THE EFFECT OF 6-METHOXY-2-BENZOXAZOLINONE (6-MBOA) ON INDOLEAMINE REGULATION AND ITS POSSIBLE ROLE IN DEPRESSION

THESIS

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ABBREVIATIONS

aMT	Melatonin
BBB	Blood - brain - barrier
5-HIAA	5-Hydroxyindole acetic acid
HIOMT	Hydroxyindole-O-methyltransferase
5-HT	Serotonin / 5-hydroxytryptamine
5-HTOH	5-Hydroxytryptophol
6-MBOA	6-Methoxy-2-benzoxazolinone
5-MIAA	5-Methoxyindoleacetic acid
5-MTOH	5-Methoxytryptophol
(L)-5-HTP	(L)-5-Hydroxytryptophan
L-Tryptophan	L-Tryptophan
MAO	Monoamine oxidase
NAS	N-Acetylserotonin
NAT	N-Acetyltransferase
NEFA	Non-esterified fatty acids
NT's	Neurotransmitters
TDO	Tryptophan 2,3-dioxygenase
TH	Tryptophan hydroxylase
TLC	Thin-Layer Chromatography

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ABSTRACT

Tryptophan is an essential amino acid that is obtained from the diet. Approximately 98 % of ingested tryptophan is metabolized by the enzyme tryptophan 2,3-dioxygenase (TDO). The metabolism of tryptophan by TDO is an important determinant of tryptophan bioavailability to the brain for serotonin (5-HT) biosynthesis, an essential amine in affective disorders such as depression. Studies done on circadian rhythmicity of the enzyme activity have shown that, TDO activity is high during the scoto-phase (dark-phase), which is attributable to the *de novo* enzyme synthesis that occurs during this phase. 6-Methoxy-2-benzoxazolinone (6-MBOA), a structural analogue of melatonin (aMT) was shown to inhibit TDO activity in both the photo-phase (light-phase) and the scoto-phase with greater potency during the light-phase.

Further studies were directed at demonstrating the effects of 6-MBOA on the brain tryptophan hydroxylase (TH) activity, which is a rate limiting enzyme in 5-HT biosynthesis and subsequently on 5-HT levels. The findings showed that, 6-MBOA induces TH activity with a concomitant rise in brain 5-HT levels.

The blockade of 5-HT re-uptake into the presynaptic neuron leads to an increase in 5-HT available for the stimulatory action of 5-HT receptors. An attempt to establish whether the administration of 6-MBOA would block the binding of 5-HT to receptors on the synaptosomal membrane showed that

6-MBOA only inhibits the binding of 5-HT at specific concentrations. In view of the positive effects imposed by 6-MBOA on brain 5-HT levels, urinary 5-hydroxyindole acetic acid (5-HIAA) excretion was measured before and after treatment with 6-MBOA. 5-HIAA excretion was found to be significantly increased after 6-MBOA treatment.

Extensive research on the biosynthesis of pineal metabolites has been conducted in the past two decades. The pineal metabolites are synthesized from the precursor tryptophan. In order to obtain an overall picture of the effect of 6-MBOA on pineal indole metabolism, an organ culture technique was employed. The results obtained showed that although 6-MBOA administration to rats caused a significant increase in aMT production, there was an insignificant increase in NAS production. This is an immediate precursor of aMT. Other pineal indoles were not affected at all by 6-MBOA administration. Furthermore, the production of pineal NAS and aMT showed an inter-individual variation with some animals producing very high, some very low and some produced average levels of these two metabolites in both photo and scoto-phase experiments.

A study undertaken to investigate the circadian rhythm in endogenous aMT production using the competitive ELISA technique showed a clear pattern with high levels of aMT produced during the dark-phase and low levels of aMT produced during the light-phase. Furthermore, the administration of 6-MBOA to rats lead to a significant rise in endogenous aMT production.

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Depressive and anxiety disorders are common and disabling. These disorders are associated with significant morbidity and mortality. However, numerous efficacious and safe drugs do exist for the treatment of these disorders. The antidepressants are structurally heterogenous with vastly different pharmacokinetics and metabolic disposition (Els, 1998). Despite increased understanding of depressive disorder, there remains a substantial clinical need for new and improved therapeutic alternatives to manage this condition and related disorders.

Depression is a disorder that is associated with feelings of sadness, apathy, inadequacy and discouragement. It affects middle and old aged people and its major cause is stress (Harper, 1998). It is one of the costly and major health concerns. It is viewed as a more physical pain as compared to hypertension, diabetes and gastrointestinal problems. Depression is rated by the World Health Organization (WHO) as a number one disorder that in developed countries leads to disability. WHO

reports that in the year 1990 in developing countries depression was rated the number four cause of disability and speculate that by the year 2020 it will be the leading cause of disability (Harper, 1998). A South African organization that deals with the disease profiles linked to medication usage ranks depression as one of the top five ailments, which include hypertension, cholesterol, diabetes and anxiety disorder (Harper, 1998).

Severe depression is speculated to be genetically linked and is caused by a combination of disorders that develop in biologically vulnerable people (Weissman *et. al.*, 1984). There is evidence that the metabolism of biogenic amines is disturbed in depressive illness (Badawy and Evans, 1981). However, there are regulatory factors for enzymes involved in metabolism of these amines and other neurotransmitters (NT's). Serotonin (5-HT) has been shown to play an essential role in the mood changes characteristic of this illness (Badawy and Evans, 1981).

Brain 5-HT has been shown by clinicians and pharmacologists to be decreased in depressed patients and that it increases after treatment with antidepressants (http://www.virtuvites.com). The enzymes, tryptophan 2,3-dioxygenase (TDO) (Rubin, 1967; Badawy and Evans, 1981) and monoamine oxidase (MAO) (Haber *et. al.*, 1981) which affect the levels of 5-HT are also known to play an essential role in depression.

The pineal hormone, melatonin (aMT) has been reported to be low in depressed subjects (Beck-Friis

et. al., 1984). aMT has been shown to have inhibitory effects on TDO (Walsh and Daya, 1994). 6-Methoxy-2-benzoxazolinone (6-MBOA), a tryptophan derivative and a aMT analogue synthesized by seedlings of winter wheat has been shown to increase pineal aMT levels (Yuwiler and Winters, 1985). These findings imply that 6-MBOA could potentially reduce depression by directly influencing the biosynthetic pathway of biogenic amines.

There are different types of antidepressants and each has its own characteristic mode of action. Antidepressants are also characterized by side effects that these impose on individuals. Extensive research is thus carried out to elucidate the improved efficacy of antidepressants and reduction of side effects, respectively.

1.2 FACTORS AFFECTING DEPRESSION

Diagnosis of depression at an early stage is very important. Depressed people can generally be treated on an outpatient basis and maintain performance levels and reduce high cost for hospitalization and treatment of other physical and psychological problems resulting from untreated depression (Haper, 1998).

Sexual dysfunction may be the result of the period of depression. Sexual dysfunction can worsen symptoms of depression if left unrecognized or untreated. However, lack of treatment of depression

is not only reflected by sexual dysfunction, but there are more untoward effects such as lowered self-esteem, poor interpersonal relationships and permanent depression episode which are reported to occur (Segraves, 1992). From the above speculations, depression appears to be a curable disorder.

Monoamines play a vital role in affective disorders and in the pathogenesis of these disorders. Noradrenergic and serotonergic systems in particular portray differences in mechanisms and pathways between depressed and normal subjects. It is important to note that both aMT and 5-HT are part of the noradrenergic and serotonergic systems and are both tryptophan derivatives. It is therefore essential to discuss the metabolism of tryptophan from ingestion to the production of these metabolites.

1.2.1 TRYPTOPHAN: AN ESSENTIAL AMINO ACID

In any normal diet, either an animal protein-based or a vegetarian-based tryptophan diet, tryptophan is the least plentiful of all 20 food amino acids. Only about 10 % of dietary tryptophan enters the brain and the rest is used to make (a) various body proteins (b) vitamin B_3 (c) 5-hydroxytryptamine (5-HT) and most is broken down through a kynurenine pathway (South, 1997).

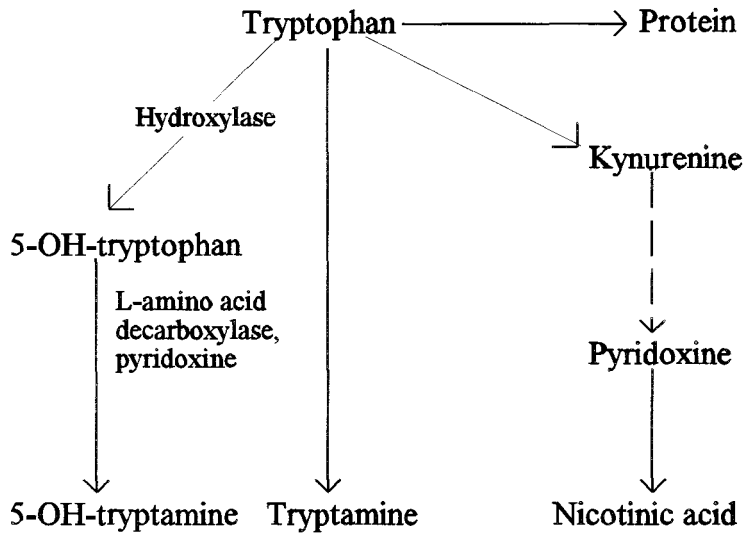

Fig. 1.1 ***The metabolic pathway of tryptophan (modified from Prange et. al., 1973)***

1.2.1.1 Biochemical metabolism of tryptophan

A quantitative important metabolic route of tryptophan starts with the oxidation / decarboxylation via liver tryptophan 2,3-dioxygenase (TDO) to formylkynurenine (Madge, 1996; Uchida *et. al.*, 1992). Tryptophan is also directly decarboxylated to tryptamine (Martin *et. al.*, 1972). A small portion of tryptophan is taken into the brain to make 5-HT (Fernstrom and Wurtman, 1973; South, 1997). In humans, tryptophan administration increases tryptamine synthesis as high as 25 % of the rate of 5-HT synthesis and the direct precursor of 5-HT, 5-hydroxytryptophan (5-HTP) does not have any effect (Awazi and Guldberg, 1978). Chouinard *et. al.* (1979) suggested that low doses of tryptophan would

increase both synthesis and release of 5-HT, while high doses would increase 5-HT synthesis, but not 5-HT release. Tryptamine on the other hand, has been shown to play a role in inhibiting firing of 5-HT neurons, thus preventing the release of 5-HT and reinforcing the detrimental effect of tryptophan at high doses.

The concentration of tryptophan in the 5-HT neuron is the rate-limiting factor for brain 5-HT synthesis (Fernstrom and Wurtman, 1971b). Fernstrom and Wurtman (1973) have shown that increments in doses of tryptophan cause an increase in 5-hydroxyindoleacetic acid (5-HIAA), an immediate metabolite of 5-HT and in 5-HT- containing raphe neurons. This suggests that 5-HT is immediately metabolized after release from neuron vesicles or is intraneurally metabolized. Sheard *et. al.* (1972) reported that, high doses of tryptophan and tricyclic antidepressants such as imipramine, amitriptyline and clomipramine, inhibit the firing of 5-HT neurons and eventually the release of 5-HT onto the postsynaptic receptors.

1.2.1.2 Tryptophan distribution

Tagliamonte *et. al.* (1971a) reported that serum and brain tryptophan levels are independent of each other. Brain tryptophan levels are also influenced by physiological and diurnal rhythms and this could partly influence 5-HT synthesis, but a drastic increase in brain tryptophan does not significantly change 5-HT levels. Wurtman *et. al.* (1967) found tryptophan values in human plasma to be lowest

at 2-4 am and increase by 50-80 % to peak values late in the morning or early afternoon. The amplitude of tryptophan and other amino acids rhythm tend to relate inversely to its availability in the body in rats and mice. Fernstrom and coworkers in 1966 observed that the peak tryptophan levels above those daily peaks reported above, occurred about 8-10 hours later than the peak levels in humans, a phenomenon probably attributable to the rat's tendency to consume most of its food during the hours surrounding the onset of daily period of darkness.

1.2.1.3 Tryptophan uptake system

The uptake of tryptophan by the brain is essential because the synthesis of useful indoleamines such as 5-HT and aMT is dependent on this amino acid. Tryptophan is unique among other amino acids as it is carried in plasma loosely bound to albumin. The binding site of the albumin is shared by non-esterified fatty acids (NEFA) which can displace tryptophan and this influences the amount of tryptophan in free solution (Haber *et. al.*, 1981). Haber *et. al.*, 1981 suggested that the non-esterified fatty acids circulating in serum are almost entirely bound to albumin and these tend to interfere with the binding of tryptophan to the protein molecule *in vivo*. This interaction permits an explanation of the paradoxical responses of serum tryptophan to insulin in rats and humans. After carbohydrate consumption and insulin secretion, NEFA levels rapidly decrease thus decreasing the levels of NEFA bound to circulating albumin. This allows more tryptophan to become bound to albumin in rats and in humans prevents the dissociation of tryptophan from albumin. In rats therefore, serum free

tryptophan levels drop, albumin-bound tryptophan rises and total tryptophan levels rise. In humans, serum free tryptophan levels also drop, albumin-bound remains unaltered and serum total tryptophan decreases slightly.

Fernstrom and Wurtman (1971b) also showed that tryptophan brain levels are not only dependent on the concentration of free serum tryptophan, but also on the concentration of other amino acids sharing the same transport system with tryptophan. To support this, Haber *et. al.* (1981) demonstrated an existence of the large neutral amino acids that share a transport system at the blood-brain barrier (BBB) which compete with each other for the carrier system.

The uptake of tryptophan by brain is stereoselective with brain to plasma ratios of approximately 0.25. The stereoselective property of the uptake of tryptophan by the brain was shown with experiments using D-tryptophan which gave little or no rise in circulating L-isomer occurring in response to an injection of the D-isomer. This therefore suggests that uptake of D-tryptophan by the brain is independent of L-tryptophan (L-tryp) (Guroff and Udenfriend, 1961).

1.2.1.4 Physiological role of tryptophan on sleep

L-tryptophan has been shown by many researchers to induce sedation and changes in sleep patterns in both humans and experimental animals. In humans, it has been found that the actions of tryptophan

on sleep are not mediated via its metabolite, 5-HT. Hartman (1967) cited that neither a tryptophan-free nor a tryptophan-rich diet significantly affects total sleep in rats. This was shown by insignificant changes in 5-HT as compared to changes seen when 5-HT inhibitors and precursors were used. It can therefore be suggested that tryptophan may influence sleep by acting via some tryptophan metabolites other than 5-HT. It is therefore important to address the control of tryptophan metabolism.

1.2.2 HEPATIC TRYPTOPHAN 2,3-DIOXYGENASE (TDO)

Tryptophan 2,3-dioxygenase (TDO) is a liver enzyme that catalyses the conversion of tryptophan into kynurenines. The formation of kynurenines is believed to be the major pathway undergone by tryptophan.

The first discovery of existence of TDO activity in the brain tissue was discovered by Gal *et. al.* (1966) when these authors studied the nature of hydroxylation of L-tryptophan (L-tryp) *in vitro* and obtained unequivocal evidence for the enzymatic formation of L and D-kynurenine from the precursors in rat and pigeon brain homogenates. Tsuda *et. al.* (1972) furthered this by demonstrating oxidative cleavage of L-5-hydroxytryptophan (L-5-HTP) to L-5-hydroxykynurenine in rat brain homogenates. Later in 1973, Hirata discovered a similar cleavage of 5-hydroxytryptamine and aMT by an enzyme partiallly purified from brain and pineal gland.

α-Methyl-DL-tryptophan which was not known as a substrate was shown to increase TDO activity *in vivo* (Civen and Knox, 1960). Schutz *et.al.* (1972) demonstrated that at certain concentration, the same substrate, α-Methyl-DL-tryptophan stimulated the activity of the enzyme *in vitro* (Gál, 1974).

1.2.2.1 Physiological and biochemical properties of TDO

Hepatic tryptophan 2,3-dioxygenase (EC 1.13.11.11 ,TDO), is a cytosolic tetrameric protein (167 kDa) containing 2 mol of protohaem IX. It catalyses the oxygenative conversion of L-tryptophan (L-tryp) to L-formylkynurenine at the expense of 1 mole of molecular oxygen (Lapin, 1980).

L-tryptophan + Oxygen $\Leftrightarrow$ L-Formylkynurenine

Stimulation of the enzyme by haem enhances the catabolism of L-tryp thus making less L-tryp to be taken up together with other amino acids such as leucine, isoleucine, valine, tyrosine and phenylalanine into the brain and other tissues. In the brain, L-tryp is converted to 5-HT and in some tissues it is utilised for protein and NAD(P)H synthesis and ultimately converted into nucleic acids. In a normal protein diet, L-tryp is the least plentiful of all amino acids, therefore it is outnumbered as much as 7 - 9 % in its competition to secure its transport through the blood-brain-barrier (BBB) into the brain (Cho-Chung and Pitot, 1967).

The enzyme exists in two forms. One form is the already active reduced holoenzyme, and the other is the inactive apoenzyme which requires the addition of haem for its activation. The ratio of holo:apoTDO indicates the degree of saturation of apoenzyme by haem (Badawy and Evans, 1975). The chronic administration of ethanol or phenobarbitone inhibits the activity of rat liver apoTDO and subsequent withdrawal of either drug enhances both holo and apoenzyme activities. The guinea-pig liver has been shown to contain only one form of TDO, the holoenzyme. The guinea-pig liver may therefore be used as a model for studying holoenzyme activity (Badawy and Evans, 1974).

TDO is subject to a diurnal rhythm with its peak activity during the pre-dark period and the lowest activity towards the end of the dark period in the rat. This circadian rhythm could be due to the endogeneous rhythm of aMT and 5-HT production which have peak levels towards the end of the dark period (Hardeland and Rensing, 1968).

1.2.2.2 Biochemical regulation of TDO

TDO activity is regulated by a number of factors. Stimulation and inhibition of the enzyme is thus dependent on these factors and on different compounds of which the substrate tryptophan is one of.

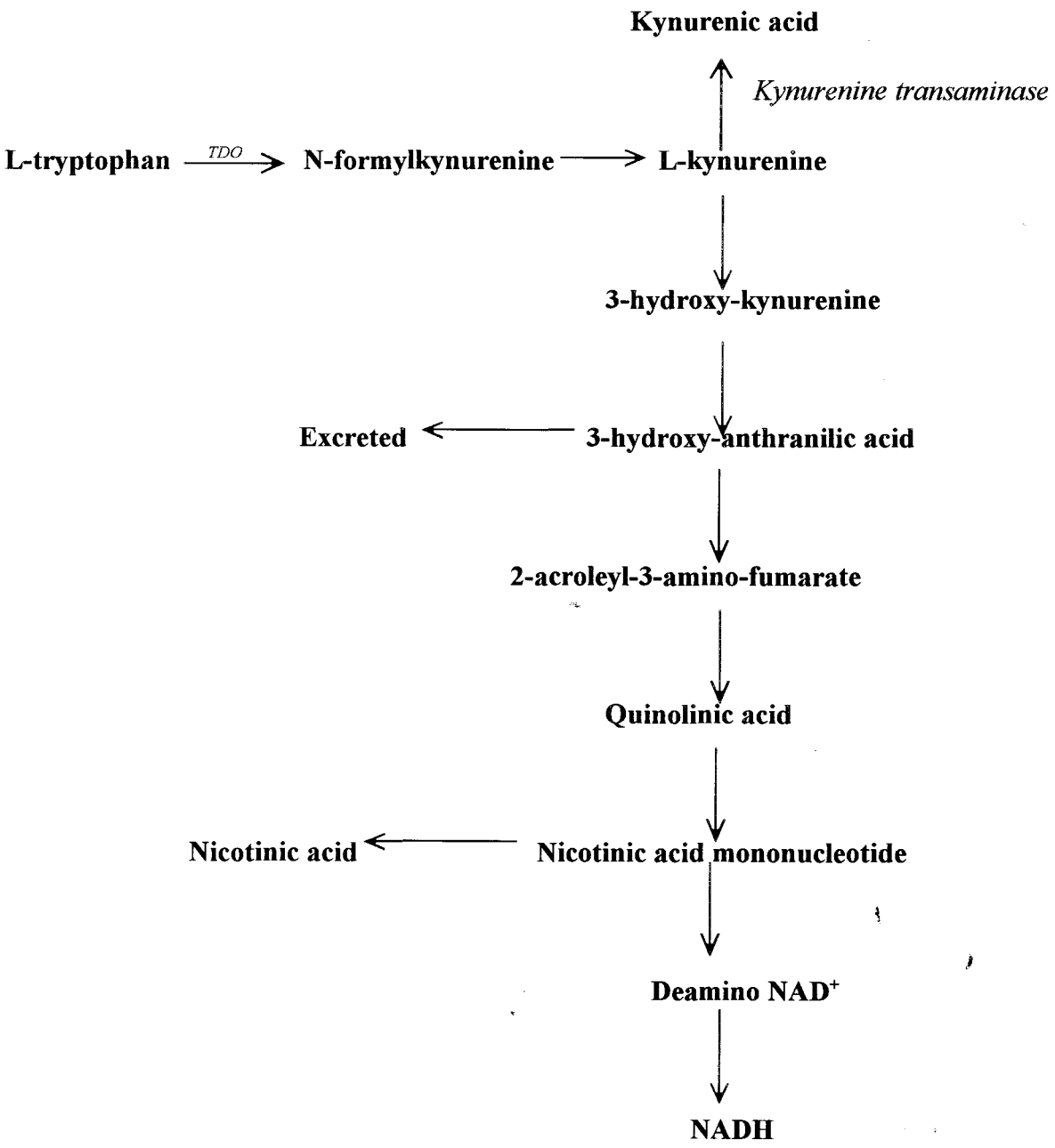

Fig 1.2 ***L-Tryptophan-Kynurenine pathway [Cho-Chung and Pitot (1967)]***

1.2.2.2.1 Biochemical regulation of TDO by tryptophan

An increase in availability of circulating amino acid to the brain may be enhanced by one of the three peripheral mechanisms: (a) Decrease in TDO activity leading to a deficient hepatic tryptophan catabolism. (b) Increased release of protein-bound plasma tryptophan. (c) A decrease in circulating plasma neutral amino acids which compete with tryptophan for cerebral uptake system. There is an inverse relationship between hepatic TDO activity and brain 5-HT concentration, whereby the latter is affected by the availability of tryptophan which is catabolised by the enzyme TDO. An inverse relationship between TDO and 5-HT synthesis was proven by experiments in which TDO activity was enhanced by acute administration of cortisol or ethanol or by chronic administration of dieldrin, ethanol or nicotinamide which resulted in inhibition of the enzyme activity (Badawy *et. al.*, 1981).

Knox (1966) hypothesised that the enhancement of activity after tryptophan addition *in vitro* involve the promotion of the conjugation of the apoenzyme with haem and the subsequent reduction of the oxidised holoenzyme. Tryptophan loading appears to be more effective than haem addition since the addition of L-tryp leads to a more stable enzyme and enhances protection of TDO against degrading enzymes. In addition to this, L-tryp loading does not interfere with the rate of synthesis of the enzyme. During haem loading, none of this is observed. It was also suggested that there might be two different mechanisms by which TDO activity is enhanced, but that has not been investigated in depth (Badawy and Evans, 1974; Badawy and Evans, 1975).

The enhancement of TDO activity by exogenous L-tryp does not depend on RNA synthesis. This was found when actinomycin D did not prevent L-tryp effect on TDO activity. On the other hand, ongoing protein synthesis does moderately prevent the L-tryp-induced increase in the enzyme activity. This was obtained by addition of cycloheximide (protein synthesis inhibitor) (Badawy and Evans, 1973a&b).

The guinea-pig holoenzyme activity has been shown to be enhanced by treatment with L-tryp (Badawy and Evans, 1974). Allopurinol, a specific apoenzyme inhibitor in the rat completely prevents L-tryp enhancement of TDO activity and increases brain L-tryp concentration (Badawy and Evans, 1973b). Guinea-pig TDO holoenzyme activity is however blocked by this inhibitor after L-tryp administration. This therefore suggests that L-tryp may activate the enzyme by promoting the rapid conjugation of an apoTDO protein with its haem activator. Furthermore, large doses of L-tryp do not cause an early activation of the rat enzyme whereas smaller doses do so in the guinea-pig liver. It has been reported in literature that guinea-pig liver and serum concentrations of L-tryp are lower than those in the rat which could partly explain the higher sensitivity of the former animal to L-tryp treatment (Badawy and Evans, 1974).

1.2.2.2.2 Biochemical regulation of TDO activity by haem

Administration of haem or its precursor 5-aminolaevulinate enhances the activity of the enzyme (Badawy *et. al.*, 1987). Initially, the mode of action of these compounds is to increase the saturation

of the endogeneous apoenzyme with haem. Badawy and Evans (1975) have shown that haem is slowly taken up by the liver because of binding to plasma proteins. These researchers obtained this finding after injection of rats with haem and only observed the effect i.e. appearance of saturation of apoenzyme by haem, after two hours. The effect was measured by an increase in apoenzyme activity.

After administration, haem immediately binds to plasma proteins. This binding is then followed by an overall increase in the activity of the apoenzyme of which the majority is haem-saturated. TDO plays an important role in haem utilisation, therefore any drug which increases TDO activity will eventually increase haem utilisation. Activation of TDO by exogeneous haem is not prevented by actinomycin D or by puromycin, which are inhibitors of RNA synthesis. These agents block pyrollase activity only when 5-aminolaevulinate is administered. This suggests that the steps in haem synthesis beyond 5-aminolaevulinate synthetase requires the continuation of mRNA and protein synthesis for activation to occur. One such step that has been proven to require mRNA and protein synthesis is the one that involves 5-aminolaevulinate dehydratase (Badawy and Evans, 1981). Badawy and Evans (1975) and Knox (1966) have showed that L-tryp enhances activity of rat liver 5-aminolaevulinate synthetase which is the rate limiting enzyme in the biosynthetic pathway of porphyrins and haem.

1.2.2.2.3 Biochemical regulation of TDO activity by glucocorticoids

Glucocorticoids have been shown to regulate TDO activity. The administration of a stress hormone,

cortisol which is produced by adrenal glands does not show any effect on the ratio of holo:apoTDO. The ratio resembles that of the basal enzyme (less than 1), thus suggesting about half of the newly synthesized apoenzyme becomes conjugated with haem whereas on the other hand L-tryp activated enzyme exhibits a larger degree of haem saturation. Moreover, cortisol does not cause any initial saturation of the apoenzyme with haem but produces a great rise in the total TDO activity while maintaining holo:apoenzyme ratio close to that of the basal enzyme (Badawy and Evans, 1975).

1.2.2.3 Inhibition of TDO activity

Most of the literature has reported that TDO substrate at acidic and at basic pH with concomitant shifts in substrate maximum (S_{max}) and the substrate concentration beyond the maximum activity (V_{max}), substrate inhibition of TDO activity is observed. This shows that the L-tryp-kynurenine pathway can be regulated through substrate inhibition.

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

aMT alone is the most potent inhibitor of TDO. Badawy and Evans (1981) showed that MAO inhibitors are weak inhibitors of TDO activity.

Binding of substrates and inhibitors to the enzyme has been extensively studied by Uchida *et. al.* (1985) using the infrared carbon monoxide (IR-CO) stretch band(s) of compound-bound carbonmonoxy TDO. The infrared spectra for TDO-CO in the presence of 5-hydroxytryptophan (5-HTP: reported as a non-competitive inhibitor) was similar to that of the substrate bound TDO-CO, while the one in the presence of tryptamine (competitive inhibitor) was different. The infrared spectra analysis therefore indicate that there are two types of catalytic site-binding inhibitors. The first type to which 5-HTP was found to belong to, binds to the catalytic site with a correct configuration similar to that of substrates and the second type e.g. tryptamine, with an inaccurate configuration with the enzyme.

Nitrogen in the indole ring of the substrate, L-tryp, appears to be indispensable for a molecule to bind to the catalytic site because the catalytic site-binding analogs studies thus far conserve the NH group whereas a substitution of NH by N-CH_3 prevents the molecule from binding to the catalytic site. This suggests that the NH group plays an essential role in positioning a molecule at a certain point near the Fe-CO group via a specific and presumably strong hydrogen-bonding with an acidic amino acid side chain (Uchida *et. al.*, 1992).

It has been demonstrated that an enzyme reaction proceeding via a hydro-peroxide, the dissociation of the N-H that is expected to occur, would be facilitated by an interaction with a strong hydrogen attracting group. Conservation of an acid side chain, $CH(NH_2)COOH$ is considered a very important component for binding into the catalytic site and to make a correct configuration suitable for the reaction to occur, but variation of protein side chains might lead to different efficiencies of enzyme reactions. If an acidic side chain of a molecule is incomplete, the NH group of the indole ring and the hydrogens in the fifth and sixth position exist, the molecule will be pinned to the catalytic site but no reaction will occur (Nakagawa *et. al.*, 1976).

Sugiyama *et. al.* (1967) and Beer *et. al.* (1954) reported that, generally, derivatives with an electron donating substituent i.e. hydroxy (OH) or methoxy (CH_3O) at position 5, are more labile to oxygen than the ones without it. It has been suggested that the structural flexibility needed for the catalytic reaction is significantly reduced. From this report, it seems that there are two types of catalytic-site binding inhibitor. The first type is a substrate analogue that lacks an accurate configuration appropriate for the catalytic reaction to occur due to an absence of functional groups for proper interactions with the protein part at the haem pocket of the catalytic site. The second type is the substrate analogue containing the correct functional groups and take a correct configuration for the reaction and is potentially reactive as the first type but cannot act as a proper substrate. The functional groups of this analogue make additional interactions with the protein side chain(s) in the catalytic site and that leads to reduction in catalytic potential of the haem pocket (Uchida *et. al.*, 1992).

1.2.3 SEROTONIN

1.2.3.1 The biosynthetic pathway of serotonin

5-HT is synthesised from tryptophan. Tryptophan from the circulation is taken up to the brain and the enzyme tryptophan hydroxylase converts tryptophan to 5-hydroxytryptophan (5-HTP). TH is a rate limiting enzyme in 5-HT synthesis (Carlson *et. al.*, 1972). Under normal conditions, the saturation of brain TH portrays regional differences (Meek and Lofstrandh, 1976) and is usually not saturated with its substrate tryptophan, thereby very sensitive to minor alterations in tryptophan concentration (Bengtsson *et. al.*, 1991). Synthesis of 5-HT is not only subjected to availability of tryptophan but also depends on the redox state of the essential cofactor tetrahydrobiopterin (BH_4) (Gal and Patterson, 1973; Bengtsson *et. al.*, 1991).5-HTP is then converted to 5-hydroxytryptamine (5-HT: serotonin) by the enzyme amino acid decarboxylase which uses pyridoxine as a cofactor.

1.2.3.1.1 Discovery of serotonin precursors: Tryptophan and 5-hydroxytryptophan

The first experimental demonstration that tryptophan is the dietary precursor of 5-HT came from the studies done by Udenfriend and colleagues in 1956. These researchers demonstrated that the administration of radioactive tryptophan to *Bufo marinus* and the rabbit, led to the formation of radioactive 5-HT. An investigation for enzymatic hydroxylation of tryptophan to 5-hydroxytryptophan

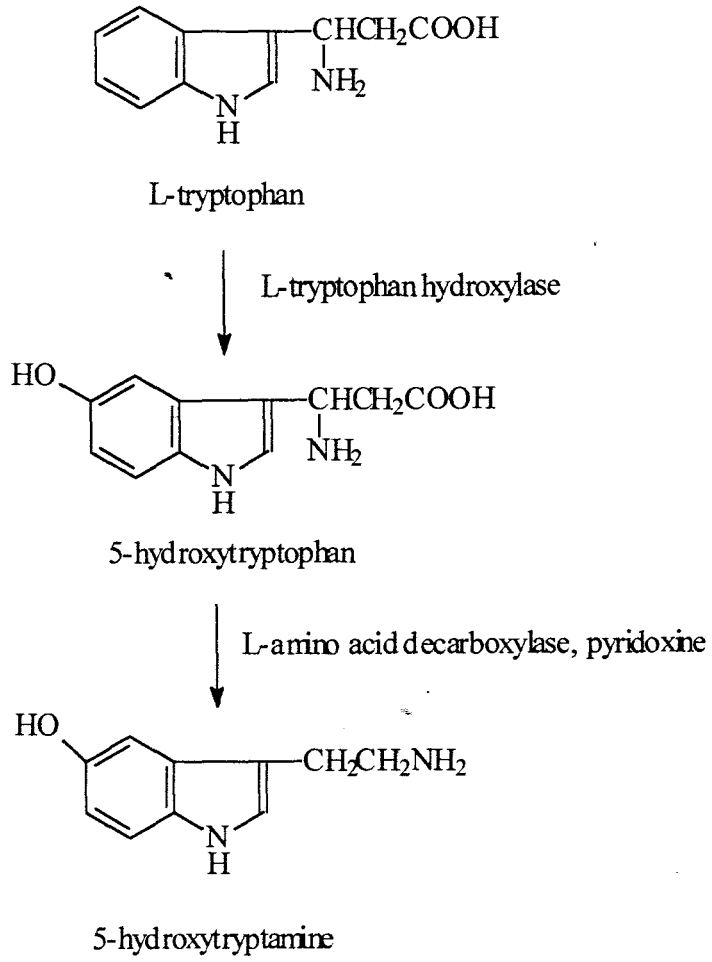

Fig. 1.3 The biosynthesis of serotonin (5-HT)

(5-HTP), the direct precursor of 5-HT was first directed towards a microorganism called *Chromobacterium violaceum* because chemical analysis of violacein, a purple pigment produced by this organism had revealed the existence of a 5-hydroxyindole moeity (Fernstrom and Wurtman, 1973).

Mitoma and coworkers in 1956 managed to isolate 5-HTP from the growth medium. Resting cells of this microorganism were then incubated with L-tryp and produced sufficient amounts of 5-HTP to be detected by the nitrosonaphthol colorimetric assay designed by Udenfriend and his colleagues in 1955. Hydroxylation of L-tryp in a cell-free system was reported for the first time by Cooper and Melcer (1961) in mammalian intestinal mucosa. This hydroxylation was found to be an artifact by Renson and colleagues in 1962 since this was a non-enzymatic hydroxylation i.e. of no physiological importance. The first unequivocal demonstration of enzymatic formation of 5-HT using a cell-free system was achieved by using rat liver homogenate in the presence of large concentrations of L-tryp (1.6×10^{-2} M) by Freedland and coworkers in 1961 (Fernstrom and Wurtman, 1973).

1.2.3.2 Serotonin function

5-HT, dopamine (DA) and nor-adrenaline (NA) are the three main monoamine neurotransmitters (NT's). Tryptophan is the precursor of 5-HT whereas both DA and NA are derived from tyrosine. 5-HT is used by body parts such as the brain, blood platelets and some intestinal lining cells is produced in the neurons. Serotonin cannot pass the BBB, therefore, 5-HT synthesized in the brain cannot be taken outside the brain. The precursor of 5-HT, tryptophan, can pass the BBB through a carrier protein whereas 5-HTP crosses BBB independently (South, 1997).

5-HT is one of the major brain NT. Studies with humans and experimental animals have shown that

5-HT nerve circuits promote feelings of well being such as calmness, confidence and concentration. 5-HT neural circuits also help counterbalance the tendency of brain dopamine (DA) and nor-adrenaline (NA) to encourage overousal, fear, anger, tension, anxiety, aggression and sleep disturbances (South, 1997).

1.2.3.3 Physiological factors affecting brain serotonin levels

Brain tryptophan content is the most essential factor in determining the rate of 5-HT synthesis because the rate limiting enzyme in 5-HT synthesis, tryptophan hydroxylase (TH) is not always saturated with tryptophan. It has been shown that both small and large changes in concentration of brain tryptophan alter the rate of synthesis of 5-HT. In rats, after tryptophan administration, a slight increase in 5-HT concentration is observed but the brain 5-HT turnover increases markedly and the evidence for that is the rise in brain 5-HIAA production (Moir and Eccleston, 1968).

1.2.4 MITOCHONDRIAL MONOAMINE OXIDASE (MAO)

Monoamine oxidase (MAO) is a mitochondrial membrane that is involved in deaminating 5-HT to 5-hydroxyindoleacetic acid (5-HIAA).

1.2.4.1 Physiological and biochemical properties of MAO

MAO (EC 1.4.3.4), an oxygen oxidoreductase deaminates a monoamine into an aldehyde (Barman, 1969). The enzyme catalyses the following reaction:

$$RCH_2NR' + O_2 + H_2O \longrightarrow RCHO + NHR' + H_2O_2$$

MAO is an integral protein embedded in a phospholipid structure required for activity in the outer-mitochondrial membrane (Sottocasa *et. al.*,1967; Edelstein and Breakfield, 1986). The enzyme appears to be a dimeric containing polypeptide chains of similar molecular weight (± 60 000), only one of which contains a covalently bound FAD cofactor (Oreland *et. al.*,1973; Salach, 1979; Edelstein and Breakfield, 1986). Bonnefil *et. al.* (1981) showed that sialic acid residue may be required for the activity of MAO, indicating that glycosylation of the molecule may occur (Houslay and Marchmont, 1980). Edelstein and Breakfield (1986) supported those studies by showing that MAO subunits appear to be glycoproteins. Youdim (1976) and Salach (1979) hypothesized that MAO may contain and depend on iron for activity, but Weyler and Salach (1981) showed that MAO only requires iron for its activity but does not contain it. These authors found that highly purified active MAO molecules do not contain iron but iron deficiency *in vivo* results in a loss of activity (Bonnefil *et.al.*, 1981).

Dixon (1958) showed that alkaline pH destroys the enzyme activity. This author discovered a linear

increase of MAO activity in pig liver between pH 5 - ±7.5 and that the activity is time dependent when tyramine is used as a substrate. The optimum temperature was found by Sumner and Myrbäck to be between 30 -39 ^{0}C. The Michaelis constants of MAO with substrates, benzylamine and octylamine from human placenta have been found to be 3.3 x 10^{-5} and 4.3 x 10^{-5} respectively at pH ranges 8.2 - 8.5 at physiological temperatures (Barman, 1969). From these two authors it can be hypothesized that the pH for optimum MAO activity depends on the species used. Wyatt *et. al.* (1973b) reported that mitochondrial MAO activity in human platelets has some similarities in substrate and inhibitor characteristics to MAO activity in brain and other tissues.

Zeller (1951) discovered that the good substrates are amines with an aromatic ring. In general, primary, secondary and tertiary amines, tryptamine derivatives and catecholamines are oxidised (Barman, 1969). Phenylethylamine, tyramine, hordenine, adrenaline and indolethylamine shown in fig 1.4 are also oxidised. In the case of tyramine, substitution in the fourth position leads to a higher degradation velocity than in the third and second positions and the opposite is true for the corresponding methoxyl derivatives (-OCH_3) (Zeller, 1951). The descending order of the best substrate for the enzyme is from primary >secondary > tertiary amines. The quaternary structure is not oxidised at all by MAO. However, the enzyme isolated from human placenta is found to attack primary amines only and an increase in chain length of simple alkyl amines results in increased affinity (Barman, 1969). The action of MAO is prevented by a methyl group on carbon one of the substrate and that is of considerable pharmacological importance (Dixon, 1958).

MAO activity increases with age in human brain, plasma and platelets (Robinson *et. al.*,1972; Nies *et. al.*1973a; Robinson, 1975; Grinna, 1977) proposed that this increase may be due to an increase in cholesterol levels of mitochondrial membranes during aging. In contrast to this, in rats, for the first weeks, following birth, the central catabolism of 5-HT is much faster than during the adult life and that this is due to an increase in MAO activity in tissues from young rats (Haber *et. al.*, 1981).

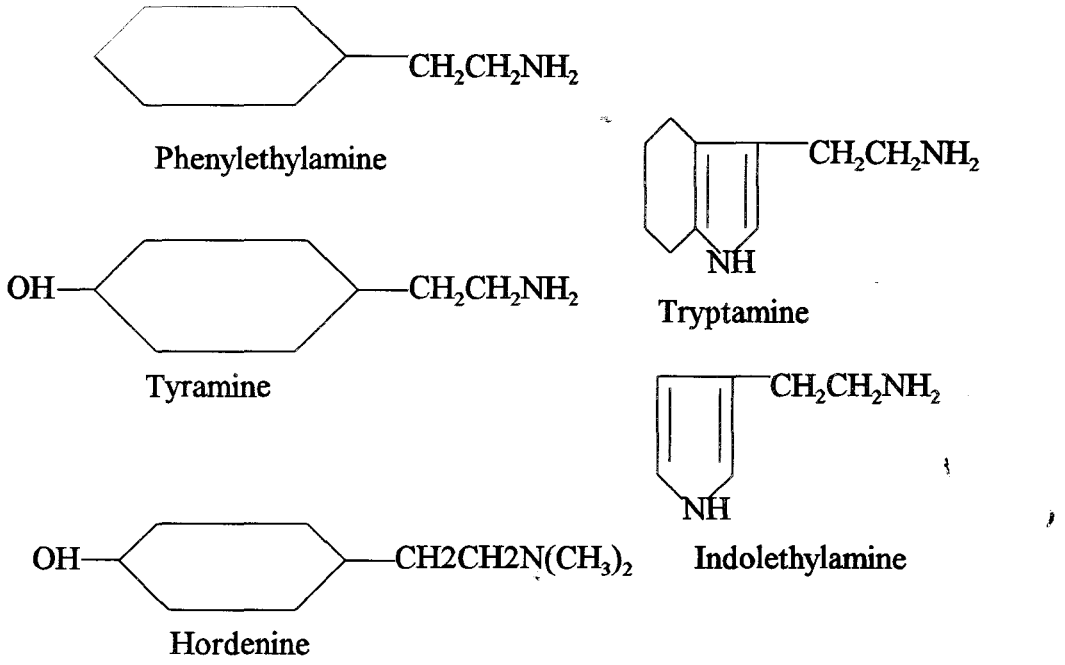

Fig.1.4 ***Amines with aromatic rings (good substrates for MAO)***

1.2.4.2 Mode of action of MAO

After the release of 5-HT from storage sites, it is immediately metabolised by MAO to an unstable metabolite, 5-hydroxyacetaldehyde. This metabolite is converted to 5-hydroxytryptophol and 5-hydroxyindoleacetic acid (5-HIAA) by the enzyme alcohol dehydrogenase (Cardinali, 1981).

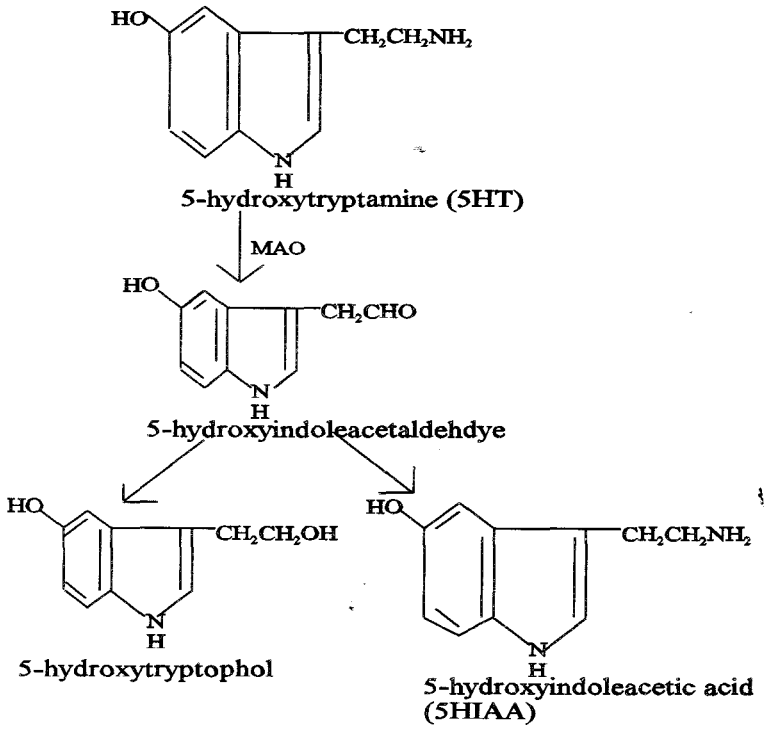

Fig. 1.5 The catabolic pathway of serotonin (5-HT)

1.2.4.3 MAO isoenzymes classification

Existence of two types of active MAO have been identified. One type is termed MAO-A and the other MAO-B (Reiter, 1989).

1.2.4.3.1 Biochemical and physiological differences between MAO isoenzymes

The two forms of active enzyme differ in optimum pH optima, sensitivity to various compounds and treatments, dependence on phospholipids, degree of glycosylation, immunoreactivity and distribution in the mitochondrial membrane. In addition, the two forms of MAO have been physically separated by ion-exchange and immunoaffinity chromatography and the flavin-containing subunits have been shown to differ in molecular weight and 1-dimensional peptide maps by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Furthermore, these two forms of MAO appear to be independently regulated. Within a single tissue, the two forms are found in different concentrations (Edelstein and Breakefield, 1986).

The two forms of MAO can be distinguished on the basis of their substrate specificity, inhibitor sensitivity, kinetic parameters and protein structures. Furthermore, phospholipids have been implicated as playing important roles in the activity, inhibitor specificity and heterogeneity of the enzyme (Youdim, 1972 and Kroon and Veldstra, 1972). These properties were used to study the molecular

mechanism(s) by which glucocorticoid hormones and aging alter MAO activity *in vivo*. Roth *et. al.* (1976) and Groshong and Baldessarini (1977) showed that fibroblasts, predominantly contain MAO-A and Donnely *et. al.* (1976) reported that also neurons are predominated by MAO-A. Cellular aging in the presence of these hormones resulted in inhibition of MAO activity, and this effect was only shown in MAO-A (Tabor, 1954).

Both forms have been shown to readily deaminate tryptamine and tyramine *in vitro* and inhibited by similar concentrations of pargyline. MAO-B metabolises dopamine but not serotonin or other catecholamines and deprenyl is a selective inhibitor of MAO-B both *in vitro* and *in vivo*. MAO-A on the other hand deaminates serotonin and noradrenaline, but not dopamine and is selectively inhibited by clorgyline (Edelstein and Breakefield,1986).

1.2.6 5-HYDROXYINDOLE ACETIC ACID (5-HIAA)

Tryptophan is a dietary precursor of 5-HT and 5-hydroxyindole acetic acid (5-HIAA) respectively. 5-HIAA was discovered in the plant, *Piptadenia peregrina* which produces large amounts of N,N-dimethyl-5-hydroxytryptamine and other 5-hydroxyindoleamines. Mammalian tissues which are rich in monoamine and aldehyde oxidases indicate that oxidation of 5-HT to 5-HIAA might be the major route of its metabolism (Udenfriend *et. al.*, 1956). 5-HIAA is a normal constituent of mammalian urine. An increase in 5-HIAA in urine (milligram) indicates an appreciable portion of tryptophan being

metabolised via the 5-hydroxyindole route (Udenfriend *et. al.*, 1956).

Administration of 5-hydroxytryptophan (5-HTP), 5-HT and 5-HIAA leads to excretion of these compounds as 5-HIAA in the urine. 5-HIAA may either be derived from these amines or from decarboxylation of 5-hydroxyindolepyruvic acid (5-HIPA) which may arise by transamination of 5-HTP (Udenfriend *et. al.*, 1956). Contractor (1966) reported that 5-HIPA is very unstable and is unlikely to be present in the urine. If large amounts of this acid excreted in the urine are from 5-HT, then the amounts excreted reflects the secretion of the amine (Udenfriend, (1956). Eccleston *et. al.* (1964) suggested that inhibition of 5-HTP decarboxylation results in accumulation of the substrate (5-HTP) and subsequent reduction in 5-HT and 5-HIAA respectively.

Considerable increase in tryptophan and 5-HIAA in different CNS compartments such as the whole brain, CSF, striatum and striatal extracellular space after injections of L-tryp (50 mg/kg) have been shown (Sarna *et. al.*, 1991). Commensurate accessions in both tissues and extracellular compartments on tryptophan levels and in 5-HIAA concentrations are subsequently reported and these increases are hypothesized to be predominantly of endothelial cell 5-HT metabolism origin (De Simonie *et. al.*, 1987).

1.3 THE PINEAL GLAND

1.3.1 The anatomy of the pineal gland

The mammalian pineal gland is a glandular structure derived as an evagination of the neural tube (Kappers, 1965). In the rat, the pineal appears as a neuroepithelial protrusion from the roof of the diencephalon, the area between the habenular and posterior commissures. Its connection with the commisural region is through the pineal stalk. The gland is composed of two parenchymal cell types, the pinealocytes and intestitial cells (Wartenberg and Gusek, 1965), both types are of neuroectodermal origin. The pinealocytes are the neurosecretory cells of the gland. The rat pineal is highly vascularised, its major blood supply being provided by branches of the posterior choroidal (Gladstone and Wakely, 1940) and posterior cerebral (Hodde, 1979) arteries. Venous drainage occurs via the distal end of the great cerebral vein into the superior sagittal sinus (Hodde, 1979).

The pineal gland is also known as the "corpus pineale" has been called a neuroendocrine transducer because of its important role in photoperiodism. Within the pineal, the neural (photic) information is transduced into a hormonal input (Reiter, 1980). The mammalian pineal gland responds to the photic information via the lateral eyes whereas in some reptilian and avian species photoreception is via penetration through the scalp. Mammals depend on neural connection between the eyes and the gland that assimilates the photic information. The neural connection between the eyes and the gland

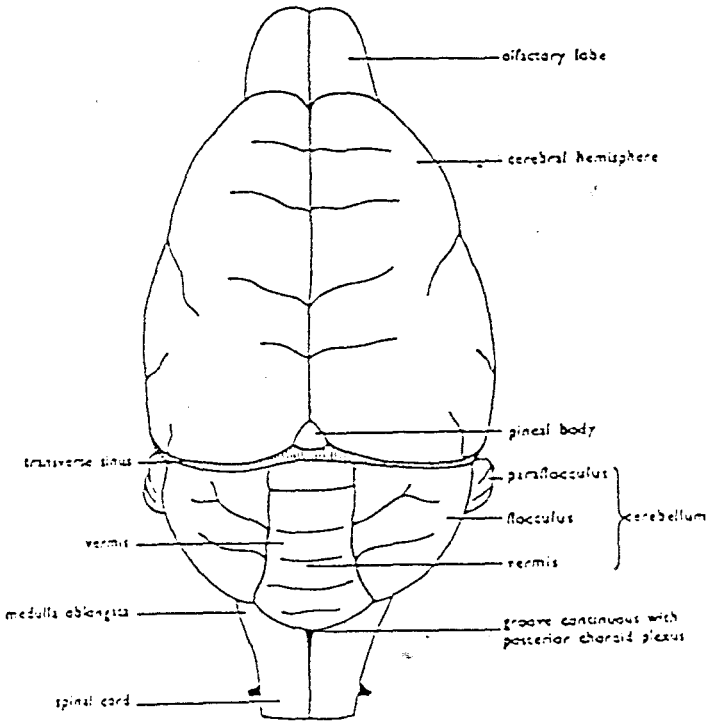

Figure 1.6 The dorsal view of the rat brain showing the location of the pineal gland (Rowett, 1960)

include peripheral sympathetic axons which form pineal distinct nervii conarii that penetrate the apex of the gland (Kappers, 1960 and 1965). In some few species such as the monkey, the parasympathetic innervation predominates (Kenny, 1961). The pineal gland is mainly dependent on autonomic neural information derived from the sympathetic nervous system for its hormonal input (Wurtman *et. al.*, 1965; Reiter and Hester, 1966b; Reiter, 1980; Moore, 1978).

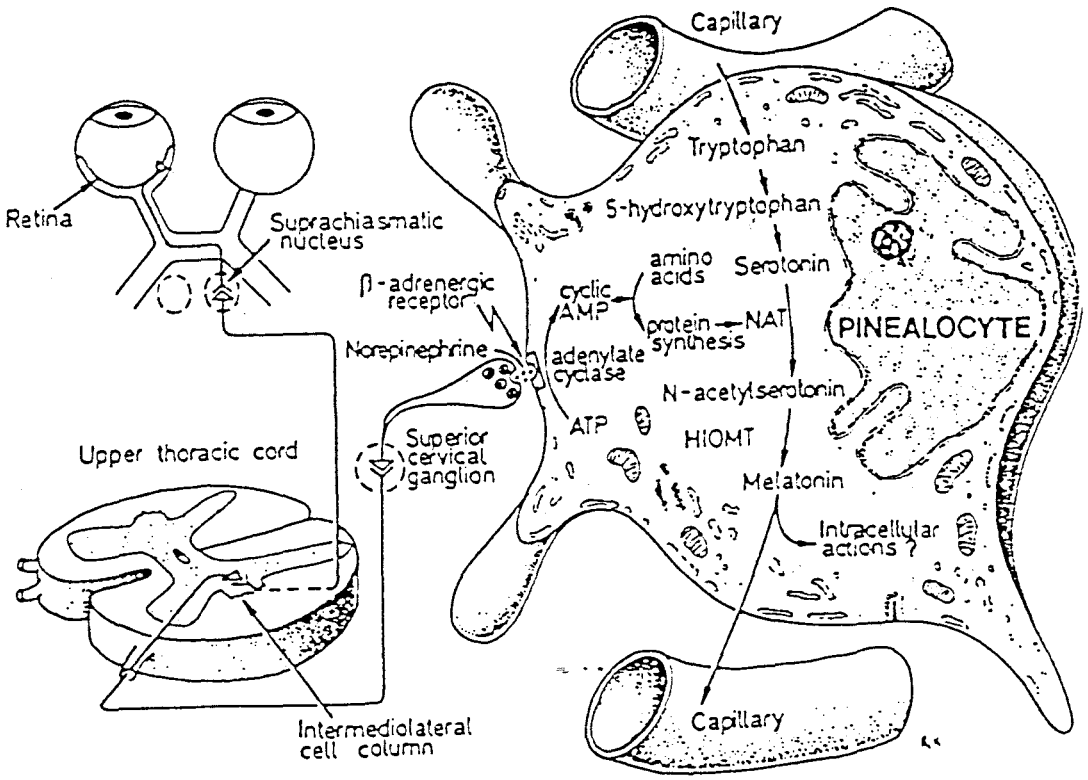

Figure 1.7 Schematic representation of the sensory input pathway and the effect of light and dark on the synthesis of the various indoleamines in the pineal gland (Reiter, 1989)

1.3.2 THE PINEAL INDOLES BIOSYNTHETIC PATHWAY

The pineal gland is an outgrowth of the ectodermally derived CNS (Reiter, 1980). The metabolism of indoleamines in the pineal gland has reviewed enormous interest after the past three decades. The major compound of the pineal gland, aMT has attracted a lot of attention.

aMT is synthesized from tryptophan, via 5-HT (Axelrod, 1974; Klein, 1974; Quay, 1974). Tryptophan is taken up from the blood and is hydroxylated in the presence of the tryptophan hydroxylase (TH) enzyme (Lovenberg *et. al*, 1967) to tryptophan hydroxylase and decarboxylated by aromatic amino acid decarboxylase (Lovenberg *et. al.*, 1962) to form 5-HT as shown in figure 1.3. 5-HT has been previously proposed as the pineal hormone (Albertazzi *et. al.*, 1966) because of its high concentrations in the pineal gland (Quay and Halevy, 1962) but it was found later not to be the case. It was discovered that although high concentrations of 5-HT were obtained, the amount released was negligible in terms of total amount available in the body (Reiter, 1980).

5-HT is then taken up by pinealocytes and is subsequently acted upon by N-acetyltransferase (NAT) resulting in formation of N-acetylserotonin (NAS) (Klein and Weller, 1970). NAS is then O-methylated by hydroxyindole-O-methyltransferase (HIOMT) to form aMT (Axelrod and Weissbach, 1960). The methyl group for the last enzymatic step is provided by S-adenosylmethionine. There are some 5-HT derivatives produced in the pineal gland. MAO enzyme acts on 5-HT and results in formation of an unstable metabolite, 5-hydroxyindole acetylaldehyde. This metabolite is either oxidised to 5-hydroxyindole acetic acid (5-HIAA) or reduced to 5-hydroxytryptophol (5-HTOH) (Quay, 1974). HIOMT methylates 5-hydroxytryptophol to 5-methoxytryptophol (McIsaac *et. al.*, 1965).

1.3.3 Metabolic fate of pineal indoles

aMT (Rollag and Niswender, 1976) and possibly 5-MTOH (Wilson *et. al.*, 1978) are released from

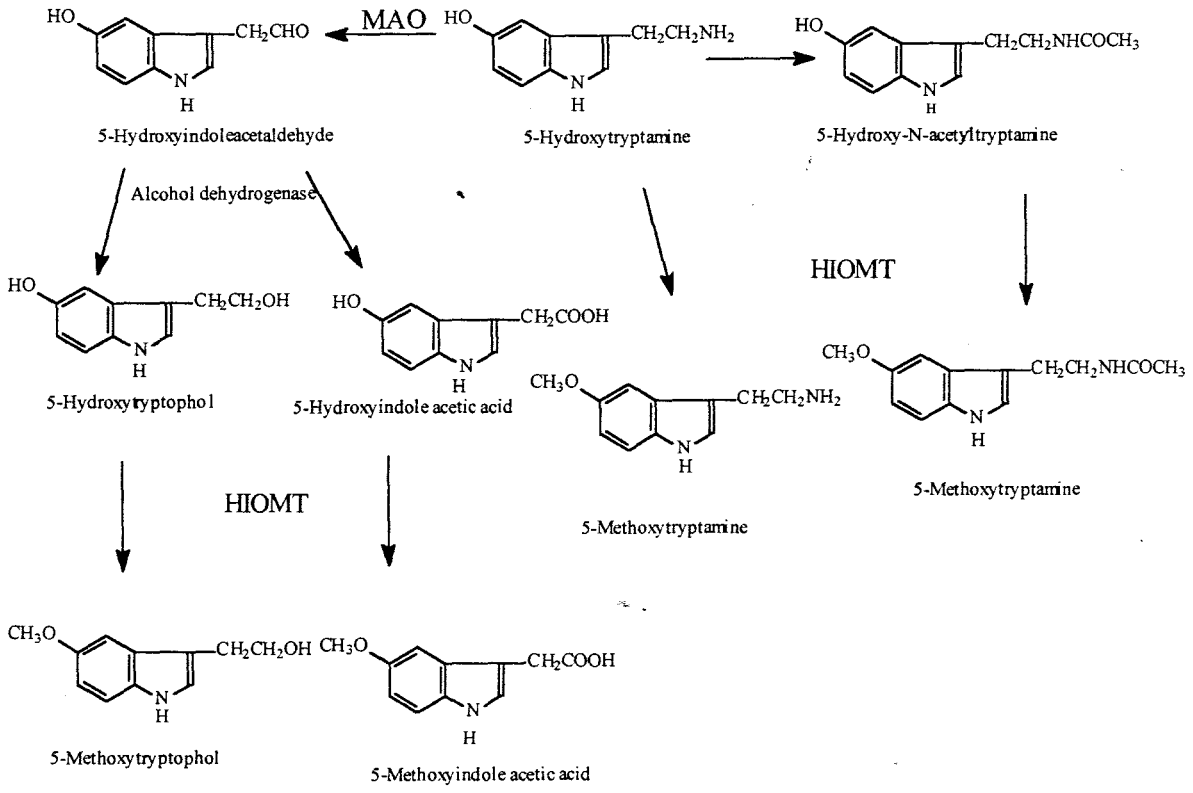

Figure 1.8 The biosynthetic pathway of the pineal indoles (Reiter 1981)

the pineal gland into the systemic circulation. However, if the pineal indoles have the primary site of action on the brain, then another potential route of transfer becomes apparent, namely the cerebrospinal fluid (CSF).

The diurnal aMT rhythms in the blood are paralled by a similar pattern of urinary excretion (Lynch *et. al.*, 1975). The rhythm in the CSF is more pronounced than in the plasma. This may imply that aMT is normally released either directly or indirectly into the CSF, but further investigation is required on this hypothesis (Hedlund *et. al.*, 1977). However, Rollag *et. al.* (1978) suggested that blood is the normal route of release of pineal aMT. In view of this, CSF appears to be the more favorable reservoir for aMT because via this route the problem of rapid deactivation of these substances by the liver is circumvented.

aMT, is metabolised in the liver to 6-hydroxymelatonin which is conjugated to either glucuronide (20 – 30 %) or to sulphate (60 – 70 %). The main metabolite to be excreted in the urine is 6-sulphatoxymelatonin. It appears that there is significant correlation between nighttime aMT peak levels and 6-hydroxymelatonin excreted from the human urine (Markey et. al., 1985). From this, one can conclude that excretory aMT can be a measure of aMT production.

1.3.4 Endocrinology of the pineal gland

The pineal gland regulates endocrine organs mostly via the neuroendocrine axis of the photoperiod and adjusts the activity of the hypothalamo-hypophyseal system accordingly. This interaction is made possible by means of hormonal input produced and secreted by the pineal gland (Reiter, 1980). Two hormones have been identified to play a role in the endocrine function of the pineal gland. These are indoleamines and polypeptides. Both hormones are synthesized and secreted from the pineal gland. The indoleamines are derived from tryptophan and polypeptides from various amino acids.

The pineal gland has very high concentrations of tryptophan hydroxylase (TH) and due to this, there is a greater potential for the pineal gland to produce hydroxyindoles than any other tissues in the mammalian body (Reiter, 1980). Furthermore, the gland contains high levels of 5-HT per mg tissue, as compared to any other body organs (Quay, 1962 and Saavedra *et. al.*, 1973). One other factor that makes the pineal gland to be unique is the N-acetylserotonin (NAS) which is the direct precursor of aMT. aMT was discovered by Lerner and colleagues in the late 1950's when these authors were exploring the effects of pineal principles on amphibian pigment cells and attempted to extrapolate these findings to treatment of human pigmentary disorders (Lerner and Case, 1959). During the course of their study, these scientists identified the chemical structure of the pineal substance that aggregated amphibian melanosomes, N-acetyl-5-methoxytryptamine and named this compound melatonin (aMT) (Lerner *et. al.*, 1958) as it is known to date.

1.3.5 Regulation of pineal aMT production

The pineal principal hormone, aMT , is regulated by light:dark cycles acting via the hypothalamic suprachiasmic nucleus (SCN). Pineal regulation is independent of sleep. In mammals, light and darkness perceived by the eyes synchronises the circadian activity of the gland whereas in birds light directly penetrating the skull influences pineal function (Reiter, 1980). The pinealocytes receive

sympathetic nerve endings which release the neurotransmitter (NT) nor-epinephrine (NE) during darkness. The NT acts on adrenergic receptors and determines the uptake of tryptophan and the synthesis of aMT from the precursor 5-HT after different enzymes have been activated. Adrenergic receptors have been shown to potentiate the adrenergic receptors which are linked to adenylate cyclase (Klein *et. al.*, 1983a), via a stimulatory guanine-nucleotide binding protein (G_s protein) (Helmreich and Pfeuffer, 1985). However, α-adrenergic receptors are coupled to adenylate cyclase system which in turn is stimulated by α-agonists. In contrast, α-adrenergic receptors can be inhibitory to adenylate cyclase in the presence of an appropriate agonist (Lefkowitz and Caron, 1985) and via an inhibitory guanine nucleotide binding protein (Helmereich and Pfeuffer, 1985). After NE stimulation, the synthesis of intracellular secondary messenger, cyclic AMP (cAMP) is amplified leading to aMT synthesis (Reiter, 1991b).

During light periods, circulating catecholamines are augmented and α-adrenergic receptors tend to lose sensitivity, i.e. α-adrenergic receptors tend to be down-regulated by very high concentrations of catecholeamines. Furthermore, Sweat *et. al.* (1986) postulated that high aMT levels at night may restore the lost α-adrenergic receptor sensitivity caused by catecholamines.

Apart from sympathetic innervation, the pineal also receives nerve fibers from the CNS. In this case the NT's are either peptides e.g.neuropeptide Yor acetylcholine. The cell bodies of these neurons are located in different nuclei of the brain (Korf and Moller, 1983). The function of this central innervation

is currently being investigated and is probably related to additional brain modulation of pineal function (Moller *et. al.*, 1992).

1.3.6 Role of circadian rhythm on pineal aMT production

There has been extensive research done on the effect of photoperiod on pineal aMT production and most findings indicate that light suppresses the endocrine activity of the pineal gland whereas darkness stimulates the production. Both light and darkness play a vital role in the biosynthetic activity and endocrine capability of the pineal gland. High concentrations of aMT in the blood and in the pineal during the dark period has been observed.

Reiter (1980) totally agrees with the studies pursued for the last two to three decades that the vertebrate pineal gland is required for photoperiodic responses that adjust physiological strategies during different seasons of the year and that daily injections of aMT at a proper circadian time can account for the pineal activity (Stetson and Watson-Whitnyre, 1986).

Klein (1978) observed that in mammals, excluding man, aMT rhythm is disturbed by constant light exposure but persists when animals are kept under constant darkness for prolonged periods. Reiter (1980) reported an increase in aMT production during dark periods.

Lewy and Sach (1992) reported that during and immediately after a change to night-shift work or travel across time zones, secretion of aMT depends more on the biological clock than on the light:dark cycles. Improvement of performance and synchronization of the onset of sleep in shift workers is

currently being investigated.

1.3.7 Distribution of aMT

For several years after the discovery of aMT, aMT was believed to be secreted solely from the pineal gland, but this was proven wrong. aMT was found to be present in body organs such as the gut and the brain (Raikhlin *et. al.*, 1975). Administration of large doses of tryptophan leads to an increase in serum aMT levels but not NAT and HIOMT activities (Yaga *et. al.*, 1993).

aMT is found in significant concentrations in several body fluids (Ozaki and Lynch, 1976; Rollag and Niswender, 1976) and small amounts of unmetabolised aMT are excreted in the urine (Lynch *et. al.*, 1975).

1.3.8 Pineal aMT on gonadotropic system

Small animals such as the rodents generally reproduce when environmental conditions are favorable. Light: dark cycles have been shown to influence reproduction of many animals including rodents. The laboratory rat has been used as the model for mammalian research experiments, but as opposed to other laboratory animals, it responds minimally and unconclusively to the maneuvering of the pineal gland and this therefore makes it to be a poor animal of choice for testing the pineal-gonadal interactions (Reiter, 1974). Syrian hamsters are regarded as excellent models for measuring alterations in reproduction system due to manipulations of the pineal gland (Reiter, 1974). The earliest reports on anti-reproductive effects of aMT were shown in early 1960's. Tamarkin in 1976 made a

breakthrough by showing that the inhibitory effect of aMT on gonadotropic system depends on time of day of administration of aMT. Tarmakin discovered that, aMT injection is effective when done late during the the light phase at 14 hours light: 10 dark hours cycle. The insensitivity of laboratory animals on early morning injections was due to endogeneous peak levels of aMT occuring at 04:00 during the dark phase of the light:dark cycle (Panke *et. al.*,1978; Panke *et. al.*, 1979; Tamarkin *et. al.*, 1979). Reiter (1980) suggested that when aMT acts on its receptors, it down regulates these to any additional aMT for a certain period of time. Hence early in the light phase, aMT is ineffective in altering reproductive physiology since its receptors are down regulated. However, by late afternoon, receptors have regenerated their potential to respond to aMT. Tamarkin *et. al.* (1979) reported that aMT receptors appear to regenerate at about 6.5 hours after lights are switched on. Animals receiving aMT injections should also possess an intact and sympathetically innervated pineal gland (Tamarkin *et. al.*, 1976).

Pinealectomy (Reiter, 1974) or light deprivation (Reiter, 1971) have been shown to have no effect on growth of reproductive organs in rats.

1.3.10 The pineal and MAO

The pineal parenchymal cells and sympathetic nerves that innervate the gland contain 5-HT. 5-HT within the nerves probably originates in the parenchymal cells and enters the nervous system via the amine reuptake mechanism. Most of the 5-HT synthesized within the pineal gland is probably

converted to aMT and its metabolites. Apparently, only the sympathetic nerves that innervate the pineal contain MAO-A which specifically metabolises 5-HT and norepinephrine (NE). Experiments on this hypothesis were done following sympathectomy of the rat pineal gland, the activity toward 5-HT was reduced by 70 % while only minimal changes toward tyramine metabolism were observed. Moreover, MAO activities of human sympathetic trunk and pineal gland were evaluated for the presence of MAO-A and MAO-B enzymes with tyramine as the substrate, two forms were evident. Sympathetic nerves contained mainly the clorgyline sensitive enzyme (MAO-A), the pineal gland on the other hand contained MAO-B which is resistant to inhibition by clorgyline. These two observations had two implications. Firstly, the transmitter NE is preferentially deaminated within the adrenergic nerve endings not within the pineal gland since the required enzyme for deamination is absent. Moreover, the adrenergic nerve endings within the pineal also have the capacity to metabolise 5-HT that finds its way into the neuron. Secondly, the virtual absence of MAO-A in pineal cells is consistent with the view that aMT and not 5-HT is the end product of tryptophan metabolism in the pineal cells. It appears that 5-HT metabolism would be unaltered in the pineal gland cells after either inhibitor, since deamination is not a major pathway for 5-HT in this tissue (Neff *et. al.*, 1974).

1.4 6-METHOXY-2-BENZOXAZOLINONE (6-MBOA)

6-Methoxybenzoxalinone (6-MBOA) was first isolated from corn and was originally considered as an insect *antifeedant* (Smissman *et. al.*, 1957). It was subsequently isolated from sprouted grass (Sanders *et. al.*, 1981) when studied to find dietary constituents which were gonadotropic in the vole *Microtus montanus* (Negus and Berger, 1977; Negus *et. al.*, 1977). The compound was then found to be gonadotropic using intraperitoneal injections under laboratory conditions.

1.4.1 Biochemical synthesis of 6-MBOA

6-MBOA is derived from 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA). DIMBOA is a defensive factor of maize and wheat seedlings against microorganisms and insects. It is stored as its glucoside and released at wounded sites by a glucosidase (Niemeyer, 1988; Wahlroos and Virtanen, 1959). In an aqueous solution, near neutral pH, DIMBOA has been reported to rapidly decompose *in vitro* to form an anti-insect substance, 6-MBOA and formic acid (Woodward *et. al.*, 1978; Kumar and Chilton, 1994). At longer photoperiods (summer), the conversion rate of DIMBOA to 6-MBOA in maize tissue is increased.

DIMBOA is abundantly present in the primary glucoside in young seedlings and vegetatively growing plants (Berger *et. al*, 1981). Plant injury coincident with grazing evokes the release of enzymes that rapidly convert DIMBOA to 6-MBOA (Nelson and Blom, 1992). Other detection of 6-MBOA in the

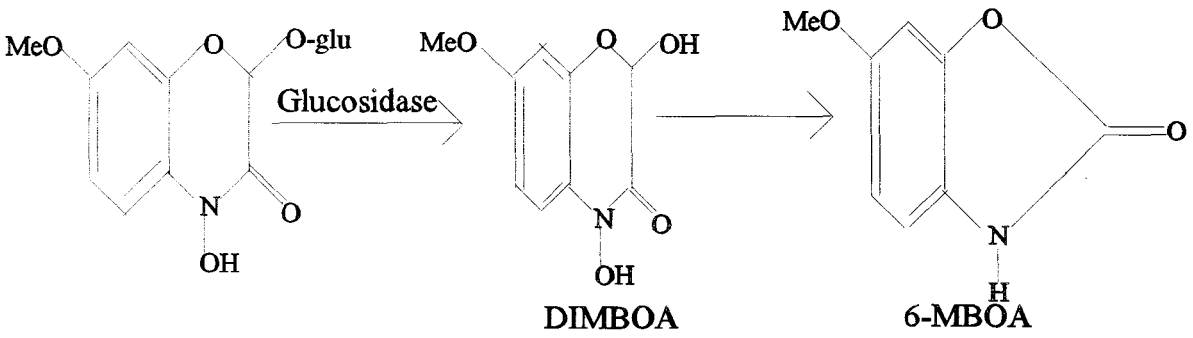

Fig. 1.9 Biosythesis of 6-MBOA

diet is an indication that the growing season has initiated and that food is readily available (Nelson and Blom, 1992).

1.4.2 Effect of 6-MBOA on gonadal function

Most research on 6-MBOA has concentrated on determining the effect of this compound on gonadal function. Seasonal breeding involves the availability of food to animals. Most seasonal breeders rely on photoperiod to link season and breeding (Sadleir, 1973) and the transduction mechanism is mainly the pineal gland (Reiter, 1980).

The synthesis of aMT exhibits a circadian rhythm controlled by the NE stimulation of $ß_1$-adrenoreceptors in the pineal gland leading to stimulated activity of the enzyme activity N-acetyl-5-hydroxytryptamine (NAT) (Axelrod, 1974; Klein, 1974; Deguchi, 1982). Animals living in

environments in which light is not closely related to food and warmth must use other factors to control reproductive activity (Yuwiler and Winters, 1985).

The mountainous habitat of the short lived Montane vole, *Microtus montanus* is characterized by overcast skies, low levels of lighting and a snowpack (Yuwiler and Winters, 1985). In 1981, Berger *et. al.* reported that it is rather the ingestion of the young seedlings of winter wheat during spring time (Klun and Robinson, 1969) not light which triggers the reproductive cycle of the vole.

6-MBOA, a chemical derived from a component of winter wheat has been reported to be responsible for the gonadal stimulation (Sanders *et. al.*, 1981). 6-MBOA does not only increase the rate of sexual maturation in the vole *Microtus montanus*, but in other species as well, such as the rabbit (Rex), kangaroo rat (Dipodomys ordii) (Rowsemitt, 1984) and the albino rat (Butterstein *et. al.*, 1985).

There is a similar structural conformation between 5-HT, aMT and NE and this suggests that 6-MBOA may be responsible for blockade of aMT gonadal activity inhibition.

One of the routes of 6-MBOA action on gonadal function is through interference with aMT synthesis in the pineal gland, 6-MBOA antagonism of aMT receptors that control gonadal function or direct 6-MBOA inhibition of gonadal function. The structural similarity between 6-MBOA and NE suggests a possibility that 6-MBOA might act as a receptor antagonist and thus prevent catecholaminergic stimulation of NAT, a regulatory enzyme in aMT synthesis. Furthermore, structural similarity between

5-HT and 6-MBOA could lead to competition of 6-MBOA with 5-HT for the active site on NAT. Both possibilities would lead to decreased synthesis of aMT, an antigonadotropic hormone.

The antigonadal effects of aMT have been correlated with decreases in follicle stimulating hormone (FSH) and luteunising hormone (LH) in the rat (Martin *et. al.*, 1980; Lang *et. al.*, 1983). NE is a positive regulator at these sites so the binding of aMT to adrenoreceptors supports the speculation that it may compete directly with NE. If this is true, 6-MBOA reproductive effects could be due to its ability to antagonize these antigonadal effects of aMT.

It has been estimated that field grass in early spring contains about 100 - 200 μg of 6-MBOA / g wet weight and Sanders *et. al.* (1981) have shown that this concentration of 6-MBOA in the laboratory food promotes gonadal growth in young voles weighing 25 g and consuming 5 g of chow per day. This dose has been shown to stimulate gonadal growth in the mouse. Yuwiler and Winters (1985) assumed that 6-MBOA may also influence synthesis of other pineal indoles.

1.4.3 Effect of 6-MBOA on pineal function

α_1 and α_2 of the adrenergic system enhance the production of aMT. NE released by postganglionic nerve terminals within the pineal gland acts on α-adrenoreceptors on the pineolocyte membrane to increase cAMP production thus enhancing the activity of NAT and aMT concentration subsequently. 6-MBOA has been shown to influence this pathway and its effects will be reviewed below.

1.4.3.1 Effect of 6-MBOA on ß- adrenoreceptor binding and cyclic AMP (cAMP)

Adenylate cyclase activity is stimulated in tissues innervated by sympathetic fibers including the pineal gland after 6-MBOA supplementation (Sweat and Berger, 1988). 10 µM 6-MBOA has been shown to be as potent as 1µM isoproterenol in stimulating adenylate cyclase activity (Sweat and Berger. 1988).

Yuwiler and Winters in 1985 suggested that 6-MBOA stimulates ß-adrenoreceptors in the same manner as does NE and this induction is blocked by a ß-adrenoreceptor antagonist, propanolol. The property of 6-MBOA as the ß-adrenergic agonist is shown by its stimulation of adenylate cyclases from the pineals and other tissues. Furthermore, aMT, a 6-MBOA analogue (Sweat *et. al.*, 1988) binds to ß-receptors like 6-MBOA (Sweat *et. al.*, 1986a,b). However, unlike 6-MBOA, aMT does not stimulate adenylate cyclase *in vitro*, but it does decrease the effects of ß-adrenergic agonists (Sweat *et. al.*, 1987).

Yuwiler and Winters in 1985 reported unexpected findings that 6-MBOA is a weak inducer of NAT activity at relatively high concentrations of 0.02 mM or more. Moreover, they showed that 6-MBOA stimulated NAT activity postsynaptically. These authors observed no alteration in subsequent steps in aMT biosynthesis. This was evidenced by the fact that pineals exposed to 6-MBOA converted more radioactive tryptophan to NAS and aMT as compared to controls *in vitro* (Yuwiler and Winters, 1985). It also appeared that 6-MBOA has very weak affinity for the receptor site and is easily displaced from it by other $ß_1$ agonists, NE in particular (Yuwiler and Winters, 1985).

1.4.3.2 Effect of 6-MBOA on pineal NAT and HIOMT activities

Yuwiler and Winters (1985) reported that 6-MBOA is a postsynaptic weak inducer of NAT activity at concentrations of 0.02 mM and above. Stimulation of NAT is reported to be cAMP dependent and the stimulation of NAT was expected to occur at 10^{-5} since cAMP is increased at such concentrations by 6-MBOA, however, Daya *et. al.*, (1990) observed stimulation at 1 mM. Parallel observations on NAT induction were reported by Yuwiler and Winters (1985).

HIOMT activity appears to be unaltered by 6-MBOA regardless of concentrations used (Yuwiler and Winters, 1985; Daya *et. al.*, 1990).

1.4.3.3 Effect of 6-MBOA on aMT levels

aMT production in the pineal gland originates with the of stimulation of ß-adrenoreceptors by NE which is cAMP dependent. This is followed by stimulation of NAT resulting in the formation of aMT. Daya and Tandt (1993) showed that 6-MBOA stimulates pineal aMT synthesis with a concomitant rise in pineal cAMP. Daya *et. al.* (1990) reported a significant elevation of pineal aMT at concentrations of 1 mM and above of 6-MBOA which is in accordance with the concentration required to stimulate NAT activity, a regulatory enzyme in aMT biosynthesis. Lower concentrations appear to have no effect on both pineal enzymes and on aMT production.

1.5 ANTIDEPRESSANTS

1.5.1 TRYPTOPHAN AS AN ANTIDEPRESSANT

Rubin (1967) found that the reduced levels of indoleamines in depression is caused by an increase in TDO activity which is followed by raised levels of plasma glucocorticoids. These observations were confirmed by tracer experiments done by Weil-Malherbe (1972) who showed that one of the few unequivocal observations in depression is the change in the activity of TDO which probably accounts, at least in part, for some of the changes of indole metabolism and 5-HT biosynthesis.

The mode of action of antidepressants on TDO activity is not regulated by glucose or insulin respectively. This finding strongly suggests that antidepressants increase brain tryptophan levels by increasing tryptophan availability to the brain secondarily to the inhibition of liver TDO activity (Badawy and Evans, 1981).

Tryptophan has been used for many years as an antidepressant, but its efficacy is limited by its rapid catabolism. The major site of tryptophan catabolism is the kynurenine pathway in the liver and the key controlling enzyme is TDO. Consequently, inhibition of TDO should decrease the catabolism of systemic tryptophan, increasing its concentration in the blood and in the brain and thereby increasing synthesis and subsequent release of 5-HT (Madge *et. al.*, 1996).

It is known that brain tryptophan concentration is increased when TDO activity is inhibited by allopurinol, glucose, nicotinamide or chronic administration of ethanol (Badawy and Evans, 1981).

Coppen *et. al.* (1963) observed that, tryptophan can be beneficially used with tricyclic antidepressants (TCA's) at very low doses, enough to potentiate the action of these antidepressants. Coppen *et. al.* (1967) showed that L-tryp administration to depressives with or without a concomitant treatment with monoamine oxidase inhibitors (MAOI) are effective antidepressants. These researchers claimed that tryptophan given alone is as effective as electroconvulsive therapy in the treatment of depression.

Several drugs and vitamins have been given in combination with tryptophan in clinical use because of their effects on peripheral tryptophan metabolism. The most common of these is pyridoxine. It is usually used as pyridoxal phosphate as a coenzyme in pathways involving tryptophan metabolism. Pyridoxine does not have direct influence on tryptophan metabolism, although it may increase decarboxylation of tryptophan to tryptamine by aromatic amino acid decarboxylase. This pathway is quantitatively minor and would not influence tryptophan levels (Haber *et. al.*, 1981) and inhibition of the enzyme amino acid decarboxylase will not increase the availability of tryptophan in the brain (Young *et. al.*, 1978). However, pyridoxine may increase decarboxylation of 5-HTP to 5-HT in the brain. Coppen (1976) supported this hypothesis because this author discovered that antidepressant effects of tryptophan did not differ significantly when it was given with or without pyridoxine. The administration of pyridoxine and tryptophan helps prevent accumulation of tryptophan metabolites such as kynurenine, 3-hydroxykynurenine (3-OH-K) and xanthurenic acid (XA) which may interfere with 5-HT synthesis in the brain (Wolf, 1973).

1.5.2 L-5-HYDROXYTRYPTOPHAN (L-5-HTP)AS AN ANTIDEPRESSANT

Over the past 30 years, a thorough investigation on a massive body of psychobiology has shown 5-HT deficiency to be the major cause in conditions such as depression, anxiety, suicide, alcoholism, migraine headaches, insomnia and obesity. L-5-hydroxytryptophan, an immediate precursor of 5-HT is thus hypothesised as the preferable antidepressant hence its administration leads to increased 5-HT concentration.

L-5-HTP is extracted from seeds of *Griffonia* plant. When neurons convert tryptophan to 5-HT, neurons first use a vitamin B_3-dependent enzyme, tryptophan hydroxylase (TH) to convert tryptophan to 5-HTP. A vitamin B_6- dependent enzyme, L-5-HTP decarboxylase is then used to convert L-5-HTP to 5-HT. L-5-HTP can easily cross BBB and is not a substrate for TDO. It is also not incorporated into proteins nor is used to make vitamin B_3. The enzyme L-amino acid decarboxylase found outside the brain has its highest activity in liver kidney and intestinal lining. It converts L-5-HTP into 5-HT which cannot cross BBB. Thus, only L-5-HTP is effective to be implemented to increase brain 5-HT levels and consequently used as an antidepressant (South, 1997).

1.5.3 L-TRYPTOPHAN-KYNURENINE PATHWAY IN DEPRESSION

The major pathway of tryptophan metabolism is the kynurenine pathway. It is known as the "kynurenine shunt" because the pathway initially, tended to be ignored in studies on depression. In

depression, kynurenine levels increase slowly and then remain elevated for a long time, because of "self-induction" due to activation of TDO by circulating kynurenines (Lapin, 1976). This effect can lead to a prolonged hypothesis which results in asthenic depression (Lapin, 1980).

Xanthurenic acid output in depressed patients is increased as compared to manic and control subjects and that leads to a suggestion that this increase is due to an elevated activity of TDO enzyme (Rubin, 1967). Mangoni (1974) suggested that the increase is due to decreased activity of pyridoxal phosphate-dependent kynureninase. This rearcher also showed a decrease in excretion of N'-methyl-nicotinamide and hypothesized it to result from pyridoxal phosphate-dependency of this metabolite perhaps secondary to decreased activity of kynureninase.

Monoaminergic kynurenines are essential for thymoanaleptic (antidepressant) action of antidepressants and kynurenines possess largely similar activity in humans. Kynurenines obstruct the effects of antidepressants, considering that the concentration of kynurenines might be increased in depression. A study about this interference was done and it was found that after tryptophan treatment (50 mg/kg), manic depressives excreted less kynurenines than controls (Lapin, 1980). In depressive patients, resistant to therapy, three times more kynurenine was excreted when compared to controls (Lapin, 1980). Furthermore, L-tryp administration may cause toxicity by producing an accumulation of products in the kynurenine pathway or simply by overloading the liver with ammonium ion since tryptophan induces TDO which may inhibit 5-HT synthesis which in turn may inhibit the effectiveness of tryptophan on depression (Van Praag and Korf, 1971c).

1.5.4 SELECTIVE SEROTONIN REUPTAKE INHIBITORS (SSRI's)

The leading antidepressants are selective 5-HT reuptake inhibitors (SSRI). By blocking the reuptake of 5-HT, SSRI increase the 5-HT available to stimulate 5-HT receptors. While SSRI's may be very effective for the treatment of depression, these drugs do have side effects e.g. loss of sex drive. SSRI's are also expensive and only available by prescription (http://www.virtuvites.com/5-htp_100mg.htm). The most studied and widely used SSRI is fluoxetine (Klipin, 1998).

SSRI's have high affinity for 5-HT transport site and low affinity for other NT receptors *in vitro*. The majority of these SSRI's are metabolised by N-demethylation. Fluoxetine and its metabolite, norfluoxetine are potent SSRI's both *in vitro* and *in vivo* (Wong *et. al.*, 1975a). These drugs have many adverse side effects. Fluoxetine and norfluoxetine have very few if any anticholinergic side effects commonly caused by tricyclic antidepressants (TCA's). Moreover, postural hypotension and weight gain have not been reported in these drugs. However, SSRI's have been associated with an increased frequency of gastrointestinal side effects including nausea, vomiting, abdominal cramps and diarrhoea and effects such as restlessness, insomnia, anxiety. These side effects have been shown to decrease as treatment continues or with a certain dosage for a particular individual (Lemberger *et. al.*, 1985, Mendels, 1987 and Burrows *et. al.*, 1988).

1.5.5 TRICYCLIC ANTIDEPRESSANTS

The clinical efficacy of tricyclic antidepressants has been related to the ability of these drugs to inhibit active reuptake of NE. The therapeutic effect of imipramine discovered in 1958 by Kuhn led to the development of a number of tricyclic compounds with antidepressant properties. Chronic and or acute administration of tricyclic antidepressants was shown to inhibit total activity of TDO. The antidepressants used were, amitriptyline, clomipramine, imipramine or tranylcypromine. Acute doses of imipramine can also increase pineal aMT production possibly by direct adrenergic stimulation (Wirz-Justice *et. al.*, 1980). Conversely, repeated imipramine injections lead to a decrease in pineal adrenergic sensitivity (Moyer *et. al.*, 1981).

TCA's have adverse side effects. TCA's are also associated with cardiovascular effects such as tachycardia and occasional heart block which have been mainly reported in overdosed patients.

1.5.6 MONOAMINE OXIDASE INHIBITORS (MAOI) ANTIDEPRESSANTS

There is an important link between the metabolism of monoamines and human disorders. MAOI inhibit the catabolism of monomanines thus augmenting the levels of monoamines in the sypnaptic cleft. In 1965, Bunney and coworkers observed that there is a high excretion of 17-hydroxycorticosteroids in urine of depressed patients with a high risk of suicide thus suggesting a decreased MAO activity. To support this report, Buchsbaum and coworkers in 1976 showed a low MAO activity in blood platelets

of suicidal people. Low brain MAO activity has also been discovered in alcoholic suicides. Wyatt *et. al.* (1973b) demonstrated a low platelet MAO activity in some individuals with a high susceptibility to schizophrenia. All these reports show that MAO inhibition is definitely associated directly or indirectly with psychiatric disorders and is beneficiary in the treatment of these disorders.

MAOI's were the first clinically effective antidepressants, but their use is now limited because of their side effects. MAO-A inhibitors interact with sympathomimetic drugs and with tyramine, commonly found in cheese ("cheese reaction"). This has limited the use of MAO-A inhibitors in treatment of depression. MAO-B does not undergo this interaction and it was thus thought as a more preferable antidepressant (Quitkin *et. al.*, 1984b), but this hypothesis was proven wrong when MAO-B inhibitors were found to be effective only at high doses and MAOI-B are likely to lose their specificity at such doses (Sunderland *et. al.*, 1985). A more preferable MAO-A inhibitor is clorgyline. Clorgyline has been shown to have mild side effects as compared to TCA's (Murphy *et. al.*, 1987a) even at low doses in patients with bipolar affective disorders (Robinson *et. al.*, 1985).

1.5.7 MELATONIN (aMT) AS AN ANTIDEPRESSANT

Pineal function and its main hormonal output has been ignored by many clinicians. In the last three decades, tremendous developments in the understanding of biochemistry and physiology of the pineal gland have occurred. Up to date, it is evidently shown by many researchers that the pineal gland interacts with many endocrine as well as non-endocrine tissues to influence their metabolic activity. The most studied modulation of organs and their functions by pineal aMT has been done on

neuroendocrine –reproductive axis. More research is still to be elucidated on neuroendocrine function on psychiatric disorders.

Low nocturnal aMT has been suggested to be a trait marker for major depressive illnesses and depressive states with abnormalities in the hypothalamic pituatory adrenal axis (Beck-Friss *et. al.*, 1985). In rodents and humans, depression treated with antidepressants such as monoamine oxidase inhibitors (MAOI) increases the pineal content of aMT precursors 5-HT, NAS and aMT respectively in both blood and CSF by enhancing NAT activity. However, tricyclic antidepressants reduce aMT production and secretion in rodents. Other psychotic drugs that disturb monoamine pathway also affect the stimulatory action of adrenergic system and yet reduce aMT production (Lewis *et. al.*,1990).

Lewis *et. al.* (1990) has reported a link between diurnal and seasonal rhythmicity of aMT production and seasonal affective disorders and various types of endogenous depression. The persistent winter depression known as seasonal affective disorder, also characterised by weight gain, carbohydrate craving and hypersomnia was found to improve significantly after treatment with bright light (Rosenthal *et. al.*, 1984). It was clearly shown that the inhibitory effect of bright light on pharmacological suppression of aMT does not improve their depression. Therefore light itself is the prerequisite to reduce winter depression (Rosenthal *et. al.*, 1986).

aMT has been shown to be reduced in depressed patients. An increase in the cortisol:aMT ratio together with inverse correlation between aMT and cortisol rhythms have been reported to exist indepressed individuals (Claustrat *et. al.*, 1984). However in non-affective disorders such as chronic

schizophrenia, abnormally low levels of aMT have been observed (Ferrier, 1993).

1.6 SUMMARY

Tryptophan is an essential amino acid. It is a precursor of biogenic amines 5-HT and aMT which are known to play important roles in affective disorders such as depression. 98 % of circulating tryptophan is oxidised by the liver enzyme, TDO and only about 1 % or less of the remaining tryptophan goes to the brain for the biosynthesis of 5-HT. TDO is induced by glucocorticoids, tryptophan and haem. It is inhibited by aMT and 5-HT both *in vitro* and *in vivo*. Reduced levels of 5-HT and to a lesser extent, aMT, are reported to exist in depression, hence TDO is the key regulatory factor in therapeutic effect of depression as the enzyme limits the bioavailability tryptophan. 6-MBOA, a plant alkaloid is an analogue of 5-HT and aMT. 6-MBOA is reported to increase aMT levels *in vitro* by inducing NAT and adenylate cyclase activity and by acting as a weak β-adrenoreceptor agonist. Drugs which enhance 5-HT and aMT levels are imperative in treatment of depression. These drugs include MAOI, SSRI's, tryptophan, 5-HTP, 5-HT and aMT supplements.

CHAPTER 2

EFFECT OF 6-METHOXY-2-BENZOXAZOLINONE (6-MBOA) ON HEPATIC TRYPTOPHAN 2,3-DIOXYGENASE (TDO) ACTIVITY

2.1 INTRODUCTION

Tryptophan 2,3-dioxygenase (EC 1.13.11.11,TDO) is a haem-dependent hepatic enzyme that catalyses the conversion of its substrate, L-tryptophan (L-tryp) into N'-formylkynurenine. It does this by incorporating two atoms of molecular oxygen (O_2) into L-tryp. TDO exists in two forms in the rat and human livers whereas only one form exist in other animals. One form exists in an active state (holoenzyme) and the other form requires exogenous haem for its activation (apoenzyme). Activation of the apoenzyme *in vitro* involves its conjugation with haem to form the oxidised (ferrihaem) holoenzyme which is then reduced to an active holoenzyme in the presence of tryptophan (Badawy and Evans, 1975; Uchida *et.al.* 1992).

TDO activity can be regulated by glucocorticoids and tryptophan. Glucocorticoids induce TDO activity by causing synthesis of new apoenzyme whereas the substrate tryptophan causes the decreased degradation of pre-existing apoenzyme during the normal rate of synthesis (Badawy

and Evans, 1973).

It has been shown that haem, a TDO cofactor may also act as a regulating agent in TDO activity. Addition of exogenous haem or haem precursors leads to induction of conjugation of the apoenzyme with haem and reduction of oxidised holoenzyme to an active form (ferrihaem). In contrast, addition of substances which inhibit haem synthesis cause opposite effects (Badawy and Evans, 1975).

Many antidepressants inhibit TDO activity both *in vitro* and *in vivo*. Antidepressants have been shown to inhibit total but not holoenzyme activity i.e. apoenzyme activity. The fact that these drugs exert a direct inhibitory effect on TDO activity *in vitro* suggests that the apoenzyme synthesis is not affected by these drugs but inhibition might be caused by interferance in haem saturation of the apoenzyme and therefore depends on the endogeneous liver haem content. This could be true since haem is dissociable from the apoenzyme (Badawy and Evans, 1981).

This study is aimed at investigating the effect of 6-MBOA on TDO activity *in vivo*. 6-MBOA is a tryptophan metabolite and aMT and 5-HT analogue. It has been shown that both aMT and 5-HT inhibit hepatic TDO activity. It has also been shown in our laboratory that 6-MBOA inhibits the enzyme activity *in vitro*.

The assay employed in determination of TDO activity in this study was performed as described by Badawy and Evans, 1973, 1975 and 1981. Both the holo- and the apoenzyme activities were measured. The apoenzyme activity was taken as the difference between the total activity (added

exogeneous haem) and holoenzyme activity (no haem added). The activities were expressed as the nmoles of L-kynurenine formed per milligram of protein. The absorbance was read at 365nm with an extinction coefficient of 4540 L/mol.cm.

2.2 ANIMALS AND INJECTIONS

Male Wistar rats of the albino strain (200-250g) were used for all the experiments. The animals were housed in groups of 5 (n=5) in opaque rectangular cages with metal floors and covers. Water and Epol food were supplied *ad libitum.* The animal room was windowless and the temperature of the room was regulated at 22-25 ^{0} C. The room was artificially lighted and lights were automatically regulated to maintain a 12 hr dark (18:00-06:00) and 12 hr light (06:00-18:00) cycle. The intensity of the illumination was 300 μWatts/cm^2. The animals were killed swiftly by cervical dislocation and rapidly decapitated. For experiments conducted during the photo-phase, animals were sacrificed between 12h00 and 13h00 and for the scoto-phase experiments, animals were sacrificed between 00h00 and 01h00 under a dim red light (60 Watts).

2.2.1 ADMINISTRATION OF 6-MBOA TO RATS

For all the *in vivo* studies, the route used for injections was intraperitoneal (i.p). Controls were injected with 0.9 % NaCl in 50 % EtOH and the treated animals injected with 10 mg / kg 6-MBOA dissolved in vehicle. Animals were injected twice at one hour interval and sacrificed one hour after the last injection by cervical dislocation. For the photo-phase experiments, the injections were initiated between 10h00 and 11h00 and animals were sacrificed between 12:00 and 13:00.

For the scoto-phase experiments, injections were initiated between 22h00 and 23h00 and the animals were sacrificed between 00:00 and 01:00 under a dim red light (60 Watts).

For the removal of 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. A careful removal of livers was done and the livers were dissected free of blood vessels and connective tissue. The livers were excised and perfused with cold 0.9 % NaCl to remove blood haem that can interfere with conjugation of exogenous haem with apoenzyme. Livers were either homogenised immediately after perfusion or immediately stored in liquid nitrogen and kept frozen at -70 ^{0}C until required.

2.3 CHEMICALS AND REAGENTS

2.3.1 CHEMICALS

All chemicals were of the highest purity available. Potassium chloride (KCl), Phosphate buffer, Copper sulphate ($CuSO_4 \cdot 5H_2O$), Sodium tartrate, Sodium carbonate (Na_2CO_3), Folin-Ciocalteu (F.C) reagent, Sodium hydroxide (NaOH) and Trichloroacetic acid (TCA) were all purchased from Saarchem Limited, Krugersdorp, South Africa. Sodium chloride was purchased from Holpro Analytical Division, Midrand, South Africa. Haematin chloride, 6-Methoxybenzoxazolinone (6-MBOA) and L-Tryptophan (L-tryp) were purchased from Sigma Chemical Co., St Louis, USA. Ethanol (Abs) was obtained from Chemistry Dept., Rhodes University, South Africa.

2.3.2 REAGENTS

Milli R/Q System, Millipore water (deionised water) was used for preparing all the solutions. L-tryp (0.03 M) was prepared in 4 mM NaOH just prior to the start of the experiment. TCA (0.9 M) and NaOH (0.6 M) was dissolved in deionised water. Sodium phosphate buffer (0.2 M, pH 7.4) and 0.14 M KCl - 2.5 mM NaOH were prepared in deionised water and refrigerated until use. Haematin chloride, final concentration 2 μM was freshly prepared in 0.1 M NaOH just before commencing with the experiment.

2.4 HOMOGENATE PREPARATION

The liver was either used immediately after removal or stored at -70 ^{0}C as described in section 2.1.3. The frozen liver was slowly thawed and dried with a paper towel to remove any excess water that might interfere with the weight of the liver. The liver was then weighed and homogenized in a mixture of 10 % w/v 0.2 M sodium phosphate buffer, pH 7.4 and 10 % w/v 0.14 M KCl - 2.5 mM NaOH. Homogenisation was done in a Waring blender for a period of 1 minute at 1000 rpm. The homogenate was sonicated to free the enzyme from the cells. Sonication was done for a period of two minutes at 30 second intervals. All the above steps were done on ice since both the blending and especially sonication are exothermic and could lead to reduction in enzyme activity.

2.5 DETERMINATION OF TDO ACTIVITY

Table 2.1 below summarises the procedure for TDO assay. The samples containing 10 % w/v homogenate (15 ml) were added to 12.5 ml distilled H_2O and the solution was gently swirled. For total activity determination, 100 μl haematin chloride (final concn 2 μM in 0.1 M NaOH, optimum for enzyme activity) was added. Furthermore, 2.5 ml of 0.03 M-L-tryp in 4 mM NaOH was added to both total and holoenzyme reactions. The mixtures were gently swirled. Samples of 3 mls of the assay mixture were transferred to test tubes. The mixtures were stoppered in an atmosphere of carbogen (95 % O_2:5 % CO_2) and incubated at 37 ^{0}C in an orbital shaker for a period of one hour. The reaction was terminated by an addition of 2 ml 0.9 M TCA and tubes were further shaken at 37 ^{0}C for 4 minutes then filtered through Whatman no.1 filter paper. To a measured volume (2.5 ml) of the filtrate, 1.5 ml of 0.6 M NaOH was added and the L-kynurenine formed was determined by measuring the E_{365} using a spectrophotometer with an extinction coefficient (ε) = 4540 L/mol.cm.

2.6 HOMOGENATE PROTEIN DETERMINATION

The homogenate protein concentration was determined by using the method of Lowry *et. al.*(1951). An aliquot of 0.05 ml of homogenate was added to 0.095 ml of H_2O. To this, 6 ml of alkaline copper reagent solution prepared by mixing 1 ml of a 1 % $CuSO_4 \cdot 5H_2O$ solution, 1 ml of a 2 % sodium tartrate solution and 98 ml of 2 % Na_2CO_3 in 0.1 N NaOH in order. The mixtures were vortexed and left to stand at room temperature for 10 minutes. Following that, 0.3 ml of

Folin-Ciocalteau (F.C) reagent was added to each of the tubes and left to stand at room temperature for 30 minutes in the dark. After incubation, the absorbance was measured at 500 nm. Protein standards containing 0 - 150 μg / ml of bovine serum albumin (BSA) were assayed as above and the procedure is summarised in Table 2.2. A typical standard curve obtained is shown in fig. 2.1

Table 2.1 Summary for determination of TDO activity

Reagents	Volume (ml)
Distilled H_2O	12.5
Homogenate	15
Haematin	0.1 (Total activity)
L-Tryptophan	2.5
Transfer 3 ml to glass test tubes, bubble the mixture with carbogen and incubate @ 37 ^{0}C in a rotary shaker for 1 hour	
Terminate with 2 ml 0.9M TCA and incubate for a further 4 min	
Filter through no.1Whatman filter paper	
To 2.5 ml filtrate add 1.5 ml 0.6M NaOH	
Vortex and read Abs at 365_{nm} (Blank : 2 ml 0.9 M TCA + 1.5 ml 0.6 M NaOH)	

Table 2.2 Protein determination using Folin-Lowry method:

BSA stock solution = 300 μg / ml

Concentration (μg / ml)	Volume protein (ml)	Volume H_2O (ml)	Alk.Cu. reagent (ml)
0	0	1	6
25	0.025	0.975	6
50	0.075	0.95	6
100	0.1	0.925	6
150	0.15	0.85	6
Stand for 10 min. at room temperature after addition of protein, water and Alk.Cu reagent.			
Add 0.3 ml F.C. reagent into each tube and leave at room temperature for 30 min.			
Read absorbance at 365 nm			

2.7 RESULTS

Absorbance readings from the triplicate determinants were pooled and averaged. The concentration of L-kynurenine was calculated by applying Beer-Lambert Law and measuring the increase in absorbance at 365 nm.

2.7.1 Statistical analysis

All results were analysed by one-way analysis of variance (ANOVA). Statistical differences between groups were determined using the Student t-test. Values of $P < 0.05$ were taken as statistically significant. For determination of post tests, the Bonferroni multiple range test was employed.

2.7.2 Time-course of TDO activity

Enzyme activity was measured either in the presence (total enzyme) or in the absence (holoenzyme)of haematin and the difference between the two (apoenzyme) showed a time-dependency profile (fig. 2.2). For the apoenzyme activity, a linear increase was observed from T_{15} - T_{60} which remained constant from T_{60} -T_{90}. The enzyme was induced from T_{90} - T_{105}. The other two activities, the total and the holoenzyme appeared to show a linear increase from T_0 - T_{60}. From T_{60} it followed the same trend as the apoenzyme. One hour incubation period was thus used for all other experiments.

2.7.3 Effect of circadian rhythm on TDO activity

The total and holoenzyme activities appeared not be influenced by light-dark variations, however, the apoenzyme activity was significantly altered by these variations ($P < 0.05$) as shown in fig. 2.3 with higher activity occurring during the photo-phase.

2.7.4 Sensitivity of TDO to 6-MBOA treatment

The sensitivity of TDO to 6-MBOA is shown in fig. 2.4 and fig. 2.5. In both photo- and scoto-phase samples, 6-MBOA caused a significant inhibition of apoenzyme activity and it was more effective during the photo-phase ($P < 0.01$ and $P < 0.05$). Moreover, in photo-phase, 6-MBOA did not only alter apoenzyme activity, the total enzyme activity was also significantly reduced ($P < 0.001$). In contrary to this, holoenzyme activity was not altered by 6-MBOA in both phases. The ratio of apoenzyme / holoenzyme activity indicates the magnitude of conjugation of the enzyme with haem and it was found to be more than 1 in both the control and 6-MBOA treated photo-phase samples (2.25; 1.25). In scoto-phase samples this ratio was found to be less than 1 (098; 0.41). In both phases the ratio for treated animals is more or less twice the ratio for untreated animals.

2.8 DISCUSSION

In the rat, TDO activity is subjected to circardian rhythm. TDO activity demonstrates a diurnal oscillation from 09h00-18h00 with the highest activity observed during the pre-dark period and the lowest activity towards the end of the dark period. This hypothesis is reinforced by the results obtained from this study (fig.2.3),which showed a significant augmentation in apoenzyme activity during the photo-phase (12h00-13h00) as compared to the scoto-phase (00h00-01h00). The increased TDO activity could be attributable to the *de novo* enzyme synthesis. TDO synthesis is initiated between 08h00 and 09h00 and increases until it reaches its maximum at18h00.Readily

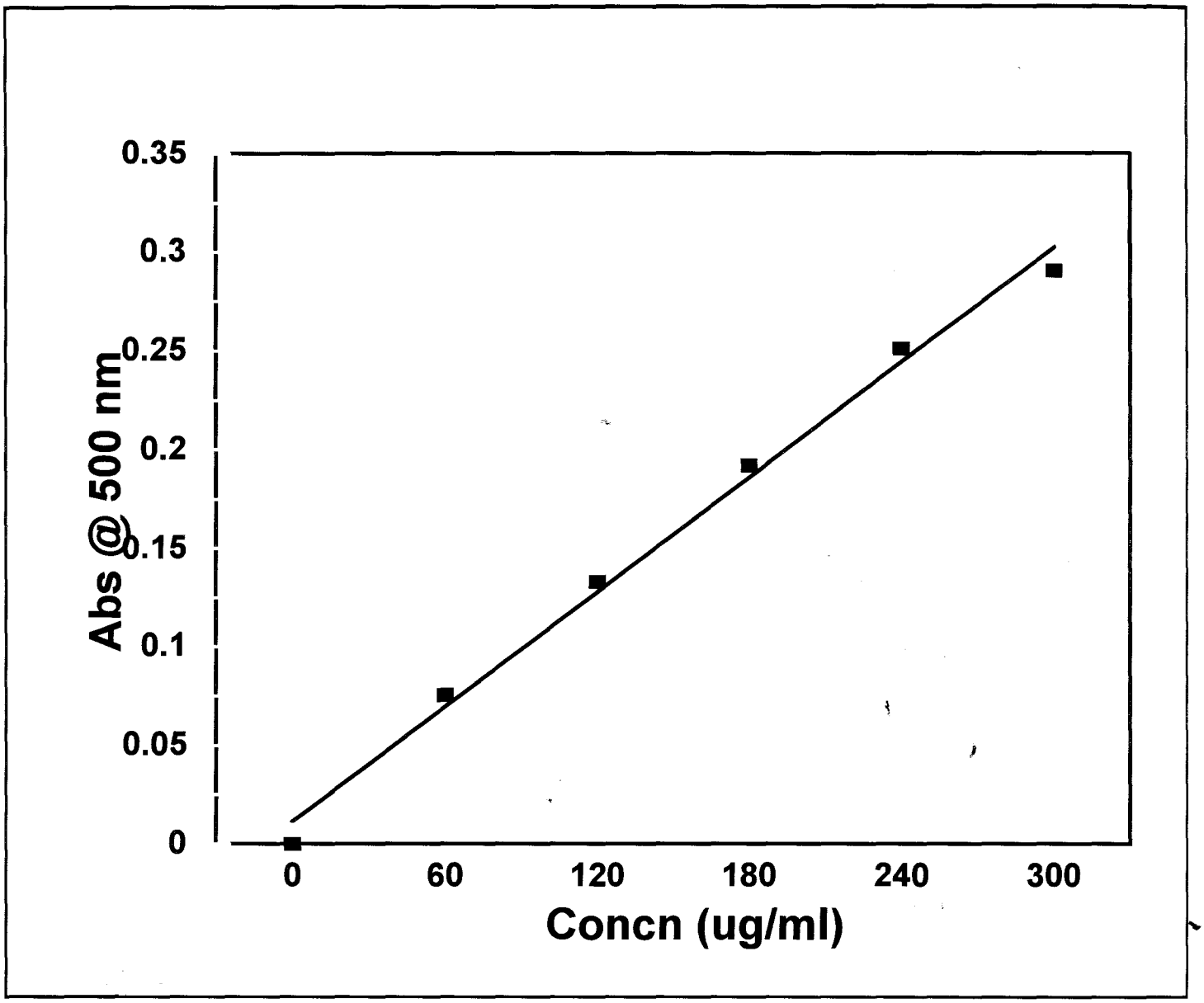

Figure 2.1 Protein standard curve using bovine serum albumin (BSA)

[Data points represent mean of triplicate determinants (r^2 = 0.997)]

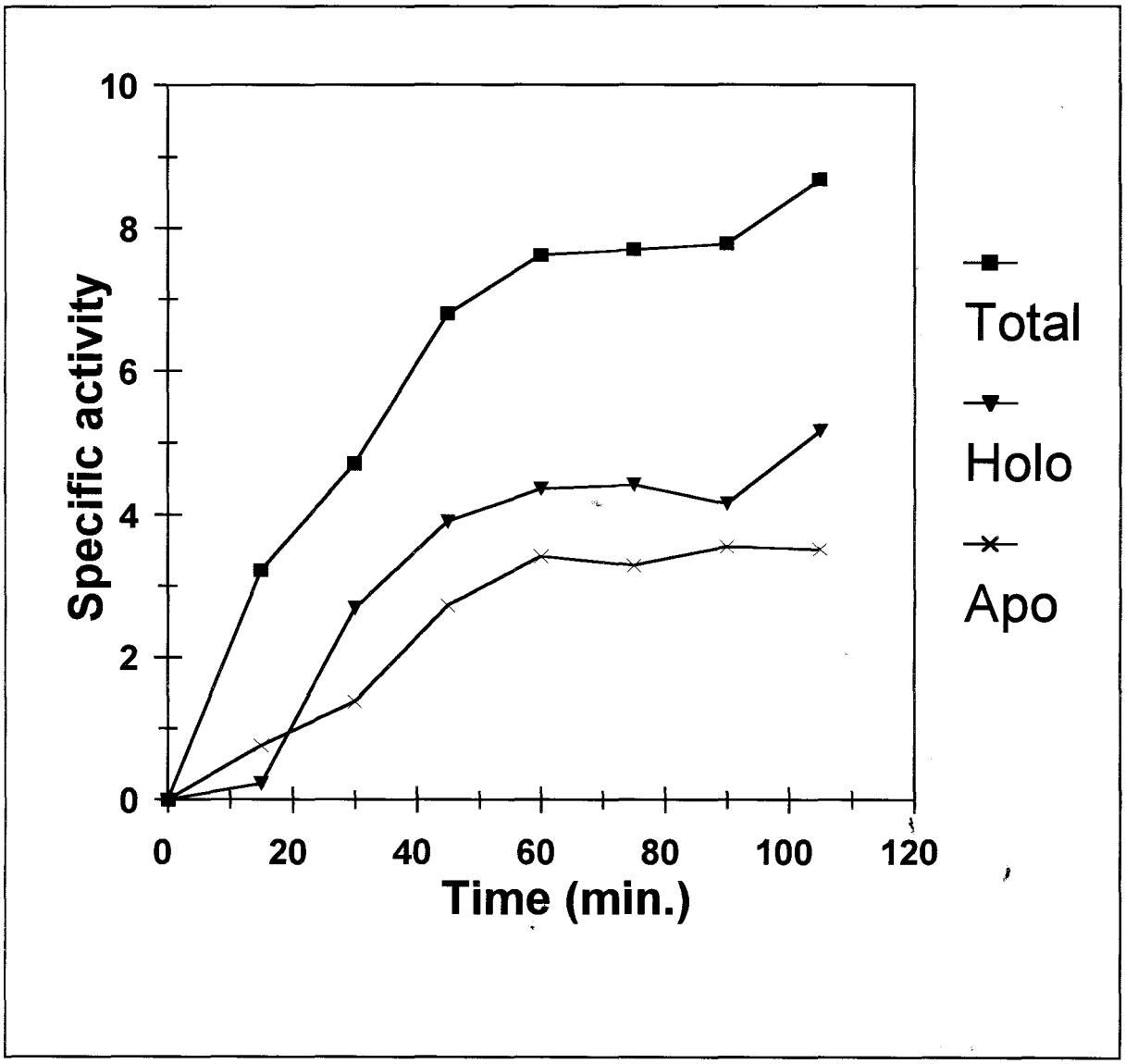

Figure 2.2 Time-course of TDO activity in the presence (total enzyme) and the absence (holoenzyme) of haem and the difference between the two (apoenzyme).

Specific activity is expressed as nmoles of L-kynurenine formed per mg protein per min.
[Data points represent mean of triplicate determinants]

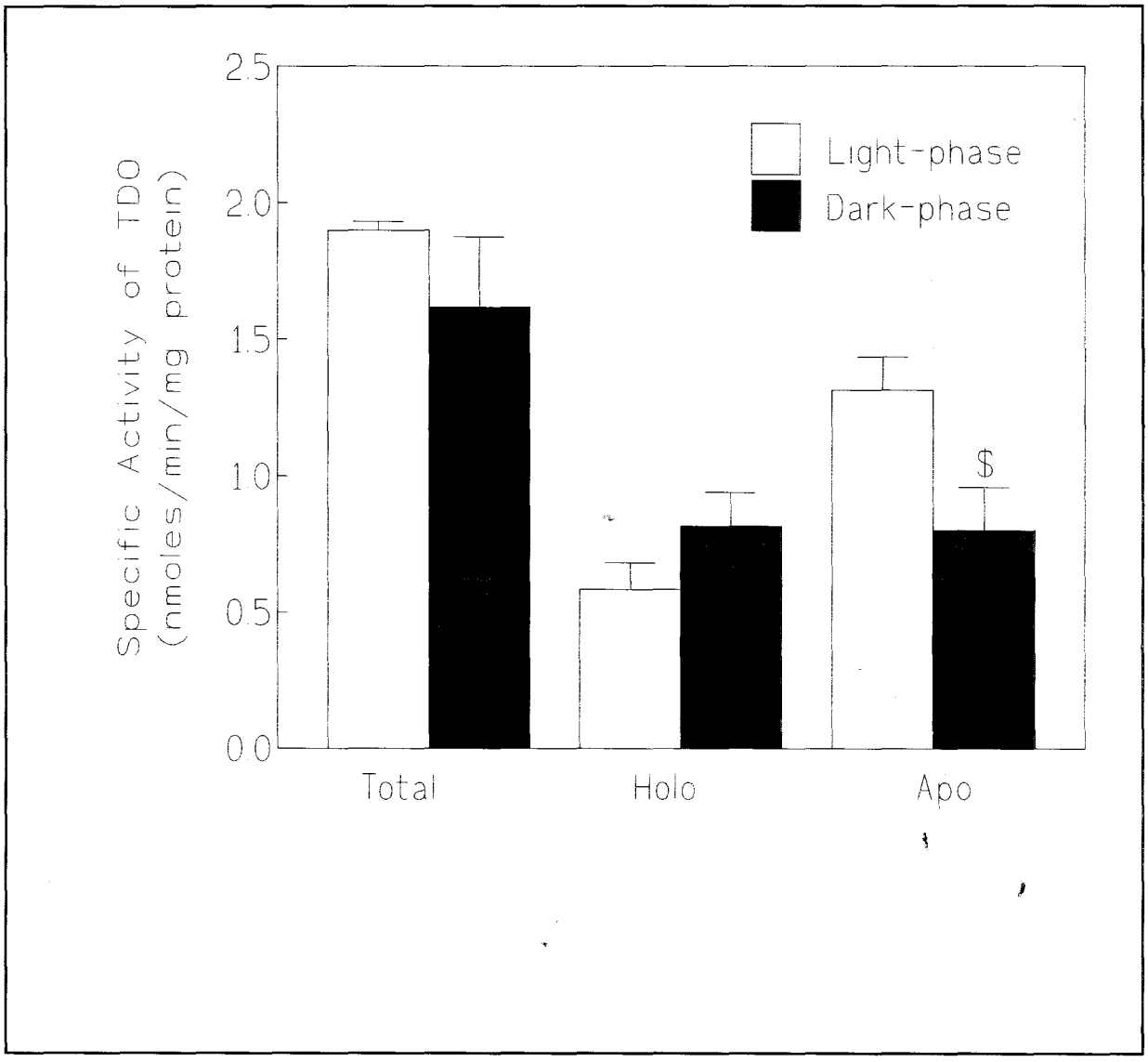

Figure 2.3 Assessment of light-dark variations of hepatic TDO activity

[Data represents mean ± SEM; n = 5; ($): P < 0.05; Light - phase: TvsH: P < 0.001; TvsA: P < 0.01; HvsA: P < 0.001; Dark - phase: TvsH: P < 0.05; TvsA: P < 0.05; HvsA: P > 0.05]

T: Total enzyme activity
H: Holoenzyme activity
A: Apoenzyme activity

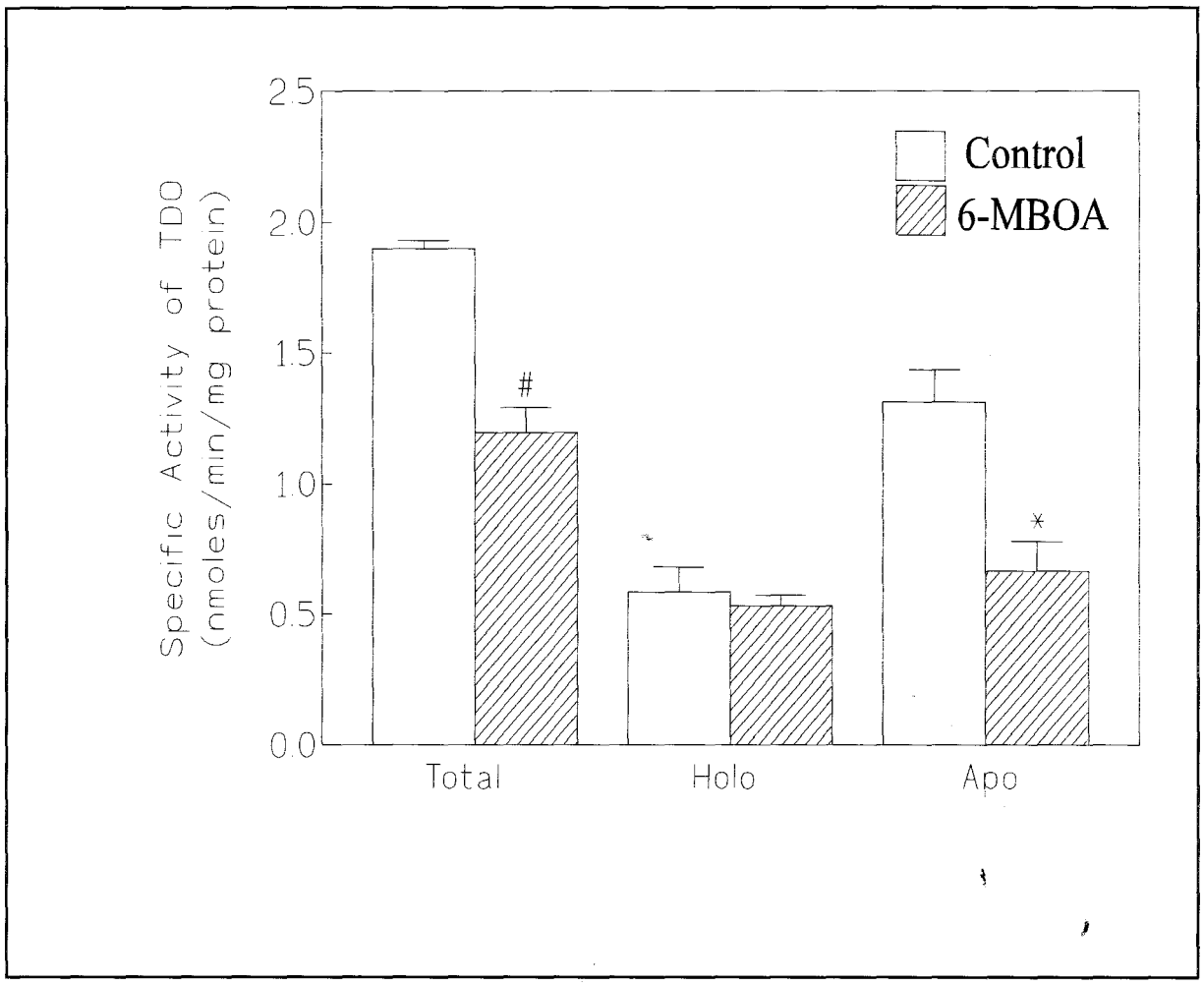

Figure 2.4 Determination of the effect of 6-MBOA on hepatic TDO activity: Photo-phase samples

[Data represents mean ± SEM; n = 5; (*): P < 0.01; (#): P < 0.001 TvsH: P < 0.001; TvsA: P < 0.01; HvsA: P > 0.05]

T: Total enzyme activity
H: Holoenzyme activity
A: Apoenzyme activity

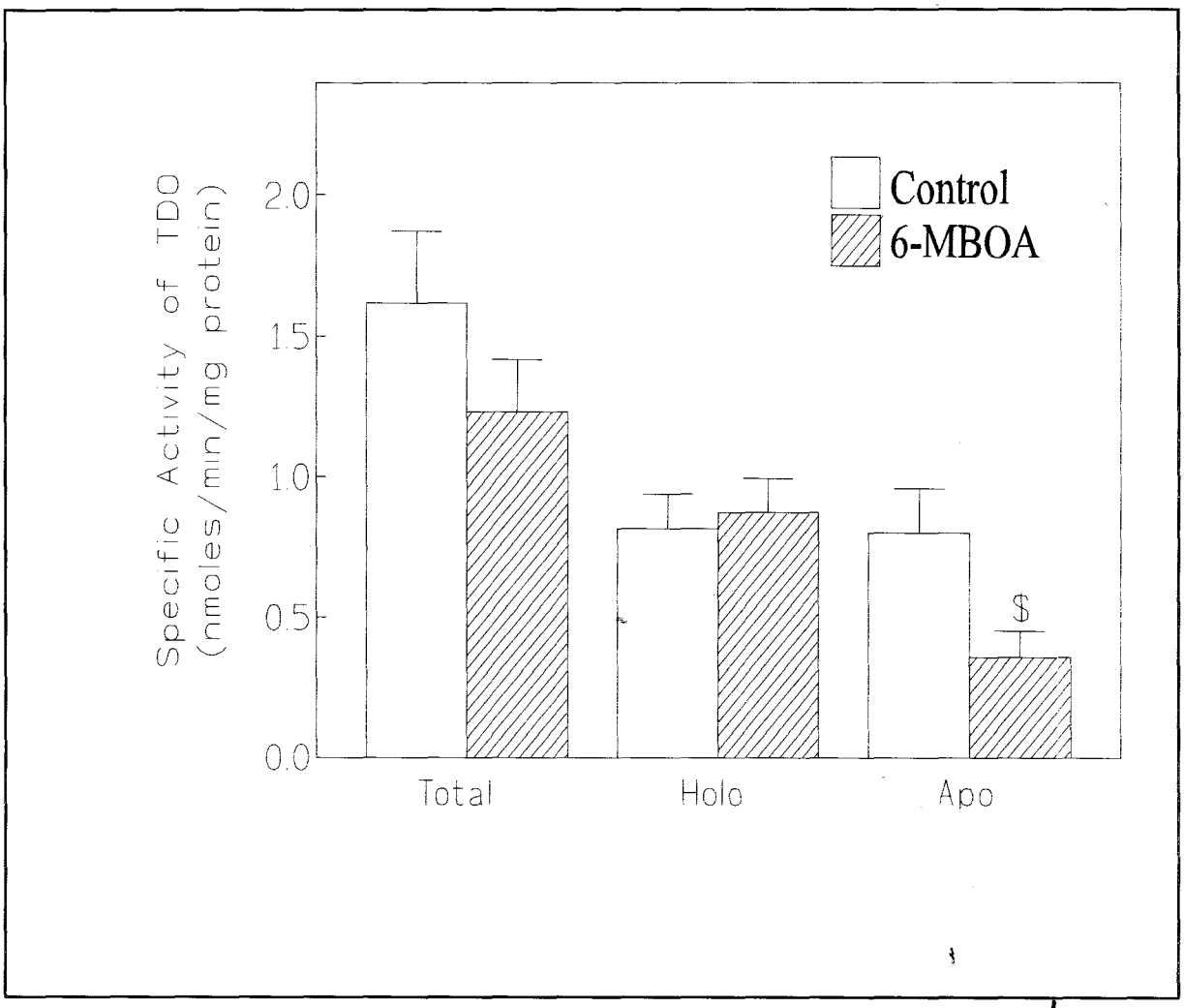

Figure 2.5 Determination of the effect of 6-MBOA on hepatic TDO activity: Scoto - phase samples

[Data represents mean ± SEM; n = 5; ($): P < 0.05; TvsH: P > 0.05; TvsA: P < 0.01; HvsA: P > 0.05]

T: Total enzyme activity
H: Holoenzyme activity
A: Apoenzyme activity

Table 2.3 Percentage reduction caused by 6-MBOA on hepatic TDO activity

PHOTO - PHASE	
Activity	**% Inhibition**
Total	**63**
Holo	**9.1**
Apo	**51**
SCOTO - PHASE	
Activity	**% Inhibition**
Total	**24**
Holo	**-**
Apo	**45**

after18h00, enzyme synthesis starts to be reduced. TDO activity thus decreases until synthesis is re-initiated (Hardeland and Rensing, 1968). The significant reduction of apoenzyme activity during the scoto-phase is in accordance with an inhibitory effect of nocturnal aMT at the level of haem induction (Walsh and Daya, 1997). Total activity showed an insignificant (Student's t-test) reduction during the scoto-phase, however, there was a trend towards a substantial increase in holoenzyme activity during this period, but non-significant (Student's t-test). The increase in holoenzyme activity could suggest that most TDO apoenzyme is not saturated with tryptophan during the scoto-phase and thus the apoenzyme remains oxidised. Alternatively, conjugation of the apoenzyme with haem is disturbed during this phase which also results to an inactive apoenzyme, hence holoenzyme activity is elevated during this phase.

TDO is a key enzyme that regulates the availability of tryptophan to the brain since it initiates the

catabolism of tryptophan. Tryptophan is crucial for the biosynthesis of biogenic indoleamines including the neurotransmitter (NT) 5-HT and aMT which play a vital role in affective disorders such as depression. aMT has been shown to be a more specific competitive inhibitor of the liver TDO *in vitro* as compared to other indoleamines (Walsh *et. al*, 1994). Preliminary studies done in our lab have shown that 6-MBOA, a structural analogue of aMT that is a more potent inhibitor of this enzyme than aMT *in vitro*.

In this study, 6-MBOA is shown to cause a significant inhibition of TDO apoenzyme activity *in vivo* with more potency occurring during the photo-phase (figure 2.4 and figure 2.5). No significant change was observed in both scoto- and photo-phase holoenzyme activity, but there was an insignificant increase in holoenzyme activity during the scoto-phase as seen in vehicle treated animals. The low inhibition of apoenzyme activity by 6-MBOA during the scoto-phase could be due to difficulty of 6-MBOA to overcome the increase in basal TDO (holoenzyme) activity.

The possible explanation for this difference could be that since during the scoto-phase the enzyme activity is at its minimum, the effect of 6-MBOA is thus not as apparent as it is during the photo-phase. Another possibility could be that 6-MBOA does not have an effect on the enzyme activity but other indoleamines such as aMT and 5-HT which have highest production during the dark-phase and also reported to inhibit the enzyme activity are causatives of this inhibition. TDO substrate, L-tryp at high concentrations is reported to have inhibitory effects on TDO activity. During the scoto-phase, TDO activity is reduced thereby increasing circulating tryptophan which can in turn inhibit TDO activity. Another possible mechanism for the smaller reduction in TDO

activity in scoto-phase samples may be due to an enhanced holoenzyme activity as explained above.

Two binding sites of the enzyme to the substrate are known to exist. One site is catalytic and the other is regulatory (Ishimura *et. al.*, 1970; Uchida *et. al.*, 1985). At low substrate concentrations, binding takes place only in the catalytic site and with increasing substrate concentrations, additional binding can occur at the regulatory site (Ishimura *et.al.*, 1970). The NH group is critical for the molecule to be capable of acting as a substrate for TDO. NH forms a very strong hydrogen bonding (H-bonding) with the acidic amino acid side chain. Conservation of the $CHNH_2COOH$ in the carbon, is essential for the NH-fastened molecule in the catalytic site to take a configuration acquired for the reaction.

Mode of inhibition in the catalytic-site of TDO can be exerted in one of the three possible ways. Firstly, by binding of the substrate analogue which cannot act as a substrate although it has a reactive potential, because it cannot take the correct configuration due to a lack of functional group(s) ($RCHNH_2COOH$) for proper interactions with the protein part of the catalytic site. Secondly, the substrate analogue that contains appropriate functional groups and takes a correct configuration and is potentially reactive but contains other functional group(s) (OH or CH_3) that may interact with protein side chain by acting as electron donors, leading to a marked reduction in the catalytic ability of the haem pocket (Uchida *et. al.*, 1992).

From the above insight concerning the catalytic status of TDO, 6-MBOA appears to qualify as a substrate analogue of TDO as it contains the NH group that is indispensable for the molecule to

act as a substrate. However, NH requires the functional group $CHNH_2COOH$ for proper interactions with the catalytic site to be competent and 6-MBOA lacks that particular group. 6-MBOA also contains CH_3 in position 5 which forms H-bonding with protein side chains that may lead to distortion and stiffening of the protein structure in the haem pocket thus leading to reduction in flexibility required for the catalysis to occur. Therefore, inhibition of TDO activity by 6-MBOA probably occurs via one of the mechanisms discussed above.

CHAPTER 3

3.1 THE EFFECT OF 6-MBOA ON BRAIN SEROTONIN (5-HT) LEVELS

3.1.1 INTRODUCTION

5-HT is a biogenic amine which was first discovered in the intestine (Vialli and Espamer, 1983) and has been characterised in the brain for the last five decades. This has prompted intense investigation on the physiological roles of 5-HT. 5-HT is an intermediate product of tryptophan metabolism and the rate limiting enzyme is tryptophan hydroxylase (TH). 5-HT is primarily located in the enterochromaffin cells of the intestine, serotonergic neurons in the brain, platelets of the blood, and in the CSF. Central serotonergic neurons have been associated with physiological functions such as sleep, endocrine regulation and cardiovascular control. Most 5-HT that is found in circulating blood is concentrated in platelets (http://www.IBL-Hamburg.com).

The concentration of 5-HT is decreased in brains of subjects with affective disorders such as depression (http://www.virtuvites.com). However, pathological conditions such as chronic tension, headache, schizophrenia and hypertension are also reported to manifest alterations in 5-HT concentrations. Therefore, drugs which enhance 5-HT production are very important in

treatment of such disorders. Hence this study will evaluate whether 6-MBOA exhibits potential antidepressant effects or not. This will be achieved by quantifying brain 5-HT levels before and after 6-MBOA treatment.

In this study, a serotonin-ELISA technique was employed to quantitatively measure brain 5-HT levels. The technique is used to measure chemically derivatized serotonin in serum, plasma, urine, tissue homogenates and tissue culture supernates. Here, the forebrain tissue homogenate was used as the sample. The sample preparation (derivatization) was achieved by dilution of the sample followed by incubation of the respective sample with the 'Acylation Reagent'.

Serotonin determination is mainly done by using High Performance Liquid Chromatography (HPLC). The reason for choosing Serotonin-ELISA technique is because there are a number of difficulties relating to the application of HPLC method such as pump malfunctioning or column blocking. This in turn interferes with the final result and can give broad peaks which are difficult to interprete.

The assay procedure conforms to the basic principle of competitive ELISA whereby competition between a biotinylated and a non-biotinylated antigen for a fixed number of antibody binding sites transpires. The amount of biotinylated antigen bound to the antibody is inversely proportional to the concentration of the anaylate of the sample. When the system reaches equilibrium, the free biotinylated antigen is removed by a washing step and the antibody bound biotinylated antigen is determined by use of an anti-biotin alkaline phosphatase as a marker and p-nitrophenyl phosphate

as a substrate. Unknowns are quantitatively measured by comparing the enzymatic activity of unknowns with a response curve prepared by using known standards (http://www.IBL-Hamburg.com).

3.1.2 ANIMALS

Male Wistar rats of the albino strain weighing 200 - 250 g were randomly assembled into two groups of five and maintained as described in section 2.2. Administration of 6-MBOA to rats was done as described in section 2.2.1. Animals were sacrificed between 12h00 and 13h00.

For removal of forebrain, an incision using a pair of scissors was made through the bone from the foramen magnum to near to orbit. The top of the skull was lifted with a pair of forceps exposing the forebrain for removal with tweezers. Care was taken to remove all adhering tissue and visible traces of blood from the forebrain. Immediately after removal, the forebrain was frozen in liquid nitrogen and stored at -70 °C until required.

3.1.3 CHEMICALS AND REAGENTS

3.1.3.1 CHEMICALS

All chemicals were purchased from the Research Diagnostics Inc., Pleasant Hill Rd, Flanders and were of the highest purity available.

3.1.3.2 REAGENTS

ASSAY BUFFER: 15 ml of the Assay Buffer Concentrate was diluted 1:10 with bidistilled water up to 150 ml. The buffer was stored at 2 - 8 °C until use.

WASH BUFFER: 15 ml of the Wash Buffer Concentrate was diluted 1:20 with bidistilled water up to 300 ml. The buffer was stored at 2 - 8 °C until use.

CONTROL SERA: Added 0.5 ml of bidistilled water to each vial and let stand for 15 minutes. The mixture was then gently swirled.

ANTI-BIOTIN-AP: Diluted 60 μl of the Enzyme Conjugate Concentrate in 6.0 ml of Assay Buffer (ready for use). This was prepared freshly prior to use.

PNPP SUBSTRATE SOLUTION: Dissolved 3 PNPP Substrate Tablets in 8 ml of PNPP Substrate Buffer. The solution was prepared 10 minutes prior to use.

3.1.4 HOMOGENATE PREPARATION

The frozen forebrains were removed from -70 °C and thawed. Thereafter, the forebrains were dried with a paper towel and weighed. The forebrains were homogenised in 10 % w/v of 0.9 %

saline with a glass homogeniser. 20 μl of the homogenate was used for quantification of forebrain 5-HT. Each sample was done in duplicate.

3.1.5 SAMPLE PREPARATION

After removal of assay reagents from the refrigerator, reagents were allowed to reach room temperature before pipetting. To 20 μl of 10 % w / v tissue homogenate prepared in 0.9 % saline, 100 μl of Assay Buffer was added. 25 μl of Acylation Buffer 1 (3 %) was added to the mixture and immediately vortexed. The mixture was then incubated for 15 minutes at 37 °C. Following incubation, 4 ml of Assay Buffer was added and the mixture was vortexed and centrifuged in a benchtop centrifuge for 10 minutes at 1.500 x g.

3.1.6 ASSAY PROCEDURE

To 50 μl each of standard and acylated tissue homogenate, 50 μl of Serotonin-Biotin and 50 μl Antiserum were added and the plate was carefully shaken. The mixture was incubated for overnight at 2 - 8 °C. Thereafter, the mixture was washed 3 times with Wash Buffer and 150 μl of Anti-Biotin-AP was added to it. This was followed by incubation for 120 minutes at room temperature on an orbital shaker (200 rpm). After the incubation, the mixture was washed 3 times with Wash Buffer followed by an addition of 200 μl of PNPP Substrate Solution. The mixture was incubated at room temperature for 60 minutes on an orbital shaker (200 rpm) as before and the reaction terminated by an addition of 50 μl of PNPP Stop Solution. The contents were briefly

mixed by gently shaking the plate and the optical density was read at 405 nm within 60 minutes after termination of the reaction.

3.1.7 RESULTS

The logarithmic concentrations of the standards were plotted against their corresponding optical densities. Each sample was done in duplicate and the concentrations of the samples were read directly from the standard curve by using their average optical density. The sample preparation lead to a 207.25 fold dilution, hence the values read from the standard curve had to be corrected by being multiplied by 207.25. Furthermore, a 10 fold dilution of tissue homogenate was also taken into consideration for calculation of the final concentration of 5-HT.

Results were analysed by one-way analysis of variance (ANOVA) and the statistical differences between the groups were determined using the student t-test. A significant rise in brain 5-HT production was observed after 6-MBOA administration ($P < 0.05$) as shown in figure 3.2.

3.1.8 DISCUSSION

The initial step in brain 5-HT biosynthesis involves hydroxylation of L-tryptophan by the enzyme tryptophan hydroxylase (TH), a rate limiting enzyme (Boadle-Biber, 1982). However, the concentration of tryptophan usually present in neurons that produce 5-HT may not be sufficient to saturate TH. Thus, brain serotonin synthesis may normally be limited by the availability of

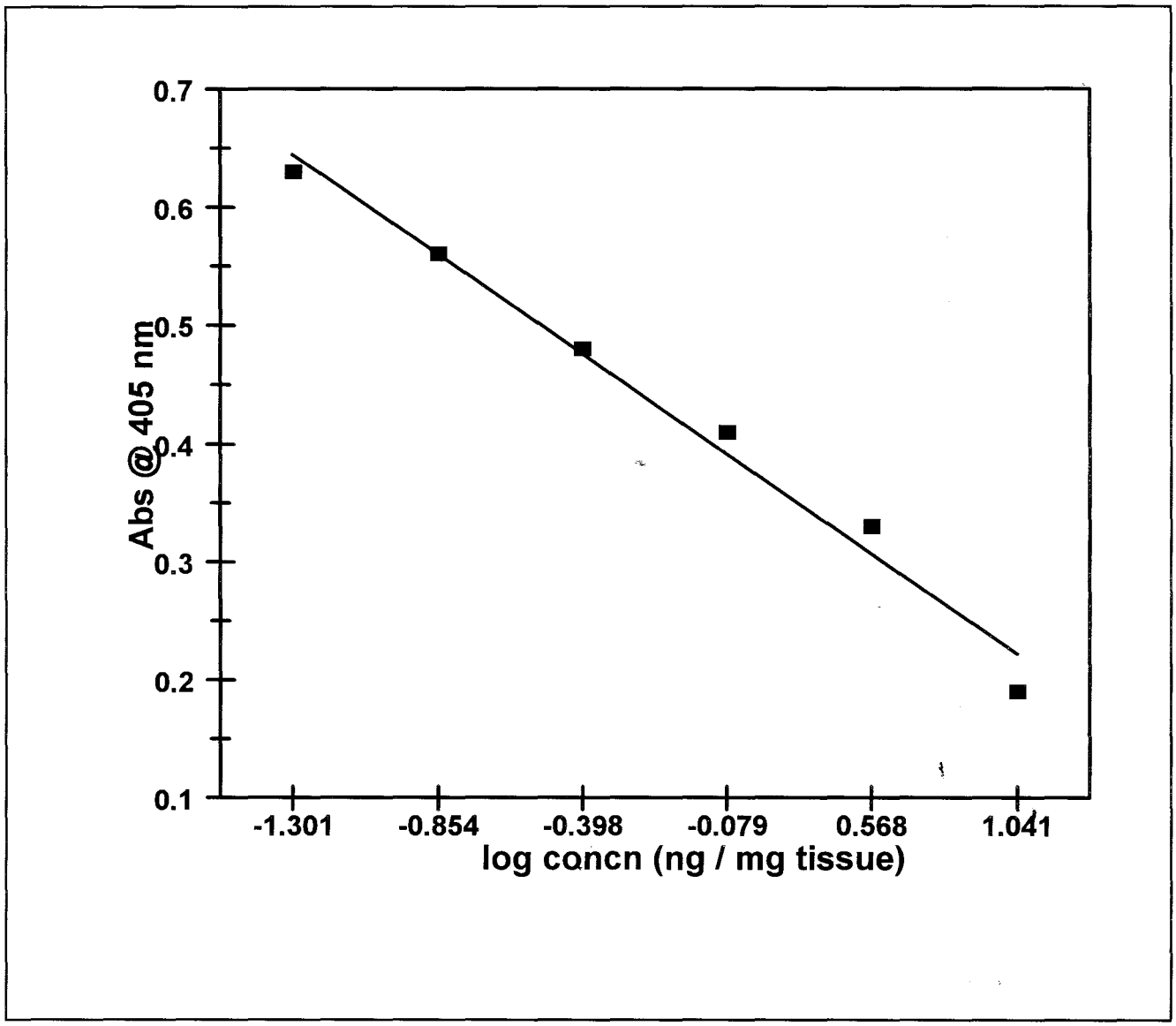

Figure 3.1 **Brain serotonin standard curve using the competitive ELISA technique**

[Each data point represents mean of triplicate determinants, $r^2 = 0.987$]

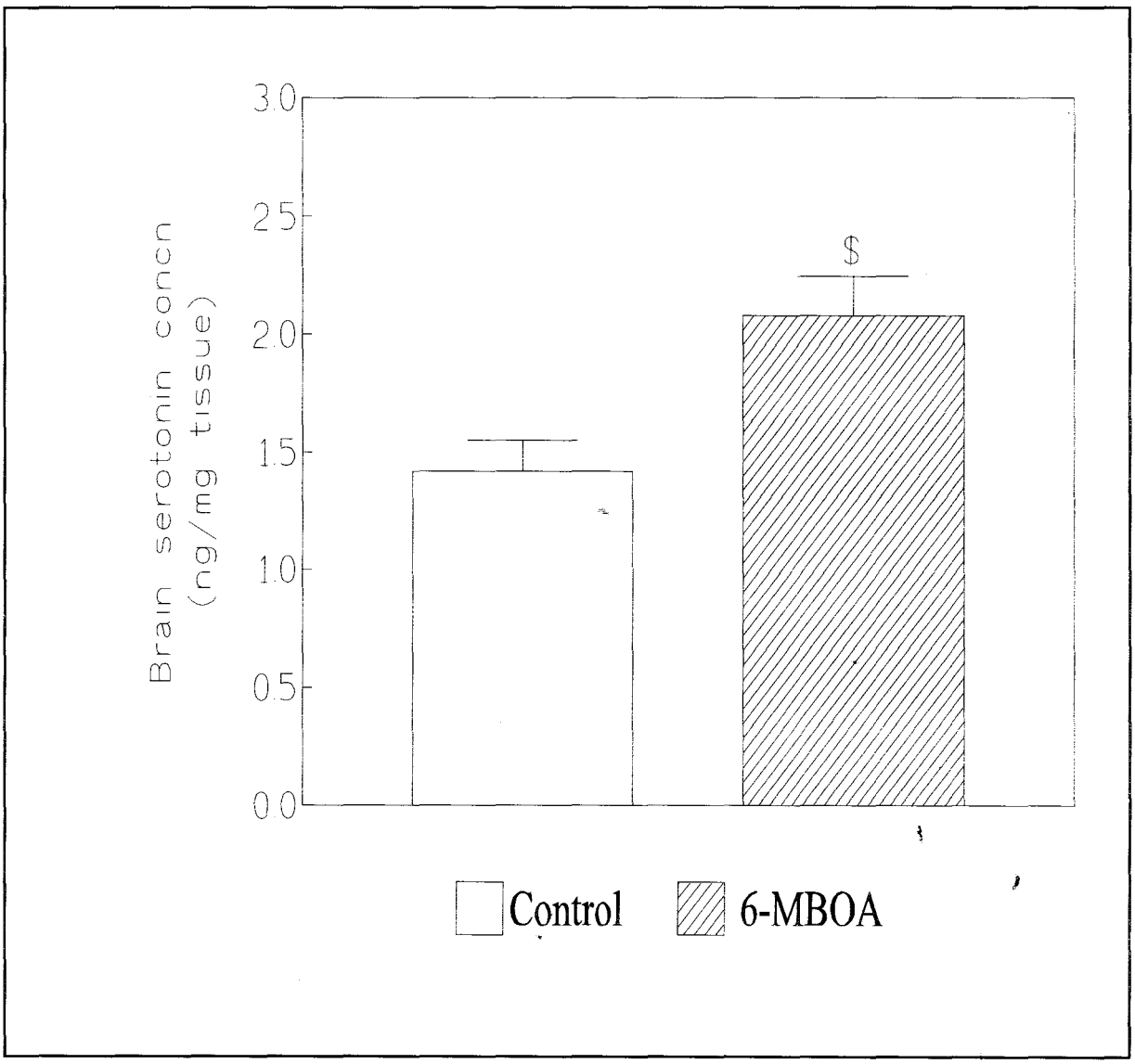

Figure 3.2 Determination of the effect of 6-MBOA on serotonin (5-HT) production using the competitive ELISA technique

[Data represents mean ± SEM: n = 5; ($): P < 0.05]

tryptophan. (Mckean *et. al.*, 1967). A variety of drugs and treatments thought to accelerate brain 5-HT biosynthesis have been shown by many researchers to induce parallel changes in brain tryptophan content. Hence, any drug which increases tryptophan availability to the brain is essential for the biosynthesis of 5-HT.

6-MBOA, a structural analogue of 5-HT has been shown in this study to augment brain 5-HT levels. An increase in 5-HT biosynthesis could be due to an increase in tryptophan availability which can result to stimulation of TH. Another possible explanation could be that 6-MBOA stimulates the release of stored 5-HT within nerve terminals into the synapse.

3.2 THE EFFECT OF 6-MBOA ON TRYPTOPHAN HYDROXYLASE (TH) ACTIVITY

3.2.1 INTRODUCTION

Having established that 6-MBOA enhances 5-HT production, it was necessary to investigate whether the effect was in par with the stimulation of TH, a rate limiting enzyme in 5-HT biosynthesis. Furthermore, a limiting factor in 5-HT biosynthesis is the bioavailability of tryptophan and TH is usually not saturated with tryptophan, which is its substrate. But interestingly, the previous study has shown that 6-MBOA inhibits hepatic TDO activity thus leading to a rise in tryptophan available to the brain, therefore this lead to a more positive

apprehension for stimulation of the enzyme.

Tryptophan hydroxylase, a brain monooxygenase catalyses the rate limiting reaction in the synthesis of 5-hydroxy-indoles, including 5-HT.

$$\text{L-Tryp} + BH_4 + O_2 \Rightarrow \text{5-HTP} + BH_2 + H_2O$$

The substrate for this enzyme is L-tryp and the natural cofactor is tetrahydrobiopterine (BH_4). However, there are some aspects which affect the enzyme activity and one of them is the diurnal rhythm of circulating L-tryp and 5-HT. It has been suggested that an average of 5 μg / g of brain content of the amino acid is obtained but higher values have also been reported, ranging from 2.5 - 4.5 x 10^{-5} M (McKean *et. al.*, 1968). An accepted km value of 3 x 10^{-4} M L-tryp for both crude and partially purified enzymes have been reported (Jequier *et. al.*, 1969; Ichiyama *et. al.*, 1970). This km is somewhat an incautious conclusion because the km is much higher than the free typtophan in the brain thus leaving the enzyme unsaturated. This leads to an intriguing concept that tryptophan concentration in the brain may be the limiting factor in the synthesis of 5-HT, but *in vivo* it is clear that TH does not operate under substrate limitation.

TH in the brain was first demonstrated by Grahame-Smith in 1964 . Grahame-Smith showed that homogenates from the dog and rabbit brain stems hydroxylate L-tryp to produce 5-HTP (Grahame-Smith, 1964). Investigations on the partial purification and some properties of the monooxygenase rabbit brain stem have been briefly described (Nakamura *et. al.*, 1965). However, the relatively low activity of this enzyme and the lack of sensitive and convenient assay have hindered further studies on purification and properties of the enzyme as well as the reaction

mechanism involved.

The present study undertaken follows the reaction of the radioactive L-tryp as the substrate for the enzyme and tritiated water used as a measure of TH activity. When ^{3}H-L-tryp is hydroxylated, 5-^{3}H is replaced by a hydroxyl group. The displaced tritium exchanges with water and one mole of tritiated water is formed per mole of 5-HTP formed.

3.2.2 ANIMALS

Male Wistar rats of the albino strain weighing 200 - 250 g were randomly assembled into two groups of five and maintained as described in section 2.2. Injections were done as described in section 2.2.1. The animals were sacrificed between 12h00 and 13h00.

The forebrains were removed as described in section 3.1.2. Immediately after removal of forebrains, the brain stems were separated by a spatula, weighed and homogenised with 20 % w/v 0.01 M phosphate buffer. 4 ml of crude enzyme homogenate was used for determination of TH activity. Each sample was done in triplicate.

3.2.3 CHEMICALS

All chemicals were of the purest quality. DL-5-^{3}H-tryptophan (36 μCi / mmol), L-tryptophan and DL-5-hydroxytryptophan (DL-5-HTP) were purchased from Amersham, Life Sciences, England.

3.2.4 CRUDE TH ASSAY PROCEDURE

0.95 ml of DL-^{3}H-Tryp - L-Tryp (final concentration of 0.2 mM) was added to 4 ml of crude enzyme homogenate. 0.05 DL-5-HTP was then added as 5-HTP decarboxylase inhibitor and the mixture was aerated with carbogen (95 % O_2 and 5 % CO) and sealed. This step was followed by incubation at 37 °C for 1 hour thereafter the reaction was terminated by the addition of 3 ml of 30 % TCA. The tubes were then left to stand at room temperature for 1 hour to allow complete extraction of product. Tritiated H_2O was removed by distillation. To 1 ml of distillate, 3 ml of scintillation fluid was then added to ^3H_2O and the radioactivity was counted in a LS-2800 Beckmann scintillation counter.

3.2.5 RESULTS

The results for determination of TH activity were expressed as dpm's ^3H_2O / ml distillate. Statistical difference between the two groups was determined by use of Student's t-test. Figure 3.3 indicates a significant increase ($P < 0.05$) in TH activity effectuated by *in vivo* administration of 6-MBOA.

3.2.6 DISCUSSION

TH a rate limiting enzyme in 5-HT biosynthesis utilises tryptophan as a substrate(Reiter, 1980).Para-chlorophenylalanine (PCPA), a powerful depletor of tissue 5-HT *in vivo* appears to

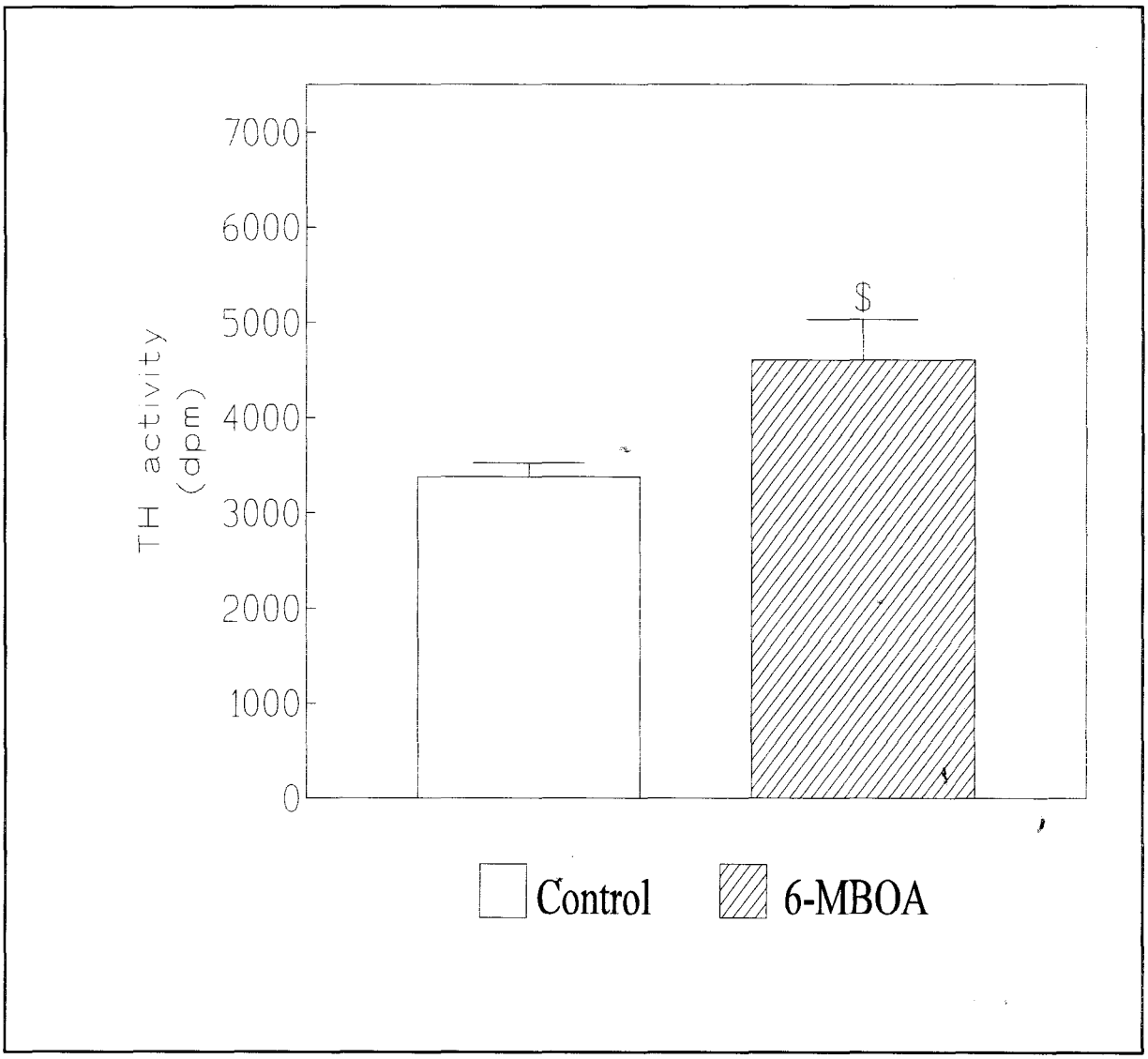

Figure 3.3 Determination of the effect of 6-MBOA on brain-stem TH activity

[*Data represents mean ± SEM; n = 5; ($): P < 0.05*]

be a potent inhibitor of TH (Reiter, 1989). 6-MBOA on the other hand appears to enhance TH activity. The crucial prerequisite for TH catalytic activity is suggestive to be the amino group that contains no bulky side chain that may lead to distortion or reduction of the catalytic activity of the enzyme as is the structure of PCPA. It can thus be suggested that 6-MBOA might enhance TH activity by acting as a substrate for the enzyme. Alternatively, the increased bioavalability of tryptophan caused by inhibition of hepatic TDO activity by 6-MBOA might lead to the saturation of the enzyme and consequently enhancing the enzyme activity.

3.3 DETERMINATION OF THE EFFECT OF 6-MBOA ON BINDING OF RADIOACTIVE SEROTONIN ON SYNAPTOSOMAL MEMBRANE

3.3.1 INTRODUCTION

Brain cells communicate with each other by sending NT's to each other. The NT is released from the nerve terminals located at the end of the axons. After diffusing across the synapse, the NT binds a receptor located on the surface of the post-synaptic cell. Binding of NT to receptor induces, inhibits or modulates currents of ions across the cell membrane (South, 1997).

Human mood disorders such as depression are effectively treated with drugs which specifically

block the reuptake of 5-HT (a NT essential in the treatment of depression) into the presynaptic axon terminal. 5-HT is synthesized within the nerve cell from tryptophan. When 5-HT is synthesized, it can be stored within the presynaptic or membrane vesicles within the nerve terminals. Most of the released 5-HT is reabsorbed by the nerve terminals for repackaging and reuse. The re-uptake site (carrier or transporter), a specialised proteinaceous structure located in the nerve terminal membrane is responsible for this process. 5-HT uptake sites can be quantified by either measuring the rate of the uptake itself or by binding of radioactively labelled ligands that bind specifically to the uptake carrier and subsequently measuring the radioactivity (http://www.ecstasy.org/leon.html).

An important aspect of the membrane preparation is to be able to obtain high density binding sites and eliminate a large portion of endogenous contaminants which may interfere with the assay. Subcellular fractionation techniques are often designed to isolate synaptic membrane based on one or two morphological or biochemical criteria. The major drawback for the use of these techniques is their inability to isolate the total synaptic membrane fraction. However, the use of crude preparations leads to relatively high levels of non-specific binding. The choice of a synaptic membrane preparation should entail prevention of the loss of membranous fractions thereby reducing the extent of non-specific binding.

In the present study, the technique of binding of radioactive 5-HT to synaptosomal membrane was employed. The procedure for the preparation of cerebral synaptosomal membrane was followed as described by Foster *et. al*,.1981, Cotman and Taylor, 1972 and Brose *et. al.*, 1990. This

procedure involves the removal of endogenous compounds which interfere with the binding of the ligand to the membrane. A crucial step in this procedure involves the lysis of synaptosomes and membrane vesicles by osmotic or mechanical shock and subsequent washing by repeated centrifugations to rid the membrane of any contaminants. The aim of this study was to investigate the effect of 6-MBOA on binding of 5-HT to synaptosomal membrane *in vivo*.

3.3.2 ANIMALS

Male Wistar rats of the albino strain weighing 200 - 250 g were randomly assembled in two groups of five and maintained as described in section 2.2. Injections were done as described in section 2.2.1. The animals were sacrificed between 12h00 and 13h00.

The forebrains were removed as described in section 3.1.2 and immediately frozen in liquid nitrogen and kept at -70 °C until required.

3.3.3 CHEMICALS

All chemicals were of the highest purity. Hydroxy-[side-chain-2-^{14}C]tryptamine creatine sulphate (56 mCi / mmol) was purchased from Amersham, Life Sciences, England, Sucrose purchased from Riedel-deHaën, Trizma and acetate from Sigma Chemical Co., Canada.

3.3.4 PREPARATION OF CEREBRAL SYNAPTOSOMAL MEMBRANE

The forebrains were rapidly removed from the rats after sacrifice as described previously in section 3.1.2. All the following steps were performed on ice and centrifugations perfomed at 4 °C.The forebrains were pooled together and homogenised in ice cold 10 % (w / v) 0.32 M sucrose in a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 1500 x g for 5 minutes in a Beckman centrifuge with a JA 21 rotor. The supernatant was put aside on ice while the pellet was washed by resuspending it in a similar volume of ice cold sucrose and re-centrifuging as before. The supernatants from both steps were pooled together and centrifuged at 17 000 x g for 10 minutes in a Beckman L8 -80M ultracentrifuge with a 70 Ti rotor. The supernatant was discarded while the pellet was resuspended in 20 ml of 200 μM tris acetate buffer, pH 7.2 and incubated for 15 minutes at 4 °C.. Thereafter, the suspension was centrifuged at 50 000 x g for 25 minutes in a Beckman L8 - 80 ultracentrifuge with a 70 Ti rotor. The pellet as recovered and washed four times by resuspending it in 200 μM tris acetate buffer, pH 7.2 andre-centrifuging as before. The final pellet was resuspended in 10 % sucrose and layered onto a discontinous sucrose density gradient which consisted of 10 ml of each 0.85 M, 1.0 M and 1.3 M sucrose. As the centrifuge tubes were made non-wettable polyallomer, the gradient was layered by adding the lightest sucrose solution first. The solutions were applied to the apex of the tube and the lighter concentration was allowed to float up from the bottom.The gradient was centrifuged in a Beckman L8 - 80M ultracentrifuge with a SW 28 rotor. The synaptic plasma membrane fraction at the 1.0 - 1.3 M sucrose interface was collected, diluted with 200 μM tris acetate buffer, pH 7.2 and centrifuged at 50 000 x g for 20 minutes in a Beckman L8 - 80M ultracentrifuge with

a 70 Ti rotor. The resulting pellet was resuspended in 50 mM tris acetate buffer, pH 7.2 to give a protein concentration of 1 mg / ml.

3.3.5 PROTEIN DETERMINATION OF CEREBRAL HOMOGENATE

The protein concentration of the cerebral homogenate was determined by the method of Lowry *et. al.* (1951) as described in section 2.6.

3.3.6 DETERMINATION OF BINDING OF SEROTONIN ONTO SYNAPTOSOMAL MEMBRANE

The binding procedure is summarized in Table 3.1. The procedure was initiated by addition of 200 μl aliquots of the prepared cerebral homogenate to tubes containing 40 μl of varying oncentrations of [^{14}C]5-HT (0 - 1 μM) made up to a final volume of 250 μl with 50 mM tris acetate buffer, pH 7.2. Non-specific binding was assessed by replacing 10 μl of the tris acetate buffer with 10 μl of 2.5 mM cold 5-HT solution. The tubes were incubated at 20 °C for 30 minutes. The incubation was terminated by the addition of 4 ml ice cold buffer. The samples were then rapidly filtered through pre-wetted Whatman GF/C glass microfilters in a 12 place Millipore sampling manifold. Each filter was washed three times with 4 ml cold buffer. The filters were transferred to scintillation vials containing 3 ml of scintillation fluid and shaken for an hour. Radioactivity was quantified in a Beckman LS 2800 scintillation counter.

3.3.7 RESULTS

The results are expressed as dpm's of specifically bound radioactive 5-HT. Specific binding was calculated as the total binding minus the non-specific binding determined in the presence of 2.5 mM cold 5-HT. 10 mg/kg 6-MBOA was shown to have no effect on specific binding of radioactive 5-HT on forebrain synaptosomal membrane from 0.1 nM to 10 nM and from 800 nM to 1 mM. An insignificant inhibition of 5-HT specific binding was only shown to occur from 50 nM up to 600 nM concentration (figure 3.3).

Table 3.1 Summary for [^{14}C]5-HT Binding Assay

REAGENTS	TOTAL BINDING		NON-SPECIFIC BINDING	
	CNTRL	6-MBOA	CNTRL	6-MBOA
Buffer (μl)	10	10	-	-
[^{14}C]5-HT (μl)	40	40	40	40
5-HT (μl)	-	-	10	10
Homogenate (μl)	200	200	200	200
Incubate at 20 °C for 30 minutes				
Buffer (ml)	4	4	4	4
Filter through Whatman GF / C glass fibres then wash filters 3 x with 4 ml ice cold buffer				
Scintillation fluid (ml)	3	3	3	3
Shake for 1 hour and count radioactivity				

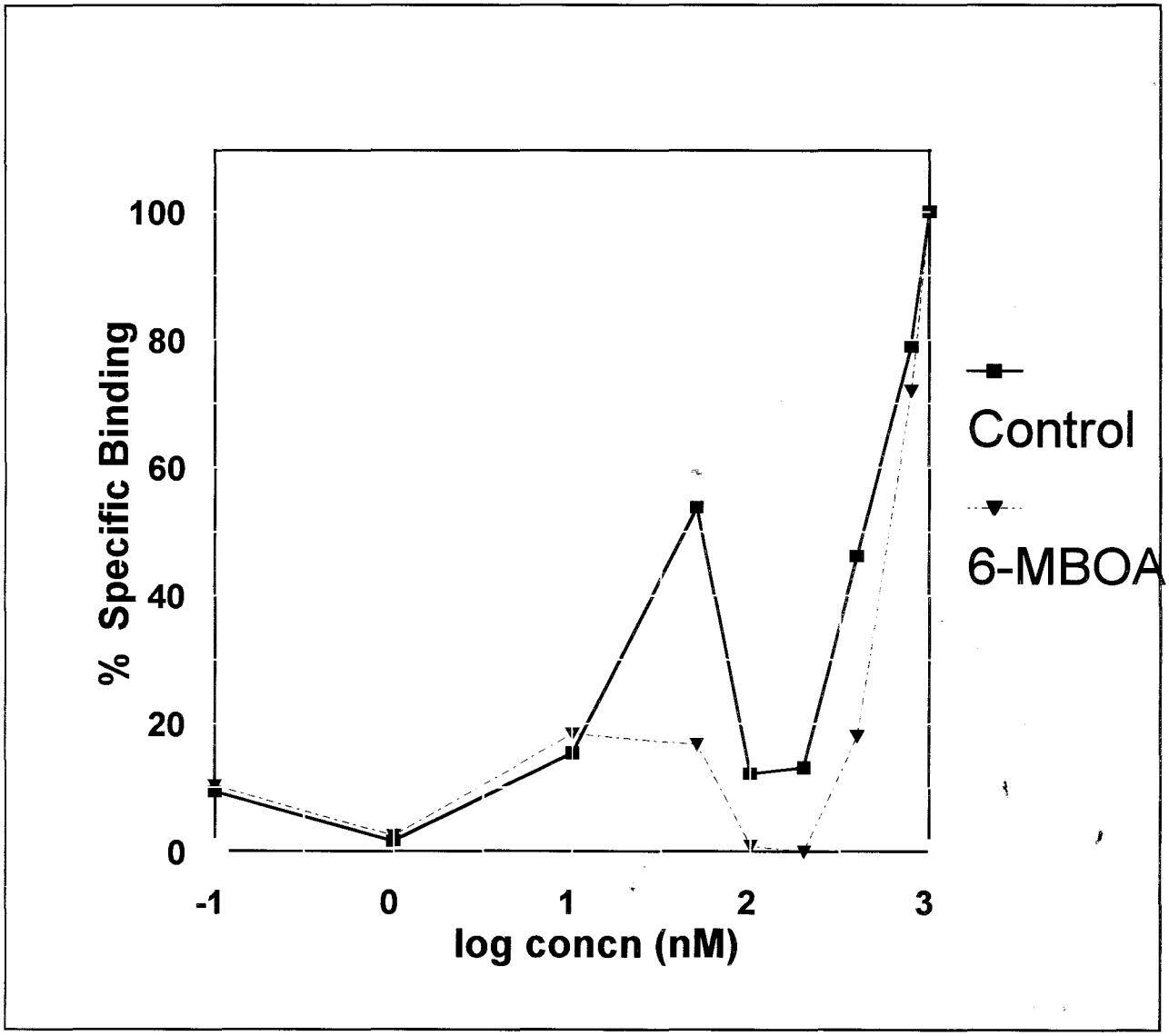

Figure 3.4 Determination of the effect of 6-MBOA on specific binding of radioactive 5-HT on cerebral synaptosomal membrane

[*Data represents mean of triplicate determinants; n = 5*]

3.3.8 DISCUSSION

From the results, 6-MBOA is shown to cause an insignificant reduction of binding of 5-HT to the synaptosomal membrane. Reductions in ligand binding are very difficult to interpret as they may be caused by either reduction in nerve terminals as a result of degeneration or by down regulation of the uptake transporter. Loss of 5-HT nerve terminals is suggested by the reduction in the density of 5-HT uptake carriers as indicated by reduced 5-HT binding of specific 5-HT uptake ligands (Battaglia *et. al.*, 1988). Another possible explanation for the reduction could be that 6-MBOA is a structural analogue of 5-HT so it might compete with 5-HT for binding to the carrier protein that is responsible for the uptake process.

Inhibition of 5-HT uptake is very essential in affective disorders hence drugs which specifically block the uptake of 5-HT are used for the treatment of depression. However, the resultant enhanced 5-HT activation leads to a cascade of reactions which ultimately result in a reduced sensitivity of presynaptic autoreceptors for 5-HT synthesis. This suggests a dysregulation of NT existence in depressive disorders (http://www.csuchico.edu/psy/BioPsych/serotonin.html).

3.4 DETERMINATION OF THE EFFECT OF 6-MBOA ON URINARY 5-HIAA EXCRETION

3.4.1 INTRODUCTION

Tryptophan is a dietary precursor of 5-HT and 5-HIAA respectively. 5-HIAA is an essential metabolite of 5-HT catabolism. An increase in 5-HIAA in urine (mg) indicates an appreciable portion of tryptophan being metabolized via the 5-hydroxyindole route. 5-HIAA excreted in the urine may be derived from 5-HTP, an immediate precursor of 5-HT and 5-HT itself.

Many researchers have reported differences in 5-HIAA concentrations in the CSF of depressed and non-depressed subjects. Bulat and Zivkovic (1971) stated that 5-HIAA in the CSF is of spinal cord origin. Curzon *et. al.* (1980) showed that it is only lumbar CSF from depressed patients that has lower 5-HIAA levels than the controls, ventricular 5-HIAA values reflect reduced spinal-neuronal activity which could occur as a result of the motor retardation which commonly occurs in depressed patients. However, Åsberg *et. al.* (1976a) observed that there are two groups that exist. One group showed decrease in 5-HIAA levels and the other group retained its normal 5-HIAA levels. The former group was very much more likely to be suicidal than the latter group (Åsberg *et. al.*, 1976b) and the latter group was more likely to show an increased tendency to develop some episodes of depression (Van Praag,1976). From these observations, it can be concluded that changes in 5-HIAA concentrations may be an indication of some sort of a

depression episode.

The purpose of this study was to determine whether administration of 10 mg/kg of 6-MBOA could affect urinary 5-HIAA excretion. This was prompted by the results obtained from the previous studies which showed that 5-HT is increased after 6-MBOA administration, because 5-HIAA is an essential metabolite of 5-HT.

There are a number of different techniques which are used to quantitavely measure the amounts of 5-HIAA in the urine. These techniques include gas chromatography, HPLC and spectroflourimetry. All three mentioned techniques require specialised equipment and are expensive. A technique used for the estimation of 5-HIAA in this study employed a method modified from Udenfriend *et. al.* (1956). This is a very old and time consuming method but the technique is sensitive (mg 5-HIAA obtained), specific and cost effective. The technique involves the pretreatment of urine with dinitrophenylhydrazine (DNPH) to react with keto acids that might be present in urine. The technique is colorimetric and the keto acids are said to interfere with the final colour if not removed and that may give false positives in the final results. Most urine samples contain a lot of keto acids therefore the precipitate formed (keto acids-DNPH) should be removed by centrifugation. The acidic urine is then extracted with chloroform ($CHCl_3$) twice. Furthermore, 5-HIAA is extracted with ether and sodium chloride/ NaCl is used to saturate the aqueous layer so that 5-HIAA can be extracted into ether because of the low distribution coefficient of 5-HIAA between ether and water. Ether extract is returned to neutral pH (pH 7) for colorimetric assay because high pH values lead to instability of the compound. Ether is evaporated and the aqueous

layer is reacted with nitrosonaphthol and nitrous acid reagent. A colour reaction with this reagent (violet chromophore) is characteristic of 5-HIAA. More than 20 phenolic compounds including tryptamine and some 7-hydroxyindoles do not respond to this reagent to give the authentic colour. 5-HIAA is then extracted twice with ethyl acetate and the aqueous phase is transferred to a microcuvette and the absorbance read at 540 nm.

3.4.2 ANIMALS

Male Wistar rats of the albino strain weighing 200 - 250 g were used. Tap water and Epol food were supplied *ad libitum*. The temperature and lights of the animal room were maintained as described in section 2.2. The route for adminitration of 10 mg/kg 6-MBOA was intraperitoneal (i.p.). Two injections initiated between 10h00 and 11h00 were given at one hour interval. Immediately after the first injection, rats were individually placed in catabolic cages and the urine samples were collected for a duration of 24 hours and analysed.

3.4.3 CHEMICALS AND REAGENTS

3.4.3.1 CHEMICALS

Chemicals used were of the highest purity available. The chemicals were purchased from the Chemical Store, Rhodes University, South Africa.

3.4.3.2 REAGENTS

1-Nitrosonaphthol reagent was prepared by dissolving 1-nitrosonaphthol in 0.1 % absolute ethanol, nitrous acid reagent prepared by mixing 2.5 % $NaNO_2$ (0.2 ml) and 2N HCl (5 ml) and 2,4-dinitrophenylhydrazine (2.4-DNPH) was dissolved in 5 % in 2N HCl.

3.4.4 METHOD FOR THE ESTIMATION OF 5-HIAA IN THE RAT URINE USING A COLORIMETRIC ASSAY

To 1 ml urine, 1 ml 2,4-DNPH reagent was added. The mixture was incubated for 30 minutes at room temperature and was gently shaken at 10 minutes intervals for complete removal of keto acids. 4.2 ml $CHCl_3$ was added and the mixture centrifuged at 3 000 x g for 5 minutes. To an aqueous layer, 4.2 ml $CHCl_3$ was added. The mixture was centrifuged at 3 000 x g for 5 minutes. 1.7 ml aqueous layer was added to a test tube containing 0.6 g NaCl and 4.2 ml ether. The mixture was vortexed and centrifuged for 5 minutes. 3.3 ml ether aliquot was transferred to a tube containing 0.25 ml phosphate buffer, pH 7.0. The mixture was shaken and centrifuged for 5 minutes at 3 000 x g. Ether was evaporated in a fume hood under a gentle stream of nitrogen (N_2). 0.17 ml of aqueous layer was transferred to a tube containing 0.08 ml nitrosonaphthol reagent. Nitrous acid was added to this mixture (0.08 ml). The mixture was vortexed and warmed at 37 °C for 5 minutes. 0.83 ml ethyl acetate was added and the mixture vortexed to separate the two layers. Ethyl acetate was carefully removed and fresh ethyl acetate (0.83 ml) was added and ethyl acetate was then carefully removed as before. The aqueous layer containing 5-HIAA was

transferred to a cuvette and the optical density read at 540 nm. The standards of concentrations ranging from 10 - 200 μmoles were treated the same way as urine samples. Water was used to replace urine for blanks.

3.4.5 RESULTS

The concentration of urinary 5-HIAA was read from the standard curve and converted to μmoles / ml urine. In figure 3.4 it is shown that 6-MBOA significantly increases the urinary excretion of 5-HIAA ($P < 0.001$). To determine if the correct metabolite was extracted, a thin layer chromatography (TLC) plate of the authentic 5-HIAA and that of the urine extract was run and the respective Rf values obtained were 0.27 and 0.26 which shows that an accurate metabolite was obtained in the final product.

3.4.6 DISCUSSION

5-HIAA is a normal constituent of mammalian urine. Van Praag *et. al.* (1973) and Van Praag and De Hans (1979) observed that there is a drastic increase in 5-HIAA in depressed patients. An increase in 5-HIAA excretion suggests that the major route for tryptophan metabolism is through the 5-HT biosynthesis since 5-HIAA is a major metabolite of 5-HT metabolism and is reported to be quite stable in urine as compared to other indoleamines.

6-MBOA has been shown to significantly increase urinary excretion of 5-HIAA (fig.3.4). Shopsin

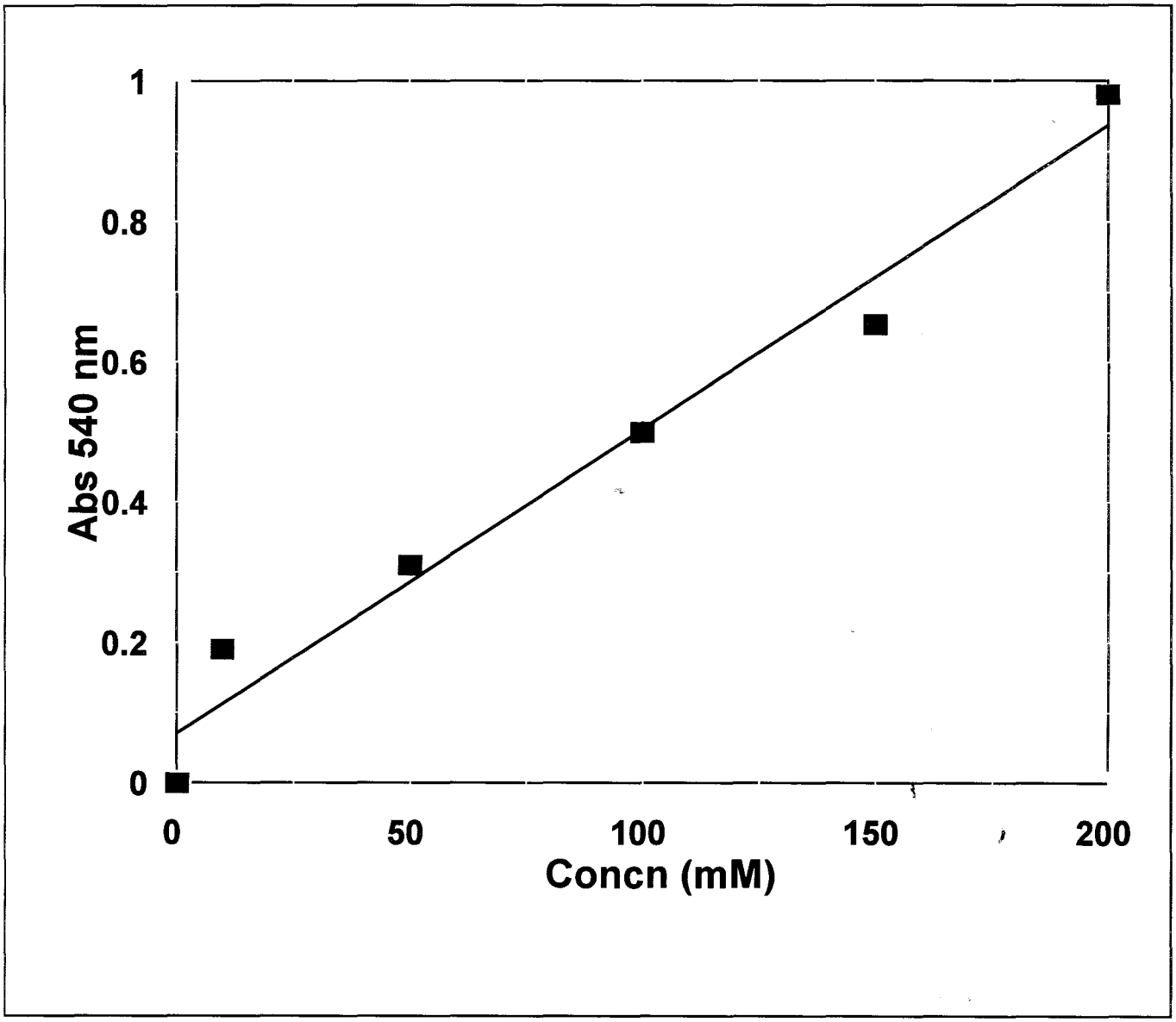

Figure 3.5 5-HIAA standard curve using the colorimetric assay

[*Data represents the mean of triplicate determinants;* $r^2 = 0.971$]

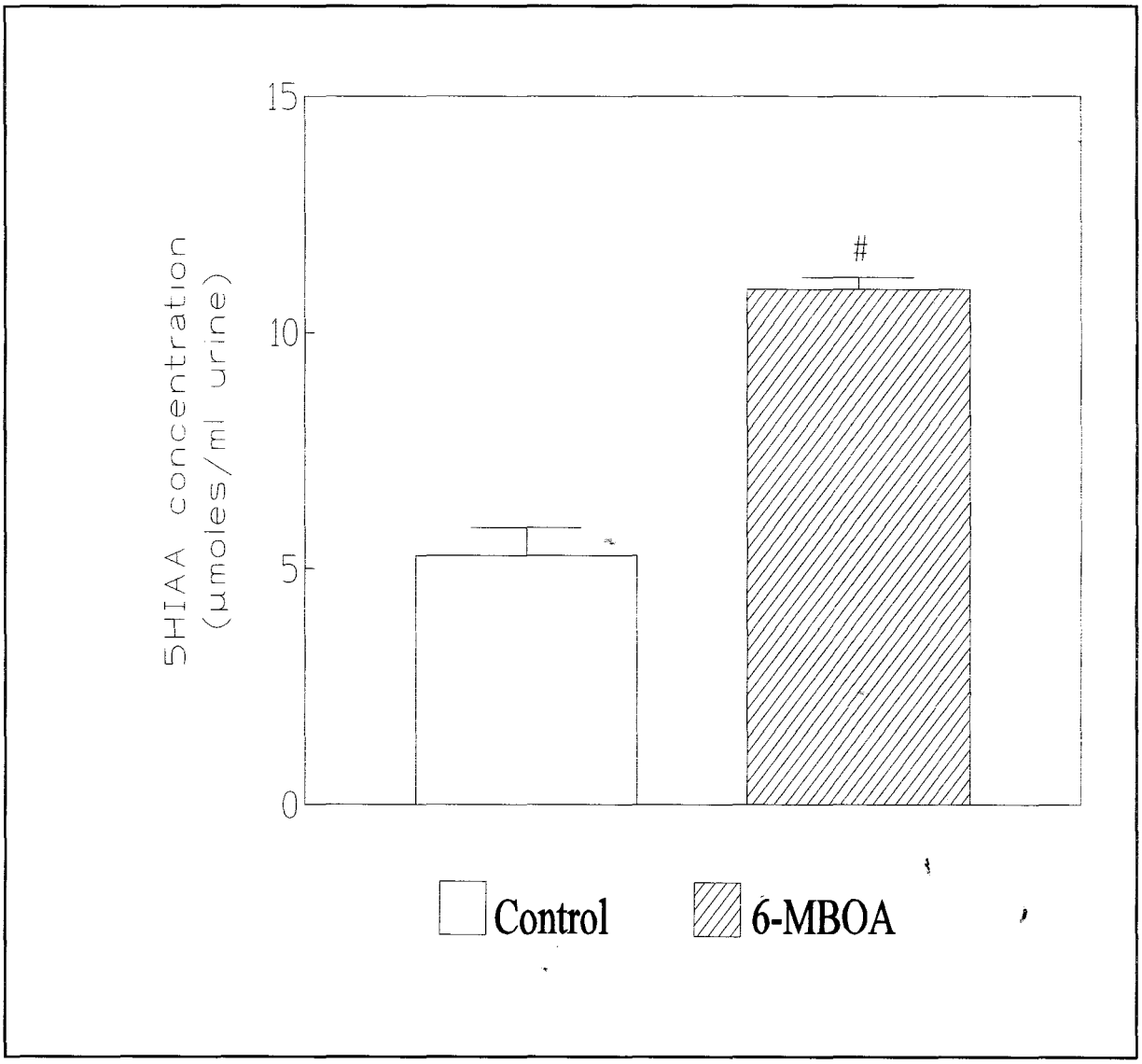

Figure 3.6 Determination of the effect of 6-MBOA on urinary 5-HIAA excretion.

[*Data represents mean ± SEM; n = 5; (#): P < 0.001)*]

et. al. (1972) and Wilk and Green (1973) have shown that a single dose of tryptophan slightly increases the amount of 5-HT and this is reflected by increased levels of 5-HIAA, but when tryptophan is combined with MAOI or pretreated with MAOI 5-HIAA levels are decreased since MAOI block the catabolism of 5-HT to 5-HIAA. From this study it may be proposed that increases in urinary excretion of 5-HIAA caused by 6-MBOA administration might be due to an increase in brain and CSF 5-HT and that *in vivo*, 6-MBOA does not have any significant effect on MAO activity.

Whereas studies on CSF and urinary metabolites may be indicative of abnormalities in central amine neurotransmission, further experiments need to be employed to verify the amine hypothesis of depression.

CHAPTER 4

4.1 INTER-INDIVIDUAL VARIATION IN NAS AND aMT PRODUCTION

4.1.1 INTRODUCTION

The levels of aMT have been reported to change in patients suffering from depressive illness (Beck-Friss *et. al.*, 1984). However, studies about the existence of inter-individual differences in the production of this indoleamine has been limited. Lynch *et. al.* (1975) showed that there are two distinct groups of aMT and NAS producers, the high and the low producers. These authors detected this by taking healthy volunteers and measuring each individual's aMT and NAS levels. Bergianniki *et. al.* (1995), made a sequel on this study by measuring the urinary excretion patterns among healthy volunteers for a year on a monthly basis. Healthy volunteers were used as controls since they showed a clear distinction between these two groups.

The aim of this study was to investigate whether rats can be divided into these respective groups of NAS and aMT producers and the mean used as the reference point. The activity of animals which rate above the mean be regarded as high producers (HP) and the animals below the mean will be rated as low producers (LP) of NAS and aMT. The animals which

have scores that are very close to the mean will be an indication of the existence of the third group of animals, which can be called the intermediate group of producers.

The pineal NAS and aMT levels were determined using the pineal organ culture technique. The organ culture technique was discovered in the early 1920' s. It was mainly used for the culture of embryonic rudiments. To date, organ culture systems are employed in the study of the physiological and biochemical properties of many living tissues. Trowell (1959) developed a technique which allowed a number of fully differentiated organs, or parts thereof to remain viable *in vitro* without either growth or dedifferentiation thus opening a path to many experimental studies.

Pineal organ culture technique is a very sensitive technique. It measures trace quantities of indole metabolites ranging from picomole to micromole concentrations. It is also a useful technique in that it eliminates the complexities of organ interaction and allows for direct pharmacological manipulation. Klein (1969) reported that the rat pineal glands cultured in adequately aerated medium and incubated at 37 °C tend to stay for at least six days.

the pineal gland in culture medium is able to utilize an exogenous radioactive precursor (tryptophan, 5-HTP and 5-HT) to synthesize different radioactive indoles. It is believed that approximately 95 % of the synthesized radioactive indoles are secreted into the culture

medium during the incubation period (Klein and Rowe, 1970; Skene, 1985). The radioactive indoles are then separated using a bi-dimensional thin-layer-chromatography (Klein and Notides, 1969; Daya and Potgieter, 1982).

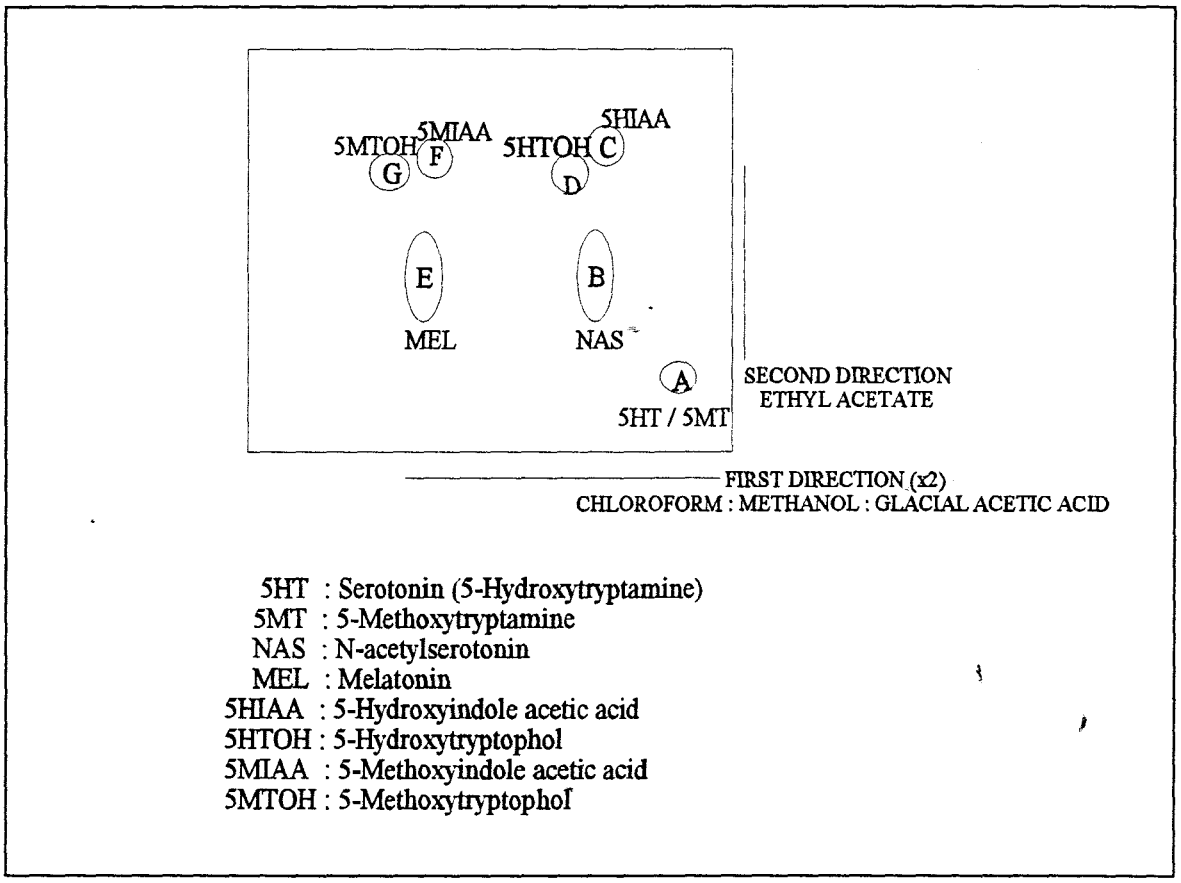

Figure 4.1 A typical bi-dimensional thin-layer chromatogram of the pineal indoles (Modified from Klein and Notides, 1969)

4.1.2 ANIMALS

Male Wistar rats of the albino strain weighing 200 B 250 g were randomly assembled into test groups of five and maintained as described previously in section 2.2. For photo-phase experiments, animals were sacrificed between 12h00 and 13h00 and for scoto-phase experiments, animals were sacrificed between 00h00 and 01h00 under a dim red light (60 Watts).

For removal of pineal gland, the top of the skull was removed by making an incision through the bone on either side of the head from the foramen magnum to near the orbit. The skull was lifted with a pair of forceps exposing the pineal gland for removal with tweezers. All adhering tissue and visible traces of blood were carefully removed.

4.1.3 CHEMICALS AND MATERIALS

BGJB medium (Fitton-Jackson modification) was obtained from GibcoBRL, Life Technologies. Absolute alcohol from Chemistry Dept. Rhodes University, South Africa. Novopen (600 mg ~ 1 million units) = Sodium Benzylpenicillin B.P., Streptomycin sulphate B.P., Fungizone^{R} = Amphoterin B and non-radioactive 5-HT, NAS, aMT, 5-HTOH, 5-HIAA, 5-MTOH, 5-MIAA and Dimethylbenzaldehyde (DMBA) were obtained from Sigma Chemical

Co., St Louis, USA. 5-Hydroxy-[side-chain-2-^{14}C]tryptamine sulphate creatine sulphate (56 mCi / mmol) was obtained Amersham, Life Sciences. TLC plates (20 x 20 cm silica gel, $60F_{254}$) were obtained from Merck, Germany. Emulsifier Scintillation Plus was obtained from Parkard. Glacial acetic acid was obtained from uniVAR. Chloroform HiPerSolv for HPLC and methanol were obtained from BDH Laboratory Suppliers.

4.1.4 REAGENTS AND METHODOLOGY

4.1.4.1 PREPARATION OF CULTURE MEDIUM AND REAGENTS

Culture medium preparation was perfomed under a laminar flow hood with UV utilized as the light source for sterilization. 5 g of sodium benzylpenicillin B.P., streptomycin sulphate B.P. and amphoterin B were added into the BGJB medium to prevent any bacterial and fungal infections that may contaminate the medium. The culture medium was then stored at 4 °C until use.

4.1.4.2 PREPARATION OF UNLABELLED INDOLES FOR TLC

Of each unlabelled indole, 4 µg was dissolved in 1 ml of 95 % ethanol containing 1 mg /f ml ascorbate (ascorbate was used as an antioxidant). The mixture was kept away from light and room exposure to prevent photo-oxidation and thereafter stored at 4 °C until use.

4.1.4.3 COMPONENTS OF TLC SOLVENTS

Mobile phase A - chloroform : methanol : glacial acetic acid (97 : 7 : 1)

Mobile phase B - ethyl acetate

4.1.4.4 PREPARATION OF VAN URK'S REAGENT

Van Urk's reagent was used for visualising the indoles after these have been separated by TLC. 1 gram of dimethylbenzaldehyde (DMBA) was dissolved in 50 ml of 25 % Hcl and 50 ml of 95 % ethanol.

4.1.4.5 THE PINEAL ORGAN CULTURE TECHNIQUE

The pineal organ culture technique was followed as described by Morton[1](1990). The rats were sacrificed invasively. The intact pineal gland was then rapidly excised and incubated at 37 °C in medium containing 8 μl [^{14}C]5-HT for 24 hours. During the incubation period, the pineal gland metabolised the radioactive 5-HT and this was followed by release of the radiolabelled metabolites into the culture medium. After the incubation period, the pineal

Table 4.1 The composition of the BGJB culture medium modified from Fitton Jackson

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

CONTENTS	CONCENTRATION (mg / ml)
Sodium bicarbonate	3500.00
Sodium chloride	5300.00
OTHER COMPONENTS	
Calcium Lactate	555.00
Glucose	10000.00
Phenol red	20.00
Sodium acetate	50.00
VITAMINS	
α-Tocopherol phosphate	1.00
Ascorbic acid	50.00
Biotin	0.20
Calcium pantothenate	0.20
Choline pantothenate	50.00
Folic acid	0.20
Inositol	0.20
Nicotinamide	20.00
ρ-Aminobenzoic acid	2.00
Pyridoxal phosphate	0.20
Riboflavin	0.20
Thiamine HCl	4.00
Vitamin B_{12}	0.04

glands were flicked out of the medium and stored at -20 °C. An aliquot of the medium which contained the radioactive metabolites of the pineal gland was analysed using TLC and quantified by liquid scintillantion technique.

4.1.4.6 SEPARATION OF PINEAL INDOLES BY TLC

TLC plates (10 x 10) were used for the separation of indoles. Using a pencil, an arrow pointing forward and the other pointing to the right were drawn on top right hand corner of the plate. The first arrow showed the direction of movement of mobile phase A and the latter to the direction of mobile phase B. 10 μl of the medium was then spotted 1 cm from the bottom and from the left hand side of the plate with gentle continual stream of nitrogen to form a 4 - 5 mm spot. When the spot was dry, 5 μl of the standard solution was spotted on top of the first spot and dried as before.

The plates were then developed in the darkness in solvent A to about two thirds of the first direction. Plates were rapidly dried with nitrogen on both sides and immediately placed again in the darkened developing tank for the second development in solvent A. The plates were dried with nitrogen and placed in solvent B for development in the darkness in the second direction pointing to the right and allowed the solvent to migrate half-way up the plate. The plate was dried and sprayed with van Urk's reagent to visualise the indoles. The plates were then placed in a 60 °C oven for two minutes and thereafter the spots were identified. The

silica gel containing the radioactive metabolites was scraped off the plate and placed in counting vials containing 1 ml 95 % ethanol. The vials were put in a shaker for an hour and 3 ml of scintillation fluid was then added to the cocktail and left to stand for 30 minutes. The products were counted in a Beckmann LS 2800 scintillation counter.

4.1.5 RESULTS

The results were expressed as dpm's of radioactive NAS and aMT. The solid line represents the mean and the dotted line represents the standard errorof the mean (SEM). Figure 4.2 and figure 4.4 show that there are clear inter-individual differences in scoto-phase NAS and aMT production. However, the intermediate group of producers is not prominent during the scoto-phase. In photo-phase samples, the distinction between high and low producers of NAS and aMT is not as substantial as in scoto-phase (figure 4.3 and figure 4.5). It is the intermediate group that is distinctive during the photo-phase.

4.1.6 DISCUSSION

Pineal indoleamines exhibit a distinctive and well described circadian rhythmicity. Pineal NAS levels are low during the day and elevated during at night. This rhythm is due to the activity of NAT (Klein and Weller, 1970). The pineal aMT circadian rhythm is in phase with that of

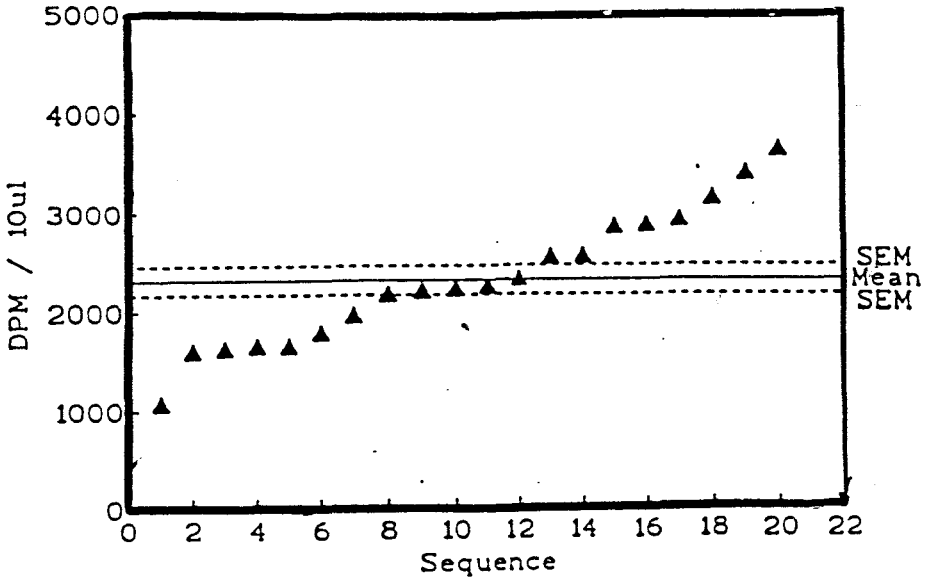

Figure 4.2 Pineal aMT levels analysed using the organ culture technique and TLC:

Scoto-phase samples were taken from a mixed population; n = 20

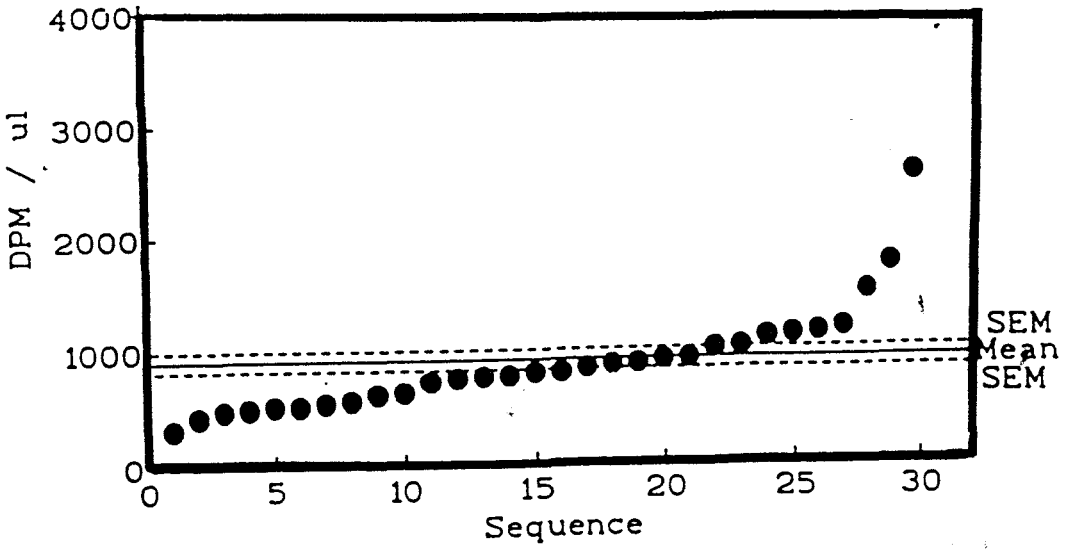

Figure 4.3 Pineal aMT levels analysed using the organ culture technique and TLC:

Photo-phase samples were taken from a mixed population; n = 20

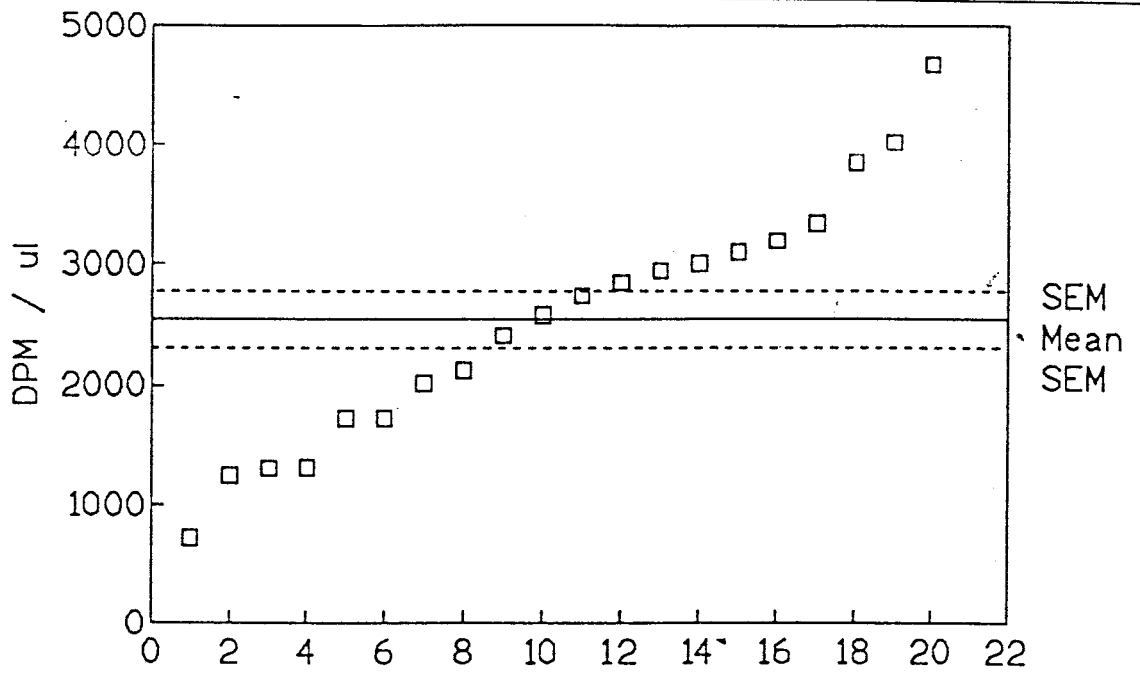

Figure 4.4 Pineal NAS levels analysed using the organ culture technique and TLC:

Scoto-phase samples were taken from a mixed population; n = 20

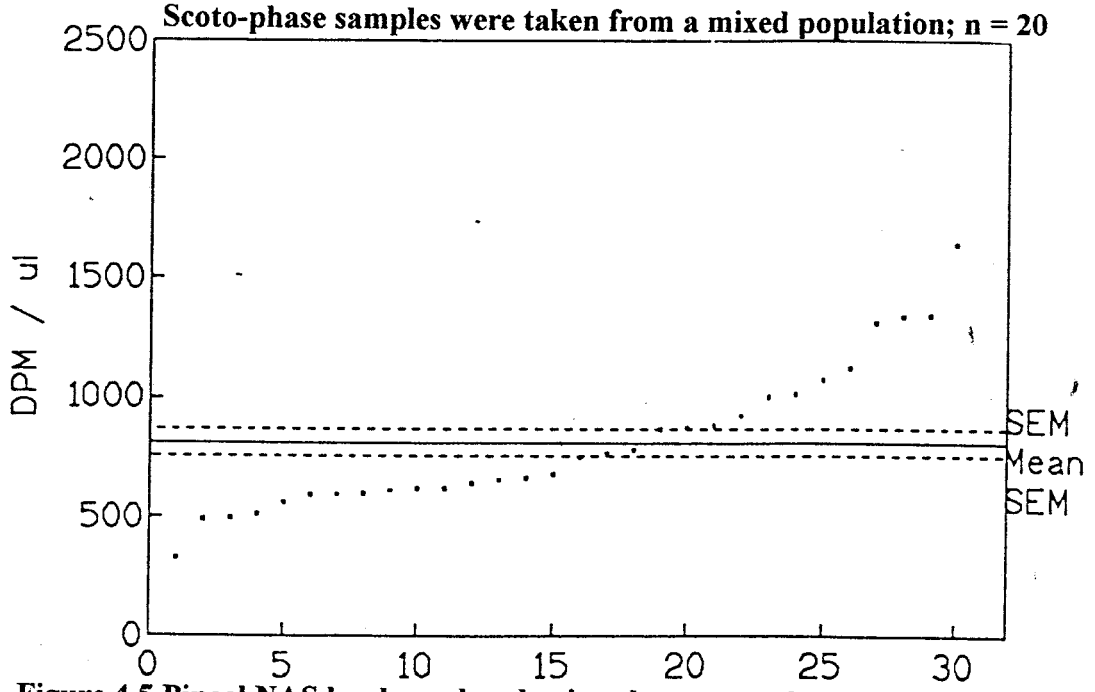

Figure 4.5 Pineal NAS levels analysed using the organ culture technique and TLC:

Photo-phase samples were taken from a mixed population; n = 20

NAT and NAS respectively.

The magnitude of the nocturnal rise in pineal aMT varies among species (Reiter, 1988). The results obtained from this study were in agreement with this report. The inter-individual variation of NAS and aMT was shown to exist in both the scoto-phase and the photo-phase samples (figure 4.2 and figure 4.5), with a more pronounced effect during the scoto-phase. The possible explanation for the clear distinction between high and low producers during the scoto-phase is the rise in pineal aMT and NAS production during the scoto-phase. An existence of an intermediate group was apparent during the photo-phase since NAS and aMT production are low during this period.

4.2 DETERMINATION OF THE EFFECT OF 6-MBOA ON PINEAL INDOLE BIOSYNTHESIS

4.2.1 INTRODUCTION

The objective of this experiment was to determine whether 6-MBOA would have an effect on the biosynthesis of the pineal indoles *in vivo*. This was prompted by the observation made by Daya *et. al.* (1990) and Daya and Tandt (1993), which showed that 6-MBOA induces NAT activity and aMT biosynthesis with a concomitant rise in cAMP levels at high

concentrations (1 mM). However, no effect was shown on the activity of HIOMT by these authors.

The pineal organ culture and TLC technique were employed for quantifying the pineal indoles as described by Morton (1990).

4.2.2 ANIMALS

Male Wistar rats of the albino strain weighing 200 - 250 g were used and maintained as described in section 2.2. Animals were sacrificed between 12h00 and 13h00. The pineals were removed as described in section 4.1.2.

4.2.3 CHEMICALS AND MATERIALS

Chemicals and reagents used were as described in section 4.1.3.

4.2.4 REAGENTS AND METHODOLOGY

Reagents were prepared as described from section 4.1.4.1 to section 4.1.4.4. The methodology was followed as described from section 4.1.4.5 to section 4.1.4.6.

4.2.5 RESULTS

The results for the determination of the effect of 10 mg / kg on pineal indole biosynthesis were expressed as dpm's / 10 μl medium. Statistical differences were determined using the Student's t-test. P values less than 0.05 were taken as significant. The radioactive 5-HT added as the precursor for the pineal indoles tested was significantly reduced in 6-MBOA treated animals ($P < 0.05$). NAS production was insignificantly elevated. However, aMT production was significantly increased after 6-MBOA administration ($P < 0.01$). No significant change was observed in other indoleamines, 5-HIAA, 5-HTOH, 5-MIAA and 5-MTOH.

4.2.6 DISCUSSION

Pineal indole biosynthesis starts by uptake of tryptophan form the blood circulation. Within the pinealocyte, tryptophan is hydroxylated to 5-HTP by the enzyme TH. Thereafter, the enzyme aromatic amino acid decarboxylase decarboxylates 5-HTP to form 5-HT. Once 5-HT is formed, 5-HT is biotransformed in one of three possible routes:

(i) Some 5-HT is deaminated to 5-HIAA and 5-HTOH by the enzyme MAO. These hydroxyindoles may be methylated by HIOMT to form 5-MIAA and 5-MTOH.

(ii) Some 5-HT may be directly methylated by HIOMT to form 5-methoxytryptamine

(iii) The major route of 5-HT is its conversion to aMT via acetylation by the enzyme 5-HT NAT to yield NAS which is then methylated by HIOMT to form aMT. This study dealt only with routes (i) and (iii).

6-MBOA, structural analogue of 5-HT and aMT has been reported to increase aMT production *in vitro* at 10^{-3} M concentrations (Daya and Tandt, 1993). The results of this study somehow confirm this finding in that, aMT production was significantly increased proportionally with the decrease in exogenous 5-HT and an insignificant increment in NAS production, an immediate precursor of aMT. The possible explanation for the considerable reduction in exogenous 5-HT could be that, 6-MBOA has been shown to stimulate NAT activity, a rate limiting enzyme in aMT biosynthesis *in vitro* at pharmacological concentrations (Daya *et. al.*, 1990).

From the results, the pathway of 5-HT metabolism which involves deamination of 5-HT to 5-HIAA and 5-HTOH by MAO and the subsequent methylation of these hydroxyindoles to 5-MIAA and 5-MTOH appeared not to be affected by 6-MBOA treatment. One optional explanation could be that, 6-MBOA does not have an effect on pineal MAO. Another possible explanation could be that, since the aMT route for 5-HT metabolism is stimulated by 6-MBOA and aMT biosynthesis being the major route on its own for 5-HT metabolism, the other routes are outcompeted for the substrate thus leading to maintenance of the normal biosynthesis of the other indoleamines.

CHAPTER 5

DETERMINATION OF THE EFFECT OF 6-MBOA ON PINEAL MELATONIN (aMT) PRODUCTION EMPLOYING COMPETITIVE ELISA TECHNIQUE

5.1 INTRODUCTION

Melatonin (aMT), a major hormone of the pineal gland is synthesized from the amino acid tryptophan. aMT has its highest levels during the night time. Circulating aMT is almost exclusively of pineal origin since in some cases, plasma concentrations at night in particular are depressed and undetectable after pinealectomy. Human plasma melatonin levels show a marked individual variation. Daytime levels are very low and are in the range of 10 pg / ml in the morning hours whereas night time levels exceed 30 pg / ml. aMT is metabolised in the liver to 6-hydroxymelatonin which is conjugated to either glucuronide (20 - 30 %) or sulphate (60 - 70 %) (http://www.IBL-Hamburg.com).

Several researchers have proposed that circadian rhythms play a vital role in the etiology of affective

disorders (Halberg, 1967; Wehr and Goodwin, 1983). Depression symptomology exhibits a diurnal variation with exacerbation early in the morning and improvement in the evening. Depressive symptoms also vary seasonally. aMT rhythm thus provides a useful marker in depressive illness. The secretion of aMT in blood and urine follows a 24 hour rhythm. The majority of studies indicate that aMT secretion is reduced in depressed patients although conflicting results have been reported. A functional deficiency of NA, 5-HT or both has the potential to lower pineal aMT production (Arendt, 1989).

There are a number of methods employed to measure aMT blood plasma levels and these include radioimmunoassay (RIA) (Rollag and Niswender, 1976), gas chromatography (GC) and mass spectrometry (MS) (Lewy and Markey, 1976). In this study, the circadian pattern and the effect of 6-MBOA in endogenous pineal aMT production was measured and the method of competitive ELISA was employed.

aMT ELISA is a competitive enzyme immunoassay for the quantitative analysis of aMT in serum, plasma and tissue homogenate which initially requires extraction. The assay procedure follows the basic principle for competitive immunoassays whereby there is competition between a biotinylated and non-biotinylated antigen for a fixed number of antibody binding sites. Bound biotinylated antigen to the antibody is inversely proportional to the analyte concentration of the sample. When equilibrium is reached, the free biotinlyated antigen is removed by a washing step and the bound antibody to the

biotinylated antigen is determined by employing an anti-biotin alkaline phosphatase enzyme as a marker and p-nitrophenylphosphate as a substrate. The quantification of unknown samples is obtained by comparing the enzymatic activity of unknowns with a response curve prepared by using known standards (http://www.IBL-Hamburg.com).

5.2 ANIMALS

Male Wistar rats of the albino strain weighing 200 - 250 g were randomly assembled into two groups of five and maintained as previously described in section 2.2. Injections of 10 mg/kg of 6-MBOA were done as described in section 2.2.1. For photo-phase experiments, animals were sacrificed between 12h00 and 13h00 and for scoto-phase experiments animals were sacrificed between 00h00 and 01h00 under a dim red light. Pineal glands were removed as described in section 4.1.2. Immediately after removal, the pineals were immediately stored in liquid nitrogen and kept frozen at -70 °C until required.

5.3 CHEMICALS AND REAGENTS

5.3.1 CHEMICALS

All chemicals were purchased from Research Diagnostics Inc., Pleasant Hill Rd, Flanders and were

of the highest purity.

5.3.2 REAGENTS

ASSAY BUFFER	: Dilute 50 ml phosphate buffer with tween and stabiliser then dilute1:10 with bidistilled water.
ANTISERUM	: Dissolve contents of each vial in 2 ml of bidistilled water.
MELATONIN-BIOTIN	: Dissolve contents of each vial in 2 ml of assay buffer.
STANDARD A-F	: Dissolve contents of each vial in 2 ml of bidistilled water and store aliquotes at -20 °C.
CONTROL SERUM 1 & 2	: Dissolve contents of each vial in 2 ml of bidistilled water and store aliquotes at -20 °C.
ENZYME CONJUGATE	: Dilute 250 μl anti-biotin-alkaline phosphatase in TRIS buffer with stabilizers and dilute 1:81 with Assay Buffer

5.4 PREPARATION OF THE PINEAL HOMOGENATE

The frozen pineal glands (-70 °C) were slowly thawed on ice and sonicated in 500 μl of ice cold 0.9 % saline for two seconds.

5.5 aMT EXTRACTION PROCEDURE

Extraction columns for each sample were placed into glass tubes. Two x 1 ml of absolute methanol were added to the columns and centrifuged for one minute at 200 x g. After centrifugation, two x 1 ml of distilled H_2O were added to columns and centrifuged for 1 minute as before. Immediately after centrifugation, the pineal homogenate was applied to avoid the columns from getting dry and centrifuged for 1 minute at 200 x g. Thereafter, the columns were washed with two x 1 ml of 10 % methanol in distilled water (v/v) and the columns centrifuged for 1 minute at 500 x g. The columns were then placed into clean glass tubes and 1 ml of undiluted methanol was added to the columns (undiluted methanol was used for extraction of the product) and centrifuged for 1 minute at 200 x g. Subsequently after centrifugation, the columns were removed from the glass tubes. Methanol was evaporated to dryness and the sample was reconstituted with 0.15 ml of distilled H_2O. The mixture was vortexed for 1 minute and immediately assayed afterward.

5.6 ASSAY PROCEDURE

To 50 μl of each extracted standard, controls and samples, 50 μl of melatonin-biotin and subsequently 50 μl of antiserum were added. The plate was sealed with adhesive foil and incubated overnight at 2 - 8 °C. The wells were washed three times with assay buffer. 200 ml of PNPP substrate solution was pipetted into the wells followed by incubation at room temperature for 20 to 40 minutes. The

substrate reaction was stopped by adding 50 μl pf stop solution. Optical density was read at 405 nm within 60 minutes after termination of the reaction.

5.7 RESULTS

The logarithmic concentrations of the standards were plotted against the corresponding optical densities. Each sample was done in duplicate. The concentration of the samples were read directly from the standard curve by using the average optical density of each sample. Statistical differences between the two groups were determined using the Student's t-test.

The results obtained from this study showed that the pineal aMT production is circadian with peak levels occurring during the scoto-phase (figure 5.2). The values obtained for photo-phase samples were ± 2.48 pg/ml and for scoto-phase samples only ±17.67 pg/ml. Administration of 10 mg/kg 6-MBOA lead to a significant augmentation of aMT production in both phases (figure 5.3 and figure 5.4).

5.8 DISCUSSION

aMT is synthesized from 5-HT, which is present in the pineal gland at higher concentration than any other tissue, with the possible exception of the raphe nucleus, the location of serotonergic cell bodies

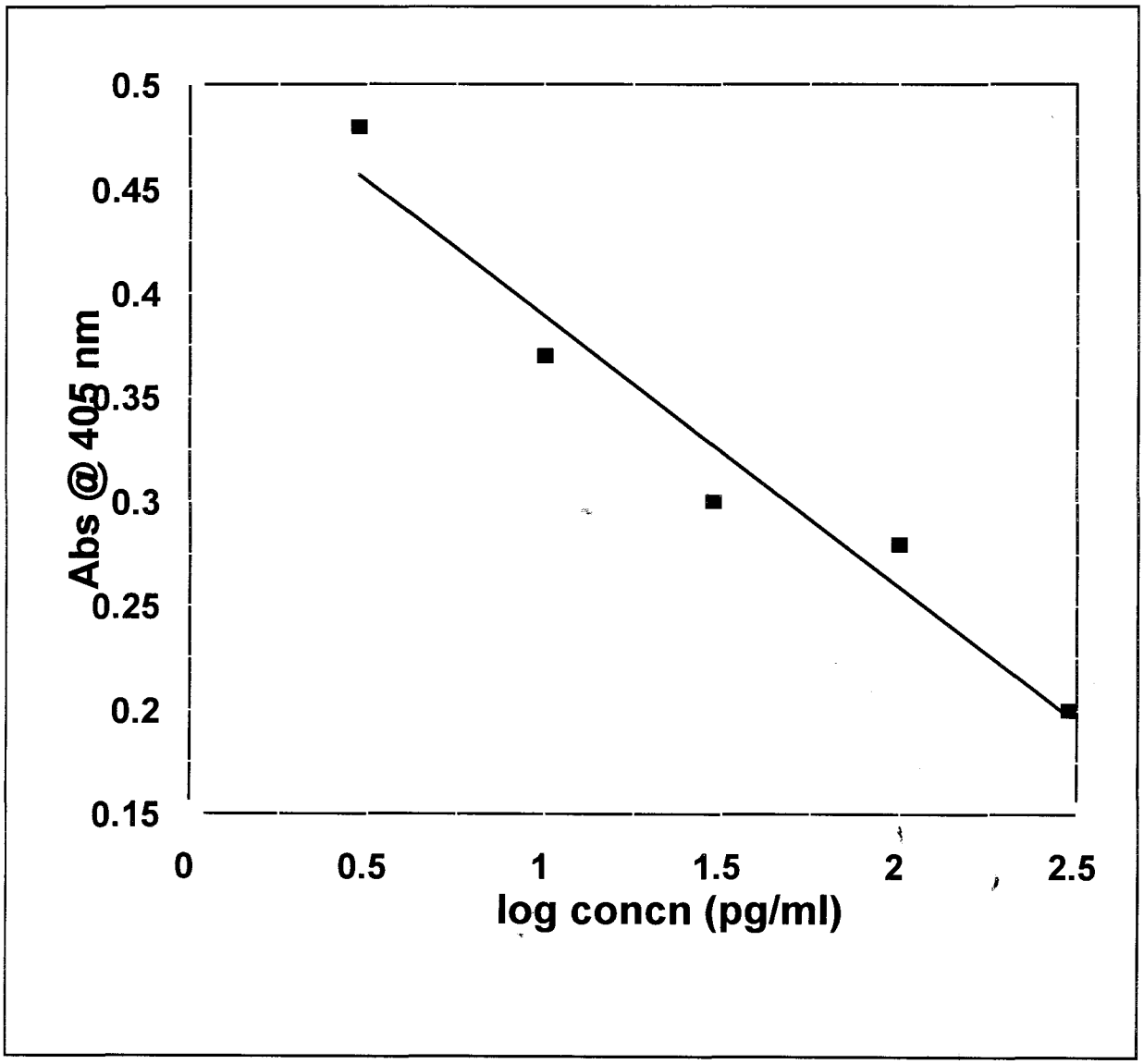

Figure 5.1 Melatonin standard curve using competitive ELISA

[*Data represents mean of triplicate determinants;* $r^2 = 0.953$]

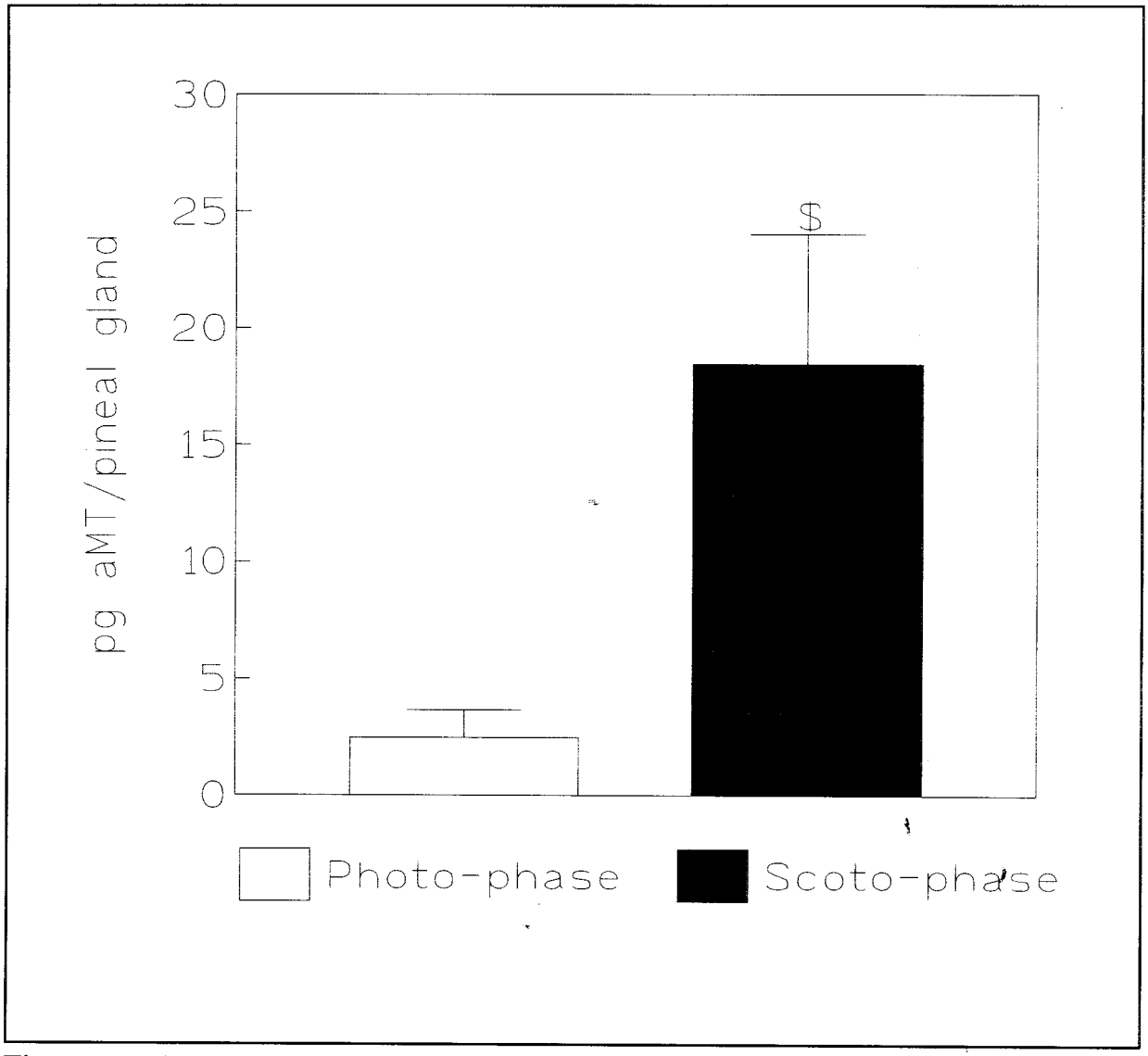

Figure 5.2 Assessment of light-dark variations in pineal aMT production

[*Data represents mean ± SEM; n = 5; ($): P < 0.05*]

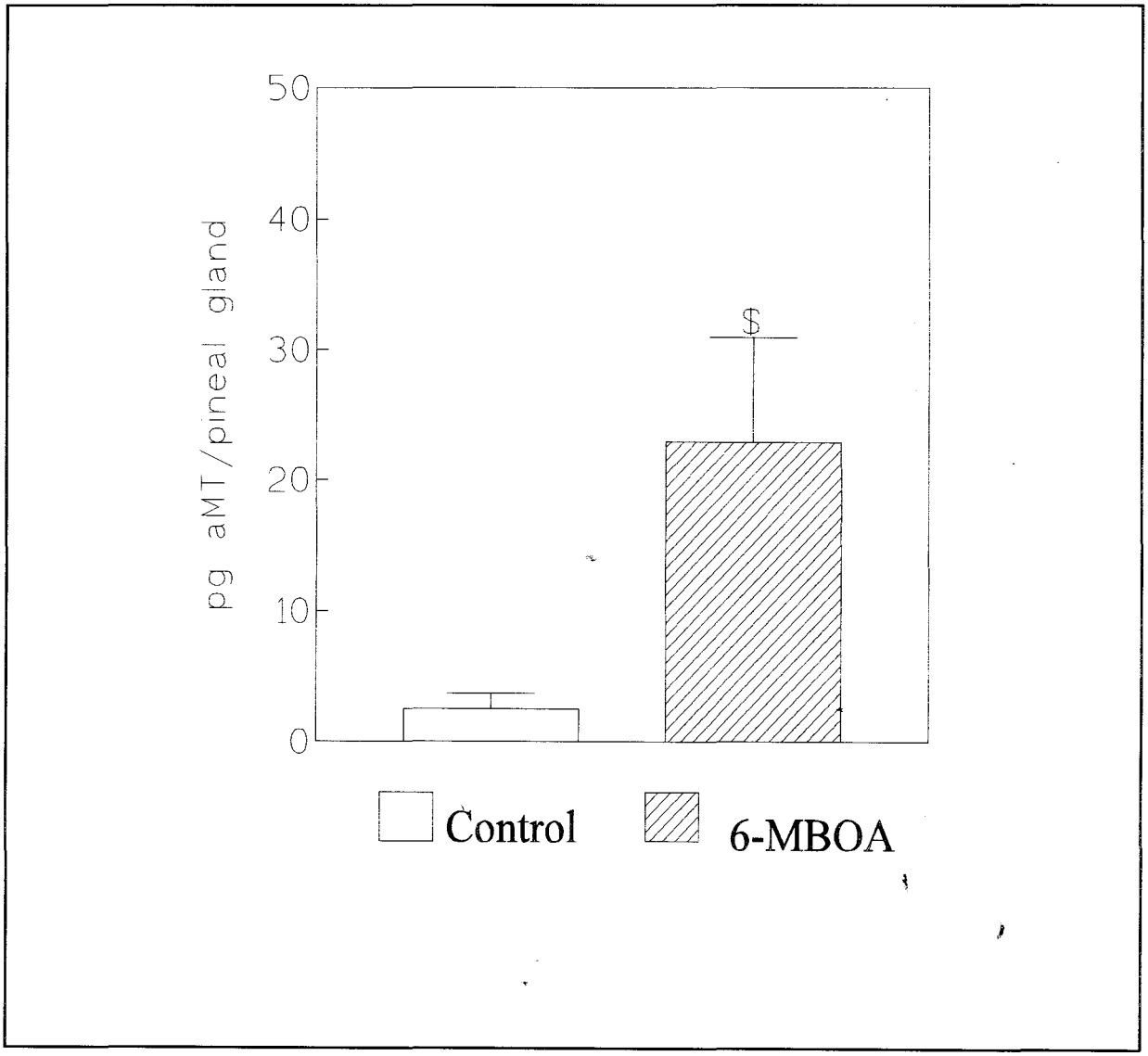

Figure 5.3 Determination of the effect of 6-MBOA on pineal aMT production: Photo-phase samples

[*Data represents mean ± SEM; n = 5; ($): P < 0.05*]

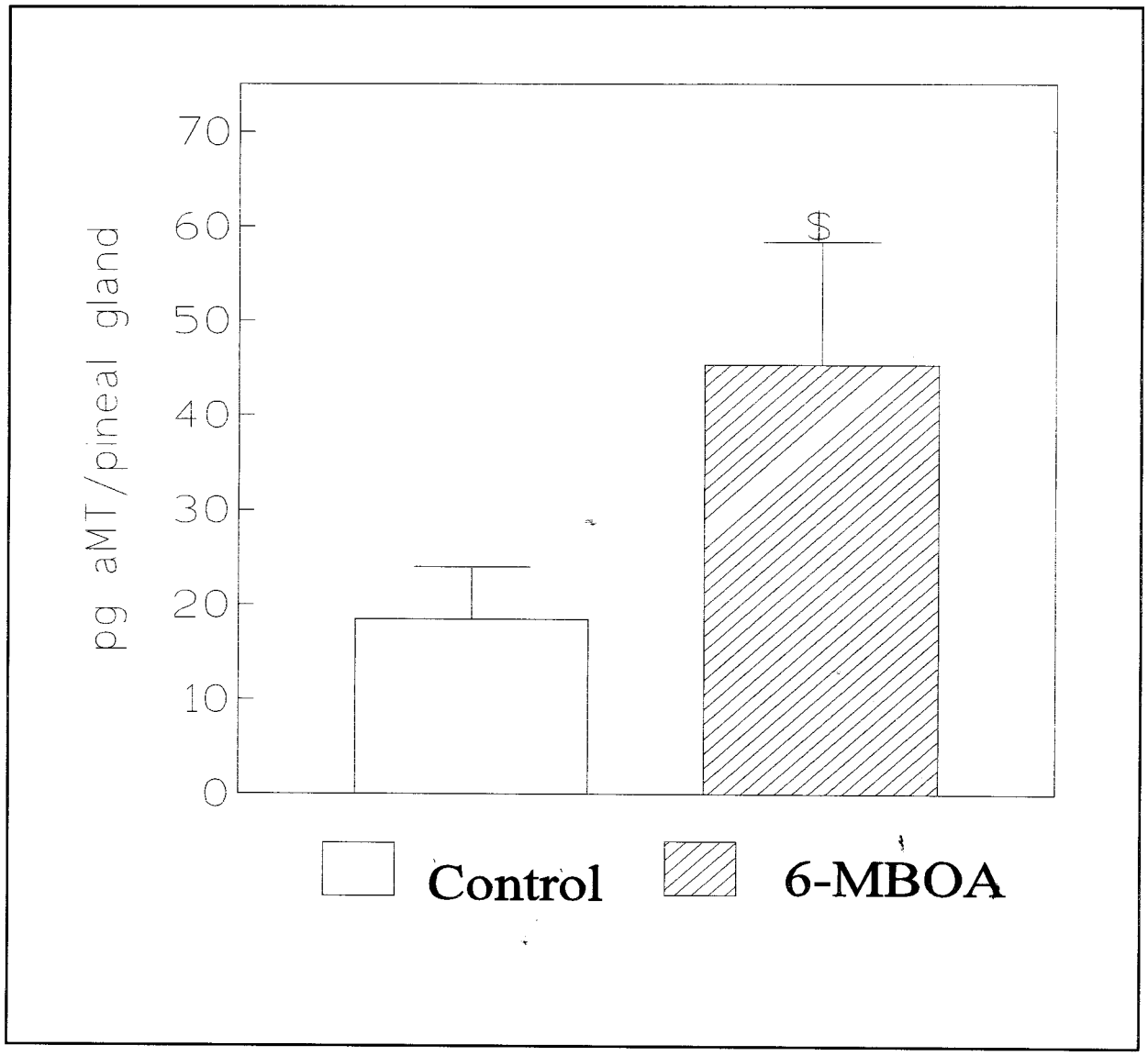

Figure 5.4 Determination of the effect of 6-MBOA on pineal aMT production: Scoto-phase samples

[Data represents mean ± SEM; n = 5; ($): P < 0.05]

(Klein *et. al.*, 1997). The conversion of 5-HT to aMT involves two enzymes, NAT and HIOMT. The first enzyme N-acetylates 5-HT to form NAS and the second enzyme O-methylates NAS to form aMT (Klein *et. al.*, 1997). The rhythm in aMT biosynthesis is generated by a rhythm in the NAT activity (Klein and Weller, 1970). An increase in NAT activity at night decreases the pineal 5-HT and increases the concentration of NAS (Klein *et. al.*, 1997). Changes in the activity of HIOMT have little influence on the large changes in aMT production that drive the aMT rhythm (Klein, 1985).

The results of this study validate the above reports as more aMT was produced during the scoto-phase. 6-MBOA has been shown to possess properties of a weak β-adrenergic agonist *in vitro* (Sweat and Berger, 1988). 6-MBOA has also been shown to stimulate NAT activity and aMT respectively. The present study indicates that, 6-MBOA further enhances aMT production *in vivo*.

CHAPTER 6

SUMMMARY AND CONCLUSION

6.1 SUMMARY

6.1.1 CHAPTER 2

Hepatic TDO activity was determined using a method described by Badawy *et. al.* (1973, 1975 and 1980). The principle for the assay involves the incubation of the crude enzyme with its substrate, L-tryptophan (0.03 M). For determination of the total enzyme activity, haem was added to activate the non-active form of the enzyme. The difference between the total enzyme and the holoenzyme activity was used as a measure of the apoenzyme activity. The product formed, L-kynurenine was used as a measure of TDO activity.

The results obtained show that hepatic TDO activity is subject to a circadian rhythm. The highest enzyme activity occurs during the photo-phase and the minimum activity during the scoto-phase. This circadian variation was observed in apoenzyme activity.

6-MBOA inhibited hepatic TDQ activity *in vivo* in both phases with a more pronounced effect

during the photo-phase. Holoenzyme activity was shown to be increased during the scoto-phase in both treated and untreated samples. From these results it can be deduced that the mode of inhibition of 6-MBOA is through:

(i) the interference in the conjugation of the apoenzyme with haem

(ii) improper interactions between 6-MBOA and the catalytic site of TDO caused by the lack of the $CHNH_2COOH$ functional group and

(iii) H-bonding formed between CH_3 and haem pocket leading to loss of catalytic activity of TDO.

It can therefore be concluded that 6-MBOA is a potential antidepressant as it could increase the availability of L-tryptophan to the brain by inhibiting hepatic TDO activity which is a prerequisite for the biosynthesis of an essential biogenic amine, 5-HT, in depression.

6.1.2 CHAPTER 3

5-HT is synthesised from tryptophan by the enzyme TH which is rate limiting in 5-HT biosynthesis (Reiter, 1981). After the biosynthesis and release of 5-HT from presynaptic vesicles, 5-HT is either:

(i) oxidised by MAO and converted to 5-HIAA, an essential metabolite of 5-HT or

(ii) taken into the presynaptic vesicles by re-uptake or

(iii) binds to the postsynaptic receptors.

Competitive ELISA was used for measuring brain 5-HT levels. This technique is very sensitive

as concentration of ng / mg tissue of 5-HT were obtained. The results of the present study show a rise in 5-HT levels induced by *in vivo* administration of 6-MBOA in rats.

It has also been shown that an increase in 5-HT biosynthesis caused by 6-MBOA does not only enhance 5-HT production and TH activity, however, 6-MBOA also causes inhibition of 5-HT binding to synaptosomal membrane at specific 5-HT concentrations. 5-HIAA production was also significantly increased after 6-MBOA administration.

Drugs which increase 5-HT concentration, including 6-MBOA can be clinically used for treatment of affective disorders However, the possibility of development of side effects should be taken into consideration.

6.1.3 CHAPTER 4

Relative proportion of metabolites formed during the 24 hour incubation of [^{14}C]5-HT with rat pineal glands in culture medium was assessed by separation of the respective indoles using TLC and the radioactive metabolites quantified using a Beckman LS-2800 scintillation counter.

Results obtained from this study show at 6-MBOA has variable effects when administered to the rat. The only significant change caused by 6-MBOA in the production of pineal indoles is thesynthesis of aMT. This finding is in accordance with the *in vitro* studies done on the assessment of the pineal indoles NAS and aMT production, whereas NAS did not show any significant

change. Furthermore, a study to investigate the existence of inter-individual variation in pineal NAS and aMT production showed that the scoto-phase samples which portray high levels of the two indoles can be used as the models for dividing animals into high and low NAS and aMT producers. The third group of NAS and aMT producers, the intermediate group can be clearly distinguished in photo-phase samples.

6.1.4 CHAPTER 5

Competitive ELISA technique was employed for estimation of endogenous pineal aMT production. This technique is very sensitive and specific as concentrations of aMT in picogram levels can be detected. The rhythm in circulating aMT is closely related to the environment, specifically to light and darkness (Arendt, 1995). The results obtained from this study showed a circadian production of aMT with higher levels occurring during the scoto-phase. 6-MBOA administration lead to an increment in aMT production. It can be concluded that 6-MBOA has potential as a possible antidepressant since low levels of aMT are reported in patients suffering from winter depression, which is known as seasonal affective disorder (Rosenthal *et. al.*, 1984).

6.2 CONCLUSION

Depression is associated with abnormal sensitivity of serotonergic and noradrenergic neuronal receptors (Johnson and Lydiard, 1998). Surveys in the USA over the past fifteen years have shown that fewer than half of those who develop depressive illness seek treatment for the illness

(Bucholz and Dinwiddie, 1989; Dew *et. al.*, 1991). Because nearly all the literature describing affective disorders is based on treated samples, very little is known about the larger group with untreated depression.

Antidepressant medications are the main pharmacological treatment for acute severe depression. The efficacy of these medications in patients with unipolar disorders is well established and the benefit of continued treatment for prevention of future relapse is well documented (Prien *et. al.*, 1984). However, these medications have been associated with the onset of mania (Prief *et. al.*, 1973; Prien *et. al.*, 1984) or cycle acceleration (Kukupulos *et. al.*, 1980) in some but not all (Angst, 1985) reports.

The present study has provided support on the hypothesis that 6-MBOA may act as a possible antidepressant. This has been shown by the results which indicate the inhibition of the liver TDO activity with subsequent rise in 5-HT biosynthesis. However, modification needs to be done on the enzyme TH which is the rate limiting enzyme in 5-HT biosynthesis. TH requires pretreatment with dithiothreitol-Fe^{2+} for maximum activity and it was not included in this study. Further studies on the effect of 6-MBOA on the re-uptake system of 5-HT also needs to be done since more attention with regards to the use of SSRI=s for treatment of depression is currently implemented.

There is also an increasing likelihood that 6-MBOA may be a potential antidepressant as animals produced more pineal aMT after treatment with this drug. Pineal aMT biosynthesis has been proposed as a measure of the overall effect of antidepressant drugs on nor-adrenergic NT

(Checkley *et. al.*, 1986). aMT biosynthesis depends on NA release which stimulates nor-adrenergic receptors that stimulate adenylate cyclase which subsequently stimulates NAT activity, which is the rate limiting enzyme in aMT biosynthesis. 6-MBOA has potential as a possible antidepressant and therefore should be investigated for antidepressant activity.

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