SEROTONIN-MELATONIN INTERACTIONS IN ACETAMINOPHEN AND N,N-DIMETHYLFORMAMIDE TOXICITY

Thesis

Submitted in Fulfilment of
the Requirements for the Degree of

MASTER OF SCIENCE (BIOCHEMISTRY)

OF

RHODES UNIVERSITY

BY

SHAILENDRA ANOOPKUMAR-DUKIE

DECEMBER 1999

"Formerly, when religion was strong and science weak, men mistook magic for medicine, now, when science is strong and religion weak, men mistake medicine for magic"

Thomas Szasz in *The Second Sin,* (1973)

Contents

Chapter One

Literature Review

Chapter Two

Photolytic Degradation of Acetaminophen and Melatonin

Chapter Three

Melatonin and DMF-Induced Liver Damage

Chapter Four

Lipid Peroxidation of the Liver

Chapter Five

The Effect of Acetaminophen on Liver Tryptophan-2,3-dioxygenase Activity

Chapter Six

The Determination of Rat Pineal Melatonin and Forebrain Serotonin Levels Using ELISA

Chapter Seven

Organ Culture Studies: The effect of acetaminophen administration on rat pineal indole metabolism

Chapter Eight

The Measurement of Urinary 5-HIAA: The effect of acetaminophen administration on rat urinary 5-HIAA levels

Chapter Nine

Summary of Results: Conclusions and Recommendations for Future Work

Acknowledgments

I would like to express my sincerest gratitude to my supervisor, Professor Santy Daya for his expert supervision, encouragement, and guidance throughout this study. I would also like to express my gratitude to Prof Daya for all the help with the animals, and for his enthusiasm towards my project. It was truly a privilege to work with someone who is in my opinion, a leader in his field. **Thank you!**

I am also extremely grateful to the following people:

Moira Poigrund, Sharon Richner, Sagran Aboo, Michael Nqweniso, Rueben Molefe, and Sally and Dave Morley for always making sure that I had all the materials for this Study.

Dr Rod Walker, Aileen Boshoff, and Helen Skilling for their expertise on the HPLC.

Prof Beverly Glass for all her help and enthusiasm.

My two heads of department: John Duncan and Peter Rose for all their support.

Prof Chris Whiteley for his help and support.

The academic staff from the Department of Microbiology and Biochemistry.

Dr K.P. Mohanakumar and the CSIR, Calcutta, India.

My Family for their support and encouragement, without which this work would not have been possible.

My lab mates (Clint, Sindi, Paresh, Linda, Sam, and Franz) for all their help.

My best and dearest friend Cathy Hornby, for her expert editing of this thesis, and her unfailing support and encouragement.

The Electron Microscopy Unit, Rhodes University, Grahamstown.

I would also like to thank the NRF, MRC, and Rhodes University for financial assistance towards this study.

Lastly to, Sathya Sai Baba, who makes everything possible.

Abbreviations

aHT	–	N-acetylserotonin
aMT	–	Melatonin
AMMC	–	N-acetyl-S-(N-methylcarbamoyl)-cysteine
ATP	–	Adenosine triphosphate
AUFS	–	Absorbance units full scale
BSA	–	Bovine serum albumin
BHT	–	Butylated Hydroxytoluene
^{o}C	–	Degrees Celsius
cAMP	–	5' cyclic adenosine monophosphate
Ca	–	Calcium
CNS	–	Central nervous system
CSF	–	Cerebrospinal fluid
Cu	–	Copper
DHBA	–	Dihydroxybenzoic acid
DMF	–	N,N-dimethylformamide
DMPO	–	5,5-dimethylpyrroline-oxide
DNA	–	Deoxyribonucleic acid
DNPH	–	Dinitrophenylhydrazine
DSS	–	Dextran sodium sulphate
E. Coli	–	*Escherichia coli*
EDTA	–	Ethylenediaminetetraacetic acid
ELISA	–	Enyme-linked immunosorbent assay
Fe^{2+}	–	Iron
g	–	Gram
G_s	–	Stimulatory guanine regulatory protein
GTP	–	Guanine triphosphate
µg	–	Microgram
HA	–	5-HIAA
4HDA	–	4-hydroxyalkenal
5-HIAA	–	5-hydroxyindole acetic acid
HIOMT	–	Hydroxyindole-O-methyltransferase
H_2O	–	Water
HCl	–	Hydrochloric acid
H_2O_2	–	Hydrogen peroxide
HL	–	5-hydroxytryptophol
HMMF	–	N-(hydroxymethyl)-N-methylformamide
HPLC	–	High Performance Liquid Chromatography
5-HT	–	Serotonin
i.p.	–	Intra peritoneal
kg	–	Kilogram

kHz	–	Kilohertz
LD_{50}	–	Dose at which 50% of the animals die
LPS	–	Lipopolysaccharide
µl	–	Microlitre
M	–	Molar
MA	–	5-methoxyindole acetic acid
MAO	–	Monoamine oxidase
MDA	–	Malodialdehyde
mg	–	Milligram
MIC	–	Methylisocyanite
MIH	–	3-methyl-5-isopropyl-hydantoin
ML	–	5-methoxytryptophol
ml	–	millilitre
µM	–	Micromolar
n	–	Sample size
NAB	–	N-acetyl-benzoquinoneimine
NAD	–	Nicotinamide adenine dinucleotide
NAD(P)H	–	Nicotinamide adenine dinucleotide (phosphate)
NaCl	–	Sodium chloride
NaOH	–	Sodium hydroxide
NAT	–	N-acetyltransferase
nm	–	nanometre
NMU	–	N-methylnitrosurea
NOS	–	Nitric oxide synthetase
O_2	–	Oxygen
$O_2^{\cdot}$	–	Superoxide anion radical
p	–	Probability
^{32}P	–	Radiolabelled phosphorous
PNPP	–	Para-nitrophenol phosphate
$^{\cdot}OH$	–	Hydroxyl radical
Rf	–	Rate of flow
ROO.	–	Peroxyl radical
rpm	–	revolutions per minute
SEM	–	Standard error of the mean
SCN	–	Suprachiasmatic nucleus
SSRI	–	Selective serotonin reuptake inhibitor
TBA	–	Thiobarbituric acid
TCA	–	Trichloroacetic acid
TDO	–	Tryptophan-2,3-dioxygenase
TLC	–	Thin layer chromatography
USA	–	United States of America
UV	–	Ultraviolet
w/v	–	Weight per volume

List of Figures

List of Tables

Publications

The articles published/in press/submitted thus far as a result of this work are listed below.

1. Anoopkumar-Dukie, S., Cross, R., Mohanakumar, K. P. and Daya, S. (1997) Melatonin Protects Against Dimethylformamide-induced Hepatotoxicty in Rats. *Microscopy Society Of Southern Africa- Proceedings*. **27**, 124.

2. Daya, S., and Anoopkumar-Dukie, S. Acetaminophen Inhibits Liver Tryptophan-2,3-dioxygenase Activity with a Concomitant Rise in Brain Serotonin Levels and a Reduction in Urinary 5-Hydroxyindole Acetic Acid. *Life Sciences*. **In Press**.

3. Anoopkumar-Dukie, S., Glass, B. D., Walker, R. B. and Daya, S. Melatonin Alters the Photodegradation of Paracetamol. *Pharmacy and Pharmacology Communications*. **In Press**.

4. Walker, R. B., Daya, S. and Anoopkumar-Dukie, S. HPLC Analysis of Melatonin. *Pharmacy and Pharmacology Communications*. **Submitted**.

ABSTRACT

Acetaminophen and N,N-dimethylformamide (DMF) are compounds which are extremely toxic to the liver. Acetaminophen is a drug which is well known for its analgesic and antipyretic properties. However, the abuse potential of this agent as a non-narcotic analgesic in alcoholics is well known. It is also the leading cause of overdose in England. DMF toxicity results mainly from occupational exposure. At present there are no known reports of an antidote for DMF poisoning, while N-acetylcysteine, the antidote for acetaminophen poisoning, is known to produce adverse effects. The present study evaluates the potential of melatonin as an antidote for acetaminophen and DMF poisoning. This study also investigates the mechanism underlying acetaminophen addiction and abuse.

Initial studies involved *in vitro* techniques in an attempt to remove the complexities of organ interactions. The photodegradation studies, using ultraviolet (UV) light, revealed that melatonin accelerates the rate of acetaminophen degradation in the presence of air, and reduces the rate of degradation in the presence of nitrogen. This study also revealed that melatonin is rapidly degraded in the presence of air, following UV irradiation. The effect of DMF on hydroxyl radical generation was also determined. DMF was shown to act as a free radical scavenger, rather that a generator of free radicals.

The *in vitro* studies were followed by lipid peroxidation determination. DMF (0.4ml/kg and 0.8ml/kg) did not produce any significant increases in lipid peroxidation in the liver. Three different doses of acetaminophen (30mg/kg, 100mg/kg, and 500mg/kg) were administered to rats for seven days. Acetaminophen (500mg/kg) was shown to significantly increase ($p<0.05$) lipid peroxidation in the liver. Melatonin (2.5mg/kg) was not able to significantly reduce the damage. The lower doses of acetaminophen (30mg/kg and 100mg/kg) did not increase lipid peroxidation. Electron microscopy studies showed that DMF adversely affects the liver, and in particular, the endoplasmic reticulum. Co-

administration of melatonin (2.5mg/kg) was able to reduce the damage. Further experiments need to be performed before an accurate assessment can be made on the ability of melatonin as an antidote for DMF and acetaminophen poisoning.

Several experiments were done in an attempt to uncover the biochemical mechanism underlying acetaminophen addiction and abuse. The first experiment targeted the liver enzyme tryptophan-2,3-dioxygenase (TDO). This enzyme is the major determinant of tryptophan levels *in vivo*. Acetaminophen administration (100mg/kg for three hours) was shown to significantly inhibit ($p<0.05$) the activity of TDO, indicating increased peripheral levels of tryptophan. This experiment was followed up with determination of brain serotonin and pineal melatonin. Brain serotonin was determined using the ELISA technique. Melatonin was estimated using this technique as well as with pineal organ culture. Acetaminophen administration (100mg/kg for three hours) significantly increased ($p<0.05$) brain serotonin levels. Using organ culture where exogenous (^{3}H) tryptophan is metabolised to (^{3}H) melatonin, acetaminophen (100mg/kg for three hours) was shown to significantly increase ($p<0.05$) pineal melatonin concentrations. However, the ELISA technique did not reveal any changes in endogenous pineal melatonin levels.

The final experiment was the determination of urinary 5-hydroxyindole acetic acid (5-HIAA), the major metabolite of serotonin, following acetaminophen administration (100mg/kg for three hours). Acetaminophen was shown to significantly reduce 5-HIAA levels ($p<0.05$) suggesting reduced catabolism of serotonin. The findings of this study indicate that acetaminophen mimics the actions of an antidepressant. This compelling finding has important clinical implications, and needs to be examined further.

Chapter 1

Literature Review

1. Introduction

Painkillers, such as acetaminophen, are generally available over-the-counter. Acetaminophen is a very effective analgesic and antipyretic. It is relatively non-toxic at therapeutic doses, but can produce potentially fatal hepatic necrosis when large quantities are ingested. This necrosis is caused by its metabolite, N-acetyl-benzoquinoneimine (Insel, 1996), which does so via a proposed free radical mechanism. Incorrectly stored acetaminophen is also susceptible to degradation with the possibility of a toxic compound being formed. In some countries such as the United States, acetaminophen accounts for 70% of the annual reported poisoning incidents, while in England, acetaminophen is the leading cause of death from drug overdose (Awang, 1997).

N-acetylcysteine is currently used in the treatment of acetaminophen toxicity. However the use of N-acetylcysteine is not without problems. Side effects associated with its use include bronchospasm and life threatening anaphylaxis (Insel, 1996). The possibility of using a non-toxic free radical scavenger for the treatment of acetaminophen-induced hepatotoxicity thus needs to be investigated. Melatonin fits these criteria. It is a known potent free radical scavenger of both hydroxyl radicals and superoxide anions at physiological concentrations (Daya, 1999). Before such studies can be undertaken, it is important to determine the behaviour of melatonin and acetaminophen under conditions in which the complexities of organ interactions are absent. One such system involves the use of a photoreactor and the subsequent analysis of the photodegradant products using a validated high performance liquid chromatography (HPLC) analytical method. This investigation of the chemical interactions between melatonin and acetaminophen, in a purely chemical environment, is an important initial investigation, albeit far removed from

an *in vivo* situation.

N, N-dimethylformamide (DMF), like acetaminophen, is also an extremely toxic agent. It is commonly used for vinyl-based polymers in the manufacture of films, fibres and coatings (Angerer *et al*, 1998). DMF toxicity results mainly from occupational exposure (Scailteur *et al*, 1984). DMF commonly affects the liver, and at present there are no known reports of an antidote for DMF poisoning. The mechanism of DMF-induced liver damage is not fully understood. This report hypothesises that DMF-induced liver injury is via a free radical mechanism, which can be confirmed using electron spin resonance and HPLC with electrochemical detection. If this hypothesis is correct, melatonin would be the perfect candidate as an antidote, as it is a potent free radical scavenger.

The *in vitro* studies can be compared to *in vivo* techniques using the rat as a model. The quantification of lipid peroxidation products is a very reliable indicator of oxidative stress. Lipid peroxidation can be measured using the Thiobarbituric acid (TBA) test, as this test is the single most commonly used test used for measuring lipid peroxidation (Gutteridge and Halliwell, 1990).

As stated earlier, acetaminophen is a drug which is well known for its analgesic and antipyretic properties, however the abuse potential of this agent as a non-narcotic analgesic in alcoholics is well known. This drug is also known to cause mood changes at high doses (Payan and Katzung, 1995). Although at present there are no reports to suggest that this agent alters neurotransmitter levels in the brain, which could contribute to this phenomenon, the precursor of this drug, phenacetin, is also known to cause euphoria in humans and sedation in animals. Phenacetin, although an analgesic, has been compulsively misused in the past as an anti-anxiety agent (Meyers *et al*, 1978). It is therefore crucial to gain insight into the biochemical events that underlie this addiction. It is apparent after surveying the literature available on mood, that tryptophan metabolism plays an important role in the synthesis of the neurotransmitter, serotonin (Walsh and Daya, 1998). Curtailed serum and cerebral tryptophan levels and brain serotonin turnover

as a consequence of an enhanced tryptophan-2,3-dioxygenase rate of catalysis, are linked to mood changes. Tryptophan-2,3-dioxygenase (TDO) thus has the potential to be a key regulatory site in the modulation of certain aspects of central nervous system activity. Alterations in TDO activity could also affect behaviour. This study examines the activity of this enzyme and its effect on brain and pineal indoleamine metabolism, using the rat as a model, in an attempt to explain the biochemical parameters involved in acetaminophen addiction and abuse.

In the following sections the relevant aspects of the vast literature pertaining to this study, are presented. Chapters Two and Three deal with the *in vitro* studies involving acetaminophen and DMF respectively. Chapter Four examines the effect of both these compounds on lipid peroxidation, using *in vivo* techniques. Chapters Five through to eight deal with tryptophan metabolism as well as brain and pineal indoleamine metabolism. In Chapter Nine, the results obtained are summarised and conclusions are drawn. Recommendations for future experiments are also reported in the final chapter.

1.1 The Pineal Gland

1.1.1 The history of the pineal gland

After centuries of disregard and unfounded philosophical ideologies, the pineal gland has, over the past decade, finally been acknowledged as an important functional neuroendocrine gland. The human pineal gland was discovered by the famous anatomist Herophilos (325-280 BC). The pineal gland was implicated as the regulator of the flow of the spirit, and this lead the philosopher, René Descartes, to proclaim that the pineal gland was the seat of the soul. During this period, physiological studies of the brain were eclipsed by researchers' preoccupations with the spiritual significance of the brain.

Between the mid 18th and 19th, centuries methodical and thorough investigations into the pineal gland were carried out. Cytological studies revealed that the pineal glands of lower

vertebrates functioned as photosensory organs. This led to the pineal gland being referred to as 'the third eye'. Subcellular granules and clear vesicles were also identified. These structures were consistent with a secretory function, and indicated that the pineal gland had glandular functions. By the end of the 19th century there were suggestions that the pineal gland had an endocrine role. In 1898, Heubner described a boy suffering from a pinealoma, and showing signs precocious puberty. Later, in 1930, Marburg also thought that precocious development of the primary and secondary sex organs was caused by hypopinealism, due to pineal degeneration (Pevét, 1984). It wasn't, however, until the 1950s, that scientists conducted serious research on the pineal gland. Subsequent research confirmed this endocrine role and also uncovered a neural connection between the pineal gland and the hypothalamus.

The pineal gland is presently recognised as an endocrine gland and its major metabolite, melatonin, as a hormone. The full extent of the functions of the pineal is not yet known. This has stimulated vast research worldwide in an attempt to elucidate the role of the pineal gland in the mammalian body.

1.1.2 Pineal Anatomy and Location

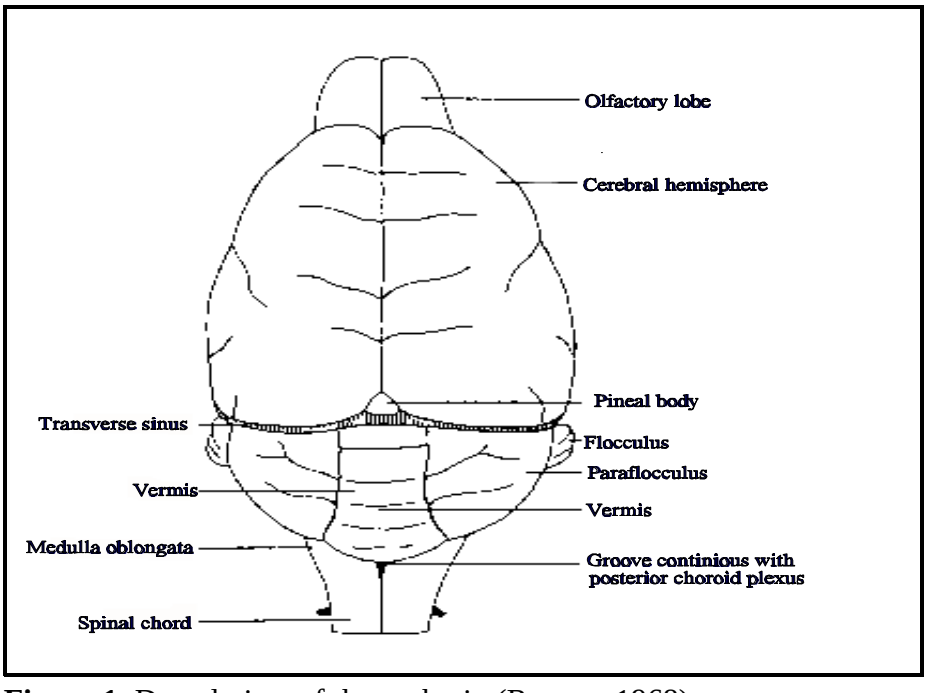

Figure 1: Dorsal view of the rat brain (Rowett, 1968).

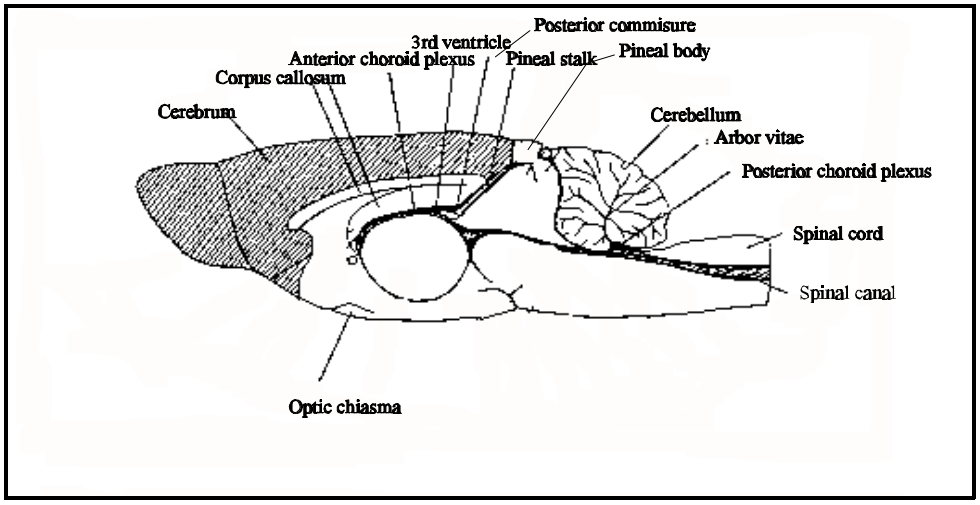

Figure 2: Median Sagittal view of the rat brain (Rowett, 1968).

The pineal gland develops from an evagination of the region of the neural tube which becomes the diencephalon (Ariens Kappers, 1971). The size, shape, and location of the gland varies in different species. It is superficially located between the cerebral hemispheres in the rat (Figures 1 and 2). The pineal gland is attached to the brain by the pineal stalk. The stalk consists of pinealocytes, pinealoblasts, and fibrocytes.

1.1.3 Indole metabolism in the pineal gland

Indole metabolism in the pineal gland occurs in the pinealocytes. The different stages are outlined in Figure 3. It commences with the uptake of tryptophan from the blood stream. Some of the tryptophan is utilized in the synthesis of pineal proteins, but the majority is converted to 5-hydroxytryptophan by the enzyme tryptophan hydroxylase (Lovenberg *et al*, 1967). This step occurs in the presence of oxygen, ferrous iron, and a reduced pteridine cofactor (Snyder and Axelrod, 1964). The enzyme has a low affinity for tryptophan, which is its substrate. Tryptophan availability is therefore the rate-determining factor for this enzyme.

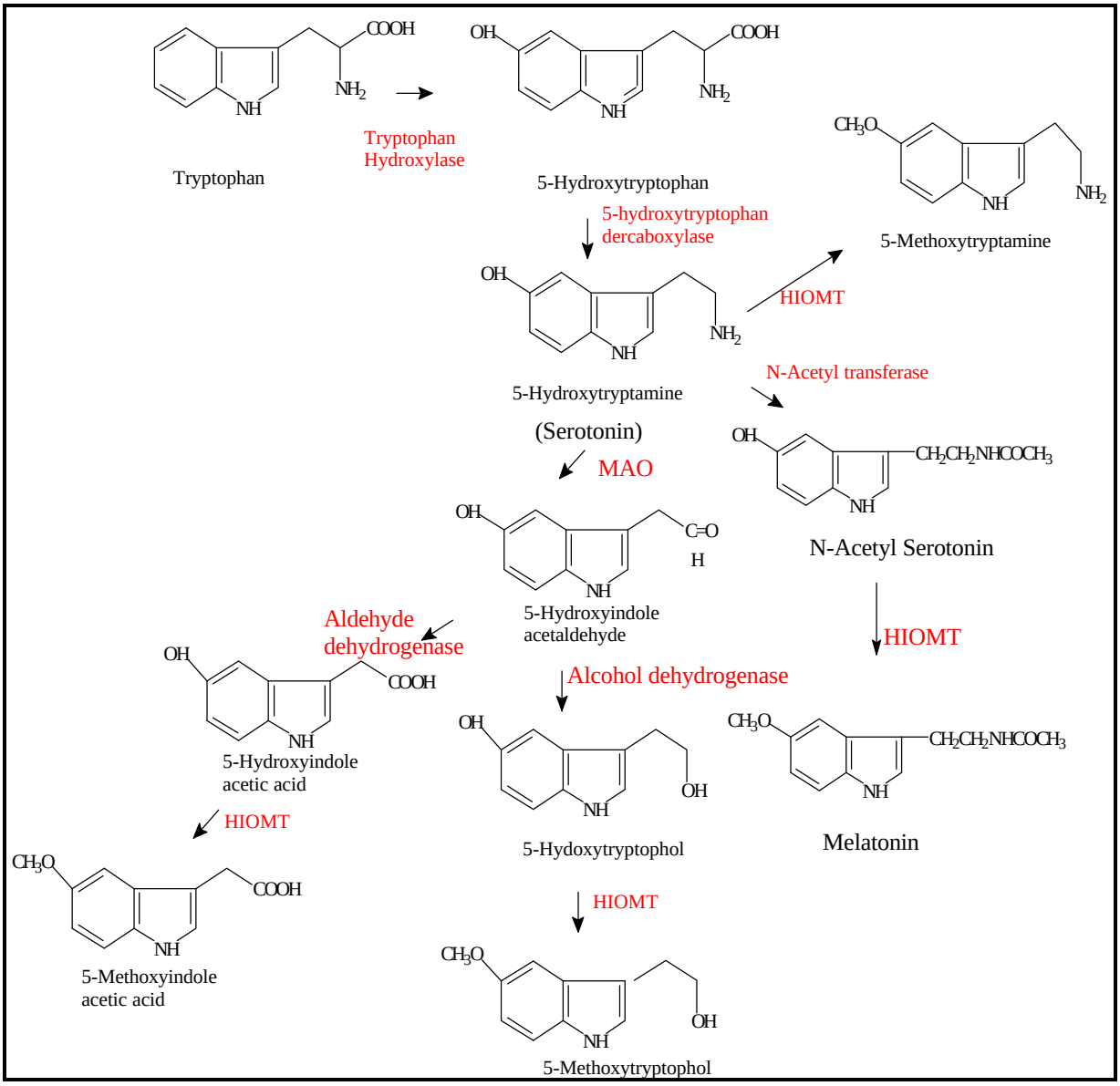

Figure 3: The pathway of indole metabolism (modified from Young and Silman,1982).

5-Hydroxytryptophan is then decarboxylated by L-aromatic amino acid decarboxylase. This leads to the formation of 5-hydroxytryptamine or serotonin (Lovenberg *et al*, 1962). Serotonin can then be metabolised in three different ways:

(1) A fraction of the serotonin undergoes methoxylation by hydroxyindole-O-methyltransferase (HIOMT) to form 5-methoxytryptamine. The methyl group is donated by S-adenosylmethionine.

(2) Monoamine oxidase oxidises serotonin to 5-hydroxyindole acetaldehyde (Axelrod *et al*, 1969). This is an unstable intermediate and is converted to either one of two metabolites:

(a) it may be converted to 5-hydroxyindole acetic acid and then to 5-methoxyindole acetic acid. These two steps are catalysed by aldehyde dehydrogenase and HIOMT respectively (Lerner and Case, 1960; Wurtman and Larin, 1968)

(b) alternatively, 5-hydroxyindole acetaldehyde may be converted to 5-hydroxytryptophol and 5-methoxytryptophol. These steps are catalysed by alcohol dehydrogenase and HIOMT respectively (Wurtman and Larin, 1968).

(3) The major fraction of serotonin is converted to melatonin. This is a two step process. First, N-acetlytransferase transfers an acetyl group from acetyl coenzyme A to the amino group of serotonin to form N-acetylserotonin. N-acetylserotonin is the precursor of melatonin (Klein *et al*, 1971). N-acetylserotonin is then O-methylated by HIOMT to form melatonin. Melatonin is the principal pineal hormone and has been the focus of the larger part of pineal research.

1.1.4 Pineal peptides

A variety of pineal peptide constituents exist in the pineal gland. This does not necessarily mean that these are produced by the pineal gland. These peptides could be synthesised in other organs and taken up by the pineal gland. These peptides can be classified into three different groups (Table 1). Various pineal peptide hormones act on the reproductive

system and on the pituitary-gonadal axis.

Due to the difficulty in specifically isolating these peptides, some of them have not been characterised. The physiological actions of the peptides are extremely complex and the variation in biorhythms also raises difficulties in assigning a biological role to these (Feuer, 1990).

Table 1: Various types of hormones and derivatives produced by the pineal gland (Adapted from Feuer, 1990).

METHOXYINDOLES	PEPTIDES
Melatonin Serotonin N-Acetylserotonin 5-Methoxyindoleacetic acid 5-Methoxytryptamine 5-Hyroxytryptophol 5-Methoxytrytophol N–Acetyl-5-methoxytryptophol	**Identified peptides and proteins**: a) Neurohypophysial peptides: oxytocin, vasopressin, vasotocin neurophysins. b) Hypothalamic hormones: Somatostatin, TRH, etc. c) ACTH, angiotensin 1 and 2, substance P, enkephalin, â-endorphins **Partially identified peptides and proteins:** Pinealin, E5 and NH2.

1.2 Melatonin

1.2.1 History

Melatonin is phylogenetically very old. Melatonin is known to have existed in the dinoflagellate *Gonyaulax polyedra* (Reiter *et al*, 1994). Melatonin is known to be produced in virtually all organisms in the animal kingdom. In 1917, McCord and Allen reported that extracts of bovine pineals produced a dramatic blanching of the skins in amphibians (Pevét, 1984). Forty-two years later, in 1959, the compound responsible for this blanching was isolated from the mammalian pineal gland by Lerner and colleagues, and chemically analysed for the first time (Reiter, 1997). This compound was identified as

N-acetyl-5-methoxytryptamine. Since it caused aggregation of melanin granules with melanophores, Lerner and colleagues named the compound melatonin (Pevét, 1984).

1.2.2 Chemical structure of melatonin

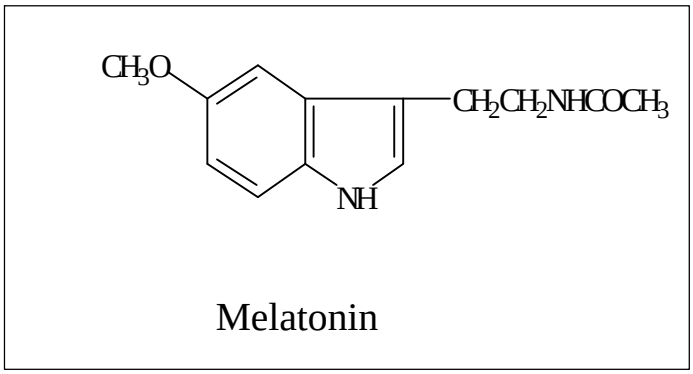

Figure 4: The chemical structure of the pineal hormone, melatonin (Feuer, 1990).

1.2.3 Synthesis of melatonin

Melatonin is primarily synthesised in the pineal gland and this process is described in section 1.1.3 above.

1.2.4 Other sites of melatonin synthesis

Melatonin synthesis is not confined to the pineal gland. Presently melatonin is known to be synthesised in the retina, the Harderian gland, the intra-orbial lacrimal glands (Reiter, 1989), and the enterochromaffin cells of the gastro-intestinal tract (Raikhlin and Kvetnoy, 1976). Pineal melatonin, is however, predominately responsible for the melatonin circulating in the blood. It is possible that melatonin produced in non-pineal sites may exhibit a compensatory increase in melatonin synthesis in pinealectomized animals (Reiter, 1989).

1.2.5 Circadian variation in melatonin synthesis

In vertebrates, melatonin production in the pineal gland exhibits a circadian rhythm, with the highest levels of melatonin being produced during the night. Fiske *et al* (1960), reported that the pineal gland decreases in size and mass following continuous exposure to light. This was the first report of the influence of light on the pineal gland.

The rhythm of melatonin synthesis depends primarily on the activity of the enzyme serotonin-N-acetyltransferase (NAT). This enzyme is considered to be the rate-limiting enzyme in the synthesis of melatonin (Reiter, 1994). The circadian rhythm in NAT activity is responsible for the circadian rhythm in melatonin production. Activation of NAT is controlled by signals from photoreceptor cells. Melatonin synthesis in the mammalian pineal gland is regulated by endogenous oscillators and photoreceptors in the retina of the eye. The eye is functionally and anatomically connected to the pineal gland by a neural network. In mammals the endogenous oscillator is believed to be located in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN has been shown to contain melatonin receptors named the ML1 receptor (Hagan and Oakley, 1995). The effect of light on pineal function is attributed to the inhibitory effects of light on the sympathetic nerves to the pineal gland (Klein, 1973).

Neural pathways from the SCN, where glutamate is the neurotransmitter, relay episodic sympathetic nerve impulses via the superior cervical ganglion, terminating at the postganglionic sympathetic fibres within the pineal gland (Petterborg *et al*, 1991). Norepinephrine is released from the nerve terminals. This leads to a stimulation of postsynaptic â1-adrenergic receptors which are coupled to adenylate cyclase. â-adrenergic stimulation of the pinealocyte activates adenylate cyclase via a stimulatory guanine nucleotide-binding regulatory protein, G_s , (Reiter, 1991). Adenylate cyclase catalyses the conversion of ATP to cAMP (Strada *et al*, 1972).

The increase in intracellular levels of cyclic AMP (up to 60-fold in the rat pineal) results in

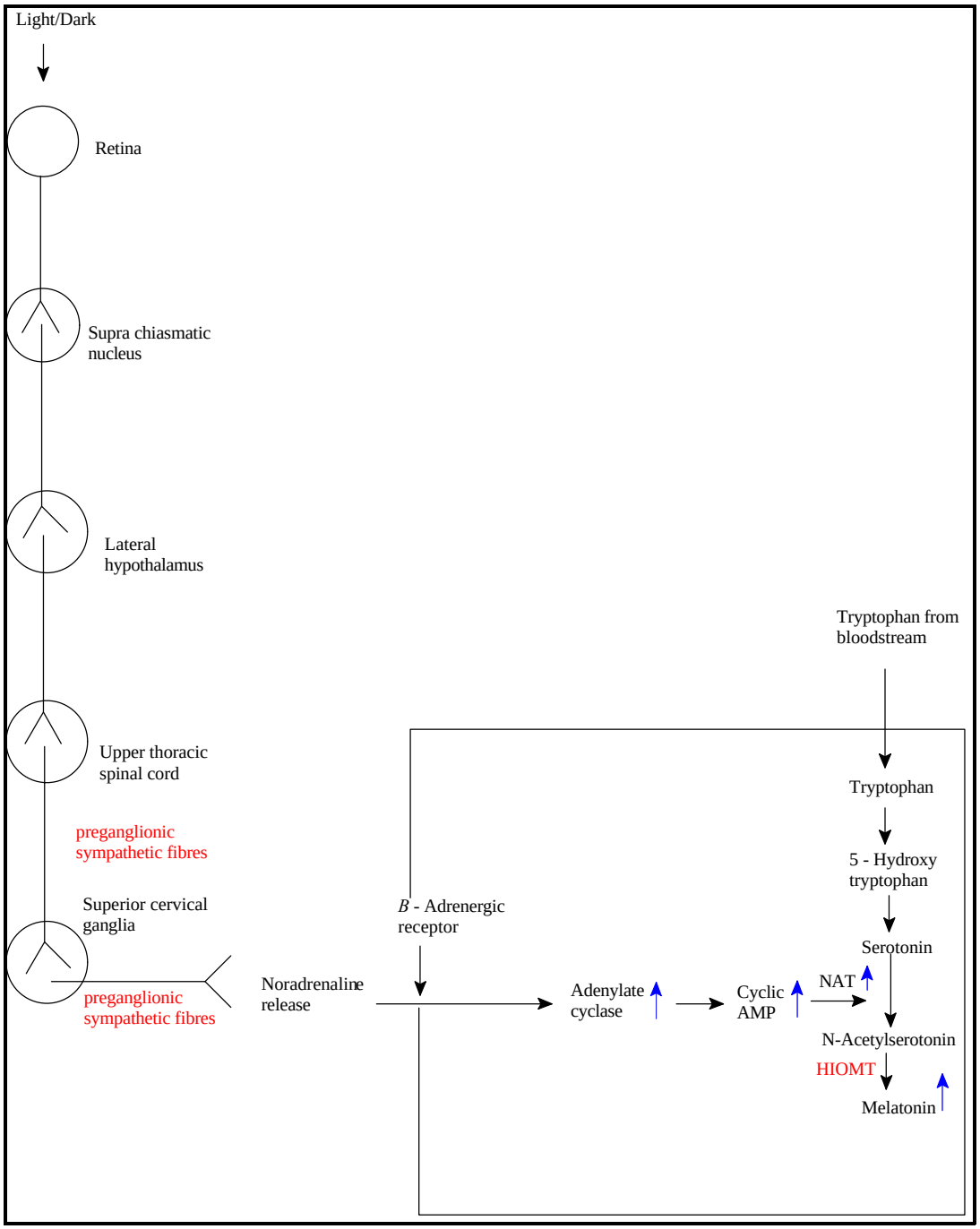

Figure 5: The schematic representation of light and innervation on melatonin synthesis in the pineal gland (Feuer, 1990).

elevated NAT activity. Cyclic AMP serves as a second messenger in the nocturnal elevation of melatonin biosynthesis. It elevates a cyclic AMP dependant protein kinase, increases the transcription of mRNA, and eventually results in a rise in NAT (Reiter, 1991). NAT then converts serotonin to N-acetylserotonin which is converted to melatonin. This is depicted in Figure 5. NAT activity has been shown to be lower during the day (Reiter, 1997), resulting in lower melatonin production during the day. At night, NAT is activated as described earlier, and melatonin production increases. The activity of NAT is 30-70 fold greater at night than during the day (Arendt,1988; Daya,1999). As a result of this circadian rhythm, the physiological levels of melatonin in body fluids and tissues vary according to the light/dark cycle. At night, blood melatonin levels reach values of 150pg/ml, which is ten times higher than daytime values.

1.2.6 Melatonin secretion and distribution

Melatonin is not stored in large pools in the pineal gland. Due to its high lipophilicity, melatonin is secreted from the pinealocytes by diffusion. Melatonin is released directly into the blood vascular system, and secondarily into other body fluids which include blood and saliva (Lewis *et al*, 1990). The normal route of melatonin secretion comprises the pineal capillaries draining into surrounding venous sinuses. This view is based on animal experiments as well as human data (Feuer, 1990). The majority of circulating melatonin in the bloodstream is plasma bound (60-70%), while melatonin is present in its free form in the cerebrospinal fluid (Feuer, 1990). Melatonin has a relatively short life, and during a single passage through the body, approximately 90% is taken up into the tissues (Lewis *et al*, 1990).

1.2.7 Catabolism of melatonin

The turnover of melatonin is fairly quick. In the rat, the half-life of melatonin is twenty minutes. Approximately 75% of the melatonin taken up by the liver is inactivated by hepatic microsomal enzymes, via hydroxylation, to form 6-hydroxymelatonin. This

product is then conjugated either with sulphate, to form 6-sulphatoxymelatonin, or with glucuronic acid. These products are excreted in urine. In the brain, excess melatonin is converted to N-acetyl-5-N-methoxykynurenamine by the enzyme indoleamine-2,3-dioxygenase. This is then converted to N-Acetyl-5-methoxykynurenine by hydrolysis (Figure 6).

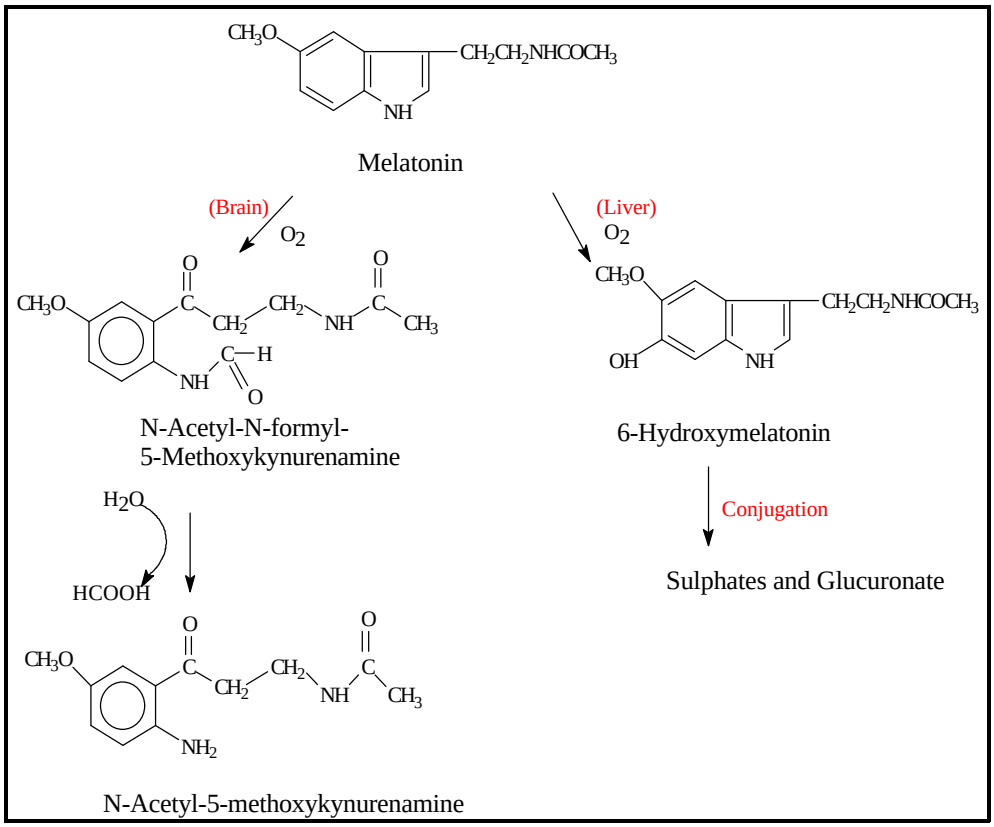

Figure 6: The catabolism of melatonin in the brain and liver (Feuer, 1990).

1.2.8 Free radicals and melatonin

1.2.8.1 Production of free radicals

Oxygen is vital for the survival of aerobic organisms. However, it is this very oxygen that can lead to their destruction. Under normal physiological conditions aerobic organisms utilize approximately 98% of cellular oxygen at cytochrome a_3, which is the terminal

cytochrome in the electron transport chain in the mitochondria (Reiter *et al*, 1994). Oxygen is reduced by four electrons without partially reduced intermediates being released. However, not all oxygen follows this pathway, and a small percentage is mono- and divalently reduced to produce either the superoxide anion radical, or hydrogen peroxide (H_20_2).

The generation of free radicals from oxygen is depicted in Figure 7. Free radicals contain unpaired electrons and are highly unstable. The complete reduction of one molecule of O_2 to water is a four-electron process as mentioned earlier. The superoxide anion radical ($O_2^{\cdot}$) is produced by the addition of a single electron to O_2. The superoxide anion is then enzymatically reduced by superoxide dismutase to H_2O_2. Although H_2O_2 is not a free radical, at high concentrations it can be toxic to cells. Neither $O_2^{\cdot}$ nor H_2O_2 is particularly reactive. H_2O_2 can be further reduced in the presence of transition metals such as Fe^{2+} to the highly toxic hydroxyl radical ($^{\cdot}OH$) (Reiter *et al*, 1995). This last step is known as the Fenton reaction.

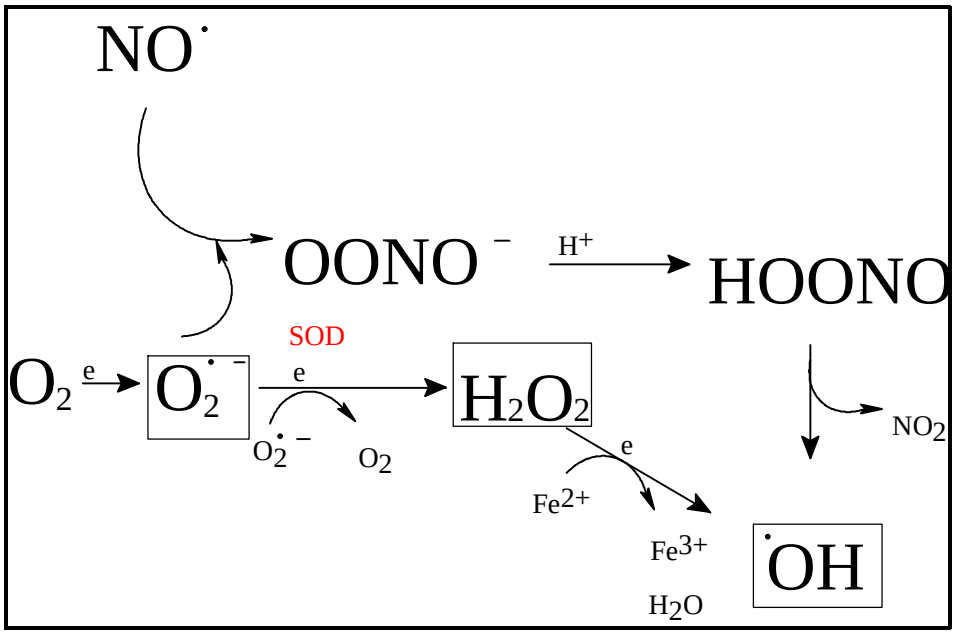

Figure 7: Generation of free radicals and reactive oxygen species from molecular oxygen (Reiter *et al*, 1994).

Various physiological disturbances of cellular homeostasis, such as UV light and chemical toxins, can lead to a dramatic increase in the production of $O_2^{\cdot}$, H_2O_2 and $^{\cdot}OH$ (Freeman and Crapo, 1981). These radicals react with, and frequently irreversibly damage, a variety of biological molecules including phospholipids, proteins, and nucleic acids. Cellular damage to cells by free radicals is referred to as oxidative stress, and can adversely affect cellular functions. This can ultimately lead to cell death. Inflammation, tissue ischemia, and hypoxia are examples of oxidative stress. The ageing process is also believed to result from accumulated free radical damage.

1.2.8.2 Melatonin as a free radical scavenger

1.2.8.2.1 *In Vitro* evidence

Tan and colleagues were the first to convincingly establish melatonin as a free radical scavenger (Reiter *et al*, 1994). In these studies, the authors exposed H_2O_2 to UV light to produce $^{\cdot}OH$. The hydroxyl radical has a very short half-life and cannot, therefore, be easily detected. Using 5,5-dimethylpyrroline-oxide (DMPO), a spin trap agent, to trap the hydroxyl radicals producing DMPO-$^{\cdot}OH$ adducts, these authors separated the adducts using HPLC. The adducts were further confirmed using electron spin resonance spectroscopy.

Tan and colleagues then compared melatonin, mannitol, and glutathione as free radical scavengers by measuring the reduction of the DMPO-$^{\cdot}OH$ adducts. The results of this study show melatonin to be the most effective free radical scavenger.

Tan then went on to investigate the structure-activity relationships of melatonin and other indoles in terms of their free radical scavenging ability. Melatonin was compared with serotonin, N-acetylserotonin, and 5-methoxytryptamine. Using the system outlined earlier, these authors again showed that melatonin was the most effective scavenger. These authors concluded that the methyl group, at position five in the nucleus, is responsible for

the ability of melatonin to scavenge the hydroxyl radical (Reiter *et al*, 1994).

In 1995, Vijayalaxmi and colleagues studied the effect of melatonin on free radical-induced DNA damage (Reiter, 1997). Human lymphocytes were incubated with various concentrations of melatonin. Treated and untreated lymphocytes were then exposed to gamma radiation, and the resulting damage was estimated using a variety of cytogenetic parameters, such as exchange aberrations. Melatonin was shown to reduce damage, once again confirming its ability to scavenge free radicals *in vitro*, and thereby providing tissue protection.

1.2.8.2.2 *In Vivo* evidence

Following the *in vitro* evidence, confirming melatonin as a free radical scavenger (Tan, 1993), Reiter undertook studies to determine its effectiveness as an antioxidant *in vivo* (Reiter, 1997). Safrole, an extract of sassafras oil, was used to induce DNA damage. Safrole is known to generate large numbers of oxygen-centred radicals (Reiter *et al*, 1994). Reiter proposed that prior administration of melatonin would assuage the damage produced by safrole.

Rats were injected with safrole (300mg/kg). Some animals were treated with melatonin (0.2 and 0.4 mg/kg) prior to the administration of safrole. After 24 hours the rats were killed and the livers removed. The DNA was isolated and labelled with ^{32}P. The damaged DNA adducts were separated and autoradiographed. The autoradiograms were then quantified using a Blot analyser (Reiter *et al*, 1994). The lower dose (0.2mg/kg) of melatonin reduced DNA damage by 41% while the higher dose (0.4mg/kg) reduced damage by 99%. This illustrated the ability of melatonin to scavenge free radicals *in vivo*.

Tan *et al* (1994) followed up these studies. Tan and colleagues injected rats with safrole (100mg/kg) during the early light, and dark phases. Rats injected with safrole at night had reduced DNA damage as compared to the animals injected during the day. This is due to

higher physiological levels of melatonin at night (Reiter, 1997).

In 1995, Melchiorri and colleagues exposed the lungs of rats to the herbicide paraquat. Paraquat was used to induce oxidative damage. The end products of lipid peroxidation, malondialdehyde (MDA) and 4-hydroxyalkenals (4HDA), were measured. When administered in conjunction with paraquat, melatonin totally overcame the rise in lipid peroxidation products (Reiter and Fujimori, 1996). This showed melatonin to scavenge the peroxyl radical (ROO.) which propagates and re-initiates lipid peroxidation.

This study conclusively confirmed melatonin as a free radical scavenger. The *in vivo* findings were also consistent with the *in vitro* findings.

1.2.8.2.3 Additional evidence supporting the antioxidative function of melatonin

Giusti and colleagues (1995) tested the ability of melatonin to protect cultured neurons from the excitotoxic effects of glutamate and kainic acid. Melatonin was shown to increase cellular viability, thereby protecting the neurons from damage. Melatonin was however not effective against NMDA excitotoxicity, and was only effective against kainic acid when administered simultaneously to the culture medium.

In another assessment of the antioxidative action of melatonin, Pentney and Bubenik (1995) induced colitis in mice by adding dextran sodium sulphate (DSS) to their drinking water (Reiter, 1997). DSS induces the proliferation of *Escherichia coli* in the intestine. This leads to an excessive production of the endotoxin lipopolysaccharide (LPS) which generates free radicals. These free radicals damage the mucosa lining the intestine, leading to diarrhoea. The daily administration of melatonin was shown to significantly reduce the severity of the colitis.

1.2.8.2.4 Free radical scavenging mechanism of melatonin

Melatonin detoxifies free radicals by electron donation. Melatonin is oxidised to produce the indolyl cation radical (Reiter *et al,* 1996). The indolyl cation radical is believed to scavenge both the superoxide anion and the peroxynitrous radical, thereby becoming N-acetyl-N-formyl-5-methoxykynuramine, a non-toxic urinary by-product. It still needs to be established whether or not the indolyl cation radical can be reduced back to melatonin. Unlike the indolyl intermediate, which is easily oxidised upon exposure to O_2·G, melatonin is inert, and specifically interacts with ·OH. This results in melatonin being a potent and selective free radical scavenger.

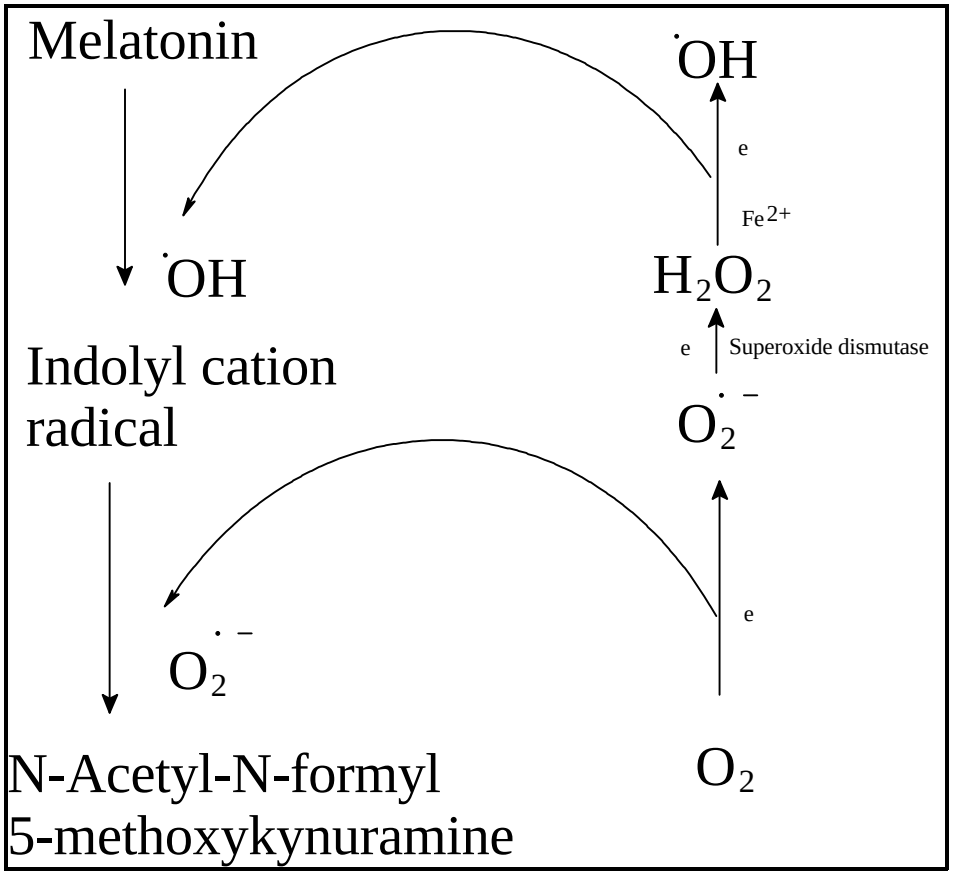

Figure 8: The presumed mechanism by which melatonin detoxifies and reduces the formation of the highly toxic ·OH (Reiter, 1996).

1.2.8.2.5 Effect of melatonin on the antioxidative defence system

In addition to the direct free radical scavenging actions of melatonin, melatonin has been shown to stimulate the major antioxidative enzyme, glutathione peroxidase (Reiter, 1997). This enzyme metabolises H_2O_2 to H_2O (Figure 8), thereby reducing the generation of $^{\cdot}OH$. In addition, according to Pierrefiche and Laborit (1995), melatonin also stimulates hepatic and cerebral glucose-6-phosphate activity in mice, thereby increasing NADPH levels (Reiter, 1997). This indirectly promotes the enzymatic production of glutathione via glutathione reductase. Glutathione is a necessary cofactor of glutathione peroxidase. Glutathione is required for, and oxidised by, innate cellular defences against free radicals (Johnson *et al*, 1993). In addition, glutathione is also responsible for the detoxification of acetaminophen (Correia, 1995).

Prozo *et al* (citied in Reiter *et al*, 1996) further reported that melatonin suppresses the activity of nitric oxide synthase (NOS). Nitric oxide interacts with O_2 to generate the peroxynitrite anion, which can degrade to produce $^{\cdot}OH$. The inhibitory effect of melatonin on NOS may contribute to its antioxidative capability.

1.2.9 Other important functions of melatonin

This report suggests that the most important function of melatonin is that of a free radical scavenger. However melatonin has been shown to have other important functions. Melatonin has been shown to effectively inhibit mammary tumorigenesis, induced by N-methylnitrosurea (NMU), when administered prior to the carcinogen (Blask *et al*, 1989). Melatonin is believed to block estrogen-induced mitogenesis.

Another important study showed melatonin to complex metals thereby possibly reducing metal toxicity (Limson *et al*, 1998). Using electrochemical techniques, Limson and colleagues showed that melatonin was able to form complexes with toxic metals, such as aluminium and copper. The ability of melatonin to bind to aluminium may ultimately have

serious implications in the treatment of Alzheimer's disease, as this disease is characterised by the accumulation of aluminium.

1.2.10 Melatonin and depression

The studies of melatonin patterns in depression have been conflicting. One study, using eight patients who were suffering from depression, showed no significant differences in melatonin levels when compared to normal controls (Lewis *et al*, 1990). Melatonin levels were measured in serum and CSF, obtained by lumbar puncture. Other studies have shown higher melatonin levels in depressed individuals (Lewis *et al*, 1990). However recent studies indicate that depressed individuals are more sensitive to light than normal individuals resulting in the inhibition of nocturnal melatonin synthesis. A study involving 30 acutely ill patients, and 24 patients with a history of unipolar or bipolar major affective disorder, showed lower nocturnal serum melatonin levels compared to healthy individuals (Lewis *et al*, 1990).

Mendlewicz and colleagues (1979) reported a lowered amplitude of the nocturnal rise in melatonin in three of four severely depressed women. The lowered melatonin levels were still apparent six weeks following recovery (Branchey *et al*, 1982). Another study by Wetterburg (1983), also reported lower nocturnal levels in 17 depressed patients. Reduced night time levels of melatonin may therefore represent a marker for depression.

Melatonin production and secretion is known to exhibit a circadian rhythm. Depression is characterised by a diurnal variation in mood associated with alterations in circadian rhythms. This ‘dysregulation’ hypothesis of depression claims that there is chronobiological instability in the depressed state (Maurizi, 1984). Thus malfunctioning of the biological clock is symptomatic of the depressed state.

Biochemical indices of the depressed state due to dysregulation of the circadian rhythm include earlier appearance of reduced night-time temperature, and phase advances in

melatonin. Melatonin has the ability to re-entrain the biological clock. The biological clock has been accepted to reside in the SCN, which is rich in melatonin receptors. Clinical data suggests that regular doses of melatonin result in the re-entrainment of circadian rhythmicity in depressives (Maurizi, 1984). However melatonin administration in healthy individuals causes a drop in basal body temperature. Thus, there appears to be differential melatonin metabolism in healthy and depressed individuals (Daya, 1994).

1.2.11 Melatonin and antidepressants

The most promising and effective antidepressants are those whose action is based on the 'biogenic amine' hypothesis of depression (Daya, 1994). Studies on the effect of antidepressants on melatonin production have yielded inconsistent results.

Wirz-Justice and Arendt (1980) reported on the effect of maprotiline treatment on the morning plasma melatonin levels in three depressed patients. Melatonin was undetectable in all three patients and this remained so during two to four weeks of therapy. Murphy *et al* (1986), examined the morning plasma melatonin levels in 27 depressed patients before and after treatment with monoamine oxidase inhibitors, such as clorgyline and deprenyl. Melatonin levels increased with clorgyline but not with deprenyl (Murphy *et al*, 1986). Similarly, studies involving patients on lithium and tricyclic antidepressant therapy showed no significant changes in melatonin levels (Wetterburg, 1983).

The noradrenaline uptake inhibitor, desipramine, administered as a single dose of 100mg at 16h00, advances the onset of melatonin secretion, resulting in prolonged melatonin secretion and therefore increased levels of melatonin (Daya, 1994). However the question of whether the antidepressant action of these drugs is attributable to increased levels of melatonin remains open.

1.3 Serotonin

1.3.1 History

Serotonin was discovered approximately a century ago. Physiologists discovered a vasoconstrictor compound in serum after blood clotting. This led to the compound being named vasotonin. This substance was a frequent nuisance in experiments requiring defibrinated blood (Douglas, 1975). In the early 1940s, during research into hypertension, the "nuisance" proved to be an obstruction and had to be removed. In 1948 researchers isolated this vasoconstrictor substance as a crystalline complex, and named it serotonin. A year later it was discovered that the active moiety of serotonin was 5-hydroxytryptamine (5-HT). Synthetically manufactured 5-HT possesses all the properties of naturally produced serotonin (Douglas, 1975).

During this period independent studies led to another substance being isolated in the enterochromaffin cells of the gastrointestinal mucosa. This substance was a gut stimulating factor and was named enteramine. This substance was later identified as 5-hydroxytryptamine or serotonin. Subsequent research has uncovered several important functions of serotonin which are discussed later in the report.

1.3.2 Chemical structure of serotonin

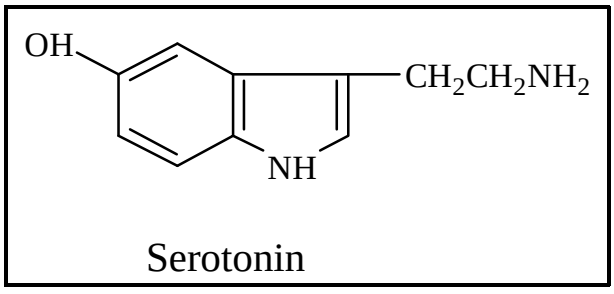

Figure 9: The chemical structure of serotonin.

1.3.3 Synthesis of serotonin

The synthesis of serotonin has been discussed in section 1.1.3 above.

1.3.4 Distribution

In mammals, approximately 90% of the serotonin present in the body is located in the gastrointestinal tract. The major fraction of this is in the enterochromaffin cells. The remaining serotonin is found in platelets and the central nervous system (CNS). Some serotonin has been identified in the mast cells of rodents (Douglas, 1975).

1.3.5 Catabolism of serotonin

The principal route of the catabolism of serotonin involves monoamine oxidase (MAO). The catabolism process is outlined in Figure 10. MAO converts serotonin to 5-hydroxyindole acetaldehyde (Sanders-Bush and Mayer, 1996). The enzyme, aldehyde dehydrogenase, then catalyses the formation of 5-hydroxyindole acetic acid (5-HIAA) from the aldehyde.

5-HIAA is the major metabolite of serotonin and is excreted in the urine, mainly as glucuronide or sulphate conjugates. Alternatively, 5-hydroxyindole acetaldehyde may be reduced by alcohol dehydrogenase to produce 5-hydroxytryptophol. The production of 5-hydroxytryptophol is quite insignificant (Sanders-Bush and Mayer, 1996).

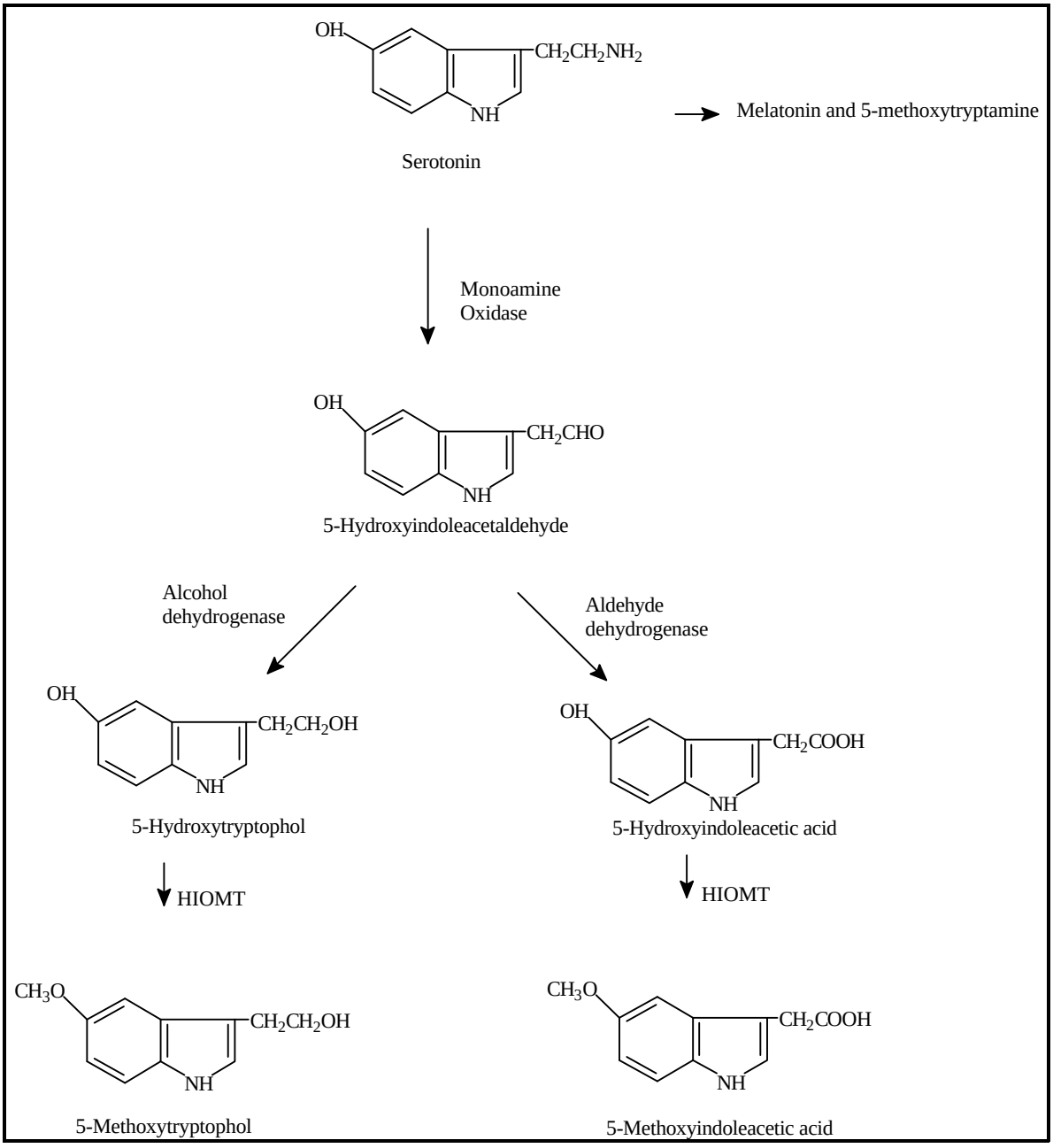

Figure 10: The catabolism of serotonin (modified from Young and Silman, 1982).

1.3.6 Functions of serotonin

1.3.6.1 Enterochromaffin cells

The enterochromaffin cells contain the highest proportion of serotonin. The physiological functions of the enterochromaffin cells are presently unclear. Intestinal cells release serotonin which is augmented by mechanical stretching, such as by food (Douglas, 1975). It is speculated that serotonin plays an additional role in stimulating motility via the myenteric network of neurons which are located between the layers of smooth muscle (Sanders-Bush and Mayer, 1996). Serotonin may either enhance or inhibit gastric and intestinal motility.

1.3.6.2 Platelets

Platelets do not synthesize serotonin. Serotonin is actively accumulated in platelets and is stored in specialized intracellular organelles. When isolated, these organelles catalyse the uptake of serotonin (Rudnick *et al*, 1980). The main function of platelets is to repair holes in damaged endothelial cells. This is facilitated by aggregation of the platelets. It is believed that serotonin potentiates this aggregation. This effect is however very small (Sanders-Bush and Mayer, 1996).

1.3.6.3 C.N.S

Serotonin influences a multitude of brain processes and functions, including sleep and appetite. Serotonin is also believed to be implicated in violent and compulsive behaviour (Sanders-Bush and Mayer, 1996).

1.3.6.3.1 Serotonin as a neurotransmitter

Most serotonin pathways originate from neurons in the raphe or midline regions of the

pons and upper brainstem (Nicoll, 1995). Myelinated fibres that innervate most regions of the CNS contain serotonin. The density of the innervation does vary. Serotonin has an inhibitory action in most regions of the CNS. This inhibitory action is mediated by the 5-HT_{1A} receptors and is associated with an increase in potassium conductance. This leads to membrane hyperpolarization. Serotonin does excite some cells by blocking the potassium channels. This is mediated by the 5-HT_2 receptors. Both inhibition and excitation can occur on the same neuron (Nicoll, 1995).

1.3.6.3.2 The role of serotonin in depression

Research into biological determinants of depression has two focal points, namely psychopathological and biochemical (van Praag, 1982). The role of serotonin in depression is best explained by the 'biogenic amine' hypothesis, which is a biochemical hypothesis. This theory predicts that disturbance in the biogenic amines, specifically, noradrenaline, dopamine, and serotonin, underlies the pathology of depression and mania. The first suggestion of the role of serotonin in depression was made in the 1950s when it became apparent that reserpine induced depression when used in the treatment of hypertension. When analysed, it was later revealed that reserpine inhibits the storage of serotonin and noradrenaline in the vesicles of presynaptic nerve endings. It is therefore reasoned that depression is associated with reduced serotonin levels and decreased amine-dependant nerve transmission (Hollister, 1995).

Another impressive clinical finding in depressed patients, was an abrupt reversal of the therapeutic response to antidepressant drugs by treatments that reduce the level of serotonin, such as p-chlorophenylalanine. This clinical finding supported a role for serotonin in the pathogenesis of depression (Sanders-Bush and Mayer, 1996). However, as important as this theory was, it did not fully explain the aetiology of mood disorders, for example, cerebrospinal fluid (CSF), and urinary and serum transmitter metabolites did not exhibit a consistent pattern of abnormality in depressed individuals (Harvey, 1997). The theory was simplistic, assuming that depression was caused by a synaptic deficiency

of indoleamines such as serotonin.

The critical involvement of serotonin in mood regulation is, however, well supported. Reduced platelet uptake of serotonin is observed in depressives, and the cerebrospinal fluid of suicide victims reveals a reduction in 5-hydroxyindole acetic acid, the major metabolite of serotonin (Harvey, 1997). A more sound anatomical and neurochemical theory identifying the role of serotonin in depression and mania is the 'permissive' hypothesis. This theory emphasised the importance of serotonin as a neuro-modulator and therefore a target for antidepressant therapy. It predicted that a fall in central nervous system serotonin would allow an effective state which is regulated by noradrenaline. Consequently depression arises due to decreased serotonin and noradrenaline levels. The presence of anatomical, as well as functional receptor interactions, typifies the way in which serotonin may act as a 'permissive' modulator of neurotransmitter function. Heteroreceptors (serotonin neurons) enable synaptic connections between serotonergic pathways and dopaminergic, cholinergic, and adrenergic pathways. This enables the serotonergic pathway to loosely modulate the functions of the other pathways. Conversely, release of both serotonin and noradrenaline is controlled by alpha-2 adrenergic neurons (autoreceptors) and serotonin neurons (heteroreceptors). Inhibition of these receptors results in increased release of serotonin and noradrenaline, thereby alleviating depression (Harvey, 1997).

1.3.6.3.3 Antidepressant drugs targeting serotonin

Monoamine oxidase inhibitors, such as isocarboxazid (marplan®) and phenelzine (nardil®), block the degradation of serotonin. This permits more serotonin accumulation in presynaptic stores and therefore allows more to be released. These antidepressants act more specifically on monoamine oxidase A. Monoamine oxidase B is responsible for dopamine catabolism (Hollister, 1995).

Another class of antidepressants that targets serotonin are the selective serotonin

re-uptake inhibitors (SSRIs). Examples of this group of antidepressants include fluoxetine (prozac®) and paroxetine (paxil®) (Hollister, 1995). These drugs inhibit the inactivation of serotonin by active reuptake. This potentiates the action of serotonin released by neuronal activity (Sanders-Bush and Mayer, 1996). Antidepressant agents, such as mianserin and mirtazepine, inhibit the serotonin neurons (heteroreceptors) in the CNS, thereby resulting in enhanced serotonin release (Harvey, 1997).

1.3.6.3.4 The onset of antidepressant action by serotonin targeting drugs

The onset of therapeutic activity is dependant on extracellular and intracellular dynamics. Due to prolonged starvation of serotonin amongst other neurotransmitters, various receptors in the CNS of depressed individuals become upregulated in order to maintain normal neurotransmission (Harvey, 1997). Antidepressants that elevate the synaptic levels of serotonin, activate the synaptosomal 5-HT_{1A} auto receptors, thereby inhibiting the release of serotonin. It is only once these receptors are downregulated, due to changes in subcellular elements, that the antidepressant can exert its full action.

Once serotonin has activated its specific receptor, a cascade of events is set in motion. The extracellular signal (receptor stimulation), is conveyed through the cell membrane into the nucleus, where cell function is regulated. For the serotonin receptors, 5-HT_1 and 5-HT_2, the transmembrane signal is dependant on an interaction between the receptor and a transducer G-protein which binds GTP. This complex is responsible for increasing or decreasing the activity of adenylate cyclase and phospholipase C, which is responsible for the synthesis of cyclic AMP and inositol triphosphate. The secondary messengers phosphorylate critical regions in the DNA, leading to the encoding of transcription factors which control transcription and translation of modified proteins such as receptors (5-HT, etc.).

Once antidepressant drug therapy is initiated, upregulated receptor proteins have to be disposed of, and new receptors have to be synthesised and incorporated into the

membrane. Receptor stimulation can occur within six hours, while the resetting of the cellular response can take up to three weeks. This is the main reason for the observed delay in antidepressant action (Harvey, 1997).

1.3.6.3.5 Serotonin syndrome

Serotonin syndrome results from the interaction between the SSRIs and monoamine oxidase inhibitors. This occurs when the two classes of antidepressants are used in conjunction to treat depression. This increases stores of serotonin and increases serotonin in the synapses (Hollister, 1995). The serotonin syndrome is typified by mental status changes, diarrhoea, shivering, and incoordination (Brown *et al*, 1996).

1.4 Tryptophan-2,3-dioxygenase

1.4.1 Tryptophan-2,3-dioxygenase (TDO)

The haem-dependent enzyme, tryptophan-2,3-dioxygenase (EC 1.13.11.11), is a major determinant of tryptophan levels *in vivo*. The enzyme (molecular weight 167 000) is a tetramer consisting of two identical subunits, $\acute{a}_2\hat{a}_2$, held together non-covalently (Tanaka and Knox, 1959). Changes in circulating levels of tryptophan can be achieved by altering the activity of the enzyme. This liver cytosolic enzyme catalyses the oxidative cleavage of the pyrrole ring of L-tryptophan to produce N-formylkynurenine during the first step in the kynurenine-nicotinic acid pathway of tryptophan degradation (Badawy and Evans, 1975). This first step is also the rate limiting step in the degradation of tryptophan. Approximately 90% of the body's total tryptophan undergoes degradation via this pathway (Figure 11).

The enzyme exists in at least two forms in certain animal species. These two forms are:
(1) the active reduced holoenzyme, and,
(2) the predominant, inactive apoenzyme which requires the addition of exogenous

haematin for demonstration of its activity (Badawy and Evans, 1974).

Both forms of the enzyme are present in the livers of rats, mice, chickens, and human beings. The apoenzyme is absent from the livers of guinea pigs, hamsters, frogs, cats, and

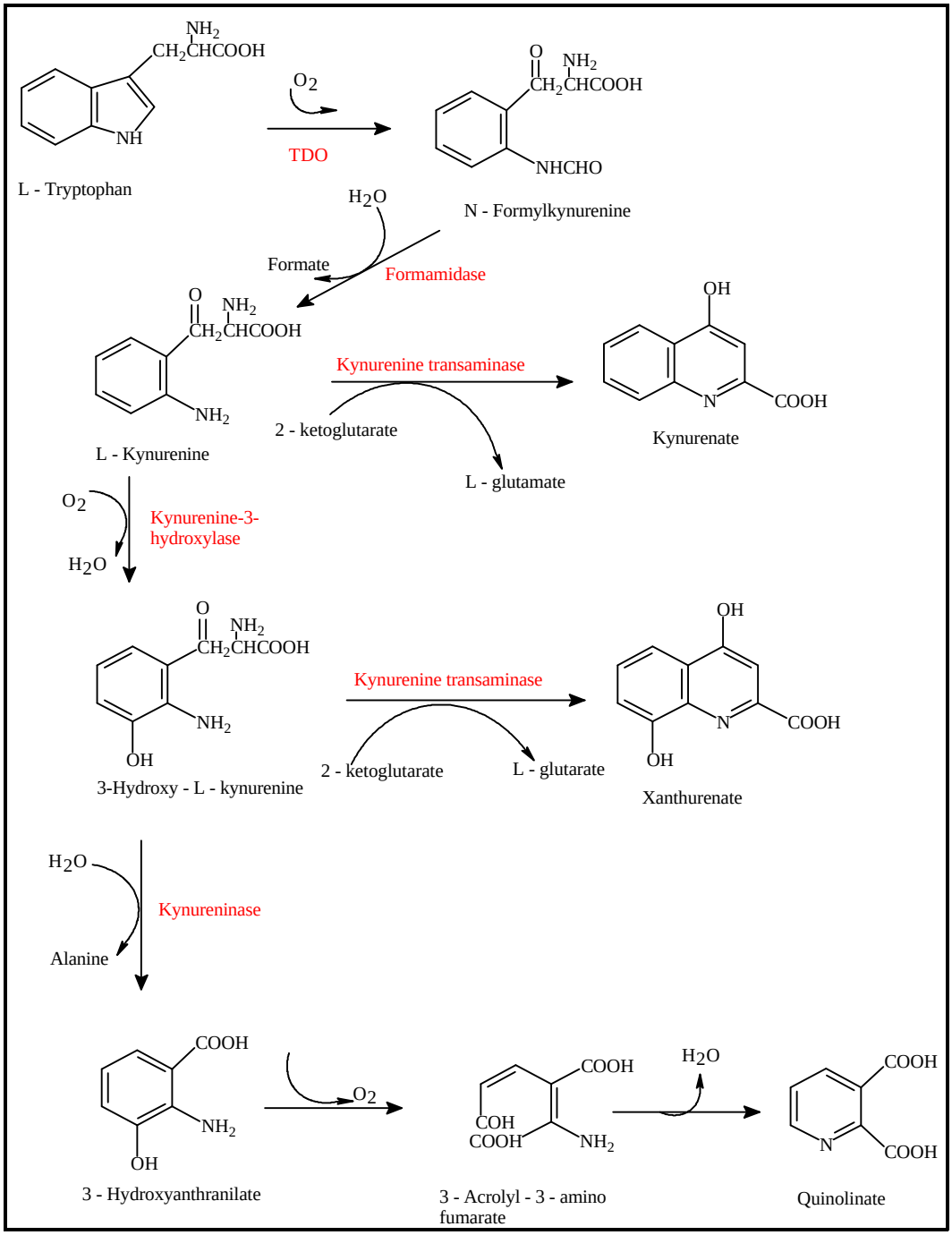

Figure 11: The kynurenine pathway (Martin and Beal, 1992).

the gerbil. Activation of the apoenzyme *in vitro* involves the conjugation of the apoenzyme and haem to form the oxidised (ferrihaem) holoenzyme. The holoenzyme is then reduced to the active form in the presence of tryptophan. Hepatic cellular levels of tryptophan ultimately determine the rate of apo- to holoenzyme, and the distribution of haem between the two forms and other haem binding proteins such as albumin. Two thirds of the total enzyme in the cell exists as the apoenzyme (Knox and Piras, 1965).

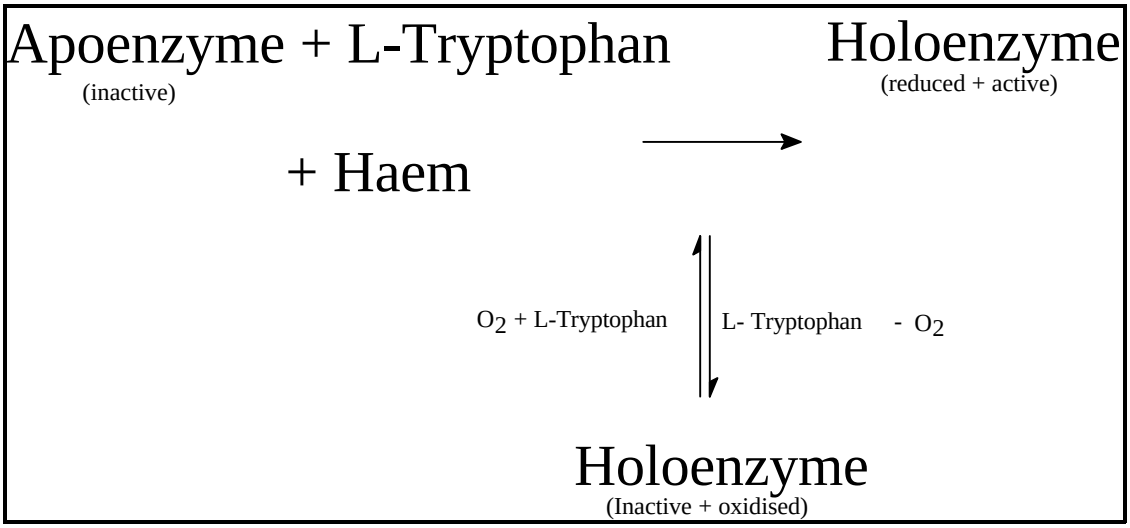

Figure 12: The activation of TDO (modified from Walsh, 1996).

1.4.2 Regulation of tryptophan-2,3-dioxygenase

Rat liver TDO is controlled by four mechanisms. These regulating factors are glucocorticoids, tryptophan, haem, and NADPH (Badawy, 1979). The glucocorticoids, hydrocortisone and corticosterone, cause a hormonal induction of TDO. This involves the synthesis of a new apoenzyme (Knox and Auerbach, 1955; Young, 1981). TDO exhibits a diurnal rhythm which correlates with plasma corticosteroid levels (Wurtman, 1974). The basal capacity of the liver to metabolize tryptophan is however unaffected by the absence of glucocorticoids. The metabolism of tryptophan is affected in adrenalectomised rats. This is possibly due to the fact that these animals are unable to initiate the steroid-induced

increases in TDO activity.

Tryptophan induces substrate activation of TDO. This involves decreased degradation of the pre-existing apoenzyme in conjunction with its normal rate of synthesis. Substrate activation of TDO is also accompanied by increases in haem saturation and stabilization of TDO (Badawy and Evans, 1975). Agents that inhibit protein synthesis inhibit the substrate activation of TDO.

Tryptophan-2,3-dioxygenase conjugation with haem, its cofactor, is the initial step in the activation of the apoenzyme. The saturation of the apoenzyme with haem is modified by several agents. This results in inhibition or enhanced synthesis of haem in the liver. The administration of haematin has been shown to increase the activity of TDO (Badawy and Evans, 1975). Utilization of endogenous haem induces negative feedback inhibition on the enzyme 5-aminolaevulinate synthase. This rate-limiting enzyme is responsible for the production of haem from its precursor 5-aminolevulinic acid. This leads to further synthesis of haem and increased activity of TDO. Drugs, such as the barbiturates, increase haem turnover and thereby increase TDO activity. The daytime administration of the haem precursor 5-aminolevulinic acid, has also been shown to increase TDO activity (Daya *et al*, 1989). This is also due to saturation of the enzyme with haem.

1.4.3 TDO and its role in depression

The 'biogenic amine' hypothesis states that depression is characterised by a decrease of serotonin at its synapse (Walsh and Daya, 1998). Several researchers have shown that in depressed individuals, changes in serotonin metabolism affect mood. TDO activity is the major peripheral determinant of tryptophan levels (Badawy *et al*, 1981). The availability of tryptophan to the brain is also an important factor in the synthesis of serotonin (Daya *et al*, 1989). There is an inverse relationship between TDO activity and the rate of serotonin synthesis (Walsh and Daya, 1998). Activation of TDO has been shown to reduce rat forebrain serotonin levels (Daya *et al*, 1989; Van Wyk *et al*, 1991).

Several inducers of TDO activity have been associated with depressive disorders. The 'kynurenine' hypothesis claims that a link exists between abnormal serotonin and adrenocortical metabolism (Curzon and Green, 1969). As stated earlier, hepatic TDO concentrations are increased by corticosteroids. High levels of plasma corticosteroid, which stimulate TDO activity, have been found in depressed individuals (Hullin *et al*, 1967). Stress, which leads to an increase in the levels of corticosteroids has also been shown to increase TDO activity and subsequently reduce brain serotonin levels (Curzon and Green, 1969). It has also been demonstrated that a large number of antidepressant drugs, for example, desipramine, increase brain tryptophan as a result of the inhibition of TDO activity. This inhibition appears to be as a result of the prevention of the conjugation of the apoenzyme with haem (Badawy and Evans, 1981).

1.5 Acetaminophen

1.5.1 History of acetaminophen

The first observations about the analgesic and antipyretic properties of acetaminophen were made back in the late nineteenth century when alternative compounds were being sought as antipyretic agents in the treatment of infections (Insel, 1996). The antipyretics which were used at the time were preparations of natural compounds such as cinchona bark, from which quinine is derived. These natural sources declined, and cheaper synthetic substitutes were required. Between 1886 and 1887, two alternative antipyretic agents, acetanilide, and phenacetin, were developed. Both these compounds possessed analgesic and antipyretic actions resulting in them being more advantageous than quinine. However both these compounds proved to be very toxic.

In 1893, another compound, acetaminophen, was first introduced into medicine. It was shown to be present in the urine of patients who had taken phenacetin and acetanilide. Subsequent research in 1949 established that acetaminophen was the major metabolite of both phenacetin and acetanilide. It was believed that the action of these two compounds

was due to their rapid conversion to acetaminophen. It was later established that acetaminophen formation was not the mode of action of phenacetin. Acetaminophen was introduced in the United Kingdom in 1956, and its popularity increased rapidly. Acetaminophen has become one of the most widely used and accepted analgesic and antipyretic agents worldwide.

1.5.2 Structure of acetaminophen

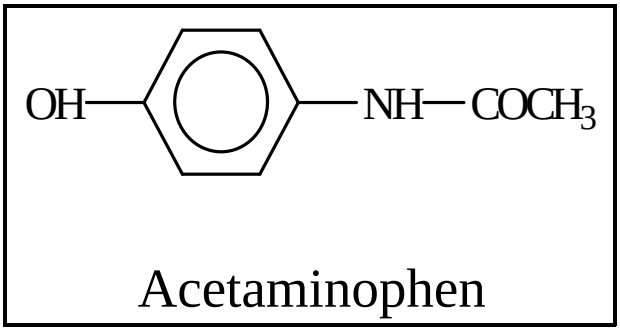

Figure 13: The chemical structure of the analgesic and antipyretic agent, acetaminophen.

1.5.3 Pharmacological effect of acetaminophen

Acetaminophen has analgesic and antipyretic agents similar to those of aspirin. However, it only possesses weak anti-inflammatory actions. This is mainly due to acetaminophen being a weak inhibitor of cyclooxygenase in the presence of peroxides that are found in inflammatory lesions (Insel, 1996). Acetaminophen, like aspirin, is indicated in the relief of mild to moderate pain, such as headaches. Acetaminophen reduces fever by inhibiting the action of endogenous pyrogen, which is released from leukocytes, on the hypothalamic heat-regulating centres.

1.5.3.1 The antinociceptive action of acetaminophen

The antinociceptive action of acetaminophen is believed to be related to the serotonergic

system (Pini *et al*, 1996). Srikiatkhachorn *et al* (1999) examined the antinociceptive effect of both acute and chronic administration of acetaminophen, using tail flick latency measurements in the rat. A significantly reduced tail flick latency was observed in acutely treated rats but not in chronically treated rats (Srikiatkhachorn *et al*, 1999). The same researchers then investigated the plasticity of receptors at the postsynaptic membrane by employing a radioligand binding method on the frontal cortex and brainstem membrane. Srikiatkhachorn and colleagues found a significant decrease in serotonin binding sites on the frontal cortex after acetaminophen administration. These results are consistent with other results which show a decrease in serotonin receptors (5-HT_2 and 5-HT_{1A}) following acetaminophen treatment. These results suggest that the downregulation of serotonin receptors in response to serotonin release is a major step in the mechanism underlying the analgesic effect produced by acetaminophen (Srikiatkhachorn *et al*, 1999).

1.5.4 Absorption and distribution of acetaminophen.

Acetaminophen is rapidly and completely absorbed from the gastrointestinal tract. Peak plasma concentrations are reached within an hour (Payan and Katzung, 1995). The plasma half-life is between one to three hours. Acetaminophen is evenly distributed throughout most body fluids, and between 20% to 50% is plasma bound. Approximately 90% of acetaminophen may be recovered in urine after the administration of therapeutic doses.

1.5.5 Metabolism of acetaminophen

As stated earlier, approximately 90% of acetaminophen administered therapeutically is recovered in the urine. There is a significant first pass metabolism of approximately 20%. Approximately 60% undergoes hepatic conjugation with glucuronic acid. Another 35% of the drug undergoes conjugation with sulphuric acid. A small amount (3%) is also

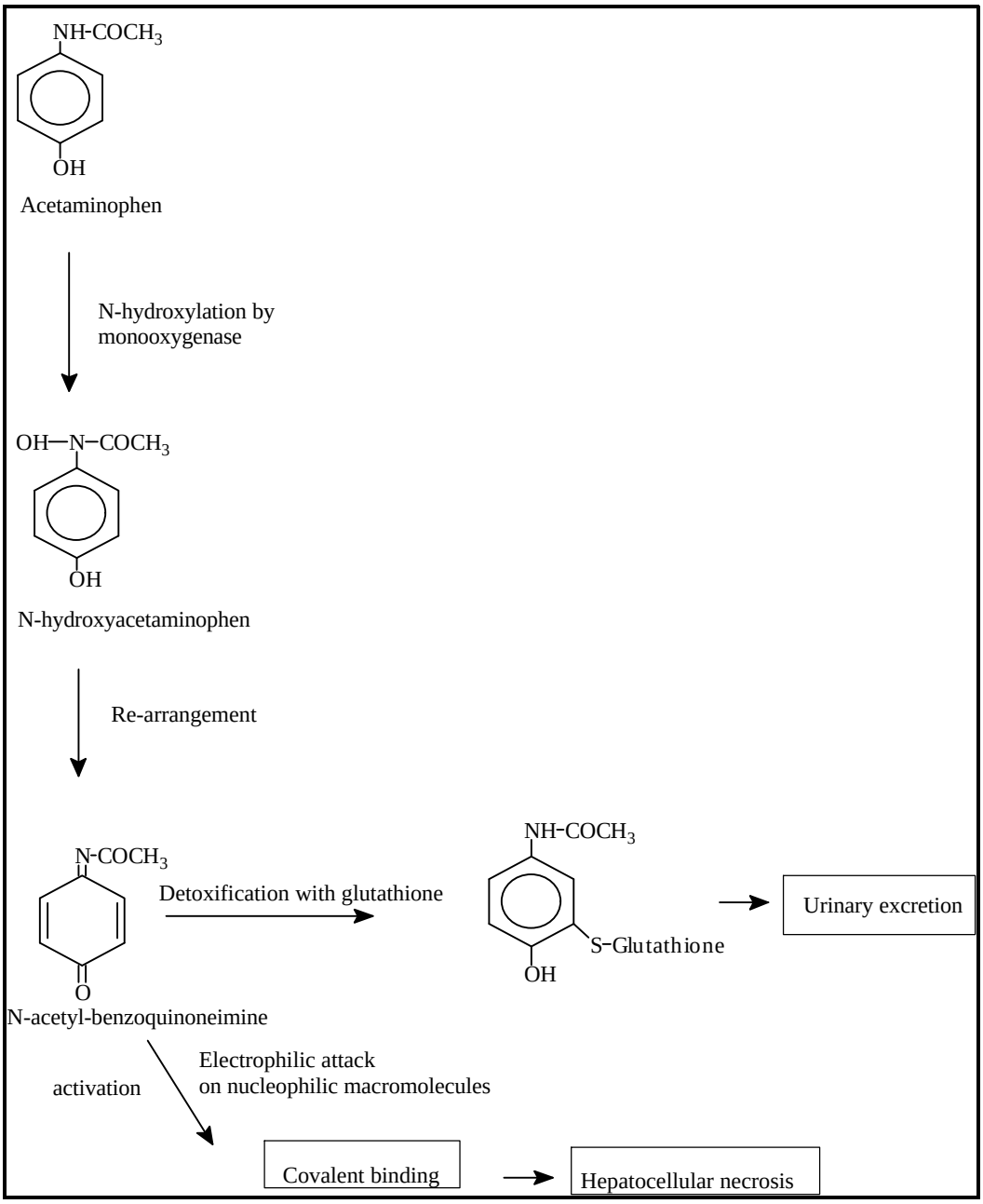

Figure 14: The role of metabolism in the toxicological activation of acetaminophen (Gibson and Skett, 1986).

conjugated with cysteine. Hydroxylated and deacetylated metabolites have also been detected in urine. A small proportion of acetaminophen undergoes cytochrome P450 mediated N-hydroxylation to form N-acetyl-benzoquinoneimine. This is a toxic metabolite and is detoxified by binding to the sulphydryl groups of glutathione (Figure 12). This

complexation is catalysed by the enzyme gluthathione-S-tranferase (Gibson and Skett, 1986) and the complex is then excreted in the urine (Insel, 1996).

1.5.6 Toxicity of acetaminophen

In the recommended therapeutic dose, acetaminophen is well tolerated. Skin rashes, which are usually urticarial, may occur occasionally. Renal tubular necrosis is also consistent with chronic use of acetaminophen (Bach *et al*, 1998). The most serious adverse effect of acetaminophen overdosage is a dose-dependant, potentially fatal, hepatic necrosis.

1.5.6.1 Acetaminophen and free radicals

The studies on acetaminophen and free radical generation have been conflicting. Acetaminophen is metabolised to N-acetyl-benzoquinoneimine (Figure 12). This compound is then metabolised by liver microsomes to produce the p-aminophenoxyl radical (Mason and Fischer, 1986). The acetaminophen free radical is highly reactive. Lores Anaiz *et al* (1995) analysed the effect of acute acetaminophen administration on oxidative stress. These researchers administered a single dose (375mg/kg) of acetaminophen, and then determined the effect of the dose on several parameters. These authors found a significant decrease in the activity of catalase and glutathione peroxidase after 15 minutes. This was also found to result in an increased steady state level of H_2O_2 and hydroperoxides. Microsomal superoxide production was also found to increase two-fold following the administration of acetaminophen (Lores Anaiz *et al*, 1995).

However, there are reports which indicate that acetaminophen acts as a free radical scavenger, rather than generating free radicals. Dinis *et al* (1994), showed acetaminophen to be an effective peroxyl radical scavenger *in vitro*. These authors also found that acetaminophen protected the sarcoplasmic reticulum membranes against Fe^{2+}/ascorbate-induced lipid peroxidation. Nakamoto *et al* (1997) also demonstrated the antioxidant properties of acetaminophen. These authors showed that acetaminophen reduced lipid

peroxidation caused by the hydroxy radical. This was authenticated by Tsujimoto *et al* (1998). Using electron spin trap resonance, these authors showed that acetaminophen scavenged the hydroxyl radical generated by Cu^{2+}/H^2O^2. Acetaminophen has therefore been shown to generate and scavenge free radicals.

1.5.6.2 Hepatotoxicity

In therapeutic doses, acetaminophen produces a toxic metabolite. This metabolite, N-acetyl-benzoquinoneimine (NAB), is detoxified by glutathione. However when large doses of acetaminophen are ingested, there is sufficient NAB produced to deplete hepatic glutathione levels. This is due to glutathione being depleted faster than it can be regenerated. Under these circumstances, NAB covalently binds with sulphydryl groups in hepatic proteins, leading to hepatic necrosis (Figure 12). This necrosis is believed to result from the accumulation of Ca^+, the activation of Ca dependent endonuclease, and resultant DNA fragmentation (Insel, 1996). Clinically, hepatotoxicity can be diagnosed by measuring the increased levels of the serum transaminases.

1.5.6.3 Toxic dose

In adults, hepatotoxicity may occur after the ingestion of 10g to 15g of acetaminophen. Administration of a single dose of 15g or more is potentially fatal (Payan and Katzung, 1995).

1.5.6.4 Symptoms of overdosage

Symptoms that manifest during the first two days of acute poisoning of acetaminophen may not reflect the potential seriousness of the intoxication. Symptoms that occur during the first 24 hours include nausea, vomiting and abdominal pain, and this may persist for a week. Hepatic damage is evident within two to four days after ingestion. Plasma aminotransferases are elevated and prothrombin time is prolonged. Approximately 10% of

poisoned patients who do not receive treatment develop liver damage. Between 10% and 20% of the patients who do not receive treatment eventually die due to hepatic failure (Insel, 1996).

1.5.6.5 Treatment of overdosage

Early diagnosis of overdosage is vital in the treatment. Gastric lavage should be performed within four hours of ingestion. The principal antidote used in the treatment of acetaminophen overdosage is N-acetylcysteine which is effective if administered within eight hours. N-acetylcysteine is a glutathione precursor and is also a source of sulphydral groups. This facilitates the conversion of toxic metabolites to non-toxic metabolites. N-acetylcysteine may also act as a free radical scavenger and prevent lipid peroxidation. N-acetylcysteine offers complete protection if administered within eight hours. Treatment between 8 to 24 hours lowers mortality but does not offer complete protection.

However, N-acetylcysteine administration may also lead to adverse reactions such as anaphylactoid reactions, nausea, and vomiting. This is partly due to half the dose, 150mg/kg, being administered immediately, and may worsen the situation.

1.6 Dimethylformamide

1.6.1 Introduction

N,N-dimethylformamide (DMF) is an industrial chemical with an estimated worldwide production capacity of approximately 250 000 tons in 1989 (Angerer, *et al*, 1998). It is commonly used for vinyl-based polymers in the manufacture of films, fibres, and coatings. DMF is also used as a solvent for making polyurethane lacquers for clothing and accessories made of synthetic leather (Angerer *et al*, 1998). DMF is an excellent lipid and water soluble solvent. Occupational exposure results mainly from inhalation of vapour and from contact with skin (Scailteur *et al*, 1984). Although there is inadequate evidence for

the carcinogenicity of DMF in experimental animals, DMF is possibly carcinogenic to humans according to the International Agency for Research on Cancer of the World Health Organisation (Angerer *et al*, 1998).

1.6.2 Chemical structure of DMF

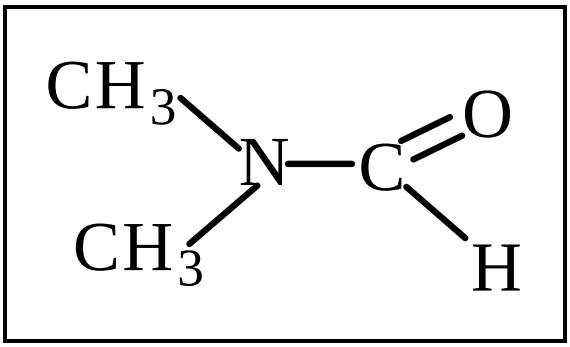

Figure 15: The chemical structure of DMF (Angerer *et al*, 1998).

1.6.3 Metabolism and toxicity of DMF

Current scientific evidence suggests that the main target organ, following acute or long-term exposure to DMF, is the liver (Scailteur *et al*, 1984). The primary metabolite of DMF is N-(hydroxymethyl)-N-methylformamide (HMMF). The metabolic end product after oxidation of the formyl group of HMMF, is N-acetyl-S-(N-methylcarbamoyl)-cysteine (AMMC) (Angerer *et al*, 1998). It is believed that the formation of AMMC may occur via the pathway which may include the reactive intermediate methylisocyanate (MIC) (Angerer *et al*, 1998). This reasoning is attributed to the Bhopal incident where N-methylcarbamoyl adducts were found at the N-terminal valine end of haemoglobin in the post-mortem blood of victims following acute poisoning by MIC (Angerer *et al*, 1998). The end product following the analysis of this blood was found to be 3-methyl-5-isopropyl-hydantoin (MIH).

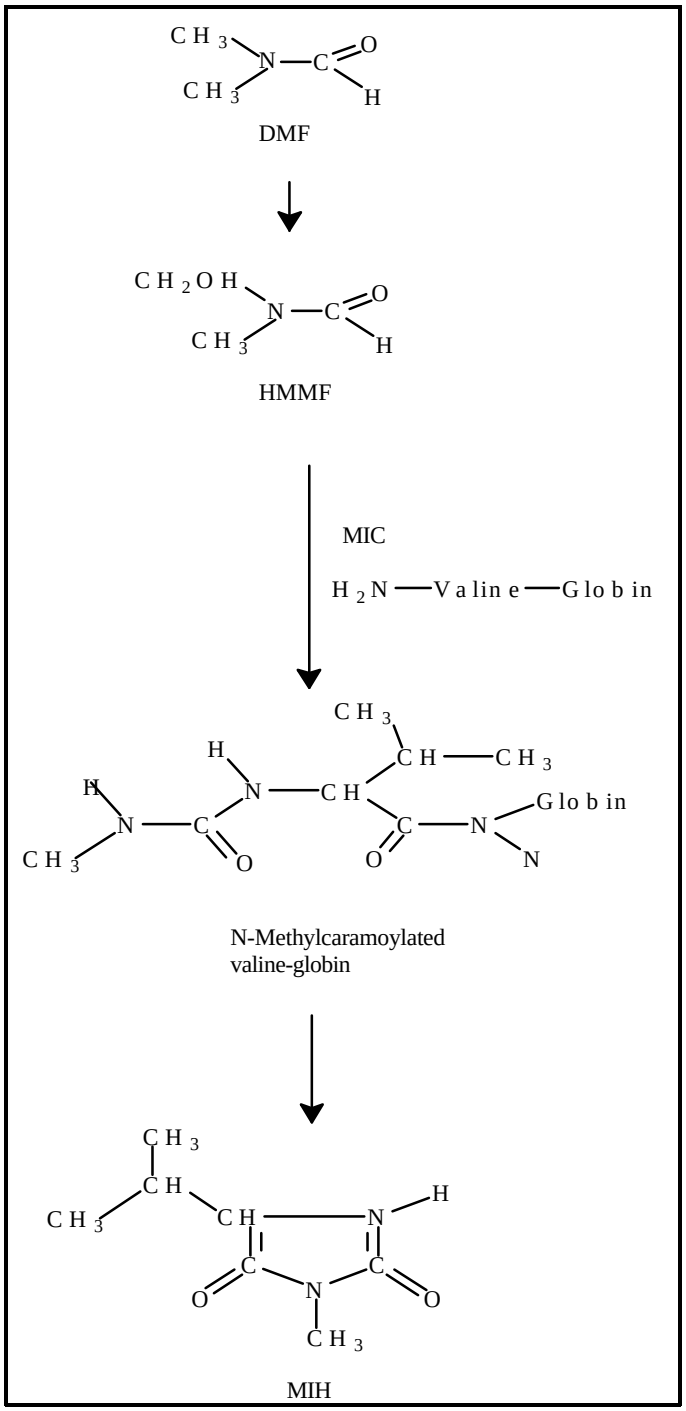

Figure 16: The metabolism of DMF in humans, with the suspected involvement of MIC (Angerer *et al*, 1998).

Using capillary gas chromatography, Angerer *et al* (1998) investigated whether similar haemoglobin adducts were present in workers after chronic exposure to DMF. The workers chosen were those exposed to DMF (2.2ppm - 53,7ppm) in the polyacryl fibre industry. Angerer and colleagues found identical haemoglobin adducts as those found after exposure to MIC. This is illustrated in Figure 14. In a similar study, Wrbitzky (1999) examined the effects of DMF exposure on liver function. Once again workers in a synthetic fibre factory were chosen. The DMF concentrations in the air ranged between 0.1ppm and 37.9ppm. These authors found a statistically significant toxic influence on liver function (Wrbitzky, 1999). Malley *et al* (1994) studied the toxicity and oncogenicty of chronic DMF exposure on male and female rats. The results obtained indicate severe hepatocellular injury. These studies undeniably illustrate the hazardous nature of DMF, especially from occupational exposure.

1.6.4 Symptoms of DMF toxicity

The symptoms of acute and chronic intoxication by DMF are the same as those usually associated with liver damage (Amatimaggio *et al*, 1998). The main symptoms of DMF poisoning are gastrointestinal disturbances such as diarrhoea and nausea. There are also numerous reports documenting alcohol intolerance following exposure to DMF (Amatimaggio *et al*, 1998; Wrbitzky, 1999).

1.6.5 The monitoring of DMF exposure

Chang and Lin (1991) investigated a non-invasive method of monitoring DMF exposure. Using a colorimetric method, HMMF was measured in rat urine following a single exposure to DMF. The researchers found a linear relationship between the total excretion of HMMF in two days, versus the exposure ranging from 1% (47.2mg/kg) to 20% (944mg/kg) of the LD_{50} of DMF. This method was proposed as a non-invasive biological method for monitoring exposure to DMF, and as a possibility in the prevention of the occupational toxicity of DMF (Chang and Lin, 1991).

1.7 Research objectives

The first objective of this study was to investigate the hepatoprotective properties of melatonin against acetaminophen and DMF-induced toxicity. This part of the study is based on the assumption that melatonin might be able to protect the liver against acetaminophen and DMF-induced damage. The first objective was to determine the toxic doses of acetaminophen and DMF, and the mechanism of this damage. The investigation of the mechanism of toxicity was to be carried out *in vitro* and *in vivo* using the rat as an animal model. A determination of lipid peroxidation in the liver is a reliable indicator of free radical-induced damage. Several *in vitro* techniques, such as free radical generation and quantification using HPLC with electrochemical detection, would also be useful in determining the mechanism of toxicity. Melatonin could be the perfect candidate as a hepatoprotectant against acetaminophen and DMF induced damage if the damage is due to free radical generation, since melatonin is a known potent free radical scavenger. To date there have been no reports on an antidote for DMF poisoning. Melatonin is also known to increase glutathione production. Glutathione is responsible for the detoxification of the toxic metabolites produced by acetaminophen metabolism, and free radicals in general.

The second objective was to determine the effect of acetaminophen on rat brain and pineal idoleamine synthesis. The precursor of acetaminophen, phenacetin, is known to produce euphoria after ingestion. This would also possibly unlock the secret of analgesic addiction. Most mood states depend on the levels of brain serotonin and pineal melatonin. As discussed earlier, reduced serotonin and melatonin levels are a reflection of an altered mind state. This change is usually manifested as depression. Therefore serotonin and melatonin levels could possibly be indicators of mood states. By determining the effect of acetaminophen on these indoles, an insight into the mind-altering capabilities of acetaminophen can be obtained. This can be confirmed by determining the activity of TDO, which is known to play a role in depression by altering the synthesis of serotonin.

1.7.1 Proposed experiments

1.7.1.1 Evaluation of melatonin as a hepatoprotectant (toxicology)

The effectiveness of melatonin as a hepatoprotectant would be established by:

- the effect of melatonin on acetaminophen degradation, using a photoreactor and HPLC (*in vitro)*;
- Melatonin stability in solution (*in vitro)*;
- the quantification of free radical generation by DMF (*in vitro*), in collaboration with the CSIR, Calcutta, India;
- Electron microscopy; and
- the determination of lipid peroxidation following the chronic administration of acetaminophen and DMF (*in vivo*).

1.7.1.2 The effect of acute acetaminophen administration on brain and pineal indole metabolism

Establishing the effect of acute acetaminophen administration on brain and pineal metabolism would comprise:

- the determination of TDO activity following acute administration of acetaminophen *(in vivo)*;
- the determination of brain serotonin levels using ELISA (*in vivo*);
- the determination of urinary 5-HIAA levels (*in vivo*); and
- the determination of pineal melatonin levels using ELISA and Pineal organ culture (*in vitro).*

Chapter Two

Photolytic Degradation of Acetaminophen and Melatonin

2.1 Introduction

Photodegradation studies can be performed either on an analytical or on a preparative scale. Analytical apparatus enables the reaction vessels to be moved varying distances from the light source, thereby controlling the rate of the reaction. This technique also enables the use of a shutter, placed between the lamp and vessel which facilitates the analysis of the solution without the lamp having to be switched off (Moore, 1987). Preparative scale apparatus results in higher yields of photoproducts. Vigorous stirring is essential, as the majority of absorption of light takes place in the layer of solution closest to the light source.

The immersion-well photoreactor (Figure 17) is a useful instrument for performing photodegradation studies. This instrument consists of an outer Pyrex vessel and an inner removable double-jacketed immersion-well. The lamp is contained in the double-walled immersion-well, which is made of either quartz or borosilicate glass. This allows water cooling, and filtering of excitation radiation. Due to the lamp being surrounded by the solution being irradiated, the immersion-well photoreactor is among the most efficient reactors used for photochemical reactions (Photochemical Reactors, Applied Photophysics).

Since the absorption of light is necessary to effect a photochemical change, the choice of light is important (Moore, 1987). The most widely used sources of ultraviolet and visible light for conventional photochemical experiments are the mercury and xenon lamps. All irradiations carried out in this study involved using a 400W high pressure mercury lamp, emitting over the ultraviolet-visible range. According to the ‘first law of photochemistry’,

the generation of free radicals by acetaminophen is possible in this case, since the excitation wavelength is such that radiation absorbed by the molecule may result in a chemical reaction (Gilbert & Baggott 1991).

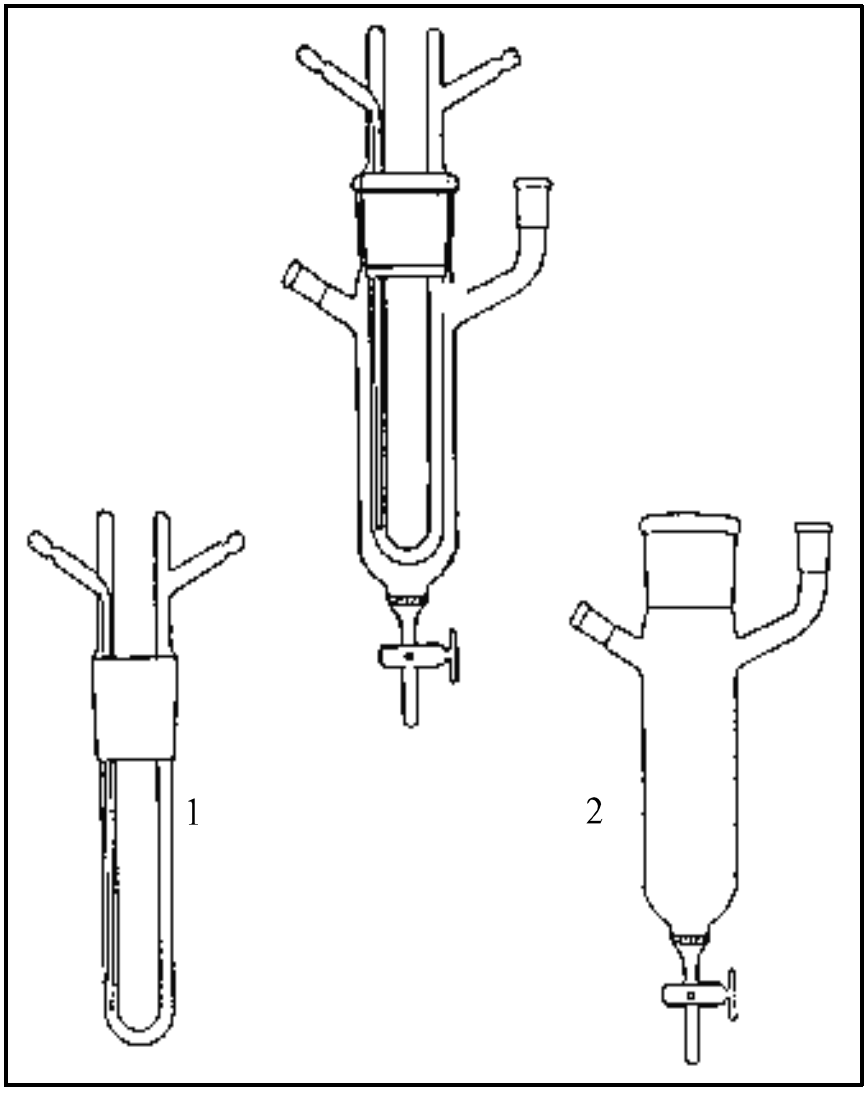

Figure 17: The Immersion well photoreactor with detail of the double-walled immersion well (1), and the outer borosilicate flask (2) (Photochemical Reactors, Applied Photophysics)

2.2 Materials and methods

2.2.1 Chemicals and reagents

Melatonin was purchased from the Sigma Chemical Co, St Louis, MO, USA. Acetonitrile (HPLC grade) was purchased from BDH Laboratory Supplies, Poole, England. Absolute ethanol (HPLC grade) was purchased from Saarchem (PTY) Ltd, Krugersdorp, South Africa.

2.2.2 Instrumentation

Samples were analyzed on a modular, isocratic high performance liquid chromatographic (HPLC) system. The chromatographic system used consisted of a Spectraphysics Iso Chrom LC Pump, a Linear UVIS 200 Detector, and a Perkin Elmer 561 Recorder. Samples were introduced into the system using a Rheodyne fixed loop injector, fitted with a 20µl loop. The photodegradation apparatus consisted of a quartz immersion-well photoreactor (Applied Photophysics), and a 400W high pressure mercury lamp.

2.2.3 Chromatographic conditions

Separation was achieved using a C_{18} (Waters Spherisorb, 5µm, 250×4.6mm i.d.) column. The mobile phase composition for the analysis was acetonitrile:water (10:90), and acetonitrile:water (40:60), for acetaminophen and melatonin respectively. The mobile phase flow rate was 1ml/min and the chart speed on the recorder was 5mm/min. The detector sensitivity was set at 0.5 AUFS (absorbance units full scale). Acetaminophen was detected at 254nm and melatonin at 304nm.The analytical procedure was validated by assessment of peak purity and selectivity, linearity of calibration (0.01-0.1mg/ml), repeatability, accuracy, precision, and limits of quantitation and detection.

2.2.4 Sample preparation

Separate solutions of acetaminophen (0.1mg/ml) and melatonin (0.1mg/ml) were made up in 10% absolute ethanol in Millipore water. A combination of acetaminophen and melatonin was also prepared using 0.1mg of each per ml of 10% absolute ethanol in Millipore water. A calibration curve was obtained using 0.01mg/ml - 0.1mg/ml of both acetaminophen and melatonin.

2.2.5 Method

Acetaminophen (0.1mg/ml) was placed in the immersion-well photoreactor and irradiated continuously for six hours using a 400W UV lamp, whilst bubbling air or nitrogen through the solution. Melatonin (0.1mg/ml) was placed in the immersion-well photoreactor and irradiated continuously with a 400W lamp for one and a half hours, whilst bubbling air or nitrogen through the solution. The selected times for UV irradiation were predetermined. In addition, the combination of acetaminophen and melatonin was irradiated continuously with the same intensity of UV light either in the presence of air or nitrogen. Aliquots of 5ml were removed every 20minutes for the first hour, and thereafter every 30minutes. These aliquots were analysed using the HPLC system described above.

2.3 Results

The height of the peak at time (0) was taken to represent 100% of the drug. The decrease of the peak height during the study was taken to be and indication of the percentage of drug present. The mobile phase was optimized for a rapid and interference-free chromatogram. As shown in Figure 18, the chromatogram obtained from the acetaminophen and melatonin standard solutions demonstrates sharp, symmetrical, and well resolved peaks. The peaks were also well removed from the solvent front. The retention times for acetaminophen and melatonin were approximately 8.6 minutes and 7.2 minutes respectively. As shown in Figure 19, the rate of melatonin photodegradation is

accelerated when the solution is aerated, compared to purging with nitrogen only. In air, only 20% of the melatonin remained after 20 minutes of irradiation, whereas under nitrogen, 50% remained after the same period. The rate of acetaminophen photodegradation was similar in air and nitrogen, with 50% of the drug remaining after six hours of irradiation. In the experiment in which the two drugs were combined, melatonin retarded the degradation of acetaminophen up to six hours in the presence of nitrogen, with 10% more of the drug remaining at the end of six hours. However, in the presence of air, melatonin rapidly enhances the photodegradation of acetaminophen, with total degradation in two hours, with the degradation at 45% remaining stable from 20-60 minutes before declining to zero at two hours.

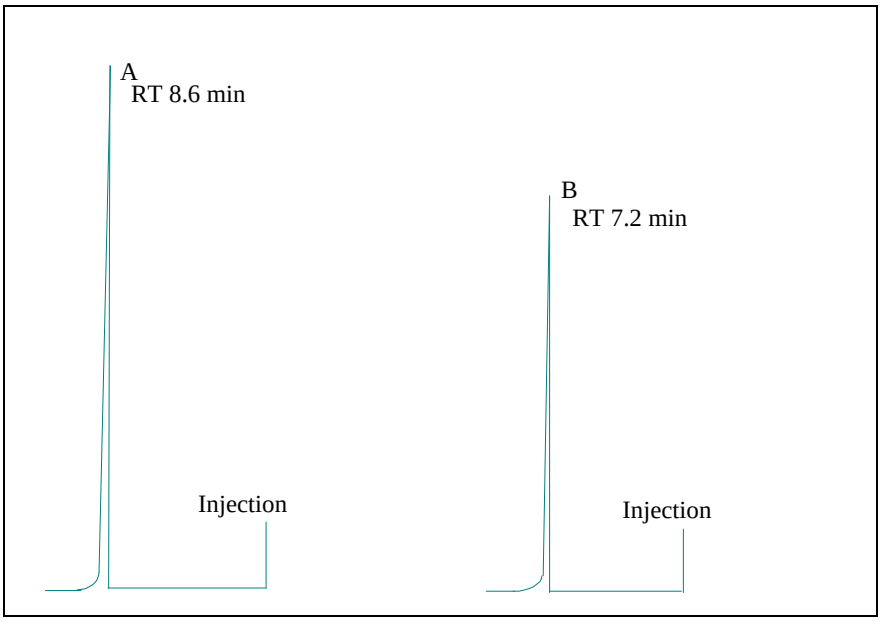

Figure 18: A typical chromatogram of acetaminophen (A) and melatonin (B)

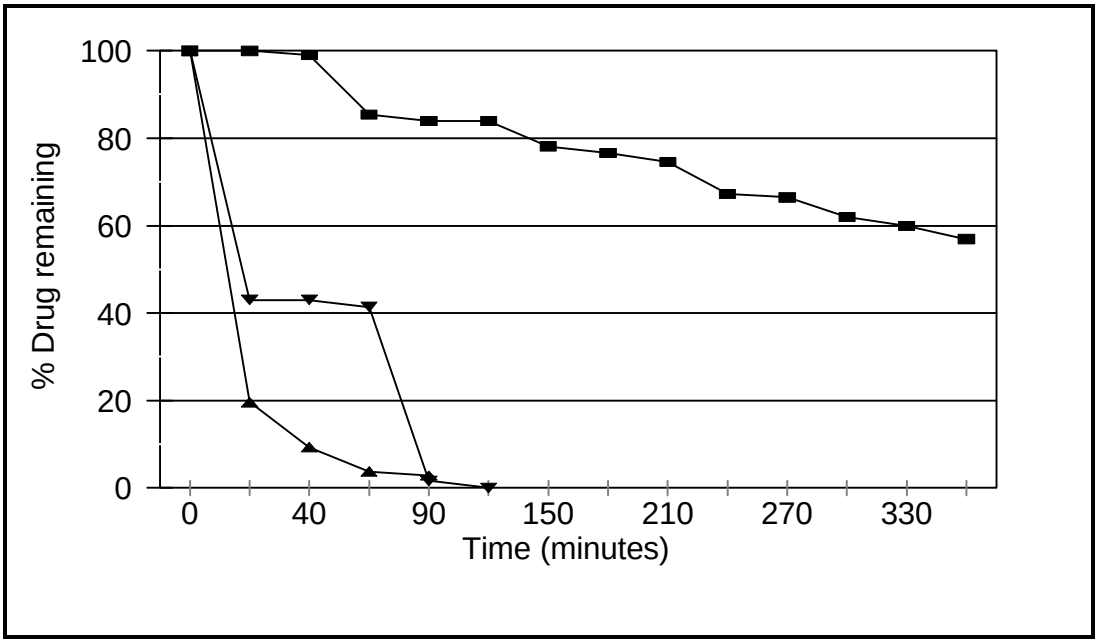

Figure 19: The effect of 400W UV irradiation in the presence of air on the photodegradation of melatonin alone (>), and acetaminophen alone (■), and on acetaminophen combined with melatonin (?).

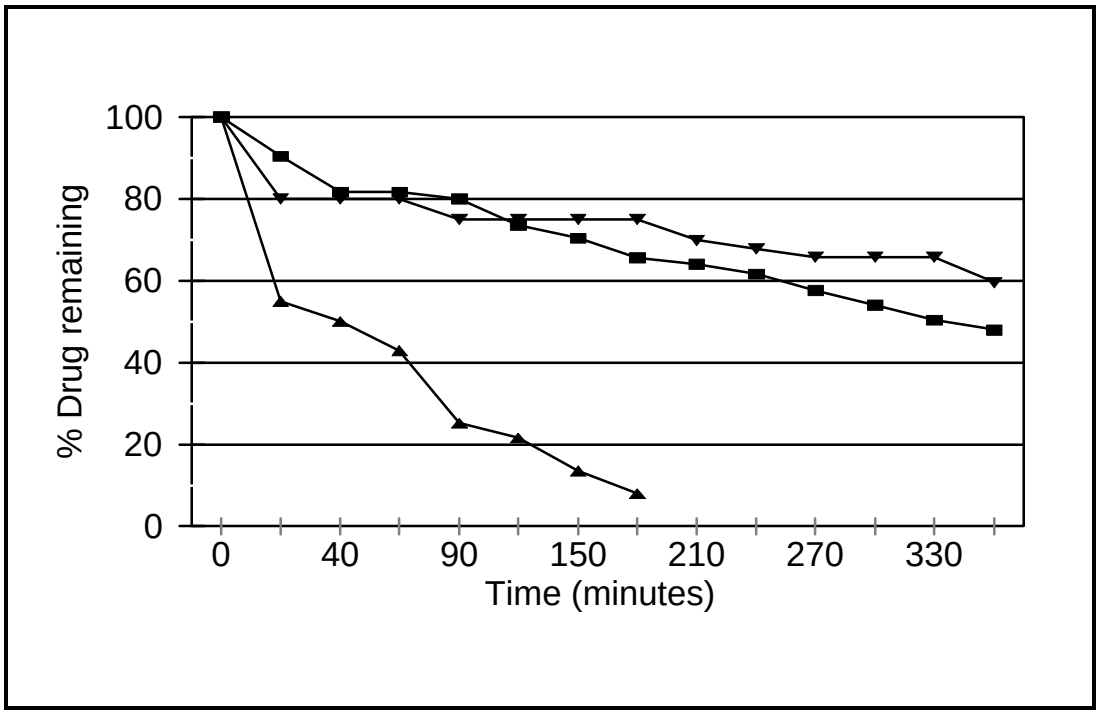

Figure 20: The effect of 400W UV irradiation in the presence of nitrogen on the photodegradation of melatonin alone (>), and acetaminophen alone (■), and on acetaminophen combined with melatonin (?).

2.4 Discussion

The possible use of melatonin as a potential antidote for acetaminophen toxicity is a novel approach. However, before such studies can be undertaken it is important to determine the behaviour of both acetaminophen and melatonin under conditions in which the complexities of organ interaction are absent. The present system used in these experiments, allows the investigation of chemical interaction between these agents in a purely chemical environment, albeit far removed from an *in vivo* situation.

UV irradiation of acetaminophen is known to result in photodegradation with the possibility of free radical generation. Melatonin, the principal hormone of the pineal gland, now known to be a potent free radical scavenger at physiological concentrations (Tan *et al*, 1993; Daya, 1999), is an ideal candidate for scavenging such radicals, whether these are formed as degradants of acetaminophen, or whether these assist in the photodegradation of acetaminophen (Anoopkumar-Dukie, In Press).

The results of the present report show that the presence of oxygen accelerates the photodegradation of melatonin, as opposed to purging with nitrogen. Such photodegradation occurs with a complexity of photoproducts as is customary in the presence of oxygen. Our finding that these photoproducts are more polar than melatonin, suggests the incorporation of oxygen. The rate of acetaminophen degradation was much slower than that of melatonin, as shown in Figures 19 and 20. It appears that this rate of degradation is similar in air and in nitrogen, with 50% of the drug remaining after six hours of UV irradiation. The photodegradants in this case, although more polar than the parent drug, could not be identified as the major degradant of acetaminophen, p-aminophenol, ruling out á-cleavage as the mechanism of photodegradation. This was confirmed by comparison with a known sample of p-aminophenol. The results show that the presence of melatonin retards the degradation of acetaminophen in nitrogen. However, in the presence of air, melatonin dramatically enhances the photodegradation of

acetaminophen, suggesting that the reaction does not occur via a triplet state, since molecular oxygen is an effective quencher of triplet excited states (Gilbert and Baggot 1991).

It is known that solutions of melatonin, maintained in sterile, pyrogen-free conditions, may be stored at 4^{o}C for six months without degradation (Cavallo & Hassan 1995). The present study shows that exposure of a melatonin solution to UV irradiation causes rapid degradation of melatonin. In the presence of air, melatonin also induces rapid photodegradation of acetaminophen. This implies that the combination of melatonin and acetaminophen, in the presence of air and UV irradiation, could lead to rapid inactivation of both agents. One of the objectives of the present study (Section 1.7) was to determine the stability of melatonin. The results from this study raise important concerns about the medical use of melatonin in sunscreens, since it is rapidly inactivated by UV light. Further studies need to be conducted to determine the exact nature of the photodegradants of both acetaminophen and melatonin.

Chapter Three

Melatonin and DMF-Induced Hepatotoxicity

3.1 Introduction

Due to the ultra short half-life of oxygen free radicals, it has been extremely difficult to demonstrate the formation of ·OH in biological systems (Chiueh *et al*, 1993). The same phenomenon is responsible for the ability of ·OH radicals to produce site-specific oxidative tissue damage *in vivo* (Chiueh *et al*, 1993). Hydroxyl free radicals (·OH) react with salicylate, and generate 2,3- and 2,5-dihydroxybenzoic acid (DHBA). These compounds can then be measured in picomole quantities by HPLC (Chiueh *et al*, 1992) and could provide an assay for the measurement of ·OH radicals.

DHBA formation, following the systemic administration of salicylate, is currently being used as an index of ·OH generation in the heart and brain tissues during ischemia and reperfusion (Chiueh *et al*, 1992). However, it was recently discovered that 2,5-DHBA can be formed not only by ·OH adduct, but also by hydroxylation, catalyzed by liver cytochrome P450 and microsomal enzymes. 2,3-DHBA can be nonenzymatically formed by ·OH aromatic hydroxylation, and can provide a more reliable assay for ·OH formation (Chiueh *et al*, 1992).

This technique (2,3- and 2,5-DHBA formation) has been adapted for the detection of ·OH formation in the extracellular fluid of the brain. Using a microdialysis probe, it is possible to infuse salicylate into the brain by intracranial microdialysis brain perfusion. The formation of 2,3-DHBA, the nonenzymatic ·OH product of salicylate, is a reflection of ·OH generation. 2,3-DHBA is measured using HPLC coupled to an electrochemical detector (Chiueh *et al,* 1993).

3.2 Materials and methods

3.2.1 Chemicals and reagents

Due to the unavailability of very expensive equipment required for the hydroxyl radical determination, it was done in collaboration with the CSIR in Calcutta, India. All materials, including chemicals and reagents, were provided by the CSIR. The chemicals required for electron microscopy was provided the Electron Microscopy Unit, Rhodes University, Grahamstown.

3.2.2 Hydroxyl radical generation and determination

This work was undertaken in collaboration with Dr. K. Mohanakumar at the CSIR. An *in vitro* cell-free system was used in this study The radicals were generated based on the method of Mohanakumar (1994). The radicals were measured by employing the salicylate hydroxylation procedure of Chiueh *et al* (1992). Briefly, the reaction mixture contained 1ml Fe^{2+}-citrate complex (2.1nmol), 1mM sodium salicylate and various concentrations (0%, 0.1%, 1%, and 5.0%) DMF. 10µl of the samples were injected into an HPLC equipped with an electrochemical detector. 2,3- and 2,5-DHBA were oxidized at +0.7V and the peaks were measured in relation to the known standards of 2,3- and 2,5-DHBA.

3.2.3 Electron microscopy

3.2.3.1 DMF administration

Male Wistar rats weighing 250g-300g were used in the experiment. The rats were maintained as described in Appendix 1. The control group (n=5) received ethanol:0.9%saline (40:60) i.p. Another two groups (n=5) received 0.4ml/kg and 0.8ml/kg DMF i.p. respectively. The final two groups received the same doses of DMF in

conjunction with 2.5mg/kg melatonin i.p. The rats were injected for seven days and were killed on day eight, and the livers were removed as described in Appendix 2.

3.2.3.2 Transmission Electron Microscopy

Small cubes of liver (1mm^3) were placed in buffered gluteraldehyde (2.5% gluteraldehyde in 0.1M sodium phosphate buffer), and incubated at 4^{o}C overnight. The gluteraldehyde was replaced the following morning. After 10 minutes the gluteraldehyde was replaced by osmium tetroxide (1% in 0.1M sodium phosphate buffer) and left at room temperature for an hour. Following this, the osmium tetroxide was decanted, and replaced with gluteraldehyde for 10 minutes. The gluteraldehyde was decanted, and replaced with 30%, 50%, and 100% ethanol for three minutes each. This was followed with the addition of propylene oxide for 15 minutes. The propylene oxide was replaced with propylene oxide:araldite (75:25, 50:50, 25:75) for an hour each. Following the addition of the last propylene:araldite mixture, the tissue was placed in pure resin overnight. The tissue was then transferred to moulds containing pure resin and was left to polymerize at 60^{o}C for 36 hours. After this period, the capsules were cooled and thin samples were cut on an ultra microtome for examination.

3.3 Results

The hydroxyl radicals generated were expressed as pmol/ml. The data is the average for three estimations, with the variations being less than 2%. As shown in Figures 21 and 22, there is a decrease in the hydroxyl radical formation (2,3- and 2,5-DHBA) with increasing concentrations of DMF. At 5%, DMF is able to completely reduce the formation of 2,3- and 2,5-DHBA.

As shown in Figure 23, the control livers contained no visible structural changes. DMF was shown to affect the integrity of the endoplasmic reticulum in the rat liver (Figures 24

and 25). As shown in Figures 26 and 27, melatonin was able to reduce the changes caused by DMF.

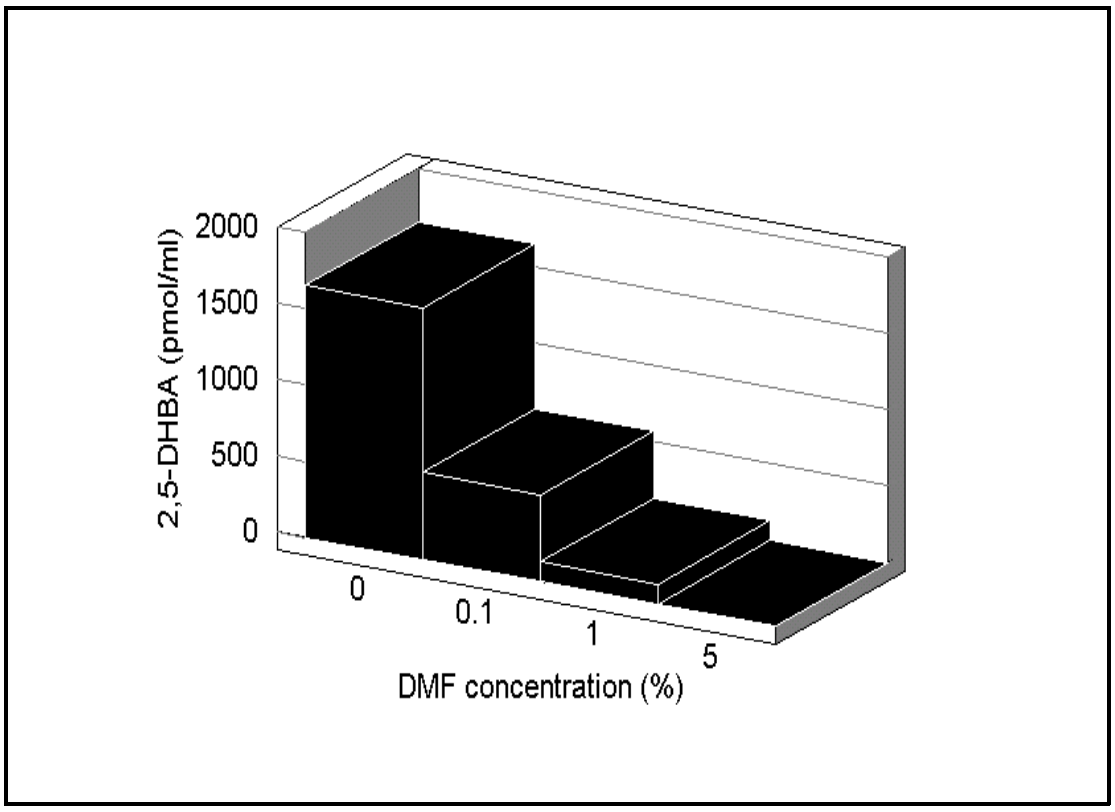

Figure 21: The effect of DMF on 2,5-DHBA generation. Each bar represents the mean of three estimations.

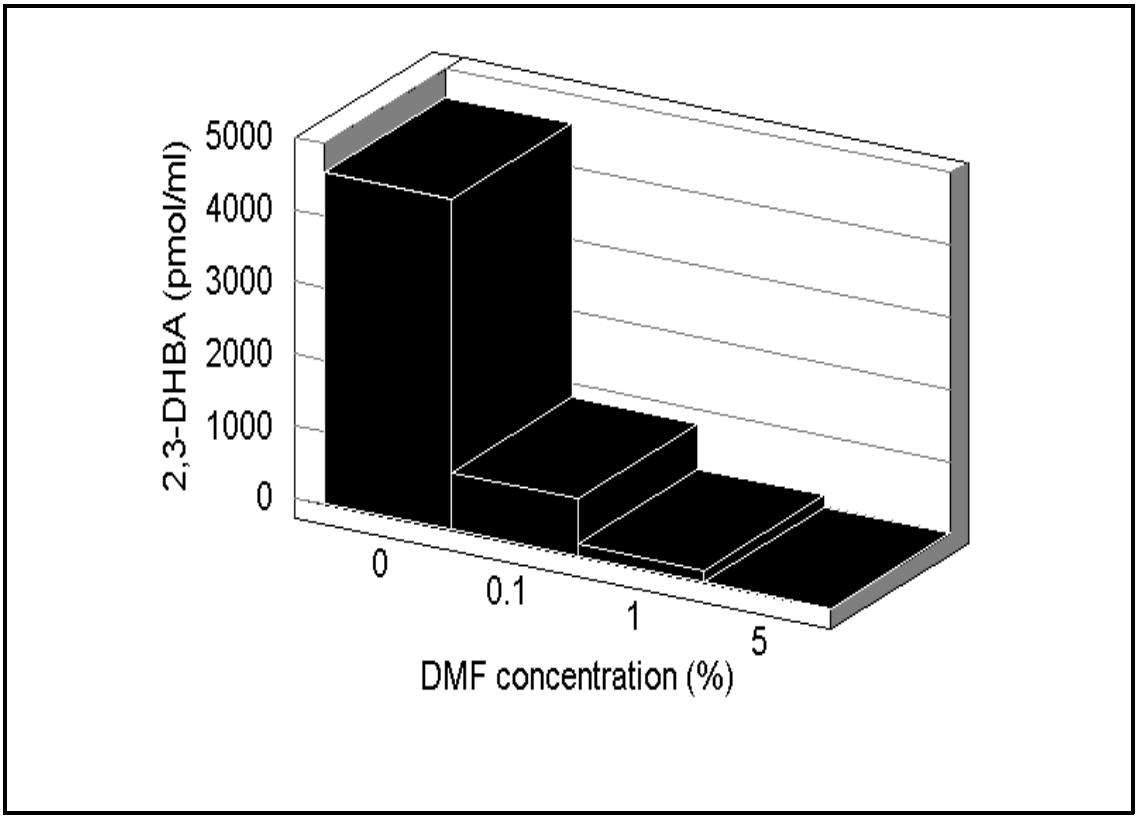

Figure 22: The effect of DMF on 2,3-DHBA generation. Each bar represents the mean of three estimations.

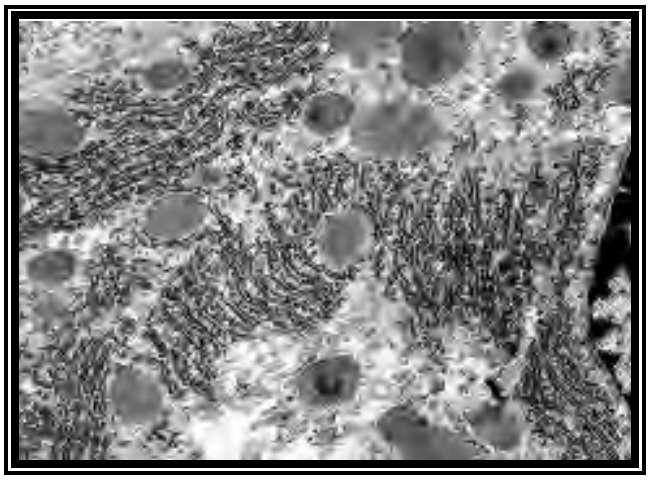

Figure 23: Electron micrograph of control rat liver (15000× magnification).

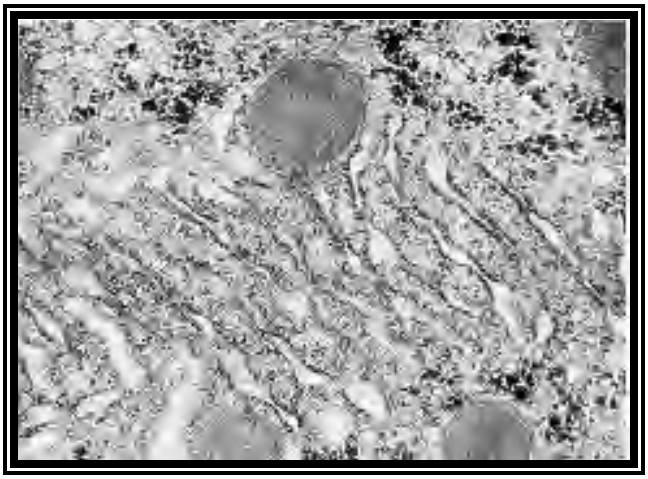

Figure 24: Electron micrograph of DMF (0.4ml/kg) treated rat liver (16000× magnification).

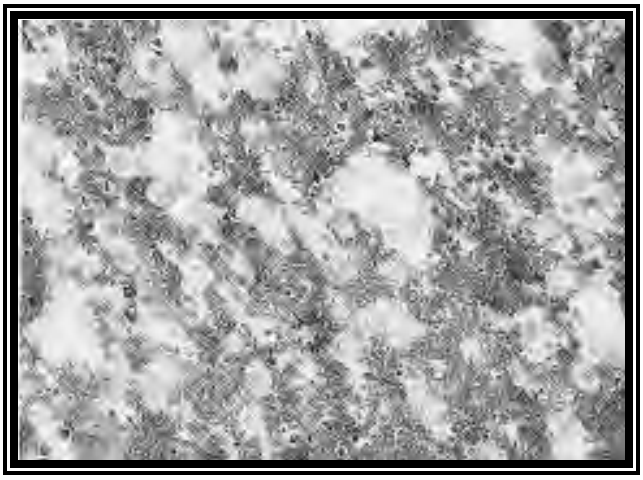

Figure 25: Electron micrograph of DMF (0.8ml/kg) treated rat liver (16000× magnification).

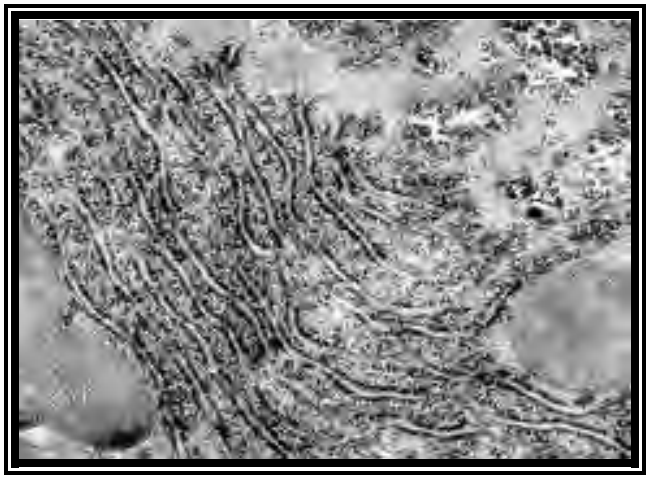

Figure 26: Electron micrograph of DMF (0.4ml/kg) and melatonin (2.5mg/kg) treated rat liver (16000×magnification)

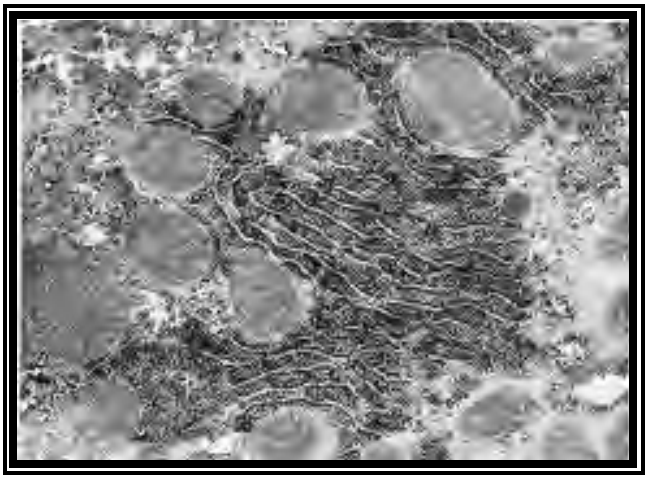

Figure 27: Electron micrograph of DMF (0.8ml/kg) and melatonin (2.5mg/kg) treated rat livers (16000× magnification)

3.4 Discussion

N,N-dimethylformamide is an organic solvent used in several industrial processes. This solvent is toxic, and exposure to it leads to adverse effects. Several studies have shown that DMF is harmful to human beings, with the liver being most adversely affected (Amatimaggio *et al*, 1998). Injury to the liver is predominately due to its being the site of metabolism of DMF. The exact mechanism of this damage is presently unclear. This study hypothesizes that the damage is as a result of free radical generation, and in particular hydroxyl radicals. DMF is known to generate peroxyl radicals *in vitro* (Misik and Riesz, 1996). These authors showed peroxyl radical formation by exposing DMF to 50kHz ultrasound. The peroxyl radicals are then measured using electron spin trap techniques (Misik and Riesz, 1996).

The hydroxyl radicals in the *in vitro* cell-free system used in this study, are generated by the Fe^{2+}-citrate complex. This author speculates that Fe^{2+} itself may be involved in the electron transfer reaction with molecular oxygen or with citrate, forming Fe^{3+} and superoxide, leading to the formation of hydroxyl radicals by the Fenton reaction (Mohanakumar *et al*, 1994). As shown in Figures 21 and 22, increasing concentrations of DMF reduce the formation of both 2,3- and 2,5-DHBA, indicating that it has a negative effect on hydroxyl radical generation. It is also evident that at a concentration of 5% (DMF), there is no formation of these $^{\cdot}OH$ adducts of salicylate. Hydroxyl radicals react with salicylate to generate 2,3- and 2,5-DHBA. These adducts are taken to be reliable indicators of hydroxyl radical generation (Chiueh *et al,* 1992). The results obtained in this experiment refute the hypothesis that DMF induced hepatotoxicity is due to free radical, and in particular hydroxyl radical, generation. DMF has rather been shown to be a hydroxyl radical scavenger.

However, the electron micrographs show that DMF (0.4ml/kg and 0.8ml/kg) disrupts the structure of the rat liver endoplasmic reticulum (Figures 24 and 25). Structural changes observed include endoplasmic reticulum swelling and distortion. At 0.8ml/kg of DMF there is evidence of disappearance of the endoplasmic reticulum (Figure 25). It is also evident that melatonin (2.5mg/kg) protects against the DMF-induced morphological changes.

The electron micrographs clearly indicate that DMF is hepatotoxic. However, the spin trap studies show that DMF does not generate hydroxyl radicals. Thus, the mechanism by which DMF induces changes in the endoplasmic reticulum, and the prevention of these changes by melatonin, need to be investigated further (Anoopkumar-Dukie, 1997).

Chapter Four

Lipid Peroxidation of the Liver

4.1 Introduction

Biological membranes function as compartmentalizing structures, and are essential for cell functioning. Biological membranes function as important barriers, protecting cells from possible harmful compounds in the surrounding medium. The structure of these membranes has been described by Singer and Nicholson as the 'Fluid Mosaic Model' (Mathews and Van Holde, 1991). According to this model, biological membranes are dynamic, irregular lipid mixtures of phospholipids and cholesterol, with globular proteins embedded within the membrane (Mathews and Van Holde, 1991). The membranes possess machinery for the transportation of molecules across them, and are also the site of metabolic activities such as electron transport. Membranes are also the site of cell-cell and cell-organelle interactions, such as hormone-cell interactions (Clark and Switzer, 1977). Changes in the membrane fluidity, by either physical or chemical disturbances, alter membrane structure, and therefore membrane functions. This could lead to increased to permeability to ions such as Ca_2^+, ultimately leading to cell destruction. Free radicals are able to cause such alterations in membrane integrity resulting in lipid peroxidation.

Peroxidation of lipids is initiated by the attack of any chemical species that has sufficient activity, or produces metabolites that have sufficient activity, to abstract a hydrogen atom from a methylene carbon in the side chain of the lipid. The hydrogen atom (Figure 28) is a free radical, and its removal leaves behind an unpaired electron on the carbon atom to which it was previously attached. The resulting carbon-centred radical can undergo molecular rearrangement in aerobic cells, followed by a reaction with O_2 to produce a peroxyl radical. Peroxyl radicals can either combine with each other or they can attack membrane proteins. The peroxyl radicals are also capable of initiating peroxidation in

adjacent fatty acid side chains. This leads to a propagation of lipid peroxidation (Gutteridge and Halliwell, 1990).

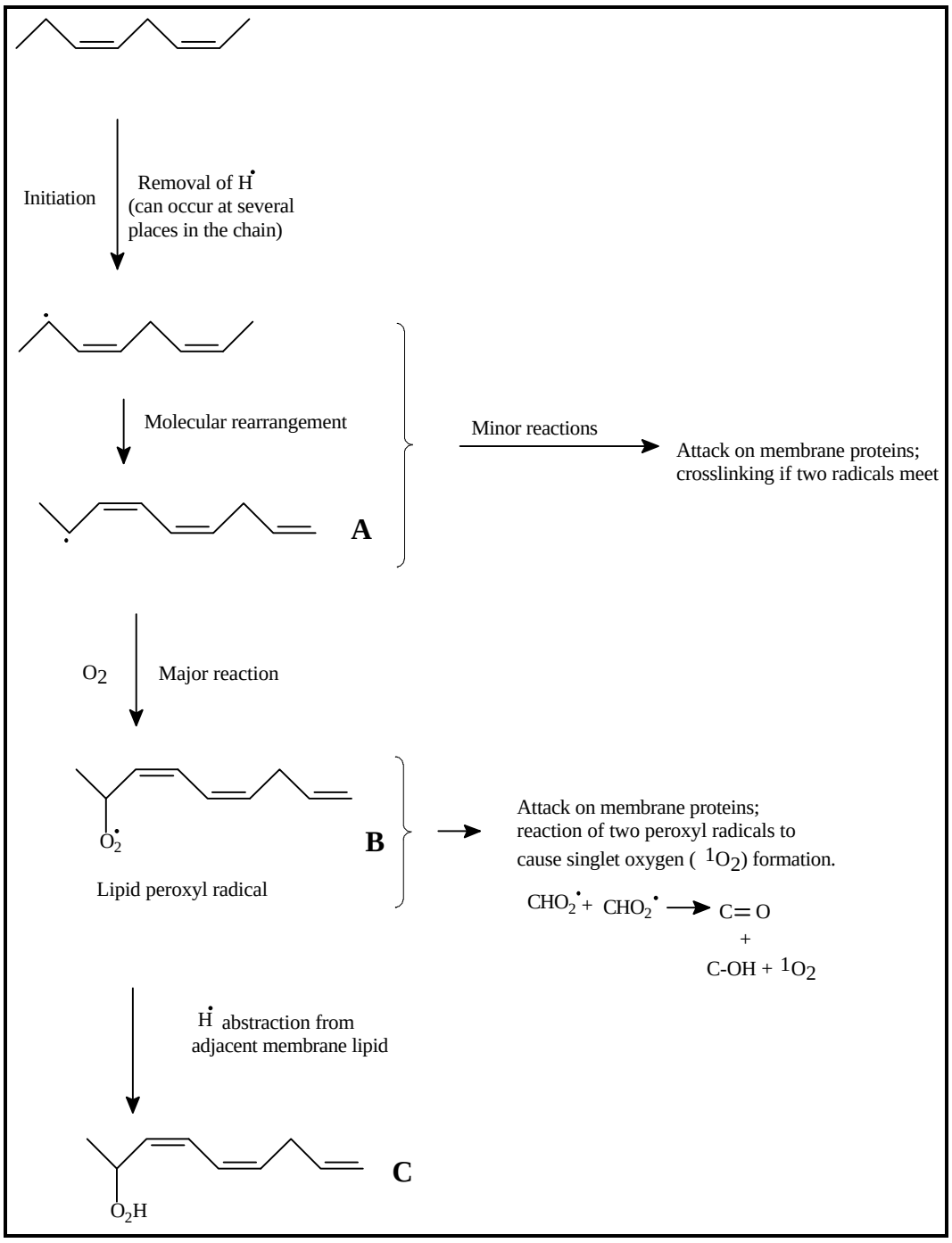

Figure 28: An outline mechanism of lipid peroxidation (Gutteridge and Halliwell, 1990).

MDA and 4-HDA are degraded lipid products in cell membranes and are reliable indicators of free radical-induced damage (Reiter *et al*, 1995). The most widely employed technique, used in the determination of peroxidation in biological materials, is the TBA test. This test is based on the reaction of one molecule of MDA with two molecules of TBA, resulting in the formation of a pink chromogen (Figure 29). This complex has an absorption maximum at 532nm. Hot acid hydrolysis, usually TCA, is required for the formation of the complex and the release of protein-bound MDA.

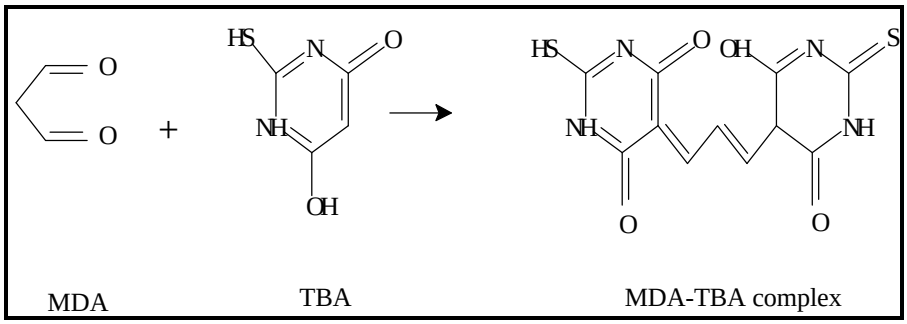

Figure 29: The reaction of MDA with TBA to yield a pink TBA-MDA complex (Mead *et al*, 1986).

4.2 Materials and methods

4.2.1 Animals

Male Wistar rats weighing 250g-300g were used in the experiment. The rats were randomly assembled into groups of five, and were maintained as described in Appendix 1. The control group (n=5) received the drug vehicle, ethanol:0.9%saline (40:60). Another three groups (n=5), received 30mg/kg, 100mg/kg, and 500mg/kg of acetaminophen i.p. The final three groups (n=5), received the same doses of acetaminophen in conjunction with 2,5mg/kg of melatonin i.p. The rats were injected (i.p.) for seven days and were sacrificed on day ten, and the livers were removed as described in Appendix 2. The DMF treated animals (two groups of five) were injected (i.p.) for seven days with 0.4ml/kg and 0.8ml/kg of DMF respectively. The control animals (n=5) in this group received 0.9%

saline. The final two groups (n=5) received 2.5mg/kg melatonin in conjunction with the DMF i.p. These animals were injected for seven days and were sacrificed on day eight. The livers were removed as described in Appendix 2.

4.2.2 Chemicals and reagents

1.1.3.3-Tetramethoxypropane (MDA) was purchased from Fluka AG, Switzerland. Butylated hydroxytoluene (BHT), melatonin, and 2-thiobarbituric acid (TBA) were purchased from Sigma Chemical Co, St Louis, MO, USA. Trichloroacetic acid and butanol was purchased from Saarchem (PTY) Ltd, Krugersdorp, South Africa. All other reagents were obtained from local sources and were of the highest purity available.

4.2.3 Methods

4.2.3.1 Preparation of the liver homogenate

The livers were removed immediately and placed at -70^{o}C. Prior to homogenisation, the livers were thawed to room temperature. A 10% w/v homogenate was then prepared using 0.01M Tris-HCl buffer, pH 7.4. 1ml aliquots of the homogenate were then incubated at 37^{o}C for 30 minutes.

4.2.3.2 The TBA test

A modification of the method of Sagar *et al* (1992), was used in this experiment. After the incubation period 0.5ml BHT (0.5g/litre of ethanol) was added to each tube. This was followed by the addition of 1ml TCA (10g/100ml distilled water). The sample was then centrifuged at 2000 rpm for 20 minutes to remove insoluble proteins. 2ml of the supernatant was then transferred to a clean set of tubes. 0.5ml TBA (0.33g/100ml distilled water) was then added to the tubes. The tubes were boiled for 1 hour at 95^{o}C. The tubes were then cooled and the TBA-MDA complex was extracted using 2ml butanol. The

absorbance of the butanol fraction was determined at 532nm. A series of standards (0-20 nmoles/ml) was also prepared using 1.1.3.3-tetramethoxypropane and a standard curve was generated (Appendix 4). This was done exactly as described above, except that the standards were made up to 1ml using distilled water. Protein concentration was also determined according to the method of Lowry *et al* (1951), described in Appendix 3.

4.3 Results

The final results were corrected for dilutions and expressed as nmoles MDA produced/mg protein. The data represented is the mean of five determinations. The mean ± SEM is represented, and the differences in the means were statistically analysed using the Tukey-Kramer multiple comparisons test. $P<0.05$ was determined to be statistically significant. Range finding tests were carried out to optimize the assay. The effect of incubation time and temperature on lipid peroxidation was determined. As shown in Figures 30 and 31, the optimum incubation time and temperature was 60 minutes and 37^{o}C, respectively. It is also apparent from Figures 32, 33, and 34, that acetaminophen induces peroxidation ($p<0.05$) at a dose of 500mg/kg only. It is also evident from Figures 35 and 36, that DMF does not induce lipid peroxidation.

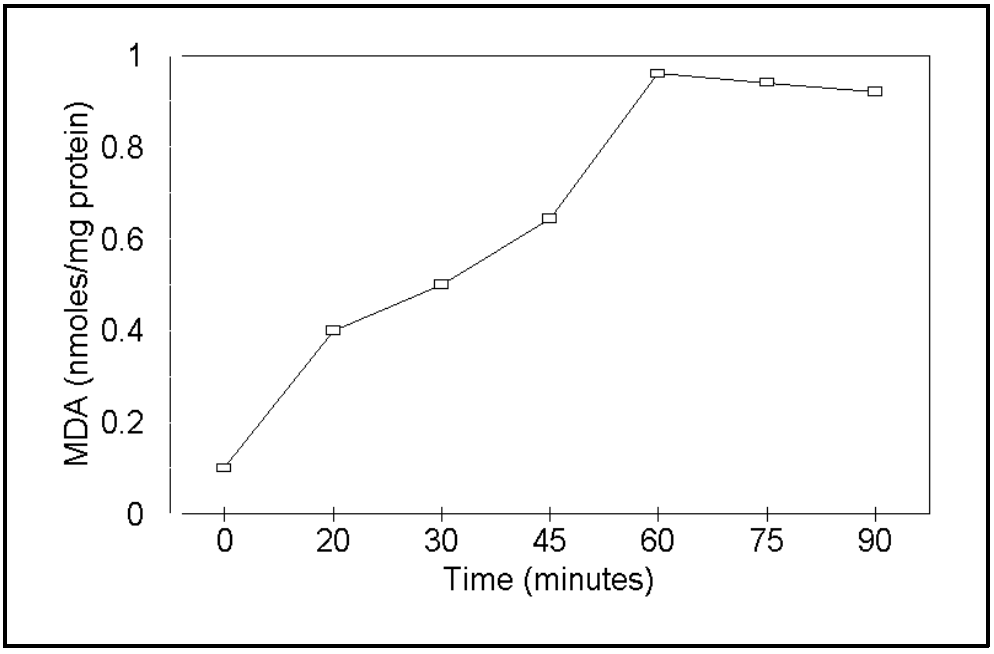

Figure 30: The effect of incubation time on lipid peroxidation. Each point represents the mean of triplicate determinations.

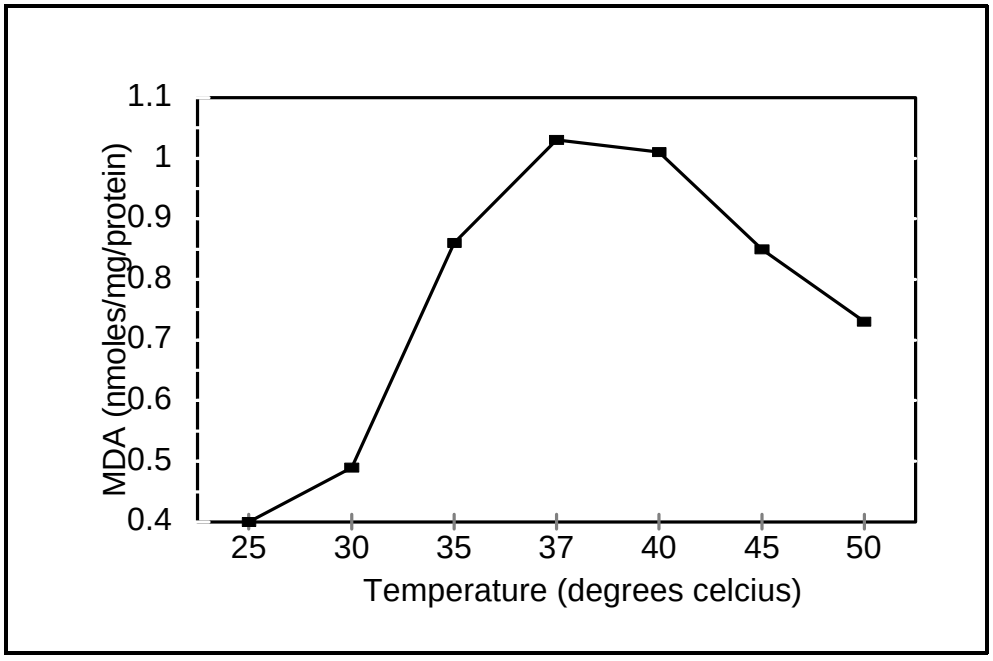

Figure 31: The effect of incubation temperature on lipid peroxidation. Each point represents the mean of triplicate determinations.

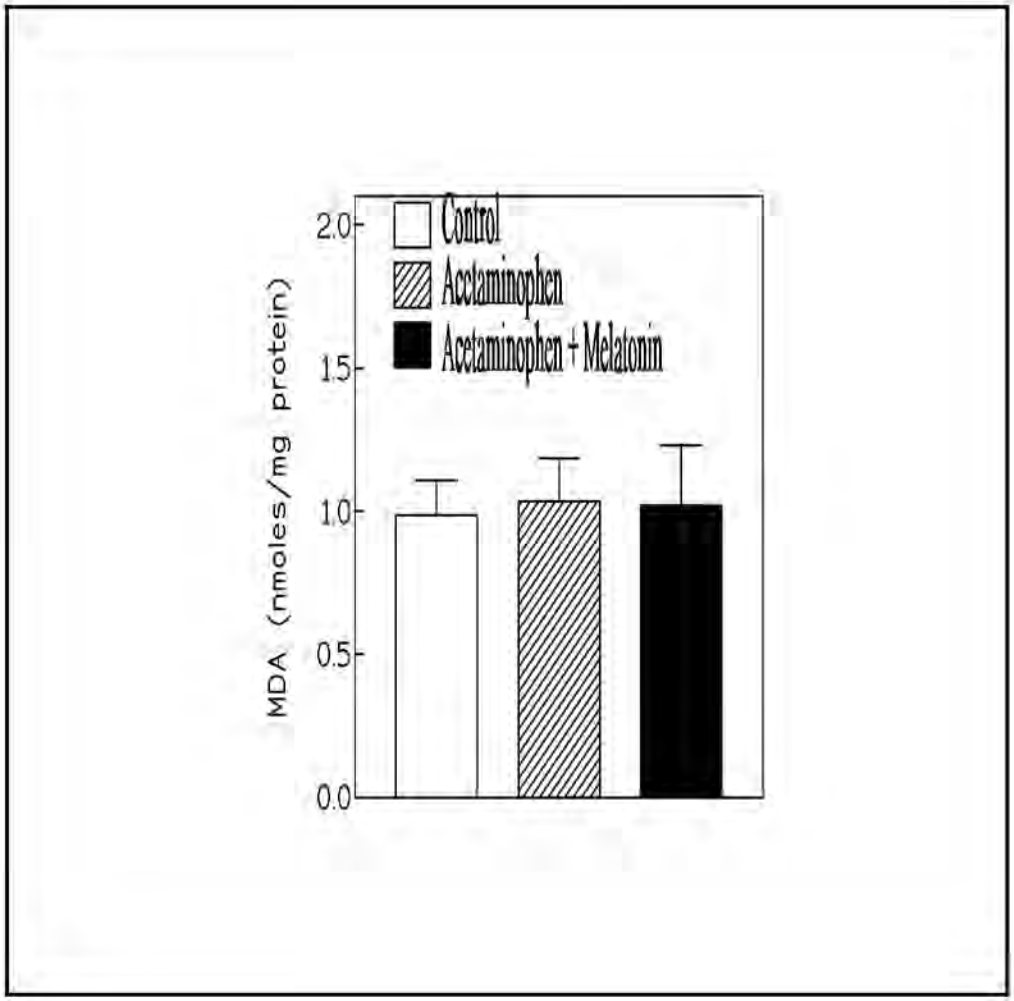

Figure 32: The effect of acetaminophen administration (30mg/kg) on lipid peroxidation in rat liver. Each bar represents the mean ± SEM; n=5.

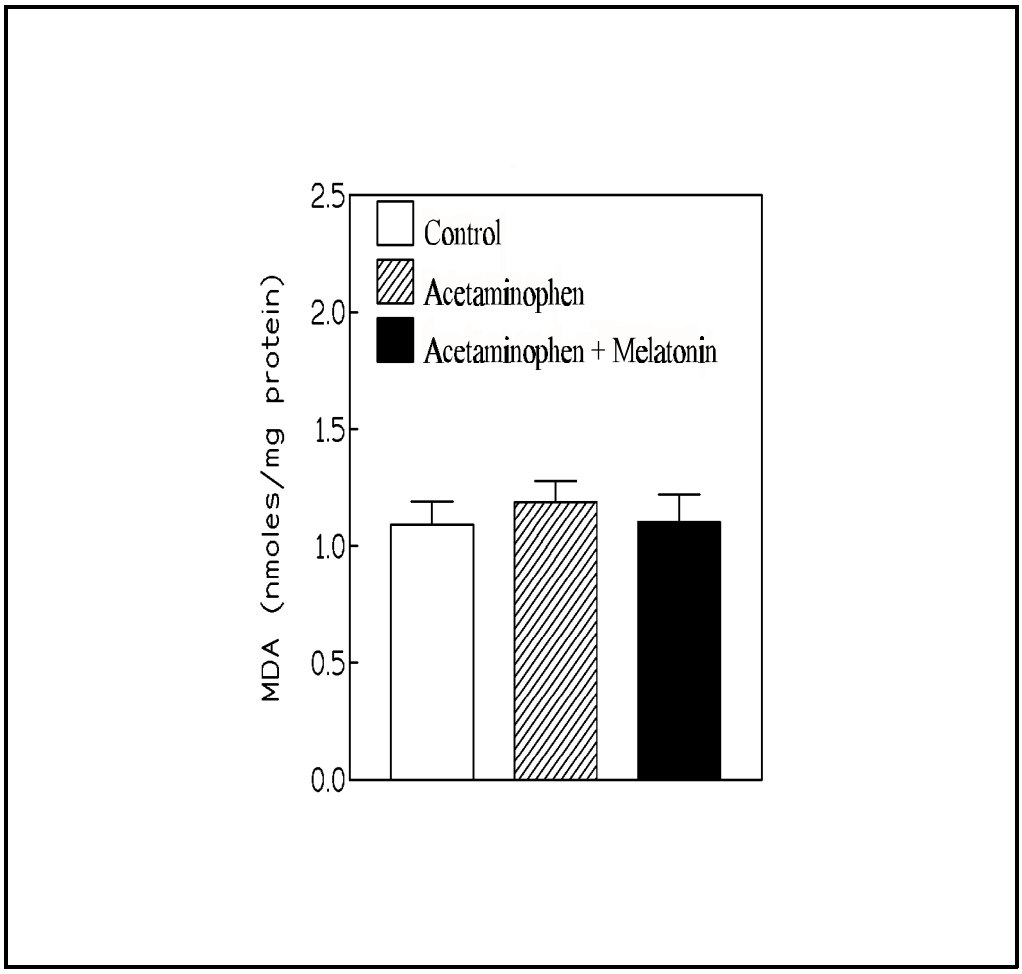

Figure 33: The effect of acetaminophen administration (100mg/kg) on lipid peroxidation in rat liver. Each bar represents the mean ± SEM; n=5.

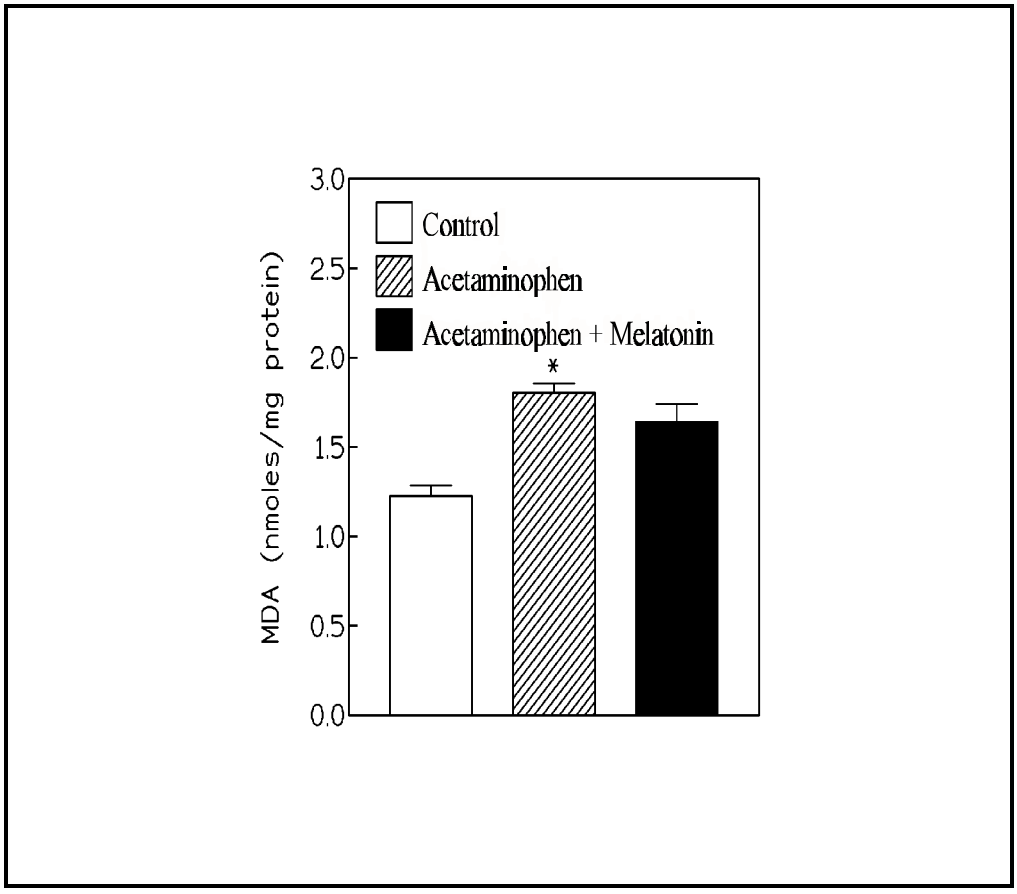

Figure 34: The effect of acetaminophen administration (500mg/kg) on lipid peroxidation in rat liver. Each bar represents the mean ± SEM; n=5. * ($p<0.05$). Tukey-Kramer Multiple Comparisons test.

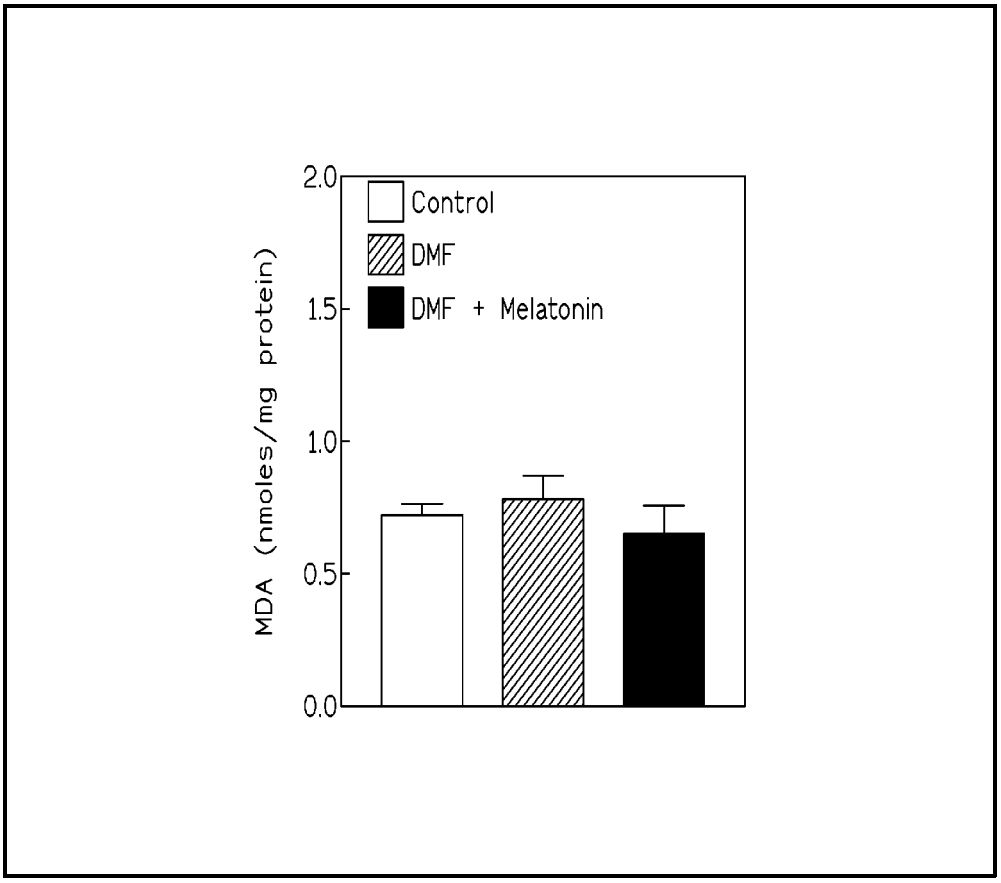

Figure 35: The effect of DMF administration (0.4ml/kg) on lipid peroxidation in rat liver. Each bar represents the mean ± SEM; n=3.

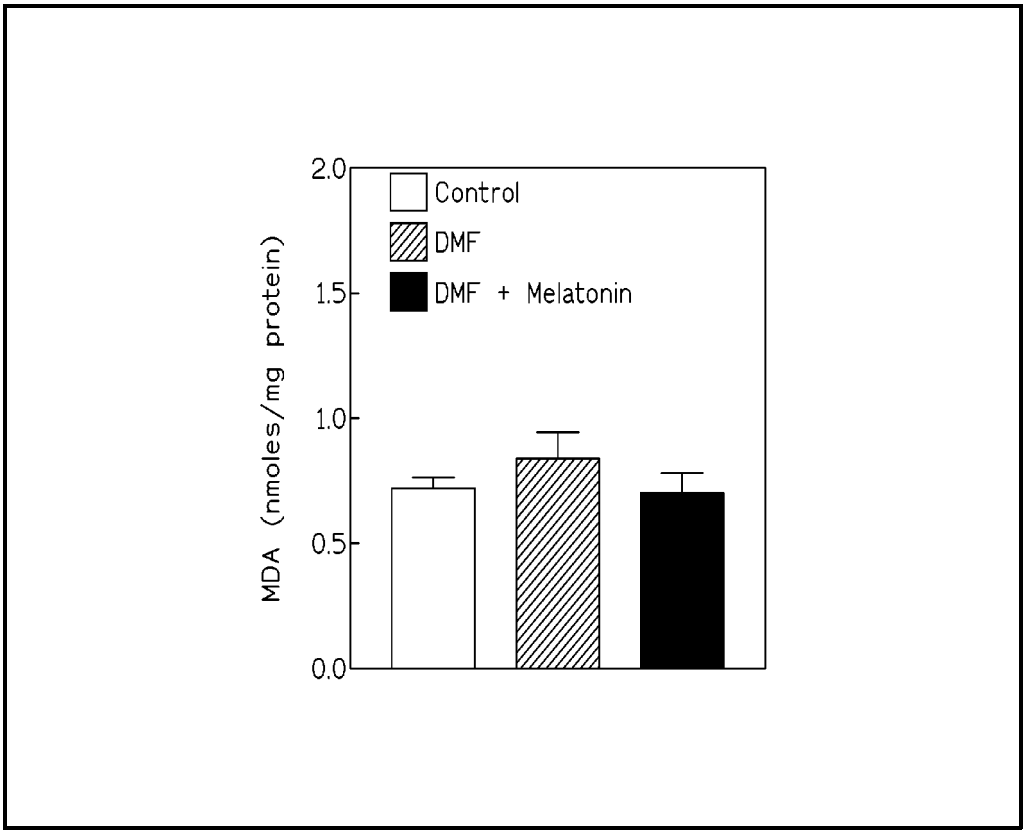

Figure 36: The effect of DMF administration (0.8ml/kg) on lipid peroxidation in rat liver. Each bar represents the mean ± SEM; n=5.

4.4 Discussion

There is considerable interest in the role played by lipid peroxidation, and other free radical reactions, in human and animal disease, as well as in toxicology (Gutteridge and Haliwell, 1990). Indeed, elevated end products of lipid peroxidation in human and animal material, are probably indicative of the involvement of free radical reactions in tissue damage (Gutteridge and Halliwell, 1990). Early research on lipid peroxidation involved halogenated hydrocarbons, such as carbon tetrachloride and bromobenzene. These studies indicate that free radical damage does not necessarily accompany an increase in peroxidation of cell membrane lipids. The rise in intracellular levels of Ca^{2+}, with the consequent activation of proteases and nucleases, and the resultant damage to DNA, are often more important toxic events than bulk lipid peroxidation (Halliwell, 1987). Lipid peroxidation is often a late event, accompanying, rather than causing final cell death (Kappus, 1987).

This study employs the use of the TBA test to measure the effect of DMF and acetaminophen on lipid peroxidation. This test is the single most widely used assay for measuring lipid peroxidation. The test measures the levels of MDA, which is a product of lipid peroxidation, and together with 4-HDA are taken to be reliable indicators of oxidative stress (Reiter *et al*, 1995). As shown in Figure 32, at a dose of 30mg/kg, acetaminophen does not induce any peroxidation. This is also shown to be the case at a dose of 100mg/kg. As stated earlier, this does not necessarily indicate an absence of free radical damage. As shown in Figure 34, at a dose of 500mg/kg, acetaminophen does induce peroxidation of cell membranes. It is possible that at the lower doses of acetaminophen there is free radical damage, but there is no increase in peroxidation of cell membranes. At these doses, it is likely that the cell destruction process has just been initiated. However, at 500mg/kg, it is evident that acetaminophen significantly ($p<0.05$) increases lipid peroxidation. This step often accompanies cell death (Kappus, 1987).

As shown in Figure 34, melatonin is unable to significantly reduce the damage inflicted by

acetaminophen. The slight reduction in peroxidation could indicate that melatonin is able to partially thwart the free radical-induced damage caused by acetaminophen. Melatonin is a known, potent free radical scavenger. The antidotes commonly used to treat acetaminophen toxicity are N-acetylcysteine and methionine. These compounds possess sulphydral groups which enables these agents to detoxify the toxic metabolite of acetaminophen (Payan and Katzung, 1995). This compound, N-acetyl-benzoquinoneimine, binds to the sulphydral groups of cell proteins and this leads to hepatocellular necrosis. N-acetyl-benzoquinoneimine, the toxic metabolite of acetaminophen, is metabolised to a phenoxyl radical by the liver microsomes (Mason and Fischer, 1986). It is plausible to suggest that melatonin, a compound known to scavenge hydroxyl and peroxyl radicals, is not able to detoxify the phenoxyl radical. Melatonin does not possess sulphydral groups, and this could partially explain the reason for it not being able to reduce acetaminophen-induced lipid peroxidation.

N-acetylcysteine is a precursor for glutathione, which is the chief agent responsible for detoxifying acetaminophen and its metabolites. Melatonin is known to promote the enzymatic production of glutathione (Reiter, 1997), and this property could enable it to partially detoxify N-acetyl-benzoquinoneimine, thereby reducing the amount of phenoxyl radicals generated. This could also be a reason for the slight reduction in peroxidation by melatonin. The antioxidant properties of melatonin are well documented. Despite this property, it is strikingly evident from this study that it is unable to detoxify the free radicals generated by acetaminophen and its metabolites.

As shown in Figures 35 and 36, DMF does not induce lipid peroxidation at the doses administered. Like acetaminophen, DMF is known to adversely affect the liver. The exact mechanism of DMF induced liver damage is not clearly understood. It is speculated that DMF toxicity could possibly be related to the fact that DMF and/or its metabolites form adducts with haemoglobin (Angerer *et al*, 1998). This study suggests that the liver damage could also occur via a free radical mechanism. The results depicted in Figures 35 and 36, show that DMF does not induce lipid peroxidation. As was the case with the lower doses of acetaminophen, it is not unlikely that DMF does not produce free radicals. Lipid

peroxidation is often the last stage accompanying cell death (Kappus, 1987). However, the results from Chapter Three, as well as the from this experiment, clearly suggest that DMF does not induce liver damage via a free radical mechanism.

The results (Figures 35 and 36) obtained in this study imply that it is unsuitable to evaluate melatonin as hepatoprotectant against DMF. The results shown in Figure 34, clearly suggest that melatonin is unable to reduce the peroxidation caused by acetaminophen (500mg/kg). It can also be hypothesised that this highly reactive metabolite (N-acetyl-benzoquinoneimine) produced by acetaminophen, is also able to disrupt the structure of melatonin, thereby rendering it inactive against free radicals. The results obtained in this experiment also suggest that acetaminophen is an antioxidant at low doses (0-100mg/kg). However, at a higher dose (500mg/kg), acetaminophen acts as a prooxidant. Acetaminophen has been reported to possess both these abilities (Mason and Fischer, 1986; Nakamoto *et al*, 1997). This needs to be examined further.

Chapter Five

The Effect of Acetaminophen on Liver Tryptophan-2,3-dioxygenase Activity

5.1 Introduction

Tryptophan-2,3-dioxygenase (TDO) is a haem-dependant liver cytosolic metalloenzyme that catalyses the irreversible insertion of molecular oxygen into the pyrrole moiety of L-tryptophan to produce N-formylkynurenine (Walsh *et al*, 1994).This is the most important peripheral determinant affecting plasma tryptophan levels. Changes in circulating levels of tryptophan can be achieved by altering the activity of the enzyme, tryptophan-2,3-dioxygenase (Daya *et al*, 1989). This also governs the availability of tryptophan to the brain. In the brain, tryptophan is used to synthesize serotonin, an important neurotransmitter implicated in depression (van Praag, 1982). Some antidepressants such as Desipramine, have been shown to inhibit TDO thereby increasing tryptophan availability to the brain (Walsh and Daya, 1998).

In the livers of some animal species, TDO exists in at least two forms: the active reduced holoenzyme which does require the presence of a cofactor for the demonstration of its activity, and the inactive apoenzyme which is dependant on exogenous haem for the demonstration of its activity (Badawy and Evans, 1974). The conjugation of the enzyme with haem results in its activation, forming the oxidised holoenzyme which is then reduced in the presence of tryptophan to form the active form.

It is important to measure the activities of both the holoenzyme and apoenzyme when assessing TDO activity. This elucidates the site of action of drugs which are able to alter the activities of TDO. The assay employed in this study is a modification of the method described by Badawy and Evans (1975).The activity of the enzyme is determined by

measuring the formation of kynurenine from L-tryptophan. The holoenzyme is measured in the absence of exogenous haematin and the total enzyme activity in the presence of exogenous haematin. The apoenzyme is then calculated as the difference between the two.

5.2 Materials and methods

5.2.1 Animals

Male Wistar rats weighing 250g-300g were used in the study. These were randomly assembled into groups of five and maintained as described in Appendix 1. The control group (n=5), received 0.25ml 0.9% saline:ethanol (60:40), which was also the vehicle in which acetaminophen was dissolved. The remaining group (n=5) received 100mg/kg acetaminophen every hour for three hours. After three hours the rats were sacrificed as described in Appendix 2, and the livers were removed and perfused with 0.9% saline. The livers were homogenised and TDO activity was measured immediately.

5.2.2 Chemicals and reagents

L-Tryptophan and haematin were purchased from Sigma Chemical Co, St Louis, MO, USA. All other chemicals were obtained locally and were of the highest chemical purity available.

5.2.3 Preparation of the liver homogenate

A 10 % (w/v) homogenate was made using equal volumes of 140mM KCl/2.5mM NaOH buffer, pH 7.0 and 0.2M sodium phosphate buffer, pH 7.0. The liver was homogenised using a waring blender followed by sonication.

5.2.4 Determination of TDO activity

A scheme of the assay is outlined in Table 2. To determine the activity of the apoenzyme, a 15ml aliquot of homogenate was added to a solution containing 12.5ml distilled water and 2.5ml 0.03M L-tryptophan. 0.1ml of 1.2M haematin was added to determine the activity of the total enzyme. The assay was carried out in triplicate. 3ml aliquots of the reaction mixture were transferred to clean test tubes.

Table 2: Scheme of the Tryptophan-2,3-dioxygenase enzyme assay

	Holoenzyme	**Total Enzyme**
Distilled water	12.5ml	12.5ml
⇩		
Homogenate (10% w/v)	15ml	15ml
⇩		
Haematin (1.2M)		100µl
⇩		
Tryptophan (0.03M)	2.5ml	2.5ml
Continue in triplicate		
Transfer to clean tubes	3ml	3ml
Incubate in an atmosphere of carbogen for one hour at 37^{o}C		
⇩		
TCA (0.9M)	2ml	2ml
Shake for two minutes and filter through Whatman No.1 filter paper and transfer 2.5ml filtrate to another set of tubes		
⇩		
NaOH (0.6M)	2.5ml	2.5ml
Read absorbance at 365nm		

The mixture was saturated with carbogen (95% O_2:5% CO_2) and sealed. The tubes were incubated at 37^{o}C for 60 minutes in an oscillating water bath. The reaction was terminated by the addition of 2ml 0.9M trichloroacetic acid (TCA). The mixtures were shaken for a further two minutes and then filtered using Whatman No. 1 filter paper. 2.5ml of the filtrate was transferred to another set of test tubes containing 1.5ml of 0.6M NaOH. The absorbance was measured at 365nm using a Shimadzu UV-160A UV-visible spectrophotometer with 2ml TCA and 1.5ml NaOH as the blank.

5.3 Results

The concentration of kynurenine was calculated by applying Beer-Lamberts Law. The molar extinction coefficient of kynurenine is 4540 l/mole.cm. The results (average of five estimations) were expressed as nmoles kynurenine formed/minute/mg protein. Extant holoenzyme activity is taken to be that activity present in the hepatocyte in the absence of added haematin, while total enzyme activity is that activity measured in the presence of added haematin. The apoenzyme activity is the difference between the two. The data were analysed by one-way analysis of variance, and the statistical variance among specific means was determined using the Tukey-Kramer multiple comparison test. A $p<0.05$ between groups was accepted as being statistically significant. As shown in Figure 39, acetaminophen administration did not alter either total enzyme or holoenzyme but significantly reduced ($p<0.05$) apoenzyme activity.

The ëmax for kynureine was determined (Figure 37). The value was determined to be 365nm, and is consistent with the values reported in the literature. The optimum incubation time for enzyme activity was also determined (Figure38). The formation of kynurenine was linear for the first 60 minutes. There was no difference in product formation between 60 minutes and 90 minutes, and 60 minutes was chosen as the optimum incubation time as it indicates maximum product formation.

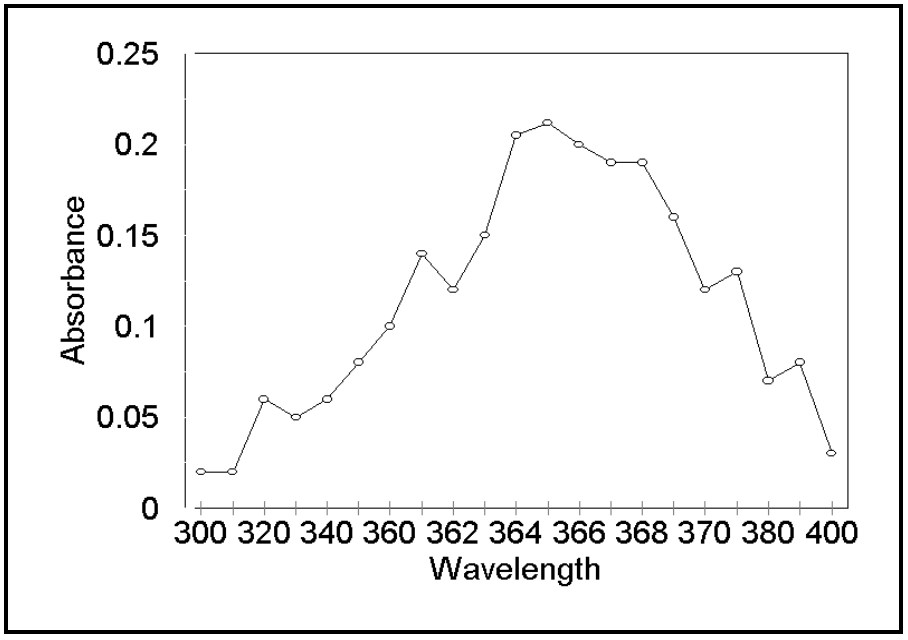

Figure 37: Determination of ëmax for kynurenine. Each point represents the mean of duplicate determinations.

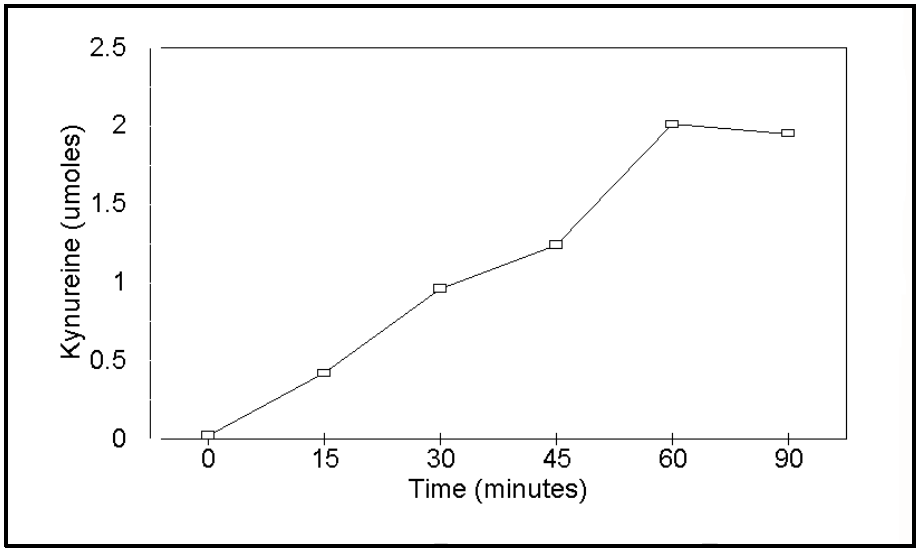

Figure 38: The effect of incubation time on TDO activity. Each point represents the mean of triplicate determinations.

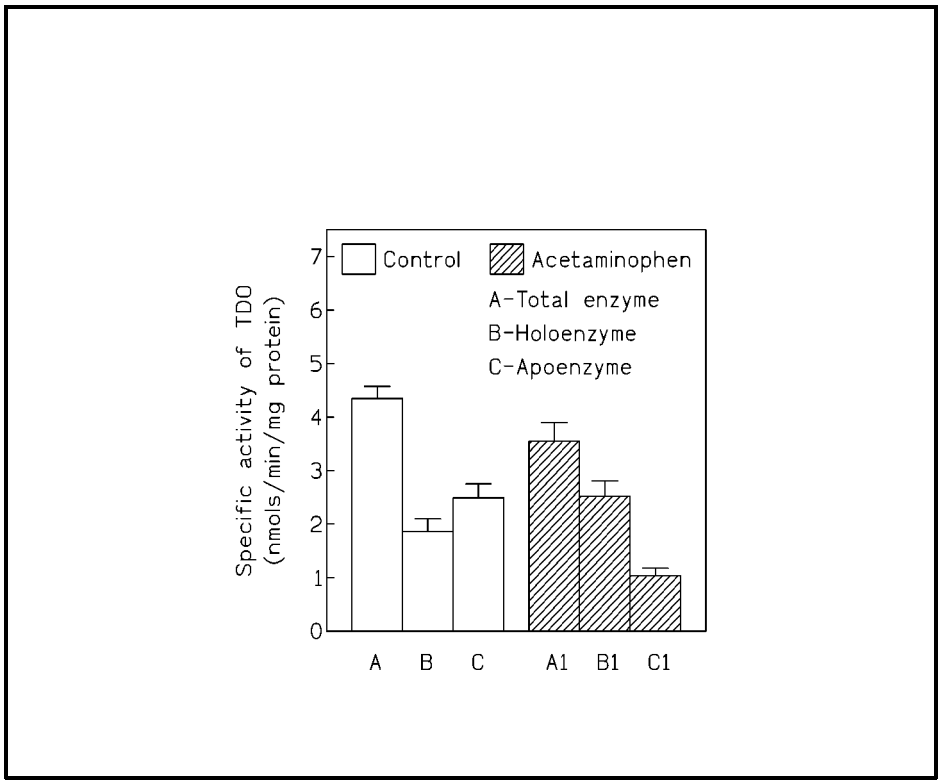

Figure 39: The effect of acetaminophen administration on rat liver TDO activity. Each bar represents the mean ± SEM; n=5. C vs C1 ($p<0.05$). Tukey-Kramer Multiple Comparisons test.

5.4 Discussion

Liver tryptophan-2,3-dioxygenase plays a very important role in determining the levels of circulating tryptophan in the blood (Badawy *et al*, 1981). An increase in the activity of this enzyme enhances the conversion of tryptophan to
N-formylkynurenine, thus reducing the amount of tryptophan available for uptake into the brain (Badawy, 1979). Therefore, agents which inhibit the activity of this enzyme could increase plasma levels of tryptophan, and subsequently induce a rise in brain tryptophan and serotonin levels (Daya *et al*, 1989). The tricyclic antidepressants are known inhibitors of this enzyme and this effect could partially explain the rise in brain serotonin levels following the administration of these drugs.

Phenacetin, the precursor of acetaminophen, is known to produce central effects such as mild euphoria (Payan and Katzung, 1995). To date the mechanism underlying this phenomenon has not been explained. Acetaminophen, a derivative of phenacetin, is widely used as an analgesic. The mood changes observed with high doses of acetaminophen (Payan and Katzung, 1995), could be related to central nervous system effects such as alterations in neurotransmitter levels, for example, serotonin. As shown in Figure 39, the results of the present experiment indicate that acetaminophen administration results in inhibition of tryptophan-2,3-dioxygenase activity. The inhibition appears to be at the level of the apoenzyme. This implies that acetaminophen could exert its inhibitory action by partial inhibition of the binding of this enzyme component to its cofactor haem. This conjugation is essential for the activation of the apoenzyme. This inhibition indicates that acetaminophen administration increases the plasma tryptophan levels and in turn the tryptophan available for uptake into the brain. This also affects serotonin and melatonin production, and is discussed in Chapter Six.

It is also known that the binding of tryptophan to TDO induces a change, which is an increase in the haem ligand binding affinity of the enzyme (Walsh *et al*, 1994). The enzyme has a regulatory and catalytic site. At low concentrations of tryptophan binding of

tryptophan only occurs at the catalytic site. At higher doses, binding occurs at both sites. Acetaminophen administration could also possibly prevent the binding of tryptophan to TDO thereby reducing its ability to bind to haem. This would therefore prevent apoenzyme synthesis, and reduce the activity of TDO (Walsh *et al*, 1994). Acetaminophen is also a poor substrate for the enzyme as it lacks the correct functional groups for proper interactions at the catalytic site. Acetaminophen administration may therefore inhibit the activity of TDO by one of the ways discussed above, or by an unknown mechanism. This needs to be investigated further.

Chapter Six

The Determination of Rat Pineal Melatonin and Forebrain Serotonin Levels Using Enzyme-linked Immunosorbent Assay (ELISA)

6.1 Introduction

The use of the ELISA technique is widespread. It has been rapidly adapted to a wide range of applications such as indole determination, and to screen B-cell hybridomas for antibody production. The ELISA technique can be modified depending on whether antigen or antibody is being detected. If antibody is being detected, then the wells of the microtiter plate are coated with antigen. In most cases the antigen will adhere spontaneously to the wells. However, in certain instances the antigen may have to be coupled to the wells chemically. The samples are then incubated in the antigen coated wells for a certain period of time, after which unbound sample is removed by washing. A second antibody, coupled to an enzyme such as alkaline phosphatase and horseradish peroxidase, is then added to the wells. A major requirement of the enzyme is that it still functions, when coupled, to produce a coloured product using colourless substrate. This antibody also reacts with the sample. The unbound antibody, as well as cofactors, are then washed away and the colourless substrate for the particular enzyme is then added to the wells. If the specific antibody is present in the sample, then the second antigen will bind to the well, ultimately leading to a colorimetric reaction. If antigen is being detected then the procedure is the opposite to the one described (Clark, 1991). A clever ploy for amplifying the alkaline phosphatase reaction is to use NADP as a substrate to generate NAD which is then able to serve as a coenzyme for the second enzyme system. Generally, phosphatase from *E. coli* is used as it is not present in any tissues. In addition, the enzyme from this source is stable and generates a good colour reaction (Roitt, 1991).

ELISA plates are now available commercially and can be purchased precoated with either antigens or antibodies. The sensitivity of ELISA, despite its ease of use, has made this

technique one of the most commonly used immunoassay systems, particularly in clinical laboratories.

6.2 Materials and methods

6.2.1 Animals

Male Wistar rats weighing between 250g-300g, were used in the experiment. These were randomly assorted into two groups of five and were maintained as described in Appendix 1. The control group (n=5) received 0.25ml 0.9% saline:ethanol (60:40), which was the vehicle. The remaining group (n=5) received 100mg/kg acetaminophen i.p. every hour for three hours. The rats were killed after three hours, and the forebrains and pineal glands were removed, as described in Appendix 2, and stored at
-70^{o}C until use.

6.2.2 Chemicals and reagents

All chemicals and reagents used in the ELISA determination were provided with the diagnostic kit from Research Diagnostics INC, Flanders, NJ, USA. All other chemicals and reagents were obtained locally and were of the highest purity available.

6.2.3 Serotonin determination

6.2.3.1 Specimen preparation

The forebrains were homogenized individually (10% w/v), using 0.9% saline. 20µl of the homogenate was placed in a set of clean test tubes, followed by the addition of 100µl of the assay buffer (Phosphate buffer with tween). 25µl of acylation reagent (acetic acid anhydride in acetone) was added to the tubes which were then incubated for 15 minutes at 37^{o}C. After incubation 4ml of the assay buffer was added to the tubes which were then centrifuged at 1500×g for ten minutes.

6.2.3.2 Test procedure

50µl of the acylated samples and standards were placed in the wells of the microtiter plate. This was followed by the addition of the serotonin-biotin complex (50µl) and the antiserum (50µl). The plate was then shaken and incubated overnight at 2ºC - 8ºC. The wells were then washed three times with the wash buffer (1/10 dilution of phosphate buffer). 150µl of the anti-biotin-alkaline phosphatase complex was then pipetted into each well. The plate was then incubated for two hours at room temperature on an orbital shaker (200 rpm). Following this, the plates were washed three times with the wash buffer. 200µl of the substrate, para-nitrophenol phosphate (PNPP), was added to each well and the plate was incubated at room temperature for an hour. The reaction was then terminated with the addition of 50µl stop solution (1N NaOH with 0.25 M EDTA). The optical density was measured at 405nm (reference wavelength 600-650nm) using a microtiter plate reader (Powerwave) within an hour. A standard curve (Appendix 4) was also generated in the same manner.

6.2.4 Melatonin determination

6.2.4.1 Extraction

The pineal glands were individually homogenised (10% w/v) in 0.9% saline. The extraction columns (C_{18} reverse phase) were individually placed in extraction tubes followed by the addition of two volumes (1ml) of undiluted methanol. The tubes were then centrifuged for one minute at 200×g. The same procedure was repeated with distilled water. Following this 0.5ml of the samples and standards were added to the tubes, which were then centrifuged for one minute at 200×g. Subsequently the columns were washed with two volumes (1ml) of 10% methanol and centrifuged for one minute at 500×g. The columns were then placed in clean tubes and the melatonin was eluted with 1ml undiluted methanol, followed by centrifugation for one minute at 200×g. Centrifugation in this step is important to avoid any melatonin being left in the column. The methanol was then evaporated to dryness, using a speed-vac evaporator centrifuge, and the melatonin was

reconstituted in 0.15ml distilled water.

6.2.4.2 Test procedure

50µl of each sample and standard was pipetted into the appropriate wells. This was followed by the addition of the melatonin-biotin (50µl) and antiserum (50µl). The plates were shaken and incubated overnight at 2^{o}C - 8^{o}C. The next day the plates were washed three times using the wash buffer (1/10 dilution of phosphate buffer). 150µl of the enzyme conjugate was then added to the wells and the plates were sealed and incubated at room temperature for two hours on an orbital shaker (200 rpm/min). After the incubation period the plate was washed three times using the wash buffer. 200µl of the para-nitrophenol phosphate substrate solution was then added to each well. The plate was then incubated at room temperature for 20 minutes on an orbital shaker (200 rpm/min). The reaction was terminated using 50µl of the stop solution (1N NaOH with 0.25 M EDTA) provided, and the plate was gently shaken. The optical density was measured at 405nm (reference wave length 600-650nm) using a microtiter plate reader (Powerwave) within an hour. A standard curve (Appendix 5) was also generated in the same manner.

6.3 Results

The concentrations of the samples were obtained from the standard curve (log concentration vs absorbance at 405nm). The values from the serotonin determination were corrected due to dilution and acylation of samples. The data shown is an average of duplicate values, and the values are expressed as the mean ± SEM. Values from the serotonin determination are expressed as ng/mg tissue, while the melatonin values are expressed as pg/ml. The data was analysed by the Student's t-test. A $p<0.05$ between the groups was accepted as statistically significant. As shown in Figure 40, acetaminophen significantly increased ($p<0.05$) rat forebrain serotonin levels. The results shown in Figure 41, show that there was no significant ($p>0.05$) alterations in pineal melatonin levels following acetaminophen administration.

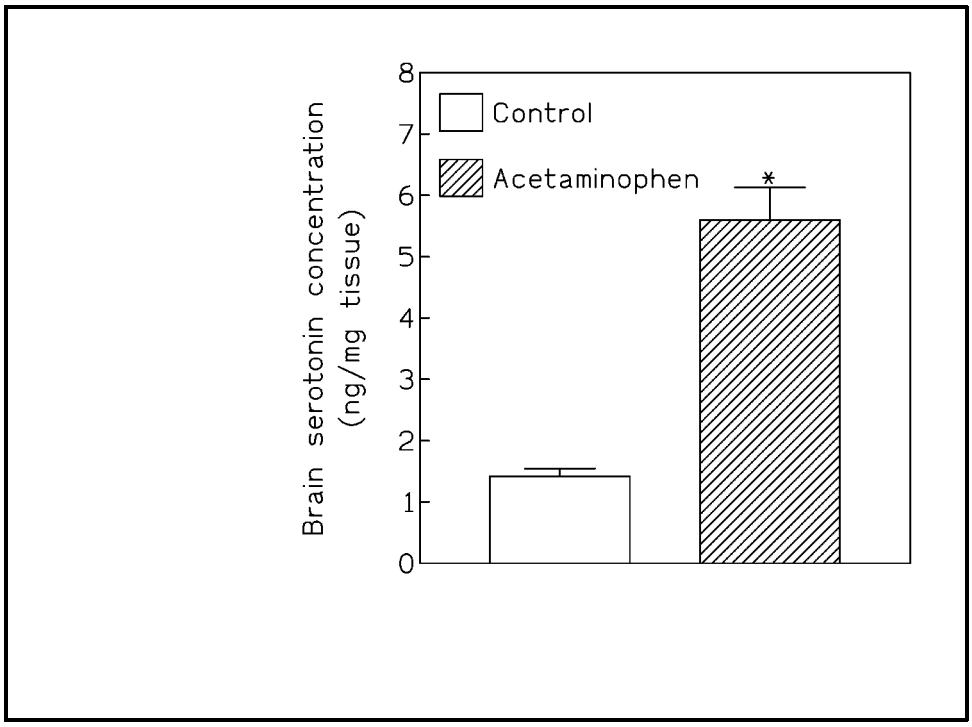

Figure 40: The effect of acetaminophen administration on rat forebrain serotonin levels. Each bar represents the mean ± SEM; n=5. * ($p<0.05$). Student's t-test.

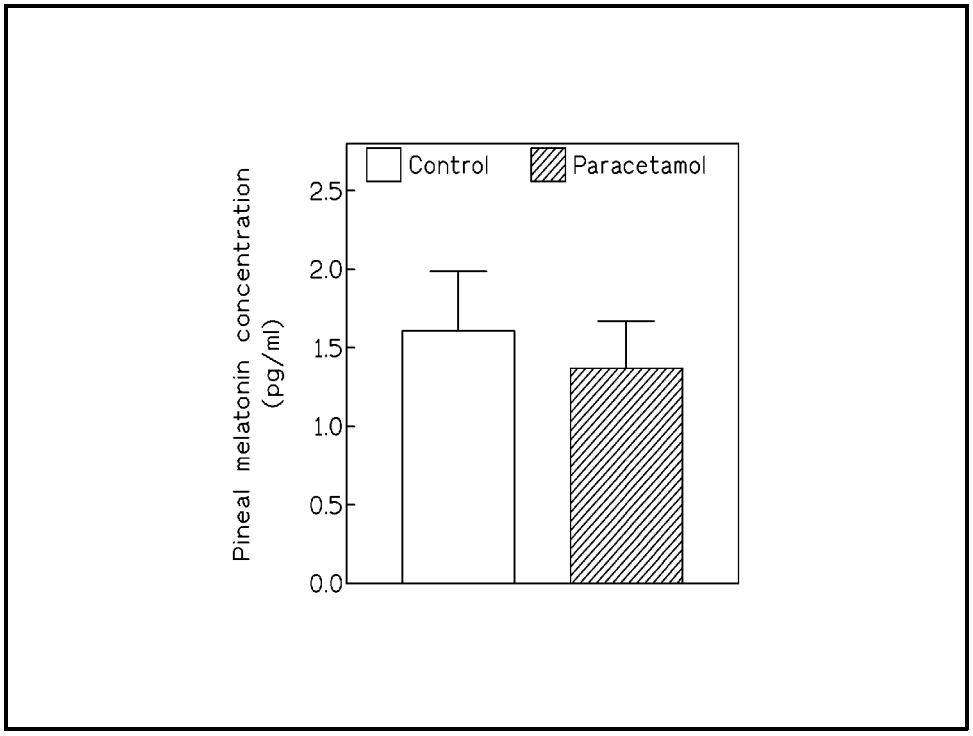

Figure 41: The effect of acetaminophen administration on rat pineal melatonin levels. Each bar represents the mean ± SEM; n=5.

6.4 Discussion

Alterations in brain serotonin levels are known to result in mood disorders, particularly depression (van Praag, 1982). The main function of serotonin is that of a neurotransmitter. Interest in correlations between serotonin and depression was stimulated by the finding that first generation antidepressants enhance the availability of serotonin at central receptors (van Praag, 1982). As shown in Figure 40, acute acetaminophen administration significantly ($p<0.05$) increases rat forebrain serotonin levels. This increase could possibly be due to the inhibition of the enzyme tryptophan-2,3-dioxygenase, discussed in Chapter Five. This inhibition is known to induce a rise in circulating tryptophan levels, which in turn results in a concomitant rise in brain serotonin levels. This increase follows the increase in tryptophan which is the precursor of serotonin (Daya *et al*, 1989). This result is consistent with the theory that the antinociceptive activity of acetaminophen is linked with the serotonergic system (Pini *et al*, 1996). The antinociceptive activity of acetaminophen is believed to result from serotonin receptor downregulation and an increase of serotonin levels in the pontine and cortical areas of the brain (Pini *et al*, 1996).

Acetaminophen administration may also affect the enzyme, tryptophan hydroxylase. This enzyme is the rate-limiting step in serotonin synthesis (Carlson *et al*, 1972). This enzyme has a poor affinity for its substrate and is normally not fully saturated with its substrate. This makes tryptophan hydroxylase very sensitive to alterations in tryptophan concentrations, which ultimately affects serotonin synthesis. The results obtained from Chapter Five show that acetaminophen administration significantly inhibits TDO activity, suggesting increased peripheral tryptophan levels. This increase could result in increased tryptophan hydroxylase activity and ultimately increased serotonin levels. This needs to be investigated further.

The increase in serotonin levels could also be due to another mechanism. Acetaminophen administration could inhibit the activity of monoamine oxidase B. This enzyme is responsible for the inactivation of serotonin. The enzyme is also the target for antidepressant drugs such as isocarboxazid (marplan®). These monoamine oxidase

inhibitors prevent the catabolism of serotonin and therefore increase its concentration in the CNS (Hollister, 1995). It is possible that acetaminophen inhibits the enzyme and therefore increases serotonin levels.

There is also a possibility that acetaminophen administration mimics the action of fluoxetine, a potent serotonin reuptake inhibitor. This would prevent the reuptake and inactivation of serotonin thereby potentiating the action of serotonin released by neuronal activity (Sanders-Bush and Mayer, 1996).

As shown in Figure 41, the acute administration of acetaminophen does not alter pineal melatonin levels. Results from the previous experiments suggest an increase in peripheral tryptophan levels, followed by a rise in brain serotonin levels. It would therefore be accurate to assume that melatonin levels would also increase, as serotonin is the precursor of melatonin. However, this was not found to be the case. This lack of a significant change in pineal melatonin levels could be attributed to the finding that pineal serotonin concentrations are 100-fold higher than the forebrain. Changes in forebrain serotonin will not have a dramatic effect on pineal serotonin, and thus melatonin (Daya *et al*, 1989). The pineal gland is protected from changes in circulating tryptophan by the high concentration of serotonin within it. As a result, melatonin synthesis in the pineal gland is normally not affected by increases in peripheral tryptophan and increased brain serotonin levels (Daya *et al*, 1989).

The results from this set of experiments authenticate the implication in the previous chapter, that acetaminophen could have mild antidepressant activity. This implication is analysed and discussed further in the chapters that follow.

Chapter Seven

Organ Culture Studies: The effect of acetaminophen administration on rat pineal indole metabolism

7.1 Introduction

The pineal gland is recognised as a fully functional organ that is responsible for the synthesis of indoleamines. The pineal gland is an integral and important component of the neuroendocrine system (Reiter, 1989). The quantification of the pineal indoles and metabolism, requires a sensitive technique that is able to mimic normal physiological processes and conditions as closely as possible.

The organ culture technique is one technique which provides the neurobiochemist with an invaluable tool. This technique enables the researcher to finely control the experimental conditions and avoid the complications of *in vivo* interactions. Due to its size, the pineal gland is suitable for the organ culture technique. Pineal organ culture systems have been utilized and optimised by a number of researchers (Klein and Rowe, 1970; Daya *et al*, 1989). The pineal gland in organ culture is able to remain viable for as long as six days under optimum conditions. It is able to utilise exogenous radioactive (^{14}C)serotonin and (^{3}H)tryptophan to produce various indoles including melatonin and serotonin (Daya *et al*, 1989). As much as 95% of the synthesised indoles are secreted into the culture medium which can then be isolated and quantified.

Isolation of the pineal indoles are achieved by using a bi-dimensional thin layer chromatography system (Klein and Notides, 1969). This technique employs the use of two organic solvents. The first solvent, chloroform:methanol:glacial acetic acid (93:7:1), is used to separate melatonin (aMT) from N-acetylserotonin (aHT), and the 5-hydroxyindoles from the 5-methoxyindoles. The second solvent, ethyl acetate, is used to separate 5-methoxyindole acetic acid (MA) and 5-methoxytryptophol (ML) from aMT, as

well as 5-hydroxyindole acetic acid (HA) and 5-hydroxytrytophol (HL) from aHT. Tryptophan, serotonin (HT), 5-hydroxytryptophan, and 5-methoxytrptamine are not affected by either of the solvents and remain at the origin. The thin layer chromatographic technique is rapid, simple, and effectively separates trace quantities of the pineal indoles.

7.2 Materials and methods

7.2.1 Animals

Male Wistar rats weighing 250g-300g were used in the experiment. These were randomly assembled into groups of five and maintained as described in Appendix 1. The control group (n=5) received 0.25ml ethanol:0.9% saline (40:60). The remaining group (n=5) received 100mg/kg acetaminophen for three hours. The rats were killed after three hours as described in Appendix 2, and the pineal glands were removed.

7.2.2 Chemicals and reagents

(^{3}H)tryptophan (specific activity 55mCi/ml) was obtained from Amersham International, England. The culture medium, BGJb culture medium, was purchased from Gibco, Europe, and fortified with the antibiotics streptomycin and benzyl penicillin (Hoechst, South Africa). The composition of this medium is represented in Table three. The aluminium TLC plates coated with silica gel 60, Type F254 (0.25mm), were purchased from Merck, Darmstadt, Germany. Beckman Ready-Sol multipurpose liquid scintillation fluid was purchased from Beckman RIIC Ltd, Scotland. The indole standards, MT, HA, HL, MA, ML, aMT, and aHT were purchased from Sigma Chemical Co, St Louis, MO, USA. All other reagents and chemicals were obtained locally and were of the highest purity available.

Table 3: Composition of the BGJb culture medium

Contents	Concentration (mg/litre)
Amino acids	
L-Alanine	250.0
L-Arginine	175.0
L-Aspartic acid	150.0
L-Cysteine (HCl)	90.0
L-Glutamine	200.0
Glycine	800.0
L-Histidine	150.0
L-Isoleucine	30.0
L-Leucine	50.0
L-Lysine (HCl)	240.0
L-Methionine	50.0
L-Phenylalanine	50.0
L-Proline	400.0
L-Serine	200.0
L-Threonine	75.0
L-Tryptophan	40.0
DL-Valine	65.0
Vitamins	
á-Tocopherol phosphate	1.0
Ascorbic acid	50.0
Biotin	0.2
Calcium pantothenate	0.2

Contents	Concentration (mg/litre)
Vitamins	
Choline chloride	50.0
Folic acid	0.2
Inositol	0.2
Para-aminobenzoic acid	2.0
Pyridoxal phosphate	0.2
Riboflavin	0.2
Thiamine HCl	4.0
Vitamin B_{12}	0.04
Inorganic salts	
Dihydrogen sodium ortho-phosphate	90.0
Magnesium sulphate	200.0
Potassium Chloride	400.0
Potassium dihydrogen phosphate	160.0
Sodium bicarbonate	3500.0
Sodium chloride	5300.0
Other compounds	
Calcium lactate	555.0
Glucose	10000.0
Phenol red	20.0
Sodium acetate	50.0

7.2.3 Organ culture of the rat pineal gland

The pineal glands were placed individually into sterile 75×10mm Kimble tubes containing 52µl BGJb culture medium. 8µl of (^{3}H)tryptophan (specific activity 55mCi/ml) was added to each tube. The tubes were then saturated with carbogen and sealed. The tubes were the placed at 37^{o}C in the dark for 24 hours in a Forma Scientific model 3028 incubator. At the end of the 24 hour incubation period, the reaction was terminated by the removal of the pineal glands from solution.

7.2.4 Separation of the indoles using TLC

A modification of the technique employed by Klein and Notides (1969), was used to separate the radio labelled indoles. The TLC plates were activated by placing them in an oven at 100^{o}C for 10 minutes. 5µl of the culture medium was spotted on a 10×10cm TLC plate. The spot was dried using a gentle stream of nitrogen. 10µl of the standard solution was spotted onto the culture medium. The standard solution was prepared as follows: 0.1mg of each standard indoleamine was dissolved together in a test tube containing 95% ethanol and 1% ascorbic acid. The ascorbic acid serves as an antioxidant. The second spot was also dried using a gentle stream of nitrogen. It is important to note that the plates were spotted in subdued light to prevent photo-oxidation of the indoleamines.

The spotted plates were then placed in a TLC tank containing chloroform: methanol: glacial acetic acid (93:7:1). The plates were developed twice in this direction, and were allowed to develop until the solvent front had migrated approximately 9cm. The plates were then allowed to dry, after which they were developed once in ethyl acetate. This was done at right angles to the first direction of development. Following this the plates were dried and sprayed with Van Urks reagent. Van Urks reagent is prepared by adding 1g 4-dimethylamino-benzaldehyde to 50ml 25% HCl, followed by the addition of 50ml 95% ethanol. The plates were subsequently dried in an oven at 60^{o}C for 10 minutes to allow for

colour development of the spots. The spots were then cut and individually placed in scintillation vials. 3ml Beckman Ready-Sol scintillation fluid was added to each vial. The vials were vortexed on a Vortex Rotor-mixer for 30 seconds, and the radioactivity of each metabolite was then measured in a Beckman LS 2800 scintillation counter.

7.3 Results

A typical bi-dimensional thin layer chromatogram of the pineal indole metabolites was obtained. This is represented in Figure 42. Excellent separation of the indoles was achieved. Results (average of three estimations) obtained from these studies are expressed as DPM/10µl/pineal gland (mean ± SEM) for each indole. The background counts were negligible. The data were statistically analysed and the difference between the control and acetaminophen groups was determined using the Student's t-test. $P<0.05$ was determined to be statistically significant. As shown in Figure 43, acetaminophen administration significantly increased ($p<0.05$) pineal (^3H) melatonin synthesis.

Table 4: The effects of acetaminophen on pineal indole metabolism

Pineal Metabolites	**Control (DPM/10µl/pineal) Mean ± SEM**	**Acetaminophen (DPM/10µl/gland) Mean ± SEM**
Serotonin	112100 ± 5485	105654 ± 2971
N-acetylserotonin	35963 ± 3035	38175 ± 3466
5-HIAA	1930 ± 222	2442 ± 238
5-Hydroxytryptophol	6119 ± 566	8784 ± 752
Melatonin	5813 ± 542	11155 ± 665
5-Methoxyindole acetic acid	24428 ± 3791	23080 ± 1233
5-Methoxytryptophol	30390 ± 652	31632 ± 3289

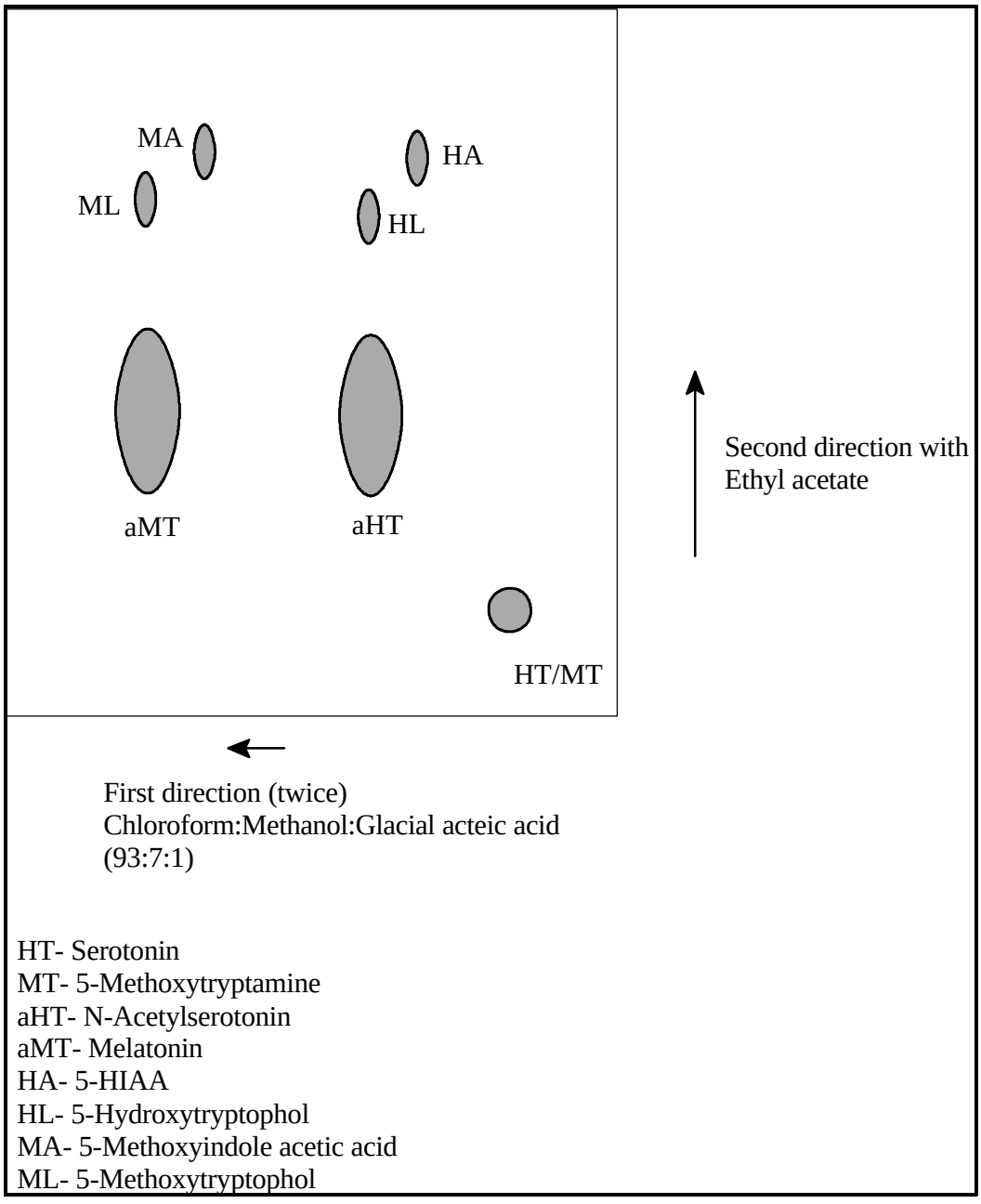

Figure 42: A trace of the TLC plate illustrating the direction in which the plate was run and the location of the pineal indole metabolites (Klein and Notides, 1969).

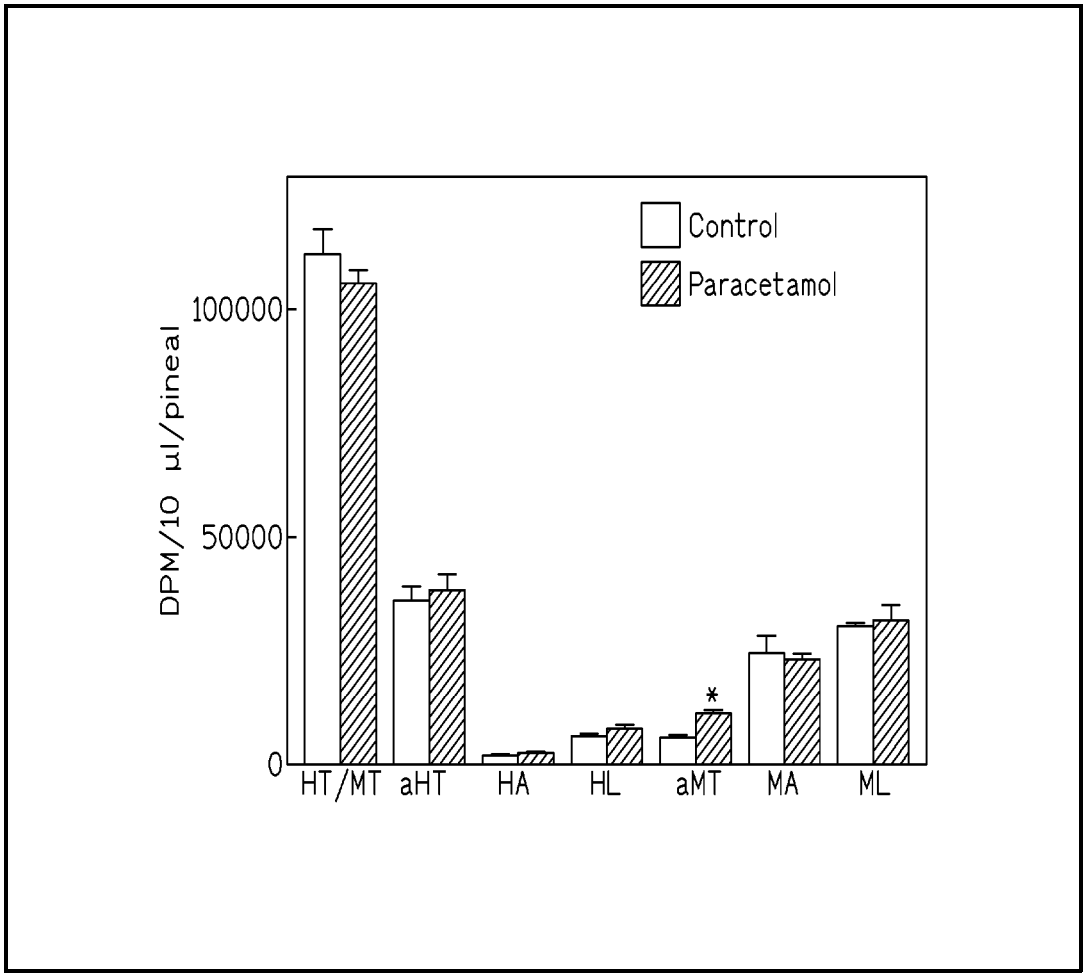

Figure 43: The effect of acetaminophen administration on rat pineal indole metabolism.Each bar represents the mean ± SEM; n=5. * ($p<0.05$). Tukey-Kramer Multiple Comparisons test.

7.4 Discussion

Indole metabolism in the pineal gland occurs in the pinealocytes. The different stages are outlined in Figure 3. As described in section 1.1.3, pineal indole metabolism commences with the uptake of tryptophan from the bloodstream. Tryptophan is converted to 5-hydroxytryptophan by the enzyme tryptophan hydroxylase. A decarboxylase enzyme then converts 5-hydroxytryptophan to serotonin. Serotonin is then N-acetylated by NAT to form N-acetylserotonin. The acetyl group is provided by acetyl CoA. The formation of melatonin from N-acetylserotonin is catalysed by the enzyme HIOMT. The methyl group donor in this step is S-adenosylmethionine (Reiter, 1981).

The rat pineal gland in organ culture is able to metabolise radiolabelled tryptophan to various indoles (Olivieri *et al*, 1990). As shown in Figure 43, the results of this experiment show that acetaminophen administration significantly increases melatonin levels ($p<0.05$). Olivieri *et al* (1990) showed that exogenous radiolabelled tryptophan is converted to melatonin in the organ culture system. The increased synthesis of radiolabelled melatonin by pineals from rats which received acetaminophen could possibly be due to enhanced uptake of (^{3}H)tryptophan into the pineal parenchymal cells. This needs to be investigated further.

The acetaminophen-induced increase in pineal (^{3}H) melatonin biosynthesis could be due to increased N-acetylation of (^{3}H) serotonin by NAT, resulting in elevated (^{3}H) N-acetylserotonin levels as a substrate for conversion to (^{3}H) melatonin. Acetaminophen may also mimic the action of the antidepressants, desipramine and maprotiline. Both drugs have been reported to inhibit pineal monoamine oxidase activity *in vitro* (Nir and Hirschmann, 1983). This would increase (^{3}H) serotonin levels for conversion to (^{3}H) melatonin.

Alternatively, the increase in (^{3}H) melatonin synthesis could be attributed to the increase in

brain serotonin levels following acetaminophen administration (Chapter Six, Figure 40). Acetaminophen could act either as a monoamine oxidase inhibitor, or as a serotonin reuptake inhibitor in the brain, and thereby increase serotonin levels. The results from Chapter Six, show that acetaminophen administration significantly increases forebrain serotonin levels. This increases the levels of serotonin available for melatonin production, and subsequently melatonin levels. Studies have demonstrated that selective serotonin reuptake inhibitors increase melatonin synthesis in rats as well as in healthy human volunteers (Wirz-justice *et al*, 1980). It is a possibility that the presynaptic inhibition of serotonin, possibly by acetaminophen, may increase the availability of serotonin within the pinealocyte as substrate for conversion to melatonin. However, Daya *et al* (1989), showed that pineal serotonin and pineal melatonin are unlikely to be affected by changes in brain serotonin because the levels of brain serotonin are 100-fold lower than in the pineal gland. Thus, the relationship between brain serotonin levels, and pineal serotonin and melatonin levels needs to be examined in greater detail.

The increase in melatonin levels following acetaminophen administration could also be due to increased stimulation of the â-adrenergic receptors. Skene (1985) showed that the antidepressants mianserin and trazodone, increase â-adrenergic receptor sensitivity. The monoamine oxidase inhibitors also appear to increase melatonin production via â-adrenergic receptor stimulation (McIntyre *et al*, 1985). It is possible that acetaminophen is also able to do this. As described in section 1.2.5, these receptors are coupled to adenylate cyclase, which is responsible for cAMP production. Skene (1985), showed that chronic isoprenaline administration increases basal and pineal cAMP levels. Acetaminophen administration could possibly increase adenylate cyclase activity, thereby increasing cAMP levels, and consequently melatonin levels. This needs to be investigated further.

Chapter Eight

The Measurement of Urinary 5-HIAA: The effect of acetaminophen administration on rat urinary 5-HIAA levels

8.1 Introduction

Most of the data on central serotonin metabolism in depression has been obtained by measuring the cerebrospinal fluid concentrations of 5-HIAA (van Praag, 1982). The production of 5-HIAA from serotonin is illustrated in Figure 10. 5-HIAA is the major metabolite of serotonin. Since tryptophan is the dietary precursor of serotonin, it is also correct to state that it is also the precursor of 5-HIAA (Udenfriend *et al*, 1955). Due to large quantities of 5-HIAA being present in urine it is believed that an appreciable amount of tryptophan is metabolized via the 5-hydroxyindole route (Udenfriend *et al*, 1955). It has been reported that 5-hydroxytryptophan, serotonin, and 5-HIAA, are excreted in the urine when administered (Udenfriend *et al*, 1955). It is probable that 5-HIAA is derived solely from serotonin, but it is possible that some of it is derived from the decarboxylation of 5-hydroxyindolepyruvic acid which is derived from the transamination of serotonin (Udenfriend *et al*, 1955).

Since 5-HIAA accounts for nearly 100% of the metabolism of serotonin in the brain, the amount of the acid excreted reflect the secretion of serotonin (Sanders-Bush and Mayer, 1996). 5-HIAA from the brain, and peripheral sites of serotonin storage, is excreted in the urine, together with small amounts of 5-hydroxytryptophol, and sulphate or glucuronide conjugates. Probenicid, a drug used in the treatment of gout, inhibits the transport of 5-HIAA from the central nervous system to the bloodstream. The resulting accumulation of 5-HIAA in the brain provides a yardstick for serotonin metabolism in the CNS. Most investigators have found the Probenicid-induced accumulation of 5-HIAA in the brain and

cerebrospinal fluid to be diminished in depression, indicating diminished serotonin levels (van Praag, 1982). Since urinary 5-HIAA is an indication of brain serotonin, the levels of the metabolite present in urine may be important in the diagnosis of depression. Depression is characterized by the reduction of serotonin levels in the brain (Walsh and Daya, 1989). Low cerebrospinal fluid levels of 5-HIAA have also been linked to violent and impulsive acts such as suicide (Sanders-Bush and Mayer, 1996). The large amounts 5-HIAA, excreted in the urine of patients with malignant carcinoid, provide a valuable diagnostic test for the disease (Sanders-Bush and Mayer, 1996). The enhanced excretion of 5-HIAA in the urine is due to increased levels of serotonin in the tumor itself (Udenfriend *et al*, 1955).

The alternative pathway for the metabolism of serotonin, rather than the oxidation to 5-HIAA, is the reduction of 5-hydroxyindoleacetaldehyde to 5-hydroxytryptophol. The ingestion of large amounts of alcohol results in elevated amounts of NADH, which diverts 5-hydroxyindoleacetaldehyde from the oxidative pathway to the reductive pathway. This leads to an increase in the excretion of 5-hydroxytryptophol, with a corresponding decrease in 5-HIAA excretion (Sanders-Bush and Mayer, 1996).

8.2 Materials and methods

8.2.1 Animals

Male Wistar rats weighing 250g-300g were used in the experiment. The rats were housed individually in metabolic cages, and were maintained as described in Appendix 1. The control group (n=5) received 0.25ml 0.9% saline:ethanol (60:40) every hour for three hours i.p. This was also the vehicle in which the drug was dissolved. The second group received 100mg/kg of acetaminophen every hour for three hours i.p. Only urine collected in the last hour (three to four hours after the first injection) of the experiment was analyzed.

8.2.2 Chemicals and reagents

1-Nitrosonapthol reagent was purchased from Sigma Chemical Co, St Louis, MO, USA. 2,4-Dinitrophenylhydrazine was purchased from Merck, Darmstadt, Germany. All other reagents were purchased from local sources and were of the highest purity available.

8.2.3 5-HIAA determination

A modification of the method used by Udenfriend *et al* (1955) was used in this experiment.1ml urine and 2,4-dinitrophenylhydrazine (2,4-DNPH) (0.5% in 2N HCl) were mixed together in a clean test tube. This mixture was incubated for 30 minutes at room temperature with occasional, gentle shaking. This was done to facilitate the removal of keto acids. An aliquot of 4.2ml chloroform was added, and the mixture centrifuged at 3000×g for five minutes. The organic layer was removed, and 4.2ml chloroform was added to the aqueous layer. This mixture was centrifuged at 3000×g for five minutes. After centrifugation, 1.7ml of the aqueous layer was removed and added to a test tube containing 0.6g NaCl and 4.2ml ethyl ether. The mixture was vortexed for approximately five minutes, and then centrifuged at 3000×g for a further five minutes. 3.3ml of the ether aliquot was then transferred to a tube containing 0.2ml phosphate buffer, pH 7. This mixture was shaken and centrifuged at 3000×g for five minutes. The ethyl ether layer was then evaporated in a fume hood under a gentle stream of nitrogen. 0.17ml of the aqueous layer, 0.08 ml nitrosonapthol (0.1% in absolute ethanol) reagent, and 0.08 ml nitrous acid were then mixed together and incubated at 37^{o}C for five minutes. 0.83ml ethyl acetate was added to the tube which was subsequently vortexed to separate the layers. Following this procedure, the ethyl acetate then carefully removed and the aqueous layer transferred to a plastic cuvette. This layer contained the 5-HIAA and its optical density was measured at 540nm, using a Shimadzu UV-160A UV-visible spectrophotometer. A standard curve (10-200µmoles/ml) was generated using a 5-HIAA standard (Appendix 6).

In order to determine whether 5-HIAA was present in the urine extract, and if the correct metabolite was being analyzed, a TLC chromatogram was run. The method used is described in section 7.2.4. The Rf values were measured for both the sample and the 5-HIAA standard.

8.3 Results

The data obtained were expressed as µmol/ml of urine and were the mean of three determinations. The data were analyzed by the Student's t-test and a $p<0.05$ between the groups was accepted as being statistically significant. As shown in Table 5, 5-HIAA was present in the urine extract. The Rf values were the mean of three determinations and were calculated measuring the distance migrated by the sample divided by the distance migrated by the solvent front. It is also evident from Figure 44, that acetaminophen administration significantly reduces ($p<0.05$) the urinary concentration of 5-HIAA.

Table 5: The results (Rf values) of TLC analysis of the urine extract.

Compound	Mean Rf values
Urinary extract	0.27
5-HIAA standard	0.26

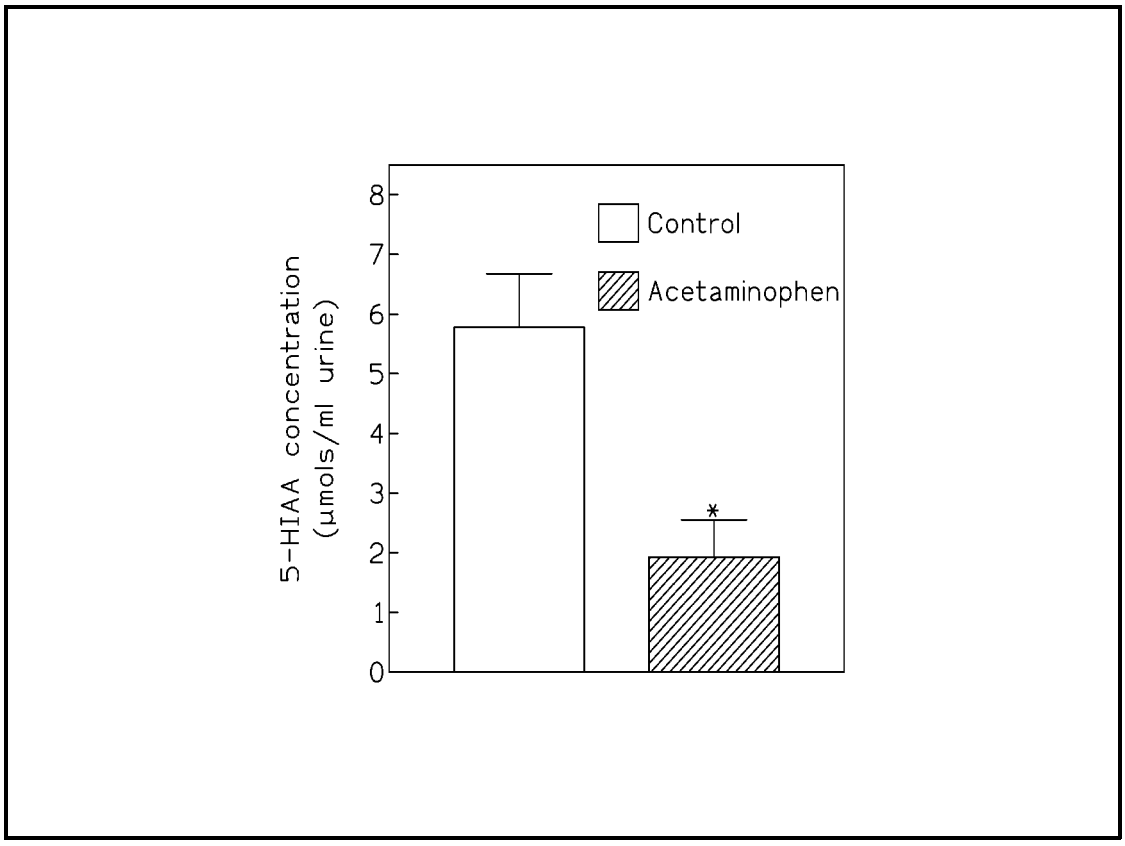

Figure 44: The effect of acetaminophen administration on rat urinary 5-HIAA levels. Each bar represents the mean ± SEM; n=5. * ($p<0.05$). Student's t-test.

8.4 Discussion

5-HIAA is the major metabolite of serotonin metabolism. The enzyme, monoamine oxidase, catalyzes the formation of 5-hydroxyindoleacetaldehyde from serotonin. This is an unstable intermediate, and is converted to 5-HIAA by aldehyde dehydrogenase (Figure 10). Reduced levels of 5-HIAA in the CNS and urine are an indication of reduced serotonin turnover (van Praag, 1982). Several researchers have shown that reduced serotonin levels in the CNS are associated with depression (Walsh and Daya, 1998). Therefore, a reduction in the 5-HIAA levels are also an indication of depression (van Praag, 1982).

The results obtained by the chromatographic analysis of the urine extract indicate that the metabolite being analyzed was 5-HIAA (Table 5). The Rf values obtained by TLC analysis were almost identical, indicating that the 5-HIAA was successfully extracted from the rat urine samples. The results obtained from the colorimetric analysis of the urine extract show reduced levels of 5-HIAA (Figure 44), suggesting that acetaminophen administration induces depression. However the results obtained from previous experiments (Figures 39 and 40) illustrate a decrease in TDO activity with a concomitant rise in forebrain serotonin levels. These changes would normally be accompanied by a rise in 5-HIAA levels. The decrease in urinary 5-HIAA levels obtained in this experiment therefore contradict the suggestion that acetaminophen has the potential to alleviate depression.

However, the reduction of urinary 5-HIAA levels can be interpreted differently. The reduction of this metabolite could suggest that there is reduced breakdown of serotonin in the CNS, making more of it available to perform its functions. This explanation is consistent with the previous findings, and supports the ability of acetaminophen to alter mood by acting as an antidepressant.

Chapter Nine

Summary of Results: Conclusions and Recommendations for Future Work

9.1 Summary of results

<u>Chapter Two</u>: The photodegradation of acetaminophen and melatonin

This study examined the effect of melatonin, a known free radical scavenger, on the degradation of acetaminophen following irradiation. The results show that melatonin is able to alter the photodegradation of acetaminophen in the presence of air and nitrogen. Melatonin retards the rate of degradation of acetaminophen in the presence of nitrogen, but enhances the rate of degradation of acetaminophen in the presence of air. The photodegradation studies also revealed that melatonin is also rapidly degraded in the presence of air.

<u>Chapter Three</u>: Melatonin and DMF-Induced Hepatotoxicity

DMF is hypothesized to initiate liver damage via a free radical mechanism. This study was done in collaboration with the CSIR in India, using a cell-free system. Hydroxyl radicals were generated using Fe^{2+}-citrate, in the presence of salicylate. This leads to the formation of 2,3- and 2,5-DHBA adducts which are reliable indicators of hydroxyl radical generation. DMF was shown to reduce the formation of both these adducts, and thereby act as a hydroxyl radical scavenger, proving the hypothesis incorrect.

However, the electron microscopy studies clearly indicate that DMF is toxic to the liver, and that melatonin is able to reduce the damage.

<u>Chapter Four</u>: The effect of DMF and acetaminophen on lipid peroxidation

The results from this experiment show that DMF administration (0.4ml/kg and 0.8ml/kg for seven days) did not significantly increase lipid peroxidation in the rat liver. This *in vivo* finding verifies the *in vitro* finding that DMF does not generate hydroxyl radicals. Three different doses of acetaminophen (30mg/kg, 100mg/kg, 500mg/kg) were administered to rats for a seven day period. Acetaminophen (500mg/kg) was found to significantly increase ($p<0.05$) lipid peroxidation in the liver. This does not conclusively prove that the lower doses of acetaminophen (30mg/kg and 100mg/kg) do not generate free radicals, as lipid peroxidation is often a late process accompanying cell death, rather than initiating it. Melatonin was not able to significantly reduce the peroxidation induced by acetaminophen (500mg/kg), thereby indicating that melatonin alone would not be an effective antidote for acetaminophen poisoning.

<u>Chapter Five</u>: The effect of acetaminophen administration on TDO activity

The haem-dependant enzyme, TDO, is a major determinant of tryptophan levels *in vivo*. Acetaminophen administration (100mg/kg every hour for three hours) was found to significantly inhibit ($p<0.05$) the activity of TDO at the level of apoenzyme, indicating that acetaminophen prevents the conjugation between the holoenzyme and haem. Acetaminophen did not alter the activity of the holoenzyme and the total enzyme.

<u>Chapter Six</u>: Acetaminophen administration and rat forebrain and pineal melatonin levels

This study involved the use of the ELISA technique. Acetaminophen administration (100mg/kg every hour for three hours) was found to significantly increase ($p<0.05$) rat forebrain serotonin levels, but had no effect on pineal melatonin levels. The increase in serotonin levels are consistent with the finding that acetaminophen administration inhibits

TDO. The unchanged melatonin levels are however not consistent with TDO inhibition.

<u>Chapter Seven</u>: The effect of acetaminophen administration on pineal melatonin levels

This study employed the use of organ culture and thin layer chromatography. Acetaminophen administration (100mg/kg every hour for three hours) was shown to significantly increase ($p<0.05$) pineal melatonin levels, using radiolabelled tryptophan as a precursor.

<u>Chapter Eight</u>: The effect of acetaminophen administration on rat urinary 5-HIAA levels

This study was performed in order to substantiate the results from Chapters Five, Six, and Seven. However, acetaminophen administration (100mg/kg every hour for three hours) was found to reduce urinary 5-HIAA levels. Reduced 5-HIAA levels are consistent with increased TDO activity and reduced serotonin levels. The results could however, also indicate reduced serotonin catabolism, which is indicative of increased brain serotonin levels.

9.2 Conclusions

Section 1.7 outlines the two main research objectives of this study. The first objective was to evaluate the effectiveness of melatonin, a non-toxic free radical scavenger, as a hepatoprotectant against DMF and more importantly, acetaminophen poisoning. Both these compounds are known to induce liver damage. Although melatonin appears to be an effective antidote for DMF poisoning, the results indicate that DMF is not a hydroxyl radical generator, and does not induce lipid peroxidation in the rat liver. The electron

microscopy studies tentatively suggest that melatonin would be able to reduce the extent of the damage caused by DMF. However, the results obtained from the spin trap studies, electron microscopy, and lipid peroxidation, do shed some light on the mechanism by which DMF induces liver injury. This study hypothesizes that this damage is via a free radical mechanism. The experimental data obtained refutes this hypothesis, indicating that DMF-induced liver damage is not inflicted by free radicals.

The results obtained tentatively suggest that acetaminophen generates other free radicals besides the phenoxyl radical. Melatonin was also found to be ineffective against the peroxidation induced by acetaminophen administration (500mg/kg for seven days). This suggests that melatonin alone would not be an effective antidote for acetaminophen poisoning. However, the effectiveness of melatonin as a potent free radical scavenger cannot be ignored. A combination of melatonin and methionine may prove useful in the treatment of acetaminophen poisoning. This, as well as the exact mechanism of DMF and acetaminophen induced organ damage, needs to be further investigated.

The photodegradation studies also revealed important insight into the stability of both acetaminophen and melatonin. This also needs to be investigated thoroughly. However, the results obtained thus far indicate that both these compounds need to be stored in the absence of sunlight, and in the case of melatonin, in an airtight bottle. This is important, especially in medical institutions, where melatonin is used as a therapeutic agent. This study revealed the adverse effects of melatonin on acetaminophen in the presence of UV light, indicating that melatonin which if incorrectly stored, can be potentially hazardous if co-administered with drugs such as acetaminophen.

The second objective of the study was to investigate the increasing abuse of and addiction to acetaminophen in an attempt to outline the biochemical basis of such addiction. The inhibition of TDO, accompanied by the rise in brain serotonin levels induced by acetaminophen, implies that this drug has the potential to alter mood states, which could in

turn explain why this drug causes mood changes at high doses. The antinociceptive action of acetaminophen is already known to be linked to the serotonergic system. Certain antidepressants have been shown to inhibit TDO, followed by a rise in brain serotonin levels. Acetaminophen, therefore, mimics the action of an antidepressant. Criticisms may be aimed at the previous statement due to the short period of acetaminophen administration. However, it is well documented that antidepressant action can be exerted within six hours (Harvey, 1997), adding credibility to the findings of this study. The actions of this drug's ability to alter the levels of this important neurotransmitter need to be investigated further.

In addition to the increased serotonin levels, the organ culture studies revealed that acetaminophen administration increased pineal melatonin levels. Increased melatonin levels have been reported following antidepressant therapy. This finding supports the idea that acetaminophen mimics the action of an antidepressant. Acetaminophen administration was also shown to reduce the urinary 5-HIAA levels indicating diminished serotonin catabolism, thereby indicating increased serotonin levels in the brain.

The results obtained in this part of the study have important clinical applications and could possibly explain acetaminophen addiction and abuse. Acetaminophen, could possibly be used as an effective, mild antidepressant in cases where cost of therapy is an issue. Since acetaminophen possibly acts as an antidepressant, care should be taken when it is administered in conjunction with known antidepressant drugs as it could result in the potentially dangerous serotonin syndrome. The findings from this study have generated several possible areas of research.

9.3 Possible future areas of work cultivated by this study

The toxicology part of this study needs to be further investigated. Several more experiments on DMF and acetaminophen toxicology have to be performed before an

accurate assessment can be made on the ability of melatonin as a hepatoprotectant against these compounds. The measurement of the liver transaminases (alanine and aspartate) could prove valuable. A rapid rise in the activity of these enzymes has been reported in cases of serious liver damage. The effect of melatonin on the activity of these enzymes would be indicative of its ability, or lack of ability, to reduce the liver damage. Hepatoprotectants such as N-acetylcysteine are able to reduce the activity of these enzymes.

Another enzyme that can be studied is cytochrome P450, as this enzyme is responsible for formation of the toxic metabolite N-acetyl-benzoquinoneimine. Inhibitors of this enzyme can reduce the severity of organ damage. It would be interesting to determine if melatonin could alter the activity of this enzyme.

The lipid peroxidation technique employed in this study is widely used. However, there are more reliable and sensitive methods available for determining peroxidation of cell membranes. These techniques include HPLC analysis, and antibody techniques, and will definitely produce more accurate and reliable results. The only negative aspect of using these techniques is the cost involved.

In vitro chemical studies can also be performed to ascertain whether or not melatonin is able to form complexes with acetaminophen, and its metabolite, N-acetyl-benzoquinoneimine, and by doing so possibly inactivate these compounds. This complexation can be studied using techniques such as infra-red and UV spectroscopy.

The antidepressant action of acetaminophen can also be further investigated. The action of acetaminophen on monoamine oxidase activity could clarify the antidepressant mechanism of acetaminophen, as several antidepressants are known to inhibit this enzyme. There are several short and relatively simple assays to determine the activity of monoamine oxidase.

Acetaminophen should also be compared with known antidepressants, in terms of their ability to inhibit TDO activity, and increase brain serotonin levels. This would determine the potency of acetaminophen as an antidepressant. The effect of acetaminophen on tryptophan hydroxylase could also be determined. This enzyme is the rate-limiting step in brain serotonin synthesis, and could be a target for acetaminophen, thereby clarifying the antidepressant action of acetaminophen.

The possible antidepressant activity of acetaminophen has important clinical implications, and should definitely be examined in greater detail.

Appendix 1

Animals

All the work involving the use of animals was approved by the Rhodes University animal ethics committee. The animals used throughout this study were male wistar rats weighing 250-300g. The animals were housed in opaque plastic cages with metal grid floors and covers, under a diurnal lighting cycle 12 light:12 dark with food and water *ad libitum*. The intensity of the light guring the 12 hour light phase was approximately 300 µWatts/cm^2. The temperature of the animal room was maintained between 20^{o}C and 25^{o}C. The cages were cleaned daily.

Appendix 2

Sacrificing and dissection of the animals

The rats were killed swiftly by cervical dislocation and rapidly decapitated. The top of the skull was removed by making an incision through the bone on either side of the head. Using a pair of forceps, the skull was lifted, exposing the pineal gland and brain. The pineal glands and brains were either used immediately or stored at -70^{o}C until needed.

To remove the livers, a mid-ventral incision was made through the abdominal musculature from the pelvic region to the posterior edge of the sternum. A transverse cut was made anteriorly to expose the liver, which was removed carefully. The livers were either used immediately or stored at -70^{o}C until needed.

Appendix 3

Protein determination

Materials

Folin & Ciocalteu's reagent was purchased from Saarchem (PTY) LTD, Krugersdorp, South Africa. The Bovine serum albumin (BSA) was supplied by Sigma Chemical Co, St Louis, MO, USA. All other chemicals and reagents were obtained locally and were of the highest available purity.

Protein Determination

A modification of the method employed by Lowry et al, 1950 was used throughout this study. 6ml of alkaline copper reagent (1ml 1% copper sulphate, 1ml 2% sodium tartrate, and 98ml 2% sodium carbonate) was added to 1ml homogenate in a set of clean test tubes. The tubes were mixed and allowed to stand at room temperature for ten minutes. Following this, 0.3ml of the Folin-Ciocalteu reagent was added to the tubes. The tubes were then mixed and allowed to stand in the dark for 30 minutes at room temperature. After this period the absorbance was read at 500nm using a Shiamdzu UV-160 A UV-visible spectrophotometer. A standard curve (0-300µg/ml) was also generated in the same manner, using 1ml BSA instead of homogenate.

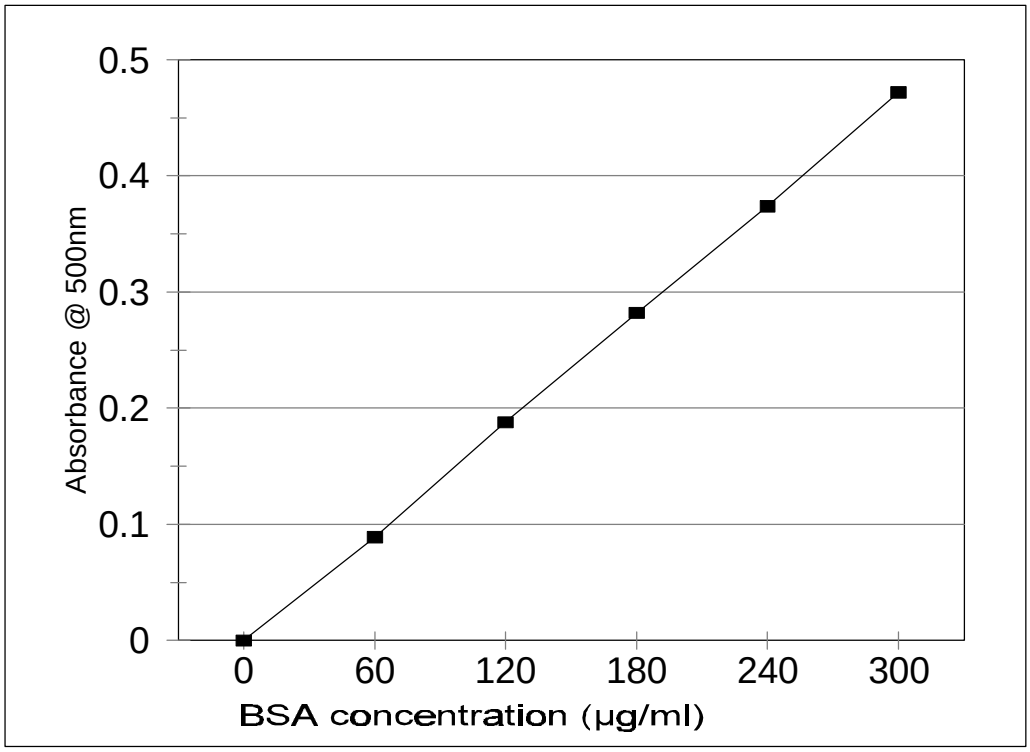

Figure 45: Protein standard curve (r^2 = 0.999).

Appendix 4

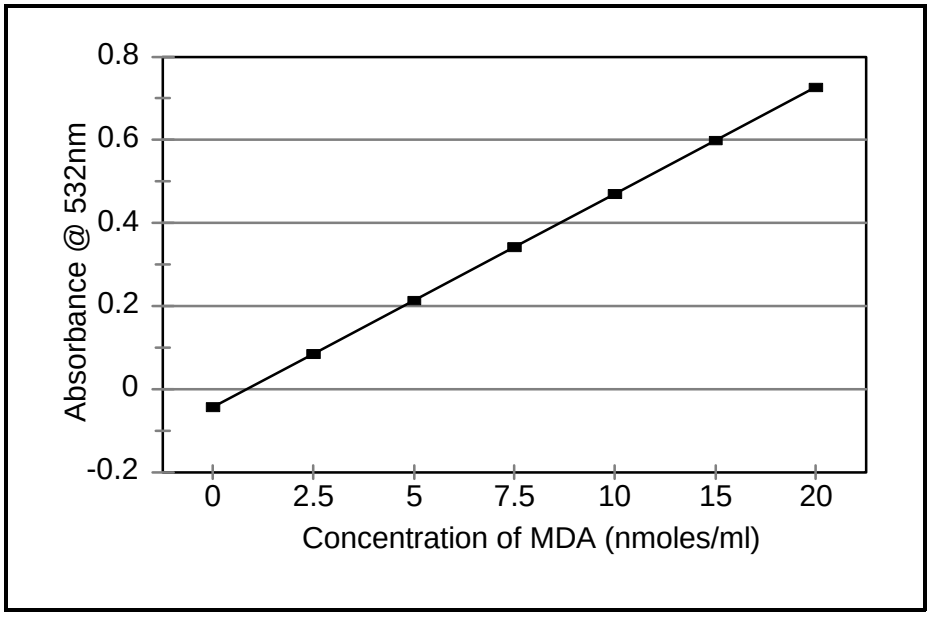

Figure 46: Lipid peroxidation standard curve ($r^2 = 0.999$).

Appendix 5

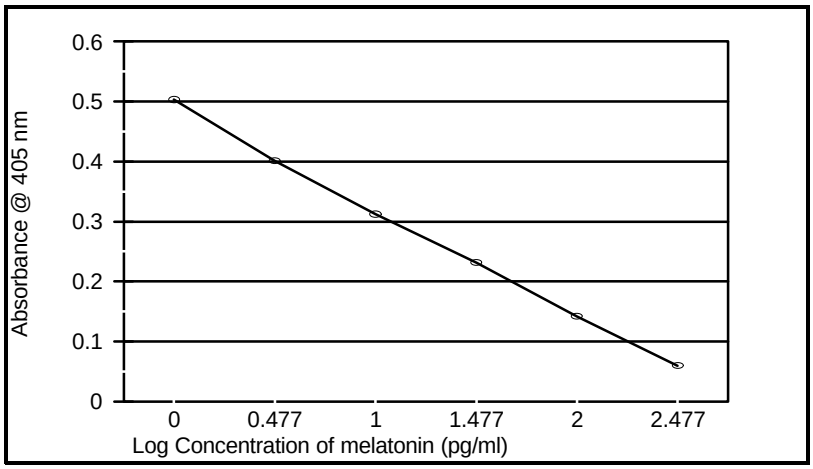

Figure 47: Melatonin standard curve ($r^2 = 0.998$)

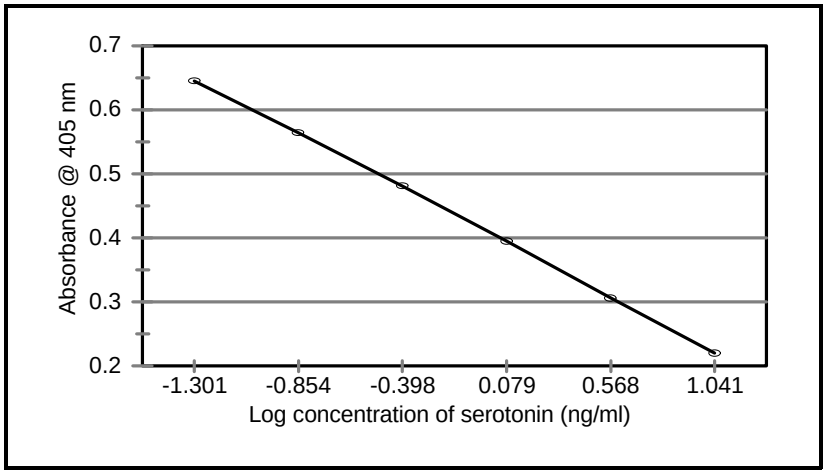

Figure 48: Serotonin standard curve ($r^2 = 0.991$).

Appendix 6

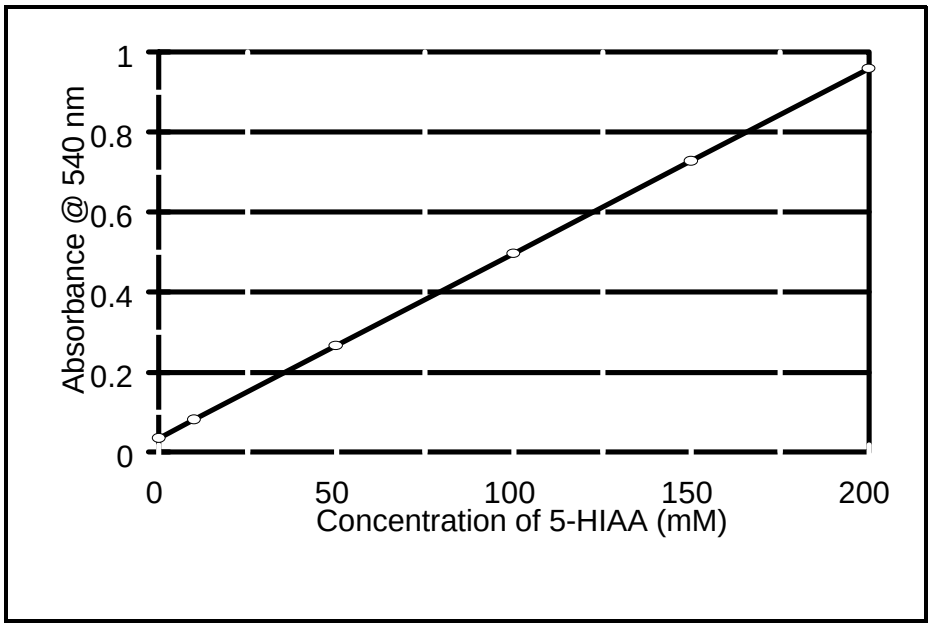

Figure 49: 5-HIAA standard curve ($r^2 = 0.996$).

References

Amatimaggio, F., Calistri, S., Venturs, F., Margheri, M., Niglio, F., Perico, A. & Perissi, S. (1998). *Med. Lav.* **89**, 533-537.

Angerer, J., Goen, T. & Kramer, A. (1998). *Arch. Toxicol.* **72**, 309-313.

Anoopkumar-Dukie, S., Cross, R., Mohanakumar, K. P. & Daya, S. (1997). *Microscopy Society of Southern Africa - Proceedings*. **27**, 124.

Anoopkumar-Dukie, S., Glass, B. D., Walker, R. B. & Daya, S. *Pharmacy and Pharmacology Communications*. In Press.

Arendt, J. (1988). *Clinical Endocrinolgy*. **29**, 205-229.

Ariens Kappers, J. (1971). In G. E. W. Wolstenholme & J. Knight (Eds.), *The Pineal Gland*. London: Churchill Livingstone, 3-33.

Axelrod, J., Shein, H. M. & Wurtman, R. J. (1969). *Proc. Nat. Acad. Sci.* **62**, 544-549.

Awang, R. (1997). *Review of Paracetamol Poisoning*. Internet. World Wide Web. http://prn.usm.my/bulletin/1997/prn14.html (3 May, 1998).

Bach, P. H., Berndt, W. O., Delzell, E., Dubach, U., Finn, W. F., Fox, J. M., Hess, R., Miechielsin, P., Sandler, D. P., Trump, B. & Williams, G. (1998). *Renal Failure*. **20**, 749-762.

Badawy, A. A. B. & Evans, M. (1974). *Biochem. J.* **138**, 445.

Badawy, A. A. B. & Evans, M. (1975). *Biochem. J.* **150**, 511.

Badawy, A. A. B. (1979). *Biochem. Soc. Trans.* **7**, 575-583.

Badawy, A. A. B., Welch, A. N. & Morgan, C. J. (1981). *Biochem. J.* **248**, 309-314.

Banoo, S. (1991). Neuropharmacological interaction in the rat pineal gland: A study of antidepressant drugs. *PhD Thesis*, Rhodes University.

Blask, D. E., Hill, S. M., Pelletier, D. B. Anderson, J. M. & Lemus-Wilson, A. (1989). In R. J. Reiter and S. F. Pang (Eds.), *Advances in Pineal Research*, Vol. 3. London: John Libbey, 259-263.

Branchey, L., Weinberg, U., Branchey, M., Linkowski, P. & Mendlewicz, J. (1982). *Neuropsychobiology*, Vol. 8. 225-232.

Brown, T. M., Skop, B. P. & Mareth, T. R. (1996). *Ann. Pharmacotherap*. **30**, 527-528.

Carlson, A., Davis, J. N., Lindquist, W. & Attack, C. V. (1972). *Arch. Pharmacol.* **275**, 153.

Cavallo, A. & Hassan, M. (1995). *J. Pineal. Res.* **18**, 90-92.

Chang, M. J. & Lin, R. S. (1991). *Drug. Chem. Toxicol.* **14**, 173-184.

Chiueh, C. C., Krishna, G., Tulsi, P. Obata, T., Lang, K., Huang, S-J. & Murphy, D. L. (1992). *Free. Rad. Biol. Med.* **13**, 581-583.

Chiueh, C. C., Miyake, H. & Peng, M-T. (1993). *Adv. Neurol.* **60**, 251-258.

Clark, J. J. & Switzer, R. L. (1977). *Experimental Biochemistry* (2nd edn.). New York: Freeman, 23-45.

Clark, W. R. (1991). *The Experimental Foundations of Modern Immunology* (4th edn.). Singapore: John Wiley & Sons, 150-153.

Coreia, M. A. (1995). In Bertram G. Katzung (Ed.), *Basic and Clinical Pharmacology* (6th edn.). Norwalk, CT.: Appleton & Lange, 54.

Curzon, G. & Green, A. R. (1969). *Biochem. J.* **111**, 15.

Daya, S., Nonaka, K. O., Buzzell, G. R. & Reiter, R. J. (1989). *J. Neurosci. Res.* **23**, 304-309.

Daya, S. (1994). *Specialist Medicine*. September 1994, 58.

Daya, S. (1999). *Specialist Medicine*. August 1999, 529.

Dinis, T. C., Maderia, V. M. & Almeida, L. M. (1994). *Arch. Biochem. Biophys.* **15**, 161-169.

Douglas, W. W. (1975). In L. S. Goodman and A. Gilman (Eds.), *The Pharmacological Basis of Therapeutics* (5th edn.). New York: Macmillan, 613-623.

Feuer, G. (1990). In G. Feuer (Ed.), *Review of Drug Metabolism and Drug Interactions*, Vol. 8. London: Freund, 203-217.

Fiske, V. M., Bryant, K. & Putman, J. (1960). *Endocrinology*. **66**, 489-491.

Freeman, B. A & Crapo, J. D. (1981). *J. Biol. Chem*. **256**, 10986-10992.

Gibson, G. Gordon & Skett, P. (1986). *Introduction to Drug Metabolism*. London: Chapman & Hall, 77-193.

Gilbert, A. & Baggot, J. (1991). *Essentials of Molecular Photochemistry*. London: Blackwell, 5-177.

Giusti, P., Gusella, M., Lipartiti, M., Milani, D., Zhu, W., Vicini, S., & Manev, H. (1995). *Exp. Neurol*. **131**, 39-46.

Gutteridge, J. M. C. & Halliwell, B. (1990). *TIBS.* **15**, 129-134.

Halliwell, B. (1987). *FASEB J.* **1**, 358-364.

Hagan, R. M. & Oakley, N. R. (1995). *TiPS.* **16**, 81-83.

Harvey, B. H. (1997). *S. Afr. Med. J.* **87,** 540-544.

Hollister, L. E. (1995). In Bertram G. Katzung (Ed.), *Basic and Clinical Pharmacology* (6th edn.). Norwalk, CT.: Appleton & Lange, 448-458.

Hullin, R. P., Bailey, A. D., Macdonald, R., Dransfield, G. A. & Milne, H. M. (1967). *Brit. J. Psychiat.* **113**, 593-600.

Insel, P.A. (1996). In P. B. Molinoff & R. W. Ruddon (Eds.), *The Pharamacological Basis of Therapeutics* (9th edn.). New York: McGraw-Hill, 617-633.

Johnson, M. E., Sill, J. C., Uhl, C. B. & Van Dyke, R. A. (1993). *Anesthesiology*. **79**, 1061-1069.

Kappus, H. (1987). *Chem. Phys. Lipids.* **45**, 105-115.

Klein, D. C. & Notides, A. (1969). *Anal. Biochem.* **31**, 480.

Klein, D. C., Weller, J. L. & Moore, R. J. (1971). *Proc. Natl. Acad. Sci.* **68**, 3107-3110.

Klein, D. C. (1973). In F. O. Scmitt and F. C. Worden (Eds.), *The Neurosciences*. Cambridge: MIT, 509-516.

Knox, W. E. & Auerbach, V. H. (1955). *J. Biol. Chem.* **214**, 307.

Knox, W. E. & Piras, M. M. (1965). *J. Biol. Chem.* **242**, 764.

Lerner, A. B. & Case, J. D. (1960). *Fed. Proc.* **19**, 590-593.

Lewis, A. J., Kerenyi, N. A. & Feuer, G. (1990). In G. Feuer (Ed.), *Reviews of Drug Metabolism and Drug Interactions*, Vol. 8. London: Freund, 247-312.

Limson, J., Nyokong, T. & Daya, S. (1998). *J. Pineal. Res.* **24**, 15-21.

Lores Anaiz, S., Llesuy, S., Cutrin, J. C. & Boveris, A. (1995). *Free. Rad. Biol. Med.* **19**, 303-310.

Lovenburg, W., Jequier, E. & Sjoerdsma, A. (1967). *Science*. **155**, 217-219.

Lovenburg, W., Weisbach, H. & Udenfriend, A. (1962). *J. Biol. Chem.* **193**, 89-93.

Lowry, O. H., Rosenburg, M. J., Farr, A. L. & Randall, R. J. (1951). *J. Biol. Chem.* **193**, 256.

Malley, L. A., Slone, T. W., Van Pelt, C., Elliot, G. S., Ross, P. E., Stadler, J. C. & Kennedy, G. (1994). *Fundam. Appl. Toxicol.* **23**, 268-279.

Martin, J. B. & Beal, F. M. (1992). *Ann. N. Y. Acad. Sci.* **648**, 169.

Mason, R. P. & Fischer, V. (1986). *Fed. Proc.* **45**, 2493-2499.

Matthews, C. K. & Van Holde, K. E. (1991). *Biochemistry*. Reading, Mass.: Benjamin/Cummings, 456-459.

Maurizi, C. P. (1984). *South. Med. J.* **77**, 1516.

McIntyre, I. M., McCauley, R., Murphy, S., Goldman, H. & Oxenkrug, G. F. (1985). *Biochem. Pharmacol.* **34**, 3393-3394.

Mead, J. F., Alfin-Slater, R. B., Howton, D. R. & Popjak, G. (1986). *Lipid Chemistry, Biochemistry, and Nutrition*. New York: Plenu, 422-431.

Mendlewicz, J., Linkowski, P., Branchey, L., Weinberg, U., Weitzman, E. D., Branchey, M. (1979) *Lancet*. **2**, 1362.

Meyers, F. H., Jawetz, E. & Goldfin, A. (1978). *Review of medical pharmacology*. Los Altos, CA.: Lange, 289.

Misik, V. & Riesz, P. (1996). *Free. Rad. Biol. Med.* **20**, 129-138.

Mohanakumar, K. P., De Bartolomeis, A., Wu, R-M., Yeh, K. J., Sternberger, L. M., Peng, S. Y., Murphy, D. L. & Chiueh, C. C. (1994). *Ann. N. Y. Acad. Sci.* **738**, 392-399.

Moore, D. E. (1987). *J. Pharm. and Biomed. Anal.* **5**, 441-453.

Maurizi, C. P. (1984). *South. Med. J.* **77**, 1516.

Murphy, D. L., Garrick, N. A., Tamarkin, L., Taylor, P. L. & Markey, S. P. (1986). *J. Neural. Transm.* **21**, 291-309.

Nakamoto, K., Kamisaki, Y., Wada, K., Kawasaki, H. & Itoh, T. (1997). *Pharmacology*. **54**, 203-210.

Nicoll, R. A. (1995). In Bertram G. Katzung (Ed.), *Basic and Clinical Pharmacology* (6^{th} edn.). Norwalk, CT.: Appleton & Lange, 323-332.

Nir, I. & Hirschmann, N. (1983). *Biochem. Pharmacol.* **32**, 2139-2141.

Olivieri, G., Welman, A. & Daya, S. (1990). *Med. Sci. Res.* **18**, 99-100.

Payan, D. G. & Katzung, B. G. (1995). In Bertram G. Katzung (Ed.), *Basic and Clinical Pharmacology* (6th edn.). Norwalk, CT.: Appleton & Lange, 551-552.

Petterborg, L. J., Kjellman, B. F., Thalen, B. E., Wetterberg, L. (1991). *J. Pineal. Res.* **10**, 9-13.

Pevet, P. (1984). *Euro-Diagnostics Newsletter*. May 1984, No. 1.

Photochemical Reactors, London: Applied Photophysics, 1-3.

Pini, L. A., Sandrini, M. & Vitale, G. (1996). *Eur. J. Pharmacol.* **308**, 31-40.

Raikhlin, N. T. & Kvetnoy, I. M. (1976). *Acta. Histochem.* **55**, 19-24.

Reiter, R. J. (1981). *Am. J. Anat.* **162**, 287.

Reiter, R. J. (1989). In R. J. Reiter and S. F. Pang (Eds.), *Advances in Pineal Research*, Vol. 3. London: John Libbey, 165-173.

Reiter, R. J. (1991). *Mol. And Cell. Endocrinol.* **79**, 153-158.

Reiter, R. J., Tan, D. X.., Poeggeler, B., Menendez-Pelaez, A., Chen, L. D. & Saarela, S. (1994). *Ann. N. Y. Acad Sci.* **719,** 1-50.

Reiter, R. J., Melchiorri, D., Poeggeler, B., Barlow-Walden, L., Chuang, J., Oritz, G. G. & Acuna-Castrviejo, D. (1995). *J. Pineal. Res.* **18**, 1-11.

Reiter, R. J., Pablos, M. I., Agapito, T. T. & Guerrero, J. M. (1996). *Ann. N. Y. Acad. Sci.* **786**, 362-376.

Reiter, R. J., Oh, C. & Fujimori, O. (1996). *TEM*. **7**, 22-27.

Reiter, R. J. (1997). *Adv. Pharmacol.* **38**, 103-116.

Roitt, I. (1991). *Essential Immunology* (7th edn.). Oxford: Blackwell, 94-96.

Rowett, H. G. Q. (1968). *Dissection Guides: The Rat, with notes on the mouse*. London: John Murray, 42-44.

Rudnick, G., Fishkes, H., Nelson, P. J. & Schuldiner, S. (1980). *J. Biol. Chem.* **255**, 3638-3641.

Sagar, P. S., Das, U. N., Koratkar, R., Ramesh., G., Padma, M., & Kumar S. G. (1992). *Cancer. Lett.* **63**, 189-198.

Sanders-Bush, E. & Mayer, S. E. (1996). In P. B. Molinof and R .W. Ruddon (Eds.), *The Pharmacological Basis of Therapeutics* (9th edn.). New York: McGraw-Hill, 249-262.

Scalteur, V., de Hofmann, E., Buchet, J. P. & Lauwerys, R. (1984). *Toxicology*. **29**, 221-234.

Skene, Debra Jean. (1980). The evaluation of melatonin as a possible antidepressive. *MSc Thesis*, Rhodes University.

Snyder, S. H. & Axelrod, J. (1964). *Biochem. Pharmacol.* **13**, 805-806.

Srikiatkhachorn, A., Tarasub, N. & Govitrapong, P. (1999). *Neurochem. Int.* **34**, 491-498.

Strada, S., Klein, D. C., Weller, J. & Weiss, B. (1972). *Endocrinology.* **90**, 1470-1475.

Tanaka, T. & Knox, W. E. (1959). *J. Biol. Chem.* **234**, 1162.

Tsujimoto, Y., Saitoh, K., Kashima, M., Shiozawa, A., Kozuka, M., Hashizume, H., Kimura, K., Yamazaki., M., & Fujii, A. (1998). *Gen. Pharmacol.* **31**, 405-408.

Udenfriend, S., Titus, E. & Weissbach, H. J. (1955). *Biol. Chem.* **215**, 499-505.

Van Praag, H. M. (1982). *Lancet*. **ii**, 1259-1264.

Van Wyk, E. J., Daya, S. & Briceland, F. (1991). *Med. Sci. Res.* **19**, 159.

Van Wyk, Elizabeth J. (1993). Pineal-adrenal gland interactions: In search of an anti-stressogenic role for melatonin. *MSc Thesis*, Rhodes University.

Walsh, H., Daya, S. & Whitely, C. G. (1994). *J. Pineal. Res.* **16**, 188-192.

Walsh, H. (1996). Regulation of tryptophan-2,3-dioxygenase and pineal indoleamines by selected tryptophan derivatives and antidepressants. *PhD Thesis*, Rhodes University.

Walsh, H. & Daya, S. (1998). *Life. Sci.* **62**, 2417-2423.

Wetterburg, L. (1983). *Psychoneuroendocrinology*. **8**, 75-80.

Wirz-Justice, A. & Arendt, J. (1980). *Lancet*. **1**, 425.

Wrbitzky, R. (1999). *Int. Arch. Occup. Environ. Health.* **72**, 19-25.

Wurtman, R. J. & Larin, F. (1968). *Nature*. **217**, 953-954.

Wurtman, R. J. (1974). *Life Sci.* **15**, 827-847.

Young, I. M. & Silman, R. E. (1982). In R. J. Reiter (Ed.), *The Pineal Gland: Extra-Reproductive Effects*, Vol. 3. Florida: CRC, 189-218.

Young, S. N. (1981). *Br. J. Pharmacol.* **74**, 695.