REGULATION OF TRYPTOPHAN-2,3-DIOXYGENASE AND PINEAL INDOLEAMINES BY SELECTED TRYPTOPHAN DERIVATIVES AND ANTIDEPRESSANTS

THESIS

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In the **BOOK** of ISIAAH:

"The **LORD GOD** sits enthroned high above the **circle** of the **earth**".

Elsewhere in the **BIBLE:**

"Lean not unto your own understanding".

"The wisdom of man is foolishness to **GOD**".

"In the last days knowledge will increase".

DECLARATION

I, HAROLD ARCHIBOLD WALSH, hereby declare that the work submitted herein is original and neither the complete thesis nor any part thereof has been submitted for another degree at this or any other university.

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H. A. WALSH

CONTENTS

CHAPTER 1

LITERATURE REVIEW

CHAPTER 2

TDO AND ITS EFFECTORS

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ABBREVIATIONS

AC	=	adenylate cyclase
Ach	=	acetylcholine
5-ALA	=	5-aminolevulinate
ACTH	=	adrenocorticotropin hormone
aMT	=	melatonin
AT	=	amitriptyline
ATP	=	adenosine triphosphate
BF_3	=	boron trifluride
BH_4	=	tetrahydrobiopterin
BSA	=	bovine serum albumin
Ca^{2+}	=	calcium
cAMP	=	5'cyclic adenosine monophosphate
CCB	=	calcium channel blockers
CHO	=	carbohydrate
CI	=	clomipramine
Cl^-	=	chloride ion
CNS	=	central nervous system
CO_2	=	carbon dioxide
CSF	=	cerebrospinal fluid
DA	=	dopamine
DCMI	=	desmethylchlomipramine
DHGL	=	dihomo-γ-linoleic acid
DMI	=	desipramine
DNA	=	deoxyribonucleic acid
ECD	=	electrochemical detector
ECT	=	electro convulsive therapy
FBI	=	feedback inhibition
Fe	=	iron
FIG.	=	figure
GABA	=	γ-aminobutyric acid
GC	=	gas chromatography
GDP	=	guanine diphosphate

G_i	=	inhibitory guanine regulatory protein
G_s	=	stimulatory guanine regulatory protein
GTP	=	guanine triphosphate
h	=	hour
H	=	histamine
^3H	=	tritium
5-HIAA	=	5-hydroxyindole acetic acid
HIOMT	=	hydroxyindole-O-methyltransferase
HPLC	=	high performance liquid chromatography
H_2O	=	water
5-HT	=	serotonin
5-HTOH	=	5-hydroxytryptophol
5-HTP	=	5-hydroxytryptophan
5-MIAA	=	5-methoxyindole acetic acid
5-MTOH	=	5-methoxytryptophol
HVA	=	homovanillic acid
IMI	=	imipramine
$I.C._{50}$	=	concentration of inhibitor at which there is 50% inhibition
i.p.	=	intra peritoneal
K^+	=	potasium
K_i	=	inhibitor constant
K_s	=	sigmoidal constant
K_m	=	Michaelis constant
kda	=	kilodalton
kg	=	kilogram
KH_2PO_4	=	potasium dihydrogen phosphate
kyn	=	kynurenine
LHRH	=	lutenizing hormone releasing hormone
LNAA	=	large neutral amino acid
MAO	=	monamine oxidase
MAOI	=	monamine oxidase inhibitor
mg	=	milligram
MHPG	=	4-methoxy-3-hydroxyphenyl glycol
mM	=	millimolar

mRNA	=	messenger ribonucleic acid
N^+	=	sodium ion
NA	=	noradrenalin(e)
NAD^+	=	nicotinamide adenine dinucleotide
NAD(P)H	=	nicotinamide adenine dinucleotide (phosphate)
NaH_2PO_4	=	sodium dihydrogen phosphate
NaOH	=	sodium hydroxide
NAT	=	N-acetyl transferase
NAS	=	N-acetylserotonin
NT	=	nortriptyline
p	=	probability
O_2	=	oxygen
OPT	=	o-phthaldehyde
PD	=	pars distalis
PKA	=	protein kinase A
PKC	=	protein kinase C
PT	=	pars tuberalis
r	=	correlation coeffcient
RNA	=	ribonucleic acid
REM	=	rapid eye movement
SA	=	serum albumin
SAD	=	seasonal affective disorder
S_{opt}	=	substrate concentration at maximum velocity
SCG	=	superior cervical ganglion
SCN	=	supra chiasmatic nucleus
SD	=	standard deviation
SEM	=	standard error of the mean
SNS	=	sympathetic nervous system
SSRI	=	selective serotonin reuptake inhibitor
TAD	=	tricyclic antidepressant
TDO	=	tryptophan-2,3-dioxygenase
TLC	=	thin layer chromatography
TRP	=	tryptophan
μM	=	micromolar

μg	=	microgram
μl	=	microlitre
V	=	velocity
V_{max}	=	maximum velocity
VOC	=	voltage operated channel

LIST OF FIGURES

APPENDICES

LIST OF TABLES

PUBLICATIONS

The articles published in recognised journals thus far as a result of this work are listed below.

1. Walsh, H.A and Daya, S. (1991). Administration of the heme precursor 5-aminolevulinic acid increases rat forebrain serotonin concentrations with a concomitant rise in liver tryptophan pyrrolase activity. *Med. Sci. Res.* 19:695
2. Walsh, H.A. and Daya, S. (1991). Melatonin displaces L-tryptophan from bovine serum albumin *in vitro*. *Med. Sci. Res.* 19:745
3. Walsh, H.A., Daya, S. and Whiteley, C.G. (1991). Inhibition of rat liver tryptophan pyrrolase by melatonin *in vitro*. *Med. Sci. Res.* 19:849
4. Walsh, H.A., Daya, S. and Whiteley, C.G. (1994). Mode of inhibition of rat liver tryptophan 2,3-dioxygenase by melatonin. *J. Pineal Res.* 16:188

ABSTRACT

The regulation of tryptophan-2,3-dioxygenase (TDO) (EC 1.13.1.12) and, to a lesser extent, pineal indoleamines, both *in vitro and in vivo,* is examined in this study.

Rat liver TDO is a cytosolic enzyme which plays a crucial role in the regulation of circulating tryptophan (TRP) levels. Stimulation of this enzyme by heme enhances the catabolism of TRP, making less TRP available for uptake into the brain and other tissues, and for protein synthesis. At pH 7, the enzyme has an approximate K_m of 100μM, is subject to substrate inhibition immediately beyond S_{opt} ([S] at V_{max}), and response of the enzyme is cooperative in both uninhibited and inhibited regions. Hill analysis of the uninhibited region reveals a biphasic plot and two classes of binding sites. Negative cooperativity is brought about through deprotonation of the enzyme. Substrate inhibition also occurs at both acidic and basic pH values with concomitant shifts in S_{opt}. The results obtained indicate that substrate inhibition could be an additional mechanism whereby the flux through the TRP-kynurenine pathway is regulated.

TDO is subject to a diurnal rhythm, with peak activity during the pre-dark period and the lowest activity towards the end of the dark period. It is possible that the enzyme controls the synthesis of the neurotransmitter serotonin (5-HT), and that the circadian rhythm in TDO activity is due to the endogenous rhythm of melatonin (aMT) production by the pineal gland. In the present study, aMT displaces TRP from bovine serum albumin (BSA) *in vitro*, and it is therefore possible for the indoleamine to regulate the availability of TRP for uptake into the brain for conversion to its derivatives.

Chronic intraperitoneal administration of aMT affects physiological hepatic parameters in rats, such as TDO activity and stromal fatty acid composition, whilst no observable effect is demonstrable with respect to protein synthesis, nucleic acid metabolism, membrane fatty acid composition and pineal indole biosynthesis. On the other hand, chronic treatment of rats with antidepressants, the tricyclic desmethylimipramine (DMI) and the selective serotonin reuptake inhibitor (SSRI), fluoxetine, reveals significant negative alterations in TDO concentrations and pineal indoleamine synthesis. Combining aMT with any of these two drugs normalises the activity of the hepatic enzyme.

DMI is found to be an effective inhibitor of TDO in the micromolar range *in vitro,* and also affects total enzyme concentrations *in vivo*. Fluoxetine has no effect on TDO *in vitro*, but *in vivo* also reduces total enzyme levels in the liver. However, the SSRI does not affect conjugation between apo- and holoenzyme. Instead, it decreases extant holoenzyme levels. Indoleamine synthesis by the pineal gland, in organ culture,

is altered by both antidepressants, although in different ways. DMI increases N-acetylserotonin levels and reduces the output of methoxyindole acetic acid and methoxytryptophol. Fluoxetine treatment markedly reduces aMT concentrations and also brings about high levels of the 5-HT catabolites, 5-hydroxytryptophol and 5-hydroxyindole acetic acid.

Insulin also lowers aMT synthesis significantly in pineal organ cultures, via a mechanism that involves inhibition of the enzyme, N-acetyl transferase, that regulates aMT synthesis. The effects of insulin on pineal indole metabolism are due to the observation that a carbohydrate rich diet which induces insulin release elevates plasma TRP and brain 5-HT, but has no effect on pineal TRP and indoleamine synthesis. It could thus be possible for insulin to have an effect on the pineal,since the latter is outside the blood brain barrier.

The findings of this study support the biogenic amine deficiency hypothesis, implicating some of the major biogenic amines such as noradrenaline (NA), 5-HT and aMT in depression. There is believed to be a deficiency of NA and 5-HT at their respective synapses in the depressed state. The drug DMI could act, firstly, by inhibiting TDO and thus increasing plasma TRP levels, and could, secondly, stimulate NA release and inhibit NA reuptake at the pineal membrane. The combined effect would be to enhance aMT synthesis, with eventual remission. Fluoxetine, on the other hand, appears to utilize a slightly different mode of action to DMI, which seems to focus on the preservation of 5-HT. The fact that aMT counteracts the effects of both antidepressants, and restores the activity of TDO to that of the controls, is also consistent with the observation that the therapeutic action of drugs such as these coincides with the restoration of normal plasma levels of the neurohormone in depressives.

In view of the biogenic amine deficiency hypothesis of depression and the contentious claim that TDO is the major peripheral determinant of brain TRP, brain 5-HT and ultimately aMT, the regulation of TDO is investigated and discussed. The study concludes that TDO activity is regulated by a number of endogenous compounds which are mainly derivatives of TRP, such as aMT and oxidized nicotinamide adenine dinucleotide and exogenous substances, of which DMI and fluoxetine are but two. In addition, modulation of TDO activity in depression appears to be an important aspect of antidepressant action. aMT, the product of the pineal gland, also has the potential to increase plasma TRP and hence forebrain TRP levels, and ultimately 5-HT concentrations, firstly by displacing TRP from serum albumin and secondly by inhibiting TDO.

CHAPTER 1

LITERATURE REVIEW

1 INTRODUCTION

Hepatic enzyme tryptophan-2,3-dioxygenase (TDO) is a branch point in the metabolic pathway of L-tryptophan (TRP) metabolism. The activity of this enzyme determines firstly how much of the essential amino acid is converted to nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) and ultimately to nucleic acids, and secondly the amount of TRP available for uptake into the brain to be converted to serotonin (5-HT). Intermediates and end products of both pathways are crucial in the proper functioning of any organism. It is therefore conceivable that the relative concentrations of these catabolites of TRP need to be finely regulated to achieve that objective. Excesses and deficiencies of TRP and its metabolites, such as the neurotransmitter 5-HT, the neurohormone melatonin (aMT), the vitamin niacin etc, could result in affective disorders and diseases such as pellagra.

One such affective disorder is depression, for which various theories have been proposed in an attempt to define the underlying neurobiological aberrations that precipitate this potentially debilitating illness. The disease is a major source of loss of productive individuals in a rapidly expanding and competitive society with its myriad of challenges and needs. Some of the negative aspects of the depressive episode are, amongst others, destruction of the family, alienation from society, loss of a sense of responsibility, drug abuse and in extreme instances suicide.

Thus there is a crucial need to gain insight into some of the sociological and biochemical events that precipitate depression if there is to be a rational strategy to deal with the problem both on the psychological and neurobiological levels. Any attempt at effectively treating this disorder requires knowledge of the pathophysiology of the disease, ie the biochemical changes that accompany the appearance of the symptoms of depression. One of the hypotheses which have been proposed to account for depression symptomatology asserts that TDO is the major peripheral determinant of brain TRP, and ultimately 5-HT concentrations, the latter being implicated in depression. Dysregulation of this hepatic enzyme's activity consequently results in imbalances in the serotonergic neurobiological system, and eventually in aMT levels. aMT, the major indolealkyleamine product of the endocrine gland, the pineal, is generally accepted as the endogenous circadian rhythm generator, besides having other purported roles which include that of an antioxidant and a neuroprotectant.

The biological rhythm of this neurohormone is known to be disturbed in depression, as is that of a number

of other important biological substances such as 5-HT and glucocorticoids. Efficacious antidepressants such as the tricyclic desipramine (DMI) and the selective serotonin reuptake inhibitors (SSRIs) (such as fluoxetine) used to treat major depression have as part of their mechanism of action the ability to inhibit TDO, and thus increase plasma TRP concentrations, in addition to influencing aMT and 5-HT synthesis by the pineal gland. Thus, knowledge about the precise regulation of TDO *in vivo* is of fundamental importance.

It is apparent after surveying the vast literature available on depression and its therapy that the unifying factor in almost all subtypes of depression is TRP. Curtailed serum and cerebral TRP levels and brain 5-HT turnover, as a consequence of an enhanced TDO rate of catalysis, are causally linked to the pathogenesis of the clinically characterised depressive episode in humans. TDO thus has the potential to be a key regulatory site in the modulation of certain aspects of central nervous system activity, and alterations in the functioning of TDO could influence the individual's behaviour. In this study an effort is made to examine some of the supposed biochemical parameters involved. The questions to be addressed are as follows:

i) How is the activity of TDO regulated *in vitro*?

ii) To what extent is brain 5-HT altered when TDO is activated?

iii) Do some antidepressant drugs such as DMI and fluoxetine, whose mode of action is not completely known, exert their therapeutic effects by altering hepatic TDO activity?

iv) Does the pineal gland hormone aMT, a structural analogue of TRP, regulate TDO activity and have an influence on plasma TRP levels and how does this inhibition differ from that effected by 5-HT?

v) What effects do DMI, fluoxetine (*in vitro* and *in vivo*) and the pancreatic hormone insulin (*in vitro)* have on pineal indole metabolism?

Both male and female Wistar rats are used as models to evaluate the effects of various TRP derivatives and antidepressants *in vitro* and *in vivo*, and as a source of TDO. In Chapter 1, the literature survey overviews the relevant written material relating to some of the pertinent aspects of TRP metabolism, the biological determinants of depression, primarily TDO and, to a minor extent, TRP-hydroxylase. The various hypotheses regarding depression are also reviewed, however the indoleamine hypothesis and the roles of 5-HT and various antidepressants are covered in greater detail. Finally some of the biological functions of aMT in metabolism and its role in depression and neuroprotection are briefly overviewed. In Chapter 2 the phenomenon of substrate inhibition of TDO by TRP is examined *in vitro*. The *in vitro*

effects of selected TRP derivatives and the *in vivo* consequences of the heme precursor 5-aminolevulinic acid (5-ALA) on TDO activity are examined, as well as the influences of 5-ALA on forebrain 5-HT levels. Chapter 3 outlines the experimental work performed with the tricyclic antidepressant (TAD) DMI and the SSRI fluoxetine, either individually or in combination with aMT, to observe their effects on TDO and on indoleamine metabolism in pineal organ cultures *in vivo*. The effect of insulin on pineal indole metabolism in pineal organ culture is investigated in Chapter 4. Finally, Chapter 5 examines the *in vivo* effects of aMT on TDO, pineal indole metabolism and various other physiological parameters such as hepatic nucleic acid synthesis, protein metabolism and fatty acid status. The effects of melatonin and selected antidepressants on bovine serum albumin (BSA)-bound TRP are also experimentally ascertained *in vitro*. The concluding chapter (6) presents a summary of the research findings, and suggests possible future areas of research.

1.0 TRYPTOPHAN: ESSENTIAL AMINO ACID

L-TRP, one of the eight essential amino acids encoded by ribonucleic acid (RNA), has to be obtained from the diet in human beings. This molecule is the only indole of all the amino acids and has the greatest molecular weight of the twenty known amino acids incorporated into protein. TRP derivatives constitute a number of physiologically important molecules such as the neurotransmitter 5-HT, the neurohormone aMT, the vitamin niacin, proteins, and pyridine nucleotides (Maurizi, 1990) such as nicotinamide adenine dinucleotide (NAD^+) (Feigelson *et al.*, 1951; Williams *et al.*, 1950; Nishizuka and Hayaishi, 1963) (figs. 1 & 2).

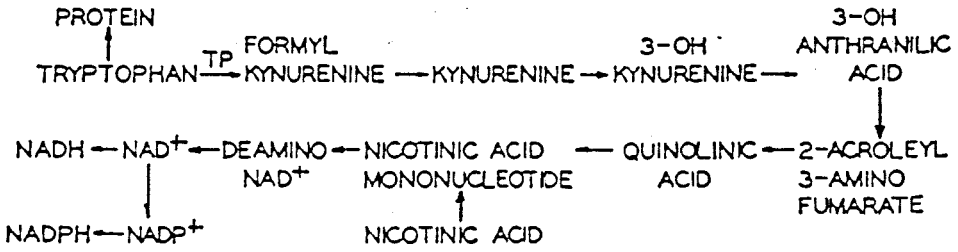


FIG. 1: The TRP - kynurenine pathway [Cho-Chung and Pitot, 1967].

The D-isomer is poorly absorbed and metabolised in both humans and animals (Langer and Berg, 1955; Price and Brown, 1956). In mice, exogenous TRP increases, *in vitro*, hepatic protein synthesis (Sidransky *et al.*, 1967; Sidransky *et al.*, 1968). Deficiencies of this essential indole result in a reduced rate of liver enzyme protein synthesis in the rat (Nishizuka and Hayaishi, 1963).

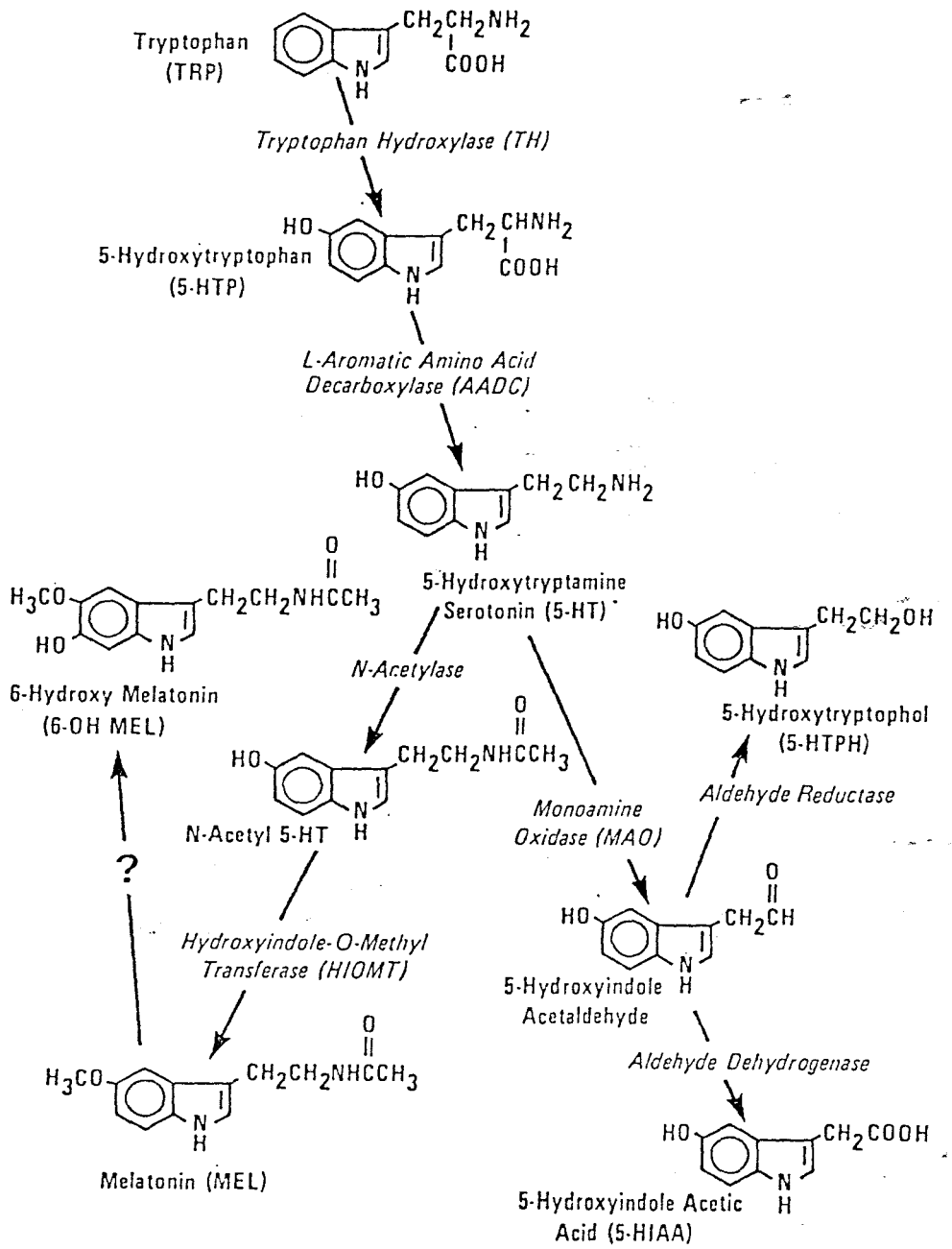


FIG. 2: Proposed synthetic route for TRP-indole metabolism in rat brain and pineal [Mefford and Barchas, 1980].

There are essentially two sources of plasma TRP in the body: increased hepatic portal output after a protein rich meal, and secretion by the tissues as a consequence of alterations in the concentrations of bound and free TRP pools in the body. The manner in which TRP exits from the blood potentially involves three mechanisms: firstly, by uptake into the tissues, secondly, due to hepatic TDO catabolism and, thirdly, a small proportion is eliminated in the urine (Fernstrom and Wurtman, 1971). TRP is transported in the blood either free or ionically bound (70-90%) to serum albumin (SA) (Mcmenamy and Oncley, 1958; Smith, 1991). This binding is highly stereospecific, saturable and reversible (Mcmenamy and Oncley, 1958). The ionic nature of the association is demonstrated by the fact that a reduction in pH of the environment causes a release of TRP into the surrounding medium (Mcmenamy *et al.,* 1958). Human serum albumin has two specific binding sites (known as site one and site two (Muller *et al.,* 1994)) one for warfarin and another for indoles (Sjoholm and Ljungsteadt, 1973; Noctor *et al.,* 1992) and benzodiazepines, as well as a number of non-specific binding sites (Geisow, 1992). Receptor binding sites also exist for the amino acid on hepatic nuclear envelopes in rats, where stimulation of these receptors by TRP could enhance protein synthesis (Sidransky *et al.*, 1994). The binding to these receptor sites is also saturable, stereospecific and highly specific. Other indolic compounds, such as 3-methylindole, apparently compete with the parent indole for binding to these receptors (Sidransky *et al.*, 1992).

On the other hand, non-essential fatty acids (Curzon *et al.*, 1973; Badawy *et al.*, 1984) and certain drugs (Badawy *et al.*, 1984) increase free TRP levels through displacement of TRP from serum albumin both *in vitro* and *in vivo* (Badawy *et al.*, 1984; Smith and Poison, 1980). Generally, compounds with large hydrophobic groups could readily displace TRP from serum albumin due to the presence of expansive nonpolar regions on the albumin molecule that straddle the specific binding site of TRP (Mcmenamy and Oncley, 1958). However, for any compound to have an effect on increasing the free serum TRP levels by displacement from SA, the rate of displacement would have to be more rapid as opposed to the rate of TRP passage through the liver. In addition there would have to be a large hepatic fractional extraction. The latter is necessary so as to assist in maintaining the equilibrium between bound and free TRP (Salter *et al.*, 1989). TRP levels in plasma can be measured in the picogram range using high performance liquid chromatography (HPLC) coupled to an electrochemical detector (ECD) on a reverse phase column (Mefford and Barchas, 1980) or using HPLC coupled to a fluorometric detection system (Anderson and Purdy, 1979). Hallucinogenic derivatives of TRP such as lysergic acid, bufotenine, methoxybufotenine, psilocybin, dimethyltryptamine and harmaline have their biosynthetic origin in plants. These indoles can act as inhibitors of monoamine oxidase (MAO) (Maurizi, 1990). The major factor generally accepted to be important in determining brain TRP concentrations is the plasma free TRP level (Bloxam and Curzon, 1978; Curzon *et al.*, 1975). In the brain, conversion to indoleamine derivatives such as the

neurotransmitter 5-HT occur (Wurtman, 1982). Uptake of TRP into the brain occurs to a limited extent, passively, via diffusion, and albumin inhibits TRP uptake (Etienne *et al.*, 1976; Curzon *et al.*, 1973). However an active uptake system is present that transports the amino acid into the brain in competition with other large neutral amino acids (LNAAs) (Wurtman, 1982; Goldstein and Betz, 1983). In rats, high levels of the dietary amino acid TRP, increases brain concentrations of the indole (Block and Harper, 1991).

For any dietary precursor molecule to have an influence on increased neurotransmitter synthesis in the brain five sequential biochemical phenomena are required:

i) A pronounced rise in the plasma levels of the precursor after the ingestion of a meal rich in the starter molecule is necessary.

ii) A positive correlation must exist between plasma and brain levels of the precursor nutrient.

iii) The transport mechanism at the blood/brain barrier should be acutely sensitive to alterations in plasma levels of the relevant substrate.

iv) In addition, the neuronal enzyme catalysing the transformation of the substrate should be of the low affinity type.

v) The neuronal enzyme should not be subject to feedback inhibition.

These conditions have to be met in order to demonstrate the reliance of brain neurotransmitters such as 5-HT, acetylcholine and the catecholamines on their plasma precursors. This points to a real precursor-neurotransmitter relationship which implies an adaptive value. However, it should be emphasised that although there is both an increase in substrate levels and an enhanced rate of neurotransmitter synthesis, there is not a concomitant rise in neuronal firing rate (Wurtman, 1982).

Increasing consumption of protein would appear to be a logical way of ensuring an adequate supply of TRP. However, the TRP content of good quality protein is relatively small when compared with other LNAAs. These LNAAs are also necessary for synthesis of certain neurotransmitters and protein (Etienne *et al.*, 1976; Yokogoshi *et al.*, 1987). The LNAA's compete with TRP for cellular uptake and are present in much higher levels in protein. On the other hand TRP has a greater molecular weight than any of its uptake competitors and, in addition, its numerical disadvantage on a stoichiometric basis is even more than the listed gram weight ratios. In the light of these factors it is not surprising that the intake of good quality protein paradoxically lowers brain TRP (Goldstein and Betz, 1983; Yokogoshi *et al.*, 1987). However, another dietary paradox is that consumption of a protein-poor carbohydrate-rich meal increases brain levels

of TRP (Goldstein and Betz, 1983; Brindley *et al.*, 1984). This is because carbohydrate induced secretion of insulin decreases the serum levels of the amino acids that compete with TRP for brain uptake, due to the fact that TRP is the only amino acid that is transported in the blood bound to serum albumin, while the LNAAs are free and therefore readily taken up into the tissues (Goldstein and Betz, 1983). The continued uptake of carbohydrate alone will obviously also precipitate a TRP deficiency (Bloxam and Curzon, 1978), and large carbohydrate meals do not increase 5-HT or 5-hydroxyindole acetic acid (5-HIAA) levels in the rat brain (Brindley *et al.*, 1984).

Brain TRP levels, however, appear to be influenced to a greater extent by changes in free plasma TRP levels than by changes in the serum concentrations of competing amino acids (Bloxam and Curzon, 1978) or total plasma TRP. More specifically it is the ratio of TRP to LNAA that ultimately determines the translocation of TRP into the brain (Wurtman, 1982). It is this ratio that depicts more accurately the concentrations of brain TRP and 5-HT and not the total basal TRP value (Green *et al.*, 1962, Delgado *et al.*, 1990). In the blood, however, the diet seems to have a greater influence on TRP levels (Delgado *et al.*, 1990) than on the concentrations of other amino acids. The magnitude of this elevation depends on the time of day that the protein is ingested, given that the plasma TRP is subject to a circadian rhythm that is possibly due to the intrinsic diurnal rhythm of the hepatic enzyme TDO which catabolises TRP to N'-formylkynurenine (Fernstrom and Wurtman, 1971). Plasma TRP levels peak at midday and the troughs occur at night (Sugden, 1983) (fig. 3).

Diets free from or low in TRP cause a lowering of plasma levels of TRP in healthy humans. However, this is not accompanied by any adverse or serious medical or psychological manifestations if the period involved is kept to a minimum (Delgado *et al.*, 1990). TRP is also metabolised by the non-specific, ubiquitous indoleamine-2,3-dioxygenase found in both animals and bacteria (Shimizu *et al.*, 1978; Taylor and Feng, 1991). Research data supports the notion that the availability of the amino acid constitutes a major factor controlling the rate of conversion to brain 5-HT (Fernstrom and Wurtman, 1971; Green *et al.*, 1962, Delgado *et al.*, 1990) and also enhances the production of aMT. Thus physiological changes in plasma TRP appear to cause parallel alterations in brain 5-HT synthesis. Plasma TRP has a negligible effect on the pineal TRP and thus there appears to be differential metabolism of 5-HT and TRP (Sugden, 1983). Concentrations of central 5-HT display a diurnal rhythm that is possibly due to the rhythm in plasma and brain TRP levels (fig. 3), which in turn is dependent on the activity of TDO (Fernstrom and Wurtman, 1971). A recent report, however, indicates that administration of fatty acids to rats results in an accumulation of brain TRP, but no concomitant enhanced brain 5-HT synthesis until ± 5 hours later (Yokogoshi *et al.*, 1993). There appears to be no relationship between the circadian rhythm of pineal TRP

(fig. 3) and the bio-rhythm of serum TRP. These two events seem to be independent of one another. Pineal TRP levels rise prior to any increase in serum free or total TRP. It is possible that this event could take place in order to prime the pineal for the night-time elevation in aMT concentrations when high levels of TRP are required for neurohormone synthesis. This phenomenon seems to indicate that an active TRP uptake mechanism exists within the pineal, in addition to passive diffusion, that transports the amino acid into the endocrine gland against a concentration gradient. The independence of pineal TRP concentration from serum TRP levels is further demonstrated by the fact that the end of the light period is accompanied by a rapid reduction in pineal TRP (which could probably be ascribed to enhanced transformation to 5-HT (Sugden, 1983)), and this decrease in TRP is not parallelled in blood.

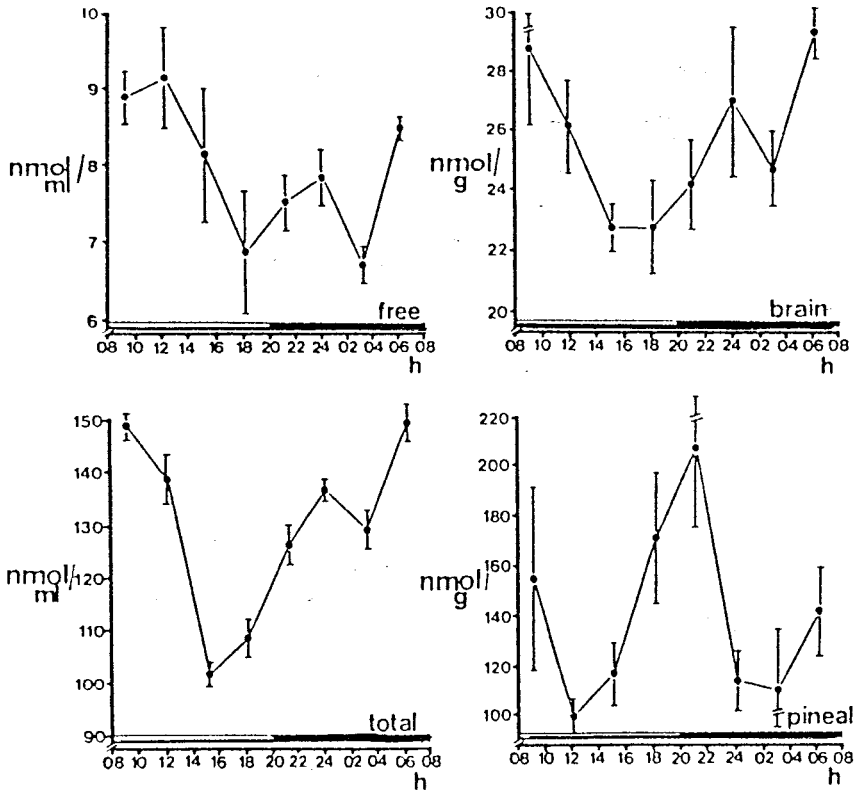


FIG. 3: Circadian changes in serum free, serum total, brain and pineal TRP levels of the rat. (The mean (±SEM) value for five rats is shown at each time point) [Sugden, 1979].

1.1 FUNCTIONS OF TRP DERIVATIVES

Due to stress and the dietary limitations of TRP, lack of this essential amino acid with concomitant secondary deficiencies of its derivatives 5-HT and aMT could be common. Brain function, with respect to second messengers, receptor synthesis, peptides and a variety of interrelated metabolic phenomena, can

be adversely altered by acute TRP depletion. Interactions between the serotonergic and other neuronal systems can be similarly disrupted. The finding that remitted depressed patients relapse within a very short time interval after acute TRP withdrawal implicates TRP and its derivatives specifically in the pathophysiology of depression (Delgado *et al.*, 1990). However, it does not exclude the involvement of factors unrelated to TRP metabolism in the depressive episode. Thus the process of becoming depressed possibly encompasses a whole range of interrelated phenomena including genetic factors. It suffices to state that attempts to understand the underlying neurobiological and biochemical events that occur in the depressed state depend almost entirely on studying the mechanism of action of the range of antidepressant drugs in use at present.

Proposed roles of 5-HT include, *inter alia*, neural development, sleep, feeding, temperature regulation, pain, maternal behaviour and adrenocorticotrophic hormone (ACTH) secretion (Soubrie, 1986; Lopez-Ibor, 1988). aMT on the other hand is implicated in circadian rhythmicity (Axelrod, 1974) and seasonal reproduction (Reiter, 1982) in certain animal species, but the role of this neurohormone has not been adequately clarified in humans. Possible roles with regard to humans have, however, been postulated. aMT is implicated in a number of diseases particularly with respect to its role as the natural antidepressant, neuroprotectant (Blickenstaff *et al.*, 1994) and free radical scavenger (Hardeland *et al.*, 1993a), and its involvement in human behaviour (Maurizi, 1990).

1.2 DYSFUNCTION OF TRP DERIVATIVES

1.2.0 Mental depression

Evidence indicates that TRP deficiencies and excesses are common and have a role to play in human disease (Vaughan, 1984). TRP depletion appears to have a negative effect on learning ability in individuals (Park *et al.*, 1994). Excess TRP has been implicated in hepatocyte lipid accumulation in rats by certain researchers. However, large doses of TRP administered to rats could not demonstrate such an effect on fatty livers or any morphological alterations. The lipid accumulation in this instance is believed to be related to the imposed food deprivation (Mathies and Jacobs, 1993). In another report, TRP loading promoted lipid peroxidation in rats. However, this effect is thought to be due to the anticipated concomitant increase in 5-HT plasma levels (Aviram *et al.*, 1991). Pellagra, a disease caused by a shortage of TRP and niacin, is linked to severe depression and dementia. However, it was recently discovered that the disease is also due to a lack of iron in the diet which reduces TRP utilization. This is understood to be a result of the fact that two enzymes in the pathway of TRP metabolism, one of which is TDO, require iron (Fe) for activity. Thus a lack of iron in the diet would restrict conversion of TRP to niacin (Oduho *et al.*, 1994).

A negative correlation exists between free plasma TRP levels and depression during acute TRP depletion (Mcdougle *et al.*, 1993), where a lack of the amino acid has a pronounced effect on the depressive symptoms (Curzon and Bridges, 1970). There is considerable evidence that TRP metabolism is disturbed during depression, namely:

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

b) Low levels of 5-HIAA, the excretory product of 5-HT metabolism, implies a decreased level of 5-HT, and is also indicative of dysregulated TRP metabolism.

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

Abnormal 5-HT levels in the brain and cerebrospinal fluid (CSF) could be due to a number of biochemical variables of which high TDO activity is one. Although animal studies indicate a negative correlation between 5-HT levels and TDO activity (Curzon and Bridges, 1970; Litman and Correia, 1985), results obtained from clinical studies reveal elevated TDO activity even after recovery from the depressive episode in some patients. Thus, in human subjects, there appears to be no consistent temporal relationship between excreted metabolites or the severity of the depressive illness and enzyme activity (Curzon and Bridges, 1970).

Most antidepressant drugs involve either noradrenaline (NA) or 5-HT, or both neurotransmitters. It is believed that the therapeutic effect of certain antidepressant drugs could be dependent on the availability of 5-HT (Delgado *et al.*, 1990). 5-HT is implicated in a variety of functions by virtue of the anatomy of the serotonergic system.

The 5-HT system originates in the raphe nuclei of the brain stem from where it branches into diverse cerebral areas such as the cortex, hypothalamus, basal ganglia and the limbic system (specifically the septohippocampalsystem and the amygdala). The limbic system is associated with mood and emotions, and harbours 5-HT receptors. Thus, specific antidepressants targeted at these receptors are likely to profoundly affect the patient's mood (Eison, 1989). In addition, the regions of the locus ceruleus and the dorsal raphe nucleus lack a blood brain barrier (Felten and Crutcher, 1979) and are therefore likely to be extremely sensitive to fluctuations in the serum levels of the amino acid (Maurizi, 1990). Dysfunction of these neurons is implicated in depression because they are important elements of the sleep/wake mechanism, and disruptions of this cycle are characteristic of depression of the bipolar variety (Jouvet,

1972). Cellular losses in the locus ceruleus (Zwieg *et al.*, 1988; Zubenko and Moossy, 1988), dorsal raphe nucleus, and serotonergic central superior nucleus (Zubenko and Moossy, 1988) are linked to symptoms of depression in Alzheimer's disease.

In addition to its dietary disadvantage, TRP levels are also subject to depletion by stress. Cortisol stimulates RNA and protein metabolism in rat liver (Feigelson *et al.*, 1962) through direct interaction with deoxyribonucleic acid (DNA). Increased cortisol sequestration by the adrenal glands is stimulated by stress resulting in an enhanced catalytic rate of hepatic TDO (Young, 1981) with consequent elevated TRP catabolism and kynurenine production. Cortisol release is normally regulated by vasotocin, a hormone whose excretion is in turn curtailed by aMT input (Pavel and Goldstein, 1979; Pavel *et al.*, 1977). The resultant effect of elevated cortisol concentrations is inhibition of aMT synthesis, thereby effectively removing the modulatory influence of the indolealkyleamine. It is thus reasonable to assume that prolonged stress would institute an undesirable cyclical chain of events in the following sequence: raised cortisol → reduced TRP → lowered 5-HT → decreased aMT → elevated cortisol (Maurizi, 1990).

Identifying the determinants which control brain TRP thus becomes an important issue, given that brain TRP has a major influence on 5-HT synthesis and ultimately on the activity of a population of central nervous system neurons. Acute or chronic diseases of the liver are normally characterised by changes in the levels of the monoamines, 5-HT and dopamine (DA), and also in the activities of hepatic enzymes such as TDO (Litman and Correia, 1985). There are accompanying increases in the levels of certain metabolites such as 5-HIAA, homovanillic acid (HVA), neurotransmitters, and also TRP and tyrosine in the brain and CSF. These changes are important for two reasons: firstly, they cause neurological disturbances (Bloxam and Curzon, 1978; Litman and Correia, 1985) and secondly, the mechanism of hepatic TRP elevation in the diseased state is unclear. Thus a means is provided whereby the regulatory mechanisms involved in the control of TRP levels can be studied. In acute liver failure in animals and man these changes can be explained by an increase in plasma free TRP. However in chronic liver failure in man and portocaval anastomosis this is not the case (Bloxam and Curzon, 1978; Hirayama, 1971).

Nonetheless, given that TRP levels in plasma and brain are important, the question arises as to what ultimately determines these circulating levels. Are they determined by the enzyme TDO, which is believed to be the major peripheral determinant of circulating TRP, or is the regulatory decision made in the brain via the rate limiting TRP-hydroxylase? It is speculated that it is specifically the ratios and absolute levels of the aromatic amino acids, TRP, tyrosine and phenylalanine, which are crucial for uptake into the brain for biogenic amine generation. Thus, alterations in the activities of the so-called 'rate limiting hepatic

enzymes' - TDO, tyrosine amino transferase and phenylalanine hydroxylase - could secondarily manipulate neurotransmitter synthesis via amino acid extraction from plasma. It is inconceivable that any one particular enzyme could have absolute control over a specific pathway, because the metabolic regulatory motif invariably involves a 'sharing' between various components of any particular pathway. Although the fact that the activity of one enzyme can play a major role is not discounted, it is highly likely that in addition to competition for transport into the brain, there is also a scramble for uptake into the hepatocytes (Salter *et al.*, 1986). It is thus possible that imbalances in certain amino acid concentrations could give rise to alterations in biocatalyst activity - for example, in the TRP-nicotinamide pathway (Shibata *et al.*, 1993).

1.2.0.1 Biochemical theories of depression

1.2.0.1.1 Biogenic amine deficiency hypothesis

The biogenic amine deficiency hypothesis (Schildkraut, 1965; Bunney and Davis, 1965; Lapin and Oxenkrug, 1969) states that there is an absolute or relative deficiency of monoamines (Schildkraut, 1965). According to this theory, two subtypes of depression prevail. Firstly, there could be a lack of NA at noradrenergic receptor sites (or DA at DA receptor sites) or secondly, there could be a deficiency of 5-HT at serotonergic receptors in the brain (Bunney and Davis, 1965; Schildkraut, 1965; Coppen, 1967; Lapin and Oxenkrug, 1969). In the case of mania or elation there is a surplus of the neurotransmitter at the synapse. Support for this hypothesis stems from clinical observations that efficacious antidepressants act by supplementing the available neurotransmitter levels at the post-synaptic receptors via either a blockade of monoamine reuptake (as is the case with most TADs) or, alternatively, through inhibition of monoamine catabolism by the enzyme MAO. Results obtained from studies thus far have failed to demonstrate the hypothesised randomness of biogenic metabolism in the depressed state. In addition, these empirical observations have neglected to illustrate a clearcut relationship between alterations in monoamine levels and antidepressant therapy (Charney *et al.*, 1981). Further criticisms against the simplistic nature of this hypothesis are the following:

a) The efficacious atypical antidepressants, mianserin and iprindole, have a different mode of action (Fan *et al.*, 1972 cited in Skene, 1979).

b) The therapeutic action of antidepressant drugs involves a lag period of approximately 2 to 3 weeks, but inhibition of reuptake and MAO occurs within minutes (Blier and de Montigny, 1994).

c) Compounds ineffective as antidepressants, such as cocaine and amphetamines (Hanson, 1967), raise the concentrations of catecholamines at their respective synapses.

1.2.0.1.2 Dopamine hypothesis

A major paradox of the indoleamine hypothesis of depression is the fact that the levels of the DA metabolite HVA are lower than those of the NA metabolite 4-methoxy-3-hydroxyphenyl glycol (MHPG) as opposed to normal controls in the central nervous system (CNS) (Syvälahti, 1994). This observation could imply that DA has a major role to play in depression (Syvälahti, 1994; Blier and de Montigny, 1994). Support for the involvement of DA in depressive illness comes from the observation that TADs and some of the recently developed antidepressants are potent inhibitors of DA uptake in rat brain synaptosomes (Randrup and Braestrup, 1977). On the other hand, a reversal of the symptoms of depression occurs in some patients treated with a DA agonist, piribedil (Post *et al.,* 1978) . Nomifensine, which is also an inhibitor of NA reuptake, has a similar action (Costall *et al.*, 1975; Schacht *et al.*, 1977).

1.2.0.1.3 Histamine hypothesis

This theory claims that a blockade of central histamine (H) receptors by antidepressants such as the TADs mianserin, iprindole and viloxazine both *in vitro* and *in vivo* (Tran *et al.,* 1978, 1981; Hall and Ögren, 1981) are responsible for remission in depressives. H_1 receptors are acutely blocked by a range of antidepressant drugs (Figge *et al.,* 1979). However the degree of blockade is unrelated to the concentration of the blockers. Instead, the potencies correlate well with their ability to induce sedation (Peroutka and Snyder, 1980a).

1.2.0.1.4 Permissive amine deficiency hypothesis

Here the hypothesis states that a central 5-HT deficiency increases the risk of depression but is not in itself sufficient to precipitate a depressive episode (Kety, 1971; Prange *et al.*, 1974). What is also required is alteration in catecholamine levels, and this would produce the clinical syndrome, depression being associated with reduced levels of catecholamines and mania with high levels. The model claims that mania and depression are extremes of the same biological dysfunction, with mania being characterised by a more pronounced deviation from the normal mood (Court, 1968). This model is supported by the fact that both ailments respond to the same antidepressant treatment, for example with imipramine (IMI) (Akimoto *et al.*, 1960 cited in Skene, 1979) or lithium (Goodwin *et al.*, 1969). Furthermore, drugs such as cortisone can precipitate depression of the bipolar variety (Bunney, 1967).

1.2.0.1.5 Kynurenine hypothesis

This hypothesis claims that a link exists between abnormal 5-HT and adrenocortical metabolism (Lapin and Oxenkrug, 1969; Curzon, 1969). Hepatic TDO concentrations are increased by hydroxycorticosteroids (Knox, 1951; Knox and Auerbach, 1955). This observation provides a basis for the theory, which

maintains that in depression there is an increase in plasma cortisol which stimulates TDO synthesis at the level of the gene, thereby increasing TRP catabolism and diverting free plasma TRP away from 5-HT formation in the brain (Curzon, 1969; Curzon and Bridges, 1970). It is thus possible that kynurenine and other tryptophan metabolites such as 3-hydroxykynurenine are responsible for the symptoms of depression. Experimentally, hydrocortisone has been shown to reduce brain 5-HT and CNS 5-HIAA levels indirectly (Curzon and Green, 1968). In addition, increased levels of 3-hydroxykynurenine have been noted in individuals suffering from endogenous depression (Curzon and Bridges, 1970). Added support for this hypothesis comes from the fact that efficacious TADs IMI and DMI inhibit TDO in mice (Parachi, 1970).

1.2.0.1.6 Tryptamine hypothesis

This hypothesis (Dewhurst, 1968) has been proposed as an alternative to the monoamine theory of depression. It attempts to explain the functions of 5-HT and NA in terms of central receptors. Dewhurst asserts that two types of monoamine receptors prevail: an excitatory type, of which the 5-HT receptor (Type A) is an example, and a depressive type, such as the NA adrenergic receptor (Type C).

Experimental findings obtained from studies on immature chicks after stimulation of the respective receptors produced behavioural and physiological excitation (Type A) on the one hand and sedation (Type C) on the other (Dewhurst, 1968). The theory supposes that depression is a result of the lack of a monoamine or hyposensitivity at a particular receptor, with mania due to excess neurotransmitter or supersensitivity of the relevant receptor (Dewhurst, 1968). The hypothesis has two major drawbacks:

a) The first is the claim that NA and 5-HT have opposite effects. In humans NA is excitatory and not inhibitory.

b) The theory does not account for other neurotransmitters which are known to be implicated in the pathophysiology of depression such as DA (Skene, 1979).

1.2.0.1.7 False transmitter theory

Examples of false transmitters are α-methyl-dopa and octopamine (which has 1/100th of the efficacy of NA), as these compounds produce very little or no excitation when released. This theory (Murphy, 1972) proposes a role for such molecules in depression and states that the reduced central noradrenergic activity in depression could be a result of these false neurotransmitters. The overall result is a depletion of central biogenic monoamines similar to that effected by reserpine, as proposed by Schildkraut (1965). In patients with a history of depression, administration of the precursor of α-methyl-dopa gave rise to depressive

symptoms and suicidal behaviour (Smirk, 1963 cited in Skene, 1979), while in other depressed patients with reduced MAO activity, an accumulation of octopamine occurred in blood platelets (Murphy, 1972). Thus far, a limited amount of evidence has been accumulated in support of this hypothesis, and the exact role of these substances in depression requires further clarification (Skene, 1979).

1.2.0.1.8 Cholinergic - adrenergic hypothesis

This theory (Janowsky *et al.*, 1972a, 1972b) not only implicates the monoamines in affective disorders but also other biological amines such as acetylcholine. There is believed to exist a balance between cholinergic and monoaminergic transmissions. Disruption of this equilibrium precipitates a major affective disorder. Mania results from hyperactivity of the central monoaminergic system and depression is due to central cholinergic predominance. In other words, high monoamine and low acetylcholine levels give rise to mania while low monoamine and high acetylcholine concentrations result in depression.

This hypothesis has a strong empirical basis, and major support comes from studies with cholinomimetic agents in humans such as physostigmine. Administration of physostigmine to already depressed patients promoted depression symptomatology (Janowsky *et al.*, 1972b; Modestin *et al.*, 1973). Furthermore an acute dose of physostigmine had a sedative-like effect in a person suffering from mania, which eventually resulted in a state of psychomotor retarded depression (Janowsky *et al.*, 1972a; Carroll *et al.*, 1973). The indirectly acting cholinomimetic compound, reserpine, produces depression when administered to hypertensive patients (Goodman and Gilman, 1975). More evidence in support of this theory stems from the fact that the efficacious TADs block central cholinergic activity with a concomitant increase in adrenergic transmission (Janowsky *et al.*, 1972b; Sulser *et al.*, 1964). However, α-methylparatyrosine, a specific blocker of catecholamine synthesis, does not affect acetylcholine levels when given to hypertensive patients, nor does it precipitate a depressive episode (Charalampous and Brown, 1967 cited in Skene, 1979).

1.2.1 THE BIOLOGICAL DETERMINANTS OF DEPRESSION

Research into the biological determinants of depression focuses mainly on two areas: the psychopathological and the biochemical (Van Praag, (1982). In most instances depression involves the interaction of both biological and psychosocial factors (Blier and de Montigny, 1994; Syvälahti, 1994). Depression is not just due to a shortage of some vital brain neurotransmitter, but rather to a disrupted equilibrium between different regulatory systems with resultant neuronal hyperactivity in certain cerebral areas. Thus far, a definite causal relationship between the various determinants has not been established. There could however be a variety of different types of biological depressions which would require widely

differing biological treatment regimens to effect remission (Blier and de Montigny, 1994). There exists a relationship between monoamines and depression that is characterised by disturbances in the normal levels of certain neurotransmitters. Disruptions in monoaminergic systems are believed to be more a result of depression than the cause, and the therapeutic effects of antidepressants apparently involve these systems. Principal monoamines are the indoleamine, 5-HT, and the catecholamines, NA and DA. It is possible that chronic treatment with antidepressants causes alterations in other neurobiological systems which require monoaminergic input for effective operation. Withdrawal of monoamines in the interim would cause a collapse of those systems and result in a relapse with consequent reappearance of the symptoms of the depressed state (Delgado *et al.*, 1993; Sulser, 1978).

The pathophysiology of depression is also believed to include, *inter alia*, alterations in the effects of second and third messenger systems, protein kinases, phosphoproteins and phospholipids. Therapeutic action of antidepressants could depend on restoration of functional aberrations which pertain to all of the above (Lui *et al.*, 1985).

1.2.1.0 5-HT and depression: Indoleamine hypothesis

The indolealkyleamine, 5-HT, was first discovered in the intestine (Vialli and Erpsamer, 1933), in serum (Rapport *et al.*, 1947) and eventually also in peripheral tissues such as the gastrointestinal tract and heart (Twarog and Page, 1953). Ten years after its initial discovery, the neurotransmitter 5-HT's distribution profile was histochemically characterised in the brain and this observation prompted intense investigation with respect to its localised physiological roles (Frazer *et al.*, 1990).

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

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

b) The effects of antidepressants were either sensitisation of post-synaptic 5-HT_3 receptors (De Montigny, 1981) or alterations in receptor number (Cowen *et al.*, 1986).

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

Several lines of clinical and preclinical evidence implicate the 5-HT system in the therapeutic mechanism of action of antidepressants (Delgado *et al.*, 1990; Blier and de Montigny, 1994). Firstly, all selective 5-

HT reuptake inhibitors are effective in major depression. These drugs belong to different families, and the only property that they have in common is the ability to inhibit 5-HT uptake. Secondly, inhibition of 5-HT synthesis in remitted patients results in an immediate relapse, with symptoms identical to those of the earlier depressed state. It is also possible that a component of plasma plays a role in depression via inhibition of 5-HT binding to its receptors. A peptide from bovine forebrain is known to inhibit 5-HT receptor binding by the selective antidepressants mianserin and ketanserin (Roth *et al.*, 1985).

However the notion that a number of different neurobiological systems are involved in the pathophysiology of depression is supported by the following observations:

i) Not all patients suffering from major depression depict a reduced noradrenergic and serotonergic output (Blier and de Montigny, 1994).

ii) It has not been possible to bring about a clinically certifiable depressed state in healthy individuals by inducing a lack of 5-HT or NA either through TRP depletion (in case of 5-HT) (Park *et al.*, 1974) or with the use of a catecholamine inhibitor (α-methyl-*para*-tyrosine) for NA.

Nevertheless, a substantial body of evidence has thus far been collected in support of the 30 year old indoleamine hypothesis, which asserts that modifications in 5-HT neuronal function are a core feature of depression. The traditional monoamine hypothesis asserts that there is a metabolic and functional aberration in depression, and drugs which curtail the availability of 5-HT and NA precipitate depression while those which enhance their abundance are therapeutic (Coppen, 1967). This, however, was modified once it was realised that some monoaminergic receptors could be downregulated (Sulser, 1979). It is now hypothesised that there is monoaminergic hyperfunction in depression, and antidepressants operate so as to downregulate post-synaptic monoamine receptors (Van Praag, 1982).

Several research strategies have been employed to acquire empirical evidence to validate this theory. These include:

a) measuring peripheral indices of monoamine metabolism,

b) post-mortem determinations of the biogenic amine levels and identification in brain tissue of suicide and/or depressed individuals whose death was not due to unnatural causes,

c) analysis of CSF monoamine metabolites,

d) evaluation of the efficacy of drugs on central monoamine metabolism in depressives, and

e) endocrine research stimulated by the knowledge that the release of hypophyseal hormones is to

a certain extent affected by biogenic amines and brought about through hypothalamic inhibiting and releasing factors, and thus disturbances in hormone release could be related to dysregulation of monoamine metabolism (Van Praag, 1982).

Some of the major findings are, *inter alia*:

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

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

c) Chronic clinically efficacious antidepressant treatment enhances 5-HT neuronal transmission in the laboratory rat (De Montigny and Aghajanian, 1984).

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

e) Post-mortem brain tissue of depressed suicide victims (Stanley and Mann 1983; Mann *et al.*, 1986), drug free depressed patients (Yates *et al.*, 1990) and platelets of drug free depressed cases, display definite increases in the levels of 5-HT_{2A} receptor binding sites (Arora and Meltzer, 1989; Pandey *et al.*, 1990).

f) There are lowered levels of the 5-HT transporter binding sites in the frontal cortex and hypothalamus of depressed suicide victims (Stanley *et al.*, 1982), depressed patients (Leake *et al.*, 1991) and in the platelets of drug free depressed individuals (Nemeroff *et al.*, 1988). This reduction in transporter binding sites in platelets is not due to prior drug treatment or hypercortisolemia and is also not present in mania, Alzheimer's disease, schizophrenia, panic disorder, atypical depression or fibromyalgia. This implies a depression peculiarity (Mann *et al.*, 1989).

g) Reserpine, which is a depleter of monoamines from synaptic vesicles and also prevents vesicular amine transport, produces depression-like symptoms in rats (Curzon, 1969).

1.2.1.0.1 The significance of 5-HT disturbances

The question now arises as to whether these abnormalities in 5-HT levels are necessary events for depression to occur, or whether these are disturbances that could make a particular individual susceptible to depression. Thus far, research findings point to the likelihood of aberrant 5-HT metabolism increasing the risk of depression. There are a number of factors in support of this hypothesis which are, *inter alia*, decreased levels of both 5-HIAA and MAO plus high TDO activity persisting after remission in certain

subtypes of depressives. By implication, increasing 5-HT concentrations should be prophylactic, because if 5-HT plays an important role in the pathogenesis of depression then factors that could potentially affect 5-HT synthesis should be effective in treating depression. The molecular substances generally used normally increase 5-HT availability at the 5-HT receptors. Of the endogenous compounds, 5-hydroxytryptophan (5-HTP) has been more successful than TRP in increasing 5-HT levels (Van Praag, 1981; Jimerson *et al*, 1990). The most efficacious antidepressants clinically are fluoxetine and fluvoxamine. How effective a particular antidepressant therapy is ultimately appears to depend, to a certain extent, on dietary factors involved in 5-HT synthesis, as well as other changes in the implicated monoaminergic neurobiological system (Jimerson *et al*, 1990).

1.2.1.0.2 Biochemical studies

The accumulation of the 5-HT metabolite 5-HIAA provides a crude yardstick of CSF 5-HT metabolism in clinical depression. Low levels of 5-HIAA in CSF are a common denominator in approximately 50% of all cases of vital depression (Ashberg *et al.*, 1976). It is unclear whether there is a correlation between these two variables. However post-mortem studies on certain raphe nuclei revealed diminished 5-HT and 5-HIAA concentrations. Thus far no consistent link can be shown to exist between low CNS 5-HIAA levels and major depression, but in suicidal and aggressive depressives there appears to be a causal relationship (Twarog and Page, 1953). Concentrations of 5-HT and 5-HIAA have reportedly been reduced in certain rat cerebral areas following chronic treatment with the TAD, IMI. In addition, the levels of DA were changed while those of NA displayed only minor alterations (Fernstrom *et al.*, 1971). There could also be a link between low cholesterol and low 5-HIAA levels (Gross *et al.*, 1991). Another index of CSF 5-HT turnover in clinical depression is the reduced plasma ratio of TRP to LNAA in depressives. The clinical efficacy of a SSRI such as fluvoxamine has been evaluated using this specific amino acid profile in individuals suffering from major depression. This plasma TRP/LNAA ratio was found to serve as a good indicator of cerebral concentrations of TRP and 5-HT, and could function as a predictive biological index response to treatment with efficacious SSRIs (Carlson *et al.*, 1972).

1.2.1.0.3 Peripheral studies

Peripheral serotonergic markers of a depressed patient's clinical state are platelets and the neuroendocrine system. The function of 5-HT in platelets is believed to be in blood coagulation where it plays a role in platelet activation, alterations in shape and aggregation (Worral and Williams, 1994). Both peripheral indices, platelets and the neuroendocrine system studies indicate that there is serotonergic receptor dysfunctioning in depression. The availability of specific markers for the variety of 5-HT receptor subtypes should facilitate further clarification of the extent and nature of these proposed irregularities (Stahl, 1992).

1.2.1.0.4 5-HT receptors

Several different subtypes of the 5-HT receptor are believed to exist, namely 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2B}, 5-HT_{1D}, 5-HT_{1P}, 5-HT_2 and 5-HT_3 (Zemlan *et al.*, 1988). These subtypes are classified pharmacologically according to the drug that blocks the normal expression of the signal transducers and second messengers. As is the case with other biogenic receptors (cholinergic, dopaminergic and adrenergic), the various 5-HT receptor subtypes are linked to a number of different second messengers (Frazer *et al.*, 1990). Three clearly defined 5-HT receptor subtypes have been found to be linked to adenyl cyclase in the vertebrate brain - namely, 5-HT_{1A}, 5-HT_{1B} and 5-HT_D . Even though these receptors are known to activate or stimulate AC via G-protein couplings, it is not as yet possible to discern whether the individual receptor subtypes are linked to G_s or G_i. Thus far, in the rat hippocampus, the 5-HT receptor has been found to be linked to both inhibitory and stimulatory G-protein. Two receptor subtypes 5-HT_{1C} and 5-HT_2 are believed to be linked to phosphoinositide hydrolysis via phospholipase C activity (Hanson *et al.*, 1987).

1.2.1.0.5 The 5-HT Transporter

Four classes of neurotransmitter transport vesicles are recognised - namely, Ach, glutamate, GABA/glycine and the monoamine transporters. These transporters are highly specific for their respective substrates (Mann *et al.*, 1989).

The mode of action of both the TADs and certain 5-HT reuptake inhibitors involves binding to a 5-HT transporter protein. The TADs bind specifically to this transporter with the most potent being IMI and citalopram (Worral and Williams, 1994). Biochemical and pharmacological studies reveal distinct areas of specific functions (Giros *et al.*, 1994), and SSRIs such as fluoxetine, paroxetine and citalopram bind almost to exactly the same site as 5-HT (Owens and Nemeroff, 1984). TAD binds to transmembrane domains 6 to 8, while 9 to 12 through the COOH terminus are responsible for stereoselectivity and high affinity for substrate (Helmeste and Tang, 1994). The 5-HT transporter is known to have two binding sites for 5-HT in addition to two sites for TADs (Worral and Williams, 1994).

Molecular characterisation reveals that all known neurotransmitter transporters (5-HT, NA, DA, GABA etc.) display significant sequence homology within their 12 membrane spanning domains. They also exhibit - between the transmembrane domains 3 and 4 - glycosylation sites on the extracellular loop with tentative phosphorylation sites on the intracellular side (Owens and Nemeroff, 1984) (fig. 4). The 5-HT transporter has been isolated in three forms of 45-, 56- and 68kda (Tamir *et al.*, 1994). Little is known about physiological transporter regulation with respect to activity, stability or lipid requirement.

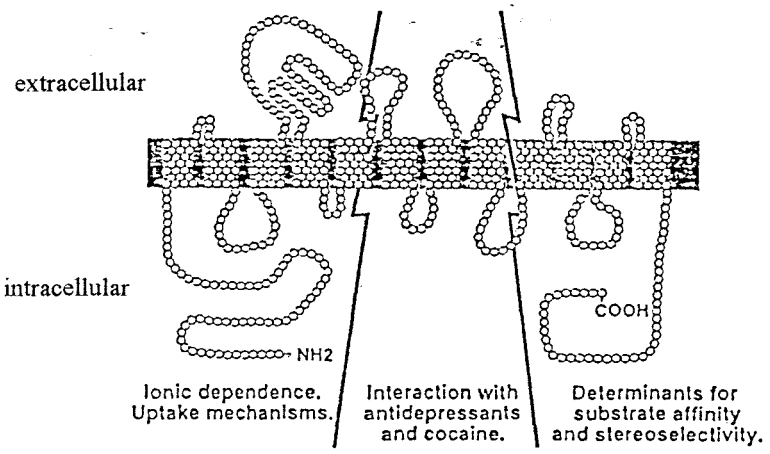


FIG. 4: Working model of the various putative functional domains of Na^+/Cl^-- dependent transporters [Giros *et al.*, 1994].

Certain transporters such as the GABA and glutamate require cholesterol with a high specificity and potency. The 5-HT binding protein and related members have, as previously stated, potential phosphorylation sites for cAMP dependent protein kinase A and protein kinase C. Protein kinase C is also possibly involved in regulation of transport (Worral and Williams, 1994; Helmeste and Tang, 1994). It has been reported that 5-HT transport in human blood platelets is reduced by protein kinase activators (Worral and Williams, 1994). Marked reductions in transporter mRNA occurred after chronic antidepressant treatment with the non-specific 5-HT/NA reuptake inhibitor IMI and the selective 5-HT reuptake inhibitor fluoxetine. This effect, however, could not be duplicated with the MAO inhibitor clorgyline or a 5-HT agonist such as 8-hydroxydipropylaminotetralin (Lesch *et al.*, 1993). However, experimental evidence does appear to support the notion of 5-HT transporter synthesis regulation at the level of mRNA transcription (Owens and Nemeroff, 1984).

1.2.1.0.6 Pharmacological studies

Pharmacologically, the biogenic amine deficiency hypothesis appears to be the most pertinent with respect to defining the underlying neurobiochemical phenomena responsible for depression symptomatology. This view is supported by the fact that remission in clinically depressed individuals is accompanied by a restoration of normal biogenic amine levels. Antidepressants such as the TADs, MAOIs and SSRIs, whose mode of action forms the basis of this theory, are at present the most effective in clinical treatment of

depression. These antidepressant drugs raise the synapse concentrations of 5-HT and catecholamines such as NA (Daya, 1994). Overall, however, the conventional therapeutic strategy in the investigation of the events occurring during depression involves use of 5-HT precursors TRP, 5-HTP, the TADs, SSRIs and post-synaptic 5-HT agonists. Thus far the results obtained implicate 5-HT in mood regulation (Van Praag, 1982), and most antidepressants enhance serotonergic function (Lui *et al.*, 1985). Drugs which selectively facilitate 5-HT mediated neurotransmission are d-fenfluramine, citalopram, femoxetine, fluoxetine, zimelidine and fluvoxamine (fig. 5).

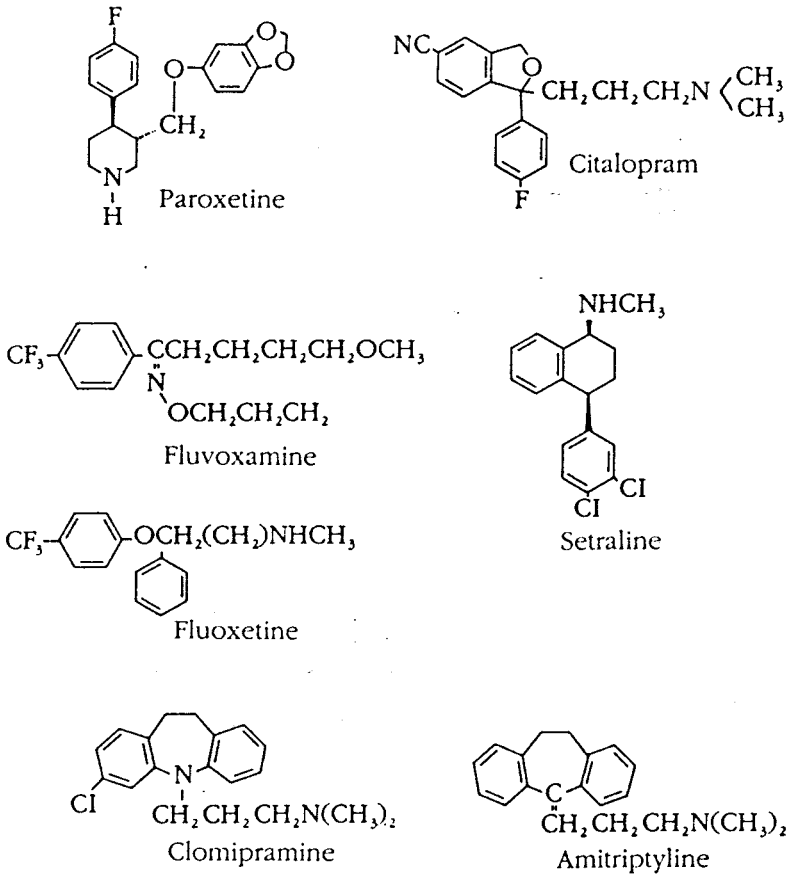


FIG. 5: Structural formulae of some selective SSRIs and TADs [SmithKline Beecham Pharmaceuticals: Paroxetine monograph, 1992].

Effective recovery due to antidepressant therapy depends on an intact and functional 5-HT neuronal system (Lui *et al.*, 1985). Antidepressants that interact with neurotransmitters other than 5-HT induce carbohydrate (CHO) craving and weight gain (Wurtman and Wurtman, 1988). Not all antidepressants are

amine uptake inhibitors and these are called atypical antidepressants which are effective in treating atypical depression. Atypical depression does not express the conventional symptomatology of depression (such as insomnia, appetite loss and psychomotor agitation) and is characterised by hyperphagia, excessive sleeping, persistant fatigue, mood alterations and chronic sensitivity to rejection (Horwath *et al.*, 1992). Examples of atypical antidepressants are iprindole and mianserin which block pre-synaptic α_2-receptors thereby raising NA synaptic junction levels (Pinder *et al.*, 1982) in addition to inhibiting TDO. Three major groupings characterise antidepressant drugs pharmacologically: TADs and MAOIs (both of which are termed first generation antidepressants), and the newer second generation antidepressants which differ both structurally and pharmacologically with respect to the TADs and MAOIs (see fig. 5). These so-called second generation antidepressants are further separated according to whether they prevent NA or 5-HT reuptake. Within this particular group are the previously mentioned atypical antidepressants and the SSRIs (such as fluoxetine) (Banoo, 1991).

Most antidepressant drugs are thought to mediate their specific action through the 5-HT_{1A} receptor sites located in the raphe of the hippocampus (Blier *et al.*, 1987). First generation antidepressant drugs increase the availability of 5-HT and NA at their central receptors (Kopin, 1981). The mechanisms involved in this process are:

a) reuptake inhibition by TADs mainly of NA and in certain instances 5-HT, and

b) inhibition of degradation by MAOI. The TADs such as DMI, nortryptaline, IMI, doxepin and amitryptaline are relatively selective NA reuptake inhibitors *in vitro*.

The first clinically effective antidepressants were the MAOIs (eg tranylcypromine), but they were superceded by the TADs for a number of reasons which mainly revolve around the undesirable side effect profile. The primary mechanism of action involves inhibition of MAO by binding to the active site on the enzyme (ie competitive inhibition) in brain and peripheral tissue (Kline, 1958). These antidepressants are effective in treating atypical depression, specifically the older type of non-selective MAOIs (Liebowitz *et al.*, 1988).

Antidepressants exert part of their therapeutic effect through enhancing serotonergic and noradrenergic activity at their respective synapses. The efficacy of the SSRIs such as fluoxetine is believed to depend on 5-HT while that of TADs is more dependent on the availability of NA. This inference is based on the fact that remitted patients on fluoxetine experienced an immediate relapse during acute TRP withdrawal, while remitted patients on DMI hardly responded when subjected to similar treatment (Delgado *et al.*, 1993).

Research data indicate that most antidepressants do not have a common mode of action: some such as the TADs may affect the 5-HT neuronal system and the noradrenergic system (Lemberger *et al.*, 1985) while others affect the serotonergic system only (eg SSRIs) (Delgado *et al.*, 1993a; Lemberger *et al.*, 1985).

The second generation antidepressants have a similar mode of action and are selective reuptake inhibitors of catecholamines (maprotilene and nomifensine for NA, and nomifensine for DA). TADs also inhibit hepatic TDO in order to increase plasma TRP levels and ultimately brain TRP and 5-HT (Badaway and Evans, 1981).

The atypical antidepressants, mianserin and iprindole, are drugs which are structurally and pharmacologically very diverse, have a limited ability to prevent reuptake of NA and 5-HT, but are known to inhibit TDO (Van Praag, 1982). The tricyclic DMI is also effective as an analgesic in diabetic neuropathy, acting via a noradrenergic dependent mechanism (Courteix *et al.*, 1994). However, even though the TADs have widespread use and are efficacious in clinical psychiatry, their use has a number of drawbacks. Due to their nonspecificity, these drugs have a broad pharmacological spectrum and affect a number of other physiological systems.

Clinical data obtained from depressives indicate that chronic treatment with tricyclics, such as DMI, elevates plasma NA (Veith *et al.*, 1994). This effect could be primarily due to two reasons: firstly, there could possibly be desensitisation of CNS pre-synaptic α_2-adrenergic receptors and, secondly, there might be enhanced sympathetic nervous system (SNS) activity. It was also experimentally determined that the increase in serum NA is not due to a reduced plasma clearance rate. On the other hand, contradictory findings have been reported which claim that TADs blunt SNS neurotransmission in both healthy and depressed subjects. Nevertheless, this latter effect can be ascribed to an initial brief transitory α_2-adrenergic receptor downregulation, and thus this manifestation could probably be an important aspect of the mode of action of the TADs (Veith *et al.*, 1994).

However, chronic DMI therapy has been found to result in a reduction in the number of post synaptic β-adrenergic receptors (Goodnough and Baker, 1994). In rats, chronic DMI causes β-adrenoreceptor downregulation in a dose dependent manner, an effect which is potentiated by fluoxetine. The SSRI fluoxetine on its own, however, does not induce a similar effect on either β-adreno- or 5-HT receptors (Banerjee and Kung, 1977; Goodnough and Baker, 1994).

5-HT plays a role in controlling food intake and macronutrient selection. Stimulation of paraventricular

and ventromedial nuclei of the medial hypothalamus by 5-HT leads to satiety and termination of eating (Pijl *et al.*, 1991). Fluoxetine has proven to be effective in treating obesity in humans, which provides evidence for its action on serotonergic pathways (Wurtman and Wurtman, 1989). In rats and mice, the antidepressant also causes a dose dependent reduction in food intake (Goodnough and Baker, 1994).

The high specificity of the two SSRIs, fluoxetine and norzimelidine, has been aptly demonstrated in humans. In depressed individuals the ratio of zimelidine to its N-methylated derivative norzimelidine is 1:7 in the CSF, but with no accompanying reduction in CSF MHPG concentrations. DMI levels, however, correlated well with a reduced count of MHPG, and this highlights the specificity of newer SSRIs such as zimelidine/norzimelidine and fluoxetine, and the noradrenergic orientation in the mechanism of action of a tricyclic such as DMI (Potter *et al.*, 1981). Further evidence, in support of the assertion that norzimelidine and fluoxetine selectively inhibit 5-HT uptake, is based on the fact that they both significantly lower the concentrations of the 5-HT metabolite 5-HIAA in the CSF (Meltzer *et al.*, 1979). Chronic and acute treatment of depressed patients with SSRIs had almost no effect on the neuroendocrine system, and these results contrast with those obtained from using the rat as a model where significant changes are brought about when they are subjected to similar treatment regimes as humans (Syvalahti, 1979).

All SSRIs share the same pharmacological profile but have different physicochemical properties, and thus have a varied physiologic disposition with respect to absorption, distribution, metabolism and excretion. However they are generally well absorbed, requiring from 4 to 8 hours to reach a plasma peak. The treatment regime of the SSRIs depends to a large extent on their half life in the body (Lemberger *et al.*, 1985). The half life of fluoxetine is 24-60h (Lemberger *et al.*, 1978) and of citalopram is 33h (Overo, 1982). These compounds require a once daily dose, whereas zimelidine (8h) and fluvoxamine (15h) have to be administered in multiple daily doses (Lemberger *et al.*, 1985).

A major criticism against the marketing of these SSRIs is that the clinical drug trials have been conducted over too short a time period - namely, less than 6 weeks - and have only involved out-patients with moderate degrees of depression (Ferrier, 1993). Recent chronic studies with fluoxetine, over a period of six months, indicated a loss of efficacy with prolonged use. Fluoxetine metabolism is not affected by age, obesity or renal impairment. However the dosage administered is important, given that high doses of $+500\mu g/l$ result in loss of efficacy (Altamura, 1994). This is ascribed to the fact that SSRIs such as fluoxetine depend on the presence of endogenous 5-HT to exert their therapeutic effect. Proof of this dependence comes from the observation that combined therapy with a partial agonist of the 5-HT_{1A}

receptor subtype, buspirone, whose efficacy is independent of 5-HT, restored antidepressant activity (Fabre *et al.*, 1990).

In conclusion it can be stated that the SSRIs, because of their neurochemical specificity, have contributed significantly towards an understanding of some of the underlying mechanisms of depression (Ferrier, 1993). However, as is the case with all other antidepressant therapies, short term treatment is not effective in preventing a relapse, and maintenance dosing regimens are required. There is now general consensus that depression necessitates prolonged therapy, since discontinuation of interim treatment results in re-emergence of the earlier depressive symptoms in 50% of all clinical cases (Delgado *et al.*, 1993).

1.2.1.0.7 The 5-HT syndrome

This disease arises due to elevated toxic concentrations of 5-HT which can occur in both animals and humans. However most of the available literature on the 5-HT syndrome has been acquired from studies done on animals (Brennan *et al.*, 1988) where the symptomatology includes, *inter alia*, tremor, rigidity, hypertonicity, hind-limb abduction, rigidly arched tail, lateral head shaking and generalised seizures (Gerson and Baldessarini, 1980). In humans the disease is not recognised and is generally known as neuroleptic malignant syndrome (Brennan *et al.*, 1988). The most common manifestations of the syndrome in clinical subjects are psychological disturbances, agitation, myoclonus, hyperflexia, diaphoresis, shivering and tremor (Oates and Sjoerdsma, 1960).

1.2.1.0.8 Why the three week delay in antidepressant action?

A major criticism against the monoaminergic hypothesis of depression is its inability to explain the one to three week lag period before the therapeutic effects of antidepressants are manifested. Possible reasons for the delay which have been proposed are, firstly, adrenoreceptor downregulation (Reisine, 1981), secondly, tyramine accumulation which would result in desensitisation of adrenergic receptors (Edwards, 1982) and, thirdly, the initial disorienting caused by the antidepressant drugs to dysregulated monoaminergic systems (Waldmeier, 1982). An alternative explanation proffered to account for the delay suggests that this period is necessary to allow for re-entrainment of the endogenous biological clock (Wehr and Wirz-Justice, 1982). However, this lag period could also indicate that receptor modification is a prerequisite that is necessary or sufficient for antidepressant activity (Stahl, 1992; Charney *et al.*, 1981).

A paradox in the treatment of depressives with SSRIs is the fact that they inhibit the reuptake system within minutes but require the usual lag period of up to three weeks to exert their therapeutic effect. This phenomenon clearly implies that inhibition of reuptake is only part of the mechanism of action of the SSRI

and that additional adaptive biological changes in serotonergic receptor system operation underlie their action (Blier and de Montigny, 1994).

1.3 MELATONIN

The neurohormone melatonin is a ubiquitously acting biological substance within the mammalian body (Reiter, 1991a), and can be found in both pineal and extra-pineal tissue. At first, aMT was believed to originate in the pineal only, but now the hormone is known to be synthesised in other tissues as well, particularly in the gut where its concentration can be several-fold (as much as 100 times) greater than in the pineal (Raikhlin *et al.*, 1975). Rats appear to have extra-pineal aMT synthesising machinery due to the fact that large TRP doses increase serum aMT levels, but not pineal N-acetyl transferase (NAT) or hydroxyindole-O-methyltransferase (HIOMT) activity (Yaga *et al.*, 1993). In human beings no such effects are observed and no extra-pineal plasma aMT producing centres have been demonstrated as yet (Lewy, 1983). However, human pineals have thus far been shown to contain the highest concentration of 5-HT for any neural structure (Gairman and Day, 1958). Nocturnal aMT levels in humans appear to be subject to large variations with no correlation between the concentrations and age, anxiety, depression or sleep ratings (Smith *et al.*, 1991).

There appears to be differential aMT metabolism in different organisms. Organ culture experiments indicate that in certain species, such as the Syrian hamster, a long lag period of ±7 hours after noradrenergic stimulation is required for mRNA transcription prior to aMT synthesis, unlike in the rat where pineal aMT synthesis can occur within 1 to 2 hours of enhanced nocturnal NA release (Santana *et al.*, 1990). The pineal gland is the interface between the photoperiodic environment and the internal milieu, where it functions as a transducer of the photic signal into a chemical response via neural input in the form of NA (Reiter, 1991a).

It is speculated that most body organs are subject to the influences of the pineal gland through the release of the neuroendocrine modulator, aMT. The aMT molecule is released from the pineal irrespective of whether the individual is diurnal or nocturnal and, in humans, aMT has a well developed circadian rhythm, peaking at subjective night while remaining basal during the subjective day (Lynch *et al.*, 1975) (fig. 6).

aMT secretion by the rat pineal gland provides a realistic assessment of noradrenergic neurotranmission within this species (Thompson *et al.*, 1985). Denervation of the pineal gland prevents the nocturnal elevation in both brain and plasma aMT levels, which clearly demonstrates that the nightly rise in the neurohormone is a consequence of pineal secretion. Nocturnal increases in pineal aMT display species differences, and there is a proportional relationship between the photoperiod and nightly aMT levels. A relatively minor

there is a proportional relationship between the photoperiod and nightly aMT levels. A relatively minor number of animals do not display the nocturnal increase in pineal aMT even though the integrity of biochemical components, believed to be instrumental in aMT circadian rhythmicity and synthesis, is retained (Reiter *et al.*, 1987). Blood and pineal aMT concentrations are very similar which seems to imply that there is a rapid output from the pineal into the bloodstream, apparently utilising an active secretory mechanism and short term retention that could be characteristic of both diurnal and nocturnal animals. Circadian rhythms in body fluids also appear to be directly dependent on plasma aMT derived from the pineal gland (Reiter, 1988), and the aMT rhythm is constant and reproducible for any particular individual (Arendt, 1988). The levels of pineal aMT and 5-HT appear to be regulated independently of blood TRP, given the finding that a pronounced reduction in serum TRP had a negligible effect on pineal aMT and 5-HT (Daya, 1989).

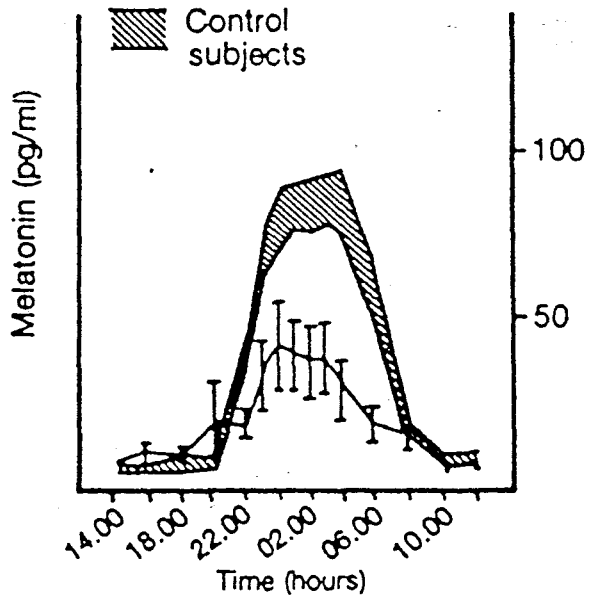


FIG. 6: Twenty-four hour plasma aMT concentrations in psychologically normal individuals (cross-hatched area) and in depressed patients (line) [Reiter, 1991].

aMT is transported in the blood mainly bound to SA and the neurohormone has a half life of between 10 and 40 minutes. Liver aMT metabolism clears approximately 90% in a single passage through the hepatic portal system (Pardridge and Mietus, 1980). Of this 90%, microsomal enzymes convert 75% to 6-hydroxy-aMT with subsequent conjugation of about 70% to 6-sulphatoxy-aMT and the rest to glucuronide (Kopin *et al.*, 1961) (see fig. 7). Urinary 6-sulphatoxy-aMT is a reliable indicator of pineal aMT output (Brown *et al.*, 1991) (fig. 7). Of the total aMT released by the pineal, only approximately 1% escapes

catabolism in the liver. Metabolism of the indoleamine in the brain follows a different route. Here the molecule is transformed into N-acetyl-5-methoxykynurenamine and small amounts of other minor metabolites. All catabolic products are ultimately excreted in the urine with an obvious higher nocturnal than diurnal rate of urinary elimination. During TRP depletion there is a marked reduction (±80%) in 6-hydroxy-aMT sulphate, which is coupled to a much reduced serotonergic tone in the presence of low levels of 5-HT (Zimmermann *et al.*, 1993b).

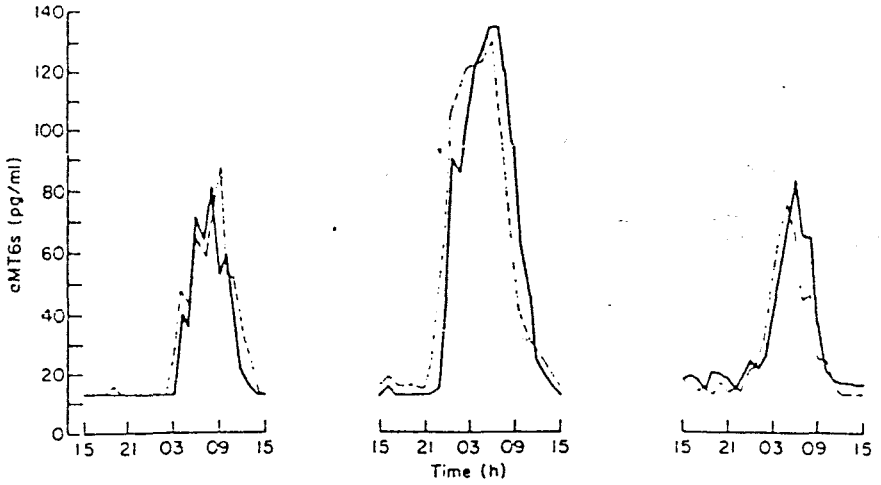


FIG. 7: One hourly 6-hydroxy-aMT sulphate levels after aMT administration to rats [Reiter, 1988].

1.3.0 aMT receptors

aMT receptors have been identified and characterised in a large number of species such as the rat, Syrian hamster, bat, ostrich etc. These receptor sites are ubiquitous in lower vertebrate brain where they are predominantly found in retinal, auditory and limbic systems (Rivkees *et al.*, 1989). In the mammalian brain they are to be found at a much lower density and are mainly limited to individual brain nuclei. No sex differences with respect to receptor density have as yet been demonstrated (Morgan *et al.*, 1994). The most generously endowed area has been found to be the pars tuberalis (PT) (Morgan and Williams, 1989) of the pituitary followed by the supra chiasmatic nucleus (SCN) (Reppert *et al.*, 1988) and the pars distalis (PD) (Williams *et al.*, 1981). Receptors are also found in the gonads and gut. At present the physiological and functional significance of the distribution of aMT receptors is not clear. These receptors were identified using 2-[^{125}I]iodomelatonin in both brain and peripheral tissues such as the gut and retina. The

structural analogues. Specific binding sites have only been demonstrated for those receptors of the PT and PD (Morgan *et al.*, 1994). So far, research findings indicate that only high affinity receptors yield a consistent response when stimulated, and these are either linked to the second messengers cAMP or phosphoinositide, as has been shown in ovine PT (Morgan *et al.*, 1990). It is possible that the entry of aMT into the cell does not necessarily require a receptor protein in all instances. The indoleamine could move freely into the cell due to its hydrophobic nature. Recent studies, however, demonstrate that the neurohormone is soluble up to 5mM in aqueous media (Shida *et al.*, 1994).

1.3.0.1 Structure-activity relationships

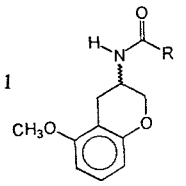
The elucidation of the structure-activity relationship of the aMT receptor involves not only pharmacological binding studies, but also functional bioassays. Little attention has, however, been paid to the relationship between binding activity and pharmacological efficacy, except in the retina where excellent correlation exists between these two parameters (Morgan *et al.*, 1994). The high affinity of indolic compounds such as aMT and its derivatives depicts a large degree of consistency between different tissues and species. Amongst the indoleamine derivatives of aMT, N-acetyl-5-methoxytryptamine with a halogen substitution at the C_2-atom displays the highest activity, surpassing even that of aMT when tested in functional bioassays (Sugden and Chong, 1991).

1.3.0.2 Agonists of the aMT receptor

The biological activity of aMT is not dependent on the 5-methoxy group, and aMT derivatives lacking the 5-methoxy group have proven to be potent agonists of the aMT receptor (Sugden, 1991a). In the hamster brain the compound which lacks the 5-methoxy group, N-acetylserotonin, has also been shown to be as potent an agonist as aMT (Duncan *et al.,* 1986). However the 5-methoxy group is generally important for binding (Sugden, 1994a). Synthetic aMT analogues can be created with modifications to the N-acetyl side chain or the C_5-atom on the indole ring of the parent molecule aMT. Side chain modifications beyond five carbons and homologation resulted in a significant loss of activity (Sugden, 1991a). The indole nucleus was found not to be essential for activity or initiation of receptor mediated events. Substitution of the indole nucleus with naphthalene had no significant adverse effects, and cyclisation of the side chains of both naphthalene and aMT profoundly enhanced binding to the receptor protein (Sugden, 1994b) (fig.8).

In hamster brains, prazosin, a non-indole and α_1-adrenergic selective blocker, has been reported to bind with high affinity to aMT receptors (Niles, 1989). Other agonists include the amidotetralines that cause Ca^{2+}-dependent dopamine release in humans (Dubocovich *et al.*, 1992).

1



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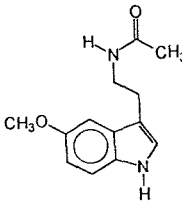


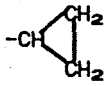
COMPOUND	ABBREVIATION	R-SIDE CHAIN
Melatonin	aMT	
N-acetyl-3-amino-5-methoxychroman	aMCh	$-CH_3$
N-propionyl-3-amino-5-methoxychroman	pMCh	$-CH_2CH_3$
N-butanoyl-3-amino-5-methoxychroman	bMCh	$-(CH_2)_2CH_3$
N-cyclopropyl-3-amino-5-methoxychroman	cyclopMCh	
N-chloroacetyl-3-amino-5-methoxychroman	ClaMCh	$-CH_2Cl$

FIG. 8: Structures of the chroman analogues (1) and aMT (2) [Sugden, 1994b].

1.3.0.3 Antagonists of the aMT receptor

The most potent naturally occurring antagonist of the aMT receptor *in vivo* has been found to be 6-methoxybenzoxazolinone (Sanders, *et al.,* 1981). However this effect could not be reproduced *in vitro* (Sander *et al.*, 1981). Luzindole, a competitive antagonist, inhibits Ca^{2+}-dependent release in rabbit retina and melanophores, but there is a lack of consistency in the molecule's affinity for the aMT receptor, given the observation that binding by the antagonist to aMT receptors is negligible in ovine PT (Niles, 1987). Generally, side-chain additions of more than five carbons function as antagonists (Sugden, 1991a). At present, studies pertaining to the development of effective agonists and antagonists of the aMT receptor are still in their infancy, and thus preclude a definitive classification system based on the properties of these synthetic ligands (Morgan *et al.*, 1994).

1.3.0.4 Regulation of the aMT receptor

Numerous experimental findings indicate that aMT operates via a cAMP dependent mechanism, particularly in melanocytes (White, *et al.,* 1987; Sugden, 1989; Sammak, *et al.,* 1992). However other observations indicate that the indoleamine could act through alternative second messenger signalling systems (Morgan *et al.*, 1994; Sugden, 1991b). This inference is based on the fact that phorbol esters,

which activate PKC, reverse or inhibit the aMT initiated pigment aggregation in melanocytes. In the PD of the pituitary gland, aMT appears to act exclusively by inhibiting Ca^{2+} by means of a voltage dependent L-type channel. The neurohormone could thus influence membrane potential via hyperpolarisation, repolarising the depolarising effects of lutenizing hormone releasing hormone (LHRH) (Sugden, 1994a; Vanacek and Klein, 1992a, 1992b) which involves the induction of calcium flux into the cell (Vanacek and Klein, 1992b).

1.3.1 Regulation of aMT synthesis

The rate limiting enzyme in aMT biosynthesis is accepted to be N-acetyltransferase, as has been shown empirically in the rat and the majority of other animals (Klein, 1985). However, a few exceptions exist where no correlation is noticeable between NAT activity and pineal aMT output (Reiter, 1991a). The activity of this enzyme changes positively during aMT synthesis (fig. 6).

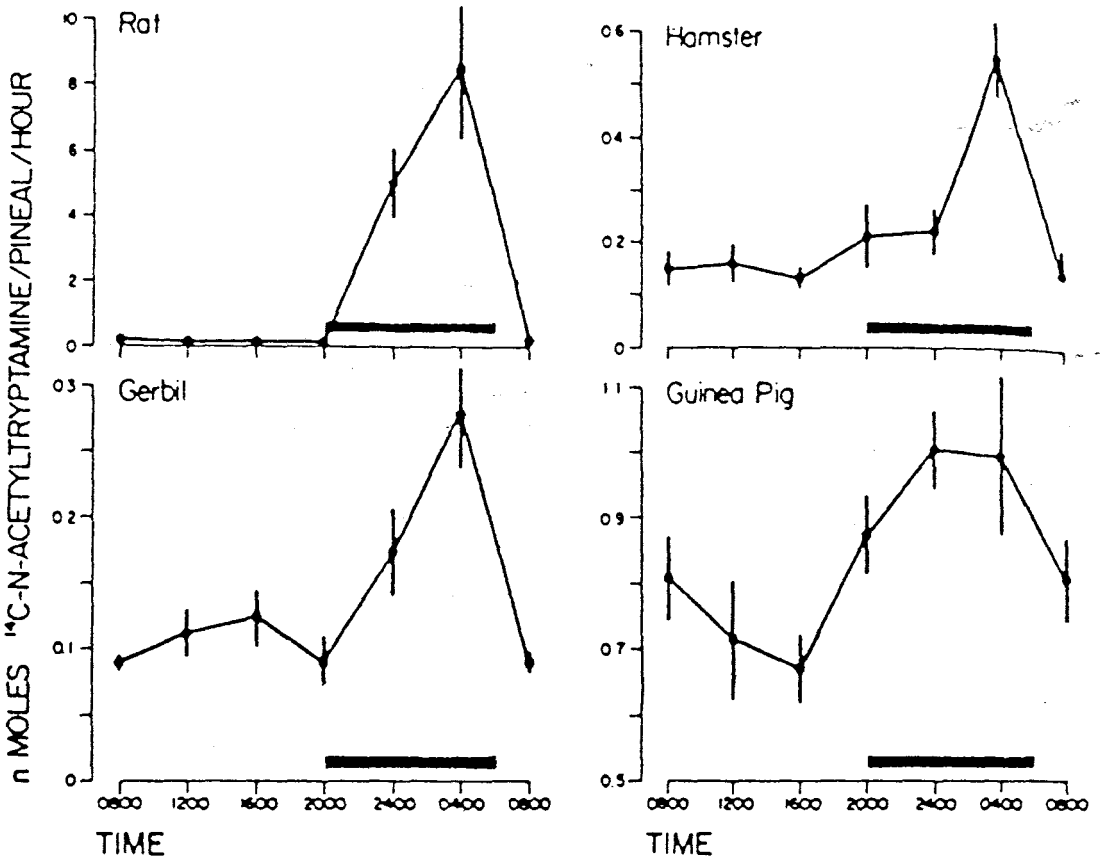


FIG. 9: Activity of NAT over a twenty-four hour period in different species [Reiter, 1991b].

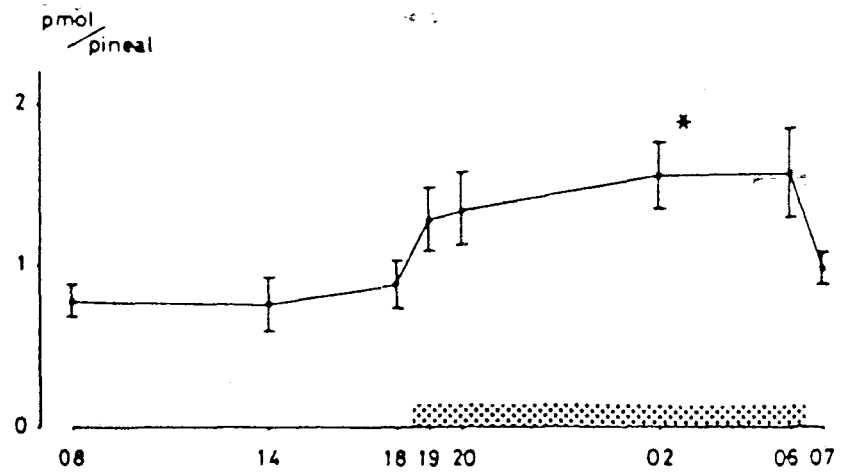


FIG. 10: Twenty four-hour variations in the cAMP content of the rat pineal gland. The speckled area represents the dark period and the *= $p < 0.02$ at the time 14:00h [Reiter, 1991b].

Stimulation of NAT is initiated by NA interacting with adrenergic receptors of the pinealocyte membrane (Klein *et al.*, 1981) which activate AC via G_s, with consequential increases in cAMP levels (Smith *et al.*, 1979) (fig.10).

A variety of factors influence the levels of circulating plasma aMT, and the major effector is believed to be the photoperiod. This regulatory influence holds for both humans and animals, and alterations in aMT blood patterns have parallel physiological modifications. The specific component of the aMT rhythm which imparts the message relating to the identity of the photoperiod to the organism, in order to effect the appropriate physiological changes, is still a matter of controversy.

Three major hypotheses (fig. 68) have been proposed in an attempt to explain this phenomenon which pertains to that aspect of the neurohormone biological rhythm involved in conveying the neuroendocrine message to a particular organ. These are:

i) the duration hypothesis (Carter and Goldman, 1983),

ii) internal and external coincidence theory (Reiter, 1987), and

iii) the amplitude hypothesis (Berga *et al.*, 1988).

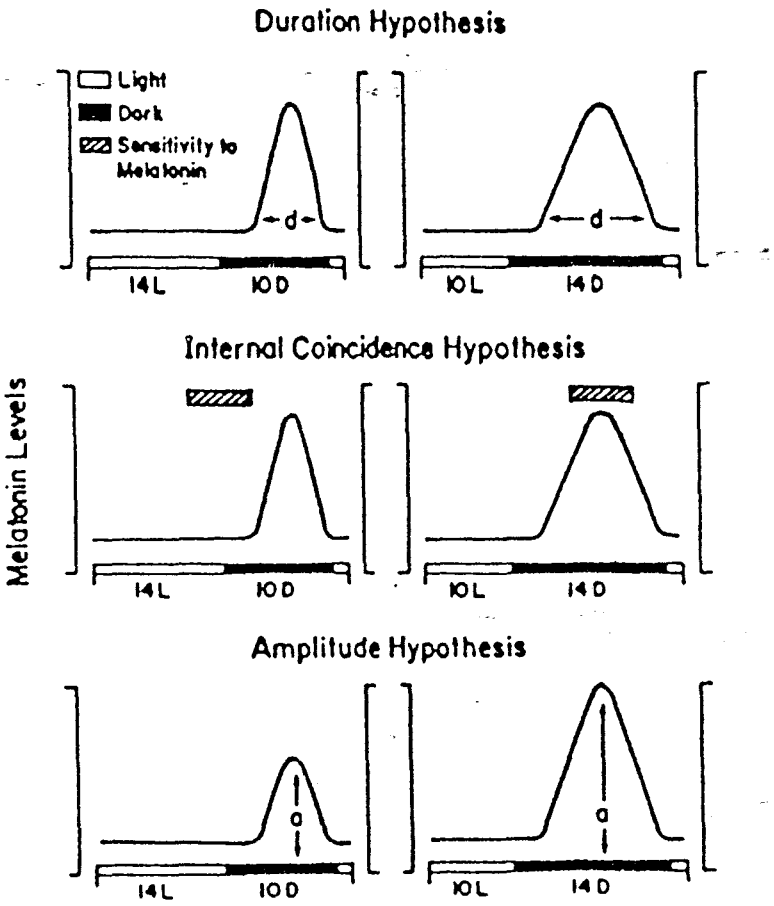


FIG. 11: Diagrammatic representations of the three hypotheses to explain the actions of aMT [Reiter, 1991b].

In the duration model, the aMT peak is set by annual oscillations in the daily photoperiod which exact appropriate physiological adaptations in the organism, as is the case with seasonal reproduction in animals such as sheep. On the other hand, the internal and external coincidence hypothesis asserts that there exists a window of sensitivity, equivalent to a time period at night within which the organism is sensitised to respond to the aMT peak. This time slot of susceptibility can either be determined by the environmental lighting conditions or by aMT itself. It is only when the peak of the indoleamine coincides with this time frame that a physiological change will be effected. Autoregulation of its receptor by aMT is possible. However, it would have to be dependent on the internal milieu of the organism, as a particular set of environmental conditions is a prerequisite for such a phenomenon to occur (Reiter, 1991a). The amplitude theory is widely accepted as being appropriate for describing the events that occur in human beings. Here, the amplitude of the nocturnal rise in aMT is the parameter which imparts the message that will induce a physiological change (Berga *et al.*, 1988).

In humans the physiological aMT dose is 20μg for three hours when administered intravenously (Zaidan *et al.*, 1994), and the time of aMT administration affects the onset, acrophase and offset of the circadian rhythm. These observations support the contention that the neurohormone is the endogenous diurnal rhythm synchroniser. Exogenous aMT is also able to synchronise its own rhythm, and this harmonisation is subject to entrainment (Zaidan *et al.*, 1994). aMT can cause either a phase delay or phase advance in humans and rats, depending on the time and dosage of administration, in both central and peripheral neural systems. The neurohormone has been effective in the treatment of jet lag, insomnia and synchronising the sleep/wake cycle in blind subjects (Lars *et al.*, 1991; Arendt, 1994).

The indolealkyleamine may also be useful as an immunostimulant, regulator of blood pressure, and in potentiation of cancer therapy (Arendt, 1994).

1.3.1.0 Assays for aMT

Clinically, aMT levels can be assayed in blood and urine using two methods: by gas chromatography-mass spectroscopy for the 6-hydroxy-aMT or by radioimmunoassay to detect 6-sulphatoxy-aMT. These two methods are straightforward, reliable and non-intrusive markers of pineal aMT output (Tetsuo *et al.*, 1981; Bojkowski *et al.*, 1987). However, determining total aMT concentration is difficult due to the fact that the indices of aMT turnover used, such as the urinary 6-hydroxy-aMT and 6-sulphatoxy-aMT levels, only yield a fractional value of approximately 80%. In addition, aMT can be reconverted to 5-methoxytryptamine (Hardeland *et al.*, 1993a) which points to a constantly fluctuating pool of the indoleamine that further complicates the determination.

1.3.2 The noradrenergic system: regulation of pineal indole metabolism

Studies pertaining to the neural route regulating the functions of the neuroendocrine gland, the pineal, have mainly been performed using the rat as a model. The rat has been an invaluable tool in the development of our understanding of the role that the SCN plays as mediator of the activity of the pineal gland (Klein, 1978).

Ultimately pineal indole metabolism is regulated by the effects of catecholamines such as NA on β-adrenergic receptors (Klein *et al.*, 1983) (fig. 12). However other neurotransmitters also have affinity for these receptor binding sites, but with little or no effect. Stimulation of pineal GABA receptors has been shown to inhibit aMT synthesis. The pineal gland contains the highest concentration of 5-HT in the body, and stimulation of α_1-receptors promotes release of 5-HT from the pineal.

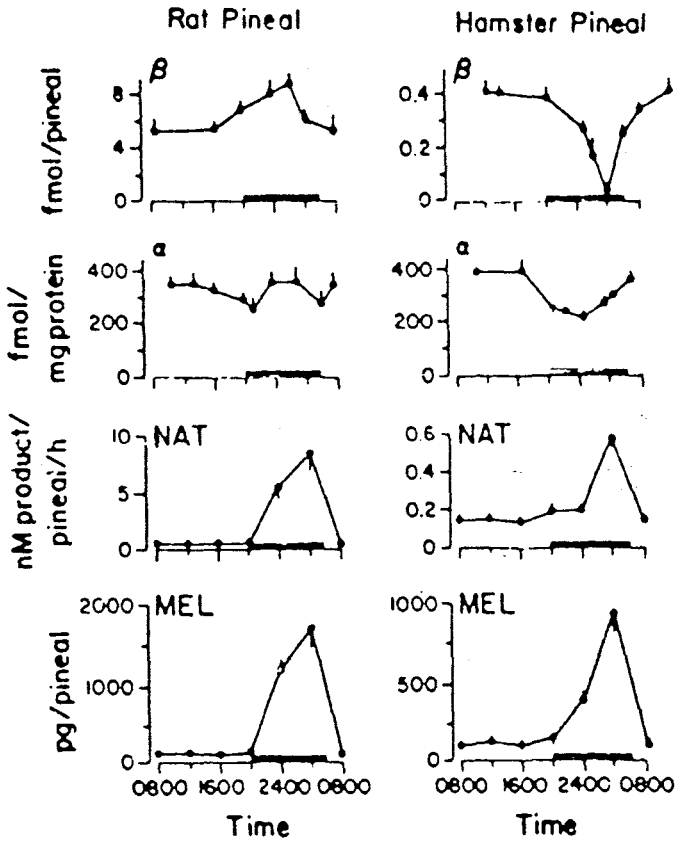


FIG. 12: β- and α-adrenergic receptors, pineal NAT activity and aMT concentrations in rat and Syrian hamster pineal over 24 hours. The dark bar on horizontal axis indicates the dark period [Reiter, 1991b].

There is a distinct possibility that other neurobiological systems could influence pineal aMT synthesis and release, due to the presence of other binding sites (glutamate, DA_2, Ach, and substance P) on the neuroendocrine gland. Functional muscarinic$_1$ Ach receptors, independent of the noradrenergic system, and linked to the phosphoinisitide signalling system, are also present on the neuroendocrine gland (Laitinen *et al.*, 1989). The precise roles of these receptor sites are as yet undefined. Neuropeptides such as the vasoactive intestinal polypeptide, neuropeptide Y and vasotocin are involved in pineal aMT biosynthesis (Ebedi *et al.*, 1989). Binding studies indicate that the adrenergic receptors have the greatest affinity for the catecholamines, NA and adrenaline, plus the β_1-agonist, isoproterenol.

It is apparent that regulation of aMT synthesis is a complex process, but the most important controlling factor is the noradrenergic system without which the rhythmic secretion of the neurohormone would be lost (Klein, 1978) (figs. 12 & 15). A deficiency in NA has been shown to be linked to aMT, and a reduction in the levels of the neurohormone in major depression is associated with a noradrenergic deficiency (Checkley *et al.*, 1986). Other researchers, however, failed to demonstrate such a link (Waldhauser *et al.*, 1993). The initiation of aMT synthesis in all probability depends on the level of activity of the noradrenergic system, while the availability of 5-HT and/or aMT turnover could in turn determine the peak of the aMT rhythm (Skene *et al.*, 1994).

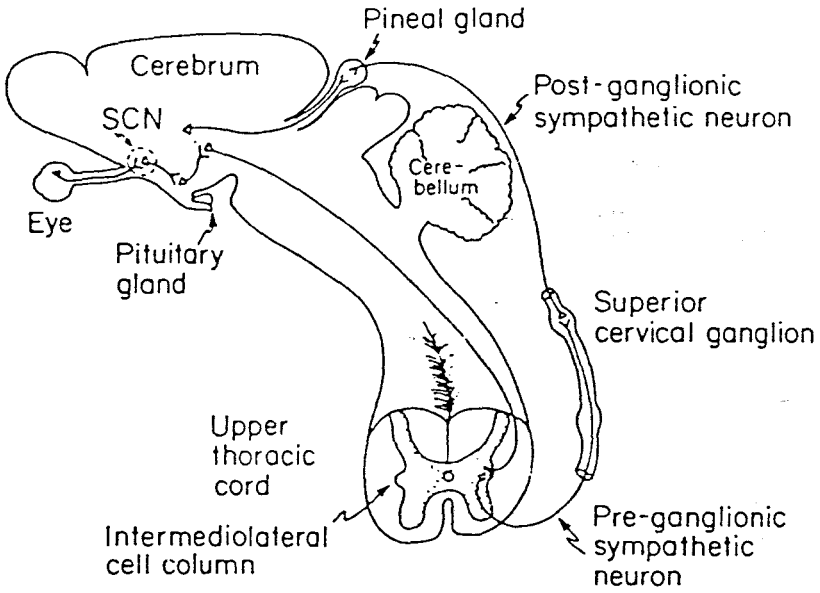


FIG. 13: Diagrammatic representation of the neural links between the eyes and the pineal in rat [Reiter, 1981].

The transfer of photic signals, received from the environment, involves a multisynaptic pathway, originating in the retina, through the CNS via the superior cervical ganglion (SCG) and the SCN, and ceasing as postganglionic sympathetic neurons within the pineal (Reiter, 1981) (fig. 13 & 14). This system imparts information concerning to the environmental lighting conditions to various regions of the brain and related tissues (Petterborg *et al.*, 1991). The activity of the retinohypothalamic tract, which originates in the retina and ends in the suprachiasmatic nucleus, determines the extent of the changes effected by lighting with respect to the circadian rhythm and pineal indoleamine metabolism (Reiter, 1991a). Normal activity is characterised by low aMT, high 5-HT, and low NAT during the subjective day, and high aMT, low 5-HT coupled with high NAT at night (Klein, 1978). The function of aMT appears to be in the transduction of the light signal and the conveyance of this data to inner areas of the brain and periphery (Short, 1993).

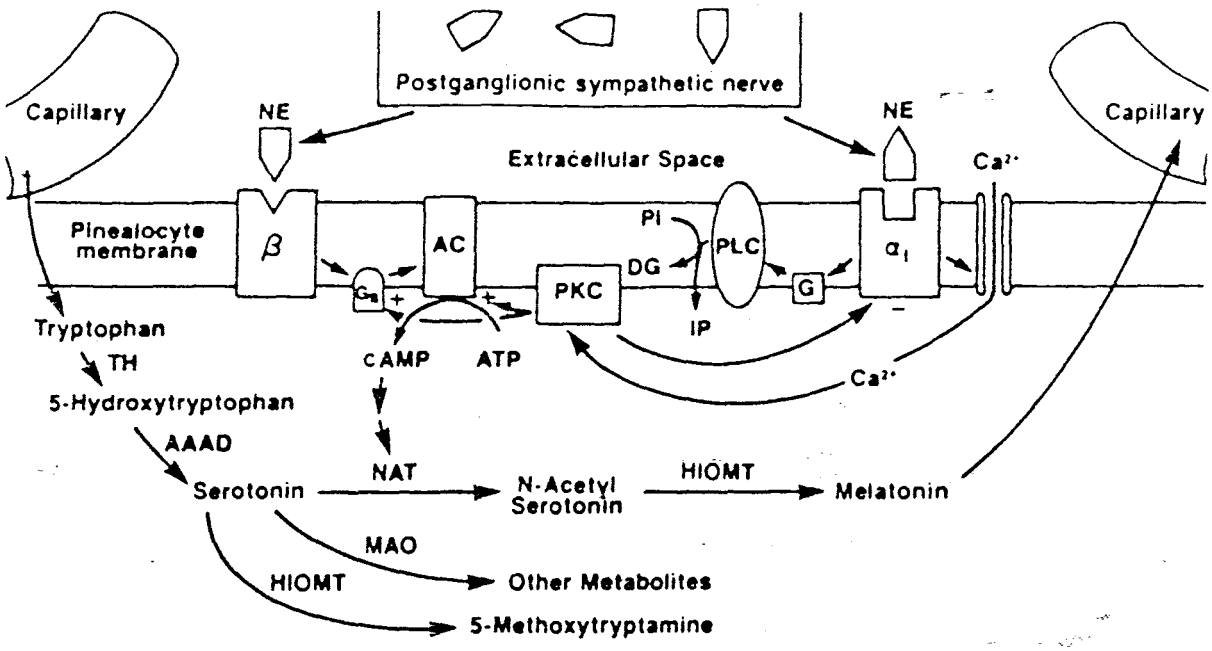


FIG. 14: Diagrammatic representation of potential signal transduction mechanisms between the postganglionic sympathetic neurons and the mammalian pinealocyte. KEY: AAAD=Aromatic amino acid decarboxylase; AC=adenylate cyclase; DG=diacylglycerol; G=guanine nucleotide-binding protein; IP=inositol phosphate; PI=phosphotidylinositol [Reiter, 1991a].

HIOMT is the enzyme involved in the conversion of N-acetyl serotonin (NAS) to aMT, and is present in the pineal. This enzyme has a high degree of specificity for NAS and does not display wide variations in concentration due to its circadian rhythmicity. The magnitude and duration of the NA signal, via cAMP, is apparently finely modulated, and the initial burst in cAMP probably coincides with the aMT peak and window of sensitivity (Moore and Klein, 1974).

The enzymes involved in the synthesis of aMT and its intermediates are subject to a circadian rhythm which is under the control of the SCN. Destruction of the SCN results in a loss of the NAT rhythm, and thereby implicates the SCN as harbouring the elements of the endogenous biological timing mechanism which

regulate the secretion of pineal aMT. The activity of the SCN appears to be entrained, a supposition which is based on the fact that this circadian rhythm generator can operate, to a limited extent, in the absence of environmental lighting (Lynch *et al.*, 1973). Severing of the neural tract between the retina and SCN fails to abolish the endogenous circadian rhythm in NAT activity. It is speculated that this entrainment of the SCN is possibly brought about by aMT (or 5-HT) because the SCN is rich in aMT receptors.

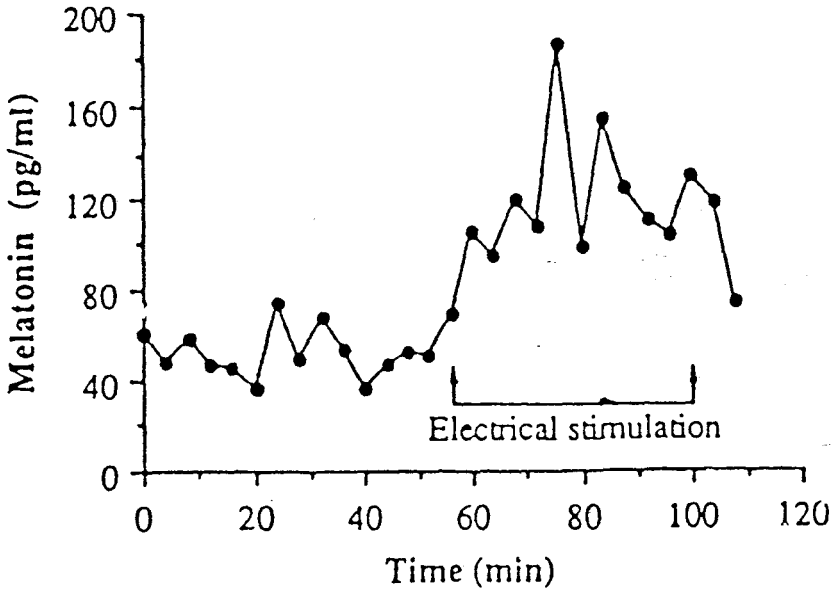


FIG. 15: aMT concentrations before and after stimulation of NA nerve fibres [Reiter, 1991b].

1.3.2.0 The NA transporter

This neurotransmitter binding protein is ubiquitous with respect to the mammalian brain, albeit in low levels. The fact that the mammalian CNS is sparsely populated with these transporters makes isolation and purification difficult. The transporter is sodium ion dependent and inhibited by TADs, the most potent being DMI, nortriptyline (NT), cocaine and amphetamines. The NA binding protein shares certain drug binding specificity with the 5-HT transporter (Worral and Williams, 1994).

1.3.3 aMT in depression

The neurohormone aMT is rhythmically secreted by the pineal gland. Circadian rhythmicity and noradrenergic neuronal activity are believed to be the mechanisms involved in regulation and control of aMT metabolism (Reiter, 1991a). Depression is characterised by diurnal variation in mood associated with

alterations in other circadian rhythms such as those of:

a) salivary secretions,

b) urinary electrolytes,

c) plasma cortisol, and

d) TRP metabolism (Curzon and Bridges, 1970).

The dysregulation hypothesis of depression claims that in the depressed state there is chronobiological instability, and that disruption in circadian rhythmicity could take the shape of either a phase advance or a phase delay (Maurizi, 1984; Piletz, 1994). Thus, malfunctioning of the biological clock is symptomatic of the depressed state, and would arise due to the irregular firing rate of the neurons at the locus coeruleus as a result of loss of specificity (Piletz, 1994; Faravelli *et al.*, 1985). Well- documented clinical observations allude to circadian alterations in the classical symptoms of depression such as mood and overall performance (Wirz-Justice and Arendt, 1980).

Biochemical indices of the depressed state manifesting dysregulated bio-rhythms include, *inter alia*, earlier appearance of the reduced night-time temperature, lessened rapid eye movement (REM) sleep latency, phase advances in prolactin, ACTH-cortisol, thyroid stimulating hormone, NA, and aMT. In these instances there is a disruption in circadian rhythmicity by way of a phase advance (Maurizi, 1984). aMT has the ability to re-entrain the biological clock in a number of species including humans. This biological clock, universally accepted to reside in the SCN, is richly populated by aMT receptors, and clinical data indicates that regular physiological doses of the hormone result in re-entrainment of circadian rhythmicity in depressives. However, in healthy individuals similar regular doses cause a fall in basal body temperature (Short, 1993; Maurizi, 1984). Chronic DMI treatment in depressives results in an increase in the intensity of the acrophase, but not the duration of aMT concentrations. In unaffected individuals, chronic DMI therapy causes an initial rise followed by a return to normal baseline levels (Waldhauser *et al.*, 1993). Thus there appears to be differential regulation of aMT metabolism in healthy and depressed individuals (Daya, 1994).

In rats and humans, aMT synthesis is contingent on noradrenergic output, so factors that influence NA release will invariably affect the release or synthesis of aMT. Antidepressants are believed to have an effect on monoaminergic systems, and should therefore have a bearing on aMT levels in blood. Clinical research indicates that depressed patients exhibit a reduced secretion of aMT, which can be corrected by chronic antidepressant therapy (Banoo, 1991). However it has been reported that chronic treatment with clomipramine (CI) reduced aMT levels in depressed patients. The reduced aMT output in depression can thus

be maintained by chronic antidepressant treatment. This apparent phenomenon is possible if the question of the time of dosage has not been taken into consideration, given the finding that antidepressants retard the biological clock and therefore the normal aMT peak would appear later in the circadian cycle. Sampling for analysis at the usual time could yield a false result of reduced aMT concentrations. Thus, according to proponents of the dysregulation hypothesis of depression, an important parameter in depression therapy is the timing of dosage as opposed to the quantity of drug (Nyback *et al.*, 1975).

Initially (as outlined in Chapter 3), the therapeutic efficacy of antidepressants was believed to be exclusively due to stimulation of the monoaminergic system. However, the fact that neither 5-HT nor NA is essential for maintenance of normal mood implies that additional disturbances in other neurobiological systems underlie depressive symptomatology (Sulser *et al.*, 1978). This is further supported by the finding that the TAD iprindole, even though it has been proven to be effective clinically, did not affect monoaminergic neurotransmission in the conventional manner via a blockade of NA uptake, modification of NA metabolism or the turnover of NA and 5-HT. In addition, it prevented spontaneous noradrenergic neural transmission in the locus coeruleus, and thus did not elevate NA concentrations at post-synaptic receptor sites (Coppen *et al.*, 1976). Thus, there is a growing belief that the biogenic amine hypothesis only focuses on a particular aspect of the phenomenon of depression and does not adequately cater for all the associated symptoms (Sulser *et al.*, 1978).

The efficacious antidepressant mianserin-HCL which inhibits TDO also apparently does not affect the uptake of biogenic amine 5-HT and NA (Palazidou *et al.*, 1992). These abovementioned observations raise questions that are difficult to reconcile with the classical biogenic amine hypothesis. However, the information does not eliminate associations at the level of the receptor with the various neuronal systems (Sulser *et al.*, 1978) or involvement of other factors. With respect to the noradrenergic system, there is no relief of the core symptoms of depression when treated with antidepressants that prevent re-uptake of NA, such as desipramine (DMI) and maprotilene (Sylvalahti, 1994; Blier and de Montigny, 1994).

Chronic DMI increases NA release at the pineal gland which acts on post-synaptic β_1-adrenergic receptors (Sack and Lewy, 1985) and indirectly on α_2-adrenoreceptors (Petterborg *et al.*, 1991) by causing re-uptake inhibition in depressed individuals. In animals, DMI and related antidepressants cause retardation of aMT secretion, ie a phase delay, while substances with known adrenergic activity, such as β_1-blockers, potently affect aMT synthesis (Sack and Lewy, 1985). Fluvoxamine increases aMT levels in plasma, possibly via inhibition of aMT catabolism since there is a lack of correlation between urinary 6-sulphatoxy-aMT and aMT concentrations in blood (Skene *et al.*, 1994). Acute DMI and fluvoxamine both increase the time period of

aMT secretion, though in different ways: fluvoxamine retarding offset time and DMI causing a phase advance in peak aMT release (Skene *et al.*, 1994).

A controversial issue with respect to the effects of TADs (specifically DMI) in humans on aMT secretion, is whether TADs actually cause down- or upregulation of post-synaptic β-adrenoreceptors (Peet, 1994). Drug therapy causes a variety of adaptive changes in receptor status throughout the noradrenergic system. It has been reported that chronic antidepressant treatment results in β-adrenoreceptor downregulation which manifests itself via a reduction in the number of β-receptors, the levels of cAMP and other associated intracellular second messenger concentrations in depressed individuals (Sulser *et al.*, 1978). However, acute DMI treatment in depressives resulted in upregulation after day one, an effect that was maintained throughout six weeks of therapy (Preskorn, 1994). This finding casts doubt on the hypothesis which claims that the key event in TAD mechanism of action is β-adrenoreceptor downregulation (Sulser, 1978). Nevertheless, the phenomenon of increases in receptor number does not preclude β-receptor downregulation, but could imply that other adaptive modifications, such as α_1-adrenoreceptor downregulation, counteract the β-adrenoreceptor downregulation, and thus β-receptor upregulation is sufficient to alter net noradrenergic neurotransmission. However, if downregulation does occur, it is not pronounced enough to counteract the primary effects of the re-uptake blockade imposed by DMI which increases aMT output in depressed patients (Carter and Goldman, 1983; Preskorn, 1994).

1.3.3.0 Seasonal affective disorder and aMT

Seasonal affective disorder (SAD) is another form of depression which is characterised by annually recurring episodes of depression coupled with symptoms such as hypersomnia, anergia, fatigue, carbohydrate craving and weight gain (Wurtman and Wurtman, 1988; Waldhauser *et al.*, 1993). This type of depression is of the bipolar variety with winter depression and summer mania (Waldhauser *et al.*, 1993). Seasonal alterations in aMT secretory patterns are related to changes in the photoperiod and SAD appears to be intricately associated with dysregulation of aMT circadian rhythms. In SAD there is a phase delay of the aMT peak, and morning phototherapy of a few hours is effective in alleviating the symptoms by synchronising the circadian oscillator (Petterborg *et al.*, 1991; Waldhauser *et al.*, 1993). Phototherapy is highly effective in treating SAD, but is apparently unrelated to the quantity and secretory pattern of aMT (Waldhauser *et al.*, 1993). Phototherapy regimes attempt to re-synchronise the aMT rhythm with the normal day/night bio-rhythm (Daya, 1994). It is not known whether modification of the endogenous aMT rhythm is responsible for the therapeutic effects of phototherapy in the treatment of SAD, but it is clear that it is not a necessary event for remission (Waldhauser *et al.*, 1993). However, it is accepted, based on experimental data, that bright light of sufficient intensity reduces nocturnal aMT secretions, as has been shown in rat pineals (fig. 16).

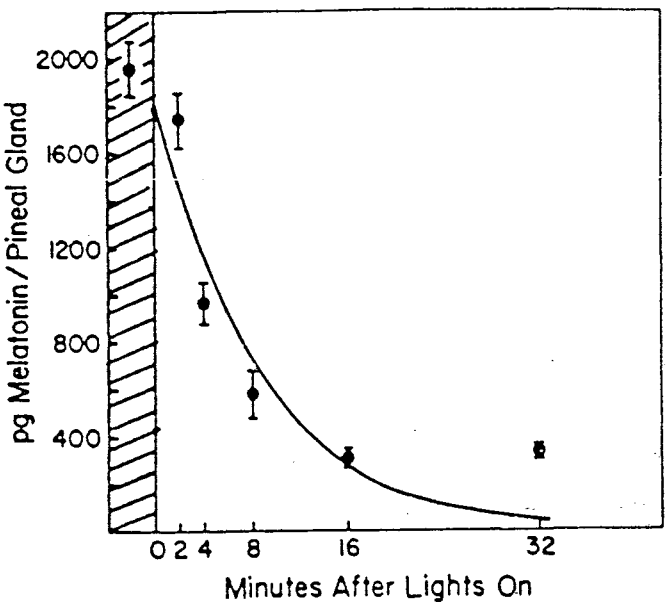


FIG. 16: Depression in rat pineal aMT content after acute exposure to light at night. The cross-hatched area indicates darkness [Reiter, 1991b].

In healthy individuals, brief exposure to bright light for approximately 15 minutes suppresses aMT synthesis transiently, but not cortisol production. This reduction in synthesis is apparently due to a decrease in the rate of NAT activity in the pineal gland. Serum aMT is an easy and reliable biological index in evaluating the influence and efficacy of phototherapy (Welman and Daya, 1990). aMT can have a negative effect in patients with abnormal, irregular aMT rhythms, such as those found in SAD. Here the neurohormone exaggerates the depressive symptoms when administered during the day in certain depressed patients (Van Praag, 1982). The fact that phototherapy is effective in suppressing aMT levels in humans can be clinically advantageous, as this would allow for accurate regulation through control of the degree and intensity of the light.

1.3.3.1 Mania

It is hypothesised that in mania there is noradrenergic hyperfunction characterised by increased levels of NA in the synapse as a result of dysregulation of the endogenous circadian rhythm generator. aMT levels in this type of affective disorder appear to be abnormally high and acutely sensitive to bright light (Wurtman and Wurtman, 1988). This feature could serve as a state marker for mania, given that the ultrasensitivity to suppression by light is maintained during depression. However, phototherapy does not always reduce the

aMT levels in manic depressives (Daya, 1994).

1.3.3.2 Abnormal sleep chemistry

Abnormal sleep is a characteristic feature of depression and is accompanied by a deficiency of aMT. Based on this association, it is possible that aMT is partly responsible for this condition (Maurizi, 1990). The lipophillic nature of aMT allows the molecule to effectively traverse the blood brain barrier. In the brain the neurohormone is involved in numerous physiological manifestations (Birkeland, 1982). One of the roles of the indolealkyleamine is in REM sleep where release of the neurohormone is synchronised with the REM sleep biorhythm (Yogman and Zeisel, 1983). The brain centre believed to be involved in regulating REM sleep, the SCN of the hypothalamus, is rich in aMT receptors, and in depression REM sleep is accompanied by an abnormal aMT circadian rhythm. Experimental findings indicate that REM sleep can be prolonged if dietary TRP concentrations are increased, while a LNAA rich meal reduces the amount of REM sleep (Weaver *et al.*, 1988).

The manner in which aMT could influence REM sleep is by inducing the release of the nonapeptide vasotocin (Pavel and Goldstein, 1979), which is present in the CSF of humans during REM sleep but absent during non-REM sleep (Rupfer *et al.*, 1994). Therapeutic action of the TADs is accompanied by improved sleep, and this could possibly be due to aMT, either directly or indirectly (Haimov *et al.*, 1994), given the fact that TADs potentially elevate aMT synthesis in depressives (Skene *et al.*, 1994). In insomniac elderly people there is a good correlation between low aMT and low sulphatoxy-aMT concentrations. Disruptions in the circadian rhythm characterised by retarded aMT peaks are prevalent (Adey *et al.*, 1968).

1.3.3.3 aMT and stress

There is believed to be a correlation between the blood aMT levels and stress, with below basal levels linked to stress. However, conclusive evidence in this regard is lacking. It has been shown in a few cases that aMT has an ameliorating effect on stress via vasotocin induced by elevated corticosteroid levels on the hippocampus. The manner in which the neurohormone brings this about is by reducing the affinity of corticosterone for its receptor and thus preventing neuronal damage (Thompson *et al.*, 1985), as well as stimulating vasotocin release which in turn inhibits cortisol secretion by the adrenals (Zimmermann *et al.*, 1993).

1.3.3.4 Proposed regime for treatment of depression

A rational treatment therapy based on the relationship between precursor TRP and its derivative aMT for depression would have to include both substances. The reason for this is that conventional antidepressant

treatment with a TAD such as DMI enhances aMT synthesis but does not provide the precursor TRP. By supplying the nutritional substrate it is thus possible to counteract the vicious cycle imposed upon the body by stress and its associated high levels of cortisol. This régime would thus allow for raised TRP → elevated 5-HT → increased aMT → reduced cortisol → lowered TDO activity → increased TRP (Maurizi, 1994). The treatment scheme, if instituted, could eliminate the three week lag period associated with the therapeutic response of efficacious antidepressants, and remission could be induced within 3 to 4 days, as with phototherapy for SAD. This proposed therapy is dose and time dependent given the fact that both TRP and aMT promote sleep and could be potentially toxic in very high doses (Maurizi, 1990).

Given the finding that acute TRP depletion in humans causes a marked reduction in nocturnal aMT secretion (Young and Silman, 1982), the opposite approach - increasing the levels of TRP - should theoretically be effective in elevating the levels of aMT. In this regard, however, there has been limited success (Naamboodiri *et al.*, 1983). It has nevertheless been possible to increase daytime serum aMT to the levels achieved nocturnally, with exogenous 5-HTP administration. 5-HTP given to sheep during the day caused a sevenfold increase in aMT levels two hours later (Naamboodiri *et al.*, 1983). This effect of 5-HTP on aMT is possible because 5-HTP promotes the synthesis of NA and DA in addition to enhancing the production of 5-HT (Van Praag, 1983). Thus there could be an increase in noradrenergic neurotransmission due to elevations in synaptic NA concentrations that would stimulate NAT synthesis and ultimately raise aMT levels.

1.3.4 aMT as free radical scavenger and neuroprotectant

As stated earlier, aMT can be found in extra-pineal tissue such as the retina, (Yu *et al.*, 1981) Harderian gland (Hofman *et al.*, 1989), gut (Bubenik, 1980) and leucocytes (Finochario *et al.*, 1988). Secretion in these tissues does not necessarily follow a circadian rhythm as is the case with the pineal. Thus it appears that the role of aMT does not purely reside in its ability to convey information about the photoperiod but possibly covers a wide spectrum of alternative activities (Hardeland *et al.*, 1993c). Other functions include its role as a neuroprotectant (ie a free radical scavenger) (Hardeland *et al.*, 1993a) (fig. 17) and the activities of its active metabolites, such as the methoxyindoles, β-carbolines (Fenerty and Lindup, 1991) and kynurenamines (Kelly *et al.*, 1984). The non-polar nature of aMT confers the ability to penetrate most tissues - hence its ubiquitous distribution both inside a number of tissues and in various body fluids including neuronal and non-neuronal tissue. This physicochemical property of aMT makes quantification of the number of aMT binding sites in any particular tissue difficult. (Hardeland *et al.*, 1993c).

The molecular nature of aMT imparts a number of unique biochemical features such as enhanced free radical trapping by the 5-methoxy group Uemura *et al.*, 1984, eliminating dimerisation of phenol radicals (Huether,

1990) and also formation of quinone imine structures at physiological pH (Pérez-Reyes and Mason, 1981). aMT, nevertheless, can interact with membrane receptors such as the 5-HT receptor in an agonistic or antagonistic manner. The N-acetyl group has an attenuating effect on the binding of aMT to the 5-HT receptor, in addition to its role in preventing degradation by MAO. That the N-acetyl group inhibits MAO catabolism of aMT is borne out by the fact that an analogue, 5-methoxytryptamine, is broken down by MAO (Galzin *et al.*, 1988). The primary function of the N-acetyl group concerns free radical scavenging (cited in Hardeland *et al.*, 1993c).

It should be emphasised, however, that the most important and reactive part of the molecule is the pyrrole ring, given that ring opening results in the formation of a series of potent biogenic amines (Hayaishi and Yoshida, 1979). Ring cleavage could be due to one of two mechanisms, either non-enzymatic (fig. 17), via free radicals (Hardeland *et al.*, 1993c), or enzymatic by the enzyme indoleamine-2,3-dioxygenase (Hayaishi and Yoshida, 1979).

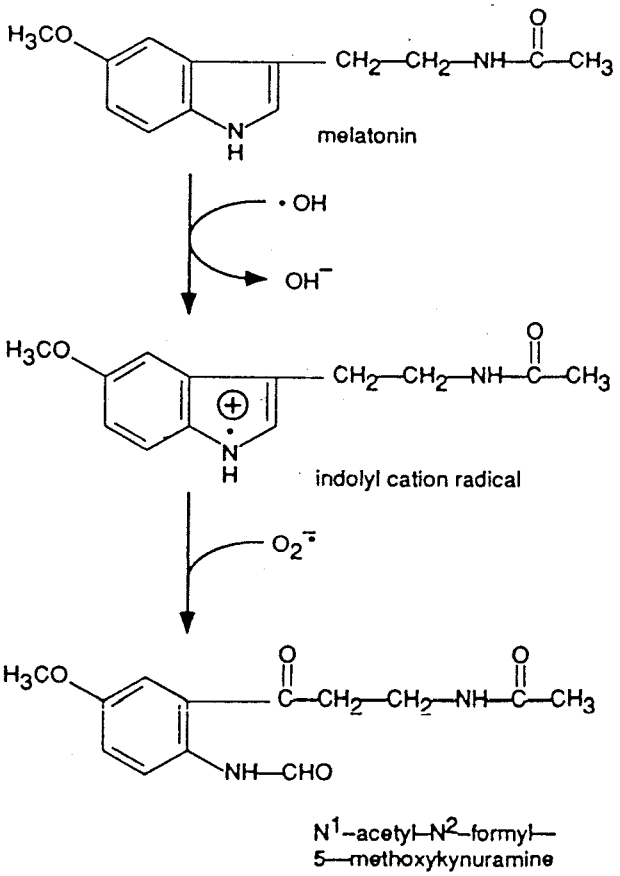


FIG. 17: Presumed mechanism of direct free-radical trapping by aMT and its indolyl cation radical. OH:hydroxyl radical; $O_2^{\cdot-}$:superoxide anion [Hardeland *et al.*, 1993c].

When considering the physiological properties of aMT and its derivatives it is important to take cognisance of the biological rhythm of the molecule. Circadian rhythmicity in most instances is a prominent feature of aMT and is quite pronounced in certain tissues such as the pineal (Reiter, 1988), but is virtually non-existent in others such as the Harderian gland (Pevet *et al.*, 1980). In all species studied to date, the neurohormone peaks at subjective night with a much lower basal level during the subjective day. The intensity and duration of the pineal peak ultimately determines the quantity of aMT present in each particular tissue, and also the circadian pattern (Reiter, 1980).

Research pertaining to the relative potencies of aMT and its metabolites indicates that for a particular effect at a common binding site, the parent molecule has a superior efficacy. However there are exceptions to the rule, as in the unicellular microorganism *Gonyaulax polyedra* where 5-methoxytryptamine shows a greater potency than aMT with respect to stimulating bioilluminescence (Balzer and Hardeland, 1991). Nonetheless this group of biogenic amines can be separated on the basis of the reactions in which they are involved, and the derivatives of aMT mainly differ from one another by virtue of modifications to their side groupings. A more pronounced change that involves the aliphatic side chain is cyclisation through the formation of an additional 5-atom ring structure coupled with hydroxylation and saturation of the 2,3-C double bond with the subsequent synthesis of a 2-hydroxymelatonin (Vakuri *et al.*, 1987) (fig. 18). This hydroxylated aMT, which possesses psychomimetic properties, is structurally similar to β-carboline and has been shown to have skin-blanching qualities (Frohm *et al.*, 1980). The molecule has been observed in human and rat urine from where it has been isolated. β-Carbolines are normally present in a variety of unrelated tissues that synthesise copious amounts of aMT (Vakuri *et al.*, 1987; Kari, 1981)

A possible alternative route of β-carboline production is via the addition of a carbon atom to aMT which allows cyclisation (fig. 18). The mechanism of synthesis is still a mystery, but it is possible that β-carboline is an artifact of purification. However the compound could be formed via peroxidation of 5-HT by the superoxide anion. The structure of aMT (specifically, its N-acetyl group) precludes β-carboline formation along this route, but is possible with aMT derivatives such as 5-methoxytryptamine. In rats, diets rich in TRP stimulate synthesis of a β-carboline derivative called 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (Adachi *et al.*, 1993).

β-Carboline derivatives have a number of physiological effects within the cell which include, *inter alia*, binding to both benzodiazepine and 5-HT receptors. The molecule shares similar structure-activity relationships with TRP, benzodiazepines and 5-HT. It also binds to albumin (Adachi *et al.*, 1993) and thus

has the potential to displace TRP from SA and elevate plasma TRP levels. Given the fact that the molecule has psychomimetic properties, it can be inferred that it could have significant effects on these two neurobiological systems. The substance is known to affect an endocrine marker of serotonergic function, namely, prolactin, whose secretion is enhanced (Rovescalli *et al.*, 1986). In addition to its effects on the neuroendocrine system, β-carboline also inhibits MAO-B activity in 5-HT releasing neurons. A derivative of β-carboline, pinoline (5-methoxytryptoline) (fig. 18), a much more potent molecule, is known to be present in certain tissues and is effective at nanomolar concentrations. Pinoline can be synthesised indirectly from aMT via 5-methoxytryptamine (Airaksinen *et al.*, 1993).

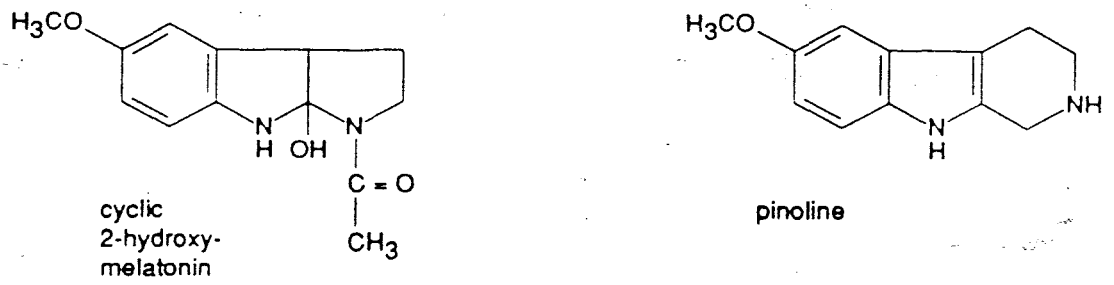


FIG. 18: Tricyclic aMT metabolites [Hardeland *et al.*, 1993c].

Binding sites for pinoline have been found on the pineal gland and in certain brain regions such as the adrenal cortex and medulla. The unicellular microorganism *Gonyaulax polyedra* harbours pinoline, aMT and 5-methoxytryptamine, and is sensitive to IMI in a dose-dependent manner. This sensitivity manifests itself either through an increase or decrease in bioluminescence (Pöggeler, 1992).

As stated earlier, aMT and some of its derivatives share a similar structure-activity relationship pertaining to the trapping of superoxide anions and hydroxyl radicals. In fact aMT has been proven to be the most efficient hydroxyl radical scavenger ever discovered (Pöggeler, 1992). In this process aMT is non-enzymatically degraded, which effectively terminates the radical chain reaction. The mechanism of action ascribes a crucial role to cofactors such as hemin or other porphyrins, and is thought to involve the abstraction of an electron, forming an indolyl cation radical that can eventually trap a superoxide anion. The fact that aMT, besides its high affinity for the hydroxy radical, gives rise to an indolyl cation that reacts directly with another superoxide anion in the absence of a catalyst, imparts the unusually pronounced free

radical trapping ability (fig. 17). Another feature that makes aMT such a unique radical scavenger is that it does not require regeneration as do other free radical trappers, such as ascorbate, α-D-tocopherol and glutathione (Pöggeler, 1992).

aMT's role as the mediator of the photoperiod is in all likelihood secondary to its function as a free radical scavenger (Hardeland *et al.*, 1993c; Pöggeler, 1992). This inference stems from the fact that in the Harderian gland the circadian oscillation in aMT levels is negligible, and high concentrations of the neurohormone are related to increased levels of porphyrins in females. This state of affairs exists possibly to counteract the large amounts of hydroxyl radicals and superoxide anion transformers.

An example that illustrates the protective function of aMT in the cell involves the carcinogen safrole which generates hydroxyl radicals in DNA. Concentrations of aMT at physiological levels 1000 times less than the carcinogen practically abolish the negative effects of safrole, which include DNA adduct formation. The mechanism by which aMT achieves this is facilitated by the fact that aMT binding sites exist on chromatin. Binding sites for aMT have been shown to be present on both neuronal and non-neuronal tissue. The association of aMT with such binding sites affords DNA direct on site protection (Tan *et al.*, 1994). A recent study involving aMT and homologues revealed that these compounds can offer protection against radiation. The homologues were prepared by acylation of 5-methoxytryptamine, and tested in mice. It was found that homologues with increased side chain lengths were more effective in preventing radiation damage than aMT (Blickenstaff *et al.*, 1994). However the mechanisms and factors involved in the radioprotection still require clarification.

Given these observations, it is clear that the indoleamine aMT is unusually efficacious as a free radical scavenger that has the potential to offer protection against a range of highly reactive, potentially mutagenic, carcinogenic and cytotoxic hydroxyl radicals. Thus the therapeutic application of aMT could have significant implications for the physical well-being of individuals, particularly with respect to age-related neuro-degenerative processes (such as Alzheimer's disease) which are linked, for example, to MAO-B (Tan *et al.*, 1993). Ageing human beings have lower levels of aMT (Sack *et al.*, 1986; Maurizi, 1987), increased MAO activity, and show signs of mental depression (Maurizi, 1987). The activity of MAO serves as a prime generator of free radicals, and aMT could counteract this effect (Hardeland *et al.*, 1993c). Oral aMT has been shown to reduce spontaneous mammary tumour proliferation in susceptible mice in a dose-dependent manner (Subramanian and Kothari, 1991).

The biological significance of aMT covers a broad spectrum, particularly with respect to the synthesis of

biologically active metabolites that could potentially play a role in the regulation of GABA-dependent chloride channels and serotonergic receptor function. The neurohormone has an inhibitory effect on the hepatic mixed function oxidase cytochrome p-450 which is also involved in heme metabolism (Tan *et al.*, 1994). Potential advantages of aMT, if used as a natural pharmacological tool, include (amongst others) non-toxicity, free radical scavenging, neural system protection, and action as a natural antidepressant and photoperiod mediator (Hardeland *et al.*, 1993c).

1.4 DETERMINANTS OF TRP AND ITS METABOLITES' LEVELS IN BLOOD AND BRAIN

1.4.0 TRP-hydroxylase

TRP-hydroxylase is the rate limiting step in 5-HT synthesis in the brain (Carlson *et al.*, 1972). This points to a possible area of control (Boadle-Biber, 1982) (fig.6).

Normally, *in vivo*, the saturation of TRP-hydroxylase displays regional differences (Meek and Lofstrandh, 1976), the rat brain enzyme is not fully saturated with substrate because of its poor affinity for TRP and thus is very sensitive to minor alterations in TRP concentrations which could have significant effects on 5-HT synthesis (Bengtson *et al.*, 1991; Wallen and Rissanen, 1994). The TRP-hydroxylase enzyme of the pineal gland, on the other hand, is saturable (Sitarem and Lees, 1978 cited in Skene 1979). Both these enzymes are subject to a diurnal rhythm, with maximal activity occurring during the subjective day (Hardeland and Rensing, 1968).

The hydroxylation of TRP is an inducible phenomenon, and it is possible that the synthesis of 5-HTP does not only depend on the levels of TRP acquired via the diet but is intricately linked to pineal levels of the indole. This inference is based on the fact that TRP-hydroxylase activity and 5-HIAA levels are markedly reduced in the amygdala of male rats after pinealectomy. Thus by implication the serotonergic system of the amygdala could be crucial in the expression of pineal function within this endocrine system (Miguez *et al.*, 1991).

It is hypothesised that the supply and redox state of the essential cofactor tetrahydrobiopterin (BH_4) could be the mechanism whereby the activity of TRP-hydroxylase and consequently circulating TRP levels and 5-HT synthesis is controlled with super sensitivity. It is therefore conceivable that regulation of BH_4 supply and redox state could be one of the ways whereby 5-HT synthesis in neurons is controlled (Bengtson *et al.*, 1991; Gal and Patterson, 1973) (fig. 19).

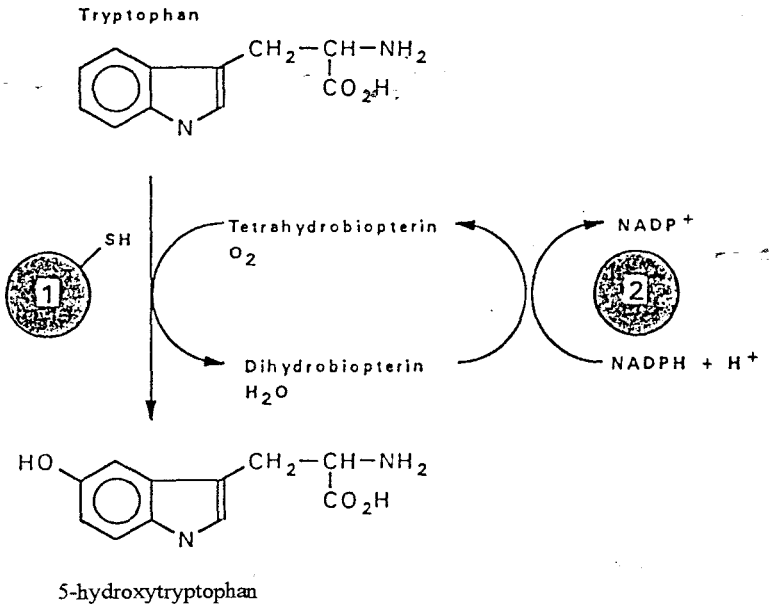


FIG. 19: Schematic illustration of the major step in the biosynthesis of 5-HTP (note shuttle between reduced (1) (active) and oxidised (2) (inactive) pterin cofactor [Rattray, 1992].

1.4.1 Catalyst: TDO

In depression there is increased pituitary-adrenal gland activity with a consequent rise in plasma cortisone concentrations, coupled with an induction of TDO synthesis (Curzon, 1969). Given the fact that only a small proportion of total serum TRP is utilised in 5-HT production, enhanced TDO activity would result in diversion of most of the available TRP towards the TRP-kynurenine pathway (fig. 1). This results in increased urinary levels of kynurenine, an event which is consistent with TRP loading in depressives (Curzon and Bridges, 1970). In depression there is:

i) early morning depression, associated with high plasma corticosteroid levels which are subject to a circadian rhythm (Bridges and Jones, 1966),

ii) pituitary-adrenal hyperactivity in a large number of depressives (Rubin, 1967), and

iii) lack of feedback inhibition of ACTH on the pituitary gland, and therefore no regulatory mechanism to control the output of glucocorticoids by the adrenal cortex (Gibbons, 1964).

Hydrocortisone causes a marked reduction (±30%) in 5-HT levels due to increased synthesis of TDO (Curzon and Green, 1969). This effect is abolished in the presence of inhibitors such as allopurinol (Becking and Johnson, 1967) and yohimbine (Sourkes *et al.*, 1969). An analogue of TRP, α-methyltryptophan, a dose-dependent activator of TDO (Schutz *et al.*, 1972), also causes a reduction in brain 5-HT (Curzon, 1969).

Empirical studies seem to indicate that the peak of enzyme activity induced by hydrocortisone coincides with the rapid transient fall in plasma TRP (Curzon, 1969). These observations imply that it is possible that the reduction in brain 5-HT levels is related to increased activity of TDO and that adrenocorticoid hyperactivity could be an important factor in depression (Curzon, 1969).

It is also known that immobilisation stress induces cortisone release with a consequent elevation in TDO. However, chronic administration of hydrocortisone or prolonged periods of stress do not cause the expected 5-HT decrease (Curzon and Green, 1969). This initial enhanced TDO rate of catalysis with consequent reduction in 5-HT levels could be explained in terms of the time required to mobilise the presently unknown cellular components involved in opposing the rise in enzyme activity effectively (Curzon, 1969). Increased TDO activity is not always associated with depression, but is also implicated in a number of other illnesses linked to stress (Altman and Greengard, 1966) of which schizophrenia (Faurebye and Pind, 1964) is but one.

However this does not preclude a crucial role for TDO in the depressed state and could be explained in a number of possible ways:

i) In depression the availability and turnover of TRP could be more critical than the actual activity of the enzyme itself.

ii) It is possible that, in depression, the reduction in 5-HT levels is not the sole contributory factor. An accompanying factor is possibly the inability to mount the appropriate countermeasures that would prevent the decline in 5-HT. These supplementary adaptations could involve elevated cofactor levels, and/or an increased rate of TRP hydroxylase activity. In rats, depletion of 5-HT levels by the NA depleter, reserpine, results in an increase in TRP-hydroxylase activity that increases 5-HT levels.

iii) 5-HT regulation of hydrocortisone secretion by the pituitary-adrenal glands is affected by lowered 5-HT concentrations, and this precipitates an increase in cortisone release which would stimulate TDO (Curzon, 1969). Pituitary-adrenal activation takes place in subjects treated with the drugs p-chlorphenylalinine (Rosecrans, 1968) and reserpine (Westermann *et al.*, 1962) that cause a lowering of brain 5-HT.

It is therefore possible for there to be an inverse relationship between 5-HT levels and TDO activity. Thus low brain 5-HT concentrations and a high level of enzyme activity coupled with disturbances in the pituitary-adrenal system are associated with depression in certain subtypes of depressives (Curzon, 1969).

1.4.1.0 TDO: Properties

TDO, a heme dependent cytosolic enzyme, catalyses the irreversible oxidative ring cleavage of L-TRP to yield, as a reaction product, N-formylkynurenine (Schutz *et al.*, 1972; Mehler and Knox, 1954; Schutz and Feigelson, 1972). The enzyme (molecular weight 167000) is a tetramer consisting of two sets of identical subunits, $\alpha_2\beta_2$, held together non-covalently (Schutz and Feigelson, 1972; Tanaka and Knox, 1959). Enzymes from both rat liver and *pseudomonas* have been purified to homogeneity (Knox *et al.*, 1970; Uchida *et al.*, 1985). These were found to contain 2 mole heme + 2 mole Fe (Schutz and Feigelson, 1972; Makino and Ishimura, 1976; Feigelson *et al.*, 1965), and TDO is the only oxygenase with organically bound Fe. Two forms of the enzyme are present within the cell, an inactive apoenzyme (no heme) (Feigelson *et al.*, 1965; Greengard and Feigelson, 1962; Mehler *et al.*, 1958) and an active metalloenzyme (apoenzyme + heme) (Feigelson *et al.*, 1965; Greengard and Feigelson, 1962; Knox and Ogata, 1965) (fig. 20) which is specific for TRP and the most stable form at optimum pH 7.2 (Uchida *et al.*, 1985).

APOENZYME + L-TRP + HEMIN → HOLOENZYME [reduced + active]

(inactive)

$$\mathbf{O_2 + L\text{-}TRP} \downarrow\uparrow \mathbf{+ L\text{-}TRP - O_2}$$

HOLOENZYME [inactive + oxidised]

FIG. 20: Mechanism of activation and inactivation of TDO by substrate TRP and cofactor hemin [Knox and Tokuyama, 1964].

There is a loose association between the apo-enzyme and heme (Greengard and Feigelson, 1962). In the rat, activity of the enzyme TDO increases with age (Curzon, 1969). Hepatic cellular levels of TRP ultimately determine the rate of apo- to holoenzyme and the distribution of hemin between the two forms and other heme binding proteins such as albumin, while two thirds of the total enzyme in the cell exists as apoenzyme (Knox and Piras, 1965).

Catalyst TDO is a sigmoidal enzyme with interacting catalytic and regulatory sites (Makino and Ishimura, 1976; Maeno and Feigelson, 1968), the catalytic site(s) being highly specific for substrate TRP, while the non-specific allosteric site(s) binds a range of different ligands including TRP. Intermediates such as 3-hydroxyanthranilate (Wagner, 1964), as well as end product NAD(P)H (Cho-Chung and Pitot, 1967), are also examples. The extent of inhibition/activation by effectors in general is tempered by substrate TRP and, to a lesser degree, by the concentration of the regulator. The major peripheral determinant of circulating blood

TRP levels in the body is believed to be hepatic TDO (Williams and Elvehjem, 1950; Salter and Pogson, 1985). TRP serves as the only substrate of the enzyme and thus determines its activity. TDO determines the fate of TRP with respect to uptake by the brain (Lucca *et al*, 1994; Green *et al.*, 1962; Fernstrom and Wurtman, 1974; Hillier *et al.*, 1975) and tissues, and for conversion to the nicotinyl moiety of the pyridine nucleotides (Williams *et al.*, 1950; Cho-Chung and Pitot, 1967; Schutz and Feigelson, 1972) (fig. 1).

1.4.1.1 TDO: Regulation

As stated earlier, TDO is subject to hormonal and substrate induction as well as a number of other effectors (Knox and Auerbach, 1955). The substrate TRP increases activity, synthesis and stabilisation of the enzyme. Adrenal glucocorticoids also increase synthesis in rat liver (Civen and Knox, 1959) but via direct interaction with the genome which results in raised levels of mRNA leading to increased apoenzyme synthesis (Feigelson *et al.*, 1961; Greengard *et al.*, 1963; Greengard *et al.*, 1966) (fig. 21).

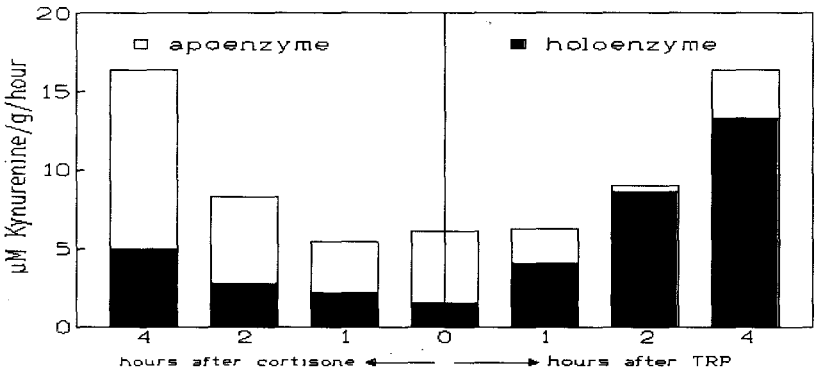


FIG. 21: Change in the degree of saturation of TDO with its cofactor during induction by TRP, but not by cortisone [Knox, 1963].

Rat TDO *de novo* activity reflects a circadian rhythm with an increase in synthesis from 09:00h to 18:00h and decreasing from 24:00h to 09:00h. There appears to be a relationship between the diurnal rhythms of glucocorticoids and enzyme activity. However there is no correlation between the maximum peaks of TRP, glucocorticoids and enzyme peak activity (Hardeland and Rensing, 1968). The importance of glucocorticoids in maintaining basal plasma TRP levels is aptly demonstrated by the fact that adrenalectomised rats have a reduced ability to cope adequately with increased TRP levels. Adrenal aphasia abolishes the hormonal induced elevation in enzyme levels, but not that induced by substrate TRP (Civen and Knox, 1959; Schimke

et al., 1965; Knox *et al.*, 1966). TDO levels could thus be increased in two ways: firstly, elevated synthesis initiated by glucocorticoids (Civen and Knox, 1959) and secondly, a reduced rate of degradation due to stabilisation by substrate (Schimke *et al.*, 1965) (fig. 22).

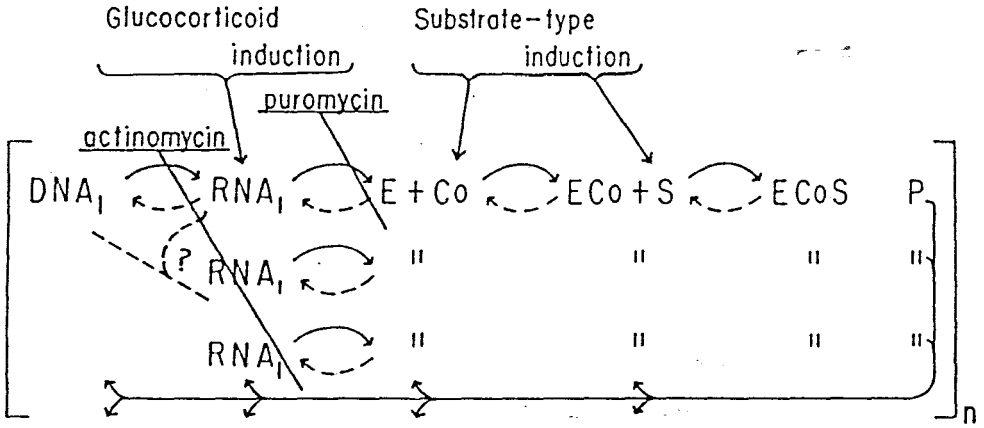


FIG. 22: Model of the two known mechanisms of enzymatic adaptation in animal tissues: substrate-type induction, which accumulates more total enzyme by combining with the free enzyme in effective equilibrium with its precursors, and hormone type induction, which provides more of the limiting RNA species for the specific enzyme synthesis [Knox, 1963].

Substrate TRP stimulates enzyme synthesis via a mechanism that disrupts the reversible equilibrium between apo- and holoenzyme. The induction manifests itself through promotion of conjugation between heme and apoenzyme, effectively depleting the levels of naked apoenzyme, and thus drawing protein synthesis through extant template RNA species (Feigelson and Greengard 1961a). This induced phenomenon then results in increased template RNA biosynthesis to restore the disturbed equilibrium. An additional effect emanating from substrate induction of enzyme synthesis is a reduction in the rate of TDO catabolism due to substrate stabilisation of the enzyme which enhances resistance to breakdown by the intracellular proteolytic enzymes (Knox, 1963). Glucocorticoids on the other hand 'push' enzyme protein synthesis by stimulating increased template RNA production (Knox, 1958). These two modes of enzyme induction can be aptly demonstrated in the presence of inhibitors such as puromycin, an inhibitor of protein synthesis, and actinomycin D, which prevents RNA formation (Greengard *et al.*, 1963)(fig. 22).

In experimentally induced diabetes, TDO activity is altered positively (Schor and Frieden, 1958). This effect could be due to a blockade in the synthesis of nicotinic acid which would precipitate an accumulation of TRP that would stimulate the enzyme. This inferred mechanism is based on results obtained from studies

performed using alloxan-induced diabetic rats. The pancreatic hormones glucagon and insulin also alter TDO activity, but glucagon can only induce enzyme synthesis in the presence of TRP. It is possible that the hormone exerts its effect at the level of translation (Schor and Frieden, 1958). Insulin inhibits the effects of glucagon, and stimulation of the enzyme by insulin is potentiated by TRP. This enhancement is not always dependent on the adrenals (Nakamura *et al.*, 1980). Other compounds such as histidine, adrenaline and histamine also stimulate TDO either through direct or indirect action on the adrenal glands. Adrenaline and histamine act on the pituitary that releases ACTH which in turn stimulates the adrenal glands to release hydrocortisone. Histidine, on the other hand, acts directly on the adrenal glands (Knox and Auerbach, 1955).

A large intake of TRP results in an increased production of hepatic pyridine nucleotides giving rise to an increased rate of enzyme protein synthesis. However, this induction occurs in a dose dependent manner. Smaller doses are more effective than larger doses (Jago *et al.*, 1964). TRP (both D and L forms) could also inhibit enzyme degradation *in vivo* which could raise tissue enzyme levels (Feigelson *et al.*, 1961). The amino acid is known to have a stabilizing effect because in its absence there is rapid degradation of TDO while in its presence there is an increase in enzyme levels. Another way in which enzyme activity and levels can be raised is through increasing conjugation of extant apoenzyme with heme in the cell, thus not elevating total enzyme levels but merely active enzyme concentration via conversion of apoenzyme to holoenzyme (Knox and Auerbach, 1955). A relationship is believed to exist between heme saturation and increase in total enzyme concentration in the cell, and also between saturation and total enzyme activity. Saturation of enzyme with activator results in a concomitant increase in active holo-enzyme and a decrease in inactive apoenzyme levels. In the absence of substrate inducer TRP there is a consequent reduction in the heme saturation that eventually leads to the basal level of activity. Experimental findings indicate that the extent of the heme saturation of TDO is an important determinant of active holoenzyme concentrations in the cell (Feigelson *et al.*, 1961). Cortisone increases apoenzyme levels only, and thus, in the absence of added TRP and activator hemin, there will not be a marked increase in enzyme activity but a rise in total enzyme concentration only manifested as an increase in apoenzyme levels. The question now arises as to how TRP actually causes an increase in enzyme levels via a substrate and hormonal mechanism. It is proposed that the manner in which it is executed is based on the presupposition that there exist on the genome enzyme forming sites that are normally occupied by inhibitory apoenzyme molecules. These apoenzyme molecules are in a dynamic equilibrium with the free apoenzyme molecules within the cell. Raising the levels of TRP induces conjugation between hemin and apoenzyme to allow for production of more active holoenzyme, with the resultant effect being the disruption of this dynamic equilibrium. To restore the balance, genome bound apoenzyme is released, thus lifting the blockade and allowing synthesis of additional apoenzyme via raised mRNA levels. Reducing the levels of circulating plasma TRP precipitates a dissociation between hemin and apoenzyme,

effectively raising the levels of apoenzyme that would now bind at the enzyme synthesising sites on the DNA, thereby preventing additional apoenzyme synthesis (Feigelson *et al.*, 1961). Cortisone increases enzyme synthesis by binding to the enzyme template on the DNA and controlling enzyme protein synthesis via mRNA. It forms a steroid-receptor complex with steroid binding proteins that allows it to penetrate into the nucleus and associate with the chromatin from where it then directs the genetic machinery to transcribe RNA. Cortisone also delays mineralocorticoid activity independent of TRP. Hydrocortisone and TRP potentiate one another (Civen and Knox, 1959) while TRP alone induces the conversion of apo- to holoenzyme (Feigelson *et al.*, 1961). These compounds also enhance TDO activity independently (Civen and Knox, 1959). It can thus be stated that hormones appear to mediate the rate of enzyme synthesis (Civen and Knox, 1959) while the rate of catalyst degradation and activation is modulated by TRP (Civen and Knox, 1959; Schimke *et al.*, 1965; Dubnoff and Dimick, 1959).

1.4.1.2 TDO: Effects of 5-ALA

A large number of hepatic porphyrias are characterised by increases in both porphyrins and TDO. It is possible that the elevated activity of the enzyme is due to stimulation by certain porphyrins and this could contribute to the symptomatology of certain porphyrias (Feigelson and Greengard, 1961b). Saturation of rat liver TDO is promoted by the administration of the heme and porphyrin precursor, 5-ALA, and this is coupled with enhanced enzyme activity (fig. 23).

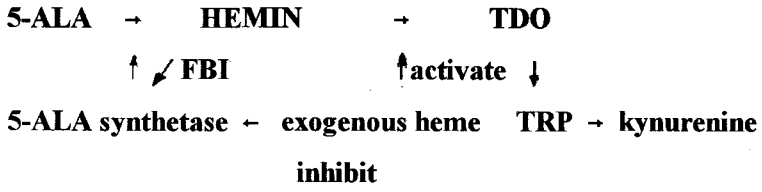


FIG. 23: Relationship between heme and TDO activation.

It has been reported, however, that saturation of the rat hepatic enzyme with exogenous hemin did not cause any major changes in activity (Jago *et al.*, 1964). Nevertheless it is possible, based on experimental findings, that there could be a relationship between TRP metabolism and porphyria, and factors that affect heme metabolism could have an effect on TDO activity (Badaway and Evans, 1973a; Badaway and Evans, 1975). The rate limiting enzyme of heme biosynthesis, 5-ALA synthetase, is, under normal conditions, inhibited by excess heme and saturation of TDO. In porphyria there is also enhanced excretion of the intermediates in the pathway between TRP and kynurenine. Inhibition of the enzyme could be brought about by salicylate which displaces TRP from SA and also binds heme, thereby effectively decreasing the concentrations of the

of porphyrin (Badaway and Smith, 1971). An inverse relationship exists between 5-ALA synthetase activity and the levels of holoenzyme. The degree of saturation of TDO apparently determines the catalytic rate of 5-ALA (Welch and Badaway, 1980), and TRP potentiates this effect (Badaway *et al.*, 1981).

1.4.1.3 TDO: Allosteric effectors

Feedback inhibition (FBI) of TDO by the allosteric inhibitor, NAD(P)H, is thought to be due to dissociation of the respective subunits to a monomeric form, or alternatively induction of a conformational change which gives rise to an inactive catalyst. These effects are mediated through a specific inhibitory binding site on the enzyme (Cho-Chung and Pitot, 1967). Rat TDO is inhibited by chronic drug treatment with nicotine (Cho-Chung and Pitot, 1967) and pentobarbitone (Badaway and Evans, 1973a), and this inhibition is thought to be as a result of FBI by NAD(P)H (Badaway and Evans, 1975). Some nicotinic acid derivatives are acute inhibitors of TDO, as are the phenolic intermediates such as 3-hydroxy-anthranilate and 3-hydroxy-kynurenine. In these instances the inhibitory site on the enzyme is either the same as the substrate binding site or is very close to it, due to the competitive nature of the inhibition.

The inhibition of TDO by intermediates in the pathway between nicotinic acid and NAD/NADP could indicate how nicotinic acid supplementation allows for conservation of plasma TRP (Wagner, 1964). Other potent phenolic inhibitors are the neurotransmitters 5-HT and adrenaline, which are believed to bind at the heme association site of both the bacterial and mammalian enzyme, thereby effectively preventing conjugation (Frieden *et al.*, 1961). TADs such as DMI inhibit TDO by preventing conjugation between the apoenzyme and its cofactor heme. Thus it appears that the ability of the administered antidepressant to inhibit the enzyme could depend on the extent of the heme saturation of the enzyme *in vivo*. On the other hand, antidepressants could block substrate activation by TRP completely. However, studies with TDO have been performed with animal models, using very high concentrations of the antidepressant drug. It is not clear whether a similar mechanism of action is operative in humans, although MAOIs weakly inhibit TDO (Badaway and Evans, 1981).

1.4.1.4 TDO: Mechanism of action (mammalian and rat)

The mode of action of TDO is based on three experimentally determined observations:

i) TRP alone can activate the enzyme,

ii) hemin is essential for activity and binds to the enzyme in a reversible manner, and

iii) the heme iron is indispensable for activity (the valence state of Fe changes during the reaction due to the method of activation of TRP and oxygen (Feigelson *et al.*, 1964a).

TDO is inducible. The inactive form of the enzyme can be converted to the fully active form by the substrate TRP (Greengard and Feigelson, 1961), and the prosthetic group hemin (ferroprotoporphyrin (ix)) can activate the enzyme in the presence of TRP irrespective of the valence state of the iron in the hemin. TRP binds directly to the enzyme and not to the prosthetic heme. During the catalytic process, the heme iron is subject to a cyclical chain of events brought about by TRP and oxygen. The binding of L-TRP to the enzyme induces a fundamental change in the ligand binding affinity of the catalyst in addition to increasing the reactivity of the heme iron towards the substrate (Makino *et al.*, 1980). Two clearly defined sequential events are implicit in enhanced enzymatic catalysis due to substrate induction (Frieden *et al.*, 1961). These are:

i) enzyme saturation by activator heme, and

ii) increases in the levels of total enzyme concentrations.

Empirical evidence obtained from a number of investigations suggests the following model to explain the mode of action of TDO. This mechanistic model requires that TRP reduces the Fe of heme to its divalent state, $Fe^{3+} \rightarrow Fe^{2+}$, because the ferrous state is necessary for the initiation of catalysis (Hayaishi *et al.*, 1957, Ishimura *et al.*, 1970). The exact mechanism of action is not absolutely clear and the overall reaction involves no electron transfer, but it is highly likely that the individual reactions could involve redox reactions which function so as to activate the substrates for interactions (Knox and Ogata, 1965).

It is generally accepted that a cationic TRP free radical (nucleophile) and a superoxide anionic free radical (electrophile) are generated during enzymatic catalysis. Furthermore, a ternary complex consisting of TRP---HEMIN---O_2 is apparently also created. The L-TRP catalytic binding site is close to the iron of heme to allow interaction between O_2 and TRP, thereby facilitating the cooperative movement of electrons from TRP via hemin to oxygen. This hypothesis requires that the transitory, intermediate free radicals generated remain enzyme bound throughout catalysis (Uchida *et al.*, 1985; Ishimura *et al.*, 1970). This postulated scenario could allow for the regeneration of the original catalytically active ferric site of the enzyme (fig. 24).

i) $\mathbf{TRP + HEMIN\text{---}Fe^{3+} \rightarrow HEMIN\text{---}Fe^{2+} + TRP^{+}(ox)}$

ii) $\mathbf{O_2 + HEMIN\text{---}Fe^{2+} \rightarrow HEMIN\text{---}Fe^{3+} + O_2^{-}(red)}$

iii) $\mathbf{TRP(ox) + O_2(red) \rightarrow N'\text{-}FORMYLKYNURENINE}$

$\mathbf{TRP + O_2 \rightarrow N'\text{-}FORMYLKYNURENINE}$

FIG. 24: Proposed sequence of events in the cyclical reduction of TDO [Feigelson *et al.*, 1964b].

Thus the mode of action involves a cyclical reduction and re-oxidation of the metallic-organic factor from protoporphyrin (ix) cofactor, encompassing a two step process:

i) reduction of Fe^{3+} → Fe^{2+} by TRP, and

ii) oxidation of Fe^{2+} → Fe^{3+} by oxygen,

which allows free radical formation that would facilitate interaction at the surface of the enzyme (Feigelson *et al.*, 1964b; Feigelson, 1964). The reaction is characterised by direct oxygenation of substrate with molecular O_2 by the ferrous enzyme (Hayaishi *et al.*, 1957; Feigelson, 1964), with the oxygenated form being the obligatory intermediate of catalysis (Ishimura *et al.*, 1970) (fig. 25). An alternative catalytic mechanism involves the donation of a hydride ion from TRP to the enzyme to create a carbonium ion instead of a cationic free radical (Feigelson, 1964).

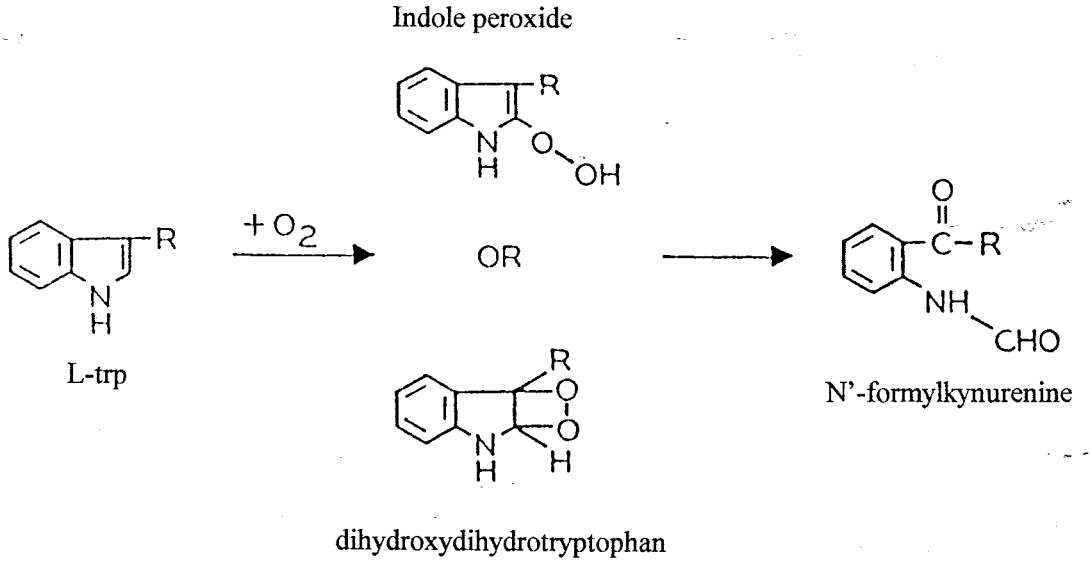


FIG. 25: Hypothesised reaction mechanisms of TRP oxidation [Hayaishi *et al.*, 1957].

1.5 CONCLUSION

In the context of the literature survey just completed, which covers experimental and clinical data accumulated over the past few decades, and links TDO, aMT and 5-HT to the pathophysiology of affective disorders, such as depression, it is clear that TDO occupies a central role in regulating tryptophan levels in the body, and ultimately synthesis of tryptophan derivatives in the brain. Thus, further study of the interactions between TDO, tryptophan derivatives, aMT and 5-HT, the pineal, albumin and popular antidepressants such as DMI and fluoxetine would appear to be useful.

CHAPTER 2

TDO AND ITS EFFECTORS

EXPERIMENT 1

2.0 THE NATURE AND EXTENT OF SUBSTRATE INHIBITION OF TDO

2.0.0 Introduction

The antagonistic interplay that exists between TRP and its various metabolites regarding the enzyme TDO ultimately determines the direction of flow of the amino acid (Schutz *et al.*, 1972). Plasma levels of the indole and, in particular, the total TRP concentration in the body, could thus be crucial for the finely balanced control of protein synthesis, and possibly brain indoleamine concentrations (Green *et al.*, 1962). In view of these roles, specifically the branch point TDO occupies in TRP metabolism, it is highly likely that the enzyme could be a site for metabolic control. Persistent negative alterations in the required levels of this essential amino acid have been shown to lead to a reduction in the rate of hepatic protein synthesis in mice (Sidransky *et al.*,1968). However the inactivation of the enzyme in the absence of substrate is readily reversible (Knox and Piras, 1965). Regulation of the enzyme takes numerous forms, including hormonal (glucocorticoids) (Feigelson *et al.*, 1961), feedback inhibition (NAD(P)H) (Wagner, 1964), 3-hydroxyanthranilate (Schutz *et al.*, 1972), and 3-hydroxykynurenine (Wagner, 1964), substrate TRP (Williams and Elvehjem, 1950; Feigelson *et al.*, 1961) and cofactor heme (Feigelson and Greengard, 1961a; Feigelson and Greengard, 1962b) activation and stabilization. The enzyme is also inhibited by chronic glucose, sucrose, high fat diets (Smith and Pogson, 1980) and by chronic ethanol administration in rats, with a consequent increase in plasma free TRP and brain 5-HT (Badaway *et al.*, 1979).

A common deviation from the predictions of the Michaelis-Menton theory is substrate inhibition. This phenomenon occurs at relatively high substrate concentrations and has been observed with various types of enzymes including hydrolases, transferases and oxidoreductases (De Arragia *et al.*, 1984). Previous work in this laboratory, with TDO revealed that the enzyme is inhibited by substrate TRP. The objective of the study was to investigate the nature of this inhibition, whether it could possibly have any physiological relevance, and also to characterise the kinetic properties of the enzyme at pH 7.

2.0.1 Materials and methods

Female rats of the Wistar strain weighing 200-250 g were purchased from the South African Institute for Medical Research, Johannesburg. The animals were housed four per cage under a diurnal lighting cycle 12L:12D with food and water *ad libitum*. Twenty-five animals were injected three times intraperitoneally

with 100 mg/kg TRP and 5 mg/kg of hydrocortisone to induce TDO. The animals were injected at the following times: 11:00h, 14:00h and 17:00h. Ten hours after the first injection at 21:00h, the animals were killed by neck fracture and the livers were removed and stored at -70°C until further use. The livers were thawed in 3 volumes of 0.14 M potassium chloride containing 0.01 M TRP (pH 7), homogenized briefly in a blender, sonicated and centrifuged (137,500 x g, 1 hr). Since the enzyme is exclusively present in the soluble part of the cell, the supernatant was used for further purification. The purification procedure followed was that described by Schutz and Feigelson (1972), with slight modifications. This particular method gives a thousand fold increase in purification as opposed to the cell sap, and the total yield routinely varies between 10 to 20 percent. The addition of 0.01M TRP to all solutions in this purification procedure was to stabilise and hence protect TDO. The stability of the enzyme is markedly affected by ionic strength and protein concentration, with low levels of either resulting in a reduction of the catalytic rate.

2.0.1.0 Heat treatment (for the removal of unwanted protein)

Hemin was added to the supernatant to give a final concentration of 46.5μM. The resultant solution was manually stirred in a warm water (H_2O) bath that was kept at 55°C for 3 minutes, rapidly cooled in an ice bath and centrifuged at 10K x 40 minutes in a Beckman JA-10 centrifuge to precipitate denatured protein.

2.0.1.1 pH 5 precipitation (to precipitate the enzyme)

The supernatant was titrated to pH 5 with acetic acid, kept at 4°C for 10 minutes, and subsequently spun at 13200 rpm x 30 minutes (70Ti rotor). Potasium phosphate buffer (0.1M) containing 0.01M TRP (40mls) was used to dissolve the resulting precipitate, which was then clarified at 6000 rpm x 10 minutes (JA-21) by centrifugation.

2.0.1.2 Diethylaminoethyl-sephadex (DEAE) chromatography (absorption of enzyme by anion exchange)

A DEAE-sephadex A-50 column (3 x 30cm) equilibrated with 0.01M KH_2PO_4/pH 7 containing 0.01M TRP was used for the application of the sample (45mls). This anion exchanger was set up with a UV FRAC-300 collector. All solutions used in this particular step were equilibrated with 100% N_2 prior to use to create an anaerobic environment and prevent oxidative damage of enzyme. Subsequent to passage of all the sample through the column, the exchanger was washed with 3 to 4 volumes of 0.3M KH_2PO_4/pH7/0.01M TRP and then subjected to a salt gradient ranging from 0.3M to 1.0M KH_2PO_4. The enzyme was displaced at approximately 6M KH_2PO_4 and the fractions containing the coloured product were pooled. Removal of the enzyme from solution at pH 7 involved an equal volume of amonium

sulphate. This solution was centrifuged at 12500 rpm x 20 minutes (70 Ti rotor) and the resultant precipitate re-dissolved in 3 to 4mls of KH_2PO_4/pH 7/0.01M TRP. This fraction was then used for the activity assays.

2.0.1.3 Activity assays

The activity of TDO was determined spectrophometrically by continuous measurement at 321nm of the rate of appearance of N'-formylkynurenine, using a Shimadzu spectrophotometer. The specific activity of the enzyme was expressed in terms of μM N'-formyl/minute/mg protein at 30°C. Hemin was added to give a final concentration of 2μM, and buffer instead of water was added to the reaction mixture. The assay mixture consisted of 1ml of 0.2M NaH_2PO_4/pH 7, 0.3ml of 0.03M TRP, 0.2ml of 4.6μM hemin (fresh), enzyme solution and 0.1M KH_2PO_4 to give a final volume of 3ml. For all activity assays, the value present at the start of each experiment was taken to be zero and therefore subtracted from all subsequent photometric measurements for each particular assay. Protein concentrations were determined by the method of Lowry *et al* (1951), using whole BSA as standard.

2.0.2 Results

Maximum activity was obtained with a pH of 7 at a substrate concentration of 5mM (S_{opt}), and the activity decreased with increasing levels of substrate. Graphical representation of the data acquired for the uninhibited region by means of a Hanes-Woolf plot (Hanes, 1932) (fig. 26), for estimates of K_s (K_s = substrate concentration that gives $0.5V_{max} = S_{0.5}$) and V_{max}, in the presence of variable TRP concentrations ranging from 0.0625 to 5mM, resulted in a K_m of 100μM and V_{max} of 15.8 μM N'-formylkynurenine/min/mg protein respectively. The Hill plot (Cornish-Bowden, 1979) (fig. 27) is biphasic (n=1.1 and n=2.0) with a transition region where n = 0.5 (inset fig. 27). Given the finding that TRP behaves both as a substrate and as an inhibitor, a theoretical activity curve (fig. 28) was constructed incorporating the approximations of V_{max} and K_s obtained from the Hanes-Woolf plot (in fig. 26), using the Hill equation to approximate the velocity.

$$\mathbf{V = V_{max} \cdot [S]^n / K_s + [S]^n}$$

Assuming that the values associated with the theoretical activity are those that would be present if no inhibition occurred, calculation of the percentage inhibition due to the increase in TRP levels beyond S_{opt} is possible (De Arragia *et al.*, 1984; Bernstein *et al.*, 1978). A plot of percentage inhibition against S was sigmoidal (fig. 29). The Hill plot (fig. 30) graphed from the data for the inhibited region using the relationship (De Arragia *et al.*, 1984):

$$\log (V/V_{max}-V) = \log K_s - n \log I$$

gave an $I.C._{50}$ = 18.80mM and $n^{app.}$ = 1.9.

2.0.3 Effects of pH on substrate inhibition

Assays were performed at four different pH values 5, 6, 7 and 9. As stated previously, maximum enzyme activity occurs at pH 7. Raising the pH to 9 results in a drop in V_{max} to 13.3μM N'-formylkynurenine/min/mg protein (fig.31). This is coupled with a shift in the S_{opt} from 5mM to 2mM, and an unchanged K_m value of 100μM. The profile of this graph over the total concentration range, however, remains the same as the graphs obtained at pH 7 and pH 6 (fig. 31). The Hill plot (inset fig. 32) reveals $n^{app.}$ of 0.8. In the region where there is substrate inhibition $n^{app.}$ changes from 0.8 to 0.4. (fig. 32) indicating negative homotropic cooperativity at pH 9. The graph of percentage inhibition against [S] (inset fig. 29), is also sigmoidal in shape.

At pH 6 there is a change in K_s to 144 μM TRP $_{max}$V remains unaltered at 15.8 μM N'-formylkynurenine/min/mg protein and S_{opt} shifts to 4mM. The data obtained at pH 5 depicts a 47 per cent reduction in V_{max}, a fivefold increase in $S_{0.5}$, and S_{opt} shifts to 10mM (fig. 31). The activity at 20mM is only 3.53 μM N'-formylkynurenine/min/mg protein (22.32%$V_{max\ (pH7)}$), and maximum velocity is approximately twice as high at pH 9 than at pH 5 for the enzyme. The response of the enzyme at pH 6 and 9 (fig. 31) is very similar to that at pH 7, exhibiting positive cooperativity (albeit to a lesser degree) which extends over the whole concentration range, and is subject to substrate inhibition. Enzyme activity at pH 5, 6, 7, 8 and 9 (fig. 34), at the inhibiting TRP level of 16mM, shows that the least inhibition occurs at pH 7, the maximum at pH 5, while the inhibition at pH 6 is less than at pH 8 and pH 9.

2.0.4 Discussion

With reference to the Hill plot (pH 7, fig. 27), it is apparent that initially, at low concentrations of substrate, there is very little or no cooperativity (n^{app} =1.1). The enzyme is displaying hyperboloidal kinetics with only the catalytic site binding TRP (Cho-Chung and Pitot, 1967). As the substrate concentration increases the slope becomes less than unity (see inset), which is indicative of the involvement of two classes of binding sites (Van Holde, 1985). Increasing the levels of substrate results in all the binding sites on the enzyme being filled. The slope approximates two, and clearly demonstrates positive homotropic cooperativity. It is apparent that the enzyme is subject to differential kinetics of a complex nature (Cho-Chung and Pitot, 1967; Schutz *et al.*, 1972), Michaelis - Menten kinetics at levels of TRP in

the vicinity of the K_s and sigmoidal kinetics at higher concentrations (Schutz *et al.*, 1972).

TDO is inhibited at all concentrations of substrate immediately beyond S_{opt}. The TRP concentration at which substrate inhibition is discernible not only depends on the pH of the environment (fig. 31) but also on the enzyme concentration (fig. 35). Here it should be noted that an increase in enzyme concentration results in a concomitant reduction in S_{opt}, even at TRP levels as low as between 800-900μM, while, on the other hand, decreasing the enzyme concentration gives rise to an increase in S_{opt}. Thus, given the observation that substrate inhibition occurs immediately beyond V_{max} at both high and low enzyme levels, as tested in this laboratory, it can be inferred that this type of inhibition could be an additional mechanism whereby the flux through the TRP-N'-formylkynurenine pathway is regulated in the rat.

Plots of percentage inhibition against [S] (fig. 29) illustrate the sigmoidal mode of inhibition at pH 7 and pH 9 (inset), which is also a characteristic feature at all other pH values. However, at pH 7 there is gross positive homotropic cooperativity, while at pH 6 and 5, this response is marginal. At pH 9, on the other hand, negative homotropic cooperativity occurs. This apparent pH dependence of cooperativity (Schutz *et al.*, 1972) could imply that the allosteric behaviour of the enzyme is mediated through changes in the ratios of protonated to deprotonated side chain amino acids. In this instance it is obvious that the transition from positive to negative cooperativity is due to the removal of hydrogen ions from the catalyst, TDO. It is, however, clear (fig. 34) that the activity of the enzyme is strongly dependent on the hydrogen ion concentration of the environment (Schutz *et al.*, 1972).

Substrate inhibition could be caused by the additional binding of substrate to either the regulatory or catalytic TRP binding sites. The latter site is the most likely candidate, for if any interference occurred at the regulatory site, it is very likely that there would be loss of cooperativity between the individual subunits, and the graphical response would take the form of a rectangular hyperbola. Graphs of velocity against [S] (fig. 31) initially depict sharp reductions in activity of 12% at pH 7, 21.35% at pH 9 and 19.31% at pH 6, which subsequently become more gradual. At both pH 7 and pH 6, the positive cooperative effect remains relatively constant in the transition to the substrate inhibited region, while at pH 9, there is a pronounced enhancement of the negative modulation. This behaviour of the enzyme - namely, retention of the ability to respond cooperatively at all the indicated pH values in the presence of substrate inhibition - not only implicates the catalytic site as the affected area which causes the significant reduction in the catalytic rate, but also effectively excludes the regulatory site from any possible involvement.

The mechanism whereby substrate inhibition is brought about is unclear, but it could occur in one of the following ways: firstly, there could be formation of a dead end complex between enzyme, substrate and product, preventing release of the product. Alternatively, two molecules of substrate could each get partially lodged in the active site, giving rise to an inactive enzyme-S_2 complex (Cleland, 1979). It could be argued that substrate inhibition in this instance is of no consequence, given that a particular phenomenon is of physiological relevance only if it can be shown to take place under physiological conditions. It can also be claimed that the apparent inhibition observed is due to interactions between the hydrophobic side chain of TRP and non polar regions of the enzyme that would affect its integrity as a biological catalyst.

All work done with purified TDO in this laboratory repeatedly demonstrated inhibition beyond S_{opt} at values of TRP between 800μM and 5mM (fig. 35). Clearly then, if an increase in enzyme concentration coupled with a reduction in S_{opt} also results in substrate inhibition at TRP levels approximately sixfold less than 5mM, it is unlikely that substrate inhibition could be due to non-specific interactions between enzyme and substrate. On the other hand, the Hill plot of the inhibited region (fig. 30) points to the presence of only two binding sites for substrate TRP, namely a catalytic site and a regulatory site.

2.0.5 Conclusion

In the major part of this study, TRP did not inhibit the reaction until an approximate concentration of 5mM was reached. However it must be emphasized that the substrate concentration at which inhibition occurs appears to be related to the maximum velocity of the reaction, ie the rate of product formation, which ultimately depends on the enzyme concentration at saturating levels of substrate. The higher the enzyme level, the greater the specific activity and vice versa. Thus the crucial marker seems to be V_{max} , for once V_{max} is reached any further increases in TRP gives rise to partial inhibition of TDO.

In the light of these observations, it appears that the phenomenon of substrate inhibition in this instance might have a significant role to play in the regulation of TDO activity at a physiological level.

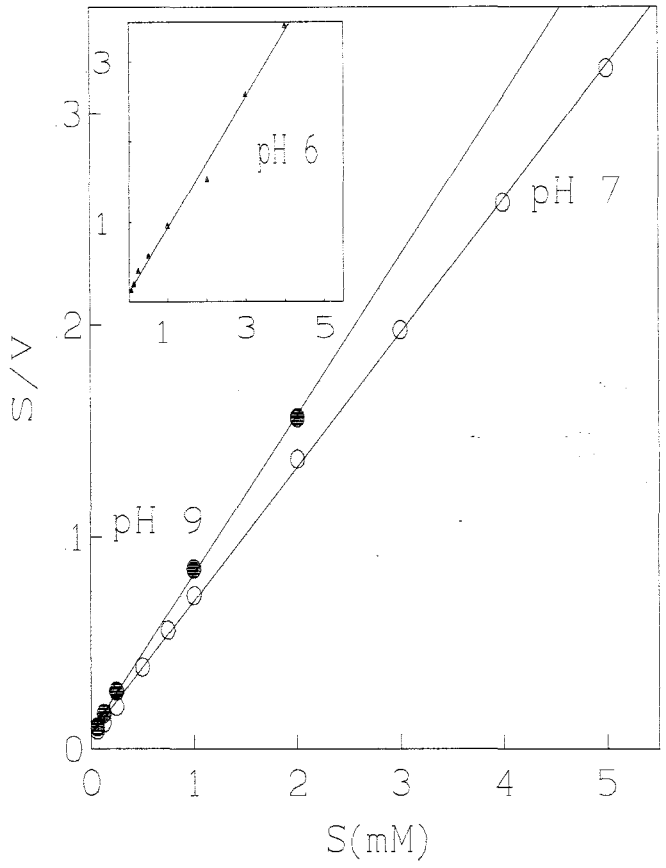


FIG. 26: Hanes Woolf plots to determine K_m and $V_{max.}$ for uninhibited tryptophan-2,3-dioxygenase at pH 7, 6 and 9. S = [tryptophan] and V = velocity expressed as μM N'-formylkynurenine/minute/mg protein. r = 0.99 for both regression lines.

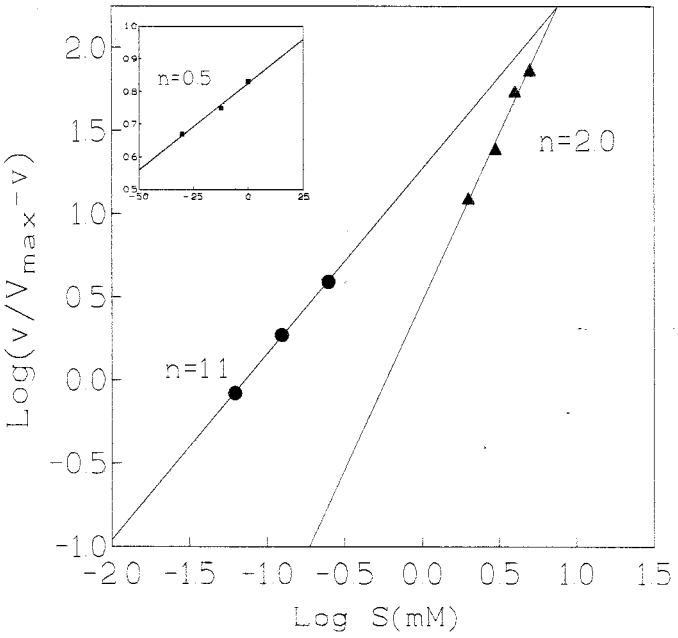


FIG. 27: Hill plot to determine the number of binding sites for the uninhibited tryptophan-2,3-dioxygenase at pH 7. Note the inset depicting the transition from catalytic to regulatory binding site. n = Hill coefficient, r = 0.99, S = [tryptophan], V = velocity expressed as µM N'-formylkynurenine/minute/mg protein.

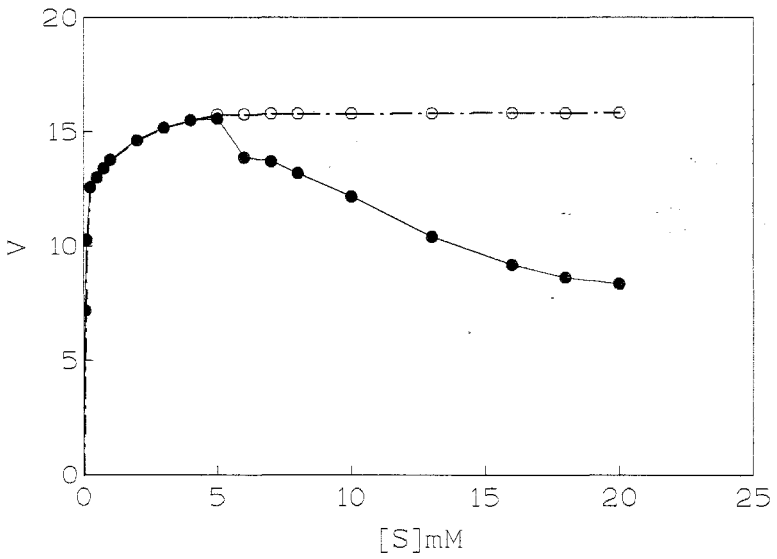


FIG. 28: Theoretical activity curve at pH 7 of tryptophan-2,3-dioxygenase. The dashed line represents the supposed activity profile in the absence of any inhibition. V = velocity expressed as μM N'-formylkynurenine/minute/mg protein, S = [tryptophan].

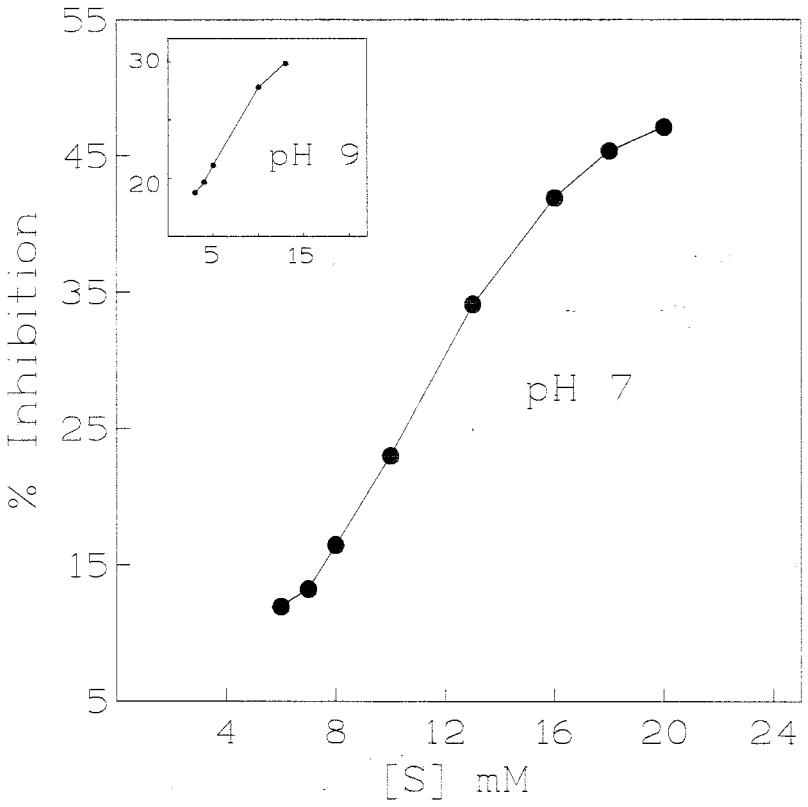


FIG. 29: Graph of % inhibition against substrate [tryptophan] inhibited tryptophan-2,3-dioxygenase at pH 7 (note inset for pH 9). The percentage inhibition was determined from the data plotted in fig. 15. [S] = [tryptophan] and activity is expressed as μM N'-formylkynurenine/mg protein/minute.

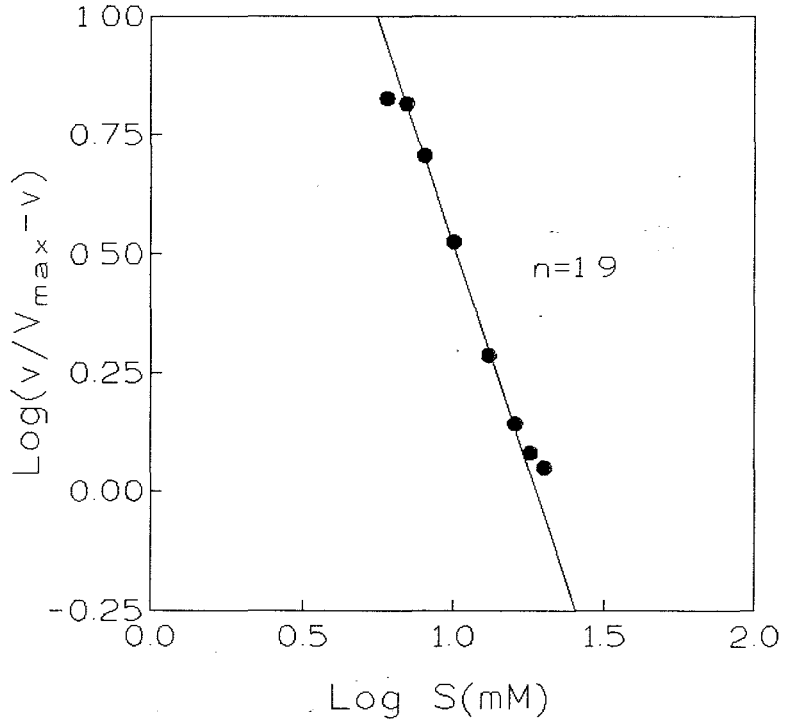


FIG. 30: Hill plot of substrate inhibited region at pH 7. This graph clearly shows the presence of only two binding sites on the enzyme tryptophan-2,3-dioxygenase. V = velocity in μM N'-formyl-kynurenine/mg protein/minute, V_{max} = maximum velocity, S = tryptophan concentration in mM, n = Hill coefficient, r = 0.99.

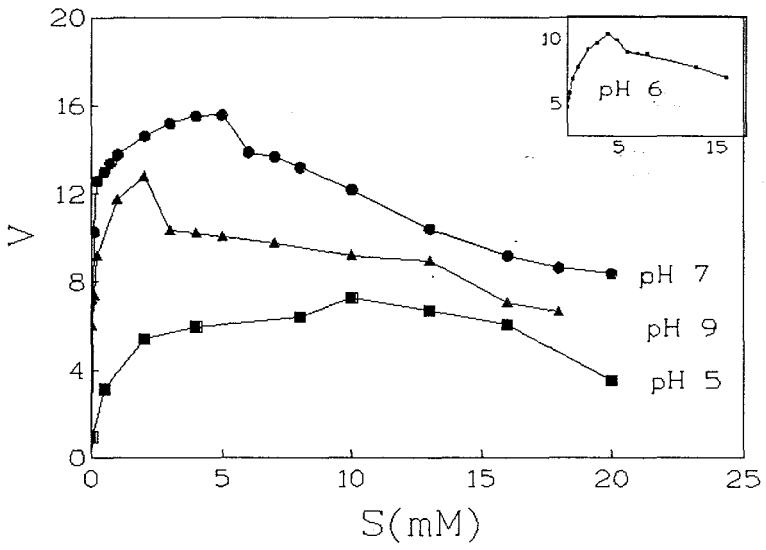


FIG. 31: Specific activity curves at pH 5, 6, 7 and 9 for tryptophan-2,3-dioxygenase (note S_{opt} values). V = velocity in μM N'-formylkynurenine/mg protein/minute, S = tryptophan concentration in mM.

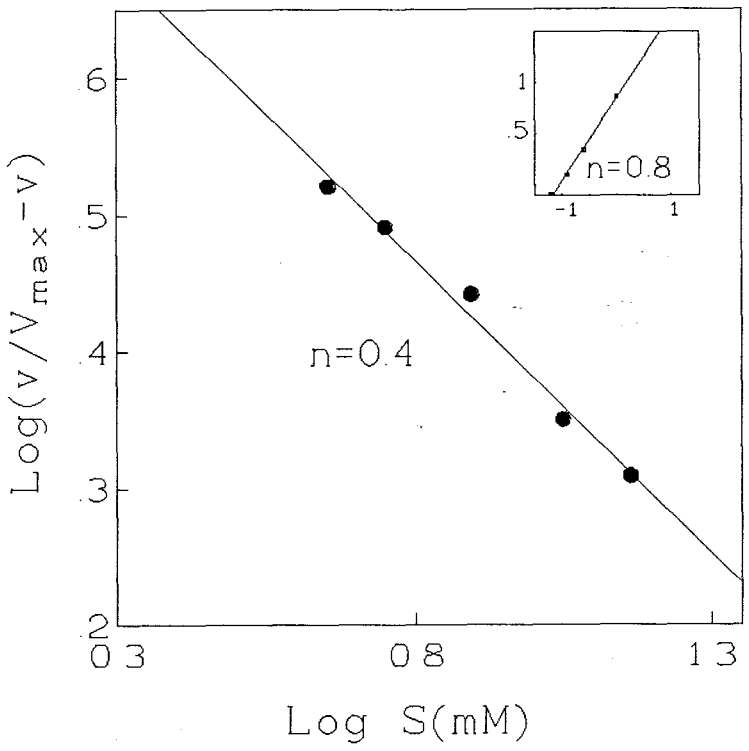


FIG. 32: Hill plots at pH 9 (note inset for substrate inhibited region) for tryptophan-2,3-dioxygenase. r = 0.996, n = Hill coefficient, V = velocity in μM N'-formylkynurenine/mg protein/minute, S = tryptophan concentration in mM.

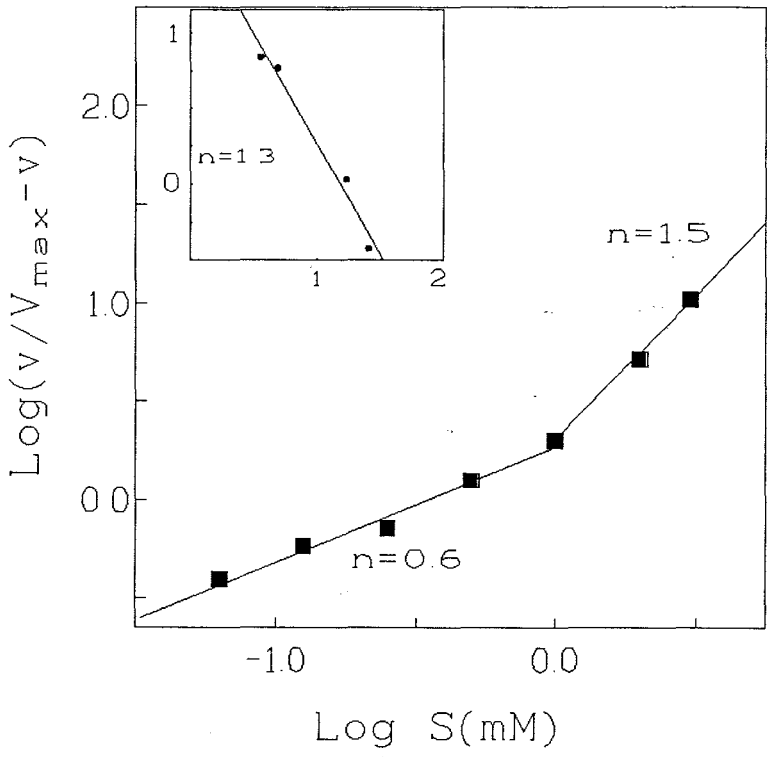


FIG. 33: Hill plots at pH 6 (note inset for substrate inhibited region) for tryptophan-2,3-dioxygenase. n = Hill coefficient, V = specific activity in μM N'-formylkynurenine/mg protein/minute, S = tryptophan concentration in mM, r = 0.99 (inset r = 0.95).

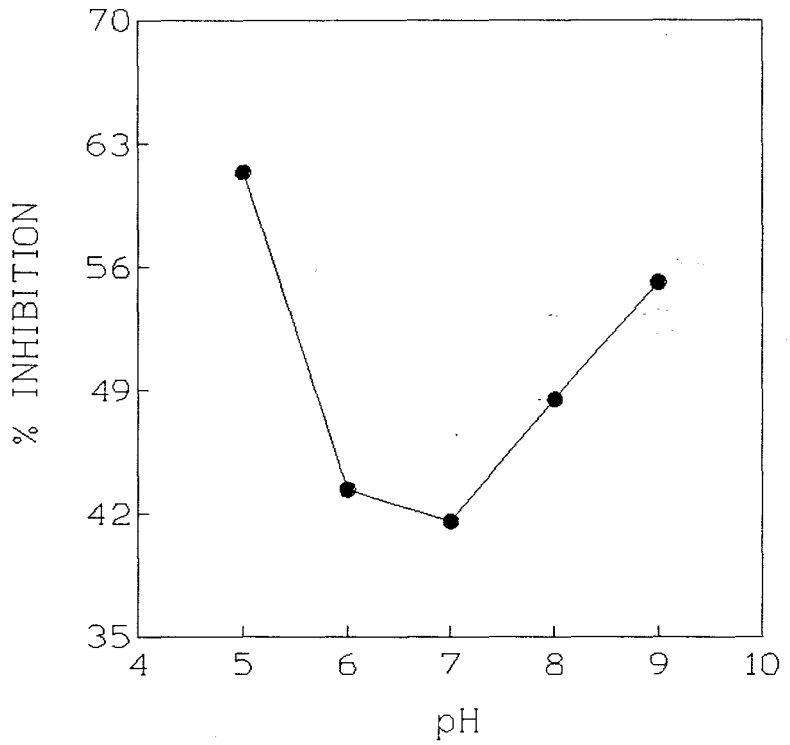


FIG. 34: Graph of % inhibition of activity plotted against pH at 16mM TRP. The effects of pH on tryptophan-2,3-dioxygenase activity are shown as pH decreases the inhibition increases drastically (<6) while at pH values above the optimum of 7 the inhibition is more gradual. Activity is expressed as μM N'-formylkynurenine/mg protein/minute.

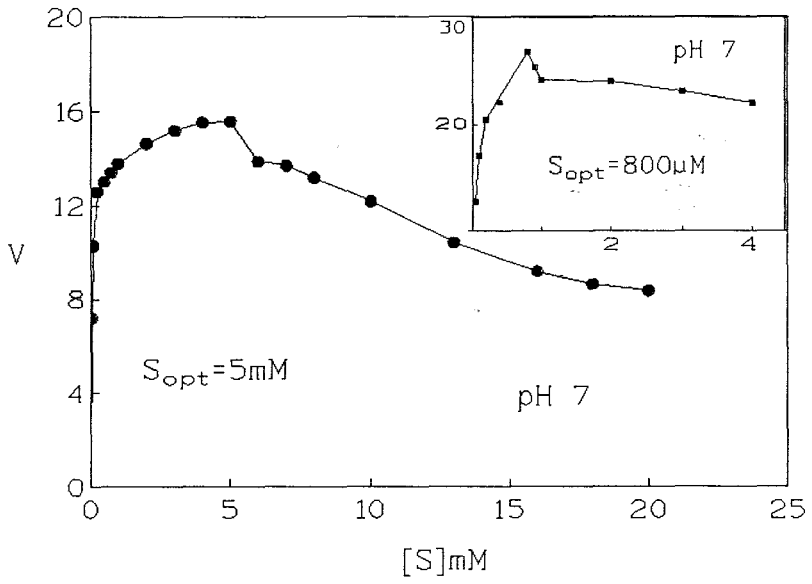


FIG. 35: Substrate inhibition at different enzyme concentrations. Tryptophan-2,3-dioxygenase had an approximate K_m of 100μM and specific activities of 15.8 (S_{opt} = 5mM) and 26.7 (S_{opt} = 800μm, see inset). (See reference 72 where S_{opt} = 2.5mM). S_{opt} = tryptophan concentration at which there is maximum velocity (V), V = μM N'-formylynurenine/minute/mg protein (specific activity), S = tryptophan concentration in mM.

EXPERIMENT 2

2.1 – THE INHIBITION OF CRUDE RAT LIVER TDO BY MELATONIN *in vitro.*

2.1.0 Introduction

Metabolism in living tissue is controlled by enzymes, which are regulated by other factors, mainly hormones and substrates. The rat liver enzyme TDO is an example where such control is exercised. This enzyme is also characterised by circadian rhythms in the rat, the highest activity being observable during the late hours of the light period and the lowest activity towards the end of the dark period (Hardeland and Rensing, 1968).

TDO is responsible for the catabolism of TRP, which possibly makes it one of the most important peripheral determinants of brain and plasma free TRP concentration (Badaway *et al.*, 1980). Therefore the availability of TRP to the brain for the synthesis of 5-HT ultimately appears to be dependent on the level of activity of TDO. It can be assumed that an inverse relationship exists between TDO activity and 5-HT synthesis (Badaway and Evans, 1981). This relationship has been demonstrated previously (Green *et al.*, 1962).

Stress causes rat TDO activity to rise and brain 5-HT levels to fall. Normally, rat TDO activity rises throughout the day while brain 5-HT levels are at a minimum during early darkness (Hardeland and Rensing, 1968). It is generally accepted that three peripheral mechanisms are involved in controlling the elevation of brain TRP concentrations. These are: lower TRP catabolism, resulting from a decrease in the activity of hepatic TDO; elevation of free plasma TRP concentrations; and a decreased level of circulating LNAAs, which compete with TRP for uptake into the brain (Green *et al.*, 1962).

Rat brain TRP concentrations have been reported to be elevated after acute administration of TADs such as IMI and DMI (Hardeland and Rensing, 1968). Secretion of the pineal hormone aMT is enhanced by TADs and MAOIs. During stress, normal aMT levels are disturbed and treatment with TAD and MAOIs restores normal aMT levels after $\pm$ 3 weeks. This period is comparable to the time required for antidepressants to exert their clinical effect. Thus aMT could be responsible for relieving the symptoms of depression (Arendt *et al.*, 1986).

Present therapy to alleviate depression appears to involve inhibition of TDO, either directly or by factors which enhance the ability of TRP to increase brain 5-HT levels (Curzon, 1969).

This study was undertaken on the basis of a number of observations. These included findings that both aMT and TDO display diurnal variations although inversely under normal conditions in the rat, and that the activity of TDO is at its diurnal minimum at 12 o'clock (Hardeland and Rensing, 1968). aMT and TRP have a number of apparent structural similarities, and the natural rise in pineal aMT coincides with the normal increase in blood aMT.

2.1.1 Materials and methods

aMT and hemin were purchased from the Sigma Chemical Co. St. Louis, MO, USA. Twenty male rats of the Wistar strain weighing 200-292 g were purchased from the South African Institute for Medical Research, Johannesburg, and maintained in a light:dark cycle of 12:12 (lights on from 07:00h-19:00h). Food and water were provided *ad libitum*. The animals were killed by cervical dislocation at 09:00h and the livers were removed immediately and frozen at -70°C using liquid nitrogen until assayed.

TDO activity was determined in triplicate according to the method described by Badaway and Evans, (1975). The livers were homogenised in 0.14M KCL/2.5mM NaOH for 2 minutes with an electric driven homogeniser at 0°C. 15mls of this homogenate were added to a beaker kept at 0°C containing 5 mls of 0.03M TRP/4mM NaOH at pH 7.2, 25mls of 0.2M NaH_2PO_4 (pH 7.2). These solutions were thoroughly mixed and 3mls immediately added to boiling tubes which were kept on ice. After rapidly adding 95%O_2/CO_2 the tubes were stoppered and incubated in a shaking incubator oscillating (120/min) at 37°C for the desired time. To determine the appropriate time to measure TDO activity, a graph showing the time course of TRP catabolism was constructed both in the absence and the presence of hemin (fig. 36). A time point (60 minutes) on the linear portion of the graph was then chosen for all subsequent determinations. Each reaction was stopped through the addition of 2mls of 0.9M tricarboxylic acid and shaken for a further 2 minutes. The resultant suspension was filtered using Whatman No 1 filter paper in a Buchner funnel. To 2.5mls of the filtrate 1.5mls of 0.6M NaOH were added and the absorbance read at 365nm. The enzyme activity was determined in the absence and presence of hemin in order to evaluate the activity of the holo- and apoenzymes of TDO. In addition, TDO activity was measured in the presence of 50 μM, 100 μM and 200 μM aMT, in the presence of hemin. The antidepressant DMI was also tested in the same concentration range.

Protein concentration was determined according to the method described by Lowry *et al.*, (1951). The data were analysed by one-way analysis of variance, and the statistical significance among specific means was determined using the Newman-Keuls multiple range test. A p value of < 0.05 between groups was accepted as being statistically significant.

2.1.2 Results

As shown in figure 37, TDO activity was enhanced significantly ($p < 0.05$) in the presence of hemin. aMT caused a dose-dependent inhibition of the hemin-induced rise in TDO activity ($p < 0.05$) to levels approximating TDO activity in the absence of added hemin. DMI caused a similar effect, although to a lesser extent (fig. 38), while fluoxetine had no observable impact (fig. 39).The mean counts obtained for the tested concentrations 1.5×10^{-3} M, 1.5×10^{-5} M and 1.5×10^{-6} M showed no significant difference ($p > 0.05$), and thus only the group mean was plotted against the control (fig. 39).

2.1.3 Discussion

Liver TDO is one of the most important peripheral determinants of brain and plasma TRP levels (Badaway *et al.*, 1980). Increased catabolism of TRP results when liver TDO activity is high (Badaway *et al.*, 1981). Thus, when TDO is activated, less TRP becomes available for uptake by the brain, resulting in reduced cerebral concentrations of this indole and its metabolite 5-HT. TDO is a heme-dependent enzyme, the apoenzyme being dependent on hemin as a cofactor (Feigelson and Greengard, 1962b).

The addition of hemin produced an expected rise in TDO activity in this study. aMT, a hormone secreted by the pineal gland and implicated in the pathology of depression (Arendt *et al.*, 1986), caused a dose-dependent inhibition of hemin-activated TDO, with the highest concentration reversing the hemin-induced activation of TDO. Whether aMT interferes with the binding of hemin to the apoenzyme remains to be investigated.

A number of TADS have been shown to inhibit TDO activity in a similar fashion, thus causing enhanced levels of TRP and 5-HT in the brain (Badaway and Evans, 1981). aMT appears to have a similar action to these agents in that it causes inhibition of liver TDO activity. However it has been shown previously that acute administration of aMT to rats in a dose of 25 μg reduces forebrain TRP concentrations but does not alter forebrain 5-HT concentrations (Daya *et al.*, 1989).

2.1.4 Conclusion

aMT inhibits crude TDO competitively at micromolar concentrations *in vitro*. Whether chronic aMT administration results in increased brain TRP and 5-HT levels due to inhibition of TDO and whether aMT is responsible for the diurnal variation in TDO activity has yet to be determined.

The SSRI fluoxetine *in vitro,* unlike the tricyclic DMI, does not inhibit TDO. It can be concluded that fluoxetine has no direct effect on the hepatic enzyme.

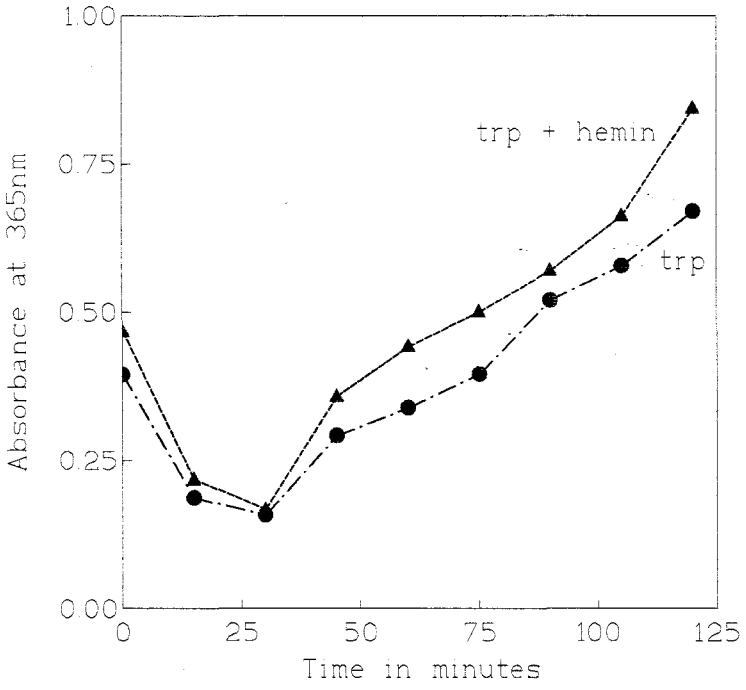


FIG. 36: Time course of tryptophan catabolism by tryptophan-2,3-dioxygenase in the presence (▲) and absence (o) of hemin (2μM) at 30°C/pH 7. V = μM kynurenine/minute/mg protein; tryptophan (trp) concentration at 0.03M.

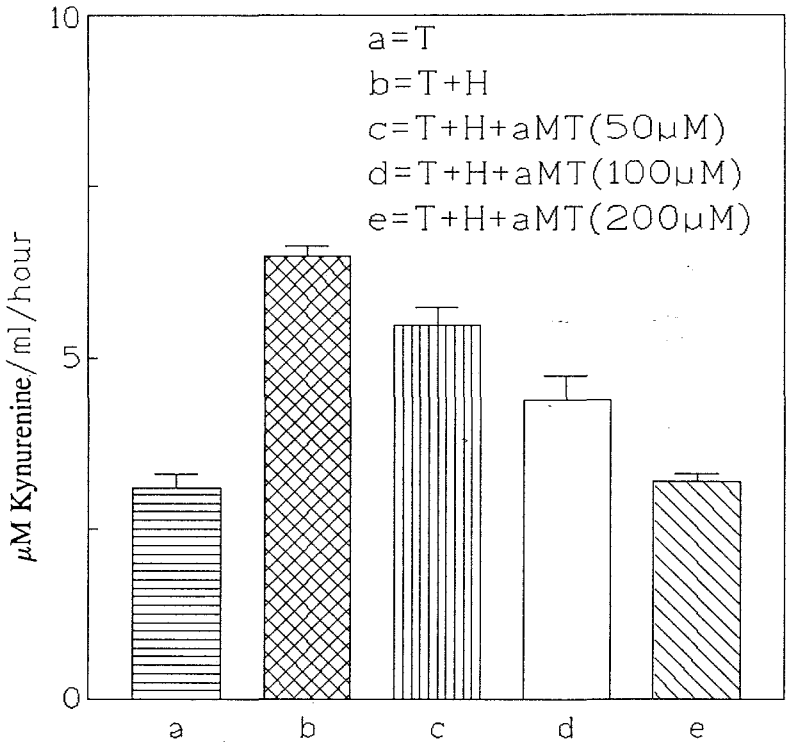


FIG. 37: Effect of melatonin (aMT) on the hemin (H) induced rise in crude rat liver tryptophan-2,3-dioxygenase activity (means ± SEM; n = 4 and $p < 0.05$ for all individual assays, $p < 0.05$ as compared with control for b,c and d). Control = tryptophan (T). Specific activity = μM kynurenine/mg protein/minute at 365nm.

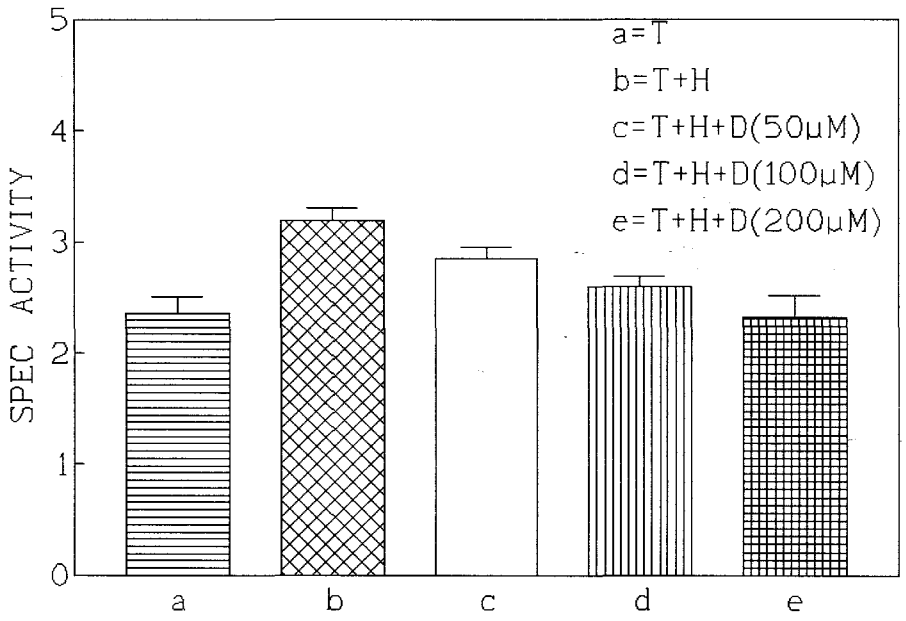


FIG. 38: Effect of desipramine (D) on the hemin (H) induced rise in rat liver tryptophan-2,3-dioxygenase activity (means ± SEM; n = 4, $p < 0.05$ for each assay (a,b,c,d,e), At D= 50, $p < 0.05$ as compared with control). Control = TRP (T).

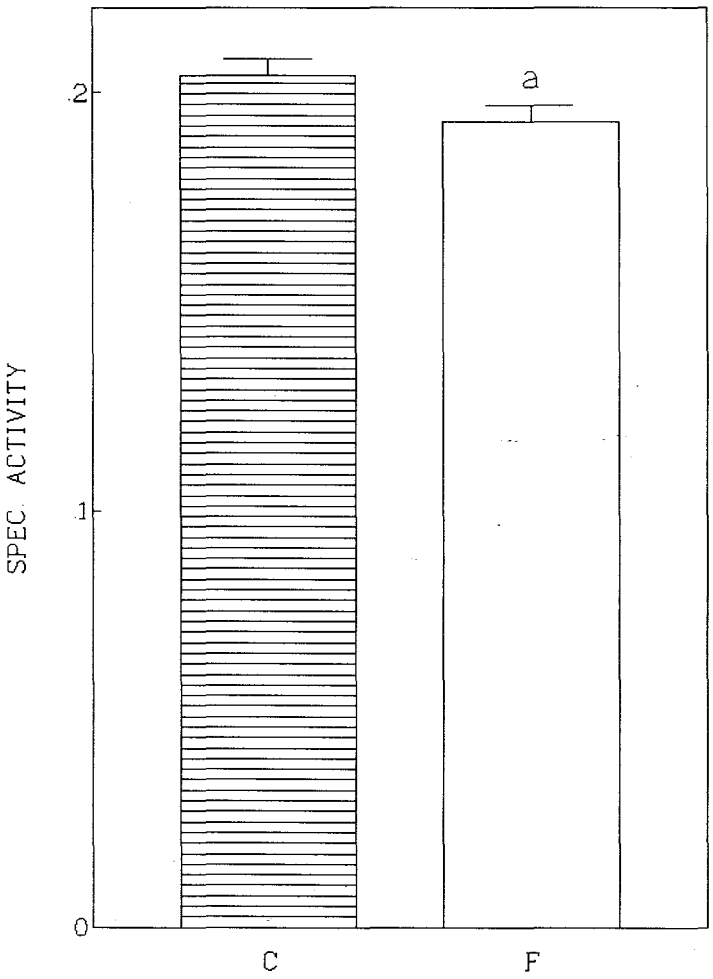


FIG. 39: Effect of different concentrations (a = 1.5×10^{-6}M, 1.5×10^{-5} M, 1.5×10^{-4} M) of fluoxetine (F) on the hemin induced rise in rat liver tryptophan-2,3-dioxygenase activity (means $\pm$SD; n = 4 for C and n = 12 for F). $p > 0.05$ for all tested concentrations within a and when compared to control (C).

EXPERIMENT 3

2.2 THE MODE OF INHIBITION OF RAT HEPATIC TDO BY EFFECTOR MELATONIN.

2.2.0 Introduction

Changes in the activity of TDO could affect the synthesis of related biogenic amines in the brain such as the neurotransmitter (5-HT) and possibly the neurohormone aMT. A reduction of circulating TRP in the blood could thus indirectly affect the synthesis of brain indoleamines (Mehler and Knox, 1954; Hayaishi *et al.*, 1957; Daya *et al.*, 1990). It is possible that the enzyme TDO could be a site for metabolic control since it initiates the catabolism of TRP to the pyridine moiety of NAD^+ and related nucleotides (Fernstrom *et al.*, 1971; Frieden *et al.*, 1961).

An inverse relationship exists between the levels of aMT and TDO activity. In the previous *in vitro* experiment performed in our laboratory, using a crude extract of TDO, it was demonstrated (fig. 23) that the pineal hormone aMT causes a dose-dependent inhibition of the enzyme at concentrations of 50 μM, 100 μM and 200 μM (Walsh *et al.*, 1991b). The purpose of the present study was to investigate the nature and degree of this inhibition using purified TDO.

2.2.1 Materials and methods

Male rats of the Wistar strain weighing 200-250 g purchased from the South African Institute for Medical Research, Johannesburg, were used in the experiment. The animals were housed four per cage under a diurnal lighting cycle 12L:12D with food and water *ad libitum*. The method used for obtaining the enzyme was identical to that outlined in Experiment 1. The enzyme activity was determined both in the absence and in the presence of hemin in order to evaluate the activity of holo- and apoenzymes.

2.2.2 Results

Graphical analysis of the plotted data is as follows: V_{max} = 1.50 μM kyn/min/mg protein, K_i = 100 μM and K_i = 2.70 μM. As shown in figure 40, a plot of V against [S] yields lines that are sigmoidal at all concentrations of substrate (S). Replotting this data S/V against I (Eisenthal and Cornish-Bowden, 1974) results in parallel lines indicative of competitive inhibition (fig. 41). Figure 42 shows the same data plotted on a Hanes Woolf plot (S/V against [S]) depicting parallel lines with a positive slope. The effect of substrate inhibition can be seen at concentration 3 mM. A secondary plot of Y- intercept against I gives a straight line (fig. 43) with a K_i of 2.70 μM. Figure 44 shows the effects of high inhibitor concentration (60 μM) on low (1 mM) substrate concentration. The specific activity of the enzyme varies with the

concentration of TRP and the highest specific activity was observed at 2.5 mM.

2.2.3 Discussion

aMT, a structural analog of TRP, appears to be a competitive inhibitor of liver TDO (fig. 41). The kinetic behaviour of the enzyme has been ascribed to the presence of two binding sites for the substrate L-TRP, namely a catalytic and a regulatory site (Schutz and Feigelson, 1972; Uchida *et al.*, 1985; Makino *et al.*, 1980; Ishimura *et al.*, 1970). It is known that the binding of TRP to the enzyme induces a change in the heme ligand binding affinity of the enzyme. At low concentrations of substrate, binding only occurs at the catalytic site. With an increase in the concentration of TRP, additional binding takes place at the regulatory site.

It is postulated that the binding of the second molecule of substrate influences the affinity at, and interaction between, the two binding sites. This behaviour of the enzyme is explained in terms of negative and positive homocooperativity. TRP appears to give rise to positive homotropic cooperativity (Schutz and Feigelson, 1972). The binding of aMT, on the other hand, apparently negatively modifies the catalytic efficiency of the enzyme, with the degree and nature of the inhibition depending on the concentration of TRP (figs. 41 and 44). From figure 41 it can be seen that the sigmoidal mode of inhibition is very pronounced at high substrate concentration ($>$ 1 mM). This effect is almost absent at 1 mM. Figure 44, on the other hand, depicts aMT (60 μM) affecting basal enzyme (holoenzyme) activity at low concentrations of TRP (1 mM).

Thus, the homotropic cooperative interaction of the negative effector aMT is expressed when the enzyme becomes progressively saturated with substrate. Schutz and Feigelson (1972) observed a similar effect on the enzyme in the presence of 3-hydroxyanthranilate. Maximum velocity is reached at a substrate concentration of 2.5 mM. However, as the concentration increases above this value, substrate inhibition becomes a factor, as indicated graphically in figure 42. This is particularly apparent at a concentration of 4 mM. The effect is characterised by a sharp increase in the positive slope of the curve at a concentration of 3 mM. The manner in which substrate inhibition is brought about is not known, and remains to be examined (see Experiment 2). At the micromolar concentrations used, aMT appears to be highly specific when compared to other indoleamines. These TRP derivatives (eg tryptamine, 5-HT and 5-HTP) were marginally effective only at millimolar levels *in vitro* (Uchida *et al.*, 1992). Whether the methoxyindoles such as 5-MTOH and 5-methoxytryptamine have a similar potency remains to be determined.

The question now arises as to why a global regulatory mechanism, such as that resulting from the aMT rhythm, leads to a decrease in enzyme activity when the level of enzyme protein is relatively high. TDO

occupies a branchpoint in the pathway of TRP metabolism. A fraction of the free circulating amino acid is required for indoleamine synthesis, while a large proportion of the remainder is catabolized by TDO to ultimately yield nicotinate and related compounds such as picolinate and quinolinate. The onset of darkness necessitates an increase in the levels of free serum TRP, primarily for two reasons. On one hand, the requirements of the enzyme with respect to the TRP-nicotinate pathway have to be met, and secondly, TRP is needed for increased indoleamine synthesis by both the brain and pineal. aMT could bring this relatively high serum TRP concentration about by displacing TRP from serum albumin. Concomitant modulation of TDO by aMT would then allow sufficient TRP to escape catabolism for uptake across the blood-brain barrier.

The mode of aMT inhibition could be exerted in one of three possible ways. Firstly, the molecule may not be able to take a configuration appropriate for the catalytic reaction because of a lack of functional group(s) ($RCHNH_2COOH$) for proper interactions with the protein part at the catalytic site. Secondly, it might have the correct functional groups and adopt a pertinent configuration to allow catalysis in addition to being potentially reactive, but may not be able to serve as a substrate because other functional group(s) (5-methoxy) make additional interactions with a protein side chain which greatly reduces the catalytic ability of the heme pocket (Uchida *et al.*, 1985). Alternatively, aMT could act as a free radical scavenger (Poegeler *et al.*, 1993) of the superoxide anion (O_2^-) that would be generated during the enzymatic reaction (Feigelson *et al.*, 1964a, 1964b) effectively preventing the formation of N'-formylkynurenine.

The finding that a binding site for aMT exists on an enzyme found exclusively in the cytoplasm of hepatocytes supports the notion that the neurohormone does freely penetrate the liver cell in the absence of cell surface aMT receptors. That aMT does enter the cell is further borne out by the presence of nuclear protein binding sites in purified rat hepatic nuclei (Acuña-Castroviejo *et al.*, 1993). The mechanism involved with movement into the nucleus is at present still a mystery. However the lipophilic nature of the molecule could also allow for crossing of the nuclear envelope. Alternatively, a transport protein could facilitate traversal into the nucleus.

2.2.4 Conclusion

The results of the present study indicate that inhibition of liver TDO activity by aMT in supraphysiological concentrations could inhibit circulating TRP catabolism, resulting in greater availability of the amino acid for uptake into the brain (Daya *et al.*, 1990). However, as stated earlier, the effect of aMT on the enzyme is ultimately dependent on TRP concentration, and further work in this regard is required to clarify the biological relevance of this finding.

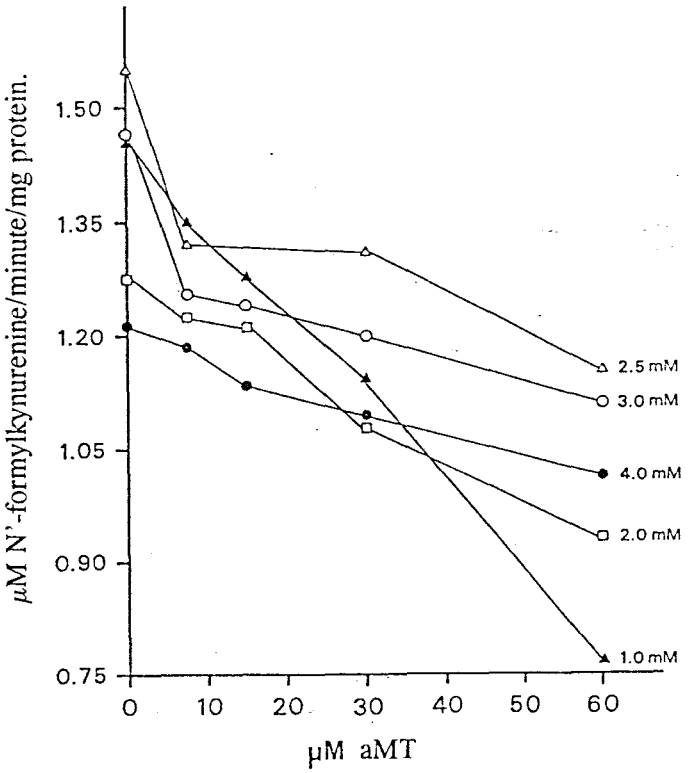


FIG. 40: The inhibitory effect of different concentrations of melatonin (aMT) on rat liver tryptophan-2,3-dioxygenase activity at varied substrate tryptophan concentrations (1 - 4mM). Velocity in μM N'-formylkynurenine/minute/mg protein.

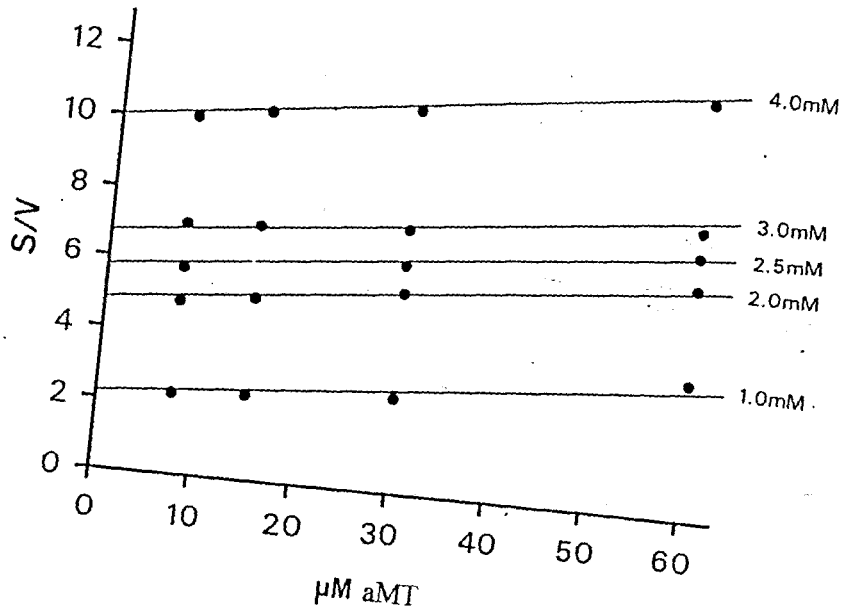


FIG. 41: Cornish-Bowden plot depicting competitive inhibition of rat hepatic tryptophan-2,3-dioxygenase by melatonin (aMT). S = tryptophan concentration in mM, V = velocity in μM N'-formylkynurenine/minute/mg protein.

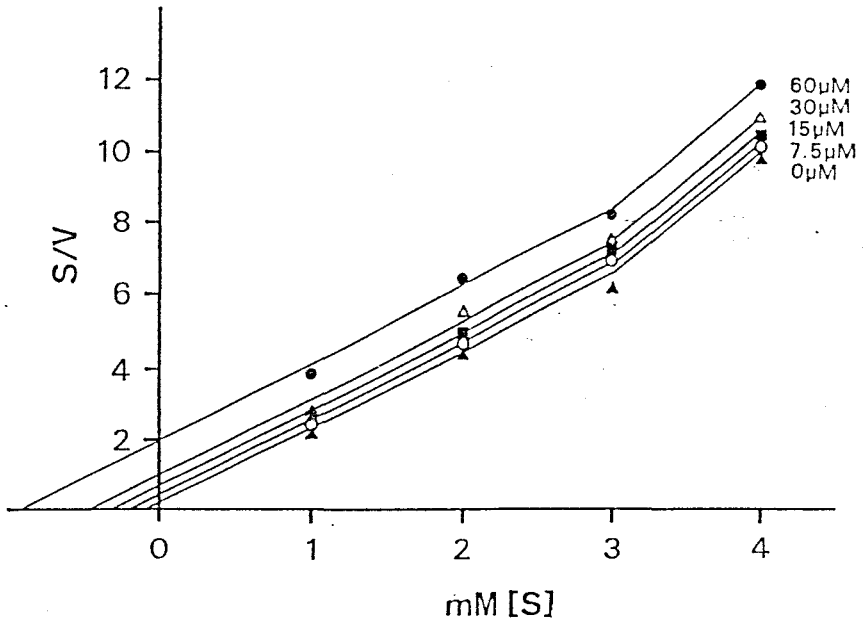


FIG. 42: Hanes-Woolf plot showing substrate inhibition of rat liver tryptophan-2,3-dioxygenase at high tryptophan (S) levels in the prescence of varied melatonin concentrations (0 - 60μM). V = velocity in μM N'-formylkynurenine/minute/mg protein.

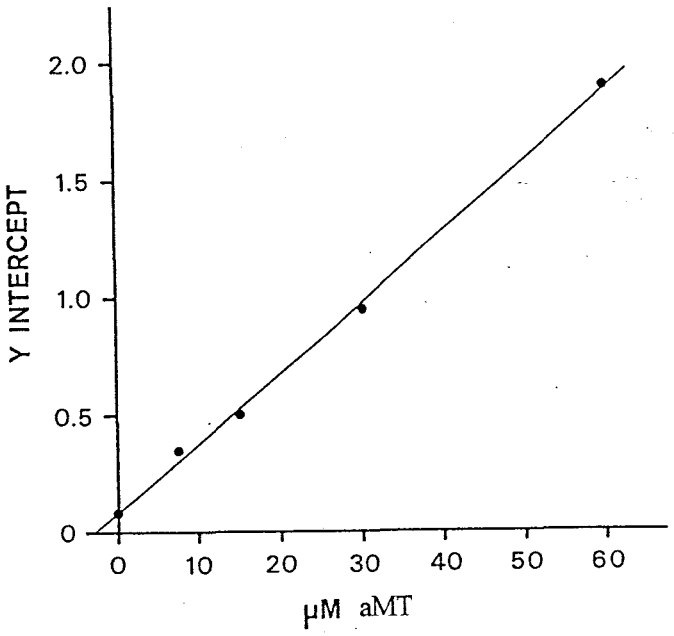


FIG. 43: A Dixon plot (Dixon, 1953) of the effect of various concentrations of aMT (melatonin) on rat liver TDO (tryptophan-2,3-dioxygenase) activity to determine the K_i ($r = 0.997$).

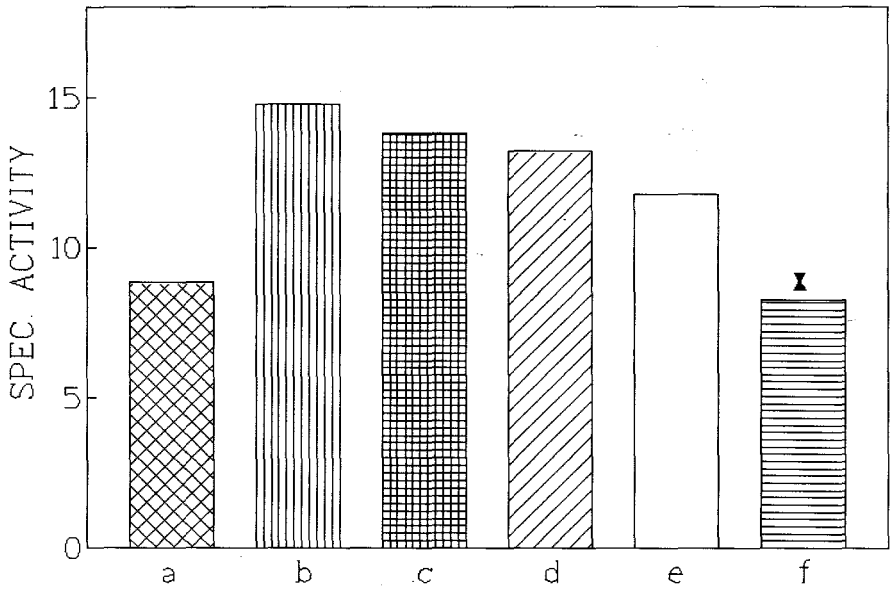


FIG. 44 Effect of substrate TRP (1mM) on the degree of inhibition of TDO by aMT at varying concentrations (note inhibition at 60μM ie substrate dependence of inhibition). Key: a = extant holoenzyme activity; b = total enzyme activity; c = b+aMT (7.5μM); d = b+aMT (15μM); e = b+aMT (30μM); f = b+aMT (60μM). ♦= Basal level of activity affected

EXPERIMENT 4

2.3 REVERSIBILITY OF TDO INHIBITION BY MELATONIN, THE NATURE OF 5-HT AND NAD^+ INHIBITION AND A COMPARISON OF THE RELATIVE POTENCIES OF MELATONIN AND SEROTONIN ON TDO (*in vitro*).

2.3.0 Introduction

Inhibition of TDO by 5-HT is both non-competitive and reversible (app. 4.1) (Frieden *et al.*, 1961). The initial studies on the relationship between TDO and 5-HT were performed with a crude extract; however, subsequent investigations using a purified form of TDO revealed that 5-HT is a weak inhibitor in the millimolar range (Uchida *et al.*, 1992). aMT, on the other hand, has been shown to be quite a potent competitive inhibitor of TDO (Walsh *et al.*, 1994), and this inhibition should therefore theoretically also be reversible in the presence of excess substrate.

It was on the basis of these observations that it was decided to investigate whether the inhibitory effects of the neurohormone on TDO are reversible and to what extent the neurotransmitter 5-HT inhibits the enzyme.

aMT is hailed as a possible therapeutic agent in the treatment of certain affective disorders such as SAD and depression. The two indoleamines also have opposing circadian rhythms, and it was therefore of interest to note the combined effect of these biogenic amines on TDO.

NAD^+ is one of the end products of TRP catabolism (fig.1) and a key metabolic intermediate with respect to nucleotide synthesis and adenosine triphosphate (ATP) production. It was thus also decided to examine the possibility of interactions between TDO and NAD^+.

2.3.1 Materials and methods

Purification of the enzyme and the experimental procedure were performed as outlined in Experiment 1. To determine whether the inhibition of TDO by aMT is reversible, both TRP and negative effector aMT concentrations were kept constant while TDO levels were increased. The results were plotted as specific activity of TDO against volume of enzyme (fig. 45).

2.3.2 Results

Reversibility of the aMT inhibition is depicted in the graph of figure 45 (compare with app. 4.1). The non-competitive nature of the inhibition by 5-HT and the dose dependence are clear, in the light of the graph

obtained in figure 46. However, this phenomenon of non-competitive inhibition only manifests itself at relatively low 5-HT concentrations. At higher levels, the inhibition becomes mixed, also being competitive, as can be seen from the Lineweaver-Burk plot (Lineweaver and Burk, 1934) of the same data (fig. 47).

It is apparent from figure 48 that aMT is a more potent inhibitor compared to 5-HT at equivalent concentrations (6μM). The combined treatment, however, reveals that 5-HT neutralises the effects of aMT (fig. 48). NAD^+, on the other hand, appears to be an allosteric effector of TDO, inhibiting the enzyme at relatively high concentrations (6mM) and stimulating it at low levels (3mM) (fig. 49).

2.3.3 Discussion

5-HT seemingly has a greater affinity for the non-competitive binding site. It is apparent from the Lineweaver-Burk plot that, at relatively low 5-HT concentrations, there is non-competitive binding. This is evidenced by the fact that V_{max} changes with no change in K_m (100μM). At higher levels of 5-HT, a mixed type of binding takes place, given that both the y- and the x-intercepts shift. This implies competitive and non-competitive inhibition. Thus at 6- and 12μM, 5-HT first binds to the non-competitive high affinity site. With subsequent elevations in inhibitor levels (24μM), binding also occurs at an additional site, which is in all likelihood the catalytic TRP binding site.

The combination of 5-HT and aMT (fig. 48) clearly shows that 5-HT eliminates the effects of aMT on the enzyme, apparently by inducing a conformational change which probably precludes the binding of aMT at the active site. The fact that the degree of inhibition is significantly greater for the individual neurohormone at the same concentration (6μM) could indicate that the influence of 5-HT involves a downregulation of TDO activity, as opposed to a total inhibition. This reduction in the catalytic rate, in contrast to an inhibition of catalysis via displacement of substrate TRP from and subsequent occupation of the active site (as is the case with aMT), appears to be a form of ultramodulation of enzyme activity. The type of inhibition brought about by aMT, on the other hand, effectively reduces the concentration of active enzyme at any one time, and appears to be more decisive.

It is apparent that, at high levels of NAD^+ (6mM) *in vitro*, the nucleotide acts as an inhibitor via the mechanism of feedback inhibition, but at relatively low (3mM) concentrations, the compound acts as an allosteric activator of the enzyme. This phenomenon could be related to the link between energy requirements within the cell and oxidative enzyme activity. Whether this event manifests itself *in vivo* in the rat still remains to be explored.

According to Frieden *et al.*, (1961), 5-HT apparently prevents conjugation between cofactor hemin and the apoenzyme. However the results obtained in this study seem to indicate that the neurotransmitter binds at the allosteric site initially, at low concentrations, where it induces dissociation of the respective subunits, as is the case with the known allosteric effector NAD(P)H.

2.3.4 Conclusion

The inhibition of TDO by aMT, as is the case with 5-HT, is reversible in the micromolar range. 5-HT behaves as an allosteric inhibitor of the enzyme *in vitro* in the same manner as NAD(P)H. At low concentration the inhibition by 5-HT is non competitive, becoming mixed at higher levels. In combination with aMT, the inhibitory effect of the neurohormone on TDO is nullified. Individually, however, aMT has a much higher potency at equimolar levels. The coenzyme NAD^+ is also an allosteric inhibitor of TDO, but in the millimolar range.

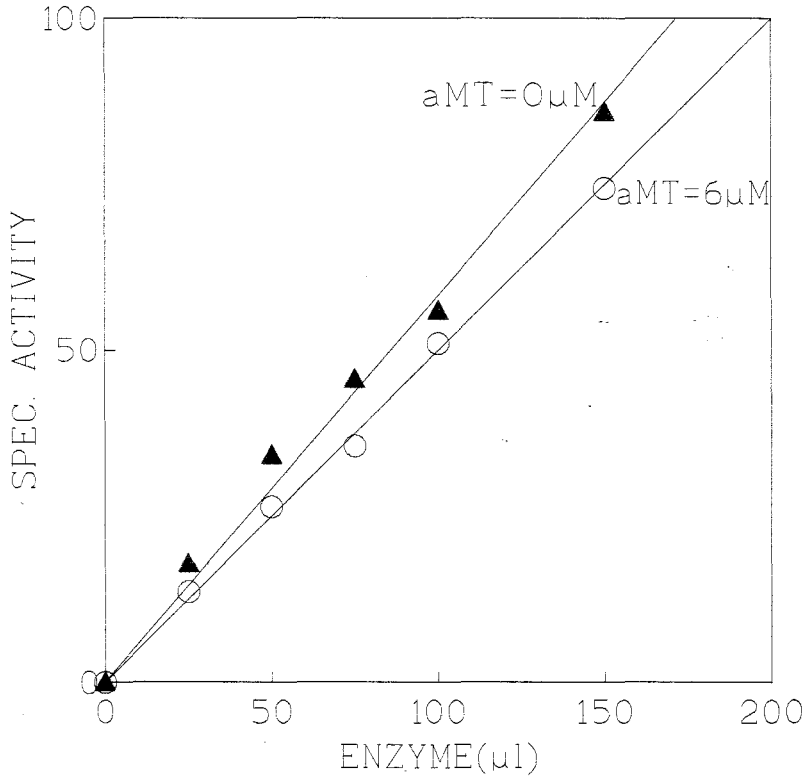


FIG. 45: Reversibility of melatonin (aMT) inhibition of purified tryptophan-2,3-dioxygenase at 1mM tryptophan, pH 7 and at 30°C (r = 0.99). At aMT = 0; r = 0.96, for aMT = 6μM; r = 0.98. The specific activity is expressed as N'-formylkynurenine/mg protein/min at 321nm.

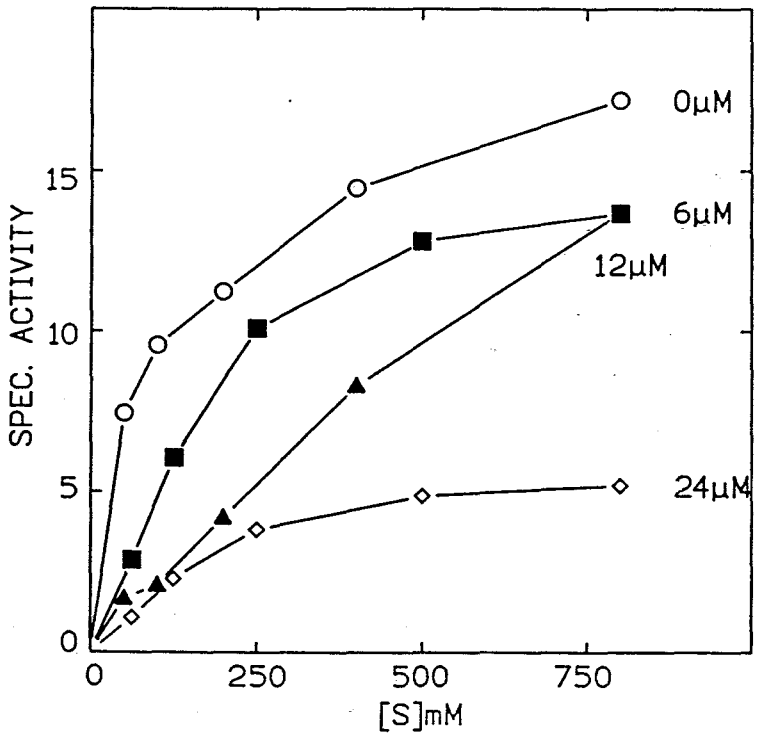


FIG. 46: Effects of different serotonin concentrations (0 - 24μM) on purified rat liver tryptophan-2,3-dioxygenase activity. [S] = TRP concentration and specific activity is expressed as μM N'-formylkynurenine /mg protein/minute at 321nm.

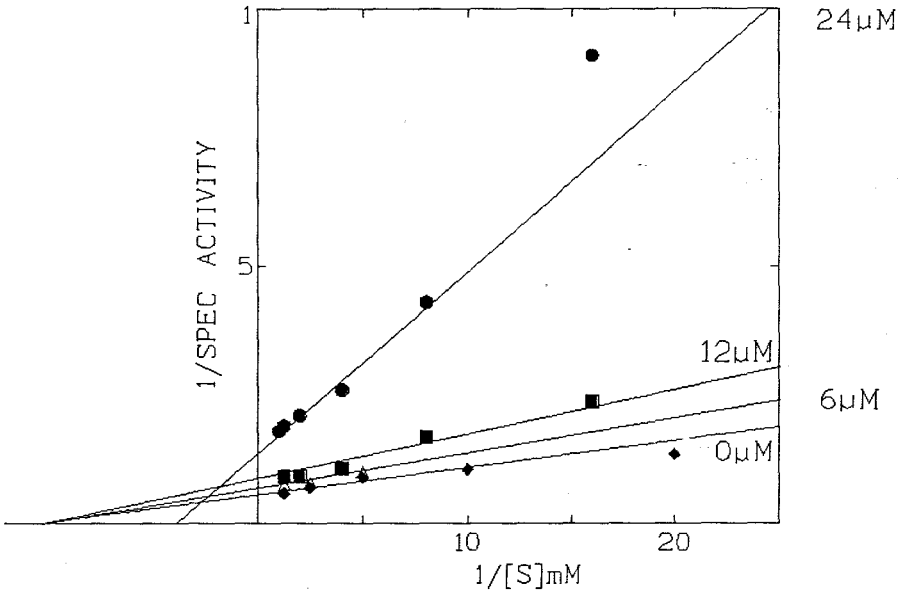


FIG. 47: Graph of inhibition of tryptophan-2,3-dioxygenase by serotonin (5-HT) at different concentrations (0 -24μM) (note the shift in the inhibition pattern from non-competitive at 6 & 12 μM 5-HT to mixed at 24 μM). [S] = TRP concentration.

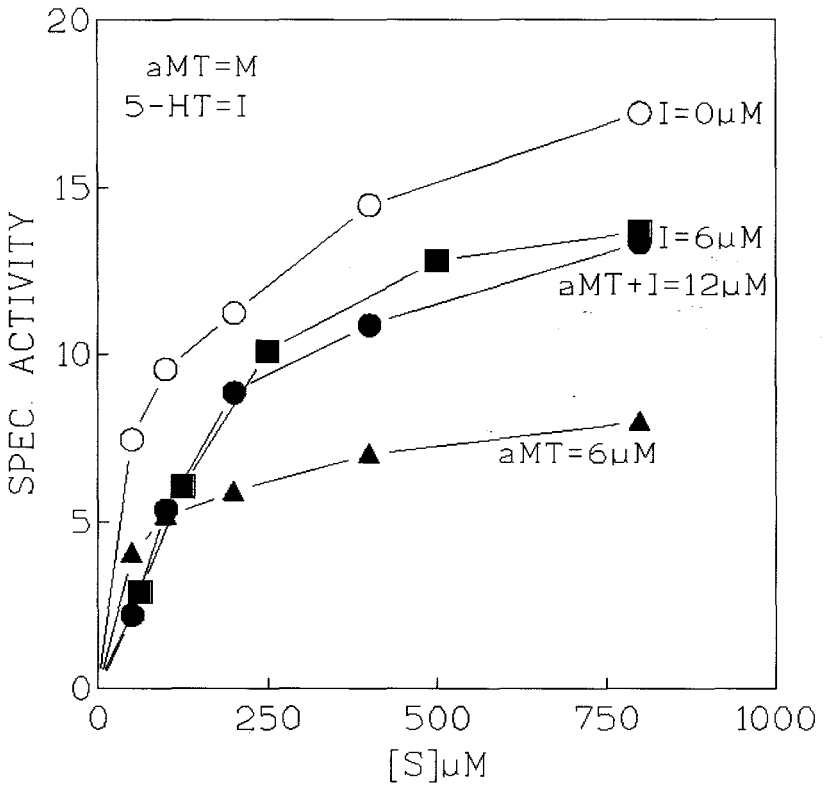


FIG. 48: Inhibition of TDO by aMT (6μM), 5-HT(6μM) and a combination of both (6μM + 6μM). Note the superior potency of aMT independent of 5-HT but also observe the effects of the combined treatment where 5-HT eliminates the influence of aMT. [S] = TRP concentration.

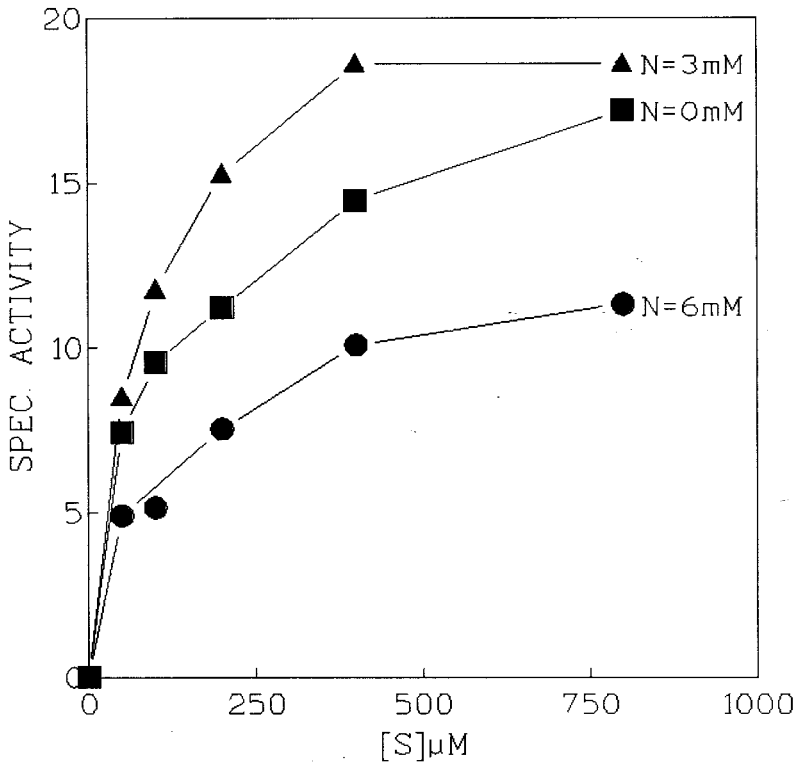
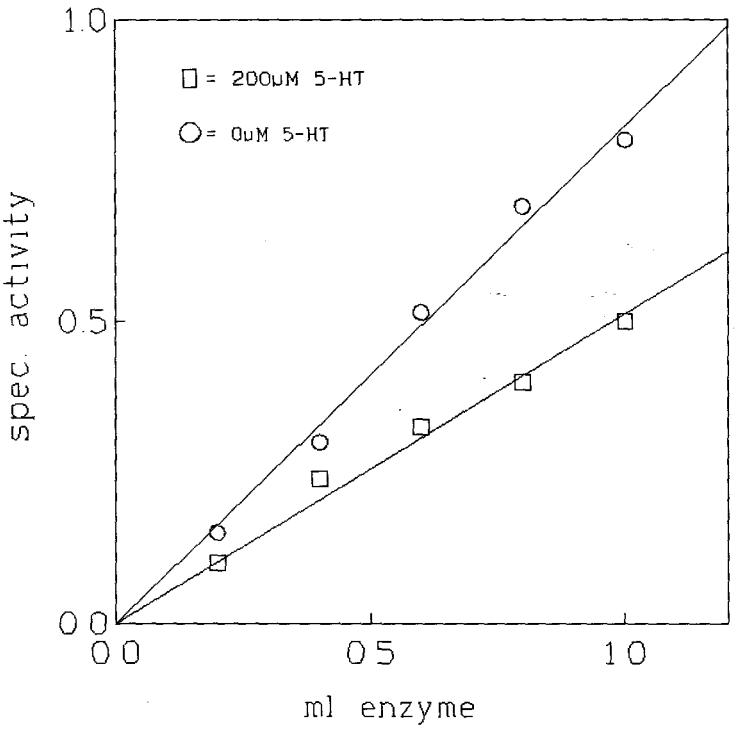


FIG. 49: Effects of NAD^+(mM) on rat hepatic TDO activity *in vitro*. [S] = TRP concentration.



APPENDIX 4.1: Reversibility of serotonin (5-HT) inhibition of tryptophan-2,3-dioxygenase (5% liver homogenate in 3ml reaction volume). Velocity expressed as μM kynurenine/mg protein/minute at 365nm (spec. activity) is plotted against volume of crude enzyme (modified from Frieden et al., 1961).

EXPERIMENT 5

2.4 EFFECTS OF ACUTE 5-ALA ADMINISTRATION ON RAT TDO ACTIVITY AND FOREBRAIN 5-HT METABOLISM

2.4.0 Introduction

The heme precursor 5-ALA and hemin induce saturation of hepatic TDO (Daya *et al.*, 1989). Activation of this enzyme results in the enhanced catabolic conversion of TRP to N'-formylkynurenine during the first and rate limiting step of the kynurenine-nicotinic acid pathway of TRP degradation (Badaway, 1979) (fig. 1).

To investigate the relationship between TDO and forebrain 5-HT requires a direct determination of brain 5-HT levels. Recently it has been shown that the acute administration of 5-ALA to rats reduces the cerebral concentrations of both 5-HT and TRP due to reduced availability of circulating TRP for uptake by the brain brought about by activation of liver TDO (Badaway *et al.*, 1987). These findings were confirmed (Daya *et al.*, 1989,) but it was also shown that if the 5-ALA treated animals were killed at night, in the presence or absence of light, they had significantly higher forebrain levels of TRP and 5-HT (Daya *et al.*, 1989; Daya *et al.*, 1990).

In an attempt to explain this rise in forebrain indoles, it was decided to study the effect of longer-term administration of 5-ALA to rats. Firstly, it was investigated whether the rise in forebrain indoles would persist in animals treated repeatedly with 5-ALA. Secondly, the state of activity of liver TDO was also determined in the same animals by measuring both the activity of the apoenzyme as well as the holoenzyme (Badaway and Evans, 1975).

2.4.1 Materials and methods

Ten male Wistar rats weighing 200-250g obtained from the South African Institute for Medical Research were maintained in a light:dark cycle of 12:12 (lights on from 07:00-19:00 h). Food and water were provided *ad libitum.* The animals were divided into two groups of five animals each. The first group served as the controls and were injected subcutaneously with saline (1 ml kg^{-1}) at 10:00h and 20:00h for two consecutive days, followed by a further injection at 10:00h on the third day. The second group received 5-ALA (15 mg kg^{-1}) in the same order.

Two hours after the last injection, the animals were killed by cervical dislocation and forebrain tissue and livers were collected and frozen immediately on solid CO_2 and stored at -70°C until assayed. The

forebrain consisted of a piece of tissue taken from the frontal pole of one cortical hemisphere and extending 5-10 mm caudally.

Forebrain 5-HT was measured according to the flourimetric method described by Maickel (1981) (fig. 50). TDO activity was determined according to the method described by Badaway and Evans (1975), as explained in Experiment 2 and the activities of both the holoenzyme and apoenzyme were determined. Protein concentration was determined by the method of Lowry *et al* (1951).

2.4.1.0 Fluorometry

This method was developed by Maickel (1981). Both 5-ALA treated and untreated forebrains weighing between 200 - 300mg were homogenised using a dounce homogeniser in 4ml of cold acidified n-butanol. The resultant suspension was then centrifuged at 2000 rpm x 5 minutes to sediment protein. To 2.5ml of the butanol phase were added 5ml n-heptane plus 0.4ml of a 0.1N HCL in a boiling tube. The tubes were stoppered and shaken on a mechanical shaker for 5 minutes, and then centrifuged at 2000 rpm x 5minutes. The two phases obtained, upper organic and lower aqueous, then separated. Fractions of 0.2ml of the aqueous phase containing the 5-HT were used for the o-phthaldehyde (OPT) reaction.

2.4.1.0.1 OPT reaction

This quantitative method of 5-HT determination is non-specific and thus requires isolation of the substance of interest from tissues such as the forebrain. Four different separation techniques are reported in the literature: both column and paper chromatography, acidified acetone, and liquid - liquid extraction. The use of column chromatography for the separation of 5-HT was not practical, as excessive dilution with consequent loss of sensitivity occurs. In the case of paper chromatography the procedure is too lengthy and tedious, while the end results of the acidified acetone extraction have proven to be inconsistent and unsatisfactory. The method of liquid - liquid extraction was selected because of the high degree of reproducibility (small standard deviation; less than 10 percent of the mean values for twenty animals) (Maickel *et al.*, 1975).

Preparation of the OPT solution required taking between 4 - 10mg of the substance and dissolving it in 100mls of 10N HCL (OPT has a very poor solubility in H_2O). Volumes of 0.6ml were added to the fractions containing 5-HT, and thoroughly mixed (for the blank, 0.1ml distilled H_2O replaced the 5-HT) in a boiling H_2O bath for 15 minutes, and subsequently cooled with tap H_2O for 10 minutes. The mixture was then tranferred to a cuvette, and the fluorescence measured at an excitation wavelength of 360mμ and with an emission filter of 470mμ.

Here the high energy is associated with the shorter wavelength. When a molecule absorbs energy in the form of radiation, its energy level is increased. Some of this absorbed energy is converted to vibrational energy while the remainder is emitted as light of a lower energy (longer wavelength) than that which is absorbed. In the system employed, linearity was achieved for concentrations of 5-HT between 0.01 - 2.5μg. Recoveries were prepared by adding 0.1ml of a 10μg/ml solution of 5-HT to 3mls of acidified butanol, and the procedure already described followed. The percentage recovery for 5-HT was 97, and the blank consisted of 0.1ml OPT + 10.1ml distilled H_2O.

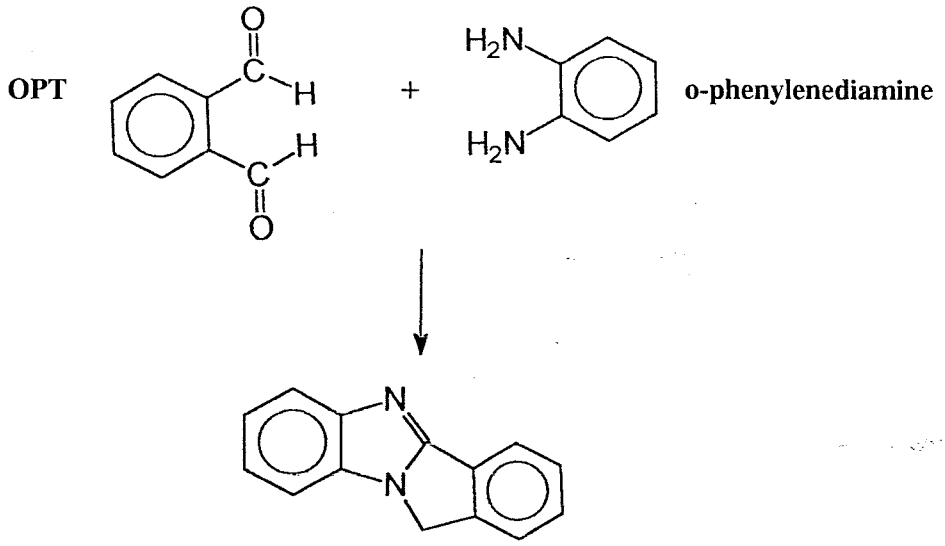


FIG. 50: An example of the proposed OPT reaction involving an ionizable amino group (Amos and Gillis, 1964).

Results are expressed as means $\pm$SEM. A student *t*-test was used to determine statistical significance. A *p* value of < 0.05 was regarded as being statistically significant.

2.4.2 Results

As shown in figure 51, the administration of 5-ALA induced a rise in forebrain 5-HT levels ($p < 0.001$). Figure 52 shows the saturation of TDO with heme, determined by measuring the ratio of holoenzyme:apoenzyme (Badaway *et al.*, 1987). 5-ALA administration induced a rise in saturation of the enzyme. As shown in figure 53, the holoenzyme activity was significantly elevated ($p < 0.05$) in livers of 5-ALA treated rats.

2.4.3 Discussion

Acute administration of 5-ALA to rats increases liver TDO activity (Badaway *et al.*, 1987) and reduces brain TRP and 5-HT (Daya *et al.*, 1990; Uchida *et al.*, 1992). However, if the rats are killed several hours after acute treatment with 5-ALA, the levels of these indoles rise markedly (Daya *et al.*, 1990). In the present study, chronic administration of 5-ALA induced a similar rise in forebrain 5-HT. This rise was, however, accompanied by a commensurate rise in saturation of the apoenzyme as well as in the activity of the holoenzyme of TDO.

Ordinarily, an inverse correlation could have been expected between liver TDO activity and brain indoleamine levels (Daya *et al.*, 1990). If TDO is the major peripheral determinant of the availability of TRP to the brain (Badaway *et al.*, 1981), and consequently 5-HT synthesis in the brain, a high level of enzyme activity should theoretically be accompanied by low levels of brain 5-HT. The reasons for high levels of brain 5-HT accompanied by increased activity of TDO in the present study are not clear. One reason could be that the high affinity TRP uptake system in the brain could be activated, since this system can compensate to a certain extent for fluctuations in extracellular TRP concentrations (Loizou and Redfern, 1988).

Alternatively, it is possible to speculate that a large percentage of this displaced TRP could escape its catabolic fate in the liver (Salter *et al.*, 1989). A high level of free TRP resulting from this mechanism could account for greater uptake of this indole into the brain and its subsequent conversion to 5-HT (Wurtman and Fernstrom, 1975). Increases in the levels of TRP in the blood are known to result in increased synthesis of 5-HT in the brain (Badaway *et al.*, 1987; Wurtman and Fernstrom, 1975).

2.4.4 Conclusion

The precursor of the iron-porphyrin cofactor hemin, 5-ALA, stimulates the activity of TDO *in vivo,* but paradoxically increases rat forebrain 5-HT levels.

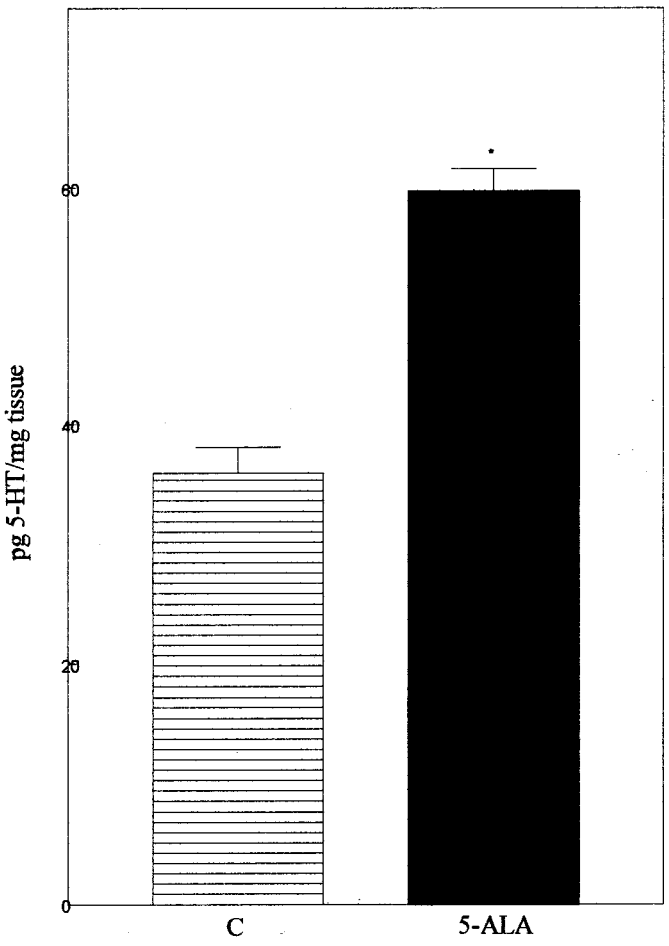


FIG. 51: Effect of 5-ALA (5-amino levulinic acid) on forebrain serotonin (5-HT) levels (means ± SEM; n=5). As compared with control (C). * $p < 0.01$.

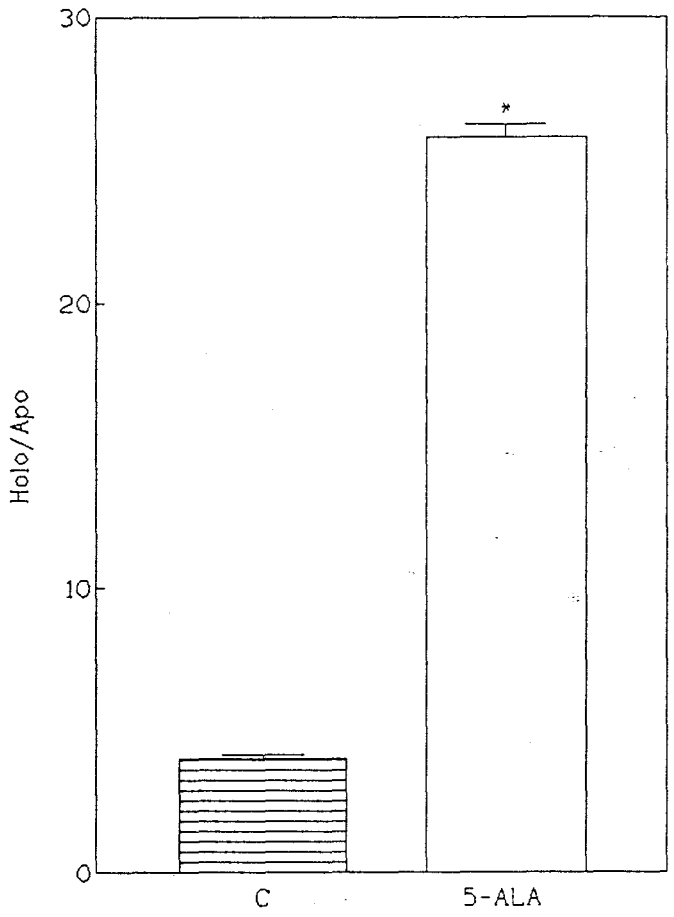


FIG. 52: Effect of 5-ALA (5-amino levulinic acid) on the saturation of liver TDO (tryptophan-2,3-dioxygenase) as determined by the ratio of holoenzyme: apoenzyme (means ±SEM; n=5). As compared with control (C) * $p < 0.01$.

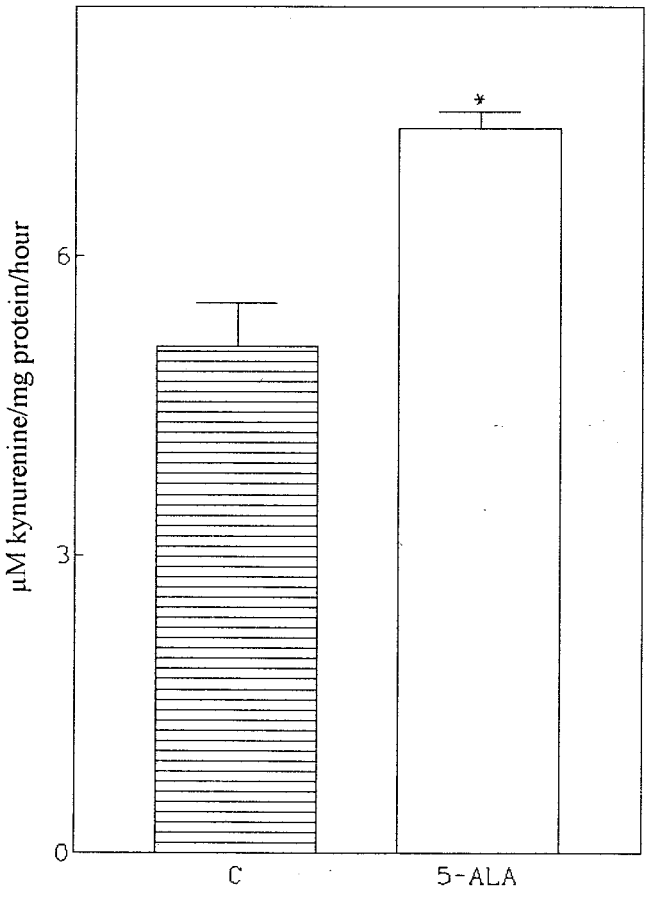
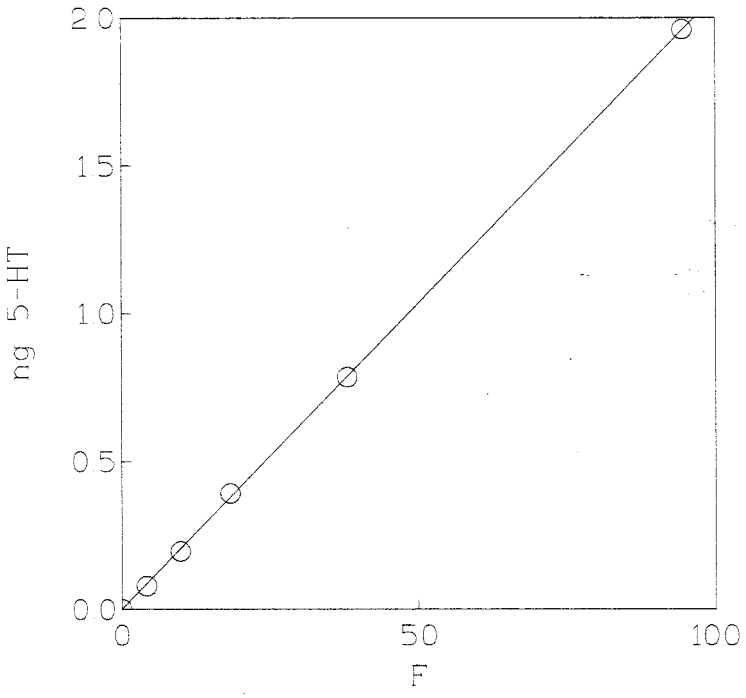


FIG. 53: Effect of 5-ALA (5-amino levulinic acid) on the activity of the holoenzyme of TDO (tryptophan-2,3-dioxygenase) (means ± SEM; n=5). As compared with control (C), * $p < 0.05$.



APPENDIX 5.1: serotonin (5-HT) fluorescence (F) standard curve (r = .998)

CHAPTER 3

aMT, DMI, FLUOXETINE, TDO AND THE PINEAL

EXPERIMENT 6

3.0 THE EFFECTS OF CHRONIC ADMINISTRATION OF DESIPRAMINE, FLUOXETINE AND MELATONIN ON TDO ACTIVITY AND PINEAL INDOLEAMINE METABOLISM

3.0.0 Introduction

aMT, given its implication in stress, affective disorders (such as depressed mood, loss of interest, hypersomnia, fatigue and weight gain or loss), as well as in ageing, has generated a lot of interest in psychiatric circles (Daya, 1994). One of the reasons for this is that there could be a link between aMT and mood, given that oral aMT results in a worsening of depression (Wurtman and Wurtman, 1988). Experimental evidence indicates that during major depression a reduction in the peak of the daily biological aMT rhythm occurs. The ratio of blood cortisol to aMT in depressives differs from those in healthy subjects and, in bipolar depression, mania is associated with elevated levels of aMT (Lewy *et al.*, 1989). Major depression, on the other hand, is characterised by altered diurnal rhythms in body temperature and REM sleep (Wehr *et al.*, 1979). However these findings are inconclusive, and thus far experimental data linking low aMT levels to depression are inconsistent, and the dysregulation hypothesis is not regarded as important in the etiology and treatment of depression (Waldhauser *et al.*, 1993). This theory cannot, however, be discarded as it has relevance with respect to certain subtypes of depressives.

The secretion of aMT is enhanced by TADs and MAOIs. In depression there is an apparent reduced aMT secretion, and the frequency of this affective disorder is more pronounced in spring and autumn when aMT levels are lowered. Rat pineal and plasma aMT are significantly elevated with antidepressants that have a different pre-synaptic effect such as maprotilene and CI (Nyback *et al.*, 1975). The mode of action of clinically efficacious antidepressants, such as MAOIs and TADs, is characterised by a restoration of normal plasma aMT concentrations at approximately three weeks. This time frame coincides with the period necessary for antidepressants to exert their therapeutic effects, and implicates aMT in the remission of the depressed state.

Experimentally, aMT has been shown to behave as a MAOI (Urry and Ellis, 1975) and to increase brain levels of 5-HT (Anton *et al.*, 1968) and NA (Wendel *et al.*, 1974) in the rat. The aim of this experiment was to determine whether aMT potentiates the effects of chronic antidepressant (DMI and fluoxetine)

treatment on TDO and also on pineal indoleamine metabolism.

3.0.1 Materials and methods

25 Male Wistar rats, weighing between 200 and 250 g, were used in this study, and divided into 5 groups. These animals were subjected to an 8 day treatment in the following manner:

i) control - 0.9% NaCl in absolute ethanol,

ii) DMI (2.5mg/kg/day),

iii) DMI (2.5mg/kg/day) + aMT (0.25mg/kg/day),

iv) fluoxetine (2.5mg/kg/day),

v) fluoxetine(2.5mg/kg/day) + aMT (0.25mg/kg/day).

All treatments lasted 8 days except for the combination of group (v) which lasted 3 days only, due to the fact that these animals (group v) became acutely anaemic and started perishing. The excised livers were immediately stored in liquid N_2 until assayed while the pineals from groups (I), (ii) and (iv) were organ cultured immediately after removal. On the eighth day the last dose was administered 2 hours before sacrifice. The time of the daily dosage was 10.30 am.

3.0.1.0 Pineal organ culture

Rat pineal glands, obtained from the animals, were immediately placed into sterile Kimble glass tubes, one per tube, containing 52μl of BGJ culture medium supplemented with 8μl of radiolabelled [^{14}C] 5-HT (0.4μCi), in an atmosphere of carbogen (95%/5%, O_2/CO_2), sealed with parafilm and incubated in the absence of light at 37°C for 24 hours. Subsequent to incubation the pineals were removed and the culture medium assayed for pineal indoles using two dimensional thin layer chromatography (TLC). To prevent oxidation of the indoleamines, care was taken to limit exposure to O_2 and light. The tubes containing the culture medium were then stored at -20°C until further use. This particular technique was developed by Klein and Notides (1969) and improved by Daya and Fata (1986).

3.0.1.1 Thin layer chromatography (TLC) for analysis of radiolabelled indoleamines

This method is essentially that engineered by Klein and Notides (1969) with minor adaptations, and encompasses the following steps to allow identification of the various metabolites of the radiolabelled 5-HT:

a) 10 x10 cm of TLC plates (from Merck, West Germany) were spotted with 10μl aliquots of the culture medium and evaporated with a gentle stream of nitrogen.

b) Spots of "cold" indoleamine standards (10μl) were superimposed on a) and subjected to the same treatment.

c) The prepared TLC plates were subsequently developed twice in the same direction in a solvent mixture containing chloroform, methanol and acetate in a ratio of 97:3:1, until the solvent front reached a distance of 9cm up the TLC plate. The plates were then evaporated to dryness with N_2 after each run.

d) The final development was prepared in a solvent of ethyl acetate at right angles to the direction of the first, and the solvent front allowed to migrate 6cm up the alumina plates. The plates were removed and the solvent was evaporated in the same manner as before.

e) To facilitate visualisation of the compounds the spots were sprayed with Van Urcks reagent (1% 4-dimethylaminobenzaldehyde solution in 12.5% HCl and 50% ethanol). Development of the spots was achieved by placing the plates in an oven at 60°C for 5 minutes.

f) The spots were excised individually and placed into vials containing 3ml scintillation cocktail (from Beckman, USA) plus 1ml absolute ethanol. The vials were then agitated for 20 minutes and counted in a Beckman LS 2500 scintillation counter with a counting efficiency of 96% for the ^{14}C-window.

The preparation of the standards involved dissolving 4μg of each indole in a minimal amount of 100% ethanol containing a 1%(w/v) vitamin C, to limit oxidation, and kept in the dark at 4°C.

3.0.1.2 TDO activity

Refer to Experiment 3 for the method.

3.0.2 Results

3.0.2.0 Organ cultures

Three sets of pineals, controls, DMI and fluoxetine, were incubated to investigate the effects of both DMI and fluoxetine on pineal indoleamine biosynthesis. All results are given with respect to the values obtained for the controls.

Table 1: Effects of antidepressants DMI and fluoxetine (FLU) on the metabolism of various pineal indoleamines by pineal organ culture. Values are expressed as percentage increase (↑) or decrease (↓) compared to control (saline treated animals).

	NAS	aMT	5-THOH	5-HIAA	5-MIAA	5-MTOH
DMI	↑ (600)	(0)	↓ (50)	(0)	↓ (1000)	↓ (350)
FLU	↓ (30)	↓ (70)	↓ (90)	(0)	↑ (1000)	↑ (350)

3.0.2.1 TDO Activity

TDO activity appears to be affected by the tricyclic DMI and the SSRI fluoxetine to a similar extent, although in different ways (figs. 64-71). DMI affects the ratio of apo- to holoenzyme in the cell by reducing the extant holoenzyme concentrations and increasing the apoenzyme levels (figs. 64, 65). Fluoxetine, on the other hand, apparently reduces the extant apoenzyme levels that are available for conjugation with hemin in a manner which involves raising the levels of active holoenzyme in the cell (fig. 66, 67). In the presence of aMT, these effects are abolished, as can be seen from figures 68-71. Both antidepressants, however, reduce the total concentration of TDO in the hepatocytes (fig. 72).

3.0.3 Discussion

The effects of DMI and fluoxetine on pineal indole metabolism appear to be dissimilar, particularly with respect to aMT metabolism. This points to widely differing modes of action. aMT synthesis is apparently unaffected by DMI, but is inhibited by fluoxetine (figs. 56, 57). That DMI has an influence on noradrenergic neurotransmission is clear given the manifested elevations in NAS levels which demonstrate that DMI potentiates NA effects in the synapse, given the fact that NAS synthesis is dependent on stimulation of the noradrenergic system. The TAD also seems to channel 5-HT towards aMT synthesis, as indicated by the reduction in the concentrations of the methoxyindoles (fig. 58). The hydroxyindole pathway of 5-HT catabolism has a reduced turnover rate, indicated by the decrease in the levels of 5-HIAA (figs. 62, 63). This probably occurs due to the fact that most of the 5-HT is channelled towards aMT synthesis.

The results obtained in this experiment regarding DMI's influences on pineal indole metabolism are thus consistent with what is known about the mode of DMI action on the noradrenergic system - specifically, β-adrenergic receptor stimulation coupled with NA reuptake inhibition.

Fluoxetine, on the other hand, seems to have a negative effect on aMT synthesis, judging from the observation that aMT synthesis is apparently reduced by 70% (fig. 57) coupled with an enhanced 5-HT catabolic rate probably stemming from elevated 5-HT levels, and in the light of the observed increased 5-methoxyindoles concentrations (3.5-fold) (fig. 59). In addition, there is a reduction of about 30% in NAS levels (fig. 55) in the presence of fluoxetine. However, fluoxetine is also known to downregulate β-adrenergic receptors, and this could in part account for the results obtained. That fluoxetine has an effect on the enzymes involved in the conversion of 5-HT → aMT, and 5-HT → 5-methoxyindoles is clear, but the site of specific action of the antidepressant requires clarification. The low concentrations of 5-HTOH (fig. 61) further support the contention that fluoxetine affects 5-HT metabolism.

Given the experimentally determined ratio of apo- to holoenzyme within the cell for DMI treated rats, it can be observed that the relative apoenzyme levels are very high. There is thus an apparent increase of 155% in the catalytic rate of the enzyme in the presence of hemin. This observation is consistent with the proposed role of DMI, which asserts that it acts to prevent conjugation between apoenzyme and the iron-porphyrin (ix). However, an important additional effect is the reduction in total enzyme levels of approximately 58% (fig. 65). Fluoxetine, on the other hand, has no significant effect on the activity of the catalyst upon heme supplementation, which indicates a higher degree of heme saturation coupled with significantly lower apoenzyme levels (fig. 67). The SSRI apparently reduces total enzyme concentration by about 48%. These results seem to indicate that the antidepressants interact with the genome, affecting enzyme protein synthesis, since there is a reduction in total enzyme levels (fig. 72).

aMT counteracts the effects of both antidepressants when administered in combination (figs. 68 - 71), and this could be brought about via prevention of binding to the enzyme and/or binding to the genome by the antidepressants. It is possible that the phenomenon of chemical antagonism is manifesting itself between the individual substances aMT, the apoenzyme and either DMI or fluoxetine.

3.0.4 Conclusion

DMI and fluoxetine both significantly reduced total TDO concentrations. In addition, the two antidepressants also affected the available apoenzyme concentrations in the hepatocyte, DMI causing an increase and fluoxetine a reduction. In the pineal, DMI stimulated NAS synthesis, and fluoxetine inhibited aMT production coupled with enhancement of 5-HT turnover.

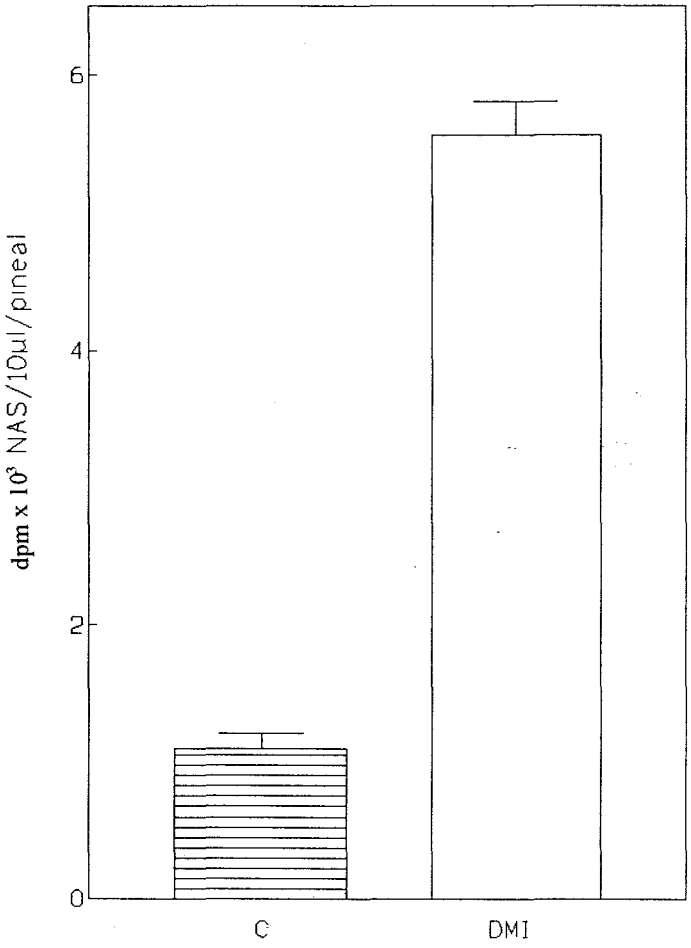


FIG. 54: The effects of intraperitoneally administered desipramine (DMI) on N-acetylserotonin (NAS) biosynthesis in rat pineal organ culture (n=4, $p < 0.05$, ±SD). Control = C.

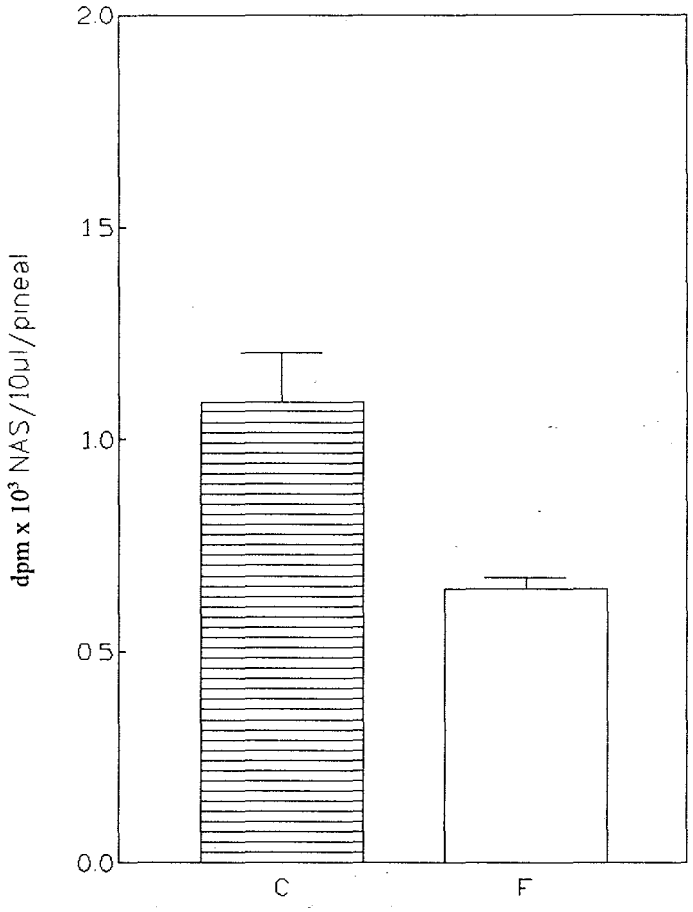


FIG. 55: The effects of intraperitoneally administered fluoxetine (F) on N-acetylserotonin (NAS) biosynthesis in rat pineal organ culture (n=4, $p < 0.05$, ±SD). Control = C.

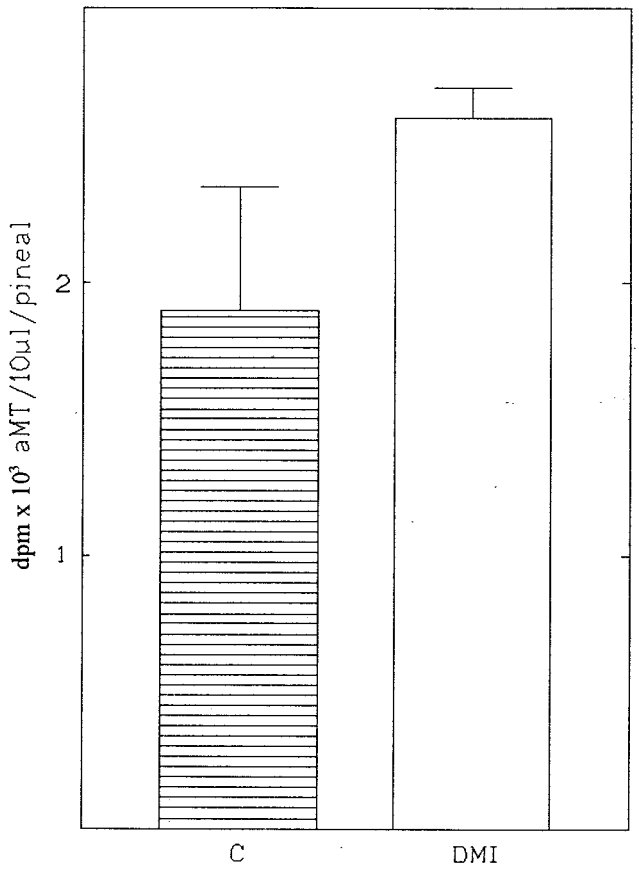


FIG. 56: The effects of intraperitoneally administered desipramine (DMI) on melatonin (aMT) biosynthesis in rat pineal organ culture (n=4, $p > 0.05$ (ns) $\pm$SD). Control = C.

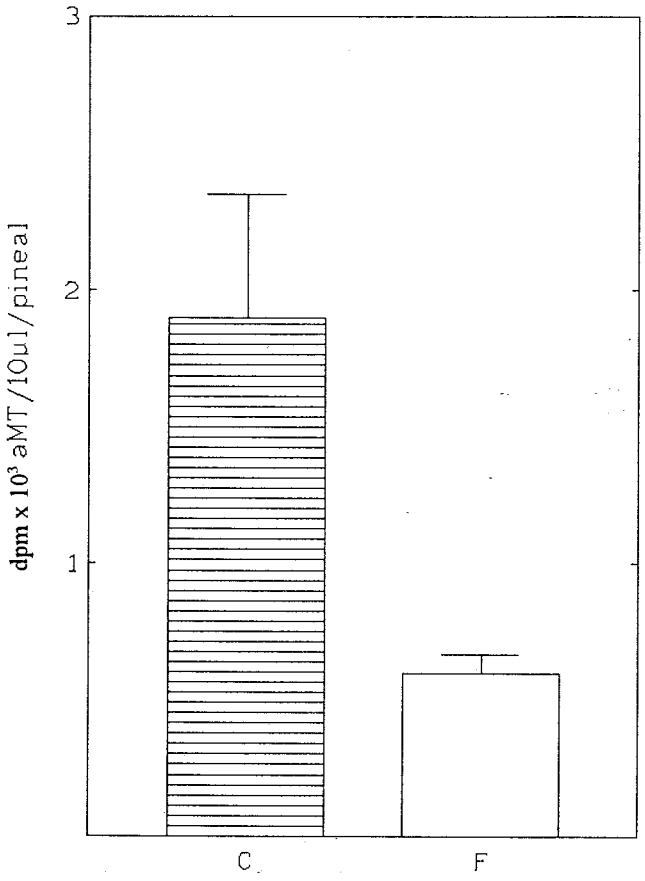


FIG. 57: The effects of intraperitoneally administered fluoxetine (F) on melatonin (aMT) biosynthesis in rat pineal organ culture (n=4, $p < 0.05$, ±SD). Control = C.

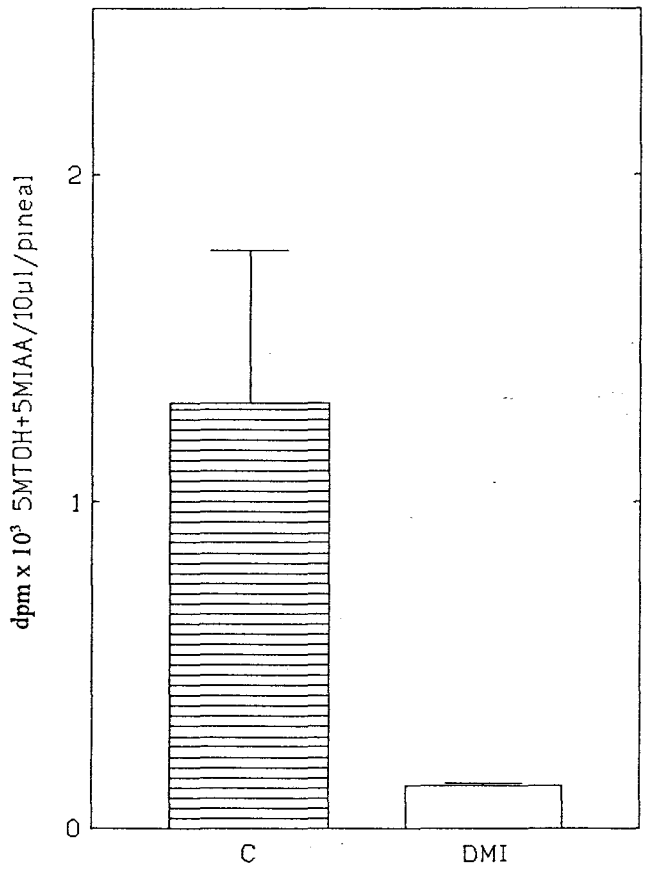


FIG. 58: The effects of intraperitoneally administered desipramine (DMI) on 5-methoxytryptophol and 5-methoxyindole acetic acid (5-MTOH + 5-MIAA) biosynthesis in rat pineal organ culture (n=4, $p < 0.05$, $\pm$SD). Control = C.

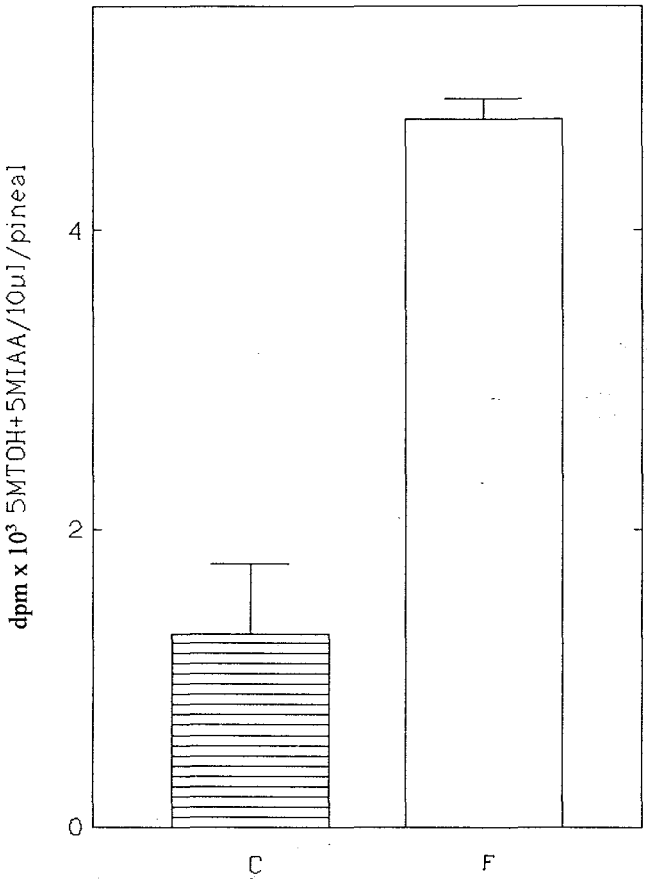


FIG. 59: The effects of intraperitoneally administered fluoxetine (F) on 5-methoxytryptophol and 5-methoxyindole acetic acid (5-MTOH + 5-MIAA) biosynthesis in rat pineal organ culture (n=4, $p < 0.001$, ±SD). Control = C.

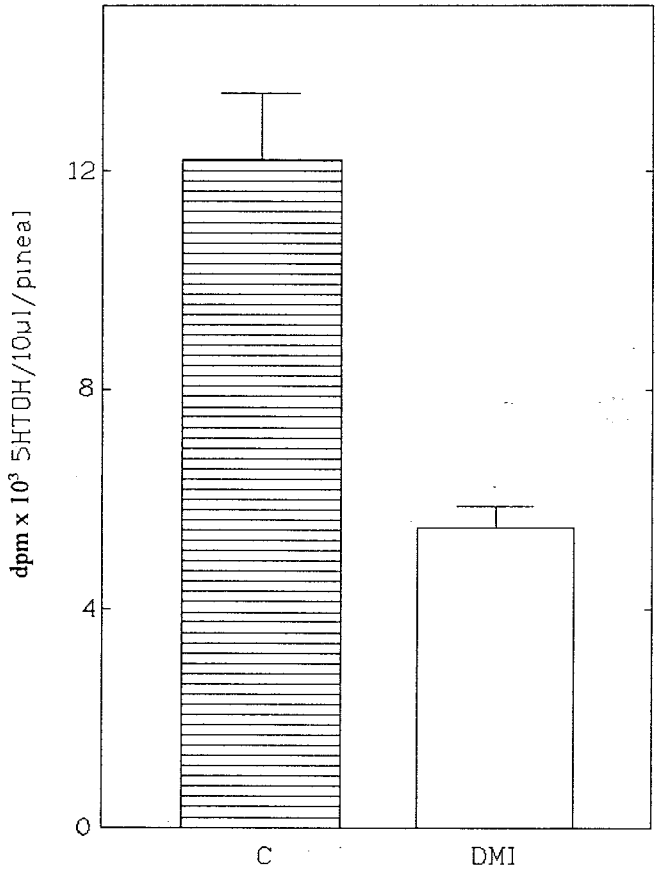


FIG. 60: The effects of intraperitoneally administered desipramine (DMI) on 5-hydroxytryptophol (5-HTOH) biosynthesis in rat pineal organ culture (n=4, $p < 0.05$, ±SD). Control = C.

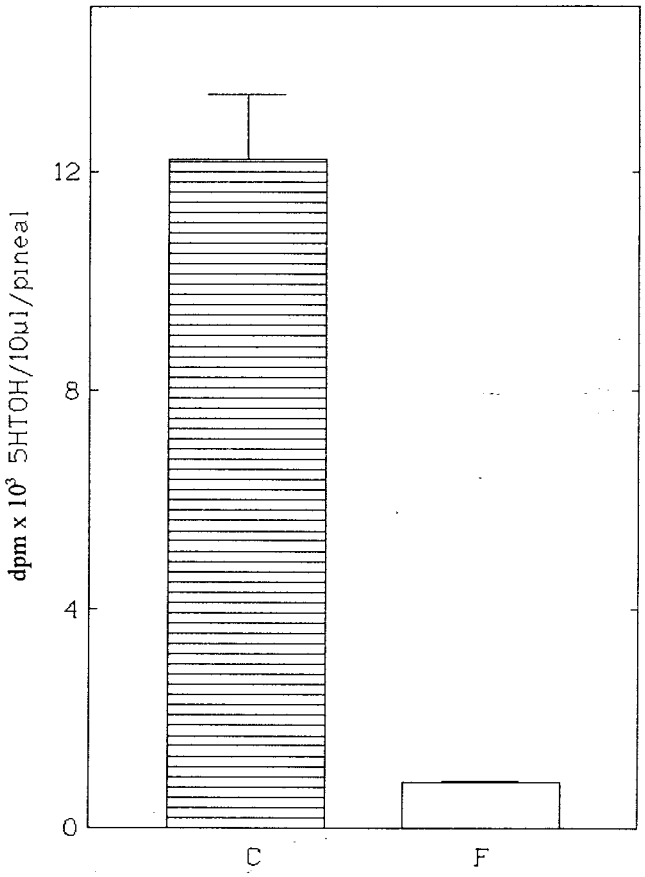


FIG. 61: The effects of intraperitoneally administered fluoxetine (F) on 5-hydroxytryptophol (5-HTOH) biosynthesis in rat pineal organ culture (n=4, $p < 0.001$, ±SD). Control = C.

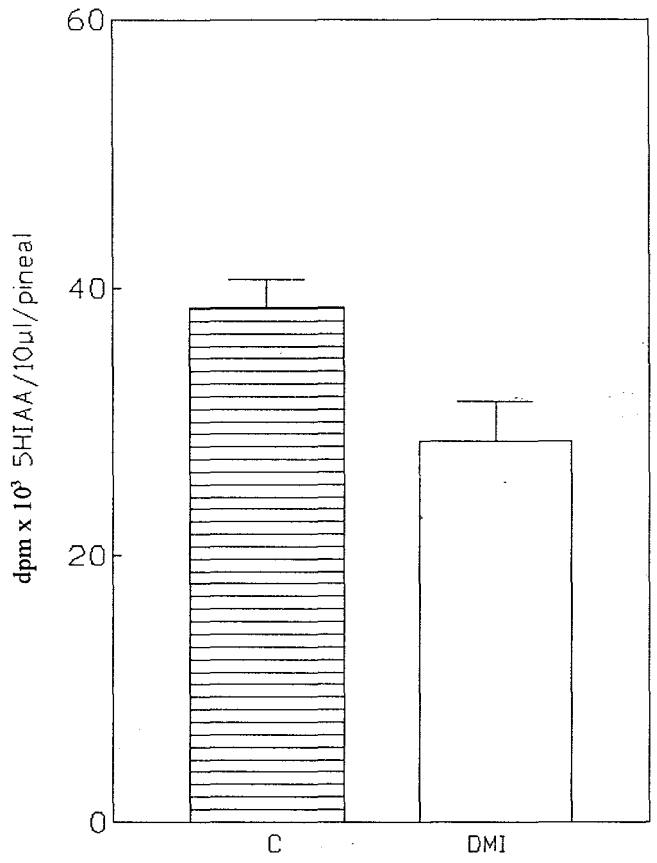


FIG. 62: The effects of intraperitoneally administered desipramine (DMI) on 5-hydroxyindole acetic acid (5-HIAA) biosynthesis in rat pineal organ culture (n=4, $p > 0.05$ (ns), ±SD). Control = C.

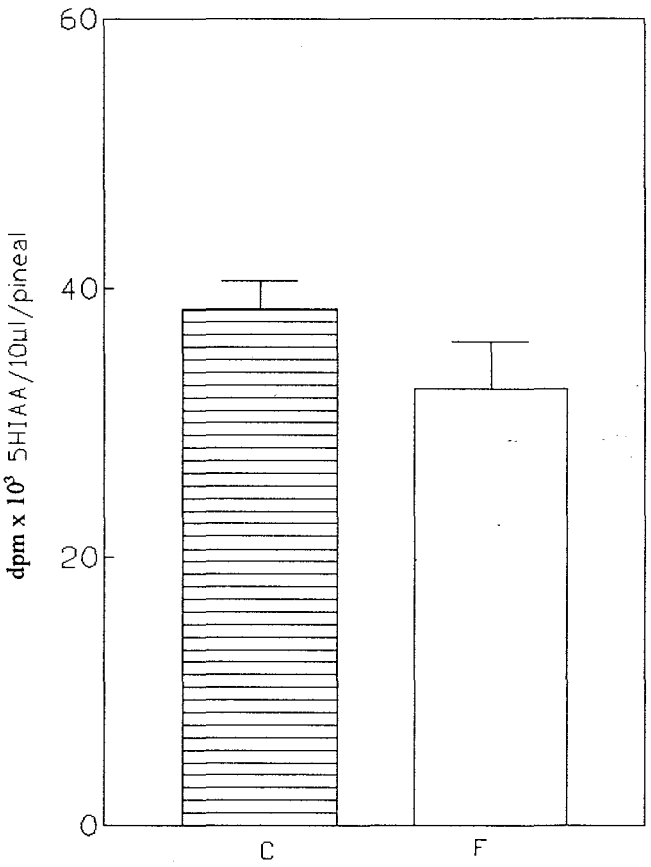


FIG. 63: The effects of intraperitoneally administered fluoxetine (F) on 5-hydroxyindole acetic acid (5-HIAA) biosynthesis in rat pineal organ culture (n=4, $p > 0.05$ (ns), $\pm$SD). Control = C.

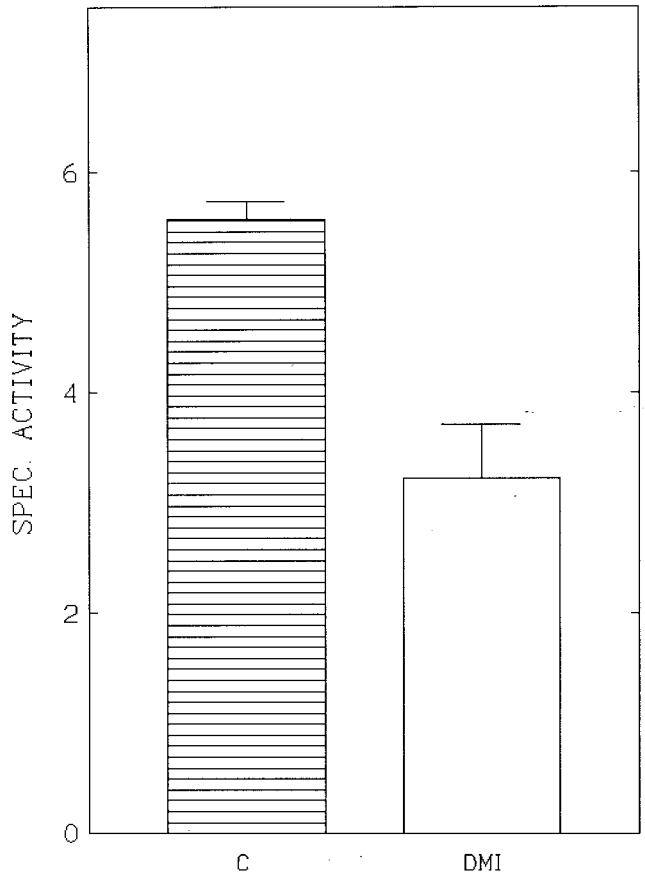


FIG. 64: The effects of intraperitoneally administered desipramine (DMI) on crude tryptophan-2,3-dioxygenase extant holoenzyme activity in rat liver (n=4,$p < 0.05$, ±SD). Control = C.

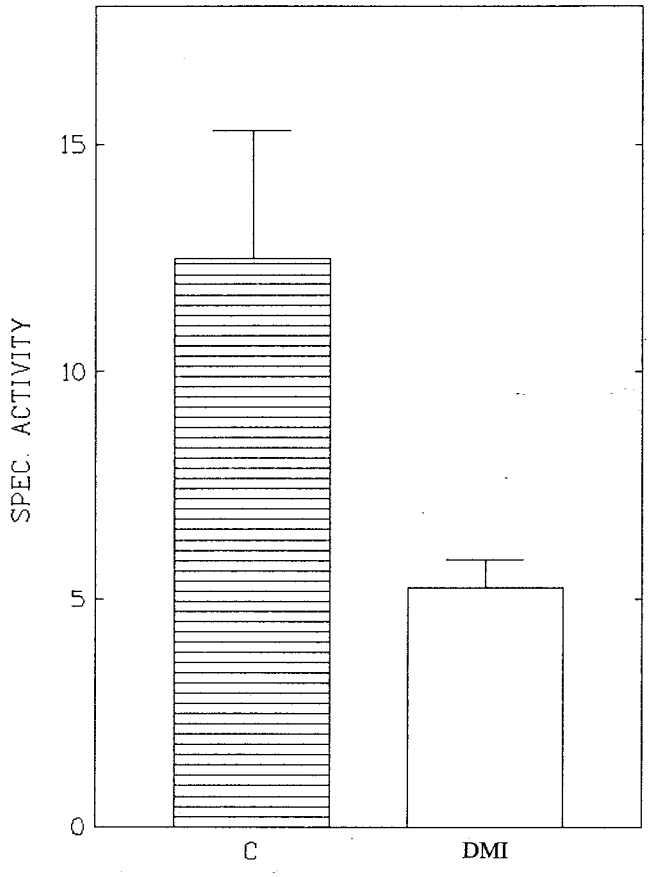


FIG. 65: The effects of intraperitoneally administered desipramine (DMI) on crude TDO (tryptophan-2,3-dioxygenase) total enzyme activity in rat liver (n=4, $p < 0.05$, $\pm$SD). Control = C. Velocity in μM N'-formylkynurenine/minute/mg protein.

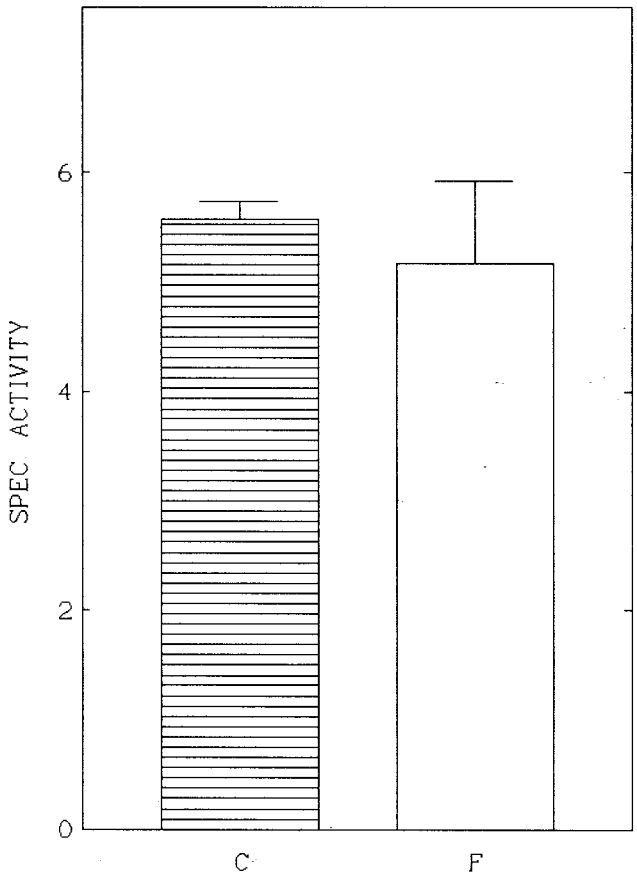


FIG. 66: The effects of intraperitoneally administered fluoxetine (F) on crude tryptophan-2,3-dioxygenase extant holoenzyme activity in rat liver (n=4, $p > 0.05$ (ns), ±SD). Control = C.

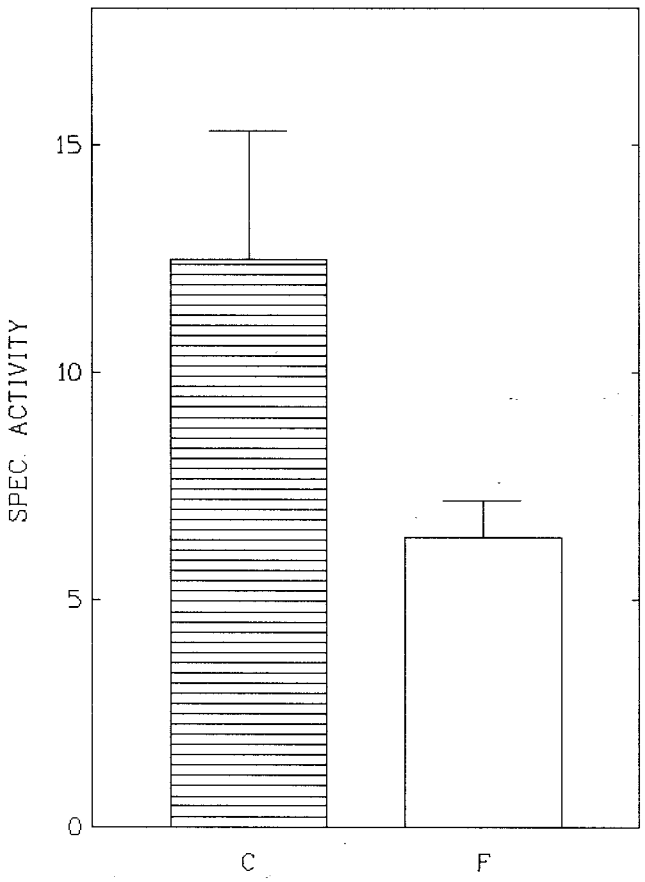


FIG. 67: The effects of intraperitoneally administered fluoxetine (F) on crude tryptophan-2,3-dioxygenase total enzyme activity in rat liver (n=4, $p < 0.05$, ±SD). C = Control.

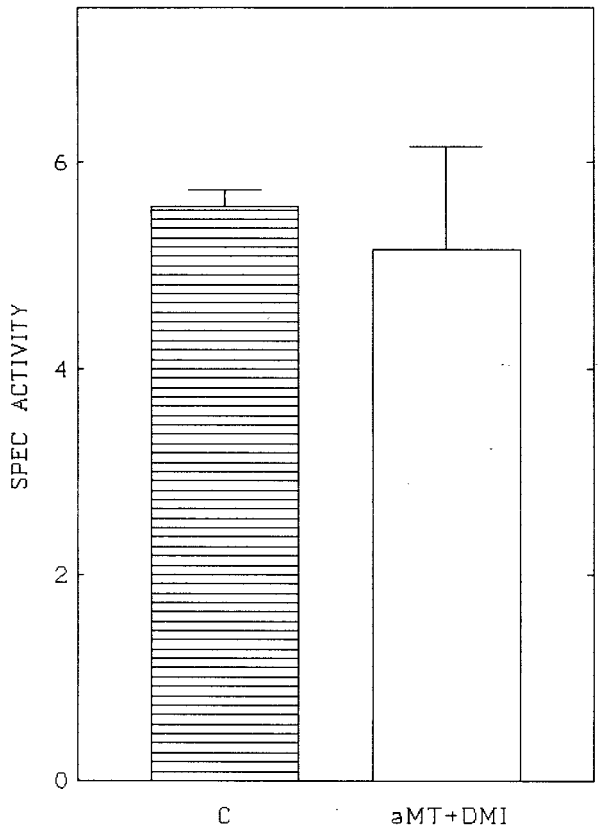


FIG. 68: The effects of intraperitoneally administered desipramine (DMI) +melatonin (aMT) on crude tryptophan-2,3-dioxygenase extant holoenzyme activity in rat liver (n = 4, $p > 0.05$ (ns), ±SD), C = Control.

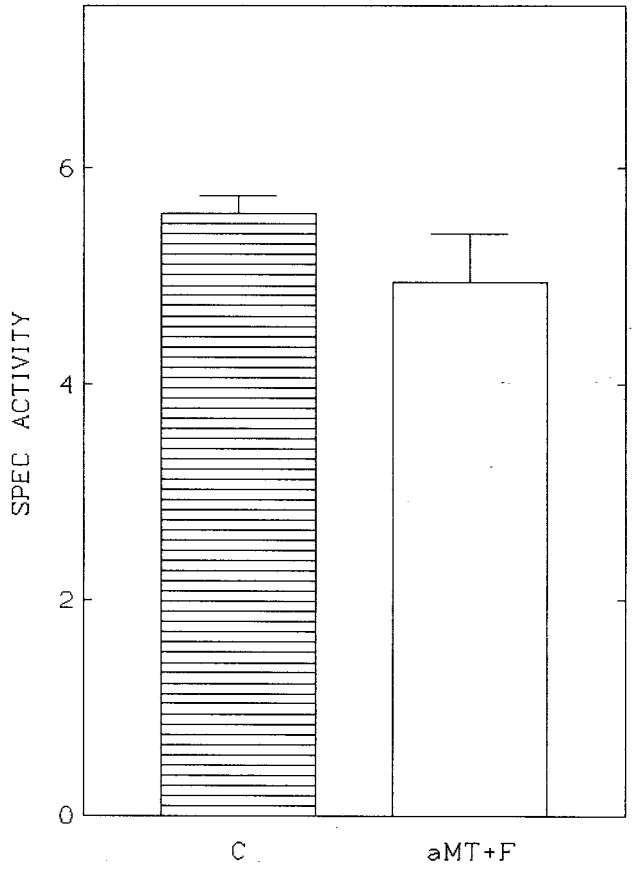


FIG. 69: The *in vivo* effects of intraperitoneally administered melatonin (aMT) + fluoxetine (F) on crude tryptophan-2,3-dioxygenase extant holoenzyme activity in rat liver (n=4, $p > 0.05$ (ns), ±SD). C = Control.

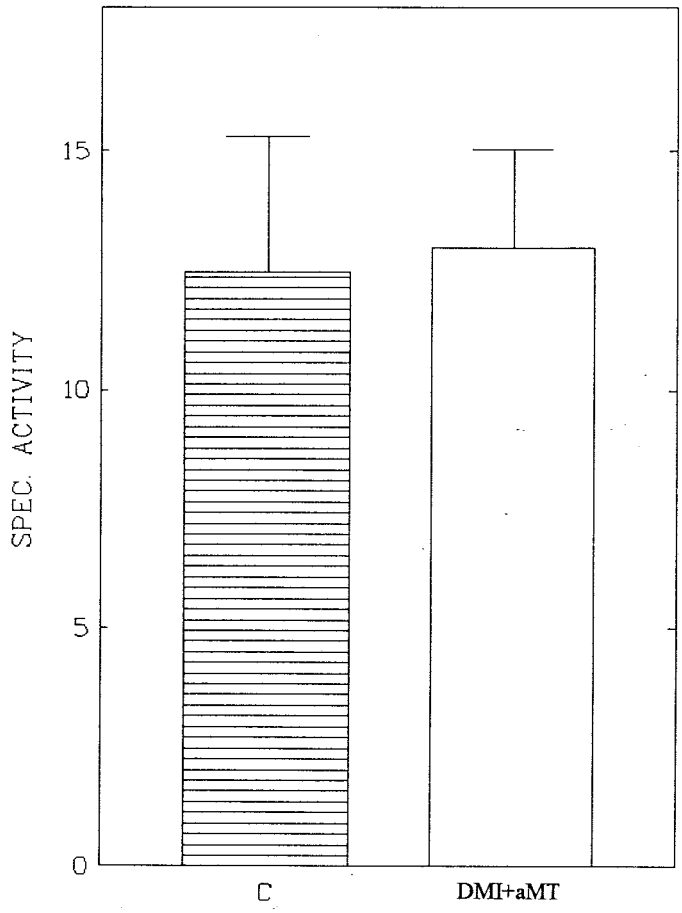


FIG. 70: The effects of intraperitoneally administered desipramine (DMI) + melatonin (aMT) on crude TDO (tryptophan-2,3-dioxygenase) total enzyme activity in rat liver (n = 4, $p > 0.05$ (ns), ±SD). C = Control.

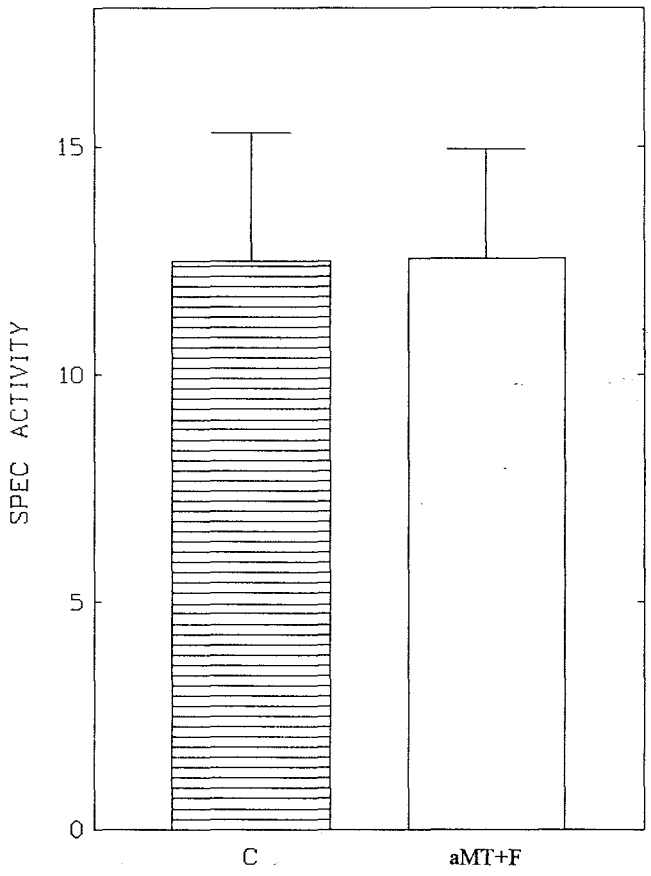


FIG. 71: The *in vivo* effects of intraperitoneally administered fluoxetine (F) + melatonin (aMT) on crude TDO (tryptophan-2,3-dioxygenase) total enzyme activity in rat liver (n = 4, $p > 0.05$ (ns), ±SD), C = Control.

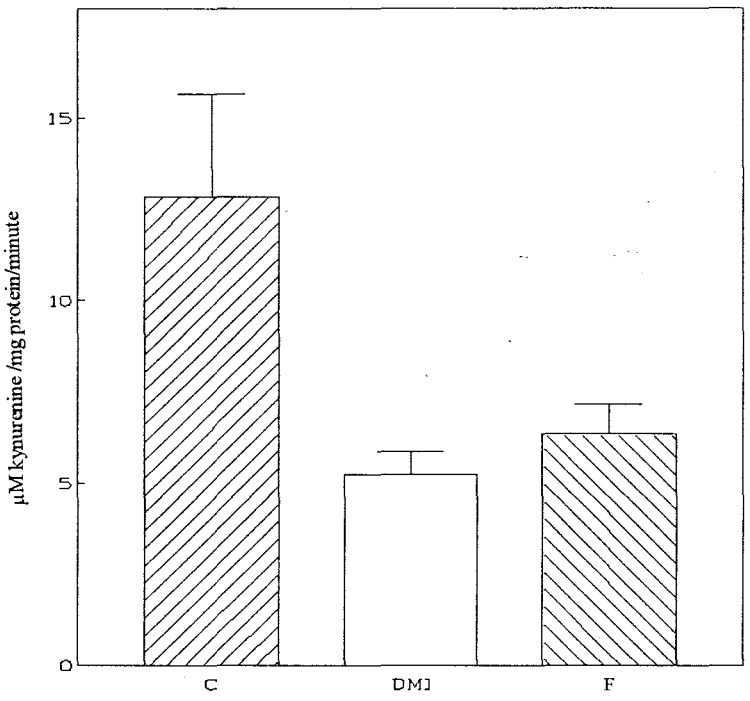
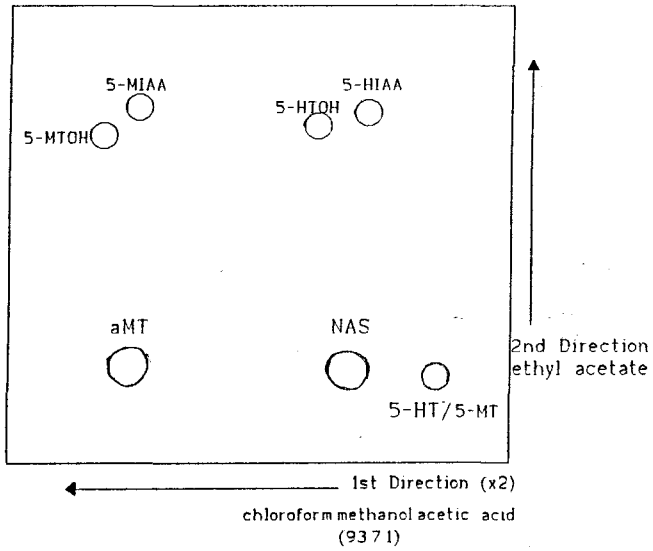


FIG. 72: Effects of desipramine (DMI) (n = 4) and fluoxetine (F) (n = 3) on total crude tryptophan-2,3-dioxygenase activity (for control (C) n = 5). There is a statistically significant difference ($p < 0.05$) between DMI and C and also between F and C but no significant difference between the two antidepressants. Velocity = μM kynurenine/mg protein/minute at 365nm.



KEY: NAS= N-acetylserotonin — aMT=melatonin

5-MT=5-Methoxytryptamine — 5-HT=Serotonin

5-HTOH=5-Hydroxytryptophol — 5-HIAA=5-Hydroxyindole acetic acid

5-MTOH=5-Methoxytryptophol — 5-MIAA=5-Methoxyindole acetic acid

APPENDIX 6.1: A typical bi-directional pineal indole thin layer chromatogram (modification of Klein &Notides, 1969).

CHAPTER 4

INSULIN AND PINEAL INDOLE METABOLISM

EXPERIMENT 7

4.0 EFFECTS OF INSULIN ON PINEAL INDOLE METABOLISM IN PINEAL ORGAN CULTURE

4.0.0 Introduction

As mentioned in Chapter 1, a carbohydrate rich diet has the effect of elevating serum TRP, brain TRP and brain 5-HT levels (Sulser *et al.*, 1978), but has no effect on pineal TRP and indoleamines. The effects of the carbohydrates are known to be related to the release of insulin (Wurtman, 1982). However, it is not clear what the direct effects of insulin on the pineal gland are, given the fact that the pineal gland is located outside the blood brain barrier. It was thus decided to examine the effects of insulin on pineal indole metabolism in organ culture, and to investigate whether insulin inhibited NAT and consequently aMT synthesis. Regulation of aMT biosynthesis is (as stated previously) believed to be at the rate limiting enzyme NAT. However, the manner in which this occurs is still controversial. One of the ways in which inactivation of NAT could occur is proposed to be via a disulfide-containing compound. This idea is based on the following observation: in broken pineal cell preparations, the hormone insulin inhibited the activity of NAT via the oxidation of the hydrogen sulfide group on the enzyme (Naamboodiri *et al.*, 1981):

ENZYME----S--H + X----S---S----X ⇌ ENZYME----S---S----X + X----S--H

(active) (inactive)

4.0.1 Materials and methods

The organ culture protocol that was followed was outlined in Experiment 7. 1 IU of insulin, Actraphane HM (ge), supplied by Novo Nordisk (Pty) Ltd. South Africa, was added to the incubation mixture to give a constant total volume of 52μl.

4.0.2 Results

As can be seen from table 2 and the constructed histograms, the hormone insulin only affected pineal aMT metabolism. There was a significant reduction in aMT levels (fig. 77) by approximately 42%, while NAS levels (fig. 75) were unaffected, as was the concentration of 5-HIAA (fig. 73). The 5-MTOH (fig. 74) and 5-MIAA (fig. 76) in addition to 5-HTOH (fig. 78) concentrations also revealed no significant changes when compared to the controls.

Table 2: Results of the effects of insulin on pineal gland indole metabolism in organ culture.

Indole	aMT	5-HIAA	5-HTOH	5-MIAA	5-MTOH
% reduction	42 ($p<0.05$)	ns ($p>0.05$)	ns ($p>0.05$)	ns ($p>0.05$)	ns ($p>0.05$)

4.0.3 Discussion

The results obtained (table 2) (figs. 73-77) clearly show that insulin has a significant effect on pineal gland aMT metabolism, and that the overall effect is a significant reduction in aMT synthesis (fig. 77). It could therefore be inferred that insulin has a negative effect on the anabolism of aMT. Judging from these results, it is evident that insulin has affinity sites on the pineal gland which are linked to inhibitory intracellular factors that affect aMT synthesis.

The manner in which insulin brings about this effect is not clear, given that entry of insulin into the pinealocytes poses a major problem considering the size of the polypeptide. For the proposed hypothesis to be applicable, traversion of the cell membrane would be a prerequisite. However, it is speculated that insulin could cause a redox shift through an alteration in the redox potential of the cell membrane which is normally required for noradrenergic manipulation of NAT activity (Naamboodiri *et al.*, 1981). Alternatively, the hormone could have selective receptors on the pinealocyte that are perhaps linked to inhibitory G-proteins or voltage operated calcium (Ca^{2+}) channels. At present these assumptions are all rather speculative and require further investigation. However, the rise in serum and brain TRP and ultimately brain 5-HT following increases in circulating plasma insulin levels (Goldstein and Betz, 1983) could not only be due to the tissue uptake of the competing LNAAs caused by insulin. It could also be a result of the insulin-induced raised aMT plasma titres, which would have the effect of displacing albumin bound TRP, thereby increasing plasma free TRP and hence brain TRP, with a consequent elevation of forebrain 5-HT levels. The results obtained in this study differ from those obtained by Champney *et al* (1985), where insulin-induced stress caused an increase in NAT activity and aMT biosynthesis in the rat. However, these authors also found that, under the same conditions, insulin caused a marked reduction in aMT levels in the hamster.

4.0.4 Conclusion

The known inhibitor of pineal NAT enzyme *in vitro*, insulin, had a significant effect on pineal aMT metabolism in organ culture. The pancreatic hormone was shown to inhibit aMT synthesis markedly, an effect which is possible *in vivo*.

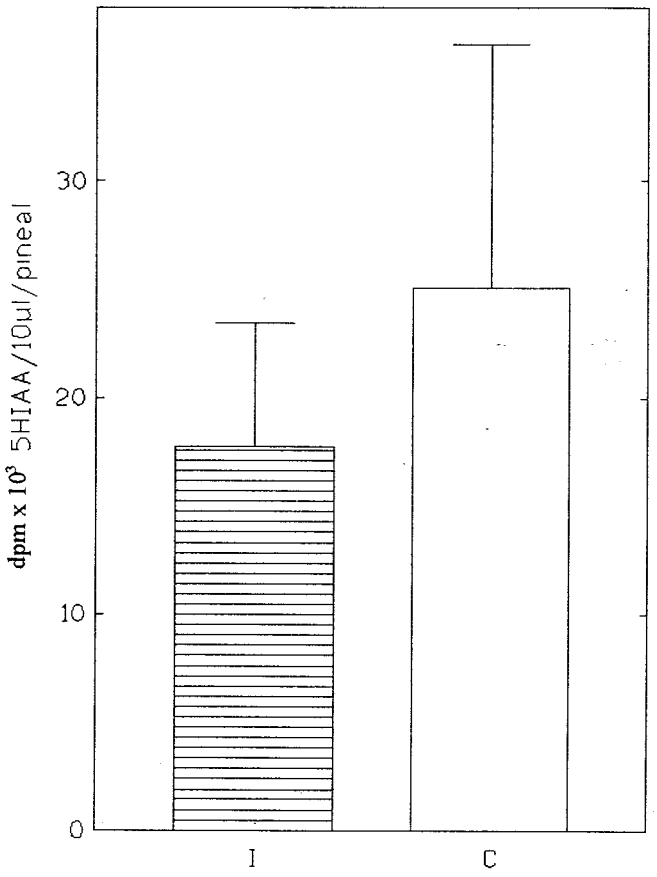


FIG. 73: Effects of insulin (I) (1 IU/52μl) on 5-hydroxyindole acetic acid (5-HIAA) synthesis in pineal organ cultures (n = 4, $p > 0.05$ (ns), $\pm$SD). C = control, IU = international unit.

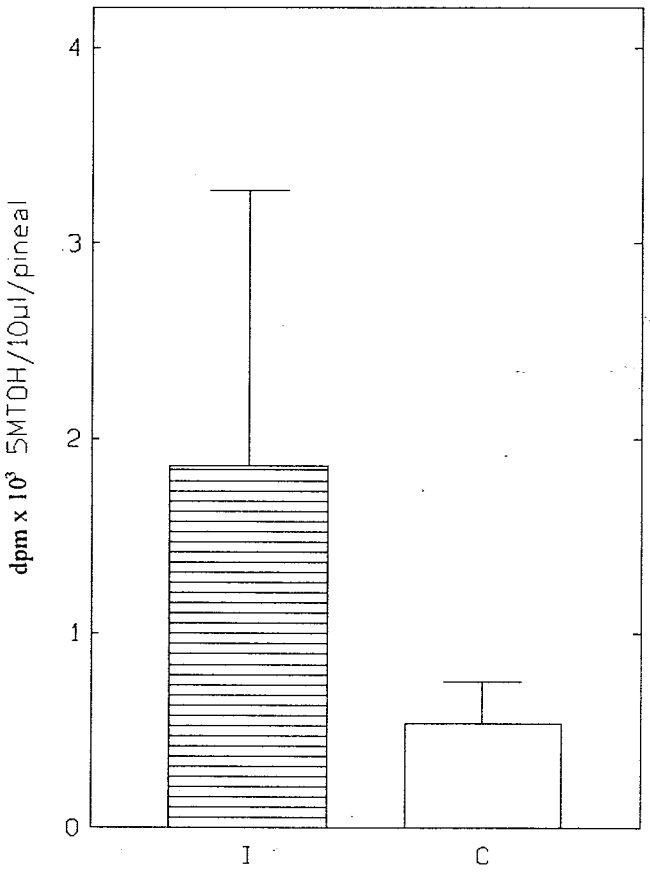


FIG. 74: Effects of insulin (I) (1 IU/52μl) on 5-methoxytryptophol (5-MTOH) synthesis in pineal organ cultures (n = 4, $p > 0.05$ (ns), $\pm$SD). C = control, IU = international unit.

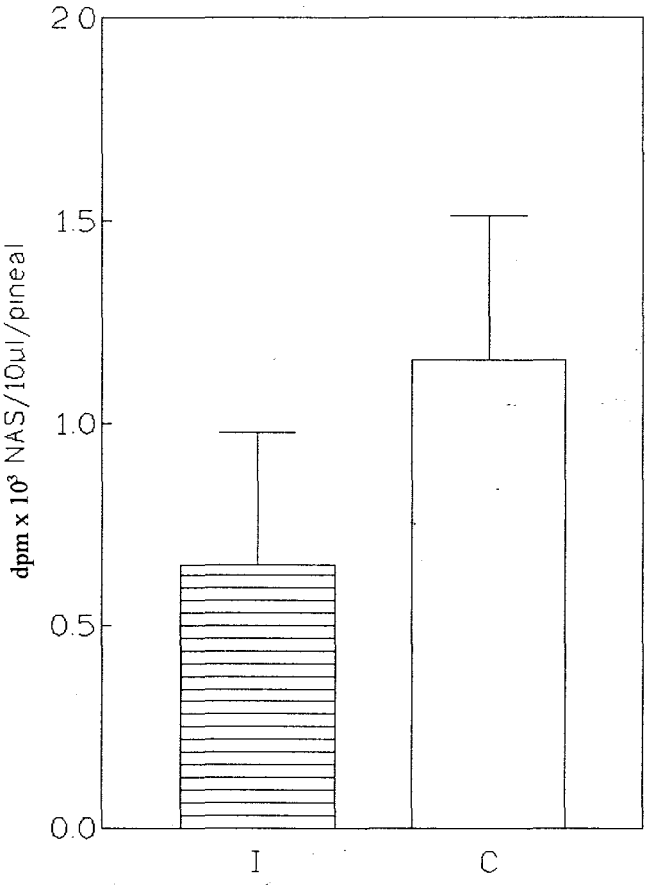


FIG. 75: Effects of insulin (I) (1 IU/52μl) on N-acetyl serotonin (NAS) synthesis in pineal organ cultures (n = 4, $p > 0.05$ (ns), $\pm$SD). C=control, IU = international unit.

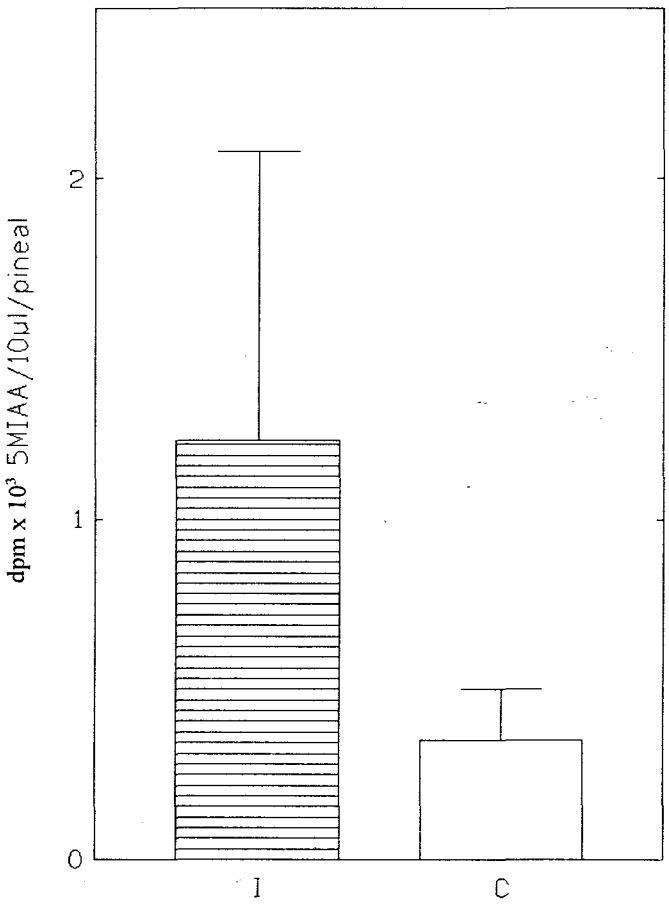


FIG. 76: Effects of insulin (I) (1 IU/52μl) on 5-methoxyindole acetic acid (5-MIAA) synthesis in pineal organ cultures (n = 4, $p > 0.05$ (ns), ±SD). C = Control, IU = international unit.

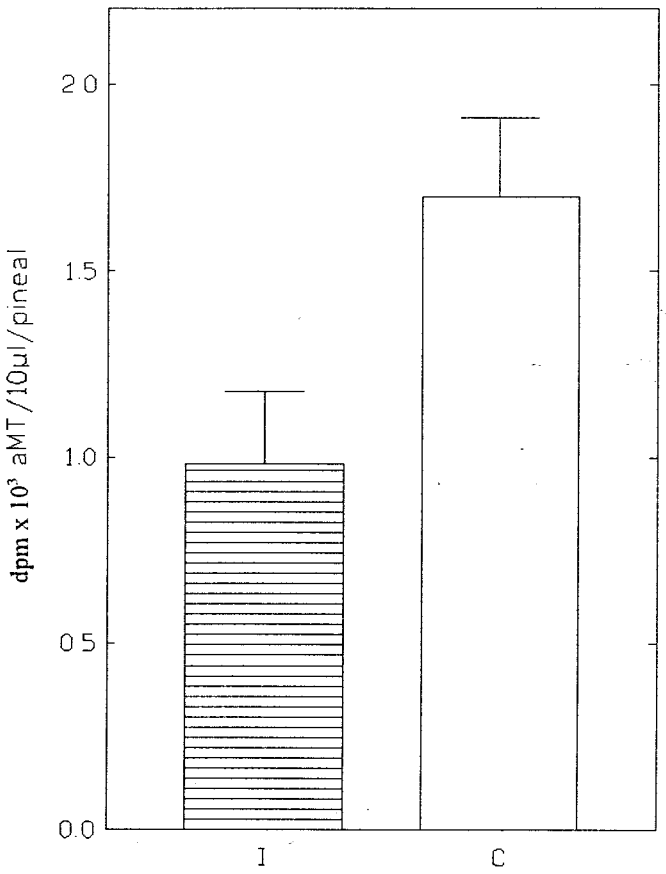


FIG. 77: Effects of insulin (I) (1 IU/52μl) on melatonin (aMT) synthesis in pineal organ cultures (n = 4, $p < 0.05$, $\pm$SD). C = control, IU = international unit.

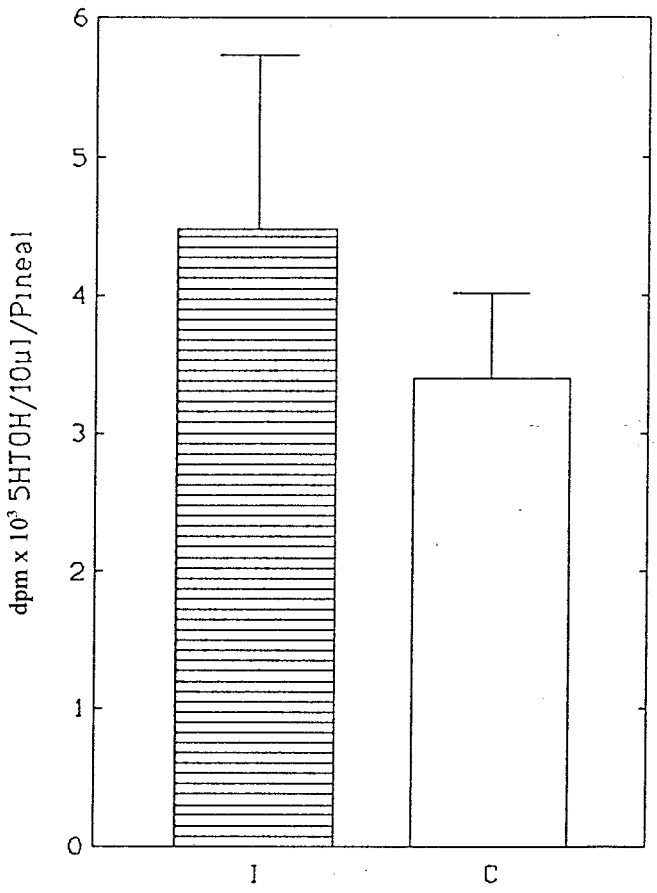


FIG. 78: Effects of insulin (I) (1 IU/52μl) on 5-hydroxytryptophol (5-HTOH) synthesis in pineal organ cultures (n= 4, $p > 0.05$ (ns), $\pm$SD). C = control, IU = international unit.

CHAPTER 5

PHYSIOLOGICAL EFFECTS OF MELATONIN INCLUDING DISPLACEMENT OF TRP FROM BSA BY VARIOUS AGENTS

EXPERIMENT 8

5.0 THE EFFECTS OF CHRONIC MELATONIN ADMINISTRATION ON RAT HEPATIC FATTY ACID, NUCLEIC ACID AND PROTEIN METABOLISM, PINEAL INDOLE METABOLISM AND TDO ACTIVITY.

5.0.0 Introduction

A survey of the available literature on aMT reveals that this neurohormone has a number of other functions within the vertebrate body besides being a circadian rhythm generator (Reiter, 1980) or a modulator of endocrine secretions (Arendt and Broadway, 1987). The compound has been demonstrated to be an effective oncostatic agent both *in vitro* and *in vivo* in rats (Demoss *et al.*, 1981) and humans (Hill and Blask 1988).

An example that illustrates the protective function of aMT in the cell involves the carcinogen safrole which generates hydroxyl radicals in DNA. Physiological concentrations of aMT 1000 times less than safrole practically abolished the negative effects of safrole, which include DNA adduct formation. It is believed that aMT inhibits the hepatic mixed function oxidase cytochrome p-450 (Kothari and Subramanian, 1992) that is activated by safrole (Lewandowski *et al.*, 1990). This enzyme generates a highly reactive electrophillic molecular species of safrole which causes biotransformations within the DNA. The inhibitory effects of aMT on nucleic acid synthesis have also been found to occur in a protozoan *Stentor Coerrulus* (Bannerjee *et al.*, 1980).

The metabolism of fatty acids involves a heme-containing enzyme cytochrome b_5 that is involved in redox reactions similar to the holoenzyme of TDO and the function oxidase mentioned above. aMT has been shown to inhibit TDO *in vitro* (Walsh *et al.*, 1994) probably by scavenging the free radical generated during enzymic catalysis. By implication, aMT theoretically should also have an effect on the activity of cytochrome b_5, which could take the form of either increasing the levels of saturated fatty acids or decreasing the concentrations of unsaturated fatty acids in the hepatocytes. In this context, and given the ubiquitous presence of aMT in the rat body, it was decided to examine the *in vivo* effects of the neurohormone after chronic treatment on a number of different metabolic aspects such as hepatic fatty acid metabolism, nucleic acid and protein turnover, pineal indoleamine metabolism and TDO activity.

5.0.1 Materials and methods

Twelve rats weighing between 200-250mg were given a dose of 0.25 mg/kg aMT intraperitoneally once daily for 6 days at 11:00h. On day 6 the animals were sacrificed 2 hours after treatment. Tissues (blood and liver) were rapidly removed and frozen rapidly in liquid N_2 until further use. The pineal glands were subjected to culture immediately, and the effects of aMT observed after incubation.

5.0.1.0 TDO activity

This assay was performed according to the method of Badaway and Evans (1975) (as described earlier) using crude liver homogenates. Enzyme activity was determined at two different substrate concentrations, 1mM and 2.5mM, in the presence and absence of cofactor hemin. The incubation period was 65 minutes.

5.0.1.1 Extraction of nucleic acids

The two classical quantitative colorimetric assays generally employed for nucleic acids are the orcinol (Biels) test for RNA and the diphenylamine (Dische) assay for DNA. Both tests are performed in an extreme acid environment that allows for quantitative depurination of the nucleic acid. In the case of RNA, the acid liberated D-ribose is dehydrated to furfural. Furfural then condenses with orcinol in the presence of ferric ions to produce a solution consisting of a variety of compounds with an intense blue-green colour, which absorbs maximally at 665nm (see fig. 79 for the reaction).

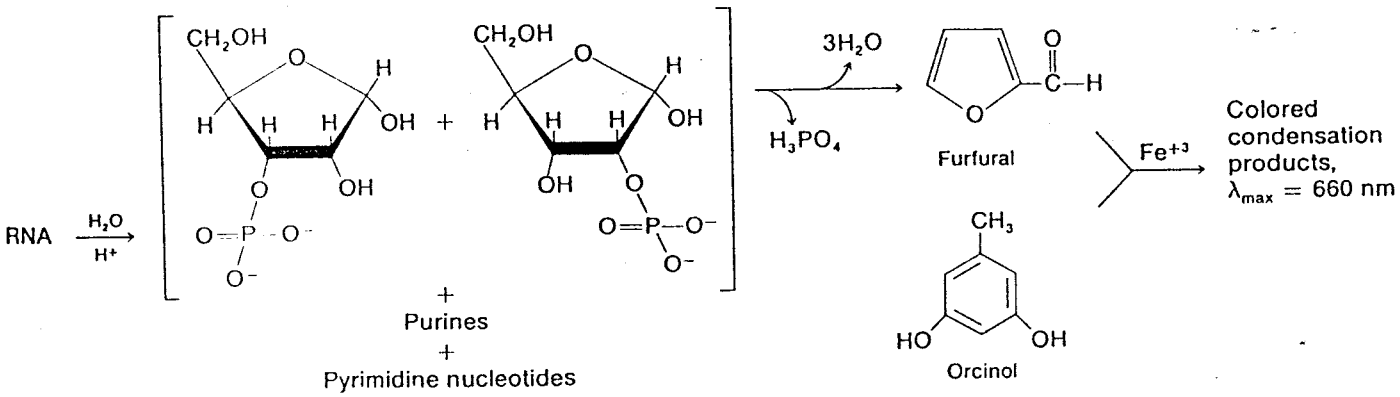


FIG. 79: The orcinol test for RNA (Clarke and Switzer, 1977).

DNA on the other hand, yields upon concentrated acid hydrolysis, 2-deoxy-D-ribose, which dehydrates to ω-hydroxylevulinylaldehyde that condenses with diphenylamine to produce a combination of coloured

products with a distinctive hue (blue) and a wavelength maximum of 600nm (see figure 80 for outline of reaction). For both RNA and DNA the resultant colour produced is proportional to the concentration of the nucleic acid.

Both experimental and control livers were homogenised using a Waring blender and the resultant homogenate centrifuged at 2000g for 10 min. The supernatant was retained and the pellet discarded. 5ml of 5% TCA solution was added to the supernatant to precipitate the crude nucleic acid. The resultant suspension was centrifuged at 2000g for 10 min. and the sediment treated with 8ml of a 5% TCA solution. This mixture was boiled in a H_2O bath at 100°C for 15 min. To prevent evaporation, the test tubes were covered with marbles. The solution was allowed to cool, and then centrifuged at 2000g for 10 minutes. The supernatant containing the purified nucleic acids was kept for analysis (Plummer, 1978).

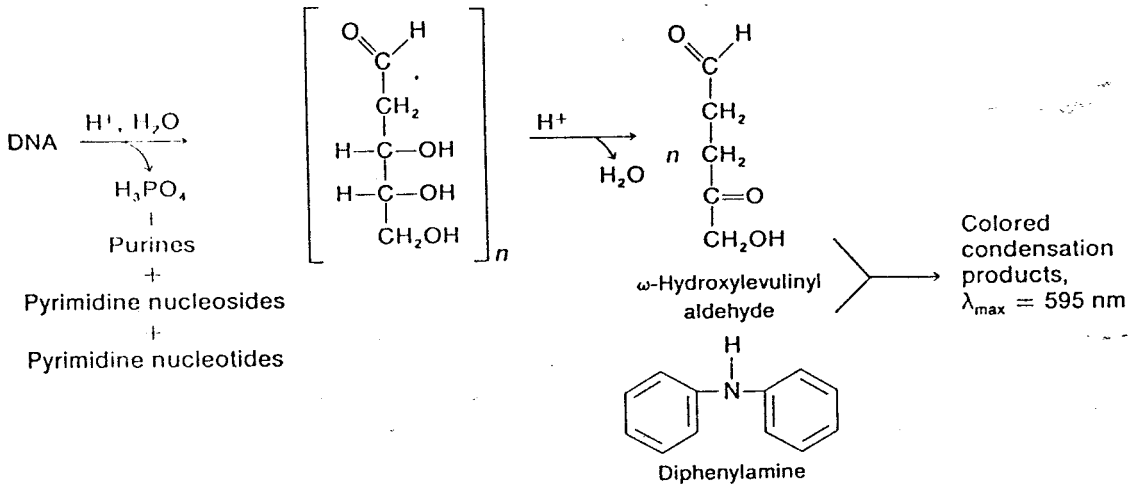


FIG. 80: The diphenylamine assay for DNA (Clarke and Switzer, 1977).

5.0.1.2 RNA determination

Orcinol reaction

For the preparation of the standard curve (appendix 3), 2ml fractions of the appropriate dilutions of the yeast extract RNA (0.2mg/ml) were added to 6ml of orcinol reagent, boiled for exactly 15 min, cooled, and the extinction read at 665nm against an orcinol blank. 3mls of the test samples were subjected to the same treatment after an initial purification step outlined above (Plummer, 1978).

5.0.1.3 DNA determination

Diphenylamine reaction

For the DNA standard curve (appendix 2), 10 mg of the nucleic acid was dissolved in 50ml of borate buffer at a pH of 8.5. To appropriate dilutions of 2mls, 4mls of diphenylamine reagent was added. This solution was then heated on a boiling H_2O bath, cooled, and the extinction read at 600nm against a H_2O blank. 3mls of the experimental and control samples were treated similarly after an initial purification step outlined above (Plummer, 1978).

5.0.1.4 Protein determination

This was determined according to the Folin-Lowry (1951) protocol.

5.0.1.5 Fatty acid determination

The saponification, esterification and extraction of fatty acids were performed according to a modification of the methods of Catala *et al.*, (1975), Carreau *et al.*, (1980), Faas and Carter (1981; 1982), De Schriver and Privett (1982), and Alam *et al.*, (1984).

Hepatocytes were homogenised using, firstly, a Warring blender, followed by a dounce homogeniser. All tubes were rinsed with a minimal amount of a 10% methanolic KOH solution. For the stromal fatty acid determination the cells were only homogenised 30 times with a tight plunger in order to rupture the cell and release the free fatty acids.

The lipids were then saponified by heating with reflux and under nitrogen for 45min. at 85°C. These were then acidified to free fatty acids by the addition of 1ml of 7M HCl. The fatty acids were extracted twice (shaking vigorously for 2min each time) with 3ml of petroleum ether (bp 40-60°C). The pooled aliquots for each sample were then 90% evaporated under nitrogen at 60°C. The residual fatty acids were then methylated by heating with BF_3-methanol reagent (0.3ml) under nitrogen for five minutes at 100°C. The fatty acid esters were extracted twice, as outlined before, with 1ml of petroleum ether. These were then 90% evaporated at 60 °C under nitrogen, reconstituted to the desired volume, and stored at -20°C under nitrogen and protected from light to prevent oxidation.

The capillary-column gas chromatographic system was coupled to a flame ionisation detector. During analysis of the volatilised fatty acids, the samples were kept on ice to limit evaporation and oxidation. The carrier gas used was nitrogen and the program parameters are tabulated in table 3.

Table 3: Program parameters for the gas chromatography.

column	Quadrex 007-23	sample volume	0.5μl
program	gradient	oven temperature	190°C
initial temperature	120°C	initial time	15min
final temperature	190°C	final time	30min
injector B	210°	detector A	210°
oven temperature$_{max}$	190°	equilibrium time	0.5min
flow rate (ml/min)	14.5	rate (°C/min)	3.0

The fatty acid standards were supplied by Sigma chemicals (USA) and the gas chromatograph can be found in appendix 8.4. Examples of chromatographs for both stromal and membrane fatty acids appear in appendix 8.5. To determine the ratio of one particular fatty acid relative to others, the following fatty acids were used: palmitoleate, stearate, oleate, linoleate, linolenate, arachidate, arachidonate, erucate and lignocerate.

5.0.1.6 Organ culture

8 Pineals (2 sets: 4 controls and 4 experimentals) were incubated for 24 hours according to the procedure outlined in Experiment 7.

5.0.2 Results

5.0.2.0 TDO activity

At low substrate (1mM) levels (figs. 90, 91) there were no significant differences between the aMT treated and control livers, as was the case at the higher concentration of 2.5mM for both apo- and holoenzymes (figs. 92, 93).

5.0.2.1 Nucleic acid determination

The effect of aMT on both DNA and RNA levels (figs. 87, 88) for the aMT treated rats as compared to the controls was not significant. See appendices 8.1 (DNA) and 8.2 (RNA) for standard curves.

5.0.2.2 Protein determination

The neurohormone had a negligible effect on hepatic protein synthesis when the controls were compared to the experimentals (fig. 89). Note appendix 8.3 for protein standard curve.

5.0.2.3 Fatty acid metabolism

There appeared to be a significant degree of modification in hepatic fatty acid composition of only some of the stromal fatty acids (figs. 94 - 102) and none in the membranes (figs. 103 - 111) when compared to the controls. Chronic aMT administration brought about changes in hepatic fatty acid synthesis in the cytoplasm. These alterations were manifested either by increases or decreases in the fatty acid ratios. In the stroma, there were increases in the level of arachidonate (fig. 101) and the saturated stearate (fig. 95).

5.0.2.4 Organ culture

The experimental pineals revealed no significant difference in aMT (fig. 83) and NAS (fig. 84) levels after the incubated period. A similar result was obtained with respect to 5-HIAA (fig. 85) and 5-HTOH (fig. 86), when compared to the controls. 5-MIAA (fig. 81) and 5-MTOH (fig. 82), on the other hand, manifested the most marked alterations, depicting statistically significant increases of 3.5- and 2.5- fold respectively. Table 4 below shows the tabulated results.

Table 4: The results of the effects of aMT on pineal indoleamine metabolism in pineal organ culture. Results are expressed as percentage increase (↑) relative to the controls (ns = not significant at $p > 0.05$).

	aMT	NAS	5-HIAA	5-HTOH	5-MTOH	5-MIAA
aMT	ns	ns	ns	ns	(↑) 350	(↑) 250

5.0.3 Discussion

5.0.3.0 Hepatic TDO activity

There was no statistically significant difference in enzyme activity for both aMT treated and control animals (figs. 90 - 93).

5.0.3.1 Hepatic nucleic acid synthesis

aMT had no significant effect on hepatic nucleic acid synthesis, as can be seen from figures 87 and 88.

5.0.3.2 Hepatic fatty acid metabolism

There does seem to be a trend in the modifications caused by aMT on hepatocyte fatty acid metabolism. It can be observed that the neurohormone has an effect on certain pathways such as the one which involves the desaturation of unsaturated fatty acids from the results obtained. The ratio of the unsaturated fatty acid

stearate is significantly elevated in both the membrane and the cytoplasm, while that of oleate is not altered. Conversion of stearate to oleate involves an enzyme system that is potentially susceptible to the antioxidant effects of the neurohormone. The mechanism of action of this microsomal fatty acyl-CoA desaturase and cytochrome b_5 reductase involves redox reactions utilising an iron porphyrin factor, O_2, and the transfer of two electrons (Mathews and Van Holde, 1990). Thus, given the hypothesised mode of action of aMT in free radical scavenging (Hardeland *et al.*, 1993), it is possible that the neurohormone could negatively influence this reaction.

Cytoplasmic ratios of arachidonate depict statistically significant increases while those of the membranes reveal none. The ratios of the potential arachidonate precursor linolenate is unaffected. However, there could be stimulation of this pathway which involves the delta-6 desaturase enzyme system. When taken in conjunction with the supposed effects on the oleate → stearate pathway there could, on the one hand, be inhibition of the cytochrome b_5 reductase and, on the other hand, stimulation of Δ^6 desaturase. These influences manifest themselves as an increase in ratios of stearate and arachidonate respectively. It may be speculated that these modifications in fatty acid concentrations are possibly due to direct effects of melatonin on some of the previously discussed enzyme systems involved in fatty acid biosynthesis, or on another factor such as a hormone which is involved in regulating the synthesis of hepatic fatty acids.

5.0.3.3 Pineal organ culture

The results obtained with the organ culture experiment reveal no changes with respect to NAS and aMT synthesis, but marked elevations in the 5-HT catabolites such as the 5-MTOH and 5-MIAA, which is consistent with what would be expected if the levels of a particular metabolite (5-HT) are increased within the cell and/or there is a channelling of precursor away from one branch in a particular pathway. It is also possible that the pineal is saturated with aMT (due to the imposed treatment), which subsequently gives rise to a type of feedback inhibition by the indolealkyleamine on some of the enzymes (NAT and/or HIOMT) in the relevant biosynthetic pathway of 5-HT to aMT.

5.0.4 Conclusion

Chronic aMT treatment of rats had no significant effect on DNA, RNA, protein and membrane fatty acid metabolism. Similar treatment, however, brought about marked alterations in cytoplasmic fatty acid composition. Here levels of stearate and those of arachidonate were both significantly increased. The activity of TDO was also significantly curtailed, and this inhibition could be ameliorated by exogenous TRP. Thus aMT does seem to have an effect *in vivo* on both iron porphyrin utilising enzymes.

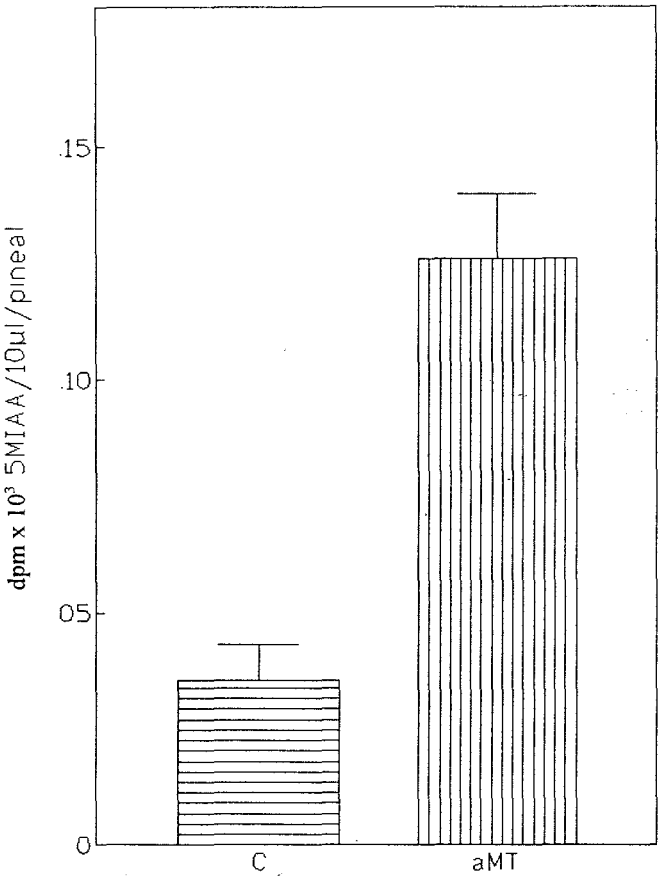


FIG. 81: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on 5-methoxyindole acetic acid (5-MIAA) biosynthesis by the pineal (n = 4, $p < 0.05$, ±SD). C = control.

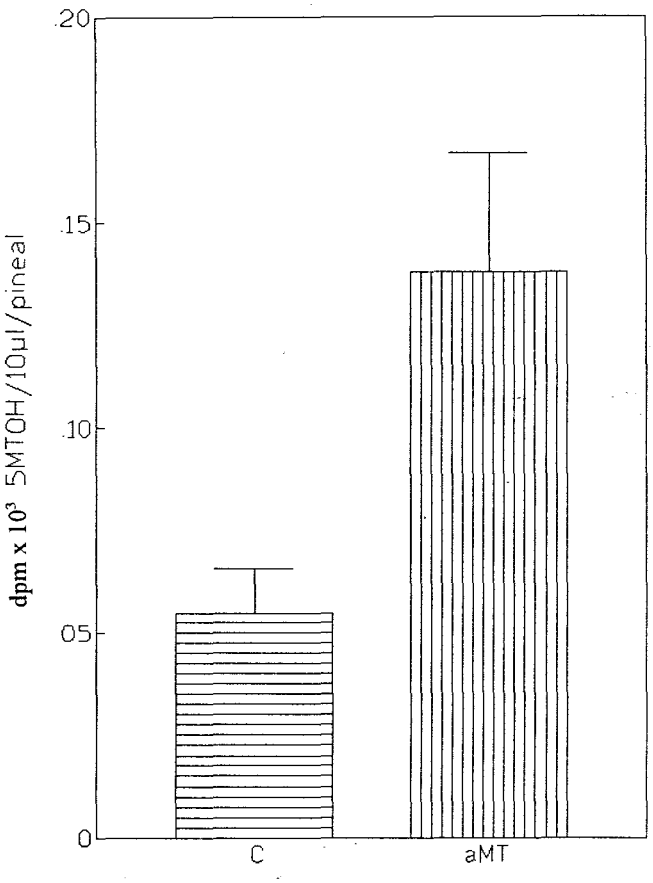


FIG. 82: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on 5-methoxytryptophol (5-MTOH) biosynthesis by the pineal (n = 4, $p > 0.05$, ±SD). C = control.

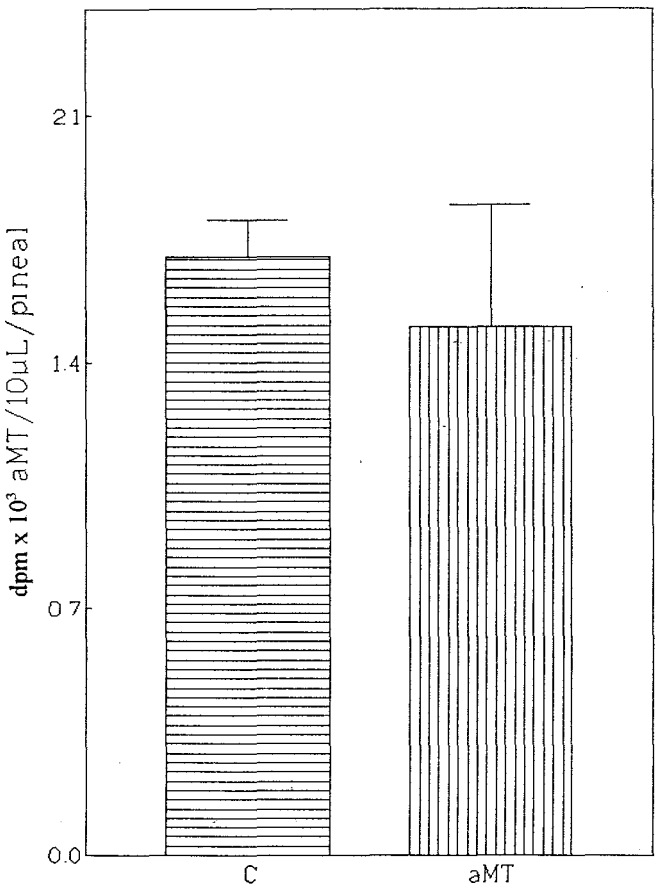


FIG. 83: Effects of chronic intraperitoneally administered melatonin (aMT)(0.25mg/kg) on endogenous pineal aMT synthesis (n = 4, $p > 0.05$, ±SD). C = control.

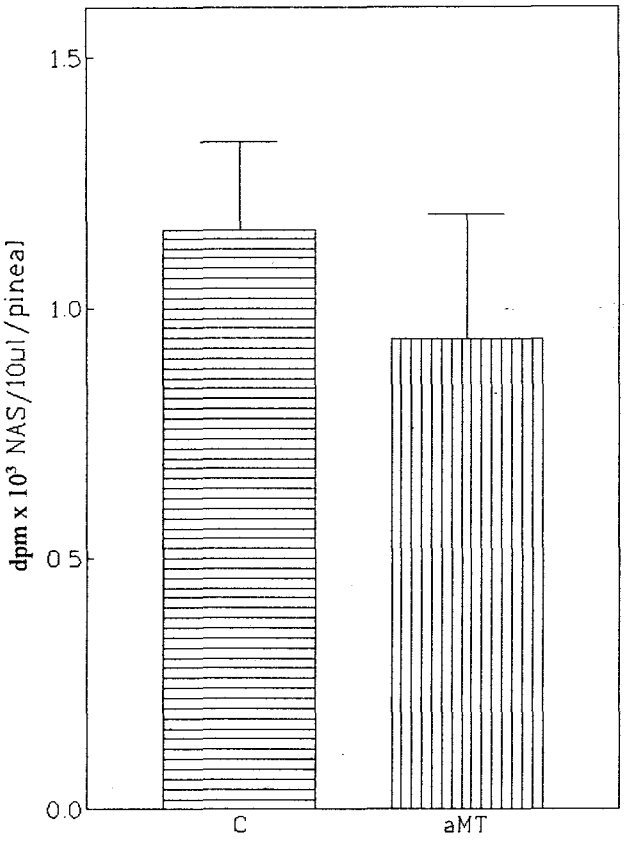


FIG. 84: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on N-acetylserotonin (NAS) metabolism in rat pineal organ culture (n=4, $p > 0.05$ (ns), ±SD). Control = C.

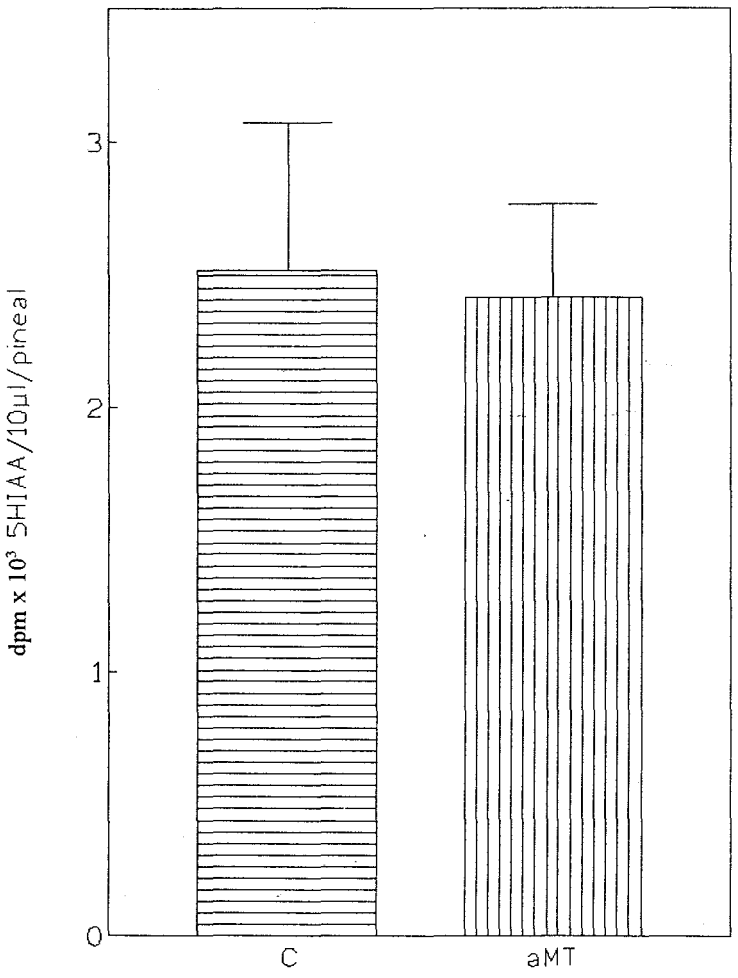


FIG. 85: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on 5-hydroxyindole acetic acid (5-HIAA) metabolism in the pineal (n = 4, $p > 0.05$ (ns), ±SD). C = control.

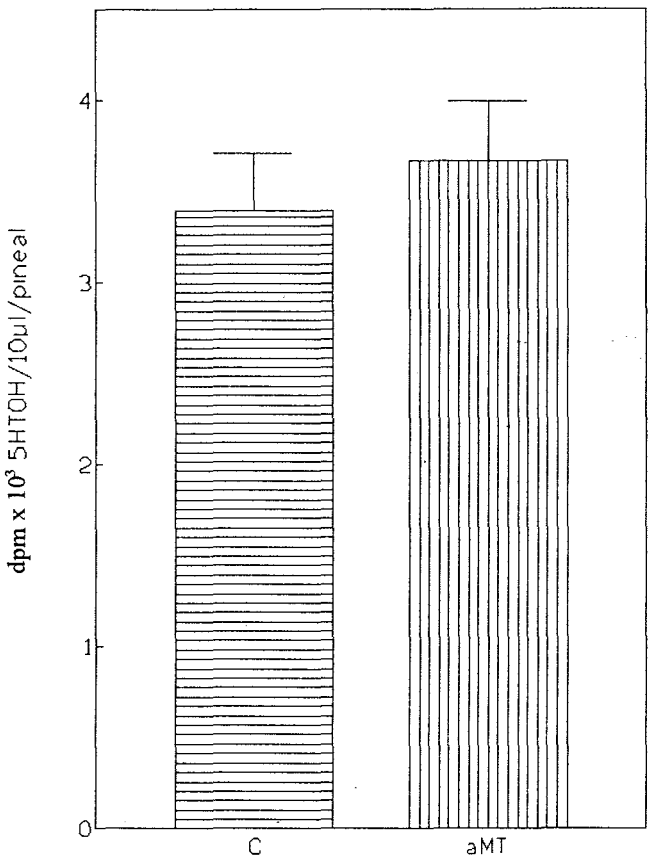


FIG. 86: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on pineal 5- hydroxytryptophol (5-HTOH) metabolism. (n = 4, $p > 0.05$ (ns), ±SD). C = control.

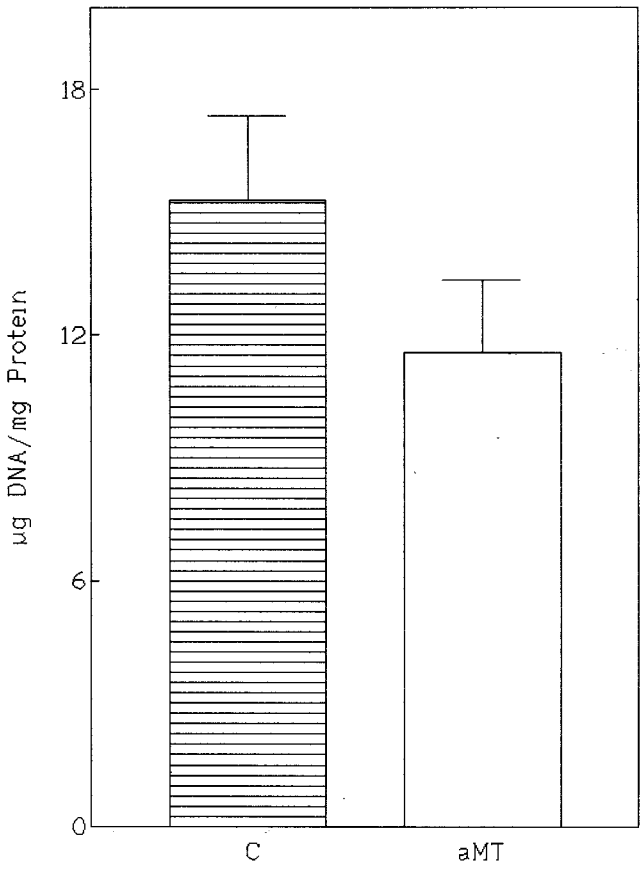
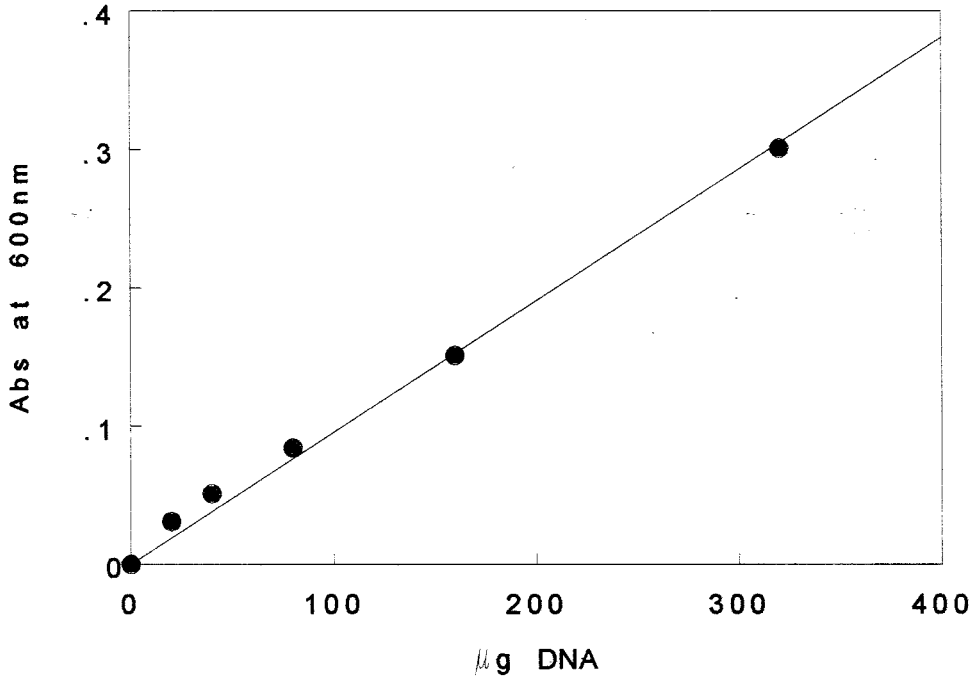


FIG. 87: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on hepatic deoxyribonucleic acid (DNA) synthesis (n = 4, $p > 0.05$ (ns), ±SEM). C = control.



APPENDIX 8.1: Deoxyribonucleic acid (DNA) standard curve (r = .997). Absorbance = Abs.

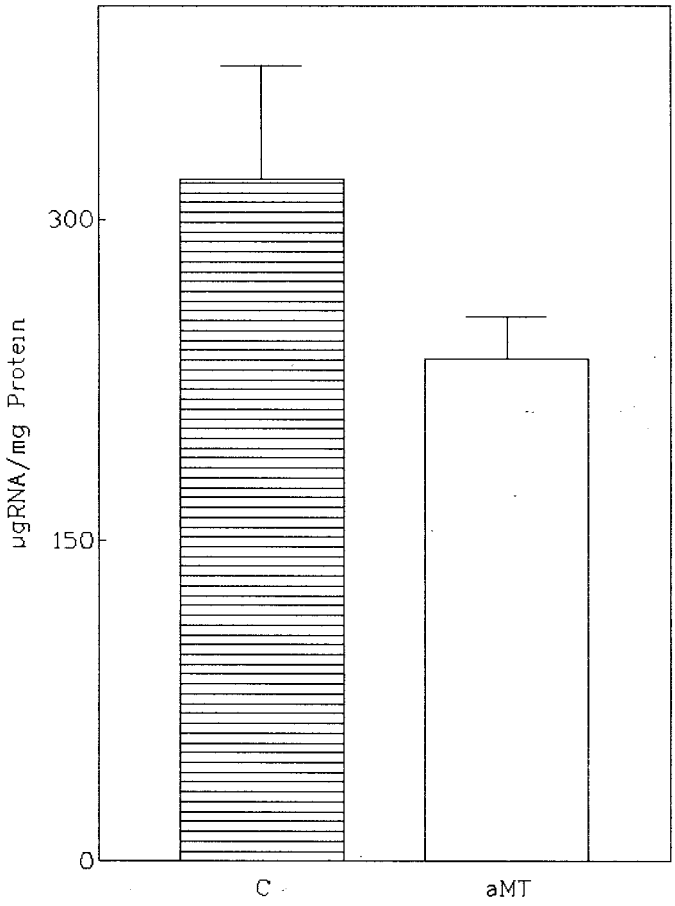
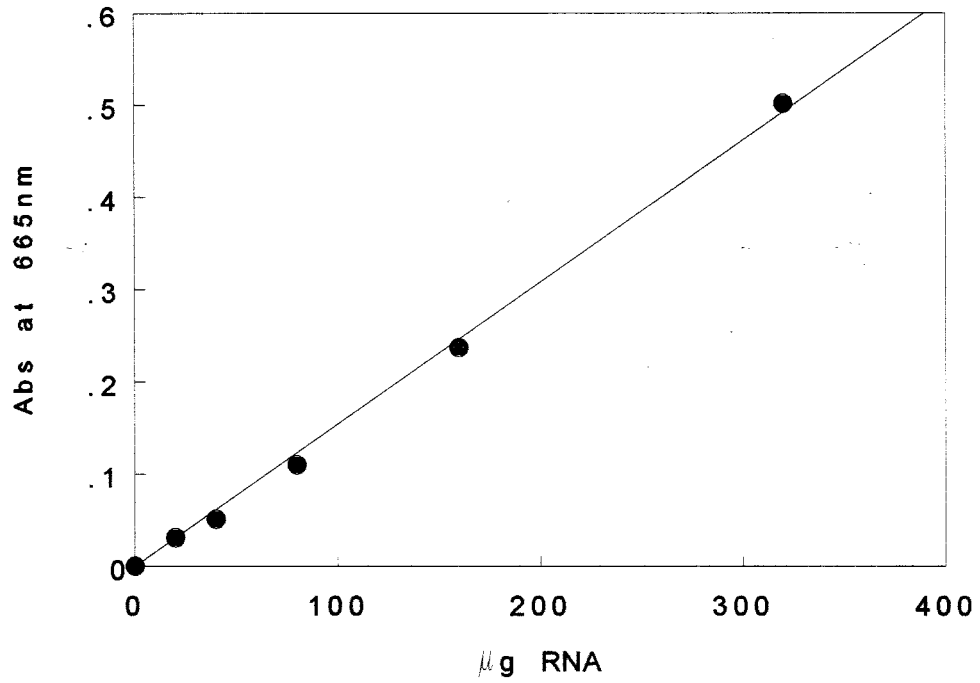


FIG. 88: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on hepatic ribonucleic acid (RNA) synthesis (n = 4, $p > 0.05$ (ns), ±SEM). C = control.



APPENDIX 8.2: Ribonucleic acid (RNA) standard curve (r = .995). Absorbance = Abs.

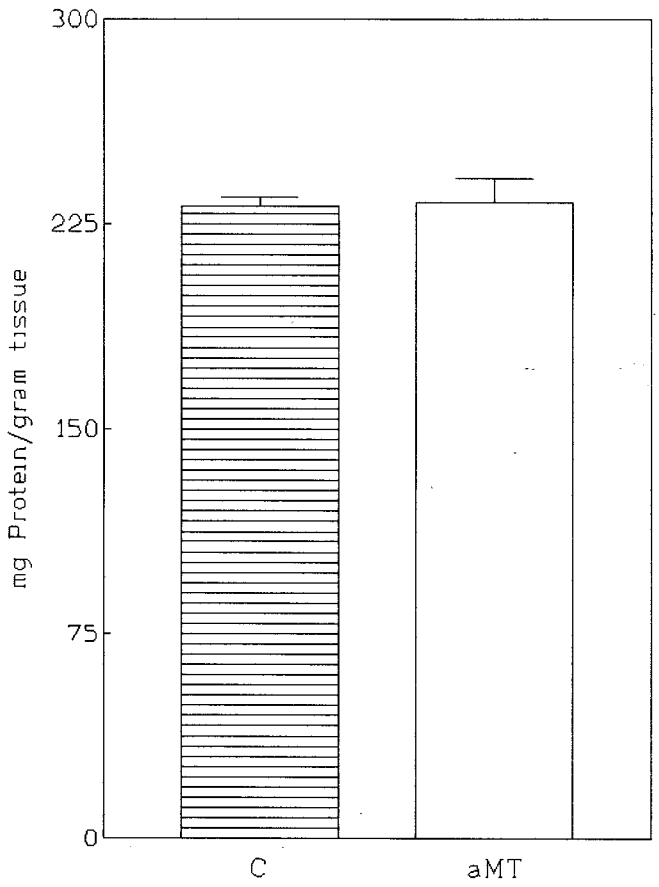
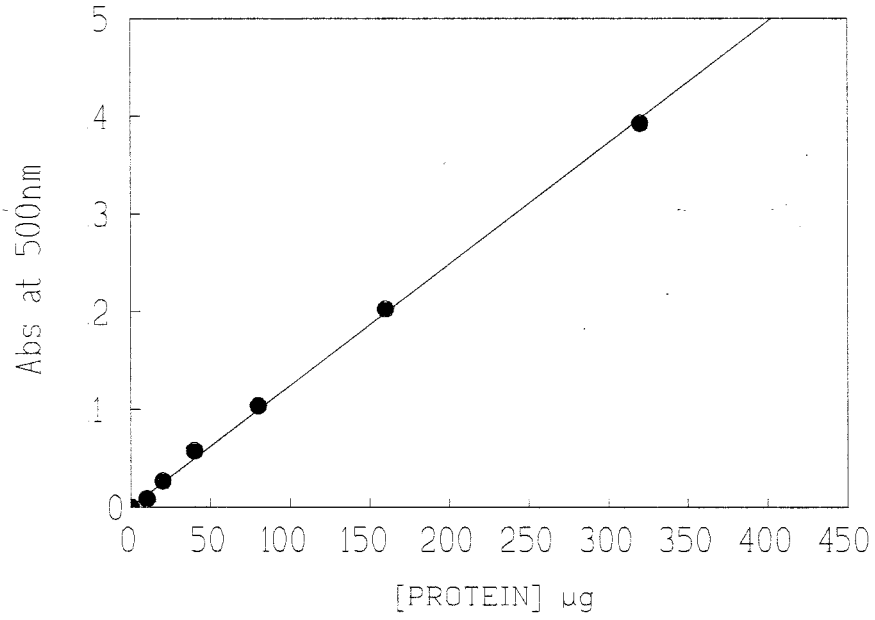


FIG. 89: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on hepatic protein metabolism (n = 4, $p > 0.05$ (ns), ±SEM). C = control.



APPENDIX 8.3: Protein standard curve (r = .998). Absorbance = abs.

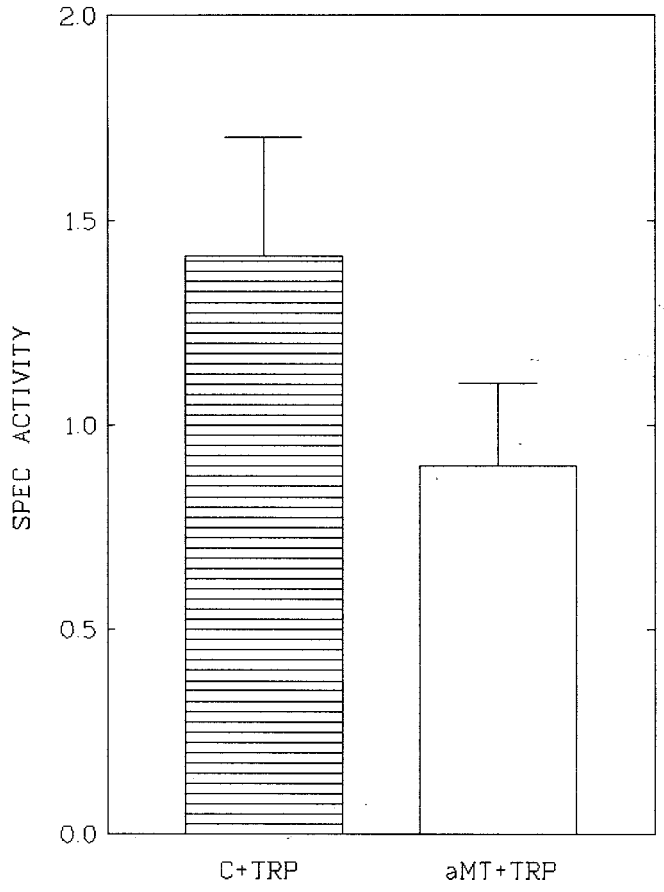


FIG. 90: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on hepatic tryptophan-2,3-dioxygenase extant holoenzyme activity (n = 4, $p > 0.05$ (ns), ±SD) at 1mM tryptophan (TRP). C = control.

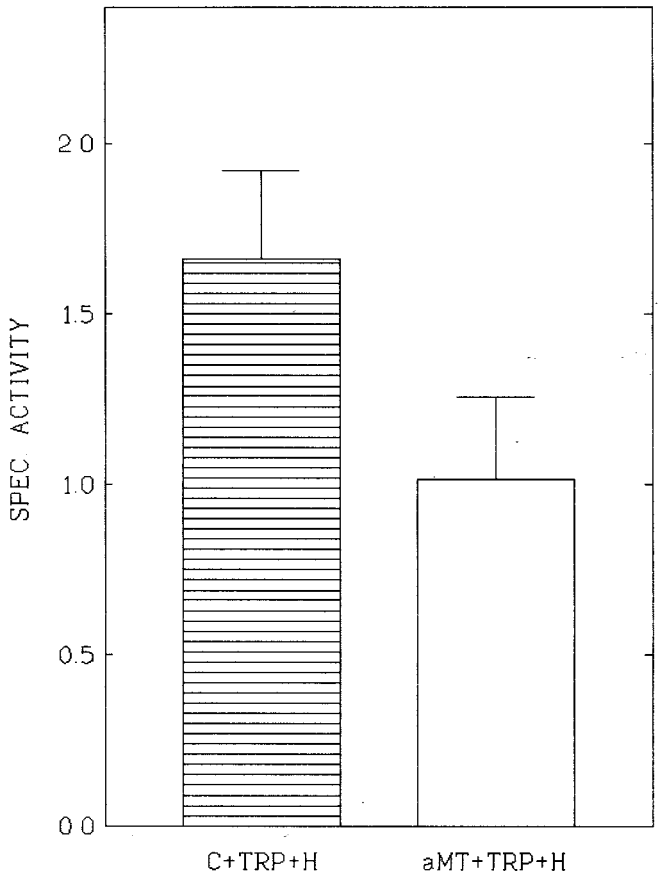


FIG. 91: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on hepatic tryptophan-2,3-dioxygenase total enzyme activity (n = 4, $p > 0.05$ (ns), ±SD) at 1mM tryptophan (TRP) + hemin (H). C = control.

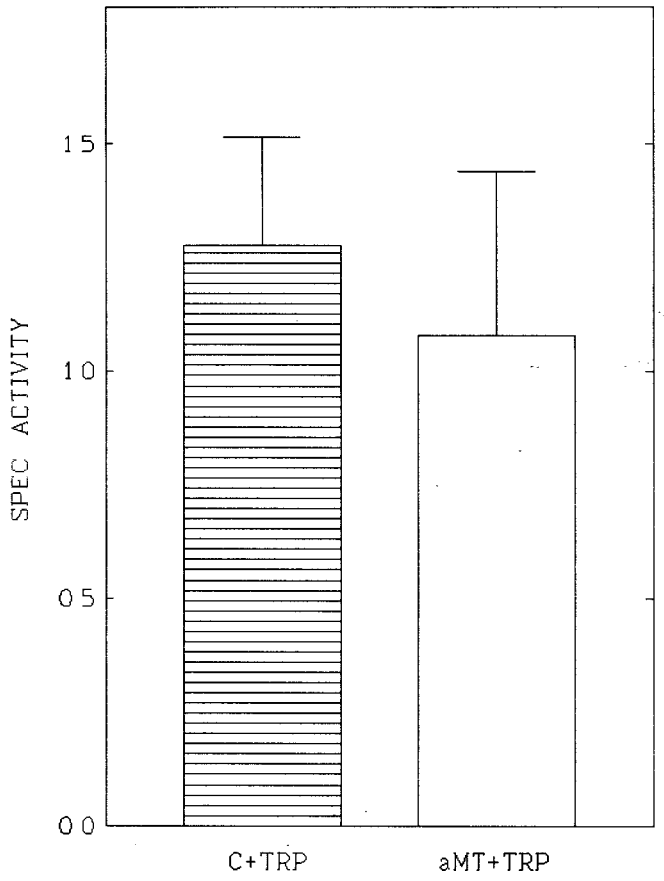


FIG. 92: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on hepatic tryptophan-2,3-dioxygenase extant holoenzyme activity (n = 4, $p > 0.05$ (ns), ±SD) at 2.5 mM tryptophan (TRP. C = control.

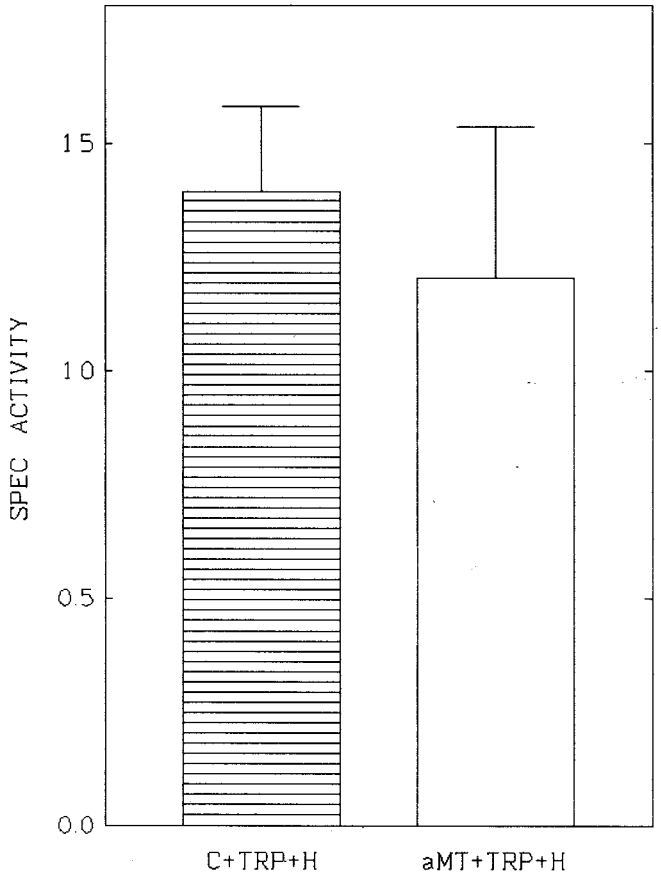


FIG. 93: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on hepatic tryptophan-2,3-dioxygenase total enzyme activity (n = 4, $p > 0.05$ (ns), ±SD) at 2.5 mM tryptophan (TRP) + hemin (H). C = Control.

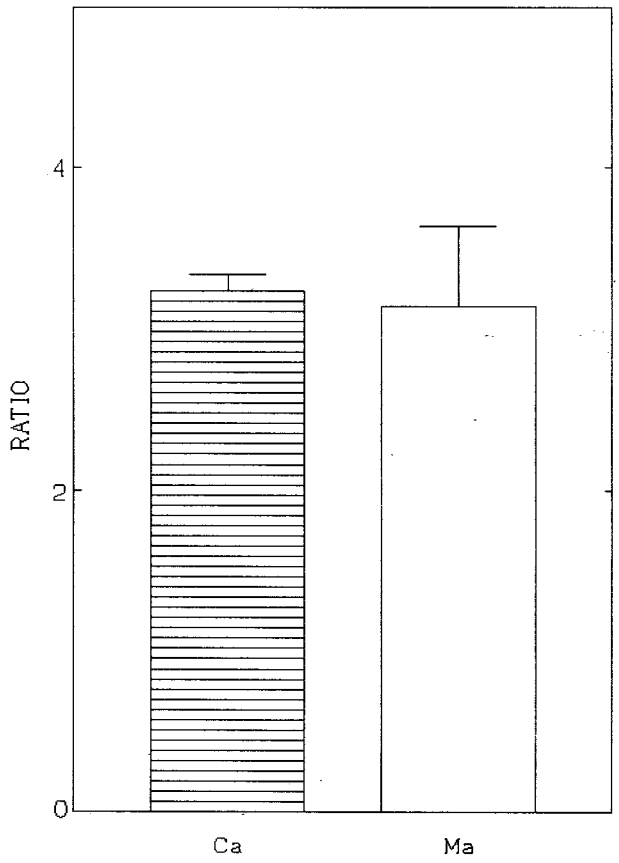


FIG. 94: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on stromal fatty acid composition (n = 4, $p > 0.05$ (ns), ±SD): Ma = aMT + palmitoleate. Ca = control.

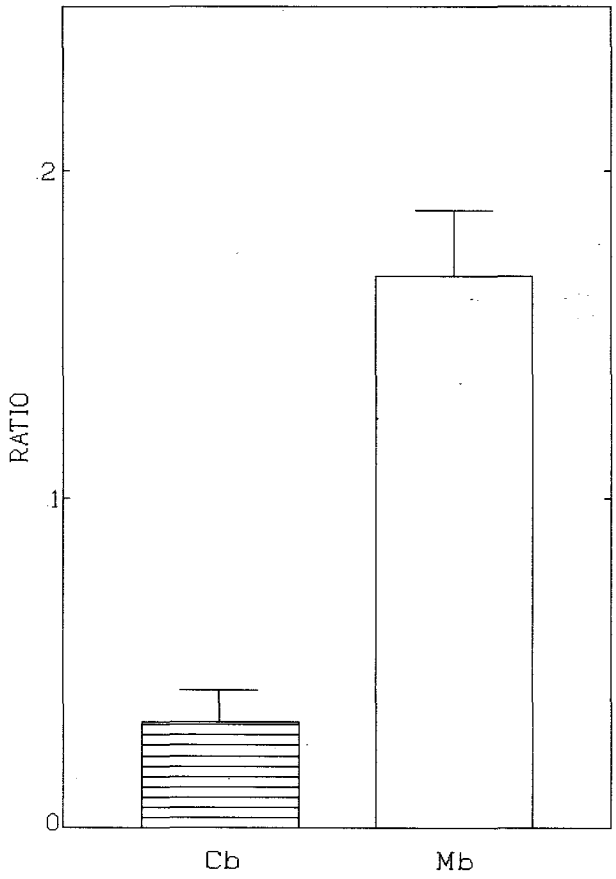


FIG. 95: Effects of chronic intraperitoneally administered melatonin (0.25mg/kg) on stromal fatty acid composition (n = 4, $p < 0.001$, ±SD): Mb = aMT + stearate. Cb = control.

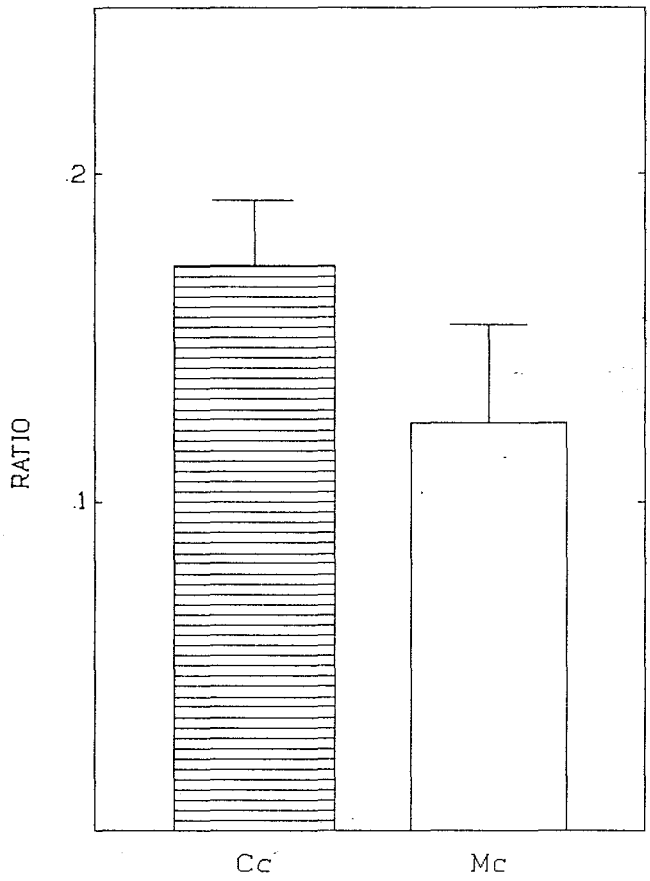


FIG. 96: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on stromal fatty acid composition (n = 4, $p > 0.05$ (ns), ±SD): Mc = aMT + oleate. Cc = control.

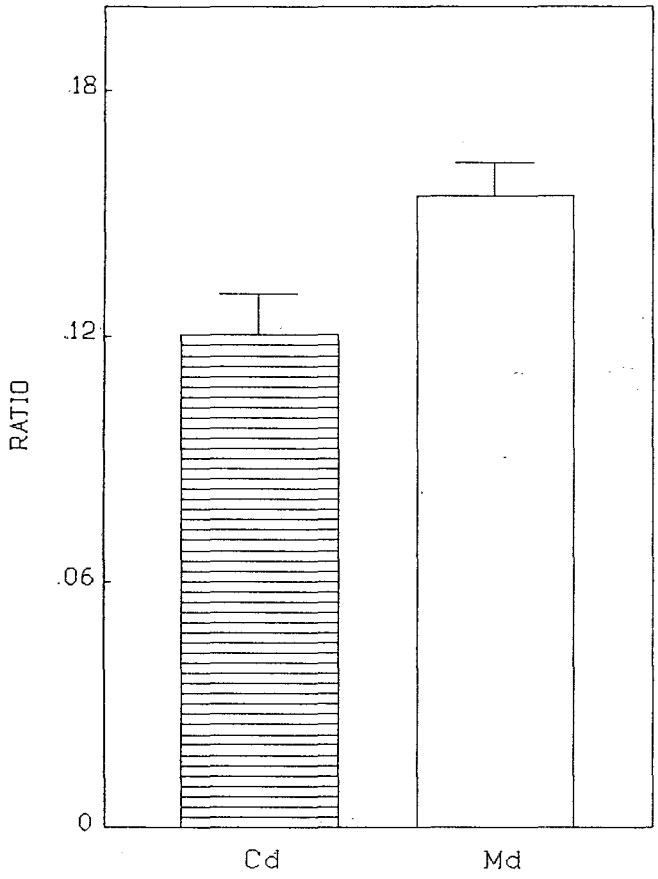


FIG. 97: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on stromal fatty acid composition (n = 4, $p > 0.05$ (ns), ±SD): Md = aMT + linoleate. Cd = control.

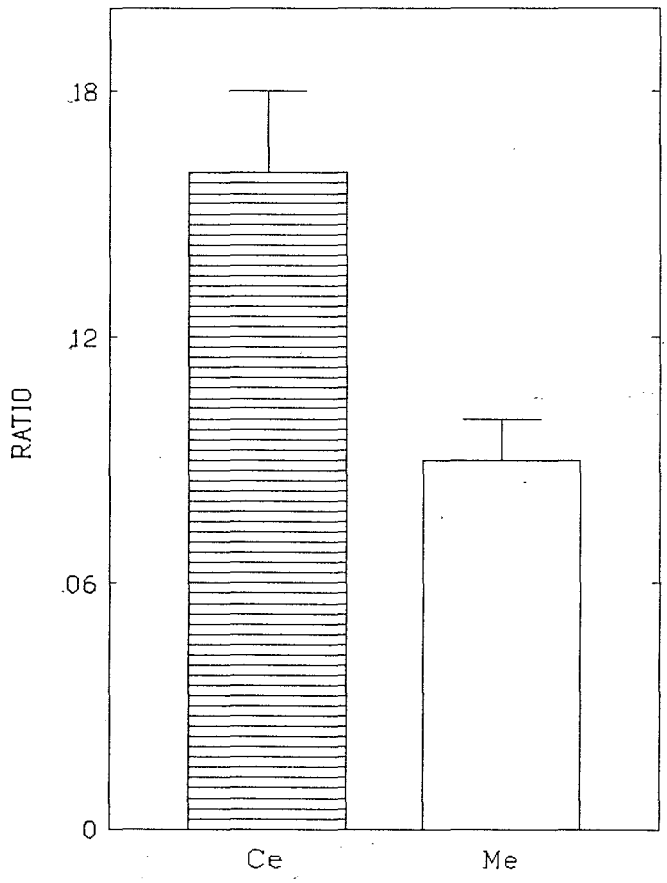


FIG. 98: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on stromal fatty acid composition (n = 4, $p > 0.05$ (ns), ±SD): Me = aMT + linolenate. Ce = control.

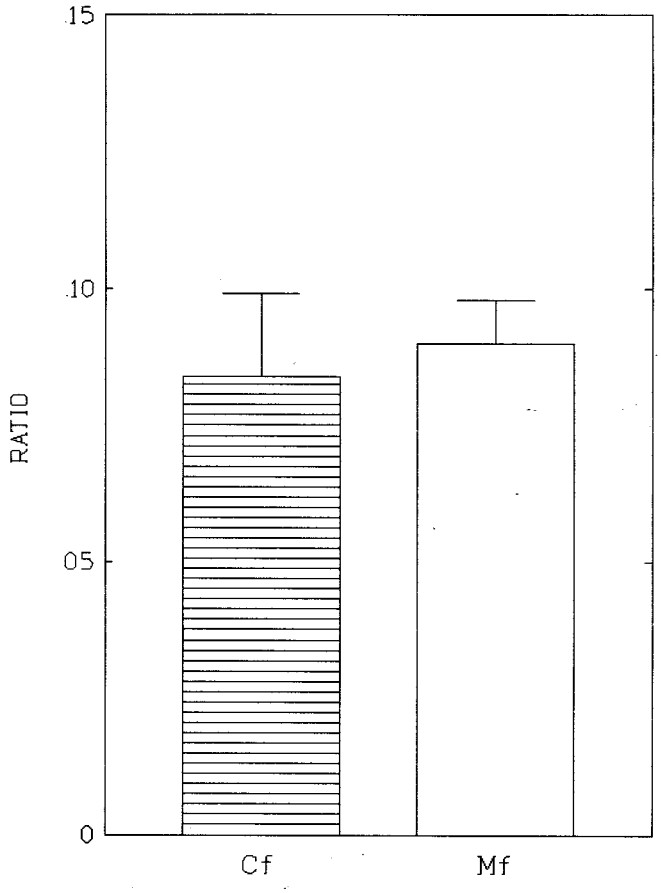


FIG. 99: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on stromal fatty acid composition (n = 4, $p > 0.05$ (ns), ±SD): Mf = aMT + erucate. Cf = control.

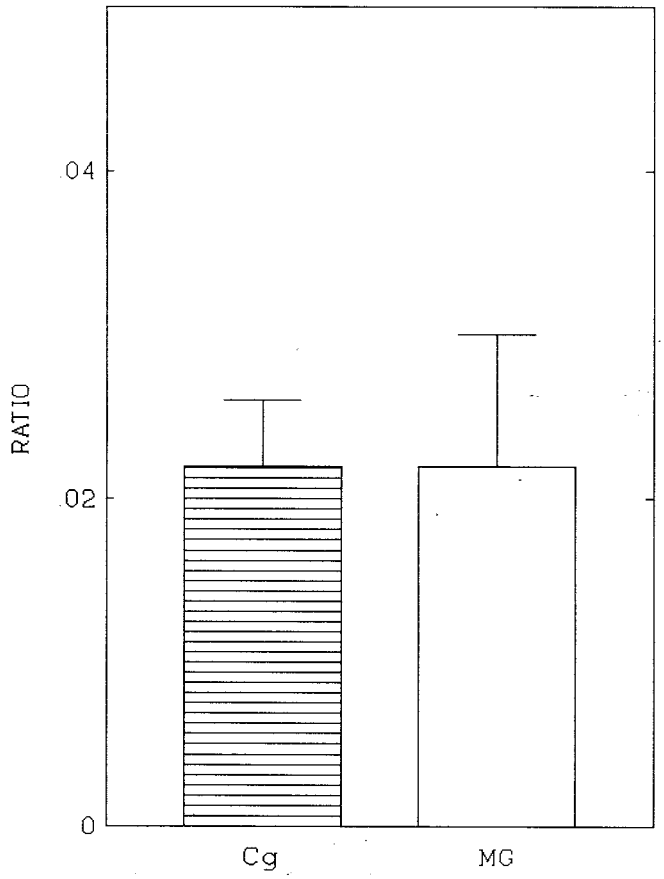


FIG. 100: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on stromal fatty acid composition (n = 4, $p > 0.05$ (ns), ±SD): Mg = aMT + arachidate. Cg = control.

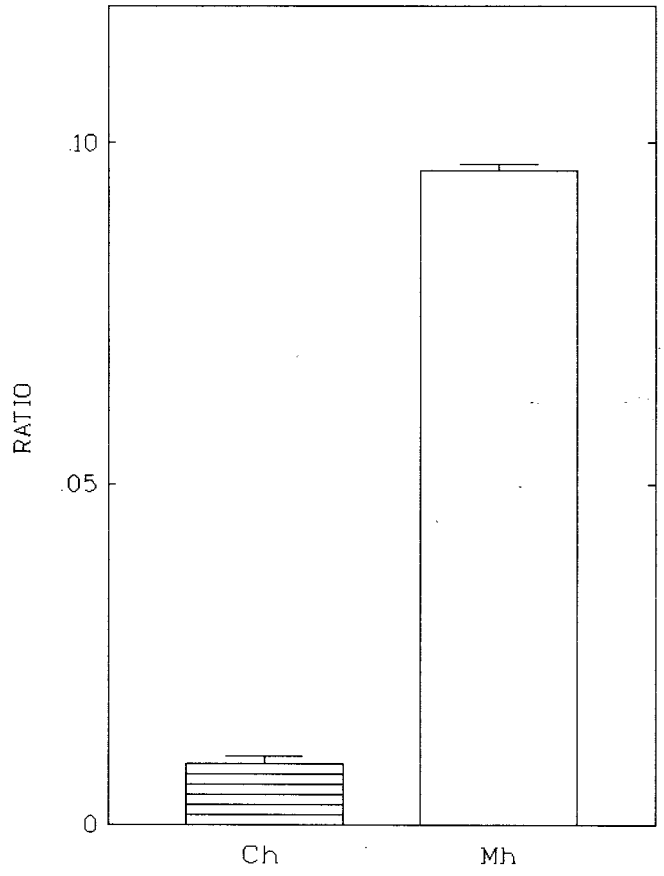


FIG. 101: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on stromal fatty acid composition (n = 4, $p < 0.001$ (ns), ±SD): Mh = aMT + arachidonate. Ch = control.

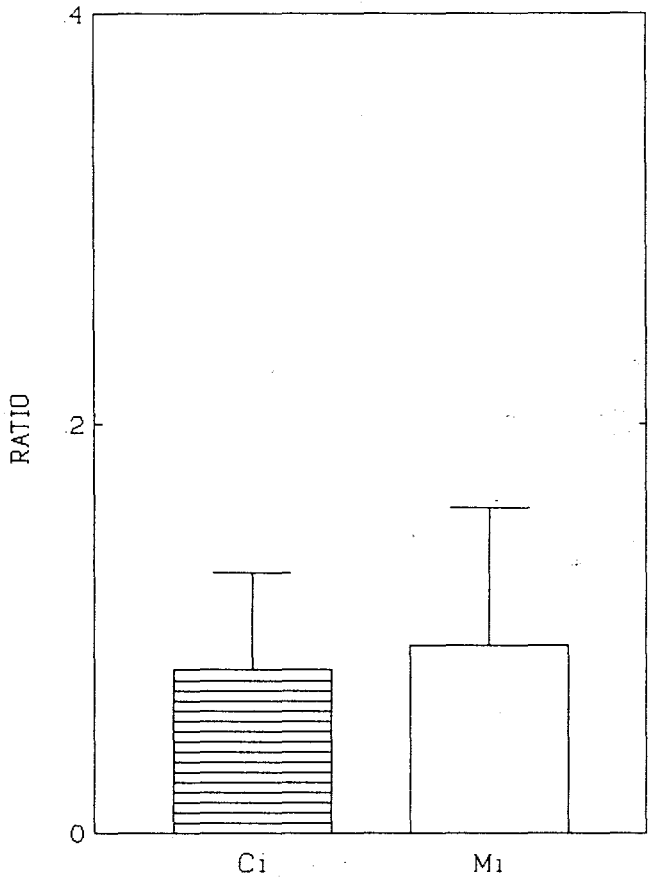


FIG. 102: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on stromal fatty acid composition (n = 4, $p > 0.05$ (ns), ±SD): Mi = aMT + lignocerate. Ci = control.

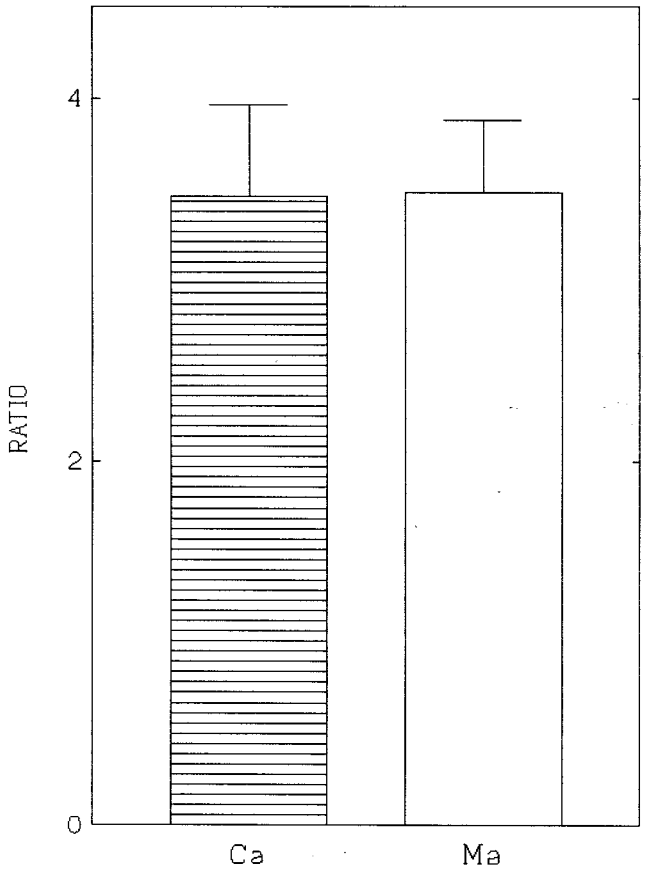


FIG. 103: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on membrane fatty acid composition (n = 4, $p > 0.05$ (ns), ±SD): Ma = aMT + palmitoleate. Ca = control.

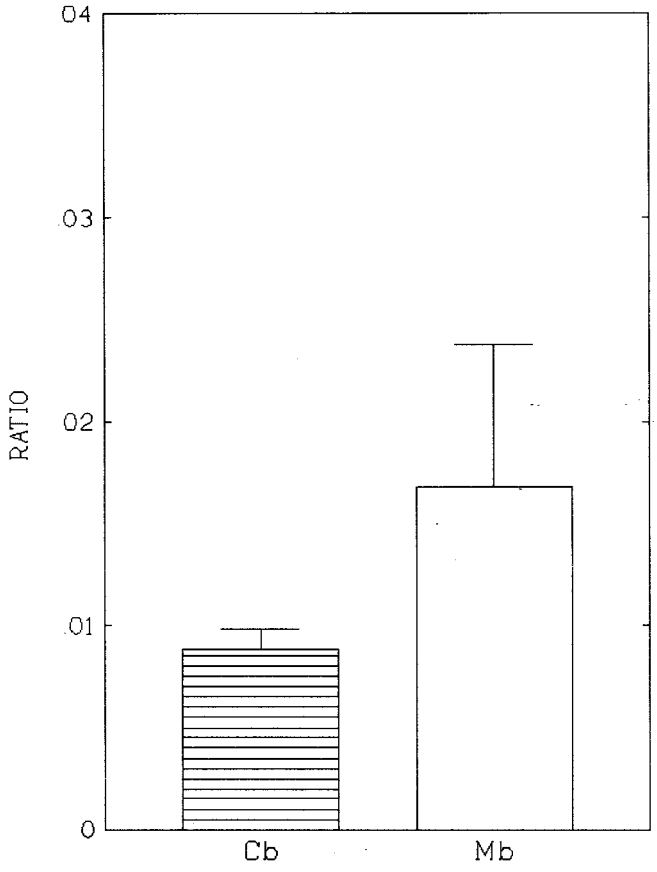


FIG. 104: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on membrane fatty acid composition (n = 4, $p > 0.05$ (ns), ±SD): Mb = aMT + stearate. Cb = control.

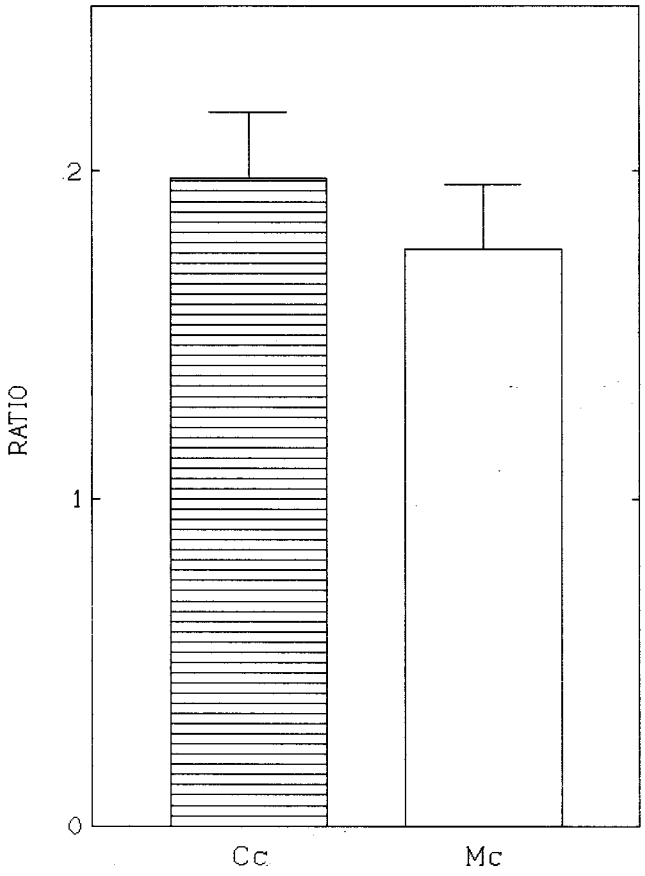


FIG. 105: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on membrane fatty acid composition (n = 4, $p > 0.05$ (ns), ±SD): Mc = aMT + oleate. Cc = control.

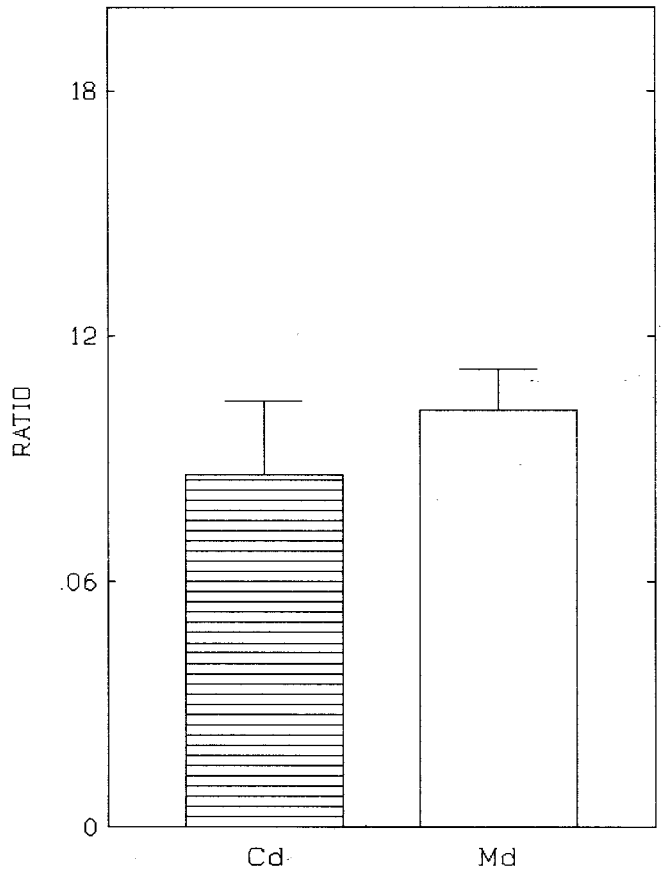


FIG. 106: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on membrane fatty acid composition (n = 4, $p > 0.05$ (ns), ±SD): Md = aMT + linoleate. Cd = control.

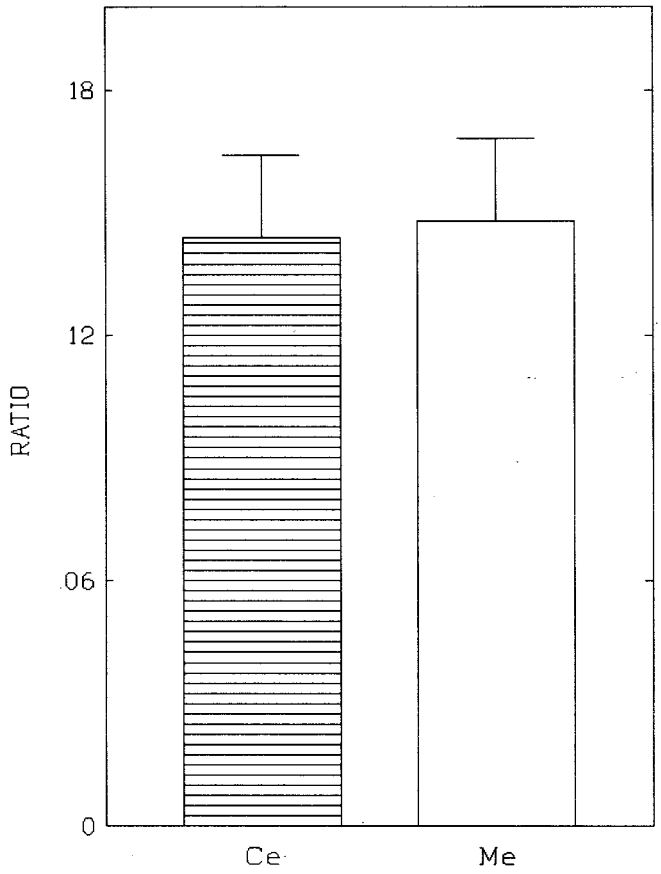


FIG. 107: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on membrane fatty acid composition (n = 4, $p > 0.05$ (ns), ±SD): Me = aMT + linolenate. Ce = control.

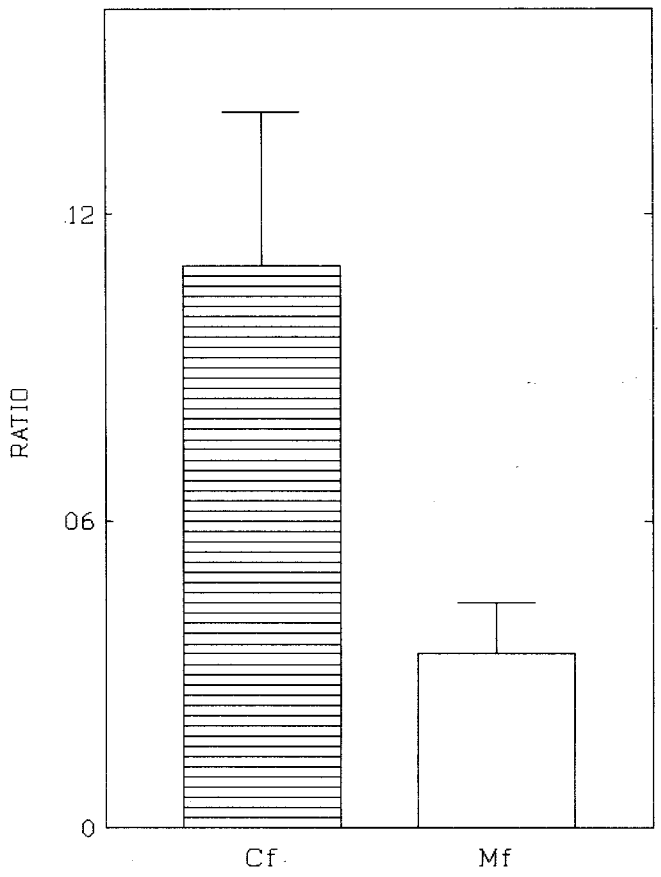


FIG. 108: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on membrane fatty acid composition (n = 4, $p > 0.05$ (ns), ±SD): Mf = aMT + erucate. Cf = control.

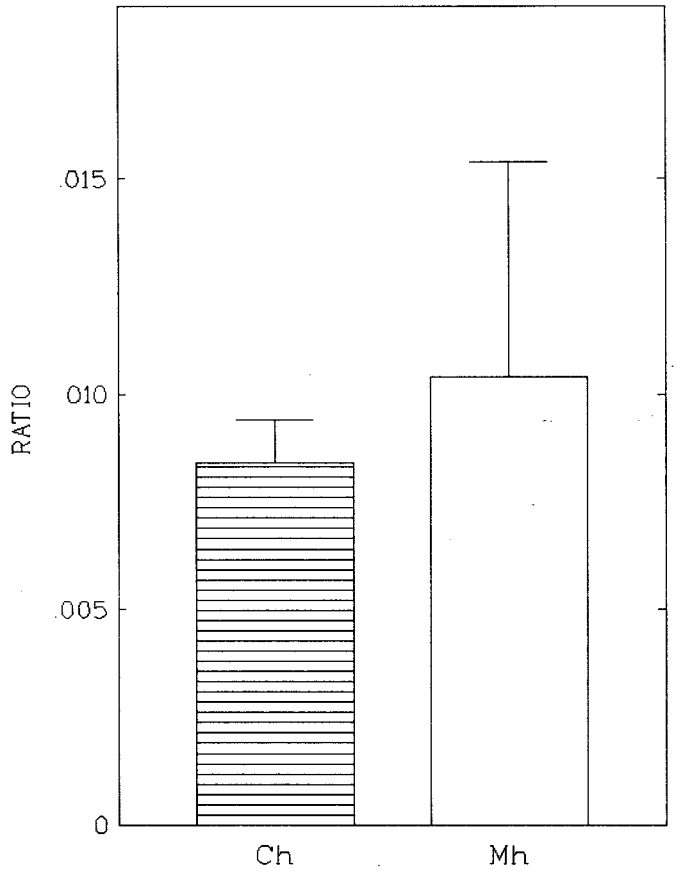


FIG. 109: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on membrane fatty acid composition (n = 4, $p > 0.05$ (ns), ±SD): Mg = aMT + arachidate. Cg = control.

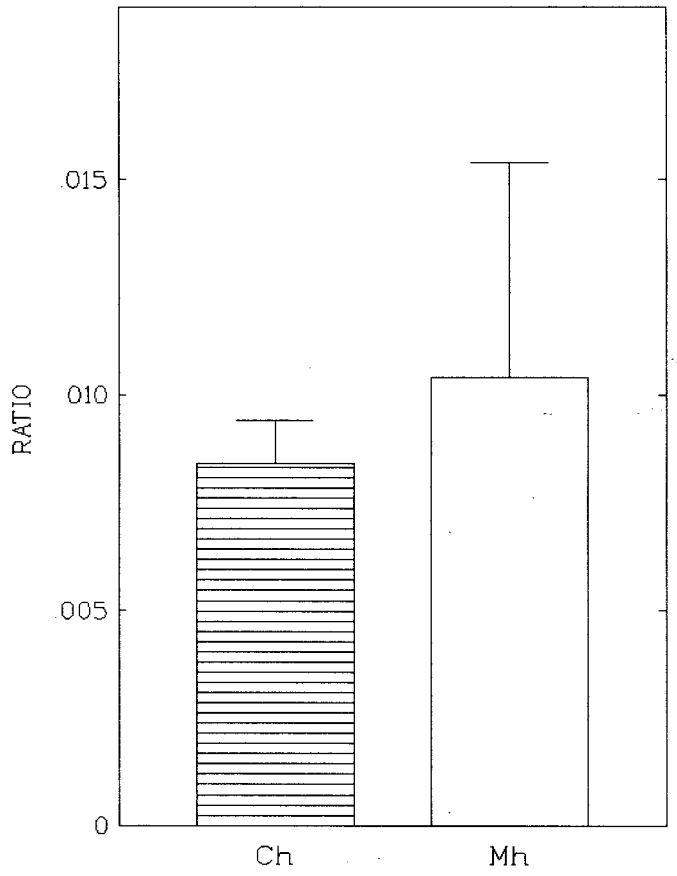


FIG. 110: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on membrane fatty acid composition (n = 4, $p > 0.05$ (ns), ±SD): Mh = aMT + arachidonate. Ch = control.

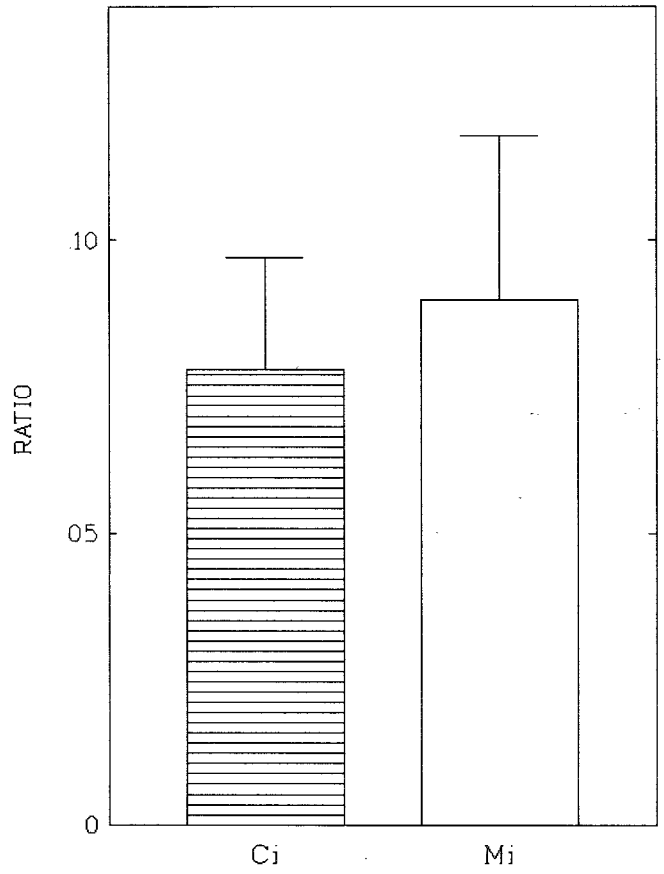
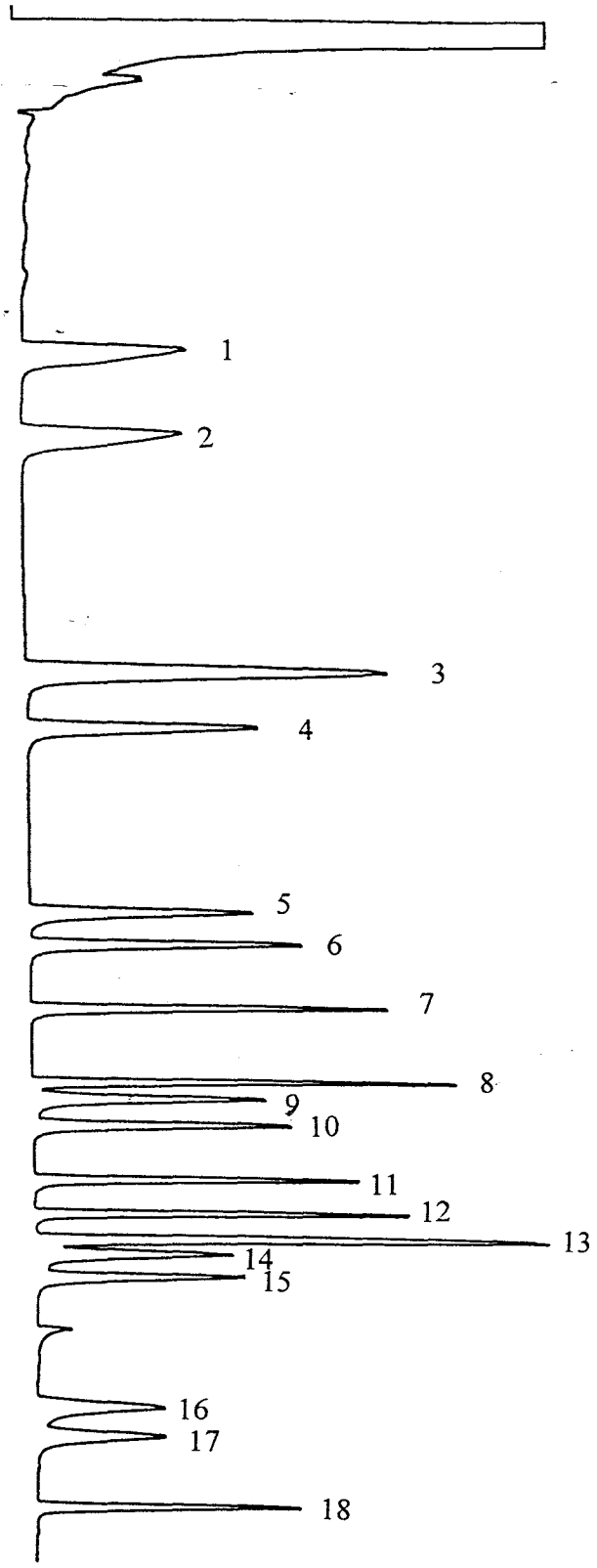
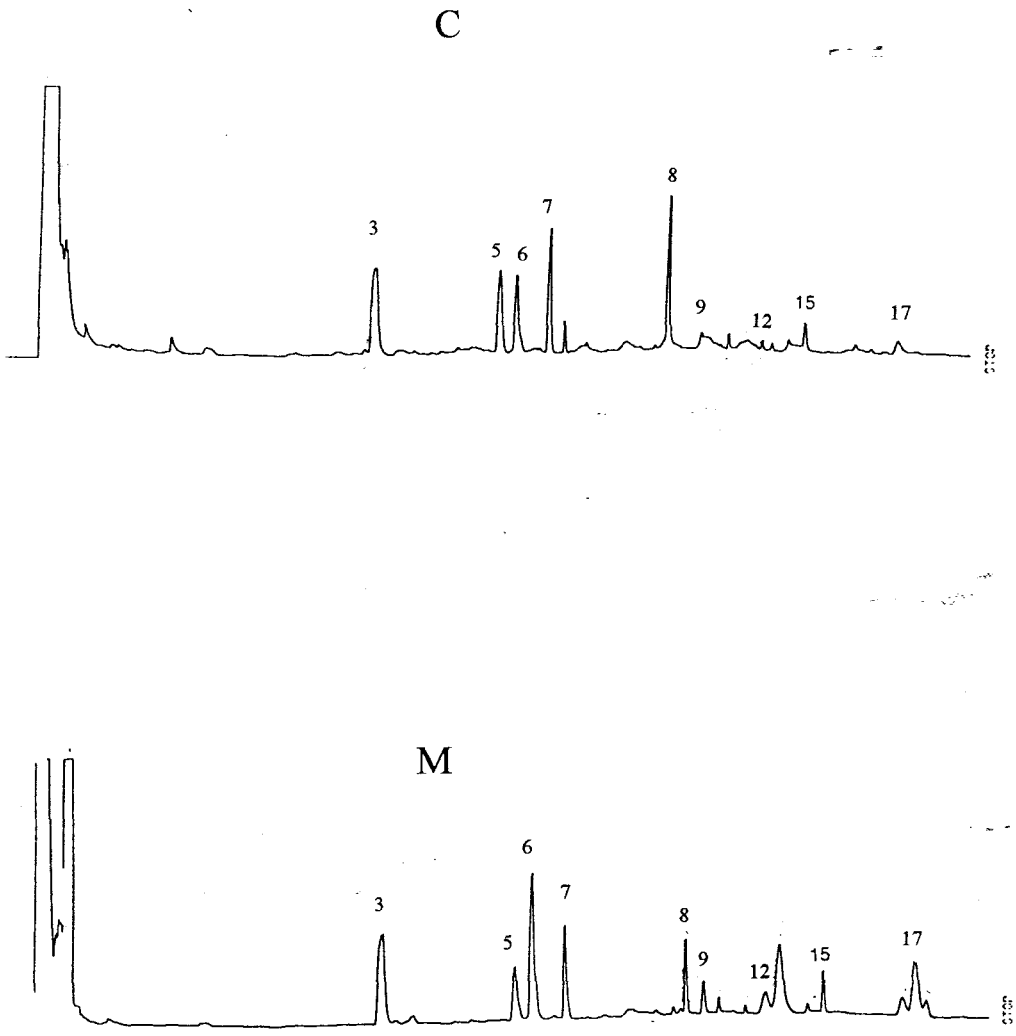


FIG. 111: Effects of chronic intraperitoneally administered melatonin (aMT) (0.25mg/kg) on membrane fatty acid composition (n = 4, $p > 0.05$ (ns), ±SD): Mi = aMT + lignocerate. Ci = control.



C:dble bond	(CH_3-)Fatty acid	RT
12:0	Laureate (1)	11.76
14:0	Myristate (2)	14.13
16:0	Palmitate (3)	20.90
18:0	Palmitoleate (4)	22.48
18:1	Stearate (5)	27.71
18:2	Oleate (6)	28.80
18:3	Linoleate (7)	30.47
20:0	Linolenate (8)	32.53
20:1	Arachidate (9)	33.01
20:2	Eicosanoate (10)	33.76
20:3	Dihomo-γ-linoleate.(11)	35.28
20:4	Arachidonate (12)	36.27
20:5	Unidentified (13)	37.03
22:0	Behenate (14)	37.43
22:1	Erucate (15)	38.01
22:4	Docatetranoate (16)	41.73
24:0	Lignocerate (17)	42.50
24:1	Nervonate (18)	44.52

APPENDIX 8.4: Fatty acid standard (STD) gas chromatogram (RT = retention time; C:dble = number of carbons (C) and double bonds (dble)).



APPENDIX 8.5: Chromatograms of control (C) and experimental (M) traces of membrane fatty acid samples (to identify the individual fatty acids refer to appendix 6).

EXPERIMENT 9

5.1 DISPLACEMENT OF TRP FROM BSA BY MELATONIN AND VARIOUS DRUGS *IN VITRO*

5.1.0 Introduction

A number of studies have shown that depression is associated with depressed day-time and night-time levels of aMT (Vaughan, 1984).

It has been demonstrated that TRP can be displaced from SA by a number of agents which are known to bind to SA, such as salicylates (Tagliamonte *et al.*, 1973) and non-esterified fatty acids (Curzon *et al.*, 1973; Badawy *et al.*, 1984). The purpose of the present study was to investigate whether aMT, a compound structurally related to TRP and known to bind non-covalently to SA (Reiter, 1981), could possibly displace TRP from SA. The effect of certain antidepressants such as DMI, AMI and fluoxetine was also examined. The drug sulphanilamide, a known displacer of TRP from SA, was used as a reference standard.

5.1.1 Materials and methods

Defatted BSA and aMT were purchased from the Sigma Chemical Company, St. Louis, Mo, USA. The radiolabel, L-[methylene-^{14}C]TRP, specific activity 54 mCi mmol^{-1} , was purchased from Amersham International, Amersham, UK.

Displacement studies were performed according to a modification of the method described by Smith and Pogson (cited in Fernstrom *et al.*, 1971) using equilibrium dialysis. Briefly, 950 μl aliquots of Krebs-Henseleit bicarbonate buffer was supplemented with BSA to a final concentration of 0.003% (w/v) and pre-equilibrated with 0.1 μCi of radiolabelled (^{14}C) TRP for 30 minutes. This was followed by the addition of aMT to this compartment in final concentrations of 5 x 10^{-8} M, 5 x 10^{-7} M and 5 x 10^{-5} M.

Following this, each compartment was dialysed against Krebs-Henseleit bicarbonate buffer while shaking in a H_2O bath at a rate of 120 oscillations min^{-1} for 3.5 h at 37° C. After the 3.5 h incubation, 100 μl aliquots of the dialysate was assayed for the presence of radioactivity using radiospectrometry.

The experiment was performed in triplicate for each concentration of aMT and the experiments were repeated four times. The results are expressed as the means ± SEM. The data were analysed by one-way analysis of variance and the significance among specific means was determined using the Newman-Keuls

multiple range test. A p value of less than 0.05 between groups was accepted as statistically significant.

This particular method was chosen after initial attempts with affinity chromatography involving Affi Gel Cibracon blue. The TRP failed to bind SA in this system, probably because the binding of SA to the Affi Gel altered the TRP binding site. More importantly, equilibrium dialysis yielded consistent results.

5.1.2 Results

As shown in figure 112, the addition of aMT at a concentration of 5 x 10^{-8}M caused a significant displacement of radiolabelled (^{14}C) TRP ($p < 0.05$) and an even more significant displacement ($p < 0.001$) at concentrations of 5 x 10^{-7}M and 5 x 10^{-5}M.

None of the tested antidepressants had any effect on TRP displacement at the concentration range applied (μM to mM) (figs. 113, 114 and 115). For the drugs AMI, DMI and fluoxetine, there were no significant differences between the individual assays within a group or between the various groups with reference to the control ($p > 0.05$) level. Thus the mean values for each group were plotted against the control.

The drug sulphanilamide on the other hand (fig. 113), at a concentration of 5 x 10^{-7}, gave a statistically significant displacement ($p < 0.001$) of TRP from BSA.

5.1.3 Discussion

aMT, a hormone of the pineal gland secreted in a circadian rhythm (Reiter, 1981), is derived from TRP. TRP is the only amino acid which is non-covalently bound to SA (Curzon *et al.*, 1975). The concentration of circulating TRP is an important factor regulating the uptake of TRP in the brain (Fernstrom *et al.*, 1971; Curzon *et al.*, 1975), and consequently the synthesis of 5-HT in the brain (Smith, 1991). High concentrations of 5-HT in the brain, in turn, are known to result in the elevation of mood (Lucca *et al.*, 1994). Thus, agents which displace bound TRP *in vivo* would have the potential to raise 5-HT levels in the brain, thereby eliciting antidepressant effects.

The SSRI fluoxetine is known to be transported in blood bound to SA, and 94% of orally administered fluoxetine is associated with the protein in plasma. However, the antidepressant had no observable effect on TRP binding to BSA at the concentrations tested. This finding, raises the possibility that the antidepressant could bind to a distinct site which is clearly separate from the TRP affinity site.

5.1.4 Conclusion

The results of the present study show that the pineal hormone, aMT, is able to displace TRP from BSA *in vitro* at the micromolar level, suggesting the possibility that aMT has the potential for displacing TRP *in vivo*. However, this possibility requires further investigation. The antidepressants DMI, AMI and fluoxetine had no statistically significant effect at the range of concentrations tested and it can be concluded that their mode of action does not involve displacement of TRP from SA.

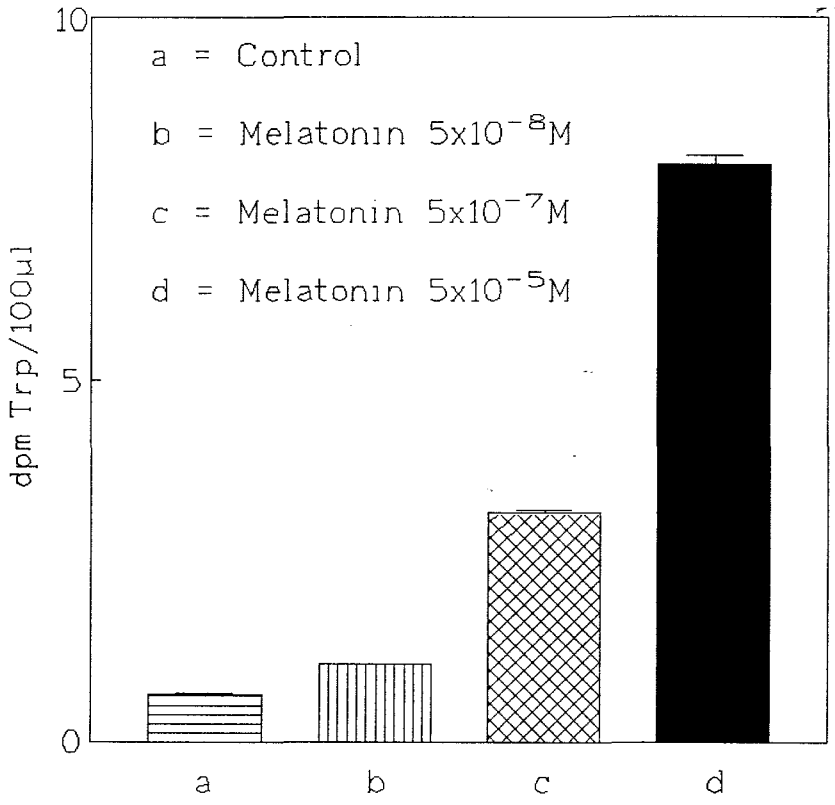


FIG. 112: Effect of different concentrations of melatonin on radiolabelled tryptophan (TRP) bound to bovine serum albumin (means ±SEM; n = 4). b as compared with a, $p < 0.005$; c as compared with a, $p < 0.001$; d as compared with a, $p < 0.001$.

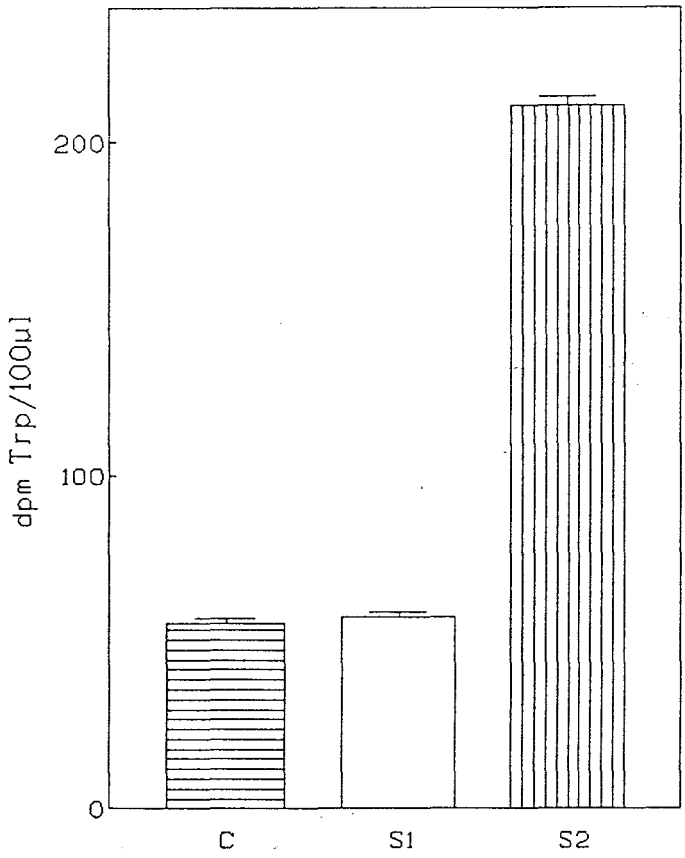


FIG. 113: Effect of reference standard sulphanilamide (S1 = 5 x 10^{-8} M and S2 = 5 x 10^{-7}) on the binding of tryptophan (TRP) (^{14}C) to bovine serum albumin *in vitro*. (means ±SEM; n = 4). S1 as compared with control (C), $p > 0.05$; S2 as compared with C, $p < 0.001$.

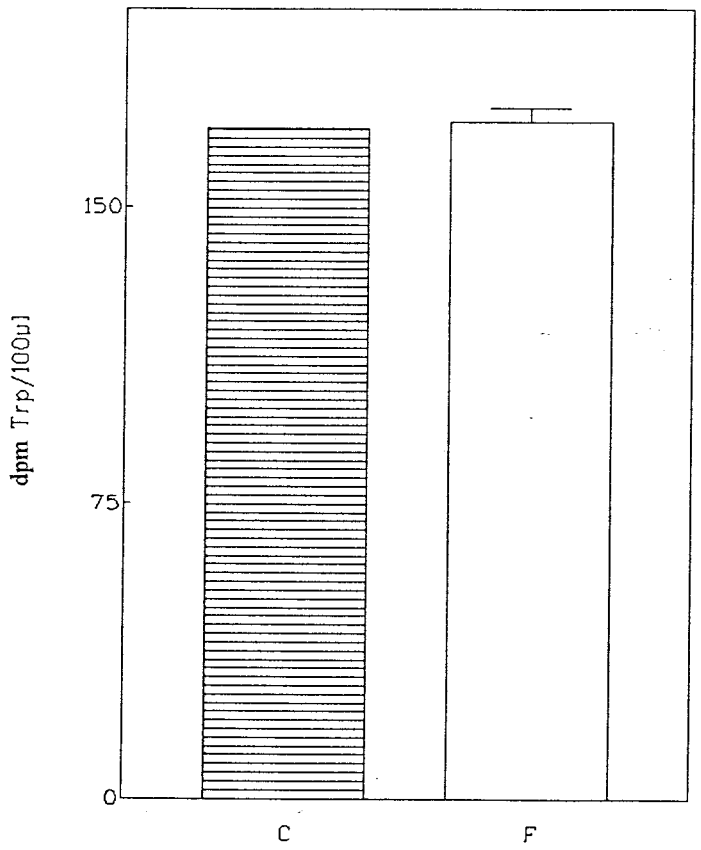


FIG. 114: Effect of different concentrations (1 x 10^{-6}M, 1 x 10^{-5}M, 1 x 10^{-3}M) of fluoxetine (F) on radiolabelled tryptophan (TRP) (^{14}C) bound to bovine serum albumin (means ±SEM; n = 4). $p > 0.05$ for all tested concentrations within the group and also with respect to control (C).

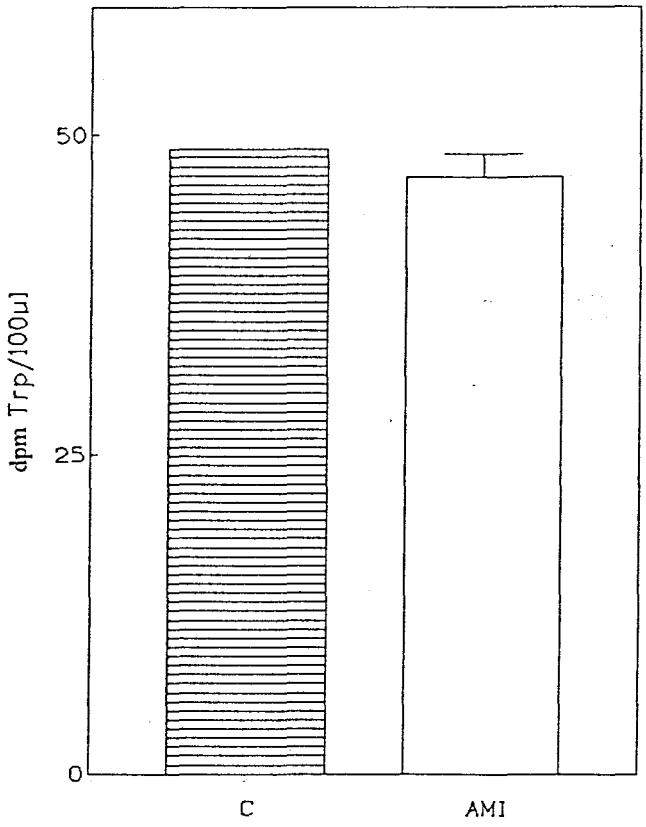


FIG. 115: Effect of different concentrations (1.5 x 10^{-6}M, 1.5 x 10^{-5} M, 1.5 x 10^{-3} M) of amitriptyline (AMI) on radiolabelled tryptophan (TRP) (^{14}C) bound to bovine serum albumin (means ±SEM; n = 4). $p > 0.05$ for all tested concentrations within the group and also with respect to control (C).

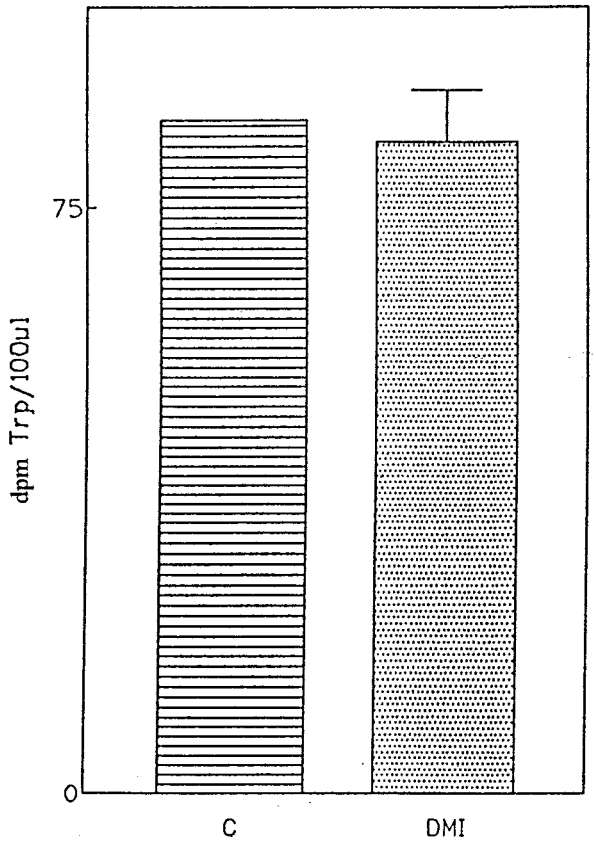


FIG. 116: Effect of different concentrations (1.5 x 10^{-6}M, 1.5 x 10^{-5}M, 1.5 x 10^{-3}M) of desipramine (DMI) on radiolabelled tryptophan (TRP) (^{14}C) bound to bovine serum albumin (means ±SEM; n = 4). $p > 0.05$ for all tested concentrations within the group and also with respect to C. C = control.

CHAPTER 6

FINAL SUMMARY AND CONCLUSIONS

6.0 FINAL SUMMARY

Figure 117 provides a diagrammatic summary of the research findings of this study.

6.0.0 Chapter 1: Literature review

This chapter reviews the physiological role of the amino acid TRP with respect to its fate in the body in relation to uptake into the brain, transport in blood and catabolism by hepatic TDO. TRP shares a common transport system for uptake into the brain with other LNAAs, and it is the ratio of TRP/LNAA which ultimately determines the amount of TRP that will be taken up by the brain. The potential roles of TRP and its derivatives in depression are reviewed, as are the peripheral determinants of brain TRP, specifically TDO. A brief overview of the literature on TRP-hydroxylase is given, but the focus is mainly on the regulation and mechanism of action of TDO. In addition, the different depression theories are outlined, although the biogenic amine deficiency hypothesis is covered in greater detail, with specific reference to the role of 5-HT, the TAD DMI, the SSRI fluoxetine and aMT. The physiological aspects of the function of aMT in humans and animals, particularly the neurohormone's role in depression and its neuroprotective role, are also dealt with.

6.0.1 Chapter 2: TDO and its effectors

At pH 7, the biocatalyst, rat hepatic TDO, show a constant K_s of ±100μM, and is subject to inhibition by substrate TRP at high TRP concentrations. aMT inhibits both the crude and purified enzyme competitively *in vitro* in a reversible fashion. 5-HT, at low concentrations, inhibits the enzyme non-competitively, becoming competitive at high levels. The inhibition of TDO by aMT is more potent than that of 5-HT at equimolar concentrations, however in the presence of 5-HT the effects of aMT are abolished.

The coenzyme NAD^+ affected TDO in an opposing manner *in vitro*. On the one hand, relatively low concentrations of the coenzyme (3mM) stimulate the enzyme, while relatively high levels (6mM) inhibit the enzyme. This molecule thus also appears to be an allosteric effector of TDO, similar to its phosphate derivative NAD(P)H. The precursor of the iron-porphyrin cofactor hemin, 5-ALA, stimulates the activity of TDO *in vivo,* but paradoxically increases rat forebrain 5-HT levels.

6.0.2 Chapter 3: aMT, DMI, Fluoxetine, TDO and the pineal

Both antidepressants inhibit TDO but in combination with aMT this effect is abolished. DMI increases NAS levels in the pineal while fluoxetine inhibits aMT synthesis.

6.0.3 Chapter 4: Insulin and pineal indoleamine metabolism

The pancreatic hormone insulin is shown to inhibit aMT synthesis and also to stimulate 5-HT breakdown to a significant degree.

6.0.4 Chapter 5: Physiological effects of aMT including displacement of TRP from BSA by various agents

Chronic aMT treatment had no significant effect on DNA and RNA levels, and no observable effect on protein metabolism and the fatty acid composition of hepatocyte membranes *in vivo*. However, significant alterations in the composition of liver cell cytoplasm fatty acids occurs. TDO activity *in vivo* is curtailed significantly, but this inhibition can be ameliorated by supplementation of exogenous substrate TRP.

aMT is shown to displace TRP from BSA *in vitro* at micromolar concentrations. The antidepressants AMI, DMI and fluoxetine had no effect on the binding of TRP to BSA *in vitro* at the concentrations tested .

6.1 CONCLUSIONS

An increasing body of experimental evidence acquired from studies on animals indicates that, in depression, aberrations in indoleamine metabolism are metabolically related to TDO. The findings of this study further support this assertion, particularly the results obtained with the antidepressant drugs used and aMT. A common denominator in the mode of action of these therapeutically efficacious antidepressants, DMI and fluoxetine, is their ability to influence TDO behaviour negatively. Inhibition of TDO resulted in accompanying changes in events in the pineal, specifically 5-HT metabolism. Stimulation of TDO by 5-ALA was also followed by alterations in forebrain 5-HT levels.

Another implication of the results of this research is the potential role of aMT as the natural antidepressant given the neurohormone's potential role in displacing bound TRP from TDO and inhibiting TDO. The net effect of these phenomena result in sufficiently elevated blood free TRP levels with a concomitant increase brain 5-HT and aMT synthesis. Additional experimental support for a possible therapeutic role by aMT in depression is the observation that the disruptive effects of DMI and fluoxetine on TDO activity are abolished in the presence of the neurohormone.

The potential role of aMT as a free radical scavenger is a distinct possibility given the proposed mechanism of action of TDO. The study demonstrates that enzymic catalysis is inhibited by aMT. Whereas allosteric effectors such as 5-HT and NAD(P)H exert their inhibitory effects on TDO via induction of a conformational change, the mode of action of the neurohormone aMT involves the TDO active site. It is highly likely that

either one or both of the potential free radicals (superoxide anion or the electrophillic TRP) generated during the reaction is scavenged by aMT, thereby causing effective inhibition of the reaction. A similar motif could account for the neurohormone's effect on fatty acid composition in rat hepatocytes, which is manifested by a reduction in the desaturated fatty acids.

The findings of this study, namely:

i) a restoration of normal TDO activity by aMT in the presence of DMI and fluoxetine,

ii) the inhibition of TDO by aMT,

iii) displacement of TRP from BSA

implicate aMT in both depression and as a possible neuroprotectant. These experimental observations, when viewed in combination, point to a function for aMT as a potential neuropsychiatric probe, in addition to having potential effects on a number of other physiological parameters.

The findings of this study support the biogenic amine deficiency hypothesis, implicating some of the major biogenic amines such as NA, 5-HT and aMT in depression. The drug DMI could act firstly by inhibiting TDO and thus increasing plasma TRP levels, and secondly stimulate NA release and prevent NA uptake at the pinealocyte membrane. The overall effect would be to enhance aMT synthesis with eventual remission. Fluoxetine appears to employ a different pharmacological mode of action in comparison to DMI. This SSRI appears to focus on the preservation of 5-HT. The fact that aMT counteracts the effects of both antidepressants, and restores the activity of TDO to that of the controls, is also consistent with the observation that the therapeutic action of drugs such as these coincides with the restoration of normal plasma levels of the neurohormone in depressives.

6.2 POSSIBLE FUTURE AREAS OF RESEARCH GENERATED BY THIS STUDY.

i) Examine specifically the interaction between aMT and fluoxetine also aMT and DMI, the experimental animals, particularly the older rats, had a low survival rate when treated with this combination . The possibility of chemical antagonism appears to be a distinct possibility and studies in this regard should definitely be pursued.

ii) Determine if any binding sites on the rat genome exist for DMI and fluoxetine, and if so, whether aMT has an affinity for the receptor site. This should be done in an attempt to explain the neutralising effect of aMT on TDO inhibition by both antidepressants.

iii) Ascertain the effects of aMT on fatty acid synthesising enzymes, particularly those early in the biosynthetic pathway, to try and understand how the indoleamine causes alterations in hepatocyte fatty acid composition in both the membrane and cytoplasm.

iv) Determine whether the experimental findings with NAD^+ have any physiological significance *in vivo.*

v) Attempt to identify the mechanism of insulin action on aMT synthesis, whether insulin receptors are present on the pinealocytes, and if inhibition of NAT and enhancement of 5-HT turnover is glucose-dependent.

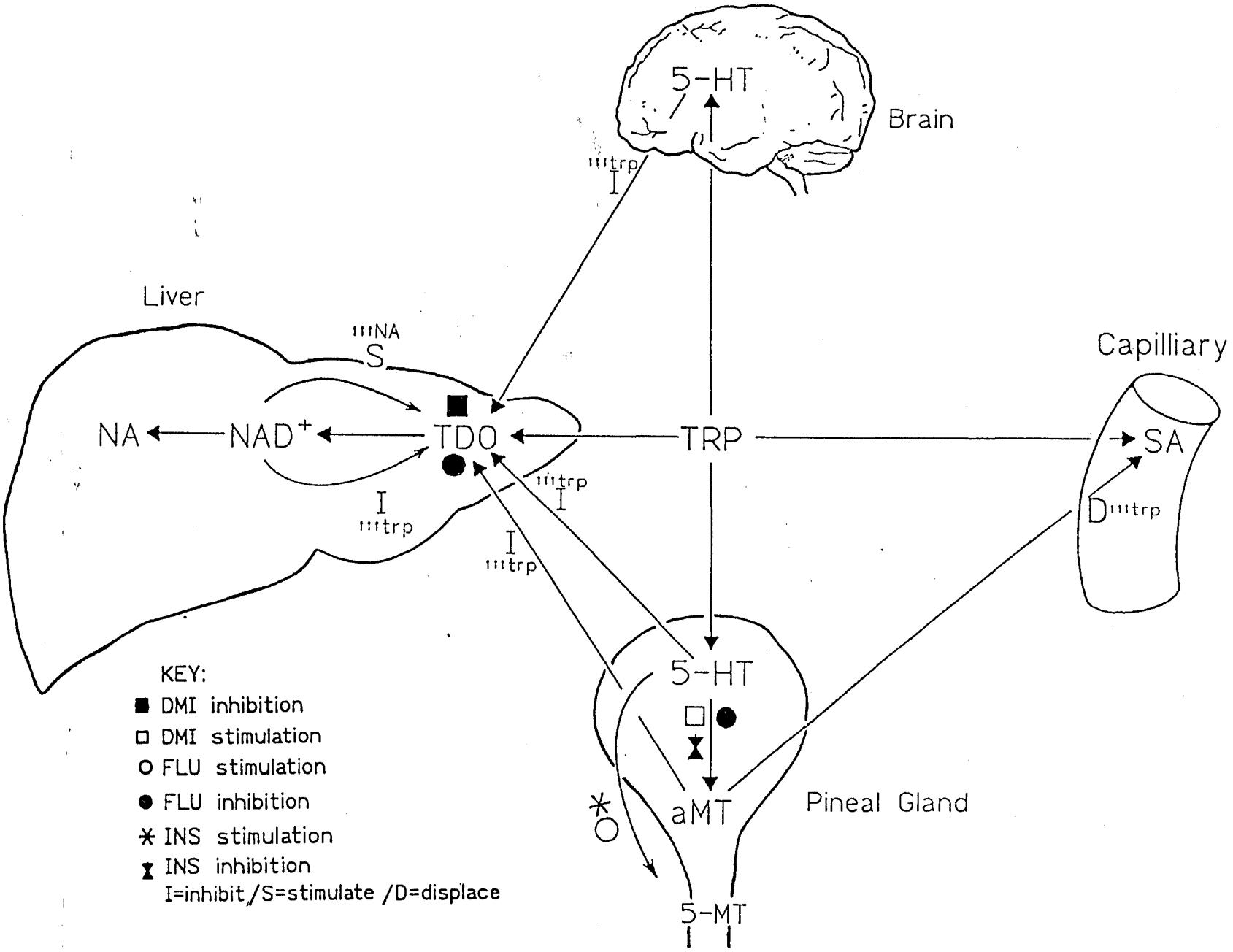


FIG. 117: Diagrammatic summary of research findings

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