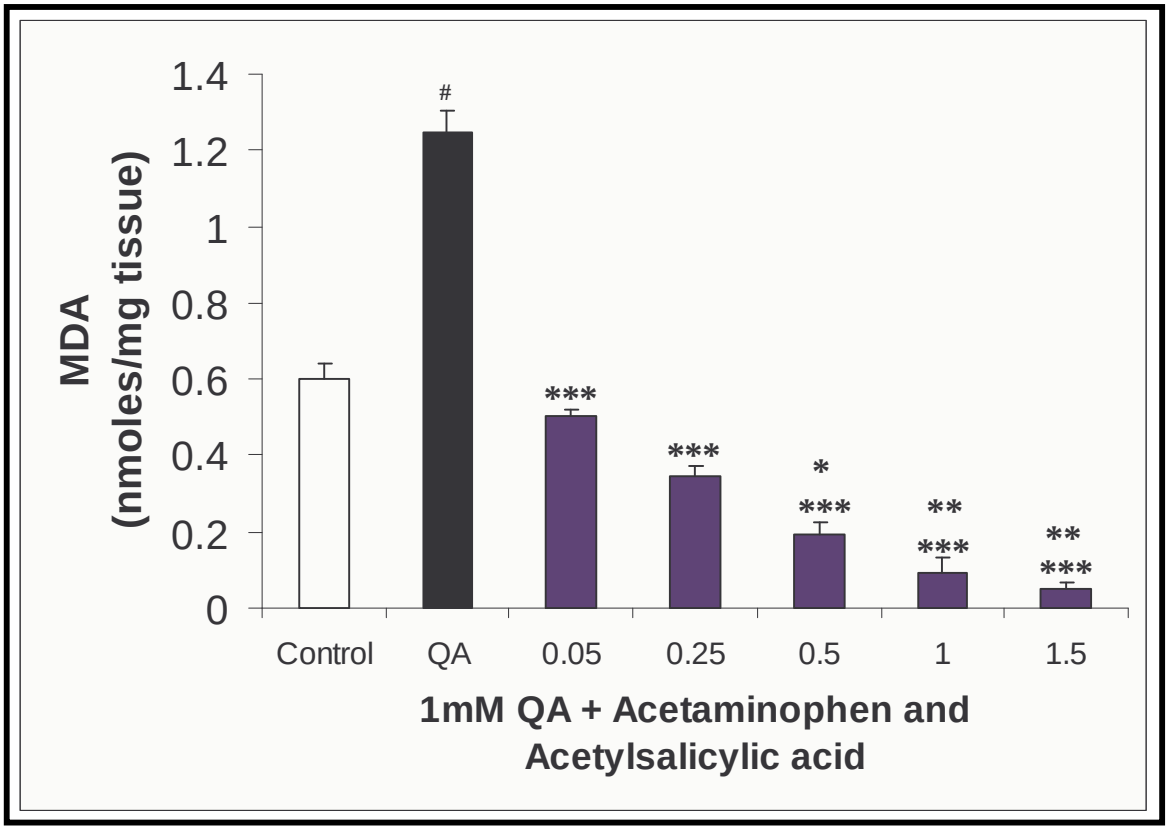


Figure 6.9. The effect of different concentrations of Acetaminophen and Acetylsalicylic acid on 1mM QA-induced lipid peroxidation in rat whole brain homogenate *in vitro*. Each bar represents the mean ±SEM; n=5. $^{\#}(p<0.001)$ vs. control, ***($p<0.001$) vs. QA, **($p<0.01$) vs. control and *($p<0.05$) vs. control. Student Newman-Keuls Multiple Range Test.

6.4. EFFECT OF ACETYLSALICYLIC ACID AND ACETAMINOPHEN IN QA-INDUCED LIPID PEROXIDATION IN RAT HIPPOCAMPUS *IN VIVO*.

6.4.1. INTRODUCTION

Quinolinic acid is a neuroactive metabolite of the kynurenine pathway (Stone, 1993) that has been implicated in the etiology of human neurological diseases. Quinolinic acid is an endogenous glutamate agonist with selectivity for the NMDA receptor (Tsuzuki *et al*., 1989; McLarnon and Curry, 1990), which causes neuronal loss in vulnerable regions in the mammalian brain (Foster and Schwarcz, 1989).

It has been reported that QA activates the NMDA ion channel complex, followed by the cytosolic accumulation of free Ca^{2+} (Daniel, 1991), which is believed to promote lipid peroxidation (Gutteridge, 1977; Barbizhayev, 1988), which consequently induces nerve cell death (Keilhoff *et al*., 1990). Quinolinic acid is able to reproduce the pathological features of Huntington's disease, such as γ-aminobutyric acid (GABA) depletion and striatal spiny cell loss. Zaleska and Floyd (1985) found a linear relationship between a susceptibility of various rat brain regions to undergo lipid peroxidation *in vitro* and their content of endogenous iron. Iron plays a crucial role in initiating and propagating lipid peroxidation (Halliwell and Gutteridge, 1995).

The aim of this study is to investigate whether ASA and acetaminophen either individually or in combination could offer protection against lipid peroxidation following an intrahippocampal injection of QA.

6.4.2. MATERIALS AND METHODS

6.4.2.1. Chemicals and Reagents

As described in section 5.4.2.1.

6.4.2.2. Dosing of Animals

The animals were separated into five groups of five animals each. The animals in group 3, 4 and 5 received a dose of 100mg/kg/day of ASA, acetaminophen and a combination of ASA and acetaminophen in 100µl sweet oil, injected subcutaneously, 20 mins prior to intrahippocampal QA respectively. The animals in groups 1 and 2 received the vehicle for these drugs, viz. sweet oil. On day one, 20mins after dosing the animals with the respective drug or vehicle, the animals of groups 2-5 were injected bilaterally into the hippocampal regions with QA. Quinolinic acid was dissolved in PBS made up to pH 7.4. A dose of QA (120nmol) was used to induce neurotoxicity as this concentration of QA is known to cause severe behavioural disturbances and total loss of hippocampal neurons (Lekieffre *et al*., 1990; Schwarcz *et al*., 1984).

Following the intrahippocampal injections of QA the animals, groups 3, 4 and 5 received subsequent daily doses of ASA, acetaminophen and a combination of ASA respectively. The injections were administered each day for seven days. The animals in groups 1 and 2 received daily doses of sweet oil for seven days.

6.4.2.3. Surgical Procedures

Rats were anaesthetized with diethelyether as described in chapter five, section 5.4.2.3.1.

6.4.2.3.1. Bilateral Intrahippocampal QA Injections

Quinolinic acid was dissolved in phosphate buffered saline (PBS), pH= 7.4. Quinolinic acid (120nmol in 2µl PBS) was infused bilaterally into the hippocampii employing a rat brain stereotaxic apparatus (Stoelting, IL, USA) (figure 8.8). The same procedure as described in chapter eight, section 5.4.2.3.2 was used.

6.4.2.3.2. Sham Lesioned Rats

The rats used as controls were subjected to the same surgical procedures described in section 5.4.2.3.3. However, bilateral stereotaxic injections into the hippocampal regions were free of QA and comprised solely of PBS.

6.4.2.3.3. Dissection of the Hippocampus

On the eighth day following the intrahippocampal injection of QA, the brains were removed as described in 5.4.2.4 and the hippocampii rapidly dissected according to a modified method of Glowinski and Iversen (1966).

6.4.2.4. Homogenate Preparation

Rats were sacrificed by cervical dislocation and then decapitated as described in appendix II. The brains were rapidly removed and placed on ice. Each brain was homogenized as described in section 6.2.2.5.

6.4.2.5. Lipid Peroxidation Assay

A modified method of Sagar *et al*., (1992) and Das *et al*., (1990) was used in this assay. The procedure as described in section 6.2.2.7 was followed. Final results were expressed

as nmoles MDA/mg tissue and analyzed for statistical significance as demonstrated in section 6.2.2.8.

6.4.3. RESULTS

The infusion of 120nmoles QA into the rat hippocampal region induced a significant rise in lipid peroxidation ($p<0.001$) when compared to the control as shown by figure 6.10. The intrahippocampal injection of QA resulted in a ±78% increase of MDA levels in comparison to the control.

As shown in figure 6.10, the postoperative treatment of QA lesioned rats, with ASA significantly attenuated the peroxidation effects of the neurotoxin in the rat hippocampus. Furthermore, ASA treatment was more effective in reducing the QA-induced lipid peroxidation in the rat hippocampal tissue in comparison to the acetaminophen treatment group ($p<0.05$).

Similar treatment with 100mg/kg acetaminophen also blocked the effects of QA in the rat hippocampus as shown in figure 6.10.

The combination of ASA and acetaminophen, which had a potentiating effect when compared to either agent used alone, also significantly inhibited QA induced *in vivo* lipid peroxidation, $p<0.001$ (figure 6.10.). In addition, the combination treatment was effective in reducing the MDA levels below basal control levels.

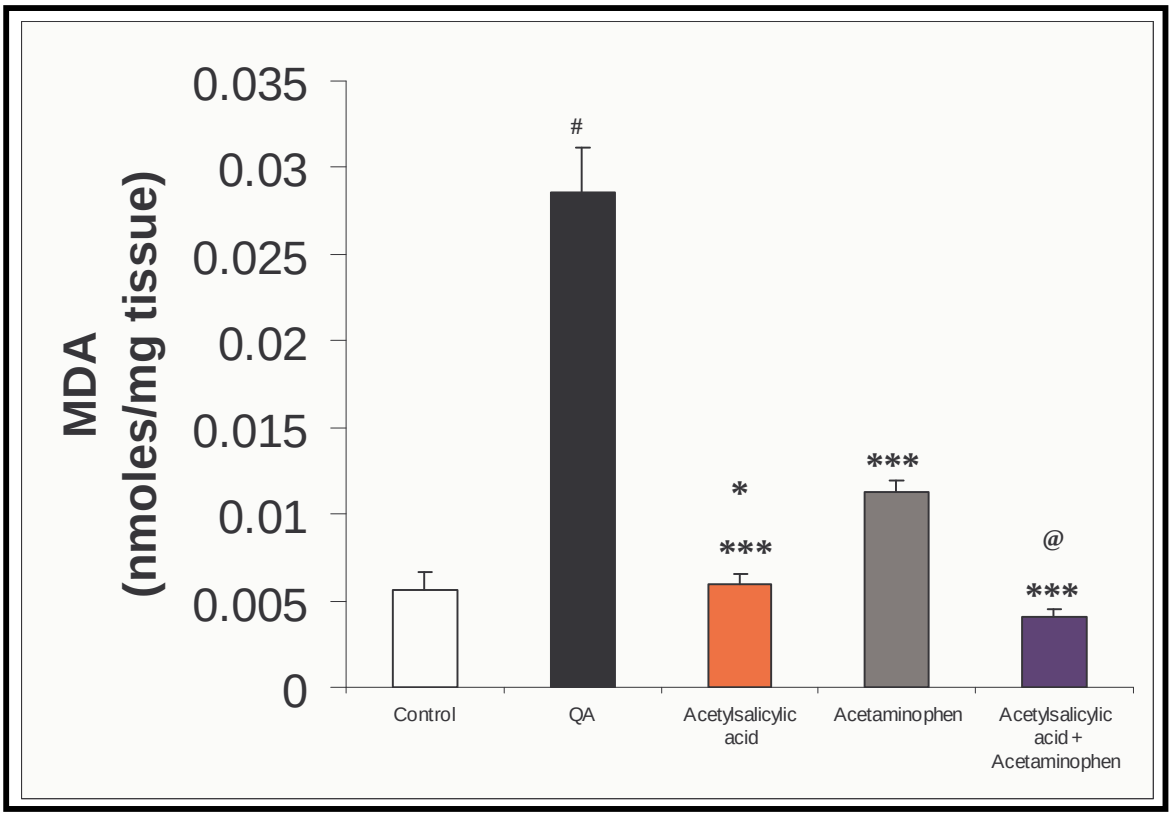


Figure 6.10. The effect of the *in vivo* administration of acetaminophen and acetylsalicylic acid alone or in combination on intrahippocampally injected QA-induced lipid peroxidation in rat hippocampal homogenate. Each bar represents the mean ± SEM; n=5; #p<0.001 in comparison to control, ***(p<0.001) in comparison to QA; *(p<0.05) in comparison to the acetaminophen group and $^{@}$(p<0.05) in comparison to the control value. Student-Newman-Keuls Multiple Range Test.

6.4.4. DISCUSSION

The administration of QA into the rat hippocampus resulted in an increase in the lipid peroxide product, MDA, in the rat hippocampus. The results clearly demonstrate the ability of QA to induce lipid peroxidation in rat brain homogenate. The potent *in vivo* induction of lipid peroxidation by QA is in agreement with the *in vitro* study discussed in section 6.2. which shows QA to induce severe lipid peroxidation in rat brain homogenate.

The results obtained from figure 6.10 correspond with the *in vitro* study, performed in section 6.3. and indicated that ASA and acetaminophen are capable neuroprotective agents against QA-induced lipid peroxidation *in vivo*.

Quinolinic acid is known to be an endogenous agonist of the NMDA receptor, which causes neuronal damage due to an influx of calcium into the cell, resulting in the production of free radicals. It is therefore possible that these drugs are preventing lipid peroxidation via a free radical scavenging mechanism.

Acetylsalicylic acid is a non-narcotic analgesic drug with a wide spectrum of pharmacological activities and multiple sites of action. Data from recent studies have suggested mechanisms of action when explaining the neuroprotective effects of ASA. These mechanisms include the reduction of oxidative stress (Pekoe *et al*., 1982a;b) and the inhibition of the activation transcription factor, NF–κB (Kopp and Gosh, 1994; Grilli *et al*., 1996). It has also been reported that ASA inhibits oxygen-glucose deprivation–induced glutamate release by recovering the fall of ATP levels in cortical neurones (De CristÓbal *et al*., 2001).

Furthermore it has been shown that ASA rectifies calcium (Ca^{2+}) homeostasis and therefore decreases ROS in human endothelial cells, *in vitro* (Simionescu *et al*., 2004). The alteration in Ca^{2+} levels by QA which results in the generation of ROS could therefore be attenuated by ASA which would also explain the potent action exhibited by this agent in figure 6.10.

In addition, Stipêk *et al* (1997) demonstrated that quinolinate does not have a direct peroxidative effect, but that it modulates lipid peroxidation via its interaction with Fe^{2+}. Acetylsalicylic acid has been shown to complex with Fe^{2+} and Fe^{3+} (Kotrly and Sucha, 1985). Thus, the chelation of ASA with iron would serve to further explain the protective effects of this agent against QA-induced lipid peroxidation.

At this juncture it is of interest to mention that, the mode of action of ASA in prevention of stroke is due to irreversible inhibition of the enzyme COX-1 by acetylation in platelets, resulting in blockade of thromboxane A_2 production (Patrono, 1994). Furthermore, COX-1 inhibition has been shown to be conducive to attenuating the $^{\bullet}OH$ free radical production during the metabolism of arachadonic acid, which is released from membrane phospholipids under conditions of neuronal death. $^{\bullet}OH$, as mentioned in section 6.1, play an important role in the peroxidation of lipids and thus COX-1 inhibition may be the primary mechanism by which ASA prevents QA induced lipid peroxidation (Patrono, 1994).

These results provide novel information regarding acetaminophen antioxidant activity with one other report of such activity, wherein acetaminophen had been shown to attenuate glutamate induced neurotoxicity in cultured primary rat embryonic neurons from mesencephalon (Casper *et al*., 2000). Furthermore, the potent inhibition of lipid peroxidation by ASA provides novel knowledge on the mechanism by which this agent can offer neuroprotection. The actions of these agents as free radical scavengers in the brain provides an alternative use of these, commonly used drugs in neurodegenerative disorders such as PD.

6.5. EFFECT OF ACETYLSALICYLIC ACID AND ACETAMINOPHEN IN MPP^+ INDUCED LIPID PEROXIDATION IN RAT STRIATUM *IN VIVO*.

6.5.1. INTRODUCTION

Parkinson's disease is a progressive neurodegenerative disease characterized by the loss of dopaminergic neurons in the nigrostriatal pathway. Several factors including inflammation are believed to be involved in the pathogenesis of this disease. It has been demonstrated that the enzyme cyclooxgenase (COX) as well as inflammatory mediators are increased in PD (Iravani *et al*., 2002; Knott *et al*., 2000). In the MPP^+ animal model of PD it has been shown that inflammatory mechanisms contribute to the neuronal damage. There have been conflicting reports as to whether COX inhibitors are effective in protecting neurons against MPP^+ -induced neurotoxicity (Aubin *et al*., 1998; Thomas and Mohanakumar, 2000).

Oxidative stress, with an increased production of activated oxygen species, such as superoxide anion and $^{\bullet}OH$ radicals, has also been suggested to play an important role in nigral neuron death in PD. Oxidative stress in nigral neurons in patients with PD increases iron content (Dexter *et al*., 1989), SOD activity and lipid peroxidation of neuronal cells but decreases the levels of the reduced form of glutathione (GSH). Thus, oxygen free radicals, especially $^{\bullet}OH$ may be a causative factor in the onset and/or progression of PD and may finally contribute to neuronal cell death (Dexter *et al*., 1989).

The aim of this experiment is to therefore demonstrate the induction of lipid peroxidation by MPP^+ and to determine if ASA and acetaminophen are capable of preventing the MPP^+-induced lipid peroxidation in rat striatum *in vivo*.

6.5.2. MATERIALS AND METHODS

6.5.2.1. Chemicals and Reagents

As described in section 5.5.2.1.

6.5.2.2. Dosing of Animals

Adult male rats of the Wistar strain were housed in separate cages, in a controlled environment as described in appendix I. The animals were separated into five groups of five animals each. Animals were dosed as described 5.5.2.2.

6.5.2.3. Surgical Procedures

Rats were anaesthetized with diethyl ether as described in chapter five, section 5.4.2.3.1.

6.5.2.3.1. Unilateral Intrastriatal Injections

Adult male Wistar rats were administered intratstriatal injections of MPP^+ as described in section in chapter 5, section 5.5.2.3.2.

6.5.2.3.2. Sham Lesioned Rats

Sham lesioned rats were subjected with surgical procedures as described in section 5.5.2.3.3.

6.5.2.3.3. Dissection of Rat Striatum

Striatum was removed as described in section 5.5.2.4.

6.5.2.4. Lipid Peroxidation Assay

A modified method of Sagar *et al*., (1992) and Das *et al*., (1990) was used in this assay. The procedure as described in section 6.2.2.7 was followed. Final results were expressed as nmoles MDA/mg tissue and analyzed for statistical significance as demonstrated in section 6.2.2.8.

6.5.3. RESULTS

The results obtained from figure 6.11 illustrates that the potent parkinsonian, neurotoxin MPP^+ induced a significant rise ±73%, in lipid peroxidation in rat striatum when compared to the control group (figure 6.11).

ASA was administered following MPP^+ infusion in the adult male rats and, as shown in figure 6.11, the non-narcotic analgesic completely blocked the potent lipid peroxidation effect of the toxin in rat striata as there was no significant difference in the MDA concentration obtained for ASA treatment group and the control group.

Figure 6.11, also demonstrates the effectiveness of acetaminophen at attenuating the lipid peroxidation when compared to the toxin group.

The combination of the ASA and acetaminophen significantly reduced MPP^+-induced lipid peroxidation as shown in figure 6.11 ($p<0.001$).

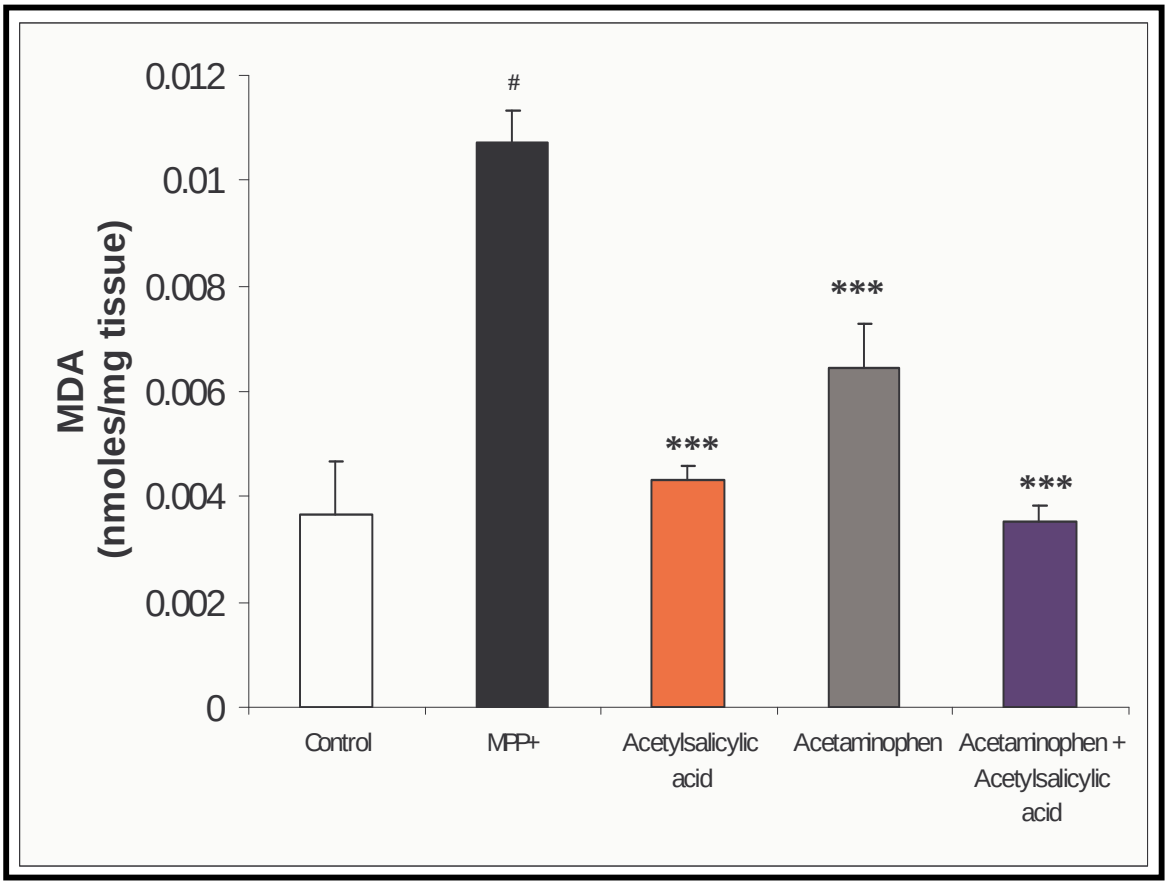


Figure 6.11. The effect of the *in vivo* administration of acetaminophen and acetylsalicylic acid alone or in combination on intrastriatal injected MPP^+-induced lipid peroxidation in rat striatal homogenate. Each bar represents the mean ± SEM; n=5; #p<0.001 in comparison to control and ***p<0.001 in comparison to MPP^+. Student-Newman-Keuls Multiple Range Test.

6.5.4. DISCUSSION

The important observations made from this study are the significant induction of lipid peroxidation in rat striatum caused by the intrastriatal administration of MPP^+ and the potent antioxidant activity of the non-narcotic analgesics, ASA and acetaminophen, in attenuating the lipid peroxidation induced by MPP^+. The neuroprotection offered against MPP^+, *in vivo*, by these two agents has not been previously reported and is therefore novel.

These results also indicate that the neuroprotective effects of these agents against MPP^+-induced lipid peroxidation are independent of MAO inhibitory activity since MPP^+ was administered directly into the rat striatum.

Generation of free radicals is considered to be one of the major factors in the pathogenesis and progression of PD (Adams and Odunze, 1991; Chiueh *et al*., 1994). Primary cause of dopaminergic neuronal death in the substantia nigra following MPP^+ exposure has been reported to be the generation of the $^{\bullet}OH$ radical (Chiueh *et al*., 1994; Obata, 2002). The use of antioxidants, such as L-deprenyl and pargyline, has been reported to protect dopaminergic neurons against MPTP or MPP^+-induced neurodegeneration (Leret *et al*., 2002; LeWitt, 1994).

Kalivendi *et al*. (2003) demonstrated that Fe^{2+} plays an important role in MPP^+-induced $^{\bullet}OH$ formation which ultimately results in lipid peroxidation as shown in figure 1.17. Blum *et al* (2001) reported that MPTP/MPP^+ administration results in enhanced Fe^{2+} levels in the brain which propagates the formation of ROS resulting in neurodegeneration. Since ASA has been shown to chelate with Fe^{2+} and Fe^{3+} ions (Kotrly and Sucha, 1985), it can be postulated that the binding of ASA with iron could be responsible for the results obtained from this study.

Acetylsalicylic acid and acetaminophen have been shown, in the present study, to possess potent antioxidant activity at inhibiting MPP^+-induced lipid peroxidation in rat striatum.

There is therefore potential that these drugs may have an important role to play at inhibiting the progression of the pathogenesis of PD since free radicals are widely implicated in neurodegeneration in PD.

6.6. CONCLUSION

The results obtained from this chapter would serve to encourage the use of these agents as free radical scavengers in neurodegenerative disorders such as Alzheimer's disease and PD since ASA and acetaminophen are capable of reducing the effect of the toxic free radical inducers i.e. QA and MPP^+, respectively. This study provides substantial and novel information regarding the potential of these agents as free radical scavengers in animal models mimicking neurodegenerative disorders. Furthermore ASA has been shown to have neuroprotective properties however the mechanism by which this has been achieved is controversial. These results show ASA to be a potent inhibitor of lipid peroxidation in the presence of QA thus maintaining structural integrity of neuronal cells.

The use of commonly used non-narcotic analgesics, such as ASA and acetaminophen, in MPP^+ induced PD would be a novel and cost effective approach in treating this neurodegenerative disorder. ASA and acetaminophen have been shown to be potent inhibitors of MPP^+-induction of free radicals in the rat striatum. The potential therapeutic benefits that these agents may possess in preventing MPP^+-induced neurotoxicity were investigated in further chapters.

Thus the investigation into the neuroprotective effect of these agents was taken further by performing histological and immunohistochemical studies in chapters seven and eight respectively.

CHAPTER SEVEN

HISTOLOGICAL STUDIES

7.1. INTRODUCTION

Histology is derived from the Greek word *histos* for web or tissue, and *logia*, meaning "the study of" or knowledge and involves the examination of preserved, sectioned and stained tissues. It therefore refers to the science of tissues of both plant and animal. Vast knowledge of the internal structures of tissue from plants and animals has been gained from this field of study (Hodgson & Bernard, 1992). There are two basic types of cells within the nervous system, which are referred to as neurons and supportive cells.

Neurons are highly specialized cells that easily conduct nerve impulses and are easily excited to produce them. A typical neuron exhibits a large cell body with a large central nucleus and many cytoplasmic extensions of which there are two types: dendrites and axons. These features are also visible on a stained section of nervous tissue observed under the microscope depicted in figure 7.1. Nervous tissues have a wide distribution throughout the body, innervating most visceral and peripheral tissues.

The neuronal damage that characterizes neurodegenerative disorders such as AD and PD is believed to result from, in part, over-activation of glutamate receptors such as the NMDA, which leads to a rise in intracellular Ca^{2+}, which promotes cell damage by both activating destructive enzymes and the generation of reactive oxygen species. Quinolinic acid, which is relatively selective for the NMDA receptor (Tsuzuki *et al*., 1989; Mclarnon and Curry, 1990), causes neuronal loss in vulnerable regions in the mammalian brain (Foster and Schwarcz, 1989). When introduced directly into the mammalian CNS, QA is a potent excitotoxin acting through excitatory amino acid receptors to cause neuronal excitation (Stone and Perkins, 1981), seizure activity (Lapin, 1981), and neuronal degeneration (Schwarcz *et al*., 1984).

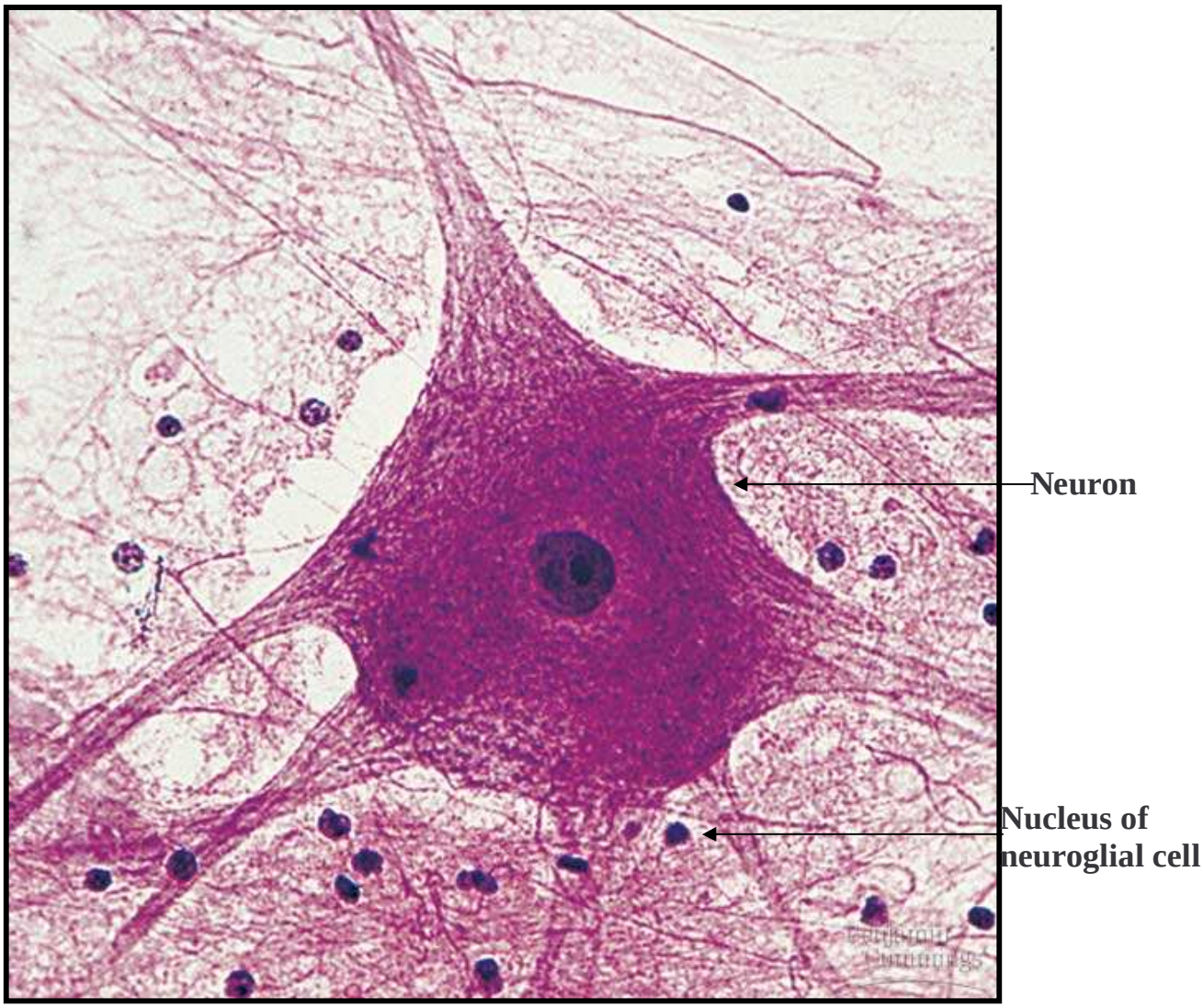


Figure 7.1. A high power magnified section of nervous tissue showing large star shaped neuronal cell body and the nerve cell processes (35.9.122.184/.../ 40-03-Neuron.jpg).

In chapter six, it was shown that ASA, acetaminophen and the combination of these agents, protects against QA-induced lipid peroxidation both *in vivo* and *in vitro*. Since, these studies were only a measure of the MDA, which is a degraded lipid product, and even though the decline in the formation of this lipid peroxidation product is indicative of decreased cell damage, it is still important to examine the structural changes in cells following QA administration and treatment with ASA and acetaminophen.

Histology

The present study aims to examine the hippocampal neurons following an intrahippocampal injection of QA, and treatment with subcutaneous injections of the ASA and acetaminophen either alone or in combination. Two different histological techniques were employed and the hippocampal neuronal cells were examined under a light microscope attached with an Olympus camera.

7.2. HISTOLOGICAL ANALYSIS OF THE EFFECT OF ACETYLSALICYLIC ACID AND ACETAMINOPHEN AGAINST QA-INDUCED NECROTIC DAMAGE TO HIPPOCAMPAL NEURONS USING CRESYL VIOLET STAIN.

7.2.1. INTRODUCTION

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. This stain is extremely useful since it distinguishes neurons and glia from one another and allows histologists to study the arrangement or cytoarchitecture of neurons in different parts of the brain (Bear *et al*., 2001).

This study was performed to investigate the hippocampal neuronal structural events that intrahippocampal injections of QA induce in the CA1 and CA3 regions of the hippocampus. Acetylsalicylic acid and acetaminophen has been shown to afford protection against QA mediated oxidative stress in chapters five and six, it was decided to determine whether ASA and acetaminophen offers neuroprotection against QA-induced induced intrahippocampal lesions in the rat brain. After treatment, the brains of the rats were sectioned and the hippocampus was stained with cresyl violet stain and examined microscopically to detect changes in neuronal structure and size, for evidence of any morphological changes.

7.2.2. MATERIALS AND METHODS

7.2.2.1. Chemicals and Reagents

Quinolinic acid was purchased from Sigma St. Louis, MO, U.S.A. Paraffin wax was obtained from Lasec (South Africa). Cresyl violet stain was purchased from BDH Chemicals Ltd (England), while DPX was purchased from Philip Harris Ltd (England). Haupt's adhesive consisted of the following: 1g gelatine, 100ml water, 2g phenol and 15ml glycerol. All other chemicals were of the highest quality available and were purchased from commercial distributors.

7.2.2.2. Animals

Adult male rats of the Wistar strain housed in separate cages, in a controlled environment as described in appendix I.

7.2.2.3. Surgical Procedures

The injection of QA into the hippocampus was carried out as in chapter five, section 5.4.2.3.2.

7.2.2.4. Treatment Regime

The rats were dosed s.c. as described in chapter five, section 5.4.2.2. Care was taken to rotate the injection site as to allow for optimum absorption of the drug.

7.2.2.5. Histological Techniques

The histological techniques were performed according to the methods described by Southgate (1999).

7.2.2.5.1. Fixation of brain tissue

The animals were sacrificed and the brains removed as described in appendix II. Neural tissues are extremely fragile and easily subjected to rapid anoxic and postmortem changes. Immediately after death, animal tissues begin to break down as a result of autolysis and bacterial attack. To prevent this, immediate fixation of these tissues is required (Chang, 1995). This process usually entails submerging the tissues in stabilizing or cross– linking agents or perfusing them with these substances in order to preserve as much as possible of the morphological and molecular characteristics. The role of the fixative is to maintain the morphology of the tissues as close to *in vivo* morphology as possible and to prevent post-sampling necrosis. A recommended fixative used for the study of brain tissue is Davidson's solution. The ratio of fixative to tissue volume should be at least 10:1 to ensure good fixation. Thus, fixation functions to chemically stabilize proteins, and thus preserve structures (Southgate, 1999).

There is no universal fixative and choice should be made taking into account later use of fixed material as well as practical aspects of fixative use (price, component availability, etc). Davidson's solution is an excellent choice for preserving the structure of the tissues (Lighter, 1996). In addition, tissue sections fixed with Davidson's solution can be stained later by different immunohistochemical methods, as well as *in situ* hybridization with DNA proteins. Davidson's alcohol formalin acetic acid fixative consists of 220ml of formalin (100%), 115ml of glacial acetic acid, 330ml of ethanol, and 335ml of water. This mixture is stored at room temperature prior to use (Lighter, 1996).

Brains were rapidly fixed in the Davidson's fixative mixture for 48 hours. After fixation, a slice of brain, 2mm thick was prepared to exclude the location of the injection track, which was normally apparent from the residual dimpling of the cortical surface produced by the needle penetration. Exclusion of the tissue directly below the site of the injection ensured that all damage was due to neurotoxin or drug and not due to the physical damage caused by the cannula needle. The 2mm block of brain tissues were then stored in 70% ethanol.

7.2.2.5.2. Specimen Preparation and Embedding

In order to be cut, the slices need to be supported. Embedding involves the infiltration and orientation of tissue in the paraffin wax support medium. Moisture was extracted from the tissue fragments by bathing them successively in a graded series of mixtures of ethanol. This step was followed by the clearing process, which involves the removal of ethanol by immersing the tissue in xylene twice for one hour each. The tissue was then submerged in molten paraffin wax at 57^0C twice for one hour each, which facilitated the removal of xylene and while infiltrating the tissue without encountering water. This stage provides the hardness and support that the tissue requires for sectioning. The method used is shown in Table 7.1

Table 7.1. Procedure for embedding brains in paraffin wax.

Step	Processing Agent	Time (Hours)
1	70% Ethanol	1
2	90% Ethanol	1
3	Absolute Ethanol	1
4	Absolute Ethanol	1
5	Xylene	1
6	Xylene	1
7	Melted Paraffin Wax	1
8	Melted Paraffin Wax	1

7.2.2.5.3. Blocking Out

The brain material was fixed into a block and this procedure was performed in order to form a support that would facilitate sectioning using the rotary microtome. The mould used was a plastic ice tray and this was coated with ethanol-glycine to prevent the block sticking to the mould. The brain was removed from the final molten wax stage (previous section) and placed into the mould with warmed forceps. The brain was then completely

covered in molten wax. Air was gently blown over the surface of the wax until the top solidified. The 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.

7.2.2.5.4. Sectioning

Sectioning is a technique performed using a microtome. This is an instrument, which consists of a sharp metal knife held in a fixed position, and a chuck in which a block of wax with the tissue is held. Depending on the type of microtome, a particular mechanism oscillates the chuck up and down and with each oscillation; the chuck is brought closer to the knife by a fixed distance. In this way sections are cut from the wax block (Hodgson & Bernard, 1992). The wax block was trimmed with a razor blade so that two of the sides were parallel, while the other two converged slightly (figure 7.2). The sides were cut so as to leave about 2 mm of wax around the tissue. The wax block was attached to a small wooden block with a small amount of molten wax.

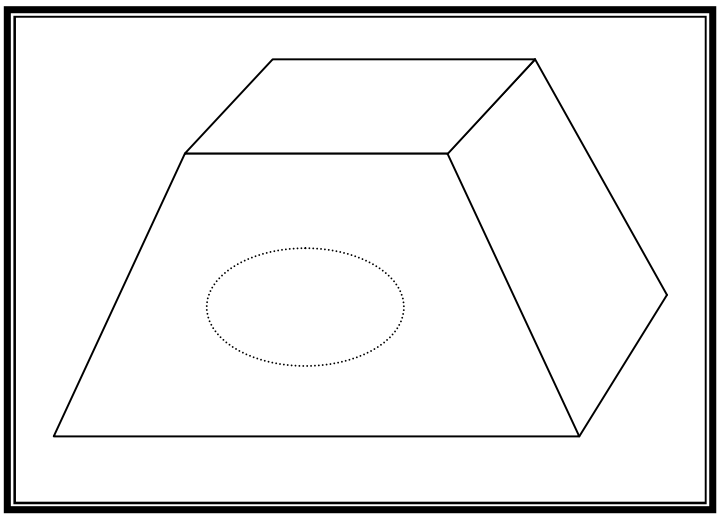


Figure 7.2. Diagram of wax block ready for sectioning with rat brain embedded in the centre (Southgate, 1999)

Sectioning was done using a RMC MT-7 rotary microtome. The microtome was set to cut sections of 10µm thickness. As sections were cut these would stick to one another, so as to form long ribbons. When the part of the brain containing the hippocampus was reached, every second section was removed and placed in a water bath (40° C) using forceps, which smoothens out the wrinkles.

7.2.2.5.5. Transferring Sections to Slides

Three sections at a time were removed from the water bath and placed onto glass microscope slides using a thin paintbrush. The glass slide was initially brushed with a thin layer of Haupt's adhesive before the sections were mounted. The slides were left overnight in an oven at 40^{o}C to enable the section to adhere to the slide.

7.2.2.5.6. Staining

This stain, stains the Nissl substances intense purple and the nuclei purple. The background is left clear (Bauer *et al*., 1974). Figure 7.3 shows a Nissl-stained coronal section through the caudal telencephalon of a rat brain depicting the hippocampus.

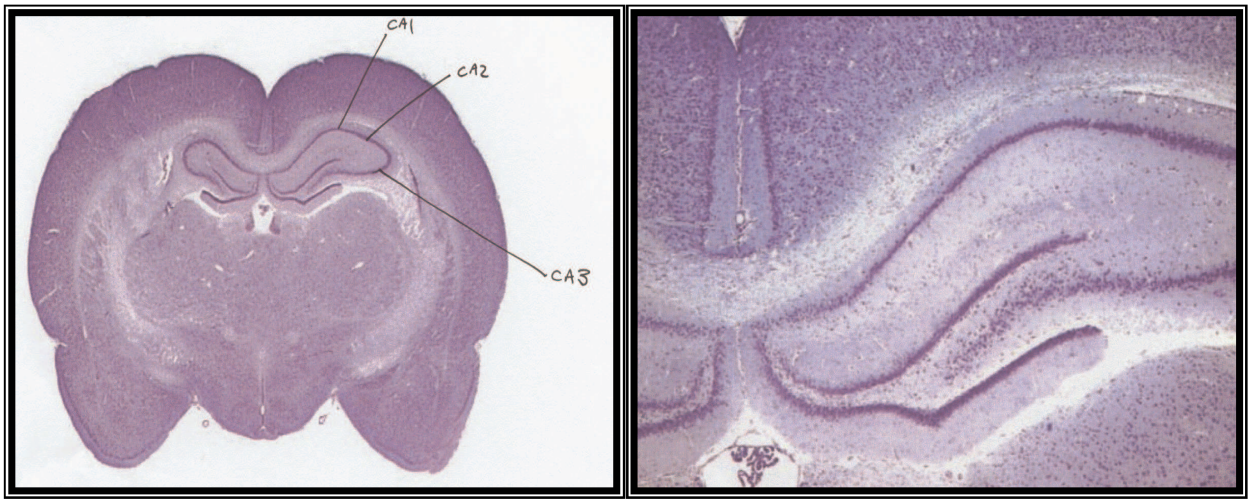


Figure 7.3. (Left) A coronal section through the caudal telencephalon of a rat brain displaying the hippocampal structure and three subdivisions. (Right) A magnified view of the rat hippocampus.

Since this dye is water soluble, the entire embedding process had to be reversed in order to remove the paraffin wax from the tissue and allow penetration of the dye. The paraffin was removed by running the slides through xylene twice for five minutes each, followed by immersion in a mixture of xylene and absolute ethanol (1:1) for three minutes. This step was followed by immersion in absolute ethanol for five minutes, and then re-immersion in absolute ethanol overnight. This was done as per Table 7.2.

Table 7.2. Procedure for dewaxing and rehydrating brain sections

Step	Processing Agent	Time (minutes)
1	Xylene (dewaxing)	5
2	Xylene	5
3	Xylene / Absolute Ethanol (1:1)	3
4	Absolute Ethanol	5
5	Absolute Ethanol	Overnight at 30°C

Sections were stained by placing in a 0.1% cresyl violet solution for 2 hours. The cresyl violet solution contained 0.25g cresyl violet, 250 ml Milli-Q water, 0.75 ml glacial acetic acid and 0.0512g sodium acetate. The pH was adjusted to 3.5 before use. The slides were differentiated rapidly in 95% ethanol by rinsing in a flat dish until the background was clear. Sections were then dehydrated again as shown in Table 7.3.

Table 7.3. Procedure for dehydrating brain sections after staining

Step	Processing Agent	Time (minutes)
1	Absolute Ethanol	5
2	Absolute Ethanol	5
3	Xylene	5
4	Xylene	5

7.2.2.5.7. Mounting of 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 like scratching, and to provide better optical quality for viewing under the microscope. While the slides were kept moist with xylene, enough DPX was added to just cover the tissue. A cover slip was placed over the tissue. The slides were allowed to dry on a flat surface for 48 hours.

7.2.2.5.8. Photo-microscopy

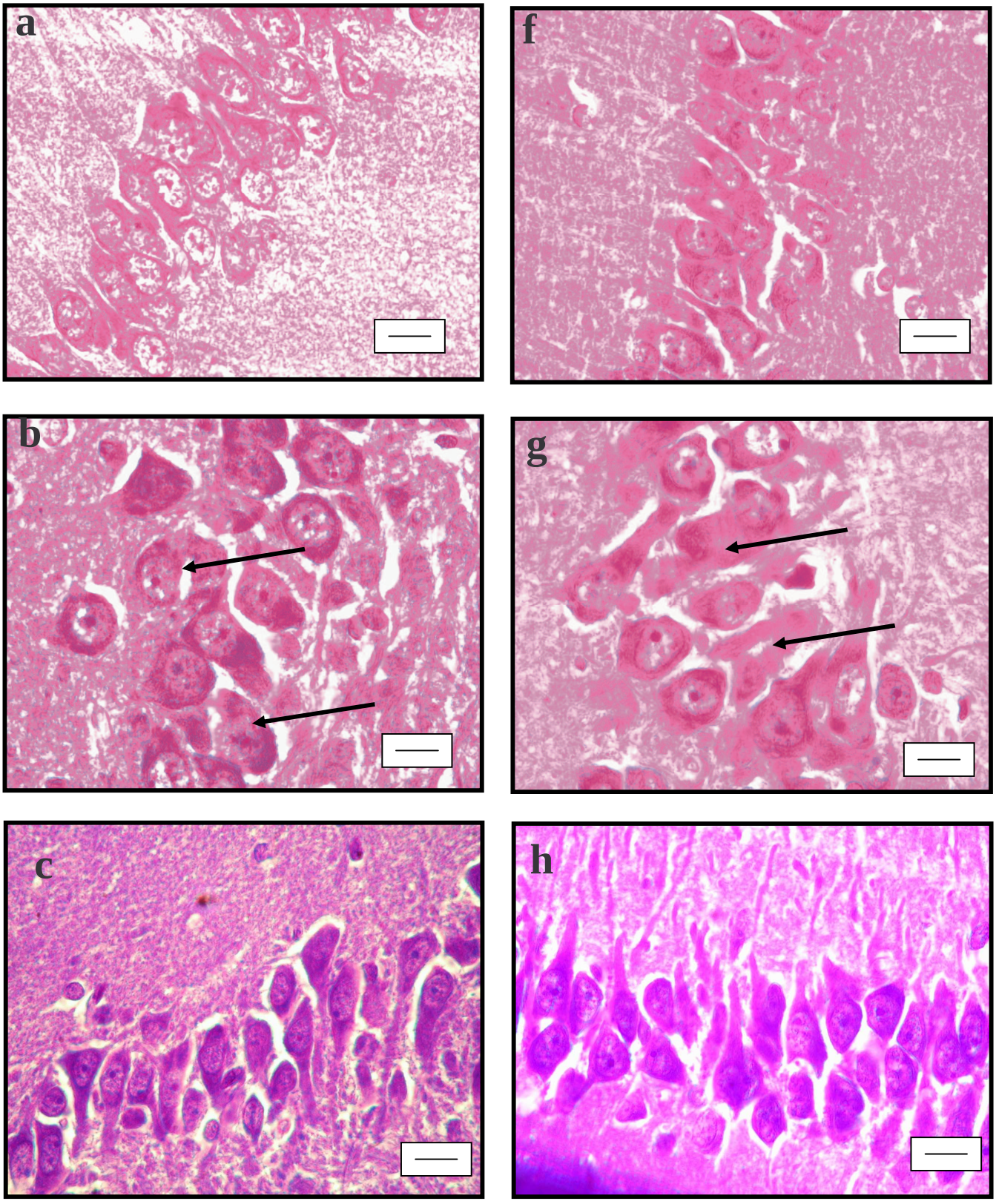
The slides were photographed using a combination Olympus camera and light microscope.

7.2.3. RESULTS

Neurons in the CA1 and CA3 regions of the hippocampus were examined microscopically. Sections of the CA1 and CA3 regions of the control treated rats (figure 7.4, photomicrographs a and f) showed optimally sized, pyramidal shaped neuronal cells with a clearly observable cell nucleus and continuous cell membrane. The cells are grouped closely together to form a band – like appearance, which is characteristic of both the CA1 and CA3 regions. Thus the neurons in both the CA1 and CA3 region appear to be undamaged. The neurons and nuclei were stained an intense purple colour while the background appeared light purple to a pink colour.

The hippocampal neuronal cells of both regions of the QA treated animals (figure 7.4 photomicrographs b and g) show extensive degeneration (as shown by the arrows). This is evident by virtue of their roundness and swelling. The hippocampal cells appear scattered with little integrity of cell membrane and appearance of dense nuclei. Necrosis of the neuronal cells in many areas is also evident (as indicated by arrows).

It is evident from figure 7.4 that the CA1 and CA3 regions of the rats treated with QA and with either ASA (photomicrographs c and h), acetaminophen (photomicrographs d and I) or the combination of these agents (photomicrographs e and j), that the neurons show significant protection in comparison to the neurons of the QA only treated rats. The cells maintained their structural integrity following exposure to QA and post surgical treatment with ASA and acetaminophen.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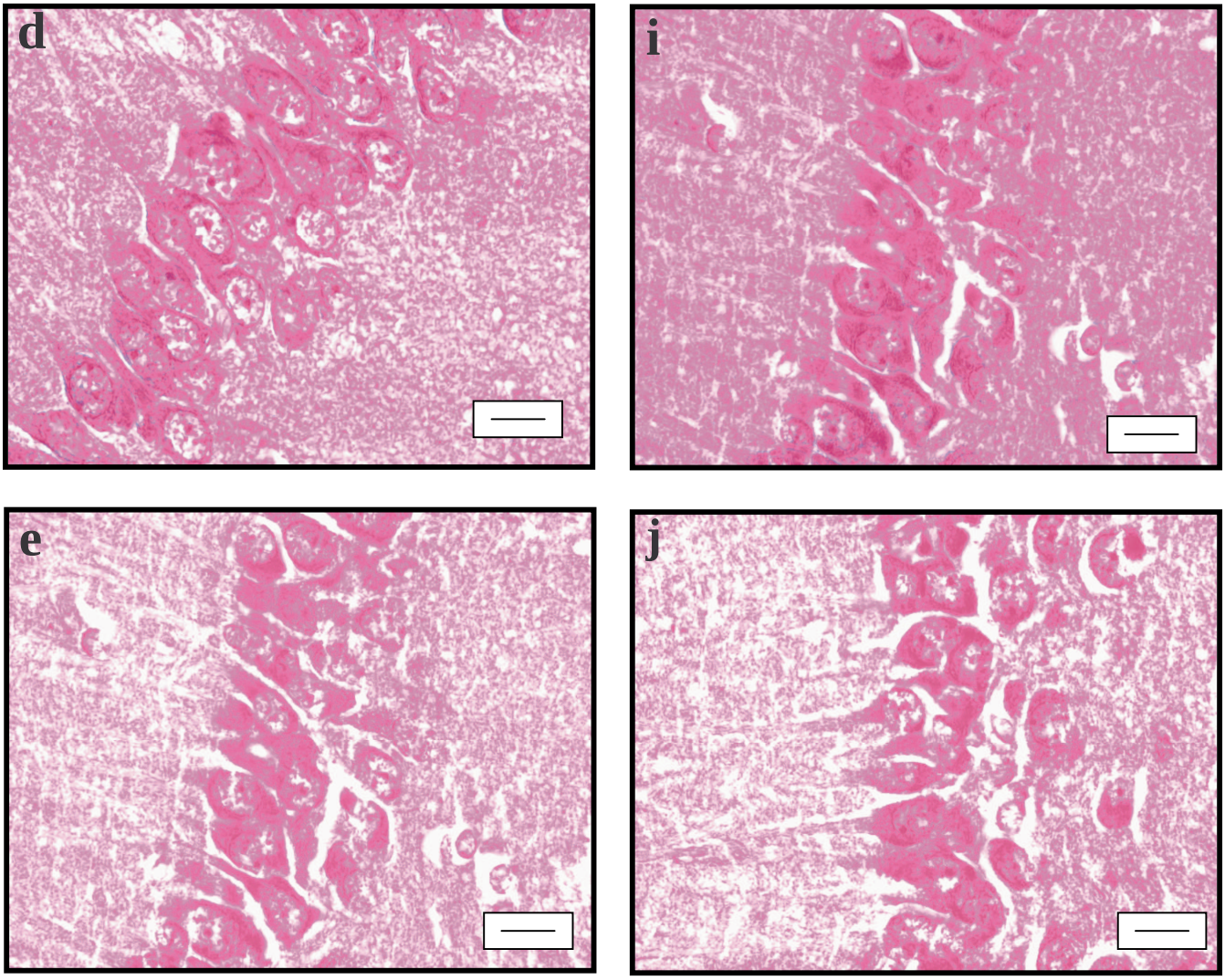


Figure 7.4. QA toxicity and the protective effects of acetylsalicylic acid and acetaminophen alone or in combination on rat hippocampal neurons using cresyl violet stain. Micrographs (a-e) indicate cells in the CA1 region of the hippocampus from a control animal (a), an animal treated with QA (b), animal treated with QA and acetylsalicylic acid (c), animal treated with QA and acetaminophen (d) and animal treated with QA and acetaminophen and acetylsalicylic acid (e). Micrographs (f-j) indicate cells in the CA3 region of the hippocampus from a control animal (f), an animal treated with QA (g), animal treated with QA and acetylsalicylic acid (h), animal treated with QA and acetaminophen (i) and animal treated with QA and acetaminophen and acetylsalicylic acid (j). Bar = 10µm.

7.2.4. DISCUSSION

The results of the present study clearly illustrate that QA is a potent neurotoxin, which damages the structural integrity of cells resulting in cell death. The cell damage induced by QA is dependent on the NMDA receptor. However, in order for NMDA receptors to be activated, another non-NMDA glutamate receptor such as AMPA must be activated. This activation results in an influx of Na^+ ions into the cell causing a depolarization of the membrane. The depolarization results in the removal of the magnesium block, allowing the opening of the channel once QA has bound. Once opened, Ca^{2+} ions move through and into the neuron. In cases where there are high concentrations of glutamate within the synapse, excessive activation of glutamate receptors occurs; resulting in a Ca^{2+} dependent rise in free radicals and acute toxicity follows. Acute toxicity occurs because of the rapid influx of Na^+ ions into the neurons, which causes passive water and Cl^- entry via osmotic pressure. This toxic process may be associated with abnormalities in membrane permeability and may be lethal, via osmotic lysis (Southgate, 1999).

The process that takes place may be direct, by over-stimulation of the neuron leading to prolonged depolarization and depletion of energy reserves, or exchange. In addition, the activation of the NMDA receptor by QA results in a further influx of Ca^{2+} ions. This together with the water uptake results in the swelling of the cells evident in the photomicrographs (figure 7.4 b and g).

The damage produced by QA is also partly dependent on the gliosis and inflammatory reaction, which occurs in response to excitotoxic challenge (Behan *et al.*, 1999). Activated microglia as well as activated macrophages which infiltrate the CNS in the aftermath of insults or lesions are known to produce ROS which could account for some of the neuronal damage *in vivo*. Quinolinic acid could act synergistically with the ROS produced from this source to cause damage, which is dependent on both the activation of NMDA receptors and the oxidative stress imposed by free radical generation. However, QA must also be able to generate ROS independently of such cells, in view of the $O_2^{-\bullet}$ generation and lipid peroxidation which was noted *in vitro* and *in vivo* in chapter five and

chapter six. The neurons damaged due to the cannula needle were excluded ensuring that all damage was of an excitotoxin-induced nature and not due to physical damage caused by the needle tract.

The results provide visual evidence that ASA and acetaminophen reduce QA damage in the hippocampus of the rat. These results also serve to confirm that free radical generation, as observed in chapters five and six, induced by QA plays an important role in cell degeneration. The ability of ASA and acetaminophen to maintain cell integrity further enhances the importance of the use of novel antioxidants in the treatment of neurodegenerative disorders such as AD and PD.

7.3. HISTOLOGICAL ANALYSIS OF THE EFFECT OF ACETYLSALICYLIC ACID AND ACETAMINOPHEN AGAINST QA-INDUCED NECROTIC DAMAGE TO HIPPOCAMPAL NEURONS USING ACID FUCHSIN STAIN.

7.3.1. INTRODUCTION

Acidophilia is considered as one of the hallmarks of acute neuronal damage and death in brain ischemia, excitotoxic and traumatic lesions and epileptic seizure (Victorov *et al*., 2000). Acidophilic ("red") neurons stain intensively with acidic (anionic) dyes, such as acid fuchsin. Histochemical analysis of the staining by these anionic dyes shows that nuclear and cytoplasmatic acidophilia of degenerating neurons is due to proteins rich in arginine and lysine (Kiernan *et al*., 1998). The principal features of damaged neurons expressing acidophilia were initially described by Spielmeyer (1922) in autopsy material, which included shrinkage of neuronal somata, deformation and displacement of strongly stained nuclei, chromatolysis, and acidophilia of the cytoplasm.

In this experiment it was decided to investigate whether ASA and acetaminophen either alone or in combination offer neuroprotection against QA -induced intrahippocampal toxicity in the rat hippocampus. After treatment, the brains of the rats were sectioned and the hippocampus was examined microscopically for evidence of acidophilic and hyperchromatic shrunken neurons.

7.3.2. MATERIALS AND METHODS

7.3.2.1. Chemicals and Reagents

Quinolinic acid, ammonium metavanadate, borax (sodium tetraborate), sodium acetate, acid fuchsin, and toluidine blue were purchased from Sigma St. Louis, MO, U.S.A. Acetic acid glacial was purchased from Saarchem (PTY) Ltd., Krugersdorp, South Africa. All other chemicals were of the highest quality available and were purchased from commercial distributors.

7.3.2.1. Solution Preparation

a) Ammonium metavanadate solution. 500mg of ammonium metavanadate was dissolved in 100ml of hot (80-90^{o}C) distilled water with constant stirring.
b) Vanadium acid fuchsin (VAF). 100mg of acid fuchsin was dissolved in 75ml of distilled water and to this solution, 25ml of 0.5% ammonium metavanadate solution and 1ml of glacial acetic acid was added.
c) Acetic acid –sodium acetate buffer (pH 3.3). 130mg of sodium acetate was dissolved in 100ml of distilled water; to this solution 1.2ml of glacial acetic acid was added.
d) Toluidine blue. 25mg of toluidine blue was dissolved in 100ml of acetic acid-sodium acetate buffer.
e) 0.01% solution of borax. This solution needs to be changed regularly.
f) Acid alcohol (1% hydrochloric acid in 70% alcohol).
g) 1% solution of sodium bicarbonate. This solution needs to be changed regularly.

7.3.2.3. Treatment Regime

Adult male rats of the Wistar strain housed in separate cages, in a controlled environment as described in appendix I. Surgical procedures were conducted according to the method described in 5.4.2.3, and the rats were dosed s.c. as described in section 5.4.2.2.

7.3.2.4. Histological Techniques

The histological techniques were followed according to the methods described by Victorov *et al*., (2000).

7.3.2.4.1. Fixing the brain

The animals were sacrificed and the brains removed as in section 2.2.2.4., and immediately fixed as described in section 7.2.2.5.1.

7.3.2.4.2. Specimen Preparation and Embedding

Dehydration of tissue fragments and embedding of tissue in paraffin wax was conducted as described in section 7.2.2.5.2.

7.3.2.4.3. Blocking Out

The brain material was fixed into a block as described in section 7.2.2.5.3.

7.3.2.4.4. Sectioning

Sectioning was done using a rotary microtome and 8-10 μm thickness sections were cut as described in section 7.2.2.5.4.

7.3.2.4.5. Transferring Sections to Slides

Sections placed onto glass microscope slides using a thin paintbrush as described in section 7.2.2.5.5.

7.3.2.4.6. Staining

Paraffin wax was removed from the tissue to allow penetration of the dye. The paraffin was removed by running the slides through xylene for five minutes, followed by dehydrating the slides in a graded ethanol solutions (100%, 96%, 70%) for 5 min each. Thereafter the slides were washed in distilled water. The slides were then stained with VAF for 1 min (maximum of 2 min) and thereafter rinsed in distilled water. The slides were then rinsed with 0.01% borax solution for 20-30sec until a light red colour of the section was obtained and then rinsed again in distilled water. The slides were immersed in acetate buffer for 30s and then counterstained with toluidine blue for 20-30s (maximum of 1 min).

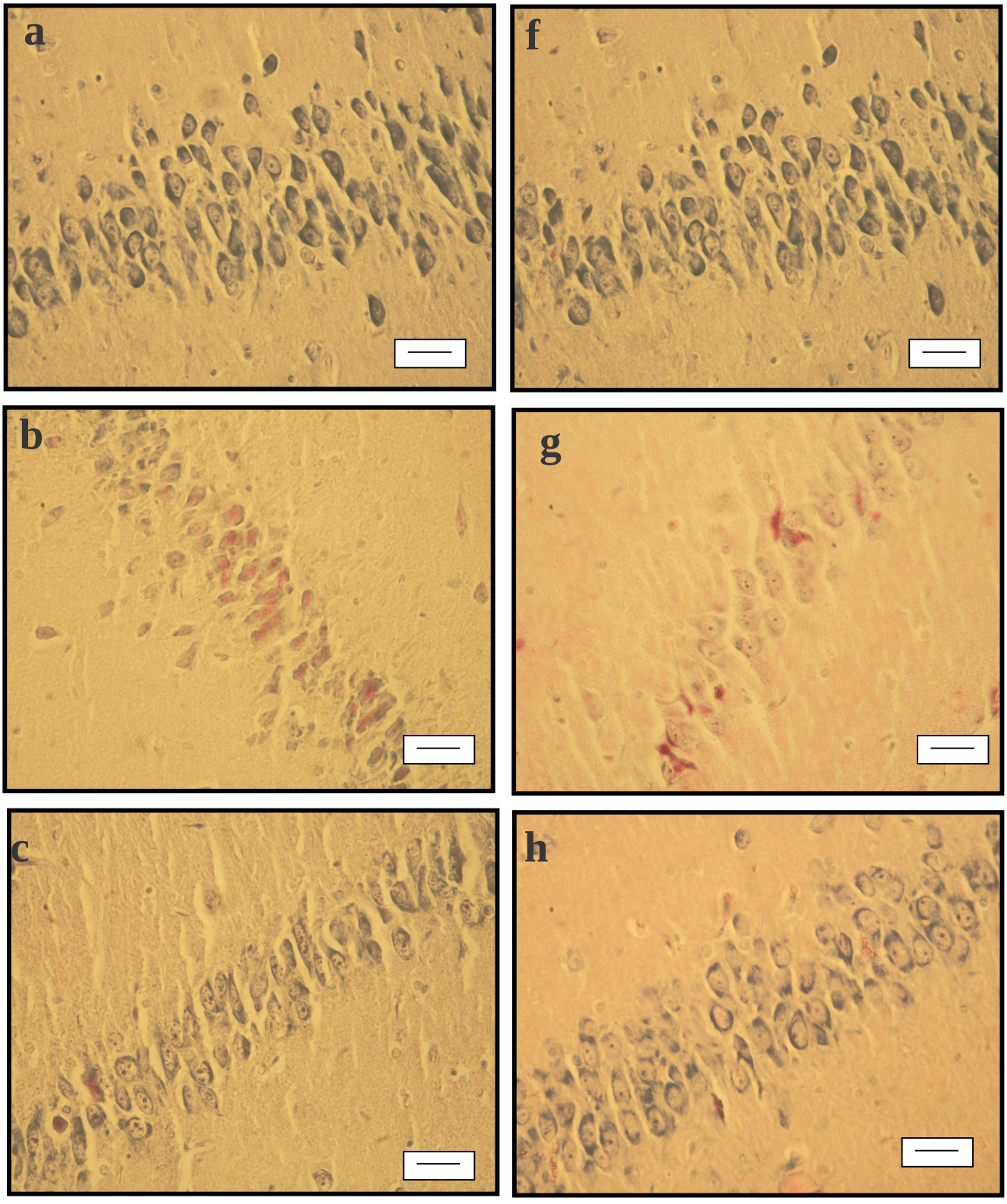
The slides were then rinsed with acetate buffer for 30 s until the sections were a pale sky blue colour with at pale reddish brown background. The slides were examined using a light microscope to determine if staining was satisfactory. Abnormal neurons are stained a deep red colour while healthy neurons stain a dark blue colour. Once staining has been achieved satisfactorily the slides were rinsed with distilled water, blotted gently dry with filter paper and dehydrated as described above in Table 7.3.

The stained sections were mounted with cover slips as described in section 7.3.2.5.6 and photographed using a combination Olympus camera and light microscope.

7.3.3. RESULTS

From the photomicrographs, of the CA1 and CA3 regions of control group, it was evident that the nuclei and cell membrane of healthy, undamaged neurons were stained blue while abnormal hippocampal neurons stood out as bright red objects against a brownish pink background (figure 7.5, photomicrographs a and f). It is evident from figure 7.5. (photomicrographs b and g), by the extensive red staining of damaged neurons, that QA caused excessive damage to the CA1 and CA3 regions of the hippocampus. The abnormal neurons were variable in appearance and distribution. The dose (100mg/kg) of

ASA (photomicrographs c and h), acetaminophen (photomicrographs d and i) and the combination of these agents (photomicrographs e and j) used was effective in protecting the neurons of the CA1 and CA3 regions of the hippocampus from QA induced neuronal damage. This is due to the lack of acidophilic neurons that were visible. In order to obtain a fair assessment of the effects of these agents the same region (throughout the rostrocaudal extent) of the hippocampus was viewed as compared to the toxin and control groups.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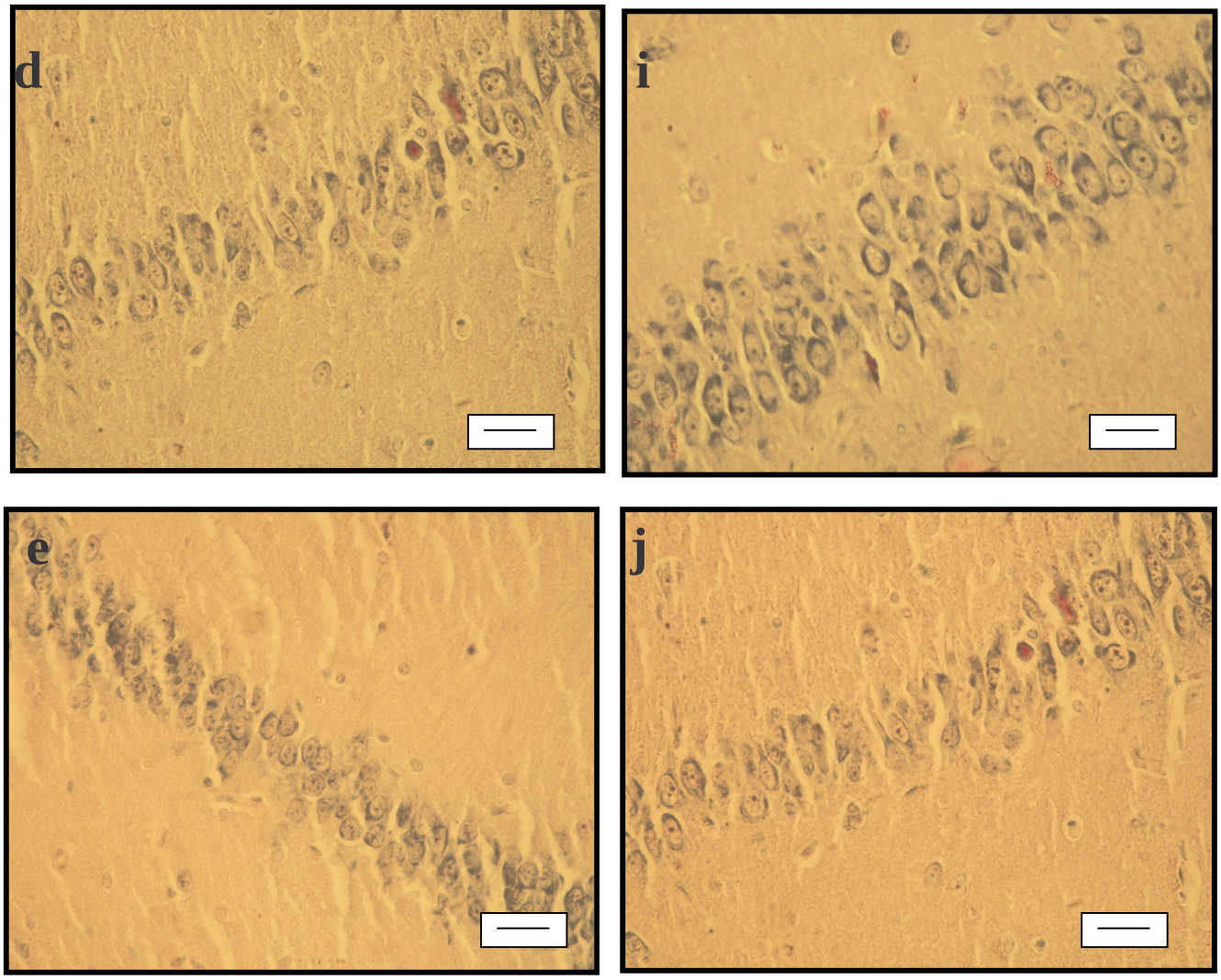


Figure 7.5. QA toxicity and the protective effects of acetylsalicylic acid and acetaminophen alone or in combination on rat hippocampal neurons using acid fuchsin stain. Micrographs (a-e) indicate cells in the CA1 region of the hippocampus from: a control animal (a), an animal treated with QA (b), animal treated with QA and acetylsalicylic acid (c), animal treated with QA and acetaminophen (d) and animal treated with QA and acetaminophen and acetylsalicylic acid (e). Micrographs (f-j) indicate cells in the CA3 region of the hippocampus from: a control animal (f), an animal treated with QA (g), animal treated with QA and acetylsalicylic acid (h), animal treated with QA and acetaminophen (i) and animal treated with QA and acetaminophen and acetylsalicylic acid (j). Bar = 10µm.

The combination of the acetaminophen and ASA was also conducive to protecting the structural integrity of the hippocampal neurons. The sections from photomicrographs e and j of the CA1 and CA3 regions respectively, figure 7.5, clearly demonstrates the neuroprotective effects of the combination of ASA and acetaminophen.

7.3.4. DISCUSSION

Acid fuchsin, a biological stain, has been utilized in cell death of hypoglycaemia, traumatic injury and excitotoxicity (Auer *et al*., 1985). In any necrotic tissue the cells that remain recognizable commonly exhibit increased affinity for anionic dyes. In the CNS, acidophilic neurons are a useful indicator of the extent of damage in incomplete lesions such as the dispersed damage caused by the administration of an excitotoxin (Tanaka & Simon, 1994).

Acidophilia is generally considered as a sign of irreversible (necrotic) neuronal damage (Kirino *et al*., 1985). Shrunken neurons displaying ischemic cell changes are necrotic, but they differ from swollen "truly" necrotic cells (Garcia *et al*., 1995; Rosenblum, 1997). In subsequent studies, acidophilic (red) neurons were observed in experimental brain (Garcia & Conger, 1986; Garcia *et al*., 1995; Kirino *et al*., 1985). This type of acute neuronal damage is not unique to ischemia (Kirino *et al*., 1985), but was also found in hypoglycaemia (Auer *et al*., 1984), epilepsy (Chang & Baram, 1994), excitotoxic neuronal lesions (Kiernan *et al*., 1998), and brain trauma (Sutton *et al*., 1993).

Initially, necrotic neurons were considered to be neurons removed from brain tissue in separate experiments studying the time course of the tissue damage. However, it soon became apparent that all neurons, which demonstrated a pronounced affinity for acid dyes, were moribund. However, reversible acidophilia was observed in the hippocampus of rats after epileptic seizures induced by kainic acid (Chang & Braham, 1994).

Excitotoxins such as QA are known to cause oxidative damage to the hippocampus of rats as shown in chapter five and chapter six. In the present study it is evident that this

toxin brought about hippocampal neuronal death in both the CA1 and CA3 region. These results further support the Nissl stained sections.

These results further confirm the findings from the Nissl staining, which indicate that ASA and acetaminophen afford protection against QA induced cell damage. This confirmation further enhances the role of these agents as potent free radical scavengers in the brain.

7.4. CONCLUSION

From the results obtained in this chapter, it is evident that ASA and acetaminophen attenuate QA insult in the rat hippocampus. Heyes (1996) reported that microglia and macrophages might be an important source of QA neurotoxicity. The protection elicited by ASA and acetaminophen as well as that shown against the increase in lipid peroxidation in chapter six, could be due to its antioxidant property. However it was therefore decided to investigate whether QA can induce apoptotic cell death in the rat hippocampus. QA is known to induce apoptosis in the striatum of rats (Qin *et al*., 2001; Nakai *et al*., 1999). Furthermore any effects that these agents may have on potential QA-induced apoptotic cell death, would be investigated.

CHAPTER EIGHT

APOPTOSIS

8.1. INTRODUCTION

Apoptosis is defined as a physiological and regulated mode of cell death. Cells undergoing apoptotic cell death exhibit several morphological characteristics, such as chromatin condensation, nuclear fragmentation, blebbing of plasma membrane, cell shrinkage, cytoplasmic condensation, nucleosomal DNA fragmentation (DNA ladder), and formation of apoptotic bodies. During the apoptosis process, organelles especially mitochondria, remain morphologically intact until late stages. Apoptotic cells are phagocytosed by macrophages or microglial cells thereby preventing inflammation that occurs during necrosis. This may be a short process *in vivo* (Kerr *et al*., 1972; Clarke, 1990).

Apoptosis can be induced by a number of stimuli as shown in figure 8.1. In some cases apoptosis is initiated following intrinsic signals that are produced following cellular stress. Cellular stress may occur from exposure to radiation **(2)** or chemicals or to viral infection **(3)**. It might also be a consequence of growth factor deprivation or oxidative stress. In general intrinsic signals initiate apoptosis via the involvement of the mitochondria **(5)**. The relative ratios of the various bcl-2 proteins can often determine how much cellular stress is necessary to induce apoptosis. In some cases the apoptotic stimuli comprise extrinsic signals such as the binding of death inducing ligands to cell surface receptors **(1)** or the induction of apoptosis by cytotoxic T-lymphocytes by granzyme **(4)**. The latter occurs when T-cells recognise damaged or virus infected cells and initiate apoptosis in order to prevent damaged cells from becoming neoplastic (cancerous) or virus-infected cells from spreading the infection.

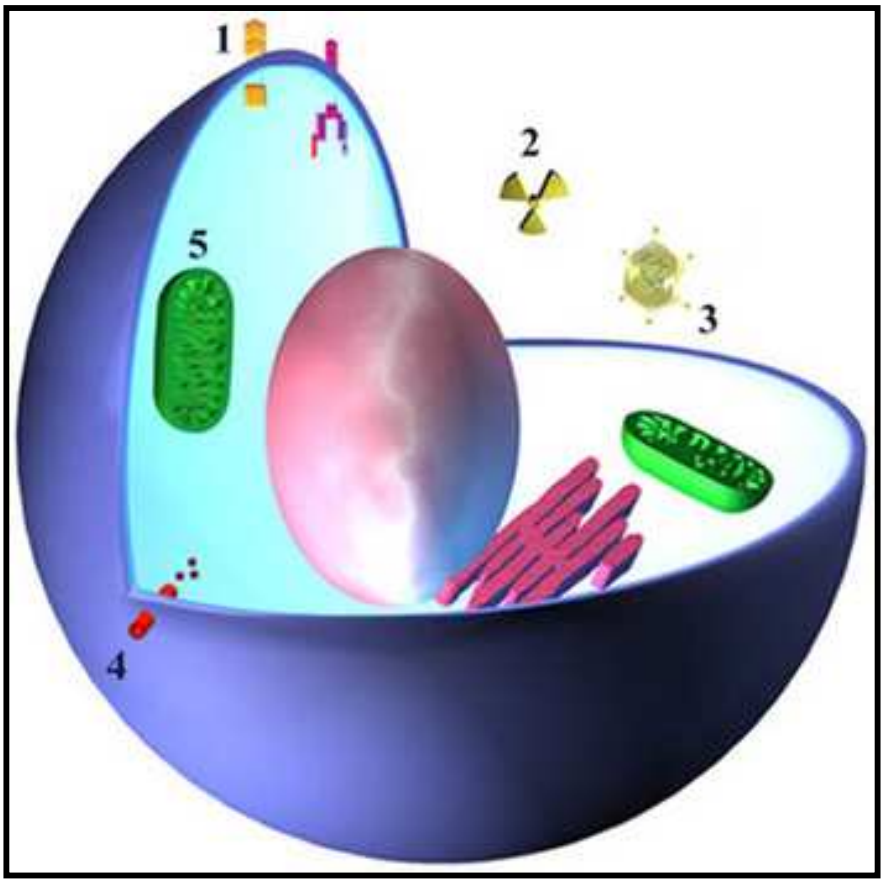


Figure 8.1. Some of the major stimuli that can induce apoptosis (www.sghms.ac.uk/depts/immun.html). Cellular stress may occur from exposure to radiation **(2)** or chemicals or to viral infection **(3)**. In general intrinsic signals initiate apoptosis via the involvement of the mitochondria **(5)**. In some cases the apoptotic stimuli comprise extrinsic signals such as the binding of death inducing ligands to cell surface receptors **(1)** or the induction of apoptosis by cytotoxic T-lymphocytes by granzyme **(4)**.

Conversely necrosis is characterized by cytoplasm and nuclear swelling, loss of plasma membrane integrity and release of cellular content hastening the immune response (Clarke, 1990). Apoptotic cells exhibit apoptotic bodies containing the cellular material and are recognized and removed by phagocytes. Activation of a cascade of proapoptotic proteins, known as caspases, precedes the morphological changes. Eventually, activated caspase-3 cleaves an inhibitory protein of the caspase-activated DNase which then act as endonuclease to digest DNA (Enari *et al*.,1998; Sakahira *et al*., 1998).

One of the hallmarks of apoptosis is fragmentation of DNA, which is visible as 'laddering' on an electrophoresis gel. However, not all forms of apoptosis appear to involve the same amount of laddering (Wyllie *et al*., 1984), or this phenomenon may occur at a relatively late stage in apoptosis. Additionally, this method does not allow for the identification of individual apoptotic cells. Therefore, labelling of DNA-strand breaks *in situ* is useful for identification of cells undergoing apoptosis, particularly for the detection of early stages of apoptosis. Figure 8.2 shows the processes leading to DNA cleavage and nuclear changes in the cell

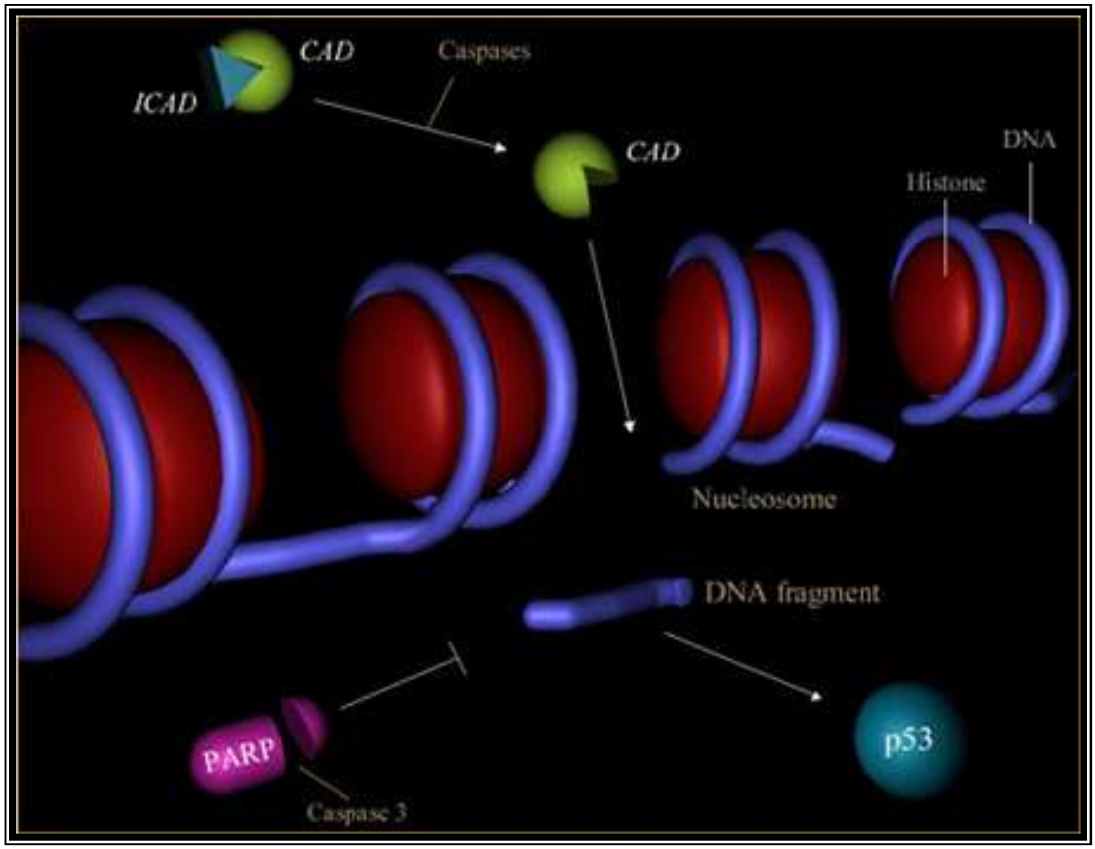


Figure 8.2. Processes leading to DNA cleavage and nuclear changes in the cell. (**CAD**) caspase-activated deoxyribonuclease, (**ICAD**) caspase-activated deoxyribonuclease inhibitor, (**PARP**) poly(ADP-ribose) polymerase, and **p53** is a pro-apoptotic gene. (www.sghms.ac.uk/depts/immunology/%7Edash/apoptosis/intro.html).

PARP is involved in repair of DNA damage and functions by catalyzing the synthesis of poly (ADP-ribose) and by binding to DNA strand breaks and modifying nuclear proteins.

The ability of PARP to repair DNA damage is prevented following cleavage of PARP by caspase-3 as shown in figure 8.2.

An enzyme known as CAD, as shown in figure 8.2, causes the fragmentation of DNA into nucleosomal units, or caspase activated DNase. Normally CAD exists as an inactive complex with ICAD (inhibitor of CAD, also known as DNA fragmentation factor45). During apoptosis, ICAD is cleaved by caspases, including caspase 3, to release CAD. Since CAD is a DNase with a high specific activity (comparable to or higher than DNase I and DNase II) rapid fragmentation of the nuclear DNA follows.

The TUNEL method was designed as a histochemical technique to detect internucleosomal DNA fragmentation at the level of individual cells (Gavrieli *et al*., 1992; Ben-Sasson *et al*., 1995). The TUNEL reaction preferentially labels DNA strand breaks generated during apoptosis and this allows discrimination of apoptosis from necrosis and from primary DNA strand breaks induced by cytostatic drugs or irradiation (Gorczyca *et al*., 1993). Several methods have been described to identify apoptotic cells (Afanasyev *et al*., 1993; Bryson *et al*., 1994; Darzynkiewicz *et al*., 1992).

Endonucleolysis is considered as the key biochemical event of apoptosis, resulting in cleavage of nuclear DNA into oligonucleosome-sized fragments. Therefore, this process is commonly used for detection of apoptosis by the typical ‘DNA ladder” on agarose gels during electrophoresis. This method, however, cannot provide information regarding apoptosis in individual cells nor relate cellular apoptosis to histological localization or cell differentiation. This can be done by enzymatic *in situ* labelling of apoptosis induced DNA strand breaks.

DNA polymerase as well as terminal deoxynucleotidyl transferase (TdT) (www.roche-applied-science.com) has been used for the incorporation of labelled nucleotides to DNA strand breaks *in situ*. The tailing reaction using TdT, which was also described as ISEL (*in situ* end labelling) (Gorczyca *et al*., 1993) or TUNEL (TdT-mediated dUTP nick end labelling) (Gavrieli *et al*., 1992; Sgonc *et al*., 1994) technique, has several advantages in comparison to the *in situ* nick translation (ISNT) using DNA polymerase:

1. Label intensity of apoptotic cells is higher with TUNEL compared to ISNT, resulting in an increased sensitivity (Gorczyca *et al*., 1993).

2. Kinetics of nucleotide incorporation is very rapid with TUNEL compared to the ISNT (Gorczyca *et al*., 1993).

3. TUNEL preferentially labels apoptosis in comparison to necrosis thereby discriminating apoptosis from necrosis and from primary DNA strand breaks induced by antitumour drugs, irradiation or excitotoxins (Gorczyca *et al*., 1993).

Cleavage of genomic DNA during apoptosis may yield double stranded, low molecular weight DNA fragments (mono-and oligonucleosomes) as well as single strand breaks ('nicks') in high molecular weight DNA. Those DNA strand breaks can be identified by labeling free 3'-$^{\cdot}$OH termini modified nucleotides in an enzymatic reaction. The working procedure described below was published by Sgonc *et al*., (1994) and the TUNEL method was used, for the experiments of this chapter, to label DNA strand breaks. Stage one involves the labeling of DNA strand breaks with TdT. This catalyses polymerization of labeled nucleotides to free 3'-OHM DNA ends in a template-independent manner (TUNEL-reaction). In stage two, the fluorescein labels incorporated in nucleotide polymers are detected and quantified by fluorescence microscopy (www.roche-applied-science.com).

Apoptosis has been found to be implicated in clinical outcomes in neurodegenerative disorders, such as AD and PD (Wyllie, 1998). Thus, in this chapter it was decided to investigate the ability of QA and MPP^+ to promote apoptosis in the hippocampus and striatum, respectively, and protection offered by the treatment with ASA and acetaminophen using *In Situ* Cell Death Detection Kits. This kit is designed as a precise, fast, and simple, non-radioactive technique for the detection and quantification of apoptosis at single cell level in cells and tissues, based on labeling of DNA strand breaks (TUNEL technology) using either fluorescence microscopy or light microscopy.

8.2. THE EFFECT OF ACETYLSALICYLIC ACID AND ACETAMINOPHEN AGAINST QA-INDUCED APOPTOSIS.

8.2.1. INTRODUCTION

Cells are consistently generating ROS during aerobic metabolism. As a consequence, each cell is equipped with an extensive antioxidant defense system to combat excessive production of ROS. Oxidative stress occurs in cells when the generation of ROS overwhelms the cell's natural defense systems. There is a growing consensus that oxidative stress plays a pivotal role in regulating apoptosis, a tightly controlled form of cell death in which a cell partakes in its own demise (Cotter *et al*., 2002). The importance of the excess generation of ROS has been demonstrated to be associated with neurodegenerative disorders including PD, AD, HD and amyotrophic lateral sclerosis (Sun and Chen, 1998; Mates *et al*., 1999).

In chapter 5, QA was shown to cause extensive production of $O_2^{-\bullet}$ in the rat hippocampus *in vivo* and thus apoptosis is postulated as a possible mode of neuronal cell death. Quinolinic acid has been reported to play a role in DNA fragmentation (Ogata *et al*., 2000). Ogata *et al*., (2000) showed that QA induced apoptosis via the caspase pathway in HL-60 cells. Quinolinic acid is a natural metabolite in the NAD cycle for NAD biosynthesis and metabolism in animals and NAD is known as the substrate for PARP involved in apoptosis (Ogata *et al*., 2000). Previous studies have suggested that NAD metabolism, especially PARP participates in the processes of apoptosis induced by various stimuli (Shiokawa *et al*., 1997).

Furthermore, the excitotoxin, QA is known to induce apoptosis in the striatum of rats (Qin *et al*., 2001; Nakai *et al*., 1999). Henchcliffe & Burke (1997) showed extensive apoptosis 24 hrs after striatal QA lesion. This was further supported by recent observations that suggest that QA induced destruction of striatal cells occurs, at least in

part, by an apoptotic mechanism (Portera-Cailliau *et al*., 1995; Qin *et al*., 1996; Dihné *et al*., 2001). Quinolinic acid lesioned neuronal cells have been shown to undergo programmed cell death (Uberti *et al*., 2003).

Since the ability of QA to induce necrotic cell death in the CA1 and CA3 hippocampal neurons was shown in chapter seven, it was decided to investigate whether QA causes apoptotic cell death as well. The non-narcotic analgesics, ASA and acetaminophen, alone and in combination, have been shown to protect against QA-induced oxidative stress, excitotoxicity and necrotic cell death in the hippocampus. Thus, the aim of this study is to investigate whether lesioning of the hippocampus with QA results in apoptotic neuronal death and if co-treatment of rats with QA and ASA and acetaminophen either alone or in combination results in reducing the apoptotic neurons, using the *in situ* cell death detection kit, fluorescein.

8.2.2. MATERIALS AND METHODS

8.2.2.1 Chemicals and Reagents

Quinolinic acid, ASA, and acetaminophen were purchased from Sigma St. Louis, MO, U.S.A. Aminopropyltriethoxysilane (APES) was purchased from NT lab Fluka. Paraffin wax was obtained from Lasec (South Africa). *In situ* cell death detection kit, fluorescein, proteinase K (nuclease free) and DNase 1, grade 1 (positive control) were purchased from Roche Diagnostics, (Nonnenwald, Penzberg). Formaldehyde, glacial acetic acid, absolute ethanol, xylene, and chloroform were purchased from Saarchem, Gauteng, SA while the aqueous mountant, SHUR/MOUNT™ was purchased from Triangle Biomedical Sciences Inc, Durham, USA. All other chemicals were of the highest quality available and were purchased from commercial distributors.

8.2.2.2. Animals

Adult male rats were cared for as described in appendix I.

8.2.2.3. Surgical Procedures

Animals were dosed as described in section 5.4.2.2. while the bilateral injection of QA into the hippocampus was carried out as in section 5.4.2.3.2.

8.2.2.4. Histological Techniques for Apoptosis Detection

The histological techniques were followed according to the method described by the *in situ* cell death detection kit, fluorescein instruction manual (www.roche-applied-science/pack-insert/1684795a.pdf).

8.2.2.4.1. Fixation and Processing of Brain Tissues

Fixation of the brain tissue can have dramatic effects on the cellular morphology of histological sections. Fixation of brain tissue should be performed rapidly without unnecessary handling beforehand, because neuronal staining artifacts generated by manipulation can be misinterpreted as pyknotic nuclei in neurons. The brain tissue was fixed according to the method described in chapter seven, section 7.2.2.5.1.

The fixation process was terminated by dehydrating the brain tissue. For apoptosis detection, traditional processing and embedding techniques cannot be used as described in chapter 7, section 7.2.2.5.2. Thus, in order to reduce residual damage and improve the fluorescence of the tissue, reagents that could affect the fluorescence were eliminated from the procedure. Moisture was extracted from the tissue fragments by bathing them successively in a graded series of mixtures of ethanol, and this step was followed by the clearing and defatting process that involves the removal of ethanol by immersing the

tissue in chloroform. The tissue was then submerged in molten paraffin wax (MP 57-58^{o}C) at 60^{o}C for one hour, which facilitated the removal of chloroform and while infiltrating the tissue without encountering water. At the end of this 1 hr period the brain tissue was placed under vacuum to remove any air that was trapped in the wax for 15min. Subsequently, the brain tissues were placed in new wax, twice for a period of 1hr each. Finally the brain tissue was embedded in molten wax and this stage provides the hardness and support that the tissue requires for sectioning. The method used here is a modification of the method described by Geiger *et al*., (1997) and is described in Table 8.1. The tissues can be stored in paraffin indefinitely without visible influence on the quality of TUNEL reactions (Geiger *et al*., 1997).

Table 8.1. Fixation and Processing of Tissues for Paraffin Embedding.

Step	Processing Agent	Time (Hours)
Fixation	Davidson's Fixative solution	48 hrs
Dehydration	50% Absolute Ethanol	1 x 2 hrs
	70% Absolute Ethanol	1 x 2 hrs
	80% Absolute Ethanol	1 x 2 hrs
	90% Absolute Ethanol	1 x 2 hrs
	96% Absolute Ethanol	2 x 2 hrs
	100% Absolute Ethanol	3 x 2 hrs
Clearing	Absolute Ethanol: Chloroform (1:1)	1x 2 hrs
	Chloroform	1 x 2 hrs
	Xylene: Chloroform (1:1) at 60^{o}C	1 x 1 hr
Wax Immersion	Melted Paraffin Wax (MP 57-58^{o}C) at 60^{o}C	1x 1 hr
	Vacuum at 60^{o}C	15min
	Melted wax	2 x 1hrs
Embedding	In molten Wax	Overnight

8.2.2.4.2. Sectioning

Paraffin sections were cut by standard methods as described in chapter seven, section 7.2.2.5.4. The sections of paraffin-embedded tissue were cut 5μm thick and placed on the APES coated slides. The treatment procedure for the slides is described below.

8.2.2.4.3. Treatment of Slides

When mounting paraffin sections on slides, it is important to use an appropriate adhesive to avoid loss of sections during the subsequent washing procedures. Sections can be mounted on Superfrost slides or on glass slides that have been coated (subbed) with either aminopropyl triethoxysilane (APES) or poly-L-lysine. APES has been shown to be superior to poly-L-lysine in preventing tissue detachment from the glass (Ben-Sasson *et al*., 1995) and thus, APES was use to treat the glass slides prior to use. The slides were subbed at least 2 days prior to the application of the paraffin sections.

The method described by Herrington and McGee (1992) was used for treating the slides. Briefly, the slides were placed in a rack and cleaned by immersion for 30 min in 2% Decon 90 made in warm (60^{o}C) distilled water. This was followed by rinsing in distilled water, and then in acetone and finally air dying. The slides were then immersed into 2% APES made in acetone for 30 min. Finally the slides were rinsed with acetone, washed in distilled water and air dried at 37^{o}C. The slides were then stored in a dry place for 2 days prior to use.

8.2.2.4.4. Transferring Sections to Slides

One or two sections at a time were removed from the water bath and placed onto glass microscope slides using a thin paint brush. The paraffin slides were stored at room temperature until use to enable the section to adhere to the slide.

8.2.2.4.5. Deparaffinising Sections

Deparaffination should be as complete as possible, since remaining paraffin adversely affects the TUNEL reaction. The sections were heated 60^{o}C for 20 min (Gavrieli *et al*., 1992) and then hydrated through several baths of xylene and a graded series of ethanol at concentrations ranging from 100 to 70% with an immersion time of 3 min per bath as shown in table 8.2. Thereafter, the sections were rinsed in PBS (pH 7.4) for 30s. Care was taken to ensure that the slides did not dry out during the deparaffinising.

Table 8.2. Procedure for dewaxing and rehydrating brain sections

Step	Processing Agent	Time
1	Heat at 60^{o}C	20 min
2	Xylene	2 x 5 min each
3	100% Ethanol	2 x 3 min each
4	90% Ethanol	1 x 3 min
6	80% Ethanol	1 x 3 min
7	PBS	30 sec

8.2.2.4.6. *In situ* Cell Death Detection Kit, Fluorescein

For the TUNEL reaction, tissue sections were processed according to the procedure described below.

8.2.2.4.6.1. Deproteinisation with Proteinase K

After the PBS wash, the tissue sections were partially deproteinised by incubation with Proteinase K. Proteinase K treatment digests cross-linked proteins and thereby increases cell permeability and access to the nucleic acid targets i.e. DNA. Proteinase K is preferred because it does not require predigestion to reduce residual nucleases (Willson & Higgins, 1990). The concentration, incubation time, and temperature of proteinase K are

extremely important and have to be optimized for each type of tissue as high concentrations can cause tissue damage and increase nonspecific staining (Tornusciolo *et al*., 1995). The slides were incubated in 20µg/ml proteinase K which was made up in 10mM Tris-HCl buffer, pH 8, for 15min at 37^{o}C in a humidified chamber. Care was taken to ensure that the sections did not dry out and drying out would prevent fluorescence.

Since the final immunohistochemical stain is peroxidase-independent (Geiger *et al*., 1997), no inhibition of endogenous peroxidases that can produce high levels of background staining and interfere with the interpretation of the results, was performed because H_2O_2 weakens TdT activity (Migheli *et al*., 1995) and induces DNA breaks (Wijsman *et al*., 1993). Thus, incubation step was terminated by washing the slides four times in PBS for 3min each. The experimental slides were kept in PBS while the positive control slide was removed for DNase treatment as described below.

8.2.2.4.6.2. Positive DNase Controls

There is a substantial amount of variation in positive staining when using the TUNEL method; therefore, at least two DNase control slides should be included with each experimental run. The type, size and fixation of the tissue are contribution factors to the variation in staining. Since DNA fragmentation is characteristic of apoptosis, application of DNase I to control slides is ideal. DNase I is an endonuclease that introduces breaks by hydrolyzing double-stranded, or single-stranded DNA, preferentially at sites adjacent to pyrimidine nucleotides (Sambrook *et al*., 1989); therefore, pretreatment with DNase I results in intensive labelling of all nuclei. If the DNase I controls do not stain, staining on the experimental slides may be artifact and not positive staining.

The concentration of DNase I used was 3000U/ml prepared in 50mM Tris-HCl, pH 7.4 containing 1mg/ml BSA. After finger flicking for 10sec, sufficient DNase mixture was applied to the desired DNase control slides in order to cover the entire section and incubated for 10min at 25^{o}C. This mixture should not be made until needed, since

thawing of the DNase I causes its inactivation. After DNase I pretreatment, the positive control slides were washed thoroughly with PBS, since residual DNase activity can introduce high background.

8.2.2.4.6.3. Labelling Protocol

In the TUNEL method, TdT is used to incorporate biotinylated deoxyuridine at the sites of DNA breaks. Both single-stranded DNA and 3' overhangs of double-stranded DNA are good substrates for TdT. The TdT is generally inefficient at catalyzing the transfer of biotinylated dUTP to blunt or recessive ends (Deng & Wu, 1983). The *in situ* cell death detection kit, fluorescein (Roche) contains two vials; vial 1 is the enzyme solution which contains the TdT from calf thymus (EC 2.7.7.31) in storage buffer while vial 2 which is the label solution contains the nucleotide mixture in reaction buffer. To prepare the reaction mixture 100µl label solution is removed from vial 2 and kept away for the 2 negative controls. The total volume of the enzyme solution (vial 1) is added to the remaining 450µl of label solution (vial 2) to obtain 500µl of TUNEL reaction mixture. The mixture is mixed well to equilibrate the components. To maximize efficiency the TUNEL reaction mixture is prepared during the 10min DNase treatment step and kept on ice until use. In addition, the TUNEL reaction mixture is sensitive to light therefore it was prepared in the dark.

50µl/section of TUNEL reaction mixture containing the enzyme and digoxigneninlabeled dUTP was added to both the experimental and DNase control slides while, 50µl of the labelling solution was added to each of the negative controls. All the slides were covered with a zip-lock bag, in order to prevent the slides from drying out and this also imposes an even layering of the reaction mixture over the whole tissue section. All the slides were then incubated in a humidified chamber for 60min at 37^{o}C in the dark. TdT is temperature-sensitive; temperatures above 40^{o}C inactivate the enzyme (Geiger *et al*., 1997) therefore the temperature was constantly monitored in the humidified chamber to ensure that a temperature of 37±2^{o}C was maintained throughout the incubation period.

The reaction was then terminated by immersing the slides in PBS. The slides were washed three times in PBS.

8.2.2.4.6.4. Mounting of Slides

In order to preserve the fluorescence and prevent the drying out of the sections, the sections need to be mounted. However, normal xylene mounts such as DPX destroys the fluorescence of the tissue therefore, while the slides were still wet enough SHUR/MOUNT™ was added to them. SHUR/MOUNT™ is an aqueous mountant that also assists in preserving the fluorescence of the tissue. Tissue sections were then covered with coverslips and allowed to dry in the dark.

8.2.2.4.6.5. Photo-microscopy

To detect apoptotic neuronal death, once the mountant had dried, the tissue sections were viewed and photographed using an Olympus DX-61 motorized epifluorescence microscope (Wirsam Scientific, Gauteng) that is controlled by the Soft Imaging Systems analysis 3.2 software (SiS Systems GmbH, Munster, Germany). Photographs were taken using a Peltier cooled Colorview camera. Apoptotic cells can be detected using an excitation (ex) wavelength in the range of 450-500nm or by detection the range of 515-565nm (green) therefore, two filter cubes, i.e. U-YFP filter (excitation (ex)= 58nm and emission (em)= 527nm) and Texas Red U-MWIYZ filter (ex= 596nm and em= 620nm), that are capable of detecting fluorescence in this wavelength region were utilized to detect the apoptotic cell death. Filter cubes were obtained from Chroma Corp. (Battlebro, USA). Sections were protected from light at all times.

8.2.3. RESULTS

The rationale for the use of DNase I controls was to provide a guideline, to ensure that the staining on the experimental slides was not artifact and was actually positive staining.

The positive control slides, which were pretreated with DNase I, illustrated intensive labeling of all nuclei throughout the rat brain. The apoptotic positive cells are shown in figure 8.3 with arrows. The apoptotic cells observed were scattered throughout the tissue section and was intensely stained green by the TUNEL treatment and were easily visible.

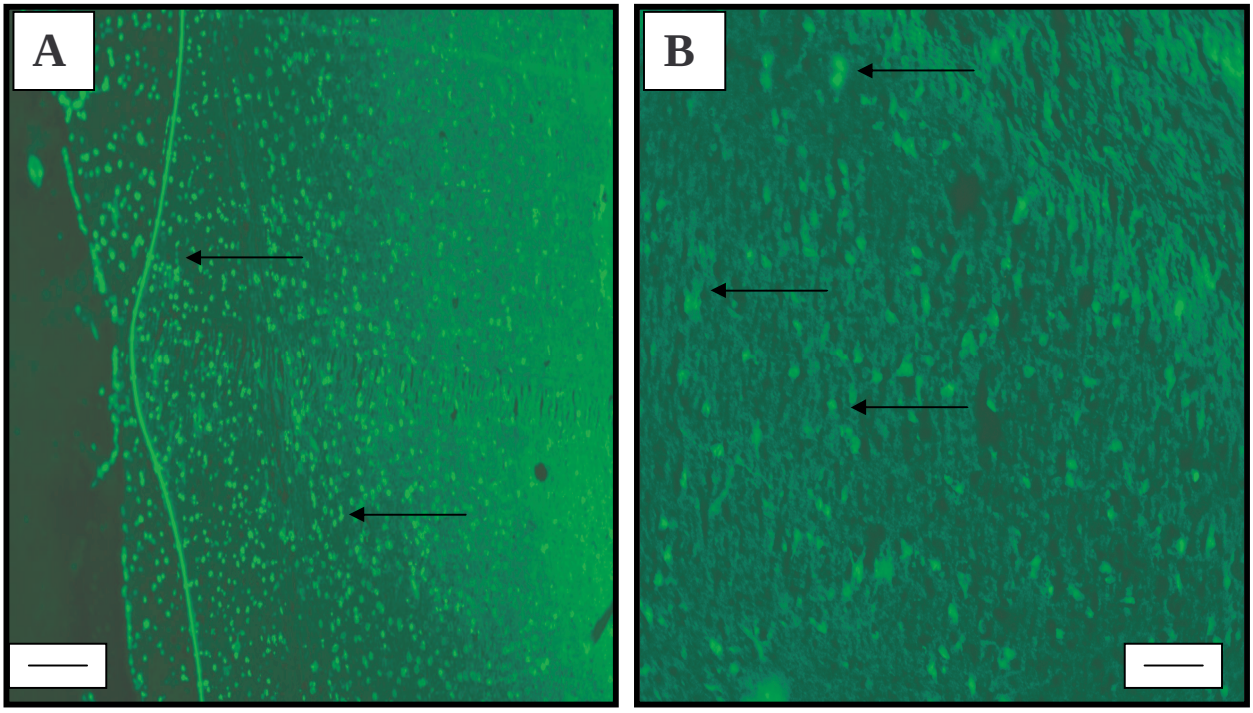


Figure 8.3. Detection of apoptotic cells (green spots) by fluorescence microscopy in the positive control tissue section from a rat brain. The section was assayed with the *in situ* cell death detection kit, fluorescein. **A**. Bar, 200µm and **B**. Bar, 100µm. The apoptotic positive cells are indicated with arrows

Figure 8.4 illustrates the absence of green fluorescence in the negative control tissues section indicates that the experimental procedure was performed correctly as no TUNEL solution was added. The arrows in figure 8.4 show the presence of healthy neurons, which have maintained normal neuronal structure and arrangement.

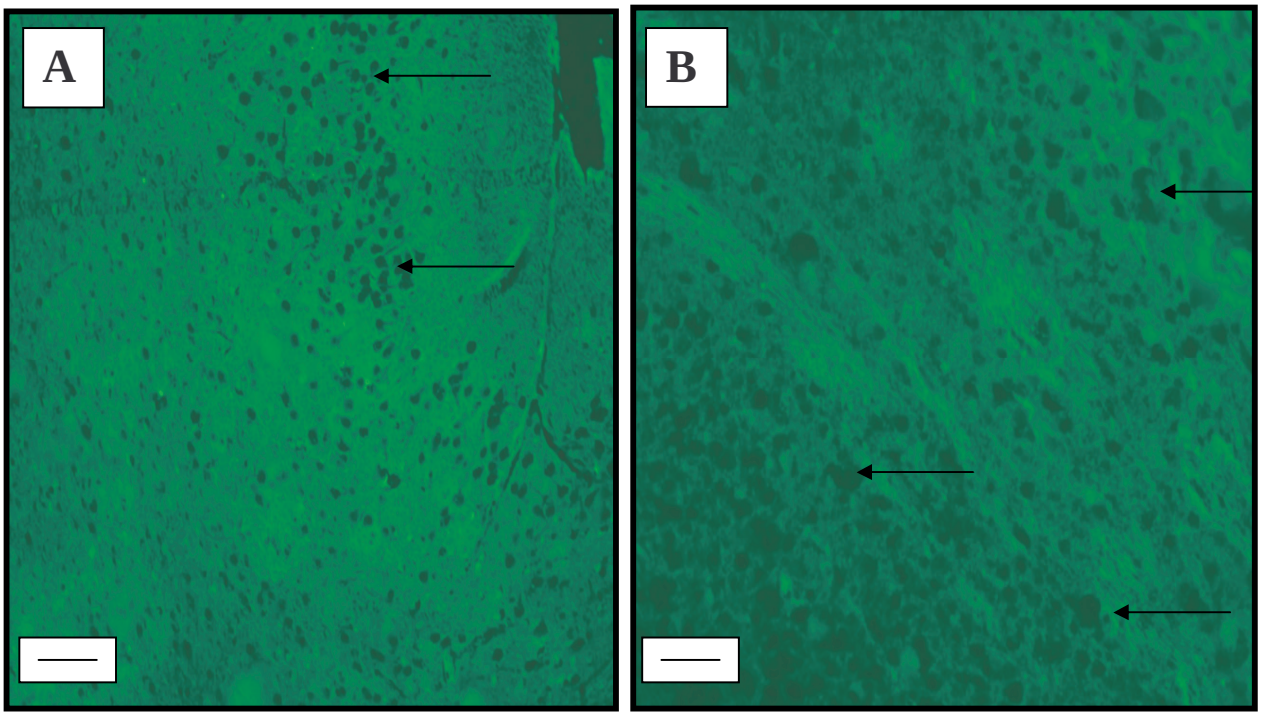


Figure 8.4. Absence of apoptotic cells (green spots) by fluorescence microscopy, indicated by arrows, in the negative control tissue section from a rat brain. The section was assayed with the *in situ* cell death detection kit, fluorescein. **A**. Bar, 200µm and **B**. Bar, 100µm.

From the experimental control sections no TUNEL positive apoptotic cells in the CA1 and CA3 regions of the rat hippocampus were observed, as evident in figure 8.5. The neurons are clearly visible with the absence of the green staining of the nucleus and thus indicate that apoptotic cell death has not occurred (figure 8.5, arrows).

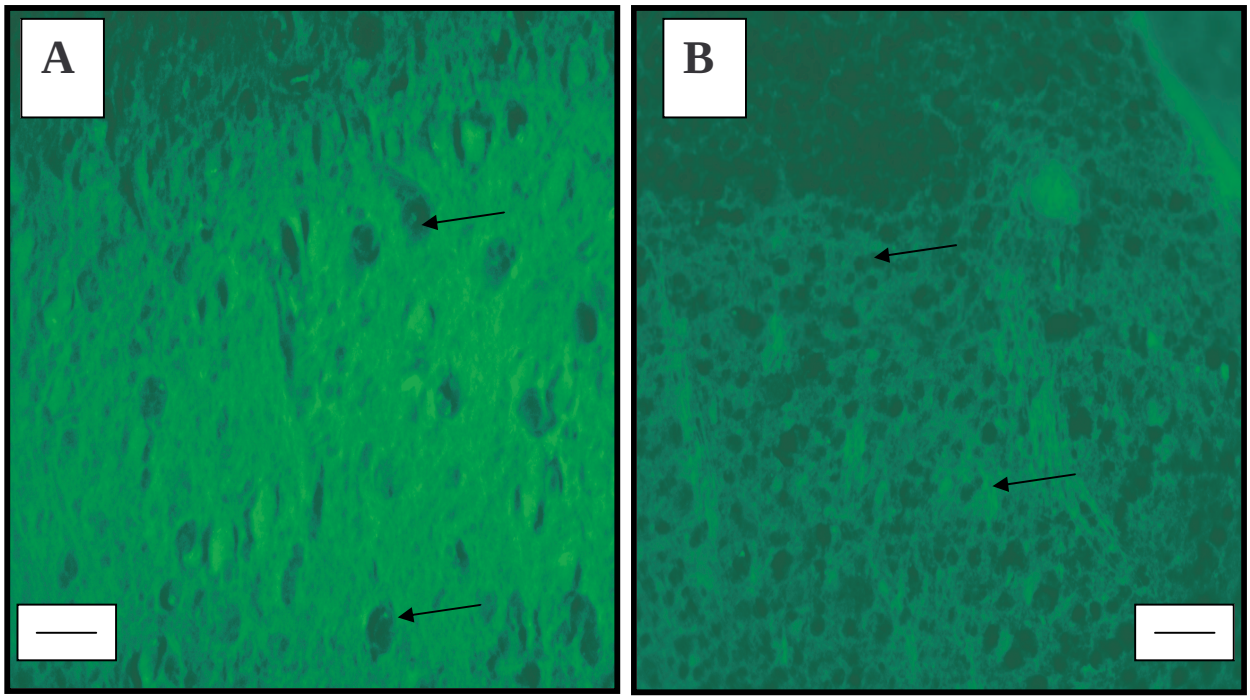


Figure 8.5. Hippocampus cells from a control treated rat. Bar 50µm. **A**. CA1 region and **B**. CA3 region of the hippocampus. Arrows show pyramidal neurons lacking the green stained nuclei indicating apoptosis has not occurred.

As evident from figure 8.6, the TUNEL positive cells appear surrounded by unstained neurons (arrows). From figure 8.6, however, it is evident that QA does cause some apoptotic cell death, which is more prominent in the CA1 region than the CA3 region of the hippocampus.

A composite picture was taken using two different filter cubes i.e. the YFP and Texas Red filter (sulfonyl chloride) cube to confirm that apoptotic cell death that was occurring, as shown in figure 8.7. It is evident from this figure that the apoptotic cell death is true as there is co-localization of the staining. The co-localized apoptotic cells appeared a yellowish orange colour. This confirmed that there was a greater presence of apoptotic cells in the CA1 region when compared to the CA3 region of the rat hippocampus.

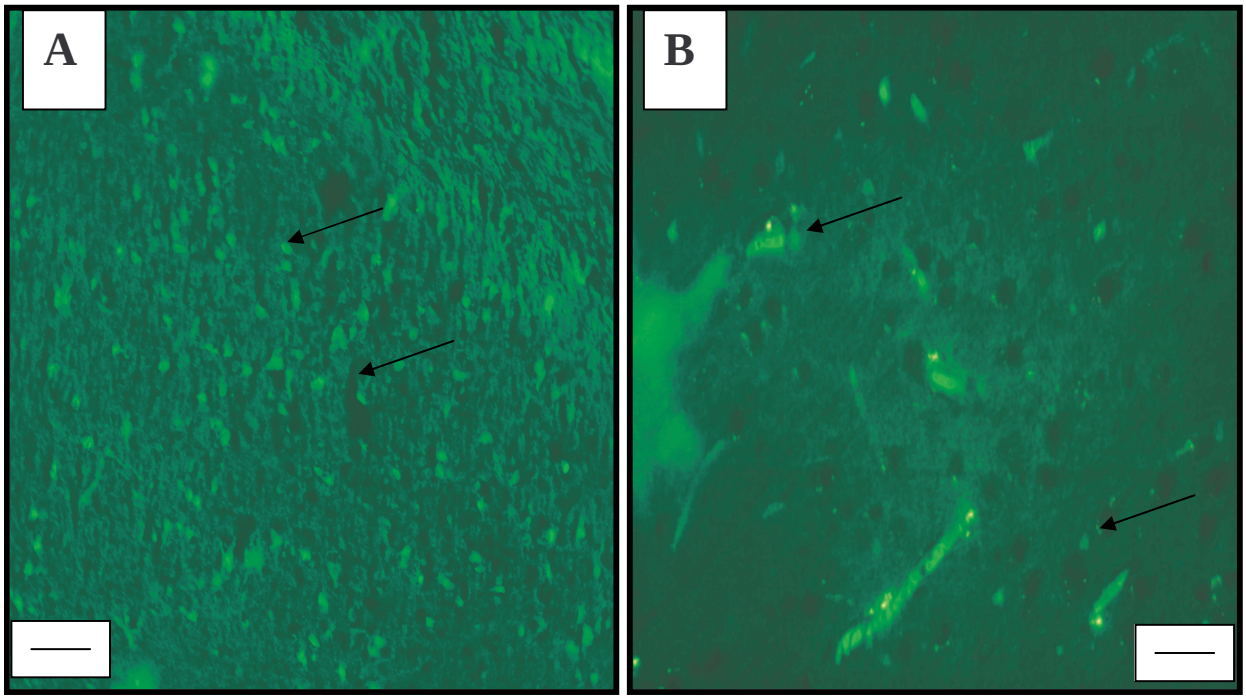


Figure 8.6. Hippocampus cells from a QA treated rat. Bar 50µm. **A**. CA1 region and **B**. CA3 region of the hippocampus. Arrows show green stained nuclei of the apoptotic positive cells.

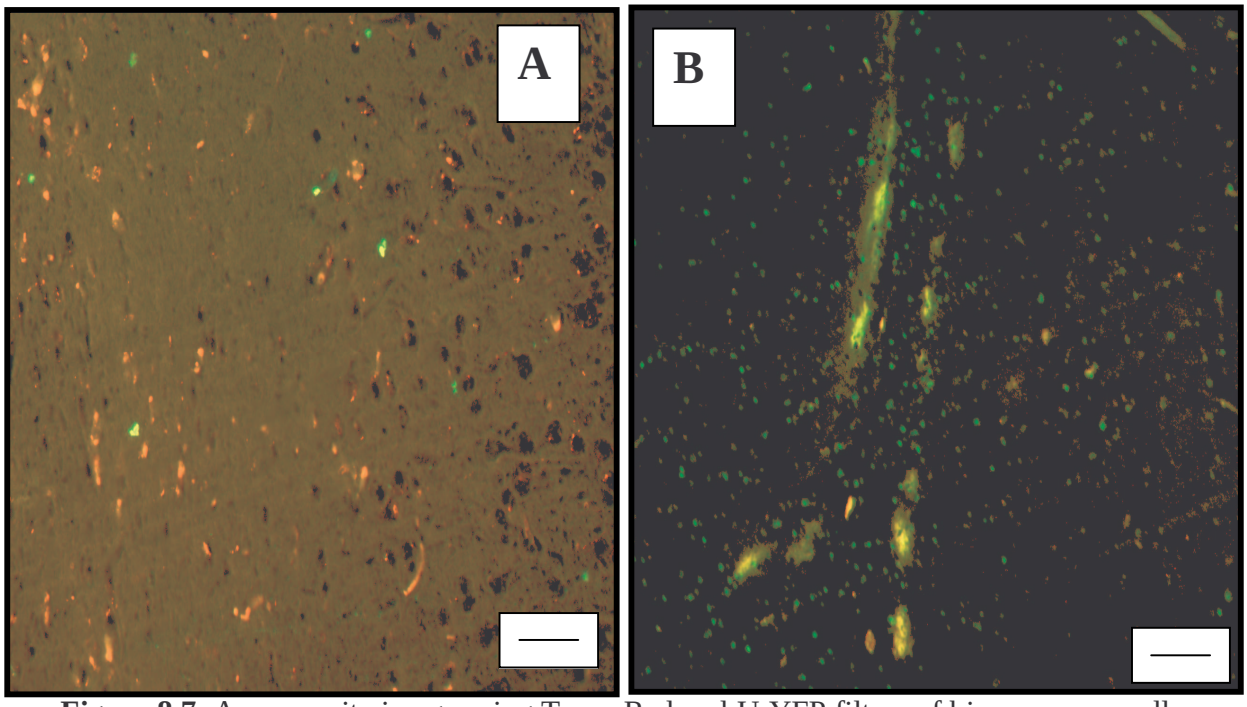


Figure 8.7. A composite image using Texas Red and U-YFP filters of hippocampus cells from a QA treated rat. Bar 50µm. **A**. CA1 region and **B**. CA3 region of the hippocampus. The yellowish orange stained nuclei of the apoptotic positive cells indicate co-localization.

The treatment of QA-lesioned rats with ASA, acetaminophen and the combination of these agents was able to prevent the QA-induced apoptotic cell death as seen in figure 8.8, 8.9 and 8.10. As evident from figures 8.8-8.10, the CA1 and CA3 regions of the non-narcotic analgesics treated rats were quiescent and lacked apoptotic positive stained cells. There was no indication of aggregation of apoptotic cells into multicellular clusters as observed in the QA treated rats indicated in figures 8.6 and 8.7. Furthermore, there is no visible difference between the hippocampal neurons of the control treated rats and the analgesic treated rats, indicating that these agents were able to protect the CA1 and CA3 region of the hippocampus against QA-induced apoptotic cell death. The neurons appeared to be tightly packed and the structures consistently exhibit normal cell morphology.

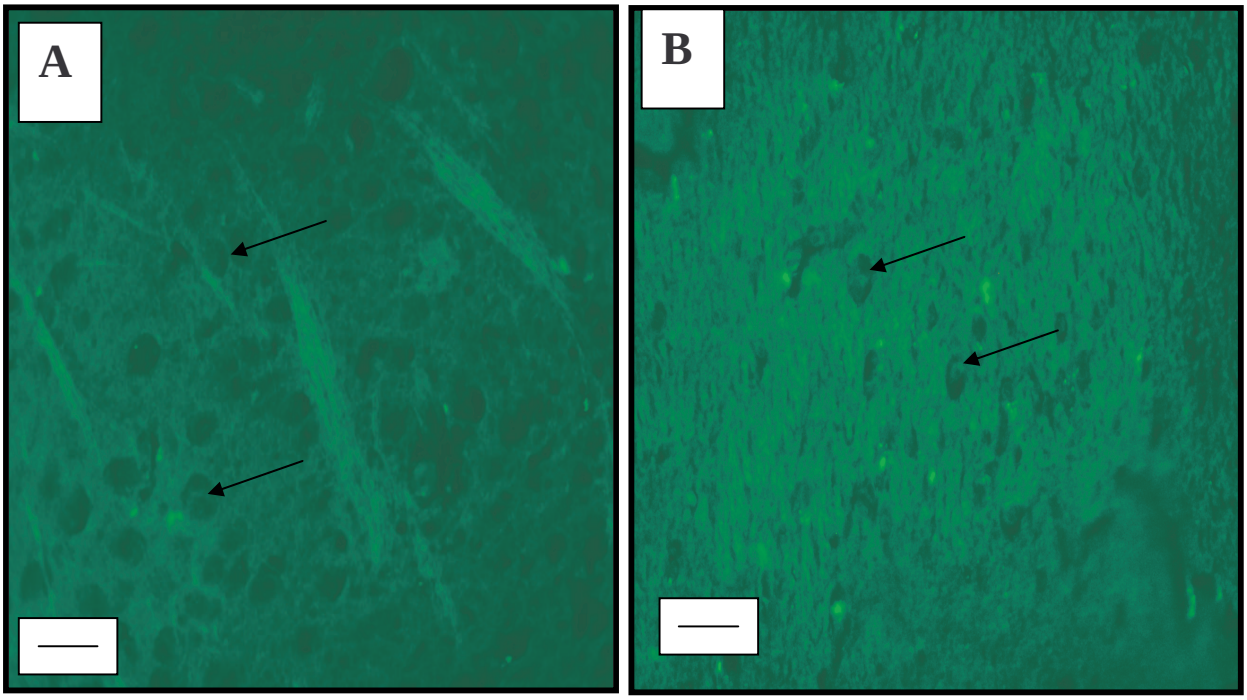


Figure 8.8. Hippocampal neurons from a QA and acetylsalicylic acid treated rat. Bar 50μm. **A**. CA1 region and **B**. CA3 region of the hippocampus. The CA1 and CA3 neurons are clearly visible with a lack of apoptotic cell death, indicated by arrows.

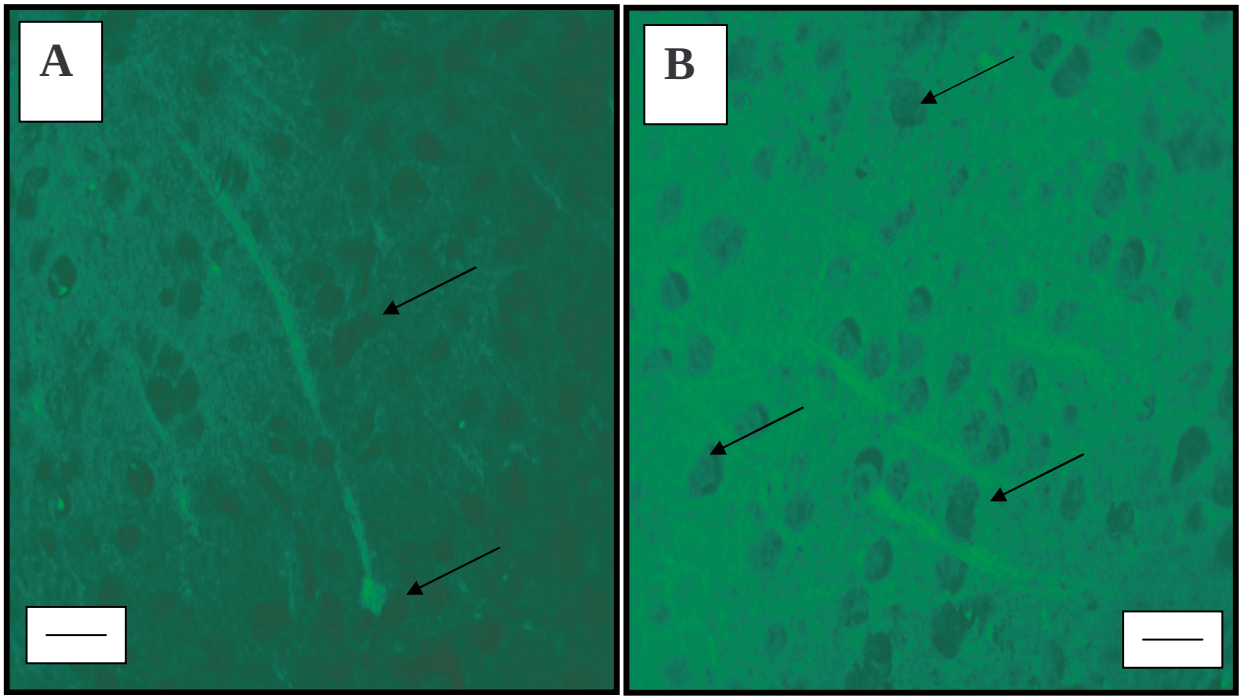


Figure 8.9. Hippocampal neurons from a QA and acetaminophen treated rat. Bar 50μm. **A**. CA1 region and **B**. CA3 region of the hippocampus. The CA1 and CA3 neurons are clearly visible with a lack of apoptotic cell death as shown by arrows.

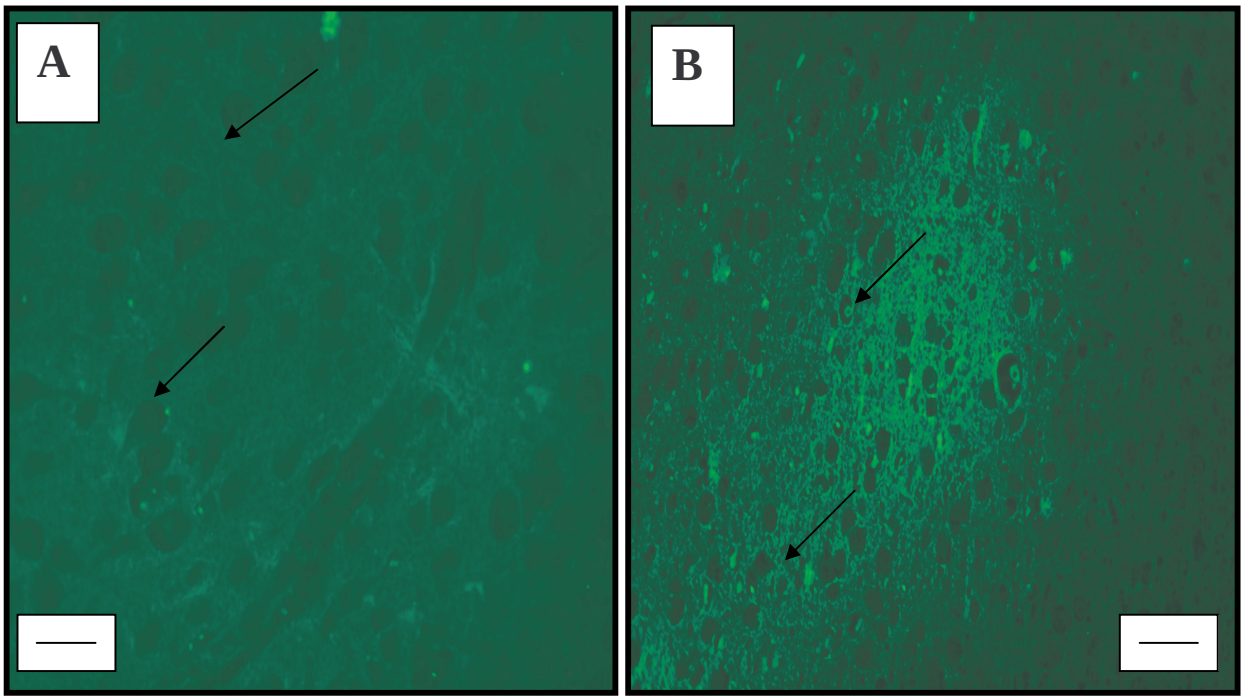


Figure 8.10. Hippocampal neurons from a QA and acetaminophen and acetylsalicylic acid treated rat. Bar 50μm. **A**. CA1 region and **B**. CA3 region of the hippocampus. The CA1 and CA3 neurons are clearly visible with a lack of apoptotic cell death (arrows).

8.2.4. DISCUSSION

The TUNEL kit used in this study was extremely sensitive, easy to use and provided a good signal-to-background ratio. The method utilized in this study allowed for specifically labelled individual apoptotic nuclei in the CA1 and CA3 regions of the rat hippocampus. The DNase treated positive control section, figure 8.3, provided visible evidence that the experimental method was carried out correctly and that any staining on the experimental sections could be attributed to apoptotic cell death, as the neurons were intensively stained. Contradictory to necrotic cell death, cells having undergone apoptosis appear scattered or follow a distinct pattern of distribution as is shown in figures 8.6 and 8.7.

Apoptotic mechanisms appear to contribute to excitotoxic neuronal injury in rat brain (Portera-Cailliau *et al*., 1995; Qin *et al*., 1996). Ogata *et al*., (2000) demonstrated that QA causes DNA fragmentation in HL-60 cells. Furthermore Ogata *et al*., (2000) suggests that since QA has two carboxyl groups in the molecule, it generally acts as a weak inducer of apoptosis. The weak ability of QA to induce programmed cell death is further confirmed by the results obtained the TUNEL reaction as shown in figures 8.6 and 8.7. This experiment confirmed that QA causes modest apoptotic neuronal death. In addition, apoptotic cell death was more predominant in the CA1 region than in the CA3 region of the hippocampus. Quinolinic acid-induced apoptosis has been suggested to occur via the caspase pathway (Ogata *et al*., 2000). This is supported by previous studies wherein it had been demonstrated that QA induced weak apoptotic neuronal death in the hippocampus and striatum cells (Portera-Cailliau *et al*., 1995; Qin *et al*., 1996; Dihné *et al*., 2001). Stimulation of NMDA receptors has been shown to initiate the QA-induced apoptotic cascade (Qin *et al*., 2001).

ASA has been reported in previous studies to possess antioxidant properties (Pekoe *et al*., 1982; Kuhn *et al*., 1995). As mentioned in chapter six, it has been reported that ASA inhibits oxygen-glucose deprivation–induced glutamate release (De CristÓbal *et al*., 2001) and acetaminophen has been shown to attenuate glutamate-induced neurotoxicity

in cultured primary rat embryonic neurons from mesencephalon (Casper *et al*., 2000). These agents could therefore be acting by preventing the consequences of QA, which stimulates the NMDA receptors to initiate apoptosis. The consequence of QA acting on the NMDA receptor involves the generation of ROS resulting in lipid peroxidation and subsequent cell death (Rios & Santamaria, 1991). In chapters five and six the antioxidant properties of these agents was aptly demonstrated where these agents prevented QA-induced superoxide anion generation and lipid peroxidation.

Hence the results obtained from this study are further indicative of the potent antioxidant properties of these agents in the presence of QA. These results and those obtained from chapter seven indicate that pre and postoperative treatment, of QA-lesioned rats, with ASA and acetaminophen either alone or in combination inhibits both forms of QA-induced cell death.

8.3. THE EFFECT OF ACETYLSALICYLIC ACID AND ACETAMINOPHEN AGAINST MPP^+-INDUCED APOPTOSIS.

8.3.1. INTRODUCTION

Apoptosis is an active, gene-directed mechanism crucially involved in the efficient removal of cells, which are either no longer needed or damaged and thus possibly dangerous (Kerr *et al*., 1972). PD is a neurodegenerative disorder characterized by a preferential loss of dopaminergic neurons of the SNpc. Although the etiology of PD is unknown, major biochemical processes such as oxidative stress and mitochondrial inhibition are largely described. However, despite these findings, the actual therapeutics are essentially symptomatical and are not able to block the degenerative process. Recent histological studies performed on brains from PD patients suggest that nigral death could be apoptotic (Blum *et al*., 2001). Since 1994, several studies have pointed out the potential involvement of apoptosis in PD. The first study was performed by Mochizuki *et al*. (1994) using the 3'-end terminal labeling of DNA. (TUNEL method) allowing *in-situ* observation of DNA fragmentation, characteristic of apoptosis, in the SN of PD patients. Using a similar method, Kingsbury *et al*. (1998) observed DNA-end labeling in 10 idiopathic PD cases.

However since, post mortem studies do not allow precise determination of the sequence of events leading to apoptotic cell death, the molecular pathways involved in this process have been essentially studied on experimental models reproducing the human disease. These latter are created by using neurotoxic compounds such as 6-OHDA, MPP^+ or DA. Extensive studies on the MPP^+ model has shown that this model mimics, *in vitro* and *in vivo*, the histological and/or the biochemical characteristics of PD and thus helps define important cellular actors of cell death presumably critical for nigral degeneration (Blum *et al*., 2001). MPP^+ has been shown to induce apoptotic and not necrotic cell death (Blum

et al., 2001) hence it was decided that investigating the histological changes using cresyl violet and acid fuchsin, chapter seven, was considered inconsequential as these stains indicate necrotic cell death.

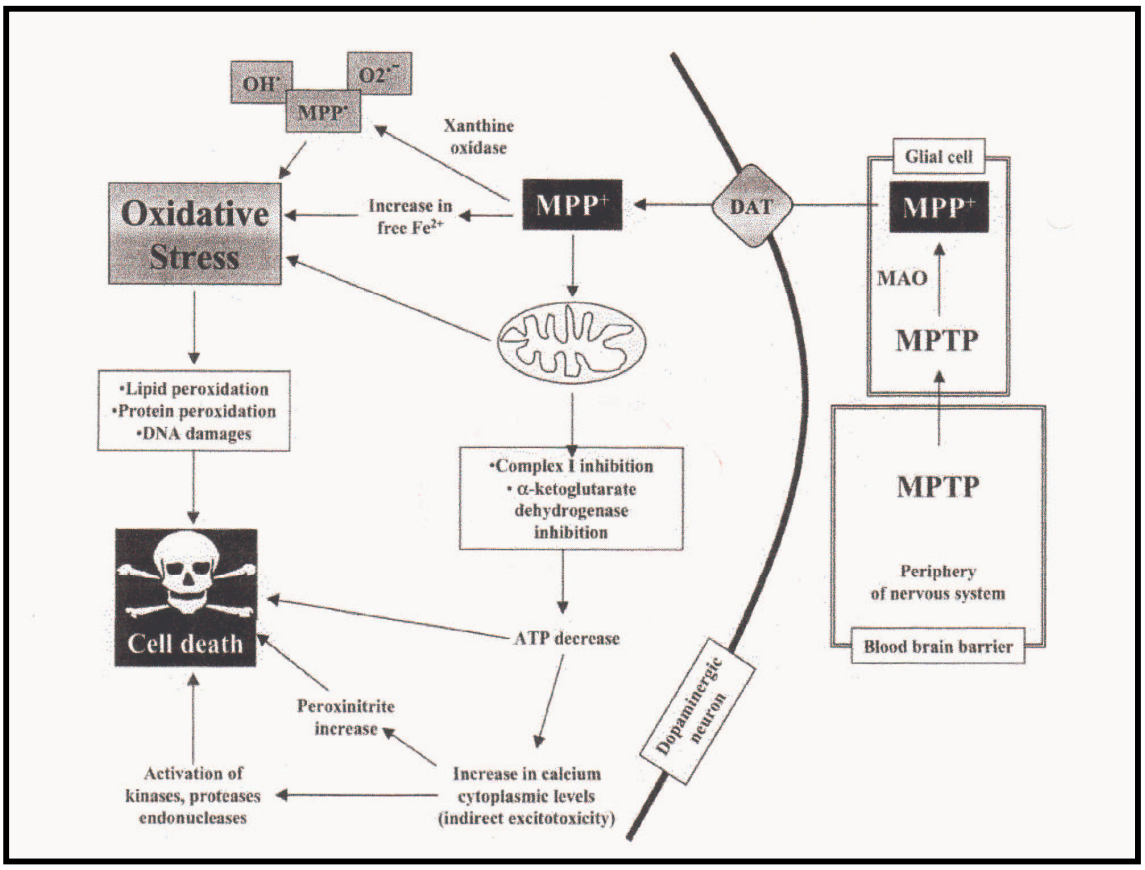


Figure 8.11. Hypothetical mechanism of MPP^+ toxicity (Blum *et al*., 2001).

Figure 8.11 illustrates the general overview of the various biochemical processes hypothesized to lead to cell death in the brain. The processes involved are major inhibition of the mitochondrial respiratory chain and the enhancement of oxidative stress. The mitochondrial inhibition provokes an ATP decrease presumably responsible for secondary excitotoxicity inducing a strong deleterious increase in cytoplasmic calcium levels. Oxidative stress generated directly by MPP^+ or subsequent to mitochondrial inhibition leads to macromolecule peroxidation and cell death.

The aim of this study is to illustrate, *in vivo*, MPP^+-induced apoptotic cell death using the *In Situ* Cell Death Detection Kit, POD. The POD kit is utilized for immunohistochemical detection of apoptosis at a single cell level, based on labeling of DNA strand breaks (TUNEL technology) analysis by light microscopy. The study also aimed at determining whether ASA and acetaminophen, alone or in combination, are capable of preventing MPP^+ -induced apoptotic cell death at single cell level.

8.3.2. MATERIALS AND METHODS

8.3.2.1. Chemicals and Reagents

In situ cell death detection kit, POD, DAB/metal substrate, peroxide buffer, proteinase K (nuclease free) and DNase 1, grade 1 (positive control) were purchased from Roche Diagnostics, (Nonnenwald, Penzberg). All other chemicals were of the highest quality available and were purchased from commercial distributors.

8.3.2.2. Animals

Adult male rats were cared for as described in appendix I.

8.3.2.3. Surgical Procedures

As described in chapter five, section 5.5.2.3

8.3.2.4. Histological Techniques for Apoptosis Detection

The histological techniques were followed according to the method described by the *in situ* cell death detection kit, POD instruction manual (www.roche-applied-science/pack-insert/1684817a.pdf).

8.3.2.4.1. Fixation and Processing of Brain Tissues

Fixation and processing of brain tissue was performed as described in section 8.2.2.4.1.

8.3.2.4.2. Sectioning

Paraffin sections were cut by standard methods as described in chapter seven, section 7.2.2.5.4. The sections of paraffin-embedded tissue were cut 5μm thick and placed on the APES coated slides.

8.3.2.4.3. Treatment of Slides

The slides were treated as described in section 8.2.2.4.3.

8.3.2.4.4. Transferring Sections to Slides

One or two sections at a time were removed from the water bath and placed onto glass microscope slides using a thin paintbrush. The paraffin slides were stored at room temperature until use to enable the section to adhere to the slide.

8.3.2.4.5. Deparaffinising Sections

Deparaffination should be as complete as possible, and was performed as described in table 8.2.

8.3.2.4.6. *In situ* Cell Death Detection Kit, POD

For the TUNEL reaction, tissue sections were processed according to the procedure described in figure 8.12.

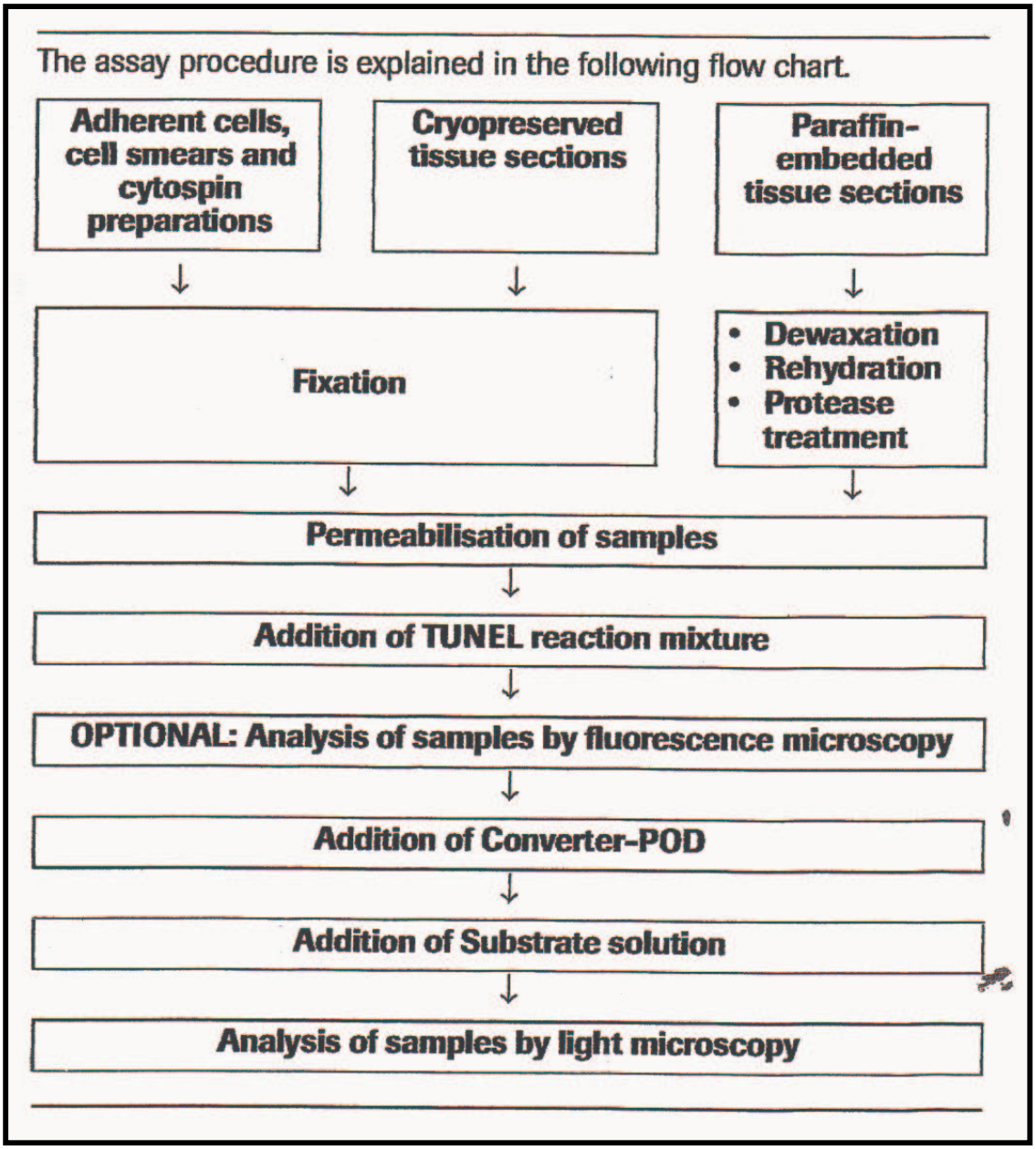


Figure 8.12. The assay procedure for the *In Situ* Cell Death Detection Kit, POD (www.roche-applied-science/pack-insert/1684817a.pdf)..

8.3.2.4.6.1. Deproteinisation with Proteinase K

After the PBS wash, the tissue sections were partially deproteinised by incubation with Proteinase K as described in section 8.2.2.4.6.1.

8.3.2.4.6.2. Positive DNase Controls

There is a substantial amount of variation in positive staining when using the TUNEL method; therefore, at least two DNase control slides should be included with each experimental run. Dnase labeling was performed as described in section 8.2.2.4.6.2.

8.3.2.4.6.3. Labelling Protocol

In the TUNEL method, TdT is used to incorporate biotinylated deoxyuridine at the sites of DNA breaks. Both single-stranded DNA and 3' overhangs of double-stranded DNA are good substrates for TdT. The TdT is generally inefficient at catalyzing the transfer of biotinylated dUTP to blunt or recessive ends (Deng & Wu, 1983). The *in situ* cell death detection kit, POD (Roche) contains three vials; vial 1 is the enzyme solution which contains the TdT from calf thymus (EC 2.7.7.31) in storage buffer while vial 2 which is the label solution contains the nucleotide mixture in reaction buffer and vial three contains anti-flourescien antibody, Fab fragmentation sheep, conjugated with horse-radish peroxidase (POD). To prepare the reaction mixture 100µl label solution is removed from vial 2 and kept away for the 2 negative controls. The total volume of the enzyme solution (vial 1) is added to the remaining 450µl of label solution (vial 2) to obtain 500µl of TUNEL reaction mixture. The mixture is mixed well to equilibrate the components. To maximize efficiency the TUNEL reaction mixture is prepared during the 10min DNase treatment step and kept on ice until use. In addition, the TUNEL reaction mixture is sensitive to light therefore it was prepared in the dark.

50µl/section of TUNEL reaction mixture containing the enzyme and digoxigneninlabeled dUTP was added to both the experimental and DNase control slides while, 50µl of the labelling solution was added to each of the negative controls. All the slides were covered with a zip-lock bag, in order to prevent the slides from drying out and this also imposes an even layering of the reaction mixture over the whole tissue section. All the slides were then incubated in a humidified chamber for 60min at 37^{o}C in the dark. Since

TdT is temperature-sensitive; temperatures above 40^{o}C inactivate the enzyme (Geiger *et al*., 1997), the temperature was constantly monitored in the humidified chamber to ensure that a temperature of 37±2^{o}C was maintained throughout the incubation period.

The reaction was then terminated by immersing the slides in PBS. The slides were washed three times in PBS.

8.3.2.4.6.4. Signal Conversion

The resultant flourescien following labeling with TUNEL reaction mixture was detected by the addition of the POD antibody and DAB substrate, which allows for the detection of apoptotic cell death under light microscopy. Figure 8.13 provides a diagrammatic representation of the reaction of fluorescein-labeled DNA strand breaks with POD and DAB substrate.

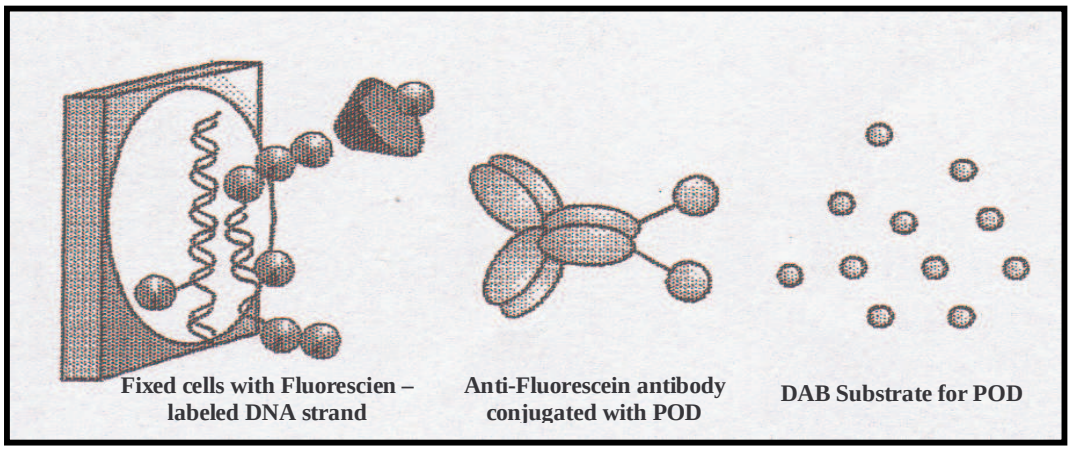


Figure 8.13. Illustration of the reaction mechanism that allows for the viewing of apoptotic cells under light microscopy, when using the *In Situ* Cell Death Detection Kit, POD (www.roche-applied-science/pack-insert/1684817a.pdf).

Following the slides being washed with PBS, the area around the sample tissue was dried with tissue paper. Thereafter 50µl converter POD was added to each tissue sample. The slides were incubated in a humidified chamber for 30 minutes at 37^{o}C. To ensure

homogenous spread of converter POD across the tissue and to avoid evaporative loss, samples were covered with parafilm during incubation. The incubation was terminated by rinsing the slide 3x with PBS.

A 10% concentration of DAB substrate was prepared by utilizing peroxide buffer. The DAB metal substrate is packaged under N_2 for long-term stability therefore after use the bottle was sealed under N_2 this enhances the life span of the substrate. The working solution of the DAB substrate was stored at 2-8^{o}C when not used. In order to stain the presence of horse-radish peroxidase (POD) 100µl of DAB substrate was added to each section of tissue and the slides were then incubated at 25^{o}C for 10 minutes in a well light room.

Thereafter the slides were washed 3x with PBS. The area around the sections was dried with tissue paper. In order to prevent the drying out of the sections and view the sections under the light microscope, the sections need to be mounted. Glycerol was used as the mountant and the tissue sections were then covered with coverslips and allowed to dry.

8.3.2.4.6.5. Photo-microscopy

To detect apoptotic neuronal death, once the mountant had dried, the tissue sections were viewed and photographed using the combination of an Olympus camera and a light microscope.

8.3.3. RESULTS

During the DAB substrate incubation period the positive apoptotic control tissue sample became stained an intense brownish colour while the negative control tissue samples appeared clear. This was an indication that the experiment had worked as the DAB substrate stains the apoptotic positive cells a brownish colour.

The apoptotic positive cells are shown in figure 8.14 with arrows. The apoptotic cells observed were scattered throughout the tissue section and was intensely stained brown by the TUNEL treatment and were easily visible. These cells exhibited apoptotic characteristics, as they appear shrunken, fragmented and scattered as shown by arrows.

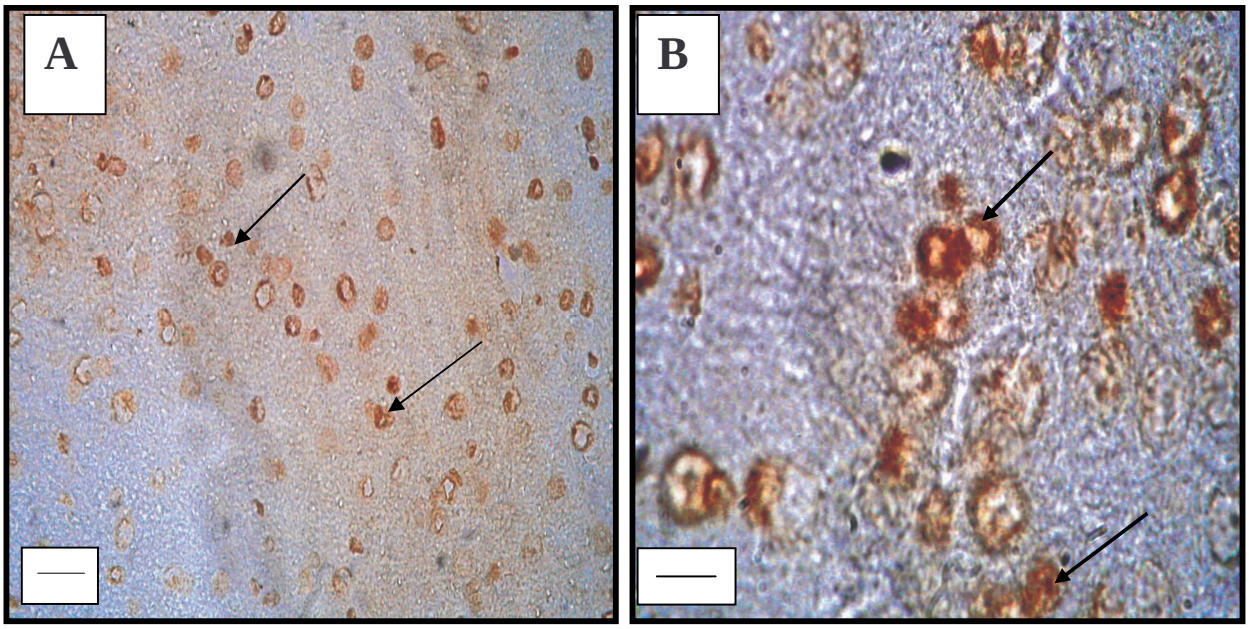


Figure 8.14. Detection of apoptotic cells (brown cells) in the striatum by light microscopy in the positive control tissue section from a rat brain. The section was assayed with the *in situ* cell death detection kit, POD. **A**. 400 X magnification and **B**. 1000 X magnification. Bar 10µm.

Figure 8.15 shows the negative control sections that exhibited no TUNEL positive apoptotic cells in the rat striatum. The neurons are clearly visible with the absence of the brown staining of the neuron and nucleus and thus indicate that apoptotic cell death has not occurred (figure 8.15, arrows). The neuronal cells have a visible nucleus and are tightly arranged. The cell wall appears intact and hence the shape of these cells are consistent with normal cellular morphology.

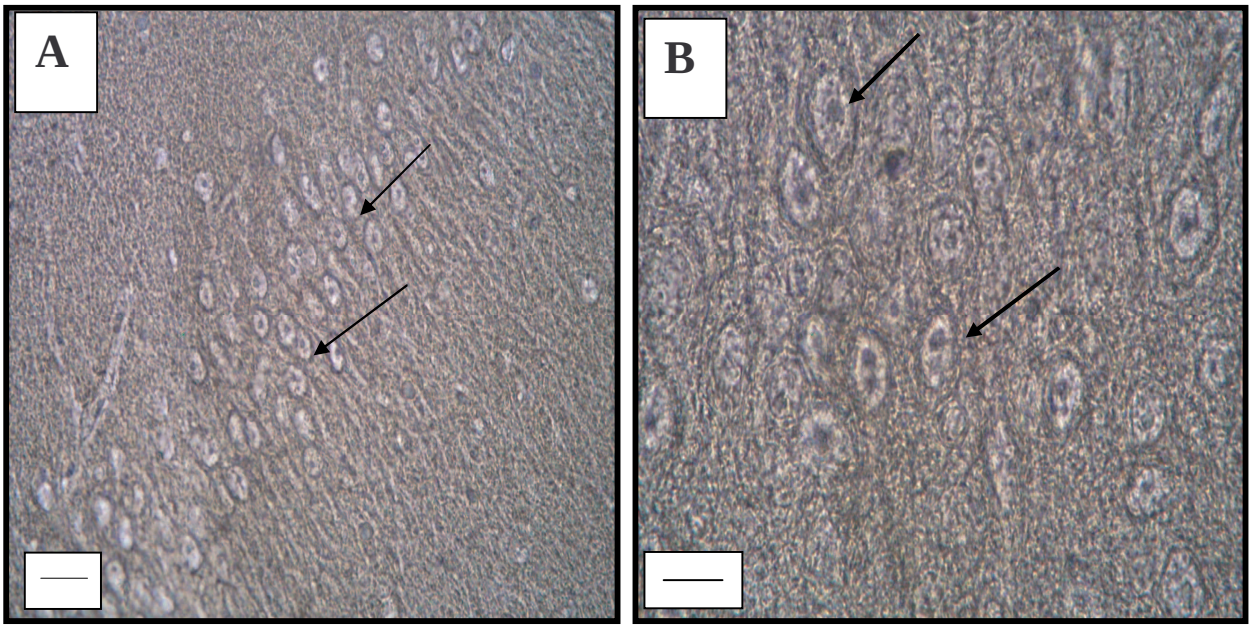


Figure 8.15. Striatal cells from a negative control treated rat brain section. **A**. 400 X magnification and **B**. 1000 X magnification. Bar 10μm. Arrows show healthy neurons lacking the brown stained nuclei which is indicative of apoptosis.

From the experimental control sections no TUNEL positive apoptotic cells in the rat striatum were observed, as evident in figure 8.16. The neurons are clearly visible with the absence of the brown staining of the nucleus and thus indicate that apoptotic cell death has not occurred (figure 8.16, arrows). The neurons resemble the neurons from the negative control section in figure 8.15. The tissue did not exhibit any color change when stained with DAB substrate.

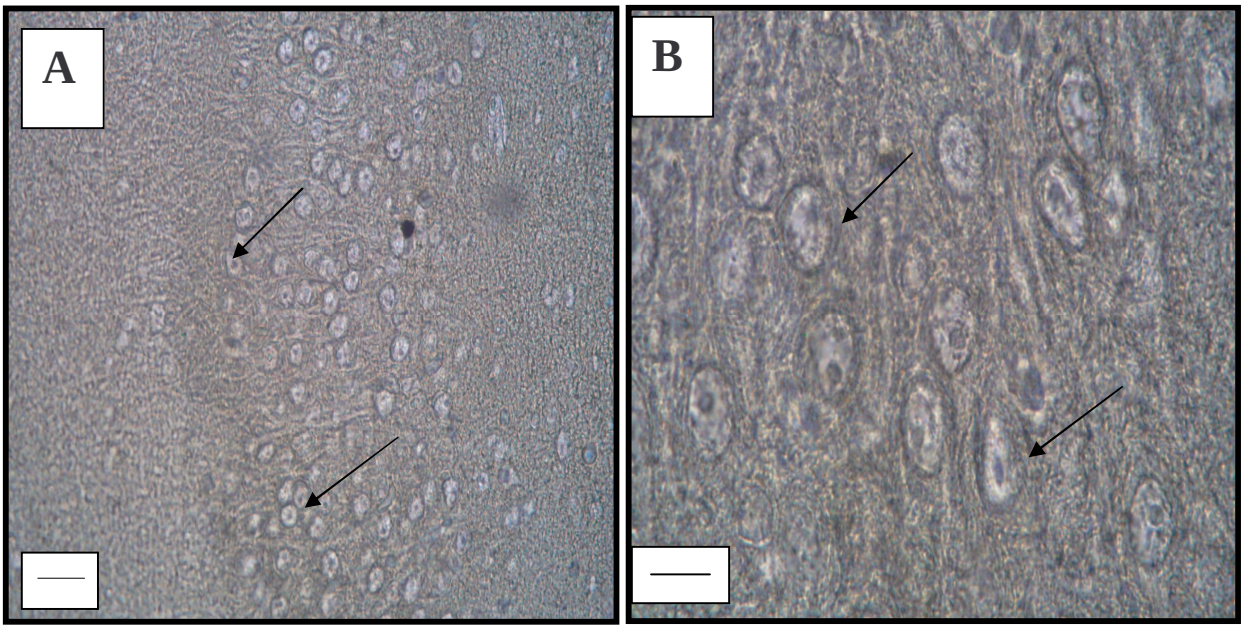


Figure 8.16. Striatal cells from a control treated rat. Bar 10μm. **A**. 400 X magnification and **B**.1000 X magnification. Arrows show pyramidal neurons lacking the brown stained nuclei.

As evident from figure 8.17, the TUNEL positive cells in the MPP^+ treated rats appeared to have an intense brownish stain. These neuronal cells have no particular uniformity in shape, size and structure. The intense brown staining is indicative of apoptotic cell death. The nucleus is absent in these cells indicating that nuclear fragmentation may have occurred. The lack of shape of these neuronal cells may result from blebbing of the cell membrane.

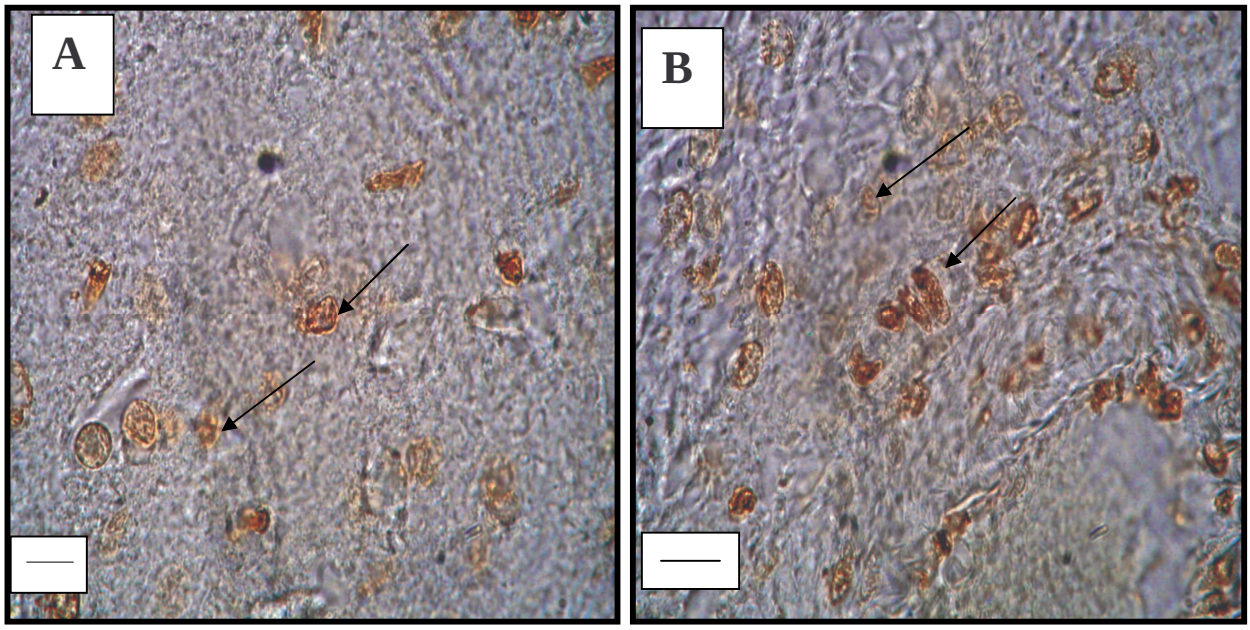


Figure 8.17. Striatal cells from a MPP^+ treated rat. Bar 10μm. **A**. 400 X magnification and **B**. 1000 X magnification. Arrows show brown stained nuclei and cell membrane of the apoptotic positive cells.

The postoperative treatment of MPP^+ rats with ASA, acetaminophen and the combination of these agents was able to prevent the MPP^+-induced apoptotic cell death as seen in figure 8.18, 8.19 and 8.20 as these lack the intense brownish staining that was visible in the MPP^+-treated group (figure 8.17). As evident from figures 8.18-8.20, the striatal regions of the non-narcotic analgesics treated rats were quiescent and lacked apoptotic positive stained cells. There was no indication of segregation of cells as observed in the MPP^+ treated rats indicated in figures 8.17. Furthermore, there is a minimal difference between the striatal neurons of the control treated rats and the analgesic treated rats, indicating that these agents were able to protect against MPP^+-induced apoptotic cell death. The neurons in figures 8.18-8.20 possess visible nuclei and intact cell membranes that are absent in the neurons of the MPP^+- treated group (figure 8.17). These neurons appear to be larger than the neurons of the MPP^+ treated group which indicates the absence/inhibition of apoptosis.

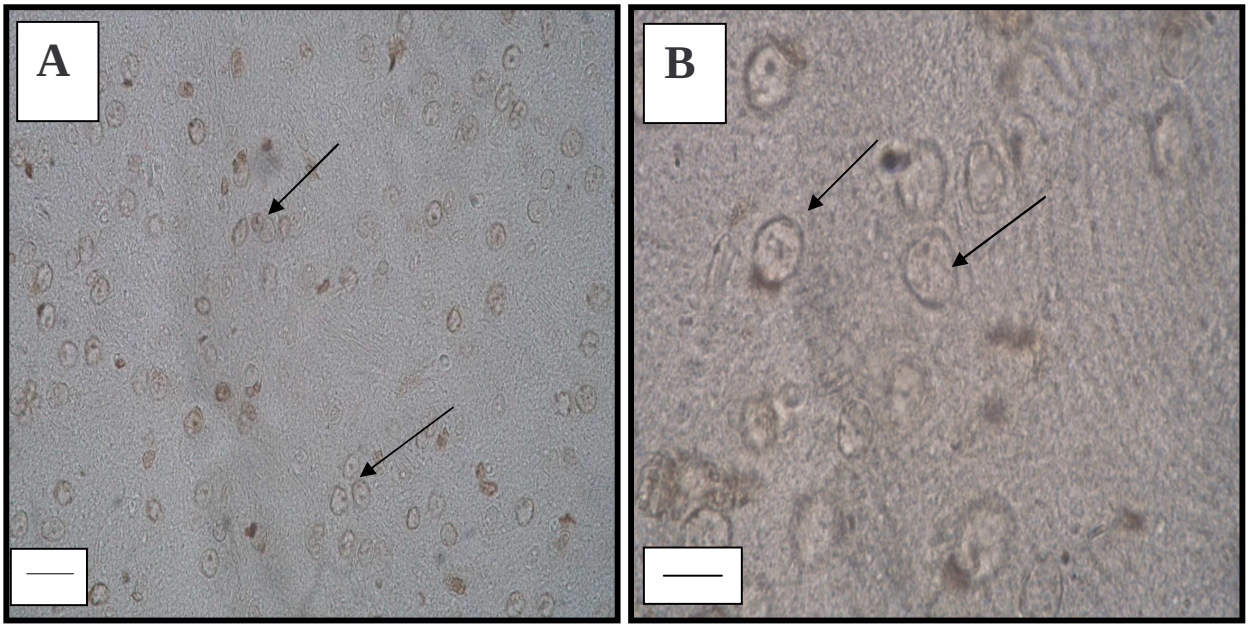


Figure 8.18. Striatal neurons from a MPP^+ and acetylsalicylic acid treated rat. Bar 10μm. **A**. 400 X magnification and **B**.1000 X magnification. The striatal neurons are clearly visible with a lack of apoptotic cell death.

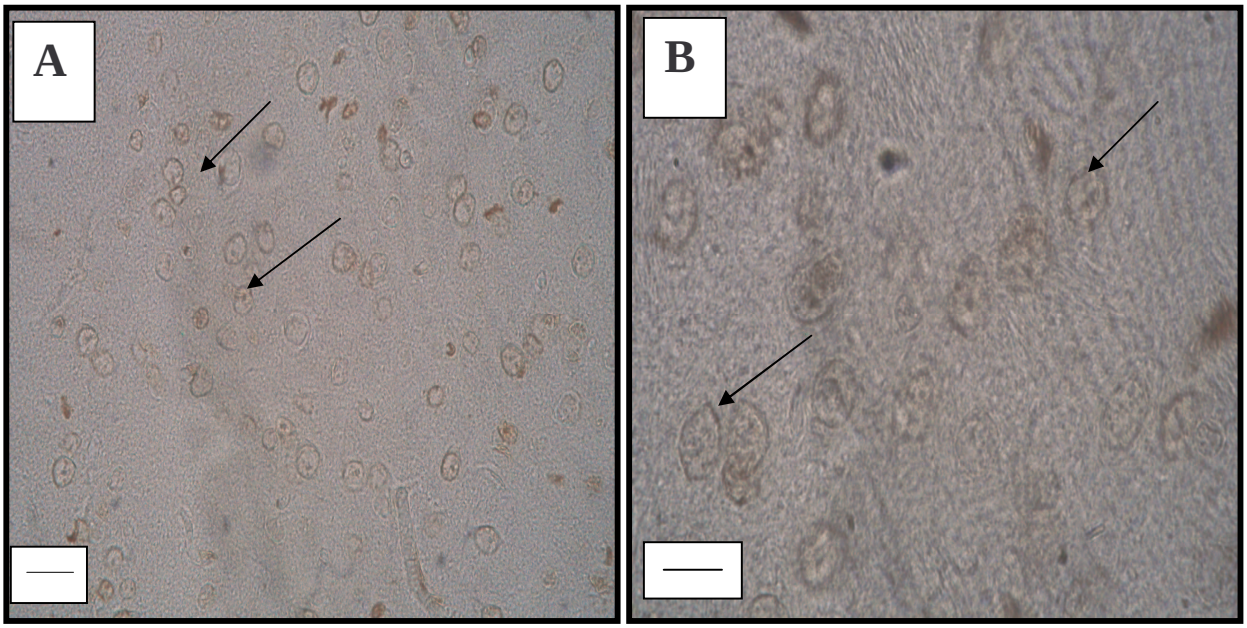


Figure 8.19. Striatal neurons from a MPP^+ and acetaminophen treated rat. Bar 10 μm. **A**. 400 X magnification and **B**.1000 X magnification. The striatal neurons are clearly visible with a lack of apoptotic cell death.

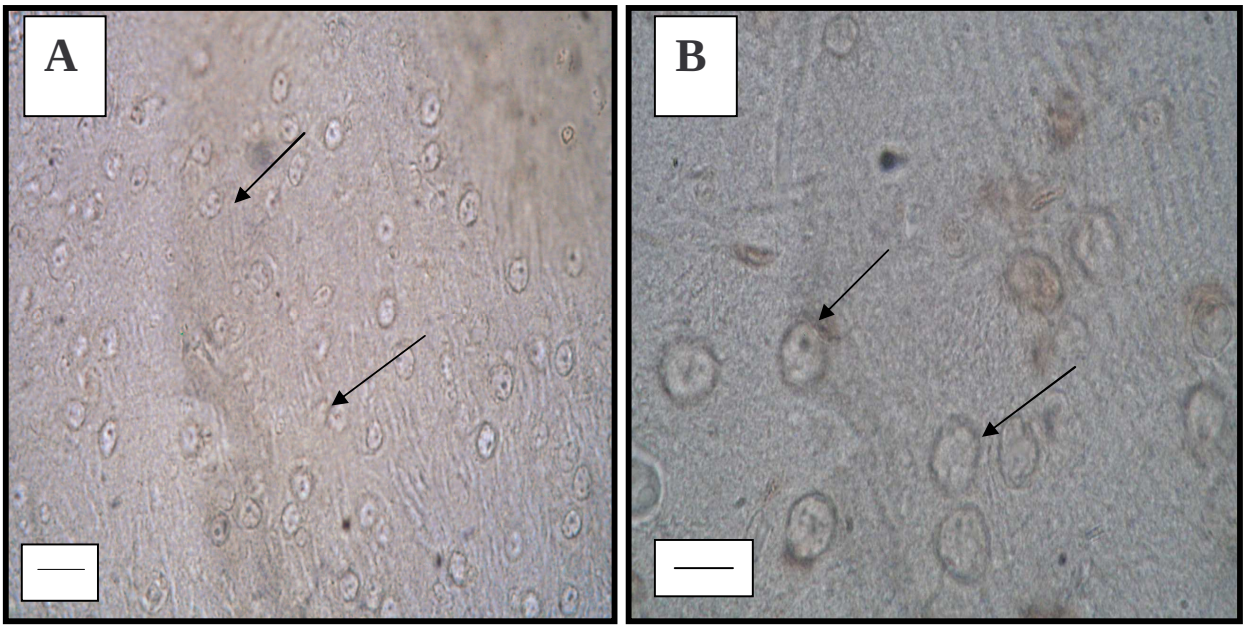


Figure 8.20. Striatal neurons from a MPP^+ and acetylsalicylic acid and acetaminophen treated rat. Bar 10μm. **A**. 400 X magnification and **B**.1000 X magnification. The striatal neurons are clearly visible with a lack of apoptotic cell death.

8.3.4. DISCUSSION

The TUNEL kit used in this study was extremely sensitive and provided a good signal-to-background ratio. The method utilized in this study allowed for the detection of apoptotic cell death, under light microscopy, induced by MPP^+. The DNase treated positive control section, figure 8.14, A and B, provided visible evidence that the experimental method was carried out correctly and that any staining on the experimental sections could be attributed to apoptotic cell death, as the neurons were intensively stained brown. The positive control provided a template for the identification of apoptotic cells in the treatment sections that were viewed under the light microscope.

Figure 8.15 illustrates that the lack of brown staining i.e. apoptotic cell death in the negative control slides and provides further confirmation that the experimental procedure was performed correctly in optimal conditions as these sections were not treated with the TUNEL reaction mixture.

Numerous studies have suggested that MPP^+ is able to induce apoptosis *in vitro* in various cell types such as cerebellar granule cells, GH3 pituitary cells, catecholaminergic cell lines such as PC12 and MES23.5 or in primary mesencephalic dopaminergic cells (Dipasquale *et al*., 1991; Hartley *et al*., 1994; and Yoshinaga *et al*., 2000) using light or electron microscopy using the TUNEL method. Concordant findings have also been established in SNpc of mice treated with MPTP (Spooren *et al*., 1998). Several recent works suggest that MPP^+ induced apoptosis may be under the control of p53 protein, Bcl-2 family genes, and caspase activity, as shown in figure 8.21 (Blum *et al*., 2001). Several lines of evidence have demonstrated that MPP^+-induced apoptosis is regulated by the Bcl-2 family. Thus, it was also described that nigral Bax mRNA and protein levels were increased in mice after MPTP exposure (Vila *et al*., 2001; Hasouna *et a*l., 1996), were as animals deficient in this pro-apoptotic gene were resistant to this neurotoxin (Vila *et al*., 2001).

Two studies support the involvement of p53 in MPP^+ neurotoxicity (Kitamura *et al*., 1998; Trimmer *et al*., 1996). The results illustrated in figure 8.17 demonstrate the dark brown staining of striatal neuronal cells indicating that MPP^+ does induce apoptotic cell death and is in agreement with the reports mentioned. It is clearly evident from figure 8.17B that the apoptotic cells differed greatly in structure and shape when compared to the control treatment group (figure 8.16). The striatal cells indicated in figure 8.16 did not exhibit apoptotic cell death characteristics such as cell shrinkage, blebbing of cell membrane and nuclear fragmentation. The intense staining of neuronal cells in the MPP^+-treated group indicated the formation of apoptotic bodies, which was absent in the control treated group.

Considering that the pathogenesis of PD involves strong oxidative stress, reduced antioxidant levels and mitochondrial defects, which can be mimicked by MPP^+ as shown in chapters five, six, ten, eleven, and twelve, it can also be suggested that these may also be contributing factors to MPP^+-induced apoptotic cell death. In particular, free radicals and GSH depletion have been shown to trigger active cell death in neurons (Merad-Boudia *et al*., 1998). The decrease in mitochondrial membrane potential (figure 8.21) has

been suggested to be one of the main phenomena leading to the apoptotic process (Jacotot *et al.*, 1999 and Martinou, 1999).

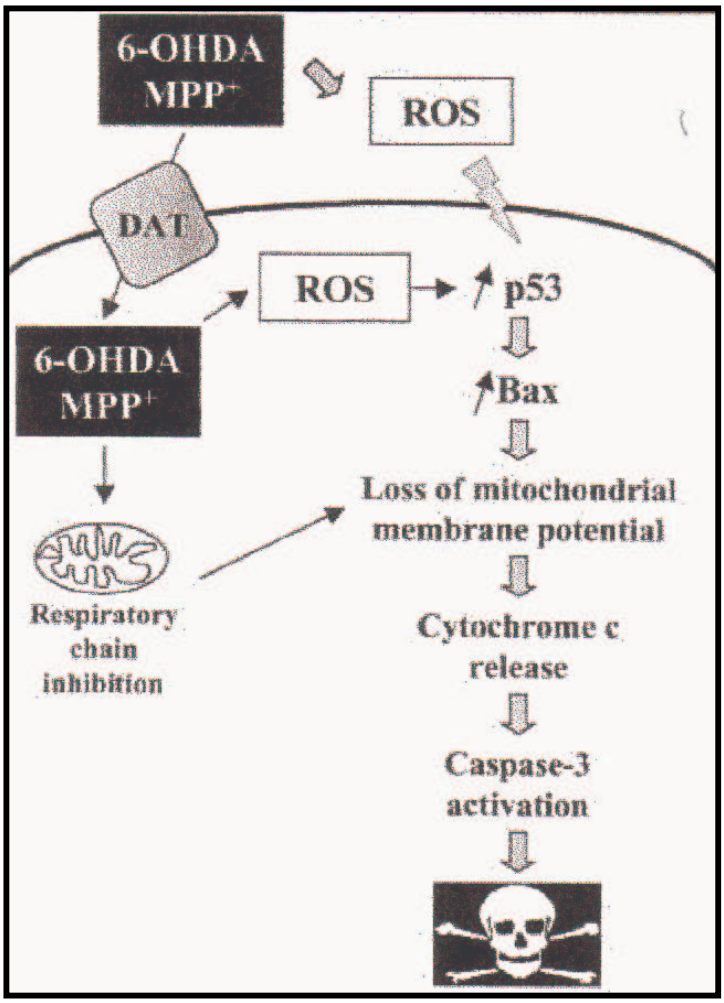


Figure 8.21. Hypothetical molecular pathways leading to apoptosis triggered by MPP^+ (Blum *et al.*, 2001)

Postoperative treatment with ASA resulted in significant attenuation of MPP^+-induced apoptotic cell death and this is clearly evident by the lack of brown staining illustrated in figure 8.18. A recent study by Aubin et al., (1998) indicates that greater amounts of hydroxylated derivates of salicylates in brain tissue following administration of ASA with MPTP than following ASA alone, suggesting that the neuroprotective effects of ASA were related to free radical scavenging. The results are confirmed by studies

performed in chapters five and six were ASA reduced MPP^+-induced $O_2^{-\bullet}$ generation and lipid peroxidation, respectively. Carrasco and Werner (2002) demonstrated the ability of ASA to protect from neuronal death in mesencephalic cell culture systems induced by MPP^+ and 6-OHDA. Furthermore, ASA has been demonstrated to prevent MPP^+-induced mitochondrial defects as was demonstrated by studies in chapter ten were it was shown that ASA prevented MPP^+ inhibition of mitochondrial respiration. The effect of MPP^+ on cellular antioxidant defense systems was also effectively negated by postoperative administration of ASA as shown in chapters eleven and twelve. The above-mentioned reasons provide an underlying mechanism by which ASA protects striatal neuronal cells against MPP^+-induced apoptotic cell death. The result from this study provides visual evidence of the potent inhibitory effect of ASA on MPP^+-induced neurotoxicity and further entrenches its role as a possible therapeutic agent in the treatment of PD.

Figure 8.19 illustrates that acetaminophen offers protection against MPP^+-induced neuronal death. The protection offered by acetaminophen can be attributed to its significant antioxidant properties. Acetaminophen has been shown to have potential neuroprotective effects. Bisaglia *et al.*, (2002) demonstrated that acetaminophen afforded protection of hippocampal neurons and PC12 cultures from amyloid β-peptides induced oxidative stress. This study suggested that the antioxidant properties of acetaminophen could be exploited as a possible therapeutic approach against neurodegenerative disorders. This hypothesis made by Bisaglia et al (2002) can be confirmed by the results shown in figure 8.19 and indicates a possible role for this agent in MPP^+-induced Parkinsonism.

The combined effect of these agents in MPP^+-induced striatal degeneration is illustrated in figure 8.20. The administration of the combination of these agents following MPP^+ infusion provides effective maintenance of cellular morphology. The cells of this treatment group illustrate cellular characteristics that were very similar to those of the control group (figure 8.16). These results further supports that these agents are indicated for MPP^+-induced Parkinsonism and that formulations containing the combination of

these agents would also be of therapeutic benefit in the presence of this potent neurotoxin.

8.4. CONCLUSION

Results from chapters five and six show that ASA and acetaminophen are potent antioxidants. However this study provides visual evidence that the potent antioxidant properties of these agents are converted to a neuroprotective role in the presence of potent neurotoxins such as QA and MPP^+. The results obtained from these studies involving QA and MPP^+ implicate the possible use of these agents in neurodegenerative disorders, which these neurotoxins are capable of mimicking i.e. AD and PD respectively. The preservation of cellular integrity in the striatal region of the rat brain, by the non-narcotic analgesics, implies that these agents could preserve the concentration of the neurotransmitter DA and therefore the effects of these agents on striatal DA levels, in MPP^+ treated rats, was investigated in chapter nine.

CHAPTER NINE

THE EFFECT OF ACETYLSALICYLIC ACID AND ACETAMINOPHEN ON MPP^+ -INDUCED DOPAMINE DEPLETION IN RAT STRIATUM *IN VIVO*.

9.1. INTRODUCTION

Parkinson's Disease (PD) is a debilitating disorder characterized by tremor, rigidity, and immobility. The biochemical basis of the disorder is a deficiency in the neurotransmitter DA in the nigro-striatal pathway in the brain due to destruction of dopaminergic neurons (Piggott *et al*., 1999). The clinical symptoms of this disorder only manifest after about 80% of the neurons are lost.

A persistent increase in free radical generation is believed to be the cause of neuronal death, which reaches more than 80% by middle age (Koutsilieri *et al*., 2002). Thus neuroprotective strategies employing antioxidants is one approach to protect neurons and to curtail the progression of this disorder. There is therefore a dire need to search for neuroprotective agents with novel mechanisms and to assess their potential benefit in such disorders.

MPTP is considered as a powerful as a neurotoxin which is used to induce nigral degeneration in animals and was shown to induce PD-like symptoms in several species of animals including rat, mouse, dog, cat and monkey. When administered to animals, MPTP crosses the BBB and is converted mainly in glial cells, into its effective form, MPP^+, by MAO-B explaining the effects of MAO-inhibitors such as L-Deprenyl against MPTP neurotoxicity (Chiba *et al*., 1984) as described in figure 9.1. MPP^+ then accumulates in dopaminergic cells after selective uptake by energy-dependent dopamine uptake sites (Chiba *et al*., 1985; Pifl *et al*., 1993). Free cytosolic MPP^+ finally enters

mitochondria by an energy-dependent mechanism (Ramsay and Singer, 1986) inhibiting the activity of this organelle and leading to a drop in cellular ATP levels and subsequent cell death as shown in figure 9.1.

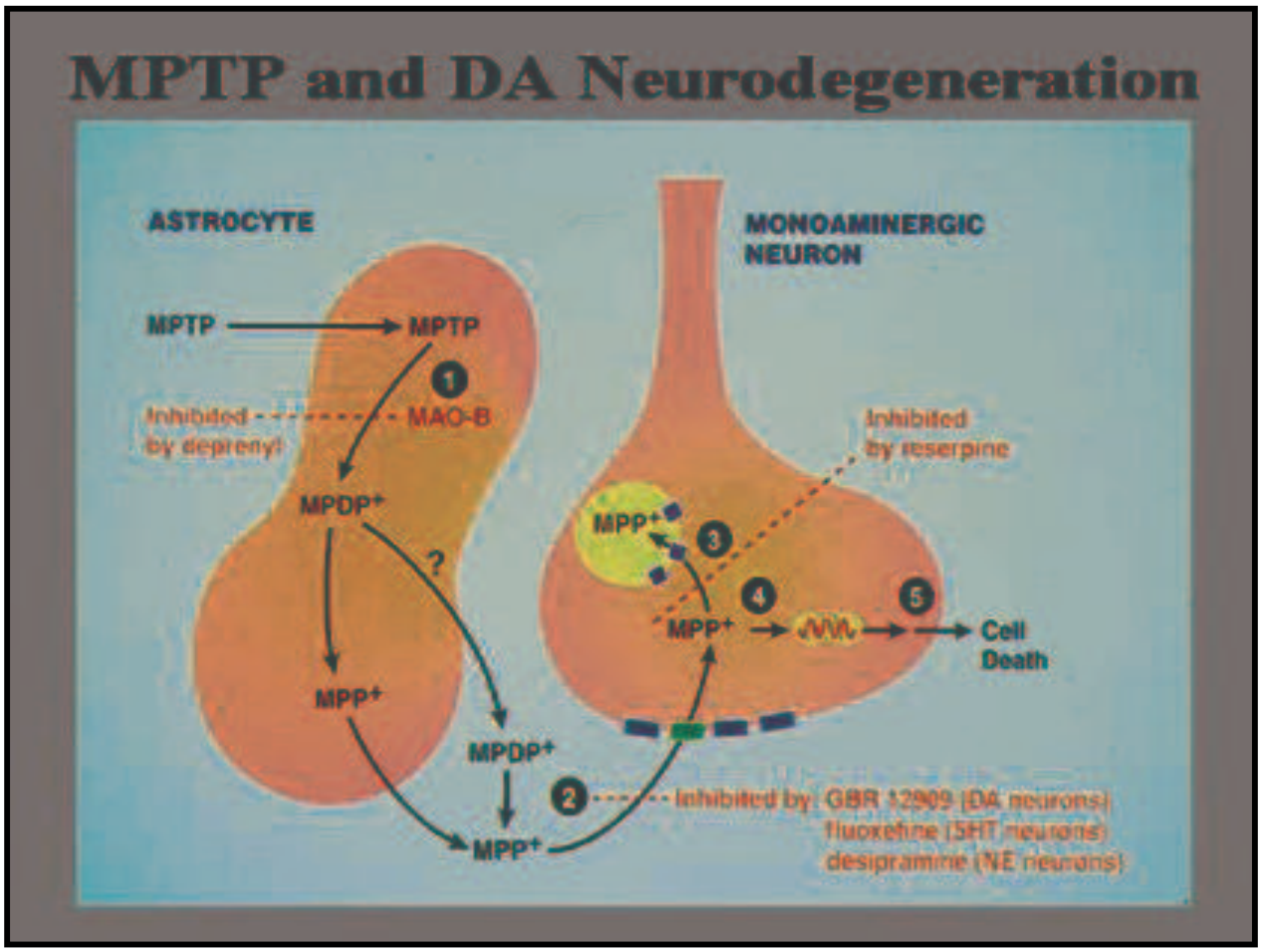


Figure 9.1. Diagram illustrating the conversion of MPTP to the active metabolite MPP^+ and the selective uptake of MPP^+ into the dopaminergic neuron (www3.utsouthwestern.edu/.../ gerimages/gerpic.jpg).

The metabolite of ASA, salicylic acid is known to protect against nigrostriatal DA toxicity induced by MPTP (Mohanakumar *et a.*, 2000) and ASA is a drug known to exhibit antioxidant effects in the brain (Daya *et al.*, 2000). Acetaminophen is often used in combination with acetylsalicylic acid in drug formulations.

The results obtained from chapters five, six, and eight confirm that ASA and acetaminophen are extremely effective at reducing the neurodegenerative effects of MPP^+. The aim of this chapter was to therefore investigate whether these agents are capable of preventing the reduction of DA, in MPP^+ induced dopaminergic neurotoxicity in rat striatum.

9.2. MATERIALS AND METHODS

9.2.1. Chemicals and Reagents

DA and DOPAC were purchased from Sigma Chemical Co., St. Louis, USA. Heptane sulphonic acid, EDTA, acetonitrile, triethylamine, sodium chloride and phosphoric acid were purchased from Merck, Darmstadt, Germany, and were of the highest chemical purity.

9.2.2. Animals

Adult male Sprague-Dawley rats were used in this study and the experimental protocol was approved by the relevent authorities described in chapter three, section 3.2.2.

9.2.3. Drug Treatment

Adult male rats of the Wistar strain were housed in separate cages, in a controlled environment as described in appendix I. The animals were separated into five groups of six animals each. Animals were dosed as described 5.5.2.2.

9.2.4. Surgical Procedures

The surgical procedure was performed as described in chapter five, section 5.5.2.3.

9.2.5. Homogenate Preparation

The rat striatum homogenate was prepared as described in chapter three in section 3.2.4.

9.2.6. Instrumentation

The instrumentation used to determine striatal DA levels was as described in chapter three, section 3.2.5.

9.2.7. Chromatographic Conditions

As described in chapter three, section 3.2.6.

9.2.8. Statistical Analysis

All results were analyzed using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test. A $p<0.05$ was considered as significant.

9.3. RESULTS

Striatal DA was reduced by 59% on the 4th day following intrastriatal administration of MPP^+ in the ipsilateral striatum as compared to contralateral side, which received saline, or the sham control (figure 9.2).

Figure 9.2, illustrates that the administration of acetaminophen (100mg/kg) four times following MPP^+ resulted in a significant attenuation of the effect of the neurotoxin on striatal DA levels.

Interestingly, similar post treatment with ASA completely blocked the MPP^+-induced striatal DA depletion in rats (figure 9.2.).

The combination of these agents resulted in the prevention of the striatal DA depletion induced by MPP^+, as shown in figure 9.2, and shows that when these agents are used in combination the effects of MPP^+ on striatal DA depletion is inhibited by their presence.

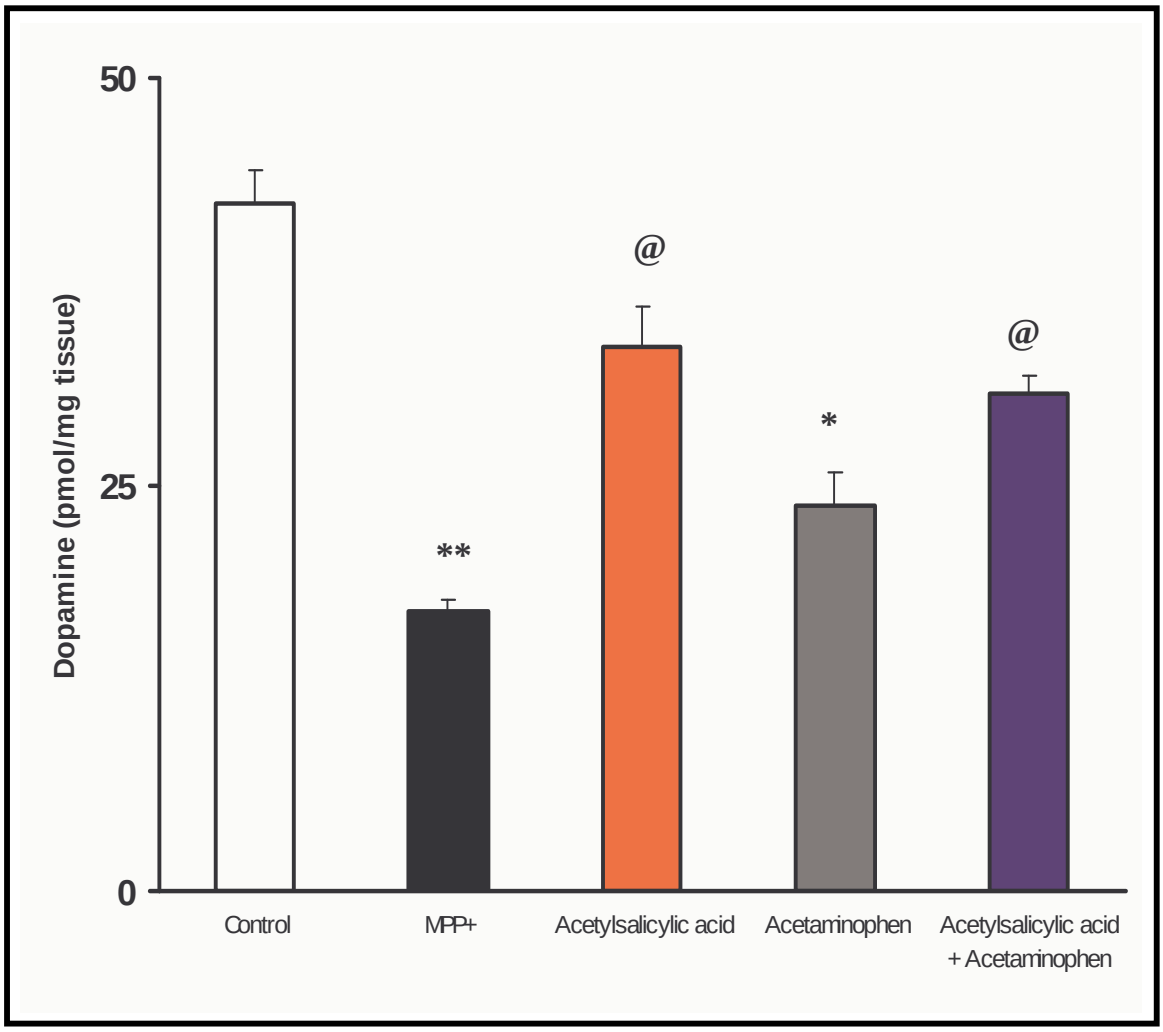


Figure 9.2. Neuroprotective effects of acetaminophen and ASA in MPP^+-induced dopaminergic neurotoxicity in rats. ASA, acetaminophen and the combination of ASA and acetaminophen were given i.p. 4 times, after intrastriatal infusion of MPP^+ in rats. Results presented are Mean ± S.E.M. n = 6. **(p<0.001) as compared to control; $^{@}$(p<0.001) as compared to MPP^+ and *(p<0.05) as compared to MPP^+. Student Newman-Keuls-Multiple Range Test.

9.4. DISCUSSION

In PD it is the free radicals, which ultimately bring about neuronal death of the dopaminergic neurons in the substantia nigra (Koutsilieri *et al*., 2002). An agent that could restore striatal DA levels would have the potential to become a useful therapeutic tool in this debilitating disorder. Logically, a drug, which possesses this capability, could be useful as an ideal adjuvant in PD therapy, to retard the progression of this disease (Gangopadhyay *et al*., 2000).

In the present study, acetaminophen was used since this analgesic in high doses is reported to induce a rise in biogenic amines, as shown in chapter three, such as serotonin (Anoopkumar-Dukie and Daya, 2000, Courade *et al*., 2000) and norepinephrine levels (Courade *et al*., 2000) in rat brain regions. Another non-narcotic analgesic, ASA, the metabolite of which (salicylic acid) has been shown to protect against DA toxicity in PD animal models (Mohanakumar *et al*., 2000; Sairam *et al*., 2003) was also included in the study.

This model has been well characterized and has been extensively used in screening anti-parkinsonian drugs (Muralikrishan & Mohanakumar 1998; Mohanakumar *et al*., 2000; Muralikrishnan *et al*., 2003). MPTP needs to be metabolized by MAO-B to produce an active neurotoxic metabolite MPP^+. Species of animals that have inherent low activity of this enzyme are shown to be resistant to MPTP (Mitra *et al*., 1994). However MPP^+ can be intracranially made available at the terminal region, the striatum or cell body region, the substantia nigra to make such animals parkinsonian (Wu *et al*., 1994; Mohanakumar *et al*., 2002). Rats are resistant to MPTP, and was infused intracranially MPP^+ to test the neuroprotective effect of acetaminophen and ASA *in vivo*, since any influence of these analgesics on MAO-B could be avoided.

The results from this study show that acetaminophen affords only a partial protection against MPP^+-induced DA depletion in the striatum in these animals. However ASA is capable of fully preventing the striatal damage caused by MPP^+. The results are in

agreement with previous reports wherein acetaminophen has been shown to attenuate glutamate-induced neurotoxicity in cultured primary rat embryonic neurons from mesencephalon (Casper *et al*., 2000). Similarly, ASA administered prior to MPTP has been shown to attenuate striatal dopamine depletion in mice (Teismann and Ferger, 2001).

In this study MPP^+-infused animals were treated post-operative and thus these results are more meaningful in a therapeutic frame. The attenuation of the neurotoxic effects of MPP^+ could well be due to the antioxidant properties of these drugs. MPTP toxicity results in oxidative stress causing lipid and protein peroxidation and DNA damage resulting in cell death (Blum *et al*., 2000). Hence a possible reason for these results could be due to the ability of these drugs to prevent free radical damage to cells.

9.5. CONCLUSION

The results obtained from this study show that ASA and acetaminophen prevent striatal DA depletion in the presence of MPP^+. These results indicated that the proven antioxidant properties of these agents, as shown in chapters five and six, induced favorable neurochemical changes in the presence of the potent PD neurotoxin, MPP^+.

CHAPTER TEN

MITOCHONDRIAL FUNCTION

10.1. INTRODUCTION

Parkinson's disease is the second most common neurodegenerative disorder after Alzheimer's disease. Several reports on the association of environmental toxins with Parkinsonism including carbon monoxide (Gordon, 1965; Grinker, 1926; Klawans *et al*., 1982), manganese, (Cooper, 1837; Mena, 1979; Huang *et al*., 1993) and, most importantly MPTP (Davies *et al*., 1979; Langston *et al*., 1983) to support a possible role for environmental agents. Both the genetic abnormalities and the environmental agents result in remarkably similar clinical and pathological syndromes. This supports the hypothesis that PD is a disease caused by more than one aetiological factor.

Research into the mitochondrial involvement in the pathogenesis of PD began with the discovery that the metabolite of MPTP, MPP^+ inhibits complex I of the mitochondrial respiratory chain (Mizuno *et al*., 1987; Nicklas *et al*., 1985; Ramsay *et al*., 1986). Within cells MPTP enters the mitochondria and selectively binds to and inhibits NADH CoQ_0 reductase, which leads to inhibition of ATP synthesis and the generation of free radicals (Krueger *et al*., 1990; Ramsay and Singer, 1986).

Mitochondria are present in every cell and provide the majority of energy in the form of ATP. Tissues with energy demands, such as brain, skeletal and cardiac muscle contain the greatest number mitochondria. Mitochondria contain the only source of extranuclear DNA. Each mitochondrion harbours 2-10 molecules of mitochondrial DNA (mtDNA) which is a 16.5kb circular double-stranded molecule consisting of a heavy (H) and a light (L) chain without any histone coating. mtDNA encodes 22 transfer RNA's (tRNA's) and 12S and 16S ribosomal RNA as well as 13 proteins, all part of the respiratory chain and OXPHOS. Of these 13 polypeptides, seven are sub-units of complex I, one is a sub unit

of complex III, three are subunits of complex IV and two are sub units of complex V (Orth and Schapira, 2002).

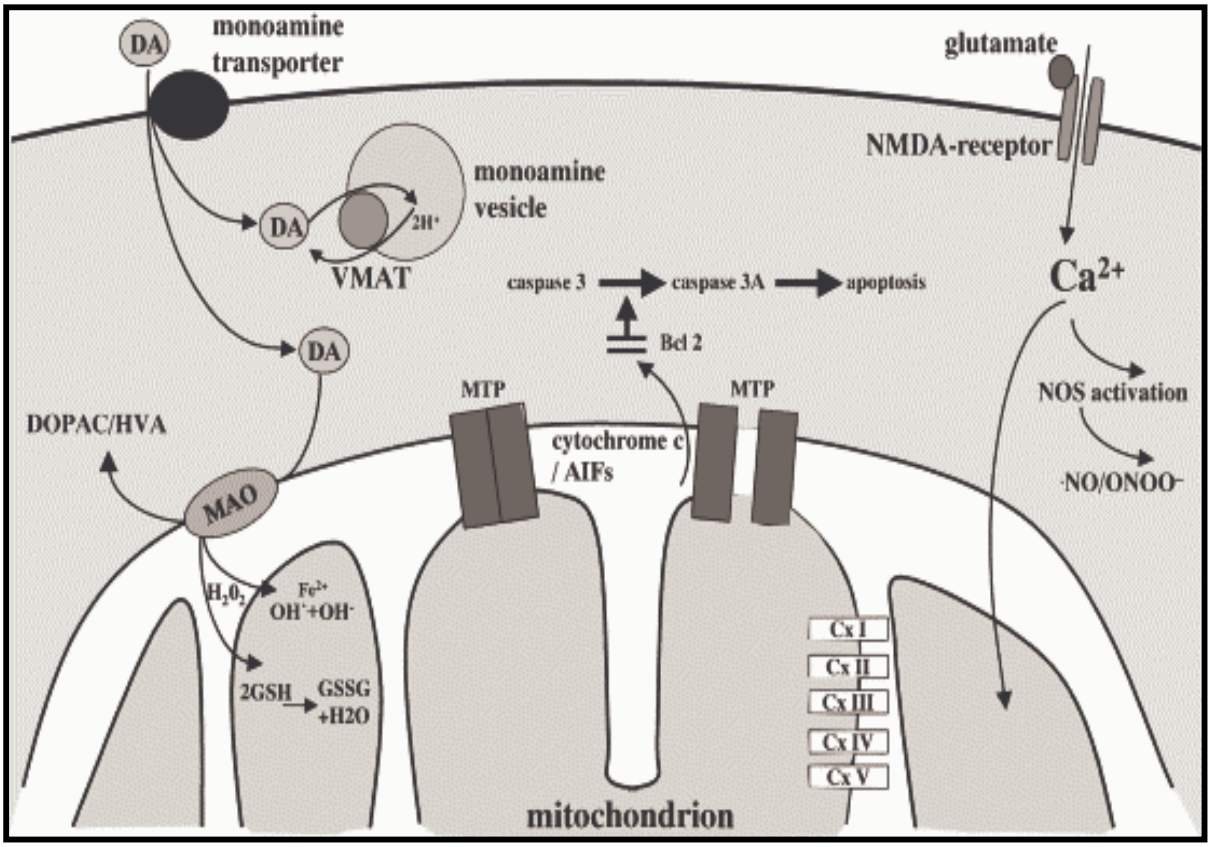


Figure 10.1. A summary of potential pathways for mitochondrial involvement in Parkinson's disease (Orth and Schapira, 2002) Abbreviations: **HVA** (homovanillic acid), **DA** (Dopamine), **MAO** (monoamine oxidase), **VMAT** (Vesicular monoamine transporter), **NOS** (nitric oxide synthase), **NMDA** (N-methyl-D-aspartate), **DOPAC** (3,4-dihydroxyphenylacetic acid) and **Cx** (complex).

Figure 10.1, illustrates the implications that dysfunctional mitochondria have in PD. Impaired complex I activity may compromise mitochondrial function in dopaminergic neurons endogenously. Mitochondria are involved in DA metabolism. DA can be compartmentalised by vesicular associated monoamine transporters (VMAT) into monoamine storage vesicles. Monoamine oxidase B is localised to the outer mitochondrial membrane and converts DA into DOPAC and HVA. Hydrogen peroxide

generated in this process is detoxified in the mitochondrial matrix. Cytochrome *c* and AIFs can activate proapoptotic proteins at the outer membane initiating apoptosis, which is inhibited by Bcl 2. Increased cytoplasmic Ca^{2+} concentrations lead to the activation of NOS and, subsequently, NO and $ONOO^-$ generation. Increased Ca^{2+} influx may occur through the NMDA receptor when the ATP and magnesium dependent blockade is lifted leading to increased excitotoxicity.

10.2. THE EFFECTS OF ACETYLSALICYLIC ACID AND ACETAMINOPHEN ON MPP^+-INDUCED IMPAIRMENT OF THE RAT BRAIN MITOCHONDRIAL RESPIRATORY FUNCTION *IN VIVO*.

10.2.1. INTRODUCTION

Mitochondria are involved in a variety of cellular reactions, which can lead to the formation of $O_2^{-\bullet}$, $^{\cdot}OH$ and H_2O_2. These reactive oxygen species can cause oxidative stress and, as a consequence, damage to cellular contents. Complexes I and III in particular are associated with $O_2^{-\bullet}$ production.

A faulty electron transfer at any point in the ETC results in an electron being accepted by atomic oxygen thus resulting in the creation of free radicals. The respiratory chain loses two to three percent of the electrons during their transfer to molecular oxygen, most of them participating in the production of $O_2^{-\bullet}$ (Boveris, 1977). Thus agents that can scavenge ROS and promote mitochondrial ETC activity can serve to protect against neurodegenerative diseases and the deleterious effects of aging.

In chapter 5, ASA and acetaminophen were shown to be effective scavengers of $O_2^{-\bullet}$ in rat brain homogenate with the combination being most effective. Since much of the $O_2^{-\bullet}$ radicals originate in the mitochondria, the following experiment was conducted to assess the effects of ASA and acetaminophen on the ETC, and whether these agents were able to offer protection against MPP^+ induced impairment of the mitochondrial respiratory functioning.

In the present study, the extent of damage that cyanide causes on rat brain mitochondrial function due to impaired respiration was investigated using a modification of the biological oxidation assay described by Plummer (1971).

This was done using a modification of the biological oxidation assay described by Plummer (1971). Briefly, the experiment used to study the electron transport chain function was done using a dye 2, 6-dichlorophenolindophenol (DPI) (blue) which acts as an artifical electron acceptor and changes colour on reduction or oxidation. In the present experiment, DPI accepts electrons from reduced flavoprotein (fp.2H) and is itself reduced to the colourless form as shown in figure 10.2. Thus, the rate of electron transfer can be measured by following the decolourization of this dye.

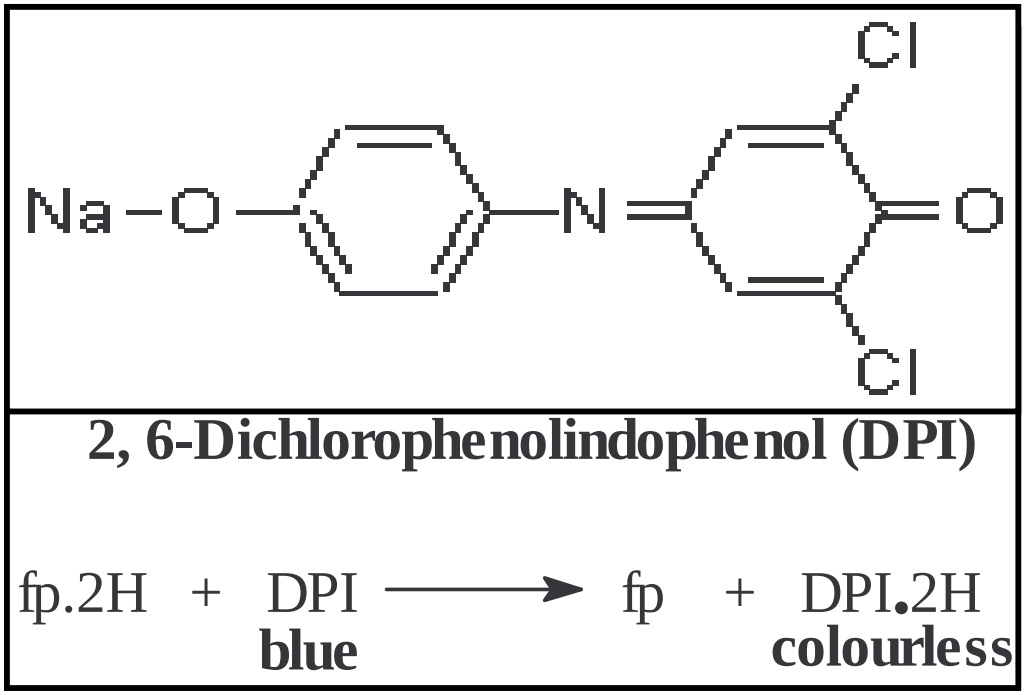


Figure 10.2. Schematic diagram of the reaction of DPI with a reduced flavoprotein (Plummer, 1971).

10.2.2. MATERIALS AND METHODS

10.2.2.1. Chemicals and Reagents

All chemicals and reagents were of the highest quality available and were purchased from commercial distributors. MPP^+, nicotinamide adenine dinucleotide (NAD), sodium chloride (NaCl), 2,6-diclorophenol-indophenol (DPI), and 3[*N*-morpholino]propanesulfonic acid (MOPS) were purchased from Sigma Chemical Corporation, St. Louis, MO, U.S.A. Sucrose was purchased from Saarchem (PTY) Ltd, Krugersdorp, South Africa. L-Malate was purchased from Eastman Organic Chemicals.

10.2.2.2. Dosing of Animals

Animals were dosed as described 5.5.2.2.

10.2.2.3. Surgical Procedures

As described in chapter five, section 5.5.2.3

10.2.2.4. Isolation of Mitochondria from Rat Brain Homogenate

The rats were sacrificed and the brains were rapidly excised as described in appendix II and chilled on crushed ice and thereafter these were rinsed in ice cold saline [0.9% (w/v) NaCl) solution to remove traces of blood. Rat brain mitochondrial suspensions were used for the biological oxidation assay of the electron transport chain. The whole brains were homogenized in 0.32M sucrose + 1M MOPS buffer at pH 7.4 in a manual glass Teflon homogenizer on ice to yield a 10% (w/v) homogenate. Mitochondrial suspensions were prepared by differential centrifugation to obtain relatively pure suspensions of intact mitochondria according to Plummer (1971). The brain homogenate was centrifuged at 600 x g for 10 minutes and then separated into supernatant and pellet. The pellet was

resuspended in half the volume of sucrose and once again centrifuged at 600 x g for 10 minutes and the supernatant obtained was combined with the previous supernatant. The combined supernatant was centrifuged at 8000 x g for 10 minutes and the pellet obtained (crude mitochondria) was washed twice in sucrose and then stored on ice until required. All procedures were carried out at 0-4^{o}C. To ensure that the crude mitochondria were obtained, transmission electron micrographs (TEM; see figure 9.3) were prepared by the standard procedures described by Cross *et al*. (2001).

10.2.2.5. Instrumentation

Samples for the biological oxidation assay were analyzed using a Shimadzu UV-160A UV-visible recording spectrophotometer.

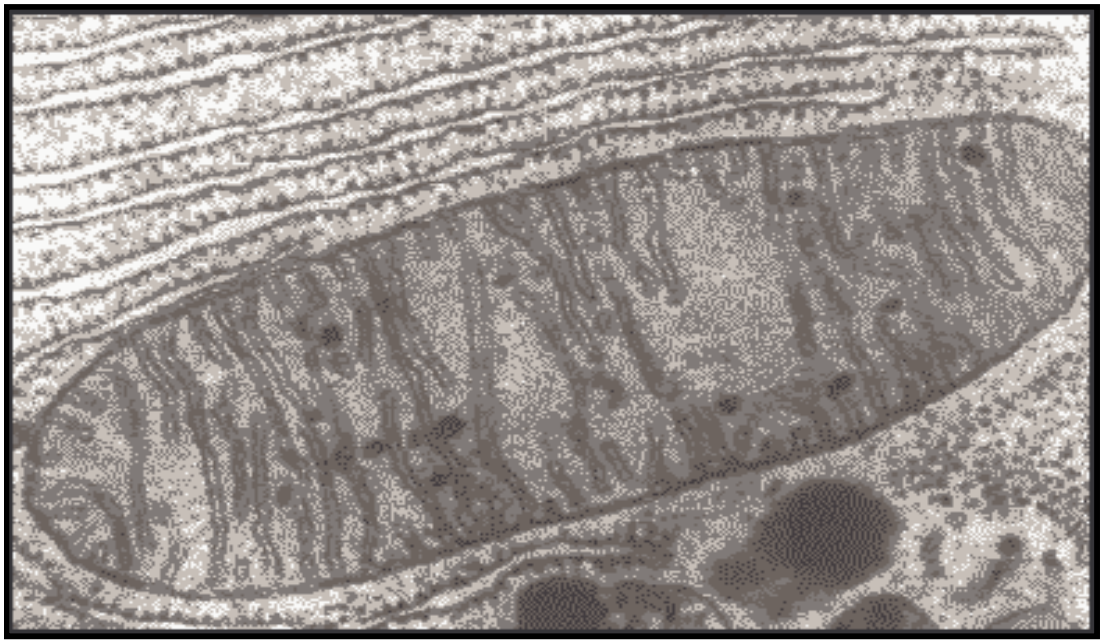


Figure 10.3. TEM micrograph showing the mitochondria that was obtained by differential centrifugation (Magnification X 100 000).

10.2.2.6. Biological Oxidation Assay

A modified method described by Plummer (1971) was used. This spectrophotometric technique was employed to determine the 'activity' of the inner mitochondrial membrane electron transport chain of the whole 'rat brain' mitochondrial suspension. The latter was determined by the rate of reduction of the synthetic electron acceptor dye 2,6-dichlorophenol-indophenol (DPI: 50µM) in the presence of L-malate (substrate). L-malate is used as an indication of the extent of 'activity' of the inner mitochondrial ETC. The substrate and NAD^+ were present in saturating final concentrations of 0.0899mM. Potassium phosphate buffer pH 7.4 (1.5ml) containing 300µl of mitochondrial suspension was incubated for 5 minutes. Following incubation 1.5ml of incubated homogenate, 500µl of substrate/buffer (control), 500µl NAD and 100µl DPI was added in that order to a quartz cuvette. This was inverted once to mix solutions and the decrease in absorbance at 600nm was read over a 5 min period at 30 s intervals. Data was expressed as ΔAbs_{600nm}/min and corrected for appropriate controls.

10.2.2.7. Statistical Analysis

The differences in the means were analyzed 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 treated and control groups. The level of significance was accepted at $p<0.05$ (Zar, 1974).

10.2.3. RESULTS

Exposure of the rat striatum homogenate to, the potent neurotoxin MPP^+, caused a significant decrease in mitochondrial electron transport activity as compared to the control group (Fig. 10.4.).

Figure 10.4, also illustrates that ASA, acetaminophen and ASA and acetaminophen significantly attenuated the reduction in electron transport activity with ASA being the most effective. Acetylsalicylic acid significantly increased mitochondrial electron transport chain activity above that of the basal control level.

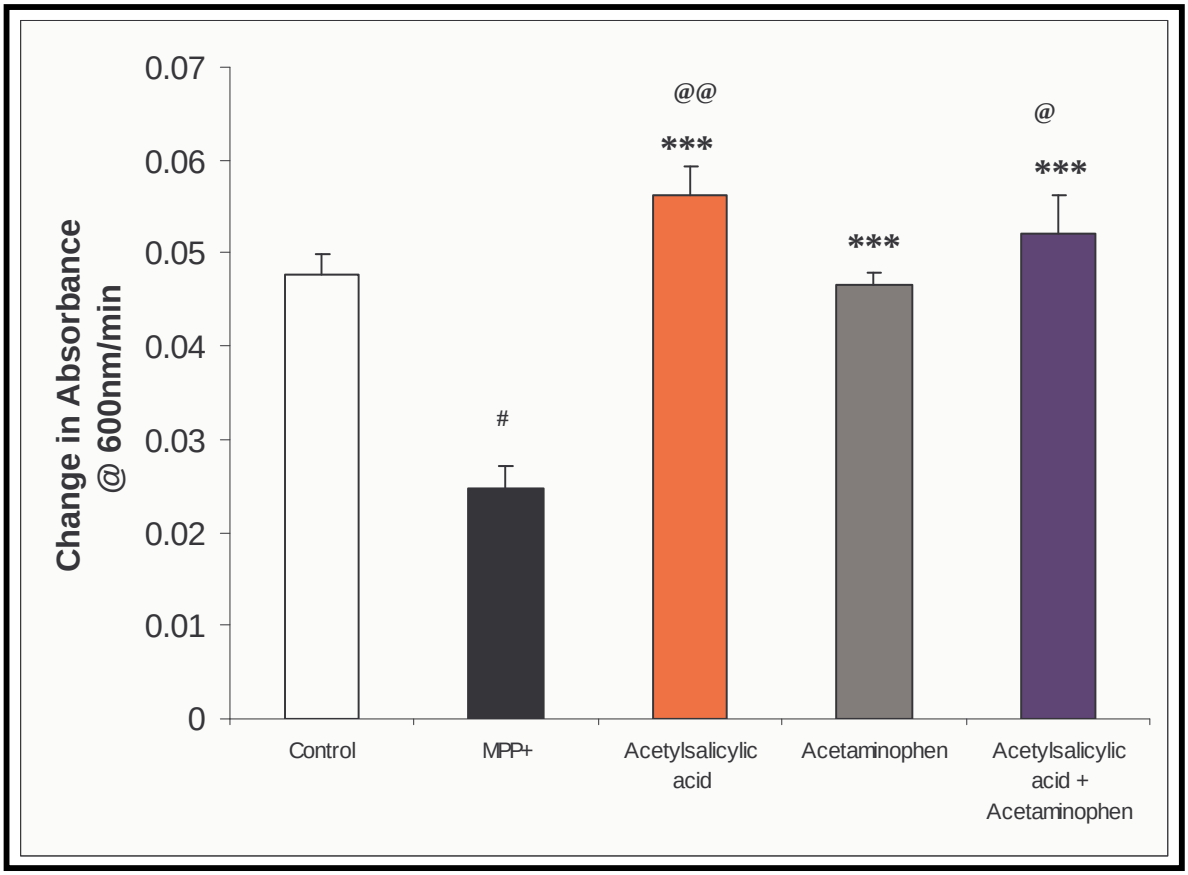


Figure 10.4. Effect of Acetylsalicylic acid, acetaminophen and the combination of acetylsalicylic acid and acetaminophen, in electron transport activity, in MPP^+ treated rats. Results are expressed are mean ± S.E.M. n= 5; $p \leq 0.05$ (ANOVA). # ($p<0.001$) MPP^+ vs control; *** ($p<0.001$) Acetylsalicylic acid vs MPP^+; Acetaminophen vs MPP^+; Acetylsalicylic acid + Acetaminophen vs MPP^+; $^{@@}(p<0.01)$ Acetylsalicylic acid vs control and $^{@}(p<0.05)$ Acetylsalicylic acid + Acetaminophen vs control.

10.2.4. DISCUSSION

It has been difficult to establish the primary step in the oxidative pathway causing neuronal death, since MPTP and MPP^+ themselves alter many of its components and manipulation of many of these components in turn alters many neurotoxin endpoints. Reactive oxygen species formation along several affected metabolic pathways does not help neuronal health. However, the key effect of electron transport inhibition at mitochondrial complex I set in motion numerous processes that seemingly send a neuron into a downward death spiral. Decreased ATP formation from MPP^+ or MPTP exposure (Mizuno *et al*., 1987) would then jeopardize the neuron's ability to maintain critical energy-dependent concentration gradients. Manipulating antioxidant systems modulates MPTP neurotoxicity. Antioxidants, such as ascorbate and *N*-acetylcysteine, etc., protect against MPTP neurotoxicity in mice (Perry *et al*., 1985)

In striatal slices and brain mitochondrial preparations, MPP^+ inhibits NADH-linked oxidation, without affecting succinate oxidation (Nicklas *et al*., 1985; Vyas *et al*., 1986), and parts of the mitochondrial electron transport cascade that generates ATP, but also generates ROS. MPTP was inactive in the mitochondrial preparation, but inhibited oxidation in brain slices, where it could be metabolized to MPP^+. These studies established that MPP^+ is a specific inhibitor of Complex I (NADH dehydrogenase or NADH ubiquinone oxidoreductase) (Ramsay *et al*., 1986) at the ubiquinone-binding site.

Mitochondria have an energy-dependent active uptake system that can concentrate MPP^+ (Ramsay *et al*., 1986; Ramsay and Singer, 1986). The MPP^+ concentrations required to inhibit mitochondrial respiration are relatively high (0.1–0.5 mM) (Vyas *et al*., 1986), but combined active neuronal plasma and mitochondrial membrane transporters might achieve more than 20mM in mitochondria (Ramsay *et al*., 1986; Ramsay and Singer, 1986).

The results from section 10.2.3, clearly demonstrates that MPP^+ adversely affects electron transport (change in absorbance at 4 minutes 0.025) as compared to the control

group (change in absorbance at 4 minutes is 0.048) (Fig. 10.4.). The results show that acetaminophen and ASA and acetaminophen alone are capable of preventing inhibition of electron transport activity in MPP^+ treated rats.

Acetylsalicylic acid however proved the most effective in attenuating the effects of MPP^+ on the electron transport activity. The increase in electron transport activity caused by ASA and acetaminophen individually or in combination would result in an increase in ATP production. In addition, ASA caused an increase in electron transport activity above that of the control value indicating that ASA possibly acts at the level of complex I and preventing the MPP^+ inhibition of complex I activity.

10.3. EFFECT OF ASA AND ACETAMINOPHEN IN MPP^+-INDUCED INHIBITION OF COMPLEX I ACTIVITY IN RAT STRIATUM *IN VIVO*.

10.3.1. INTRODUCTION

NADH-ubiquinone (coenzyme Q) oxidoreductase (EC 1.6.99.3), Complex I is a large and very complicated membrane bound multi-subunit enzyme complex that plays an important role in energy production by the mitochondrial OXPHOS system (Figure 9.6a). As evident in figure 10.5, Complex I appears to have an 'L-shape' and this unique figure is conserved between eukaryote and prokaryote Complex I (Yano, 2002). Complex I is located at the entry point of the ETC and initiates electron transfer by oxidizing NADH and the electrons are transferred to the lipid-soluble electron carrier quinone (coenzyme Q_0) as an electron acceptor.

Complex I deficiency was first identified in the substantia nigra of post mortem PD brain (Gu *et al*., 1998; Janetzky *et al*., 1994; Schapira *et al*., 1989 and 1990). The defect appeared to be restricted to both complex I in the substantia nigra and other brain areas such as the striatum. Mitochondrial dysfunction caused by inhibition of complex I leads to electron leakage, which could further lead to the formation of ROS. These free radicals are a potential source of damage to DNA, lipids, membranes and proteins (Dexter *et al*., 1989; Sian *et al*., 1994). Mitochondria contain antioxidant defense systems such as glutathione, CAT, and SOD to carefully balance free radical production and detoxification. Oxidative stress is believed to be important in the ageing process and details of how reactive oxygen species are produced from various intra- and extracellular sources as well as cellular defence mechanisms and responses to oxidative stress have recently been extensively reviewed (Finkel and Holbrook, 2000).

MPP^+ accumulates inside the mitochondria where it binds to Complex I (Javitch *et al*., 1985; Ramsay *et al*., 1986) causing an inhibition of NAD-linked mitochondrial respiration (Nicklas *et al*., 1985; Ramsay *et al*., 1986). The cellular damage caused by MPP^+ is primarily to energy depletion caused by specific binding to Complex I, a secondary effect being the production of ROS as shown in figure 10.5 (Hasegawa *et al*., 1990; Fahn and Cohen, 1992).

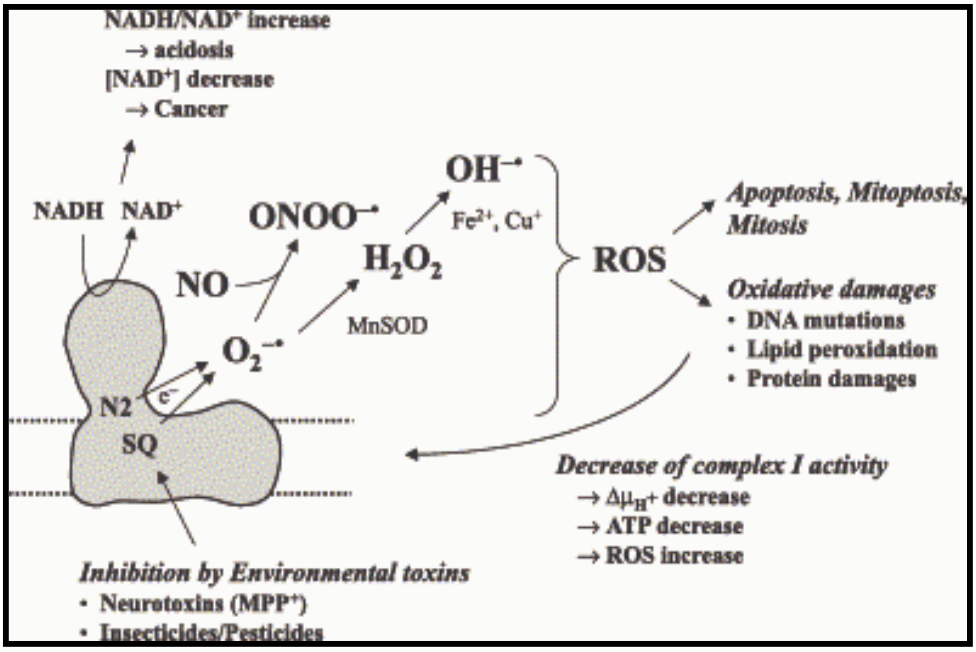


Figure 10.5. L-shaped appearance of Complex I and generation of ROS by Complex I and its implicated consequences. Superoxide free radical ($O_2^{\bullet-}$) is generated by a single electron reduction of oxygen (O_2) by SQ or cluster N2. Hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$) are formed by Manganese superoxide dismutase (MnSOD) and by Fenton's reaction in the presence of iron (Fe) or copper (Cu), respectively. Nitric oxide (NO) seems to react with $O_2^{\bullet-}$ or $SQ^{-\bullet}$ species, generating a perinitrate species, $ONOO^{\bullet-}$ (Yano, 2002).

In the previous study, ASA and acetaminophen were shown to be effective in partially reversing the inhibition of the mitochondrial ETC activity induced by MPP^+. It was therefore postulated from the above study that ASA and acetaminophen could possibly increase complex I activity in the presence of MPP^+. Thus, the present study investigates

the effect of ASA and acetaminophen, either individually or in combination, on complex I activity in MPP^+ treated rats activity *in vivo*.

10.3.2. MATERIALS AND METHODS

10.3.2.1 Chemicals and Reagents

All chemicals were of the highest quality available and were purchased from commercial distributors. Potassium dihydrogen phosphate (KH_2PO_4), potassium hydroxide, Trisaminomethane (Tris), sucrose, Coenzyme Q_0, NADH, antimycin A and rotenone were purchased from Sigma Chemical Corporation, St. Louis, MO, U.S.A.

10.3.2.2 Dosing of animals

Male Wistar rats were used for this experiment and were housed according to the conditions described in appendix I. The rats were treated as described in section 5.5.2.2. The striatum of the rat brain was homogenized and mitochondrial P_2 fraction was isolated as described below in section 10.3.2.4.

10.3.2.3. Surgical Procedures

As described in section 5.5.2.3.

10.3.2.4. Isolation of Mitochondrial P_2 Fraction

Mitochondrial P_2 fraction was isolated according to a modification of the method described by Shults *et al*., 1995. Briefly, rat whole brain was homogenized in ice cold 0.32M sucrose dissolved in 10mM potassium phosphate buffer (pH 7.2), in a Teflon glass homogenizer with clearance of 0.1cm, to yield a 10% w/v homogenate. The homogenate is then centrifuged at 1 500 x g for 10min at 4^{o}C. Thereafter, the pellet that forms is

discarded and the supernatant is spun at 10 000 x g for 30 min at 4^{o}C. At this stage, the supernatant that forms is discarded and the pellet is resuspended in the same volume of ice cold 50mM Tris in 10mM potassium phosphate buffer, pH 7.2 (1:1 v/v) and this is centrifuged at 10 000 x g for 30min at 4^{o}C. The supernatant is discarded and the pellet is resuspended in cold 10mM potassium phosphate buffer, pH 7.2. Finally the pellet is sonicated until it is uniformly dispersed. The mitochondrial P_2 fraction was used immediately or stored for 24 hours at -20^{o}C until use. Test samples revealed that storage of the mitochondrial P_2 fraction did not alter complex I activity levels compared to fresh samples. The submitochodrial particles were prepared by sonication. All procedures were carried out at 0-4^{o}C. Protein content was determined by the method of Lowry *et al*., (1951).

10.3.2.5. Complex I (NADH:ubiquinone oxidoreductase) Assay

Complex I (reduced nicotinamide-adenine dinuleotide (NADH)-CoQ reductase) was determined using a modified protocol from Shults *et al*., (1995). The oxidation of NADH to NAD^+ by complex I was monitored by a fall in absorbance over a period of 2 min. DB, is a synthetic water-soluble analogue of ubiquinone (coenzyme Q_o) (Estornell *et al*., 1993). For the *in vivo* study, the final 1ml assay mixture contained; an aliquot of the submitochondrial suspension sample (100µl), 850µl of assay buffer (10mM KH_2PO_4, pH 7.2, 5mM KCN, 2µg/ml Antimycin A, 5mM $MgCl_2$, 2.5mg/ml of defatted BSA) and CoQ_O (54.9µM). The antimycin was added in order to inhibit complex II and III activity while KCN was used to inhibit complex IV activity (figure 9.3). After adding the buffer, mitochondrial fraction sample and CoQ_o the absorbance at 340nm was recorded for 0 and 2 min. The change in absorbance was noted and an incubation time with CoQ_o of 2 min was given. Thereafter, NADH (0.12mM) was added to the above assay mixture and the absorbance at 340nm was noted at time 0 and 2 min. This assay was repeated with rotenone at a final concentration of 2µM, allowing for the calculation of the rotenone-sensitive complex I rate. The change in absorbance without NADH was deducted from the change in absorbance with NADH (ε_{NADH} = 159nmoles). The complex I activity was expressed as nmol of NADH oxidized/min/mg protein.

10.3.2.6. Statistical Analysis

The differences in the means were analyzed for statistical significance as described in section 10.2.2.7.

10.3.3. RESULTS

Sub-cellular fractions obtained from MPP^+ treated rat brain showed a significant decrease in complex I activity (Fig. 10.6.). Figure 10.6, illustrates that post treatment of MPP^+ treated rats with ASA significantly blocked the effect of MPP^+. Acetaminophen and the combination of ASA and acetaminophen also inhibited the effects caused by MPP^+ on rat brain mitochondria but were less effective than ASA. It is evident from Figure 10.6, that ASA treatment was able to significantly increase mitochondrial complex I activity in the rat striatum above that of the basal control level.

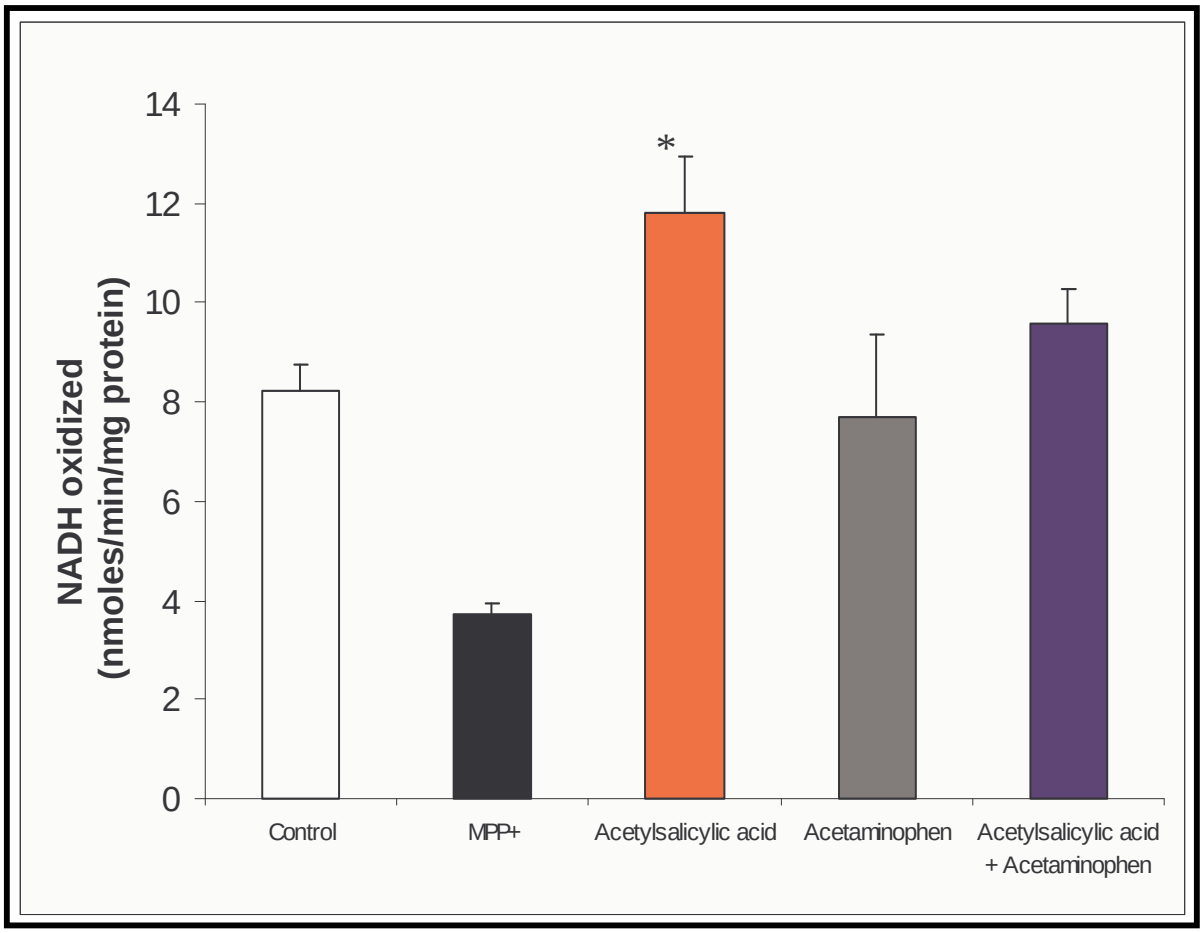


Figure 10.6. Effect of acetylsalicylic acid, acetaminophen and the combination of acetylsalicylic acid and acetaminophen, in complex I activity, in MPP^+ treated rats. Results are expresed are mean ± S.E.M. n= 5; $p \leq 0.05$ (ANOVA). $^{\#}(p< 0.001)$ MPP^+ vs. control; ***($p< 0.001$) Acetylsalicylic acid vs. MPP^+, **($p<0.01$) Acetaminophen vs. MPP^+ and Acetylsalicylic acid + Acetaminophen vs. MPP^+ and $^{@}(p<0.05)$ Acetylsalicylic acid vs. control.

10.3.4. DISCUSSION

Increasing evidence suggests that mitochondria play a role in the events leading to death of the dopaminergic cells in PD (fig. 10.1.). Normal mitochondrial function is important for the preservation of cellular antioxidant defense mechanisms and the prevention of excitotoxicity and inappropriate apoptosis.

It has been demonstrated that ASA targets mitochondrial respiratory chain complex I-III, resulting in an increased ATP production *in vitro* (De Christóbal *et al*., 2001). This mechanism has also been shown to occur *in vivo* models. De Christóbal *et al*. (2001) also determined that the ATP levels, in the brain, reduced by ischaemia is inhibited by the previous administration of ASA, and that this drug is able to increase the levels of ATP by itself when it is administered to sham-operated animals.

MPP^+ toxicity produces loss of dopaminergic neurons in a very similar distribution to the pathology of PD (Forno *et al*., 1988). MPP^+ is actively taken up into cell by monoamine transporters in the membranes of dopaminergic neurons. Within cells MPP^+ enters mitochondria and selectively binds to and inhibits NADH CoQ10 reductase (Complex I), which leads to the inhibition of ATP synthesis and the generation of free radicals (Krueger *et al*., 1990; Ramsay and Singer, 1986).

Figure 10.6 shows that intrastriatal infusion of MPP^+ in rats results in a drastic reduction in complex I activity in comparison to the control. This therefore confirms findings of other authors (Forno *et al*., 1988) that the toxin does block the oxidation of NADH to ubiquinone. The mitochondrial inhibition leads to a decrease in cellular ATP levels (Di Monte *et al*., 1986), loss of mitochondrial membrane potential, alterations of calcium homeostasis and radical formation resulting in cell death.
However the inhibition of complex I, by MPP^+, is reversed by post treatment with the non-narcotic analgesics.

ASA was again shown to be most effective at reversing the effects of MPP^+. In addition, ASA was effective in increasing complex I activity in the ASA treated group above that of the control treated rats indicating that it is preventing the MPP^+ induced inhibition of mitochondrial complex I activity and possibly competing with MPP^+ at complex I. These results correlate with those obtained in figure 10.4, and therefore demonstrate that inhibition of complex I by MPP^+ also occurs at the level of the electron transport chain.

The results in this chapter provides novel evidence that ASA inhibits processes, such as complex I activity inhibition, which results in a decrease intracellular ATP content.

10.4. CONCLUSION

Strategies aimed at preventing mitochondrial dysfunction (Kass and Lipton 1982; Galeffi *et al*., 2000) have been shown to be neuroprotective. This coupled with the potent free radical scavenging properties of ASA and acetaminophen, as was demonstrated in chapters 5 and 6, would therefore indicate the use of these drugs as neuroprotective agents in the treatment of PD.

The next level of endeavor would be to determine whether these agents possess the key role of reducing oxidative stress in the cell. The effect of ASA and acetaminophen on the cellular antioxidant defense system, which consists of CAT, SOD and glutathione was therefore investigated in chapters eleven and twelve.

CHAPTER ELEVEN

ENZYMATIC ANTIOXIDANT DEFENSE SYSTEM IN THE BRAIN.

11.1. INTRODUCTION

Free radicals are chemical entities characterized by the orbital containing unpaired electrons. These electrons confer to these molecules a strong propensity to react with target molecules giving or withdrawing one electron for them to complete their own orbital (Castagne *et al*., 1999). Excess production of these molecules can potentially damage different macromolecules such as proteins, nucleic acids and lipids thereby leading to cellular degeneration (Cohen and d'Arcy Doherty, 1987). The brain is extremely vulnerable to free radical damage as compared to other tissues, because it contains large quantities of oxidisable lipids, metals, and has comparatively less antioxidant defense mechanisms. Hence the need to preserve the already existing, antioxidant mechanisms such as SOD, CAT, GPx and other molecules in the brain which are essential in preventing neurodegeneration.

In idiopathic PD, antioxidant molecules such as GSH, SOD and CAT have been reported to be altered (Perry *et al*., 1982; Saggu *et al*., 1989; Marttila *et al*., 1988; Ambani *et al*., 1975). The mitochondria are one of the major sources of $O_2^{-\bullet}$ generation and an estimated 1-2% of the electron leak from the mitochondrial electron transport chain is utilized to form $O_2^{-\bullet}$ (Turrens and Boveris, 1980) which are dismutated by SOD (Nohl and Hegner, 1978). These involve in the dismutation of $O_2^{-\bullet}$ to H_2O_2, which is converted to H_2O and O_2 by CAT or by the oxidation of GSH in presence of GPx.

There is a delicate balance between the rise of dismutation of $O_2^{-\bullet}$ by SOD and removal of H_2O_2. Catalase has a greater affinity to this substrate than does GPx (Chance *et al*.,

1979) and CAT is therefore more inducible to the presence of higher concentrations of H_2O_2 than GPx (Jones *et al*., 1981).

The interplay of these mechanisms of free radical scavenging that exist in the brain serve as important defense systems to prevent cell death and subsequent neurodegeneration. Figure 11.1 illustrates the interplay between the various antioxidant molecules such as GSH and the enzymes responsible for scavenging free radicals in the brain. The diagram also explains the biochemical pathways by which the antioxidant molecules react with free radicals.

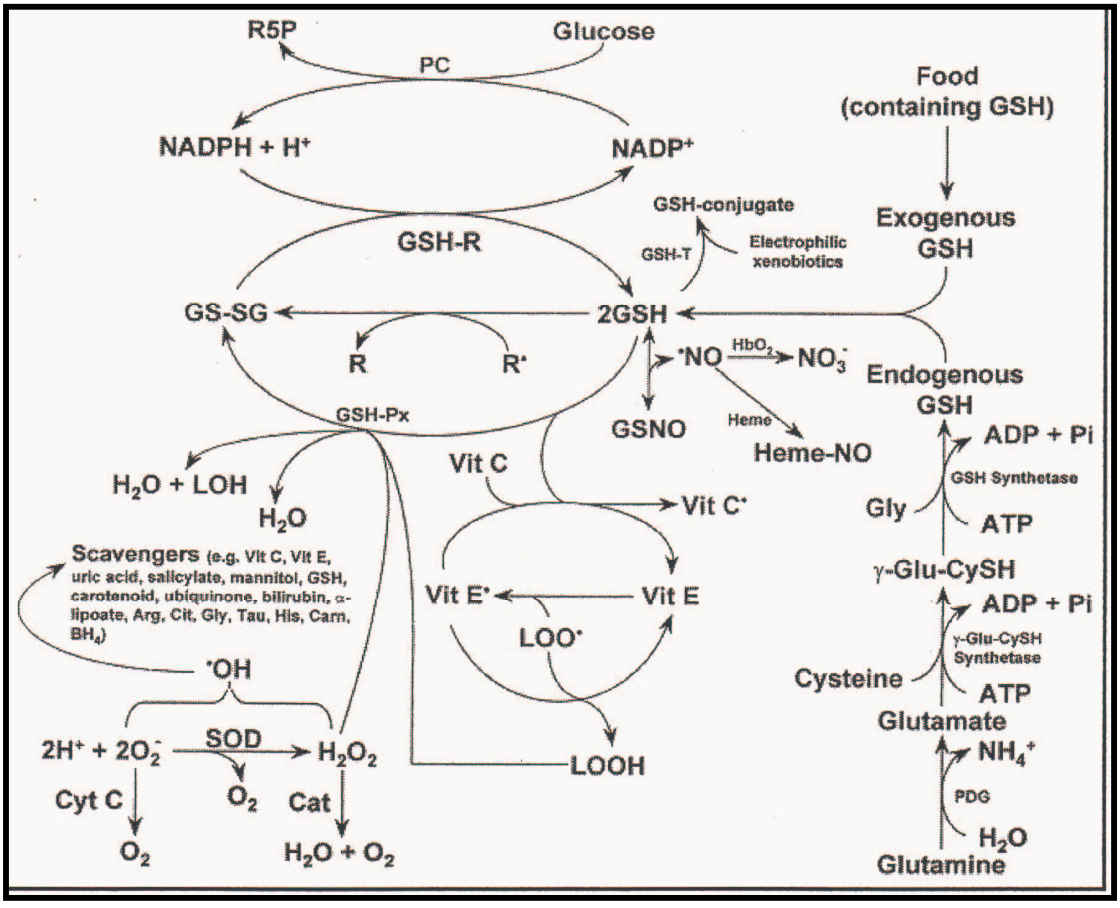


Figure 11.1. Removal of oxygen and nitrogen free radicals and other reactive species in mammalian cells by the antioxidant defense mechanisms in the brain (Fang *et al*., 2002).

11.2. THE EFFECT OF ACETYLSALICYLIC ACID AND ACETAMINOPHEN IN MPP^+-INDUCED ALTERATION OF SOD FUNCTION IN RAT STRIATUM *IN VIVO*.

11.2.1. INTRODUCTION

Superoxide anions are free radicals, which can be generated in the brain by several mechanisms, such as: (1) the inefficiency of the electron carrying components of the mitochondrial electron transport chain, (2) monoamine degradation, and (3) the xanthine oxidase reaction. Nevertheless, the produced $O_2^{-\bullet}$ can be metabolized by SOD, which is present in both the cytosol and mitochondria (Hussain *et al*., 1995). The dismutation of the $O_2^{-\bullet}$ by SOD serves as one of the antioxidant defense mechanism in the brain. Superoxide dismutase (EC1.15.1.1) is a secretory, tetrameric, copper- and zinc-containing glycoprotein with a subunit molecular weight of about 30 000 (Marklund, 1982; Tibell *et al*., 1987).

The potent parkinsonian neurotoxin, MPP^+, has been shown to retard mitochondrial function by inhibiting complex-I activity in the mitochondria as demonstrated in chapter ten and generate free radical production such as $O_2^{-\bullet}$ as shown in chapter five. These findings have been confirmed by previous reports (Nicklas *et al*., 1985; Gluck *et al*., 1994; Mohanakumar and Steinbush, 1998; Mohanakumar *et al*., 2002). In addition, authors, Muralakrishnan and Mohanakumar, 1998; Thomas and Mohanakumar, 2003, have shown MPP^+ to damage the antioxidant machinery of the mitochondria such as SOD and CAT, in the brain.

ASA and acetaminophen have been shown to be capable neuroprotective agents against MPP^+-induced toxicity with regard to the scavenging of free radicals (chapters five and

six) and effective at preventing complex-I inhibition by this potent neurotoxin (chapter ten).

Therefore the aim of this study was to investigate the effects of MPP^+ on SOD activity in rat striatum, *in vivo,* as well as to assess whether post-operative treatment with ASA and acetaminophen altered the effect of this neurotoxin on SOD activity.

11.2.2. MATERIALS AND METHODS

11.2.2.1. Chemicals and Reagents

All chemicals and reagents were of the highest quality available and were purchased from commercial distributors. Pyragallol was purchased from Sigma Chemical Corporation, St. Louis, MO, U.S.A. EDTA was purchased from Merck, Darmstadt, Germany.

11.2.2.2. Dosing of Animals

Animals were dosed as described 5.5.2.2.

11.2.2.3. Surgical Procedures

As described in chapter five, section 5.5.2.3.

11.2.2.4. Preparation of Cytosolic / Particulate fractions

The striata were homogenized in potassium phosphate buffer (pH 7.8, 0.1M) using a glass Teflon homogenizer. The homogenate was then centrifuged at 100 000 x g for 60 minutes at 4ºC. The supernatant obtained corresponds to the cytosolic fraction containing CuZn-SOD. The pellets were resuspended in the buffer, freeze thawed three times, and centrifuged at 100 000 x g for 60 minutes at 4ºC. This supernatant corresponds to the

particulate fraction containing Mn-SOD. The cytosolic and particulate fractions were mixed together in order to obtain the total enzyme fraction.

11.2.2.5. Superoxide Dismutase Assay

The method of Marklund and Marklund (1974) was employed to assay the SOD activity, with minor modifications. Pyragallol in the presence of 10mM EDTA auto-oxidizes rapidly in alkaline solution (50mM Tris-HCl; pH 8.2). The reaction mixture consisted of: supernatant (approximately 500µg protein), 300µl of 2mM pyragallol, 300µl of 10mM EDTA and 62.5mM Tris-HCl was used to make up to 3ml. Pyragallol auto-oxidation was monitored over a period of 3 minutes with and without the enzyme protein. The absorbance was read at 420nm using a SHIMADZU UV-Absorbance Spectrophotometer. The auto-oxidation of pyragallol was linear with the activity of the enzyme present. Fifty percent inhibition/(mg protein$\cdot$min^{-1}) was taken as one unit of enzyme activity.

11.2.2.6. Statistical Analysis

The results were analyzed using a one-way analysis of variance (ANOVA). If the F values were significant, the Student's Newmans-Keuls Multiple Range test was used to compare the treated and control groups. The level of significance was accepted at $p<0.05$ (Zar, 1974).

11.2.3. RESULTS

The intracranial administration of MPP^+, in adult male rats, resulted in a significant increase (± 25%) in SOD activity as compared to the control (figure 11.2).

Postoperative treatment with ASA resulted in further up regulation of SOD activity by approximately 55% and 24% when compared to the control and toxin groups, respectively as demonstrated in figure 11.2. Figure 11.2, illustrates that similar post

treatment with acetaminophen and the combination of ASA and acetaminophen resulted in a down regulation of SOD activity, on day 4, when compared to the MPP^+ treated rats. Acetaminophen had the more pronounced effect of these two treatment regimens with regards to the down regulation of enzyme activity, which was lower than the control group.

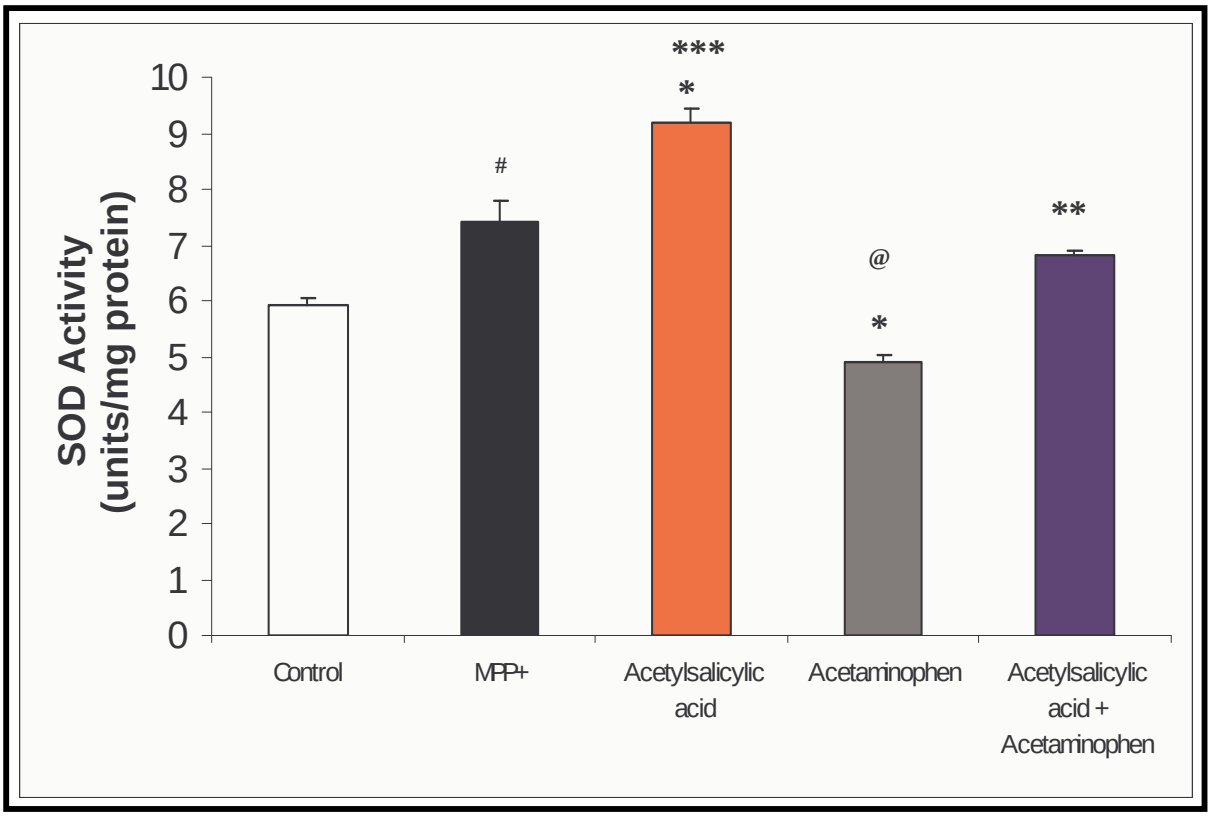


Figure 11.2. The effect of the administration of acetylsalicylic acid and acetaminophen alone or in combination on SOD activity following intracranial administration of MPP^+ in rat mitochondrial homogenate. Each bar represents the mean ±SEM, n=5. $^{\#}(p<0.001)$ control vs. MPP^+; *($p<0.001$) Acetaminophen vs. MPP^+ and MPP^+ vs. acetylsalicylic acid; **($p<0.05$), acetylsalicylic acid + acetaminophen vs. MPP^+; ***($p<0.001$) control vs. acetylsalicylic acid, and $^{@}(p<0.01)$ acetaminophen vs. control. Student Newman-Keuls-Multiple Range test.

11.2.4. DISCUSSION

Free radical generation, with increased production of activated oxygen species, such as $O_2^{-\bullet}$ and $^{\bullet}OH$ has been suggested to play an important role in nigral neuronal death in PD (Obata *et al*., 2001). Oxidative stress in nigral neurons in patients with PD results in an increase in iron content (Dexter *et al*., 1989), SOD activity and lipid peroxidation of neuronal cells.

The aim of this study was to elucidate whether ASA and acetaminophen either alone or in combination are capable of altering any possible effects of MPP^+ on SOD activity.

The salient feature of this study is consistent with an earlier report by Muralikrishnan and Mohanakumar (1998), where the prolog of MPP^+, MPTP was administered to mice and resulted in a significant upregulation of SOD activity when compared to the control group. Electron paramagnetic resonance studies have indicated that MPP^+ acts at the active site of NADH-ubiquinone (Adams *et al*., 1993; Zang and Misra, 1993), which indicated the formation of $O_2^{-\bullet}$ in mitochondria. The effect of this toxin on the mitochondria was also demonstrated in chapter ten, where a significant inhibition of complex I activity was observed. The increase in the total SOD activity that was observed in this study, following exposure to the neurotoxin, may indicate the induction of this enzyme due to the oxidative insult of the neurotoxin on the mitochondrial electron transport system which results in the generation of $O_2^{-\bullet}$ (Zang and Misra, 1993).

Acetylsalicylic acid treatment significantly enhanced SOD activity in the striatum (figure 11.2.). The protective effect of ASA, at preventing the MPP^+-induced toxicity that was investigated in chapter ten, could therefore be due to the upregulation of SOD, which is an important antioxidant molecule in the mitochondria. The results obtained from this study also indicate that ASA interferes with the initial events of the neurotoxicity of MPP^+.

Previous studies have indicated that acetaminophen partially alleviated MPP^+ induced neurotoxicity as well as the degenerative effect of the toxin on striatal DA levels (chapter nine; Maharaj *et al*., 2004; Sairam *et al*., 2003). The reduction of activity of SOD, in comparison to the toxin group, could therefore be due to the $O_2^{-\bullet}$ scavenging properties of acetaminophen (as was demonstrated in chapter five), which resulted in the low activity that was observed.

Concomitant administration of ASA and acetaminophen also resulted in a slight decrease in SOD activity when compared to the toxin group. However this decrease was not significant when compared to the control group, which indicated that the combination cancelled the neurotoxic effects of MPP^+ in rat striatum.

The results presented in this study add to the existing understanding of the free radical mechanisms involving MPP^+ induced dopaminergic neurotoxicity. In addition the study unearths the neuroprotective effects of ASA and acetaminophen (which has so far been known for the management of pain) and further implicates ASA in the treatment of PD.

11.3. THE EFFECT OF ACETYLSALICYLIC ACID AND ACETAMINOPHEN IN MPP^+-INDUCED ALTERATION OF GLUTATHIONE PEROXIDASE FUNCTION IN RAT STRIATUM *IN VIVO*.

11.3.1. INTRODUCTION

Although the etiology of PD is still ill defined, there is growing interest surrounding the phenomena underlying the degeneration process. In particular oxidative stress has been put forward as one of the major causes of the nigral degeneration. A loss in detoxification has been reported in PD patients with a reduction in GSH levels, GPx expression and CAT expression (Blum *et al*., 2001). GPx is present in large amounts in the brain during the nervous system development, decreasing during the rat adult life (Nanda *et al*., 1996). For these reasons free radicals have been pointed out as important molecules involved in the nervous system pathologies such as PD, AD and HD (Jenner, 1998; Castagne *et al*., 1999).

Glutathione reacts with free radicals both non-enzymatically and also acts as an electron donor in the reduction of peroxides catalyzed by GPx. This results in the conversion of GSH to GSSG, which induces a decrease in GSH levels (Salinas and Wong, 1999). Glutathione peroxidase plays a major role in the recycling of GSH as suggested by Klivenyi *et al*., 2000, who reported that GPx knockout mice challenged with MPTP exhibited greater depletion of DA compared to age matched control mice. Experiments involving GPx knockout mice treated with MPP^+ indicate that this toxin mediates its deleterious effects at least in part through induction of oxidative damage (Klivenyi *et al*., 2000; Przedborski *et al*., 1992).

The aim of the present study was to investigate the effects of MPP^+ on GPx activity in the rat striatum as well as to establish whether treatment with ASA and acetaminophen either individually or in combination would alter the effects of MPP^+ on GPx activity.

11.3.2. MATERIALS AND METHODS

11.3.2.1. Chemicals and Reagents

All chemicals and reagents were of the highest quality available and were purchased from commercial distributors. NADPH, reduced glutathione, glutathione reductase (Sigma type III) and *tert*-butyl-hydroperoxide (*t*-butyl-HPx) were purchased fro from Sigma Chemical Corporation, St. Louis, MO, U.S.A.

11.3.2.2. Dosing of animals

Animals were dosed as described 5.5.2.2.

11.3.2.3. Surgical Procedures

As described in chapter five, section 5.5.2.3.

11.3.2.4. Glutathione Peroxidase Activity

Glutathione peroxidase activity was measured by the method described by Sinet *et al*. (1975) using *t*-butyl-HPx as a substrate. The striata was homogenized in PBS 0.05M (pH 7.4) in buffer solution (1mg/5µl) using a Teflon homogenizer. Striatal protein (30µg) was added to 500 µl of PBS containing 10^{-3} M reduced glutathione, 2 units of yeast glutathione reductase (Sigma type III) and 2 x 10^{-4} NADPH and incubated for 10 minutes at 37 ºC. The reaction was initiated by the addition of *t*-butyl-HPx to a final concentration of 10^{-3} M, under constant agitation. The oxidation of NADPH of 6.22 x 10^3

at 340 nm and the reaction was made for 5 minutes. One enzyme unit was defined as 1µM NADPH/mU per mg protein. The results were analyzed using a one-way analysis of variance (ANOVA). If the F values were significant, the Student's Newmans-Keuls Multiple Range test was used to compare the treated and control groups. The level of significance was accepted at $p<0.05$ (Zar, 1974).

11.3.3. RESULTS

There was a significant increase in GPx activity on the 4th day following intracranial administration of MPP^+ as compared to the control group, which received saline (figure 11.3).

Figure 11.3 illustrates that postoperative treatment of adult male rats with ASA (100mg/kg) resulted in a ± 42 % down regulation in activity of GPx as compared to the toxin. ASA also significantly reduced the activity of the enzyme in comparison to acetaminophen and the combination of ASA and acetaminophen. In addition GPx activity in rats treated with ASA was reduced below basal control levels by ± 20 %.

Similar post treatment with acetaminophen and a combination with acetaminophen and ASA resulted in a significant decrease, $p<0.0001$, in activity of GPx as compared to the toxin group (figure 11.3).

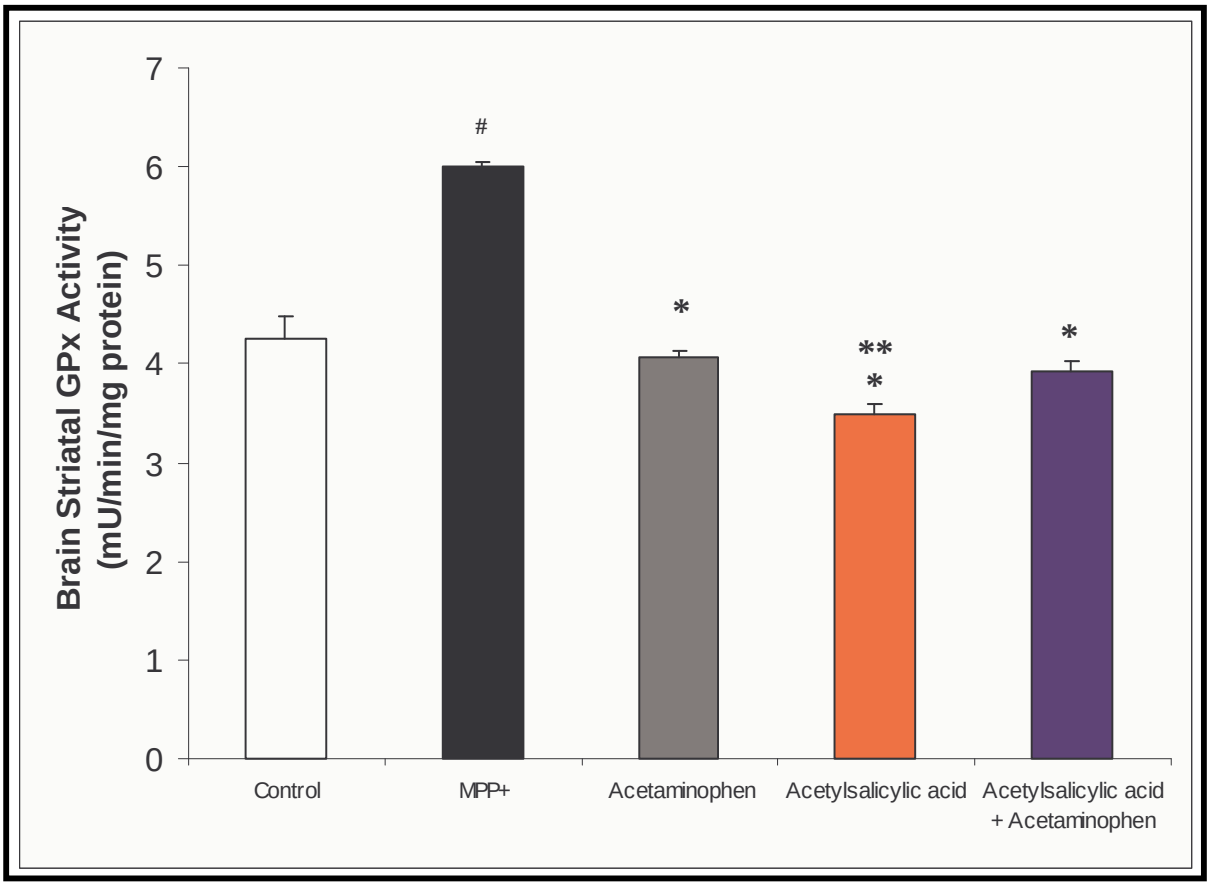


Figure 11.3. The effect of the administration of acetylsalicylic acid and acetaminophen alone or in combination on GPx activity following intracranial administration of MPP^+ in rat striatal homogenate. Each bar represents the mean ±SEM, n=5. $^{\#}(p<0.001)$ control vs. MPP^+; *($p<0.001$) Acetaminophen vs. MPP^+, acetylsalicylic acid vs. MPP^+ and acetylsalicylic acid + acetaminophen vs. MPP^+; **($p<0.05$) control vs. acetylsalicylic acid. Student Newman-Keuls-Multiple Range test.

11.3.4. DISCUSSION

Although the generation of free radicals is part of normal metabolism, overproduction or reduced efficiency of defense systems to reactive oxygen species may greatly contribute to their neurotoxicity. Hydrogen peroxide is not a free radical however it can react with $O_2^{-\bullet}$ radicals via the Haber-Weiss reaction or with iron via the Fenton reaction to produce highly reactive hydroxyl radicals (Bellissimo *et al*., 2001).

The intriguing result obtained from this study clearly demonstrates that MPP^+ infusion into the rat striatum results in a significant enhancement of GPx activity when compared to the control group (figure 11.3). This clearly demonstrates that the generation of reactive oxygen species leads to an exacerbation of the activity of GPx.

The ability of ASA and acetaminophen either alone or in combination to downregulate the activity of GPx, in comparison to the toxin group (figure 11.3) could result from their free radical scavenging properties demonstrated in chapters five and six. A lack of GPx activity can result in a greater production hydrogen peroxide, which can react with $O_2^{-\bullet}$ to form $^{\bullet}OH$ (figure 11.1.). Obata *et al.* (2001) demonstrated that the infusion of MPP^+ in rats, results in the generation of $^{\bullet}OH$. The $^{\bullet}OH$ formed during MPP^+ neurotoxicity can result in the peroxidation of lipids and subsequent cell death (Obata *et al.*, 2001). Further evidence to support this was the enhanced MDA concentration levels, a lipid peroxidation by-product, observed in chapter six in rats treated with MPP^+ alone. These non-steroidal agents reduce GPx activity in the presence of MPP^+ but they have been shown to inhibit the deleterious effects of the $^{\bullet}OH$ by significantly inhibiting MPP^+-induced lipid peroxidation (chapter six, section 6.6). Lipid peroxidation was significantly reduced in rats treated with ASA and acetaminophen, either alone or in combination, as compared to the toxin group thus implying that these agents were nullifying the effects that the hydroxyl radical may have on cells.

Chapter five demonstrates that ASA and acetaminophen are potent $O_2^{-\bullet}$ scavengers in the presence of MPP^+. Hence this provides another mechanism of action in that these agents could be preventing the availability of the $O_2^{-\bullet}$ to react with H_2O_2 to form the neurotoxic, $^{\bullet}OH$ via the Haber-Weiss reaction.

Therefore the reduced GPx activity that was observed in the groups treated with ASA and acetaminophen alone or in combination would not have any negative consequences on the integrity of the cell due to the potent antioxidant properties of these agents.

11.4. THE EFFECT OF ACETYLSALICYLIC ACID AND ACETAMINOPHEN IN MPP^+-INDUCED ALTERATION OF CATALASE FUNCTION IN RAT STRIATUM *IN VIVO*.

11.4.1. INTRODUCTION

Mammalian catalase is probably one of the best-studied enzymes in existence. It was first crystallized from beef liver by Summer and Dounce in 1937 (Summer and Dounce, 1937). Catalase has a molecular structure of about 240 000 Da and is composed of four identical subunits, each containing a protoporhyrin ring and a central iron (Fe) atom. Catalase catalyzes the destruction of H_2O_2 by following two reactions:

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O2$$

catalytic reaction

$$H_2O_2 + SH_2 \xrightarrow{CAT} 2H_2O + S$$

peroxidatic reaction

Where (S) is any one of hydrogen-donating substrates including ethanol, methanol, formate, nitrite and quinones (Halliwell and Gutteridge, 1999). H_2O_2 is produced in the cells by a number of enzymatic reactions including those catalyzed by SOD, which converts superoxide anions to water and oxygen and non-enzymatically by the autoxidation of compounds such as thiol and ascorbate. Under normal physiological conditions, catalase controls the H_2O_2 concentration so that this does reach toxic levels that could bring about oxidative damage.

Catalase is present in most aerobic cells. In many cases the enzyme is localized in a subcellular organelle such as peroxisomes of liver and kidney or in much smaller aggregates such as microperoxisomes found in a variety of other cells (Sichak and Dounce, 1986). Peroxisomes contain many of the cellular enzymes that generate H_2O_2, such as glycolate oxidase and flavoprotein dehygrogenases (Halliwell and Gutteridge, 1999). Mitochondria contain little if any catalase. Hence, any H_2O_2 diffuses to the peroxisomes (Halliwell and Gutteridge, 1999).

Catalase activity can be measured by monitoring the disappearance of H_2O_2 in the reaction system. For a given concentration of H_2O_2, the initial rate of its removal is proportional to the concentration of catalase. Decomposition of H_2O_2 can be followed by the loss of its light absorbance at 240nm, or by measuring the release of O_2 by using an oxygen electrode (Halliwell and Gutteridge, 1999). Catalase activity in tissues with relatively high activity, such as the brain, can be spectrophotometrically determined if complete lysis of all organelles and clear solutions can be obtained.

Research studies show that MPTP administration in mice results in an increase in CAT activity (Thomas and Mohanakumar, 2003). This implies that MPTP administration results in oxidative insult to the cell, which in turn results in the increased activity of the cells antioxidant defense system. Similar studies using MPP^+ demonstrate that this potent neurotoxin affects SOD and CAT activity in the human neuroblastoma cell line (Lee *et al.*, 2000).

The aim of this study was to therefore determine the effect of MPP^+ on CAT activity in the cytosolic and particulate fractions of the cell. The study also aimed to determine whether ASA and acetaminophen alone or in combination alter the MPP^+ -induced change in CAT activity, if any, in the rat striatum.

11.4.2. MATERIALS AND METHODS

11.4.2.1. Chemicals and Reagents

All chemicals and reagents were of the highest quality available and were purchased from commercial distributors. Hydrogen Peroxide was purchased fro from Sigma Chemical Corporation, St. Louis, MO, U.S.A.

11.4.2.2. Dosing of Animals

Animals were dosed as described 5.5.2.2.

11.4.2.3. Surgical Procedures

As described in chapter five, section 5.5.2.3.

11.4.2.4. Preparation of Cytosolic/Particulate fractions

The cytosolic and particulate fractions were prepared according to the procedure described in section 11.2.2.4.

11.4.2.5. CAT Assay

Catalase (EC 1.11.1.6) is a heme protein found in all living cells with the exception of certain bacteria. Functionally, it removes H_2O_2, which is toxic to the biological system by virtue of its oxidizing action. Molecular weight of this heme-protein is 240-250kD. In humans, the activity is well established in blood, liver and brain (Bergmeyer *et al*., 1983).

In the ultraviolet range H_2O_2 shows a continual increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in

absorbance at 240nm (ε_{240} = 0.00394 ± 0.0002 liters $mmol^{-1}$ mm^{-1}). The difference in absorbance per unit time is a measure of CAT activity. To avoid the inactivation of the enzyme during the assay (usually 30 seconds) or formation of bubbles in the cuvette due to the liberation of O_2, it is necessary to use low concentrations of H_2O_2 (30mM). The H_2O_2 is crucial as there is direct proportionality between the substrate concentration and the rate of decomposition.

The assay of CAT activity was based on the decomposition of H_2O_2 as described by Aebi, (1984). The rate of decrease in absorbance of H_2O_2 at 240nm for 30s in presence of CAT is taken as the enzyme activity. An assay mixture consisted of 500µl of (combined cytosolic and particulate fractions) which contained suitably diluted enzyme (approximately 100µg protein) in 50mM phosphate buffer, pH 7.0. The reaction was started by the addition of H_2O_2 (30mM), which gave an approximate absorbance of 0.5 initially. The specific activity of the enzyme is expressed as change in absorbance per minute per milligram protein.

11.4.2.6. Statistical Analysis

The results were analyzed using a one-way analysis of variance (ANOVA). If the F values were significant, the Student's Newmans-Keuls Multiple Range test was used to compare the treated and control groups. The level of significance was accepted at $p<0.05$ (Zar, 1974).

11.4.3. RESULTS

The decrease in absorbance of H_2O_2 at 240nm indicated that the CAT enzyme was active in the combined cytosolic and particulate fractions. The results from figure 11.4, demonstrate that MPP^+ significantly enhances the activity of CAT in the combined cytosolic and particulate fraction as compared to the control. The change in absorbance that was observed in the MPP^+ treated was significantly greater than the change in absorbance exhibited in the control, $p < 0.001$.

Post-operative treatment, with the non-narcotic analgesics resulted in a significant attenuation in the MPP^+ induced increase CAT activity, $p<0.05$, as illustrated in figure 11.4. There was no significant difference in CAT activity in each non-narcotic analgesic treatment group when compared to the control.

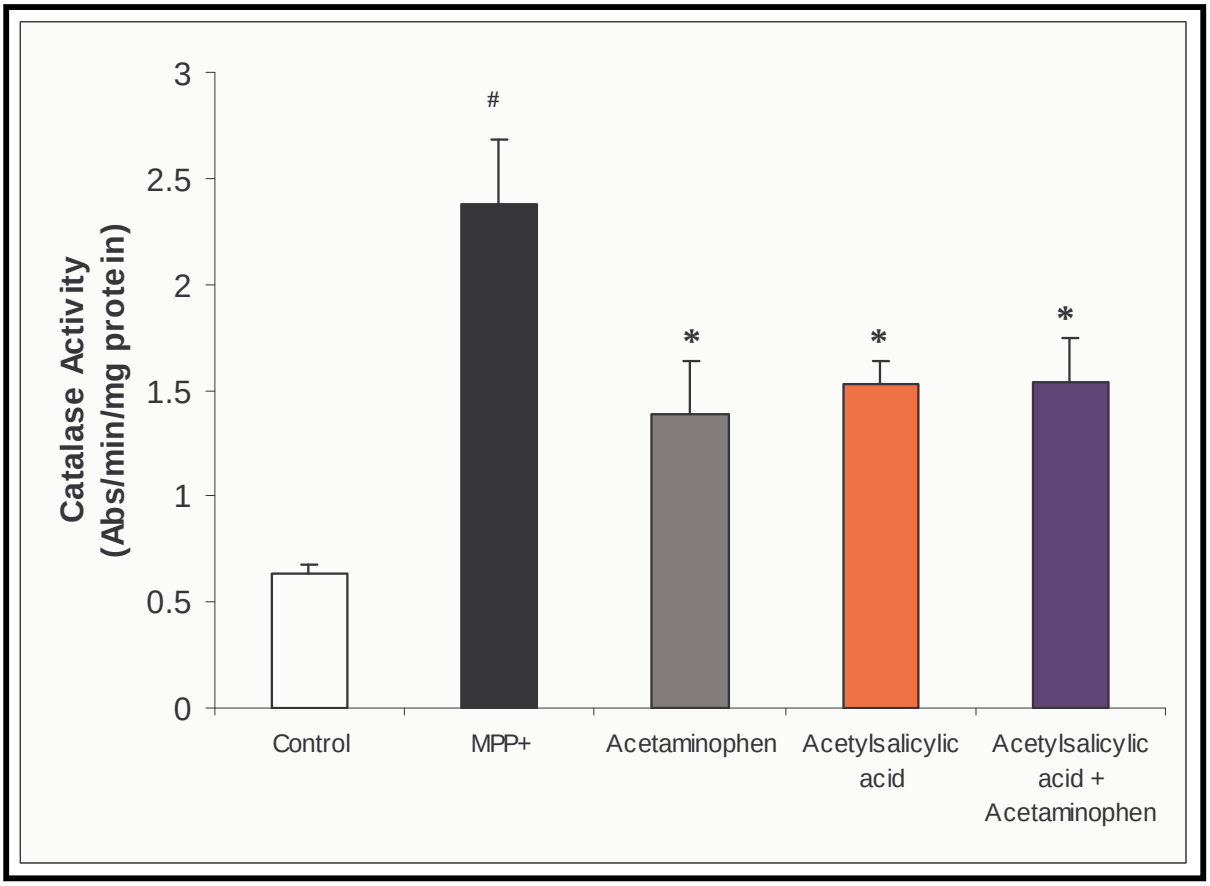


Figure 11.4. The effect of the administration of acetylsalicylic acid and acetaminophen alone or in combination on catalase activity following intracranial administration of MPP^+ in rat striatal homogenate. Each bar represents the mean ±SEM, n=5. $^{\#}(p<0.001)$ control vs. MPP^+; $*(p<0.05)$ vs. MPP^+. Student Newman-Keuls-Multiple Range test.

11.4.4. DISCUSSION

There exists a delicate balance between the rise of H_2O_2 formation via dismutation of $O_2^{\cdot}$ by SOD and its removal by CAT/GSH (Freeman and Crapo, 1982). Thomas *et al*. (2000) demonstrated that CAT is more inducible to higher concentrations of H_2O_2 and thus their findings that the increase in MPTP-induced $^{\bullet}OH$ formation in the, mouse, striatum resulted in enhanced CAT activity. Catalase may serve as a secondary defense as the GSH system becomes limiting (Erhart and Zeevalk, 2001). Erhart and Zeevalk (2001) suggest that when GSH levels are depleted, and thus insufficient to effectively oppose the generation of H_2O_2, CAT activity may subsequently become an important protective mechanism.

The results in figure 11.4 show that ASA and acetaminophen block the MPP^+-induced increase in CAT activity. The increase in SOD activity, as shown in section 11.2, coupled with the attenuation of CAT activity, following treatment with these non-narcotic analgesics in MPP^+ lesioned rats, implies that these agents interfere with the initial events of MPP^+-induced neurotoxicity. These agents have also been shown to attenuate MPP^+-induced lipid peroxidation in chapter six and therefore the lack of enhanced CAT activity in the presence of MPP^+, in the ASA and acetaminophen treated rats, suggests that these agents are acting as free radical scavengers.

As mentioned earlier, CAT activity becomes a primary antioxidant defense mechanism in the cell when GSH levels are depleted. Hence it was decided to investigate whether ASA and acetaminophen alone or in combination are capable of altering the MPP^+ -induced decrease in GSH levels in the rat striatum.

11.5. CONCLUSION

The ability of these agents to modify the antioxidant defense mechanism, SOD, Gpx and CAT, in the presence of MPP^+ further indicates that the neuroprotective effects of ASA and acetaminophen are due to more than one mechanism. These results correlate with the results obtained in previous chapters, which indicate that these agents are free radical scavengers. The effect of these agents on GPx activity warrants further investigation as this enzyme plays a critical role in the availability of the potent antioxidant molecule, GSH. The effects of the non-narcotic analgesics on these antioxidant enzymes could be secondary as free radicals play a vital role in all these mechanisms therefore this warrants further investigation.

The conversion of H_2O_2 to H_2O is catalyzed by GPx and involves GSH, which is a cofactor of this enzyme (Meister and Anderson, 1983; Meister, 1995). As mentioned earlier, GPx converts GSH to the oxidized form GSSG in the presence of free radicals. Thus, the effect of these agents in the presence of MPP^+ on GSH levels in the brain was investigated in the following chapter (chapter twelve).

Furthermore GSH was also investigated to determine whether the action of these agents on CAT activity was significant since CAT becomes of primary importance only when GSH levels are depleted (Erhart and Zeevalk, 2001).

CHAPTER TWELVE

ROLE OF ACETYLSALICYLIC ACID AND ACETAMINOPHEN IN GLUTATHIONE LEVELS IN MPP^+ TREATED RATS.

12.1. INTRODUCTION

In recent years there has been substantial evidence supporting the hypothesis that oxidative stress triggers a cascade of events leading to the death of neuronal cells during PD (Adams *et al.*, 2001). During different processes of cellular aerobic metabolism such as mitochondrial oxidative phosphorylation ROS such as $O_2^{-\bullet}$, hydrogen peroxide and $^{\bullet}OH$ are generated. Excess production of these molecules can potentially damage different macromolecules such as proteins, nucleic acids and lipids thereby leading to cellular degeneration (Cohen and d' Arcy Doherty, 1998). To counter this, the cell maintains a battery of detoxifying enzymes viz. CAT, SOD and GPx and small molecules such as GSH.

The tri-peptide GSH is the most abundant intracellular non-protein thiol compound in mammalian cells (Sies, 1999). Glutathione is also present as glutathione disulphide (GSSG), the oxidised form of GSH, and is known to function as an antioxidant with a crucial role as a scavenger of toxic free radicals and in the detoxification of xenobiotics. Recent research data suggests that GSH may also have a role in signal transduction, cell proliferation, regulation of gene expression and apoptosis (Arrigo, 1999; Sen *et al.*, 1999; Hall, 1999). Furthermore, GSH also appears to play a role in various cellular processes such as DNA metabolism, protein synthesis, activation of certain enzymes and enhancement of immune function (Lomaestro and Malone, 1995; Bains and Shaw, 1997) as illustrated in figure 12.1.

Dringen *et al.*, (2000) reported that GSH is present in the brain in millimolar concentrations. Apart from antioxidant functions in the brain, extracellular GSH has been hypothesized to have additional functions as a neurotransmitter (Janaky *et al.*, 1999), neurohomone, in the detoxification of leukotriene and glutamate metabolism.

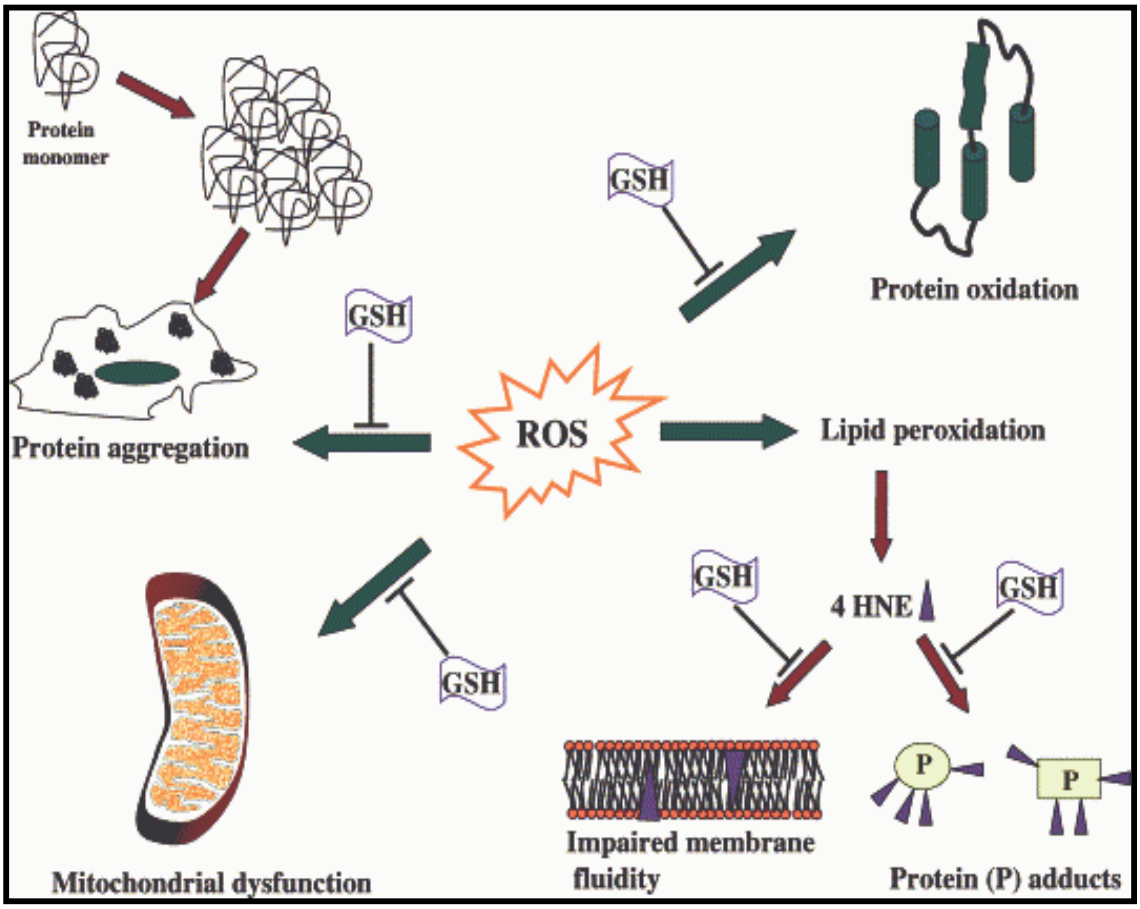


Figure 12.1. The different roles of GSH: a schematic representation of the antioxidant properties of GSH as relevant to SN dopaminergic neuronal cells in PD (Bharath *et al.*, 2002).

It has been observed that there is an age-dependent depletion in intracellular GSH of many organisms including humans (Sohal and Weindruch, 1996). In humans, there appears to be a decline in GSH levels in the cerebrospinal fluid during aging (Cudkowisz *et al.*, 1999). Research studies have shown that in aged mice, a 30% decrease in levels of

GSH compared with younger animals is evident (Chen *et al*., 1989; Hussain *et al*., 1995). Since the brain requires extensive ROS detoxification it is evident that a decrease in GSH content could increase oxidative damage making the brain more susceptible to neurological disorders such as PD.

During PD there is a further reduction in GSH levels within the SNpc (Riederer *et al*., 1989; Sofic *et al*., 1992) and GSH depletion is the first indicator of oxidative stress during PD progression suggesting a concomitant increase in ROS. Although GSH is not the only antioxidant that is depleted during PD, the magnitude of GSH depletion appears to be parallel to the severity of the disease and occurs prior to other hallmarks of the disease including decreased activity of the mitochondrial complex I (Perry and Yong, 1986; Jenner, 1998).

Furthermore, MPTP has been shown to contribute to oxidative stress by depleting the levels of GSH (Halliwell and Gutteridge, 1985; Bannon *et al*., 1984). This amplifies the damage caused because depletion of GSH causes further increase in ROS levels hereby contributing synergistically to mitochondrial dysfunction perhaps *via* direct inhibition of complex I activity. Thomas *et al*. (2000) demonstrated that MPTP administration to mice results in a significant depletion in GSH levels accompanied by a reduction in DA.

The enzymic method used in this study, as described by Tietze (1969), is a sensitive method for the quantitative determination of total and oxidised GSH in tissues in amounts as low as nanograms. The sensitivity of the method is such as to permit GSH estimations in extracellular fluids e.g., saliva, plasma and urine, which normally contain approximately 1µg/ml of the peptide, by the direct addition of the sample to the assay mixture (Tietze, 1969). Tietze (1969) demonstrated that the noninterference of cysteine at high relative ratios to GSH suggests a corresponding reliability in the presence of other nonglutathione thiol components. The method is based on the catalytic action of GSH or GSSG in the reduction of Ellman reagent or 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) in a mixture containing glutathione reductase which yields a chromophoric product with a molar absorption at 412nm. Unlike other methods of analysis the procedure described

here effectively measures the total GSH content of unknown mixtures and is not subject to appreciable interference by the presence of other thiol components (Tietze, 1969).

The aim of this study was to determine whether these analgesics, ASA and acetaminophen, have any effect on MPP^+ -induced decrease in GSH levels in rat brain striatum *in vivo*.

12.2. MATERIALS AND METHODS

12.2.1. Chemicals and Reagents

All chemicals and reagents were of the highest quality available and were purchased from commercial distributors.2-chloro-2, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), oxidised glutathione (GSSG), *N*-ethylmaleimide (NEM) and GSH reductase were purchased from Sigma Chemical Corporation, St. Louis, MO, U.S.A.

12.2.2. Dosing of Animals

Animals were dosed as described 5.5.2.2.

12.2.3. Surgical Procedures

As described in chapter five, section 5.5.2.3.

12.2.4. Measurement of Total Glutathione

Total GSH was determined by the method described by Tietze (1969). The rat striatum from each treatment group was homogenized (10%m/v) in cold 5% TCA in a glass Teflon homogenizer. The homogenate was then centrifuged at 10 000 x g for 15 minutes

at 4˚C. The pellet was discarded and the supernatant was used for the rest of the experiment. An aliquot of 1 ml of reaction mixture, which consisted of the reaction mixture: DTNB (0.6mmol/L) and GSH reductase (1 unit/ml) dissolved in 0.1M sodium phosphate-EDTA buffer (pH 7.5), was added to 1 ml of supernatant and this was incubated at 37^{o}C for half an hour in an oscillating waterbath. Thereafter, the reaction was stopped and the formation of the GSH-DTNB complex was measured at 412 nm with a Shimadzu UV 160A UV-visible recording spectrophotometer. Final results were expressed as μmoles/mg protein.

To determine the concentration of GSH a standard curve with varying concentrations of total GSH was constructed and the absorbance was read at 412 nm as described in appendix VI. The known amount of GSH, used to construct the calibration curve, was added to the reaction mixture and the absorbance measured at 412nm. Since the GSH assay method was being employed for the first time in our laboratory it was necessary to validate the method to suit the requirements of our laboratory before use. The validation results are shown in appendix VI. Validation is a process whereby the performance characteristics of an analytical method are established and the analytical method meets the requirements for its intended purpose (USP, 1999). This method was validated for linearity, precision, accuracy, limit of quantitation (LOQ) and limit of detection (LOD).

12.2.5. Measurement of Oxidised Glutathione (GSSG)

Oxidised glutathione was measured by the method described by Tietze (1969). The rapid and complete reaction of NEM with GSSG (Tietze, 1969) prevents the participation of the reduced form of GSH in the enzymic assay as well as its possible oxidation.

The experimental protocol was similar to that for the measurement of total glutathione except that the supernatant sample was first incubated at 25˚C for 20 minutes in 0.1mol/L phosphate buffer containing 10mmol/L NEM. The NEM forms a complex with GSH, which is then removed by column chromatography SEP-PAK C18 (Waters, Framingham, MA) column. The supernatant, which now contains only GSSG, was then reacted with

GSH reductase (1 unit/ml) and DTNB (0.6mmol/L) as described above in section 12.2.4. Thereafter, the GSSG was measured at 412 nm with a Shimadzu UV 160A UV-visible recording spectrophotometer. Final results were expressed as μmoles/mg protein.

To determine the concentration of GSSG a calibration curve with varying concentrations of GSSG was constructed and the absorbance was read at 412nm as described in appendix VII. The known amount of GSSG, used to construct the calibration curve, was reacted with the reaction mixture and measured at 412nm. Since the GSSG assay method was being employed for the first time in our laboratory it was necessary to validate the method to suit the requirements of our laboratory before use. was being employed for the first time in our laboratory it has been validated and validation results are shown in appendix VII. This method was validated for linearity, precision, accuracy, LOQ and LOD.

12.2.6. Calculation of Reduced GSH

In order to determine the concentration of reduced GSH in each treatment group, the difference of between the total GSH and GSSG was calculated (Tietze, 1969; Leret *et al*., 2002; Erhart and Zeevalk, 2001).

12.2.7. Statistical Analysis

The differences in the means were analyzed 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 treated and control groups. The level of significance was accepted at $p < 0.05$ (Zar, 1974).

12.3. RESULTS

It is evident from figure 12.2 that the total GSH content in the MPP^+ treated rat striatum was significantly decreased in comparison to the control treated rat striatum. The MPP^+ treated group shows a ±35% reduction in GSH levels in comparison to the control group. However, the administration of ASA and acetaminophen either alone or in combination is able to curb the MPP^+-induced reduction of total GSH. There is no significant difference between the results obtained from the three non-narcotic analgesic treatment groups when compared to each other as shown in figure 12.2.

The level of GSSG on the 4th day following MPP^+ infusion in rats was significantly enhanced as compared to the control group (figure 12.3.). However the treatment of these rats with ASA, acetaminophen and the combination of ASA & acetaminophen significantly inhibit the MPP^+-induced increase in GSSG levels in rat striatum. Acetaminophen was the least effective from all three, treatment regimens with significantly increased levels of GSSG when compared to the ASA group.

From figure 12.4, it is evident that MPP^+ treatment causes a significant decrease in reduced GSH levels in the rat striatum as compared to the control group. Figure 12.4 aptly demonstrates that ASA, acetaminophen and the combination of ASA & acetaminophen significantly inhibited the MPP^+-induced reduction of the reduced GSH levels. Furthermore, there is no significant difference when all three-treatment regimes are compared with each other and the control group.

MPP^+ administration in rats results in the overall increase in GSSG/GSH (reduced) ratio when compared to either the control or treatment groups (figure 12.5). The treatment groups are able to prevent the MPP^+-induced increase. Furthermore the overall effect of the toxin is completely blocked, as there is no significant difference in the ratios of the treatment regimes when compared to the control group as illustrated in figure 12.5.

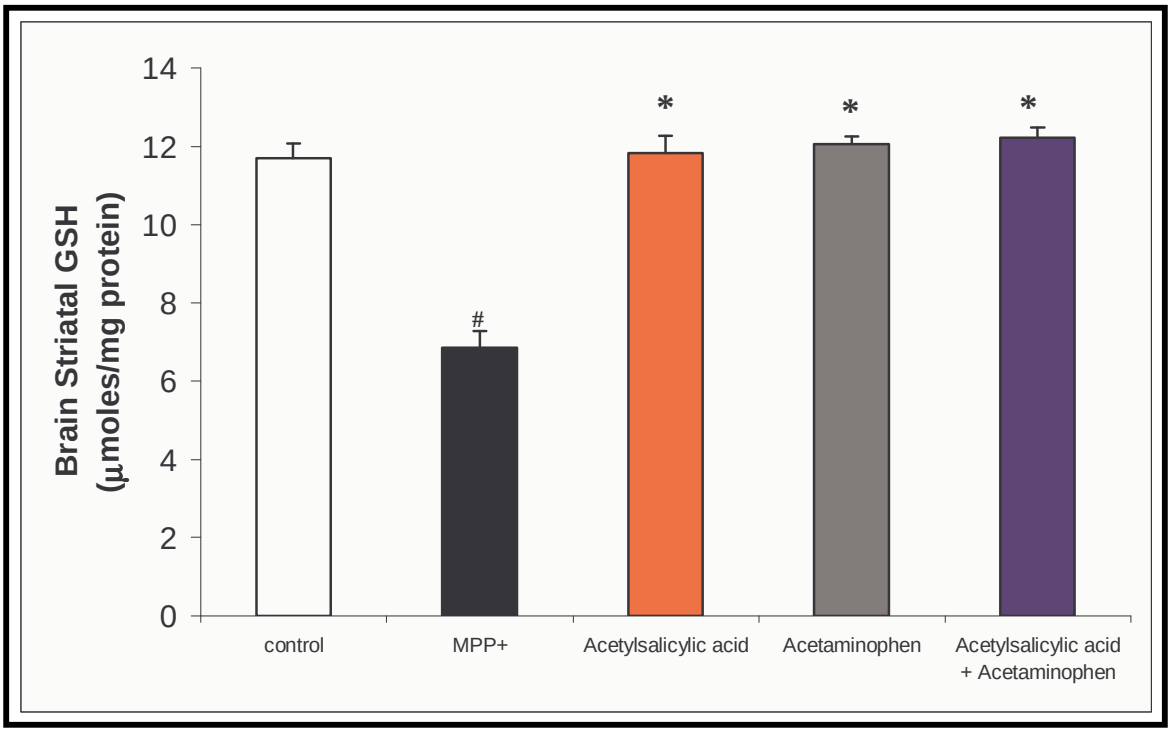


Figure 12.2. The effect of administration of acetylsalicylic acid and acetaminophen alone or in combination on total GSH activity following intracranial administration of MPP^+ in rat striatal homogenate. Each bar represents the mean ±SEM, n=5. $^{\#}(p<0.01)$ control vs. MPP^+ and *($p<0.001$) vs. MPP^+. Student Newman-Keuls-Multiple Range test.

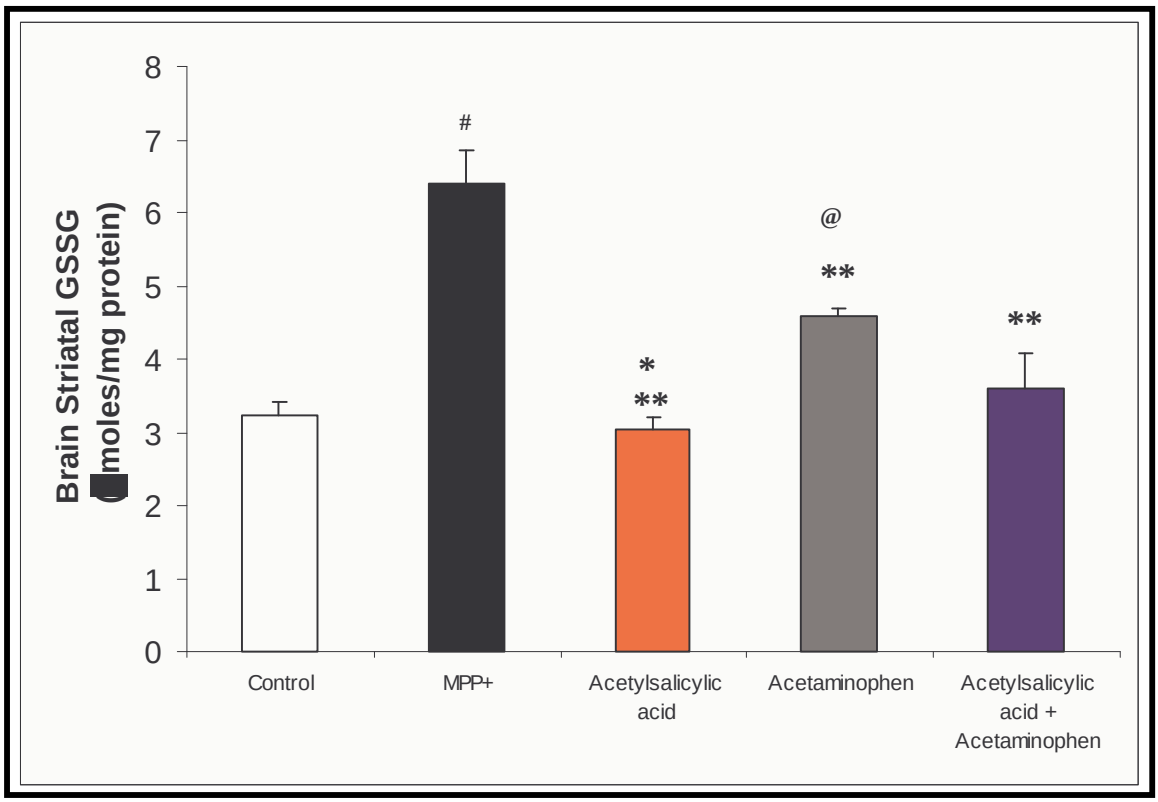


Figure 12.3. The effect of administration of acetylsalicylic acid and acetaminophen alone or in combination on oxidised glutathione levels (GSSG) following intracranial administration of MPP^+ in rat striatal homogenate. Each bar represents the mean ±SEM, n=5. $^{\#}(p<0.01)$ control vs. MPP^+; **($p<0.001$) vs. MPP^+, $^{@}(p<0.05)$ vs. control and *($p<0.05$) vs. acetaminophen. Student Newman-Keuls-Multiple Range test.

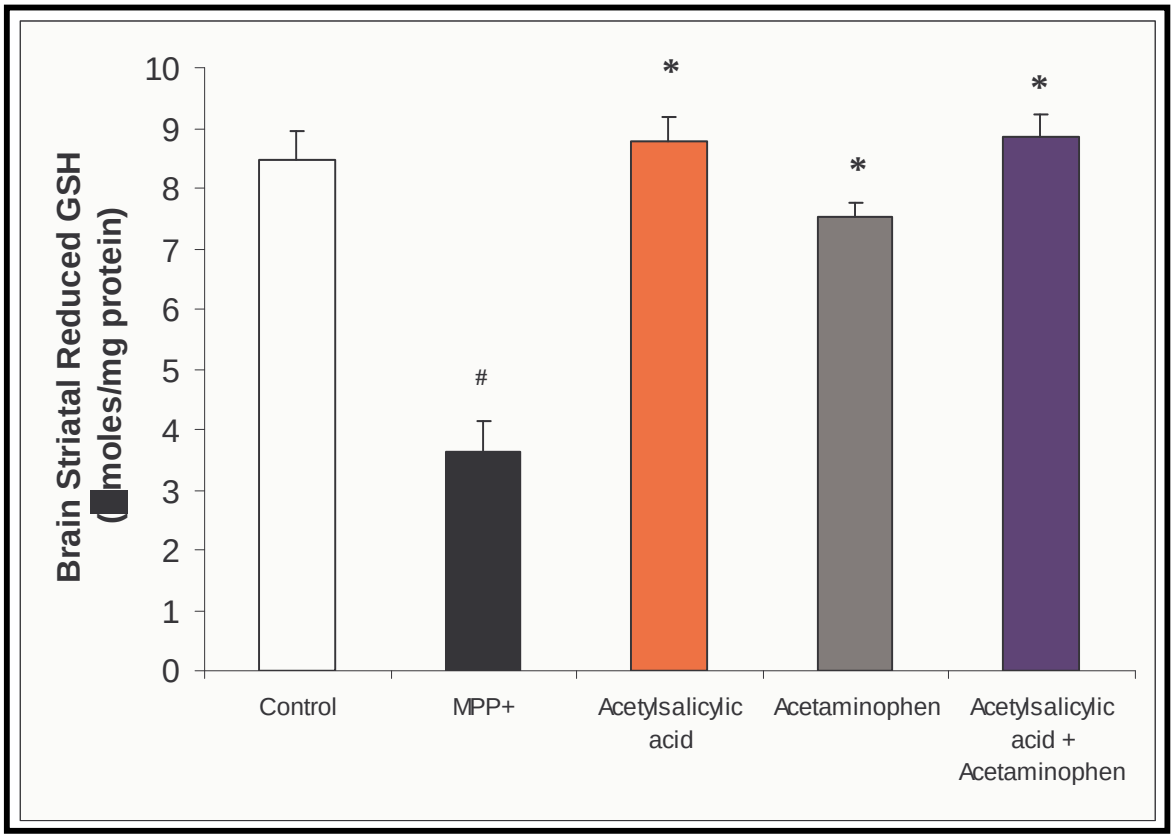


Figure 12.4. The effect of administration of acetylsalicylic acid and acetaminophen alone or in combination on reduced GSH levels following intracranial administration of MPP^+ in rat striatal homogenate. Each bar represents the mean ±SEM, n=5. $^{\#}(p<0.001)$ control vs. MPP^+ and $*(p<0.001)$ vs. MPP^+. Student Newman-Keuls-Multiple Range test.

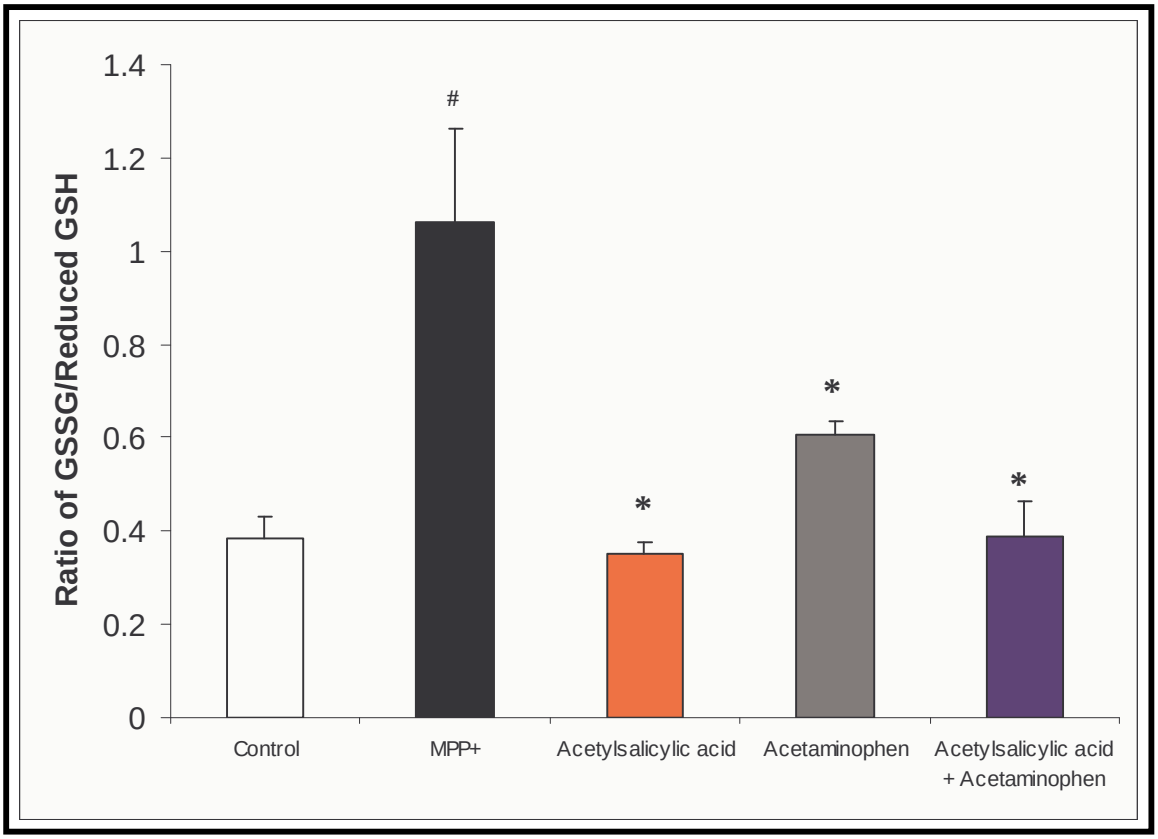


Figure 12.5. The effect of administration of acetylsalicylic acid and acetaminophen alone or in combination on the ratio of GSSG/reduced GSH levels following intracranial administration of MPP^+ in rat striatal homogenate. Each bar represents the mean ±SEM, n=5. $^{\#}(p<0.001)$ control vs. MPP^+ and $*(p<0.001)$ vs. MPP^+. Student Newman-Keuls-Multiple Range test.

12.4. DISCUSSION

The present study shows that intrastriatal administration of MPP^+ increases the GSSG content and decreases the content of reduced GSH in male rats when compared to the control group. Several authors have pointed out that an accelerated striatal metabolism of DA can lead to excess H_2O_2 radical production which can be inactivated by GSH (Leret *et al.*, 2002). However, since in this study MPP^+ significantly reduced GSH levels, it would result in an impairment of the ability of the brain to clear the dangerous H_2O_2

radicals. This implies that MPP^+ contributes to oxidative stress in PD by reducing the levels of the antioxidant defense molecule, GSH.

Literature reports indicate that GSH levels decrease following treatment with MPP^+ in rats with a concomitant increase in GSSG levels (Leret *et al*., 2002) as was evident in figures 12.2 and 12.3, respectively. Furthermore, there are no defects in the major enzymes associated with glutathione synthesis in MPP^+ treated rats (Sian *et al*., 1994) but the alteration in the glutathione redox system is consistent with an impairment of mitochondrial function (Mithöfer *et al*., 1992) as was also demonstrated in chapter ten. Studies in hepatocytes have shown that inhibition of mitochondrial respiration by MPP^+ is associated with an efflux of GSH (Davey and Clark, 1996). Enzymatic conversion or autoxidation of MPTP to MPP^+ can generate the formation of free radicals (Zang and Misra, 1993).

There exists a delicate balance between the rise of hydrogen peroxide formation via the dismutation of the $O_2^{-\bullet}$ by SOD and its removal by CAT/GPx (Freeman and Crapo, 1982). Thus GSH plays an important role in defense against endogenous membrane peroxidation and subsequent changes by reducing hydrogen peroxide via GPx. Hence levels of GSH are believed to be rate limiting process of detoxification of the hydrogen peroxide radical or other peroxides (Thomas *et al*., 2000). GSH depletion in animals has been shown to enhance MPTP -induced oxidative stress (Wullner *et al*., 1996).

These results are in correlation with the results obtained from chapter eleven, where these agents induced a decrease in activity of GPx, which is the enzyme that converts GSH to GSSG (Salinas and Wong, 1999), when compared to MPP^+ treated rats. The effects of these agents on this enzyme, GPx, resulted in decreased metabolism of GSH to GSSG, explaining the levels of GSH (reduced) and GSSG, which were observed in these treatment groups as demonstrated in figures 12.3 and 12.4. The induction of GPx was therefore not required, due to the potent free radical scavenging properties of these analgesics, as the metabolism of GSH is unaffected in the presence of MPP^+ when

compared to the control group (figure 12.2-4). In addition, the free radical scavenging ability of these drugs in MPP^+ treated rats was shown in chapters five, six and eight.

Erhart and Zeevalk (2001) demonstrated that CAT activity is not a primary defense mechanism when GSH levels are not depleted. These agents would not compromise the cellular antioxidant defense system as these agents significantly attenuate the MPP^+-induced decrease in cellular GSH levels.

The ability of ASA and acetaminophen either individually or in combination to increase GSH (reduced) levels in the presence of MPP^+ contributes to their ability to inhibit the deleterious effects of MPP^+ in the brain GSH levels. These non-narcotic analgesics reverse the MPP^+-induced reduction in total GSH levels in rat striatal tissue (figure 12.2). The results show that ASA and acetaminophen decrease the oxidation of GSH to GSSG, as is evident from figure 12.3, indicating that these common non-narcotic analgesics are potent free radical scavengers and provide novel information on the neuroprotective abilities of these agents.

12.5. CONCLUSION

The antioxidant defense mechanism in the brain is significantly compromised in MPP^+-induced Parkinsonism. The results from this study show that the administration of MPP^+ significantly reduces the amount of GSH available to the cell. However, the ability of ASA and acetaminophen to prevent MPP^+-induced reduction in total and reduced GSH levels enhances the reputation of these agents as novel neuroprotective agents in the treatment of PD.

This study is therefore important in enhancing their reputation as agents that could aid the antioxidant system in the brain. This chapter reiterates that these agents possess potent antioxidant properties and shows a mechanism by which these agents aid the cell against oxidative stress.

CHAPTER THIRTEEN

EFFECT OF ACETYLSALICYLIC ACID AND ACETAMINOPHEN IN [^{3}H] DA UPTAKE IN RAT STRIATAL SYNAPTOSOMES *IN VIVO*.

13.1. INTRODUCTION

The most important neuropathological change in PD is the degeneration of melanin-containing DA containing neurons in the brainstem. Selective nigral cell loss is associated with a specific pattern of DA depletion in the striatum (Agid *et al*., 1987). Extensive research by various groups has shown that oxidative metabolism by MAO-B with the formation of MPP^+ is an essential step in the neurotoxic process (Langston *et al*., 1984) and mainly occurs outside the dopaminergic neuron. The selectivity of MPTP for dopaminergic neurons is considered to be a consequence of the active uptake of MPP^+ into dopaminergic neurons via the neuronal DA transporter (DAT) (Nwanze *et al*., 1995).

MPP^+ toxicity is also marked by a rapid and large release of DA into the synaptic space where DA is oxidized and produces $^{\bullet}OH$ (Obata and Chiueh, 1992). In many studies, the toxicity of MPP^+ was generally based either on measurements of total DA concentrations (Alexander *et al*., 1995; Harik *et al*., 1987) and extracellular DA levels (Heikkila *et al*., 1985; Santiago *et al*., 1991), showing loss of DA in the striatum, or in binding studies showing decreased membrane expression of DAT (Alexander *et al*., 1995; Frohna *et al*., 1985). However, the DAT is the primary target for the accumulation of MPP^+ and plays a key role in the regulation of the central DA neurotransmission (Barc *et al*., 2002). By ensuring the reuptake of DA into the presynaptic neurons, it regulates the availability of the synaptic DA effectively, which interacts with pre and post synaptic DA receptors (Giros *et al*., 1996).

Dopamine transporter acts to terminate dopaminergic neurotransmission by reaccumulating released DA (Horn, 1990). Evidence has been accumulated that the process is reversible, supporting the existence of a transporter mediated release of DA (Levi and Raiteri, 1993). The activity of the DAT can be determined by different methods (Naudon *et al*., 1992; Pellmar *et al*., 1995; Ramassamy *et al*., 1995) and in different *in vitro* models: synaptosomes corresponding to synaptic nerve-endings (Horn, 1979), brain slices (Near *et al*., 1988) and transfected cells (Huff *et al*., 1997). The DAT detected in striatal-purified synaptosomes (Urwyler and Von Wartburg, 1981), of known molecular structure (Giros *et al*., 1991), is considered one of the main elements in regulating dopaminergic transmission (Jaber *et al*., 1997).

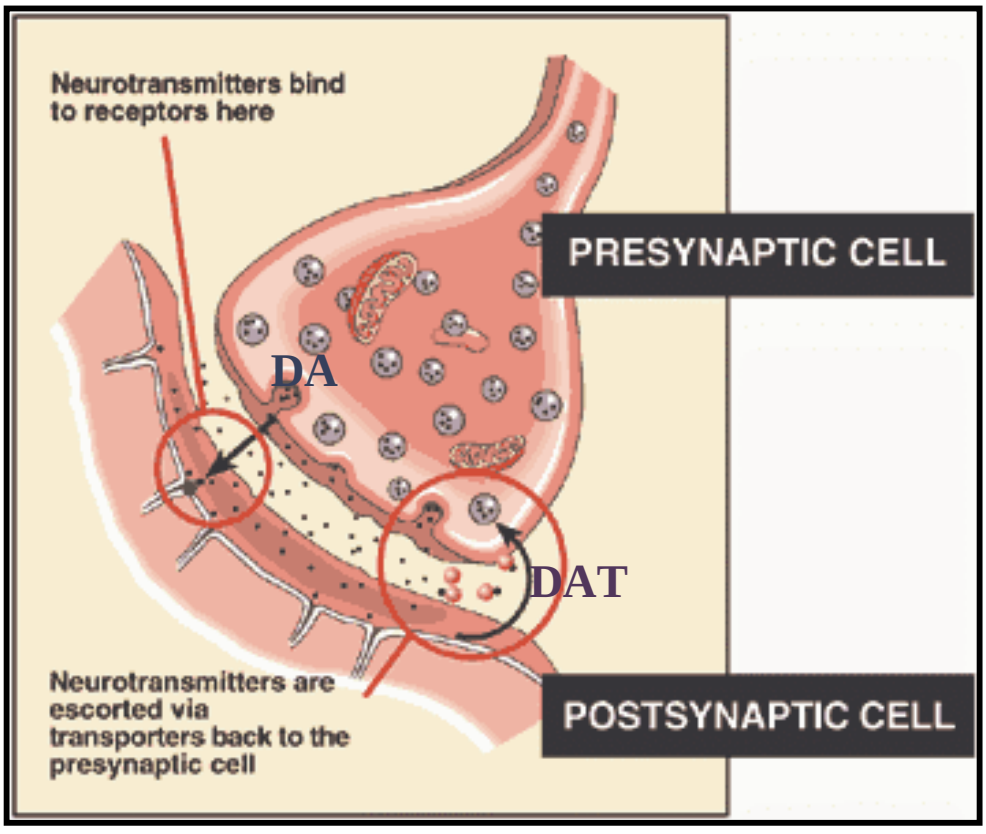


Figure 13.1 Illustration of the binding of the neurotransmitter to the receptor and the active process by which the neurotransmitter is taken up into the presynaptic cell.
Abbreviations: **DA**–dopamine and **DAT**–Dopamine transporter.
(www.medicineandbehavior.com).

Figure 13.1 provides a visual context of the role of the DA uptake system. When the receptors in the postsynaptic cell have been occupied by dopamine for a period of time and depolarization has occured, DA is then released and recycled back to its presynaptic origin. This recycling is not a passive process. The DAT protein actively participates in escorting the neurotransmitter across the synapse and back into the presynaptic cell.

The aim of this chapter is to elucidate the effects of MPP^+ on synaptosomal DA uptake in adult male rats as well as to determine the effects of ASA and acetaminophen, if any, on MPP^+ alteration of DA uptake.

13.2. MATERIALS AND METHODS

13.2.1. Chemicals and Reagents

All chemicals and reagents were of the highest quality available and were purchased from commercial distributors. [7, 8-^{3}H]- dopamine 45Ci/mmol was purchased from Amersham Biosciences, Buckinghamshire, United Kingdom. Dopamine chloride was purchased from Sigma Chemical Corporation, St. Louis, MO, U.S.A. ALL other bench reagents were of the highest quality and were purchased from Saarchem (PTY) Ltd, Krugersdorp, South Africa.

13.2.2. Dosing of Animals

Animals were dosed as described 5.5.2.2.

13.2.3. Surgical Procedures

As described in chapter five, section 5.5.2.3

13.2.4. Striatal Synaptosome Preparation

Rats were killed by decapitation, the brains were quickly removed and the striata were dissected on an ice-cooled dish according to Glowinski and Iversen (1966). Synaptosomes were prepared from the striata from each rat as previously described by Bonnet and Costentin (1989), with minor modifications. Striata were homogenized in 20 volumes of 0.32 M ice-cold sucrose in a pre-chilled manual glass Teflon homogenizer. The homogenates were then centrifuged at 1000 x g for 10 minutes at 4^{o}C to give a nuclear pellet. Supernatants were stored at 4 ^{o}C for 10 minutes and the pellet was resuspended in 20 volumes of 0.32M sucrose and centrifuged for 10 minutes at 1000xg. The two supernatants were then pooled and centrifuged 17 500 x g for 30 minutes at 4 ^{o}C, after which the supernatant is discarded and the final pellet resuspended in ice-cold Krebs Ringer Buffer, pH 7.6 (120mM NaCl, 4.8mM KCl, 1.3mM $MgSO_4$, 1.3mM $CaCl_2$, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 25mM $NaHCO_3$, 6mM glucose). To ensure that striatal synaptosomes were obtained, light microscopy micrographs (figure 13.2A) and transmission electron micrographs (TEM; figure 13.2B) were prepared by the standard procedures described by Cross *et al*. (2001).

13.2.5. [^{3}H]DA Uptake in Rat Synaptosomes

Dopamine uptake was assayed using triturated dopamine ([^{3}H]DA= 10nM) and non-radiolabelled DA to obtain a concentration of 500nM. Uptake was measured in two series of synaptosomes, one at 37^{o}C and the other 4^{o}C in order to define total and non-specific DA, uptake respectively (Holz and Coyle, 1974). The synaptosomes (3 mg tissue/ml) were incubated for two minutes with DA at the defined concentrations above. Uptake was stopped by rapid filtration under vacuum (pressure 150-200 mmHg) through Whatman GF/C filters. Vials were rinsed and filters washed three times with 3 ml of ice-cold Krebs-ringer buffer. The filters were then placed in scintillation vials containing 7.4 ml of Picofluor 15 (Packard) before being stored at room temperature overnight. Radioactivity was determined by a liquid scintillation spectrometry (Beckmann, LS

counter 2800) with efficiency of 35%. [^{3}H]DA taken up by the synaptosomes at 37^{o}C and at 4^{o}C, was expressed as fmoles/ mg protein.

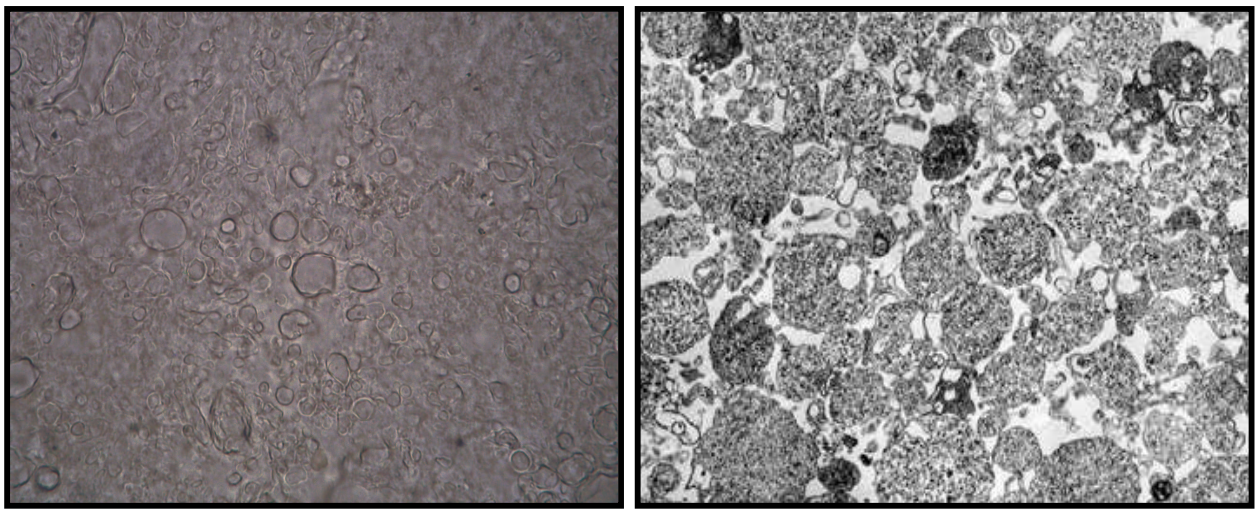


Figure 13.2. A) Light microscopy micrograph (Magnification X 1000) **B**) TEM micrograph (Magnification X 100 000) showing the striatal synaptosomes that was obtained by centrifugation.

13.2.6. Protein Determination

All protein determinations were performed using the method described by Lowry *et al*., (1952). A standard curve was generated using bovine serum albumin (BSA) as a standard at concentration intervals of 60µg/ml, described in appendix III.

13.2.7. Statistical Analysis

The differences in the means were analyzed 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 treated and control groups. The level of significance was accepted at $p<0.05$ (Zar, 1974).

13.3. RESULTS

Figure 13.3 clearly illustrates that the intrastriatal injection of 32 nmoles of MPP^+ reduces the uptake of DA in striatal synaptosomes as compared to the control. MPP^+ administration to rat striatum resulted in approximately 70% decrease in DA uptake as compared to the control ($p< 0.001$).

Dopamine uptake, in synaptosomes, of rats that were given post-surgical treatment with ASA, showed an enhanced increase of DA uptake in the presence of the neurotoxin MPP^+ as shown in figure 13.3. ASA was also capable of improving DA uptake with no significant difference to the control group and showed an approximately 4x greater increase in DA uptake as compared to the MPP^+ group.

Acetaminophen was also capable of preventing the MPP^+-induced decrease in DA uptake. DA uptake in rats treated with acetaminophen was greater when compared to the uptake achieved in the MPP^+ group (illustrated in figure 13.3.).

Concomitant administration of ASA and acetaminophen resulted in an increase in the uptake of DA as compared to the MPP^+ and control groups.

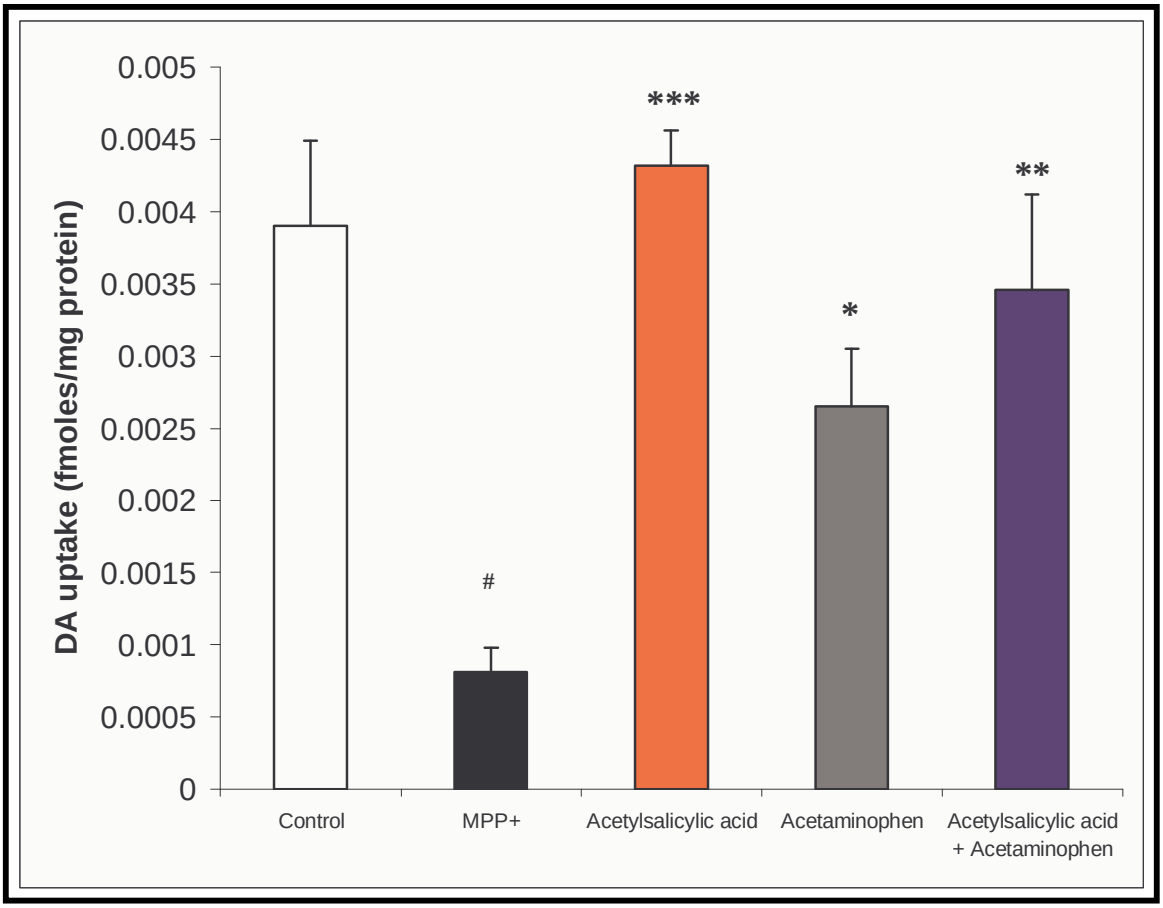


Figure 13.3. The effect of administration of acetylsalicylic acid and acetaminophen alone or in combination on [^{3}H]DA uptake following intracranial administration of MPP$^+$ in rat striatal synaptosomes homogenate. Each bar represents the mean ±SEM, n=5. $^{\#}$(p<0.01) control vs. MPP$^+$, ***(p<0.001) vs. MPP$^+$, **(p<0.01) vs. MPP$^+$ and *(p<0.05) vs. MPP$^+$. Student Newman-Keuls-Multiple Range test.

13.4. DISCUSSION

Considering that MPP^+ cannot cross the BBB, intracranial injection of this compound in the rat is often used in order to induce neurodegeneration of the nigrostriatal pathway. Furthermore, most studies have focused either on the measurements of dopamine levels or on binding experiments after administration of MPTP or MPP^+. However, an important step explaining the particular vulnerability of the nigral dopaminergic neurons projecting to striatal regions is the concentration of MPP^+ passing into the dopaminergic neurons through the active DAT (Santiago *et al*., 1995; Santiago *et al*., 1996) which plays an essential role in the modulation of dopaminergic transmission (Giros *et al*., 1996). This study was therefore performed to describe the effect of MPP^+ alone or in combination with ASA and acetaminophen on triturated dopamine uptake in striatal synaptosomes.

The results obtained from this study demonstrate that MPP^+ induced a significant inhibition of dopamine uptake in striatal synaptosomes. According to the model and experimental conditions used, the results show that the decrease in neurotoxin-induced DA uptake is due to a direct effect of the toxic agent on the transporter or from a non-specific mechanism related to other toxic events. The inhibition by MPP^+ of DA uptake has been previously shown to be dose-independent and does not result from a competitive effect with DA for the transporter (Barnes *et al*., 1990).

MPP^+ is not defined as a specific inhibitor of transporter binding sites and thus of DA uptake (Bonnet and Costentin, 1989). The MPP^+ induced decrease in DA uptake results from its active accumulation in nerve endings via the DAT (Javitch *et al*., 1985; Gainetdinov *et al*., 1997). MPP^+ is able to potentiate the release of DA which is oxidized in a chemical manner and produces dopaquinone derivatives as well as $^{\bullet}OH$, which initiate lipid peroxidation, resulting in DAT inhibition (Berman *et al*., 1996; Hasting and Zigmond, 1997). Moreover, the excess DA in the synaptic space could also undergo an enzymatic oxidation by MAO leading to the production of H_2O_2 and ROS which alter membrane lipids and proteins. To date, all studies using preventative treatment either

with an antioxidant (Grunblatt *et al*., 2000) or with a MAO-inhibitor such as L-deprenyl (Chiueh *et al*., 1994; Wu *et al*., 2000) and pargyline (Wesemann *et al*., 1993) have focused on the recovery of DA levels. It was therefore decided to investigate using ASA and acetaminophen as preventative treatments in order to determine if these agents could halt the progressive MPP^+ induced decrease in DAT activity.

The results demonstrate that ASA and acetaminophen alone and in combination in the presence of MPP^+ are able to reverse the reduction of DA uptake in striatal synaptosomes induced by MPP^+. It has been demonstrated by Barc *et al* (2002) that free radicals are continuously accumulating and gradually affecting the functionality of DAT following MPP^+ infusion into rat striatum. It can therefore be hypothesized that these non-narcotic analgesics are preventing MPP^+ induced inhibition of DA uptake by acting as potent free radical scavengers in rat striatal tissue.

The results obtained in chapters five and six demonstrate the potent antioxidant properties of these agents in the presence of MPP^+. The antioxidant properties of these agents could be a possible mode of action of attenuating MPP^+ induced DA uptake inhibition. In addition, the results from chapter six, which show that ASA and the combination of ASA and acetaminophen are most effective at reversing the MPP^+ induced lipid peroxidation of rat striatal tissue, correlates with the results obtained from this study where ASA and the combination of these agents are shown to enhance striatal DA uptake levels in striatal synaptosomes. These findings are novel and the effects of these agents on DA levels in synaptosomes in the presence of MPP^+ has not been reported in literature to date. These agents therefore provide a novel alternative in the attenuation of PD due to the ability of these non-narcotic analgesics to increase the levels of DA in the presence of MPP^+.

The results, from this study, suggest that free radicals are involved in the total inhibition of the DA transporter-mediated uptake observed on day 4 after lesion with MPP^+. These findings are in accordance with many studies showing the protective role of using antioxidants such as α-tocopherol acetate, ascorbate and pargyline to prevent MPP^+

induced inhibition of [^{3}H]DA uptake (Perry *et al*., 1985; Yong *et al*., 1986; Barc *et al*., 2002).

These results suggest the need for the use of these novel neuroprotective agents, such as ASA and acetaminophen, in the treatment of PD. Furthermore, these results aid in defining the molecular mechanisms by which these agents act in the presence of MPP^+.

13.5. CONCLUSION

The use of non-narcotic analgesics in the treatment of PD is unexplored and requires further attention. From this study it is implied that these agents possess unique mechanisms of action with regard to DA uptake, which has not been reported previously.

CHAPTER FOURTEEN

CONCLUSION

The results of the first part of this study involves an in depth investigation into the effect of the non-narcotic analgesics, ASA and acetaminophen alone and in combination, on the enzymes in the kynurenine pathway responsible for the conversion of TRP to KYN and 3-HA to QA i.e. TDO, IDO and 3-HAO, respectively. In addition, the effect of these agents on indoleamine metabolism in the pineal gland and forebrain neurotransmitters and their metabolite levels was measured. These studies were conducted in order to create an understanding of the role that these agents could play in altering indoleamine synthesis which ultimately can affect neurotransmitter levels in the brain i.e. 5-HT. These results aid in explaining the mood enhancing mechanism of these agents and assists future researchers and formulators in the pharmaceutical industry in creating an optimal analgesic preparation.

Evidence suggests that the metabolism of biogenic amines is disturbed in depressive illness, and there is no doubt there exist mechanisms by which the various biogenic amines and other neurotransmitters are mutually regulated. Hence in chapter two it was decided to determine if these enzymes TDO and IDO could be regulated by ASA and acetaminophen. In addition the effect that these agents have on 3-HAO of the kynurenine pathway would therefore be consequential in disease states such as Huntington's disease. The action of these drugs in reducing the activity of TDO and IDO decreased the formation of KYN from TRP. In addition, these non-narcotic analgesics are shown to be potent inhibitors of IDO and 3-HAO activity which could indicate their therapeutic potential in the treatment of inflammatory and infective disorders of the CNS and in the prevention of QA-induced neurodegeneration which would further aid in explaining the anti-inflammatory effects of these agents. Thus, this chapter serves to implicate the possible use of these agents in depression, HD and AD.

It is well documented that non-narcotic analgesics such as acetaminophen and ASA are abused by alcoholics and patients seeking to relieve dysphoric moods. An inverse relationship exists between liver TDO and brain 5-HT levels. Thus, in chapter three

forebrain neurotransmitter levels were investigated and the results demonstrate that there exists a correlation between the metabolism of biogenic amines and brain neurotransmitter levels. The results show that acetaminophen enhances brain NE and 5-HT levels following its systemic administration, while the effect of ASA on 5-HT turnover was also demonstrated. In addition, a reversal of acetaminophen-induced rise in the levels of NE by ASA was evident. The study also confirms the earlier findings in chapter 2 that acetaminophen has a profound inhibitory effect on liver TDO activity, which culminates in a rise in central 5-HT levels. These results demonstrate the potential of these agents to alter neurotransmitter levels in the brain. This in turn can influence mood and behaviour leading to the abuse of these commonly used agents.

The aim of chapter four was to assess the direct effect of ASA and acetaminophen on pineal indole metabolism in order to determine why ASA inhibition of TDO does not result in an increase in brain 5-HT levels. It is evident that ASA administration in rats resulted in the enhancement of 5-HT metabolites in the pineal gland. The increase in brain 5-HT levels induced by acetaminophen result in an increase in the formation of aMT. These results indicate that the mechanism of action of these agents is not confined to just the forebrain but also the pineal gland with regard to alteration of indoleamines. The resultant increase in pineal aMT levels following acetaminophen administration could have implications in neuroprotection as aMT has been demonstrated to be a potent antioxidant in the brain.

It can be concluded from the results of the studies mentioned above that ASA and acetaminophen have therapeutic potential in neurological disorders associated with KYN abnormalities such as AD and HD. Furthermore, the reported abuse of acetaminophen has been shown to be attributed to its ability to elevate brain 5-HT and NE levels. These results are important in providing a novel understanding by which these agents act on brain neurotransmitter levels since it has often been underestimated due to the lack of understanding and literature surrounding the mechanisms by which these agents act in the brain. Future recommendations would be to investigate the effect of these agents on brain QA levels due to their potent inhibitory effect on 3-HAO as shown in chapter 2. It is also suggested that the effect of these agents on the enzyme responsible for 5-HT metabolism,

MAO-A, also be studied, as this would provide a further understanding into the effects of these agents regarding the role played by these agents in altering brain 5-HT levels.

The experiments conducted for the second part of this study point conclusively to a new and emerging role of ASA and acetaminophen as neuroprotective agents by determining their antioxidant properties in the presence of potent neurotoxins.

Initial studies were conducted to investigate whether ASA and acetaminophen could scavenge, the toxic, $O_2^{-\bullet}$. The extent of protection that ASA and acetaminophen afford the brain against oxidative stress in the form of $O_2^{-\bullet}$ was investigated in chapter five. Initially, *in vitro* experimental results indicated that the cyanide acts as a potent $O_2^{-\bullet}$ generator in rat brain homogenate. It was demonstrated that ASA and acetaminophen alone and in combination significantly attenuated the cyanide-induced $O_2^{-\bullet}$ generation *in vitro*. The combination of these agents afforded significant and complete protection against cyanide- induced $O_2^{-\bullet}$ generation by reducing the levels of $O_2^{-\bullet}$ below that of the control basal value. Acetylsalicylic acid and acetaminophen was shown to alleviate the QA-induced rise in $O_2^{-\bullet}$ generation in the hippocampus *in vivo*. The combination of these agents proved more potent than either agent alone and reduced the $O_2^{-\bullet}$ levels below control levels. This indicates that in the presence of QA, the combination of ASA and acetaminophen would completely block the formation of $O_2^{-\bullet}$.

The promising $O_2^{-\bullet}$ scavenging properties of these agents prompted an investigation into the effects of these agents in protecting against MPP^+-induced $O_2^{-\bullet}$ generation in the striatum *in vivo*. Acetylsalicylic acid and acetaminophen are effective in reducing the MPP^+-induced rise in $O_2^{-\bullet}$ levels. Acetylsalicylic acid is shown to be superior to acetaminophen and the combination of ASA and acetaminophen in attenuating the MPP^+-induced $O_2^{-\bullet}$ generation and reduced diformazan levels below basal control levels. Literature reports indicate that MPP^+-induces $O_2^{-\bullet}$ generation via inhibition of mitochondrial complex I activity which results in enhanced SOD levels. Therefore, the potent activity exhibited by ASA could be attributed to possible roles in the enhancement of cellular respiration and SOD activity in the presence of MPP^+. Considering that $O_2^{-\bullet}$ generation plays an integral role in MPP^+-induced neurotoxicity, it can be postulated that ASA has potential therapeutic effects in PD. The $O_2^{-\bullet}$ scavenging ability of ASA and

acetaminophen was postulated to be due to anti-inflammatory actions that result from the inhibition of prostaglandin synthesis as well as a reduction in NF-κB as documented in literature. Due to the deleterious effects of $O_2^{-\bullet}$ in cells, it is concluded that the potent $O_2^{-\bullet}$ scavenging properties exhibited by these agents would equip them in preserving cellular integrity.

In chapter six, the neuroprotective effects of ASA and acetaminophen against QA, cyanide and MPP^+-induced neurodegeneration and oxidative stress in the form of lipid peroxidation were investigated. It was demonstrated that the neurotoxins, cyanide and QA induced a marked increase in lipid peroxidation in rat brain homogenate *in vitro* which was attenuated by ASA and acetaminophen alone and in combination. In addition these agents are shown to be effective in attenuating QA and MPP^+-induced lipid peroxidation, *in vivo*. The significance of this is obvious when considering that damage to any plasma membrane, albeit the cell or mitochondrial membrane, results in a disruption of membrane fluidity and damage to proteins. The production of ATP may also be affected. Thus the effects of lipid peroxidation may be detrimental to cell survival.

Furthermore, neuronal damage due to oxidative stress has been implicated in several neurodegenerative disorders, in which case ASA and acetaminophen may be a therapeutic advantage. It is postulated that the protection ASA offered to lipid peroxidation induced by QA is not only attributable to the antioxidant properties of this non-narcotic analgesic, but also due to a possible chelation of iron (II) and iron (III) which has been shown previously. Similarly, Fe^{2+} has been shown to be increased after MPP^+-administration in the brain which would result in the enhancement of ROS via the Fenton reaction. Since ASA chelates Fe^{2+} it would also prevent MPP^+-induced lipid peroxidation by negating the increase in Fe^{2+} levels induced by MPP^+. From this chapter it can be elucidated that the use of commonly used non-narcotic analgesics, such as ASA and acetaminophen, in QA and MPP^+-induced toxicity would be a novel and cost effective approach in treating AD and PD, respectively. The potential therapeutic benefits that these agents may possess in preventing excitotoxin-induced neurotoxicity were investigated in further chapters.

The histological studies in chapter seven were performed to determine whether ASA and acetaminophen, which exhibited antioxidant properties, are capable of maintaining the

architectural integrity of the neurons and the arrangement of the neurons in the hippocampal region i.e. the CA1 and CA3 regions. The Nissl and acid fuchsin stains were used to investigate the hippocampal neuronal structural events that occurred following intrahippocampal injections of QA. The Nissl stained cells in the CA1 and CA3 regions were damaged following QA infusion into the hippocampus. This was evident by virtue of the roundness and swelling of the neuronal cells. The hippocampal cells appeared scattered with little integrity of cell membrane and appearance of dense nuclei. Necrosis of the neuronal cells in many areas was also evident.

The principal features of the QA damaged neurons expressing acidophilia included shrinkage of neuronal somata, deformation and displacement of strongly stained nuclei, chromatolysis, and acidophilia of the cytoplasm. It was evident from both types of staining that the non-narcotic analgesics were able to translate their free radical scavenging properties into maintaining the structural integrity of the cell. The changes that were observed in untreated QA-lesioned rats were not observed in the rats that received post-operative treatment with these analgesics. The results obtained from the acid fuchsin stains confirmed the findings from the Nissl staining, which indicate that ASA and acetaminophen afforded protection against QA induced cell damage. This confirmation further enhances the role of these agents as potent free radical scavengers in the brain and their possible roles in the treatment of AD and HD.

Quinolinic acid has been reported by some authors to cause necrosis together with apoptotic cell death in the hippocampus (Uberti *et al*., 2003; Ferrer *et al*., 1995), thus, the ability of QA to induce programme cell death (apoptosis) in the hippocampus and the protection offered by both ASA and acetaminophen was investigated in chapter eight. The results of this chapter further support these findings, showing QA to induce apoptotic cell death, which was inhibited by the treatment of rats with the individual or combination of these non-narcotic analgesics. These results confirm the finding that QA induces necrotic cell death as shown in chapter seven. Furthermore chapter eight demonstrated that MPP^+ induces apoptotic cell death in the striatal region of the rat brain. The results obtained from this study are in agreement with previous reports, which describe the mode of cell death in MPP^+-lesioned animals as apoptotic. The study demonstrates the ability of ASA and acetaminophen to convert their antioxidant properties into a neuroprotective effect which resulted in the preservation of cellular integrity. Future recommendations are

that Hsp 70 studies be performed, using QA and MPP^+, since apoptosis is also characterized by caspase-independent processes, which may be inhibited by Hsp70. It can also be recommended that TH- immunostaining studies be performed using MPP^+ and ASA and acetaminophen as this would further provide evidence of the protection offered by these agents in the striatum of the rat.

The biochemical basis of the PD is a deficiency in the neurotransmitter DA in the nigro-striatal pathway in the brain due to destruction of dopaminergic neurons. A persistent increase in free radical generation is believed to be the cause of neuronal death. The aim of chapter nine was to therefore investigate whether these agents are capable of preventing the reduction of DA, in MPP^+ induced dopaminergic neurotoxicity in rat striatum. The results from this study indicate that acetaminophen affords only a partial protection against MPP^+-induced DA depletion in the striatum in these animals. However ASA was capable of fully reviving the nigral damage caused by MPP^+ thus indicating that ASA and acetaminophen, individually and in combination, prevented striatal DA depletion in the presence of MPP^+. These results indicate that the antioxidant properties, exhibited by ASA and acetaminophen, induced favourable neurochemical changes in the presence of the potent PD neurotoxin, MPP^+.

Since ASA and acetaminophen show protection against MPP^+ -induced $O_2^{-\bullet}$ generation, and considering the fact that MPP^+ is a respiratory poison which acts by blocking complex I activity of the ETC, the study in chapter ten was conducted to determine the effect of these non-narcotic analgesics, *in vivo*, on MPP^+ -induced depression of the ETC within the mitochondrial fraction isolated from the rat striatum. The results show that ASA and acetaminophen are able to reverse the MPP^+-induced inhibition of the mitochondrial ETC function. Acetylsalicylic acid causes complete abolishment of the MPP^+-induced inhibition of ETC function while acetaminophen and the combination of these agents where able to partially reverse the MPP^+-induced inhibition. This implies that ASA serves as a superior agent in protecting the mitochondrial ETC against MPP^+. The action of ASA is postulated to be due to the fact that it augments the activities of a number of antioxidant enzymes and it increases complex I activity in the mitochondrial ETC. The results show that these agents increase complex I activity in the presence of MPP^+, with the activity in the ASA group above basal control values, *in vivo*.

Furthermore, complex I deficiency has been documented to be intimately associated with the onset of several neurodegenerative disorders and the aging process (Yano, 2002; Ventura *et al*., 2002). In addition, by stimulating complex I activity, these agents can serve to possibly prevent the age and neurodegenerative disease-related decline of complex I activity eg. PD. Considering the fact that respiratory inhibition of mitochondria by MPP^+ is the basis for its $O_2^{-\bullet}$ generation, it can be concluded that the protection offered by ASA and acetaminophen alone and in combination against $O_2^{-\bullet}$ generation by MPP^+ is not due only to its antioxidant properties, but also due to the direct inhibition of MPP^+ insult and complex I activation within the ETC.

In idiopathic PD, antioxidant molecules such as GPx, SOD and CAT have been reported to be altered. The mitochondria are one of the major sources of $O_2^{-\bullet}$ generation and an estimated 1-2% of the electron leak from the mitochondrial electron transport chain is utilized to form $O_2^{-\bullet}$ which are dismutated by SOD. This reaction mechanism involves the dismutation of $O_2^{-\bullet}$ to H_2O_2, which is converted to H_2O and O_2 by CAT or by the oxidation of GSH in presence of GPx. The interplay of these mechanisms of free radical scavenging that exist in the brain serve as important defense systems to prevent cell death and subsequent neurodegneration. MPP^+ has been shown to significantly weaken the antioxidant defense system in the brain, thus further propagating it's neurotoxicity.

The aim of chapter eleven was to investigate whether ASA and acetaminophen could alter the deleterious effects of MPP^+ on SOD, GPx and CAT. Acetylsalicylic acid treatment significantly enhances SOD activity in the striatum. These results show that the protective effect of ASA could therefore be due to the upregulation of SOD, which is an important antioxidant molecule in the mitochondria. Acetaminophen administration in MPP^+ treated rats result in a reduction of activity of SOD and could therefore be due to the $O_2^{-\bullet}$ scavenging properties of this agent while the combination completely reverses any effects that MPP^+ had on rat striatal SOD activity, as there was no significant difference observed when compared to the control group. In addition, the ability of ASA and acetaminophen either alone or in combination to downregulate the activity of GPx, in comparison to the toxin group could result from their free radical scavenging properties demonstrated in chapters 5 and 6. Hence any possible free radical generation, from probable enhancement of H_2O_2 levels, that might occur would be negated by their potent

antioxidant properties. Furthermore these agents attenuated the MPP^+-induced increase in CAT activity in rat striatal tissue. The results obtained from this study show that these agents act by simply nullifying the effects of this potent neurotoxin on the enzymatic defense system of the brain. The ability of these agents to modify the antioxidant defense mechanism in the presence of MPP^+ further entrenches their neuroprotective role in PD.

During PD there is a further reduction in GSH levels within the SNpc. GSH depletion is the first indicator of oxidative stress during PD progression suggesting a concomitant increase in ROS. Research studies indicate that the magnitude of GSH depletion appears to be parallel to the severity of the disease. Furthermore, MPTP has been shown to contribute to oxidative stress by depleting the levels of GSH thus amplifying the damage caused because depletion of GSH. The aim of chapter twelve was to investigate whether ASA and acetaminophen are capable of attenuating the decrease in GSH levels are observed following MPP^+ infusion in rat striatum. The results show that the post-operative treatment with these agents result in the decreased metabolism of GSH to GSSG. The ability of ASA and acetaminophen either individually or in combination have a stimulant effect on GSH (reduced) levels contributed to the ability of these agents to inhibit the deleterious effects of MPP^+ in the brain antioxidant system. These non-narcotic analgesics proved to reverse the MPP^+ reduction of total GSH levels in rat striatal tissue. The results, in chapter twelve, show that ASA and acetaminophen decreased the oxidation of GSH to GSSG which imply that these common non-narcotic analgesics are not only potent free radical scavengers but that ASA and acetaminophen enhance the levels of antioxidant molecules such as GSH in the presence of the potent neurotoxin MPP^+.

The selectivity of MPTP for dopaminergic neurons is considered to be a consequence of the active uptake of MPP^+ into dopaminergic neurons via the neuronal DAT. By ensuring the reuptake of DA into the presynaptic neurons, DAT regulates the availability of the synaptic DA effectively, which interacts with pre and post synaptic DA receptors and thus reduces the levels of DA available for auto-oxidation in the post synaptic space. The aim of chapter thirteen is to elucidate the effects of MPP^+ on synaptosomal DA uptake in adult male rats and to determine the effects of ASA and acetaminophen on MPP^+ alteration of DA uptake. The results obtained from this study demonstrate that MPP^+ induces a significant inhibition of DA uptake in striatal synaptosomes. The results

demonstrated that ASA in the presence of MPP^+ is able to reverse the neurotoxic effects of MPP^+ on DA uptake in striatal synaptosomes. However acetaminophen and the combination of ASA and acetaminophen are less effective when compared to the ASA group. This study shows novel information that these agents possess unique mechanisms of action with regard to DA uptake, which has not been reported previously. It is suggested that further studies involving these agents and DA be performed in order to understand the mechanisms and kinetics by which these agents function on the DAT.

In conclusion, from the foregoing it is evident that ASA and acetaminophen alone and in combination possess potent neuroprotective effects. The use of these agents in neurodegenerative disorders would provide a novel approach to the treatment of PD and other neurodegenerative disorders. The unique mechanisms by which these agents function provides a variety of alternatives in attenuating various biochemical processes that are exhibited in neurodegeneration. This study highlights the potential therapeutic benefits that these agents could have in treating neurodegenerative disorders such as PD. These findings are hoped to initiate the use of these agents in PD clinical trials. Not only are these drugs effective in preventing the various biochemical processes that occur in PD but also they provide a cost effective approach for the treatment of this detrimental disorder.

APPENDIX I

Housing of Animals

All the work involving the use of animals was approved by the Rhodes University animal ethics committee. The animals used throughout this study were male Wistar rats, that were purchased from the South African Institute for Medical Research (Johannesburg, South Africa), weighing 250-300g. The animals were chosen at random and assembled into groups of five. They were housed in opaque plastic cages with metal grid floors and covers, under a diurnal lighting cycle 12 light: 12 dark with food and water *ad libitum*. The animal room was windowless with an automatic temperature and lighting controls. Lights were turned on at 6am everyday. The intensity of the light illumination during the 12 hour light phase was approximately 300µWatts/cm^2. The temperature of the animal room was maintained between 20^{o}C and 25^{o}C while an extractor fan ensured the constant removal of stale air. The cages were cleaned daily.

APPENDIX II

Sacrificing and dissection of the animals

Rats were sacrificed swiftly by cervical dislocation and rapidly decapitated. To remove the brain, the top of the skull was removed and the brain was exposed by making an incision through the bone on either side of the parietal suture, from the foramen magnum to near the orbit. Using forceps the calvarium was lifted and removed, exposing the brain which was easily removed for use in experiments. All adhering tissue and visible traces of blood was eliminated by washing the tissue in 0.9% saline solution. The brains were either used immediately or stored at -70^{o}C until needed.

APPENDIX III

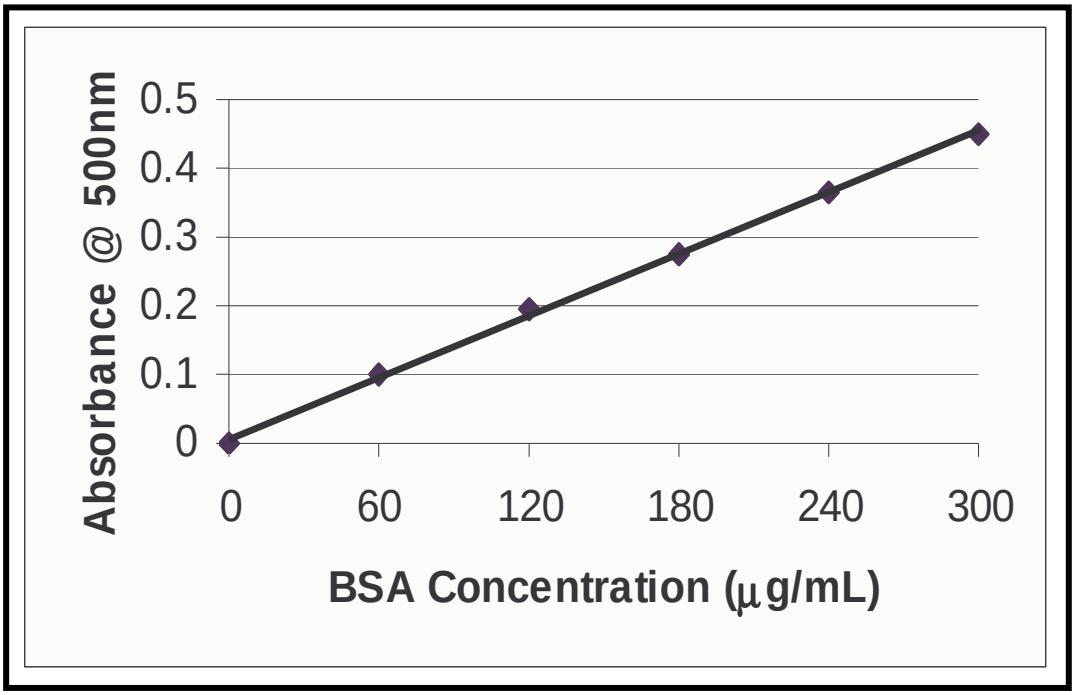
Protein Determination

Materials

Folin & Ciocalteu's reagent was purchased from Saarchem (PTY) Ltd, Krugersdorg, South Africa. The Bovine serum albumin (BSA) was supplied by Sigma Chemical CO, St. Louis, MO, USA. All other chemicals and reagents were obtained locally and were of the highest available purity.

Protein Determination

The homogenate protein determination concentration was determined by using the method of Lowry et al. (1951). An aliquot of 0.05ml of homogenate was added to 0.095ml of H_20. To this 6ml of alkaline copper reagent solution was prepared by mixing 1ml of 1 % $CuSO_4.5H_2O$ solution, 1ml of a 2 % sodium tartrate solution and 98 ml of 2% Na_2CO_3 in 0.1 N NaOH in order. The mixtures were vortexed and left to stand at room temperature for 10 minutes. Following that, 0.3 ml Folin-Ciocalteau (F.C) reagent was added to each of the tubes and left to stand at room temperatures for 30 minutes in the dark. After the incubation, the absorbance was measured at 500 nm using a Shiamdzu UV-160 A UV-visible spectrophotometer. Protein standards containing 0 – 300μg/ml of bovine serum albumin (BSA) were assayed in the same manner as described above.

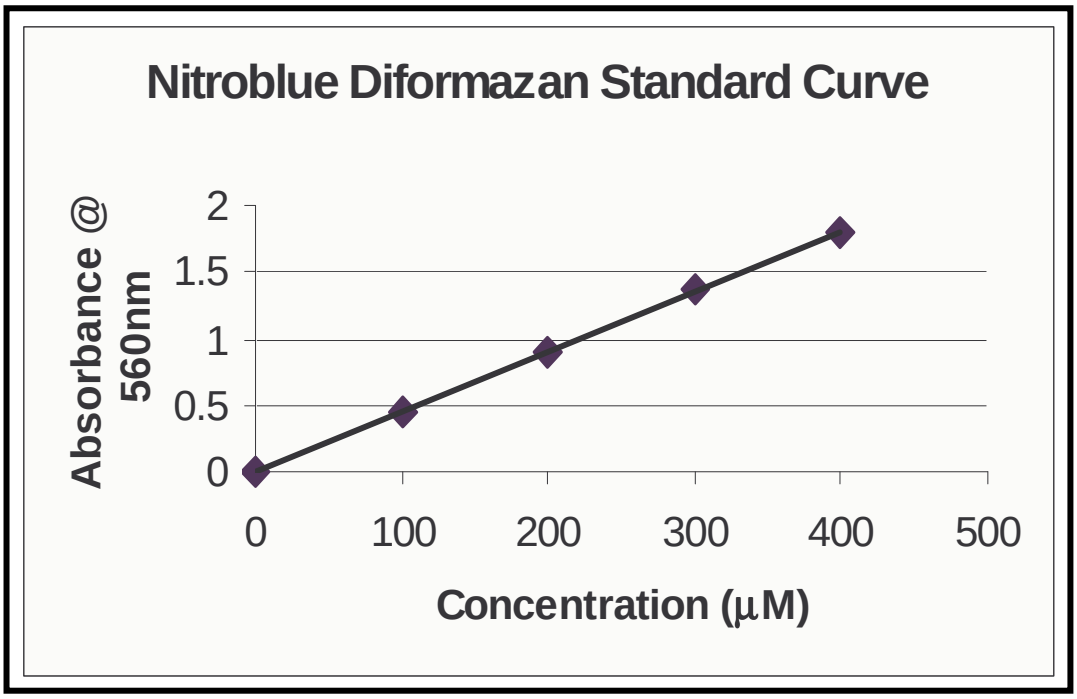


Appendix III. Protein Standard Curve Generated from BSA

($y = 0.0015x + 0.0072$, $r^2 = 0.999$)

APPENDIX IV

Nitroblue Diformazan Standard Curve

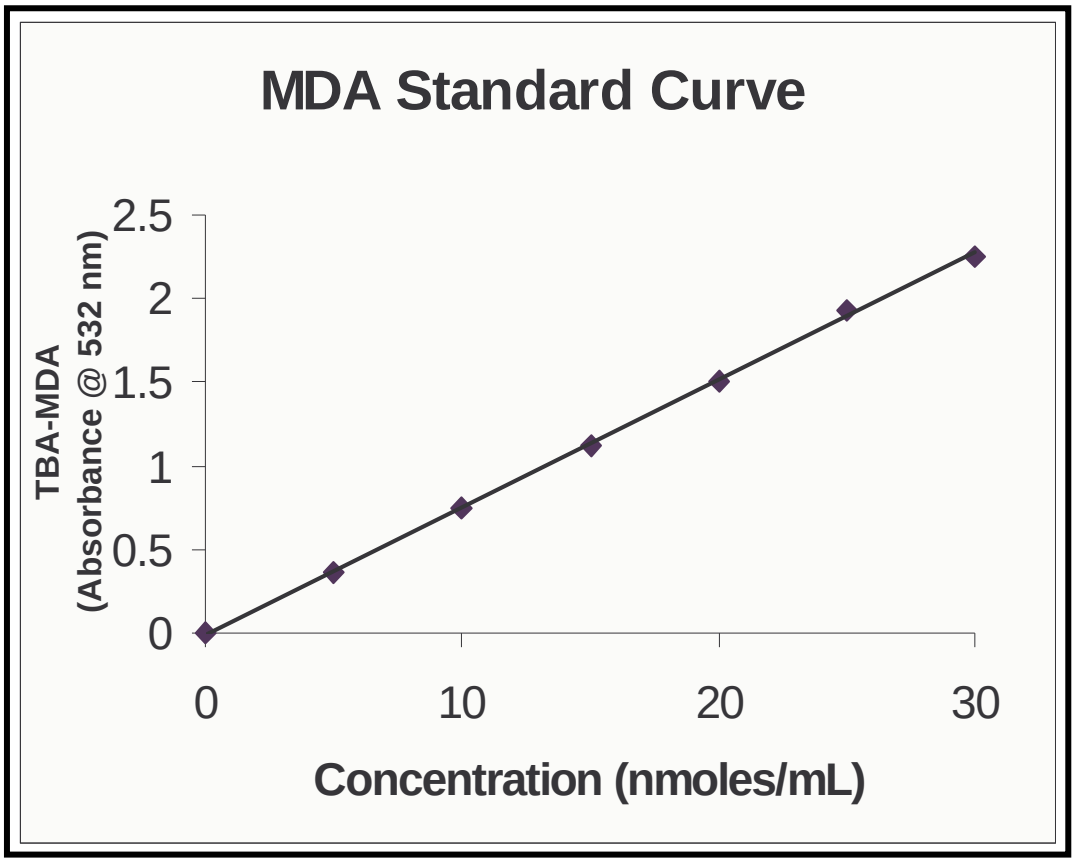


Appendix IV. Nitroblue Diformazan Standard Curve

($y = 0.0045x + 0.0033$, $r^2 = 0.9998$)

APPENDIX V

Lipid Peroxidation Standard Curve



Appendix V. MDA (malondialdehyde) standard curve
($y = 0.0761x - 0.0099$, $r^2 = 0.9994$)

APPENDIX VI

Total Glutathione Assay Validation Data

Linearity

A linearity study was used to verify that the sample solutions are in a concentration range where the analyte response is linearly proportional to the concentration (USP, 1999). Samples of total GSH were prepared by serial dilution of a stock solution to yield concentrations over the range of 0-100μmoles/ml and linearity was assessed by repeated measurements (n=20) of six concentrations over the concentration range. A known amount of GSH was added to the reaction mixture which consisted of DTNB (0.6mmol/L) and GSH reductase (1 unit/ml) dissolved in 0.1M sodium phosphate-EDTA buffer (pH 7.5), and measured at 412nm. Appendix VI graph shows a typical calibration curve for total GSH over a concentration range, with an excellent correlation coefficient of $r^2 = 0.9992$. The r^2 value is within acceptable limits set out in the USP (1999).

Precision

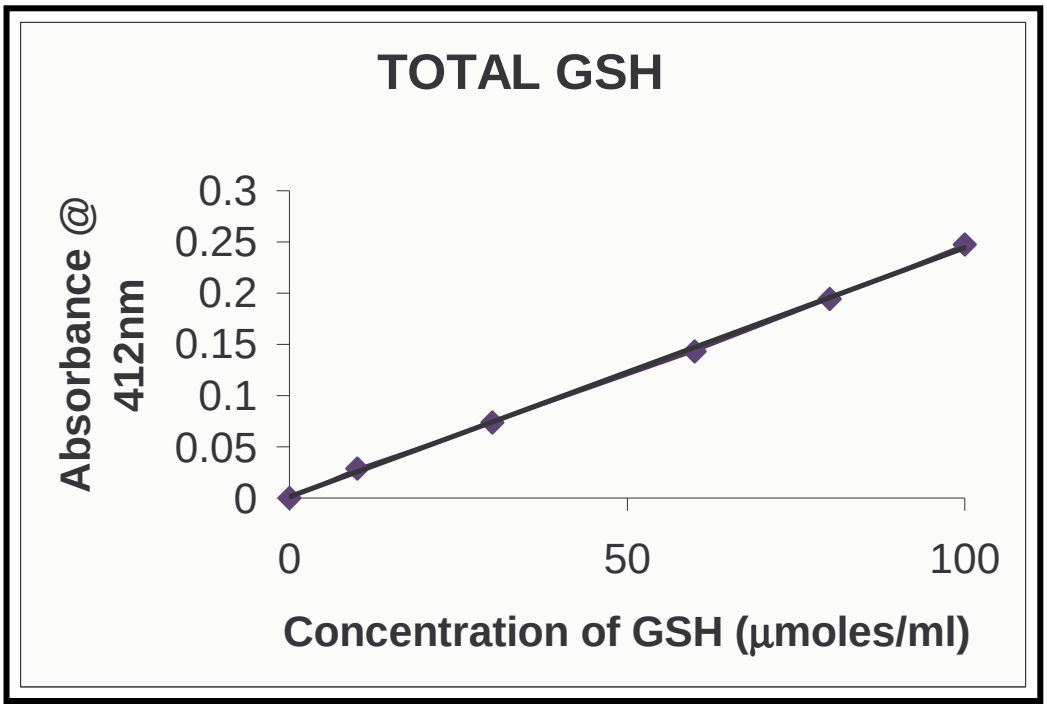
Precision is the measure of the closeness of data values to one another when a number of measurements are taken under the same analytical conditions. The total GSH validation data in shown in Table I. The intra-assay precision revealed % RSD values of 0.17-3.5% and the inter-assay precision revealed values of 0.19-3.93%. The % RSD values less than 4%, which is within the acceptable limits set out in the USP (1999) and in our laboratory.

Accuracy and Bias

Accuracy is a measure of the closeness between the true and measured values of a sample (USP, 1999). The acceptable range set out by USP (1999) for accuracy is between 98-102% of the theoretical value. The results in Table II show that the test concentrations were close to the theoretical value and fell within the acceptable range set of 98-102%. The % bias is between 0.4 to -8% and this falls within the bias acceptance criteria of ±3% set out by USP (1999) and in our laboratory.

LOQ and LOD

The sensitivity of the GSH method was evaluated by determining the lowest reproducible concentration of total GSH detectable. The LOQ is a measure of the level of analyte that can be measured with the required accuracy and precision, and LOD is the lowest analyte that is detectable above the baseline noise of the system (USP, 1999). Repeat absorbance readings of decreasing sample concentrations (n=6) yielded a LOD value of 10μmoles/ml and the LOQ value of 100μmoles/ml.



Appendix VI. GSH (Total Glutathione) standard curve

($y = 0.0024x - 0.0009$, $r^2 = 0.9991$)

Table I. Total GSH assay validation data.

	% RSD	
Concentration Total GSH (µmoles/ml)	**Intra-assay (n=6)**	**Inter-assay (n=6)**
10	3.45	3.93
30	3.5	3.76
60	1.9	1.9
80	0.17	0.19
100	0.39	0.34

Table II. Percent error obtained during determination of blinded samples of total GSH in accuracy testing.

Theoretical Concentration (µmoles/ml)	**Actual Concentration (µmoles/ml)**	**% Bias**
20	20.42	-8.42
50	50.05	-0.100
90	89.7	0.33

APPENDIX VII

Oxidized Glutathione Assay Validation Data

Linearity

Samples of oxidised GSSG were prepared by serial dilution of a stock solution to yield concentrations over the range of 0-10μmoles/ml and linearity was assessed by repeated measurements (n=20) of six concentrations over the concentration range. A known amount of GSSG was added to the reaction mixture, which consisted of DTNB (0.6mmol/L) and GSH reductase (1 unit/ml) dissolved in 0.1M sodium phosphate-EDTA buffer (pH 7.5), and measured at 412nm. Appendix VII graph shows a typical calibration curve for GSSG over a concentration range, with an excellent correlation coefficient of r^2 = 0.9991. The r^2 value is within acceptable limits set out in the USP (1999).

Precision

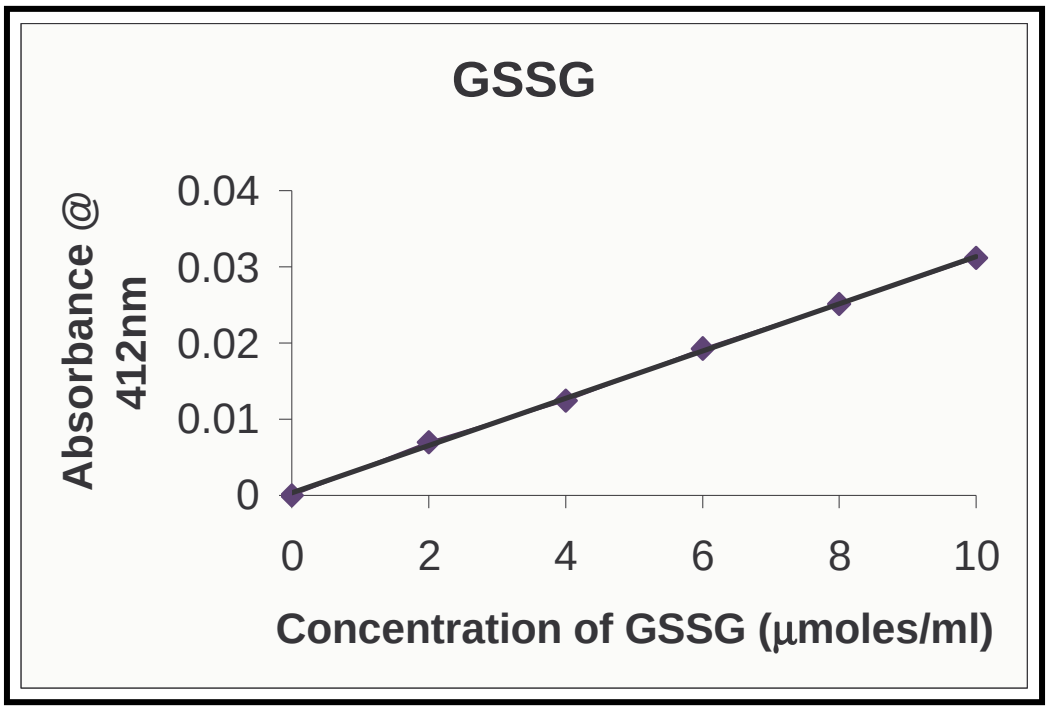
The GSSG validation data in shown in Table III. The intra-assay precision revealed % RSD values of 0.8-3.38% and the inter-assay precision revealed values of 0.86-3.03%. The % RSD values less than 4%, which is within the acceptable limits set out in the USP (1999) and in our laboratory.

Accuracy and Bias

The results in Table IV show that the test concentrations were close to the theoretical value and fell within the acceptable range set of 98-102%. The % bias is between 0.4 to -8% and this falls within the bias acceptance criteria of ±3% set out by USP (1999) and in our laboratory.

LOQ and LOD

Repeat absorbance readings of decreasing sample concentrations (n=6) yielded a LOD value of 1μmoles/ml and the LOQ value of 10μmoles/ml.



Appendix VII. GSSG (Oxidized Glutathione) standard curve

($y = 0.0031x + 0.0003$, $r^2 = 0.9992$)

Table III. GSSG assay validation data.

	% RSD	
Concentration of GSSG (µmoles/ml)	**Intra-assay (n=6)**	**Inter-assay (n=6)**
2	1.43	1.64
4	1.94	2.01
6	3.03	3.38
8	0.86	0.80
10	1.22	1.18

Table IV. Percent error obtained during determination of blinded samples of GSSG in accuracy testing.

Theoretical Concentration (µmoles/ml)	**Actual Concentration (µmoles/ml)**	**% Bias**
3	3.12	-4.42
5	4.96	0.8
9	8.93	0.77

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