

**AN INVESTIGATION INTO THE
ANTIOXIDATIVE POTENTIAL AND
REGULATORY ASPECTS OF LIVER
TRYPTOPHAN 2,3-DIOXYGENASE BY
TRYPTOPHAN AND RELATED ANALOGUES**

THESIS

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TABLE OF CONTENTS

ACKNOWLEDGEMENTSix
ABSTRACTx
LIST OF ABBREVIATIONSxii
LIST OF FIGURESxv
LIST OF TABLESxix

CHAPTER 1: LITERATURE REVIEW

1. INTRODUCTION1
1.1. TRYPTOPHAN2
1.1.1. TRYPTOPHAN CATABOLISM4
1.1.1.1. KYNURENINE PATHWAY OF TRYPTOPHAN CATABOLISM5
1.1.1.1.1. Tryptophan 2,3-dioxygenase5
1.1.1.1.1.1. Tryptophan 2,3-dioxygenase regulation8
1.1.1.1.1.2. Proposed mechanism9
a) Tryptophan induction9
b) Hydrocortisone induction10
1.1.1.1.1.3. Circadian rhythm in rat liver tryptophan 2,3-dioxygenase10
1.1.1.1.1.4. Kinetic properties of tryptophan 2,3-dioxygenase11
1.1.1.1.1.5. Structure-activity relationship of tryptophan 2,3-dioxygenase12
1.1.1.1.2. Indoleamine 2,3-Dioxygenase13
1.1.1.1.3. Kynurenine biosynthesis13
1.1.1.1.3.1. Formamidase14

1.1.1.1.3.2.	Kynurenine	14
1.1.1.1.3.3.	Kynurenine aminotransferase	16
1.1.1.1.3.4.	Kynurenic acid	17
1.1.1.1.3.5.	Kynurenine-3-hydroxylase	18
1.1.1.1.3.6.	3-Hydroxykynurenine	19
1.1.1.1.3.7.	Kynureninase	20
1.1.1.1.3.8.	3-Hydroxyanthranilic acid oxygenase	21
1.1.1.1.3.9.	Quinolinic acid	21
1.1.1.1.3.10.	Quinolinic acid phosphoribosyltransferase	26
1.1.1.2.	SEROTONERGIC PATHWAY OF TRYPTOPHAN METABOLISM	27
1.1.1.2.1.	Pineal gland	27
1.1.1.2.2.	Innervation	28
1.1.1.2.2.1.	Sympathetic	29
1.1.1.2.2.2.	Parasympathetic	30
1.1.1.2.3.	Indoleamine biosynthesis	31
1.1.1.2.3.1.	Tryptophan hydroxylase and 5-hydroxytryptophan	32
1.1.1.2.3.2.	L-Aromatic amino acid decarboxylase	32
1.1.1.2.3.3.	Serotonin	33
1.1.1.2.3.4.	Monoamine oxidase, 5-hydroxyindoleacetic acid and 5-hydroxytryptophol acid	35
1.1.1.2.3.5.	5-Methoxyindoleacetic acid and 5-methoxytryptophol	35
1.1.1.2.3.6.	N-Acetyltransferase and N-acetylserotonin	36
1.1.1.2.3.7.	Hydroxyindole- <i>O</i> -methyltransferase	38
1.1.1.2.3.8.	Melatonin	39
1.1.1.3.	6-Methoxy-2-benzoxazolinone	46
1.2.	TRYPTOPHAN 2,3-DIOXYGENASE-RELATED DEPRESSION.....	47
1.3.	LIPID PEROXIDATION	50
1.3.1.	Reduction of molecular oxygen	52
1.3.1.1.	Non-enzymatic lipid peroxidation	52
1.3.1.2.	Enzymatic lipid peroxidation	54
1.3.1.2.1.	Superoxide	54
1.3.1.2.1.1.	<i>In vivo</i> effects of superoxide	55
1.3.1.2.2.	Hydrogen peroxide	56
1.3.1.2.2.1.	<i>In vivo</i> effects of hydrogen peroxide	56
1.3.1.2.3.	Hydroxyl radicals	57
	i. Iron catalysed generation of hydroxyl radicals	57

ii.	Stimulation of peroxidation by iron complexes	58
a)	Iron-oxygen complexes	58
b)	Iron chelators	59
c)	Iron proteins	60
d)	Loosely bound iron	61
1.3.1.2.3.1.	<i>In vivo</i> effects of the hydroxyl radical	61
1.3.2.	Reactive Oxygen Species (ROS) and their biological consequences	62
1.3.2.1.	DNA damage	62
1.3.2.2.	Protein damage	63
1.3.2.3.	Lipid damage	63
1.3.3	Lipid peroxidation in neurodegenerative disorders	64
1.3.4	Defence against oxygen toxicity	65
1.3.4.1.	Antioxidant defence mechanisms	65
1.3.4.1.1.	Antioxidant enzymes	66
1.3.4.1.1.1.	Superoxide dismutase	66
1.3.4.1.1.2.	Catalase and glutathione peroxidase	68
1.3.5.	Cellular antioxidants	69
1.3.5.1.	Vitamin E	70
1.3.5.2.	Vitamin C	71
1.3.5.3.	Glutathione and uric acid	73
1.3.5.4.	Melatonin	73
1.3.6.	Antioxidants in extracellular fluids	76
1.4	CONCLUSION	77

CHAPTER 2: EXPERIMENTAL PROCEDURES

2.1.	ANIMALS	79
2.2.	DETERMINATION OF TRYPTOPHAN 2,3-DIOXYGENASE ACTIVITY..	80
2.2.1.	MATERIALS	81
2.2.1.1.	Animals	81
2.2.1.2.	Chemicals	82

2.2.2.	METHODS	82
2.2.2.1.	Chemicals	82
2.2.2.2.	Tissue preparation	83
2.2.2.3.	Tryptophan 2,3-dioxygenase determination	83
2.2.3.	RESULTS	85
2.2.4.	DISCUSSION	85
2.3.	DETERMINATION OF PINEAL INDOLE METABOLISM IN ORGAN CULTURE USING THIN LAYER CHROMATOGRAPHY	87
2.3.1.	MATERIALS	88
2.3.1.1.	Animals	88
2.3.1.2.	Chemicals	88
2.3.2.	METHODS	89
2.3.2.1.	Chemicals	89
2.3.2.2.	Pineal organ culture	92
2.3.2.3.	Separation of indoles by thin layer chromatography (TLC)	92
2.3.3.	RESULTS	94
2.3.4.	DISCUSSION	96
2.4.	LIPID PEROXIDATION DETERMINATION	96
2.4.1.	MATERIALS	99
2.4.1.1.	Animals	99
2.4.1.2.	Chemicals	99
2.4.2.	METHODS	99
2.4.2.1.	Chemicals	99
2.4.2.2.	Tissue preparation	100
2.4.2.3.	Thiobarbituric acid (TBA) test	100
2.4.2.4.	Protein determination of brain homogenates	101
2.4.3.	RESULTS	105
2.4.4.	DISCUSSION	105

CHAPTER 3: THE INFLUENCE OF KYNURENINES AND SEROTONERGIC COMPOUNDS ON TRYPTOPHAN 2,3-DIOXYGENASE106

3.1.	INTRODUCTION	106
3.2.	MATERIALS	108
3.2.1.	Animals	108
3.2.2.	Chemicals	108
3.3.	METHODS	109
3.3.1.	Chemicals	109
3.3.2.	Tissue preparation	109
3.3.3.	Tryptophan 2,3-dioxygenase determination.....	110
3.3.4.	Statistical analysis	110
3.4.	RESULTS	110
3.5.	DISCUSSION	119

CHAPTER 4: THE EFFECT OF KYNURENIC ACID AND QUINOLINIC ACID ON PINEAL INDOLE METABOLISM122

4.1.	INTRODUCTION	122
4.2.	MATERIALS	124
4.2.1.	Animals	124
4.2.2.	Chemicals	125
4.3.	METHODS	125
4.3.1.	Chemicals	125
4.3.2.	Tissue preparation	125
4.3.3.	Pineal organ culture.....	125
4.3.4.	Statistical analysis	126
4.4.	RESULTS	126

4.5.	DISCUSSION	133
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CHAPTER 5: EFFECTS OF KYNURENINES ON LIPID PEROXIDATION AND THE ANTIOXIDANT PROPERTIES OF TRYPTOPHAN ANALOGUES136

5.1.	INTRODUCTION	136
5.2.	MATERIALS	138
5.2.1.	Animals	138
5.2.2.	Chemicals	139
5.3.	METHODS	139
5.3.1.	Chemicals	139
5.3.2.	Tissue preparation	140
5.3.3.	Thiobarbituric acid (TBA) test	140
5.3.4.	Protein assay	140
5.3.5.	Statistical analysis	140
5.4.	RESULTS	141
5.5.	DISCUSSION	149

CHAPTER 6: FINAL SUMMARY AND CONCLUSION.152

Chapter 1	152
Chapter 2	153
Chapter 3.....	153
Chapter 4	154
Chapter 5	154
Conclusion	154

CHAPTER 7: FUTURE EXPERIMENTAL OBJECTIVES	157
REFERENCES	160

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ABSTRACT

The amino acid, tryptophan, obtained through dietary means, is metabolised by the enzymes tryptophan 2,3-dioxygenase (TDO), indoleamine 2,3-dioxygenase (IDO) and tryptophan hydroxylase. All the enzymes have an effect on circulating tryptophan levels, especially TDO, since it is the major site of tryptophan catabolism in the liver and results in the production of kynurenine metabolites, *viz.* kynurenine, kynurenic acid, 3-hydroxyanthranilic acid and quinolinic acid. Extrahepatically, IDO is responsible for the synthesis of the kynurenine metabolites. Tryptophan 2,3-dioxygenase and IDO activity is increased by hormones or substrates such as tryptophan, and inflammation, in the case of IDO. Tryptophan availability for serotonin (5-HT) synthesis by the enzyme tryptophan hydroxylase is primarily dependent on TDO activity. A study was attempted in order to ascertain whether any of the endogenous metabolites of the kynurenine and serotonergic pathways would be able to inhibit TDO activity. Results showed that although the kynurenines had no effect, the indoleamines, except for the indoleacetic acids, were able to reduce TDO activity. 6-Methoxy-2-benzoxazolinone (6-MBOA), a structural analogue to melatonin, was the most potent inhibitor with a reduction in activity of 55% compared with the control.

The pineal gland in the rat brain has been shown to have the highest IDO activity. With induction, the kynurenine metabolite concentrations of kynurenic acid and quinolinic acid are increased. The effects of both compounds were determined on the serotonergic pathway.

Although kynurenic acid produced no significant effect, quinolinic acid significantly reduced N-acetylserotonin and melatonin synthesis at concentrations of 10 μ M and 100 μ M respectively.

Many authors have implicated oxygen derived species as causative agents in the important neurodegenerative disorders such as Parkinson's and Huntington's disease. Increased radical generation and lipid peroxidation have been suggested to be responsible for the toxic destruction of neurons, especially in the brain because of its high lipid content and oxygen demand. The brain is therefore vulnerable to oxidative attack. During inflammatory diseases, IDO is induced with a resultant increase in kynurenines. This study was also an attempt at determining the effect of kynurenines on lipid peroxidation. All metabolites of the kynurenine pathway were able to induce lipid peroxidation significantly. The antioxidative potential of various tryptophan analogues, *viz.* serotonin, melatonin and 6-methoxy-2-benzoxazolinone, was determined using quinolinic acid-induced lipid peroxidation. Serotonin, melatonin and 6-MBOA were able to significantly reduce quinolinic acid-induced lipid peroxidation.

LIST OF ABBREVIATIONS

Al³⁺	Aluminium ion
5-ALA	5-Aminolevulinic acid
AD	Alzheimers disease
AMPA	α -Amino-3-hydroxy-5-methyl-4-isoxazoleproponoic acid
aMT	Melatonin
2APV	2-amino-5-phosphono-valeric acid
BBB	Blood brain barrier
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
C	Carbon
°C	Temperature in degrees Celsius
Ca²⁺	Calcium ion
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CH₃O	Methoxy group
Ci	Curie
CNS	Central nervous system
CO₂	Carbon dioxide
CSF	Cerebrospinal fluid
Cu²⁺	Copper ion
DNA	Deoxyribonucleic acid
DPM	Disintegrations per minute
ϵ	Extinction coefficient
EAA	Excitatory amino acids
EAE	Experimental allergic encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
Fe³⁺	Ferric ion
Fe²⁺	Ferrous ion
GSH	Oxidised glutathione
GSH-PX	Glutathione peroxidase enzyme
GSSG	Disulfide or reduced glutathione
3-HANA	3-Hydroxyanthranilic acid
3-HAO	3-Hydroxyanthranilic acid oxygenase enzyme
HCl	Hydrochloric acid
HD	Huntington's disease
HE	Hepatic encephalopathy

5-HIAA	5-Hydroxyindoleacetic acid
HIOMT	Hydroxyindole- <i>O</i> -methyltransferase enzyme
3-HK	3-Hydroxykynurenine
5-HT	5-Hydroxytryptamine (serotonin)
5-HTOH	5-Hydroxytryptophol
5-HTP	5-Hydroxytryptophan
H	Hydrogen atom
H₂O₂	Hydrogen peroxide
HO₂·	Perhydroxyl radical
IDO	Indoleamine 2,3-dioxygenase enzyme
KA	Kainic acid
KAT	Kynurenine aminotransferase enzyme
KCl	Potassium chloride
3-KH	Kynurenine-3-hydroxylase enzyme
K_m	Michaelis-Menton constant
KYN	Kynurenine
KYNA	Kynurenic acid
L·	Carbon centred radical
LH	Unsaturated fatty acids
LO₂·	Peroxy radical
LOOH	Lipid hydroperoxide
LNAA	Large neutral amino acid
MAO	Monoamine oxidase enzyme
MAOI	Monoamine oxidase inhibitors
6-MBOA	6-Methoxy-2-benzoxazolinone
MDA	Malondialdehyde
Mg²⁺	Magnesium ion
5-MIAA	5-Methoxyindoleacetic acid
mmol	Millimole
Mn²⁺	Manganese ion
mRNA	Messenger ribonucleic acid
5-MT	5-Methoxytryptamine
5-MTOH	5-Methoxytryptophol
MS	Multiple sclerosis
Na⁺	Sodium ion
NA	Nor-adrenaline
NAAC	Neutral amino acid carrier
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NAL	Nicotinylalanine
NaOH	Sodium hydroxide
NAS	N-acetylserotonin
NAT	N-acetyltransferase enzyme
NE	Norepinephrine
NMDA	N-methyl-D-aspartate

NO	Nitric oxide
NOS	Nitric oxide synthase enzyme
O₂	Molecular oxygen
O₂⁻	Superoxide anion
OH	Hydroxyl group
OH·	Hydroxyl radical
oMBA	o-Methoxybenzoylalanine
ONOO⁻	Peroxynitrite
Pb²⁺	Lead ion
PCPA	p-Chlorophenylalanine
Phe	Phenylalanine
pO₂	Partial oxygen pressure
PUFA	Polyunsaturated fatty acids
QA	Quinolinic acid
QPRT	Quinolinic acid phosphoribosyltransferase enzyme
R·	Free radical
REM	Rapid eye movement
RH	Unsaturated fatty acid
RNA	Ribonucleic acid
RO₂·	Peroxy radical
ROOR	Lipid hydroperoxide
ROS	Reactive oxygen species
SAD	Seasonal affective disorder
SAM	S-Adenosylmethionine
SCN	Suprachiasmatic nuclei
SH	Sulfhydryl group
SOD	Superoxide dismutase enzyme
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TDO	Tryptophan 2,3-dioxygenase enzyme
TEP	1,1,3,3-Tetraethoxypropane
TH	Tryptophan hydroxylase enzyme
TLC	Thin layer chromatography
TRP	Tryptophan
μCi	Microcurie
μg	Micrograms
μmoles	Micromoles
USA	United States of America
Vitamin C	Ascorbic acid
Vitamin E	α-Tocopherol
Zn²⁺	Zinc ion

LIST OF FIGURES

	Page
Figure 1.1. Structure of the essential amino acid tryptophan (TRP).....	3
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	15
Figure 1.3. A schematic representation of tryptophan catabolism in inflammatory neurological diseases in the brain with the consequent production of kynurenic acid (KYNA) and quinolinic acid (QA)	26
Figure 1.4. Diagrammatic representation of the pineal gland complex in the rat brain.....	29
Figure 1.5. 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.....	31
Figure 1.6. Tryptophan metabolism in the mammalian pineal gland	34
Figure 1.7. Schematic representation of the signal transduction mechanism between the postganglionic sympathetic neurons and the mammalian pinealocyte	37
Figure 1.8. Profiles of the various constituents of tryptophan metabolism in the pineal gland over a 24 hour period	39
Figure 1.9. Calcium entry due to stress induction by the excitatory amino acid, glutamate.....	42

Figure 1.10.	Day and night blood levels of melatonin in three different age groups.....	44
Figure 1.11.	Structure of 6-methoxy-2-benzoxazolinone, a structural analogue of melatonin.....	47
Figure 1.12.	A flow chart representing lipid peroxidation, initiated by free radicals	51
Figure 1.13.	Outline of the three main antioxidant enzymes involved in preventing the formation of free radicals	67
Figure 1.14.	Vitamin E acting as a chain-breaking antioxidant, reacting with lipid peroxy radicals to give an unreactive vitamin E radical	71
Figure 1.15.	Vitamin C (ascorbic acid) plays a protective role by reacting with free radicals including vitamin E to give semihydroascorbate, which is then able to form L-threonic acid and oxalic acid or be reduced back to ascorbate	72
Figure 1.16.	The mechanism thought to be responsible for the antioxidant effects of melatonin.....	76
Figure 2.1.	Effect of time incubation on tryptophan 2,3-dioxygenase at 37°C	86
Figure 2.2	A typical bi-dimensional thin layer chromatogram of the pineal indole metabolism	95
Figure 2.3.	The fomation of MDA by lipid peroxidation during incubation and to a much greater extent during the acid-heating stage.....	97
Figure 2.4	The mechanism which may be responsible for the decomposition of lipid peroxides to peroxy radicals	98

Figure 2.5	A typical standard curve for malondialdehyde (MDA) formation using 1,1,3,3-tetraethoxypropane (TEP) as an external standard	102
Figure 2.6.	A typical protein standard curve using bovine serum albumin (BSA)	104
Figure 4.1.	The effect of quinolinic acid and kynurenic acid at different concentrations on 5-hydroxyindoleacetic acid synthesis in rat pineal organ culture	127
Figure 4.2.	The effect of quinolinic acid and kynurenic acid at different concentrations on 5-methoxyindoleacetic acid synthesis in rat pineal organ culture	128
Figure 4.3.	The effect of quinolinic acid and kynurenic acid at different concentrations on 5-hydroxytryptophol synthesis in rat pineal organ culture	129
Figure 4.4.	The effect of quinolinic acid and kynurenic acid at different concentrations on 5-methoxytryptophol synthesis in rat pineal organ culture.	130
Figure 4.5.	The effect of quinolinic acid and kynurenic acid at different concentrations on N-acetylserotonin synthesis in rat pineal organ culture	131
Figure 4.6.	The effect of quinolinic acid and kynurenic acid at different concentrations on melatonin synthesis in rat pineal organ culture	132
Figure 5.1.	The effect of kynurenine on lipid peroxidation of rat brain homogenates	141
Figure 5.2.	The effect of kynurenic acid on lipid peroxidation of rat brain homogenates.....	142
Figure 5.3.	The effect of 3-hydroxyanthranilic acid on lipid peroxidation of rat brain homogenates.....	143

Figure 5.4.	The effect of quinolinic acid on lipid peroxidation of rat brain homogenates .	144
Figure 5.5.	The effect of melatonin as a potential antioxidant on quinolinic acid-induced lipid peroxidation of rat brain homogenates	145
Figure 5.6.	The effect of 6-methoxy-2-benzoxazolinone as a potential antioxidant on quinolinic acid-induced lipid peroxidation of rat brain homogenates	146
Figure 5.7.	The effect of serotonin as a potential antioxidant on quinolinic acid-induced lipid peroxidation of rat brain homogenates	147

LIST OF TABLES

	Page
Table 2.1. Scheme representing the method of the assay for tryptophan 2,3-dioxygenase.....	84
Table 2.2. Composition of the BGJb culture medium (Fitton-Jackson modification)	90
Table 2.2.1. Composition of the BGJb culture medium (Fitton-Jackson modification) (...continued)	91
Table 2.3. Representation of the method for pineal organ culture	93
Table 2.4. A review of the protein assay determination for brain homogenates	103
Table 3.4.1. Effect of kynurenic acid on tryptophan 2,3-dioxygenase activity	110
Table 3.4.2. Effect of 3-hydroxyanthranilic acid on tryptophan 2,3-dioxygenase activity.....	111
Table 3.4.3. Effect of quinolinic acid on tryptophan 2,3-dioxygenase activity	111
Table 3.4.4. Effect of 5-hydroxyindoleacetic acid on tryptophan 2,3-dioxygenase activity.....	112
Table 3.4.5. Effect of 5-methoxyindoleacetic acid on tryptophan 2,3-dioxygenase activity.....	112
Table 3.4.6. Effect of serotonin on tryptophan 2,3-dioxygenase activity	113

Table 3.4.7. Effect of 5-hydroxytryptophol on tryptophan 2,3-dioxygenase activity	113
Table 3.4.8. Effect of 5-methoxytryptophol on tryptophan 2,3-dioxygenase activity	114
Table 3.4.9. Effect of N-acetylserotonin on tryptophan 2,3-dioxygenase activity	114
Table 3.4.10. Effect of melatonin on tryptophan 2,3-dioxygenase activity	115
Table 3.4.11. Effect of 6-methoxy-2-benzoxazolinone on tryptophan 2,3-dioxygenase activity.....	115
Table 3.4.12. Comparison of the structure-activity relationship for the tryptophan analogues of the kynurenine pathway	116
Table 3.4.13. Comparison of the structure-activity relationship for the tryptophan analogues of the serotonergic pathway	117
Table 3.4.14. Comparison of the structure-activity relationship for the tryptophan analogues of the serotonergic pathway (...continued)	118

CHAPTER 1

LITERATURE REVIEW

1. INTRODUCTION

A nutrient differs from a drug in that the nutrient supplies energy or is a building block that the body may require. A drug is given for the sole purpose for its effect on a specific organ or cell. Nutrients or amino acids have the ability to act as drugs and so give rise to specific changes in the chemical structure of the brain, especially with regard to neurological diseases. Amino acids are the building blocks of proteins and the composition and function of the brain is dependent on specific amino acids. Such building blocks are the amino acids tryptophan (TRP), phenylalanine and tyrosine. Tryptophan is an essential amino acid because it cannot be synthesised within the body and therefore only available through dietary means. The above-mentioned amino acids are crucial to the brain because they are the precursors to many of the neurotransmitters present in the brain (Wurtman, 1982).

The concentration of the aromatic amino acids TRP, phenylalanine and tyrosine in the blood

results from a balance between dietary intake, protein synthesis and irreversible disposal through metabolism in the liver (Salter *et al*, 1986). The ratios of the aromatic amino acids are a determinant factor for neurotransmitter synthesis in the brain. The ratios and the absolute concentrations of the amino acids are important for the determination of the synthesis of biogenic amines in the brain as well as the activities of the enzymes which are responsible for their catabolism. The main enzymes which are responsible for exhibiting changes in amino acid concentrations are tryptophan 2,3-dioxygenase, tyrosine aminotransferase and phenylalanine hydroxylase and these may be indirectly accountable for a number of physiological and pharmacological conditions. The enzymes may influence neurotransmitter synthesis by removing the necessary amino acids from the blood. These changes could be interpreted as “rate-limiting” for the mentioned enzymes (Salter *et al*, 1986). It has also been proposed that competition for transport into the liver or brain is equally important. Control coefficients were introduced as a quantitative measurement for the importance of an enzyme with regard to the control of the pathway under a set of specified conditions. Salter *et al*, 1986, showed the transport of aromatic amino acids by a carrier system is slow and the catabolic flux taking place in the cells could be influenced by inhibitors of the transport system. This was previously thought to be rapid and of no consequence in controlling the metabolic flux.

1.1. TRYPTOPHAN

The amino acid is one of the eight essential amino acids. It has the greatest molecular weight

of the twenty known amino acids and is encoded by messenger ribonucleic acid (mRNA) to be incorporated into protein synthesis.

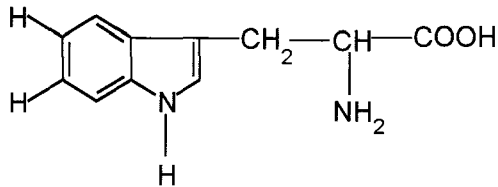


Figure 1.1. Structure of the essential amino acid tryptophan (TRP).

Following protein ingestion and the efflux of TRP from the bound and the “free” pools in the tissues, TRP enters the circulation as the overflow from the portal circulation. Tryptophan leaves the plasma via: (1) uptake into tissues, (2) metabolism in the liver by tryptophan 2,3-dioxygenase, and to an insignificant degree (3) excretion into the urine.

Passage of TRP and other amino acids between the blood and the brain is facilitated by a carrier system. Since TRP is a water-soluble molecule, transport between tissues and organs is facilitated by a diffusion process. However, TRP transportation to the brain is dependent on a carrier system to cross the blood-brain barrier (BBB). The neutral amino acids compete with each other for attachment to this carrier molecule and hence transport to the brain (Wurtman, 1982). In individuals, the metabolism of the dietary amino acid exhibits a rhythm depending on the food consumed. Peak levels have been found to occur during the latter part of the dark period or the early light period (Sugden, 1979). A high protein meal reduces the

plasma ratio of tryptophan to the competing amino acids and therefore decreases the amount of TRP crossing the BBB and consequently its availability to the brain (Wurtman, 1982; Fernstrom *et al*, 1971). A high carbohydrate intake, however, results in an opposite effect; the insulin secreted in response to the meal reduces the plasma level of competing amino acids and the ratio of TRP to the competing amino acids is now higher. Tryptophan bound to the plasma protein, albumin, is now isolated in the albumin reservoir and is immune to the effects of insulin (Wurtman, 1982). Although the ingestion of carbohydrate may elevate plasma tryptophan, the level may depend on the time the dietary meal was ingested with respect to the activity of tryptophan 2,3-dioxygenase, the enzyme responsible for catabolising this amino acid (Fernstrom *et al*, 1971). The quantity of nutrient available for metabolism is the “rate-limiting” element in this reaction. The flux of amino acids traversing tissues is influenced by a variety of hormones such as glucocorticoids, antidepressants and salicylates (Fernstrom *et al*, 1971). These compounds are able to induce the activity of tryptophan 2,3-dioxygenase and consequently shunt TRP away from the serotonin synthesis pathway in the brain and toward the production of kynurenines, consequently reducing plasma TRP levels (Delgado *et al*, 1995).

1.1.1. TRYPTOPHAN CATABOLISM

Tryptophan is degraded principally through the kynurenine pathway in the liver, and to an insignificant degree in the brain through the enzyme tryptophan hydroxylase to form serotonin. Primarily TRP is catabolised through the kynurenine pathway, the rate of catabolism of this

amino acid is regulated by the activity of the principal enzyme of this pathway, namely: tryptophan 2,3-dioxygenase, also known as tryptophan pyrrolase. Secondly, although serotonin is of considerable importance physiologically it is of minor importance with respect to the catabolism of TRP, since TRP catabolism through this pathway only accounts for 1% of the urinary metabolites (Hillier *et al*, 1975).

1.1.1.1. KYNURENINE PATHWAY OF TRYPTOPHAN CATABOLISM

1.1.1.1.1. TRYPTOPHAN 2,3-DIOXYGENASE

The rat liver enzyme tryptophan 2,3-dioxygenase (EC 1.13.11.11) is a cytosolic enzyme which has been subjected to many investigations. Enzymes that control the metabolism of living tissues are themselves subject to regulation (Fiegelson *et al*, 1961). This liver enzyme has been extensively studied with regard to both hormone and substrate regulation (Fiegelson *et al*, 1961; Badawy *et al*, 1974).

Tryptophan 2,3-dioxygenase (TDO) is a haemoprotein which catalyses the first step in the oxidative degradation of tryptophan (Mehler *et al*, 1950; Makino *et al*, 1980; Leeds *et al*, 1993). The enzyme has been extensively purified from livers of both TRP-induced and cortisone-induced rats as well as TRP-induced *Pseudomonas acidovorans* (ATCC 11299b) to homogeneity and found to have molecular weights of 122 000 and 167 000 respectively (Schutz *et al*, 1972; Brady, 1975). The enzyme has been shown to be tetrameric and contains

two copper atoms and two moles of haem per mole tetramer, the subunits consisting of identical mass (Makino *et al*, 1980; Brady, 1975). The quaternary structure is maintained by non-covalent interactions (Schutz *et al*, 1972; Makino *et al*, 1980). The complete amino acid sequence (primary structure) of the enzyme was determined from the nucleotide sequence of a full length TDO complementary deoxyribosenucleic acid (cDNA) isolated from rat liver cDNA library. According to the deduced amino acid sequence, the monomeric polypeptide of TDO consisted of 406 amino acid residues with a molecular weight of 47 796 daltons. It is comprised of twelve histidine residues around its hydrophobic region. Its homology resembles some haem proteins and oxygenases, thus suggesting that the hydrophobic region may be the core for the activity of the enzyme (Maezono *et al*, 1990).

Tryptophan is catabolized primarily by TDO in the first step of the kynurenine pathway. The reaction is believed to proceed by the addition of oxygen across the 2,3-bond of the indole ring followed by the decomposition of the resultant dihydroindole to give N-formylkynurenine. Suggestions have been made that the extraction of the indole proton is at least partially rate-determining with bond formation at C-2 becoming more rate-limiting with increasing pH (Leeds *et al*, 1993).

In the rat and humans TDO exists in two forms, while in several species only one form is present. In the rat, the holoenzyme which is already active, does not require the addition of exogenous haematin (co-enzyme), whereas the predominant haem-free apoenzyme does (Badawy *et al*, 1975). The co-enzyme-free apoenzyme occurs as two-thirds of the total liver

enzyme (Greengard *et al*, 1966). Activation of the enzyme occurs as a series of reactions, starting with the apoenzyme, which is formed in large quantities with hydrocortisone induction. The apoenzyme is conjugated with haematin (cofactor), a reaction which is encouraged by a small amount of TRP (Knox, 1966). This reaction is an interdependent process and it appears that the apoenzyme-TRP previously thought to be necessary, is not required for the attachment of the haem group (Greengard *et al*, 1962). The oxidised holoenzyme formed by the conjugation with TRP is then reduced to the ferrous form of the holoenzyme (reduced holoenzyme) (Knox, 1966; Tanaka *et al*, 1959). The reduced holoenzyme that is formed is active in the catalytic reaction which specifically requires TRP as its substrate (Knox, 1966). Haematin has a stimulatory function and findings show that the haem moiety is readily dissociable from the apoenzyme and functions rather as a dissociating coenzyme rather than a tightly bound prosthetic group (Fiegelson *et al*, 1961; Greengard *et al*, 1961). The degree of saturation of the enzyme with haematin is a primary regulator of the amount of total liver TDO, since the activity changes with the metabolism of porphyrins and haem. The saturation of the apoenzyme with haem and thus the activity of TDO is dependent on agents that destroy or inhibit (griseofulvin) the synthesis of haem and the opposite is true for treatments which increase the synthesis and thus, utilisation of haem (5-aminolevulinate, 5-ALA) (Fiegelson *et al*, 1961; Badawy *et al*, 1975).

The regulation of the amount of enzyme produced by induction and activation constitutes an essential mechanism for the regulation of amino acids, especially TRP.

1.1.1.1.1. TRYPTOPHAN 2,3-DIOXYGENASE REGULATION

Tryptophan 2,3-dioxygenase activity can be increased by the administration of cortisone or tryptophan. The portion of activity of the enzyme controlled by TRP influences the ratio of inactive apoenzyme to active holoenzyme (Knox, 1966). Both cortisone and TRP produce an increase in the concentration of the enzyme. These responses have been thought to result from different mechanisms. Puromycin, an inhibitor of protein synthesis, can inhibit cortisone-induced elevation of TDO. Actinomycin D, which inhibits RNA synthesis, abolishes the cortisone-induced rise in the level of both apoenzyme and holoenzyme but does not influence the TRP-mediated increase in the level of TDO. This suggests that there is a difference between hormonal- and substrate-induced stimulation of enzyme synthesis *in vivo* (Greengard *et al*, 1963; Greengard *et al*, 1966). A number of reports have confirmed that hydrocortisone increases the rate of enzyme synthesis by increasing the concentration of the inactive apoenzyme present in the liver (Badawy *et al*, 1975; Knox, 1966; Schimke *et al*, 1965). The apoenzyme has negligible activity unless supplemented with its cofactor, haem (Greengard *et al*, 1962; Knox *et al*, 1966a).

Tryptophan administration: (1) prevents the rapid degradation of the pre-existing apoenzyme (Badawy *et al*, 1975; Schimke *et al*, 1965), (2) enhances the conjugation of haematin with the apoenzyme, and (3) preserves the active reduced holoenzyme (in the absence of TRP the reduced holoenzyme is inactivated reversibly to the oxidised form of the holoenzyme). Therefore the rate of degradation of TRP in the rat is proportional to the amount of the

reduced holoenzyme present in the liver (Knox, 1966; Knox *et al*, 1966b).

1.1.1.1.1.2. PROPOSED MECHANISM

The proposed mechanism for substrate and hormonal induction rests on the assumption that the enzyme is in excess due to the presence of increased levels of enzyme protein synthesis and haem activation.

a) TRYPTOPHAN INDUCTION:

The molecules of the enzyme formed at the enzyme forming site are in dynamic equilibrium with enzyme molecules in the cytoplasm. Holoenzyme molecules are unable to bind at this site. Saturation of the apoenzyme with haematin by TRP would result in the enzyme forming vacant sites. This would stimulate further enzyme protein synthesis and result in the fall of the concentration of intracellular TRP. Consequently the conjugation of the apoenzyme and TRP is prevented. The apoenzyme could accumulate, occupying the enzyme forming site and inhibiting its own synthesis followed by a gradual return to basal levels would then take place (Fiegelson *et al*, 1961). The stabilisation influence could be due to the promotion of the conjugation of the apoenzyme with haem and the subsequent reduction of the oxidised holoenzyme (Badawy *et al*, 1975). Thus the biochemical, chemical and physiological conditions are appropriate for TRP to regulate its own metabolism by activating the enzyme which is able to oxidise it (Knox, 1966).

b) HYDROCORTISONE INDUCTION:

Cortisone has been found to stimulate enzyme protein synthesis by interfering with the equilibrium between the template band and soluble apoenzyme thus preventing the accumulation of apoenzyme and its binding to the template. Consequently it is able to inhibit its own synthesis (Fiegelson *et al*, 1961). The ratio of holoenzyme/apoenzyme activity in cortisol treated rats resembles basal levels at less than 1. This suggests that approximately 50% of the newly synthesised apoenzyme becomes conjugated with haem (Badawy *et al*, 1975).

Regulation of TRP fails in adrenalectomised rats. In the absence of hormones the enzyme is unable to be induced and TRP is not able to be disposed of quickly and efficiently and so excessive amounts of the amino acid accumulate and the animals die. Suggestions have been proposed that TRP may be metabolised by other pathways which may be toxic (Badawy *et al*, 1974).

1.1.1.1.1.3. CIRCADIAN RHYTHM IN RAT LIVER TRYPTOPHAN 2,3-DIOXYGENASE

The circadian rhythm in enzyme activity has been shown to be corticosteroid-dependent. However, no obvious relationship between maxima substrate, hormone and enzyme activity exists. Tryptophan 2,3-dioxygenase shows a diurnal oscillation from 09h00 and 18h00 with activity increasing by *de novo* protein synthesis. Once maximum activity is reached, the

enzyme synthesis is inhibited. As minimum activity is reached, the synthesis of the enzyme is subjected to degradation. Activity decreases from 24h00 until 09h00, until synthesis is initiated again (Hardeland *et al*, 1968).

1.1.1.1.1.4. KINETIC PROPERTIES OF TRYPTOPHAN 2,3-DIOXYGENASE

The enzyme can be strongly affected by pH. The ferric or the ferrous form of the enzyme can exist in a “low or high affinity form” for the binding of exogenous ligands depending on the presence or the absence of TRP. The spin state (high or low) of the enzyme-haem is altered by the binding of TRP which induces a great change in the ligand binding affinity of the enzyme (Schutz *et al*, 1972; Makino *et al*, 1980). Tryptophan 2,3-dioxygenase is in a high spin state at a neutral pH but still very inactive towards exogenous ligands. Activation of the haem in the enzyme occurs by the binding of TRP or by raising the pH to an alkaline pH. Therefore the thermally “high spin” state of the alkaline form is the most beneficial candidate for the “high affinity form” for the binding of exogenous ligands toward the haem of the enzyme (Makino *et al*, 1980).

The kinetic properties of the enzyme are allosteric and the activity is affected by TRP and α -methyl-TRP. Tryptophan has been found to be a competitive inhibitor of TDO. Allosteric inhibition is assumed to possess at least two distinctive, non-overlapping sites: (1) the active site- for the binding of the substrate; responsible for the catalytic activity of the enzyme, and (2) the allosteric site- it binds the allosteric effector specifically and reversibly. An example,

nicotinamide adenine dinucleotide phosphate (NADPH), binds to the allosteric site but does not affect the binding of TRP. A slight distortion of the active site is induced so that the affinity of TRP for the binding site is reduced. The independence of the two sites, *viz.* the allosteric and the active site, suggests that the TRP and NADPH binding site specificities differ greatly in size, shape and charge (Cho-Chung *et al.*, 1967).

1.1.1.1.5. STRUCTURE-ACTIVITY RELATIONSHIP OF TRYPTOPHAN 2,3-DIOXYGENASE

The basis of kinetics can be classified into substrates and inhibitors. Catalytic site inhibitors may have the appropriate configuration to act as a substrate, although the addition of a side-chain may result in the molecule having the “incorrect” configuration required for binding to the catalytic site (Uchida *et al.*, 1992).

Frieden *et al.*, 1961, mentioned that the strong electronegativity of the indole nucleus produces strong non-competitive inhibition. Alanine side-chains may also be involved in complexing directly with the enzyme. Uchida *et al.*, 1992, suggested that the NH in the indole ring is important for binding to the catalytic site and hence the amino-side chain, $\text{CH}(\text{NH}_2)\text{COOH}$, is important to make the configuration appropriate for the catalytic reaction. Even though some molecules have the pertinent NH group in the indole and the amino acid-side chain, these cannot be used as a substrate because functional groups such as the hydroxy (OH) and the methoxy (CH_3O) group at the 5th position on the benzene ring may result in the possible distortion of the protein structure in the haem pocket. This is possibly due to the strong

influence of the CH₃O and the OH group to the amino acid side-chain and thus the compounds are classified as inhibitors (Uchida *et al*, 1992).

1.1.1.1.2. INDOLEAMINE 2,3-DIOXYGENASE

Mammalian TDO has only been found in the liver whereas indoleamine 2,3-dioxygenase (IDO) is widely distributed in various tissues and organs other than the liver. It is so named because of its broad substrate specificity. It is a haem-containing oxygenase that cleaves the indole ring of TRP. It acts on both the D- and L- isomers of TRP and various other indoleamines. The two dioxygenases, TDO and IDO, differ from each other with respect to molecular properties and reaction mechanism (Watanabe *et al*, 1980).

1.1.1.1.3. KYNURENINE BIOSYNTHESIS

It was not until 1947 (Beadle *et al*, 1947) that the kynurenine pathway was recognised as a major route for the conversion of TRP to nicotinamide and its nucleotide conjugates (Stone, 1993a). One percent of dietary TRP in the peripheral tissues is converted to 5-hydroxytryptophan, while 95 % is metabolised to kynurenines (Stone, 1993a; Schwarcz, 1993). The term kynurenines is a general term and refers to the metabolites of the kynurenine pathway of TRP degradation. The term, kynurenine, is used for the specific metabolite of this pathway (Stone, 1993a; Schwarcz, 1993). Briefly, TRP is oxidatively cleaved to form L-kynurenine via N-formylkynurenine by TDO (hepatic) and IDO in the peripheral tissues and brain.

1.1.1.1.3.1. Formamidase

Tryptophan is acted on by the enzyme TDO to form N-formylkynurenine which is then deformed by the action of the enzyme formamidase (EC 3.5.1.9). The enzyme has a low substrate specificity and is able to release formate from a variety of aryl-formylamines although its greatest activity is towards N-formylkynurenine (Bender, 1975; Stone, 1993a). The resultant product is L-kynurenine.

1.1.1.1.3.2. Kynurenine (KYN)

L-Kynurenine (KYN) is (predominantly cationic) formed as an intermediary in the kynurenine pathway of TRP metabolism. The major route of TRP degradation in the mammalian periphery, which is an alternative path to the KYN metabolism in the central nervous system (CNS), is the transamination to yield kynurenic acid (KYNA) (Speciale *et al*, 1989). Approximately 5% of the TRP that enters the brain from the plasma is metabolised to KYN and is non-uniformly distributed ranging from 0.03 pmol/mg tissue to 1.05 pmol/mg tissue in the cerebellum and putamen pool respectively (Speciale *et al*, 1989). The continuous flux of KYN into the brain amounts to 60% of the cerebral pool of KYN (Gal *et al*, 1978a). Kynurenine uses a large neutral amino acid (LNAA) transporter which can readily penetrate the blood brain barrier (Schwarcz, 1993). Studies have shown that KYN is in competitive with other substrates for the high affinity, sodium-independent transporter of LNAA's. Experiments have shown that KYN uptake is sodium-independent for neural uptake and

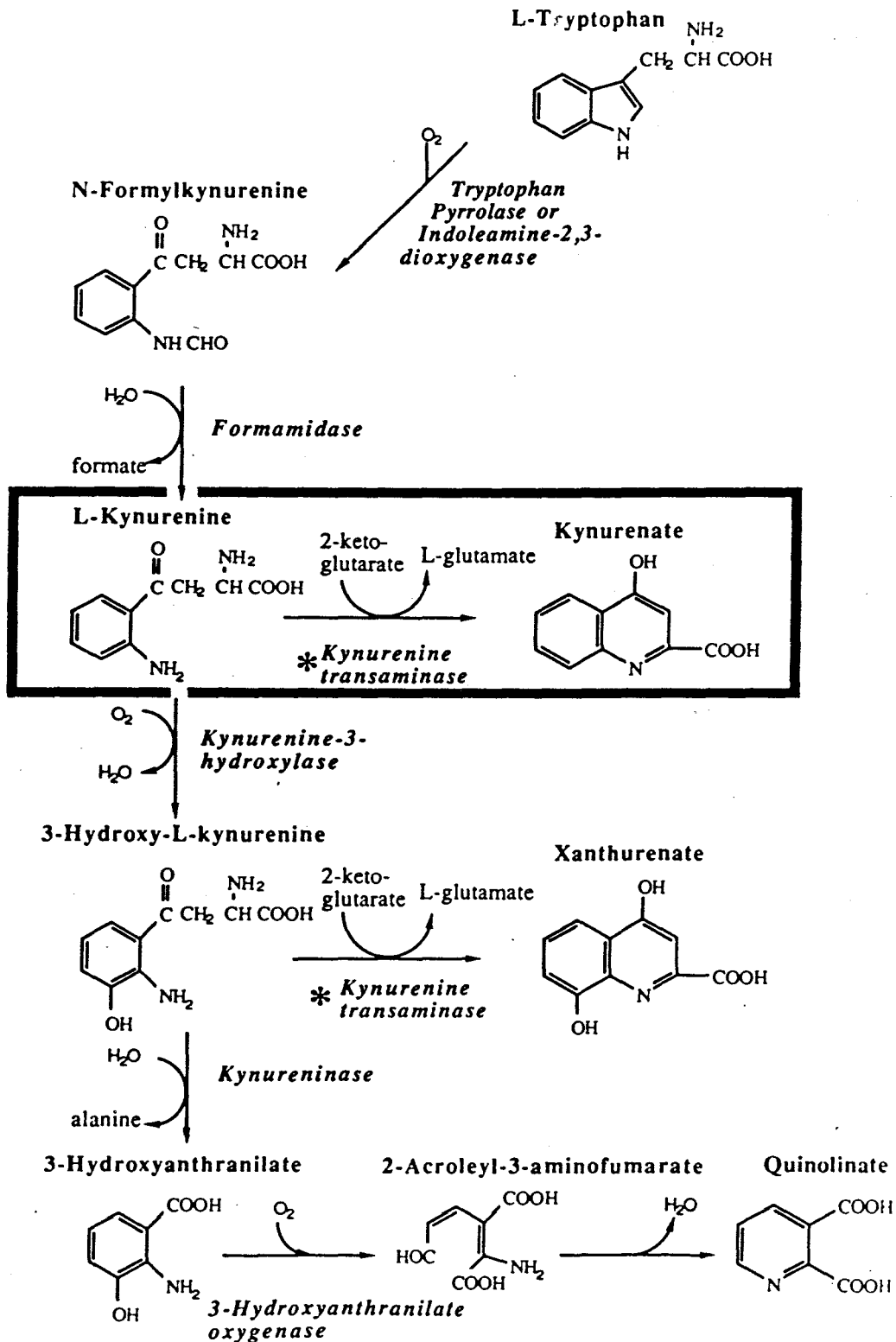


Figure 1.2. Representation of the kynurenine pathway which is responsible for the catabolism of tryptophan with the resultant production of kynurenic acid (KYNA) and quinolinic acid (QA), antagonist and agonist of the N-methyl-D-aspartate receptor respectively (Martin *et al*, 1992).

sodium-independent for glial uptake (Stone, 1993a; Speciale *et al*, 1989). The sodium-dependent transport may imply that uptake is of physiological importance because sodium-dependence is characteristic of many re-uptake processes for neuronally important transmitters and modulatory agents (Stone, 1993a). Kynurenine is transported with high affinity and stereospecificity into the astrocytes by a sodium-independent process that prefers branched chain and aromatic neutral amino acids such as TRP, phenylalanine and leucine (Schwarcz, 1993; Speciale *et al*, 1989). After KYN enters the cells it may then be stored or slowly converted to KYNA by the enzyme kynurenine aminotransferase (KAT) localised in the glial cells. After its biosynthesis KYNA rapidly exits the cell (Schwarcz, 1993). In the astrocytes KYN is not rapidly metabolised and does not exchange freely across the astrocytic membrane for metabolism elsewhere. The distinction between sodium-dependent and sodium-independent processes correspond to the movement into neurones and glial cells (Stone, 1993a). Uptake of KYN into glial cells is likely to be mediated by a neutral amino acid carrier (NAAC) (Stone, 1993a). Kynurenine is physiologically important because of its ability to exert control of the metabolic flow of the metabolites (Stone, 1993a; Speciale *et al*, 1988).

1.1.1.1.3.3. *Kynurenine aminotransferase (KAT)*

Kynurenine aminotransferase (EC 2.6.1.7) which is responsible for the metabolism of KYN to KYNA and 3-hydroxyanthranilic acid (3-HANA), has been found in the brain (Speciale *et al*, 1989). Kynurenine aminotransferase has been found to exist in both humans and rats, is vitamin B₆-dependent and mainly present in the inner membrane of the mitochondria (Turski

et al, 1989; Takeuchi *et al*, 1984). In humans KAT is present in two isoforms: KAT I and KAT II (Jauch *et al*, 1995). Immunohistochemical studies have shown that the enzyme is preferentially localised in the glial compartment which suggests that astrocytes are in close promiximity to the excitatory synapses making contact with the dendritic synapses. This allows KYNA to enter the extracellular compartment (Stone, 1993a; Schwarcz, 1993; Jauch *et al*, 1995). Its regional antogenetic distribution correlates with increased KYNA and quinolinic acid (QA) synthesis via KYN (Stone, 1993a; Chiarugi *et al*, 1995).

1.1.1.1.3.4. Kynurenic acid (KYNA)

Kynurenic acid is a broad spectrum antagonist and is able to interact with the ionotropic excitatory amino acid (EAA) receptors (Schwarcz, 1993; Chiarugi *et al*, 1995). Kynurenic acid at low micromolar concentrations shows a high affinity for the glycine recognition site at the N-methyl-D-aspartate (NMDA) receptor-ion channel complex (Schwarcz, 1993; Chiarugi *et al*, 1995). Kynurenic acid has been identified as a natural brain constituent with its concentration varying within the brain and several animal species. Kynurenic acid has also been shown to possess anticonvulsant and neuroprotective properties and therefore could possibly act as an endogenous anti-excitotoxin. Its antagonist properties could counteract the effects of naturally occurring excitatory transmitters or transmitter candidates such as glutamate, aspartate, sulphur-containing amino acids and QA (Schwarcz, 1993). It has been suggested that a concentration of $< 1 \mu\text{M}$ KYNA is able to serve a physiological role and that the intracellular KYNA is largely of glial origin (Turski *et al*, 1989). Neuronal KYNA may

be formed following the slow sodium-dependent transport of KYN into the nerve cells (Turski *et al.*, 1989). A decrease in KYNA concentration may result from overexcitation due to an imbalance between KYNA and one or more endogenous excitants (Schwarcz, 1993). Kynurenic acid, with its polar structure, is unable to penetrate the brain and the fate of KYNA under pathophysiological conditions has not been studied in great detail. It could be that altered levels of KYNA are related to changes in the activity of the enzyme KAT, or the altered penetration of circulating KYN into the brain (Schwarcz, 1993). It has been shown that increased neuronal activity or depolarisation can diminish the production of KYNA. An increased neuronal activity, hyperexcitability and seizures seen after injections of kainate and NMDA agonists could partly result from low extracellular levels of KYNA (Stone, 1993a). Wu *et al.*, 1992, showed that glutamate suppresses KYNA production by acting directly on glial cells in the lesioned hippocampus (Russi *et al.*, 1992). Kynurenine aminotransferase which is preferentially located in the glial cells, surrounds the synapses which may allow KYNA to be synthesised and released at the sites where it may reduce EAA-mediated neurotransmission (Russi *et al.*, 1992).

1.1.1.1.3.5. Kynurenine-3-hydroxylase (3-KH)

The conversion of KYN to 3-hydroxykynurenine is catalysed by the enzyme kynurenine-3-hydroxylase (EC 1.14.13.19) (3-KH) (Stone, 1993a). The enzyme appears to be present in the brain at low activity, which may raise the possibility as to whether the conversion of KYN to 3-hydroxykynurenine to 3-HANA does in fact occur. Inhibitors such as oestrogenic

compounds are able to inhibit 3-KH in a competitive manner and this could result in an increase in plasma and urinary KYN and 3-hydroxykynurenine (Stone, 1993a).

1.1.1.1.3.6. 3-Hydroxykynurenine (3-HK)

3-Hydroxykynurenine (3-HK) in the periphery is a metabolite of KYN *en route* to the formation of QA. It is however neurotoxic with the ability to produce convulsions and neuronal damage (Stone, 1993a; Schwarcz, 1993). 3-Hydroxykynurenine may play a role in brain pathology without functioning as a neuroactive brain metabolite under physiological conditions (Schwarcz, 1993). This metabolite has been shown to be elevated in severe pathological diseases such as Huntington's disease (HD) (Nakagami *et al*, 1996). 3-Hydroxykynurenine damage is related to the generation of free radicals such as hydrogen peroxide rather than the direct action on the EAA receptors (Schwarcz, 1993; Nakagami *et al*, 1996). 3-Hydroxykynurenine toxicity may result from the interaction of 3-HK and intracellular H₂O₂ (Eastman *et al*, 1990). The generation of free radicals by 3-HK and H₂O₂ is enhanced in the presence of iron (Nakagami *et al*, 1996). Antioxidants such as catalase prevent free radical formation of hydrogen peroxide by converting this reactive oxygen species into water (Nakagami *et al*, 1996; Eastman *et al*, 1990). Both sodium-dependent and -independent transport of the metabolite was detected in neurons, the former being unique to central neurons. This suggests that 3-HK may be compartmentalised into two separable pools (Stone, 1993a).

1.1.1.1.3.7. Kynureninase

Kynureninase (EC 3.7.13) catalyses the formation of 3-HANA via 3-HK (Stone, 1993a; Russi *et al*, 1992). The enzyme is cytosolic and pyridoxal phosphate-dependent and catalyses the cleavage to l-alanine with replacement of the anthranilyl group by hydrogen (Palcic *et al*, 1985). The enzyme is present in large amounts in the liver (higher in humans than rats and accounts for 1% of total liver activity), kidney and small amounts in other tissues such as the brain (Stone, 1993a). The enzyme shows highest activity towards 3-HK and is able to synthesise 3-HANA. Another pathway to form 3- or 5-HANA is from KYN via non-specific microsomal hydroxylating enzymes. The latter pathway may provide insight as to why the brain is able to synthesise 3-HANA by penetration of KYN into the brain providing, independent of TRP, an alternative route into the kynurenine pathway (Stone, 1993a). Oestrogenic compounds, nicotynylalanine (NAL) (KYN analogue) and o-methoxybenzoylalanine (oMBA) 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, 1993a). Inhibition of this enzyme enhances the levels of KYNA and prevents the metabolism of QA (neurotoxin). Increased synthesis of KYNA is accompanied by effects such as sedation and protection from seizures and audiogenic convulsions through electric shock treatments (Stone, 1993a; Chiarugi *et al*, 1995).

1.1.1.1.3.8. 3-Hydroxyanthranilic acid oxygenase (3-HAO)

3-Hydroxyanthranilic acid oxygenase (3-HAO) is an anabolic enzyme which is responsible for the synthesis of QA via an unstable intermediate 2-acroyleylaminofumarate QA (Schwarcz, 1993). The enzyme is bound to the outer and inner surfaces of the mitochondrial membrane (Stone, 1993a). Using immunoreactivity the enzyme was shown to be located in the astrocytes and contained in the thin and highly ramified astrocytic processes which engulf excitatory synapses. This suggests that the area of QA synthesis is in an excellent position to act on the NMDA receptors (Stone, 1993a; Schwarcz, 1993). Several EAA's, TRP and KYNA have no influence on the enzyme with regard to its activity (Stone, 1993a). Increased activity of 3-HAO in response to lesions could increase the production of QA which is found to be responsible for slowly progressive and neurodegenerative diseases (Schwarcz, 1993).

1.1.1.1.3.9. Quinolinic acid (QA)

Quinolinic acid (2,3-pyridine dicarboxylic acid), an endogenous metabolite of TRP metabolism, is neurotoxic when injected into rat striatum (Farmer *et al*, 1984; Stone *et al*, 1981). Ibotenic acid and kainic acid (KA) are potent neurotoxins and when injected into the CNS these agents produce lesions followed by neuronal cell loss. Excitotoxins are so termed because of the ability of these agents to cause neurodegeneration by depolarising neurons. This led to the hypothesis that the body is able to produce its own excitotoxins under abnormal conditions resulting in certain diseased states (Foster *et al*, 1983). Quinolinic acid acts

preferentially on NMDA receptors shown by the blockade by 2-amino-5-phosphono-valeric acid (2APV) (Stone *et al*, 1981; Birley *et al*, 1982). Quinolinic acid is synthesised in the liver and CNS, the latter by the activation of human macrophages (Flanagan *et al*, 1995). Quinolinic acid is found to be a potent convulsant which is able to excite neurons when iontophoretically applied and its axon-sparing lesions are similar to those produced by KA and ibotenic acid (Moroni *et al*, 1984b; Foster *et al*, 1983). Quinolinic acid causes neuronal lesions after hippocampal or striatal injections by activating the NMDA class of EAA receptors (Blight *et al*, 1995). Excitatory amino acid receptors for excitatory compounds are divided into four groups: NMDA, quisqualate, KA and α -amino-3-hydroxy-5-methyl-4-isoxazolepropanoic acid (AMPA) (Beal, 1992; Stone *et al*, 1983). The NMDA receptor regulates the calcium and sodium (Na^+) influx and is gated by magnesium (Mg^{2+}) which has been implicated in synaptic plasticity and the acquisition of memory (Beal, 1992). The NMDA site also requires the presence of glycine which is a coagonist of this receptor. Rapid NMDA receptor-mediated neurotoxicity may be mediated by excessive calcium influx (Pawley *et al*, 1996; Weiss *et al*, 1990). Increases in calcium concentrations are able to activate protein kinases, generate free radicals and mitochondrial damage (Beal, 1992). Calcium toxicity occurs in three phases; firstly, an increase in intracellular calcium lasting 5-10 minutes; a latent phase of about two hours in which the calcium level returns to normal, and finally a gradual sustained rise in intracellular calcium that reaches a plateau which eventually leads to cell death (Beal, 1992). The neurotoxic properties of a compound acting on the NMDA receptor seem to have the following properties: (1) the presence of two carboxyl groups held in an extended conformation (the neurotoxic properties are less dependent on whether the carbon structure is

of aspartic or glutamate length), (2) all compounds that possess at least one positive charge and two negative charges at physiological pH and are structurally related to NMDA have the potential to act as neurotoxins, and (3) the blockade of one carboxyl group or a substitution of the nitrogen results in the loss of neurotoxic properties (Foster *et al*, 1983; Stone, 1984).

Studies have shown that all three groups are not necessarily required for neurotoxic effects (Foster *et al*, 1983). Quinolinic acid is a rigidly planar molecule due its aromatic ring while NMDA is a flexible non-planar molecule (Stone, 1984). It may be possible that QA plays a role in the etiology of various neurodegenerative disorders such as HD, temporal lobe epilepsy, hepatic encephalopathy and Alzheimer's disease (AD). The reason for this, is that it is able to reproduce 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; Schwarcz *et al*, 1983a; Beal, 1992). Huntington's disease, first described in 1872 by George Huntington, is an inherited neurodegenerative disorder in which the striatum undergoes marked atrophic changes. The clinical features are divided into two areas: (1) progressively worsening choreoathetotic movement and (2) neuropsychiatric problems. The most important neuropathophysiologic feature is the degeneration of the medium-sized spiny projection neurons in the striatum. The abnormal movements could be the result of striatal degeneration associated with the illness; thus HD may involve an excitotoxic mechanism (Furtado *et al*, 1996). Long term lesions caused by QA closely resemble the neurochemical features of HD which include increases in 5-hydroxyindoleacetic acid, 5-hydroxytryptophan, somatostatin and neuropeptide Y concentrations. These features strengthen the possibility that a NMDA receptor-mediated excitotoxic process is involved in the pathogenesis of HD (Beal *et al*, 1991).

The excitotoxic process involves the NMDA receptors with an increase in intracellular levels of calcium to toxic proportions (Jauch *et al*, 1995). Quinolinic acid shows activity in the CNS, such as in the cerebral cortex and hippocampus, and shows almost no activity in other areas such as the spinal cord (Perkins *et al*, 1983; Birley *et al*, 1982). Quinolinic acid, at a nanomolar concentration, is able to activate the NMDA receptor of which at least two types exist. Site I is activated by NMDA only (cerebellum and spinal cord) and site II which is activated by NMDA or QA (cortex, striatum and hippocampus) (Pawley *et al*, 1996; Perkins *et al*, 1983; McLennan, 1984). The highest concentration of QA is found in the cortex and the lowest in the striatum. This may correlate with the fact that the highest concentration of excitatory receptors are present in the cortex and therefore the cortex is more susceptible to the excitotoxic effects of QA (Schwarcz *et al*, 1983a; Moroni *et al*, 1984b). Quinolinic acid toxicity is prevented with the administration of MK-801 (4 mg/kg) and its effectiveness is dose-dependent (Beal *et al*, 1988). Another agent which has provided some success in preventing QA-induced neurotoxicity is KYNA. Kynurenic acid, as already mentioned, is a non-competitive antagonist of the NMDA receptor and studies have shown that the ratio of KYNA to QA must be 1:1 for KYNA to exert protective effects (Saito *et al*, 1993a; Perkins *et al*, 1982; Moroni *et al*, 1991). Various diseased states are accompanied by an increase in the synthesis of QA. In hepatic encephalopathy (HE), increased release of glucocorticoids due to liver stress induce TDO and an increase in QA synthesis occurs. Quinolinic acid is then able to penetrate the BBB at the last stage (IV) of HE due to its poor integrity. Quinolinic acid may therefore play a role in neuronal damage in patients that exhibit seizures with hepatic failure (Basile *et al*, 1995). Immune response, due to inflammation, is prevalent in diseases

such as meningitis and autoimmune diseases such as experimental allergic encephalomyelitis (EAE) (Blight *et al*, 1995; Flanagan *et al*, 1995; Saito *et al*, 1993b). Experimental allergic encephalomyelitis is an animal model for multiple sclerosis (MS) (Flanagan *et al*, 1995). Both above-mentioned disease types are characterised by an increase in macrophages and T-lymphocytes (Flanagan *et al*, 1995). Induction of the following enzymes: IDO, 3-KH, kynureninase and 3-HAO may occur (Blight *et al*, 1995).

Induction of the enzymes IDO, 3-KH and 3-HAO is accompanied by a concomitant increase in the synthesis of QA. Quinolinic acid may be synthesised in the brain from 3-HANA within the CNS from anthranilic acid and to a lesser extent from 3-HK; therefore the 3-HK hydroxylation is bypassed and 3-HANA is converted to 2-amino-3-carboxy-muconic semialdehyde and then to QA (Saito *et al*, 1993b). The ratio of QA to KYNA is the determinant factor with respect to the pathological consequences of diseases with the ratio usually favouring QA and excitotoxicity (Heyes *et al*, 1993). Increased levels of QA allows it to penetrate the BBB due to its poor integrity in the spinal cord (Blight *et al*, 1995; Flanagan *et al*, 1995; Saito *et al*, 1993a). In MS or EAE, QA may contribute to demyelination of the spinal cord and lipid peroxidation. In studies that have been carried out to modify QA levels, NAL has been proved to be particularly effective in reducing QA production by the inhibition of the enzymes, kynureninase and 3-KH, and thus should be considered as an agent with therapeutic potential (Blight *et al*, 1995; Moroni *et al*, 1991). The level of peroxidation is correlated to the severity of the disease (Flanagan *et al*, 1995).

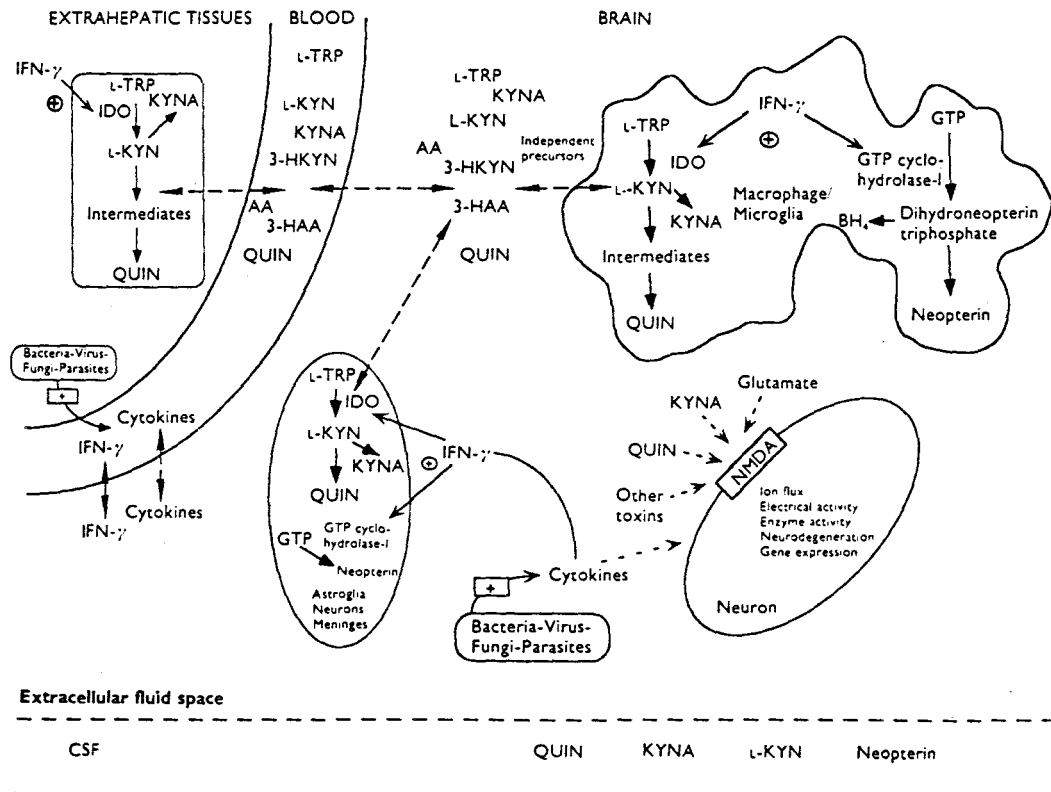


Figure 1.3. A schematic representation of tryptophan catabolism in inflammatory neurological diseases in the brain with the consequent production of kynurenic acid and quinolinic acid. Macrophages play an important role in the cerebral synthesis of kynurenes by inducing enzymes responsible for their synthesis. The following abbreviations are used: **3-HAA**: 3-hydroxyanthranilic acid, **IFN**: interferon, **QUIN**: quinolinic acid, **KYNA**: kynurenic acid, **AA**:anthranilic acid, **3-HKYN**: 3-hydroxykynurenine and **GTP**: guanosine triphosphate, **IDO**: indoleamine 2,3-dioxygenase (Heyes, 1993).

N-methyl-D-aspartate receptor activation by QA is accompanied with the influx of calcium, and delayed calcium neurotoxicity (Rios *et al*, 1991). Lipid peroxidation is enhanced with a rise in calcium entry. It may be through this mechanism that QA induces lipid peroxidation.

1.1.1.1.3.10. *Quinolinic acid phosphoribosyltransferase (QPRT)*

Quinolinic acid phosphoribosyltransferase (QPRT), is a degradative enzyme which catalyses

the condensation of QA and phosphoribosyl-pyrophosphate as well as the decarboxylation of the intermediate conjugate to nicotinic acid mononucleotide (NAD) (Stone, 1993a; Schwarcz *et al*, 1993). Quinolinic acid phosphoribosyltransferase exists as several isoforms in liver, kidney and brain and is inhibited by analogues of QA (Stone, 1993a). The enzyme is found to be in the synaptosomal fraction which suggests that QA plays a potential neuromodulatory role in the extracellular space by acting at the NMDA-sensitive glutamate receptors. V_{\max} of 3-HAO is 100 times higher than QPRT suggesting that QPRT is the rate-limiting enzyme in the formation of cerebral QA. The lack of association between the glial cells, containing 3-HAO and QPRT, shows that QA may be catabolised in cells other than those responsible for its production and therefore QA must enter the extracellular space and migrate between the two (Stone, 1993a). Quinolinic acid phosphoribosyltransferase is induced in pathological conditions, but because of its low basal activity, it is not significantly altered (Schwarcz, 1993).

1.1.1.2. SEROTONERGIC PATHWAY OF TRYPTOPHAN METABOLISM

1.1.1.2.1. PINEAL GLAND

The pineal gland was initially proposed as the “seat of the soul” by Descartes (1596-1650) and especially the seat of imagination and consciousness (Daya, 1994; Kappers, 1976). Interest in the pineal gland revived in the nineteenth century along with the development of comparative anatomy (Kappers, 1976). The pineal gland is now established as an integral component of the neuroendocrine system since it has been functionally related to virtually

every endocrine gland in the organism (Klein, 1978; Reiter, 1989; Daya, 1994). The pineal gland is highly active and light:dark sensitive (Reiter, 1989). The human pineal gland is attached to the posterodorsal aspect of the diencephalon and occupies a depression between the superior colliculi of the mesencephalon. Proximally, the pineal gland is closely associated with the third cerebral ventricle, its pineal recess in fact contains the proximal portion of the pineal gland (Reiter, 1989). The recess lies between the two laminae that form the stalk or peduncle by which the pineal is attached to the diencephalon. The habenulae commissure is present in the inferior lamina (Reiter, 1989). The pineal gland weighs about 100-150 milligrams and the chief cellular component of the pineal gland is the pinealocyte (pineal parenchyma cells) (Young *et al*, 1982). The pinealocytes have several processes that terminate near the capillary and lie adjacent to the nerve endings in the perivascular spaces. The capillary network within the pineal gland is very dense. The innervation of the pineal gland is important for endocrine processes or activity (Reiter, 1989).

1.1.1.2.2. INNERVATION

The ability of the pineal gland to convert a nervous input into a hormonal output characterises the gland as a neuroendocrine transducer. Pineal innervation comes from the autonomic nervous system as well as the parasympathetic nervous system.

1.1.1.2.2.1. Sympathetic

The starting point is light or darkness falling onto the retina (Young *et al* , 1982). The sensory information perceived is essential for the synthesis of various indoleamines such as melatonin (Reiter, 1989). Light:dark cycle, light intensity and light wavelength is a determinant in the ability of the gland to produce light-sensitive indoleamines (Reiter, 1989). After transduction into a neural signal in the photoreceptors of the retinas, the message is sent to the hypothalamus via the ganglionic cell axons which form the retinohypothalamic tract. At the level of the optic chiasma these fibres diverge from the classic optic system and terminate in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus (Reiter, 1989).

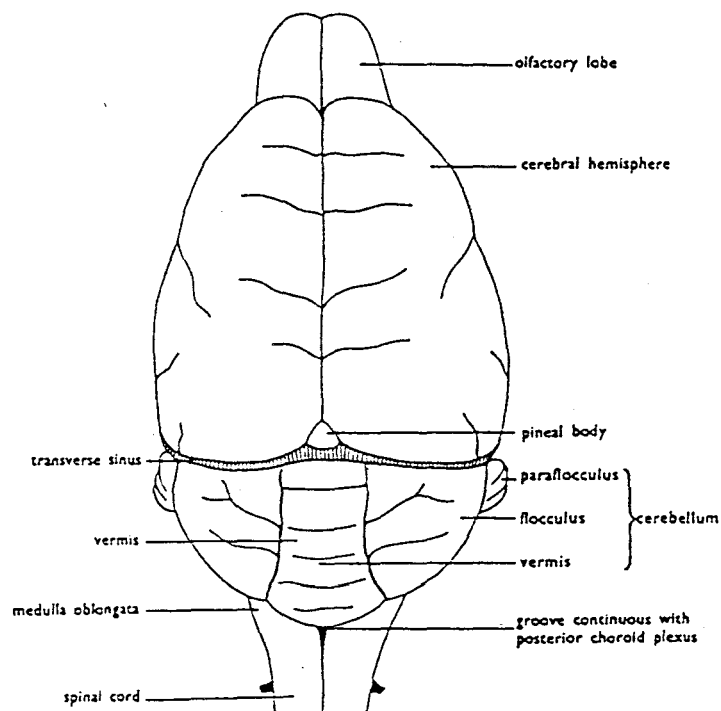


Figure 1.4. Diagrammatic representation of the pineal gland complex in the rat brain (Daya, 1982).

After the synapse in this location, fibres project to the paraventricular nuclei of the hypothalamus. Long descending fibres then supposedly carry the neural message to the intermediolateral cell column of the upper thoracic spinal cord (Reiter, 1989). Preganglionic sympathetic fibres leave the CNS and synapse with the postganglionic sympathetic fibres in the superior cervical ganglion (Young *et al*, 1982). The postganglionic nerve fibres pass along the vasculature and *nervi conarii* back into the head and among other organs and terminate in the pericapillary spaces of the pinealocytes (Young *et al*, 1982). The synaptic neural connections between the SCN and the pineal gland must remain intact in order for the pineal to function in its normal capacity, that is, if any part of the pathway is interrupted, the nervous input into the pineal gland is blocked and indoleamines, which rely on sensory input, are disrupted (Young *et al*, 1982). Postganglionic synaptic fibres release nor-adrenaline (NA) which binds to specific β -adrenergic receptors on the pinealocyte and activates adenylate cyclase to induce rapid synthesis of the enzyme N-acetyltransferase (NAT) (Young *et al*, 1982).

1.1.1.2.2. *Parasympathetic*

Preganglionic fibres arise in the superior salivatory nucleus in the medulla oblongata and leave the brainstem with the facial nerve. These run with the greater petrosal nerve and reach the pineal gland together with the sympathetic fibres in the *nervi conarii*, synapsing with ganglionic neurones along the nerves or within the pineal. The purpose of this innervation of the human pineal gland still remains to be deduced (Young *et al*, 1982).

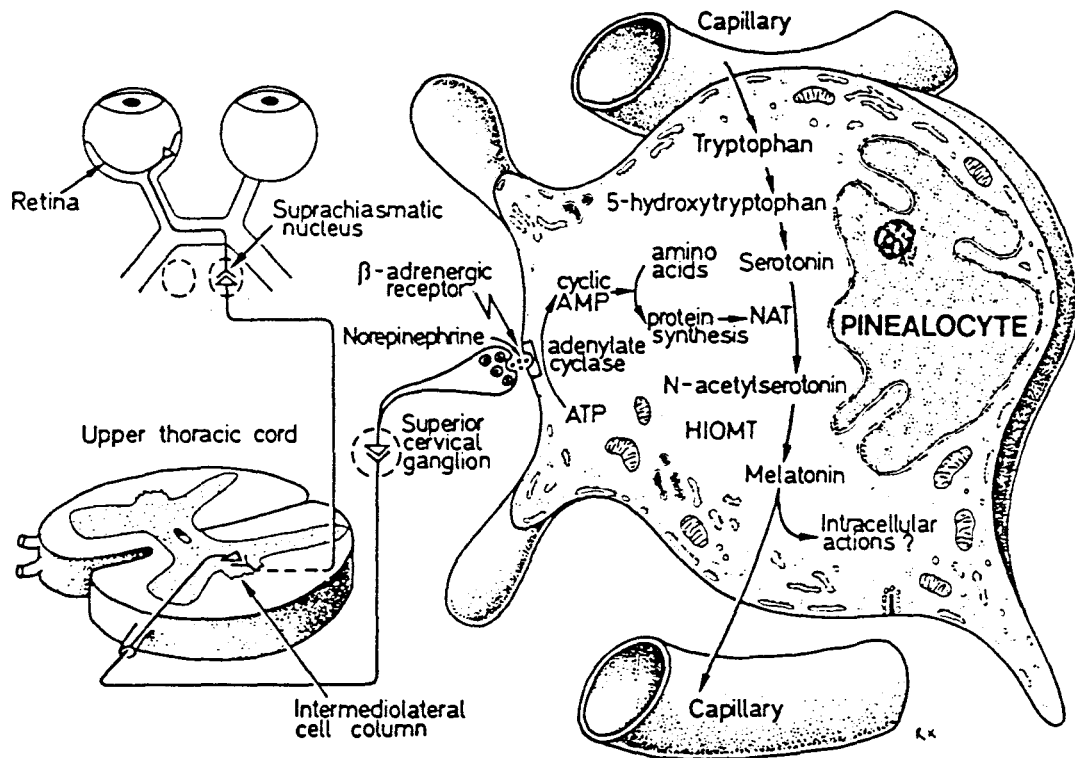


Figure 1.5. Schematic representation of the sensory input pathway and the effect of light and dark on the synthesis of the various indoleamines in the pineal gland (Reiter, 1989).

1.1.1.2.3. INDOLEAMINE BIOSYNTHESIS

Production of melatonin within the pinealocyte requires the uptake of the amino acid TRP from circulation (Klein, 1978; Reiter, 1989). Tryptophan within the blood is derived from 2 sources, *viz.* ingested protein and “free” TRP pools located in a variety of tissues. Production of the methoxyindoles may be influenced by the availability of TRP (Reiter, 1989). The ability of the pineal gland to produce large quantities of methoxyindoles is related, at least partially, to the high level of tryptophan hydroxylase enzyme within the gland (Reiter, 1981).

The uptake of TRP into the pinealocyte involves an uptake mechanism which occurs against a concentration gradient (Reiter, 1991a).

1.1.1.2.3.1. *Tryptophan hydroxylase (TH) and 5-hydroxytryptophan (5-HTP)*

Tryptophan hydroxylase (TH), is the rate-limiting enzyme in serotonin synthesis (Reiter, 1981). Tryptophan is oxidised at the fifth position to form the amino acid 5-hydroxytryptophan (5-HTP) (Reiter, 1989). This conversion depends on the activity of the enzyme TH and requires the presence of oxygen, ferrous ions and a reduced pteridine cofactor (Reiter, 1989; Reiter, 1991b). The enzyme activity is increased in darkness (Reiter, 1991a). A high substrate affinity for TH with respect to levels of TRP in the pineal gland may indicate that the enzyme is usually unsaturated and thus the rate of enzyme activity is dependent on TRP availability. Tryptophan hydroxylase is inhibited by phenylalanine and p-chlorophenylalanine (PCPA) (Reiter, 1989). The latter reduces the reserves of serotonin in the pineal gland (Reiter, 1991a).

1.1.1.2.3.2. *L-Aromatic amino acid decarboxylase*

5-Hydroxytryptophan is then converted to 5-hydroxytryptamine or serotonin (5-HT) or serotonin in the presence of the enzyme L-aromatic amino acid decarboxylase, also known as dopa decarboxylase or 5-hydroxytryptophan decarboxylase (Reiter, 1981; Reiter, 1991a; Reiter, 1989). This widely distributed enzyme is pyridoxine dependent. The enzyme activity

is highest in the pineal gland and remains stable throughout the light:dark cycle of the rat or Syrian hamster (Reiter, 1989; Reiter, 1991a). Accumulation of 5-HTP is more rapid after the enzyme is inhibited (benzeraside) during the day than at night (Reiter, 1989; 1991a).

1.1.1.2.3.3. Serotonin (5-HT)

The serotonin concentration is very high in the pineal gland and levels exceed any other organ in the body (Reiter, 1989; Reiter, 1991a; Reiter, 1981). Serotonin exhibits a 24 hour rhythm in every mammal studied. The circadian rhythm in pineal 5-HT relates to cyclic production and metabolism. Serotonin levels are always highest during light and decrease at the onset of darkness which is coincidental with the rise in aMT production (Reiter, 1991a). Extending the onset of darkness retards the decrease in 5-HT concentration (Reiter, 1991a). This may be due to the fact that increased NAT activity in the light phase is prevented. If animals such as rats are placed in a reversed light:dark cycle, 5-HT resumes the new photoperiodic regimen in about 6 days (Reiter, 1989). Thirty percent of the total 5-HT stored in the rat pineal is located in the sympathetic nerve endings; only the pinealocytes have the ability to produce 5-HT which is taken up into the adjoining nerve terminals. Serotonin is a precursor for biologically active compounds such as aMT and 5-methoxytryptophol (Reiter, 1981). Serotonin may also be converted to 5-methoxytryptamine (5-MT) by hydroxyindole-*O*-methyltransferase (HIOMT). This indole has been promoted as a pineal hormone but its activity is only one tenth of aMT in terms of its effect on the reproductive system (Reiter, 1989)

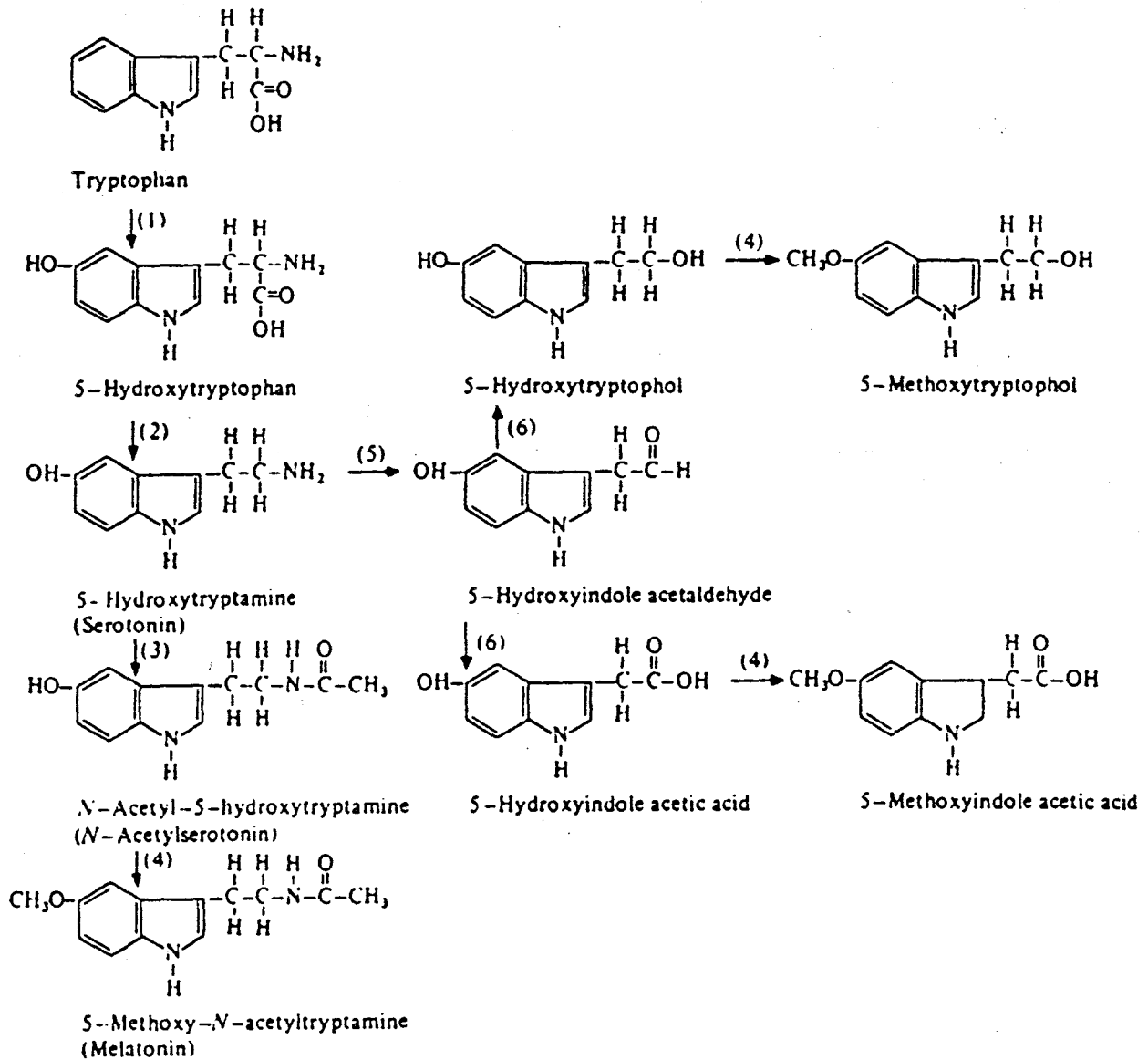


Figure 1.6. Tryptophan metabolism in the mammalian pineal gland. The numbers represent: 1: Tryptophan hydroxylase, 2: Aromatic amino acid decarboxylase, 3: N-Acetyltransferase, 4: Hydroxyindole-*O*-methyltransferase, 5: Monoamine oxidase, 6: 5-Hydroxyindole acetaldehyde (unstable intermediate) and is either oxidised to 5-hydroxyindoleacetic acid or reduced to 5-hydroxytryptophol (Reiter, 1981).

Serotonin, a precursor to several indoles, may be acted upon by several enzymes and its pineal concentration at night may decrease due to the following 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). The availability of 5-HT for these pathways depends on the granular 5-HT in equilibrium with “free” 5-HT. It is in the latter state which 5-HT is broken down or metabolised (Cardinali, 1981).

1.1.1.2.3.4. *Monoamine oxidase (MAO), 5-hydroxyindoleacetic acid (5-HIAA) and 5-hydroxytryptophol (5-HTOH)*

Serotonin is metabolised by MAO to 5-hydroxyindoleacetaldehyde, an intermediate that is either oxidised to 5-hydroxyindoleacetic acid (5-HIAA) or reduced to 5-hydroxytryptophol (5-HTOH). There are two types of MAO's: type A and type B of which one is confined to the pineal gland and the other to the sympathetic nerve terminals within the gland (Reiter, 1989). Type A enzyme activity is highly dependent upon intact sympathetic nerves and decreases sharply after superior cervical ganglionectomy, whereas type B remains unaltered (Cardinali, 1981).

1.1.1.2.3.5. *5-Methoxyindoleacetic acid (5-MIAA) and 5-methoxytryptophol (5-MTOH)*

5-Hydroxyindoleacetic acid and 5-HTOH are substrates for HIOMT which are then metabolised to 5-methoxyindoleacetic acid (5-MIAA) or reduced to 5-methoxytryptophol (5-MTOH) respectively (Reiter, 1989). The latter has been shown to exhibit antigonadotrophic

effects in that it delays the onset of puberty and in certain circumstances acting as aMT in stimulating gonadal development (Young *et al*, 1982). 5-Methoxytryptophol in the blood and the pineal gland exhibits a rhythm similar to aMT and is shown to possess endothermic properties (Reiter, 1989; Reiter, 1981). 5-Methoxytryptamine also acts a gonadotrophic hormone but less potent than aMT and 5-MTOH. It is able to inhibit light-induced oestrous in the ferret but more importantly it may disrupt conditional responses and general behaviour by exerting its effects on 5-HT receptors (Young *et al*, 1982).

1.1.1.2.3.6. *N-Acetyltransferase (NAT) and N-acetylserotonin (NAS)*

Serotonin conversion to aMT is a major pathway and involves two steps. The primary step is the N-acetylation of 5-HT by the enzyme NAT to yield N-acetylserotonin (NAS) (Reiter, 1991a). The acetyl group is provided by acetyl co-enzyme A hydrolase (Reiter, 1989). Two forms of NAT are present in the pineal gland, one of which is highly specific for arylalkylamines and exhibits a 24 hour rhythm with the highest activity at night. The activity of NAT varies between species as well as in the magnitude of the rise, but most importantly, it is responsible for the metabolism of 5-HT and aMT. The other form shows weak activity towards arylamines (aromatic compounds with amines on the ring) and remains stable over a 24 hour period. It supposedly acts as a detoxifying agent by acetylating amines (Reiter, 1981; Reiter, 1991b; Reiter, 1991a). The absence of a NAT cycle is associated with a deficient rhythm (Reiter, 1991a). The day:night difference results from neural stimulation of the pineal gland by the SCN. The mechanism of adrenergic cyclic adenosine monophosphate (cAMP)

Cyclic adenosine monophosphate then activates cAMP-dependent protein-kinase and transcription of mRNA which results in the eventual rise in NAT levels. Cyclic adenosine monophosphate is also able to prevent NAT degradation (Reiter, 1991b). N-acetylserotonin concentrations increase with increased NAT activity at the onset of darkness. The concentration of NAS in the blood follows a cycle different to that of aMT and when compared to aMT, NAS exhibits a different localisation in the brain where it presumably binds to both type I and type II 5-HT receptors (Reiter, 1991a).

1.1.1.2.3.7. *Hydroxyindole-O-methyltransferase (HIOMT)*

A methoxy group is transferred from S-adenosylmethionine (SAM) to the 5-hydroxy position of NAS yielding aMT. This conversion is catalysed by HIOMT (Reiter, 1989). Hydroxyindole-*O*-methyltransferase is a cytosolic enzyme which catalyses the *O*-methylation of 5-hydroxyindoles by the methyl donor SAM. The enzyme 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, 1991a). The enzyme does not exhibit day:night variations (Reiter 1991a; Reiter, 1981; Reiter, 1991b). 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, 1991b). The preferred substrate for HIOMT is considered to be NAS. At night as NAS levels increase, it is rapidly metabolised to aMT in the pineal gland (Reiter, 1991a). At present there are no specific drugs known to inhibit HIOMT (Reiter, 1989).

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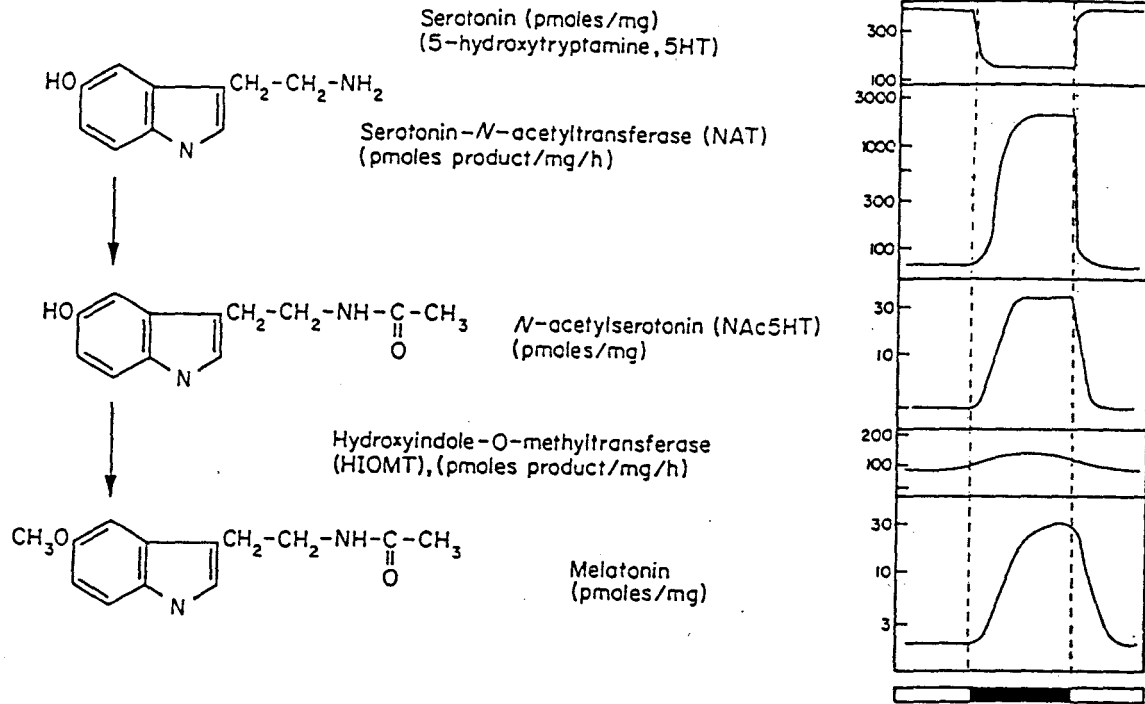


Figure 1.8. Profiles of the various constituents of tryptophan metabolism in the pineal gland over a 24 hour period. Abbreviations used: **NE**: norepinephrine, **5-HT**: serotonin, **NAT**: N-acetyltransferase, **N-Ac5-HT (NAS)**: N-acetylserotonin, **HIOMT**: hydroxyindole-*O*-methyltransferase (Arendt, 1988).

1.1.1.2.3.8. Melatonin (aMT)

Hydroxyindole-*O*-methyltransferase catalyses the conversion of NAS to aMT, a principal hormone of the pineal gland (Daya, 1994; Reiter, 1981; Cardinali, 1981). The nocturnal patterns of aMT production in the pineal gland vary between species and its significance is not clear, although it is thought that the variation is related to the ability of the pineal gland to

influence reproduction. Melatonin is not stored at any appreciable quantity and shortly after its synthesis the indoleamine is released primarily into the blood vascular system and into other fluids such as the cerebrospinal fluid (CSF) or follicular fluid by simple diffusion (Reiter, 1981; Cardinali, 1981; Reiter, 1991c). Cerebrospinal fluid and blood levels of this compound are closely related to the pineal gland levels, with the highest concentration being during the night. Following its release into the blood, aMT is bound to plasma albumin, the indole is then rapidly broken down or hydroxylated and conjugated with sulfate (70-80%) and glucuronide (5%) by hepatic microsomes. These metabolites are then excreted in the urine (Reiter, 1981; Kopin *et al*, 1961; Waldhauser *et al*, 1993).

Melatonin is a chief secretory product of the pineal gland and is responsible for conveying information to organs in the body in various species with regard to the time of day and year (Reiter, 1991a; Reiter, 1991c). Which aspect of the aMT rhythm is responsible for conveying this information remains disputed. Three theories have been postulated: (1) duration hypothesis - seasonal fluctuations in day length alter the duration of the elevated aMT and adjust the organism's physiology, especially reproduction, on an annual basis, (2) internal and external coincidence - the possibility that two rhythms must converge before any physiological consequences occur, elevated levels of aMT coincide with an increase in sensitivity of its own receptors under the conditions determined by the internal environment of the organism, and finally (3) amplitude hypothesis - the amplitude of the nocturnal rise may determine the functional importance of aMT (Reiter, 1991b). The magnitude of the nocturnal rise of aMT is dependent on the species. At middarkness, aMT levels rise rapidly for a duration of 1-2

hours followed by daytime levels before the onset of light. This is found both in rats and humans, the latter demonstrated by levels in plasma (Reiter, 1991a). Melatonin synthesis is not restricted to the pineal gland and may be found in the retina with its function being to regulate the pigment migration in the cell of this organ. Studies have shown that aMT in the brain is metabolised to N-acetyl-5-methoxy-kynurenamine via N-acetyl-N-formyl-5-methoxykynurenamine rather than the 6-hydroxy pathway (6-hydroxymelatonin) in the periphery (Cardinali, 1981). Environmental lighting acting through the eye of adult mammals and in part acting on the pineal of lower vertebrates has profound effects on the rhythms of aMT synthesis. Any manipulation causing a major activation in the sympathetic nervous system may override the inhibiting effect of light and stimulate aMT synthesis (Cardinali, 1981).

Melatonin is defined as a biochemical messenger of darkness since it is secreted by the pineal gland at night upon postsynaptic activation of β -adrenergic receptors (Maestroni, 1993). Melatonin has received much attention with regard to psychiatry and has been implicated in stress, depression, appetite, sleep, ageing, tumour inhibition and immune regulation.

Melatonin and stress

Studies have shown that the pineal gland is able to control, coordinate and regulate physiological adaptation to stress. Glucocorticoids which are released from the adrenal cortex regulate the hypothalamic pituitary-adrenal axis by interacting with the receptors in the brain and pituitary axis. The release of the adrenocorticotrophic hormone is prevented and the

negative feedback system is activated; its sensitivity is decreased due to a high level of glucocorticoids. A high level of the hormone results in the loss of receptors which are predominant in the hippocampus (Daya, 1994). Neuronal damage caused by high levels of glucocorticoids has the following consequences: (1) inhibition of ion uptake and utilisation of glucose in the hippocampus, (2) exacerbation of neurotoxins, and (3) reduction in the uptake of glutamate by glial cells which then results in the overstimulation of the NMDA receptor which in turn has the following consequences: (a) an influx of sodium, chloride and water which causes the lysis of the cell, and (b) an increase in the calcium influx through the calcium channel resulting in calcium overload in the neuron and thus death of the neuron (Daya, 1994).

Melatonin has shown promise to reduce the affinity of the ligands for the corticosterone receptors as well as glutamate affinity for the NMDA receptors (Daya, 1994).

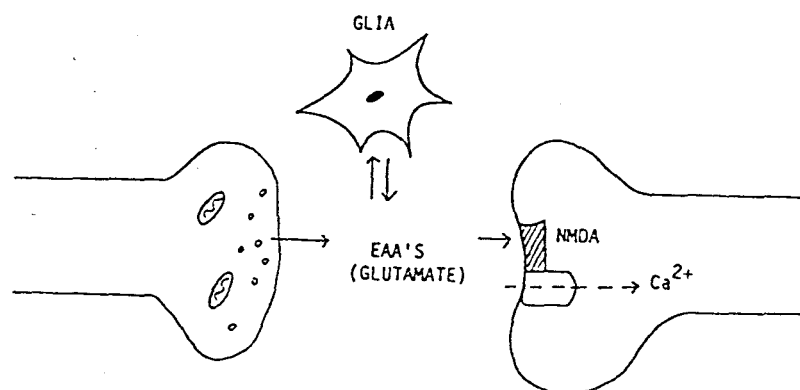


Figure 1.9. Calcium entry due to stress induction by the excitatory amino acid, glutamate (Daya, 1994).

Melatonin and depression

Seasonal moods with a strong annual constituent are known as seasonal affective disorders (SAD's). This disorder is characterised by a reoccurring depression in winter. Treatment, which reduces symptoms of SAD's such as hypersomnia and overeating, involves light therapy using artificial lighting at 2500 lux. Tryptophan, which serves as a precursor for 5-HT synthesis, is taken up by the brain since low levels of 5-HT have been shown to be indicative of depressive disorders. Changes which influence TRP levels are responsible for changes in 5-HT production. When aMT is administered orally in conjunction with the light therapy, the symptoms of SAD's partially reoccurred (Reiter, 1989). Effective antidepressants act by inhibiting MAO in the synapse, increasing 5-HT and consequently aMT production (Daya, 1994). Melatonin has also been found to inhibit the activity of the enzyme responsible for TRP catabolism (TDO) and hence increases 5-HT levels in the brain (Walsh *et al*, 1991; 1994).

Melatonin and sleep

Melatonin is able to increase the percentage time spent in rapid eye movement (REM) sleep and decrease the latency of REM onset. This may be due to the release of arginine vasotocin, however, this theory is not widely accepted (Reiter, 1989).

Melatonin and ageing

Ageing is associated with broken, fragmented sleeping patterns (Daya, 1994). Melatonin secretion is age-dependent and the nocturnal aMT serum shows a steep decline from early infancy to puberty followed by a moderate decline in old age. The theory put forward explaining this is that the the body size increases about 500 to 800% but the pineal, pineal HIOMT activity and aMT production alters only slightly. Suggestions have been made that the hormone is produced at a constant rate with respect to the increased volume of distribution after puberty (Waldhauser *et al*, 1993). Figure 1.10 shows that at puberty the aMT day:night rhythm is large, thereafter the decrease could be as a result of age. Adulthood shows a decline so that by old age, very low levels of aMT are produced (Reiter, 1989).

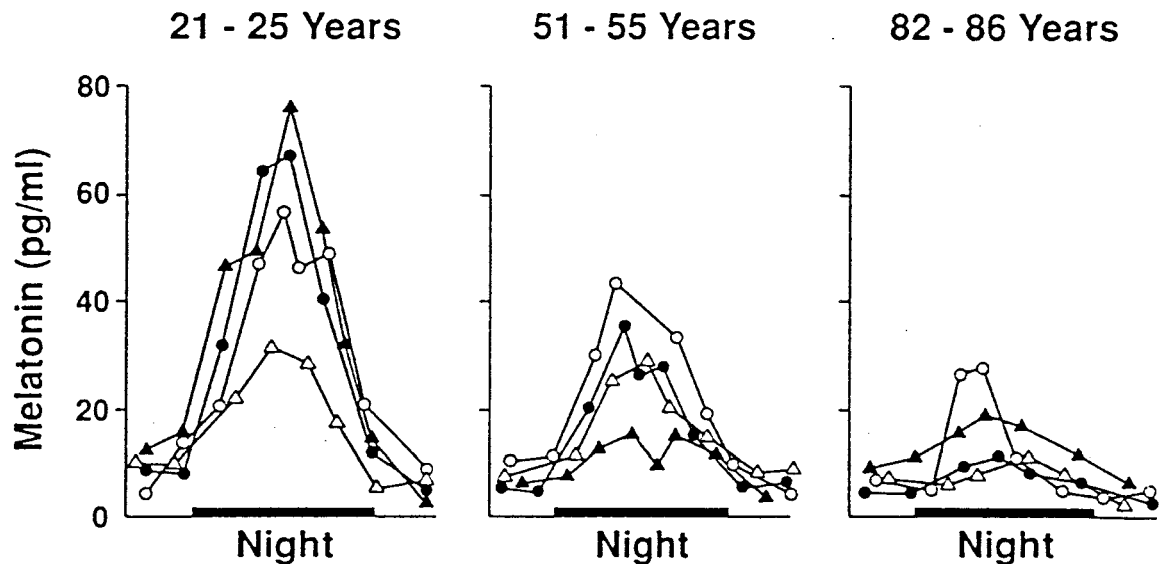


Figure 1.10. Day and night blood levels of melatonin in three different age groups. The amplitude of the melatonin rhythm decreases together with the duration of the melatonin peak as humans age (Reiter, 1995).

Amplitude of the cycle may be responsible for : (a) the stability of the cycle, (loss of aMT leads to disturbances in the circadian rhythm), (b) determination the age of the organism, and c) acting as an ageing clock (Reiter, 1995). Recently it has been put forward that free radicals accumulate within the cell and give rise to the death of the cell. Melatonin is a potent free radical scavenger whose concentration decreases as the brain ages (Reiter, 1995).

Melatonin and cancer

A vigorous aMT rhythm may prevent infections and malignant proliferation (Maestroni, 1993; Short, 1993). Patients who die of breast carcinoma have been found to have enlarged pineal glands. Decreased aMT secretions may be a predisposing factor for the development of tumours (estrogen secretion is not changed when aMT levels are decreased). Studies in rats have shown that aMT injections, timed accurately, have anti-tumour activity especially in mammary tumours. In humans, studies using aMT as a therapeutic agent are incomplete (Reiter, 1989).

Melatonin and immune regulation

Melatonin has shown activity as an antineoplastic and immunoenhancing agent. However, its activity on tumour growth and the immune system still needs to be analysed to determine whether it is seasonally dependent, as in the case of reproduction (Maestroni, 1993). Immunological studies have shown that aMT has a potential benefit for asymptomatic HIV⁺

individuals. It is possible that aMT has the same targets for CD4⁺ and T-lymphocytes (Maestroni, 1993; Waldhauser *et al*, 1993). The virus recognised by the immune system will produce an activation of immunocompetent cells which, in turn, activate the aMT response for that specific photoperiod together with other endocrine responses sensitive to aMT (Maestroni, 1993).

1.1.1.3. 6-METHOXY-2-BENZOXAZOLINONE (6-MBOA)

6-Methoxy-2-benzoxazolinone (6-MBOA) is a naturally occurring compound which is present in grasses such as winterwheat in the early spring. The seedlings are consumed by a number of rodent species and 6-MBOA is then responsible for the sexual maturation of these animals. The animal's diet, rather than the photoperiod, is responsible for the timing of the seasonal period for breeding. The structural similarity between aMT and 6-MBOA led researchers to deduce that 6-MBOA may influence progonadal effects by altering pineal function (Daya, 1989).

Thus, so far, 6-MBOA has shown to be able to induce NAT activity and aMT synthesis with a concomitant rise in cAMP levels at high concentrations (10^{-3} M). However, no effect was shown on the activity of HIOMT (Daya *et al*, 1990; Daya, 1989; Daya *et al*, 1993). 6-Methoxy-2-benzoxazolinone has also been shown to displace specific radioligands from α - and β - receptors in the pineal gland at a concentration of 10^{-4} M, at this concentration 6-MBOA is also able to stimulate aMT synthesis (Daya, 1989; Daya *et al*, 1990).

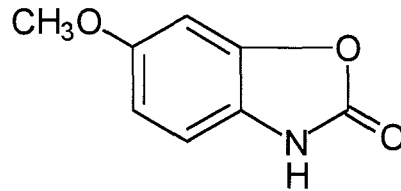


Figure 1.11. Structure of 6-methoxy-2-benzoxazolinone (6-MBOA), a structural analogue of melatonin.

6-Methoxy-2-benzoxazolinone may act as an antagonist of aMT receptors causing the desensitisation of its receptors.

1.2. TRYPTOPHAN 2,3-DIOXYGENASE-RELATED DEPRESSION

There is considerable evidence suggesting that biogenic amines are disturbed in depressive illness, even though neurotransmitters and biogenic amines are mutually regulated. Evidence has shown that serotonin may play a role in the mood changes which characterise this illness (Badawy *et al*, 1981).

Specific indication of the presence of biochemical abnormalities is provided by monoamine oxidase inhibitors (MAOI) or tricyclic antidepressants which provide therapeutic effectiveness by influencing MAO metabolism (Curzon *et al*, 1970).

Amines which are derived from TRP, such as 5-HT, have been found to play a role in the regulation of mood with depressed 5-HT levels associated with suicides (Curzon *et al*, 1970). Serotonin, as already mentioned, is formed *in vivo* from TRP by a minor pathway. Quantitatively, a more important route of TRP metabolism occurs through the kynurenine pathway which starts with the oxidation of TRP by liver TDO. Elevated cortisol levels were found to occur in depression, therefore the possibility arises that high cortisol secretion levels found in depressed patients may correlate to a link between increased adrenocortical activity and abnormal 5-HT metabolism. Antidepressants are able to decrease TDO activity whereas increased levels of this enzyme are induced by adrenocortical hormones. The raised plasma levels of cortisol induce TDO activity, generating the formation of metabolites through the kynurenine pathway and hence, diverting tryptophan from 5-HT synthesis (Curzon *et al*, 1970). Antidepressants prevent the conjugation of the apoenzyme (TDO) with its cofactor haem, which is essential for the activity of the enzyme (Badawy *et al*, 1981).

It seems that a variety of neural and behavioural functions are influenced by physiological changes in plasma tryptophan levels (Fernstrom *et al*, 1971). Subsequently, studies have shown that TRP, a precursor to neurotransmitters in the brain, may be affected by antidepressant drugs and peripheral factors (Badawy *et al*, 1981). Brain TRP may be lowered by the following factors: (1) increased TDO activity; this may occur as a result of hormonal and substrate induction, leading to decreased plasma TRP which is chronic rather than acute (Hillier *et al*, 1975; Badawy *et al*, 1981), (2) limited release of protein bound serum (plasma TRP) (the ratio of total plasma TRP to other LNAA's), the lower the ratio of TRP:LNAA the

lower the brain levels of TRP and thus its metabolite 5-HT (Wurtman, 1982; Lucca *et al*, 1994; Badawy *et al*, 1981), and finally (3) an increased plasma neutral amino acid levels which compete with TRP for the cerebral uptake mechanism (Badawy *et al*, 1981). These factors show an inverse relationship between activity of TDO and serotonin synthesis and therefore TDO is capable of affecting circulating TRP (Badawy *et al*, 1981).

There are several factors which have been noted in the regulation of the cerebral synthesis of 5-hydroxyindoles at various levels of TRP loading: (1) cerebral uptake of TRP, (2) substrate inhibition of tryptophan-5-hydroxylase (the rate-limiting enzyme responsible for the synthesis of 5-HT), (3) competitive uptake of KYN (it has been reported that KYN interferes with TRP transport for cerebral uptake, and it is essential that the ratio of TRP:KYN is 50:1 to interfere with KYN uptake to the brain), (4) cerebral synthesis of KYN, and (5) efflux rates of TRP and its metabolites (Gal *et al*, 1978b).

Thus, TRP seems to be the common denominator in the biochemistry of abnormalities and neurological information (Litman *et al*, 1985). The conversion of TRP into 5-HT may be influenced by the carbohydrate portion in the diet; a larger carbohydrate portion results in an increase in the plasma ratio of TRP and therefore, synthesis of 5-HT. In turn, 5-HT influences the diet which an individual chooses to eat, suggesting that 5-HT is able to affect neurons that control mood, appetite and sleep (Wurtman, 1982; Wurtman *et al*, 1974).

Suggestions have been made to regulate the decrease in 5-HT synthesis by administering the

amino acid, TRP, when TDO activity is at its lowest. Also, inhibitors such as antidepressants may have a direct therapeutic effect by providing an increase in the availability of plasma TRP by modulating TDO activity (Curzon, 1969).

1.3. LIPID PEROXIDATION

Living or biological systems require molecular oxygen in order to survive and therefore depend heavily on its availability. Its high oxidising potential and ability to form non-toxic agents upon final reduction makes oxygen (O_2) very important in the metabolism of many organisms. Oxygen, although indispensable, has the potential to be poisonous at a slightly higher concentration than in air (Buechter, 1988). Gerschman *et al* reported in 1954 that the toxicity is mediated by high concentrations of the partially reduced form of the oxygen. 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 *et al*, 1984). These species are collectively referred to as reactive oxygen species (ROS) and include the following: superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^{\cdot}) (Green *et al*, 1984).

Oxygen reductions may be mediated by metabolic pathways (Cytochrome P450), electromagnetic radicals or exogenous compounds. Enzymes that are involved in cellular redox reactions, such as superoxide dismutase (SOD), usually do not allow dissociation of intermediates from itself into free solution.

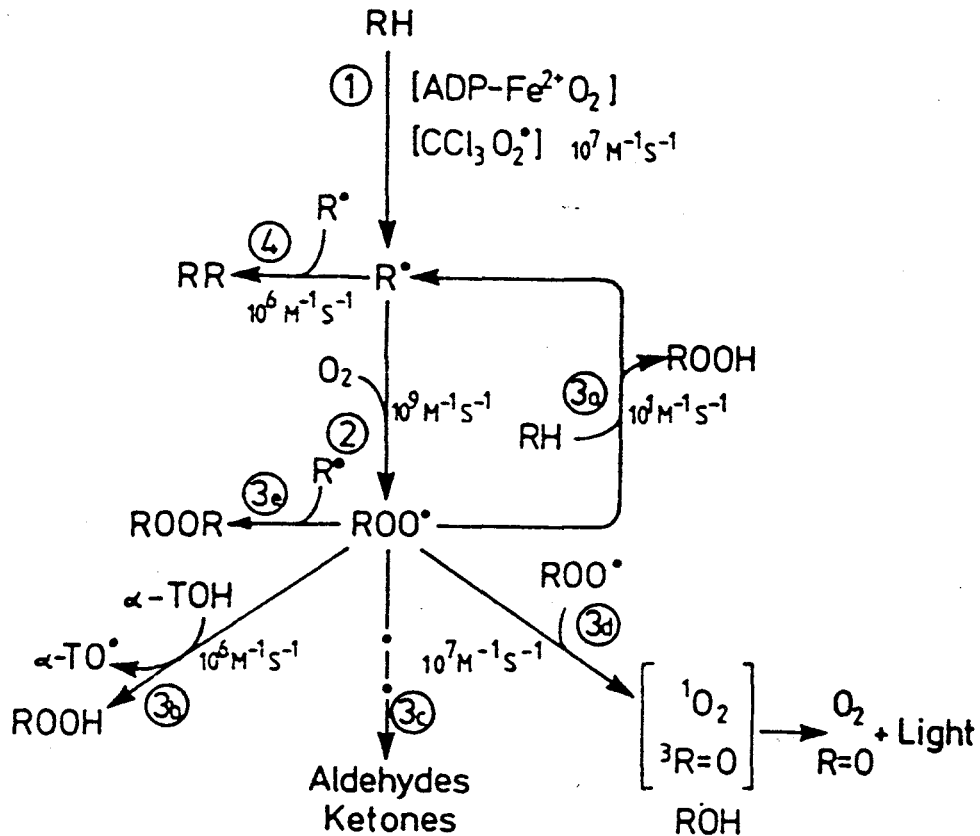


Figure 1.12. A flow chart representing lipid peroxidation initiated by free radicals. Abbreviations: **RH**- unsaturated fatty acid, **R•** - carbon-centered lipid radical, **ROO•** - lipid peroxy radical. Reaction 3b: represents the reaction of Vitamin E (Murphy *et al*, 1989).

There are many cases whereby the reactive species may “leak” into solution and the cellular contents are then exposed to these active oxygen species (Beuchter, 1988). These processes may lead to extensive cellular and membrane damage. Organisms have evolved to deal with reactive species and convert these intermediates into less reactive species using enzyme systems such as SOD, catalase and glutathione peroxidase (GSH-PX). Cellular antioxidants such as Vitamins C and E, and melatonin are found *in vivo*; the latter was only recently recognised for

its potent ability to reduce the ravages of the ROS (Beuchter, 1988).

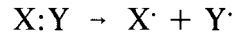
1.3.1. REDUCTION OF MOLECULAR OXYGEN (O₂)

The ground state of oxygen is a triplet rather than a singlet state. This state is paramagnetic and contains two unpaired electrons with parallel spins. Oxygen has a great oxidising potential but a divalent reduction is difficult since an insertion of a pair of electrons would result in two electrons of the same spin occupying the same orbital which violates Pauli's Exclusion Principle. Therefore if oxygen was to oxidise another molecule, the electron pair would have to be of antiparallel spin, this cannot occur if both electrons occupy the same orbital. The spin restriction is removed when: (1) the oxygen is excited to a state known as a singlet oxygen and one of the unpaired electrons moves to a higher orbital and so inverts its spin, (2) the restriction is removed enzymatically by complexing O₂ with transition metals (which have unpaired electrons) and consequently reducing oxygen, and (3) most biological reactions occur with the transfer of a single electron at a time (Beuchter, 1988; Fridovich, 1970).

1.3.1.1. NON-ENZYMATIC LIPID PEROXIDATION

Free radicals (species that possess an unpaired electron or a fragment of a molecule) may be formed in three different ways (Cheeseman *et al*, 1993):

a) haemolytic cleavage of a covalent bond, whereby each fragment retains one of the paired electrons,



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).

A free radical ($R\cdot$) has enough energy to extract a hydrogen (H) atom from a methylene carbon of an unsaturated fatty acid (LH) and initiate a chain reaction in the bulk lipid:



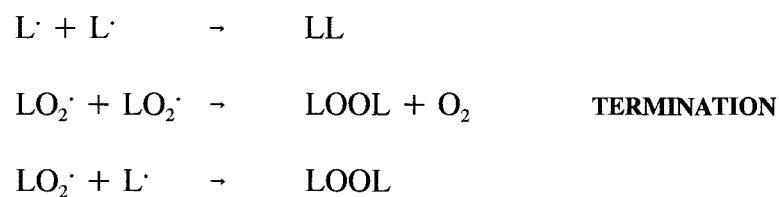
The $L\cdot$ carbon centred radical then reacts with molecular oxygen to form a peroxy radical



which can also extract or remove an H atom from an unsaturated fatty acid, leaving a carbon centred radical and lipid hydroperoxide:



The free-radical chain propagates until free radicals destroy each other to terminate the process (Gutteridge, 1987).



Hydroperoxides and cyclised endoperoxides are formed as a consequence of a chain reaction, especially in the presence of transition metals. In non-enzymatic lipid peroxidation, the peroxy radicals last long enough to attack fatty acid molecules. These radicals may be arrested and/or scavenged by enzymes or antioxidants (Gutteridge, 1987).

1.3.1.2. ENZYMATIC LIPID PEROXIDATION

1.3.1.2.1. SUPEROXIDE ($O_2^{\cdot-}$)

In biological systems, the most important free radicals are the derivatives of oxygen. Reduction of O_2 may occur by radiolysis or photosensitized oxidations. A transfer of a single electron will produce the free radical anion, superoxide ($O_2^{\cdot-}$), generated by enzymatic or non-enzymatic reactions (Fridovich, 1987).



Superoxide production was conclusively demonstrated in a biological system with the oxidations of the xanthine oxidase system (Buechter, 1988). Auto-oxidation of many biological compounds such as catecholamines and ascorbate (vitamin C) have produced $O_2^{\cdot-}$. The superoxide anion radical is usually not considered to be directly responsible for the toxic effects of O_2 but indirectly due to its production of other reactive species. Superoxide anions are able to reduce transition metals and their complexes (Buechter, 1988).

The superoxide reaction with molecular oxygen may also give rise to oxygen in the singlet state:



although this reaction is not important in biological systems. The superoxide in an aqueous solution will accept a proton to form HO_2^\cdot (perhydroxyl radical). Superoxides may also act as weak oxidants and are able to reduce haem- Fe^{3+} , free ferric iron (Fe^{3+}) and iron chelated to ethylenediaminetetraacetic acid (EDTA). Iron-mediated formation of hydroxyl radicals occur by the Fenton reaction, sometimes called the superoxide-driven Fenton chemistry (if the metal is iron) (Buechter, 1988; Halliwell, 1987).

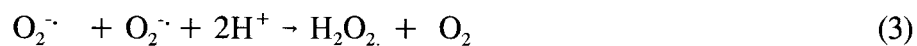
1.3.1.2.1.1. *IN VIVO* EFFECTS OF SUPEROXIDE

The superoxide exerts its effect directly *in vivo* by producing more potent oxidants (by protonation or by metal salt-catalysed interaction with H_2O_2) consequently increasing the selectivity of the damage (Fridovich, 1987). Increased levels of the superoxide may be due to the increased availability of O_2 or the decreased scavenging ability of the enzymes such as SOD, catalase and GSH-PX which are responsible for the defence against radical damage (Fridovich, 1987). The superoxide anion protonates to HO_2^\cdot with a pK_a of 4.8. Perhydroxyl radical is a stronger oxidant than O_2^- , and will directly attack polyunsaturated fatty acids (PUFA's). Protons localised in certain areas are concentrated by electrostatic forces and O_2 , when entering the microenvironment, will protonate to HO_2^\cdot thereby gaining in oxidising

potential. At physiological pH, O_2 is present at less than 1% in its protonated form (Cheeseman *et al*, 1993; Fridovich, 1987).

1.3.1.2.2. HYDROGEN PEROXIDE (H_2O_2)

Studies have shown that O_2 is indirectly damaging to biological systems of secondary reactive species. Hydrogen peroxide is not very reactive but its main significance is the provision of $OH\cdot$ radicals to the environment when in the presence of transition metals (Equation 7, page 57). Hydrogen peroxide is always present along with the superoxide anion radical because of the reaction indicated below:



Hydrogen peroxide is not a free radical, however, it has the status of a ROS in that it is a non-radical oxygen derivative that is involved in oxygen radical production (Cheeseman *et al*, 1993). Hydrogen peroxide is able to cross the biological membrane and because of its limited reactivity, H_2O_2 may diffuse a long distance from its site of production (Fisher, 1987).

1.3.1.2.2.1. *IN VIVO* EFFECTS OF HYDROGEN PEROXIDE

In lung mitochondria, H_2O_2 production increases five fold at 1 atmosphere pressure as the ambient concentration increases. Hydrogen peroxide is also produced with the electron oxidations of phenols, thiols and catecholamines (Turrens *et al*, 1982; Cohen *et al*, 1974).

1.3.1.2.3. HYDROXYL RADICALS (OH·)

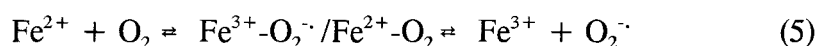
The generation of hydroxyl radicals may occur in the following ways:

i) *Iron catalysed generation of hydroxyl radicals:*

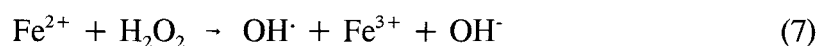
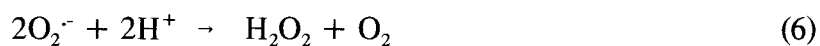
Haber and Weiss stated in 1934 that the following reaction known as the Haber-Weiss Reaction is able to produce hydroxyl radicals:



The OH· could also be part of the damage that has been attributed to O₂^{·-}. The reaction is very slow, but the addition of iron and chelated iron (which is present in biological fluids) promotes the rate of the reaction significantly. Iron ions are free radicals and may take part in electron transfer reactions with molecular oxygen.



The generation of O₂^{·-} by any source in the presence of iron can lead to formation of OH· by Fenton chemistry (Equations 4-7).

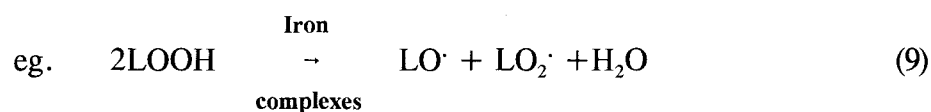


Studies have shown that OH[·] in solution is able to extract H and initiate lipid peroxidation (Gutteridge, 1987; Minotti *et al*, 1989).

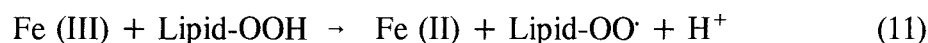
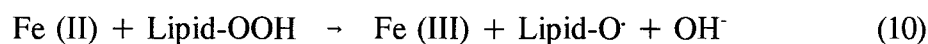


ii) Stimulation of peroxidation by iron complexes

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).



A proposed lipid peroxide decomposition mechanism catalysed by iron compounds may occur as follows (Adams Jr. *et al*, 1991):



a) Iron-Oxygen complexes

The intermediate resonance hybrid, Fe³⁺-O₂⁻/Fe²⁺-O₂ or perferryl ion (Equation 5, page 57)

has been widely implicated as the initiator of lipid peroxidation although no evidence has been found to substantiate this. The ferryl ion or FeO^{2+} is more reactive than the perferryl ion and has subsequently been noted as an alternative to the $\text{OH}\cdot$ in the Fenton chemistry (Gutteridge, 1987). Maximum stimulation of peroxidation occurs when $\text{Fe}^{2+}:\text{Fe}^{3+}$ complexes are in a ratio of 1:1 and Fe^{3+} may be reduced to Fe^{2+} by ascorbate to give the optimal ratio of 1:1 for initiation of lipid peroxidation (Andorn *et al*, 1996; Gutteridge, 1987; Halliwell, 1987; Aust, 1987). However, studies have shown that aluminium (III) and lead (II) can replace iron (III) as stimulators (Quinlan *et al*, 1988). This leaves the question as to whether or not iron is in fact necessary to stimulate lipid peroxidation (Aruoma *et al*, 1989).

b) Iron chelators

Ferric ions precipitate rapidly to form ferric hydroxides in neutral solutions. Complexing these ions with chelators such as EDTA can prevent this problem. Stimulation of lipid peroxidation with EDTA occurs with the auto-oxidation of ferrous ions to $\text{OH}\cdot$. Ethylenediaminetetraacetic acid keeps the iron in a reactive form in solution and may alter the redox potential of the iron (Gutteridge, 1987). Ethylenediaminetetraacetic acid has the potential to reduce lipid peroxidation if the molar ratio of EDTA to iron is 1:1. Evidence suggests that liposome peroxidation is inhibited by $\text{OH}\cdot$ scavengers. The $\text{Fe}^{3+}:\text{Fe}^{2+}$ ions and their chelators have different lipophilicities and thus different tendencies to penetrate the membrane and generate reactive species within them (Gutteridge, 1987).

c) Iron proteins

Biochemists in the 70's and 80's debated whether transition metals could accelerate hydroxyl radical formation *in vivo*. Haem proteins, free and bound, were found to enhance lipid peroxidation by the extraction of a H atom to form ROS (Gutteridge, 1987).

- 1) Ferritin: a spherical protein shell that encloses an iron "core". It is a complex polymer of iron holding up to 4500 moles of iron/mole protein. Iron is stored in the low affinity ferric state and this may be released in the ferrous form. The ferric iron may be reduced by $O_2^{\cdot-}$ or ascorbate and so initiate lipid peroxidation (Gutteridge, 1987; Halliwell, 1987; Gutteridge *et al*, 1983a; Aust, 1987).
- 2) Transferrin: binds 2 moles of ferric iron per mole of protein with high affinity. It may, however, lose its iron in a catalytic form at low pH values and is found in synovial fluids from arthritic patients. The ability of iron to bind gives the protein a potent antioxidant activity towards iron-stimulated lipid peroxidation. At low pH however, iron may be released (Gutteridge, 1987).
- 3) Lactoferrin: it has similar binding properties to transferrin, but is able to hold iron at low pH values. It is only partly loaded with iron and has antioxidant properties. Research has shown that the protein binds to the membranes of target cells and so labilise iron from the protein making cells very susceptible to being killed by $O_2^{\cdot-}$ and

H₂O₂ (Gutteridge, 1987; Halliwell, 1987).

The latter iron protein is a clear example of a site-specific reaction whereby a catalyst is located, OH· radical is formed, and in turn attacks molecules in the nearby region. Recent studies show that lactoferrin and transferrin are not promoters of OH· formation at pH 7.4, unless a metal chelator is used or the proteins have been incorrectly loaded (Gutteridge, 1987; Halliwell, 1987).

d) Loosely bound iron

Certain cells contain iron pools which are used to synthesise iron containing proteins. If the cell integrity remains intact, enzymes such as SOD's, catalases and peroxidases are able to remove O₂⁻ and H₂O₂ before coming into contact with the iron pool. The appearance of loosely bound iron and copper (II) ions in extracellular fluids may reflect a potential for oxidative stress (Gutteridge, 1987).

1.3.1.2.3.1. *IN VIVO* EFFECTS OF THE HYDROXYL RADICAL

Microsomes, red blood cells and liposomes are sources of hydroxyl radicals and each one can profoundly affect peroxidation. Microsomes are vesicles which encapsulate various insoluble proteins including SOD and catalases. This microsomal enzyme complex is often involved in the metabolism of xenobiotics with the resultant production of free radicals (Reiter *et al*,

1995). Induction of the cytochrome P450 system may result in an increase in the production of H_2O_2 , thus contributing to increased lipid peroxidation (van Ginkel *et al*, 1994). Red blood cells are susceptible to oxidative stresses with diseases such as sickle cell anaemia and glucose-6-phosphate dehydrogenase deficiency (Gutteridge, 1987). In the normal cells, there are high concentrations of ferrous ions, oxygen and PUFA's. The cells, however, remain resistant to oxidative damage due to the presence of antioxidative enzymes such as catalase and GSH-PX (Gutteridge, 1987). Hydroxyl radical scavengers are those agents that are able to react with the hydroxyl radical and prevent the formation of other hydroxyl radical-mediated reactions from occurring. These must react to a higher degree with the hydroxyl radical rather than other substances. In turn, these compounds must not be damaging as well. Such compounds are ethanol, mannitol and formate (Gutteridge, 1987; Carrico *et al*, 1970).

1.3.2. REACTIVE OXYGEN SPECIES (ROS) AND THEIR BIOLOGICAL CONSEQUENCES

All of the reactive oxygen species mentioned previously have had some form of consequence in the body and have been found to be important biochemical intermediates in a large number of diseases (Cheeseman *et al*, 1993). The following deleterious consequences may occur with respect to damage within the body : DNA, protein and lipid damage.

1.3.2.1. DNA DAMAGE

All components of DNA may be attacked by $OH\cdot$ and H_2O_2 but not $O_2^{\cdot-}$ (Aruoma,1994).

Damage by ROS to the bases of DNA occurs via chemical oxidation or ionising radiation, while the single strand and double strand breaks are produced by oxygen radicals or radical producing carcinogens. Chromosomal aberrations may be due to the hypoxanthine and xanthine oxidase systems which are able to generate superoxides (Sun, 1990).

1.3.2.2. PROTEIN DAMAGE

Many ROS can oxidise the sulfhydryl (SH) groups in proteins. Hydroxyl radicals attack many amino acid residues. The protein often binds transition metal ions making them a target of attack by site-specific hydroxyl generation. Oxidised methionine residues in protein may be repaired by methionine sulfoxide reductase. Other damaged proteins may be recognised and preferentially destroyed by cellular proteases (Aruoma, 1994).

1.3.2.3. LIPID DAMAGE

Biological membranes contain considerable amounts of highly unsaturated lipids and cholesterol, however, PUFA's make the membrane phospholipids, unsaturated lipids and sterols susceptible to peroxidation. Membranes are stabilised lipid species and lipophilic antioxidants that stabilise the membrane structure can reduce lipid peroxidation (van Ginkel *et al.*, 1994). A large number of chemicals are able to directly or indirectly induce radical formation and the extent may produce structural or dynamic changes. Bilayer membrane rigidity is also induced in oxidatively damaged PUFA's due to steric hindrance, restraining the

rotational movement of lipid molecules (Choi *et al*, 1995).

1.3.3. LIPID PEROXIDATION IN NEURODEGENERATIVE DISORDERS

Oxidation and lipid peroxidation of membranes may act synergistically with calcium and cause cell damage after trauma (Braugher, 1987). Once the trauma has occurred, the extracellular calcium is quickly decreased and accumulation of calcium occurs inside the cell. This results in activation of the calcium-dependent enzymes such as phospholipases and which results in “free” fatty acids being released. Calcium may finally cause cell death by: inhibiting the mitochondrial respiratory pathway, activating calcium-dependent proteases and by degrading neurofilaments and myelin proteins in neuronal tissue (Braugher, 1987). Initiation of lipid peroxidation has been shown to decrease neurotransmitter uptake by brain synaptosomes or brain slices.

Lipid peroxidation may:

- 1) increase degradation of phospholipids by two mechanisms: (a) the involvement of calcium, and (b) the presence of oxidised lipids.
- 2) increase the permeability of the membrane to calcium due to the release of fatty acids and their oxidised products.
- 3) change the membrane permeability to calcium and thus open non-specific channels within the membrane (this may result in the generalised deterioration of the membrane structure) (Braugher, 1987)

1.3.4. DEFENCE AGAINST OXYGEN TOXICITY

Cells maintain a variety of defences against oxygen toxicity. Among these are a variety of enzymes that have evolved to deal with oxidative stress and include SOD, catalase and GSH-PX. Other defence enzymes include endonucleases and DNA polymerase. Superoxide dismutase, catalase and GSH-PX are important since they are the primary defence mechanisms against cell damage by oxygen free radical toxicity (Halliwell *et al*, 1985).

There are other mechanisms which play an important role in tissue protection: (1) maintenance of low oxygen tension, (2) compartmentalisation, (3) maintenance of structural integrity, (4) miscellaneous peroxidases, and (5) cellular antioxidants (Buechter, 1988).

Biological systems are able to regulate oxygen by respiration, circulation and blood systems. The cells are able to compartmentalise oxygen in the electron transport chain until it is completely reduced to water, thus reducing the cells' exposure to oxygen. Cells that generate large amounts of reactive oxygen species (such as $O_2^{\cdot-}$) have equally high levels of antioxidant enzymes such as SOD, catalase and GSH-PX (Buechter, 1988).

1.3.4.1 . ANTIOXIDANT DEFENCE MECHANISMS

Most organisms are constantly exposed to molecular oxygen which is a necessary requirement, however, it may be toxic. The deleterious effects of oxygen are thought to be due ROS's

formed after reduction. These processes may result in extensive cellular damage and loss of membrane integrity. Organisms have evolved to deal with these ROS and effectively convert the toxic species to less reactive forms. The protective systems involved are enzymes such as SOD, catalase and GSH-PX as well as cellular antioxidants such as Vitamin E and C (Buechter, 1988).

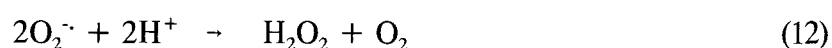
1.3.4.1.1. ANTIOXIDANT ENZYMES

As mentioned above, the enzymes responsible for the defence mechanism are SOD, catalase and GSH-PX. Each enzyme will be discussed further.

1.3.4.1.1.1. SUPEROXIDE DISMUTASE (SOD)

The superoxide, as already mentioned, may be produced *in vivo* by enzymatic, spontaneous and photochemical oxidation reactions. The primary defence mechanism found in most aerobic cells is provided by the metalloenzymes such as SOD (Fridovich, 1987).

The enzyme specifically catalyses the conversion of the superoxide radical ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) via the following reaction (Buechter, 1988, Reiter *et al*, 1995):



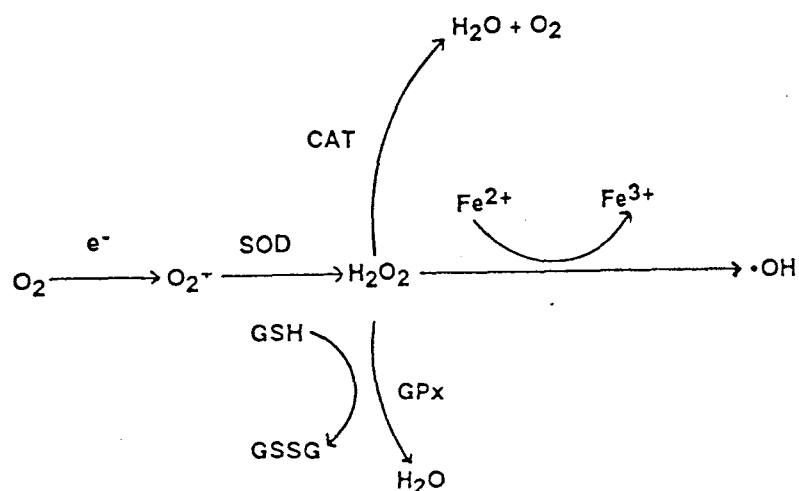


Figure 1.13. Outline of the three main antioxidant enzymes involved in preventing the formation of free radicals. Abbreviations: superoxide dismutase (**SOD**), catalase (**CAT**) and glutathione peroxidase (**G-PX**) (Reiter, 1995).

Hydrogen peroxide is a ROS. It is a derivative of an oxygen species that is involved in O_2 production (Cheeseman *et al*, 1993). Hydrogen peroxide can then be reduced to the highly reactive hydroxyl radical ($OH\cdot$) and water by another antioxidant enzyme, catalase (Buechter, 1988). Eukaryotes contain copper and zinc SOD's, which are found in the cytosol and extracellular fluid. Both show very similar sequence homology but are unrelated in the evolutionary sense to SOD's that contain iron (III) and manganese (III) at the active sites. The latter are found in the mitochondria of prokaryotes (Fridovich, 1987; Reiter *et al*, 1995)

In eukaryotes, human and bovine copper-zinc SOD's are similar in that both contain two atoms of copper (II) (Cu^{2+}) and zinc (Zn^{2+}) per molecule of enzyme. It is composed of 2 identical subunits joined by one disulfide bond. The mode of action involves the alternate

reduction/reoxidation of Cu^{2+} at the active site during interactions with O_2 (Keele *et al*, 1971; Carrico *et al*, 1970). In prokaryotes, *E. Coli*, contains both manganese (Mn) and iron (Fe) SOD's. Manganese superoxide dismutase also contains 2 subunits and the molecular weight is 40000, while FeSOD has two subunits with one Fe^{3+} molecule per molecule of enzyme and a molecular weight of 39000. Differences between both forms of the enzyme is that FeSOD is active in aerobic or anaerobic conditions, while MnSOD is under repression control and not active in anaerobically grown cells. Manganese superoxide dismutase is enhanced when the partial pressure of O_2 is elevated and thus moderates O_2^- production (Fridovich, 1987).

A compound that is able to enter the cell and induce SOD, especially MnSOD, is paraquat. Paraquat is a herbicide that is accumulated by cells is enzymatically reduced by an NADPH-dependent enzyme of the endoplasmic reticulum (ER) (Winterbourn *et al*, 1991). Auto-oxidation of the reduced compound (paraquat) generates superoxides and is the basis of the toxicity of the drug. Other redox-recycling compounds are bleomycin and dialuric acid (Buechter, 1988; Fridovich, 1987).

1.3.4.1.1.2. CATALASE AND GLUTATHIONE PEROXIDASE

Both enzymes belong to the family of enzymes which contain hydroperoxides and peroxidases, and are primarily responsible for the safe decomposition of peroxides (Cheeseman *et al*, 1993).

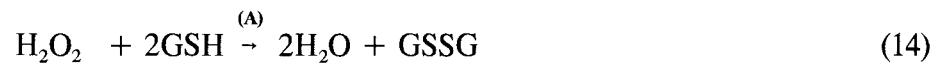
Catalase is located in the peroxisomes and acts upon H_2O_2 according to the following equation

(Cheeseman *et al*, 1993; Reiter *et al*, 1995):



Glutathione peroxidase is found mostly in the cell cytosol and is able to act upon H_2O_2

(Cheeseman *et al*, 1993; Reiter *et al*, 1995):



Enzymes responsible : (A): glutathione peroxidase.

(B): glutathione reductase.

In equation 14: the conversion of H_2O_2 to H_2O occurs via the enzyme GSH-PX which is also able to oxidise glutathione (GSH) to its disulfide form (GSSG), which in turn is returned to GSH by the enzyme glutathione reductase (Reiter *et al*, 1995).

1.3.5. CELLULAR ANTIOXIDANTS

A few questions have to be asked when choosing antioxidants for therapeutic use:

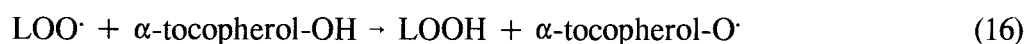
- 1) Which biomolecule is it likely to protect?
- 2) Will the compound be in sufficient concentrations near to the biomolecule in question?

- 3) How does the molecule protect?
 - i) by scavenging molecules.
 - ii) preventing their formation.
 - iii) repairing the damage.
- 4) Can the compound promote damage at sites different to the site which it protects?

Several cellular antioxidants have been thought to be involved in the scavenging of oxygen or organic radicals. One of the best known antioxidants is vitamin E, usually represented as α -tocopherol (Reiter *et al*, 1995).

1.3.5.1. Vitamin E

The oxidation of PUFA's associated with membranes may disrupt various cellular functions and cause leakage of intracellular contents (Palamanda *et al*, 1993; van Ginkel *et al*, 1994). Oxidation of membranes may be prevented by Vitamin E (Palamanda *et al*, 1993). Vitamin E or α -tocopherol (a major constituent of the vitamin E family) is an important natural antioxidant in living cells (Palozza *et al*, 1991). It is known as a "chain-breaking antioxidant" because of its ability to intercept lipid peroxy radicals ($\text{LOO}\cdot$) and terminate lipid peroxidation chain reactions (Palozza *et al*, 1991) with the formation of a relatively unreactive Vitamin E radical.



Vitamin E has the ability to scavenge free radicals and thus prevent lipid peroxidation by supplying H atoms to free radicals and inhibiting further lipid peroxidation. Tissues which lack vitamin E have shown an increase in lipid peroxidation. Vitamin E is able to reverse these effects and protect liposomes against the lipid peroxide (Buechter, 1988; Cheeseman *et al.*, 1993).

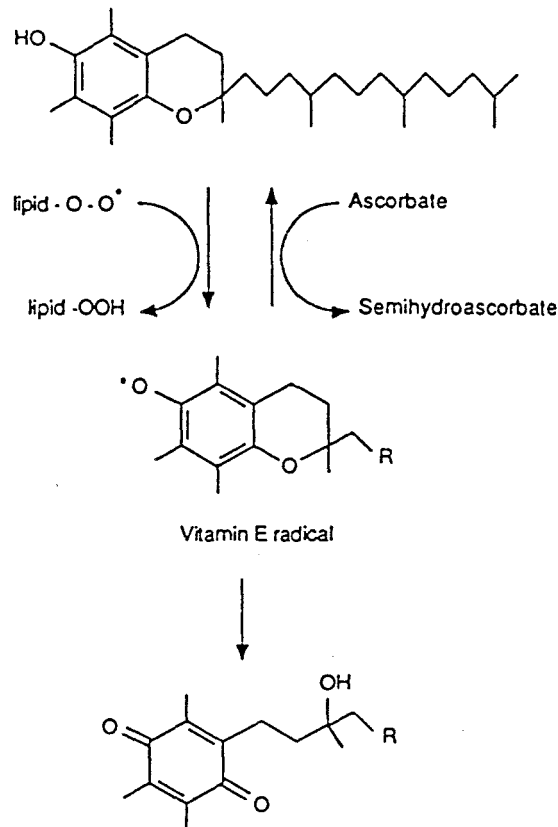


Figure 1.14. Vitamin E acting as a chain-breaking antioxidant, reacting with lipid peroxy radicals to give an unreactive vitamin E radical (Buechter, 1988).

1.3.5.2. Vitamin C (Ascorbic Acid)

Another compound which acts as an important free radical scavenger in the aqueous phase within the cells and plasma is vitamin C or ascorbic acid. It has been shown that vitamin C

is able to react with radicals such as the vitamin E radical (Figure 1.15) and form semihydroascorbate which is then able to form oxalic acid and L-threonic acid or to be reduced back to ascorbate. Evidence shows that vitamin E and C are able to react synergistically as antioxidants (Buechter, 1988)

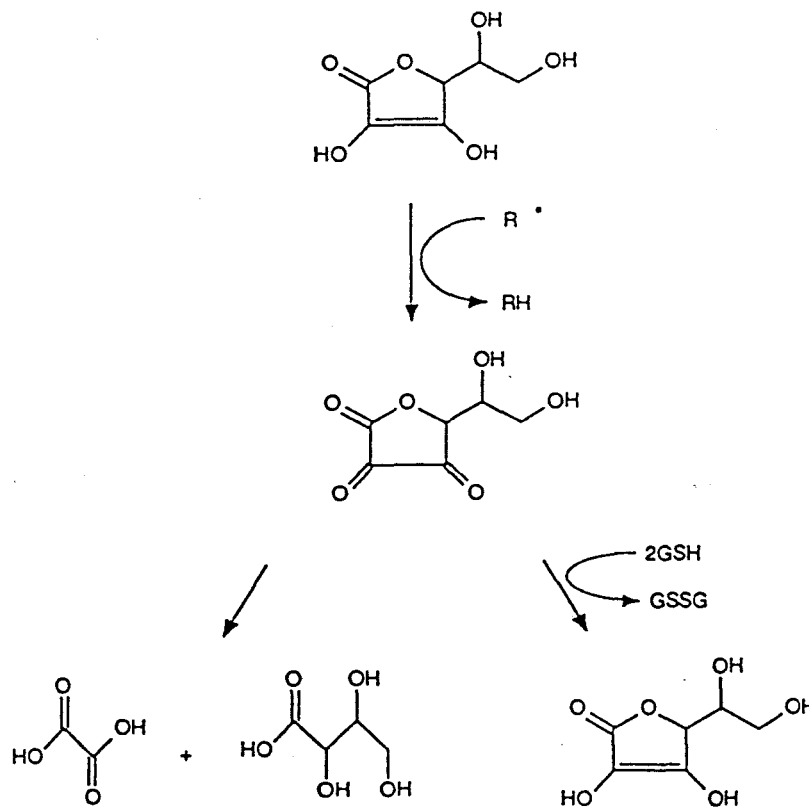


Figure 1.15. Vitamin C (ascorbic acid) plays a protective role by reacting with free radicals including the Vitamin E radical to give semihydroascorbate, which is then able to go on to form L-threonic acid and oxalic acid or be reduced back to ascorbate (Buechter, 1988).

In the presence of transition metals, however, free or loosely bound vitamin C is able to promote free radicals where ascorbate acts as a reducing agent which is able to reduce Fe^{3+} to Fe^{2+} . The ferrous ion then reacts with hydrogen peroxide and so initiates the formation of

hydroxyl radicals (Buechter, 1988). The following mechanism may also occur (Aruoma, 1994; Andorn *et al*, 1996):



1.3.5.3. *Glutathione and Uric Acid*

Glutathione, found in the cell cytosol, also possesses strong radical scavenging properties. It is found in millimolar concentrations and has important roles in xenobiotic metabolism and leukotriene synthesis. Glutathione is also important in the defence mechanism against radical formation in the lens of the eye (Reiter *et al*, 1995; Cheeseman *et al*, 1993). Addition of glutathione to tissues can inhibit the lipid peroxide by reacting to give a glutathione radical which may then react with another glutathione disulphide. With the help of glutathione reductase, the enzyme reduces the product back to glutathione (Buechter, 1988).

Uric acid, found in the plasma, also has the ability to act as a radical scavenger. The mechanism, however, is not clear (Buechter, 1988) although it seems that the antioxidant role may be attributed to its ability to bind iron and copper ions (Halliwell *et al*, 1986).

1.3.5.4. *Melatonin (aMT)*

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone synthesised almost exclusively in the

pineal gland of mammals, including humans. It exhibits a circadian rhythm with maximum production at night. Studies have shown that aMT may provide protection against damage caused by lipid peroxidation. The antioxidant effect could be provided via two pathways: (1) the compound may exert a direct antioxidant effect by scavenging free radicals or (2) inhibiting their formation. Melatonin also has the ability to increase the activity of the endogenous system's antioxidant enzymes, such as GSH-PX. Melatonin has been reported to be a potent hydroxyl and peroxy radical scavenger (Marshall *et al*, 1996). Melatonin and other structurally related indoleamines act as electron donors which detoxify endogenous electrophilic compounds. Melatonin also has the ability to combine with other chain-breaking antioxidants such as ascorbate (in the cytosol) and vitamin E at membrane level, and so improve their efficiency as free radical scavengers (Sewerynek *et al*, 1996; Reiter, 1997; Reiter *et al*, 1995). Melatonin may act as a potent scavenger because of its ability to traverse the BBB as well as other morphological barriers and is therefore accessible to every cell in the body (Reiter, 1997). The ability of aMT to be taken up into subcellular compartments is due to its high lipophilicity (lipid solubility) and hydrophilicity (water solubility). Thus it is capable of acting intracellularly without the benefit of a carrier molecule (Melchiorri *et al*, 1996; Reiter, 1997; Reiter *et al*, 1995; Reiter, 1995). Lipid peroxidation induced by kainic acid, a potent neurotoxin which is able to induce seizures and neuronal damage, is markedly reduced in the presence of aMT (Melchiorri *et al*, 1996). Also, aMT is able to reduce safrole-induced DNA damage as well as damage caused by the herbicide paraquat (Reiter, 1997; Reiter *et al*, 1995). Safrole, a chemical carcinogen, and paraquat are responsible for increased lipid peroxidation. Cytochrome P450, an enzyme responsible for xenobiotic metabolism and thus

free radical formation, is reduced by as much as 30% in the presence of aMT (Reiter *et al*, 1995). Melatonin may influence the following enzymes either responsible for increasing antioxidant activities in the cell or reducing lipid peroxidation: (1) induction of glucose-6-phosphate dehydrogenase (hepatic and cerebral) which increases NADPH levels and thus promotes the conversion of GSSG to glutathione via glutathione reductase (glutathione is important for glutathione peroxidase enzyme) (Reiter, 1997; Reiter *et al*, 1996), (2) GSH-PX activity is increased, so the conversion of H₂O₂ to water is more rapid and the formation of hydroxyl radicals is reduced and (3) the activity of nitric oxide synthase (NOS) is reduced; the enzyme responsible for the formation of the hydroxyl ion via a peroxynitrite anion (NO+O₂⁻ → ONOO⁻) in the brain (Reiter *et al*, 1996; Reiter *et al*, 1995; Reiter, 1997). Melatonin is thus able to influence enzyme activities to limit lipid peroxidation or maximise those enzymes responsible for protective effects (Reiter, 1995).

The brain, because of its lipid content and high oxygen consumption, is more susceptible to oxidative stress and it is therefore essential that the free radicals formation be minimised as much as possible. Melatonin may provide a significant defence against ROS damage (Choi *et al*, 1995; Reiter *et al*, 1995).

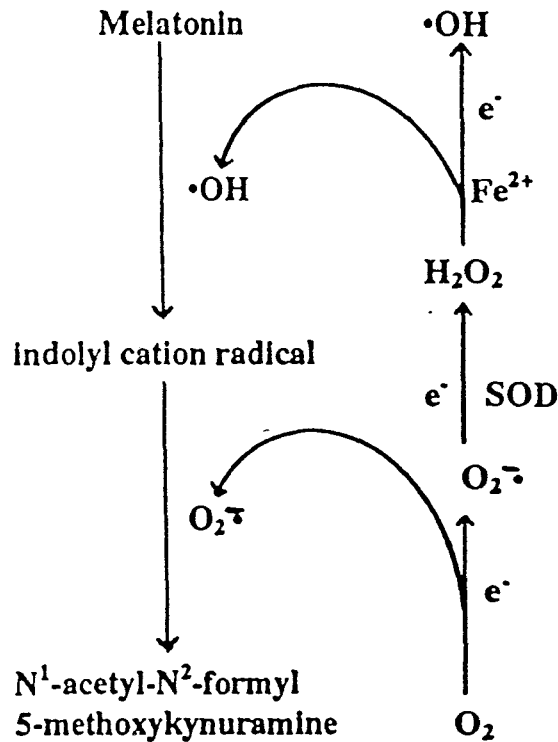


Figure 1.16. The mechanism thought to be responsible for the antioxidant effects of melatonin (Reiter *et al*, 1996).

1.3.6. ANTIOXIDANTS IN EXTRACELLULAR FLUIDS

Intracellular mechanisms are solely responsible for removing reactive oxygen species before these are able to react with the pool of iron. For this reason, enzymes such as SOD, catalase and GSH-PX are present and able to remove the free radicals (Gutteridge *et al*, 1988).

In extracellular fluids, enzymes involved in antioxidant functions do not exist. It appears that the antioxidant defence mechanisms in extracellular fluids is entirely different from those

found intracellularly. The intracellular radical reactions are limited by the conversion of pro-oxidant forms of iron and copper to a less or non-reactive form (Gutteridge *et al*, 1988). Extracellular antioxidants are acute-phase reactive proteins responding to tissue damage, since it is at this time that oxygen radical reactions are most likely to occur. Antioxidants such as ceruloplasmin (ferroxidase activity) and the iron-binding properties of transferrin and lactoferrin (iron is bound tightly) are used to inhibit iron-catalysed oxygen radical reactions (Gutteridge *et al*, 1981).

Haemoglobin and haem-transporting proteins release catalytic iron in the presence of H₂O₂ or peroxides. Haptoglobin and haemopexin have the ability to reduce the haem-stimulated lipid peroxide. The defence mechanism in extracellular fluid occurs via the transport and affinity of iron stores and is dependant on the removal or inactivation of the reactive metal complexes (Gutteridge *et al*, 1988).

1.4. CONCLUSION

Tryptophan is viewed as an important biological compound whose effects have far reaching consequences. It is primarily metabolised in the liver, by the enzyme TDO. The enzyme is induced by its substrate, TRP, and corticosterone to increase the formation of the kynurenine pathway metabolites which include the neurotoxin, QA. Consequently, the synthesis of kynurenines shunts TRP away from metabolism in the brain by the enzyme TH to form serotonergic compounds. Depleted 5-HT levels have been implicated in depressive mood

disorders and thus an inverse relationship exists between increased TDO activity and levels of 5-HT. Inhibitors of TDO could prove therapeutic by increasing the availability of TRP to the brain. Kynurenines are synthesised in the brain by the enzyme IDO and induced in broad spectrum inflammatory diseases. Diseases such as meningitis have been found to have increased levels of kynurenines in the CNS and systemic tissues, contributing to the neurological dysfunction that are associative with these disorders. Quinolinic acid has been implicated in various diseases. It has the ability to interfere with EAA transmission or NMDA receptor function by acting as an agonist or disrupting the integrity of the BBB. Much research has been focused on the kynurenine pathway products and their ability to cause deleterious effects. Quinolinic acid is also able to induce lipid peroxidation. Lipid peroxidation, caused by the reduction of O_2 to toxic products such as O_2^- , H_2O_2 and OH^\cdot , is normally increased in damaged tissues and neurological diseases such as Huntington's disease. Antioxidant enzymes, SOD, catalase and GSH-PX, and intracellular compounds such as vitamins C and E and melatonin are present to reduce the ravages of lipid peroxidation.

CHAPTER 2

EXPERIMENTAL PROCEDURES

2.1. ANIMALS

Male Wistar rats of the albino strain with a body weight of 200-250 g were obtained from the University of Durban-Westville, Durban, South Africa. The animals were chosen at random and assembled into the groups required and housed in plastic cages with metal grids and covers. The animals were maintained on Epol food and tap water *ad libitum*. The animal room was windowless and well ventilated and maintained at constant temperature of $22 \pm 2^\circ\text{C}$. The animal room was artificially lighted and automatically regulated to give a constant light:dark cycle of 12:12 with lights on at 06h00. The intensity of illumination was $300\mu\text{Watts/cm}^2$. The animals were killed swiftly by cervical dislocation between 13h00 and 14h00 and immediately decapitated. To remove the brain, the skull was removed by making an incision through the bone on either side of the head from the foramen magnum to near the orbit. The skull was lifted with a pair of forceps exposing the pineal gland and brain. Care was taken to remove the brain and pineal gland with tweezers, all adhering tissue and visible traces of blood was eliminated. The livers were isolated by a mid-ventral incision through the abdominal

musculature from the pelvic region to the posterior edge of the sternum. A transverse cut was made anteriorly to expose the liver which was carefully removed. The livers were perfused with ice-cold 0.9% sodium chloride (NaCl) to remove excess blood. The brain and the livers were immediately placed in liquid nitrogen and stored at -70°C until required. Freezing at -70°C did not affect the activity of the enzyme of interest in the liver.

2.2. DETERMINATION OF TRYPTOPHAN 2,3-DIOXYGENASE ACTIVITY

Tryptophan 2,3-dioxygenase is a haem-dependent cytosolic enzyme which catalyses the conversion of TRP to N-formylkynurenine. In rats or humans, the enzyme exists in two forms, whereas in several species, the enzyme may exist in one form only. The holoenzyme, already active, does not require the presence of exogenous haematin; while the inactive, haem-free form of the enzyme, the apoenzyme, does. The enzyme can be induced via: (1) glucocorticoids, which involve the synthesis of new apoenzyme, and (2) its substrate, TRP. Haem, a cofactor of the enzyme, is loosely associated with the apoenzyme and is therefore dissociable. Evidence has shown that the activity of the enzyme may also be regulated by a pre-existing haem pool in the body. Agents which destroy or induce haem synthesis are therefore responsible for lowering or inducing enzyme activity respectively (Fiegelson *et al*, 1961; Badawy *et al*, 1975).

Activation of the apoenzyme *in vitro* involves the conjugation with haem to form the oxidised holoenzyme (ferrihaem). The addition of TRP reduces the holoenzyme to the active form

(ferrous) which is then active in the catalytic reaction with TRP (substrate). Tryptophan prevents the degradation of the pre-existing apoenzyme (Badawy *et al*, 1975; Schimke *et al*, 1965), enhances the binding of haem to the apoenzyme, and finally, it is able to preserve the enzyme in the reduced holoenzyme form (active) (Knox,1966; Knox *et al*, 1966).

The tryptophan 2,3-dioxygenase enzyme assay employed in this study was previously described by Badawy and Evans, 1975 and 1983. The activity of the enzyme is determined by measuring the formation of kynurenine from TRP in either the absence (holoenzyme) or in the presence (total activity) of added haematin. The apoenzyme activity is calculated as the difference between the two. The kynurenine present is determined by measuring the absorbance at 365 nm using the extinction coefficient ($\epsilon = 4540 \text{ L/mol.cm.}$). The final results were expressed as μmoles kynurenine present. The haem saturation of the apoenzyme is calculated as the ratio of holoenzyme:apoenzyme.

2.2.1. MATERIALS

2.2.1.1. Animals

Male Wistar rats of the albino strain with a body weight of 200-250 g were used. The animals were randomly assembled into groups of five ($n=5$) and maintained as describe in Section 2.1. The animals were sacrificed between 12h00 and 13h00. The livers were excised and the blood and interfering haem was removed with 0.9% NaCl and livers immediately frozen in liquid

nitrogen and stored at -70°C until required.

2.2.1.2. Chemicals

All chemicals were of the highest purity available. L-Tryptophan and haematin chloride were purchased from Sigma Chemical Co., St. Louis, USA. Trichloroacetic acid (TCA), phosphate buffer, sodium hydroxide (NaOH) and potassium chloride (KCl) were purchased from Saarchem Limited, Krugersdorp, South Africa.

2.2.2. METHODS

2.2.2.1. Chemicals

All chemical solutions were prepared in deionised water (Milli R/Q System, Millipore). L-Tryptophan (0.03M) was prepared in 4mM NaOH. Trichloroacetic acid (0.9M) and NaOH (0.6M) was dissolved in deionised water. Sodium phosphate buffer (0.2M) (pH 7.4.) and 0.14M KCl - 2.5mM NaOH was prepared and refrigerated before commencing with the experiment. Haematin chloride, final concentration 2 μ M, was dissolved in 0.1M NaOH and prepared prior to the start of the experiment.

2.2.2.2. Tissue Preparation

As described in 2.2.1.1., the liver, once removed, was immediately perfused with 0.9% NaCl and rapidly frozen in liquid nitrogen and stored at -70°C. The liver, when needed, was slowly thawed on ice. The liver was then initially homogenised with 60 ml 140mM KCl - 2.5mM NaOH with a Junke and Kunkel Ultra Turrex waring blender for a period of 1 minute at 1000 rev/minute. During the homogenising procedure, care was taken not to allow excessive foaming as this results in protein denaturation. The resulting suspension was further homogenised with a hand-held glass homogeniser until a complete homogeneous solution was obtained. Thereafter, sonication for a period of 2 minutes at 30 second intervals proceeded for complete removal of enzymes from the cells. Finally, 0.2M sodium phosphate buffer, pH 7.0, was used to make up the volume required for a final 10% w/v before being gently stirred. The procedure was carried out on ice.

2.2.2.3. Tryptophan 2,3-Dioxygenase Determination

Samples containing 15 ml of homogenate (10% w/v) were added to 12.5 ml water and stirred. Where necessary, 100 μ l haematin (final concentration of 2 μ M) was added and a period of 1 minute was allowed for activation of the enzyme. The test compound (1 ml) was added to both the holoenzyme and total activity flasks; the vehicle for the test compound replaced the test compound in the control. Finally, 2.5 ml of 0.03M L-TRP was added to the overall mixture which was gently stirred. The rest of the assay was carried out in triplicate. Aliquots of 3 ml's of the assay mixture was transferred to test tubes and stoppered in a atmosphere of

CO₂/O₂ (5%: 95%) and samples incubated for a period of 0, 15, 30, 45, 60, 75, 90 and 105 minutes in a 37°C oscillating water bath. The reaction was terminated with 2 ml TCA. The mixture was further shaken for a period of 4 minutes. The resulting precipitate was filtered into another test tube through a Whatman no.1 filter paper. To a measured portion of filtrate (2.5 ml), 1.5 ml of 0.6M NaOH was added and vortexed. The kynurenine present in the solution was measured at 365 nm with a Shimadzu UV 160A UV-Visible recording spectrophotometer and using the extinction coefficient of kynurenine: $\epsilon = 4540 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$. The blank consisted of 2 ml TCA and 1.5 ml NaOH.

Table 2.1. Scheme representing the method of the assay for tryptophan 2,3-dioxygenase.

10 % w/v HOMOGENATE		
Holoenzyme	REAGENTS	Total Activity
12.5 ml	Water	12.5 ml
15.0 ml	Homogenate	15.0 ml
0 ml	Haematin (2 μM)	100 μl
1.0 ml	Test compound	1.0 ml
2.5 ml	0.03M 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		

2.2.3. RESULTS

The holoenzyme activity was determined in the absence of haematin and the total activity in the presence of exogenously added haematin. As mentioned, the difference between the two, is the apoenzyme activity. The concentration of kynurenine was calculated using the molar extinction coefficient for kynurenine: $\epsilon = 4540 \text{ L/mol.cm}$. The final results were expressed as μmoles kynurenine formed. The data obtained was analysed by one-way analysis of variance followed by the Student-Newman-Keuls test. Values for $n=5$ rats were expressed as $\pm \text{S.E. M}$.

As shown in Figure 2.1. (page 86), the enzyme produced a linear reponse with regard to the production of kynurenine over a period of one hour. The change in activity of the enzyme in frozen livers at a low temperature of -70°C was insignificant.

2.2.4. DISCUSSION

The tryptophan 2,3-dioxygenase assay, although time consuming, is effective for determining the effect of a compound on the activity of the enzyme. The enzyme is unstable and thus requires that the assay be carried out on ice. The activity of the enzyme, stored at -70°C , remains stable up to a period of 4 months.

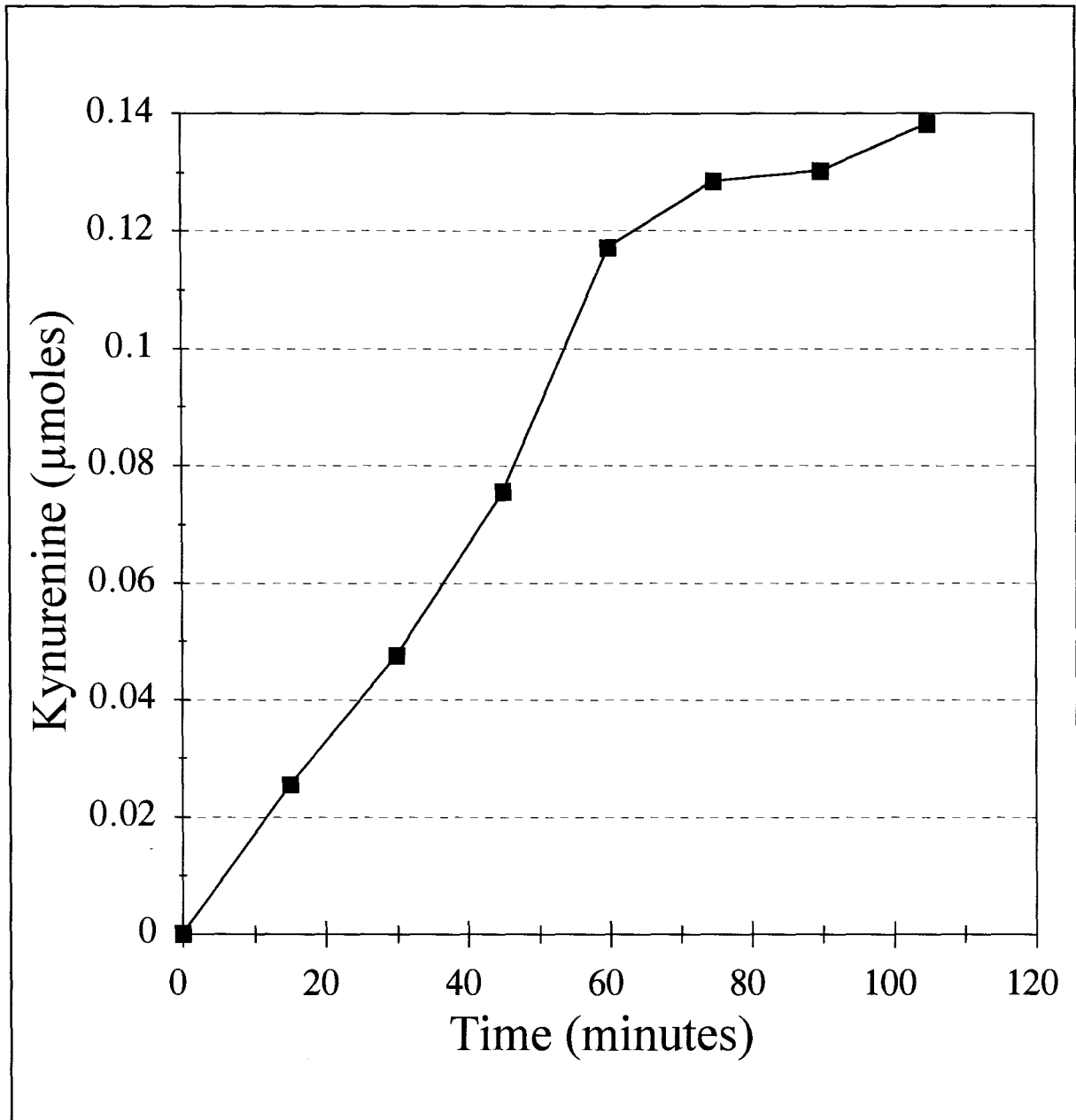


Figure 2.1. Effect of time incubation on tryptophan 2,3-dioxygenase activity at 37°C. Each point represents the mean of triplicate determinations.

2.3. DETERMINATION OF PINEAL INDOLE METABOLISM IN ORGAN CULTURE USING THIN LAYER CHROMATOGRAPHY

Organ and tissue culture techniques are used by numerous researchers since it is convenient and not time consuming (Klein *et al*, 1969; Morton, 1990; Daya *et al*, 1982; Daya *et al*, 1989). The pineal gland is especially suitable because it is small, easily accessible and able to maintain its metabolic function. It is able to synthesise various indoles by utilising a radioactive exogenous precursor such as [¹⁴C] tryptophan or [³H] serotonin. Ninety five percent of the indoles synthesised during the incubation period are secreted into the culture media. The radioactive indoles are then isolated from the media, analysed and quantified.

Earlier, the isolation of indoles was achieved by organic extractions and quantification by 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 pineal indoles. The pineal indoles are separated by two solvent systems; the first one utilises 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 NAS and the 5-hydroxyindoles from the 5-methoxyindoles. The glacial acetic acid effectively separates 5-HIAA from 5-MIAA. The second organic solvent system optimises the separation of 5-MIAA and 5-MTOH from aMT and the separation of 5-HTOH and 5-HIAA from NAS. Serotonin is unaffected by the two solvents and thus remains at the point of origin. This assay is effective and simple and allows trace quantities of indoles to be separated (1 pmole/10 μ l).

The assay employed in this study, is a modification of the method of Klein *et al*, 1969 and Daya *et al*, 1982. The radioactive metabolites were isolated using bi-dimensional thin layer chromatography (TLC) as described above and quantified using liquid scintillometry.

2.3.1. MATERIALS

2.3.1.1. Animals

Male Wistar rats of the albino strain with a body weight of 200 - 250 g were randomly assembled into groups of four (n=4) and maintained as described previously in Section 2.1. The animals were sacrificed at approximately 12h00.

2.3.1.2. Chemicals

5-Hydroxy (side-chain-2-¹⁴C) tryptamine creatine sulphate was purchased from Amersham International, United Kingdom. The concentration of the radioactivity was 50 microcurie (μ Ci) and the specific activity was 55 mCi/mmol. BGJb culture media (Fitton Jackson modification) was purchased from Gibco, Europe and aseptically fortified with antibiotics such as streptomycin, penicillin and amphotericin B. The composition of the media is schematically represented in Table 2.2. and 2.2.1. The thin layer chromatography (TLC) plates, Kieselgel 60 F₂₅₄, (20 x 20 cm) aluminium sheets coated with silica gel and a fluorescent indicator were obtained from Merck, Germany. The liquid scintillation cocktail, Packard® Scintillator 299™,

was purchased from Packard Instrument Company, Inc., Netherlands.

The indole standards: aMT, 5-HT, NAS, 5-MIAA, 5-HIAA, 5-MTOH, 5-HTOH and 4-dimethylaminobenzaldehyde were obtained from Sigma Chemical Co., St. Louis, USA.

Ethanol and hydrochloric acid (HCl) were obtained from Saarchem Limited, Krugersdorp, South Africa.

2.3.2. METHODS

2.3.2.1. Chemicals

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 2.5 ml absolute ethanol. The solution was 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°C until needed. Van Urk's reagent, used for detecting indoles, was prepared in the following manner: 2 grams 4-dimethylaminobenzaldehyde in 100 ml 25% HCl followed by the addition of 100 ml 95% ethanol. The resultant solution was vortexed and stored at room temperature until required.

Table 2.2. Composition 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 ₂ O)	200.00
Potassium chloride	400.00
Potassium dihydrogen phosphate	160.00
Sodium bicarbonate	3500.00
Sodium chloride	5300.00

Table 2.2.1. The composition of BGJb culture medium (Fitton Jackson modification)
(...continued).

CONTENTS	CONCENTRATION (mg/ml)
OTHER COMPONENTS	
Calcium Lactate	555.00
Glucose	10 000.00
Phenol red	20.00
Sodium acetate	50.00
VITAMINS	
α -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	0.04

2.3.2.2. Pineal Organ Culture

The animals were sacrificed by neck fracture and the pineals removed as described in Section 2.1. Each pineal gland was immediately and individually placed into sterile (borosilicate 10 mm x 75 mm) Kimble glass tubes. Each test tube contained 52 μl of BGJb culture medium. In those experiments that involved the addition of test compounds, a volume of 10 μl was added to the incubation medium to give the required final concentration in a total volume of 70 μl . Finally, 8 μl [^{14}C] serotonin (specific activity 55 mCi/mmol) was added. In the control test tubes, the test compounds were replaced with the vehicle of the test compound (10 μl). 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°C in the dark. The incubation was terminated after 24 hours by the removal of the pineal glands from the culture medium. The medium was then analysed by TLC.

2.3.2.3. Separation of Indoles by Thin Layer Chromatography (TLC)

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

Table 2.3. Representation of the method for pineal organ culture.

REAGENTS	CONTROL	TEST GROUP
BGJb medium	52 μ l	52 μ l
Addition of the pineal gland to the media		
Test Compound	----	10 μ l
Vehicle	10 μ l	----
[¹⁴ C] Serotonin	8 μ l	8 μ l
Gassing of the test tubes with carbogen. Incubation for a period of 24 hours at 37°C. Removal of pineal from incubation media. Stored at -20°C until further use.		

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. The plate was redeveloped in the same direction and same 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 6 cm.

Once the plates were dried under nitrogen once again, the plate was sprayed with Van Urk's reagent and dried in an oven at 60°C until the blue spots that indicated the presence of indoles were visible. The spots were cut out and placed in scintillation vials containing 1 ml absolute

ethanol. The ethanol was responsible for dissolving the silica from the aluminium plates. The vials were shaken for a period of 20 minutes. Thereafter, 3 mls of scintillation liquid was added and the vials were sealed tightly and shaken for a period of 30 minutes. The entire procedure took place under subdued light. The radioactivity was quantified by a Beckman LS 2800 scintillation counter.

2.3.3. RESULTS

Figure 2.2. (page 95) shows a typical bi-dimensional thin layer chromatogram of the pineal indole metabolites. Clear separation of the six blue-green spots could be achieved, and the positions of the metabolites were identified by following the schematic representation of the chromatogram in Figure 2.2.

The results were expressed as disintegrations per minute (DPM) / 10 μ l medium spotted for each of the indoles spotted. The data was analysed by one-way analysis of variance followed by the Student-Newman-Keuls test. Values for n = 4 rats were expressed as \pm S.E.M.

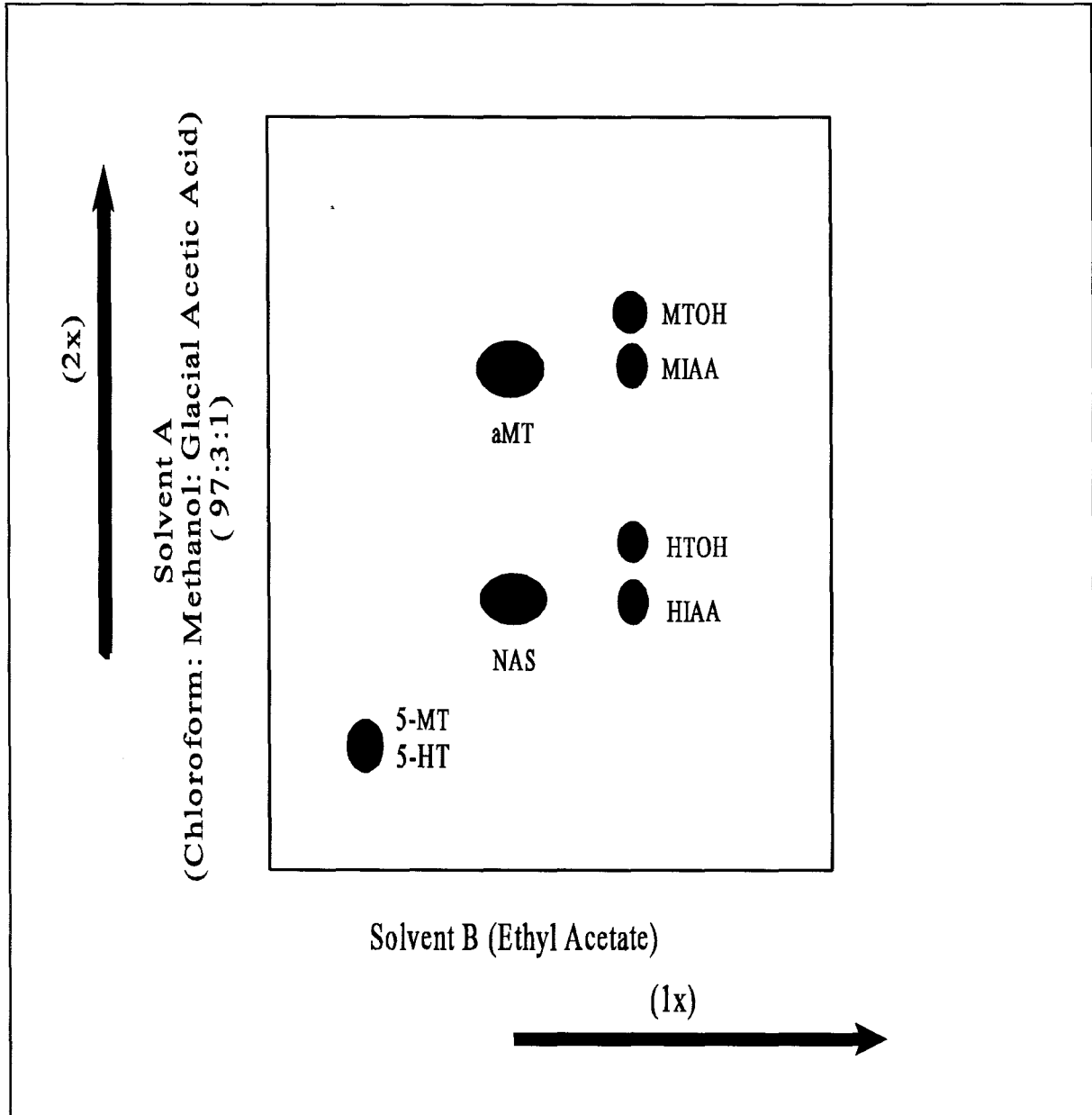


Figure 2.2 A typical bi-dimensional thin layer chromatogram of pineal indole metabolism. Abbreviations used: **5-HT**: 5-hydroxytryptamine (serotonin), **NAS**: N-acetylserotonin, **aMT**: melatonin, **5-HIAA**: 5-hydroxyindoleacetic acid, **5-MIAA**: 5-methoxyindoleacetic acid, **5-HTOH**: 5-hydroxytryptophol and **5-MTOH**: 5-methoxytryptophol.

2.3.4. DISCUSSION

The organ culture technique is simple and effective with regards to monitoring the synthesis of pineal metabolites. The resolution of this technique is dependent on several factors: (1) aseptic conditions, (2) subdued light and prevention of oxidation so that no appreciable loss of radioactivity occurs with regard to the formation of oxidative products, (3) as small a spot size that the spots, once developed, are small and sufficiently resolved from each other so that cutting the indoles out for quantification will not present a problem, and (4) the solvents must remain uncontaminated and fresh.

2.4. LIPID PEROXIDATION DETERMINATION

The thiobarbituric acid (TBA) test was introduced to biological systems for the first time by Kohn and Liversedge in 1944 (Kohn *et al*, 1944) as a measurement for lipid rancidity in the food industry (Gutteridge *et al*, 1983). Kohn and Liversedge described the reaction of TBA with an unknown secondary product of lipid peroxidation. The product was later identified as malondialdehyde (MDA) by Patton and Kurtz, in 1951. Malondialdehyde is formed when polyunsaturated fatty acids with three or more double bonds undergo lipid peroxidation. One MDA molecule reacts with 2 molecules of TBA in the acid-heating stage to form a chromogen-adduct with a maximum absorbance at 532 nm (Gutteridge *et al*, 1983).

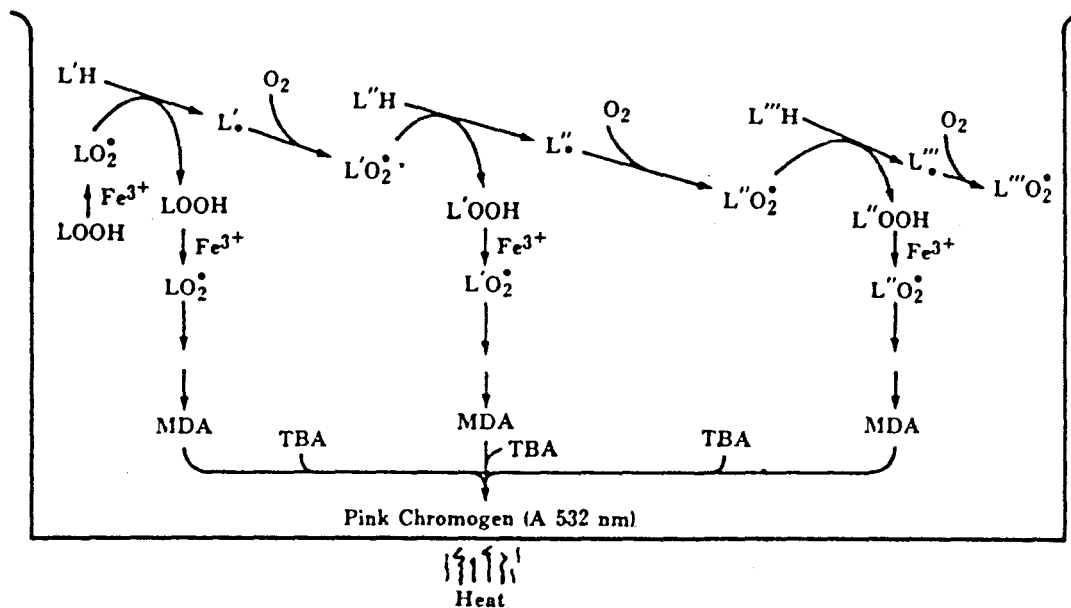


Figure 2.3. The formation of MDA by lipid peroxidation during incubation and to a much greater extent during the acid-heating stage. Enhanced chemical reactions of lipid peroxides (LOOH) and PUFA's (LH) occur in the thiobarbituric acid (TBA) reaction (Gutteridge, 1987).

There are a few factors which have influenced the development of TBA reactivity:

(1) **Iron salt concentrations:** Iron is responsible for the decomposition of lipid peroxides to form peroxy radicals which are in turn responsible for further lipid peroxidation by abstracting hydrogen from PUFA's. The continuance of lipid peroxidation once it has started, is dependent on the iron-catalysed peroxy radical formation (Figure 2.4.). Metal chelators such as EDTA, are used to lower the metal concentration within the solution but it is essential that the ratio of iron salt to chelator is kept low (Gutteridge *et al*, 1983; Esterbauer, 1990).

(2) **Acidity of the reaction:** Optimum pH's have ranged from 3 - 3.5 (Gutteridge *et al*, 1983; Okhawa *et al*, 1979).

(3) **Heating:** Time heating has varied from 15 minutes to 60 minutes depending on the various laboratories (Esterbauer *et al*, 1990; Okhawa *et al*, 1979).

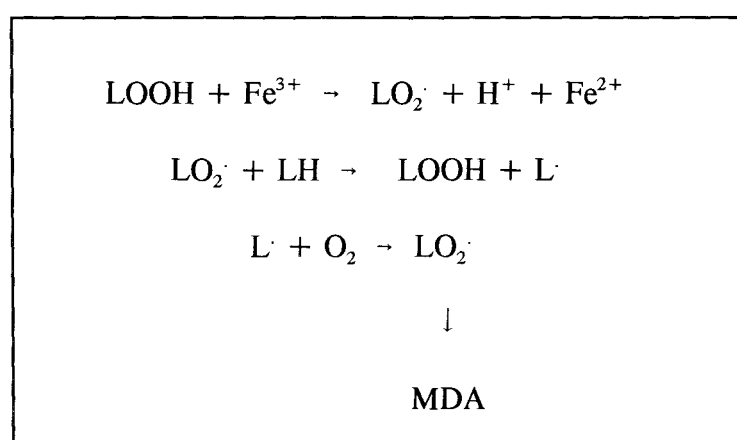


Figure 2.4: The mechanism which may be responsible for the decomposition of lipid peroxides to peroxy radicals. Abbreviations: **LOOH** - lipid peroxide, **LO₂·** - peroxy radical, **LH** - polyunsaturated fatty acid, **L·** - lipid radical and **MDA** - malondialdehyde (Gutteridge *et al*, 1983).

The method used for measuring malondialdehyde formation is a modification of the method of Sagar *et al*, 1992 and Esterbauer *et al*, 1990. The intensity of the pink chromogen formed during the acid-heating stage, is indicative of the extent of lipid peroxidation. The standard curve utilises an external standard: 1,1,3,3- tetraethoxypropane (TEP) as a measure of the lipid peroxide index (Figure 2.5.).

2.4.1. MATERIALS

2.4.1.1. Animals

Male Wistar rats of the albino strain with a body weight of 200 - 250 g were randomly assembled into test groups of five (n=5) and maintained in conditions described in Section 2.1. The animals were sacrificed between 11h00 and 12h00 by neck fracture. After decapitation, the brains were removed and the blood and adhering tissue was removed with 0.9% NaCl. Thereafter, the brains were frozen in liquid nitrogen and stored at -70°C until required.

2.4.1.2. Chemicals

Butylated hydroxytoluene (BHT), trichloroacetic acid (TCA), thiobarbituric acid (TBA) and 1,1,3,3-tetraethoxypropane (TEP) were all obtained from Sigma Chemical Co., St. Louis, USA. All other chemicals were obtained from Saarchem Limited, Krugersdorp, South Africa. Deionised water (Milli R/Q System, Millipore) was used to prepare all the solutions required.

2.4.2. METHODS

2.4.2.1. Chemicals

Thiobarbituric acid (0.33%) was prepared in deionised water. 10% w/v Trichloroacetic acid

was prepared by dissolving the compound in deionised water. Butylated hydroxytoluene (0.5 g/L) was dissolved in methanol, while TEP was dissolved in water. 1,1,3,3-Tetraethoxypropane standard was prepared by diluting 10 μ l in 10 ml and subsequently diluting the prepared solution in water to produce the required final concentration of 0 - 50 nmol MDA per ml.

2.4.2.2. Tissue Preparation

The brain tissue was thawed in a 37°C water bath. The brain was homogenised (5% w/v) in ice-cold 50mM TRIS-HCl buffer at a pH of 7.4. For each experiment n=5 rats were used.

2.4.2.3. The Thiobarbituric Acid (TBA) Test

An aliquot of 1 ml, which included 900 μ l brain homogenate and 100 μ l of the test compound (final concentration), was incubated at 37°C for a period of 1 hour. After the incubation period, the reaction was stopped by the addition of 10 % w/v TCA (1 ml). However, the addition of the antioxidant BHT (0.5 ml) preceded that of TCA so that any further hydrolysis of the fatty acids through TCA or the heating stage of the assay would be inhibited (Esterbauer *et al*, 1990; Draper *et al*, 1990). The samples were centrifuged at 4000 rpm for a period of 20 minutes. Thiobarbituric acid (0.5 ml) was added to 2 ml of the supernatant. The samples were then incubated in a 95°C water bath for a period of 1 hour. The solutions were cooled for a period of 5 minutes and 2 ml of butanol was added and vortexed for a period of 30

seconds to reduce intra-assay variability. The samples were then centrifuged at 2000 rpm for a period of 5 minutes to improve the separation between the aqueous and organic phase. The top layer was extracted and the absorbance was read at 532 nm against a blank. An external standard, 1,1,3,3-tetraethoxypropane (TEP), was used (Figure 2.5.; page 102) and the lipid peroxide level was expressed in terms of nmol MDA.

2.4.2.4. Protein Determination of Brain Homogenates

The protein concentration of the brain homogenates was determined using a modification of the method introduced by Lowry *et al*, 1951. Bovine serum albumin (BSA) was used as the external standard (Figure 2.6.; page 104).

An aliquot of 1.2 ml of protein sample (dilution 1: 50) was placed in a test tube. Copper alkaline solution (6 ml) was added to the sample and vortexed immediately. A waiting period of 10 minutes ensued. It was essential to prepare the copper alkaline solution fresh whenever it was necessary. The solution was mixed in the following order to make a total volume of 100 ml: 1ml of 1% copper sulphate, 1 ml 2% sodium tartrate and 98 ml 2% sodium carbonate in 0.1N NaOH.

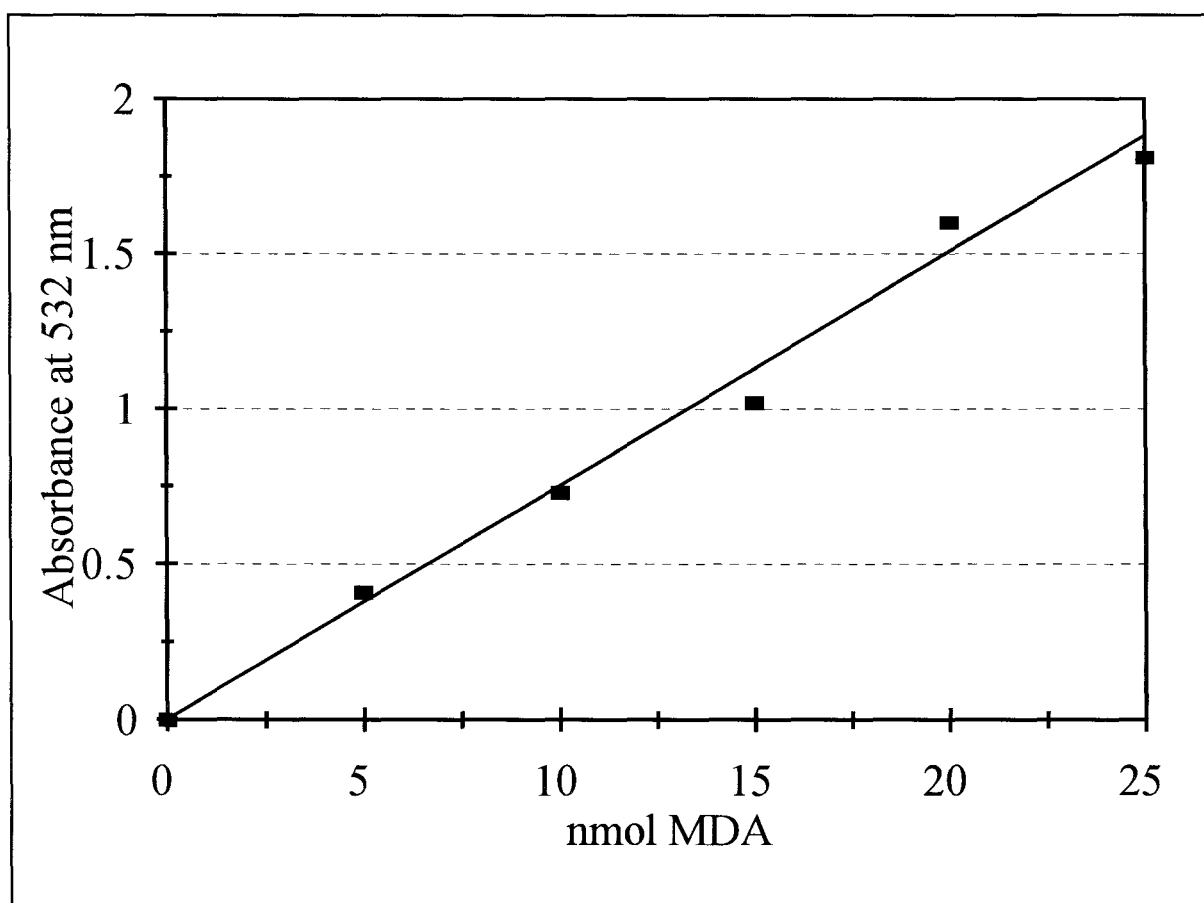


Figure 2.5. A typical standard curve for malondialdehyde (MDA) formation using 1,1,3,3 tetraethoxypropane (TEP) as an external standard. Values are a mean of triplicate determinations ($r^2 = 0.989$).

Once the ten minute waiting period was over, 0.3 mls of Folin-Ciocalteu reagent was added and the solution was immediately vortexed. The test tubes were allowed to stand for a period of 30 minutes, before the absorbance was read at 500 nm against a blank. The standard curve was prepared in the same manner, except that the test tubes contained the BSA solution (1 mg/ml) with varying concentrations ranging from 0 $\mu\text{g/ml}$ to 300 $\mu\text{g/ml}$. The protein assay is summarised in Table 2.4. All the determinations were made in triplicate.

Table 2.4. Review of the protein assay determination for brain homogenates.

PROTEIN CONCENTRATION ($\mu\text{g/ml}$)	0	50	100	150	200	250	300
BSA(1 mg/ml) (μl)	0	60	120	180	240	300	360
Deionised water (μl)	1200	1140	1080	1020	960	900	840
Alkaline Copper reagent (ml)	6	6	6	6	6	6	6
Stand at room temperature for a period of 10 minutes							
Folin-Ciocalteu reagent (ml)	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Total volume (ml)	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Stand at room temperature for a period of 30 minutes							
Read absorbance at 500 nm							

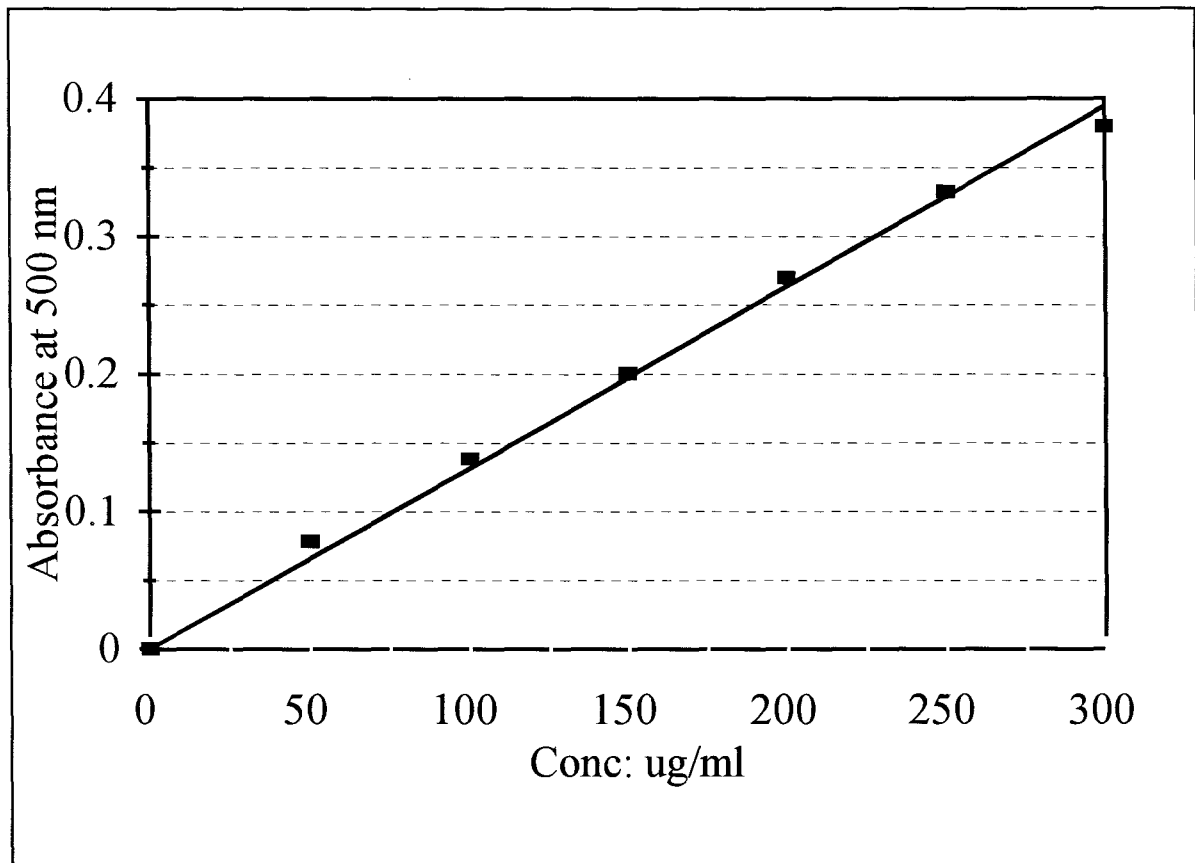


Figure 2.6. A typical protein standard curve using bovine serum albumin (BSA). Each point represents a mean value for triplicate determinations ($r^2 = 0.996$).

2.4.3. RESULTS

The results for the lipid peroxidation assay were expressed as nmol MDA using the external standard TEP (Figure 2.5.; page 102). The final results, however, were expressed as nmol MDA/mg protein. Due to high levels of lipid peroxidation in the brain, a concentration of 5% w/v for the brain homogenate was deemed appropriate. A linear response in lipid peroxidation occurred up to 1 hour incubation, whereafter it stabilised. It was decided that incubation for a period of one hour was appropriate for the incubation of the test compounds used in the study.

2.4.4. DISCUSSION

The TBA test offers sensitivity and versatility for the detection of lipid peroxidation. Many laboratories have modified this assay to suit their specific requirements. It is necessary to take the appropriate precautions to prevent any additional effects produced by the compounds added. Those may include iron and any chelators which may stimulate lipid peroxidation in the presence of reducing agents. Each of the salient points mentioned must be taken into consideration when applying the assay to a complex biological system.

CHAPTER 3

THE INFLUENCE OF KYNURENINES AND SEROTONERGIC COMPOUNDS ON TRYPTOPHAN 2,3- DIOXYGENASE ACTIVITY

3.1. INTRODUCTION

Tryptophan 2,3-dioxygenase, a key enzyme involved in TRP catabolism, is a haem-dependent liver cytosolic enzyme which is responsible for the oxidative cleavage of TRP to form N-formylkynurenine (Mehler *et al*, 1950; Uchida *et al*, 1992; Brady, 1995). The enzyme may exist in two forms in certain species such as humans and rats; the actively reduced holoenzyme which does not require the presence of haem and the non-active apoenzyme which requires the addition of exogenous haem for activity (Badawy, 1975). Activation of the apoenzyme *in vitro* occurs when the apoenzyme is conjugated with haem, the resultant oxidised holoenzyme is activated to the reduced holoenzyme (ferrous form) in the presence of TRP (Knox, 1966). Various hormones such as hydrocortisone and glucocorticoids are responsible for induction of the enzyme *in vivo*, these compounds are ultimately accountable for the rapid turnover of TRP

to further metabolites (Young, 1981).

Another pathway in which TRP is metabolised is via the enzyme TH which is rate-limiting in the formation of 5-HT (Salter *et al*, 1995; Gal *et al*, 1978). Disturbed synthesis of biogenic amines is prevalent in depressive disorders of which 5-HT is thought to be responsible for mood changes which characterise these illnesses. The two most important factors which may contribute to the decrease of TRP availability to the brain for 5-HT synthesis are: (1) an increased TDO activity which results in a decreased availability of circulating TRP levels and (2) the presence of LNAA's and KYN which compete with TRP for the same carrier system for uptake into the brain (Badawy *et al*, 1981). Thus, an inverse relationship exists between TDO activity and 5-HT synthesis since the former is able to affect circulating levels of TRP (Badawy *et al*, 1981; Salter *et al*, 1995).

Tryptophan 2,3-dioxygenase is therefore an important regulator of whole-body TRP catabolism and hence brain levels of TRP and 5-HT. Serotonin synthesis may be enhanced, to a limited extent, by inhibiting TDO activity and therefore a greater antidepressant efficiency will be achieved.

The tryptophan 2,3-dioxygenase enzyme assay employed in this study was previously described by Badawy and Evans, 1975 and 1981. The activity of the enzyme is determined by measuring the formation of KYN from TRP at 365 nm. The holoenzyme is measured in the absence and total activity in the presence of exogenous haematin. The apoenzyme is calculated

as the difference between the holoenzyme and total activity. Final results were expressed as $\mu\text{moles kynurenine}$ ($\epsilon = 4540 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$).

This study was an attempt to ascertain whether any of the endogenous compounds of the kynurenine or the serotonergic pathway were able to inhibit tryptophan 2,3-dioxygenase activity. The inhibition of the enzyme, in turn, would reduce the efficiency whereby TRP is catabolised and so increase the amino acid's uptake into the brain and consequently 5-HT synthesis.

3.2. MATERIALS

3.2.1. Animals

Eleven groups of five animals each were randomly assembled and maintained as described in Section 2.1. The animals were killed at approximately 12h00. The livers were removed as previously described in Section 2.1. The livers were perfused with 0.9% NaCl until all the blood was removed before being rapidly frozen in liquid nitrogen and stored at -70°C until required.

3.2.2. Chemicals

All reagents used were of the highest purity. Kynurenic acid, 3-HANA, QA, 5-HT, NAS,

aMT, 5-HTOH, 5-MTOH, 5-HIAA, 5-MIAA, ascorbic acid (vitamin C) and 6-MBOA were all obtained from Sigma Chemical Co., St. Louis, USA. The additional chemicals required for the enzyme assay were obtained from Saarchem Limited, Krugersdorp, South Africa.

3.3. METHODS

3.3.1. Chemicals

Deionised water (Milli R/Q system, Millipore) was used to prepare all the solutions required. Kynurenic acid, 3-HANA and QA dissolution was enhanced with a minimal amount (1 ml) of 0.1 M NaOH and made up to the final volume with deionised water. Serotonin, 5-HIAA and 5-HTOH concentrations were made up to their final volume with 0.1% ascorbic acid. Melatonin, 5-MIAA and 5-MTOH were dissolved in a 4% ethanol solution. The subsequent dilutions required were made up with 0.1% ascorbic acid-ethanol solution. 6-Methoxy-2-benzoxazolinone was dissolved in a 40% ethanol solution. The subsequent concentrations and dilutions of 6-MBOA were made up in 0.1% ascorbic acid-ethanol solution.

3.3.2. Tissue Preparation

The liver was thawed over a prolonged period in 0.2M sodium phosphate buffer on ice. The liver, once thawed, was prepared according to Section 2.2.2.2.

3.3.3. Tryptophan 2,3-Dioxygenase Determination

Activity of tryptophan 2,3-dioxygenase was determined as described in Section 2.2.2.3.

3.3.4. Statistical Analysis

All results were analysed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test to compare the different groups. Values of $p < 0.05$ were accepted as statistically significant.

3.4. RESULTS:

Table 3.4.1. Effect of kynurenic acid (KYNA) on TDO activity.

TREATMENT	TRYPTOPHAN 2,3-DIOXYGENASE ACTIVITY		
	Total Activity (%)	Holoenzyme (%)	Apoenzyme (%)
Control	100 ^a	100 ^b	100 ^c
KYNA Concentration			
10 μ M	115.8 \pm 4.6 ^d	108.8 \pm 9.9 ^e	119.0 \pm 5.3 ^f
100 μ M	108.8 \pm 1.4 ^g	114.5 \pm 3.7 ^h	104.5 \pm 1.2 ⁱ

Significance: a vs d: $p < 0.01$
 c vs f: $p < 0.01$ f vs i: $p < 0.01$

Table 3.4.2. The effect of 3-hydroxyanthranilic acid (3-HANA) on TDO activity.

TREATMENT	TRYPTOPHAN 2,3-DIOXYGENASE ACTIVITY		
	Total Activity (%)	Holoenzyme (%)	Apoenzyme (%)
Control	100	100	100
3-HANA Concentration			
10 μ M	112.2 \pm 2.8	114.0 \pm 5.5	110.2 \pm 4.5
100 μ M	112.2 \pm 8.3	116.4 \pm 5.8	112.0 \pm 6.7

Significance: none

Table: 3.4.3. The effect of quinolinic acid (QA) on TDO activity.

TREATMENT	TRYPTOPHAN 2,3-DIOXYGENASE		
	Total Activity (%)	Holoenzyme (%)	Apoenzyme (%)
Control	100	100	100
QA Concentration			
10 μ M	107.3 \pm 4.9	109.0 \pm 3.7	106.3 \pm 8.6
100 μ M	103.5 \pm 8.1	106.5 \pm 7.5	102.0 \pm 9.7

Significance: none

Table 3.4.4. The effect of 5-hydroxyindoleacetic acid (5-HIAA) on TDO activity.

TREATMENT	TRYPTOPHAN 2,3-DIOXYGENASE		
	Total Activity (%)	Holoenzyme (%)	Apoenzyme (%)
Control	100	100	100
5-HIAA Concentration			
10 μ M	99.8 \pm 2.9	101.8 \pm 5.8	100.0 \pm 9.4
100 μ M	108.3 \pm 7.4	107.5 \pm 8.7	100.0 \pm 5.7

Significance: none

Table 3.4.5. The effect of 5-methoxyindoleacetic acid (5-MIAA) on TDO activity.

TREATMENT	TRYPTOPHAN 2,3-DIOXYGENASE		
	Total Activity (%)	Holoenzyme (%)	Apoenzyme (%)
Control	100	100	100
5-MIAA Concentration			
10 μ M	98.0 \pm 7.8	97.5 \pm 7.0	100.0 \pm 9.8
100 μ M	103.3 \pm 4.0	94.8 \pm 6.5	110.8 \pm 6.5

Significance: none

Table 3.4.6. The effect of serotonin (5-HT) on TDO activity.

TRYPTOPHAN 2,3-DIOXYGENASE			
TREATMENT	Total Activity (%)	Holoenzyme (%)	Apoenzyme (%)
Control	100 ^a	100 ^b	100 ^c
5-HT Concentration			
10 μ M	105.5 \pm 8.2 ^d	116.0 \pm 3.3 ^e	97.3 \pm 9.8 ^f
100 μ M	80.5 \pm 3.2 ^g	88.3 \pm 6.7 ^h	74.8 \pm 4.5 ⁱ

Significance: a vs g: p < 0.05 d vs g: p < 0.05
 b vs e: p < 0.05 e vs h: p < 0.01
 c vs i: p < 0.05 f vs i: p < 0.05

Table 3.4.7. The effect of 5-hydroxytryptophol (5-HTOH) on TDO activity.

TRYPTOPHAN 2,3-DIOXYGENASE			
TREATMENT	Total Activity (%)	Holoenzyme (%)	Apoenzyme (%)
Control	100 ^a	100 ^b	100 ^c
5-HTOH Concentration			
10 μ M	94.3 \pm 11.7 ^d	97.0 \pm 9.5 ^e	89.0 \pm 11.5 ^f
100 μ M	70.5 \pm 3.4 ^g	95.0 \pm 2.2 ^h	55.0 \pm 5.1 ⁱ

Significance: a vs g: p < 0.05 d vs g: p < 0.05
 c vs i: p < 0.01 f vs i: p < 0.01

Table 3.4.8. The effect of 5-methoxytryptophol (5-MTOH) on TDO activity.

TREATMENT	TRYPTOPHAN 2,3-DIOXYGENASE		
	Total Activity (%)	Holoenzyme (%)	Apoenzyme (%)
Control	100	100	100
5-MTOH Concentration			
10 μ M	86.5 \pm 9.8	97.0 \pm 9.2	79.8 \pm 11.4
100 μ M	75.0 \pm 8.5	83.5 \pm 5.1	69.8 \pm 10.6

Significance: none

Table 3.4.9. The effect of N-acetylserotonin (NAS) on TDO activity.

TREATMENT	TRYPTOPHAN 2,3-DIOXYGENASE		
	Total Activity (%)	Holoenzyme (%)	Apoenzyme (%)
Control	100 ^a	100 ^b	100 ^c
NAS Concentration			
10 μ M	79.8 \pm 2.8 ^d	93.5 \pm 9.3 ^e	74.0 \pm 8.1 ^f
100 μ M	69.3 \pm 3.3 ^g	78.8 \pm 6.8 ^h	64.5 \pm 3.7 ⁱ

Significance: a vs g: p < 0.001 a vs d: p < 0.001 d vs g: p < 0.05
 c vs f: p < 0.01 f vs i: p < 0.01

Table 3.4.10. The effect of melatonin (aMT) on TDO activity.

TRYPTOPHAN 2,3-DIOXYGENASE			
TREATMENT	Total Activity (%)	Holoenzyme (%)	Apoenzyme (%)
Control	100 ^a	100 ^b	100 ^c
aMT Concentration			
10 μ M	100.2 \pm 6.5 ^d	111.4 \pm 3.4 ^e	95.6 \pm 10.3 ^f
100 μ M	78.6 \pm 5.6 ^g	101.6 \pm 8.1 ^h	69.2 \pm 3.4 ⁱ

Significance: a vs g: p < 0.01 d vs g: p < 0.05
 c vs i: p < 0.05 f vs i: p < 0.05

Table 3.4.11. The effect of 6-methoxy-2-benzoxazolinone (6-MBOA) on TDO activity.

TRYPTOPHAN 2,3-DIOXYGENASE			
TREATMENT	Total Activity (%)	Holoenzyme (%)	Apoenzyme (%)
Control	100 ^a	100 ^b	100 ^c
6-MBOA Concentration			
10 μ M	97.5 \pm 5.0 ^d	97.5 \pm 8.3 ^e	96.5 \pm 5.1 ^f
100 μ M	58.3 \pm 4.9 ^g	69.3 \pm 5.9 ^h	45.6 \pm 4.2 ⁱ

Significance: a vs g : p < 0.001 g vs d: p < 0.001
 b vs h : p < 0.01 h vs e: p < 0.01
 c vs i: p < 0.001 i vs f: p < 0.001

Table 3.4.12. Comparison of the structure-activity relationship for the tryptophan analogues of the kynurenine pathway.

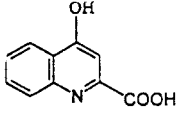
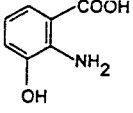
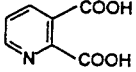
TRYPTOPHAN ANALOGUES	CHEMICAL STRUCTURE	% INHIBITION OF THE APOENZYME AT 100 μ M
Kynurenic acid (KYNA)		NONE
3-Hydroxyanthranilic acid (3-HANA)		NONE
Quinolinic acid (QA)		NONE

Table 3.4.13. Comparison of the structure-activity relationship for the tryptophan analogues of the serotonergic pathway.

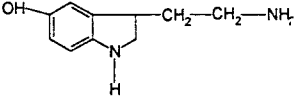
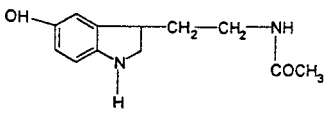
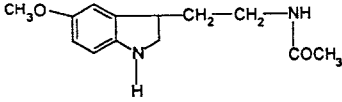
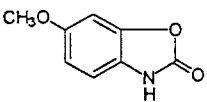
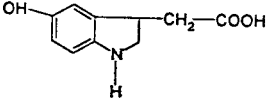
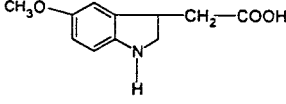
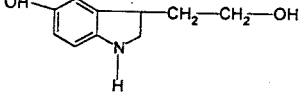
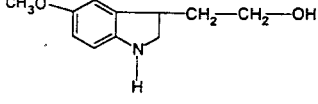
TRYPTOPHAN ANALOGUES	CHEMICAL STRUCTURE	% INHIBITION OF THE APOENZYME AT 100 μ M
Serotonin (5-HT)		25
N-Acetylserotonin (NAS)		36
Melatonin (aMT)		31
6-Methoxy-2-benzoxazolinone (6-MBOA)		55

Table 3.4.14. Comparison of the structure-activity relationship for the tryptophan analogues of the serotonergic pathway (...continued).

TRYPTOPHAN ANALOGUES	CHEMICAL STRUCTURE	% INHIBITION OF THE APOENZYME AT 100 μ M
5-Hydroxyindoleacetic acid (5-HIAA)		NONE
5-Methoxyindoleacetic acid (5-MIAA)		NONE
5-Hydroxytryptophol (5-HTOH)		45
5-Methoxytryptophol (5-MTOH)		30

3.5. DISCUSSION

The concentration of circulating TRP is important for the regulation of 5-HT synthesis in the brain. Depressed levels of 5-HT have been implicated in mood disorders. The key regulatory enzyme for TRP catabolism in the liver, is TDO. This liver enzyme is cytosolic and with the addition of its cofactor (haem) the activity rises. Tryptophan 2,3-dioxygenase is responsible for altering the availability of TRP for uptake into the brain. The accessibility of this circulating amino acid may be enhanced with decreased enzyme activity of TDO .

Inhibition of TDO activity may occur via two mechanisms: (a) interference of the test compound with the conjugation of the apoenzyme and its cofactor, haem (Badawy *et al*, 1981), or (b) the structure-activity relationship of the test compound at the active site of the enzyme (Uchida *et al*, 1992).

The kynurenines, metabolites of the enzyme tryptophan 2,3-dioxygenase, *viz.*, KYNA, 3-HANA and QA, were not able to inhibit the apoenzyme significantly at a concentration of 100 μ M with respect to the control group.

The indoleamines, excluding the indoleacetic acids of the serotonergic pathway in the brain, were all able to reduce the activity of TDO. A possible explanation for the indoleacetic acid's inability to reduce the enzyme activity is that at physiological pH (pH 7.0), the carboxyl group is likely to have ionised. The resultant anionic charge may cause repulsion at the active site preventing any interaction with the active site.

The compounds which proved to be potential inhibitors at a concentration of 100 μM are presented in ascending order: 5-HT ($p < 0.05$) < aMT ($p < 0.05$) < NAS ($p < 0.01$) < HTOH ($p < 0.01$) < 6-MBOA ($p < 0.001$). Studies by Uchida *et al*, 1992, showed that a NH group is essential for the compound to bind to the catalytic site of the enzyme and is displayed by all serotonergic compounds. The alanine side chain, $\text{CH}(\text{NH}_2)\text{COOH}$, is indispensable for the substrate to take the correct configuration for the reaction to occur at the catalytic site. Although compounds such as 5-HTP have the appropriate side chain, the functional group present at the fifth position on the benzene ring (whether it may be a hydroxyl group (OH) or a methoxy (CH_3O) group) may cause variations in the catalytic efficiency of the enzyme. The metabolites further along in the pathway have variations in their alanine side chain thereby reducing their potential as substrates. However, the groups present at the fifth position influence the enzyme with regard to its catalytic activity. The inactivity of the enzyme caused by the methoxy or hydroxyl group, especially the latter, may arise from the interactions of the OH group (the hydrogen bonding) with the surrounding amino acid side-chain(s) within the haem pocket. Indole derivatives with OH and CH_3O functional groups at the fifth position have the ability to act as electron donors and are more labile to oxygen than those without it and this reduces the structural flexibility required for the catalytic reaction to proceed. This is evident with the compounds which produced significant inhibition such as 5-HT, NAS and especially 6-MBOA which was able to reduce enzyme activity by approximately 55 percent.

Another mechanism which may explain the reduction of enzyme activity is that most of the indoleamines mentioned above may interfere with the conjugation of the haem cofactor and

the apoenzyme, thereby reducing its activity. However, studies showed that the degree of saturation of the apoenzyme did not change significantly with regard to the addition of any of the compounds mentioned. The results show that the mechanism of the structure-activity relationship of the test compound at the active site may be important with regards to the response that the indoleamines were able to achieve by inhibiting the enzyme.

6-Methoxy-2-benzoxazolinone was shown to be the most potent inhibitor of TDO. In doing so, it is capable of rendering more tryptophan available for uptake into the brain for the conversion to serotonin, although further studies are necessary.

CHAPTER 4

THE EFFECT OF KYNURENIC ACID AND QUINOLINIC ACID ON PINEAL INDOLE METABOLISM

4.1. INTRODUCTION

The pineal gland, considered to be the "seat of the soul" by Descartes (1596-1650), has received extensive attention in the past two decades. Studies have established the pineal gland as an integral and important component of the neuroendocrine system (Wainwright, 1977; Reiter, 1989). The pineal gland and its indoles have been functionally related to every endocrine gland in nonhuman organisms (Reiter, 1989). The pineal metabolites are synthesised from the precursor amino acid, TRP. The characteristic neurohormone, aMT, is formed from its precursor 5-HT. The bulk of 5-HT synthesised in the pineal gland is metabolised by the enzyme MAO from TRP; while aMT is synthesised from a minor portion of 5-HT after acetylation, by the enzyme NAT, and then methylation by HIOMT (Morton, 1990). It has been suggested that the methoxyindoles are responsible for the mediation of the

pineal function and these are exhibited in ascending order of importance: 5-MIAA, 5-MTOH and aMT (Morton, 1990).

In progressive neurodegenerative diseases, KYNA and QA are produced extrahepatically by the enzyme IDO. Kynurenic acid is synthesised from KYN which is produced in the brain or taken up by LNAA's into the CNS from the blood (Heyes, 1993). Kynurenic acid was found to be an antagonist of the NMDA receptor (Jauch *et al*, 1995). Quinolinic acid is synthesised within the CNS from 3-HANA via the enzyme 3-HAO which is located in the astrocytes (Heyes, 1993). Quinolinic acid, an agonist of the NMDA receptor, may be involved in the pathogenesis of seizures or neurodegeneration of inflammatory neurological diseases. Quinolinic acid-induced neurotoxicity produces a neurochemical profile which mimics the selectivity of the nerve cell loss which is prevalent in Huntington's disease (Beal *et al*, 1991).

During broad spectrum inflammation in neurological diseases, QA and KYNA are increased due to the induction of the enzyme IDO in the CNS or systemic tissues (Heyes, 1993). The concentration of QA has also been shown to increase with age (Moroni *et al*, 1984b). Whether QA increases with age or due to immune stimulation, it may be able to induce neurological damage by excitation of the NMDA receptors with the subsequent rise of calcium levels to excitotoxic proportions (Heyes, 1993; Jauch *et al*, 1995). The rise of calcium may be responsible for the activation of calcium-dependent protein kinases and phospholipases which may result in the degradation of neurofilament and myelin proteins in neuronal tissue (Braugher, 1987).

Melatonin levels decrease with age which correlates with the decrease in the aMT amplitude cycle (Reiter, 1995). A reduction of aMT synthesis is usually indicative of pineal failure. A loss of aMT may result in accumulated free radical damage and eventual cell death which can cause immune system failure, cancer and/or neurodegenerative diseases such as Alzheimer's disease and Huntington's disease.

In order to obtain an overall picture of indole metabolism in the pineal gland, a technique was used that would be able to monitor the indole metabolites with pharmacological and biochemical manipulations. The pineal gland in the rat is small and easily accessible for intact organ culture (Morton, 1990). The assay used in this study was previously described by Klein and Notides, 1969 and modified by Daya *et al*, 1982.

The study was done in an attempt to ascertain whether quinolinic acid or kynurenic acid produced during broad spectrum inflammation, is responsible for influencing the synthesis of the metabolites in the pineal gland.

4.2. MATERIALS

4.2.1. Animals

Male Wistar rats of the albino strain were assigned into groups of four (n=4). The animals were maintained in conditions as described in Section 2.1.

4.2.2. Chemicals

All the chemicals used were of the highest purity. Kynurenic acid and QA were obtained from Sigma Chemical Co., St. Louis, USA. All other reagents were obtained from Saarchem Limited, Krugersdorp, South Africa.

4.3. METHODS

4.3.1. Chemicals

All reagents were prepared with deionised water (Milli R/Q, Millipore system). Kynurenic acid and QA were dissolved in 0.1% ascorbic acid. Subsequent dilutions were made with 0.1% ascorbic acid.

4.3.2. Tissue Preparation

The pineal gland was removed as described in Section 2.1. After the removal the pineal gland was immediately placed in test tubes containing BJGb culture medium.

4.3.3. Pineal Organ Culture

The effect of KYNA and QA on pineal indole metabolism was determined as described in

Sections 2.3.2.2 and 2.3.2.3.

4.3.4. Statistical Analysis

All results were analysed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test to compare the different groups. Values of $p < 0.05$ were accepted as statistically significant.

4.4. RESULTS

The final results were expressed as DPM/10 μ l medium spotted/pineal gland. The data was analysed by ANOVA. Statistical differences between the means were determined using the Student-Newman-Keuls test. The radioactivity corresponding to each of the metabolites isolated from the culture medium following the incubation of the pineal glands with [14 C] serotonin is graphically represented in Figures 4.1. to 4.6.

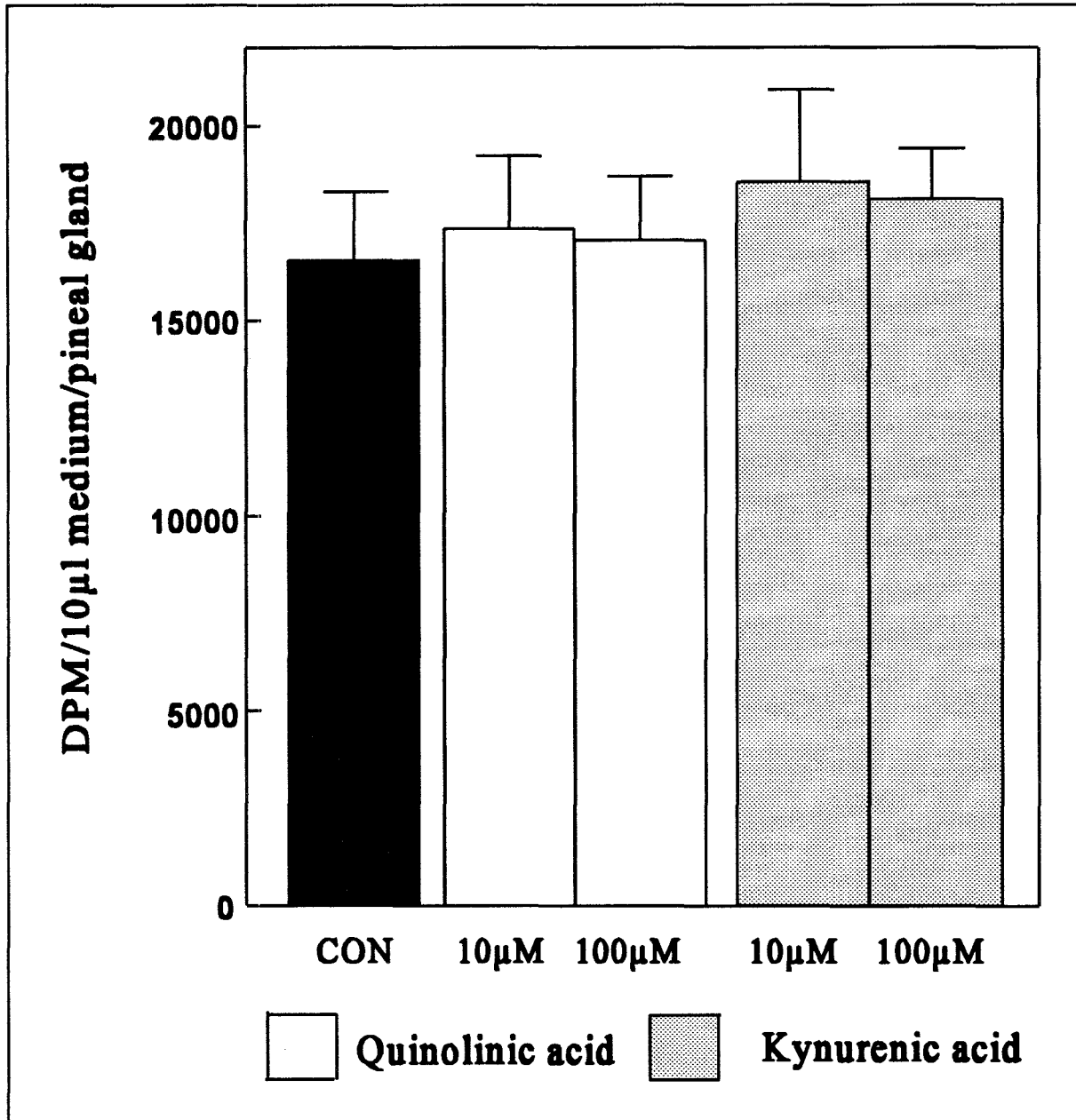


Figure 4.1. The effect of quinolinic acid (QA) and kynurenic acid (KYNA) at different concentrations on 5-hydroxyindoleacetic acid (5-HIAA) synthesis in rat pineal organ culture. Values are mean of $n=4$ rats \pm S.E.M. Significant differences were determined by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test.

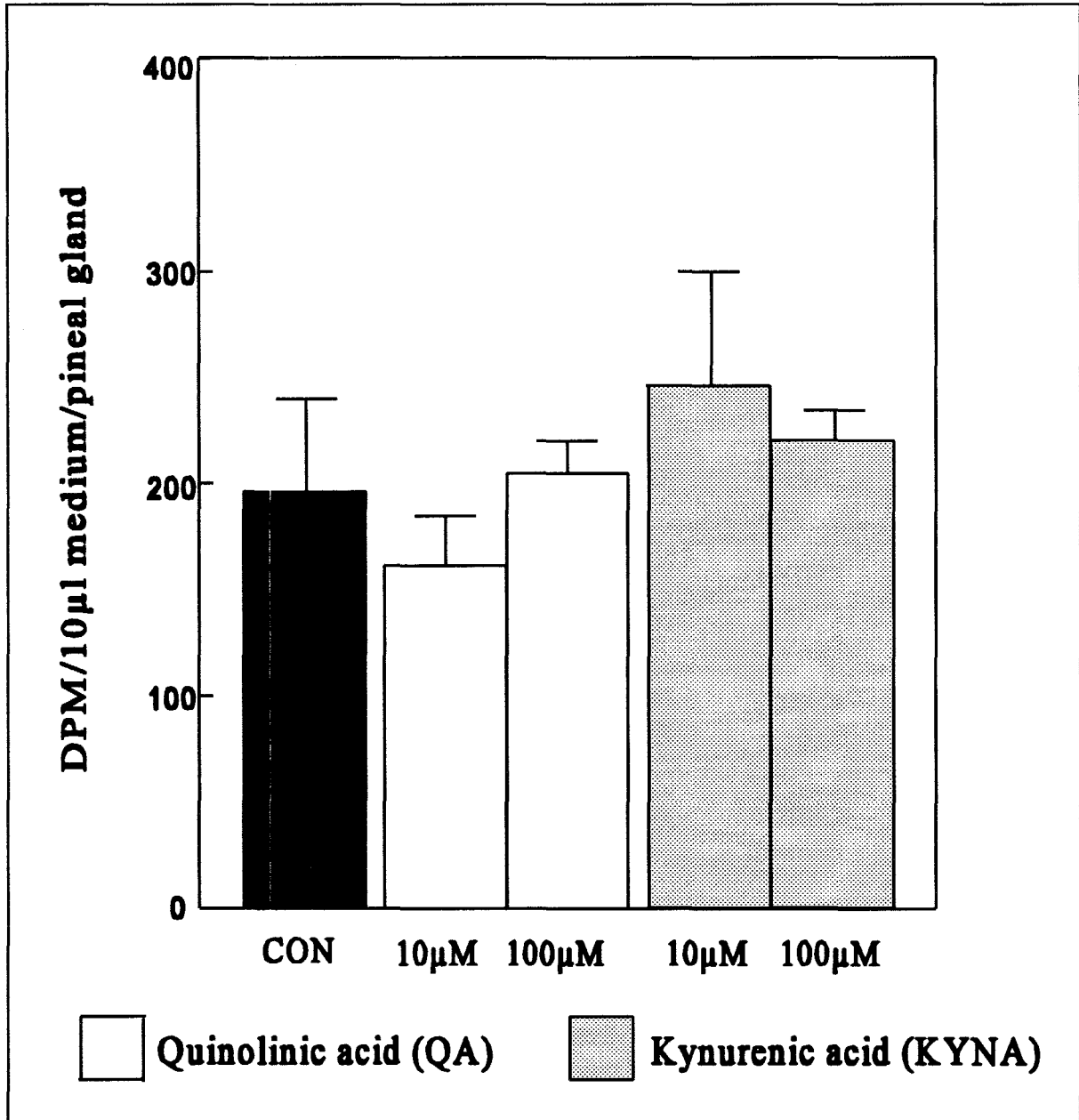


Figure 4.2. The effect of quinolinic acid (QA) and kynurenic acid (KYNA) at different concentrations on 5-methoxyindoleacetic acid (5-MIAA) synthesis in rat pineal organ culture. Values are a mean of n=4 rats ± S.E.M. Statistical differences were determined by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test.

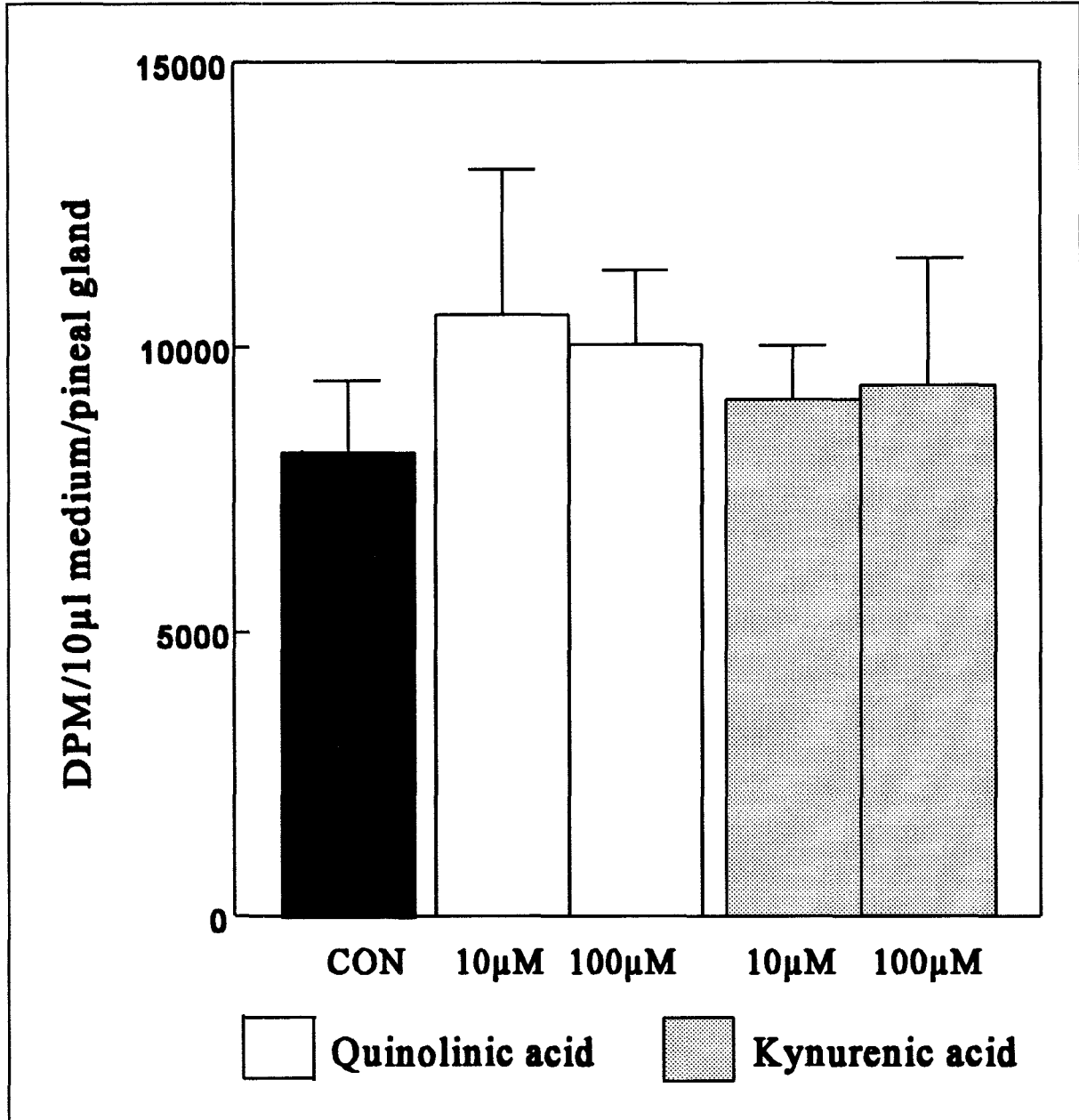


Figure 4.3. The effect of quinolinic acid (QA) and kynurenic acid (KYNA) at different concentrations on 5-hydroxytryptophol (5-HTOH) synthesis in rat pineal organ culture. Values are a mean of n=4 rats \pm S.E.M. Statistical differences are determined by the one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test.

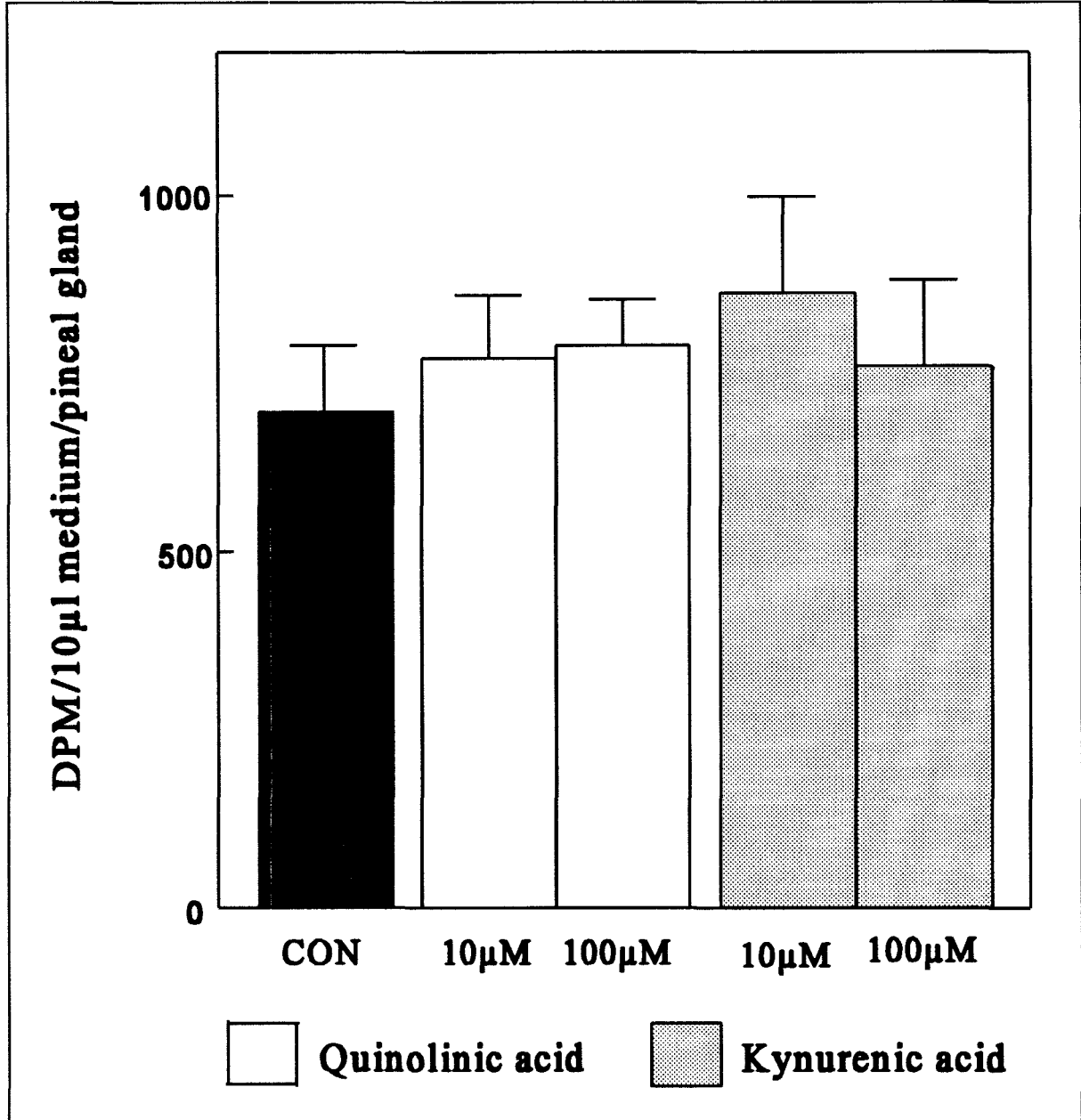


Figure 4.4. The effect of quinolinic acid (QA) and kynurenic acid (KYNA) at different concentrations on 5-methoxytryptophol (5-MTOH) synthesis in rat pineal organ culture. Values are a mean of n=4 rats. Statistical differences were determined by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test.

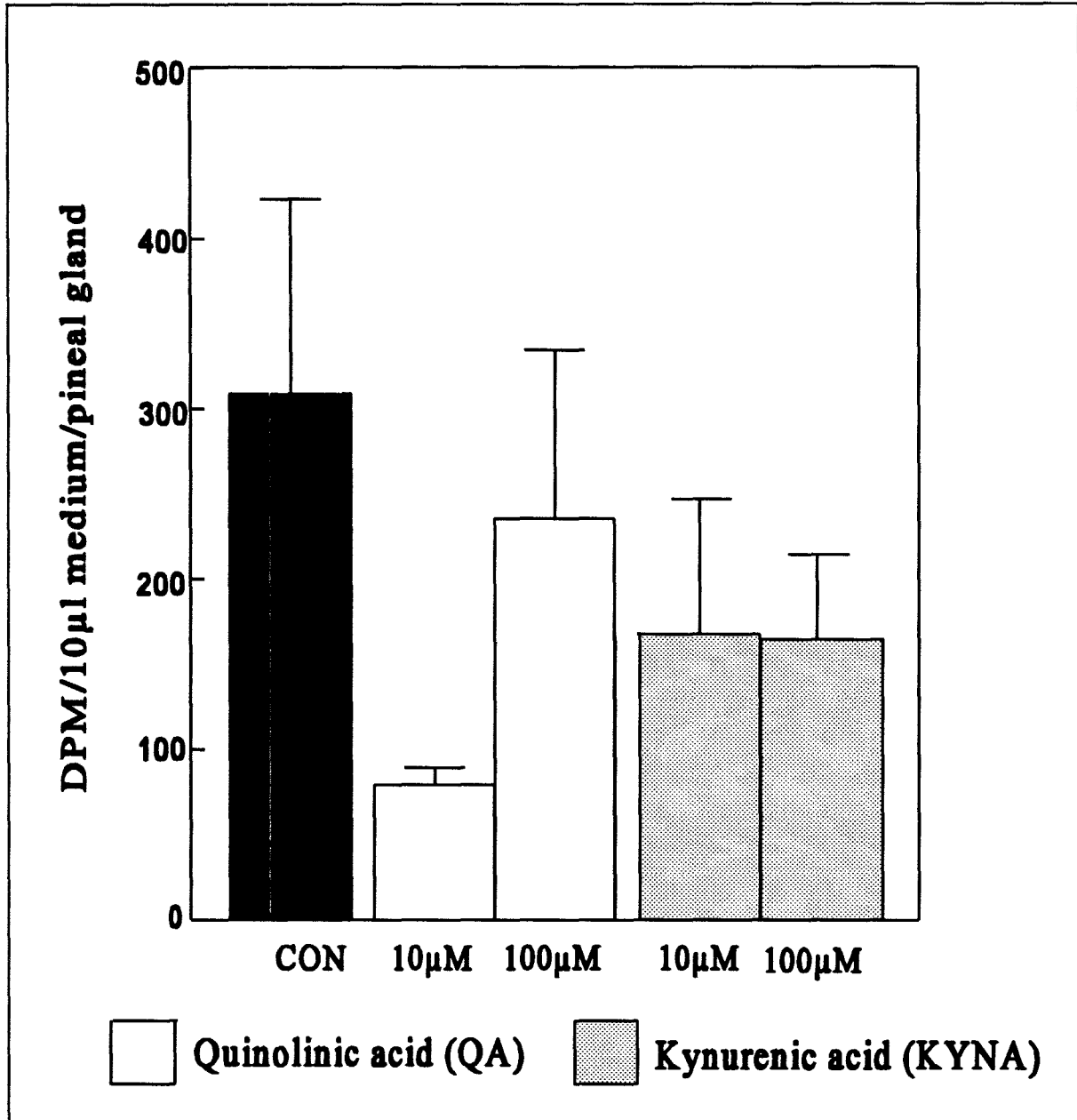


Figure 4.5. The effect of quinolinic acid (QA) and kynurenic acid (KYNA) at different concentrations on N-acetylserotonin (NAS) synthesis in rat pineal organ culture. Values are a mean of $n=4$ rats \pm S.E.M. Statistical differences are determined by the one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Significance of control (CON) versus QA at a concentration of 10 μ M is $p < 0.01$.

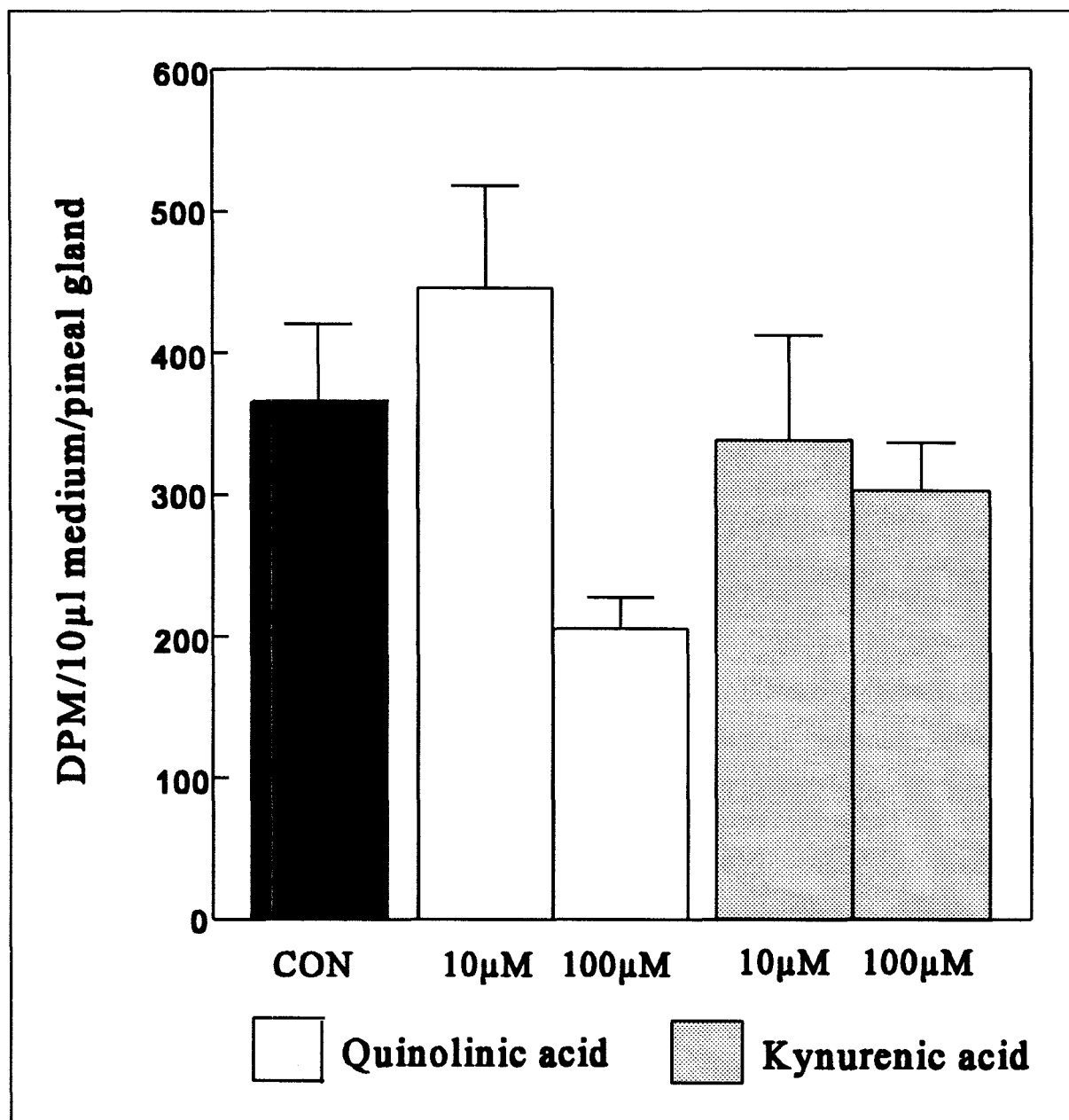


Figure 4.6. The effect of quinolinic acid (QA) and kynurenic acid (KYNA) at different concentrations on melatonin (aMT) synthesis in rat pineal organ culture. Values are a mean of $n=4$ rats \pm S.E.M. Statistical differences are determined by the one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Significance for con (CON) versus QA at a concentration 100 μ M is $p < 0.01$.

Both quinolinic acid and kynurenic acid at concentrations of 10 μ M and 100 μ M produced no significant changes with regard to the synthesis of 5-HIAA (Figure 4.1.), 5-MIAA (Figure 4.2.), 5-HTOH (Figure 4.3.) and 5-MTOH (Figure 4.4.). Quinolinic acid, at a concentration of 10 μ M, was able inhibit NAS synthesis significantly ($p < 0.01$) (Figure 4.5.). Melatonin synthesis was significantly reduced by QA at a concentration of 100 μ M ($p < 0.01$) (Figure 4.5.). Kynurenic acid, however, had no effect on NAS and aMT synthesis.

4.5. DISCUSSION

The distribution of the enzyme, IDO, within the brain has been found to be the highest in the pineal gland and choroid plexus of the rabbit. Whether this is true for the rat remains to be determined (Fujiwara *et al*, 1978). The ability of this enzyme to utilise TRP and various other substrates allows for the possibility that it may regulate indoleamine synthesis. Fujiwara *et al*, 1978, reported that 32% percent of the total metabolites of TRP was attributed to KYN and thus it appears that the major pathway of TRP metabolism in the pineal gland was through the kynurenine pathway. During inflammatory diseases, IDO, would be induced creating a potential to produce increased kynurenine metabolites instead. Indoleamine 2,3-dioxygenase may play the regulatory role of free TRP levels in the pineal gland.

Kynurenic acid and QA had no significant effect on 5-HIAA and 5-MIAA synthesis. This may suggest that the enzymes responsible for the synthesis of these compounds, *viz.*, aldehyde dehydrogenase and HIOMT respectively, were not inhibited by QA and KYNA at 10 μ M and

100 μM . For 5-HTOH and 5-MTOH synthesis, the same result was observed thus suggesting that neither enzyme, alcohol dehydrogenase nor HIOMT, responsible for their synthesis was inhibited by QA or KYNA. N-Acetylserotonin synthesis was significantly inhibited by QA at a concentration of 10 μM ($p < 0.01$). Kynurenic acid, at both concentrations, was able to reduce NAS synthesis but not significantly (Figure 4.5.). The low level of NAS observed with QA (10 μM) could be due to the inhibition of the enzyme NAT. Melatonin was significantly inhibited by QA at a concentration of 100 μM ($p < 0.01$) by suppressing the enzyme, HIOMT, responsible for its synthesis (Figure 4.6.). The reason it may have affected HIOMT, responsible for the synthesis of aMT rather than the enzymes responsible for the synthesis of 5-MIAA and 5-MTOH could possibly lie in the fact that the HIOMT enzyme has various isoforms and each one has a relatively higher affinity for the compound that it is responsible for synthesising.

The above results show that QA is able to reduce NAS synthesis and more importantly aMT synthesis, significantly. Quinolinic acid production increases with the ageing process although the amount varies, whilst aMT synthesis decreases (Moroni *et al*, 1984b; Reiter, 1995). Both KYNA and QA levels are increased during broad spectrum inflammation and the possibility may arise that QA plays a contributing factor for pathological ageing or neurodegenerative diseases (Moroni *et al*, 1984b).

The plasma concentration of TRP is responsible for the synthesis of the indoleamines in the brain. Thus with the induction of IDO, the enzyme may play a role in the regulation of 5-HT

and its metabolites. Reports have shown that 5-HT or aMT may act as a substrate for IDO and may be cleaved *in vivo*, thus limiting the half-life of both of these compounds.

Thus, a finely tuned balance exists between TRP catabolism and the synthesis of kynurenines and indoleamines in the brain. Decreasing levels of aMT and increasing levels of QA during the ageing process, or the increase of IDO during inflammatory diseases with the resultant increase of kynurenines, shunts TRP away from indoleamine synthesis and consequently the possible neuroprotective effects of indoleamines such as melatonin.

CHAPTER 5

EFFECTS OF KYNURENINES ON LIPID PEROXIDATION AND THE ANTIOXIDANT PROPERTIES OF TRYPTOPHAN ANALOGUES

5.1. INTRODUCTION

The kynurenine pathway is the major route of TRP metabolism in mammals with 90% of TRP catabolism catalysed by TDO in the liver (Reinhard Jr. *et al*, 1994). The extrahepatic enzyme responsible for the synthesis of the KYN metabolites in the brain is IDO. Indoleamine 2,3-dioxygenase has a broad substrate specificity and is maximally induced by cytokines such as interferon (Reinhard Jr. *et al*, 1994). Induction of the enzyme through viral infections elevates the synthesis of kynurenine metabolites such as QA (Saito *et al*, 1991; Reinhard Jr. *et al*, 1994).

Interest in the kynurenine metabolite, QA, arose because of its toxicity and its association with

neurological disorders (Reinhard Jr. *et al*, 1994). Intraatrial injections of QA into the brain cause convulsions and its neurotoxicity closely resembles Huntington's disease, both biochemically and anatomically. Studies have also shown that QA levels are elevated in patients with broad spectrum infections and other inflammatory diseases (Heyes *et al*, 1996). The ability of QA to exert its neurotoxic effects may occur in the following manner: QA acts as an agonist of the NMDA receptors, which is related to its "excitotoxic" properties, by inducing excessive calcium entry into the neuron (Rios *et al*, 1991). It has been proposed that membrane depolarisation occurs with the activation of the voltage-dependent calcium channels (Weiss *et al*, 1990). The influx of calcium into the neuron is the initiating factor for neurodegenerative diseases. The accumulation of intracellular calcium activates phospholipases and proteases which require the metal as a cofactor for activation (Orrenius *et al*, 1989). With the activation of the enzymes, interaction with the cytoskeleton occurs causing structural and functional injury to the cell's constituents and subsequently neuronal death (Weiss *et al*, 1990; Orrenius *et al*, 1989).

Lipid peroxidation is a process which has also been implicated in the induction of various pathologies in man and is usually associated with tissue damage after trauma and increased membrane permeability (Rios *et al*, 1991). The brain is the most vulnerable organ to oxidative stress such as free radicals because: (1) the activity of the antioxidant enzymes, such as SOD and GSH-PX, which are responsible for the conversion of the reactive oxygen species (hydroxyl radical or the superoxide anion) to less reactive components, is weak, (2) the brain possesses a high lipid content, and (3) the consumption of oxygen in the brain is high

compared to the rest of the body (Choi *et al*, 1995).

The lipid peroxidation assay used was a modification of the method previously described by Sagar *et al*, 1992 and Esterbauer *et al*, 1990. Lipid peroxidation was measured using an external standard, 1,1,3,3-tetraethoxypropane (TEP). The final results were expressed as nmol malondialdehyde (MDA) per mg protein.

In the following study, an attempt was made to ascertain whether any of the kynurenines, *viz.*, KYN, KYNA, 3-HANA or QA, were able to induce lipid peroxidation. The antioxidative abilities of various compounds such as aMT, 6-MBOA and 5-HT were compared by measuring the degree to which each one reduced a chosen kynurenine-induced lipid peroxidation.

5.2. MATERIALS

5.2.1. Animals

Adult male Wistar rats were obtained from the University of Durban-Westville (Durban, South Africa). The animals were chosen at random and assembled into groups of five. Care was taken to remove adhering tissue and all traces of blood with 0.9% NaCl. Tissue that was not used immediately was frozen rapidly in liquid nitrogen and stored at -70°C until further use.

5.2.2. Chemicals

All reagents used were of the highest purity. Quinolinic acid, KYNA, KYN, 3-HANA, aMT, 6-MBOA and 5-HT were purchased from Sigma Chemical Co., St. Louis, USA. All other chemicals were obtained from Saarchem Limited, Krugersdorp, South Africa. Deionised water (Milli R/Q System, Millipore) was used to prepare all the solutions required.

5.3. METHODS

5.3.1. Chemicals

Quinolinic acid, KYN, KYNA and 3-HANA were dissolved in a minimal amount of 0.1M NaOH and then made up to a final volume with 0.1% ascorbic acid. The final concentrations ranged from 10 nM to 100 μ M. Melatonin was dissolved in absolute ethanol (the final concentration of ethanol did not exceed 1% when added to the brain homogenate). Subsequent dilutions of aMT were prepared in 0.1% ascorbic acid-ethanol solution. 6-Methoxy-2-benzoxazolinone was dissolved in a 40% ethanol solution. The final concentration and subsequent dilutions of 6-MBOA was made with 0.1% ascorbic acid-ethanol solution. The same concentration range used for melatonin was used for 6-MBOA, i.e., 1 nM to 100 μ M final concentration. Serotonin was dissolved in 0.1% ascorbic acid and subsequent dilutions of 5-HT (1 nM - 100 μ M) were prepared using 0.1% ascorbic acid.

5.3.2. Tissue Preparation

The brain tissue was thawed in a 37°C water bath. For each experiment n=5 rats were used. The brain tissue was homogenised (5 % w/v) in ice-cold 50 mM TRIS-HCl buffer at pH 7.4.

5.3.3. Thiobarbituric acid (TBA) test

Malondialdehyde determination as a lipid peroxide index was determined as described in Section 2.4.2.3.

5.3.4. Protein Assay

Protein within the sample was measured using serum albumin as a external standard (Lowry *et al*, 1951) (Section 2.4.2.4). The final results were expressed as nmol MDA/mg protein.

5.3.5. Statistical Analysis

Results were analysed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test to compare the means. Values of $p < 0.05$ were accepted as statistically significant.

5.4. RESULTS

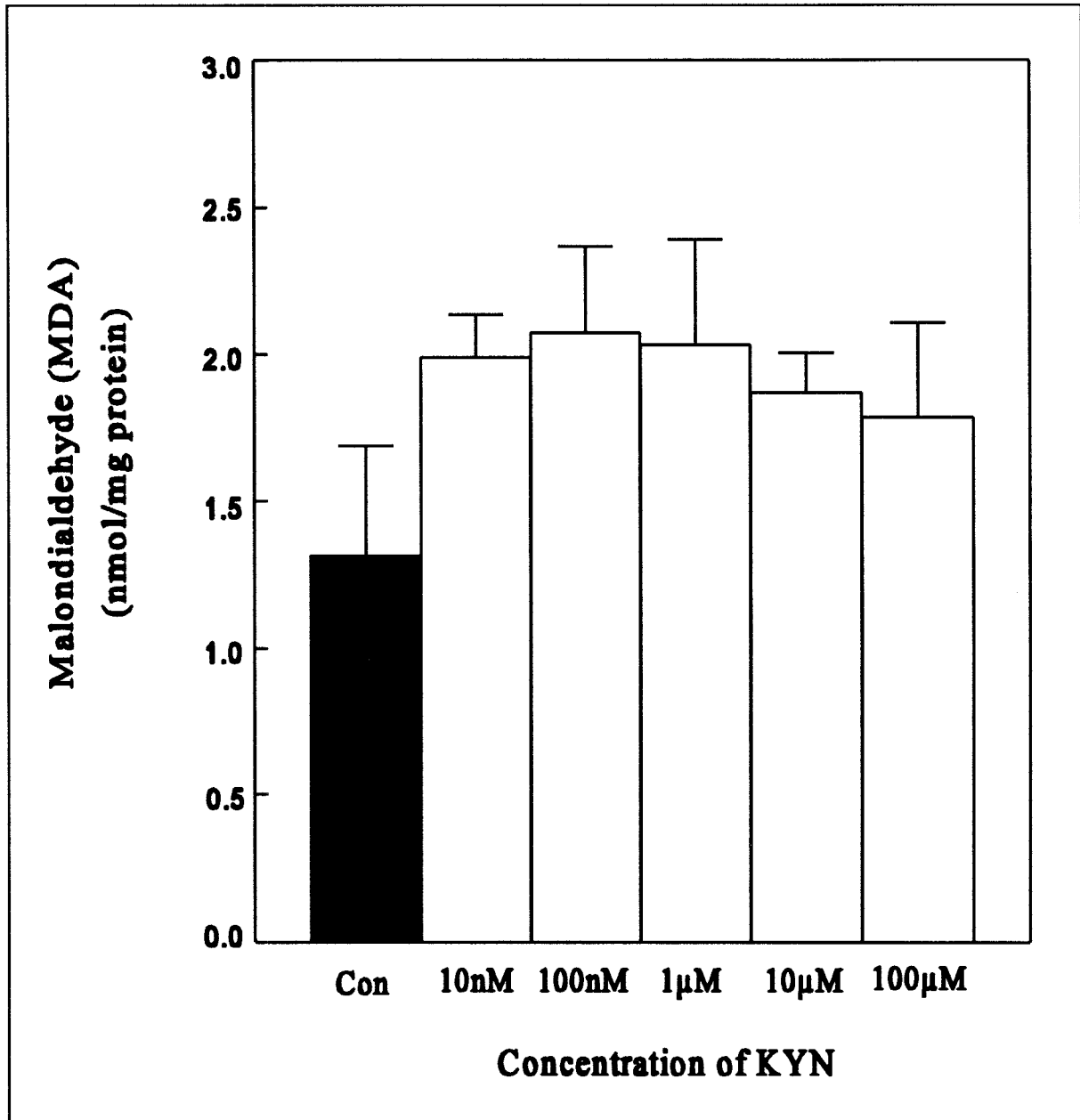


Figure 5.1. The effect of kynurenine (KYN) on lipid peroxidation of rat brain homogenates. Each point represents a mean value for $n = 5$ rats \pm S.E.M. Results were analysed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Significance for control (Con) versus all the above-mentioned concentrations is $p < 0.001$, except for 100 μ M, which is $p < 0.05$.

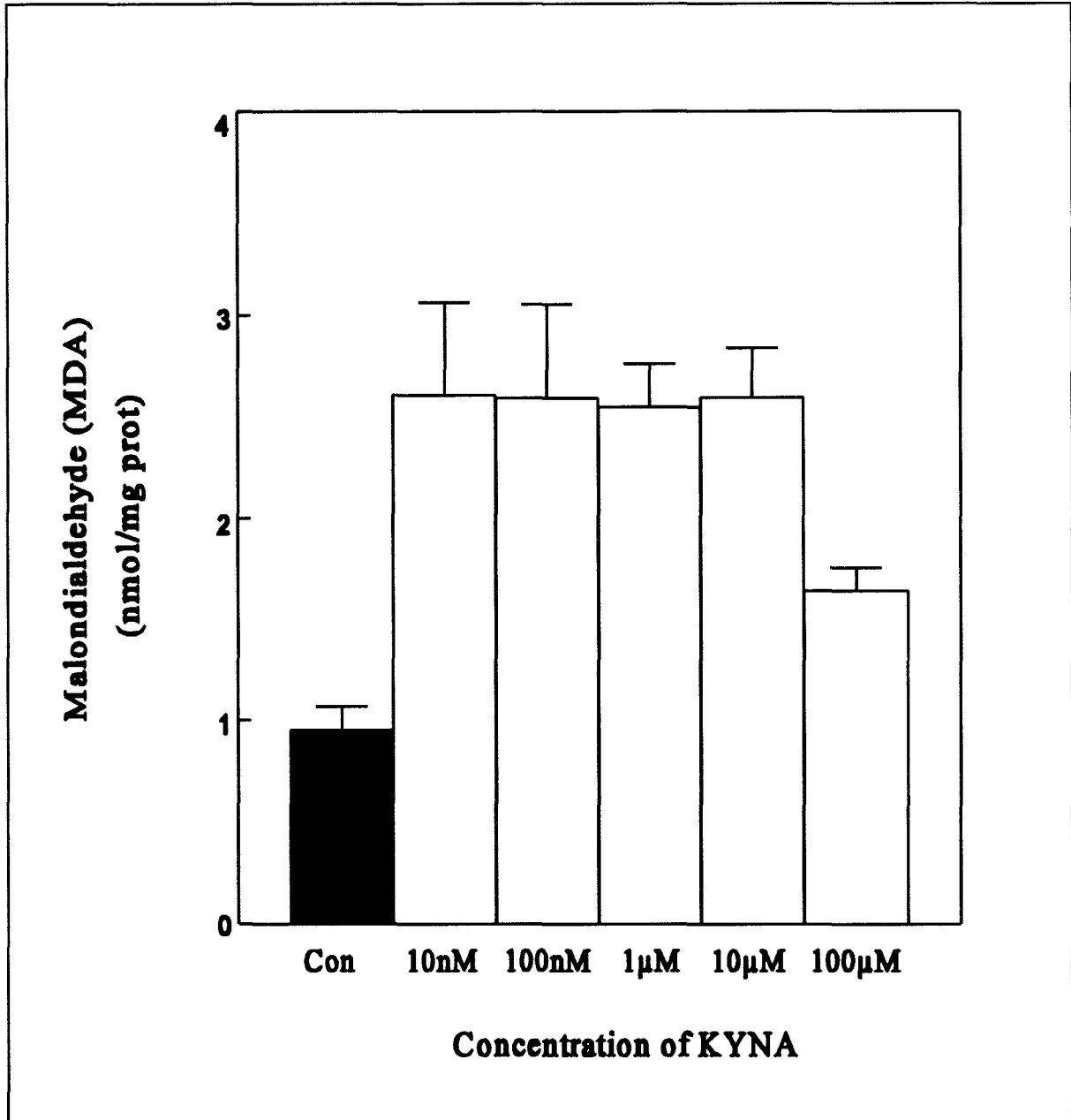


Figure 5.2. The effect of kynurenic acid (KYNA) on lipid peroxidation of rat brain homogenates. Each point represents a mean value for $n = 5$ rats \pm S.E.M. Results were analysed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Significance for the control (Con) versus all the above-mentioned concentrations is $p < 0.001$ except for $100 \mu\text{M}$, which is $p < 0.01$.

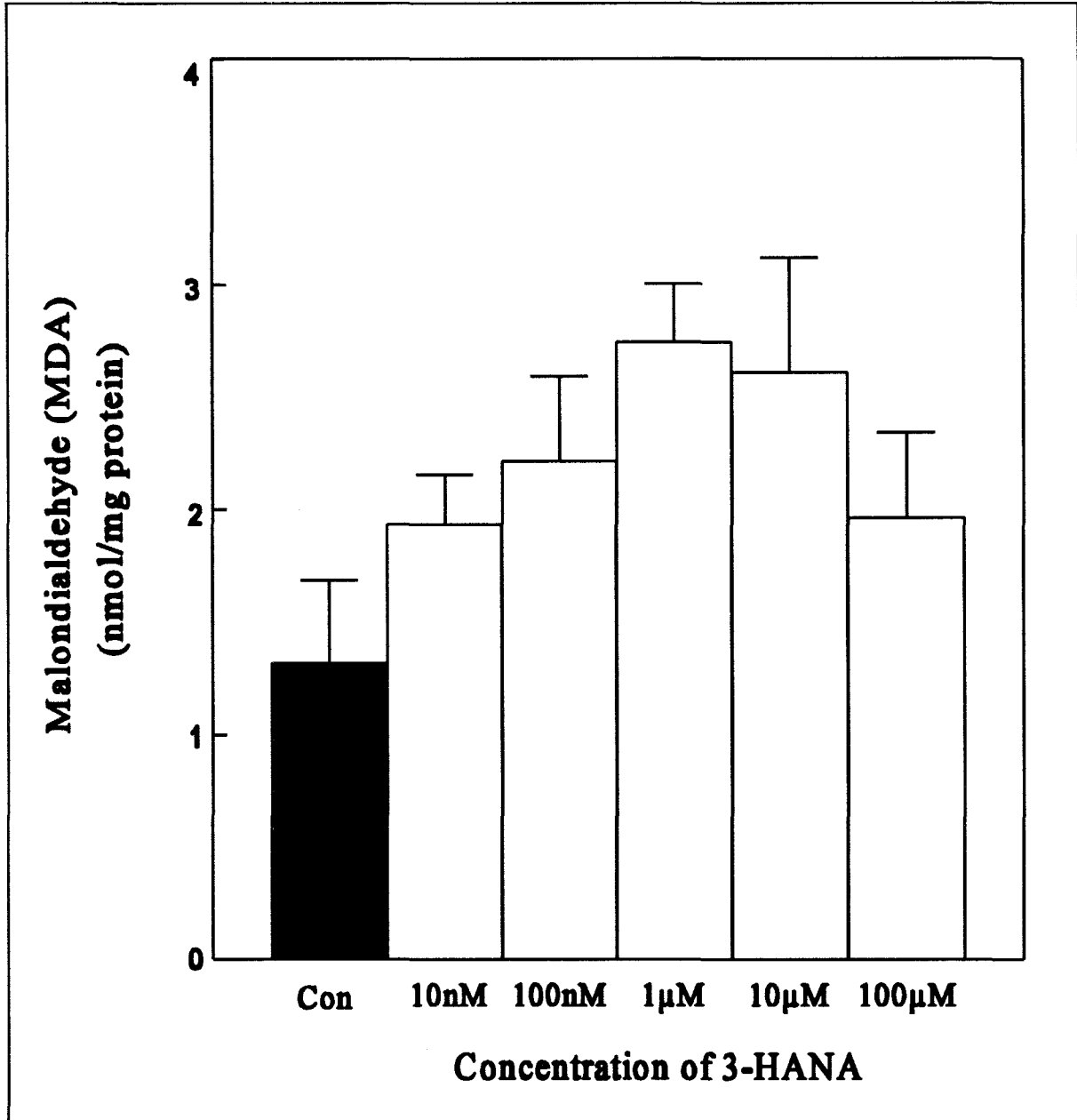


Figure 5.3. The effect of 3-hydroxyanthranilic acid (3-HANA) on lipid peroxidation of rat brain homogenates. Each point represents a mean value for $n = 5$ rats \pm S.E.M. The results were analysed by one-way analysis of variance (ANOVA) proceeded by the Student-Newman-Keuls test. Significance of the control (Con) versus all the above-mentioned concentrations is $p < 0.001$.

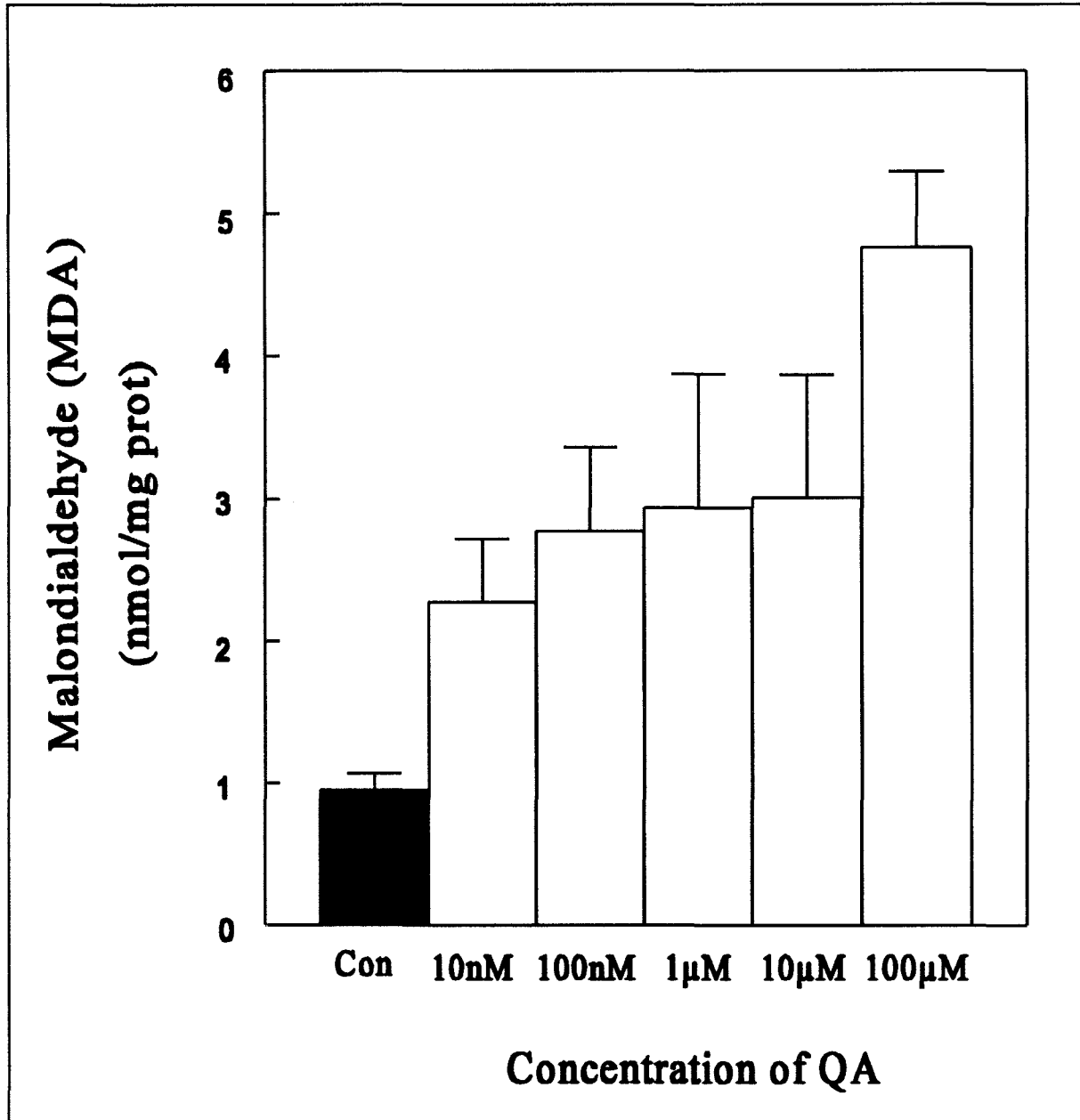


Figure 5.4. The effect of quinolinic acid (QA) on lipid peroxidation of rat brain homogenates. Values are a mean of $n=5$ experiments \pm S.E.M. Significant differences were determined by ANOVA followed by the Student-Newman-Keuls test. Quinolinic acid induced lipid peroxidation significantly for concentrations 100nM to 100 μ M ($p < 0.001$).

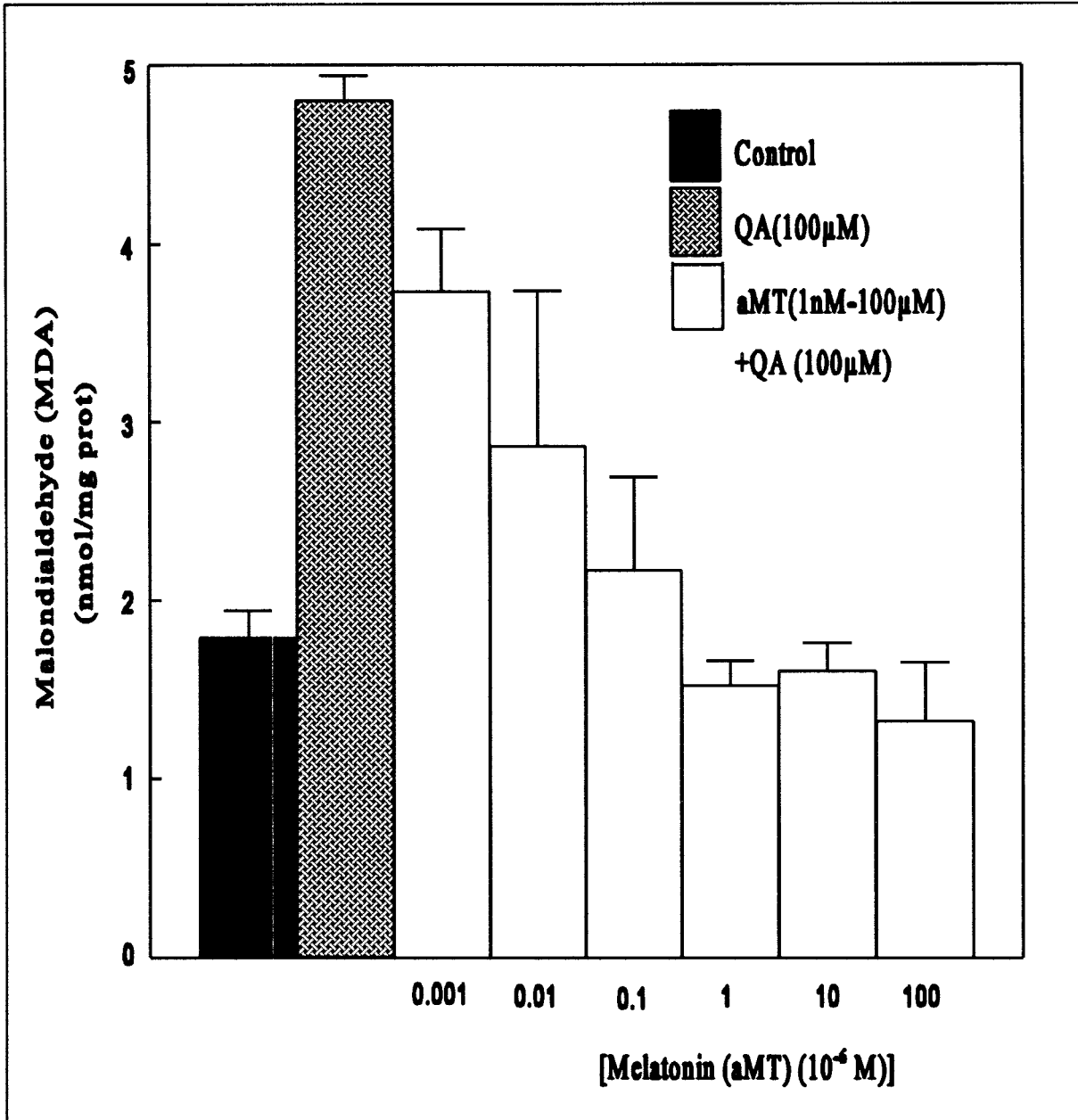


Figure 5.5. The effect of melatonin (aMT) as a potential antioxidant on quinolinic acid (QA)-induced lipid peroxidation of rat brain homogenates. Values are a mean of $n = 5$ rats \pm S.E.M. Results were analysed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Melatonin inhibited the QA-induced response very significantly for all concentrations at $p < 0.001$. Quinolinic acid was used at a final concentration of 100 μ M.

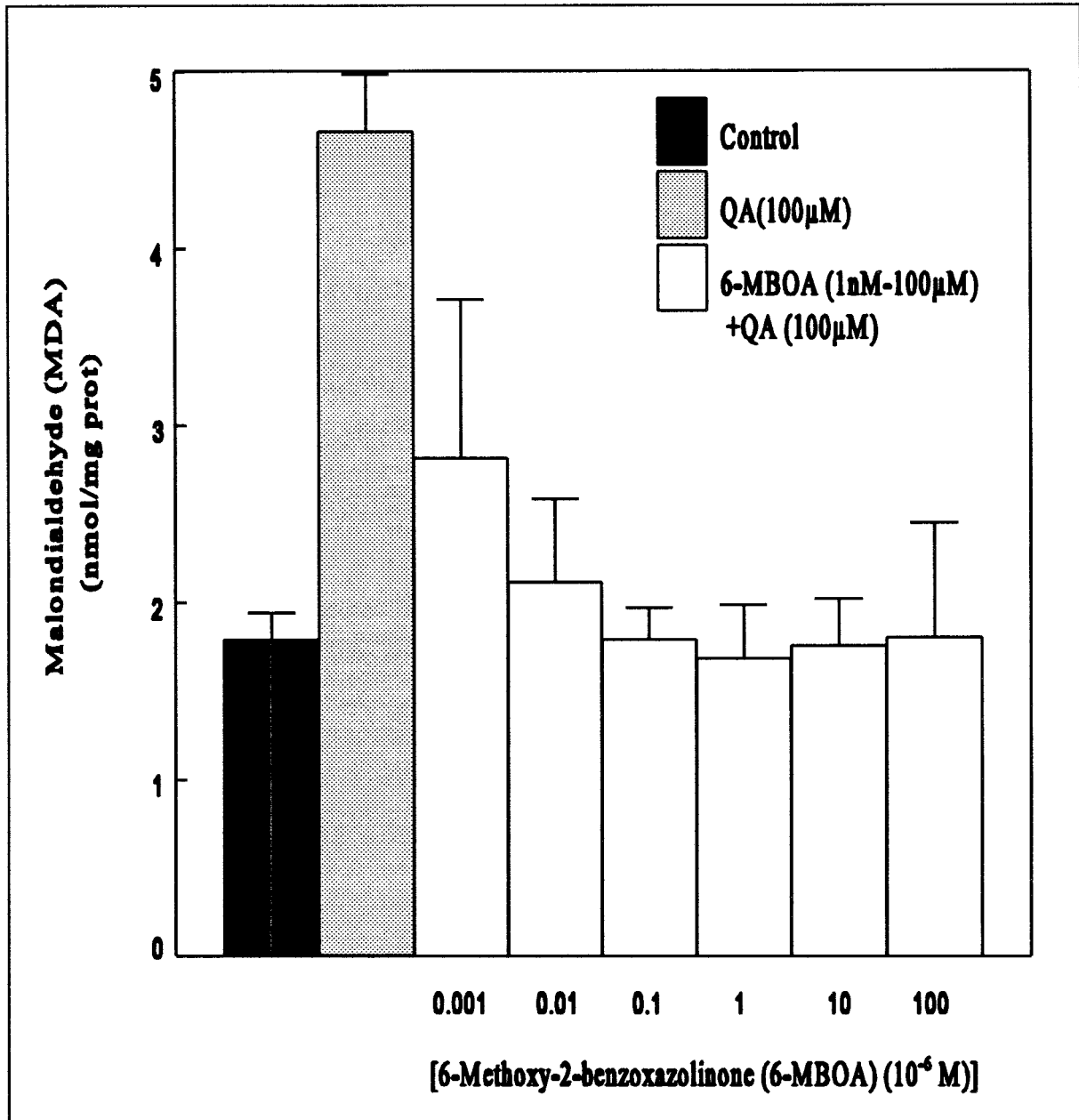


Figure 5.6. The effect of 6-methoxy-2-benzoxazolinone (6-MBOA) as a potential antioxidant on quinolinic acid (QA)-induced lipid peroxidation of rat brain homogenates. Values are a mean of $n = 5$ rats \pm S.E.M. Significant differences were determined by ANOVA followed by the Student-Newman-Keuls test. 6-Methoxy-2-benzoxazolinone was able to reduce QA-induced lipid peroxidation very significantly for all concentrations ($p < 0.001$). Quinolinic acid was used at a final concentration of 100 μ M.

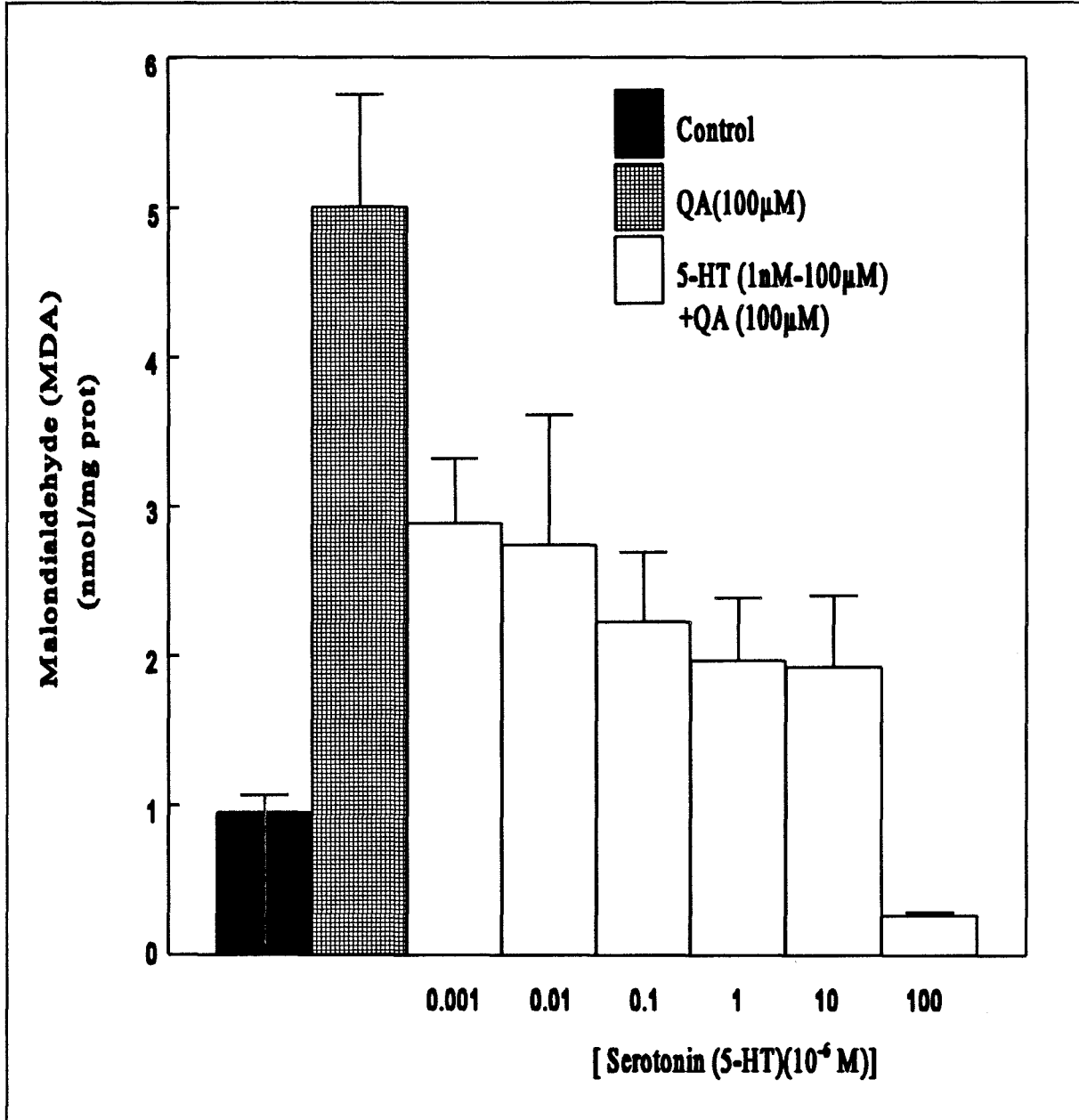


Figure 5.7. The effect of serotonin (5-HT) as a potential antioxidant on quinolinic acid (QA)-induced lipid peroxidation of rat brain homogenates. Values are a mean of $n = 5$ rats \pm S.E. M. Significant differences were determined by ANOVA followed by the Student-Newman-Keuls test. Quinolinic acid was used at a final concentration of 100 μ M. Serotonin was able to inhibit QA-induced lipid peroxidation significantly for all the concentrations used ($p < 0.001$).

The results were expressed as nmol MDA/mg protein. The amount of malondialdehyde formed was determined using an external standard, TEP (Figure 2.5.; page 102). The protein concentration was derived using bovine serum albumin (BSA) as an external standard (Figure 2.6.; page 104). The data was analysed using one-way analysis of variance (ANOVA) and statistical differences between the means were determined using the Student-Newman-Keuls test. Values are presented graphically from Figure 5.1 to Figure 5.7.

Both KYN and KYNA produced significant increases in lipid peroxidation ($p < 0.001$) at concentrations of 10 nM to 10 μ M when compared to the control group, with $p < 0.05$ and $p < 0.01$ at 100 μ M KYN and KYNA respectively. 3-Hydroxyanthranilic acid also produced very significant increases ($p < 0.001$) at all the concentrations in comparison with the control group. Quinolinic acid was able to produce significant elevations of lipid peroxide ($p < 0.001$) especially at a concentration of 100 μ M. To compare the inhibitory effects of various potential antioxidants of lipid peroxidation, QA was employed at its most potent concentration of 100 μ M. The potential antioxidants used were aMT, 5-HT and 6-MBOA; the latter being a structural analogue of melatonin. All the compounds tested were able to reduce the QA-induced response significantly ($p < 0.001$) at concentrations as low as 1 nM. However, when comparing all the antioxidants in the study, 5-HT, at a concentration of 100 μ M, was the most effective in reducing lipid peroxidation.

5.5. DISCUSSION

Organisms have evolved to survive in the presence of oxygen and for most it is a requirement in order to survive. Exposure to oxygen may be deleterious since oxygen can be reduced to form ROS such as the superoxide anion and the hydroxyl radical. These radicals are subsequently involved in tissue damage and most degenerative diseases (Buechter, 1988; Gutteridge, 1987).

The exact mechanism by which the kynurenine analogues induce lipid peroxidation is still unclear. All of the kynurenines, except for QA, limited lipid peroxidation at a concentration of 1 μ M to some extent. Quinolinic acid, acting as an agonist at the NMDA receptors may result in the entry of calcium into cells, activating proteases and phospholipases (Farber, 1981; Garthwaite *et al*, 1986; Lu *et al*, 1991; Siesjo and Wieloch, 1985; Stone, 1993; Tsuzuki *et al*, 1989; Verity, 1992). Quinolinic acid, as already known, is able to induce lipid peroxidation and the proposed mechanism is thought to be the enhancement of calcium entry into the cell. There are no reports regarding the ability of KYN, KYNA and 3-HANA to cause an accumulation of calcium in cells and consequently, lipid peroxidation.

Reiter *et al*, 1995; showed that aMT is a superior anti-oxidant when compared with the already known Vitamin E, glutathione and catalase. *In vitro* and *in vivo* experiments have shown that aMT is an excellent peroxy and free radical scavenger (Marshall *et al*, 1996). Its structure, lipophilicity and hydrophilicity may allow the protection of the cell subcompartments against

oxygen toxicity (Reiter *et al*, 1995). Figure 5.5. shows that aMT is able to reduce QA-induced lipid peroxide formation at very low concentrations.

6-Methoxy-2-benzoxazolinone, a naturally occurring compound present in grasses and winter wheat seedlings, produces sexual maturation in a number of rodent species by directly interacting with the pineal gland. It also has the ability to stimulate aMT production with a concomitant increase in pineal cAMP (Daya *et al*, 1993). Although its chemical structure is similar to aMT, no reports have shown that 6-MBOA is able to cross the blood-brain barrier. In Figure 5.6., 6-MBOA is shown to provide a similar inhibition of lipid peroxidation to that of aMT. At present, there is little information with regards to the ability of 6-MBOA to act as an antioxidant and whether it is able to provide hydroxyl and peroxy scavenging ability similar to aMT still needs to be demonstrated. It could be that the methyl group at position 5 of the indole nucleus, which is responsible for the scavenging abilities seen in aMT (Melchiorri *et al*, 1996), may also be responsible for the results observed with 6-MBOA (Figure 5.6.).

Marshall *et al*, 1996 reported that a compound without a phenolic group was more likely to inhibit lipid peroxidation. For QA-induced lipid peroxidation, 5-HT (hydroxyl group present at the fifth position) at a concentration of 1 nM to 10 μ M limited lipid peroxidation less efficiently than aMT and 6-MBOA. However, at a concentration of 100 μ M its effectiveness in reducing lipid peroxidation is more superior than aMT and 6-MBOA (Figure 5.7.).

Melatonin's ability to act as an intracellular free radical scavenger and antioxidant provides a significant defence system against oxidative damage within the body. It could be speculated that 6-MBOA is probably able to act similarly to aMT and thus may provide comparable antioxidative properties. Further characterisation of the antioxidative properties still needs to be demonstrated. As shown, aMT, 6-MBOA and 5-HT (Figure 5.5., Figure 5.6. and Figure 5.7. respectively), at a concentration of 100 μ M, are able to reduce QA-induced lipid peroxidation significantly.

The potential of these antioxidants to act as therapeutic agents is important, especially with regards to neurodegenerative diseases and ageing where life may be extended by delaying free radical and cellular structural damage.

CHAPTER 6

FINAL SUMMARY AND CONCLUSION

CHAPTER 1: LITERATURE REVIEW

Chapter 1 reviews the fate of TRP with respect to its catabolism by the hepatic enzyme, TDO, and peripheral enzyme, IDO. Another minor pathway exists to metabolise TRP in the brain to form 5-HT and aMT. The kynurenine and serotonergic pathways are described in detail. Tryptophan availability to the brain is primarily dependent on the activity of TDO and the carrier uptake system into the brain across the BBB which is competitive with other LNAA's and KYN. Depressed levels of TRP in the brain have been correlated with depressive disorders. Increased TDO or IDO results in increased synthesis of kynurenine metabolites during broad spectrum inflammation, of which QA is a neurotoxin. Quinolinic acid has also been implicated in the formation of free radicals which are known to exacerbate neurological disorders. The mechanisms which are responsible for their initiation are described in detail. Enzymes and antioxidant compounds, present in the intracellular and extracellular fluids, responsible for defence mechanisms against lipid peroxidation are also covered in great detail.

CHAPTER 2: EXPERIMENTAL PROCEDURES

All the assays used in this study and their optimisation studies are described in detail. Tryptophan 2,3-dioxygenase activity, pineal indole synthesis using organ culture, and lipid peroxidation determination is described.

CHAPTER 3: THE INFLUENCE OF KYNURENINES AND SEROTONERGIC COMPOUNDS ON TRYPTOPHAN 2,3-DIOXYGENASE ACTIVITY

Various endogenous compounds of the kynurenine and serotonergic pathway were utilised to determine whether any of the metabolites were able to inhibit TDO. None of the kynurenines inhibited TDO activity, while all the indoleamines, except for the indoleacetic acids, significantly inhibited the enzyme. Potential inhibitors of the apoenzyme, at a concentration of 100 μ M, are arranged in ascending order of inhibition: serotonin ($p < 0.05$) < melatonin ($p < 0.05$) < N-acetylserotonin ($p < 0.01$) < 5-hydroxytryptophol ($p < 0.01$) < 6-methoxy-2-benzoxazolinone ($p < 0.001$).

The structure-activity relationship of the compound at the active site is described. Inhibition of the enzyme by the above-mentioned compounds demonstrated that a methoxy or hydroxyl group be at the fifth position on the benzene group of the indoleamine. These functional groups is thought to be necessary and may reduce the structural flexibility within the haem pocket required for the catalytic reaction to proceed.

CHAPTER 4: THE EFFECT OF KYNURENIC ACID AND QUINOLINIC ACID ON PINEAL INDOLE METABOLISM

Addition of QA and KYNA to the rat pineal organ culture studies, showed that QA was able to reduce NAS and aMT synthesis significantly ($p < 0.01$) at a concentration of 10 μM and 100 μM respectively. Kynurenic acid showed no significant effect with respect to the synthesis of indoleamines.

CHAPTER 5: EFFECTS OF KYNURENINES ON LIPID PEROXIDATION AND THE ANTIOXIDANT PROPERTIES OF TRYPTOPHAN ANALOGUES

All the kynurenines, *viz.*, KYN, QA, KYNA and 3-HANA, induced lipid peroxidation significantly. Quinolinic acid produced a larger response than any other kynurenine and thus QA was employed to determine whether any of the potential antioxidants were able to reduce the QA-induced response. Quinolinic acid-induced lipid peroxidation was significantly inhibited ($p < 0.001$) by 5-HT, aMT and 6-MBOA.

CONCLUSION

Various studies have implicated indoleamine metabolism disorders in the brain to be metabolically related to the activity of the enzymes TDO (hepatic) (Salter *et al*, 1995b) or IDO (periphery) (Fujiwara *et al*, 1978). The availability of TRP to the brain may be modified by

the inhibition of the enzyme, TDO (Salter *et al*, 1995b; Litman *et al*, 1985). The findings of this research showed that endogenous compounds such as the indoleamines were capable of inhibiting the enzyme significantly. The compound that proved to be the most potent inhibitor of the enzyme was 6-MBOA which inhibited TDO activity by 55% percent. Inhibition of the enzyme may elevate 5-HT synthesis in the pineal gland thus alleviating depressive disorders which are associative with low levels of 5-HT, although further *in vivo* studies are required.

In inflammatory diseases such as HIV⁺ or neurodegenerative disorders such as Huntington's disease, high levels of cytokines induce the enzyme IDO in the brain with the highest levels of the enzyme found in the pineal gland (Fujiwara *et al*, 1978; Heyes, 1993). Induction of the enzyme results in increased synthesis of metabolites of the kynurenine pathway such as QA and KYNA (Heyes, 1993). An inverse relationship exists between quinolinic acid and melatonin levels, with QA increasing as aMT levels decrease with age (Moroni *et al*, 1984b; Reiter, 1995). Whether QA is increased due to induction of the enzyme IDO or due to ageing, QA may have causative implications in inflammatory or neurodegenerative disorders. In an intact organ culture of the rat pineal gland, QA was shown to inhibit the synthesis of NAS (precursor to aMT) and aMT. The study thus implied that the aMT concentration in the brain, as a neuroprotective effector against the ravages of the neurotoxin QA, is reduced.

As already mentioned, high levels of QA and KYNA exist in inflammatory diseases due to the increased activity of IDO in the brain (Heyes, 1993). Quinolinic acid has been shown to increase levels of lipid peroxidation (Rios *et al*, 1991). Lipid peroxidation mediates neuronal

and cell death by initiating an influx of calcium into the cell. This in turn is able to activate calcium-dependent proteases and phospholipases which are responsible for cell membrane damage (Braugher, 1987). The blood brain barrier's integrity is reduced and neurotoxic compounds are able to penetrate into the brain. The kynurenine metabolites all have the ability to initiate lipid peroxidation significantly. The potential ability of indoleamines to act as free radical scavengers was investigated. Melatonin, 6-MBOA and 5-HT were able to significantly reduce QA-induced lipid peroxidation. The findings of this study thus show that although high levels of kynurenines may exist due to various circumstances, indoleamines such as aMT and 5-HT may provide a neuroprotective role with regards to increased scavenging ability.

CHAPTER 7

FUTURE EXPERIMENTAL OBJECTIVES

The major controlling enzyme of TRP catabolism in the liver is TDO. The enzyme activity is dependent on the availability of the cofactor (haem) in the hepatic free haem pool (Litman *et al*, 1985). The activity of the enzyme may be induced by glucocorticoids and its substrate, TRP and enhanced TDO activity has been associated with reduced 5-HT turnover in the brain (Litman *et al*, 1985; Knox, 1966). Serotonin is formed by a minor pathway in the brain from the precursor, TRP, and there is considerable evidence suggesting that amines derived from TRP, such as 5-HT, play a role in the regulation of mood, appetite and depressive illnesses (Curzon *et al*, 1970). It therefore follows that inhibitors of TDO should elevate plasma TRP levels by inhibiting its activity and increasing brain TRP levels for 5-HT synthesis (Salter *et al*, 1995b). An inverse relationship therefore exists between TDO and 5-HT synthesis. In this particular study, an attempt was made to use endogenous compounds in the kynurenine and serotonergic pathway to determine whether any of the tryptophan metabolites were able to inhibit TDO activity and therefore TRP catabolism. The kynurenines were not able to inhibit TDO activity while the indoleamines, except for the indoleacetic acids, significantly inhibited

TDO activity. The most promising compound proved to be 6-MBOA, which reduced enzyme activity by 55 percent. Further investigations are necessary to obtain a clearer understanding of the indoleamines interactions that are responsible for reducing the enzymes activity. Future studies may include the following: (1) using the purified form of the enzyme and through kinetic studies characterise the type of inhibition effected by the compounds which were responsible for reducing TDO's activity, (2) establish the site of interaction through Fourier-transform infrared studies, and finally (3) establishment of the effects of 6-MBOA *in vivo* and whether it is in fact able to: i) cross the blood brain barrier, ii) cause toxicity *in vivo*, and iii) increase 5-HT synthesis in the brain and provide an alleviation to depression.

Indoleamine 2,3-dioxygenase is induced through broad spectrum inflammation via cytokines. This induction results in the increased synthesis of KYNA and the neurotoxin QA. Much attention has been given to QA because of its ability to reproduce the neurochemical profile similar to Huntington's disease. The major pathway of indoleamine synthesis in the brain is the pineal gland (Fujiwara *et al*, 1978). A converse relationship exists between QA and aMT synthesis although the concentration in the brain may vary from individual to individual. As aMT levels reduce during the ageing process, QA levels rise (Reiter, 1995; Moroni *et al*, 1984b). The research with rat pineal organ culture demonstrated that QA, although not KYNA, was able to reduce NAS (precursor to aMT) and aMT synthesis. Further characterisation is necessary to determine: (1) the exact effect of QA on the enzymes (NAT and HIOMT) that were inhibited, by determining the type of inhibition effected, (2) whether aMT inhibits the enzyme, IDO, in the rat pineal gland (aMT is already known to inhibit the

hepatic enzyme TDO) (Walsh *et al*, 1991), and (3) what effect QA may have on the circadian rhythm of the various indoleamines.

As previously mentioned, IDO activity is induced during inflammatory diseases and is responsible for the increased synthesis of QA and KYNA (Heyes, 1993). Quinolinic acid, by acting as an agonist at the NMDA receptors, is able to induce the entry of calcium into the cell activates phospholipases and protein kinases. These enzymes in turn are able to interact with the cell membrane to produce neuronal membrane damage or subsequent toxic levels of calcium which leads to eventual cell death (Stone, 1993a). However, as QA levels increase during ageing (Moroni *et al*, 1984b), the levels of aMT, a well documented antioxidant superior to those already known for their antioxidative abilities, decrease (Reiter, 1995). All the kynurenines were able to induce lipid peroxidation significantly. Quinolinic acid-induced lipid peroxidation was used to determine the degree whereby an already known antioxidant, aMT and 6-MBOA and 5-HT were able to inhibit lipid peroxidation. Melatonin, 6-MBOA and 5-HT were equally effective in reducing lipid peroxidation. Future objectives with regard to this study include the following: (1) characterise which reactive oxygen species the kynurenines are able to produce, (2) to ascertain whether the kynurenines are able to exert their excitotoxic effects through calcium influx into the cell and cause subsequent neuronal death, (3) establish which radicals 6-MBOA is able to scavenge through various experimental studies, and (4) determine why 5-HT is more effective as an antioxidant at the concentration of 100 μ M, this is contradictory to reports which suggest compounds containing a phenolic group are more likely to induce lipid peroxidation.

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